Response to Reviewers

TO: Prof. Dr. Dejan Opsenica

Editor

Journal of the Serbian Chemical Society

Dear Prof. Dejan Opsenica

Please find enclosed the revised manuscript entitled "-Opioid/D2 dopamine receptor pharmacophore containing ligands: Synthesis and pharmacological evaluation".

We would like to acknowledge Reviewers for dedicated work on our manuscript and for useful advices. In the following lines we offer detailed response to Reviewer’s comments; accordingly, changes have been made in the main text of the manuscript, the experimental section and the supporting information.

 All those revised documents/files (including corrected SUPPLEMENTARY MATERIAL with added images of the NMR spectra of the final compounds) will be uploaded together with this response to the decision letter.

 We hope that the manuscript in present form will be suitable for publishing in *Journal of the Serbian Chemical Society*

 Sincerely,

 Dr Slađana Kostić-Rajačić

The List of responses to the comments:

**Reviewer A**:

Recommendation: Minor Revision

Comments:

“While the biological results did not yield promising results, the general idea and the chemical aspect of the manuscript is thorough and well-written and as such the submitted manuscript should be suitable for publication in Journal of Serbian Chemical society, after a minor revision. Namely, H and C NMR spectra of the final compounds are missing. If the general practice of the journal is that the spectra of final compounds should be presented, it is advised for the authors to provide the H and C 13 spectra of the final compounds.

In my opinion, this manuscript should: be published after minor revision without additional review

If manuscript is suitable for publishing, referee’s recommendation: Original scientific paper”

1) “Line 99-101: Provided data on the quantity of compounds is not in accordance with the data presented in scheme 1. Please correct either entry.”

**Authors response to the comment 1:** We appreciate these observations from the Reviewer. Changes are made as suggested and these parts of the manuscript are highlighted green.

**Reviewer C**:

Recommendation: Minor Revision

Comments:

“The results of the pharmacological evaluations are not satisfactory. Starting from the results and in correspondence with the title, this study includes an evaluation of the capacity of the compounds as a pharmacological potential.

Additional comments: “They are highlighted in the manuscript ˮ

In my opinion, this manuscript should: be published after minor revision without additional review

If manuscript is suitable for publishing, referee’s recommendation: Original scientific paper ˮ

**Authors response to the comment:** We appreciate these observations from the Reviewer. However, the manuscript with Reviewer’s additional comments was not available for download from the JSCS website.

**Reviewer I**:

Recommendation: Not for publishing

Comments:

“The authors have presented a manuscript mainly with negative results. In this case, negative results can be interesting to design pharmacological therapies based on antagonists, but Ki values should be determined. The paper is well written and the research line is undoubtedly interesting. However, the experimental approach has several obscure points that should be clarified before publication.

In my opinion, this manuscript should: not be published for the reasons indicated above

If manuscript is suitable for publishing, referee’s recommendation: \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ ˮ

1) “Line 131. The authors incubate the reaction for 10 min, which is rather a short incubation time. Why? Are there any previous kinetic experiments or references regarding this short incubation time?”

**Authors response to the comment 1:** We appreciate these observations from the Reviewer. References regarding this issue are:

1. Hall, H. at all. European Journal of Pharmacology 111, (1985) 191-199
2. Tomic, M. at all. Pharmazie 58, (2003) 677-678
3. Penjišević, J. at all. Archiv der Pharmazie 349, (2016) 614-626

2) “Line 209. The authors claim that “Opioid activity of propanamides 1a-1m was estimated using in vivo tests only, since in vitro tests were not available”. This is not true, as they can perform radioligand binding (competition binding assays) with [3H]-DAMGO following the same methodology that they have used for dopamine receptors.”

**Authors response to the comment 2:** We appreciate these observations from the Reviewer. We chose in vivo antinociceptive test since it produces more clinically reliable information about the analgesic activity in the animal models than in vitro experiments in the laboratory.

Accordingly, clarification is made and this part of the manuscript is highlighted green.

3) “Besides, when they perform competition binding assays for the dopemine receptor, they use [3H]-spiperone as radioligand, and the nonspecific binding is determined with spiperone as well. Non specific binding should be performed with a different ligand.”

**Authors response to the comment 3:** We appreciate these observations from the Reviewer.

Before in vitro binding assay, the Bmax and Kd values were derived from linear-regression analysis of the Scatchard plots, using six different concentrations of 3H-spiperone, each done in triplicate. The specific 3H-spiperone binding to D2 dopamine receptors was defined as the amount of bound 3H-spiperone that is inhibited by the presence of 10 M spiperone. The values for the maximum number of binding sites (Bmax) and the dissociation constant (Kd) were determined by Scatchard analysis. This explanation is corroborated by the references cited below:

1. Penjišević, J. at all. Archiv der Pharmazie 349, (2016) 614-626
2. Penjišević, J. at all. J. Serb. Chem. Soc. 84, (2019) 925–934
3. Kessler, R. et all. M. J. Nucl. Med. 32, (1991) 1593-1600

4) “They also do not include a positive control, i.e. a ligand that yields good Ki values (e.g. spiperone itself).”

**Authors response to the comment 4:** We appreciate these observations from the Reviewer. Changes are made as suggested and these parts of the manuscript are highlighted green.

5) “Another serious issue is that they do not give a dispersion measures, as for example SD or SEM, together with the obtained Ki value for each compound. Although they indicate that the experiment was performed in triplicate, they do not indicate the replicates. Therefore I understand that the experiment was performed once in triplicate, and at least three independent experiments should be done to obtain reliable data.”

**Authors response to the comment 5:** We appreciate these observations from the Reviewer. Changes are made as suggested and these parts of the manuscript are highlighted green.

6) “line 210, the authors claim that “The complete absence of opioid activity (Table I), does not rule out the affinity 210 to MOR, as the ligands may not be able to cross brain-blood barrier”. This statement has a clear conceptual error. The authors use the tail flick assay as an antinociceptive test, which is well chosen. However, this test is based on a spinal reflex, and does not imply central processing, nor the compound has to cross the blood-brain barrier.”

**Authors response to the comment 6:** We appreciate these observations from the Reviewer.

Opioid receptors are distributed in the brain and the spinal cord. Since synthesized ligands were administered to the animals by intraperitoneal injection (i.p.), lack of the antinociceptive activity in *in vivo* tests may be due to inability of the ligands to reach the opioid receptors caused by physicochemical characteristics and/or metabolism. Due to the physicochemical characteristics ligands may not be able to cross blood-brain or in the case of tail immersion test blood-spinal cord barrier. Abolishing antinociception with -opioid antagonist, if the exanimated ligands appear to be active, is a proof of opioid receptors involvement in analgesic effect of the ligands.

Changes are made as suggested and this part of the manuscript is highlighted green.