Pergamon

Bioorganic & Medicinal Chemistry Letters 12 (2002) 21–24

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Minimal Structural Requirements for Agonist Activity of PAR-2 Activating Peptides

Vincenzo Santagada,[a] Giuseppe Caliendo,[a] Beatrice Severino,[a] Elisa Perissutti,[a] Ferdinando Fiorino,[a] Carla Cicala,[b] Vincenzo De Filippis[c,*] and Giuseppe Cirino[b]

[a]*Department of Pharmaceutical Chemistry, University of Naples 'Federico II', Via D. Montesano 49, 80131 Naples, Italy*
[b]*Department of Experimental Pharmacology, University of Naples 'Federico II', Via D. Montesano 49, 80131 Naples, Italy*
[c]*Department of Pharmaceutical Sciences and CRIBI Biotechnology Center, University of Padua, Via F. Marzolo 5, 35131 Padua, Italy*

Received 22 June 2001; revised 10 August 2001; accepted 24 September 2001

Abstract—Protease-activated receptor 2 (PAR-2) is involved in inflammatory, gastrointestinal, and vascular diseases. The aim of the present work was to elucidate the minimal structural features for PAR-2 agonist activity in short peptides. Our study resulted in the discovery of dipeptide derivatives of N^{α}-benzoyl-Arg(NO_2)-Leu-NH_2 displaying a potency comparable to that of the full-length rat PAR-2 activating peptide (Ser-Leu-Ile-Gly-Arg-Leu-NH_2).

Protease-activated receptors (PARs) represent a unique class of seven-transmembrane G protein-coupled receptors, which are activated through selective cleavage at their extracellular N-terminal domain by several endogenous serine proteases, thereby generating a new N-terminus that acts as an intramolecular tethered ligand. By virtue of this structural rearrangement, the receptor becomes activated giving rise to various signal transduction pathways at the interface between coagulation, inflammation and cell proliferation.[1–3] There are four members of this family discovered so far, three of which (PAR-1, PAR-3 and PAR-4) are activated by thrombin, while the fourth (PAR-2) is activated by trypsin, mast cell tryptase and factor Xa.[1,2] Notably, synthetic peptides incorporating the amino acid sequence of the newly generated N-terminal portion of the cleaved receptor (PAR activating peptides, PAR APs) exhibit remarkable agonist potency for receptor activation, independent of protease-mediated chain cleavage. These findings opened new avenues for the development of molecules capable to modulate PAR-mediated physiological effects, of great potential therapeutic impact in the treatment of thrombotic and inflammatory states.[4] The pathophysiological role of PAR-2 is not clearly established, and only recently this receptor has been recognized to play a key role in inflammatory,[3,5] gastrointestinal,[6] and vascular[2,7] diseases. The synthetic hexapeptide corresponding to the proteolitically revealed amino terminal sequence Ser-Leu-Ile-Gly-Arg-Leu of rat PAR-2 is able to efficiently activate the receptor.[8] Previous results indicate that Leu-2 and Arg5 are critical for PAR-2 activating potency,[8,9] even though more recent studies indicate that Arg5 can be successfully replaced by large hydrophobic amino acids.[10] Moreover, amidation of the C-terminal Leu slightly improves PAR-2 activation, whereas N- and C-terminal chain truncation dramatically decreases agonist potency.[8,9] Hence, we took the hexapeptide H-Ser-Leu-Ile-Gly-Arg-Leu-NH_2 as the starting framework for our structure–activity studies.

Here, we report the results of our study aimed to elucidate the minimal structural requirements for PAR-2 agonist activity.

Chemistry

PAR-2 AP and acetyl-PAR-2 AP were prepared by solid phase synthesis on a Rink-Amide resin according to standard fluorenylmethyloxycarbonyl chemistry. All other peptides were prepared by standard liquid phase peptide synthesis. All the peptides were purified to homogeneity (~99% purity) by RP-HPLC and

*Corresponding author. Tel.: +39-049-827-5698; fax: +39-049-827-5366; e-mail: vincenzo.defilippis@unipd.it

0960-894X/02/$ - see front matter
PII: S0960-894X(01)00636-9

analyzed on a LCQ Ion-Trap mass spectrometer, yielding mass values in agreement with the expected chemical composition within 10 ppm accuracy.

PAR-2 Activating Properties

All the synthetic peptide derivatives reported in this study were tested for PAR-2 activation in the classical rat aorta relaxation assay.[8,9] The inherent limitation of this method resides in the fact that possible cross-activation of other PARs by PAR-2 APs cannot be excluded. However, the results of SAR studies conducted by using this assay method[8,9] are reasonably comparable to those reported recently[10] and obtained on murine cell lines expressing only PAR-2.

In this study, male Wistar rats (200–250 g) were sacrificed by decapitation and exsanguinated. Thoracic aorta was rapidly removed, gently cleaned paying attention not to damage the endothelium and cut into rings of about 3 mm each. Aortic rings were placed in a 2.5 mL organ bath containing Krebs solution, pH 7.4, warmed at 37 °C, oxygenated (95% O_2, 5% CO_2), and mounted to an isometric transducer under a tension of 0.5 g. After 1 h equilibration, the tissue was pre-contracted with phenylephrine (1 μM), and the presence of a functional endothelium was ascertained by monitoring the relaxation to acetylcholine (1 μM). Tissues were then washed, pre-contracted with phenylephrine (1 μM), and the agonist peptides were added (20 μL solution) at the specified concentration directly to the organ bath. Preliminarily, relaxation responses to a submaximal concentration of peptides (10^{-4} M) were evaluated. For those peptides that showed agonistic activity, a complete concentration–response curve (10^{-7}–3×10^{-4} M) was performed and compared to the curve obtained with PAR-2 activating peptide in the concentration range 10^{-7}–10^{-5} M. All the peptides were also tested for antagonist activity at a dose that per se did not cause contractile effect. Peptides were incubated for 10 min in the organ bath and then the peptide PAR-2 AP was added as described before. The results obtained provide clear-cut evidence that the peptides investigated do not show any antagonistic effect on the relaxation induced by PAR-2 AP.

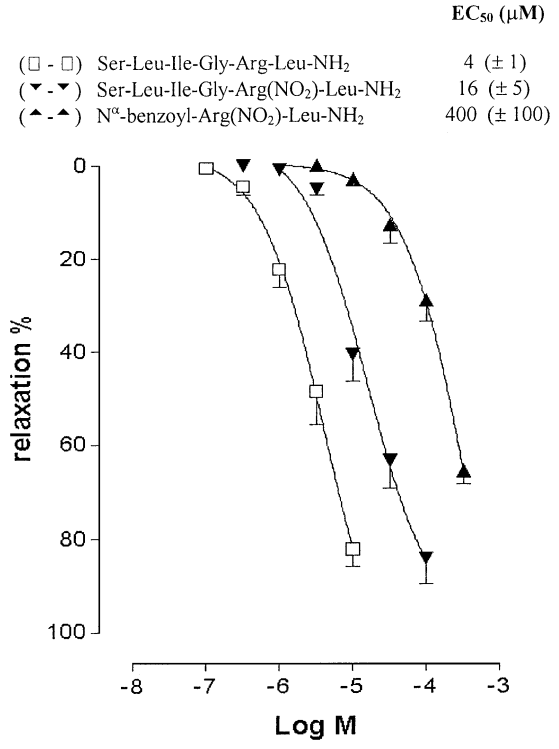

Figure 1. Concentration/effect curves for PAR-2 activating peptide Ser-Leu-Ile-Gly-Arg-Leu-NH_2 and for its derivatives. Phenylephrine-precontracted rat aorta rings were exposed to increasing concentrations (M) of agonist peptides, and the relaxation response relative to that caused by 1 μM acetylcholine was monitored. The data points are the means obtained from 6 to 10 or more independent tissue preparations coming from at least two different animals.

N^{α}-Acetylation of Ser1 abolishes activity in full-length Ser-Leu-Ile-Gly-Arg-Leu-NH_2, in agreement with previous results obtained by others.[9] Nitration of Arg5, reduces PAR-2 activating potency by 4 times ($EC_{50}=16\pm4$ μM) in respect to the unmodified PAR-2 AP ($EC_{50}=4\pm1$ μM) (see Fig. 1). This result is in line with previous ones showing that removal of the positive charge at position 5 of PAR-2 AP reduces PAR-2 activation.[8,10] In our case, the introduction of a nitro-group leads to the formation of a η^1-nitroimine derivative[11] lacking the basicity of the guanidyl-group, for example pK_a is −0.5 for nitroguanidine and 13.6 for guanidine.[12]

Proper rigidification of flexible ligands in the free, unbound state is a widely used strategy to enhance ligand–receptor interaction.[13] In this view, we replaced Ile^3-Gly^4 dipeptide backbone with more rigid difunctional scaffolds (see Fig. 2). These compounds are inactive either in the unmodified and Arg5(NO_2) form. We also tested for PAR-2 activation the corresponding N^{α}-acyl-substituted analogues of the dipeptide Arg-Leu-NH_2, which were obtained as intermediates during the synthesis of the compounds reported in Figure 2. All

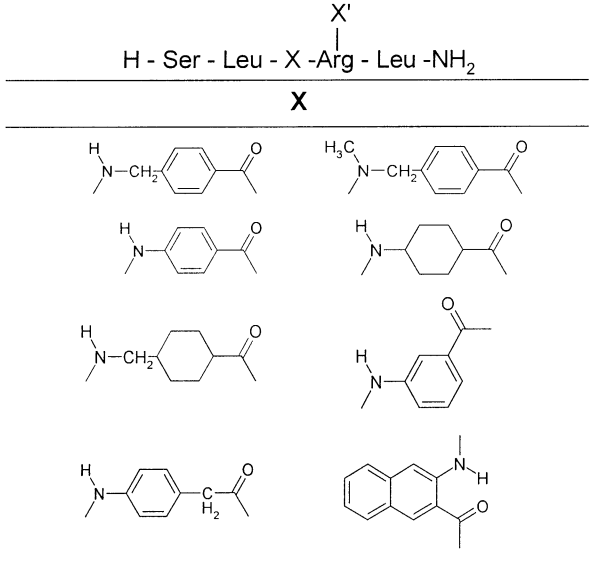

Figure 2. Chemical structure of analogues of PAR-2 activating peptides with Ile^3-Gly^4 dipeptide substituted by **X**. **X′** = –H or $–NO_2$.

these peptides, containing an amino-group attached to the aryl or cyclohexyl moiety, are inactive up to the highest concentration of agonist tested (3×10^{-4} M).

Remarkably, the N^{α}-benzoyl-Arg(NO_2)-Leu-NH_2 (compound **1** in Table 1), lacking the amino-group, displays significant PAR-2 activating potency ($EC_{50}=400\pm100$ μM), albeit two orders of magnitude lower than that of the reference PAR-2 activating peptide ($EC_{50}=4\pm1$ μM) (see Fig. 1). At this point, we synthesized several analogues of N^{α}-benzoyl-Arg(NO_2)-Leu-NH_2 containing different acyl-substituents (see Table 1). In particular, replacement of the benzoyl-group with acetyl yields the inactive compound **2**. Similar results are obtained when the phenyl ring of **1** is saturated to give compound **3**, even though phenyl and cyclohexyl moieties are almost isosteric and isophobic. In addition, if we keep the aromatic portion unchanged and substitute the amide bond with a sulfonamide, that allows almost free rotation around phenyl–sulfur and sulfur–nitrogen bonds, the inactive compound **4** is obtained. These results indicate that a rigid, conjugated π-system at the N-terminus of Arg is necessary for PAR-2 activation. Interestingly, electron-withdrawing groups (i.e., –Cl, –CN, $–CF_3$ and $–NO_2$) in the *para*-position of the phenyl-ring abolish activity (**5**–**8** in Table 1), whereas strong or weak electron-donating *para*-substituents (i.e., $–OCH_3$ and $–OCF_3$) enhance PAR-2 activating potency by 6 (**9**) or 20 times (**10**) in respect to the unsubstituted compound **1**. The presence of the bulky $–SCH_3$ group in compound **11** suppresses activity, suggesting that also steric, and/or polarizability effects strongly influence PAR-2 activating potency.

Discussion

Although caution should be used in interpreting results obtained from ex vivo assays, several conclusions can be drawn from the PAR-2 activating properties of the 30 molecules synthesized in the present study. First, the presence of a positive charge is beneficial for activity at the N^{α}-amino terminus in the full-length hexapeptide Ser-Leu-Ile-Gly-Arg-Leu-NH_2, whereas a net or partial positive charge abolishes activity when present on the phenyl ring of N^{α}-benzoyl-Arg(NO_2)-Leu-NH_2. Second, nitration of arginine reduces PAR-2 activation in the hexapeptide, but confers significant potency to N^{α}-benzoyl-Arg-Leu-NH_2, which otherwise is inactive. Third, a rigid, conjugated π-system at the N-terminus of Arg in N^{α}-benzoyl-Arg(NO_2)-Leu-NH_2 is required for PAR-2 activation. Fourth, electron-withdrawing groups on the phenyl-ring abolish agonist activity, whereas electron-releasing groups significantly enhance PAR-2 activating potency.

In general, the rising of biological activity in a flexible molecule is the result of a delicate balance between those interactions that stabilize the bioactive conformation of the ligand in the free (unbound) state, and those that actually affect the strength of interaction of the ligand on the receptor recognition sites. To possibly rationalize our results, it should be considered that nitration of Arg5 is not expected to appreciably alter the bioactive conformation of the full-length PAR-2 activating peptide, mainly dictated by the overall peptide chain fold. Hence, the reduced PAR-2 activating potency of the nitro-derivative can be reasonably assigned to the suppression of the positive charge at Arg5. Conversely, in the case of N^{α}-benzoyl-Arg-Leu-NH_2, we propose that nitration of arginine may substantially affect the conformational properties of the resulting nitro-derivative **1** in the free (unbound) state, by stabilizing its bioactive conformation. Nitration of arginine, in fact, gives a η^1-nitroimine[11] stabilized by the formation of a strong intramolecular hydrogen bond to yield a nearly planar, conjugated system (Fig. 3a), as inferred from the solution and solid-state structure of L-nitroarginine.[14,15] Hence, the electron-deficient nitroguanidyl-group of Arg(NO_2) might interact with the aromatic benzoyl moiety at the N-terminus through favorable π–π stacking interactions, shifting the equilibrium between the different conformers in the free state towards a molecular structure (Fig. 3b) able to bind and

Table 1. Vasorelaxant activity of N^{α}-benzoyl-derivative of Arg-Leu-NH_2

	X-Arg(NO_2)-Leu-NH_2	
Compd	**X**	EC_{50} (μM)[a]
1	Benzoyl-	400 (± 100)
2	Acetyl-	n.a.
3	Cyclohexyloyl-	n.a.
4	Benzensolfonyl-	n.a.
5	*p*-Cl-Benzoyl-	n.a.
6	*p*-CN-Benzoyl-	n.a.
7	*p*-CF_3-Benzoyl-	n.a.
8	*p*-NO_2-Benzoyl-	n.a.
9	*p*-CH_3O-Benzoyl-	69 (±20)
10	*p*-CF_3O-Benzoyl-	20 (±10)
11	*p*-CH_3S-Benzoyl-	n.a.

[a]Values are means of 6–10 or more independent tissue preparations coming from at least two different animals. Standard deviation is given in parentheses.
n.a. = not active up to a concentration of 3×10^{-4} M.

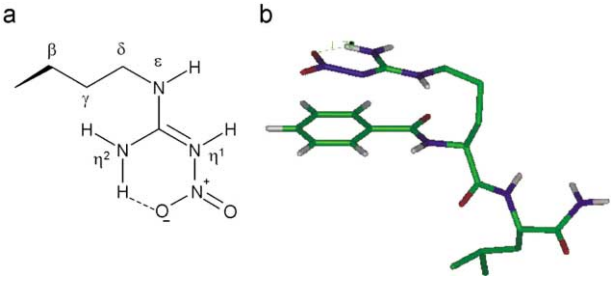

Figure 3. (a) Schematic representation of the structure of nitroarginine side chain.[14–15] The hydrogen bond between η^2 nitrogen of the guanidyl-group and one of the oxygen atoms of the nitro-group is indicated by a dashed line. (b) Proposed model for the bioactive conformation of N^{α}-benzoyl-Arg(NO_2)-Leu-NH_2. The peptide chain was set in a fully extended conformation ($\phi=-120°$, $\psi=+140°$), while the side chain of Arg(NO_2) was modelled to achieve a parallel displaced geometry of the nitroguanidyl moiety over the benzoyl group, with a centroid interplanar distance of 3.5 Å. The model was built using the program Insight-II (Biosym Technologies) run on a Silicon Graphics O2 workstation.

activate PAR-2. This view is supported experimentally by our results indicating that only the concomitant presence of an aromatic conjugated system at the N^{α}-amino terminus and of a nitro group at the η^1-nitrogen of Arg confers PAR-2 activating potency to the dipeptide H-Arg-Leu-NH_2. In fact, the N^{α}-benzoyl-Arg-Leu-NH_2 derivative, lacking the nitro-group, is inactive. Similarly, if we keep the nitro-group on the Arg side chain unchanged and saturate the phenyl ring, in order to prevent π–π stacking interaction and at the same time retain the hydrophobic and steric properties of the benzoyl group, the inactive molecule N^{α}-cyclohexyloyl-Arg(NO_2)-Leu-NH_2 (**3**) is obtained.

Statistic analysis of the geometry of π–π interactions reveals that the parallel displaced orientation is by far the most frequently represented in small organic molecules[16,17] and short peptides,[18] with a favorable free energy change in the range 0.5–2.5 kcal mol^{-1}.[17] In particular, the face-to-face arrangement is stabilized when large complementary charges or polarizations of the interacting systems lead to attractive electrostatic interactions that overcome π–π repulsion.[19] It has been found experimentally that the stacked orientation of aromatic rings in water is strengthened by the introduction of nitro-groups or nitrogen atoms into one of the interacting partners.[20,21] In this respect, the fact that electron-withdrawing *para*-substituents on the phenyl ring of N^{α}-benzoyl-Arg(NO_2)-Leu-NH_2 suppress activity, whereas electron-donating groups enhance PAR-2 activating potency (see Table 1) add more weight to our proposal.

In conclusion, we have demonstrated here that the chain-length of the hexapeptide responsible for PAR-2 activation can be shortened down to the dipeptidyl analogue **10** displaying PAR-2 activating potency only five-fold lower than that of the full-length PAR-2 AP, and very similar to that of the corresponding Arg5(NO_2) hexapeptide. The results herein reported emphasize the importance of the analogue structure-based approach in drug discovery and represent an important step in the design of novel PAR-2 modulating molecules.

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