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Original article

Ala scan analogues of HOE 140. Synthesis and biological activities

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Abstract – The role of the amino acids contained in the sequence of HOE 140 (H-DArg[1]-Arg[2]-Pro[3]-Hyp[4]-Gly[5]-Thi[6]-Ser[7]-DTic[8]-Oic[9]-Arg[10]-OH), a potent and selective bradykinin B_2 receptor peptide antagonist, has been investigated by the replacement of each original residue (one by one) with Ala. The resulting set of decapeptides has been tested for the B_2 antagonist activity as well as for competition with the binding of [^{3}H]BK to plasma membranes of the human umbilical vein (hUV). Positive correlations have been established between data obtained with the bioassay and with the binding in the hUV (same species, same tissue) and also between the two bioassays, the guinea-pig ileum (GPI) and the hUV (different species, different tissue). The structure-activity study has shown that the replacement of any of the residues that constitute HOE 140 with Ala is accompanied by a decrease of potency of at least 1 log unit. The analogues can be divided into three groups, with Ala[1] and Ala[7] showing affinities lower than HOE 140 by a factor of 10, Ala[4] and Ala[10] by a factor of 100 and Ala[2], Ala[5], Ala[6], Ala[8] and Ala[9] by a factor higher than 100 (100–1000). To verify the effect of chirality, the DAla[5] and DSer[7] analogues were synthesized and it was found that the substitution with a D-residue in position 5 is not tolerated while that in position 7 is favourable. The DSer[7] derivative is the most potent analogue found in this study: it shows potency as high as that of HOE 140 in the bioassays. © 2000 Éditions scientifiques et médicales Elsevier SAS

bradykinin antagonists / alanine scan / structure-activity studies / bioassays correlation

1. Introduction

Bradykinin (BK) and kallidin (KD) are endogenous peptides which act as natural agonists of the kinin B_2 receptor [1]. Kinin B_2 receptors of human, guinea-pig and other species have been cloned [2, 3]. Several antagonists for this receptor, either peptide [4] or non-peptide [5] in nature, have been developed. HOE 140 (Icatibant, H-DArg[1]-Arg[2]-Pro[3]-Hyp[4]-Gly[5]-Thi[6]-Ser[7]-DTic[8]-Oic[9]-Arg[10]-OH) is one of the most potent antagonists in vitro and in vivo [6], and has also been tested in phase II clinical trials for asthma [7] and rhinitis [8].

The main structural feature of HOE 140 is represented by the presence of two unnatural cyclic aminoacids, DTic and Oic, which determine conformational restraints [9] and enhance the metabolic stability as well as the affinity of the compound for the kinin B_2 receptor [6]. Several analogues of HOE 140 with different affinity and species selectivity for the kinin B_2 receptors have been described [4], including some pseudopeptides in which part of the se-

Abbreviations: Abbreviations used follow the nomenclature recommended by the IUPAC-IUB Joint Commission on Biochemical Nomenclature (Eur. J. Biochem. 9 (1984) 138). Other abbreviations: DCM: dichloromethane; Dhbt: 3-hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine; DIEA: diisopropylethylamine; DMF: *N*,*N*-dimethylformamide; FAB-MS: fast atom bombardment mass spectrometry; Fmoc: (9-fluorenylmethyl)oxycarbonyl; HOBt: 1-hydroxybenzotriazole; HPLC: high performance liquid chromatography; IsOH: isopropyl alcohol; Oic: Pfp: pentafluorophenyl; PAC: *p*-hydroxymethylphenoxyacetyl; PEG-PS: polyethylene glycol-polystyrene; PIP: piperidine; Pmc: 2,2,5,7,8-pentamethylchroman-6-sulphonyl; PyBROP: Bromo-tris-pyrrolidino-phosphonium hexafluorophosphate; TFA: trifluoroacetic acid; DTic: D-1,2,3,4,-tetrahydroisoquinoline-3-carboxylic acid; TBTU: 2-(1*H*-benzotriazole-1-yl)-1,1,3,3,-tetramethyluronium tetrafluoroborate tBu: t-Butyl; Thi: β-(2-thienyl)-L-alanine..

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quence has been replaced by non-aminoacidic portions [10].

In this study, we investigate the role and the relative importance of the side chain of each aminoacid residue in the sequence of HOE 140 by Ala scan approach, i.e. through the systematic replacement of the original residues with an Ala in each position of the decapeptide. This approach is classically used in medicinal chemistry of peptides to identify the residues that could be further replaced to enhance potency or metabolic stability. Complete Ala scan series have been reported for the BK sequence [11], but not for HOE 140, except the replacement of residues 2 through 6 with Gly [12].

The 10 peptides of the Ala scan as well as two analogues, containing either DAla in position 5 or DSer in position 7, were tested on the guinea-pig isolated ileum (GPI), a preparation extensively used for the screening of B_2 receptor antagonists [13], and on the native human vascular B_2 receptor of the human umbilical vein (hUV) both with the bioassay [14] and with the binding [15]. A comparison of the results obtained in human and guinea-pig bioassays may be useful to determine the predictive value of data obtained in guinea-pig tissues with regard to the affinity for the human B_2 receptor.

2. Chemistry

The synthesis of HOE 140 was performed on a solid phase continuous flow Milligen 9050 peptide synthesizer, starting from Fmoc-Arg(Pmc)-PAC-PEG-PS resin and following the recommended procedures (4.5 excess of protected amino acid and acylating agents, 30 min recycles). After the cleavage from the resin, the desired sequence was present in low amount (ca 5%). To avoid the formation of deletion sequences, the coupling of Pro, Ser, Thi and DTic was performed twice, using for the recouple cycle the same excess of reagents (4.5 fold) and increasing the recycle time from 30 to 60 min. For Pro coupling, the second acylation was performed using the activated OPfp ester. With this procedure, the desired compound was obtained in high yield (50%), even if the deletion of Ser was not avoided completely, since the desSer analogue was present in a 10% amount. The syntheses were therefore carried out in these conditions for all the sequences, and products in yields from 28% to 60% (*table I*) were obtained. The amount of desSer deletion sequences varied from less than 2% (compounds **5**, **6**, **9**, **10** and **12**) to 60% of the area of the main peak in the case of compound **11**. In the case of the Ala^7 analogue (com-

Table I. Abbreviated structures and analytical data of the peptides described. HOE 140: H-$DArg^1$-Arg^2-Pro^3-Hyp^4-Gly^5-Thi^6-Ser^7-$DTic^8$-Oic^9-Arg^{10}-OH.

Compound	Substitution	t_R[a] (min) (System A[c])	Purity[b] (%) (System A[c])	t_R[a] (min) (System B[d])	Purity[b] (%) (System B[d])	$[M+H]^+$[e]	Yield[f] (%)	$desAA^7$ (%)[g]
HOE 140		12.8	95	6.9	97.5	1305.0	50	10
1	Ala^1	11.95	98	11.6	>99	1219.7	58	14
2	Ala^2	11.7	94	11.4	>99	1219.7	58	7
3	Ala^3	12.4	98	11.2	>99	1278.5	51	10
4	Ala^4	12.8	95	11.3	>99	1263.0	51	23
5	Ala^5	12.8	95	11.4	>99	1319.0	59	<2
6	$DAla^5$	13.3	98	11.7	97	1319.0	36	<2
7	Ala^6	9.9	94	9.8	>99	1223.0	55	7
8	Ala^7	13.5	97	11.4	>99	1289.0	53	8
9	$DSer^7$	12.6	95	11.4	96	1304.5	33	<2
10	Ala^8	7.1	95	9.1	>99	1217.0	36	<2
11	Ala^9	8.3	>99	9.4	>99	1225.0	28	60
12	Ala^{10}	12.4	97	12.4	>99	1220.0	57	<2

[a] t_R, retention time.
[b] Purity: expressed as % of the total HPLC area.
[c] System A: for HPLC eluition systems see Section 6.
[d] System B: for HPLC eluition systems see Section 6.
[e] $[M+H]^+$: molecular ion (by FAB-MS).
[f] Yield: expressed as % of the theoretical yield calculated on the initial loading of the resin.
[g] $desAA^7$: amount of deletion peptide lacking the residue in position 7, calculated as % of the total HPLC area.

Table II. Abbreviated structures, biological activities and binding affinities for kinin B_2 receptor of HOE 140 and the peptides under evaluation in guinea-pig ileum (GPI)[18] (expressed as pK_B values ± SEM), human umbilical vein (hUV)[14,15] bioassay (expressed as pK_i values ± SEM) and hUV binding (expressed as pK_B values ± SEM). HOE 140: H-DArg[1]-Arg[2]-Pro[3]-Hyp[4]-Gly[5]-Thi[6]-Ser[7]-DTic[8]-Oic[9]-Arg[10]-OH.

Compound	Substitution	Guinea-pig B_2 receptor GPI ($pK_B \pm$ SEM)	Human B_2 receptor HUV bioassay ($pK_B \pm$ SEM)	HUV binding ($pK_i \pm$ SEM)
HOE 140		9.55 ± 0.09[a]	8.4 ± 0.13	9.8 ± 0.45
1	Ala[1]	7.55 ± 0.06[b]	7.6 ± 0.14[c]	8.3 ± 0.06
2	Ala[2]	6.9 ± 0.16[b]	6.4 ± 0.16	6.5 ± 0.08
3	Ala[3]	7.2 ± 0.18[b]	6.8 ± 0.21	8.0 ± 0.07
4	Ala[4]	7.4 ± 0.10[b]	6.7 ± 0.08	7.4 ± 0.12
5	Ala[5]	<6	<5.0	6.3 ± 0.07
6	DAla[5]	<6	5.1 ± 0.19	$6.2 \pm$
7	Ala[6]	6.6 ± 0.10[b]	6.2 ± 0.22	6.2 ± 0.06
8	Ala[7]	8.6 ± 0.10[b]	7.7 ± 0.11	8.0 ± 0.07
9	DSer[7]	9.2 ± 0.16[a]	8.5 ± 0.2	8.1 ± 0.1
10	Ala[8]	IN	<5.0	6.0 ± 0.08
11	Ala[9]	IN	5.1 ± 0.29	6.1 ± 0.08
12	Ala[10]	8.1 ± 0.17[a]	7.0 ± 0.21	7.1 ± 0.09

[a] Not competitive.
[b] $P < 0.01$ vs. HOE 140 (one-way ANOVA followed by Fisher's LSD test (least significant difference).
[c] Partial agonist.

pound **8**), an 8% of desAla[7] sequence was obtained (*table I*), proving that the formation of the byproduct is not due exclusively to the presence of the *tert*-butyl ether of Ser side chain.

The problematic coupling of the Ser residue may be attributed to the steric and conformational hindrance provided by the two cyclic aminoacids DTic and Oic. No attempts were made to synthesize the Ser-DTic dipeptide and to couple it directly.

3. Pharmacology

3.1. Functional data (GPI)

In the longitudinal smooth muscle of guinea-pig ileum, BK (1 nM/μM) produced a concentration-dependent contraction (pEC_{50} 7.7 ± 0.1, $n = 15$).

The kinin B_2 receptor antagonist HOE 140 (3–300 nM) rightward shifted the concentration–response curve to BK, and depressed the maximal response to the agonist. Thus, the highest concentration tested (300 nM) of HOE 140 reduced the maximal effect of BK to about 20% of the control maximal response. The estimated pK_B value of HOE 140 was 9.5 ± 0.1 ($n = 8$).

The antagonist activity of the Ala-substituted HOE 140 analogues (tested in the range concentration of 1 nM–10 μM) is reported in *table II*. Compounds **10** and **11** were ineffective against BK up to 10 μM. Both **5** and **6** produced some antagonist effect, but only at the highest concentration used (10 μM) (pK_B estimate < 6 for either compound). Both **2** and **7** competitively antagonized BK-induced responses with a comparable low potency (pK_B in the μmolar range: see *table II*). Compound **1** ($pK_B = 7.55 \pm 0.06$) exerted a competitive antagonism similarly to **4** ($pK_B = 7.4 \pm 0.10$), and was significantly more active than **3** ($pK_B = 7.2 \pm 0.18$, $P < 0.05$). Compounds **8** and **12** showed a pK_B of 8.6 ± 0.1 and 8.1 ± 0.17, respectively ($P < 0.01$ versus HOE 140 and all other tested compounds), and behaved as competitive antagonists. Compound **9** was the only one, besides HOE 140, to behave as an insurmountable antagonist (see Section 6.2.1), with a pK_B value of 9.2 ± 0.16. This value was comparable to that obtained with HOE 140, but statistically different ($P < 0.01$) from all the other tested compounds.

3.2. Functional data (hUV)

In the hUV without endothelium, BK induces a concentration-dependent contraction ($pEC_{50} = 8.47 \pm 0.11$, $E_{max} = 4.29 \pm 0.74$ g); desensitization occurs after the application of high concentrations (100–300 nM) of BK, but is reversible in 90 min after washing out the agonist ($pEC_{50} = 8.30 \pm 0.15$, $E_{max} = 4.69 \pm 0.97$ g). This allows one to evaluate the potential inhibitory effects of

antagonists in the same strip. 0.1 μM HOE 140 was able to displace to the right the concentration–response curve of BK without depressing the maximal response. Apparent affinity, calculated by Kenakin's equation [16] yielded a pK_B value of 8.4 in agreement with Gobeil et al. [14]. In these experimental conditions, the peptides under study (*table I*) were tested at different concentrations: compound **9** at 0.1 μM, **1**, **8**, and **12** at 1 μM; **2**, **3**, **4**, **5**, **7**, **10** and **11** at 10 μM. All the compounds had no contractile effects per se except **1** and **7**, which (at the concentration tested, see above) produced a transient contractile effect of about 1.22 ± 0.76 g and 1.88 ± 0.56 g, respectively. Compounds **5** and **10** did not exert any antagonistic effect, while all the other compounds were able to displace to the right the concentration–response curve of BK without depressing the maximal response, suggesting that, in this preparation, these compounds act as competitive bradykinin B_2 receptor antagonists in the same manner as HOE 140. The apparent affinity values are reported in *table II*. All compounds with the single exception of **9** were less potent than HOE 140, the template of the Ala scan.

3.3. Binding receptor data (hUV)

Saturation experiments of [^{3}H]BK binding to B_2 receptor in hUV membranes and the respective Scatchard plot were performed previously by Gessi et al. [15]. They showed the presence of a single class of binding sites with a K_D value of 0.51 ± 0.025 nM and a B_{max} value of 24 ± 1.14 fmol/mg of protein. These results have been confirmed in the present study. Results of displacement experiments, performed with the compounds under evaluation, are analysed in *figure 1*. The various peptides differ markedly in their affinities, but the displacements of the concentration–response curves are parallel and extend over a range of 3 log units. On the basis of their affinities, the compounds can be separated into three groups: **1**, **3**, **8**, and **9** showed high affinity ($K_i \approx 10$ nM), although about 10-fold less potent than HOE 140; **4** and **12** showed moderate affinity ($K_i \approx 100$ nM); **2**, **5**, **6**, **7**, **10** and **11** showed very low affinity (from 100 to 1000 nM). The pK_i values are presented in *table II*. All compounds that were possible to evaluate showed Hill coefficients not significantly different from unity (data not shown), suggesting competitive antagonism.

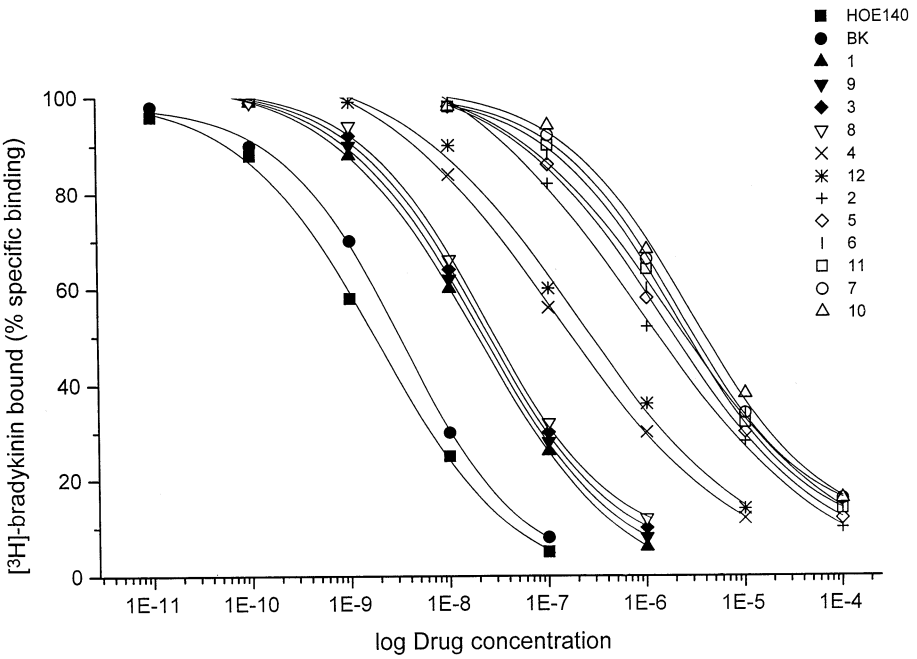

Figure 1. Inhibition curves of specific [^{3}H]BK (0.5 nM) binding to human umbilical vein membranes by HOE 140 and the 12 compounds under evaluation. Mean values of four experiments done in triplicate are shown. Non-specific binding was determined in the presence of 10 μM of BK.

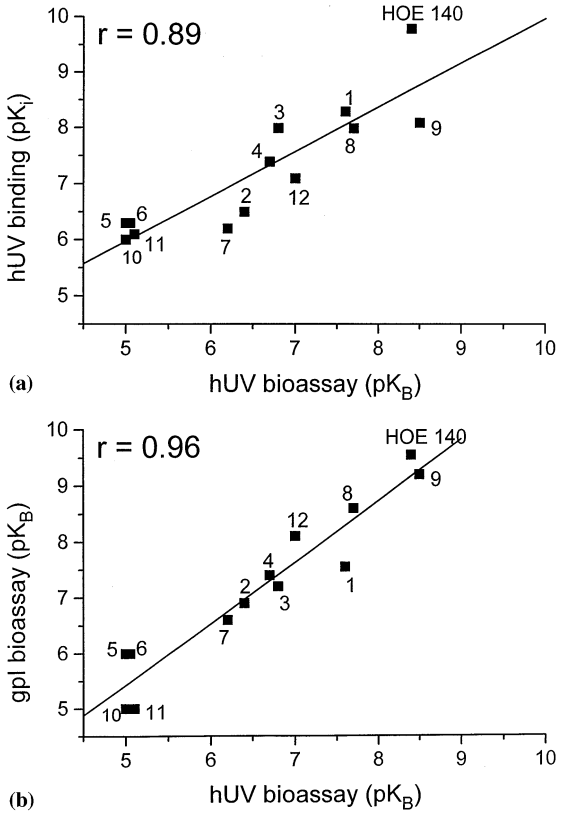

Figure 2. a) Correlation between biological activities (pK_B values) and binding affinities (pK_i values) on human vascular native bradykinin B_2 receptor carried out in the human umbilical vein; b) Correlation between biological activities (pK_B values) carried out on the vascular native bradykinin B_2 receptor of the human umbilical vein and in the guinea-pig ileum.

Apparent affinities similar to those observed on the hUV were measured in the guinea-pig ileum. Indeed all Ala scan analogues of HOE 140 were less potent than the precursor by a factor of 10 or more: the compounds could be divided in three groups and the components of each group were the same as in the hUV, namely **1**, **8**, **12** p$K_B > 7.5$ and all others pK_B lower than 7.5. [DSer^7]HOE 140 (compound **9**) was very active in this preparation, in fact as active as HOE 140.

Data obtained in biological assays performed on umbilical vein strips (expressed in terms of pK_B) have been plotted against the values of binding affinities (expressed in terms of pK_i) measured by inhibition of [^{3}H]BK binding to hUV membranes (*figure 2a*). A good correlation is observed between biological and binding data for all the compounds except for HOE 140, which in binding studies, is 10-fold more potent than in functional assays. Data obtained in the hUV and the GPI have also been plotted against each other and the fairly good correlation found is shown in *figure 2b*.

4. Results and discussion

Ala scan of short peptide agonists such as angiotensin II [17] and BK [11] has been used in the 1970s to identify the functional groups involved in receptor activation. For some peptidergic receptors, however, the switch from agonist to antagonist required major modifications in the structure, such as that conferred by the insertion of a D-residue in the peptide sequence (e.g. [DPhe^7]BK) [18]. Substantial improvements were obtained with HOE 140, which shows high affinity, long duration of action in vivo and almost absolute selectivity for the B_2 receptor. The present investigation was directed to identify the residues responsible for favourable features of this compound. The data illustrated in *figure 1* allow one to separate the Ala scan analogues into three groups: (1) compounds retaining relatively high affinity (DSer^7, Ala^1 and Ala^7); (2) compounds with intermediate affinity (Ala^4, Ala^{10}); and (3) compounds that are very weak or inactive (Ala^2, Ala^5, Ala^6, Ala^8, Ala^9). This oversimplified interpretation gives weight primarily to the side chain for the peptide-receptor interaction, although the replacement of a residue with Ala may lead to rearrangement of the whole peptide structure.

In the present study, good correlation in the rank order of potency of antagonists has been found (*figure 2b*) in the functional assays in human and guinea-pig B_2 receptors.

Although the rank order of potency of HOE 140 and its analogues is maintained when binding and functional affinity values are compared on the human B_2 receptor (*table II*, *figure 2a*), some discrepancies arise in terms of higher binding affinity for most of the compounds. This 'binding paradox' is not new in BK antagonists field [1, 19–21], and has been attributed to diffusion barriers that may be present in the complex system of the bioassay. In addition to HOE 140, relevant differences between the GPI and the hUV functional bioassays appear only for compounds **12** and **8**. It thus appears that the Arg^{10} and Ser^7 side chains may play a different role for the interaction of the antagonists with the human receptor, compared with that of guinea-pig.

The substitution of the DArg residue in position 1 with Ala determined a decrease in activity in all the tests, especially in the GPI ($pK_B = 9.55$ for HOE 140 vs. 7.55 of compound **1**). In position 2, the replacement with Ala led to a greater decrease in antagonist potency as compared to that observed in position 1 (at least 100 fold), as already reported [4]. This result suggests a more general effect of the guanidine group in position 2 on the entire ligand structure rather than a local charge interaction with the receptor [22]. The cluster of negatively charged residues that is present in the fourth extracellular domain of the B_2 receptor was supposed to form an ionic interaction with the N-terminal guanidino moieties of peptide ligands [22]. However, the binding of BK but not of HOE 140 at the rat B_2 receptor was affected by the removal of those negatively charged residues, as proved by mutagenesis studies [23, 24].

The substitution of Arg[10] produces at least a 10-fold decrease in the antagonist affinity. The drop is more evident in the hUV binding test, while in the GPI bioassay a relatively high affinity (compound **12**, $pK_B = 8.1$) was preserved. The role of Arg10 side chain is more controversial than that of Arg residues 1 and 2, since the putative bioactive conformation of HOE 140 [4] implies the orientation of the C-terminal part of the molecule in the hydrophobic cluster formed by the transmembrane domains III, IV, V and VI, where no negatively charged residues are present [23]. Recently, Fathy et al. [25] hypothesized that the positive charge of residue 9 in the BK sequence served as counterion for the C-terminal carboxylic function of the peptide.

The substitution of residues Pro[3] and Hyp[4] appears highly significant in terms of affinity (which is reduced by near 100-fold in all tests). The main effect is expected to be conformational, because of the well known turn-inducing properties of prolines [26]. In modelling of HOE 140, residues 3–6 were hypothesized to be involved in a β-turn motif, with Hyp and Gly placed at the $i+1$ and $i+2$ positions [27]. Besides this, a relevant lipophilic area is removed by substituting Hyp[4] with Ala. It is unclear, however, which of these factors is prevalent. It has been previously found that substitutions of residues 3 and 4 with Gly produced only a 3–5-fold decrease in the binding affinity to GPI membranes [12].

An interesting effect was observed by substituting Gly[5] with Ala and DAla. Other cases have been reported [28] in which the substitution of a Gly with Ala produced a strong reduction in activity, while the DAla derivative resulted more potent than the parent compound. If the conformers preferred for binding are not allowed with Ala analogue, containing an additional constraint, they will be spanned, with very few exceptions, by insertion of the complementary residue DAla. Since the number of unproductive conformers will be reduced, an increase in binding affinity is expected to occur for at least one of the two analogues. This did not happen in the case of HOE-140, nor was is true with BK itself [28]. We therefore conclude that the replacement of Gly with Ala determined a steric hindrance which affects the interaction of the ligand with the receptor in correspondence of Gly[5].

The removal of the aromatic side chain of Thi[6] appears to be relevant, since it reduced the affinity by more than three orders of magnitude. The side chain of Ser[7], whose substitution with Ala or chirality inversion causes one order of magnitude reduction in affinity, appears to produce an interaction consistent, in energy terms, with a hydrogen bond. In fact the affinity of this analogue decreased by 10–100-fold in the various assays. However, compound **8** is still a good ligand, with pK_B values of 8.6 and 7.7 in the GPI and hUV, respectively. To further investigate the role of the Ser residue, we also synthesized the DSer[7] analogue, compound **9**. By comparing the affinity of HOE 140 vs. compounds **8** and **9**, we can conclude that the Ser[7] side chain is relatively ininfluent for obtaining an antagonist with good affinity for the B_2 receptor; therefore, we designed a derivative of HOE 140, where the Ser residue was replaced with a 2,4-diaminobutyric acid (Dab) and the side chain of this amino acid formed a lactam bridge with the C-terminal carboxylic function of the parent peptide (see below and in reference 22) [20].

Regarding positions 8 and 9, it is known that the two cyclic residues DTic and Oic, which are present in the HOE 140 structure, induce a type βII′-turn in the C-terminal part of the sequence [27]. Further support to the notion that a βII-turn in the C-terminal part of HOE 140 is crucial comes from the discovery of MEN 11270 [H-DArg-Arg-Pro-Hyp-Gly-Thi-cyclo(Dab-DTic-Oic-Arg)], where the C-terminal tetrapeptide segment Ser-DTic-Oic-Arg has been constrained through the formation of a 14-members lactam bridge: this compound maintains the same affinity of HOE 140 [20]. In peptides **10** and **11** the cyclic aminoacids DTic and Oic were substituted with Ala:

on the basis of previous literature [9, 27], we speculate that a perturbation in the secondary structure may be responsible for the dramatic decrease of antagonist activity.

5. Conclusions

In conclusion, the Ala scan series of analogues of HOE 140 has shown that this compound has a critical structure which hardly tolerates substitutions in its primary sequence. The present results allow for a fairly accurate evaluation of the relative contribution of the different residues to the binding affinity of the decapeptide antagonists at the human and guinea-pig B_2 receptors. Much remains to be done to evaluate how the various residues may contribute to the metabolic resistance of HOE 140 and to the high selectivity of this compound for the kinin B_2 receptor.

6. Experimental protocols

6.1. Materials

Fmoc protected aminoacids were purchased from Bachem (Bubendorf, Switzerland) and Novabiochem (Laufelfingen, Switzerland); PyBOP and PyBROP were from Novabiochem; Fmoc-Arg(Pmc)-PAC-PEG-PS resin was from Milligen (Burlington, MA, USA). DCM, DMF, iPrOH (Merck, Milan, Italy), TFA and DIEA (Janssen, Beerse, Belgium) and Pip (Carlo Erba, Milan, Italy) were all of analytical grade and were used without further purification.

Captopril ([2*S*]-1-[3mercapto-2-methyl-propionyl]-L-proline) was purchased from Squibb (Montreal, Canada), and was dissolved in isotonic saline. [^{3}H]BK (specific activity 76.0 Ci/mmol) was from Amersham (Milan, Italy) and Aquassure was from NEN Research Products (Boston, MA, USA). All other reagents were from Sigma Chemical Co. (St Louis, MO, USA) or E. Merck (Darmstadt, Germany). Concentrated solutions (1 mM) of peptides and other agents were made in bidistilled water and kept at -20 °C. Krebs solution had the following composition (in mM): NaCl, 118.1; KCl, 4.7; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 25 and glucose, 10.

6.2. Chemistry

6.2.1. Methods

Analytical HPLC characterization of the peptides was performed on a Beckman System Gold apparatus, using a Vydac C18 218TP54 (4.6 × 250 mm) column for system A and a Waters Delta-Pack C18 (100 Å, 150 × 3.9 mm) column for system B. The solvent systems used were:

- system A: 10 mM $(NH_4)_2HPO_4$ + 0.1 M $NaClO_4 \cdot H_2O$ in distilled water (solvent A); 0.1 M $NaClO_4 \cdot H_2O$ in distilled water/acetonitrile 2/3 (v/v, solvent B); linear gradient from 31%B to 91%B in 20 min, flow rate 1 mL/min; detection at 210 nm.
- system B: 0.1% TFA in distilled water (solvent A); 0.1% TFA in acetonitrile (solvent B); linear gradient from 20%B to 80%B in 20 min, flow rate 1 mL/min; detection at 210 nm.

Purifications on a semi-preparative scale were performed on a Waters Delta Prep 3000 system, using a Waters Delta-Pack C18 (100 Å, 300 × 19 mm) column, with the proper linear gradients of acetonitrile (0.1% TFA) in distilled water (0.1% TFA); flow rate 20 mL/min; detection at 240 nm. The crude product was dissolved in 2 mL of mobile phase, then filtered through a PTFE filter (0.45 mm) and directly injected. The appropriate fractions were collected, the resultant solution evaporated under vacuum and lyophilized. Purified compounds showed an HPLC purity of at least 94%.

FAB-MS spectra were recorded on a VG Quattro mass spectrometer equipped with a FAB ion source. Ionization was achieved using a caesium gun (8 keV, 2.3 A) and thioglycerol was used as the matrix compound.

Analytical data of peptides are reported in *table I*.

6.2.2. Synthesis of peptides

The peptides were synthesized on a Milligen 9050 apparatus following the standard protocols supplied by the manufacturer for Fmoc strategy [29]. The starting material was Fmoc-Arg(Pmc)-PAC-PEG-PS resin (0.5 g, 0.170 meq/g) for all the compounds, **12** excepted, which was synthesized starting from Fmoc-Ala-PAC-PEG-PS resin (0.5 g, 0.18 meq/g). The chosen protecting groups were Pmc for Arg, tBu for Ser and Hyp. In a typical coupling step, the aminoacid and TBTU were weighted in a 4.5-fold excess and placed in the appropriate aminoacid module vial. HOBt and DIEA solutions were drawn directly from the synthesis module bottles. The recycle time was prolonged at 60 min. Similarly, the double coupling steps were performed using a 4.5-fold

excess of protected aminoacids, TBTU, HOBt and DIEA. The recycle time was prolonged at 60 min. For OPfp and Dhbt esters, no TBTU was added to the aminoacid.

6.2.3. Cleavage of peptides from the resin

The cleavage of the peptides from the resin was performed with reagent B (TFA 88%, triethylsilane 2%, phenol 5%, water 5%), 10 mL for 0.5 g of starting resin. In a typical experiment, the resin was suspended in the cleavage mixture, cooled at 0 °C and stirred under nitrogen for 7 h. The solid residue was filtered on a sintered glass funnel and washed with TFA (2×2 mL). The resultant solution was diluted with a cooled mixture of diethyl ether/petroleum ether 1/2 (v/v) and the suspension stored at -70 °C for 12 h. The solid was filtered and washed with petroleum ether, then dissolved in 50% acetic acid in water, diluted with water and lyophilized.

6.3. Biology

6.3.1. Data analysis and terminology

All the data are expressed as mean ± standard error of the mean of n experiments. pK_B values were calculated according to the classical equation [16] $pK_B = \log10[(\text{concentration ratio} - 1)/B]$. A weighed non-linear least-squares curve fitting programme LIGAND [30] was used for computer analysis of inhibition experiments. The pharmacological terminology adopted in this paper is in line with the recent IUPHAR recommendations [31, 32].

6.3.2. Guinea-pig ileum longitudinal smooth muscle: functional studies

Longitudinal muscle myenteric plexus preparations of guinea-pig ileum were prepared and set up as described by Meini et al. [33]. The preparations were put in organ baths filled with oxygenated (95% O_2, 5% CO_2) normal Krebs' solution containing indomethacin, guanethidine (3 μM each), clorpheniramine and atropine (1 μM each).

Cumulative concentration–response curves to BK were constructed in the presence of the peptidase inhibitors thiorphan, bestatin and captopril (1 μM each). The compounds under study were administered 15 min before repetition of the agonist curve, at the stated concentrations, and were tested for their ability to block the contractile response to the agonists. Agonist activity was expressed in terms of EC_{50}, or agonist concentration producing the 50% of its maximal response. Antagonists producing parallel rightward shifts of concentration–response curves to the agonist, without depression of the maximal response, were considered competitive, and their affinity (expressed in terms of pK_B) was estimated as the mean of the individual values obtained with the equation: $pK_B = \log\ [\text{dose ratio} - 1] - \log\ [\text{antagonist concentration}]$ [16]. The affinity of the antagonists causing non-parallel rightward shifts of the agonist responses, with reduction of agonist E_{max}, were considered insurmountable, and their affinity was estimated by the equation: $K_B = [B]/\text{slope} - 1$ [16].

All the values reported are mean ± SEM of at least six single experiments. Statistical analysis was performed using a one-way ANOVA, followed by Fischer's LSD (least significant difference) t-test.

6.3.3. Human umbilical vein preparations

6.3.3.1. General

Experiments on human tissues were performed under conditions and with experimental protocols reviewed and approved by the Ethics Committee of the Medical School of the University of Ferrara, Italy; written informed consent was obtained from each parturient woman.

Segments of human umbilical vessels were prepared from umbilical cords from 22–40-year-old women (bioassay studies) after spontaneous delivery at term. The cords were placed in cold (4 °C) Krebs solution. The lapse of time between the delivery and the experiment was on average 4 h (range 1–10 h). In the laboratory, the middle segment of the cord (7–8 cm long) was placed in Krebs solution at room temperature and, within 30 min, the vein was dissected free of surrounding tissues.

6.3.3.2. Organ bath studies

The vein was cut into spiral strips (2 cm long, 3 mm wide), the endothelium was rubbed off using a cotton swab. The tissues were suspended in 10 mL organ baths containing warm (37 °C), oxygenated (95% O_2, 5% CO_2) Krebs solution and stretched to a resting tension of 2 g. Changes of tension were measured isometrically with Grass FT03 force transducers and recorded on Linseis (model L2005) multichannel chart recorder. In all experiments, the kininase II inhibitor captopril was added to the Krebs solution at 1 μM concentration. Before testing any agent, the tissues were allowed to equilibrate for 180 min and the resting tension was readjusted every 20 min.

6.3.3.3. Experimental protocols

The experiments began after a 180-min period of equilibration by the application of KCl 100 mM to test the responsiveness of each preparation. For B_2 receptor studies, according to Gobeil et al. [14], BK was used as the standard B_2 receptor agonist and Lys[Leu^8]desArg^{9}BK (1 μM) was added to the Krebs solution to eliminate the interference of B_1 receptors. The antagonist activity of each compound was evaluated by measuring cumulative concentration–response curves to BK in the absence and presence of one concentration of the compound, waiting for 90 min between the first and the second concentration–response curve to BK. In all experiments, the standard B_2 receptor antagonist, HOE 140, was tested at the concentration of 0.1 μM. All the compounds investigated were applied 15 min before measuring the concentration response curve of BK in their presence.

6.3.3.4. Binding studies

Membranes were prepared according to Gessi et al. [15]. The specific binding of [^{3}H]BK to plasma membranes from hUVs was assayed in a total volume of 250 μL containing the assay buffer (see composition above) according to Gessi et al. [15]. In inhibition experiments, carried out to determine the IC_{50} values of several compounds, 0.5 nM [^{3}H]BK was incubated with 100 μL of umbilical vein membranes and 8–10 different concentrations of each of the compounds were examined. In each series of experiments, the standard B_2 receptor agonist, BK, and HOE 140, the template of the ala scan series, were tested. Inhibitory binding constants (K_i values), were calculated from IC_{50} values, according to Cheng and Prusoff equation [34], $K_i = IC50/(1 + [C^*]/Kd^*)$, where $[C^*]$ is the concentration of the radioligand and Kd^* is the dissociation constant. Non-specific binding was defined as the binding obtained in the presence of 10 μM unlabelled BK. Incubation time was 60 min at room temperature. Bound and free radioactivity were separated by filtering the assay mixture through Whatman GF/B glass fibre filters pretreated with 0.1% polyethyleneimine for at least 45 min in a Brandel cell harvester. The incubation mixture was diluted with 3 mL of ice-cold buffer (50 mM Tris-HCl) and then rapidly filtered under vacuum, and the filter was washed three times with 3 mL of buffer. The filter-bound radioactivity was counted in a Beckman LS-1800 spectrometer (efficiency 55%).

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