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Published in:
Journal of Pharmacological and Toxicological Methods

DOI:
[10.1016/j.vascn.2004.07.005](https://doi.org/10.1016/j.vascn.2004.07.005)

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2005

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Loot, A. E., van Buiten, A., Roks, A. J. M., & Henning, R. H. (2005). The suitability of iodinated Angiotensin-(1-7) peptides as pharmacological tools. *Journal of Pharmacological and Toxicological Methods*, 51(1), 51-55. <https://doi.org/10.1016/j.vascn.2004.07.005>

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Original article

The suitability of iodinated Angiotensin-(1–7) peptides as pharmacological tools

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Received 19 May 2004; accepted 7 July 2004

Abstract

Introduction: The heptapeptide Angiotensin-(1–7) [Ang-(1–7)] is a biologically active component of the Renin–Angiotensin System. Pharmacological studies often involve Ang-(1–7) radioactively labelled with ^{125}I . Given the small size of the original peptide, we investigated whether introduction of a rather bulky iodine label interferes with the biological activity of Ang-(1–7). **Methods:** Ang-(1–7) was labelled with nonradioactive iodine with the chloramine-T method. The reaction products were separated on HPLC and analysed with mass spectrometry. The products were tested for biological activity in two ways: The ability of labelled Ang-(1–7) to block Ang II-induced contraction in rat aortic rings was tested in an organ bath setup. The affinity of labelled angiotensin for ACE in rat plasma was examined in vitro. **Results:** Iodination of Angiotensin-(1–7) resulted in two main products: monoiodinated and diiodinated Ang-(1–7) that could be easily separated on HPLC. In an organ bath experiment, monoiodinated Ang-(1–7) blocked Ang II responses identical to the native compound, whereas diiodinated Ang-(1–7) had lost its ability to block Ang II responses. Likewise, monoiodinated Ang-(1–7) had retained its affinity for ACE, while the affinity of diiodinated Ang-(1–7) was greatly reduced. **Discussion:** Monoiodinated Ang-(1–7) has a biological activity identical to the native compound, whereas this is lost in diiodinated Ang-(1–7). Therefore, only the monoiodinated radioactive form seems suited for pharmacological studies.

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Keywords: Angiotensin-(1–7); Chloramine T; [^{125}I]; LC/MS; Methods; Rat aorta

1. Introduction

Angiotensin-(1–7) [Ang-(1–7)] is a biologically active metabolite of Ang I and II that counteracts the detrimental effects of Ang II in diseases that are characterised by high activity of the Renin–Angiotensin System, such as hypertension and heart failure (Collister & Hendel 2003; Iyer, Ferrario, & Chappell 1998; Loot et al., 2002). The mechanisms by which Ang-(1–7) exerts its beneficial effects are not fully understood. Amongst the possibilities identified are inhibition of the Angiotensin Converting Enzyme (ACE) and

actions at various receptors, including antagonism of the Angiotensin II type 1 (AT_1) receptor, stimulation of AT_2 and bradykinin B_2 receptors, and stimulation of a putative Ang-(1–7) receptor, resulting in the release of vasodilating prostaglandins and nitric oxide (NO; Gironacci, Coba, & Pena 1999; Porsti, Bara, Busse & Hecker, 1994; Roks et al., 1999).

Studies into the mechanisms of action of Ang-(1–7) and its significance in disease, such as radioligand binding studies, often involve [^{125}I]-labelled Ang-(1–7). Given the relative small size of the heptapeptide in relation to the iodine label, it is conceivable that the label alters its properties due to, e.g., steric hindrance. In this article therefore, we compare the biological activity of monoiodinated and diiodinated Ang-(1–7) to the native compound. We studied their effect on ACE activity and on Ang-II-induced vasoconstriction.

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2. Methods

2.1. Chemicals and reagents

Milli-Q grade water was used to prepare all stock solutions and reaction mixtures. All solvents (HPLC quality) were obtained from Merck (Darmstadt, Germany). All salts and reagents were of analytical grade and were purchased from Merck or Sigma-Aldrich (Zwijndrecht, Netherlands). Human Ang II and Ang-(1–7) were obtained from Bachem (Bubendorf, Switzerland).

2.2. Synthesis of iodinated Ang-(1–7)

Ang-(1–7) was iodinated using the chloramine-T method (Hussain, Jona, Yamada, & Dittert, 1995; Robles, Balter, Oliver, Welling, & Pauwels, 2001; Yamada, Traboulsi, Dittert, & Hussain et al., 2000). Small variations in reaction parameters may strongly influence the yield of monoiodinated and diiodinated product. Therefore, we optimised the conditions for iodination of Ang-(1–7) by using the simplex optimisation method (Andries & de Vries 2001), for which an optimal outcome was defined as a high yield of both monoiodinated and diiodinated Ang-(1–7).

After three rounds of optimisation, the following conditions for labelling were obtained: Ang-(1–7) was iodinated by incubating the peptide (178 µg) in 0.5 M sodium phosphate buffer (pH 7.4), containing 90 µg chloramine T and 150 µg sodium iodide followed by vortex mixing for 40 s at room temperature (total volume of 92.5 µl). The reaction was terminated by the addition of 175 µg of sodium bisulphate in 17.5 µl of sodium phosphate buffer.

2.3. HPLC procedure

Reaction products were separated with an HPLC-reverse phase system on a Vydac Protein & Peptides C₁₈ column (218TP54: 5×250 mm; particle size 3.9 µm, Vydac, Hesperia/California). Peptides were eluted from the column with 0.1% trifluor-acetic acid (TFA) and 12% acetonitril for 5 min followed by a linear acetonitril gradient of 12–30% developed over 25 min at a flow rate of 1.0 ml/min (Fig. 1). Peptides were detected spectrophotometrically at 214 nm, and the identity of the peaks was verified with mass spectrometry. Fractions corresponding to monoiodinated and diiodinated Ang-(1–7) were collected and lyophilised.

2.4. Animals

Approval of animal experiments was given by the institutional Animal Care and Use Committee. Twelve male Wistar rats weighing 250–300 g were obtained from Harlan (Zeist, The Netherlands). The animals were housed group-wise with free access to food and drinking water.

Fig. 1. Spectrophotometric trace of an HPLC run.

2.5. Aortic ring measurements

Under N₂O/O₂/isoflurane narcosis, the rats were heparinised and the descending thoracic aorta was excised and cleaned of adherent adipose and connective tissue. From each aorta, 16 rings of approximately 2 mm each were cut and mounted in an organ bath with Krebs solution (pH 7.5) containing (in mmol/l): NaCl (120.4), KCl (5.9), CaCl₂ (2.5), MgCl₂ (1.2), NaH₂PO₄ (1.2), glucose (11.5), and NaHCO₃ (25.0), which was kept at 37 °C and continuously gassed with 5% CO₂ and 95% O₂. We performed isotonic measurements of vascular contraction; that is, vessel rings were subjected to a constant tension of 1.4 g and changes in vessel diameter were registered in microns.

Before starting the protocol, each aortic ring was washed at least three times during a stabilization period of 45 min. To test viability of the ring, contraction was induced with 10^{−6} M phenylephrine (PE) and the rings were washed at least three times until they are stable. After a second PE challenge, endothelial function was tested with 10^{−4} M methacholine (MeCh), after which both drugs were washed out and the rings were stabilized for 45 to 60 min. If the second PE challenge evoked an absolute response of less than 100 µm contraction, the ring was not used.

When the rings were completely stabilized, we consecutively added 10^{−4} M L-N^G-monomethyl-arginine (L-NMMA), 10^{−6} M lisinopril in combination with the antagonist listed in Table 1, and finally the Ang-(1–7) peptide listed in Table 1. After a final incubation period of 20 min, responses to cumulative doses of Ang II (10^{−10} to 10^{−6} M) were assessed. A final dose of PE was added as an independent control.

Table 1
Experimental groups in organ bath experiments

	Antagonist	Ang-(1–7) peptide
1	–	–
2	–	Ang-(1–7)
3	A779	Ang-(1–7)
4	PD 123319	Ang-(1–7)
5	–	I-Ang-(1–7)
6	A779	I-Ang-(1–7)
7	PD 123319	I-Ang-(1–7)
8	–	I ₂ -Ang-(1–7)

Concentrations: PD 123319 10^{-7} M A779: 10^{-6} M, (I_x)-Ang-(1–7) 10^{-5} M. Protocols were carried out in duplicate rings.

2.6. ACE activity measurements

ACE activity was measured in rat plasma according to the method of Cushman and Cheung (1971). Briefly, rat plasma was diluted 10 times and incubated with 5 mM Hippuryl-L-Histidyl-L-Leucine (HHL) for 15 min at 37 °C. The concentration of L-Histidyl-L-Leucine (HL) was measured fluorometrically. The inhibitory effect of increasing doses (10^{-8} – 10^{-4} M) of (iodinated) Ang-(1–7) was determined in fourfold. Similarly, Lineweaver–Burk analysis was performed with varying doses of (iodinated) Ang-(1–7) (10^{-8} – 10^{-5} M) and HHL (0.5–4 mM).

2.7. Statistics

The means of the duplicate rings were calculated first. Data are expressed as a percentage of the maximal contraction to the last dose of PE. If the contraction of rings to Ang II in the control group was less than 15% of their maximum contraction to PE, the data were excluded from analysis. Differences in dose–response curves were tested by ANOVA for repeated measures with Greenhouse–Geisser correction for asphericity. Differences were considered significant at $p < 0.05$. Values are expressed as the mean $\pm$ S.E.M. For both ACE activity and Ang-II-dependent contractions, pharmacological characteristics of the dose–response curves, i.e., the maximal response ($E_{\max}$) and the concentration at which 50% of this maximal response was reached (EC_{50}) were calculated with a curve-fitting program. In fitting the Ang II contractions, we omitted the data points that reflect the desensitisation of the AT₁ receptor. $E_{\max}$ values for individual rings were based on the data points directly; individual EC_{50} values were calculated (see Alexander, Browse, Reading, & Benjamin, 1999).

3. Results

3.1. Synthesis of iodinated Ang-(1–7)

We optimised the chloramine-T reaction to yield high amounts of both monoiodinated and diiodinated Ang-(1–7). After three rounds of simplex optimisation, we found the

optimal reaction parameters as indicated in the Methods section. At these conditions, virtually all ($98.6 \pm 0.0\%$) original Ang-(1–7) was iodinated resulting in $27.5 \pm 0.4\%$ monoiodinated Ang-(1–7), $47.9 \pm 0.3\%$ diiodinated Ang-(1–7), and $23.2 \pm 0.2\%$ triiodinated Ang-(1–7).

3.2. Effect of iodinated Ang-(1–7) on Ang II dose–response curves

The ability of iodinated Ang-(1–7) to block Ang-II-induced contractions of rat aortic rings was tested. In vehicle-treated rings, Ang II elicited a concentration-dependent contraction with a $\log EC_{50}$ of -8.7 and a maximal contraction of $64.9 \pm 2.4\%$ of the PE response. Normal and monoiodinated Ang-(1–7) had identical effects: both compounds significantly inhibited this contraction in both a competitive and a noncompetitive manner, resulting in a rightward shift of the Ang II dose–response curve and a depression of the maximum by 15%, respectively. Diiodinated Ang-(1–7), however, had no effect on Ang II contractions (Fig. 2).

To distinguish between the different receptors modulating the inhibitory effect of (I-)Ang-(1–7), we performed the same experiments in the presence of the AT₂ antagonist PD 123319 (PD), or the specific Ang-(1–7) antagonist D-Ala⁷-Ang-(1–7) (A779). PD partially blocked the noncompetitive effect of (I-)Ang-(1–7) on Ang II responses, but the difference was not significant [$E_{\max}$ $44.9 \pm 5.6\%$ for (I-)Ang-(1–7) vs. $58.5 \pm 4.0\%$ for (I-)Ang-(1–7)+PD, $p = 0.056$]. Interestingly, A779 had no effect (Table 2).

Fig. 2. Ang II dose–response curves in absence or presence of iodinated or noniodinated Ang-(1–7). Data are expressed as the percentage of contraction compared to phenylephrin (PE)-evoked response $\pm$ S.E.M. and are the means of 9–12 duplicate measurements. * $p < 0.01$ vs. control.

Table 2
Pharmacological characteristics of Ang II effects

	Antagonist(s)	n	logEC ₅₀	E _{max}
1	–	12	−8.58±0.16	58.3±4.4
2	Ang-(1–7)	9	−7.62±0.14*	43.7±8.2
3	Ang-(1–7)+A779	9	−7.77±0.12*	42.1±7.2
4	Ang-(1–7)+PD	5	−7.87±0.13*	52.0±6.5
5	I-Ang-(1–7)	12	−7.52±0.12*	45.8±8.0
6	I-Ang-(1–7)+A779	12	−7.54±0.12*	42.6±5.9
7	I-Ang-(1–7)+PD	8	−7.70±0.10*	62.6±4.7

Data are expressed as mean±S.E.M.

A779: D-Ala⁷-Ang-(1–7), PD: PD 123319.

* $p < 0.05$ vs. group 1.

3.3. ACE affinity of iodinated Ang-(1–7)

The affinity for ACE was measured in vitro. Native Ang-(1–7) inhibited HHL conversion with an IC₅₀ of 3.0×10^{-6} M. The affinity of monoiodinated Ang-(1–7) for ACE was similar (IC₅₀ 3.6×10^{-6} M), but diiodinated Ang-(1–7) had a 10-fold lower affinity for ACE (IC₅₀ 5.1×10^{-5} M, Fig. 3).

Lineweaver–Burk analysis revealed that inhibition of HHL conversion by all Ang-(1–7) peptides was purely competitive (Fig. 4).

4. Discussion

Ang-(1–7) exerts its effects through several mechanisms, including ACE inhibition (Deddis et al., 1998; Roks et al., 1999), AT₁ antagonism (Mahon, Carr, Nicol & Henderson, 1994; Roks et al., 1999; Ueda et al., 2000), AT₂ agonism (Muthalif, Benter, Uddin, Harper & Malik, 1998), and agonism on a D-Ala⁷-Ang-(1–7) sensitive receptor likely to be the Mas receptor (Santos et al., 2003). A labelled counterpart of Ang-(1–7) preferably should retain all these actions to serve as a valuable pharmacologic tool. Therefore, we compared iodine-labelled Ang-(1–7) to the native peptide. To cover several of these modes of actions we

Fig. 3. Inhibition of ACE activity by native and iodinated Ang-(1–7). ACE activity is expressed as the percentage of HL produced compared to controls±S.E.M. Data points are the mean±S.E.M. of five measurements.

Fig. 4. Lineweaver–Burk plots of HHL conversion by ACE in the presence of native (A), monoiodinated (B), and diiodinated Ang-(1–7) (C). Data points are the mean±S.E.M. of four measurements.

looked at ACE activity in rat plasma and studied Ang II antagonism in rat aorta.

Monoiodinated Ang-(1–7) had fully retained the characteristics of the original compound. I-Ang-(1–7) inhibited rat plasma ACE to the same extent as Ang-(1–7). In rat aorta, both peptides had identical effects on Ang II responses, causing both a rightward shift of the Ang II dose–response curve and a lowering of the maximum response. These findings suggest that monoiodinating Ang-(1–7) does not affect its ability to bind to ACE, or to any of the angiotensin receptors that mediate its effect in rat aorta. Conversely, the addition of a second iodine had a devastating effect. I₂-Ang-(1–7) had a 10-fold lower affinity for ACE and had fully lost its ability to antagonise Ang II. These findings indicate that for studying pharmacological properties of Ang-(1–7), only the monoiodinated species is appropriate.

The pattern of Ang II antagonism by Ang-(1–7) suggests multiple modes of action. The rightward shift of the dose–response curve is indicative of competitive antagonism on the AT₁ receptor. Ang-(1–7) and its monoiodinated counterpart cause a similar change in EC₅₀ value, suggesting equal affinity for the AT₁ receptor. The lowering of the maximal response to Ang II by Ang-(1–7) indicates a noncompetitive mode of action via receptors other than the AT₁ receptor. Vasoactive properties of Ang-(1–7) have been attributed to agonism on the AT₂ receptor (Heitsch, Brovkovich, Malinski, & Wiemer, 2001) and to a D-Ala⁷-Ang-(1–7) sensitive receptor (Neves et al., 2003). Although, in the present experiments, we did observe a nonsignificant effect of the AT₂ antagonist PD123319, our observations do not allow us to draw definite conclusions on this issue, probably due to an insufficient sample size.

In conclusion, adding a single iodine label to the small peptide Ang-(1–7) does not influence its activity on ACE and angiotensin receptors in the rat aorta, whereas a second iodine almost completely disrupts its function, quite possibly due to steric hindrance. Therefore, monoiodinated Ang-(1–7), but not diiodinated Ang-(1–7), is very suitable as a pharmacological tool.

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