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Trisubstituted 1,2,4-triazoles as ligands for the ghrelin receptor: On the significance of the orientation and substitution at position 3

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Abstract—The synthesis and structure–activity relationships concerning 3,4,5-trisubstituted 1,2,4-triazoles as ghrelin receptor ligands are described. The importance of the starting aminoacid material as well as its configuration was explored and the (D) Trp residue was found to lead to the best agonist or antagonist compounds.

We recently described a new family of ghrelin receptor ligands based on a 1,2,4-triazole moiety that lead to potent agonists and antagonists of this receptor.[1] These compounds were synthesized from (D)tryptophane residue as starting material and comprised in their final structure only one asymetric carbon atom, whose configuration is controlled during all the synthetic process. In this paper, we report on the consequence of configuration and nature of the starting aminoacid side chain on the binding affinity and biological activity towards the ghrelin receptor. For this purpose, (D)Trp was successively replaced by (L)Trp, (D)Phe, (D)(Bzl)Ser and (D)(OBzl)Asp and a structure–activity relationship study was performed on the two other substituents of the triazole moiety in positions 4 and 5. From this study, it clearly appears that the (D)Trp starting material led to the best ligands for the ghrelin receptor.

Ghrelin[2] is a recently discovered hormone which binds to the Growth Hormone Secretagogue Receptor type 1a (GHS-R1a).[3] Its main biological activities were found to be stimulation of GH release and of food intake. Indeed it appears that ghrelin is involved in the initiation of food intake as the plasma level of ghrelin significantly increases just before meals.[4] Thus, ghrelin functions also as an orexigenic hormone and it can be of interest to find agonists and antagonists of its receptor.

We have recently published the synthesis of a pseudopeptide which is a potent ghrelin receptor agonist.[5] This compound, JMV 1843, currently in phase III clinical trials in the US for the diagnosis of growth hormone deficiency in adults, is active per oral route. Its structure, Aib-(D)Trp-g(D)Trp-CHO, contains a gem-diamino moiety. In a following study, we have rigidified the structure of this tripeptide by introducing heterocycles as scaffolds bearing the three major pharmacophores contained in JMV 1843: a basic amino group (in our case included in amino-isobutyric acid) and two hydrophobic regions (aromatic rings). We first conserved the Aib residue (amino-isobutyric acid) and the D configuration for Trp residue as in JMV 1843 and we found that, among different tested scaffolds, 3,4,5-trisubstituted 1,2,4-triazoles presented interesting affinities for the GHS-1a receptor. We then started a structure–activity relationship study with the general formula presented in Figure 1. Triazole derivatives were synthesized in five steps as shown in Scheme 1, starting from a *N*-Boc

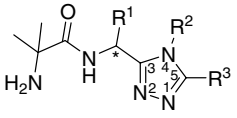

Figure 1. General formula of 3,4,5-trisubstituted 1,2,4-triazoles.

Keywords: Ghrelin; GHS-R1a; Agonist; Antagonist; 1,2,4-Triazole; Trisubstituted 1,2,4-Triazoles; Ghrelin receptor ligands.
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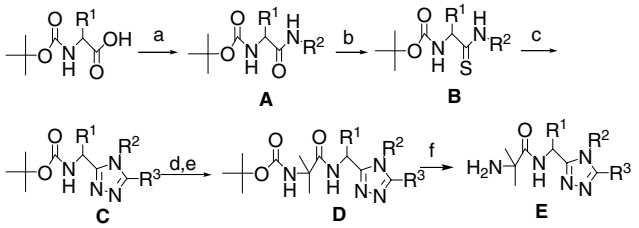


Scheme 1. Synthesis of 3,4,5-trisubstituted 1,2,4-triazoles. Reagents and conditions: (a) BOP, H_2N-R^2, NMM, DCM, (b) Lawesson's reagent, DME, 85 °C, (c) H_2N-HN-COR^3, $Hg(OAc)_2$, rt, THF, (d) HCl, AcOEt, (e) Boc-Aib-OH, BOP, DIPEA, DCM, (f) HCl, AcOEt.

protected amino acid. After coupling to an amine, the formed amide **A** was transformed into the thioamide **B** using the Lawesson's reagent.[6] The obtained thioamide **B** was then treated with 2.0 eq. of hydrazide and 1.1 eq. of mercury (II) acetate in THF, according to Hitotsuyanagi et al.[7] and Boeglin et al.,[8] to obtain the cyclized triazole derivatives. Completion of this step was monitored by reverse phase HPLC. Cyclization into triazoles **C** was achieved within two days. Compounds were purified by silica gel column chromatography. Removal of the Boc protecting group by 4 M HCl in AcOEt and coupling with Boc-Aib-OH in the presence of BOP[9] and NMM in DCM produced the N-protected desired compounds **D**. The final compounds **E** were obtained after treatment with 4 M HCl in AcOEt, purification on RP HPLC and relyophilization in a 0.1 M HCl solution. All compounds were obtained as HCl salts and checked by RP HPLC for their purity. Their structures were confirmed by MS-HPLC and NMR.

In this paper we will focus on the R^1 chain substitution and on the configuration of the carbon atom bearing the R^1 chain. We first replaced the starting (D) Trp residue by its enantiomer. Then it was substituted by (D) (Bzl)Ser, (D) Phe and (D) (OBzl)Asp residues. For R^2, 4-methoxy and 2,4-dimethoxybenzyl groups were introduced as we demonstrated that this substitution in position 4 of the triazole moiety generaly leads to ghrelin receptor antagonists.[1] R^3 was successively substituted by benzyl, phenethyl, phenylpropyl and 1*H*-indole-3-yl-ethyl groups which were found to be good substituents in a previous SAR.[1]

The synthesized compounds were tested for their ability to displace ^{125}I-His[9]-ghrelin[10] from the cloned *h*GHS-1a receptor transiently expressed in LLC PK-1 cells as previously described.[11] Binding affinities of human ghrelin and MK-0677 obtained with this model were in accordance with the literature. Their biological in vitro activity was then evaluated on $[Ca^{2+}]_i$ mobilization in GHS-R1a at a concentration of 10^{-5} M of each compound and expressed as a percentage of the maximal response induced by 10^{-7} M ghrelin (100%) (Table 1).[12] We considered as potential antagonist any compound unable to elicit an increase of intracellular calcium level superior to 10% of the maximal response elicited by 10^{-7} M ghrelin. In the case of antagonists, the IC_{50} and Kb were determined using antagonist inhibition curves in the presence of 10^{-7} M ghrelin (submaximal concentration). The IC_{50} was calculated as the molar concentration of antagonist that reduced the maximal response of ghrelin by 50% and an estimation of the Kb was made using the Cheng-Prusoff Equation.[13]

Results are gathered in Table 1. For each R^2 and R^3 substituent, both configurations of the tryptophane precursor were compared concerning their respective binding affinity to the ghrelin receptor (compounds **1**–**14**). It is obvious that the best binding affinities were obtained with compounds bearing a R configuration on the asymmetric carbon atom, with the exception of compound **2** displaying a binding IC_{50} value comparable to that of compound **1**. For all other compounds, the R configuration led to better ligands of the GHS-R1a. Compound **3** (with the R configuration) provided a binding IC_{50} value of 6 nM to compare with 140 nM for compound **4** (with the S configuration), compound **11** (R configuration) with a binding IC_{50} value of 11 nM to compare with >1000 nM for compound **12** (S configuration), compound **13** (R configuration) with a binding IC_{50} value of 12 nM to compare with >1000 nM for compound **14** (S configuration). When regarding the $[Ca^{2+}]_i$ mobilization induced by activation of GHS-R1a at a concentration of 10^{-5} M of each compound, as expected by our previous SAR study, the presence of a 2,4-dimethoxybenzyl or a 4-methoxybenzyl group in position 4 of the triazole moiety led to a poor functional activity and most of the compounds could be considered as antagonists (as we defined them), with the exception of compounds **4** and **14**, which acted as poor partial agonists, with, respectively, 31% and 17% of the maximal response induced by 10^{-7} M ghrelin (100%). Kb values were determined for the more potent antagonists and are reported in Table 1. It can be seen that compound **2** exhibited a fivefold weaker antagonist potency than compound **1**. The best Kb values were found for compounds **3** and **11** (4 and 5 nM, respectively).

We then replaced the tryptophane residue by other amino acid residues. Phenylalanine residue was chosen as it contains an aromatic side chain; serine residue, protected with a benzyl ether group on its side chain, was selected because it is often described in ligands of the ghrelin receptor.[14,15] Aspartic acid residue, protected with a benzyl ester group on its side chain, was chosen to move the aromatic group by one more carbon atom from the triazole moiety. These residues were only used in the D configuration as we demonstrated that it was the configuration yielding the best ligands. Compounds **15**–**18** were synthesized and tested for their binding affinities and their potency to elicit an increase or no activity on intracellular calcium level.

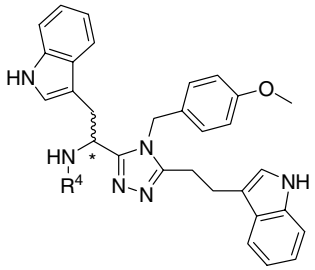
None of these new compounds exhibited high binding IC_{50} value, the better one, compound **15**, presented a 33-fold less potent binding affinity than compound **3**. Once again none of them was able to elicit $[Ca^{2+}]_i$ mobilization, due to the presence of the 4-methoxy or 2,4-dimethoxybenzyl group in R^2.

Table 1. SAR of triazoles with the general formula in Figure 1

Compound	R^1	R^2	R^3	Binding IC_{50}[a] (nM)	% of max $[Ca^{2+}]_i$ response at 10 μM[b]	IC_{50}/Kb (nM)
1	(*R*)1*H*-Indole-3-yl-methyl[c]	2,4-Dimethoxybenzyl	1*H*-Indole-3-yl-ethyl	108 ± 17	0	620 ± 100/14 ± 2
2	(*S*)1*H*-Indole-3-yl-methyl[d]	2,4-Dimethoxybenzyl	1*H*-Indole-3-yl-ethyl	83 ± 28	4	3200 ± 540/69 ± 11
3	(*R*)1*H*-Indole-3-yl-methyl	4-Methoxybenzyl	1*H*-Indole-3-yl-ethyl	6 ± 3	9	190 ± 60/4 ± 1
4	(*S*)1*H*-Indole-3-yl-methyl	4-Methoxybenzyl	1*H*-Indole-3-yl-ethyl	140 ± 3	31	ND
5	(*R*)1*H*-Indole-3-yl-methyl	2,4-Dimethoxybenzyl	Benzyl	560 ± 120	0	
6	(*S*)1*H*-Indole-3-yl-methyl	2,4-Dimethoxybenzyl	Benzyl	>1000	11	ND
7	(*R*)1*H*-Indole-3-yl-methyl	4-Methoxybenzyl	Benzyl	120 ± 30	9	870 ± 150/19 ± 3
8	(*S*)1*H*-Indole-3-yl-methyl	4-Methoxybenzyl	Benzyl	>1000	5	ND
9	(*R*)1*H*-Indole-3-yl-methyl	2,4-Dimethoxybenzyl	Phenethyl	62 ± 10	0	800 ± 360/17 ± 7
10	(*S*)1*H*-Indole-3-yl-methyl	2,4-Dimethoxybenzyl	Phenethyl	450 ± 60	4	ND
11	(*R*)1*H*-Indole-3-yl-methyl	4-Methoxybenzyl	Phenethyl	11 ± 4	2	275 ± 9/5.0 ± 0.6
12	(*S*)1*H*-Indole-3-yl-methyl	4-Methoxybenzyl	Phenethyl	>1000	0	ND
13	(*R*)1*H*-Indole-3-yl-methyl	4-Methoxybenzyl	Phenylpropyl	12 ± 3	0	600 ± 100/14 ± 4
14	(*S*)1*H*-Indole-3-yl-methyl	4-Methoxybenzyl	Phenylpropyl	>1000	17	ND
15	(*R*)Phenylmethyl[e]	4-Methoxybenzyl	1*H*-Indole-3-yl-ethyl	200 ± 85	6	ND
16	(*R*)Phenylmethyl	4-Methoxybenzyl	Benzyl	>1000	6	ND
17	(*R*)Benzyloxymethyl[f]	4-Methoxybenzyl	1*H*-Indole-3-yl-ethyl	800 ± 230	0	ND
18	(*R*)Benzyloxycarbonyl-methyl[g]	4-Methoxybenzyl	1*H*-Indole-3-yl-ethyl	450 ± 18	5	ND

[a] Inhibition of ^{125}I-His^9-ghrelin binding to membranes from *h*GHS-R1a transfected LLC cells.
[b] Maximum calcium flux activity is reported relative to ghrelin at 0.1 μM.
[c] From (D)Trp.
[d] From (L)Trp.
[e] From (D)Phe.
[f] From (D) (OBzl)Asp.
[g] From (D) (Bzl)Ser.

Table 2. SAR with various R^4 replacing Aib moieties, influence of the carbon atom configuration



Compds	C configuration	R^4	Binding IC_{50} (nM)	% of max $[Ca^{2+}]_i$ response at 10 μM	EC_{50} or Kb (nM)
19	R	Isonipecotyl	0.3 ± 0.2	93 ± 1	3 ± 1.3
20	S	Isonipecotyl	7.0 ± 0.3	100 ± 1	32 ± 3
21	R	(Pyridin-2-yl)acetyl	8.0 ± 0.1	0	12 ± 4
22	S	(Pyridin-2-yl)acetyl	820 ± 70	0	88
23	R	(L) Prolyl	9 ± 1	2 ± 1	6 ± 2
24	S	(L) Prolyl	650	0	440
25	R	(Pyridin-2-yl)carboxyl	1.9 ± 0.6	0	75 ± 1
26	S	(Pyridin-2-yl)carboxyl	175	0	1580
27	R	Tetrahydro-2H-pyran-4-carboxyl	45 ± 7	2 ± 1	24 ± 14
28	S	Tetrahydro-2H-pyran-4-carboxyl	1300	0	24

Finally, to assess these observations concerning the configuration of the carbon atom and keeping R^2 and R^3 unchanged (4-methoxybenzyl and 1*H*-indole-3-yl-ethyl groups, respectively), we attempted to replace the Aib residue (R^4 position, see Table 2) in compound **4** with moieties that were found to lead to good ghrelin receptor ligands in other SAR studies.[16,17] We then compared compounds bearing a R or S configuration on the carbon atom. Results are gathered in Table 2.

Compounds **19**–**28** were synthesized by replacing Aib by isonipecotyl, (pyridin-2-yl)acetyl, (L)-prolyl, (pyridin-2-yl)carboxyl and tetrahydro-2*H*-pyran-4-carboxyl moieties, respectively. When comparing the binding affinities between the two (*R*) and (*S*) isomers, there was no doubt that best ligands were obtained from the R configuration of the carbon atom. Compound **19** with the R configuration exhibited a subnanomolar affinity IC_{50} (0.3 nM) and its enantiomer **20** was found to have more than 20-fold less affinity for the receptor. The same loss of affinity was found for all S isomers. Concerning the biological activities, all compounds were found to be ghrelin receptor antagonists with the exception of compounds **19** and **20** which were able to elicit 100% of the response on $[Ca^{2+}]_i$ mobilization at 10 μM and could be considered as full agonists with EC_{50} values of 3 and 32 nM, respectively. Once more, in all cases, the R configuration led to more highly-active compounds than did the S enantiomer. Replacement of the nitrogen atom in the isonipecotyl moiety with an oxygen atom resulted in a switch from agonist activity to antagonist activity without regard to configuration of the carbon atom (compounds **27** and **28**). The proton which can be engaged in a hydrogen bond in para position of the piperidine ring seems to lead to agonist activity in compounds **19** and **20**. This result was already observed in previous SAR studies.[16]

In summary, we have demonstrated that the R^1 group (Fig. 1) seems to play a very important role in this new series of ghrelin receptor ligands based on 3,4,5-trisubstituted 1,2,4-triazoles. Replacement of the indole group in R^1 with different aromatic groups yielded weaker ligands. The same effect was observed when the R configuration of the asymmetric carbon atom was changed to the S configuration. So, for this part of the molecule, we have to promote the (D) Trp residue as precursor of the 1,2,4-triazole synthesis to obtain good ligands of the ghrelin receptor. With regard to the agonist/antagonist properties of the compounds, the configuration about the carbon atom does not seem to play a significant role, as assessed by the biological activity of compounds **19** and **20**, both of which were found to be agonists, while all of the other compounds are antagonists.

References and notes

1. Demange, L.; Boeglin, D.; Moulin, A.; Mousseaux, D.; Ryan, J.; Berge, G.; Gagne, D.; Heitz, A.; Perrissoud, D.; Locatelli, V.; Torsello, A.; Galleyrand, J. C.; Fehrentz, J. A.; Martinez, J. *J. Med. Chem.* **2007**, *50*, 1939.
2. Kojima, M.; Hosoda, H.; Date, Y.; Nakazato, M.; Matsuo, H.; Kangawa, K. *Nature* **1999**, *402*, 656.
3. Howard, A. D.; Feighner, S. D.; Cully, D. F.; Arena, J. P.; Liberator, P. A.; Rosenblum, C. I.; Hamelin, M. J.; Hreniuk, D. L.; Palyha, O. C.; Anderson, J.; Paress, P. S.; Diaz, C.; Chou, M.; Liu, K.; Mckee, K. K.; Pong, S. S.; Chaung, L. Y.; Elbrecht, A.; Heavens, R.; Rigby, M.; Sirinathsinghji, D. J. S.; Dean, D. C.; Melillo, D. G.; Patchett, A. A.; Nargund, R.; Griffin, P. R.; DeMartino, J. A.; Gupta, S. K.; Schaeffer, J. M.; Smith, R. G.; Van Der Ploeg, L. H. T. *Science* **1996**, *273*, 974.
4. Cummings, D. E.; Purnell, J. Q.; Frayo, R. S.; Schmidova, K.; Wisse, B. E.; Weigle, D. S. *Diabetes* **2001**, *50*, 1714.

5. Guerlavais, V.; Boeglin, D.; Mousseaux, D.; Oiry, C.; Heitz, A.; Deghenghi, R.; Locatelli, V.; Torsello, A.; Ghe, C.; Catapano, F.; Muccioli, G.; Galleyrand, J. C.; Fehrentz, J. A.; Martinez, J. *J. Med. Chem.* **2003**, *46*, 1191.
6. Clausen, K.; Thorsen, M.; Lawesson, S. O. *Tetrahedron* **1981**, *37*, 3635.
7. Hitotsuyanagi, Y.; Motegi, S.; Fukaya, H.; Takeya, K. *J. Org. Chem.* **2002**, *67*, 3266.
8. Boeglin, D.; Cantel, S.; Heitz, A.; Martinez, J.; Fehrentz, J. A. *Org. Lett.* **2003**, *5*, 4465.
9. Castro, B.; Dormoy, J. R.; Evin, G.; Selve, C. *Tetrahedron Lett.* **1975**, 1219.
10. **Receptor Binding Studies.** Isolated plasma membranes from LLC PK-1 cells (10 μg protein) were incubated in HB for 60 min at 25 °C (steady state conditions) with 60 pM ^{125}I-His^{9}-ghrelin (Amersham) in the presence or absence of competing compounds. Non-specific binding was defined using an excess (1 μM) of ghrelin and was always less than 20% of total binding. The binding reaction was stopped by addition of 4 mL of ice-cold HB followed by rapid filtration over Whatman GF/C filters presoaked with 0.5% polyethyleneimine to prevent excessive binding of radioligand to the filters. Filters were rinsed 3 times with 3 mL of ice-cold Wash Buffer (50 mM Tris (pH 7.3), 10 mM $MgCl_2$, 2.5 mM EDTA and 0.015% (w/v) Triton X-100) and the radioactivity bound to membranes was measured in a gamma counter.
11. Mousseaux, D.; Le Gallic, L.; Ryan, J.; Oiry, C.; Gagne, D.; Fehrentz, J. A.; Galleyrand, J. C.; Martinez, J. *Br. J. Pharmacol.* **2006**, *148*, 350.
12. **Intracellular Calcium Mobilization Assay.** The calcium experiments were performed using the benchtop scanning fluorometer FlexStation II machine (Pharmacologie & Screening Plateform of the Institut Fédératif de Recherche 3, Montpellier, France). CHO cells were transiently transfected with the *h*GHS-1a receptor, using electroporation, and were then plated into 96-well black-bottom plates (80,000 cells/well). Twenty-four hours later the cells were washed with 150 μL Buffer A (Hanks' balanced salt solution, 0.5% BSA, 20 mM $CaCl_2$, 2.5 mM probenecid, pH 7.4) and were then loaded with 1 μM of the fluorescent calcium indicator Fluo-4AM prepared in Buffer A, containing 0.06% pluronic acid (a mild-ionic detergent which facilitates Fluo-4AM ester loading). The cells were incubated for 1 h in the dark at 37 °C. Following the incubation, excess Fluo-4AM was removed from the cells by washing twice with 100 μL Buffer A and 50 μL of the same buffer was then added to each well. The cells were left at room temperature for 30 min to allow complete de-esterification of intracellular Fluo-4AM esters. The black-bottom plate containing the cells, as well as the plate containing the compounds to be tested, were then placed into the temperature-regulated FlexStation machine. The machine records the fluorescence output over a period of 60 s, with the compounds being automatically distributed into the wells containing the cells after 15 s. The Fluo-4AM exhibits a large fluorescence intensity increase on binding of calcium and therefore the fluorescence output is used directly as a measure of intracellular calcium mobilization. The excitation and emission wavelengths were 485 and 525 nm, respectively. The basal fluorescence intensity of dye-loaded cells was 800–1200 arbitrary units and the fluorescence peak upon maximal response was 5000–7000 U. To assess the ability of each of the compounds to induce calcium mobilization, they were tested at a concentration of 10 μM in triplicate, in at least 2 independent experiments. In each case, the change in fluorescence upon addition of the compound was compared with the basal fluorescence ouput measured with the control (addition of buffer A only). The maximum fluorescent output was equivalent to that achieved when the cells were stimulated with 0.1 μM ghrelin. For compounds behaving as agonists and displaying a high affinity binding for *h*GHS-R1a in radiolabelled binding experiments, EC_{50} (the molar concentration of the agonist producing 50% of the maximal possible effect of that agonist) was determined using a dose-response curve. In the case of high affinity antagonists, IC_{50} and Kb were determined using antagonist inhibition curves in the presence of 0.1 μM Ghrelin (submaximal concentration). The IC_{50} was calculated as the molar concentration of antagonist that reduced the maximal response of ghrelin by 50% and an estimation of the Kb was made using the Cheng-Prusoff Equation.[13]
13. Cheng, Y.-C.; Prusoff, W. H. *Biochem. Pharmacol.* **1973**, *22*, 3099.
14. Patchett, A. A.; Nargund, R. P.; Tata, J. R.; Chen, M. H.; Barakat, K. J.; Johnston, D. B.; Cheng, K.; Chan, W. W.; Butler, B.; Hickey, G. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 7001.
15. Carpino, P. A.; Lefker, B. A.; Toler, S. M.; Pan, L. C.; Hadcock, J. R.; Cook, E. R.; DiBrino, J. N.; Campeta, A. M.; DeNinno, S. L.; Chidsey-Frink, K. L.; Hada, W. A.; Inthavongsay, J.; Mangano, F. M.; Mullins, M. A.; Nickerson, D. F.; Ng, O.; Pirie, C. M.; Ragan, J. A.; Rose, C. R.; Tess, D. A.; Wright, A. S.; Yu, L.; Zawistoski, M. P.; DaSilva-Jardine, P. A.; Wilson, T. C.; Thompson, D. D. *Bioorg. Med. Chem.* **2003**, *11*, 581.
16. Moulin, A.; Demange, L.; Berge, G.; Gagne, D.; Ryan, J.; Mousseaux, D.; Heitz, A.; Perrissoud, D.; Locatelli, V.; Torsello, A.; Galleyrand, J. C.; Fehrentz, J.A.; Martinez, J. *J. Med. Chem*. 2007, DOI. 10.1021/jm0704550. unpublished results.
17. Unpublished results.