Structure-Activity Relationship Studies on Oxazolo[3,4-a]Pyrazine Derivatives Leading to the Discovery of a Novel Neuropeptide S Receptor Antagonist With Potent In Vivo Activity

Valentina Albanese¹, Chiara Ruzza²³, Erika Marzola¹, Tatiana Bernardi¹, Martina Fabbri¹, Anna Fantinati¹, Claudio Trapella¹³, Rainer K. Reinscheid⁴⁵, Federica Ferrari², Chiara Sturaro², Girolamo Calò⁶, Giorgio Amendola⁷, Sandro Cosconati⁷*, Salvatore Pacifico¹⁵, Remo Guerrini¹³ and Delia Preti.¹

¹ Department of Chemical, Pharmaceutical and Agricultural Sciences, University of Ferrara, Via Luigi Borsari 46, 44121 Ferrara, Italy. ² Department of Neuroscience and Rehabilitation, Section of Pharmacology, University of Ferrara Via Fossato di Mortara 17/19, 44121 Ferrara, Italy. ³ Technopole of Ferrara, LTTA Laboratory for Advanced Therapies, Ferrara, Italy. ⁴ Institute of Pharmacology and Toxicology, Jena University Hospital, Friedrich Schiller University, Jena, Germany. ⁵ Institute of Physiology I, University Hospital Münster, University of Münster, Münster, Germany. ⁶ Department of Pharmaceutical and Pharmacological Sciences, University of Padova, Largo Meneghetti, 2 - 35131 Padova, Italy. ⁷ “DiSTABiF”, Università della Campania “Luigi Vanvitelli”, Via Vivaldi 43, 81100 Caserta, Italy.

SUPPORTING INFORMATION

| CONTENTS                                                                 | Pag.  |
|------------------------------------------------------------------------|-------|
| Figures S1-S2: ¹H-NMR and NOE NMR analysis for compound 17             | S2-S3 |
| Table S1: Human GPCRs sharing with NPSR a sequence identity higher than 20%, a sequence coverage higher than 70%, and that were crystallized in their inactive states. | S4    |
| Figure S3: Phylogenetic tree of the human NPSR and the six selected human GPCRs used as template structures. | S5    |
| Figures S4-S9: pairwise sequence alignments of the human NPSR and the six selected human GPCRs used as template structures. | S6-S11|
| Figures S10-S15: Ligand Root Mean Square Fluctuation (RMSF) of compounds 1, 16 and 21 | S12-S14|
| Figures S16-S27: Ligand-NPSR interactions for compounds 1, 16 and 21   | S15-S26|
| HPLC traces of the final compounds 3-21                                 | S27-S36|
Figure S1. A) Full $^1$H-NMR spectrum of compound 17; B) Aliphatic region of the $^1$H-NMR spectrum of compound 17 with proton assignments.
Figure S2. NOE $^1$H-NMR analysis of compound 17. A) Red arrows indicate that, as visible in the upper trace, irradiating the CH$_3$ protons produced an enhancement of the signals of Hc (geminal proton), Hb and H0 (that have a syn relationship with the irradiated methyl group). B) The green arrows show that the irradiation of H0, produced an enhancement of the signals of the CH$_3$ protons and of He that have a syn relationship with H0 (see the bottom trace).
Table S1. Human GPCRs sharing with NPSR a sequence identity higher than 20%, a sequence coverage higher than 70%, and that were crystallized in their inactive states.

| Receptor                                      | Considered PDB | Query Coverage | Sequence Identity | ECL2 conformation |
|-----------------------------------------------|----------------|----------------|--------------------|--------------------|
| Human C5a anaphylatoxin chemotactic receptor 1| 6C1R           | 79%            | 23%                | β-hairpin          |
| Human CC chemokine receptor type 9            | 5LWE           | 73%            | 21%                | Not Present        |
| Human κ opioid receptor                       | 4DJH           | 73%            | 24%                | β-hairpin          |
| Human M2 muscarinic receptor                  | 5ZKC           | 74%            | 26%                | Random Coil        |
| Human Neuropeptide Y Y1 Receptor              | 5ZBH           | 76%            | 22%                | β-hairpin          |
| Human orexin-1 receptor                       | 6TOD           | 75%            | 22%                | β-hairpin          |
| Human type-2 angiotensin receptor             | 4ZUD           | 74%            | 26%                | β-hairpin          |
Figure S3. Phylogenetic tree of the human NPSR and the six selected human GPCRs used as template structures.
Figure S4. Pairwise sequence alignment as calculated through the gpcrd.org webserver between human M2 muscarinic receptor and the human neuropeptide S receptor.
**Figure S5.** Pairwise sequence alignment as calculated through the gpcrdb.org webserver between human type-2 angiotensin receptor and the human neuropeptide S receptor.
| c5arl_human | -------------------------- MSDFYNTTPDYGHDDDLDNTVDPKTSNTLRVIDLALVI F |
| npsrl_human | MPANFTESFDSSGTQTLDSPPPVACTETVTEVEKGEWSFYFYSF---TEQLTL |
| c5arl_human | 44 |
| npsrl_human | 56 |
| c5arl_human | AVVFLVGVLGNALVWVTAFEAKRTIAIWFNLAVADFLSCLAPILFTISIVOHHPWP |
| npsrl_human | WLVFVFTIGNSVVLFSwRRKKKSRMFTFYVTQLAITDSFTGLVNIALTDIIWRTGDFTA |
| c5arl_human | 104 |
| npsrl_human | 116 |
| c5arl_human | GGAACSILPSLILLNMYASILLATISADRFLLVFPIWCAQFRAAGHLWIAACAVAWGLA |
| npsrl_human | PDLYCRVRLQVVLYASTYVLVSLSIDRYHAIVYPMKFL--QGEQARVLIVIAWLS |
| c5arl_human | 164 |
| npsrl_human | 174 |
| c5arl_human | LLLTIPSLIFLYV---VREYFPPKVLGVDYSHDRERRAVAILRLGFLWPLTLTIC |
| npsrl_human | FLFSIPTLIIFGKRLSNGEV---QCwALWP-DDSYwTPMTIVAFLVYFIPTIISIM |
| c5arl_human | 221 |
| npsrl_human | 229 |
| c5arl_human | YTFILLRTwSRRAT--------------------------RSTTKLVVVAVASFFIFWL |
| npsrl_human | YGIVIRIwIWSKTYETVISNCSDGKLCSSYNRGLISAKIKAYIIIILAFICWSP |
| c5arl_human | 257 |
| npsrl_human | 289 |
| c5arl_human | YQVGTIMMSFLEPSSPTFLKLDLSCLVFAYINCCINPIYVYVAGGQFGQRLR |
| npsrl_human | YFLFDILDNPNFLL--PDQERFYAVSIIQNPLANSAINPLICYVCSSSSISFPCREQRSQ |
| c5arl_human | 313 |
| npsrl_human | 347 |
| c5arl_human | SLPSLLRNLTEEESVRESKSFSRTVDTMAQKTAV |
| npsrl_human | DSRMTFRERTERHEOMLISKPEFI------------------371 |
| c5arl_human | 350 |
| npsrl_human | 371 |

**Figure S6.** Pairwise sequence alignment as calculated through the gpcrdb.org webserver between human C5a anaphylatoxin chemotactic receptor 1 and the human neuropeptide S receptor.
|     | npsrl_human | npy1r_human |
|-----|------------|------------|
|     | MPANFTEGSFDSGSGTQGTDSSPVACTETVTFEVVEGKEWGSFYYSFK----TEQLIT |          |
|     | ------------- | 55         |
|     | MNSTLSQVENHSVHNFSEKNAQOLLAFENDDCDHLPLAMIFTLALA |          |
|     | ** * *   * *  * *   *  *  *   *   *   *   *   *   *   *  | 46         |
|     |               |            |
|     | LWVLFVFTIVGNSVVLSTTWRRKK-KSRMTFFVTQLAITDSFTGL-VNILTDIIWRTGD | 113        |
|     | YGAVIIICVGSGNLALIIILIQKEMIRNVINIIILNVNLFSDDLALLVAIMCLPFTFVYTLMD-H | 105        |
|     |               |            |
|     | FTAPDLVCRVVRYLQVVLYASTYVVLVSLSDRHYHAVVYPMKFLQGEKQARVALIVIAWLS | 173        |
|     | WVGFEAMCKLNPFVQSCITSVSIFSLVLIAVERHLINPRGWPNRHRAYVGIAVIVVL | 165        |
|     |               |            |
|     | SFLSISPTLIIFGKRT--------------L5NGEVCWALWPDSSYwTPYMTIVAFLVYFIPLT | 224        |
|     | AVASSLPLFYQVMDEPFVNVTDLAYKOKVCFDQFPDSDFHRLSYTTLLLQVFPLC | 225        |
|     |               |            |
|     | IIISMYGYTrVTIWKSSTYETVISNCSDGKLCSSYNRLGRISAKIAIYKYSIIIILAFI | 284        |
|     | FICIFYFYKIFYRLKRNNNNLMOKR----------DNKYSSETKRIIMLLLSIVVAFA | 273        |
|     |               |            |
|     | CCWSPYLFDFDILDNFL-PTDQERFYASVIIQNLMASPAINLIVCVFSSISFPCR | 343        |
|     | VCWLPNTIFTVFDWNHQITATCNHNNLFFLCHLTAMISTCVNPIFYGFLNKNFQRDLQF | 333        |
|     |               |            |
|     | QRSQDSRMTFRERTERHMQILSKPEF |          |
|     | FF-NCDFRSIDDDYETIAMSTMHTDVSITSLKQASPVAFKKINNDDNKEI | 384        |

**Figure S7.** Pairwise sequence alignment as calculated through the gpcrd.org webserver between human neuropeptide Y Y1 receptor and the human neuropeptide S receptor.
Figure S8. Pairwise sequence alignment as calculated through the gpcrdb.org webserver between human κ opioid receptor and the human neuropeptide S receptor.
**Figure S9.** Pairwise sequence alignment as calculated through the gpcrb.org webserver between human orexin-1 receptor and the human neuropeptide S receptor.
Figure S10. Ligand Root Mean Square Fluctuation (L-RMSF) of 1 in MD1 broken down by atom, corresponding to the 2D structure in the top panel.

Figure S11. Ligand Root Mean Square Fluctuation (L-RMSF) of 1 in MD2 broken down by atom, corresponding to the 2D structure in the top panel.
Figure S12. Ligand Root Mean Square Fluctuation (L-RMSF) of 16 in MD1 broken down by atom, corresponding to the 2D structure in the top panel.

Figure S13. Ligand Root Mean Square Fluctuation (L-RMSF) of 16 in MD2 broken down by atom, corresponding to the 2D structure in the top panel.
Figure S14. Ligand Root Mean Square Fluctuation (L-RMSF) of 21 in MD1 broken down by atom, corresponding to the 2D structure in the top panel.

Figure S15. Ligand Root Mean Square Fluctuation (L-RMSF) of 21 in MD2 broken down by atom, corresponding to the 2D structure in the top panel.
Figure S16. Plot of the ligand-protein interactions between 1 and NPSR in BM1. Protein-ligand interactions (or 'contacts') are categorized into four types: Hydrogen Bonds (green bars), Hydrophobic (violet bars), Ionic (magenta bars) and Water Bridges (blue bars). The stacked bar charts are normalized over the course of the trajectory: for example, a value of 0.7 suggests that 70% of the simulation time the specific interaction is maintained. Values over 1.0 are possible as some protein residue may make multiple contacts of same subtype with the ligand.
Figure S17. Schematic of detailed ligand atom interactions between 1 and NPSR during the MD simulation in BM1. Interactions that occur more than 30.0% of the simulation time in the selected trajectory (0.00 through 100.00 ns), are shown.
Figure S18. Plot of the ligand-protein interactions between 1 and NPSR in BM2 throughout the simulation. Protein-ligand interactions (or 'contacts') are categorized into four types: Hydrogen Bonds (green bars), Hydrophobic (violet bars), Ionic (magenta bars) and Water Bridges (blue bars). The stacked bar charts are normalized over the course of the trajectory: for example, a value of 0.7 suggests that 70% of the simulation time the specific interaction is maintained. Values over 1.0 are possible as some protein residue may make multiple contacts of same subtype with the ligand.
Figure S19. Schematic of detailed ligand atom interactions between 1 and NPSR during the MD simulation in BM2. Interactions that occur more than 30.0% of the simulation time in the selected trajectory (0.00 through 100.00 ns), are shown.
**Figure S20.** Plot of the ligand-protein interactions between 16 and NPSR in BM1 throughout the simulation. Protein-ligand interactions (or 'contacts') are categorized into four types: Hydrogen Bonds (green bars), Hydrophobic (violet bars), Ionic (magenta bars) and Water Bridges (blue bars). The stacked bar charts are normalized over the course of the trajectory: for example, a value of 0.7 suggests that 70% of the simulation time the specific interaction is maintained. Values over 1.0 are possible as some protein residue may make multiple contacts of same subtype with the ligand.
**Figure S21.** Schematic of detailed ligand atom interactions between 16 and NPSR during the MD simulation in BM1. Interactions that occur more than 30.0% of the simulation time in the selected trajectory (0.00 through 100.00 ns), are shown.
Figure S22. Plot of the ligand-protein interactions between 16 and NPSR in BM2 throughout the simulation. Protein-ligand interactions (or 'contacts') are categorized into four types: Hydrogen Bonds (green bars), Hydrophobic (violet bars), Ionic (magenta bars) and Water Bridges (blue bars). The stacked bar charts are normalized over the course of the trajectory: for example, a value of 0.7 suggests that 70% of the simulation time the specific interaction is maintained. Values over 1.0 are possible as some protein residue may make multiple contacts of same subtype with the ligand.
**Figure S23.** Schematic of detailed ligand atom interactions between 16 and NPSR during the MD simulation in BM2. Interactions that occur more than 30.0% of the simulation time in the selected trajectory (0.00 through 100.00 ns), are shown.
Figure S24. Plot of the ligand-protein interactions between 21 and NPSR in BM1 throughout the simulation. Protein-ligand interactions (or 'contacts') are categorized into four types: Hydrogen Bonds (green bars), Hydrophobic (violet bars), Ionic (magenta bars) and Water Bridges (blue bars). The stacked bar charts are normalized over the course of the trajectory: for example, a value of 0.7 suggests that 70% of the simulation time the specific interaction is maintained. Values over 1.0 are possible as some protein residue may make multiple contacts of same subtype with the ligand.
Figure S25. Schematic of detailed ligand atom interactions between 21 and NPSR during the MD simulation in BM1. Interactions that occur more than 30.0% of the simulation time in the selected trajectory (0.00 through 100.00 ns), are shown.
Figure S26. Plot of the ligand-protein interactions between 21 and NPSR in BM2 throughout the simulation. Protein-ligand interactions (or 'contacts') are categorized into four types: Hydrogen Bonds (green bars), Hydrophobic (violet bars), Ionic (magenta bars) and Water Bridges (blue bars). The stacked bar charts are normalized over the course of the trajectory: for example, a value of 0.7 suggests that 70% of the simulation time the specific interaction is maintained. Values over 1.0 are possible as some protein residue may make multiple contacts of same subtype with the ligand.
Figure S27. Schematic of detailed ligand atom interactions between 21 and NPSR during the MD simulation in BM2. Interactions that occur more than 30.0% of the simulation time in the selected trajectory (0.00 through 100.00 ns), are shown.
Compound 3

Compound 4

S27
Compound 5

Compound 6
Compound 7

![Image of Compound 7](image1.png)

Compound 8

![Image of Compound 8](image2.png)
Compound 9

Compound 10
Compound 11

Compound 12
Compound 13

![Chemical Structure of Compound 13]

Compound 14

![Chemical Structure of Compound 14]
Compound 15

Compound 16

S33
Compound 17

Compound 18
Compound 19

Compound 20
Compound 21