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Synthesis and Biological Evaluation of the Anti-Inflammatory Activity for some Novel Oxpholipin-11D Analogues

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ABSTRACT

The present study describes the synthesis of some novel analogues (I-IV) of an anti-inflammatory peptide-based drug called Oxpholipin-11 D. Chemical modifications in the peptide chain sequences were introduced to the parent compound, aiming to enhance the potency and penetration properties of the resulting peptide analogues. These novel compounds were characterized by means of their amino acid analysis, FT-IR and HPLC-ESI-mass spectra. The biological evaluation of the obtained compounds showed increased anti-inflammatory activity of the analogues I, II and IV compared to the reference drug.

Key words: Anti-inflammatory peptides, oxpholipin-11D, SPPS, voltaren, microwave irradiation

INTRODUCTION

There has been a rapid expansion in the use of peptides as drugs over the last decade, which is likely to continue. Peptides regulate most physiological processes, acting at some sites as endocrine or paracrine signals and at others as neurotransmitters or growth factors (Edwards *et al*., 1999). They are already being used therapeutically in such diverse areas as neurology (Toneff *et al*., 2013), endocrinology (Cui *et al*., 2014) and hematology (Abdel Rahman *et al*., 2014). Modifications of Solid Phase Peptide Synthesis (SPPS) including the use of polymers, microwave energy and improved activation reagents encouraged efforts toward the total chemical synthesis of large and small peptide derivatives (Merrifield, 1963, 1964; Dawson *et al*., 1994; Hackeng *et al*., 1997; Palasek *et al*., 2007; Cemazar and Craik, 2007). Recently, some of new peptide derivatives have been studied with respect to antiviral (Lin *et al*., 2011), anti-inflammatory (Ialenti *et al*., 2001) enzymatic peptide (Chen *et al*., 2010), anticancer (Abo-Ghalia and Amr, 2004) and antimicrobial activities (Naglah *et al*., 2014a, 2015; Burrows *et al*., 2006; Krishnakumari *et al*., 2006). Also, there are several successful publications of microwave-assisted solid phase peptide synthesis of various unnatural biopolymers, such as peptoids, pseudo peptides, small peptides (Bacsa *et al*., 2006; Naglah *et al*., 2014b), difficult peptides (Abdel Rahman *et al*., 2007), β-peptide libraries (Murray and Gellman, 2006) and glycopeptides (Matsushita *et al*., 2006). Evidence gathered over the past two decades has shown that inflammation is a key player in the pathophysiology of atherogenic

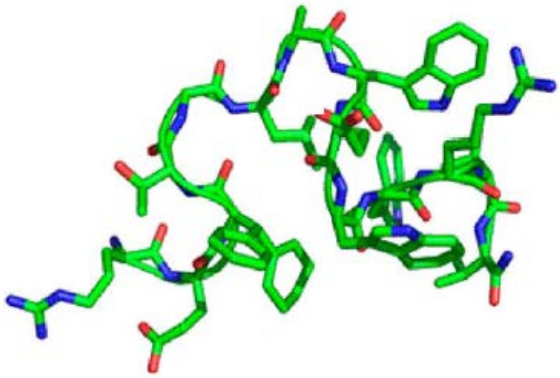

Fig. 1: Molecular illustration of the structure of Oxp-11D after 83 nsec of molecular dynamics in HFIP (Ruchala *et al*., 2010)

cardiovascular disease (CVD) (Hansson and Libby, 2006; Klingenberg and Hansson, 2009). Consequently, therapies targeting inflammatory response have already been implemented in clinical practice of CVD (Montecucco and Mach, 2009a, b, c; Moubayed *et al*., 2007). An emerging approach in treating cardiovascular disease is based on using Apo A-1 mimetic peptides (Bloedon *et al*., 2008; Navab *et al*., 2005, 2006, 2008; Sethi *et al*., 2007) with anti-inflammatory properties to sequester oxysterols and oxidized lipids (Van Lenten *et al*., 2008). These peptides have properties similar to Apo A-1 Milano (Zhu *et al*., 2005), a naturally occurring Apo A-1 mutant containing an extra cysteine disulfide bridge. Oxpholipin-11 D is an anti-inflammatory agent that binds to cholesterol and oxidized phospholipids like arachidonic acid (Ruchala *et al*., 2010). Oxpholipin-11D is a peptide in nature pore-forming exotoxin produced by Gram-positive bacteria that contains a highly conserved 12-residue domain (ECTGLAWEWWRT) (Fig. 1), this domain is usually flanked N-terminally with Arginine and C-terminally with Valine.

In view of the above facts, especially the biological importance of the Oxpholipin-11D, a series of novel derived mimetic polypeptides as Oxpholipin-11D analogues were hypothesized for systemic anti-inflammatory measurements.

MATERIALS AND METHODS

Chemistry: Peptide synthesis was carried out on a PS-PEG graft copolymer, kindly provided by Dr. W. Rapp (TentaGel-NH_2®, Rapp polymer, Tubingen). The organic solvents in this part were purchased from Sigma-Aldrich (USA) and Fluka (Switzerland) chemical companies. The used amino acids are of L- and D-configuration. All Fmoc-amino acids were purchased from Novabiochem, side chains of purchased amino acids were protected as follows: (tBu) group was used to protect Ser, Asp, Thr and Glu, the (Mtr) and (Trt) groups were used to protect Arg and Gln side chains, respectively. Infra-red spectra (KBr) were recorded on FT-IR 1650 Perkin-Elmer spectrometer, Cairo University. Purification of crude peptides using HPLC Chromatogram; Sykam S2000, 52110, National Research Centre (NRC). Amino acid analysis of the peptide sequences were carried out using the amino acid analyzer (Biotronik [LC 6000 E] with an integrator system 1), National Research Centre (NRC). Electrospray ionization-mass spectra (ESI-MS) were run on Agilent 6320, College of Pharmacy (King Saud University). The microwave-assisted syntheses were carried out in a domestic oven, LG MS-2044 W/OO, frequency is 2450 MH_Z and operating at 100 W = 10% of the total power, Faculty of Science, Zagazig University.

Coupling of TentaGel-NH_2 with HMBA using microwave irradiation: It was carried out according to the described method (Coantic *et al*., 2008).

Coupling of the first amino acid (Fmoc-Arg (mtr)-OH) to TentaGel-HMBA resin: To a swelled suspension of (0.2 g, 0.48 meq g^{-1}) HMBA-resin in 4 mL DMF, a solution of (0.5104 g, 4 mmol) Fmoc-Arg-OH (0.052 g, 4 mmol) HOBt, (0.0484 g, 4 mmol) DIC and DMAP (0.002 g, 0.3 mmol) in 4 mL DMF/CH_2Cl_2 (v/v) was added. The reaction mixture was then irradiated in microwave oven for 8 min. The resin was filtered off and washed several times with DMF, CH_2Cl_2, DMF, CH_2Cl_2, MeOH and ether.

Deprotection of Fmoc-N-protecting group using microwave irradiation: FmocA-A-HMBA-resin (0.2 g, 0.48 meq g^{-1}) was suspended in 10 mL mixture of 20% piperidine/DMF. The reaction mixture was then irradiated in microwave oven until Kaiser test (Kaiser *et al*., 1970) showed a positive result. The resin was filtered off, washed several times by DMF, DCM, DMF, DCM, MeOH and ether.

Coupling of the next Fmoc A-A using microwave irradiation: To a swelled suspension of (0.2 g, 0.48 meq g^{-1}) H-A-A-HMBA-resin in 4 mL DMF, a solution of (4 mmol) Fmoc A-A-OH, (0.052 g, 4 mmol) HOBt, (0.048 g, 4 mmol) DIC and DMAP (0.002 mg, 0.3 mmol) in 4 mL DMF/CH_2Cl_2 (v/v) was added. The reaction mixture was then irradiated in microwave oven until Kaiser test showed a negative result. The resin was then filtered off and washed several times with DMF, CH_2Cl_2, DMF, CH_2Cl_2, MeOH and ether. Peptide chain sequences were elongated according to the above mentioned methods and then cleavage the polymer to apply the spectroscopic characterization.

Anti-inflammatory activity: The present study was carried out at pharmacology department, college of pharmacy, Zagazig University; the animals were obtained from animal house of faculty of veterinary medicine. The animals were housed in metal cages, bedded with wood shavings, kept under standard laboratory conditions of aeration (room

temperature at about 20-22°C), provided with adequate rodent food and water supply. Edema was induced in the right hind paw of 36 male mice 4-6 months, weighing 200-250 g by the subcutaneous injection of 0.1 mL 2% carrageenan sodium (Sigma, USA) in distilled water (Winter *et al*., 1962). The mice were classified into 6 groups each group, consisting of 6 mice. The 1st group was kept as control group and was given the respective volume of the solvent (10% v/v of Tween 20 in distilled water). The 2nd to 5th groups were intraperitoneal injected of the synthesized compounds at a dose of 1 mg kg^{-1} before carrageenin injection. The last group was injected of Voltaren in a dose of 1 mg kg^{-1} as a reference drug. The paw volume of each mouse was measured before and after medication for a period of 3 and 6 h in groups 2-6 using Dial micrometer model (120-1206) Baty, Sussex, England. Data of anti-inflammatory study were expressed as Value±SEM. Differences between control and treatment groups were tested using one-way ANOVA followed by multiple comparisons o the Bonferroni's test.

RESULTS AND DISCUSSION

Chemistry: The major disadvantages of the application of peptides as drugs are their rapid degradation by proteases, hepatic clearance, undesired side effects by interaction of conformational flexible peptides with different receptors and low membrane permeability due to their hydrophilic character (Sewald and Jakubke, 2009). In the present work a strategy to overcome some of these problems will be applied, aiming at enhancing the potency and penetration properties of the peptides through designing and producing of some modified anti-inflammatory peptide sequences, these modifications include:

- The incorporation of non proteinogenic amino acids (e.g., D-amino acids) into the synthesized peptide chains, since D-peptides have properties that make them attractive as drugs such as: Susceptibility to be degraded in the stomach or inside cells by peptidase, easily synthesized when compared to many other drugs
- The binding of the synthesized peptides to antiflammins peptidomimetics. Those antiflammins are synthetic peptides derived from the region of highest local similarity between uteroglobulin and lipocortin (Miele, 2003)
- Binding a synthesized peptide with other anti-inflammatory drugs e.g., diclofenac acid (Voltaren®), hoping that it would increase their potency (Abo-Ghalia *et al*., 1999)
- Comprising a tail of polyarginine residues to a synthesized peptide, which may enhance its penetration through the cell membrane (Kogure *et al*., 2004)

It is evident that, the correct choice of the synthetic strategy, the protecting groups and the polymeric support could contribute most fundamentally to the improvement of the used methods. The synthesis was carried out by the use of modified Solid Phase Peptide Synthesis (SPPS) protocol with Fmoc-strategy under the application of microwave irradiation technique. 4-hydroxymethyl benzoic acid (HMBA) was used as an anchoring group, which binds the polymeric support polystyrene polyethylene glycol graft copolymer (TentaGel®-NH_2) to the first amino acid. The coupling was mediated by N,N-diisopropylcarbodiimide (DIC) and 1-hydroxybenzotriazol (HOBt) in dimethyl formamide (DMF). The prepared Oxpholipin analogues are summarized in (Table 1).

The crude peptide chains (I-IV) after cleavages of polymer were obtained by centrifugation, dissolving in distilled water, lyophilized and then characterized by amino acid analysis and were recorded in (Table 2).

The results in Table 2 show that the amino acid analyses are very close to the theoretical values expected for the same sequences. These data confirms the presence of all the amino

Table 1: Prepared oxpholipin-11D analogues (I-IV)

No.	Peptide sequence structures
I	Val-Thr-Arg-Trp-Trp-D-Glu-Trp-D-Ala-Leu-Gly-Thr-D-Phe-D-Glu-Arg
II	Ser-Asp-Leu-Val-Lys-Lys-Met-Gln-Met-Val-Thr-Arg-Trp-Trp-D-Glu-Trp-D-Ala-Leu-Gly-Thr-D-Phe- D-Glu-Arg
III	Voltaren-Val-Thr-Arg-Trp-Trp-D-Glu-Trp-D-Ala-Leu-Gly-Thr-D-Phe-D-Glu-Arg
IV	Val-Thr-Arg-Trp-Trp-D-Glu-Trp-D-Ala-Leu-Gly-Thr-D-Phe-D-Glu-Arg-Arg-Arg-Arg-Arg-Arg-Arg- Arg-Arg

Table 2: Amino acid analysis for Oxpholipin-11D analogues (I-IV)

Peptide		Amino acids													
no.	Analysis	Arg	Glu	Phe	Thr	Gly	Leu	Ala	Trp	Val	Lys	Met	Asp	Ser	Gln
I	Calculated	2.00	2.00	1.00	2.00	1	1.00	1.00	3.00	1.00	-	-	-	-	-
	Found	1.80	1.90	0.92	1.80	1	0.94	0.97	2.61	0.94	-	-	-	-	-
II	Calculated	2.00	2.00	1.00	2.00	1	2.00	1.00	3.00	2.00	2	2	1	1	1
	Found	1.75	1.74	1.02	1.84	1	2.10	0.94	2.70	1.90	1.96	1.7	0.94	1.01	0.84
III	Calculated	2.00	2.00	1.00	2.00	1	1.00	1.00	3.00	1.00	-	-	-	-	-
	Found	1.63	1.83	0.94	1.85	1	0.95	0.99	2.63	0.93	-	-	-	-	-
IV	Calculated	10.00	2.00	1.00	2.00	1	1.00	1.00	3.00	1.00	-	-	-	-	-
	Found	10.00	2.02	0.99	1.88	1	1.01	0.99	2.70	1.01	-	-	-	-	-

Arg: Arginine, Glu: Glutamate, Phe: Phenylalanine, Thr: Threonine, Gly: Glycine, Leu: Leucine, Ala: Alanine, Trp: Tryptophan, Val: Valine, Lys: Lysine, Met: Methionine, Asp: Aspartate, Ser: Serine, Gln: Glutamine

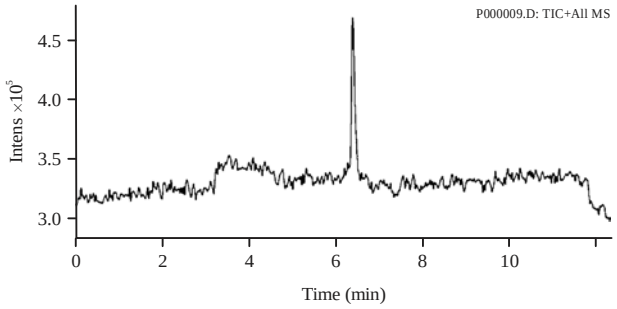

Fig. 2: HPLC chromatogram of the crude peptide chain I

Table 3: Mice hind paw edema thickness before and after medication

	Edema (Mean±SEM %)		
Groups	Before treatment	After 3 h	After 6 h
Control	31.01±1.23	30.81±1.23	30.09±1.23
Oxpholipin	29.86±0.96	23.51±1.77[ab]	23.21±0.89[ab]
Oxp-AI	29.90±1.14	23.87±1.89[ab]	22.76±1.55[ab]
Oxp-Vol	30.00±2.09	28.89±0.99[a]	28.57±2.08[a]
Oxp-Arg	30.35±2.01	24.85±2.25[ab]	22.09±1.06[ab]
Voltaren	30.06±1.09	27.37±2.03	26.05±1.01

Values represent Mean±SEM of six animals for each group, [a]p<0.001, statistically significant from control, one-way ANOVA (Bonferroni's test), [b]p<0.05, statistically significant from voltaren, one-way ANOVA (Bonferroni's test)

acids in the peptide backbone during chemical preparation. Further characterization was analyzed by HPLC and positive-mode ESI-MS (Fig. 2).

The above figure shows the HPLC profile of the crude peptide chain I, which synthesized using modified SPPS under the application of microwave irradiation. The major fraction at a retention time of 6.4 min was subjected to the mass spectroscopy, which demonstrated that the sample under investigation has correct mass value. Conformational study on the synthesized peptide chains was carried out by IR measurements. Fourier transform-infrared (FT-IR) spectroscopy is particularly useful for probing the structures of peptide and proteins; this technique can be used to study the secondary structures of proteins (Stuart and Ando, 1997). The prepared peptide chain I exhibited strong bands at (1661.84 cm^{-1}) amide I and (695.212 cm^{-1}) amide A; indicating that the peptide has an intermolecular β-structure. Also, the peptide chain II exhibited strong bands at (3400 cm^{-1}) amide A, (1621.84 cm^{-1}) amide I and (694.24 cm^{-1}) amide V; confirming the presence of an intermolecular β-structure. On the other hand, peptide chains III and IV exhibited strong bands at (1665 cm^{-1}) amide I and (1626.66 cm^{-1}) amide I, respectively; suggesting that peptide chain III has an α-Helix '3 turn and peptide chain IV has an intermolecular β-Sheet structure. Finally, from the above results of IR measurements we concluded that, the prepared peptide chains have secondary structures and exist in α-Helix and β-Sheet conformations.

Anti-inflammatory activity: Peptide chains (I-IV) were chosen as models of the synthesized Oxpholipin-11D analogues for the anti-inflammatory activity measurements, as they possess α-Helix and β-Sheet conformations similar to the original Oxph-11D according to the conformational study using IR spectroscopy. The anti-inflammatory activity for all compounds was determined via mice hind paw edema thickness before and after medication during 3 and 6 h (Winter *et al*., 1962). Percent edema inhibition was calculated in respect to control and voltaren groups and depicted in (Table 3).

Drug analogues effect comparing to control and voltaren before and after both 3 and 6 h of treatment were illustrated in (Fig. 3).

On comparing the results to control group in Fig. 3 before and after 3 h of treatment, the tested compounds showed an intelligible decrease in the edema size. On the other hand, in regard to voltaren group, the results demonstrated a moderate activity in reduction of edema size except Oxp-Vol group. Also, on comparing the results to control group after 6 h of treatment, the tested compounds were significantly higher than that of control group in minimizing the inflammation size. As well, on comparing the results to voltaren group, the tested compounds were still having better inhibitory effect more than voltaren group, but Oxp-Vol group showed no inhibitory effect. The results obtained from the biological testing confirmed that the analogues are effective and potent anti-inflammatory agents with significant effect comparable to which of Voltaren except analogue III. From the previous results it was noticed that, in peptide chain I, the presence of non proteinogenic amino acids (e.g., D-amino acids), such as (D-Glu, D-Phe and D-Ala) into the synthesized peptide chains

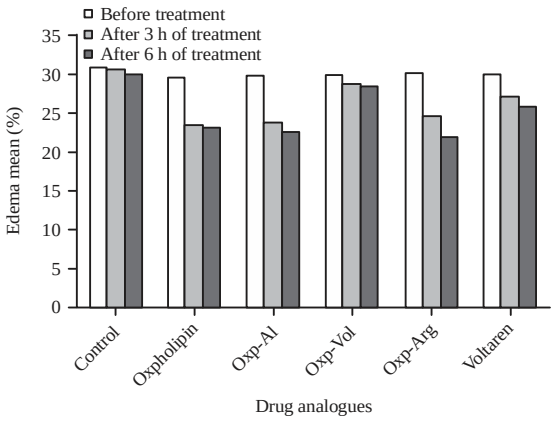

Fig. 3: Drug analogues effect comparing to control and voltaren

improved the incorporation of the peptide into cells. In case of peptide chain II (Oxp-AI), the mimetic peptide was synthesized by incorporation of Oxpholipin with AF1 caused potency as the mean of wound healing effect was increased. Concerning peptide chain III (Oxp-Vol), the synthesized peptide was bound to other anti-inflammatory drugs e.g., voltaren, with the aim to increase anti-inflammatory activity. Unfortunately the incorporation of voltaren resulted in an antagonistic, which could be explained by the α-helical structure affecting globally the bioavailability. On the other hand, the peptide chain IV (Oxp-Arg), comprised a tail of polyarginine residues, in order to enhance its penetration through the cell membrane. This modification resulted in a significant synergistic effect that exceeded Voltaren itself and gave the best anti-inflammatory effect of all the synthesized analogues. Consequently the previous results indicated that the synthesized Oxpholipin-11D analogues are potent anti-inflammatory agents compared to other drugs and are expected to be better tolerated due to their proteinogenic nature. The evaluation of potential side effect still remains to be investigated.

CONCLUSION

The objective of this study was to prepare and investigate new anti-inflammatory Oxpholipin-11D analogues. The use of a modified solid phase method with the application of microwave irradiation technique enhanced the rates of both coupling and deprotection reactions. The application of spectral analysis on the synthesized analogues was useful in the separation and characterization of the prepared peptides. The results indicated also that the most of synthesized Oxpholipin-11D analogues are potent anti-inflammatory agents compared to other drugs and are expected to be better tolerated due to its proteinogenic nature.

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