ANTIULCER AND ENZYME STABILITY STUDIES OF SYNTHESISED PALMITOYLATED DERIVATIVES

[1]ASMA SAQIB, [2]CHANDRAKANT S KARIGAR

[1]Assistant Professor, Department of Biochemistry, Maharani's Science College for Women, Palace Road, Bangalore-560001
[2]Chairman and Professor, Department of Biochemistry, Jnana Bharati Campus, Bangalore University, Bangalore
E-mail: saqhib_22@yahoo.com

Abstract - The synthesized and completely characterized palmitoylated derivatives PSA PAA, PPAP and N- PPABA were evaluated for anti ulcer activity and enzyme stability studies. The resultant activity was found to be affected by the type of NSAID attached to lipid moiety. All the palmitoylated drugs found to be anti ulcerogenic. Salicylic acid with the palmitoyl moiety was found to be the most labile to hydrolysis. In contrast, the amide prodrugs N- PAA, PPAP and N- PPABA were the least susceptible to enzymatic and chemical hydrolysis due to presence of amide bond

Key Words - Palmitoylated derivatives, NSAID's, cyclooxygenase, PSA, PAA, PPAP and N-PPABA

I. INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are cyclooxygenase (COX) inhibitors that prevent prostaglandin (PG) synthesis (1), thus exhibiting analgesic, antipyretic, and anti-inflammatory actions. However, NSAIDs have been shown to exhibit a number of adverse effects, mainly because of inhibition of the constitutive isoform of COX (1). The major adverse effects of NSAIDs are gastro-toxic effects (for example, damage of gastric mucosa, gastric bleeding and gastro duodenal ulcers, increased bleeding tendency, and delay of the birth process (3,4). It is now well known that COX exists in two isoforms, namely COX-I and COX-II, which are regulated differently. Since most of the NSAIDs used clinically inhibit both isoforms of COX, there is enough evidence that inhibition of COX-I rather than that of COX-II underlies gastric ulcer formation [1]. However, long term uses of these agents have shown some potential side effects, including ulcer exacerbation in high-risk patients, delayed GI ulcer healing, kidney toxicity and cardiovascular side effects. Hence safety of these agents is questionable on their long-term use and some of these agents have been withdrawn from the market. Thus need of safer NSAID still remains. Palmitoylation of some synthetic compounds, which have been tested in vivo and in silico for the pharmacological activities, has already been reported by us (2, 3, 4). The synthetic palmitoylated derivatives are structurally similar to COX inhibitors aspirin and paracetamol. The introduction of long-chain fatty acid moiety at the carboxylic group or amide group in existing compounds (2, 3, 4) leads to an increase in their anti-ulcer activities and increased enzyme stability.

II. MATERIAL AND METHODS

2.1 Experimental animals and laboratory maintenance

Wistar albino rats (150-230 g) and mice of both sexes were selected as test animals for experimental evaluation. The selected animals were housed in acrylic cages under standard environmental conditions at 25 ± 2 °C at a relative humidity of 45-55 %, in a well ventilated room maintained at a 12:12 h light: dark cycle and were fed a standard rodent diet and water ad libitum.

All the animals were acclimatized for a week. Animal experiments were carried out according to the guidelines of the Committee for the Purpose of Control of Experiments on Animals (Ref. GPC/CPCSEA/08/2006-07) with approval of the Institutional Animal Ethics Committee, Government College of Pharmacy, Bangalore, India.
All the compounds (200 mg kg^{-1} body mass) and standard reference NSAID; aspirin, paracetamol (100 mg kg^{-1} body mass) and omeprazole (20 mg kg^{-1} body mass) were suspended in 1ml soya oil and administered orally using an animal feeding needle.
The control group received appropriate volume of vehicle (1ml soya oil, oral).

2.2 Anti-ulcer activity of palmitoyl derivatives

Gastrointestinal toxicity of the palmitoylated ester and amide derivatives were assessed by the measuring ulcer index and percentage of inhibition of ulcer formed by ethanol using the following two approaches.

2.3 Ethanol induced gastric ulcer (cytoprotective mechanism)

Male Albino wistar rats weighing 150 - 200 g were divided into seven groups. The animals were fasted for 48h with free access to water and orally administered with test compounds and omeprazole p.o. (per oral-drug doses given to animals 10 mg, 1 mL/200g of 99% alcohol ethanol.

2.4 Gastric lesions induced by swimming stress method (proton pump inhibition mechanism)

The induction of ulcers due to swimming stress was assessed by forced swimming of rats in the glass

cylinder (height 45cm, diameter 25cm) containing water to the height of 35cm maintained at 25^0C for 3 h. Animals were fasted for 48 h prior to experiments and divided into groups with six animals each.

2. 5 Evaluation of the histopathology of drug induced ulcers

Histological evaluation was performed on the glandular stomach; the stomach was washed thoroughly with saline, dehydrated in gradual ethanol (50-100%), cleared in xylene and embedded in paraffin. Sections (4-5mm) were prepared and then stained with hematoxylin and eosin (H-E) dye for microscopic observation and photography (magnification 100 X).

2.6 Enzymatic and chemical stability studies of the palmitoylated derivatives

Hydrolysis studies of synthesized palmitoylated derivatives were carried out in simulated gastric fluid (SGF) at pH 1.2, simulated intestinal fluid (SIF) at pH 7.4 and SIF + 80 % human plasma at pH 7.4 (5). A solution of 10 mg of palmitoylated derivatives was prepared in 90 mL of SIF (pH 7.4) or SGF (pH 1.2).

An aliquot of 15 mL of this solution was withdrawn repeatedly at regular intervals and kept in test tubes maintained at 37 ± 0.5 °C. At a definite time interval (0.5 h, 1– 8 h), an aliquot was withdrawn from different test tubes and was transferred to micro centrifuge tubes, followed by addition of methanol to make up the volume.

The tubes placed in a freezing mixture in order to arrest further hydrolysis, followed by vortexing at high speed for 5min. After vortexing, the tubes were centrifuged at high speed (3000 rpm) for 5 min. 5 mL of clear supernatant obtained from each tube was read by a spectrophotometer at 230 nm. The kinetics of hydrolysis and half-life ($t_{1/2}$) were determined. The rate of hydrolysis was calculated using the equation.

$$KH = (2.303/t) \log (a/a–x)$$

where kH represents the hydrolysis constant, t is the time in min, a is the initial conjugate concentration, x is the amount of conjugate hydrolyzed, (a–x) is the amount of the remaining, conjugate

2.7 Acute toxicity and maximum tolerance studies

The acute toxicity potential of the test compounds was determined by selecting albino wistar rats of either sex (250-300 g).

The animals were divided into groups, each group containing six animals. The dosage was administered orally as a single dose of 2000 mg kg^{-1} body weight.

2.8 Statistical analysis of the data

Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by student's t-test.

III. Results and Discussion

A. Anti-ulcer activity of palmitoyl derivatives:

Gastrointestinal toxicity of the synthesized palmitoylated derivatives was measured and compared with the standard by measuring the ulcer index and percentage of inhibition of ulcer formed by ethanol induced ulcer method and swimming induced ulcer method. Results are shown in Table: 1.

Table. 1: List the anti-ulcer activity of palmitoyl ester and amide derivatives in rats

Sl no	Treatment	Ethanol induced ulcer		Swimming stress induced ulcer	
		Mean ulcer index	% inhibition	Mean ulcer index	% inhibition
1	Control	5.67 ± 0.21	-	4.667±0.333	-
2	standard	1.92 ± 0.6112	67.08*	0.83±0.2108	85.3*
3	PSA	1.25 ± 0.4031	78.57*	1.92±0.6112	78.57*
4	N-PAA	0.83 ± 0.2108	85.3*	1.0±0.2336	78.58*
5	PPAP	0.83 ± 0.2108	85.3*	0.667±0.1667	85.71*
6	N-PPABA	0.75 ± 0.250	86.77*	0.75±0.250	86.77*

B. Histopathological studies:

The microscopic investigations of gastric tissues and tissue samples of control group of rats showed normal histological findings (Fig.1a). The micrographs obtained for the standard omezaprazole group revealed a focal erosive area in gastric mucosa and a zone (clear zone) in the basal regions of the gastric glands. This zone was parallel to the surface of the stomach lumen. In this zone, the structures of the gland were destroyed. They had disintegrated from the basal lamina and fallen into the lumen. The nuclei of these cells became smaller and dense, and their cytoplasms were stained as dark eosinophilic bodies. Small hemorrhagic areas and patches of inflammatory cell infiltrations were present in the lumen of the glands and lamina propria. The omeprazole treated group has shown superficial ulcer .Normal histological findings were displayed for PSA, N-PAA, PPAP and N-PPABA revealing that the derivatives are not producing any ulceration in the gastric region (Fig.1b and 1c)

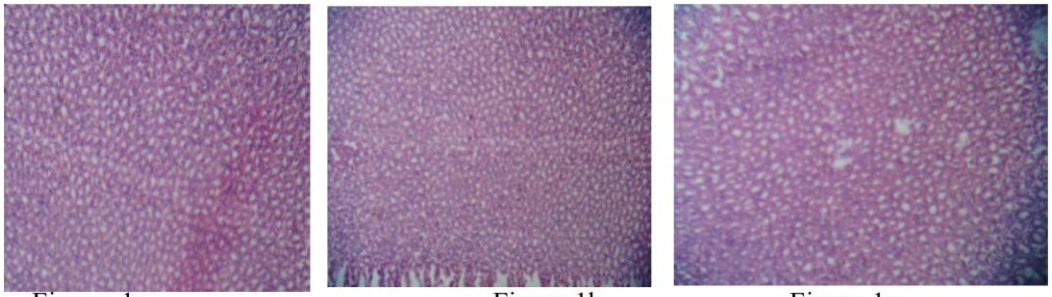


Figure. 1a Figure.1b Figure.1c

Fig.1a: Micrograph (X100) of normal gastric mucosa (control).
Fig. 1b: Micrograph (X100) of gastric mucosa not showing ulcers (palmitoyl salicylic acid treated) and mucosa appearing normal.
Fig.1c: Micrograph (X100) of gastric mucosa not showing ulcers (palmitoyl para amino phenol treated) and mucosa appearing normal.

Satisfactory hydrolysis of the palmitoyl derivatives were observed in SGF and very encouraging hydrolysis in SIF + 80 % human plasma. The latter is due to the presence of amidase and esterases (carboxyl esterases) in plasma(6) .The results suggests that all the synthesized prodrugs are sufficiently stable at pH 1.3, which is considered as a non-enzymatic simulated gastric fluid (SGF), so that no appreciable hydrolysis to the free acids might occur in the stomach. Similarly, the chemical stability of the palmitoyl derivatives pH 7.4 ($t_{½}$ - 0.8 - 24.8 h) suggests that they are absorbed almost unchanged from the intestine. This observation may be due enrichment of plasma with esterases. It is noteworthy that the susceptibility for enzymatic hydrolysis was found to be affected by the type of NSAID attached to lipid moiety. Salicylic acid with the palmitoyl moiety was found to be the most labile. In contrast, the amide prodrugs N- PAA, PPAP and N- PPABA were the least susceptible due to presence of amide bond. Gastrointestinal toxicity of the synthesized palmitoylated derivatives was measured and compared with the standard by measuring the ulcer index and percentage of inhibition of ulcer formed by ethanol induced ulcer method and swimming induced ulcer method. The rate constant (k_{obs}) for hydrolysis and the corresponding half-lives for each of the studied prodrug are listed in Table 2. The half-life ($t_{1/2}$) of PSA in SIF was found to be 0.8 h and that of

Table. 2: List the anti-ulcer activity of palmitoyl ester and amide derivatives in rats

Compound	SGF pH 1.3		SIF pH 7.4		SIF+ 80% plasma	
	k_{obs} (h^{-1})	$t_{1/2}$ (h)	K_{obs} (h^{-1})	$t_{1/2}$ (h)	K_{obs} (min^{-1})	$t_{1/2}$ (min)
PSA	0.1034	6.7	0.160	4.3	0.0018	384
N-PAA	0.026	26.8	0.028	24.8	0.0056	120
PPAP	0.044	15.8	0.036	19.3	0.0065	108
N-PPABA	0.016	43.0	0.846	0.8	0.0497	13.9

PAA 24.8 h. Satisfactory hydrolysis of the palmitoyl derivatives were observed in SGF and very encouraging hydrolysis in SIF + 80 % human plasma. The latter is due to the presence of amidase and esterases (carboxyl esterases) in plasma. The results suggests that all the synthesized prodrugs are sufficiently stable at pH 1.3, which is considered as a non-enzymatic simulated gastric fluid (SGF), so that no appreciable hydrolysis to the free acids might occur in the stomach. Similarly, the chemical stability of the palmitoyl derivatives pH 7.4 ($t_{½}$ - 0.8 - 24.8 h) suggests that they are absorbed almost unchanged from the intestine. The kinetic data (Table: 2) show that the synthesized derivatives are more susceptible to enzymes in plasma. This observation may be due enrichment of plasma with esterases. It is noteworthy that the susceptibility for enzymatic hydrolysis was found to be affected by the type of NSAID attached to lipid moiety. Salicylic acid with the palmitoyl moiety was found to be the most labile. In contrast, the amide prodrugs N- PAA, PPAP and N- PPABA were the least susceptible due to presence of amide bond. The results in Table: 2 reveals that all the synthesized prodrugs are sufficiently stable at pH 1.3, which is considered as a non-enzymatic simulated gastric fluid (SGF), so that no appreciable hydrolysis to the free acids might occur in the stomach. Similarly, the chemical stability of the palmitoylated derivatives pH 7.4 (t½ - 0.8 - 24.8 h) suggests that they are absorbed almost unchanged from the intestine. The kinetic data show that the synthesized derivatives are more susceptible to plasma than in SGF and SIF. This observation may be due to enrichment of plasma with esterases which is consistent with previously reported investigations. The rates of hydrolysis of the palmitoylated derivatives in plasma are markedly accelerated compared with those in aqueous buffers. It is noteworthy that the susceptibility for enzymatic hydrolysis was found to be affected by the type of NSAID attached to lipid moiety (6). Aspirin prodrug PSA with ester bond was found to be the most labile. In contrast, the amide prodrugs N-PAA, PPAP and N-PPABA were the least susceptible ones due to lesser electrophilicity of the carbon - nitrogen bond (7). According to the in vitro stability studies, the synthesized ester and amide derivatives were sufficiently stable at SGF pH 1.2. Hence, hydrolysis was not expected in the stomach. In addition, the

chemical stability of the compounds at SIF pH 7.8 suggested that they can be absorbed from the intestines almost intact.

CONCLUSION

The observed high anti-ulcer activities and increase in enzymatic and chemical stability studies are attributed to the presence of long lipophilic lipid chain and strong acidic functional group. Furthermore, the enzymatic stability of the palmitoyl ester and amide derivatives after 8 h of incubation in plasma establishes evidence that they may act in vivo without prior hydrolysis of the ester and amide bond and palmitoyl amides are more stable than palmitoyl esters.

REFERENCES

[1] Asma Saqib, Chandrakant.S. Karigar." Cyclooxygenase isoforms in health and biology". Internet .J. Pharmacology, 7, 1, 2009.

[2] Asma Saqib, Mohammed Afzal Pasha, Chandrakant Karigar , Metticule Shankar Naryan Rao Harish. "Synthesis, characterization and pharmacological evaluation of palmitic acid derivatives of salicylic acid and anthranillic acid". Journal of Pharmaceutical Research and Opinion 2: 35-38, 2012.

[3] Asma Saqib., M.A Pasha., C.S. Karigar.. M.S Harish. "Synthesis of palmitic acid derivatives of p-aminophenol and p-amino benzoic acid with improved pharmacodynamic profiles". Acta. Pharmaceutica. Scientia. 52: 205-212, 2010.

[4] Raghu Bhagvat, Asma Saqib, C.S Karigar, "Molecular docking studies of novel palmitoylated ligands for Cyclooxygenase-2". Chemical Biology and Drug Design, 79, 6, 1043-1048, 2012.

[5] N. W. Nielsenw and H. Bundgaard. "Glycolamide esters as biolabile prodrugs of carboxylic acid agents: synthesis, stability, bioconversion, and physicochemical properties". J. Pharm. Sci. 7, 285–298, 1998.

[6] T.A. Fadl and F.A. Omar." Paracetamol (acetaminophen) esters of some non-steroidal

[7] anti-inflammatory carboxylic acids as mutual prodrugs with improved therapeutic

[8] index". Inflammopharmacology 6, 143-157, 1998.

[9] Surender Kumar, D.K.Tyagi and Arun gupta." Synthesis and evalution of amide

[10] prodrugs of diclofenac". J. Pharm. Sci. & Res, 2, 369-375, 2010.

★★★