Spacer/Linker Based Synthesis and Biological Evaluation of Mutual Prodrugs as Antiinflammatory Agents

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Mutual prodrugs of some antiinflammatory agents were synthesized with the aim of improving the therapeutic

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index through prevention of gastrointestinal complications and to check the efficiency of release of the parent drug in presence of spacer. These mutual prodrugs were synthesized by direct condensation method using dicyclohexyl carbodiimide as a coupling agent and glycine as a spacer. The title compounds were characterized by spectral techniques and the release of the parent drug from mutual prodrug was studied in two different non-enzymatic buffer solutions at pH 1.2, pH 7.4 and in 80% human plasma. All mutual prodrugs exhibited encouraging hydrolysis profile in 80% human plasma. Biological activity of title compounds was studied by carrageenan-induced paw edema method. From the results obtained, it was concluded that these compounds retain the antiinflammatory action.

Key words: Cyclooxygenases, dicyclohexylcarbodiimide, gastro-intestinal toxicity, mutual prodrugs, NSAIDs, prostaglandin

Non-steroidal antiinflammatory agents (NSAIDs) continue to be more widely used group of therapeutic agents than any other class of medicinal agents. The action of NSAIDs is thought to involve the inhibition of cyclooxygenases (COXs), which are responsible for prostaglandins (PGs) synthesis[1,2]. The gastrointestinal (GI) toxicity of the NSAIDs is most challenging since these side effects are mostly related to mechanism of action of these agents[3,4]. They are generally attributed to direct and/or indirect mechanisms. The direct contact effect usually results from a local irritation produced by the acidic group of the NSAIDs and local inhibition of PG synthesis in the GI tract. The indirect mechanism is due to a generalized systemic action occurring after absorption and can be demonstrated by intravenous dosing[5,6]. A possible approach to solve these delivery problems may be derivatization of the carboxylic function to produce prodrugs/mutual prodrugs with adequate stability at the acidic pH of the stomach. Thus, this type of derivatization may on one hand prevent local irritation of the stomach mucosa and on the other hand be capable of releasing the parent drug/s spontaneously or enzymatically in the blood following their absorption[7]. Naproxen, ibuprofen and ketoprofen were selected as model drugs for carboxylic acid derivatization.

Melting points were determined by open capillary method and uncorrected, which were further confirmed by visual melting point apparatus (Lab India). Thin layer chromatography (TLC) was carried out on precoated silica gel plates (Merck, $60GF_{254}$) using UV light as detecting agent. UV spectra were recorded on a UV/Vis spectrophotometer (Jasco V-30). IR spectra were recorded with a FTIR-4200, Shimadzu spectrometer. ^1H NMR spectra were recorded in $CDCl_3$ using 300 MHz ^1H NMR Spectrometer (Jeol-FT-NMR-300mHz, Japan). Mass spectra were recorded on GCMS-QP-2010-Schimadzu. Elemental analyses were carried out manually by combustion method in the Indian Institute of Technology, Powai, Mumbai. Naproxen was obtained as a gift sample from M/s Themis Labs, Pvt Ltd, Thane, India. Ibuprofen and ketoproen were obtained as a gift sample from Khandelwal Laboratories, Mumbai and FDC Ltd, Goa, respectively. Paracetamol was obtained as a gift sample from Piramal Healthcare Ltd., Mumbai. Boc-glycine and DCC (dicyclohexyl carbodiimide) were purchased from Spectrochem Labs, Mumbai. Human plasma was purchased from the Blood bank of KEM Hospital, Mumbai. Prior permission was taken for the conduction of *In vivo* studies in animals. All other chemicals used were of analytical grade procured from institutional store and solvents were of synthetic grade utilized after distillation.

The synthesis of title compounds involved following steps. Boc-glycine (1.75 g, 0.01 mol) was dissolved in 15-20 ml dichloromethane (DCM) in round bottom flask with guard tube attached at the neck and stirred for 0.5 h in cold condition (0-4°). DCC (2.06 g, 0.01 mol) was dissolved in 5-10 ml of DCM in a beaker and added dropwise to a solution of Boc-glycine and stirred for 2 h in cold condition (0-4°).

Paracetamol (1.51 g, 0.01 mol) was suspended in 20 ml of DCM in a beaker and 3-4 ml of pyridine was added to dissolve the same. The mixture was stirred for 1 h in cold condition (0-4°). This solution was added dropwise to the mixture of Boc-glycine and DCC and stirred for 4 h at 0-4° followed by stirring for 16 h at room temperature. The reaction mixture was further filtered to remove the byproduct, precipitated dicyclohexylurea. The filtrate was washed with 0.05N HCl and saturated solution of $NaHCO_3$ to remove the unreacted paracetamol and concentrated under vacuum. The product of Boc-gly-paracetamol was then stirred for 2 h at room temperature with

1:1 mixture of dichloromethane and trifluoroacetic acid (TFA) to break the Boc which is a protecting group for -NH_2 functional group in Boc-glycine. After breaking Boc the compound obtained was found to be glycine-paracetamol (GlyPara).

Naproxen (1.15 g, 0.005 mol) was dissolved in 5 ml dimethyl formamide (DMF) in round bottom flask with guard tube attached at the neck and stirred for half an hour in cold condition (0-4°). DCC (1.03 g, 0.005 mol) was dissolved in 5-10 ml of dimethyl formamide in a beaker and was then added dropwise to a solution of naproxen and stirred for 2 h in cold condition (0-4°). GlyPara (1.08 g, 0.005 mole) was dissolved in 10 ml DMF in a beaker and stirred for 1 h in cold condition (0-4º). This solution was then added to the above mixture and stirred continuously for 4 h in cold condition (0-4º) and 16 h at room temperature. The reaction mixture was further filtered to remove the by product, precipitated dicyclohexylurea. The filtrate was then concentrated under vacuum. The crude product was purified by column chromatography using hexane:ethyl acetate (2:1) as a mobile phase.

The title compound, NapGlyPara (68% yield) showed the melting range 194-196^0. 1H NMR ($CDCl_3$) exhibited characteristic peaks for 7.11-7.72(m) 10H of aromatic region, 3.46(s) 3H of OCH_3, 1.50 - 1.52(d) 3H of -CH_3, 4.05 – 4.11(q) 1H of –CH, 8.46(bs) 1H of amide group, 3.91(s) 2H of CH_2, 1.91(s) 3H of –CH_3. GC-MS for title compound showed molecular ion peak at 422 and elemental analysis: $C_{24}H_{24}O_5N_2$ (Mol. Wt. 420); calculated C- 68.57%, H- 5.71%, N- 6.66%; found C- 68.84%, H- 5.54%, N- 6.91%.

The methodology for synthesis of IbuGlyPara and KetoGlyPara was similar to that of NapGlyPara. The title compound, IbuGlyPara (76% yield) showed the melting range 144-146^0. 1H NMR ($CDCl_3$) exhibited characteristic peaks for 7.08-7.26 (m) 8H of aromatic region, 1.12–1.16 (d) 6H of –CH_3, 1.43–1.45 (sept) 1H of –CH, 2.02 (d) 2H of CH_2, 1.90–1.94 (d) 3H of –CH_3, 3.87– 3.94 (q) 1H of CH, 5.51 (bs) 1H of amide group, 3.45 (s) 2H of –CH_2. GC-MS for title compound showed molecular ion peak at 398 and elemental analysis: $C_{23}H_{28}O_4N_2$ (Mol. Wt. 396); calculated C- 69.69%, H- 7.07%, N– 7.07% and found C– 69.68%, H– 7.19%, N– 7.11%.

The title compound, KetoGlyPara (75% yield) showed melting range 130-132^0. 1H NMR ($CDCl_3$) exhibited characteristic peaks for 6.96–7.72 (m) 13H of aromatic region, 1.91–1.94 (d) 3H of -CH_3, 4.05–4.11 (q) 1H of –CH, 6.32 (bs) 1H of amide group, 3.91 (s) 2H of –CH_2, 3.46 (s) 3H of –CH_3. GC-MS for title compound showed molecular ion peak at 398 and elemental analysis: $C_{26}H_{24}O_5N_2$ (Mol. Wt. 444); calculated C- 70.27%, H- 5.40%, N- 6.30% and found C- 70.42%, H- 5.55%, N- 6.70%.

The mutual prodrug synthesized was subjected to chemical hydrolysis (using buffers of physiological pH) and enzymatic hydrolysis (using 80% human plasma). The chemical hydrolysis study was carried out in two buffer systems hydrochloric acid buffer (pH 1.2) and phosphate buffer (pH 7.4). The absorbance of the aqueous layer was found to be negligible so, the mutual prodrugs did not hydrolyze in hydrochloric acid buffer solution of pH 1.2 in 5 h.

Mutual prodrug (40 mg) was dissolved in 10 ml chloroform and 1 ml of the same was added to 99 ml buffer solution in a 250 ml beaker. The solution was stirred using a magnetic stirrer and the temperature was maintained at 37°. Aliquot of 1 ml was withdrawn from the beaker and sink conditions were maintained by adding 1ml of buffer at the end of 0.5, 1, 2, 3 and 4 h. The sample solution of pH 7.4 was acidified with 0.5 ml of 9.6% phosphoric acid solution, prior to extraction. The aliquots withdrawn were extracted with 7 ml of chloroform. Each aliquot was extracted for 3 min. by using the cyclo-mixer. This was followed by centrifugation of the same at 2000 rpm for 2 minutes. 5 ml of Chloroform layer was separated from each sample and extracted into 5 ml of 0.05N NaOH solution. Absorbance of aqueous layer was recorded against 0.05N NaOH as the blank treated similarly at λ_{max} 230 nm, 224 nm and 258 nm for NapGlyPara, IbuGlyPara and KetGlyPara respectively Table 1.

Mutual prodrug (40 mg) was dissolved in 10 ml chloroform and 1 ml of the same was added to 99 ml 80% human plasma in a 250 ml beaker. The contents of the beaker were stirred continuously and the temperature was maintained at 37°. Aliquot of 1 ml was withdrawn at the end of 0.5, 1, 2, 3 and 4 h and equal aliquots of fresh 80 % human plasma was replaced in the beaker immediately to maintain sink conditions. The sample of pH 7.4 was acidified with 0.1 ml of perchloric acid solution, prior to extraction.

The aliquots withdrawn were extracted with 7 ml of chloroform. Each aliquot was extracted for 3 min by using the cyclo-mixer. This was followed by centrifugation of the same at 2000 rpm for 2 min. Chloroform layer of 5 ml was separated from each sample and extracted into 5 ml of 0.05N NaOH solution. Absorbance of aqueous layer was recorded against 0.05N NaOH as the blank treated similarly at respective λ_{max} for NapGlyPara, IbuGlyPara and KetGlyPara (Table 1).

Wistar rats weighing between 100-150 g were divided into 5 groups of 6 rats each. The rats were injected subcutaneously 0.1 ml of 1% (w/v) of carrageenin into the planter region of each hind-paw. NapGlyPara and IbuGlyPara were evaluated at single dose level; naproxen and ibuprofen were used as standard antiinflammatory drugs for comparison. The paw edema volumes were measured using plethysmometer at various time intervals like 0, 1, 2, 3, 4, 6 h after carrageenin injection. The hind paw edema inhibition at doses of test drug and standard was calculated by comparing with vehicle treated control rats shown in fig. 1

The antiinflammatory activity of test drug was studied at single dose level. The % inhibition of paw edema volume by the test compound or standard antiinflammatory drug (naproxen/ibuprofen) was calculated by the formula; (A–B)/A×100, where A represents the control value and B represents the standard and/or test drug value

All the procedures utilized for synthesis of mutual prodrugs were standardized by varying important parameters to optimize the yield of product. The structures of synthesized mutual prodrugs were characterized by spectral techniques like UV, IR, 1H NMR, and GCMS and by elemental analysis. The purity was ascertained by performing TLC and Rf values were calculated for each title compound which were found to be 0.73, 0.67 and 0.69 for NapGlyPara, IbuGlyPara and KetGlyPara, respectively. The physical constants like melting point were also recorded. UV spectra of the synthesized mutual prodrugs showed λmax values different from their parent compounds. IR spectra of the synthesized compounds showed absorption bands for functional groups of NapGlyPara (ester peak 1738), IbuGlyPara (ester peak 1735) and KetGlyPara (ester peak 1742), the characteristic feature of anticipated structures of synthesized mutual prodrugs. NMR spectra of synthesized mutual prodrugs recorded depict expected δ-values for proton whereas mass spectra of the title compounds produced molecular ion peak value and reported fragment values. Elemental analysis of title compounds also showed the expected percentage of C, H and N which were found matching with

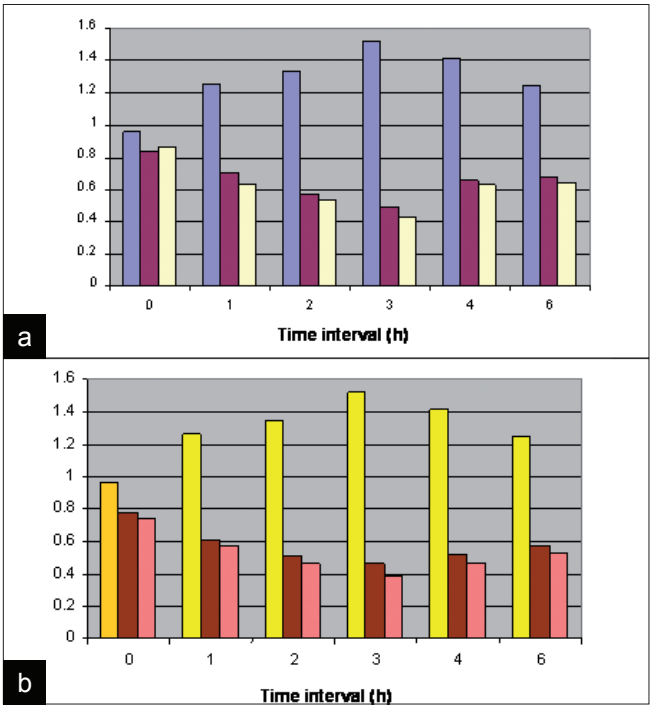
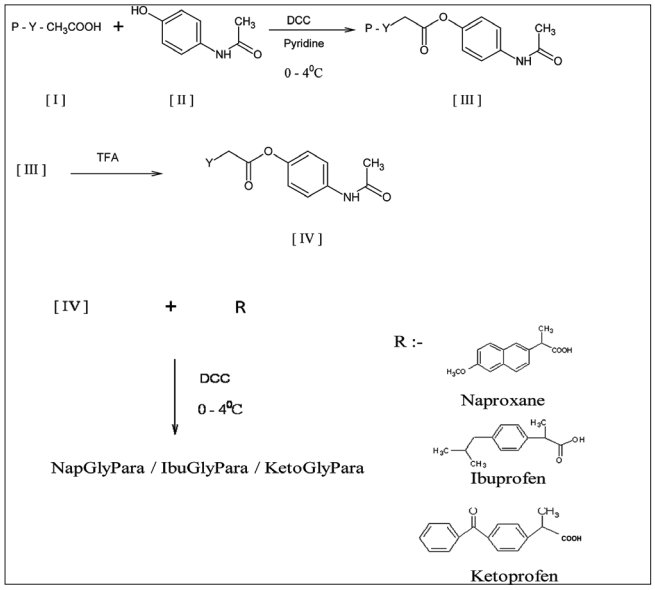


Fig. 1: Paw volume vs time interval plots for test and standard drugs (a) Control , Test 1, NapGlyPara , standard 1 is naproxen and (b) Control , Test 2: IbuGlyPara , standard 2: ibuprofen

TABLE 1: HYDROLYSIS STUDIES

Time (h)	% Release					
	In 80% human plasma			In buffer solutionof pH 7.4 (phosphate buffer)		
	NapGlyPara	IbuGlyPara	KetGlyPara	NapGlyPara	IbuGlyPara	KetGlyPara
0	-	-	-	-	-	-
0.5	11.88	12.79	15.58	3.96	3.83	-
1	23.76	26.85	28.25	10.56	8.95	7.79
2	42.25	44.76	41.89	19.80	16.62	16.56
3	67.34	63.95	57.47	34.33	31.97	30.15
4	83.19	86.97	72.08	55.32	51.16	46.76



Scheme 1: Naproxen, ibuprofen and ketoprofen mutual prodrugs with paracetamol using glycine as spacer.
P= Protecting group, Y= Spacer, TFA= Trifluoro acetic acid and DCC= Dicyclohexyl carbodimid

calculated values. All the above results positively confirm the formation of the synthesized compounds and correctness of the anticipated structures, of the synthesized mutual prodrugs.

Hydrolysis kinetics of the synthesized mutual prodrugs showed that they did not hydrolyze in hydrochloric acid buffer (pH 1.2) indicating that they would not release the parent drugs in acidic pH of the stomach. Hydrolysis of synthesized mutual prodrugs in phosphate buffer (pH 7.4) follows slow first order kinetics. While in 80% human plasma, rate of hydrolysis was found to be almost double as compared to that in phosphate buffer (pH 7.4). Also the biological activity of the synthesized compounds was found to be similar to the parent compound. The results obtained show that the synthesized mutual prodrugs of aryl propionic acid derivatives of NSAIDs with spacer are a better examples than other existing mutual prodrugs.

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