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Design of chimeric neuropeptide analogues based on galanin and substance P to obtain a possible effect in the treatment of Major depressive disorder

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Abstract: Major depressive disorder (MDD) and anxiety are two of the principal psychiatric disorders that affect humans and whose treatment is under current investigation. There are vast amounts of brain neuropeptides, which show the potential of becoming unique therapeutic targets. Some studies have reported the effects of neuropeptide structural analogues on certain depression-like behaviors.
Galanin (GAL) and substance P (SP) act as regulatory peptides that have been linked to several physiological functions, which include anxiolytic and antidepressant activities. These effects are mediated through G protein-coupled receptors (GPCRs) displaying a diversity of signal transduction pathways.
In this project the design, synthesis and effects of several chimeric and non-chimeric analogues of the neuropeptides galanin and substance P are described. Four methods were used throughout the project: microwave-assisted solid-phase synthesis (SPPS) to synthetize the chimeric analogues, high-performance liquid chromatography (HPLC) to purify the crude molecules, electrospray ionization mass spectrometry (ESI-MS) to verify the theoretical molecular weight of the analogues and radioligand competition binding studies to assess the neuropharmacological activity of the ligands.
Results showed some inconsistencies related to synthesis, purification and characterization of the ligands most likely due to technical and operational problems, so they cannot be taken as conclusive without further optimization of the techniques. After achieving optimization, the ligands tested might potentially work as therapeutic drugs for in vivo studies (using animal models) and hopefully even reach pre-clinical and clinical trials in the future.

Keywords: Chimeric Neuropeptides, Drug Design, Galanin, Major Depressive Disorder, Substance P

1. INTRODUCTION

Major Depressive Disorder. Mood disorders are among the most common psychiatric diseases from which Major Depressive Disorder (MDD or simply depression) and anxiety disorders are the principal ones. Nowadays, depression is considered the fourth major cause of disability in the world, reaching up to 20% of the population throughout lifetime and interestingly affecting with higher frequency to female subjects [1, 2].
In spite of the vast quantity of studies, there is no evidence of the exact molecular mechanisms that cause depression. However, the most accepted theory is known as the

monoamine imbalance. There are also several risk factors that can increase the likelihood of depression to appear such as: age, gender, genetics and neurobiology. Antidepressant drugs have been tested in rodents since it is assumed that they resemble human metabolism [3, 4].
People suffering from depression display an extensive range of symptoms that affect their daily activities and can worsen diseases that they already have e.g. cancer, diabetes, cardiovascular and Parkinson's disease. Moreover, depression has been classified into 12 different types according to their etiology such as major depression, persistent depressive disorder, psychotic depression, postpartum depression, seasonal affective disorder, etc. [4].

Galanin. It is a regulatory peptide that was discovered in 1983 at Karolinska Institutet by a research group led by Estonian Professor Viktor Mutt. GAL is widely distributed in both, the central nervous system (CNS) and the peripheral nervous system (PNS) of at least 15 vertebrate species where the mature peptide proteolytically processed from preprogalanin is 29 amino acids long except in humans and macaque, where GAL is formed by 30 residues whose C-end is not amidated and by 32 residues whose C-end is amidated, respectively [5]. The first 14 residues of galanin are conserved with exception of tuna and Astatotilapia burtoni [6].

Galanin receptors. Currently, three human and rodent galanin receptor types have been isolated and cloned, they are: GAL1R, GAL2R and GAL3R which belong to the family of G protein-coupled receptors (GPCRs) and lead to the activation of different signal transduction pathways. Figure 1 shows a simple description of the galanin system signaling modified from Lautatzis and Vrontakis, (2011) (7).

Substance P. Substance P is an undecapeptide member of the tachykinin family that was isolated in 1931, whose higher density has been registered in substantia nigra. SP has been the first mammalian neuroactive peptide studied within the CNS, mainly due to its binding preference towards NK_1R [9, 10]. Currently SP is considered one of the most important neurotransmitters and neuromodulators of the brain and there is an extensive amount of evidence supporting this hypothesis. Furthermore, SP's pharmacology has shown to be relevant in many neurological and psychiatric disorders, which have generated an exceptional research effort, both clinically and preclinically, to identify appropriate NK_1R antagonists that can work as effective drugs. The biologically active part of this neuropeptide is located at the C-terminus according to SAR studies [11].

Peptide design. The N-terminus of GAL has been found critical to exert biological activity, whereas the C-terminus is thought to have a protecting effect against proteases [8].
Biological activity studies of SP have indicated that in order to exert its effects is required a segment and not the complete sequence. It has been suggested that Phe8 and Leu10 are the main residues involved for binding to its receptor. Recently, the addition of Phe or Tyr residues have been tested to form SP analogues [9].

2. RESEARCH ELABORATIONS

Biological activity studies of SP have indicated that in order to exert its effects is required a segment and not the complete sequence. It has been suggested that Phe^8 and Leu^{10} are the main residues involved for binding to its receptor. Recently, the addition of Phe or Tyr residues have been tested to form SP analogues [9].

Ligand Design. In Table 1 is indicated the amino acid sequence of the original neuropeptide analogues used throughout the design and Table 2 indicates the relative affinities towards the receptors of interest, ND = Not Determined (8, 12, 13).

Table 1. Patron sequences used for the design

Name	Sequence of amino acids
Rat galanin	GWTLNSAGYLLGPHAIDNHRSFSDKHGLT- NH_2
Galanin (2–11)	WTLNSAGYLL - NH_2
Ala^5- GAL (2–11)	WTLNAAGYLL - NH_2
Galanin (1–16)	GWTLNSAGYLLGPHAI- NH_2
Substance P	RPKPQQFFGLM - NH_2

Table 2. Affinity Constant of the parental ligands

Name	Ki (nM) for Gal_1R	Ki (nM) for Gal_2R	Ki (nM) for Gal_3R	IC_{50} (nM) for NK_1R	References
Rat Galanin	1.0	1.5	1.5	ND	Webling, et al. (2012)
Galanin (2–11)	>100000	14.6±2.2	186±74	ND	Webling, et al. (2016)
Ala^5- Gal (2–11)	>100000	258 ± 68	>100000	ND	Webling, et al. (2016)
Galanin (1–16)	4.8	5.7	50	ND	Webling, et al. (2012)
Substance P	ND	ND	ND	2	Maggi and Lecci (1994)

Solid-phase peptide synthesis. The whole synthesis process deals with a stepwise C- to N-terminal assembly of a polypeptide chain immobilized on a solid support matrix, for this project the rink amide ChemMatrix resin (PCAS Biomatrix, Canada) was used as support since the peptides intended for synthesis were all designed to be amidated at the N-terminus. After swelling the resin with DMF and in order to activate and direct the reaction of peptide bond formation, the ethyl cyanohydroxyiminoacetate (Oxyma) compound was used. Then, the Fmoc group needed to be removed by applying 20% piperidine dissolved in DMF. After this, the previously activated ester of the N-protected aa residue can react with the free Nα-amine group of the aa that is attached to the resin at the C-terminus through its linker. In this way, the first Fmoc-aa was incorporated and subsequently the other aa in order to elongate the polypeptide chain. Once the peptide reached the desired length it was necessary to separate it from the solid support, namely the cleavage of the peptide and other side chain protecting groups from the resin by treatment with trifluoroacetic acid (TFA).

Throughout this project the synthesis process was implemented on an automated peptide synthesizer (Biotage® Initiator + Alstra™, Sweden); where the target sequence was typed in and the outcome was a table whose values indicated the amount of reagents needed. All the 9-Fluorenylmethyloxycarbonyl (Fmoc) protected amino acids were purchased from Iris Biotech (GMBH, Markfredwitz, Germany) except for Fmoc-Asn (Trt)-OH and Fmoc-Leu-OH, both purchased from GL Biochem (Shanghai, China). In general terms all the syntheses were performed in a 10 mL reaction vial, loading = 0.50 mmol/g, scale: 0.05 mmol. According to those values, 100 mg of the Rink amide ChemMatrix Resin were placed into the reaction vial. The total synthesis time was approximately 17 hours long in average for all the peptides except DD_5 that was the shortest.

After retrieving the synthesis product it was needed to prepare a 2-3 mL cleavage cocktail, that consisted of 95% TFA (Iris Biotech, Germany), 2.5% water and 2.5 % triisopropylsilane (TIS) (Sigma-Aldrich) acting as scavengers. The cocktail was prepared right before using it and left to react with the resin and the synthesis product in a 10 mL syringe. After a 3-hour incubation period the resulting solution was subjected to dropwise precipitation in cold

diethyl ether over an ice bench using a Falcon tube. Then, the resin was rinsed one time with 1 mL TFA and poured into diethyl ether again. Afterwards, the sample was centrifuged at 5000 G during 5 minutes and the supernatant discarded. Two more washing steps using 25 mL ether were necessary to get rid of TFA residues. Then the sample tube was left in a fume hood with its lid off until the excess of ether completely evaporated, leaving only the crude peptide that after adding a few milliliters of deionized water was left at -80ºC and then lyophilized overnight.

Mass Spectrometry. A small aliquot of each synthesis sample was dissolved in 25 μL of Acetonitrile (ACN) and sent for mass determination by ESI-MS, Voyager-DESTR (Applied Biosystems). In total, all the crude analogues were tested once and two of them DD_1 and DD_4 were tested again after purification through RP-HPLC.

Reversed-Phase High-Performance Liquid Chromatography. When the expected mass was confirmed by ESI-TOF MS, the analogues were purified by RP-HPLC BioBasic C-8 column (ThermoFisher, Sweden), where up to 5 mg of the frozen sample were weight and dissolved using 200 μL of Acetonitrile (ACN) and 800 μL deionized water inside an Eppendorf tube. Higher volumes of ACN where needed in some cases depending on the consistency of the sample given that, it is crucial to get a translucent solution before transferring it to the HPLC injection vial which was set for running during 56 minutes and the fractions collected manually in 15 mL falcon tubes. All the fractions were labeled according to their retention time (RT) along each run. The detector was set using different wavelengths where the peptide bond and aromatic groups could be identified, usually 215 nm and 238 nm. Afterwards, 25 μL of each collected fraction were taken as a sample for mass determination analysis through ESI-TOF MS one more time.

Cell Culturing. Three different cell lines expressing each type of GALR, namely: Bowe's cells for GAL_1R, CHO cells for GAL_2R and Flp-In T_REX for GAL_3R were cultured and harvested. First, the ventilation hood was started around 30 minutes before working with cell cultures. Then, the media, trypsin and other components needed during the cultivation process were placed in a water bath until they got thawed.

The work surface of the hood and all the required instruments during the procedure such as flasks, falcon tubes, strippets of different volumes and waste containers were sprayed with 70% ethanol and wiped properly before usage. Once all the material and reagents were inside the hood it was possible to cultivate and subcultivate the cells in their specific culture media. Cell cultures were stored in 75 cm^2 Corning® cell culture flasks.

When all the media was completely removed from the flasks taking care of not affecting the cells, it was needed a quick rinse with a few milliliters of trypsin that were then completely removed. After that 1 mL of trypsin were added and placed for incubation at 37ºC for a few minutes so that the enzyme can act on the cells. The incubation time is different depending on the type of cell line. Trypsin acts by cutting amino acids, specifically lysines or arginines, on their C-terminus unless these amino acids are followed by a proline. Which means that in this case the proteins that are anchoring the cells to the flask surface will be cut off, allowing cell detachment from the surface. Then 4,5 mL of fresh prepared media were added (to inactivate the enzyme) and mixed around 10 times with the pipette in order to make single cells.

In order to subcultivate two additional 75 cm^2 flasks in ratio 2:5, 2 mL of the cell suspension were added and the volume completed with fresh media until reaching a final volume of 25 mL. Finally all the cultures were properly labeled indicating the date, the type of cell line and

receptor, the passage, the name of the operator and the splitting ratio e.g. 1:10. The viability of all cell lines was verified in a Microscope Olympus Sunn 311 with software Viewfinder 3.0.1.

Radioligand competitive binding studies. Three types of solutions were prepared:

HM buffer (5X): Composed by 23.83 g HEPES + 5.08 g $MgCl_2$ in 1 L ddH_2O
HM buffer (1X): Composed by 4.77 g HEPES + 1.016 g $MgCl_2$ in 1 L ddH_2O
HM-BSA: Composed by 0.3 g BSA dissolved in 100 mL HM-buffer
PEI solution (0.3%): Composed by 1.2 mL of 50% solution in 200 mL ddH_2O

The plastic material required included silanized and unsilanized tips, silanised 15 mL tubes (for dilution of the radioactive labeled GAL), silanised 2 mL eppendorf tubes (for the dilution series of the tested ligands) a regular 96-well plate, a silanised 96-well plate and a 96-well filter plate. To make the dilution series with the ligands it was necessary to use silanised tips in order to prevent nonspecific binding. The range of logarithmic concentrations for the dilution series went from -4 M (highest) to -11 M (lowest). Each step contained 180 µL of HM buffer and 20 µL of ligand/peptide solution. Unsilanised tips were used for pipetting 200 µL of PEI to the 96-well filter plate and silanised tips for pipetting 200 µL of HM-BSA to the 96-well plate. Every well of the 96-well plate was filled with 20 µL of peptide solution in duplicates for each logarithmic concentration as indicated in Figure 1.

	LIGAND						CONTROL						
	1	2	3	4	5	6	7	8	9	10	11	12	[M]
A													-11
B													-10
C													-9
D													-8
E													-7
F													-6
G													-5
H													-4
	GAL1R		GAL2R		GAL3R		GAL1R		GAL2R		GAL3R		

Figure 1. Experimental design applied for the binding assay on a 96-well plate

Then 150 µL equivalent to 30 µg of membrane preparation were added to the unsilanized 96-well plate, the volume was adjusted by using HM-BSA and the concentrations calculated with a Bradford standard curve (Figure 2). Membrane preparations for the three types of GALRs and the purified analogue GAL (1-16) used as control were kindly provided by supervisor Kristin Webling. Afterwards, Porcine - [125I] - Galanin (2200 Ci/mmol, Perkin-Elmer Life Science) was diluted based on the next equation:

$$\textit{Volume of 125I- Galanin} = 1.25\ \mu L * \textit{number of wells used} + 2$$

Then, 30 µL of the diluted 125I-Galanin were incorporated into each well of the silanised 96-well plate.

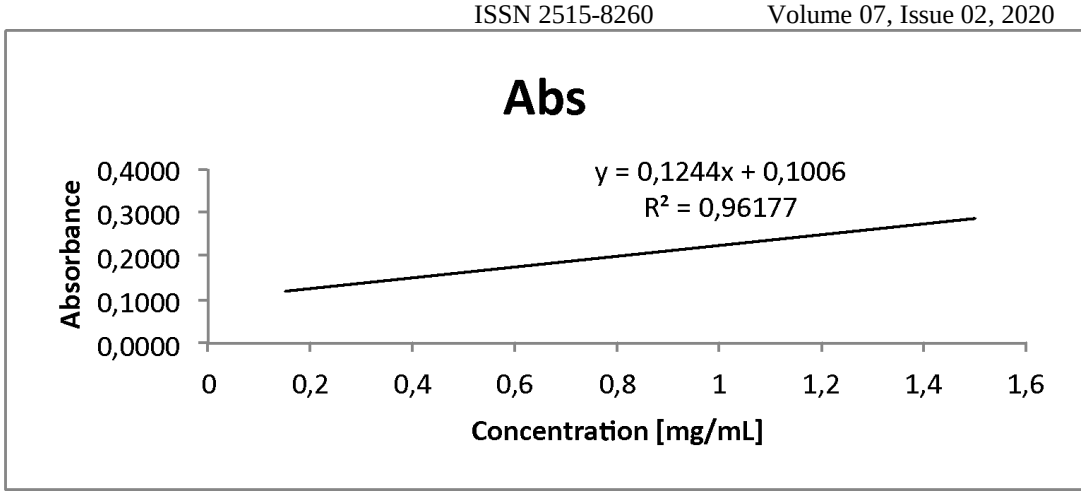

Figure 2. Standard curve to assess the concentration of membrane preparations

Everything was transferred from the unsilanised 96-well plate to the silanised 96-well plate (So that the wells contain all the components giving a final volume of 200 µL) and incubated during 30 minutes at 37 °C while shaking. Before transferring everything from the silanised 96-well plate to the filter plate, it was needed a washing step with HM-buffer and after that, 3 more washing steps with 200 µL HM-buffer, this was done while applying vacuum filtration to the plate that was left for drying a few minutes. Eventually all the filters were collected with tweezers and placed into scintillation tubes, each containing 2 mL scintillation fluid. All the tubes were carefully labeled in order to facilitate data processing.

Inside the β-counter rack the arrangement was as follows: just scintillation fluid (a background that was subtracted from all samples according to the program), a vial with 30 µL 125I-Galanin in 2 mL scintillation fluid OptiPhase Supermix Cocktail (Perkin-Elmer Life Science) for a maximum reading (to indicate the state of the radioactive mixture) followed by a gap and then the rest of the samples in duplicates according to the order of logarithmic concentration and each receptor subtype at a time. The radioactivity was measured in counts per minute (cpm) using a β-counter (Tri-Carb Liquid Scintillation Analyser, model 2500TR, Packard Instrument Company).

Statistical analysis. All the raw data in duplicates from two independent experiments were normalized and fitted to a sigmoidal dose-response with variable slope. The analysis was carried out using GraphPad™ Prism® 7.00 for MacOS Sierra (GraphPad Software, La Jolla, California USA, www.graphpad.com).

3. RESULTS

Ligand Design. Throughout this project, six syntheses were carried out in total. However, only three were successful. Their sequences and nomenclature are described in Table 3, residues marked in red are the ones that were introduced or changed as part of the modification scheme proposed for this project.

Table 3. Chimeric and non-chimeric ligands that were synthesized

Analogues	Name	Sequence of amino acids
DD_1	GAL (2–11) -SP(1-8)- F^{19}- LM -NH_2	WTLASAGYLLRPKPQQFFFLM- NH_2
DD_2	GAL (2–11) - GLEY - SP(5-11) -NH_2	WNSAGYLLGPHGLEYQQFFGLM- NH_2
DD_3	GAL (2–11) -SP(5-9) - G^{16}- LM -NH_2	WTLASAGYLLQQFFGGLM - NH_2
DD_4	GAL (1–15) - Q^{16} - SP(5-11) -NH_2	GWTLNSAGYLLGPHAQQQFFGLM- NH_2

DD_5	A^5 - a^6 - GAL (2–11) -NH_2	WTLNAaGYLL- NH_2

Five ligands were designed and synthesized, four chimeric ($DD1_4$) and one (DD_5) based only on a GAL analogue. One of the designs were tested for binding affinity towards the three GALRs since all of them have been linked with anti-depressant effects i.e. agonists targeting GAL_2R and antagonists targeting GAL_1R and GAL_3R (8). Binding affinity towards NK_1R was not tested for any of the ligands.

A summary of the main fragments encountered prior to purification is indicated in Table 4.

Table 4. Main fragments found by ESI-MS before purification process

Crudes	Expected mono. MW [Da]	Observed mono. MW [Da]	Correct Ligand
DD_1	2512,335	2515,2	YES
	1662,932	1632,8	NO
DD_2	1847,929	1860,8	NO
	1847,929	1925	NO
DD_2 V2	1847,929	1860,8	NO
	1847,929	1862,8	NO
DD_3	2001,007	2089	NO
	2001,007	2089,2	NO
DD_4	2534,242	2530	YES
	2534,242	2535	YES
	2534,242	2535,2	YES
DD_5	1119,597	1120	YES
	1119,597	1119,6	YES

Purified Ligands. Only two out of the five proposed analogues were confirmed by MS after purification: DD_1 and DD_4. Crude DD_5 was intended for purification but the attempt went wrong when using by mistake a different column.

The high byproduct concentration formed during DD_1 synthesis, required a purification step. After this process, the fractions collected containing the right analogue were minimal and thus useless for subsequent analysis. Fragments found by ESI-MS after purification are summarized in Table 5.

Table 5. Fragments found by ESI-MS after purification process

Purified	Expected mono. MW [Da]	Observed mono. MW [Da]	Correct Ligand
DD_1	2512,335	2514,9	YES
DD_4	2534,242	2535,2	YES

Radiolabeled ligand competitive binding studies. The generated curves for the first competitive binding assay are included in Figure 3, the main indicators are shown.

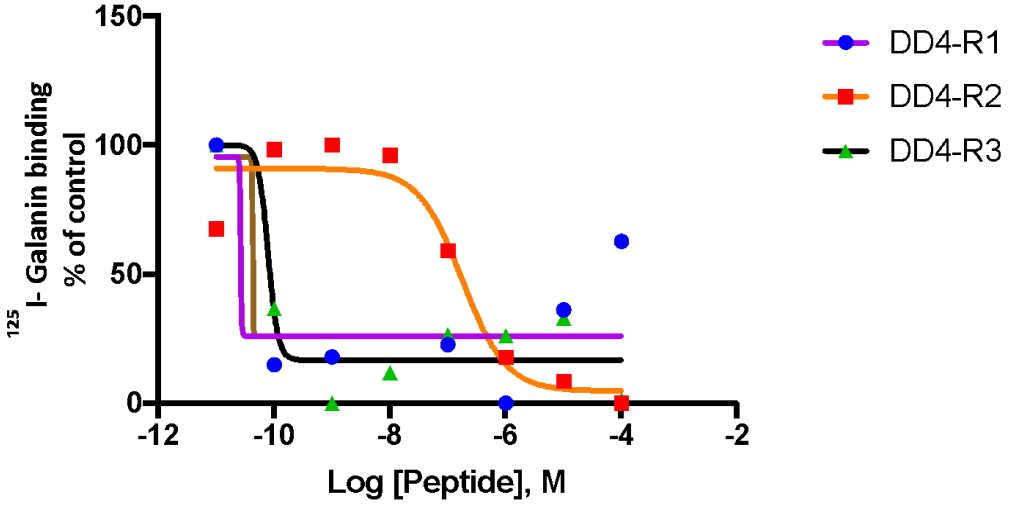

Figure 3. Curve for galanin receptor binding studies against ligand DD_4 (1st technical replicate)

4. CONCLUSIONS

At the beginning of this project five chimeric sequences derived from galanin and substance P were proposed. Three chimeric analogues out of six attempts were successfully synthesized (DD_1, DD_4, and DD_5) and two out of those ligands were purified by RP-HPLC (DD_1 and DD_4).

Only one ligand was tested for competitive binding studies (DD_4). The results for binding are preliminary, further testing and optimization of the methods are suggested. In order to avoid minimal synthesis products a change of scale should be implemented.

Once affinity values correlate within every technical replicate it is mandatory to carry out signal transduction analysis in order to determine the efficacy and potency of the ligands, namely to differentiate their behavior i.e. agonistic, antagonistic, etc.

It is required to repeat the experiment with tissue preparations that express all the types of GalRs and NK_1R simultaneously to evaluate the extent of influence shown by the intramolecular interactions within the chimeric peptides. Two possible brain regions where these ligands could be tested are the hippocampus and prefrontal cortex, since both regions are very sensitive to stress that can lead towards mood disorders.