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Design and synthesis of tryptophan containing peptides as potential analgesic and anti-inflammatory agents

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A new series of smaller peptides with tryptophan at *C*-terminal and varying *N*-protected amino acids/peptides were designed, synthesized and characterized by analytical and spectroscopic techniques. Analgesic and anti-inflammatory properties of these peptides were carried out *in vivo* using tail-flick method and carrageenan-induced paw edema method, respectively, at different doses and different time intervals. Most of the peptides synthesized displayed enhanced activity, and particularly tetra and hexapeptides 29–31 were found to be even more potent than the reference standards used. Moreover, some peptides have exhibited promising activity even after 24 h of administration, whereas the reference standards were active only up to 3 h. Further, the compounds did not present any ulcerogenic liability. Copyright © 2012 European Peptide Society and John Wiley & Sons, Ltd.

Supporting Information may be found in the online version of this article.

Keywords: indole; synthesis; peptides; analgesics; anti-inflammatory agents

Introduction

Inflammation is a reaction of a part of the body to injury or infection, characterized by swelling, heat, redness and pain. Non-steroidal anti-inflammatory drugs are among the most widely prescribed drugs worldwide [1]. Through their anti-inflammatory, antipyretic and analgesic activities, they represent a choice of treatment in various inflammatory diseases such as arthritis, rheumatisms as well as to relieve the aches and pain of everyday life [2]. However, their use is often limited by the side effects they produce, particularly in the gastrointestinal tract and the kidney [3]. Hence, the search for new therapeutic agents with high degree of safety and freedom from normally associated gastrointestinal toxic effects such as ulceration, hemorrhage and perforation has been a priority of chemists and pharmacologists [4].

The discovery of indomethacin [5] as a successful agent for clinical treatment on anti-inflammatory disorders has led to the exploration of indole moiety to obtain better anti-inflammatory agents. In particular, tryptophan (Trp) is an essential amino acid and as such is a constituent of most proteins; it also serves as a biosynthetic precursor for a wide variety of indole-containing metabolites, which are involved in a large and essential number of physiological functions [6]. Furthermore, the indole nucleus has been reported as a common dominator of psychotropism and is of great value in the field of medicine and biochemistry [7,8]. This nitrogen heterocycle and its derivatives have occupied a unique place in chemistry because of its varied biodynamic properties such as anti-inflammatory [9,10], anticancer [11,12], antidepressant [13], antimicrobial [14,15], antidiabetic [16] and antimalarial [17].

Several researchers have shown that *N*-Fmoc amino acids exert anti-inflammatory activity by recruitment of neutrophils into the inflammatory site [18–20]. In addition, these compounds also increased intracellular Ca^{2+} concentrations in Madin Darby canine kidney renal tubular cells [21]. These agents do not act by inhibiting lipid metabolic enzymes. They are not steroids, nor do they increase the circulating levels of endogenous glucocorticoids. Instead, they block recruitment of neutrophils into inflammatory lesions and, when studied *in vitro*, inhibited T-cell activation [19].

Led by the previous facts and coupled with our ongoing project aimed at investigating new bioactive molecules derived from amino acid/peptide conjugates [22–26], the present work involves the synthesis of a series of smaller peptides with Trp at the *C*-terminal as common entity and varying different protected amino acids/peptides at the *N*-terminal (Figure 1).

Materials and Methods

General

All the amino acids used except glycine were of *L*-configuration unless otherwise mentioned. All amino acids, EDCI, HOBt and TFA were purchased from Advanced Chem. Tech. (Louisville, Kentucky, USA). IBCF and NMM were purchased from Sigma Chemical Co. (St. Louis, MO). All solvents and reagents used for the synthesis were of analytical grade. Silica gel (60–120 mesh) for column chromatography was purchased from Sisco Research

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Abbreviations: *EDCI, 1-(3-Dimethylaminopropyl)-3-ethyl-carbodiimide × HCl; IBCF, Isobutyl chloroformate; Z, Benzyloxycarbonyl.*

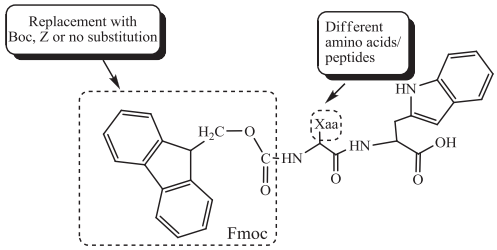

Figure 1. Design of *C*-terminal indole moiety containing peptides.

Laboratories Pvt. Ltd., (Mumbai, India). The progress of the reaction was monitored by TLC using silica gel coated on glass plates with the solvent system comprising chloroform/methanol/acetic acid in the ratio 95 : 5 : 3 (R_f^a)/90 : 10 : 3 (R_f^b). The compounds on TLC plates were detected by iodine vapors. Melting points were determined on a Thomas Hoover melting point apparatus and are uncorrected. All the HPLC analyses were performed with Lachrom-2000 (USA) Merck-Hitachi L7100 pump with a column of RP18.250–4 mm and UV detector-UV-VISL 7400. ^{1}H NMR spectra were obtained on 400 MHz instrument using $CDCl_3$, and ^{13}C NMR spectra were recorded in DMSO-d_6 using Varian 100 MHz instrument (Palo Alto, CA, USA). The chemical shifts are reported as parts per million (δ ppm) using TMS as an internal standard. Electrospray ionization mass spectrometry was performed on a Bruker electrospray mass spectrometer. Elemental analysis was performed using Vario EL III Elementar (Germany) and the values are within ±0.4% of the calculated ones.

Chemistry

*General procedure for the coupling of Y-NH-Xaa-OH where Y = Fmoc, Z, Boc; Xaa = Gly, Pro, Phe, Lys(Boc), His(Trt), Glu(OBut), GFG, GVGVP and GFGFP with HCl × H-Trp-OMe (**7**)*

To Y-NH-Xaa-OH (0.005 mol) and HOBt (0.765 g, 0.005 mol) dissolved in DMF (10 ml/g of compound) and cooled to 0 °C was added NMM (0.55 ml, 0.005 mol). EDCI (0.956 g, 0.005 mol) was added under stirring while maintaining the temperature at 0 °C. The reaction mixture was stirred for 10 min, and a pre-cooled solution of HCl × H-Trp-OMe (1.274 g, 0.005 mol) and NMM (0.55 ml, 0.005 mol) in DMF (13 ml) was added slowly. After 20 min, pH of the solution was adjusted to 8 by the addition of NMM, and the reaction mixture was stirred over night at room temperature. DMF was removed under reduced pressure, and the residue was poured into about 200-ml ice-cold 90% saturated $KHCO_3$ solution and stirred for 30 min. The precipitated peptide was taken into $CHCl_3$ and washed with 5% $NaHCO_3$ solution (2 × 30 ml), water (2 × 30 ml), 0.1 N cold HCl solution (2 × 30 ml) and finally brine solution (2 × 30 ml). The $CHCl_3$ layer was dried over anhydrous Na_2SO_4, and the solvent was removed under reduced pressure. The products (**8–19**) so obtained were recrystallized from ether/petroleum ether.

*General procedure for the hydrolysis of methyl esters of **8–19***

Each peptide (**8–19**) (0.003 mol) was hydrolysed in methanol (10 ml/g of peptide) using cold solution of 1 N NaOH (15 ml) for 2 h. The completion of the reaction was monitored by TLC, solvent was evaporated, cooled, neutralized with cold 1 N HCl, extracted with $CHCl_3$, washed with 1 N HCl followed by water and dried over anhydrous Na_2SO_4. The solvent was removed under pressure and triturated with ether, filtered, washed with ether and dried to obtain carboxyl free peptides as hygroscopic foam (**20–31**).

Pharmacology

For analgesic activity, healthy adult Swiss albino mice of either sex weighing 25–30 g were used. For anti-inflammatory activity, healthy adult wistar rats of either sex weighing between 75–125 g were used. Ibuprofen and indomethacin served as reference standards for analgesic and anti-inflammatory activities, respectively. All the animals were maintained as per the norms of the Committee for the Purpose of Control and Supervision of Experiments on Animal (CPCSEA), and the study protocols were approved by CPCSEA and Institutional Animal Ethics Committee constituted for the purpose. The significant difference between groups was statistically analyzed by one-way ANOVA followed by Dunnett's *t*-test.

Analgesic activity

The analgesic activity of the synthesized compounds was determined by the standard tail immersion method in mouse [27,28]. Albino mice of either sex weighing 25–30 g were divided into groups of five animals each. At intervals, the mouse was held so that its tail was totally immersed in a bath at the temperature of 55 ± 5 °C. The time until the typical reaction – a violent jerk of the tail was recorded and noted as the basal reaction time. The animals were administered orally with control (DMSO), test compounds (**20–31**, **24a**, **29a**) and ibuprofen (standard) at a dose of 100 mg/kg body weight. The compounds that showed potent activity at 100 mg/kg body weight were treated with low dosage such as 25 mg/kg and 50 mg/kg body weight. Time taken for tail withdrawal response was recorded at 30 min, 1, 2, 3, 4 and 24 h after administering the compounds. Increase in reaction time (interval for withdrawal of tail by the animal) was considered as an analgesic activity. The percentage analgesic activity was calculated by using the formula,

$$\%\textit{Analgesic activity} = \left[\frac{T_2 - T_1}{80 - T_1}\right] \times 100$$

where T_1 is reaction time before treatment, T_2 is reaction time after treatment and 80 is the cut-off time.

Potency of the tested compounds was calculated relative to ibuprofen 'reference standard' treated group according to the following equation.

$$\text{Potency} = \left[\frac{\%\text{analgesic activity of test compound treated group}}{\%\text{analgesic activity of ibuprofen treated group}}\right]$$

Anti-inflammatory activity

The anti-inflammatory activity was evaluated using carrageenan-induced rat hind paw edema method [29]. The animals fasted for 24 h were divided into control, standard and test groups each consisting of five rats. The first group of rats was treated with DMSO (control), second group was administered orally with a dose of 100 mg/kg of indomethacin (standard), and test groups (**20–31**, **24a**, **29a**) were treated with 100 mg/kg of test compounds orally. The compounds that showed potent activity at 100 mg/kg body weight were treated with low dosage such as 25 mg/kg and 50 mg/kg body weight. After 30 min, the animals were injected subcutaneously with 0.1 ml of freshly prepared suspension of carrageenan solution (1% in 0.9% saline) into the

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subplantar region of the right hind paw of rats. The volume of hind paw was measured using vernier calipers both in control and in animals treated with standard and test compounds at 30 min, 1, 2, 3, 4 and 24 h after carrageenan challenge. The initial paw volume was measured within 30 s of the injection. The anti-inflammatory activity was expressed as a percentage inhibition of the inflammation in treated animals in comparison with the control group [30].

$$\%\text{inhibition of edema} = \left[1 - \frac{V_t}{V_c}\right] \times 100$$

where V_c and V_t are the mean relative changes in the volume of paw edema in the control and test-treated groups, respectively.

Potency of the tested compounds was calculated relative to indomethacin 'reference standard' treated group according to the following equation.

$$\text{Protency} = \left[\frac{\%\text{edema inhibition of test compound-treated group}}{\%\text{edema inhibition of indomethacin-treated group}}\right]$$

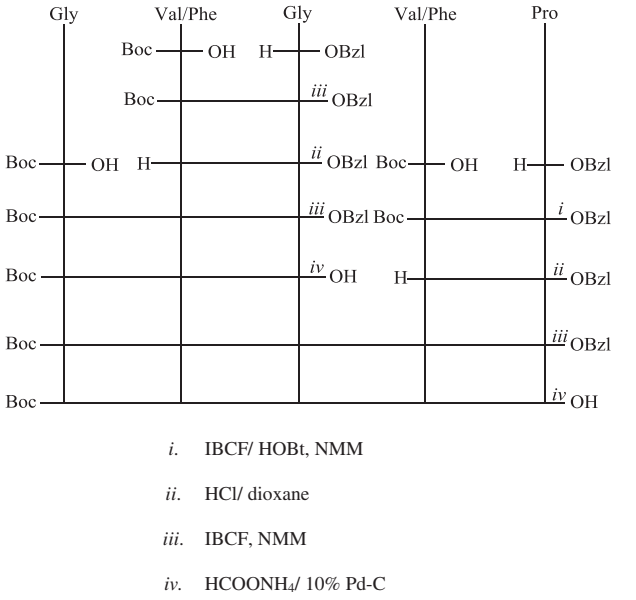
Ulcerogenic activity

Adult albino rats of either sex were fasted 24 h prior to the administration of compounds. Water was allowed *ad libitum* to the animals. The compounds (which have shown promising anti-inflammatory activity) and indomethacin were given orally and the animals sacrificed 8 h after treatment. The stomach, duodenum and jejunum were removed and examined with a hand lens for any evidence of (i) shedding of epithelium (ii) red spots below skin and bleeding and (iii) erosion or discrete ulceration with or without perforation. The presence of any one of these was considered to be an evidence of ulcerogenic activity [31].

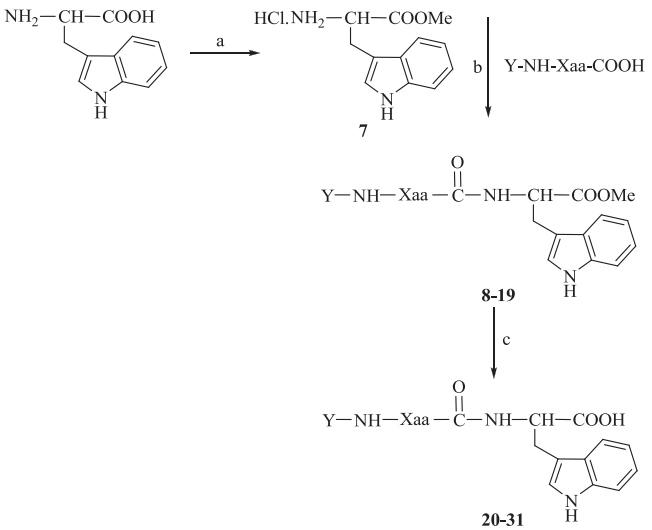
Results and Discussion

Chemistry

The peptides Boc-GFG-OBzl, Boc-GVGVP-OBzl and Boc-GFGFP-OBzl were synthesized by solution phase approach using inexpensive IBCF/HOBt as coupling agent and NMM as base (Scheme 1). Trp was converted to its methyl ester using $SOCl_2$/MeOH, and its formation was confirmed by ^{1}H NMR, which showed absence of −COOH peak and the presence of a methyl ester peak at δ 3.52. Further, this compound was used for coupling with various *N*-protected amino acids/peptides using EDCI/HOBt as coupling agent and NMM as base (Scheme 2). All the products obtained presented characteristic ^{1}H NMR signals for COOMe, $^{\alpha}$CH, NH and indole-NH at ~ δ 3.50, 4.00, 8.00 and 10.90, respectively. The analytical and spectroscopic data of the peptides (**1–3**/**8–19**) are provided in the supporting information. Further, all the peptides synthesized were confirmed by mass spectra and elemental analysis, which are in good agreement with their structure.



Scheme 1. Schematic representation of the synthesis of Boc-GFG-OH, Boc-GVGVP-OH and Boc-GFGFP-OH.



Scheme 2. Coupling of *N*-protected amino acids/peptides to **7** and saponification where Y = Boc, Fmoc, Z; Xaa = Gly, Pro, Phe, Lys(Boc), His (Trt), Glu(OBut), GFG, GFGFP, GVGVP. Include the reagents and conditions here which is found below.

Reagents and conditions: (a) $SOCl_2$/MeOH, reflux, 4–5 h (b) EDCI/HOBt/NMM, 0 °C, overnight at rt (c) 1 N NaOH/MeOH, 0 °C, 2 h at rt.

Comp. No.	Sequence	Comp. No.	Sequence
8/20	Fmoc-Gly-Trp-OMe/OH	**14/26**	Z-His(Trt)-Trp-OMe/OH
9/21	Fmoc-Pro-Trp-OMe/OH	**15/27**	Boc-Gly-Trp-OMe/OH
10/22	Fmoc-Phe-Trp-OMe/OH	**16/28**	Boc-Glu(OBut)-Trp-OMe/OH
11/23	Fmoc-Lys(Boc)-Trp-OMe/OH	**17/29**	Boc-Gly-Phe-Gly-Trp-OMe/OH
12/24	Fmoc-His(Trt)-Trp-OMe/OH	**18/30**	Boc-Gly-Phe-Gly-Phe-Pro-Trp-OMe/OH
13/25	Z-Gly-Trp-OMe/OH	**19/31**	Boc-Gly-Val-Gly-Val-Pro-Trp-OMe/OH

Pharmacology

Analgesic and anti-inflammatory activities

Analgesic and anti-inflammatory activities of the synthesized compounds were determined *in vivo* by tail-flick method in mice and acute carrageenan-induced paw edema method in rats, respectively, at a dose of 100 mg/kg body weight (Figures 2 and 3). The analgesic and anti-inflammatory properties were recorded at successive time intervals of 30 min, 1, 2, 3, 4 and 24 h and compared with that of ibuprofen and indomethacin, respectively, which were used as reference standards. When *N*-protected amino acids/peptides alone (without Trp-OMe) were subjected to analgesic and anti-inflammatory activities, they showed ~10% and 20% activity at 3-h interval, respectively. Hence, we intended to include different amino acids/peptides at Xaa region of the designed analogs (Figure 1). It was noted that the presence of methyl ester at the *C*-terminal of the peptides did not present significant results. Hence, methyl ester of the peptides was removed to obtain corresponding free acid, which were used for the activity. From the results, it was observed that the test compounds revealed significant analgesic and anti-inflammatory properties.

Among the *N*-Fmoc containing dipeptides such as Gly (**20**), Pro (**21**), Phe (**22**), Lys(Boc) (**23**) and His(Trt) (**24**), compound **24** has shown high activity, which could be due to aromaticity of His as well as trityl group, acid–base character and H-binding affinity of His. This is followed by Lys(Boc). This observation suggested that basic amino acids have pronounced effects on the activity [18], whereas the other Fmoc amino acids have shown moderate results. In contrast, when an acidic amino acid, i.e. Glu(OBut) (**28**) was present, it resulted in lower activity.

In this context, we thought of replacing Fmoc by Z and Boc groups, which are also important in peptide synthesis. It was observed that there was a slight loss in the activity when Fmoc was replaced by Z. This could be due to the presence of strong aromatic fluorene ring in Fmoc when compared with the presence of benzene in Z. Further decrease in the activity was observed when Fmoc was replaced by aliphatic Boc. This observation was supported by His(Trt) (**24** > **26**) and Gly (**20** > **25** > **27**) containing peptides, i.e. Fmoc > Z > Boc. This fact revealed that aromatic groups at the *N*-terminus play a major role. Further, to obtain insight into the essentiality of protecting group at the *N*-terminus, Boc of compound **29** was treated with TFA to obtain **29a**, which resulted in decreased activity. This fact revealed that substitution at the *N*-terminal is essential. Thus, structure–activity relationships based on the observed data explain that the substitution at *N*-terminus has a significant role in developing the pharmacological activities.

In this perspective, our next goal was to extend the length of the peptide chain. It was noticed that increasing the peptide chain length increased the pharmacological profile of the synthesized peptides [23,24]. This fact is substantiated by the evidence that the tetrapeptide GFGW (**29**) has exhibited 65% activity,

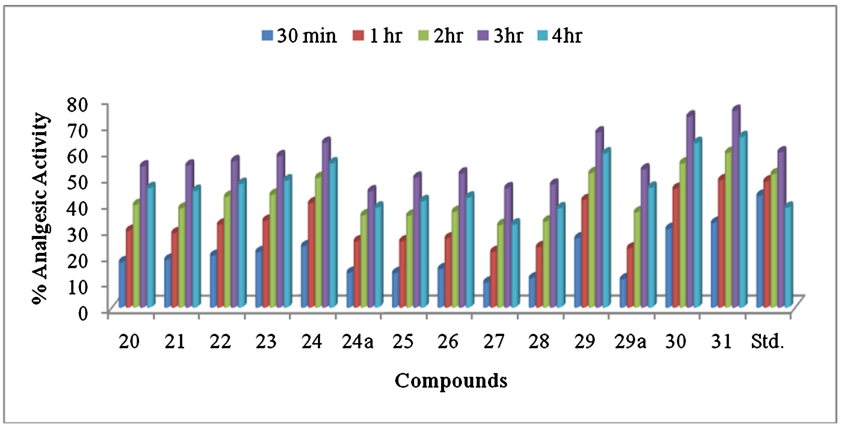


Figure 2. Diagramatic representation of the analgesic activity of the compounds.

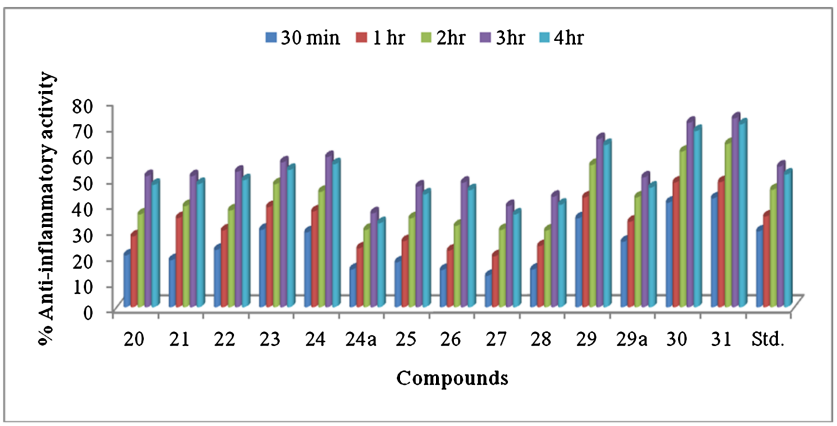


Figure 3. Diagramatic representation of the anti-inflammatory activity of the compounds.

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whereas the hexapeptides have shown 71–73% activity. Furthermore, among the hexapeptides, GVGVPW (**31**) has demonstrated better therapeutic activity over GFGFPW (**30**), although the latter contains more hydrophobic Phe units. Subsequently, we focused our attention on the importance of side-chain protecting groups. When trityl group of compound **24** was removed, it resulted in the decreased activity (**24a**). This highlighted the necessity of the side-chain protecting group in exerting better pharmacological properties.

The activity of the most potent compounds was further challenged through reduced doses such as 25 and 50-mg/kg body weight, which is in accordance with the literature [6]. It was observed that 100-mg/kg body weight is required to exhibit potent activity (Figures 4 and 5). The synthesized peptides have exhibited maximum reduction in the paw volume at 3-h interval and decreased further (from 4 h). This implies that the peptides can act against the inflammatory lesions produced and render protection up to 3 h from the time of administration. On the other hand, an additional impetus to the present investigation is that some of the synthesized peptides showed better results than the reference standards even after 24 h of administration (Figures 6 and 7), i.e. peptides have the ability to provide protection and act as better pharmacological agents compared with the available compounds in the market. A possible explanation for this observation would

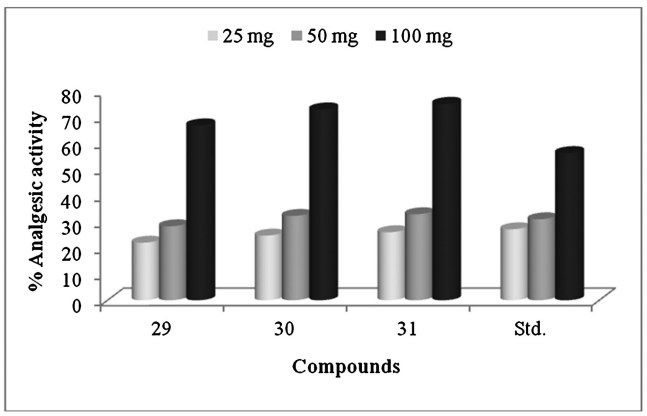

Figure 4. Diagramatic representation of the analgesic activity of the potent compounds at different doses at 3-h interval.

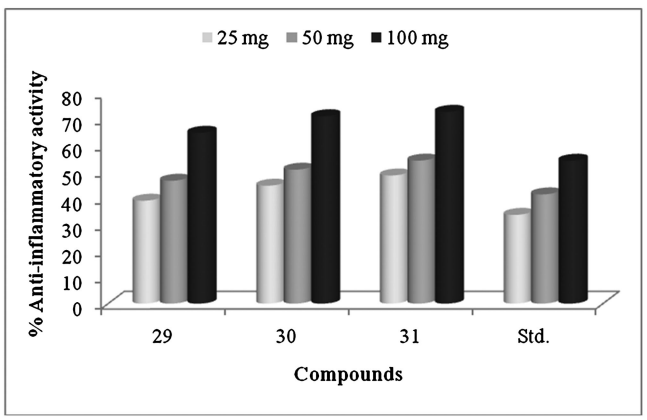

Figure 5. Diagramatic representation of the anti-inflammatory activity of the potent compounds at different doses at 3-h interval.

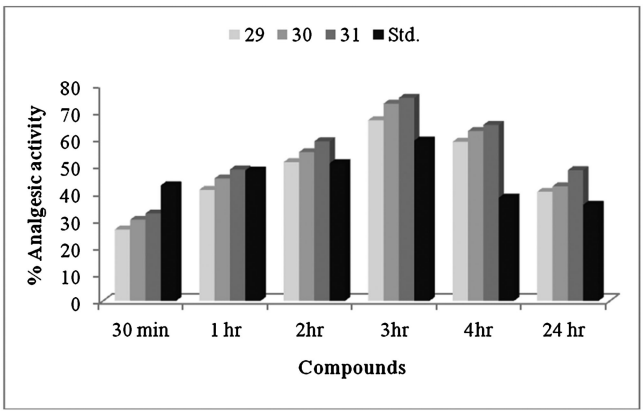

Figure 6. Comparative diagramatic representation of analgesic activity of the compounds at different time intervals.

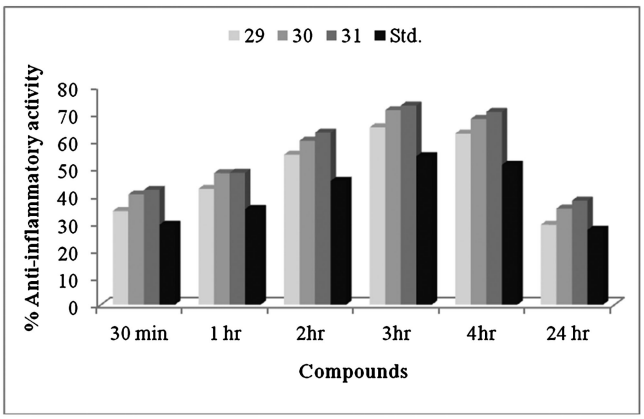

Figure 7. Comparative diagramatic representation of anti-inflammatory activity of the compounds at different time intervals.

be a better biocompatibility and bioavailability in the case of the peptides and also amino acids [32,33].

Ulcerogenic activity

Considering the potent compounds of the series, they were studied for ulcerogenic activity. The result clearly showed that the compounds were less ulcerogenic and devoid of any evidences of shedding of epithelium/red spots below skin/bleeding/erosion and so on.

Conclusion

In short, for the first time, we have developed a novel series of peptides, which demonstrated significant pharmacological properties. It was found that Fmoc group is essential for exerting potent activity. Also, presence of bulky protecting groups at the side-chain of the amino acids has a role in exhibiting the activity. This study revealed that the activity is dose as well as time dependent. The compounds showed highest activity at 3-h interval and gradually decreased, which is in correlation with the standards used. On the other hand, some peptides have exhibited promising activity even after 24 h of treatment, whereas the reference standards were active only up to 3 h. Further, as the length of the peptide chain increases, activity also increases. This article also reports that peptides containing Z/Boc groups at the

N-terminus have shown moderate activity. Several peptides *viz*., **24, 29, 30** and **31** exhibited excellent therapeutic profile with no ulcerogenic liability. Hence, these may serve as lead pharmacophores for further studies.

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