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hNK$_2$ receptor antagonists. The use of intramolecular hydrogen bonding to increase solubility and membrane permeability

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ABSTRACT

Starting from in-house capped tripeptide libraries, we have developed two series of compounds as potent antagonists of the hNK$_2$ receptor with a reduced peptide character. These two series maintained a crucial amide bond, which could not be methylated or substituted with classical isostere without a dramatic loss in binding affinity, very likely due conformational changes.

We report here the planning, synthesis and evaluation of molecules belonging to the selected chemical series, which contain a strategically placed hydrogen bond acceptor. The aim of the work was to improve membrane permeability via the formation of an intramolecular hydrogen bonding, and at the same time to maintain the structural characteristics geometry and polarity of the amide linkage so as to retain a relevant binding affinity for the biological target.

The passive permeability of a given drug molecule across a biomembrane is a particularly important physicochemical property-related characteristic because of its role in determining the extent of both oral bioavailability and drug access to certain target organs.

It is widely accepted that for a small molecule passive membrane permeability is primarily dependent on the desolvation potential of the polar functional groups, which determine the energetic price to pay for entering the lipidic bilayers. On the other hand, it is also well known that the overall lipophilicity is crucial: in fact an excessive lipophilicity may prevent the passage through the highly solvated external surface of a membrane.[1]

The high hydrogen bonding potential of the amide bond[2] is one of the reasons for the generally poor permeability of peptides. It has been evaluated that the energy cost for the transfer of a secondary amide bond from water to a non-hydrogen bonding environment is 6.12 kcal/mol.[3] Common techniques to overcome this energetic gap are either the methylation of the amide nitrogen or the substitution of the whole amide bond with a less polar isostere. Unfortunately these modifications are not always tolerated and may cause a significant drop in potency.

This was what we experienced while working on a project for the identification of oral bioavailable NK$_2$ antagonists. We selected our leads from capped tripeptide libraries (Fig. 1)[4] focusing subsequently our efforts on improving their potency and permeability through an intense work which, among others, aimed at decreasing

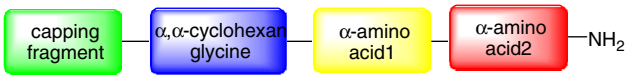

Figure 1. Scheme of the starting capped tripeptide libraries.

their peptidic character. We ended up with two chemical series, where one[5] (**B**) and two[6] (**A**) amide bonds could be substituted without any loss in potency (Fig. 2). When we concentrated our efforts on the third amide bond. the one located between the capping fragment Ar and the α,α-cyclohexaneglycine, we had less success, since methylation or isostere substitution caused a large drop in binding affinity.[7]

It was then decided to explore another strategy: the introduction of an intramolecular hydrogen bond between the amide proton and an opportunely located acceptor. This would in principle maintain the structural integrity of the peptide linkage, and at the same time reduce the energetic gap caused by desolvation, since the hydrogen bonding potential of the moiety would be partially satisfied by the intramolecular linkage.[8–10] Moreover, the addition of a polar or polarizing moiety would very likely lead to an increase in water solubility.

Having synthesized some active compounds belonging to the series **A** and **B** unable to form intramolecular hydrogen bond with the N-terminal amide (**1**, **4**, and **6**), we prepared their derivatives containing a hydrogen bond acceptor in the *ortho* position of the capping aromatic group: a heterocyclic nitrogen (pyridine, **5**, and **7**),[11] or a small polar group (fluorine, chlorine and methoxy, **2**, **3**, **8**, and **9**), Figure 2. The binding affinity (pK_i[12]) for the hNKA

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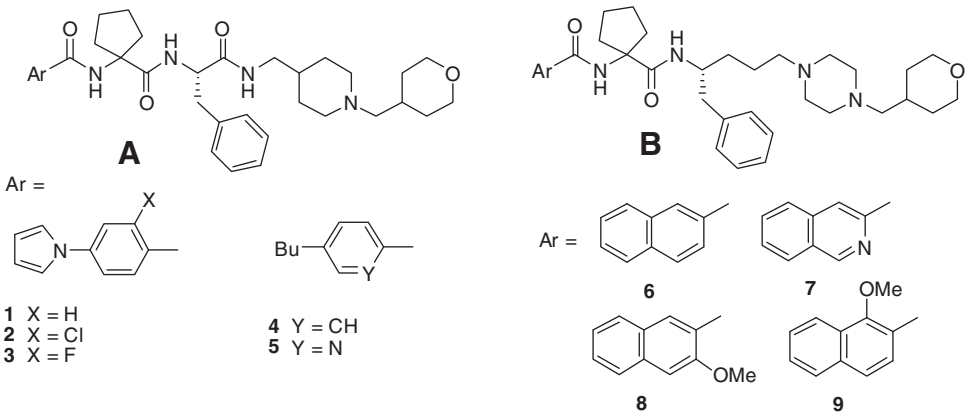


Figure 2. hNKA antagonists prepared.

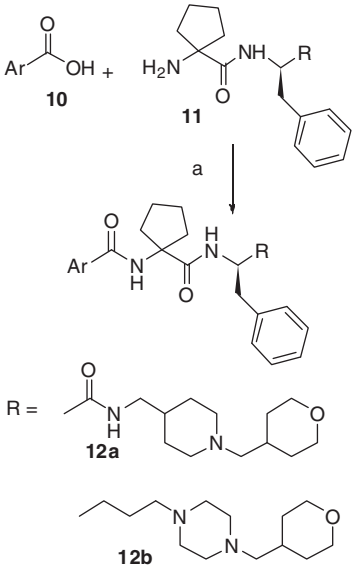
Table 1
Binding affinity, aqueous solubility, PAMPA and Caco-2 cells permeability of the NK_2 antagonists

Compds	pK_i[a]	Solubility pH 6.4 (mg/mL)[a]	Papp (PAMPA) ($\times 10^{-6}$ cm/s)[a]	Papp (CACO-2) ($\times 10^{-6}$ cm/s)[a]
1	9.63 ± 0.04	1.45 ± 0.01	nd	<1
2	8.40 ± 0.01	>2.82	0.16 ± 0.01	4.21 ± 0.88
3	8.49 ± 0.03	>2.86[b]	0.66 ± 0.02	13.80 ± 0.30
4	8.34 ± 0.02	>2.41[b]	1.60 ± 0.19	9.34 ± 1.05
5	7.57 ± 0.02	>6.60[b]	7.23 ± 0.10	17.60 ± 0.39
6	9.49 ± 0.05	1.57 ± 0.09	5.96 ± 0.48	14.65 ± 2.25
7	8.69 ± 0.02	2.80 ± 0.04	15.50 ± 0.20	25.20 ± 0.30
8	8.08 ± 0.05	3.07 ± 0.07	11.91 ± 0.03	13.55 ± 0.65
9	7.35 ± 0.03	1.91 ± 0.03	6.77 ± 0.08	11.70 ± 0.69

[a] For experimental details see the References and notes section.
[b] At this concentration saturation was not obtained.

receptor, the aqueous solubility[13] and the membrane permeability (PAMPA[14] and Caco-2 cells[15]) were evaluated for all our molecules (Table 1).

Syntheses of the planned compounds were performed according to Scheme 1. Amines **11** [4] were coupled with the opportune acid under standard conditions (EDCA, HOBt) and the resulting products were purified either by flash chromatography or crystallization. All final compounds were characterized by ^{1}H NMR and LC–MS analyzes and showed purity >97%.



Scheme 1. Reagents: (a) EDCA, HOBt and DIPEA.[16]

Two of the reference compounds (**1** and **6**) had sub-nanomolar binding affinity (Table 1), while the third (**4**) was in the low nanomolar range. We were very pleased to discover that the functionalized compounds showed only a small drop in pK_i, the lowest binder being **9** with a pK_i of 7.4.

In addition all the *ortho* substituted derivatives had an increase in water solubility in our standard test[13] when compared with the unsubstituted reference molecule. Nevertheless the most satisfying results came from the permeability measurements. Compounds **1**, **2** and **3** were prepared to evaluate the effect of a chlorine or a fluorine in the *ortho* position. Aromatic chlorine is generally known to be a poor H-bond acceptor, on the other hand a study from Huque et al. demonstrated that it decreases the H-bond donor capability of its partner when involved in intramolecular interaction.[17] Regarding fluorine, a discussion is still ongoing on its role as a H-bond acceptor, but Mecozzi[18] et al. showed that aromatic fluorine may participate in significant hydrogen bonding interactions. In our molecules the two halogens caused an increase in the permeability both the PAMPA and Caco-2 cells tests, with fluorine behaving a little bit better than chlorine.

The permeability properties related to an *ortho*-methoxy group in benzamides have been recently studied by Stahl and co-workers.[10] The Roche researchers found that *ortho*-methoxy methylbenzamide had an increased permeability in the PAMPA test when compared to the *ortho*-ethyl analog. Our results paralleled these findings. Compounds **8** and **9** were found to be somewhat more permeable than **6** in the PAMPA test.

The most impressive result was obtained with the *ortho* pyridines (**5** vs **4** and **7** vs **6**). In these cases a jump in the permeability values was observed both in the PAMPA and in the Caco-2 evaluation systems.

Following these data, we undertook NMR studies on the temperature dependence of the NH resonance value in polar hydrogen

Table 2
Temperature dependence of NH chemical shifts in water and DMSO

Compds	$d\delta/dT$[a] (H_2O)	$d\delta/dT$[a] (DMSO)	δ (H_2O)	δ (DMSO)
1	9.6	5.7	8.78	8.64
2	8.4	6.3	9.13	8.88
6	8.6	5.3	8.62	8.51
7	5.7	2.0	8.96	8.67
8	7.9	4.6	8.82	8.49
9	7.6	4.0	8.83	8.49

[a] (ppm/K $\times 10^{-3}$).

bonding solvents.[19] Generally NH resonances are shifted upfield with increasing temperature, reflecting the breaking of the solute–solvent H-bonds. Temperature dependencies are invariably linear and the *T* coefficients ($d\delta/dT$) values have been used to characterize solvent exposed and shielded NH groups. $d\delta/dT$ values <0.0003 ppm/K in solvents like DMSO are characteristic of solvent shielded NH groups, while values around 0.005 ppm/K are typical of solvent exposed NHs. Intermediate values of $d\delta/dT$ are more difficult to interpret and have sometime been taken as indicative of weakly intermolecularly H-bonded NH groups. It must be noted that this method does not allow one to make a clear distinction between intramolecularly hydrogen bonded and sterically shielded NH groups.

We evaluated the $d\delta/dT$ for compounds **1**, **2**, **6**–**9**, both in water and in DMSO and the results are reported in Table 2.[20]

Compound **7** proved the most shielded, both in DMSO and in water, which is in agreement with its permeability data, the highest of the whole group.

In conclusion, from the experimental results obtained we may say that the introduction of an H-bond acceptor as our *ortho* substituent to an aromatic amide group in the series **A** and **B**, can be used to improve membrane permeability, without altering the overall geometry of the molecule. Moreover, these functionalizations have also resulted in an increase in the solubility in our reference aqueous buffered solution. The effect is most evident for the aza-compounds, and for one of these analyzes a good shielding from solvent interactions has also been confirmed by solution NMR studies.

Acknowledgments

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11. Compounds **4** and **5** have already been described in *Bioorg. Med. Chem. Lett.* **2008**, *18*, 4705 and are here reported for completeness of the study. Data on their PAMPA permeability have been added.
12. Binding experiments were performed with membranes of CHO-K1 (hNK_2) cells stably transfected with the human NK_2 receptor. Compounds were tested for their ability to displace [^{125}I]NKA (0.15 nM) after 30 min incubation at room temperature. The affinity of the test compounds for the tachykinin NK_2 receptors determined in these competition experiments was expressed in terms of pK_i.
13. A standard solution of 0.1 mg/mL of the test compound was prepared in acetic acid/water 1:1. A standard curve was built with HPLC injections of 1, 5 and 10 μL). A suitable amount of the test compound was suspended in 500 μL of phosphate buffer pH 6.5, (in alternative, 3 mg dissolved in 20 μL DMSO, and 10 μL of DMSO solution added to 1 mL of the buffer till the permanence of solid residue) and this mixture was sonicated for 20 min at room temperature. Then it was filtered at 0.4 μm with a spin filter (GHP Nanosep—Pall Corporation) in microcentrifuge. The filtered solution (about 200 μL) was transferred in microvials and 5 and 10 μL injected in double in HPLC against the calibration curve of the same compound perfectly solubilized.
14. PAMPA permeability was determined according to the protocol reported in Millipore Application Note (AN1725EN00) *MultiScreen ™ Permeability Plates* using propanolol, warfarin and carbamazepin as references. Samples analysis was performed through HPLC. The reported values are the average from three experiments.
15. Caco-2 cells permeability was evaluated with cells differentiated in mature enterocytes measuring the passage of the compound from the apical towards the basolateral site. It was quantified in terms of apparent permeability coefficient (P_{app}). The P_{app} value, expressed in 10^{-6} cm/sec, was obtained by the formula $P_{app} = (\Delta Q/\Delta t)/(AC_0)$ where $\Delta Q/\Delta t$ is expressed in mmol/sec, A is the surface area of the cell monolayers (in cm^2) and C_0 is the initial concentration of the compound in the donor side (in mmol/cm^3). All the experiments were carried out at pH 7.4, 100 μM concentration of the tested compound and in the presence of markers of the cell monolayer integrity and of both the passive and active transport systems.
16. The acid fragment was dissolved in DMF and activated for 30 min with EDCA (1.2 equiv) and HOBt (1.2 equiv). Then a solution in dichloromethane of the preformed amine dichloridrate salt (1.0 equiv) and DIPEA (3.2 equiv) was added and left to react overnight at room temperature. Then the mixture was diluted with dichloromethane and the resulting organic layers separated and washed with 10% $NaHCO_3$, water and brine and concentrated under reduced pressure. The crude residue was purified by flash chromatography on silica gel to obtain the pure compound.
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20. NMR experiments were recorded on a Bruker Avance 400 MHz spectrometer equipped with a 5 mm inverse probe and processed using Xwin-NMR version 3.5. All experiments in H_2O have been conducted in presence of 100 mM phosphate buffer pH 6.4, 10% D_2O, with TSP as internal reference, the water signal has been suppressed by the excitation sculpting sequence [*J. Magn. Reson.* **1995**, A112, 275]. The temperature dependence of the proton spectra has been determined by acquiring spectra between 283 and 353 K in DMSO-d_6 and in H_2O. All deutered solvents have been purchased from Aldrich.