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Biological and metabolic study of naproxen–propyphenazone mutual prodrug

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Abstract

Naproxen–propyphenazone (NAP-PP) esters were synthesized as prodrugs with the aim of improving the therapeutic index through prevention of gastrointestinal irritation and bleeding. The structures of the synthesized NAP-PP hybrid esters were confirmed by IR and ^{1}H NMR spectroscopy and their purity was established by elemental analysis, HPLC and TLC. The release of NAP as well as PP derivatives, from the ester prodrugs was studied. A validated analytical HPLC method for the estimation of the NAP, and the prodrugs was developed. Also the enzymatic hydrolysis products of the ester were identified by GC–MS and in conjugation with HPLC. The kinetics of ester hydrolysis was studied in two different non-enzymatic buffer solutions, at pH 1.2, and 7.4 as well as in liver homogenates. Study of analgesic and anti-inflammatory properties in comparison with the reference compounds has shown that both analgesic and anti-inflammatory activities were present at the same doses of the investigated compounds. The ester **III** was found to be less irritating to gastric mucosal membrane than the parent drugs. These results suggest that the synthesized prodrugs are characterized by better therapeutic index than the parent drugs.

Keywords: Naproxen prodrug; Propyphenazone; Prodrugs; Ulcerogenicity; NSAIDs; Chemical and enzymatic stability

1. Introduction

For many years, several attempts have been made to develop bioreversible derivatives or prodrugs of non-steroidal anti-inflammatory drugs (NSAID) containing carboxylic acid function in order to depress upper gastrointestinal (GI) irritation and bleeding (Laporte et al., 1991; Wynne et al., 1998). Esterification of the carboxylic acid moiety of NSAIDS would suppress gastro-toxicity without adversely affecting their anti-inflammatory activity. In addition the biotransformation of the prodrugs to the parent compounds at its target site or sites of activity may be used to achieve rate and time controlled drug delivery of the active entities (Bonina et al., 2001; Rautio et al., 2000, Thorsteinsson et al., 1999). Naproxen (NAP), a potent NSAID with moderate analgesic activity associated with GI irritation was selected as a model drug for carboxylic acid derivatization (Shanbhag et al., 1992). Esterification of naproxen with different alkyl esters and thioesters lead to prodrugs with retained anti-inflammatory activity but exhibited greatly reduced gastrointestinal erosive properties and significantly reduced analgesic potency (Venuti et al., 1989). But esterification with ethylpiperazine showed that the analgesic activity was preserved whereas anti-inflammatory activity was generally reduced (Calvi et al., 1985). Propyphenazone (PP) is a non-acidic pyrazole drug and has good analgesic and antipyretic activity with no anti-inflammatory activity (Burne, 1986). The rational of this work was to couple naproxen with propyphenazone to achieve many advantages related to synergistic analgesic effect with reduced GI irritation. The metabolism of propyphenazone has been reported to proceed via the formation of 3-hydroxymethyl-propyphenazone (HMP), which is pharmacologically active as the parent drug (Goromaru et al., 1984; Neugebauer et al., 1997). Coupling of both compounds as a hybrid drug or through a spacer as a mutual prodrug results in a potent analgesic anti-inflammatory compound with reduction of the main adverse local effects related to the activity of NSAID. The preparation of naproxen esters/amides with PP derivatives

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is described. The availability of NAP and PP derivatives after hydrolysis is studied by chemical and by enzymatic means.

2. Material and methods

2.1. Materials

Melting points were recorded (uncorrected) on a melting point/MS (Stuart Scientific, UK). Infrared spectra were recorded with a Shimadzu IR-470 spectrometer (Shimadzu, Tokyo, Japan) and UV spectra on a Shimadzu UV-120-02 spectrophotometer (Shimadzu, Kyoto, Japan). Thin layer chromatography (TLC) was carried out on precoated silica gel plates (Merck, 0.25 MM, 60 F254). Column chromatography was carried out on Merck silica gel 60 (Particle size 0.063–0.200 mm). ^{1}H NMR spectra were recorded on a Jeol JNM-EX 270 MHz spectrometer (Jeol, Tokyo, Japan); all chemical shifts are given in δ ppm, relative to tetramethylsilane (TMS) as internal standard. Elemental analyses were performed at the Microanalysis Center, Chemistry Department, Faculty of Science, Assiut University.

The HPLC system consisted of a Knauer model 64, solvent delivery module (Knauer, Germany), Knauer variable wavelength UV detector, 20-μl sample loop, and a Shimadzu CR-6A Chromatopac integrator (Shimadzu, Tokyo, Japan). The column used was Du-Pont Zorbax C8 (250 mm×4.6 mm i.d., 6 μm), heated at 40 °C (Du-Pont column heater, Du-Pont, Delawara, USA). The effluent was monitored at 284 nm at a flow rate of 1 ml/min. Two mobile phases were used. Mobile A, consisting of methanol/acetonitrile/0.05 M sodium acetate (30:15:55) adjusted to pH 5.0 with glacial acetic acid was used for the estimation of HMP and NAP; Mobile B, consisting of acetonitrile/isopropanol/0.05 M sodium acetate (45:5:50), adjusted to pH 5.0 with glacial acetic acid was used for the estimation of ester. The GC–MS apparatus used was a Finnigan/MAT 1020B (Finnigan/MAT, Bremen, Germany); data system: INCOSI. Capillary column: DB-5 (J&W Scientific, Rancho, Cordova, USA), fused silica 30 m×0.25 mm i.d., 0.25 μm film thickness. Carrier gas: helium, pressure 26 p.s.i., flow rate 60 cm/s. Gerstel-Split injector, split 1:20 direct coupling. Source temperature 180 °C, ionization energy 70 eV, and multiplier voltage 2000 V. Scanning from 35 to 400 amu/s. Column port temperature 260 °C, detector (FID), temperature 320 °C, column oven temperature programmed from 150 °C (1 min) to 280 °C (5 min) to 300 °C (7 min) were used. β-Glucouronidase-arylsulfatase (from Helix Pomatia, stabilized aqueous solution, β-Glucouronidase 40 U/ml; arylsulfatase 20 U/ml) was purchased from Merck (Darmstadt, Germany). *N*-Methyl-*N*-trimethylsilyltrifluro-acetamide (MSTFA), trimethylchlorosilane (TMSCI), and NADP were purchased from Fluka (Feinchemikalien, Neu-Ulm, Germany). Naproxen was obtained as a gift from Misr Company for Pharmaceuticals (Cairo, Egypt), and propyphenazone was obtained from Kahira Company for Pharmaceuticals (Cairo, Egypt). Dimethylformamide (DMF) was purchased from El-Naser Chemical Industry Company (Cairo, Egypt), dried over phosphorus pentoxide and successively distilled under reduced pressure before use. All other chemicals and solvents were of analytical grade. The starting compound 3-bromomethyl-propyphenazone was prepared in a pure crystalline form according to the reported method (Lucius, 1907).

2.2. Chemistry

2.2.1. Preparation of 3-aminomethyl-4-isopropyl-2-methyl-1-phenyl-3-pyrazoline-5-one (AMP)

A solution of 3-bromomethylpropyphenazone (3 g, 10 mmol) in chloroform (100 ml) and hexamethylenetetramine (1.7 g, 12 mmol) was stirred at 50 °C for 3 h. The solvent was evaporated under vacuum to dryness. The yellowish crystalline adduct obtained was digested with 10 N HCl for about 2 h and evaporated under vacuum. The residue was stirred with 20 ml of water and cooled in a refrigerator for about 3 h and filtered. The product obtained was recrystallized from ethanol to give 1.9 g (65% yield) of pure 3-aminomethyl-propyphenazone hydrochloride (AMP.HCl), m.p. 164–5 °C, ^{1}H NMR ($CDCl_3$): 1.12 (d, 6H), 2.5 (s, 3H), 2.62 (m, 1H), 3.25 (m, 2H), 3.45–3.6 (m, 2H, exchangeable), 6.85–7.22 (m, 5 H). Elemental analysis: calculated (C: 59.67, H: 7.15, N: 14.91) found (C: 58.95, H: 7.25, N: 15.08).

2.2.2. Preparation of glycinyl-3-hydroxymethylpropyphenazone (Gly-HMP)

To an ice-cold solution of Boc-glycine (1.75 g, 10 mmol) in 30 ml dichloromethane were added HMP (2.46 g, 10 mmol), dimethylaminopyridine (50 mg), and DCC (2.27 g). The reaction mixture was stirred at 4 °C for 1 h, and kept overnight at room temperature. The precipitated DCU was separated by filtration and the filtrate was washed with cooled 0.05 N HCl and followed by saturated solution of $NaHCO_3$ and finally with brine, dried over anhydrous Na_2SO_4, and evaporated under reduced pressure. The product was then treated with 10 ml of 6 N HCl in dioxane and stirred for 1 h followed by evaporation under vacuum at room temperature. The resulting product was triturated with 100 ml of dry ether and left for 2 h in refrigerator. The precipitated product was filtered and vacuum dried to yield crystals of Gly-HMP (2.8 g, 82.6%), m.p. 135–7 °C, ^{1}H NMR (DMSO 6d) 1.1, 1.2 (6H, d, 9 Hz); 2.45 (3H,s); 2.5–2.65 (1H, m); 3.0–3.2 (m, 2H, exchangeable); 3.53–3.67 (m, 2H); 4.8 (2H, s); 6.7–7.2 (5 H, m). Elemental analysis: calculated (C: 56.55, H: 6.53, N: 12.37); found (C: 55.32, H: 6.52, N: 12.41).

2.2.3. Preparation of 3-[(hydroxyacetamido)methyl]-4-isopropyl-2-methyl-1-phenyl-3-pyrazolin-5-one (AcAMP)

To an ice-cold solution of *O*-tritylglycolic acid (3.5 g, 11 mmol) and AMP.HCl (2.81 g, 10 mmol) in dimethylformamide (20 ml), triethylamine (5 ml), and BOP (5 g) were added. The reaction mixture was stirred at 4 °C for 1 h, and 3 h at room temperature. The reaction mixture was evaporated under vacuum, and the residue dissolved in ethyl acetate, washed with cooled 0.01 N HCl (50 ml) and followed by saturated solution of $NaHCO_3$ and finally with brine. The solution was dried over anhydrous Na_2SO_4, and evaporated under reduced pressure; 10 ml of 6 N HCl in dioxane were added to the residue, stirred for 1 h and evaporated under vacuum at room temperature. The residue was then chromatographed on a silica column eluted with hexane/ethyl acetate (3:1) to obtain pure AcAMP. The vacuum dried crystals (2.2 g, 73%) has m.p. 127–8 °C, ^{1}H NMR ($CDCl_3$) 1.1, 1.2 (d, 6H); 2.4 (s, 3H); 2.8–3.1 (m, 1H); 3.6–3.9 (m, 2H); 4.8–5.1 (m, 3H); 6.7 (s, 1H); 6.9–7.3 (m, 5 H). Elemental analysis: $C_{16}H_{21}N_3O_3$ (FW 303.36); calculated C 63.35%, H 6.98% and N 12.85%; found C 63.0%, H 7.1% and N 13.52%.

*2.2.4. Preparation of naproxen-3-hydroxymethylpropyphenazone ester (NAP-HMP; **I**)*

A solution of CDI (0.5 g, 3.08 mmol) in anhydrous DMF (2.5 ml) was added drop-wise to a cold solution of naproxen (0.5 g, 2.17 mmol) dissolved in anhydrous DMF (3.5 ml). The mixture was kept cold for 30 min and a solution of HMP (0.52 g, 2.17 mmol) in dry DMF (10 ml) was added drop-wise. The reaction mixture was stirred at room temperature overnight. The reaction mixture was evaporated under vacuum, and the residue purified by column chromatography to yield 0.75 g (75.5%) of pure ester **I** [m.p. 143–145 °C, IR (cm^{-1}): 1773 (strong band), 1685 (strong band); 1H NMR (DMSO.d6): 1.1, 1.2 (6H, d, 9 Hz); 1.45, 1.55 (3H, d, 9 Hz); 2.6 (3H, s); 2.65, 2.70, 2.75, 2.80 (1H, q, 5 Hz); 3.7 (3H, s), 4.0–4.2 (1H, m); 4.9 (2H, s); 7.1–7.6 (11H, m)]. Elemental analysis: $C_{28}H_{30}N_2O_4.3/2\ H_2O$ (FW 485.55) calculated; C 69.26%, H 6.85% and N 5.77%; found; C 69.25%, H 6.90% and N 5.63%.

*2.2.5. Preparation of naproxen-glycine-3-hydroxypropyphenazone (Nap-Gly-HMP; **II**)*

A solution of CDI (1 g, 6.16 mmol) in anhydrous DMF (5 ml) was added drop-wise, at 4 °C, to a solution of naproxen (1 g, 4.34 mmol) dissolved in anhydrous DMF (10 ml). To the cold mixture, a solution of Gly-HMP (1.47 g, 4.34 mmol) in dry DMF (20 ml) was added drop-wise. The reaction mixture was stirred for 10 min at 4 °C, for 3 h at room temperature and then evaporated under vacuum. The residue was purified by column chromatography (hexane/ethyl acetate; 2:1) to yield 1.9 g (85%) of pure compound **II** with m.p. 115 °C. ^{1}H NMR (DMSO.d6): 1.1, 1.2 (d, 6H); 1.4, 1.5 (d, 3H); 2.5 (s, 3H); 2.4–2.6 (m, 1H); 3.7 (s, 3H), 3.9, 4.0 (d, 1H); 4.1 (s, 2H); 4.7 (s, 2H); 6.7 (s, 1H); 6.8–7.6 (m, 11H). Elemental analysis: $C_{30}H_{33}N_3O_5.H_2O$ (FW 533.62); calculated C 67.52%, H 6.56% and N 7.88%; found C 67.9%, H 6.6% and N 7.63%.

*2.2.6. Preparation of naproxen-hydroxyacetamidopropyphenazone (Nap-AcAMP; **III**)*

To a solution of thionyl chloride (0.8 g, 6.7 mmol) in dichloromethane (15 ml) was added dimethylformamide (0.5 g, 6.8 mmol) in dichloromethane (3 ml). After 10 min at room temperature, the above solution was allowed to react with a solution of naproxen (1.15 g, 5 mmol) dissolved in dichloromethane (15 ml) by reflux in the dark for about 1 h. To the cold stirred reaction mixture, a solution of AcAMP (1.5 g, 4.95 mmol) in dichloromethane (10 ml) was added drop-wise. The reaction mixture was stirred for 1 h at room temperature and then evaporated under vacuum. The residue was purified by column chromatography (hexane/ethyl acetate; 2:1) to yield 1.8 g (70%) of pure Nap-AcAMP. ^{1}H NMR (DMSO.d6): 1.1, 1.2 (d, 6H); 1.6 (d, 3H); 2.6 (s, 3H); 2.6–2.8 (m, 1H); 3.7 (m, 1H); 3.9 (s, 3H); 4.1 (m, 2H); 5.2 (s, 2H); 6.7 (s, 1H); 6.9–7.6 (m, 11H). Elemental analysis: $C_{30}H_{33}N_3O_5.1/2H_2O$ (FW 524.6) calculated C 68.68%, H 6.49% and N 8.0%; found C 68.0%, H 6.62% and N 7.83%.

2.3. In vitro hydrolysis of naproxen prodrugs

2.3.1. Chemical hydrolysis study

The hydrolysis study was carried out using a Hanson SR6 dissolution apparatus (Hanson Research, CA, USA). The test was conducted at 37±0.5 °C using apparatus II and pedal speed rotation of 50 rpm. Two hydrolysis media were used: 250 ml of non-enzymatic simulated gastric fluid (SGF) buffer solution of pH 1.2 and an isotonic phosphate buffer, pH 7.4. An accurate weight of 25.0 mg of the prodrug was used for the study. From this matrix, aliquots of 3.0 ml were withdrawn at 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 24, and 30 h and were immediately replaced with 3.0 ml of fresh hydrolysis media equilibrated at 37±0.5 °C. The samples were stored at −20 °C pending analysis. A developed HPLC assay was used for the determination of prodrug in these samples. All dissolution experiments were carried out in triplicate and the average values were reported after a correction was applied for the dilution effect.

2.3.2. In vitro enzymatic hydrolysis study

NADPH was regenerated in the liver homogenate of mice, using 30 g liver homogenate in 108 ml isotonic potassium dihydrogen phosphate (pH 7.4). The percentage recovery of the prodrugs (**I**, **II**, and **III**) were estimated from 1 ml liver homogenate, spiked with 0.50 mg of the prodrug, followed by extraction as described below.

The rest of the liver homogenate–NADPH (100 ml) was

mixed with 50 mg prodrug. The hydrolysis was carried out using Vankel dissolution apparatus (Vankel VK 7000, NJ, USA). The experiment was conducted at 37±0.5 °C, and pedal rotation speed of 50 rpm. Aliquots of 1-ml sample withdrawn at 1, 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220, 240, 260 and 280 min, were transferred to 5-ml amber colored, capped reaction vials and immediately replaced with 1 ml of fresh hydrolysis media equilibrated at 37±0.5 °C. Then, 1 ml 10% TFA was added to deactivate the enzyme in the samples, and it was kept in a refrigerator at −20 °C until the next step. The deactivated samples taken from liver homogenate treated with prodrug ester **I** (2 ml) were divided to two equal parts. The first part (1 ml), was transferred to a 5-ml centrifuge tube, mixed with 0.4 ml water, 1.1 ml acetonitrile, vortexed for 5 min, and centrifuged for 5 min at 5000 rpm. From the clear supernatant, 20 μl was injected for HPLC analysis of the prodrug content using method B. The second part (1 ml), was transferred to a capped reaction tube, and mixed with 0.35 ml acetate buffer pH 5.5, followed by 50 μl *p*-glucuronidase-arylsulfatase solution. This solution was incubated at 40 °C in a thermostatically controlled water bath for 24 h. This sample was extracted with 1.1 ml acetonitrile as described previously. From the clear supernatant, a 20-μl aliquot was analyzed by HPLC using method A, for estimation of NAP. All sample extracts were developed three times in parallel with calibration curve solutions. The recovered amount was calculated from the calibration curves of the investigated compounds, using five levels to obtain a calibration line. The calibration solutions were prepared in methanol/acetonitrile (1:1), in the range of 0.01–0.25 mmol, of each. The estimated concentrations were calculated for the final dilution, from the corresponding regression line, and corrected for the extraction factor.

2.4. Identification of the prodrug metabolites

After 280 min enzymatic hydrolysis of prodrug ester **I**, 1 ml of the sample was mixed with 2 ml phosphate buffer pH 6.0, saturated with about 1 g sodium chloride; and extracted by vortex with 5 ml dichloromethane. From the clear supernatant, 3 ml were withdrawn, transferred to a 2-ml capped reaction vial, and dried under a stream of nitrogen gas. The residue was dissolved in 0.3 ml chloroform and 50 μl MSTFA containing 10% TMSCI. The mixture was heated at about 80 °C for 30 min. The sample was then cooled to room temperature, and 1 μl was developed for GC–MS analysis. A derivative standard solution mixture of the HMP, and NAP was silylated and developed in parallel to the sample extract.

2.5. In-vivo evaluation

The prodrugs prepared were screened for analgesic and anti-inflammatory activity compared with that exerted by NAP and HMP, separately. Male Swiss mice (23–26 g) and male Sprague–Dawley rats (150–170 g) were used. The animals were starved for about 15 h before test compound administration. The tested compounds were prepared for oral administration in aqueous 0.5% carboxymethylcellulose (CMC) solution. The properties of the test compounds were compared with those of HMP and NAP. Statistical analysis was performed with $P \leq 0.05$ as the level of significance.

2.5.1. Analgesic activity

The analgesic activity was evaluated using a reported hot plate method (Jansen and Jageneau, 1957; Galina et al., 1983). this depends upon observing the normal response to a pain stimulus in untreated animals and comparing it with the response to the same stimulus after the administration of a drug at definite time intervals. The relative mouse response to the plate temperature was a convenient method, to compare the relative drug activity.

Five groups of six mice were orally treated with a solution of prodrugs (**I** or **II** or **III**), naproxen or HMP (in equivalent doses, 21.7 μmol/kg). The mice were placed individually on a preheated hot plate (54±1 °C) and the time (s) which elapsed before the mouse reacted was recorded. The reaction time (s) was determined at 1, 2, 3, 4, 6, 7 and 8 h. The differences in latencies, with respect to the control group (six mice), were used as an indication of analgesia.

2.5.2. Anti-inflammatory activity (Winter et al., 1962)

Six groups of six rats were used. Each group of animals was orally dosed with the test compound (NAP, MHP, Prodrug **I**, Prodrug **II**, or Prodrug **III**; 21.7 μmol/kg) or drug-free vehicle (CMC). After 1 h, 0.1 ml of a 0.1% carrageenan solution was injected into the sub-plantar tissue of the right paw. Swelling (volume) of the injected paw of each animal was measured at 2, 3, 4, 5, 6 and 8 h by a mercury plethysmometer.

2.5.3. Ulcerogenicity study

A Jeol, JSM-5400LV scanning electron microscope (Electron Microscope Unit, Assiut University) was used for observing mucosal injury from a scanning micrograph of stomach specimens. Four groups of four mice each were fasted for 12 h prior to the administration of the drug. The first group was administered a daily oral dose (21.7 μmol/kg) as a 1 ml suspension of prodrug **III** in 0.1% CMC solution, for four successive days. In a similar manner, the second and third groups received equivalent doses of NAP and HMP, separately. The fourth group received an equivalent amount of the vehicle (CMC) and was considered as control group. Food was withdrawn from all groups until 24 h after the last dose. The mice were then sacrificed, so that the stomach could be removed, opened along the greater curvature, investigated for appearance of lesions and prepared for scanning in the electron microscope. The

specimens were fixed by soaking in glutaraldehyde solution (5% in cacodylate buffer, pH 7.2) for 24 h followed by three washings each for 20 min with cacodylate buffer. They were then treated with osmium tetraoxide (1% solution) for 2 h and washed with cacodylate buffer as described above. Then the specimens were subjected to dehydration by ethanolic solutions of 30, 50 and 70%, for 30 min with each, followed by 90% ethanol for 1 h and finally in absolute ethanol for 2 days. After discharge of the alcohol, the specimens were soaked in amyl acetate solution for 2 days, dried under reduced pressure, and mounted on holders coated for scanning in a scanning electron microscope (SEM).

3. Results and discussion

3.1. Chemistry

Bromopropyphenazone (BMP) was used for preparation of hydroxymethyl-propyphenazone (HMP) by reflux with water, or 3-aminomethylpropyphenazone (AMP) by reaction with hexamine followed by acid treatment. Direct coupling of naproxen with HMP was carried out by three different reaction conditions as shown in Scheme 1. The carbonyldiimidazole (CDI) method gave better yield of the corresponding ester with minimal reaction by-products. In order to avoid or minimize the steric hindrance effect on the hydrolysis of naproxen-HMP ester prodrug (**I**), a spacer was introduced. The spacers chosen were glycine and glycolic acid. N-Protected glycine coupled with HMP by DCC followed by N-deprotection and coupling with naproxen using CDI yielded naproxen amide prodrug (**II**). Coupling of *O*-tritylglycolic acid with aminomethylpropyphenazone (AMP) was carried out using strong coupling reagent BOP under very mild conditions. The *O*-trityl group was removed using mild acidic conditions to yield the amide AcAMP which reacted with naproxen acid chloride to yield naproxen ester prodrug (**III**). The structures and purity of the prodrugs formed were confirmed by instrumental methods including UV, ^{1}H NMR, and elemental analysis.

3.2. In vitro hydrolysis of ester

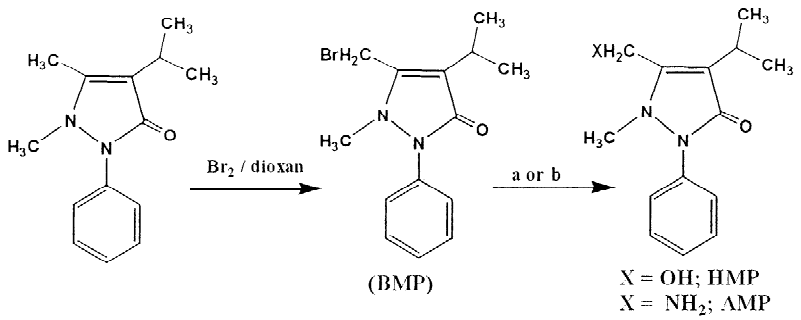
Since the carboxyl group of naproxen is essential for the therapeutic action, the prodrugs of a prolonged action were designed in a form which the biologically active moiety can be released in its original state with time. Therefore, the release of NAP from NAP–propyphenazone prodrugs was studied in vitro in order to evaluate the possible time span in which the drug could be available from different prodrugs. The prodrugs that quantitatively dissociate and release naproxen were selected for in vivo study. A validated analytical HPLC method for the estimation of the NAP and prodrugs was developed. The developed chromatographic methods were validated for selectivity, precision, accuracy, suitability, linearity and range. Also, the applied chromatographic procedures were validated to separate selectively, the ester, HMP and NAP from the biogenic materials of the hydrolyzate extract. GC–MS was used for the identification of enzymatic hydrolysis products of the prodrug (**I**). The hydrolyzate after 280 min contains naproxen and HMP in addition to *O*-desmethyl-naproxen ester with HMP and *O*-desmethyl-naproxen. These data prove the low activity of this sterically hindered ester prodrug so metabolism mainly occurs at the terminal demethylation rather than by ester hydrolysis.

The kinetics of ester hydrolysis was studied in two different buffer solutions (pH 1.2 and 7.4), without enzymes. Also, the effect of activated esterase enzymes present in liver homogenates was investigated. The data for chemical hydrolysis, presented in Fig. 1, fit pseudo-first-order kinetics (correlation coefficient >0.98) and the rate constants computed at pH 1.2 were 0.75×10^{-2} (h^{-1}), 1.02×10^{-2} (h^{-1}) and 1.15×10^{-2} (h^{-1}) for prodrugs **I**, **II** and **III**, respectively. These values correspond to half times in vitro of about 91, 67, and 60 h for Prodrugs **I**, **II** and **III**, respectively. The data for chemical hydrolysis, presented in Fig. 2, fit pseudo-first-order kinetics (correlation coefficient >0.99) and the rate constants computed at pH 7.4 were 3.54×10^{-2} (h^{-1}), 5.48×10^{-2} (h^{-1}) and 6.94×10^{-2} (h^{-1}) for prodrugs **I**, **II** and **III**, respectively. These values correspond to half times in vitro of about 19, 12, and 10 h for Prodrugs **I**, **II** and **III**, respectively. The results were the average of three runs. As expected, the rate of prodrug hydrolysis was found to be highly pH-dependent as shown in Figs. 1 and 2. While the prodrug was hydrolyzed at higher pH (7.4), it showed insignificant hydrolysis in the acidic medium (pH 1.2).

The hydrolysis of the ester was tested in the rat liver homogenate, to investigate the effect of activated esterase enzyme on the drug. Fig. 3 shows that the metabolic rate of the ester is linear. The rate constants computed were 0.8×10^{-2} (h^{-1}), 1.04×10^{-2} (h^{-1}) and 1.27×10^{-2} (h^{-1}) for prodrugs **I**, **II** and **III**, respectively. These values correspond to half times in vitro liver enzyme of about 85, 66 and 54 min for prodrugs **I**, **II** and **III**, respectively. Moreover, after a 240-min incubation period of the ester in liver homogenate, less than 10.5% of intact prodrug was recovered.

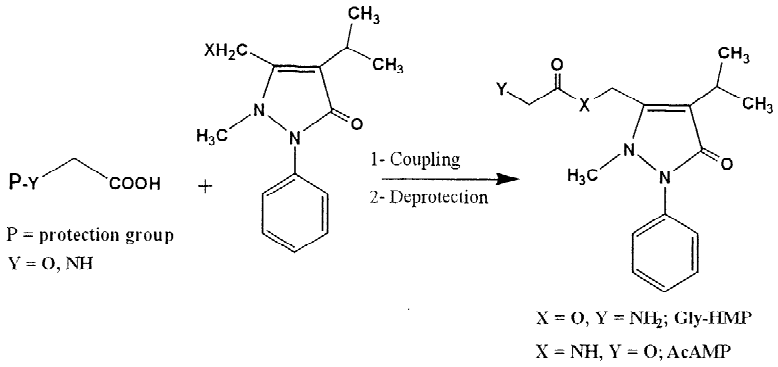
Sample stability in the liver homogenate was tested by incubating the prodrug, HMP, and NAP at $-20\,^{\circ}C$ for 24 h, followed by re-estimating the content by applying HPLC. The recovered amounts of the prodrug (**I**), prodrug (**II**), prodrug (**III**), and NAP were found to be 100 ± 2, 97 ± 2, 97 ± 1, and $85\pm2.3\%$, respectively. The extracted samples have shown non-significant difference, and typical chromatograms compared with those extracted at zero time were observed.

Synthesis of HMP and AMP



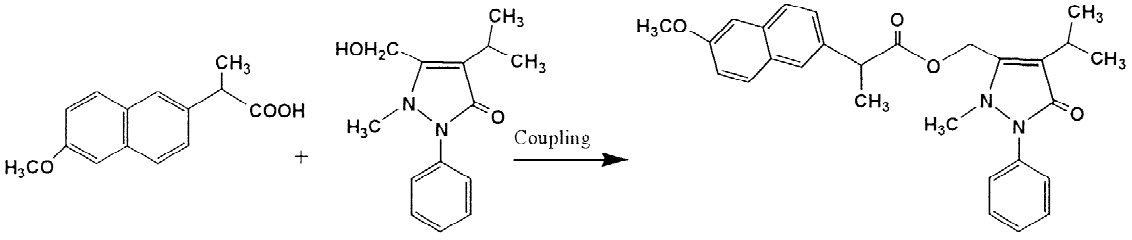
a: Boiling with water ; b: hexamine / $CHCl_3$ then HCl

Synthesis of spacer-propyphenazone derivatives



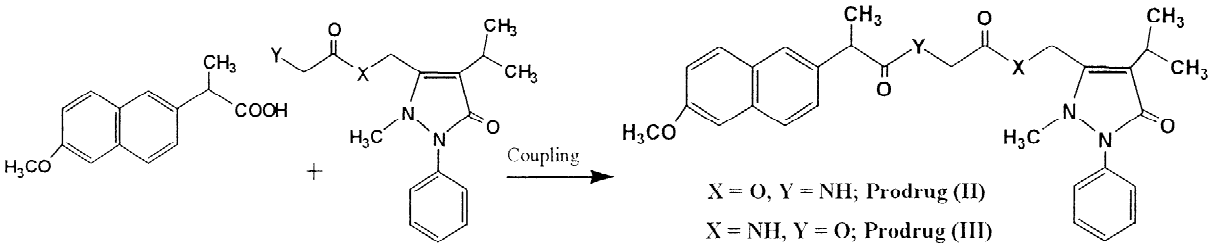
Coupling reagents used DCC / DMAP or BOP / triethylamine ; Deprotection : 6N HCl / dioxane

Synthesis of Nap-HMP prodrug (I)



Coupling reagent : carbonydiimidazole (CDI) in anhydrous DMF

Synthesis of Nap-Gly-HMP prodrug (II) and Nap-AcAMP prodrug (III)



Coupling reagent: CDI / DMF in preparation of prodrug II; SOCl2 / DCM in preparation of prodrug III

Scheme 1. General steps for synthesis of the targeted prodrugs (**I–III**).

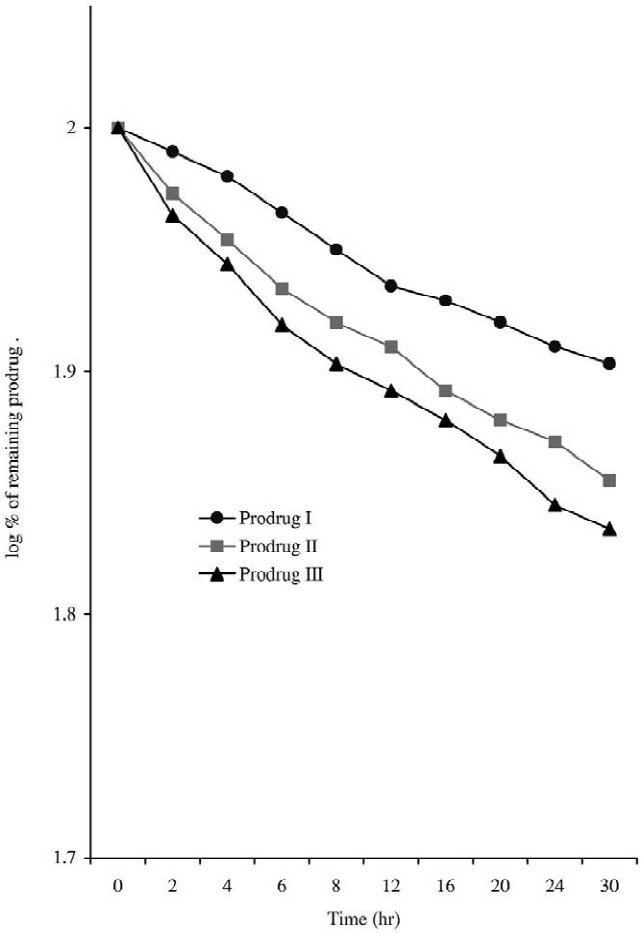


Fig. 1. Pseudo-first-order plot of the chemical hydrolysis profile of the prodrugs in SGF.

3.3. GC–MS data interpretation

The extracted prodrug **I** metabolites have shown the same GC-mass spectrum of the derivatized standard solutions of HMP, and NAP. HMP was trimethylsilylated at its hydroxyl group, and showed characteristic mass fragment representing: TMS group at $m/z=73$ (47%), abundant mass fragment of $m/z=318$ (82%) representing the molar peak (3-O-TMS) and base peak at 303 representing 3-O-TMS that loses one methyl group from the TMS part. NAP was trimethylsilylated at its carboxylic group and shows characteristic mass fragments of 302.7 (22%), 287.3 (25%), 230.2 (64%) and base peak at 215.3. However, the mass spectrum of the prodrug (**I**), was not silylated, but its desmethyl metabolite was identified. This new metabolite has shown a TMS derivative at 516 (20%) represented the molar peak of ester-OTMS (instead of ester-OCH_3), 502 (10%) represents *N*-desmethyl-ester-OTMS, 309 (20%), 230 (55%), and base peak at $m/z=215$, represent the demethylsilylated NAP. The desmethyl-ester metabolite could not be identified in the HPLC chromatogram.

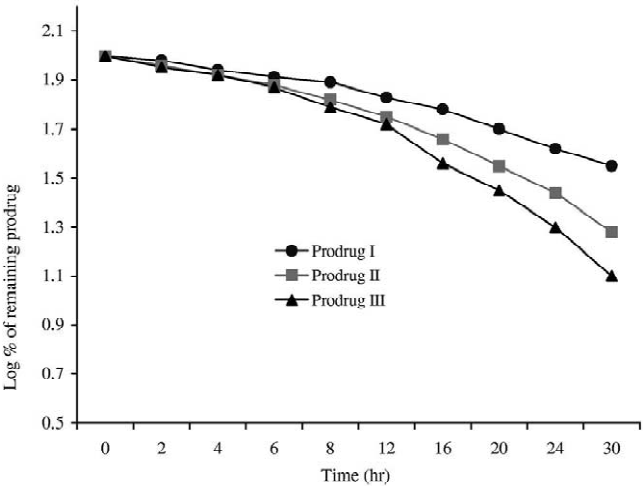


Fig. 2. Pseudo-first-order plot of the chemical hydrolysis profile of the prodrugs in SIF.

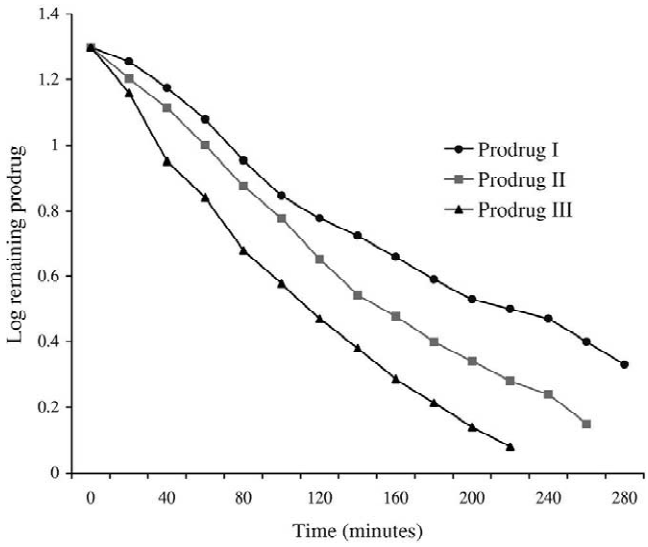


Fig. 3. Pseudo-first-order-plot of the enzymatic hydrolysis of the naproxen prodrugs.

3.4. In-vivo evaluation

Analgesic, anti-inflammatory and ulcerogenic activities of the prodrugs were studied in comparison to equivalent doses (21.7 μmol/kg) of NAP and HMP. The analgesic activity was investigated in mice according to reported methods (Jansen and Jageneau, 1957; Galina et al., 1983). In this procedure, the rodent is placed on a hot plate that is preheated to a temperature that is presumed to be aversive. The latency to lick a paw and/or jump is recorded. The differences between the latencies and those of controls are used as a measure of the degree of analgesia (Table 1). As a general pattern, the analgesic activities of the prodrugs show improvement over time. The maximum analgesic activity is reached after about 4–6 h for all prodrugs. The observed latent analgesia may result from prodrug bioavailability and/or hydrolysis to parent drugs.

Table 1
The latencies of pain threshold (seconds, s)in mice treated with the prodrugs **I**–**III**, naproxen (NAP) and hydroxymethylpropyphenazone (HMP)

Compound	Mean±S.E.[a] of the pain threshold (s)						
	1 h	2 h	3 h	4 h	6 h	7 h	8 h
NAP	85.0±3.14	72.5±3.33	61.6±2.52	50.9±1.28	45.1±2.01	36.7±1.19	30.0±0.92
HMP	137.1±9.10	125.5±8.38	109.2±8.09	84.6±5.32	60.0±3.10	56.5±3.91	45.8±6.51
Prodrug **I**	30.6±3.78	50.3±4.10	87.0±8.59	98.2±9.71	92.8±7.26	95.9±3.58	71.3±8.06
Prodrug **II**	35.0±2.18	72.5±4.10	97.6±5.70	107.6±11.95	103.1±5.72	87.5±6.12	58.4±3.29
Prodrug **III**	51.3±3.16	64.1±5.27	87.3±5.20	99.1±7.21	101.3±6.25	97.2±7.72	81.2±4.17

[a] $n=6$ mice; dose 21.7 μmol/kg; the results are significant at $P<0.01$.

The effectiveness of NSAIDs in acute inflammation can be estimated by using intraplantar injection of carrageenan in the rat. Swelling of the injected paw was measured at 2, 3, 4, 5, 6, and 8 h using a mercury plethysmometer. The pretreatment with oral naproxen (5 mg/kg) resulted in a significant reduction in swelling that lasted about 5 h post-carrageenan injection (Fig. 4). The anti-inflammatory activity of prodrugs significantly improves over time. This means that the prodrugs per se are devoid of anti-inflammatory activity and that the observed latent activity results from hydrolysis to the parent drugs. An equimolar dose of prodrug **III** (11.17 mg/kg) reduced inflammation in a pattern approximately similar to naproxen. Prodrugs **I** and **II** show moderate anti-inflammatory activity with time in comparison with prodrug **III** that may be due to the slow rate of hydrolysis of prodrugs **I** and **II** to release naproxen.

The ulcerogenic activity of the prodrug **III**, as a representative example for the synthesized oral delivery system, was tested in comparison with parent drugs (NAP and HMP) following oral administration for 4 days in mice. As could be seen from the gross observation, linear or oval-shaped lesions were found mainly in the corpus of the stomach. The group subjected to NAP prodrug **III** had significantly fewer lesions than the NAP group. The examination of the stomach specimens of the treated experimental animals under scanning electron microscope affords a highly precise method for investigation of the ulcerogenic potential of NSAIDs. Fig. 5 shows scanning electromicrographs, at a constant magnification power, for stomach specimens of mice treated with a chronic dose of prodrug **III** (Fig. 5A); the parent drugs HMP (Fig. 5B); NAP (Fig. 5C) and the control group (Fig. 5D) which receive only the vehicle. As shown in Fig. 5, the NAP-treated group (Fig. 5C) and to some extent HMP-treated group were characterized by complete damage of the mucous layer besides ulceration of the sub-mucosal cells. These effects are not observed in the prodrug-treated group

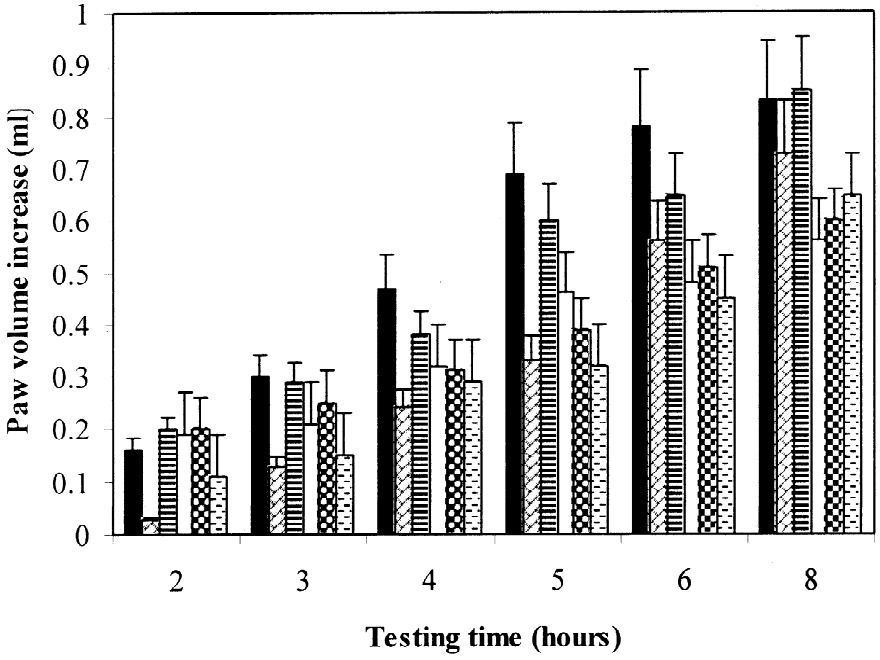


Fig. 4. The mitigation of acute inflammation by test compounds (21.7 μmol/kg) in a carrageenan model in rats. The filled boxes represent animals to whom vehicle was administered, diagonally lined boxes represent naproxen, the horizontally lined boxes represent hydroxymethyl-propyphenazone, the open boxes represent prodrug **I**, the large checker board boxes represent prodrug **II**, and the dashed horizontal lined boxes represent prodrug **III**.

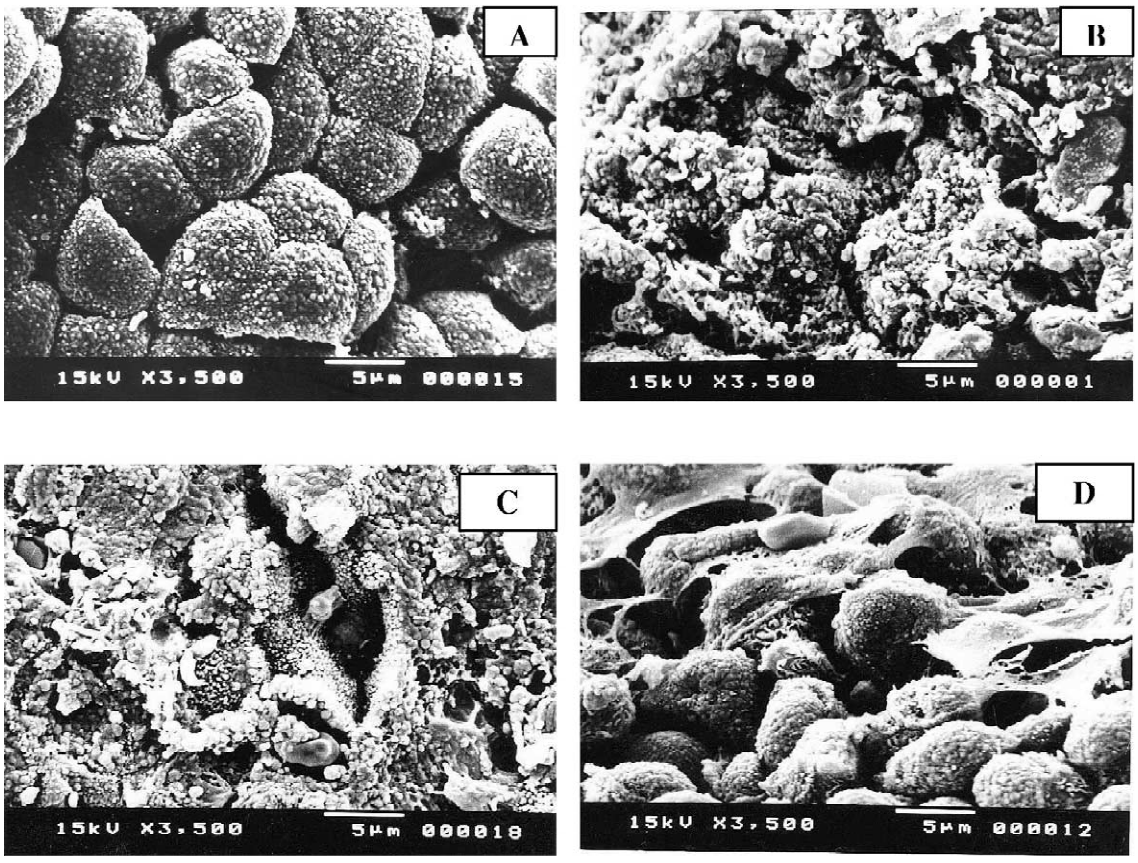


Fig. 5. Scanning electromicrographs of mouse stomach after chronic doses (4 days) of: Prodrug **III** (A), HMP (B), NAP (C), and the vehicle (D).

(Fig. 5A) or control group (Fig. 5D). The previous observations afford good evidence for the safety of the suggested oral delivery system of NSAIDs compared with the traditional use of the parent drugs.

4. Conclusion

The in vitro and in vivo evaluation of the synthesized naproxen–propyphenazone hybrid drug ester and/or amide revealed improvement in the therapeutic index of the parent drugs. The derivatives are characterized by prodrug profile, adequate chemical stability, and reduced ulcerogenic liability. The prodrugs retained the anti-inflammatory and analgesic activities of the parent drugs.

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