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Impact of polyphenols on receptor-ligand interactions by NMR : the case of neurotensin-NTS1 complex

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Impact of polyphenols on receptor-ligand interactions by NMR. : the case of neurotensin-NTS1 complex

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Abstract

Ligand-receptor interactions can be implicated in many pathological events as chronic neurogenerative diseases. Thus, the discovery of molecules disrupting this type of interactions could be an interesting therapeutic approach. Polyphenols are well known for their affinity for proteins and several studies have characterized these direct interactions. But studying the direct influence of multi-therapeutic drugs on a ligand-receptor complex relevant to a neurodegenerative disorder is a challenging issue. Solution NMR, molecular modeling and iterative calculations were used to obtain information about the interaction between a phenolic compound, α -glucogallin (α -2), and a ligand/receptor complex neurotensin (NT) and its receptor NTS1. The α -2 was shown to bind to NT and to a peptidic fragment of its NTS1 receptor, independently. Although the formation of the corresponding ligand-receptor complex did not seem to be affected, this experimental modeling protocol will enable the evaluation of other anti-amyloidogenic compounds such as blockers of NT-NTS1 binding. These types of studies help in understanding the specificity and influence in binding and can provide information to develop new molecules with a putative pharmacological interest.

1. Introduction

Polyphenols are natural substances present in plants, fruits and vegetables and beverages such as red wine and tea (Quideau et al., 2011). They are known for their ability to interact with proteins by forming macromolecular complexes (Haslam, 1974) and induce favorable or adverse nutritional properties (Perez-Gregorio and Simal-Gandara, 2017). Many articles report the affinity of polyphenols for proteins involved in different biological mechanisms, such as proteins involved in neurodegenerative diseases (Sylla et al. 2015, Coutant et al. 2007, Ono et al 2004) and salivary proteins (Faurie et al. 2016, Soares et al. 2019). Numerous chemical and physical studies have been used to characterize the interactions between these compounds (Poklar Ulrih, N., 2017; Lacroix S. et al. 2018). NMR is particularly suitable for the study of these non-covalent complexes (Delius, J., 2017; Faurie et al., 2016; Richard et al. 2013 ; Vergé et al. 2002). While direct interaction studies between polyphenols / proteins are numerous, studying the impact of polyphenols on a ligand / receptor interaction mechanism remains a challenging task. Yet understanding the mechanism of action of these compounds on a complex formation would help to develop novel molecules with a putative pharmacological interest. Recently, Faurie et al. (2016) have studied by NMR and dynamic light scattering a ternary complex and have shown that sugars play an indirect role in the phenomenon of astringency.

We recently showed that compounds from a series of gallotannins and related galloylated glucose derivatives express promising anti-amyloidogenic activities *in vitro*. In particular, α -glucogallin (α -**2**) (Figure 1), a monogalloylated glucose, was found to be quite potent (Sylla et al., 2015). This compound had a high inhibition percentage of fibrillization for the both peptides A β_{25-35} and A β_{1-42} (73 ± 13 % and 59 ± 6 % respectively), and we have already shown that this inhibition could be correlated with the formation of a complex between the peptides and two α -**2** molecules.

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Hence, considering the multifactorial pathogenicity of Alzheimer's disease (AD), we decided to investigate its putative effect on another pathological event of this disease, glutamate excitotoxicity. Indeed, it has now been clearly demonstrated that glutamate, the major excitatory neurotransmitter in the central nervous system, is an important factor responsible for neuronal injury (Crews et al., 2010; Dong et al., 2009; Vauzour et al., 2010, Esposito et al., 2013) Neuronal cell death could be due to the extracellular accumulation of glutamate and the excessive activation of glutamate receptors, particularly N-methyl-D-aspartate receptors. Emerging evidence has shown that neurotensin (NT), a tridecapeptide neurotransmitter, could play a key role in the excessive release of glutamate via its neurotensin receptor 1 (NTS1) (Ferraro et al., 2008; Ferraro et al., 2009; Wang et al., 2014). Moreover, several authors have postulated that antagonists of NTS1 could be a possible new therapeutic strategy for these neurodegenerative diseases (Antonelli et al., 2007; Ferraro et al., 2008; Ferraro et al., 2009; Wang et al., 2014). However, studying *in vitro* the release of glutamate via NTS1 is very complicated and even more so when attempting to examine inhibitors of this metabolic pathway (Antonelli et al., 2008; Chen et al., 2006; Ferraro et al., 2000; Matsuyama et al., 2003). By analogy with the results of the inhibition of fibrillization by the formation of complexes between β -amyloid peptides and α -2, we decided to study the behavior of this anti-amyloidogenic phenolic compound by investigating its interactions with both peptides NT and hNTS1, and its potential modulation effect concerning the formation of the NT:hNTS1 complex. Despite the physiological importance of the NT-NTS1 interaction, no NMR and molecular modeling studies so far have examined the influence of phenolic compounds on the ligand-receptor complexes NT:NTSs. However, such knowledge would provide insights into the specificity of NT:NTSs binding and would help in developing new molecules or selecting those with putative pharmacological interest from among compound libraries. Even if such an approach is only a first step in understanding binding mechanism, it can provide relevant structural information about the potency of phenolic compounds on the formation of the corresponding ligand-receptor complex.

2. Materials and methods

2.1. Materials

NT was purchased from Bachem (Weil am Rhein, Germany, purity 95%) and Sigma Aldrich (\geq 90% pure) and was used without any further purification. The hNTS1₃₂₁₋₃₄₄ receptor fragment contained 18 residues from the ECL3 and six residues from TM6. Its sequence is as follows: Val321-Arg322-Arg323-Leu324-Met325-Phe326-Ser327-Tyr328-Ile329-Ser330-Asp331-Glu332-Gln333-Trp334-Thr335-Pro336-Phe337-Leu338-Tyr339-Asp340-Phe341-Tyr342-His343-Tyr344. To avoid thiol group instability in solution, we replaced the Cys327 by the residue serine (Dupradeau et al., 2002). It was synthesized and purified (purity 98%) by Polypeptide (Strasbourg, France) and SynVec (Bordeaux, France). Deuterated trifluoroethanol (TFE)-*d*₃, acetic acid-*d*₄, and sodium [3-methylsilyl 2,2',3,3'-2H₄] propionate (TSP-*d*₄) were purchased from Eurisotop (Saint Aubin, France). Methanol-*d*₄, which was used to solubilize α -**2**, was purchased from Eurisotop. α -**2** was synthesized and purified according to the method we previously described (Sylla et al., 2015).

2.2. Spectroscopy experiments

All 1D and 2D NMR experiments in a 80% TFE:15% $H_2O:5\%$ CD₃OD solution were performed on a Bruker Avance 600MHz NMR spectrometer. Owing to weak overlaps at 293 K, spectra were collected at 308 K. Data were processed using TOPSPIN software (Bruker). Sodium [3-methylsilyl 2,2',3,3'-2H4] propionate (TSP-*d*₄) was used as an internal reference for proton chemical shifts. NT and hNTS1₃₂₁₋₃₄₄ were prepared as previously described (Da Costa et al., 2013).

NMR samples were prepared by dissolving NT or hNTS1₃₂₁₋₃₄₄ to a 1.0 mM concentration in 500 μ L of the solvent mixture (80% TFE/15% H₂O/5% CD₃OD). A stock solution of α -2 was prepared in 200 μ L of the same solvent mixture at a 20 mM concentration. Titration experiments were performed by addition of small amounts of α -2 to the peptide solution (NT or hNTS1₃₂₁₋₃₄₄). After each addition, 1D-¹H and 2D ¹H-¹H TOCSY spectra were acquired to examine the evolution of the amide proton chemical shifts.

For the complex structural studies, NT: α -2 and hNTS1₃₂₁₋₃₄₄: α -2, two-dimensional NMR spectra were acquired by using 1 mM of NT and 1mM of hNTS1₃₂₁₋₃₄₄ respectively and 8 mM of α -2, with an 8:1 α -2:peptide molar ratio corresponding to the high saturation of NT and hNTS1₃₂₁₋₃₄₄ peptides, as shown by the binding curves. For the study of NT:hNTS1₃₂₁₋₃₄₄: α -2, two-dimensional NMR spectra were acquired by using 1 mM of NT, 1mM of hNTS1₃₂₁₋₃₄₄ and 8 mM of α -2.

The sequence-specific assignments of the hNTS1₃₂₁₋₃₄₄ receptor fragment, NT:hNTS1₃₂₁₋₃₄₄, NT: α -2, hNTS1₃₂₁₋₃₄₄: α -2 and NT:hNTS1₃₂₁₋₃₄₄: α -2 complexes were obtained by using twodimensional total correlation spectroscopy (TOCSY) and nuclear overhauser effect spectroscopy (NOESY) experiments (Braunschweiler & Ernst, 1983; Kumar et al., 1980). TOCSY experiments were performed with the DIPSI-2 sequence (Shaka etal., 1988) and a mixing time of 80 ms. NOESY experiments were performed with a mixing time of 150, 200 ms. All 2D homonuclear experiments were carried out with 2048 data points, 400 increments and a spectral width of 6009 Hz in both dimensions, 80 scans for NOESY and 40 scans for TOCSY experiments. Water suppression was accomplished by presaturation during the relaxation delay or with excitation sculpting using the water-selective 180° (zgesgp) pulse sequence from the Bruker pulse sequence library. The ¹H–¹³C HSQC

experiment was carried out with 2048 data points, 300 increments and 400 scans, and with a spectral width of 6009 Hz and 12 073 Hz in proton and carbon dimensions, respectively.

For α -2 assignments, two-dimensional ¹H-¹H COSY, ¹H-¹³C HSQC and ¹H-¹³C HMBC experiments were performed. All 2D-experiments were carried out with 2048 data points, 256 increments and a spectral width of 7211 Hz and 31700 Hz in proton and carbon dimension respectively, 128 scans and with 1.5 s for relaxation delay.

2.3. Peptide titration

Complex stoichiometry was performed by using the chemical shifts of amide protons whose variations have been measured unambiguously without overlapping. Binding constants, number of sites and binding percentage of species in solution were determined by fitting the chemical shift changes of amide protons with previously described technique (Richard et al., 2005; Verge et al., 2002) according to the equations:

with hNTS1_{321–344} = (R), NT = (L), the monogalloylglucose α -**2** = (G) and K and K' are the association constants.

Changes in backbone chemical shifts were used to map the binding sites and the sizes of the chemical shift changes ($\Delta\delta$) were calculated using equation:

$$\Delta \delta = (\Delta H_N^2 + \Delta H_\alpha^2)^{1/2}$$

 with ΔH_N and ΔH_{α} being the difference between free and observed proton chemical shifts, amide protons and H α protons, respectively.

2.4. Competition between the three molecules

To interpret the results obtained for the effect of α -2 on the binding between the receptor and its ligand and to calculate the binding percentage of molecules in solution, we were faced with two hypotheses. Indeed, this was a very complex and totally unprecedented situation because of the interaction between three molecules with numerous binding sites (see results). The first model posited that the interaction between NT (L) and hNTS1₃₂₁₋₃₄₄ (R) prevented any binding of α -2 (G) on the RL complex, except for one NT site that could not reasonably be involved in the binding (see result). The second model hypothesized that the RL complex formation blocked only two sites, one on each molecule, leaving the other three binding sites free (see results). For both models, supplementary equations were necessary:

In model 1	R + L + G	₽	RLG				
In model 2	R + L+ 3G	₽	RLG + 2G	5	$RLG_2 + G$	₽	RLG ₃

For both hypotheses, neither the classical equilibrium nor conservation equations (Wilkinson, 2004) were relevant so they were modified (see supplementary material). With the hypothesis that the sites were non-interacting and the interaction between the three molecules had not modified the binding constant values of the bimolecular complexes, we obtained at the molecular level: $K_1 = k_a + k_b$ and $K_1K_2 = k_ak_b$ with a and b being the two sites of hNTS1₃₂₁₋₃₄₄, and $K'_1 = k_a' + k_b' + k_c'$, $K'_1K'_2 = k_a'k_b' + k_a'k_c' + k_b'k_c'$ and $K'_1K'_2K'_3 = k_a'k_b'k_c'$ with a', b' and c' being the three sites of NT. We used the binding constants calculated in this study and

in our previous publication for the constant RL (Da Costa et al., 2013), then an iterative calculation allowed us to obtain the concentration values of the free and of bound species.

2.5. Molecular modeling

Molecular modeling was conducted with the Accelrys software package. The peptides in extended form and α -2 were constructed using Biopolymers and Builder, respectively, and then were minimized. Simulated annealing (SA) and energy minimization were performed with Discover and NMR Refine using the consistent valence force field model (Dauber-Osguthorpe et al., 1988). Interproton distances were obtained from NOESY cross-peaks by integrating intensities at a 150 ms mixing time and calibrating the intensities using the nuclear overhauser effects (NOEs) between the β -methylene protons of prolines (Pellegrini et al., 1997). The NOE distance constraints between two protons were estimated using a relation of the Braun model for short flexible peptides (Braun et al., 1981). To take spin diffusion and internal motion into account, distance constraints were classified into four categories of upper distance limits rather than as strict constraints. Cross-peaks were divided into strong, medium, weak and very weak classes, corresponding to the upper limits of 2.7, 3.3, 5.0, and 6.0 Å, respectively. Pseudoatoms were used for non-stereospecifically assigned protons and the limits were adjusted with standard pseudoatom corrections (Granier et al., 1982). Using the PREDITOR software, the chemical shift data obtained from homo and heteronuclear spectra allowed us to calculate the range of dihedral angles, which were then used as restraints (Berjanskii et al., 2006).

2.6. Structure calculations

Simulation annealing (SA) calculations involved 15 separate phases. The first phase involved 100 steps of randomization and minimization of the starting structure into the steepest descent algorithm. The second phase involved additional minimization into 500 iterations of the

conjugate gradient algorithm. Dynamic phases 3–5 involved simulated annealing for 30, 10, and 10 ps, respectively, at 1000 K. Meanwhile, the force constants were increased stepwise up to their full values. Phases 6-10 involved cooling of the molecule from 1000 to 300K over 2 ps. Phases 11 and 12 involved minimization using100 steps of the steepest descent algorithm followed by 500 iterations of the conjugate gradient. Then, three minimization phases 13-15 with a distance-dependent dielectric constant to mimic the solvent effect involved 200 steps of the steepest descent, followed by 3000 iterations of a conjugate gradient. The last phase involved 200 steps of the steepest descent algorithm without the NMR constraints in order to improve the molecular relaxation.

100 initial structures were generated using the experimental distances determined from the NOE spectra. From these, we kept the conformations with the lowest total energy, discarding the conformers with more than one violation of the distance restraint above 0.3 Å and with backbone dihedral angles outside the peptide range in the Ramachandran plot. Structural statistics for the converged structures were then evaluated in terms of root mean square deviations (RMSDs) and were grouped in families. The validity of conformers was controlled by Procheck analysis (Laskowski et al., 1996).

3. Results and Discussion

In a previous work, we studied a molecular model of the interaction between NT and its receptor NTS1 (Da Costa et al., 2013). Briefly, NT principally activates three receptor subtypes, named NTS1, NTS2, and NTS3. Most of the known biological and physiological effects are mediated through the NTS1 receptor (Dobner, 2005; Dubuc et al., 1999; Kitabgi et al., 2006; Pettibone et al., 2002; Vincent et al., 1999). NTS1 belongs to the G-protein-coupled receptor (GPCRs) family with seven putative trans-membrane domains (TMs) connected by

extracellular and intracellular loops. For this receptor, the mapping of the NT-binding site shows the importance of TM6 and TM4 and the third extracellular loop (ECL3) for NT recognition (Barroso et al., 2000; Richard et al., 2001, White et al., 2012). Thus, to further our understanding of the NT ligand–receptor system, we investigated the interaction between NT and a synthetic peptidic fragment from the human receptor NTS1 (hNTS1) corresponding to the ECL3 and six residues from the adjacent TM6, which has been found to be critical for NT interaction (hNTS1_{321–344}). The results showed a ligand-receptor complex with a 3D structure between NT and the hNTS1_{321–344} receptor fragment (Da Costa et al., 2013).

Thus, we have at our disposal a powerful molecular model to study the interaction between any compound and a couple formed by a receptor and its ligand relevant to a neurodegenerative disorder such as AD.

The NT and hNTS1₃₂₁₋₃₄₄ protons were unambiguously assigned using the standard technique developed by Wüthrich (Wüthrich, 1986) and by comparison with our previous results (Da Costa et al., 2013) (Table 1). For α -2 protons, it was done classically with 1D proton NMR and HMBC sequences (Table 2). Under the experimental conditions, the self-association of molecules was not significant (data not shown). To study the interaction between α -2 and peptides, we used three parameters: (i) the chemical shift variations of amide protons, which led us to calculate the dissociation constants of complexes; (ii) the chemical shift disturbances ($\Delta\delta$) along the sequence of peptides upon addition of α -2; and (iii) the observation of intermolecular NOEs, which could lead to the three-dimensional structure of the complexes.

3.1. Complex stoichiometry between each peptides and α -glucogallin

All data indicate that α -2 is able to form a stable complexe with the both peptides NT and its receptor hNTS1₃₂₁₋₃₄₄ alones. The variation in amide proton chemical shifts, measured

unambiguously without overlapping, enabled us to calculate the complex stoichiometry (Figure 2). By fitting the chemical shift changes, the binding constant was determined. We observed two binding sites for the complex hNTS1₃₂₁₋₃₄₄: α -**2** complex with dissociation constant values equal to (2.9 ± 0.6).10⁻⁴ and (2.9 ± 1.2).10⁻³ mol.L⁻¹ and three binding sites for the NT: α -**2** complex with dissociation constant values equal to (4.0 ± 0.1).10⁻⁴, (1.2 ± 0.1).10⁻³ and (4.4 ± 1.2).10⁻³ mol.L⁻¹. The number of sites and the dissociation constant values were in agreement with the intermolecular NOEs observed (Table 3) and with the fast exchange observed in NMR spectra between the free and bound states, respectively. The observed dissociation constant values are of the same magnitude as the ones measured in others polyphenol/protein interactions (Murray, N.J. et al., 1994, Richard et al. 2006).

3.2. Structure of the complex formed between hNTS1₃₂₁₋₃₄₄ and α -2

For hNTS1₃₂₁₋₃₄₄ alone, the first observation was as expected since its structure was not altered by the small addition of methanol in our solvent condition (80% TFE:15% H₂O:5% CD₃OD). Indeed, the NOESY cross-peaks and H α CSI (Chemical Shift Index) of this peptide fragment were similar to those observed in our previous study with the solvent conditions 80% TFE:20% H₂O (Figure 3) (Da Costa et al., 2013). Thus, as in our previous work, α -helices were observed in the C-terminal domain (from residue Pro336) and, to a lesser degree, in the central part, whereas the N-terminus could indicate an extended conformation.

The interaction of α -2 with the receptor fragment was evidenced by titration curves (Figure 2), the seven unambiguous intermolecular NOEs cross-peaks between both molecules (Table 3) and the chemical shift changes $\Delta\delta$ along the hNTS1₃₂₁₋₃₄₄ sequence, showing that the N-terminus domain was the most perturbed (Figure 4). Chemical shifts of the backbone

resonances were sensitive to the surrounding environment and to structural changes. Perturbation of chemical shifts upon complexion with a phenolic compound such as α -2 is a qualitative tool for mapping residues involved in binding. However, careful examination of the H α CSI showed that the α -2 interaction did not modify the secondary structure of the peptide fragment, since similar values were observed in the presence or absence of α -2 (Figure 3). Interestingly, the interaction occurred in the less structured peptide domain, *i.e.*, the N-terminus in an extended conformation and a part of the central domain with a nascent helix.

The use of 235 unambiguous distance restraints (228 intramolecular and 7 intermolecular) derived from NOE cross-peaks evidenced 15 low-energy structures. Superimposition of the backbone atoms of parts Thr335-His343, Ile329-Trp334 and Arg323-Tyr328 showed a stabilization in these regions with RMSD values of 0.48 Å, 0.19 Å and 0.45 Å, respectively (Table 4). The low number of intermolecular NOE cross-peaks indicated that the spatial positions of the α -2 molecules in the N-terminus domain were not well defined, the α -2 molecules just surrounding this region (Figure 5). This was particularly obvious for molecules in contact with the last residues of the N-terminus domain, *i.e.*, Val321, Arg322 and Arg323, owing to the large degree of freedom of these amino acids.

3.3. Structure of the complex between NT and α -2

For NT, the interaction with α -2 was evidenced by the titration curves (Figure 2), the intermolecular NOEs (Table 3) and the chemical shift disturbances $\Delta\delta$ (Figure 6). The latter parameter showed that the N- and C-termini were disturbed by the addition of α -2. The use of 152 unambiguous distance restraints (140 intramolecular and 12 intermolecular distances), which were derived from the observation of NOE cross-peaks allowed the structures to be

calculated. Owing to the presence of 12 intermolecular NOEs, we used only the unambiguous intermolecular NOEs in the first SA calculation, *i.e.*, from protons of amino acids that correlated inevitably with two different α -2 molecules (e.g. H δ Tyr3 and H δ Leu13 with α -2 H_{arom}, see Table 3), in agreement with the extended NT structure. Thus, the first simulations explored the conformational space. From these data, supplementary cross-peaks could be unambiguously introduced one by one into new SA calculations. Indeed, whenever we introduced an unsuitable NOE attribution, we obtained large violations of the distance restraints and/or structures with the backbone dihedral angles outside the permitted regions in the Ramachandran plot. These steps were repeated as many times as necessary to attribute all intermolecular NOEs.

In the final SA, 18 low total energy structures were obtained. Superimposition of the backbone atoms over Leu2-Asn5 and Arg8-Ile12 showed a stabilization of these regions as extended structures, with RMSD values equal to 0.48 Å and 0.44 Å, respectively (Table 5 and Figure 7), which was in agreement with previous results (Da Costa et al., 2013; Coutant et al., 2007). As with the hNTS1 fragment, the spatial position of the α -2 molecules was not well defined and these molecules surrounded the N- and C-termini of NT (Figure 7).

3.4. Formation of a ternary complex between both peptides and α -2

After the formation of the NT:hNTS1₃₂₁₋₃₄₄ complex, α -2 was added. Figure 8 shows the chemical shift changes $\Delta\delta$ of the complex NT:hNTS1₃₂₁₋₃₄₄ for two ratios: peptide NT:hNTS1₃₂₁₋₃₄₄: α -2 equal to 1:1:4 and1:1:8. There was no significant chemical shift perturbation with either of the ratios. These results could be correlated with the lack of observed intermolecular NOEs between the peptide molecules and α -2. Thus, α -2 did not seem to perturb the NT:hNTS1₃₂₁₋₃₄₄ complex.

We then sought whither the complexes hNTS1₃₂₁₋₃₄₄:(α -2)₂ or NT:(α -2)₃ could prevent the formation of the complex NT:hNTS1₃₂₁₋₃₄₄. For the first complex, the hNTS1 fragment would contain five crucial amino acids involved in the binding to NT (*i.e.*, Arg322, Phe326, Trp334, Tyr339 and Tyr342) with three key interactions: an ionic interaction between Leu13 and Arg322 and a π - π and H-bond interaction between Tyr11 and Tyr342 (Da Costa et al., 2013; Kitabgi, 2006; Barroso et al., 2000). Thus, the formation of the hNTS1₃₂₁₋₃₄₄:(α -2)₂ complex in the N-terminus could block the association with NT by preventing two bindings with Arg322 and Phe326, including the key Leu13-Arg322 ionic interaction. For the second complex, it is well known that the six amino-acid-long C-terminal fragment of NT, NT(8-13), possesses all of the structural requirements to bind to NTS1 with sub-nanomolar affinity, as observed with the full-length peptide (Granier et al., 1982; Kitabgi et al., 1977). Thus, for the formation of the complex between NT and α -2, the localization of two α -2 molecules in the C-terminus.

For the hNTS1₃₂₁₋₃₄₄:(α -**2**)₂ complex, we added NT to obtain a ratio hNTS1₃₂₁₋₃₄₄: α -**2**:NT equal to 1:8:1. For the second complex NT:(α -**2**)₃we added the hNTS1 fragment to obtain the same ratio 1:8:1. The addition of NT to the complex hNTS1₃₂₁₋₃₄₄:(α -**2**)₂ strongly disturbed the proton chemical shifts of the hNTS1 fragment (Figure 9). To understand these chemical shift changes, we compared these data with those of the hNTS1 fragment in the NT:hNTS1₃₂₁₋₃₄₄ complex (Figure 10). There was no significant difference between these proton chemical shifts. Thus, the NT addition to the hNTS1₃₂₁₋₃₄₄:(α -**2**)₂ complex shifted the latter to restore a hNTS1₃₂₁₋₃₄₄ structure close to that in the NT:hNTS1₃₂₁₋₃₄₄ complex. The

results showed that the interaction between the hNTS1 fragment and both α -2 molecules was not able to prevent the NT interaction with the receptor fragment.

We obtained a similar result in the second experiment. The addition of hNTS1₃₂₁₋₃₄₄ to the NT:(α -**2**)₃ complex showed significant disturbances in NT chemical shifts (Figure 11). These changes led to an NT conformation close to that in the NT:hNTS1₃₂₁₋₃₄₄ complex (Figure 12), still implying a partial dissociation of the NT:(α -**2**)₃ complex in favor of the NT:hNTS1₃₂₁₋₃₄₄ complex. Thus, this complex was too weak to prevent the ligand-receptor binding.

These two results could be correlated with the dissociation constants of the complexes. Indeed, in our previous study on the intermolecular interactions between NT and the hNTS1₃₂₁₋₃₄₄ receptor fragment, the dissociation constant was equal to 3.10^{-5} mol.L⁻¹. The value was lower than those obtained in this study for the hNTS1₃₂₁₋₃₄₄:(α -2)₂ and NT:(α -2)₃ complexes. Thus, under our experimental conditions, the weak peptide: α -2 complexes seemed to be shifted towards a stronger ligand-receptor complex. To further illustrate this by using the dissociation constant values and the concentrations of the different partners, a percentage calculation of the various bound species is presented in Table 5. For the complex between the three molecules, the calculated values of k constants could not be attributed to one particular constant, so we permuted values and the results are presented as mean ± standard deviation. The results for the bimolecular hNTS1₃₂₁₋₃₄₄:(α -2)₂ and NT:(α -2)₃ complexes showed that more than 75% of peptides were bound. In the presence of the three molecules, model 1 (see *Competition between the three molecules* in Materials and Methods) was in disagreement with the experimental results owing to the very low percentage (6%) of binding between NT and hNTS1₃₂₁₋₃₄₄.

On the contrary, model 2 was in agreement as the percentage of the hNTS1₃₂₁₋₃₄₄:NT complex was always high (54%). Thus, the presence of the monogalloylglucose α -2 did not prevent binding between NT and hNTS1₃₂₁₋₃₄₄; it simply decreased the association, which was always substantial despite a high concentration in α -2. Lastly, these calculations could explain the lack of observed intermolecular NOEs between the peptides and α -2. Indeed, for the bimolecular hNTS1₃₂₁₋₃₄₄:(α -2)₂ and NT:(α -2)₃ complexes, the values of saturation were equal to 80% and 78%, respectively (Table 5), and the observed intermolecular NOEs were classically very weak, at the limit of the detection threshold, *i.e.*, just above the background noise. When the three molecules were present, these values dropped to 62% and 61%, thus below the background noise. Many studies have sought to study the interactions between polyphenols and proteins involved in biological processes (Lacroix S. et al., 2018). Our results show that even if the polyphenol has the ability to bind to both potential targets (ligand and/or receptor) this property alone is not sufficient to guarantee its ability to inhibit or modulate the ligand-receptor interaction. The competition between the three partners must be examined to identify positive candidates.

4. Conclusion

This work describes the development of a new model to study the impact of potential drugs on the interaction between neurotensin (NT) and its human receptor 1 (hNTS1). The antiamyloidogenic compound used in this study, α -glucogallin (α -2), was shown to form stable complexes with NT and its receptor a peptidic fragment of NTS1. However, α -2 did not affect the formation of the corresponding ligand-receptor complex, even if α -2 is added previously to the formation of the ligand-receptor complex formation. This is could be due to the competition between the three molecules and to the difference in the dissociation constants between the peptides and α -2 bindings, on one hand, and the ligand-receptor binding, on the

 other hand. Nevertheless, this experimental modeling protocol will provide relevant structural information helpful to understanding the mechanism of interaction between the NT-NTS1 complex and phenolic compounds.

The main result of this investigation thus indicates that the interaction of the (poly)phenolic compound with (either or) both peptidic partner(s) is not sufficient, and that the interaction must be strong enough to block the ligand-receptor binding. In the search for multi-therapeutic drugs among the anti-amyloidogenic agents, our molecular model should certainly prove useful in identifying candidates capable of blocking NT:hNTS1 binding to counteract the role of NT in the development of glutamate excitotoxicity in chronic neurodegenerative diseases. Subsequent *in vitro* evaluations could then be performed to confirm the role of these compounds in the decrease in the excessive accumulation of glutamate.

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	NH	Ηα	Нβ	Others
hNTS1 ₃₂₁₋₃₄₄				
V321	_	3.89	2.30	Ηγ 1.09
R322	8.41	4.47	1.95/1.90	Ηγ 1.75 ; Ηδ 3.23 ; Ηε 7.14
R323	8.21	4.35	1.92/1.87	Ηγ 1.72 ; Ηδ 3.21 ; Ηε 7.10
L324	7.79	4.36	1.69	Ηγ 1.64 : Ηδ 1.01/0.96
M325	7 88	4 40	2 07	$H_{\gamma} = 2.54 \cdot H_{\epsilon} = 2.07$
F326	7 74	4 53	3 19/3 11	Arom 7 15 7 08
S327	7 90	4 34	3 99/3 90	
¥328	7.75	4 52	3 14	H& 7 14 · HE 6 84
1320	7.75 7 77	4.02	1 92	$H_{\nu}CH_{2} = 1.64/1.26 \cdot H_{\nu}CH_{2} = 0.97 \cdot H\delta = 0.94$
S330	7.85	4.02	2 00/2 85	11yeng 1.04/1.20, 11yeng 0.97, 110 0.94
D221	7.0 <i>J</i> 8.01	4.37	2.07	
D331 E222	8.01	4.74	2.27	$H_{2} 2.40$
E332	8.00 8.00	4.20	2.14/2.07	1172.47
Q333 W224	8.02 7.74	4.28	2.04	$\Pi\gamma 2.10$, $\Pi\epsilon 0.91/0.30$ US 7.26 , $\Pi\epsilon 0.44$, others 7.59, 7.20, 7.16, 7.02
W 334	1.14	4.84	3.48/3.31	Ho /.20; HE 9.44; others /.38, /.39, /.10, /.03
1335	/.4/	4.44	4.20	$H\gamma 1.25$
P336	-	4.25	2.26/1.72	Ηγ 1.92 ;Ηο 3.60
F33/	1.25	4.48	3.22/3.17	Arom. 7.34, 7.25
L338	7.65	4.24	1.77	$H\gamma 1.64$; $H\delta 1.01/0.95$
Y339	7.87	4.37	3.17/3.05	Ηδ 7.12 ; Ηε 6.76
D340	8.11	4.57	2.98/2.87	
F341	8.01	4.32	3.02/2.98	Arom. 7.22, 6.94
Y342	7.70	4.41	3.02/2.84	Ηδ 7.16 ; Ηε 6.85
H343	7.67	4.54	3.17/2.98	Ηδ 7.08 ; Ηε 8.21
Y344	7.60	4.67	3.14/3.00	Ηδ 7.15 ; Ηε 6.85
NT				
Pca1	7.46	4.28	2.54/1.93	Ηγ 2.40/1.93
Leu2	7.68	4.33	1.63/1.58	Ηγ 1.56 ; Ηδ 0.96/0.90
Tyr3	7.52	4.54	3.07	Arom. 6.86, 7.11
Glu4	7.98	4.32	2.14/1.99	Ηγ 2.41
Asn5	7.97	4.74	2.84	ΝΗδ 7.17/6.46
Lys6	7.68	4.71	1.89/1.79	Ηγ 1.53 ; Ηδ 1.70 ; Ηε 3.05
Pro7	_	4.45	2.30/1.97	Ηγ 2.11/2.05 ; Ηδ 3.82, 3.64
Arg8	7.84	4.44	1.93/1.80	Hy 1.73 : Hδ 3.22 : NHguanid 7.12
Arg9	7.76	4.70	1.84/1.71	Hy 1.67: Hδ 3.21: NHguanid 7.04
Pro10	_	4.43	2.23/1 94	Ην 2.03 : Ηδ 3.76, 3.60
Tvr11	7 42	4 61	3 09/3 05	Arom 6 86 7 10
Ile12	7 38	4 24	1 87	HγCH ₂ 1 50/1 14 · HγCH ₂ 0 96 · Hδ 0 91
Leu13	7 42	4 44	1 70	$H_{\nu} = 1.69 \cdot H_{\delta} = 0.99/0.96$

1	2	3	4	5	6
6.32	3.82	3.99	3.63	3.80	3.80
Aromatic	galloyl protons	S			
7.28					



Figure 2: Relative chemical shift variations of peptide amide protons with increasing a-glucogallin (a-2)/peptides molar ratio. For hNTS1₃₂₁₋₃₄₄, open circles and down triangles are for Arg322 and Tyr328 respectively; black squares, circles and down triangles are for Leu324, Arg323 and Ile329, respectively. For NT, open circles and down triangles are for Glu4 and Tyr11, respectively; black squares, circles and down triangles are for Leu13, Tyr3 and Arg8, respectively.

140x54mm (300 x 300 DPI)

Tyr3 Hδ Harom Val321 Hα H ₁ Ans5 Hα Harom Arg322 Hα Harom Arg9 Hγ H ₁ Arg322 Hδ Harom Pro10 Hα Harom Arg322 Hδ Harom Pro10 Hα Harom Arg322 Hγ H ₁ Pro10 Hβ Harom Met325 Hγ H ₁ Pro10 Hδ Harom Met325 Hγ H ₁ Pro10 Hδ Harom De326 Hβ Harom Tyr11 Hβ Harom De329 HγCH2 Harom Ile12 Hδ Harom De329 HγCH2 Harom Ile12 HζCH2 Harom Leu13 Hδ Harom Leu13 Hδ Harom Harom Harom Harom	Neurotensin	α-2	hNTS1 ₃₂₁₋₃₄₄	α-2	
Asn5 H α H αrom Arg322 H α H αrom Arg9 H γ H 1 Arg322 H δ H αrom Pro10 H α H αrom Arg323 H γ H αrom Pro10 H β H αrom Met325 H γ H 1 Pro10 H δ H αrom Met325 H γ H 1 Pro10 H δ H αrom Met325 H β H αrom Ilc12 H α H αrom Ilc329 H $\gamma CH2$ H αrom Ilc12 H α H αrom Ilc329 H $\gamma CH2$ H αrom Ilc12 H $\gamma CH2$ H 2	Tyr3 Hδ	H _{arom}	Val321 Ha	H_1	
Arg9 H_Y H_1 Arg322 $H\delta$ H_{arom} Pro10 $H\alpha$ H_{arom} $Arg323$ $H\gamma$ H_{arom} Pro10 $H\beta$ H_{arom} $Met325$ $H\gamma$ H_1 Pro10 $H\delta$ H_{arom} $Met325$ $H\gamma$ H_1 Pro10 $H\delta$ H_{arom} $Met325$ $H\gamma$ H_1 Pro10 $H\delta$ H_{arom} $Ile326$ $H\beta$ H_{arom} Ile12 $H\gamma_{CH2}$ H_2 Ile12 $H\gamma_{CH2}$ H_2 Ile12 $H\gamma_{CH2}$ H_2 Ile13 $H\delta$ H_{arom} Leu13 $H\delta$ H_{arom} H_{arom} H_{arom}	Asn5 Ha	H _{arom}	Arg322 Ha	H _{arom}	
Pro10 Hα Harom Arg323 Hγ Harom Pro10 Hβ Harom Met325 Hγ H1 Pro10 Hδ Harom Phet326 Hβ H3rom Tyr11 Hβ Harom Ile329 HYCH2 Harom Ile12 Hα Harom Ile329 HYCH2 Harom Ile12 Hδ Harom Ile329 HYCH2 Harom Ile12 Hδ Harom Ile329 HYCH2 Harom Ile12 Hδ Harom Ile329 HYCH2 Harom Ile12 HζCH2 Harom Ile329 HYCH2 Harom Ile12 HζCH2 Harom Ile329 HYCH2 Harom Leu13 Hδ Harom Harom Ile329 HYCH2 Harom	Arg9 Hγ	H_1	Arg322 Hδ	Harom	
Pro10 Hβ H _{arom} Met325 Hγ H1 Pro10 Hδ H _{arom} Phe326 Hβ H _{arom} Tyr11 Hβ H _{arom} Ile329 Hγ _{CH2} H _{arom} Ile12 Hα H _{arom} Ile329 Hγ _{CH2} H _{arom} Ile12 Hδ H _{arom} Ile329 Hγ _{CH2} H _{arom} Ile12 Hγ _{CH2} H ₂ H ₂ H ₂ Ile12 Hγ _{CH2} H ₃ H _{arom} Ile329 Hγ _{CH2} H _{arom} Leu13 Hδ H _{arom} H ₂ H ₂ H ₂	Pro10 Ha	H _{arom}	Arg323 Hy	H _{arom}	
Pro10 Hδ H _{arom} Phe326 Hβ H _{arom} Tyr11 Hβ H _{arom} IIc329 H' _{7CH2} H _{arom} Ile12 Hδ H _{arom} IIc329 H' _{7CH2} H _{arom} Ile12 Hδ H _{arom} IIc329 H' _{7CH2} H _{arom} Ile12 Hδ H _{arom} IIc329 H' _{7CH2} H _{arom} Ile12 Hζ H _{arom} IIc329 H' _{7CH2} H _{arom} Ile12 Hζ H _{arom} IIc329 H' _{7CH2} H _{arom} Leu13 Hδ H _{arom} IIc329 H' _{7CH2} H _{arom}	Pro10 Hβ	H _{arom}	Met325 Hy	H_1	
Tyr11 Hβ Harom Ile329 HγCH2 Harom Ile12 Hα Harom Ile12 Hβ Ile12 Hβ Harom Ile12 HγCH2 Ile12 HγCH2 Hg Ile12 Hg Ile12 HγCH2 Hg Ile12 Hg Ile12 HγCH2 Hg Ile12 Hg Ile13 Hδ Harom Ile12	Pro10 Hδ	H _{arom}	Phe326 HB	Harom	
Ile12 Hα Harom Ile12 H ⁷ CH2 H2 Ile12 H ⁷ CH2 Harom Leu13 Hδ Harom	Tyr11 Hβ	H _{arom}	Ile329 $H\gamma_{CH2}$	Harom	
Ile12 Hδ Harom Ile12 Hγ(H2 H2 Ile12 Hγ(H2 Harom Leu13 Hδ Harom	Ile12 Ha	Harom			
Ile12 Ηγ(H2 Η2 Ile12 Ηγ(H2 Ηarom Leu13 H8 Harom	Ile12 Ho	H _{arom}			
Πε12 Ηγ(H2 Ηarom Leu13 Hδ Harom	Ile12 $H\gamma_{CH2}$	H_2			
Leul3 Hð H _{aron}	Ile12 $H\gamma_{CH2}$	H _{arom}			
or peer Review Only	Leu13 Ho	H _{arom}			

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50 51 52 53 54 55 55	





Figure 3: HaCSI of the hNTS1₃₂₁₋₃₄₄ fragment. Black bars represent CSI of our previous study in 80% TFE/20% H2O (Da Costa et al. 2013). Open bars and grey bars represent CSI in the absence or presence of a-2 in 80% TFE/15% H2O/5% CD3OD.

139x87mm (300 x 300 DPI)



Figure 4 : Chemical shift perturbation along the sequence of the hNTS1₃₂₁₋₃₄₄ receptor fragment upon addition of a-2. The hNTS1₃₂₁₋₃₄₄/a-2 ratio was 1/8. The values were calculated as $\Delta \delta = (\Delta HN^2 + \Delta Ha^2)^{1/2}$ (see text).

139x61mm (300 x 300 DPI)

	(%)	(%)	(%)
	Peptide binding	R bound sites by G	L bound sites by G
	$RL + RLG_n$	$RG_n + RLG_n$	$LG_n + RLG_n$
NT: hNTS1 ₃₂₁₋₃₄₄	82		
hNTS1 ₃₂₁₋₃₄₄ : $(\alpha-2)_2$		80	
NT:(α- 2) ₃			78
Model 1			
NT:hNTS1 _{321–344} :α -2	6 ± 3	70 ± 2	71 ± 2
Model 2			
NT:hNTS1 _{321–344} :(α -2) ₃	54 ± 8	61 ± 3	62 ± 3



Figure 5: $hNTS1_{321-344}$: (a-2)₂ complex; backbone superimposition of the 15 $hNTS1_{321-344}$ conformers. Superimposition between residues R323-Y328 (A), residues I329-W334, (B) and residues T335-H343 (C) are presented in ribbon format. For the sake of clarity, the peptide is presented without side-chains and only the aromatic ring of the a-2 molecules is displayed in ball and stick.



Figure 6: Chemical shift perturbation along the sequence of NT upon addition of a-2. The values were calculated as $\Delta \delta = (\Delta H N^2 + \Delta H a^2)^{1/2}$ (see text). The NT/a-2 ratio was 1/8.

69x43mm (300 x 300 DPI)

	hNTS1 ₃₂₁₋₃₄₄ : $(\alpha-2)_2$	NT:(α -2) ₃
Number of structures	15	18
Distance restraints		
Peptide intramol. NOEs		
Intraresidue	125	85
Sequential	84	39
Medium range	19	16
Peptide:glucogallin		
intermol. NOEs	7	12
Rmsd analysis (Å)		
R323-Y328backbone	0.45	
I329-W334backbone	0.19	
T335-H343 backbone	0.48	
Leu2-Asn5	0.10	0.48
Arg8-Ile12		0.44
		0.11
NOE violations per molecule		
Total violations ≥0.3 Å	0	0.06 ± 0.23
average (viol. ≥ 0.3 Å)	0	0.35 ± 0.01
Total violations >0.2 Å	1.5 ± 0.9	0.06 ± 0.23
Total violations $\ge 0.1 \text{ Å}$	1.1 ± 0.9	0.11 ± 0.32
Deviation from idealized		
geometrv ^a		
Bonds (Å)	0.02 ± 0.01	0.02 ± 0.01
Angles (°)	0.7 ± 2.7	0.3 ± 2.4
Ramachandran regions		
(Procheck-NMR %)		
Most favored	83.8	00.7
Additionally allowed	16.2	90.7
Generously allowed	0	9.5
Disallowed	0	0
Disanoweu	0	U

are ference bonds: NC, CO, C_AC, C_AC_B, NC_A and reference angles: CNC_A, C_ACN, C_ACO, C_BC_AC, NC_AC, NC_AC, NC_ACB, OCN.



Figure 7: NT:(a-2)₃ complex; backbone superimposition of the 18 NT conformers. Superimposition between residues Leu2-Asn5 (A) and residues Arg8-Ile12 (B) are presented in ribbon format. For the sake of clarity, the peptide is presented without side-chains and only the aromatic ring of the a-2 molecules is displayed in ball and stick.





Figure 9: Chemical shift changes along the sequence of the hNTS1₃₂₁₋₃₄₄:a-2 complex upon addition of NT. The values were calculated as $\Delta \delta = (\Delta HN^2 + \Delta Ha^2)^{1/2}$ (see text). The ratio of hNTS1321-344/a-2/NT was 1/8/1.

139x64mm (300 x 300 DPI)





Figure 11: Chemical shift changes along the sequence of the NT:a-2 complex upon addition of the hNTS1 fragment. The values were calculated as $\Delta \delta = (\Delta HN^2 + \Delta Ha^2)^{1/2}$ (see text). The ratio NT/a-glucogallin/hNTS1₃₂₁₋₃₄₄ was 1/8/1.

69x36mm (300 x 300 DPI)



Figure 12: Comparison of chemical shifts along the NT sequence between the ternary NT:a-2:hNTS1₃₂₁₋₃₄₄ complex (ratio 1/8/1) and the binary NT/hNTS1₃₂₁₋₃₄₄ complex (ratio 1/1). The values were calculated as $\Delta \delta = (\Delta HN^2 + \Delta Ha^2)^{1/2}.$

70x35mm (300 x 300 DPI)



Supplementary material

Both hypotheses may result in the scheme below, but the classical equilibrium and conservation equations (Wilkinson, 2004) were not relevant and have been modified.

$$R + L \qquad \stackrel{K}{\leftrightarrows} \qquad RL$$

$$R + 2G \qquad \stackrel{K_1}{\leftrightarrows} \qquad RG + G \qquad \stackrel{K_2}{\leftrightarrows} \qquad RG_2$$

$$L + 3G \qquad \stackrel{K'_1}{\leftrightarrows} \qquad LG + 2G \qquad \stackrel{K'_2}{\leftrightarrows} \qquad LG2 + G \qquad \stackrel{K'_3}{\leftrightarrows} \qquad LG3$$

In considering that the sites were non-interacting, we obtained at the molecular level: $K_1 = k_a + k_b$ and $K_1K_2 = k_a k_b$ with a and b the two sites of hNTS1₃₂₁₋₃₄₄, and $K'_{1} = k_{a'} + k_{b'} + k_{c'}, K'_{1}K'_{2} = k_{a'}k_{b'} + k_{a'}k_{c'} + k_{b'}k_{c'}$ and $K'_{1}K'_{2}K'_{3} = k_{a'}k_{b'}k_{c'}$ with a', b' and erier c' the three sites of NT.

Model 1

For this model, there was a supplementary equation:

R + L + G⇆ RLG

Equilibrium equations:

[RL] = K [R][L] $[RG] = (k_a + k_b)[R][G]$ $[RG_2] = (k_a k_b)[R][G]^2$ $[LG] = (k_{a'} + k_{b'} + k_{c'})[L][G]$ $[LG_2] = (k_a, k_b, + k_a, k_c, + k_b, k_c) [L][G]^2$ $[LG_3] = (k_a, k_b, k_c) [L][G]^3$ $[RLG] = K k_{c'} [R][L][G]$

Conservation equations

$$[R_{T}] = [R] + [RL] + [RG] + [RG_{2}] + [RLG]$$
$$[L_{T}] = [L] + [LG] + [LG_{2}] + [LG_{3}] + [RLG]$$
$$[G_{T}] = [G] + [RG] + 2 [RG_{2}] + [LG] + 2 [LG_{2}] + 3 [LG_{3}] + [RLG]$$

Model 2

For this model, there was a supplementary equation:

 $R + L + 3G \rightleftharpoons$ RLG + 2G $RLG_2 + G$ ⇆ ⇆ RLG₃ Equilibrium equations: [RL] = K [R][L] $[RG] = (k_a + k_b)[R][G]$ $[RG_2] = (k_a k_b)[R][G]^2$ $[LG] = (k_{a'} + k_{b'} + k_{c'})[L][G]$ $[LG_2] = (k_a, k_b, + k_a, k_c, + k_b, k_c) [L][G]^2$ $[LG_3] = (k_a, k_b, k_c) [L][G]^3$ $[RLG] = K (k_b + k_{b'} + k_{c'}) [R][L][G]$ $[RLG_2] = K (k_b k_{b'} + k_b k_{c'} + k_{b'} k_{c'})[R][L][G]^2$ $[RLG_3] = K k_b k_{b'} k_{c'} [R][L][G]^3$

Conservation equations:

 $[R_{T}] = [R] + [RL] + [RG] + [RG_{2}] + [RLG] + [RLG_{2}] + [RLG_{3}]$

 $[L_T] = [L] + [LG] + [LG_2] + [LG_3] + [RLG] + [RLG_2] + [RLG_3]$

 $[G_T] = [G] + [RG] + 2 [RG_2] + [LG] + 2 [LG_2] + 3 [LG_3] + [RLG] + 2 [RLG_2] + 3 [RLG_3]$

From these equations, the calculation of free molecules was realized by iteration, which allowed to calculate the various bound species.

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