Referee's Report on the submitted Ph. D. thesis of D. Biyashev entitled Biological Activity of Structurally Modified Opioid Ligands

D. Biyashev prepared this treatise at the Institute of Biochemistry of Biological Research Center. This thesis was supervised by Dr. Anna Borsodi.
The purpose of thesis was to study the structure activity relationships for newly synthesized morphine-like compounds and opioid peptide analogues.
Biological activities of the new substances have been determined in rat brain membranes using in vitro radioligand binding experiments and ^{35}S-GTPγS binding assays.

I guess the selection of the research project is up-to-date and the presentation of the results is good. The biochemistry and pharmacology of opioids is a very exciting research topic since the cloning of opioid receptors a large body of work has been dedicated to the identification of binding sites for opioid ligands and of regions of the receptors responsible for selectivity.

The design of the treatise is proportional. It contains 67 pages including 5 main chapters and finally the literature references. The first chapter is a good survey about opioid receptors (p. 1-16). The second chapter gives a brief (p. 17) summary about the scientific purpose of the work while the third chapter (p. 18-22) is an experimental part of the thesis. The fourth chapter presents the new results and the conclusions can be found in the last chapter.

New Scientific Results of the Thesis

1. New derivatives of naltriben and naltrindole were synthesized in order to study δ receptor selectivity and affinity. The rationale for the design of naltrindole was based

on the so-called message-address concept. Naltrindole is a δ opioid receptor antagonist with high δ affinity and good selectivity as found in bioassays.

O-alkylation of C-14-OH group has significant influence on δ receptor selectivity. A C-14 ethoxy group in indolomorphinans seems to be superior to both a C-14 methoxy or propoxy group. C-14-O-ethyl ether of naltriben shows higher δ receptor selectivity than that of the corresponding ethyl ether of naltrindole. O-aralkylation of the C-14-OH group in naltrindole and naltriben resulted in compounds with low affinity and δ selectivity.

14-O-2,6-diCl-benzylether of naltriben (compound # 6) has a low selectivity and affinity for the δ opioid receptor. What is a possible reason for this decreased affinity?

Alkylation of the C-14-OH group af naltrindole and naltriben can confer δ receptor selectivity and affinity whereas substitution at C-5 position by methyl group does not induce any significant changes in δ receptor but C-5 methyl compounds have very low affinities for μ and κ sites. The results suggest that C-5 methyl group is not necessary for high δ opioid receptor antagonism but this methyl group is obviously able to decrease antagonism at μ receptors.

There is an important point regarding the design of selective new ligands namely that a concomitant proportional decline in the potency at all three receptor types can afford highly selective ligands.

As it was expected N-allyl or N-CPM substituted compounds behave opioid antagonists on the basis of the sodium index determination. On the contrary compound # 1 has higher index than other derivatives. What is the explanation for this high value? The oxymorphindole derivative which has N-methyl substituent displays opioid agonist properties as confirmed by sodium index determination and by stimulation of GTP binding.

It was mentioned on p. 30 that the N-substitution of indole part of NTI does not seem to have much influence on delta affinity and selectivity. I think it is not suitable to draw any conclusion studying one substance and otherwise surveying the literature some interesting observation can be found out. For instance N-methyl-NTI is a potent delta antagonist while the N-benzyl derivative is also a potent delta receptor antagonist. It is noteworthy that the latter compound possesses greater in vivo δ2

selectivity and longer duration of action than that reported for the standard δ2 antagonist naltriben.

I have some remarks and questions about sodium index determination. In this method IC_{50} values of substance to be tested were determined against ^{3}H-naloxone binding in the presence and absence of NaCl. Since agonist binding was depressed by sodium, IC_{50} value of agonists was shifted to the right in the presence of sodium, yielding a ratio of IC_{50} values greater than one. Antagonists retain their potency and have ratios of approximately one. Dual agonists-antagonists have shifts intermediate between pure agonists and antagonists.

Since the elaboration of this method opioid receptor multiplicity is well established and this finding complicates the interpretation of the effect of sodium ions on single IC_{50} values. For example it was demonstrated that nalorphine is a μ antagonist and a κ3 agonist. Nevertheless, it was found that the binding of κ agonists was less sensitive to the effect of sodium present in the binding assay.

2. New 7-β-epimers of etorphine and dihydroetorphine were synthesized in order to study their affinity for opioid receptors. Methyl ethers of epimeric pairs were also examined. It can be expected that changing the configuration of C-7 would result in significant difference in potencies of epimeric pairs. Etorphine has an exceptionally high agonist analgesic potency in order of 8600x morphine in guinea pigs and although it has been demonstrated to be potent analgesic in humans it had low therapeutic index causing considerable respiratory depression in primates.

It is well known that the analgesic potency of etorphine derivatives depends on the chirality of the C-19 center.

The new compounds have high affinity in ^{3}H-naloxone binding assay and they potently stimulate ^{35}S-GTPγS binding in neuronal membranes. The rank order of the potencies of phenolic compounds was β-etorphine > etorphine > dihydroetorphine > β-dihydroetorphine. Both radioligand binding and functional studies corroborate the agonist property of each compounds tested.

On the basis of binding assay there was no significant difference in the potency etorphine analogues. However Hutchins and Rapoport (J. Med. Chem. 27 521 (1984)) proposed a lipophilic subsite to interpret the interaction of etorphine

analogues with the opioid receptor. Changing the configuration at C-7 one can expect different interaction with the receptor i.e. some change in potency.
The sodium indices are by far higher for the methyl ethers of etorphine derivatives than for the corresponding C-3 phenolic compounds. Is there any explanation for this observation?
It was demonstrated that etorphine displays high affinity for the μ, κ and δ sites as well but compared to the μ binding site etorphine is 1.8 times more potent at δ binding site and 4 times more potent at the κ binding site. (Magnan et al. Naunyn-Schmiedberg's Arch. Pharmacol. 319 197 (1982) . I think these findings account for the similar studies of the new etorphine derivatives. Were there any studies for the determination of receptor selectivity?

3. Possible degradation fragments of endomorphin 1 i.e. di-, tri- and tetrapeptides were prepared to investigate how enzymatic degradation can influence their binding to μ opioid receptors and also consequent activation of G proteins.
The peptide fragments showed significant lower opioid receptor binding and potency compared with the parent endomorphin 1. The free C-terminal amino acid containing tetrapeptide (Tyr-Pro-Trp-Phe) proved to be the most active but it showed much lower affinity than endomorphin 1. None of these peptides had any effect on GTP binding. These results proved that enzymatic degradation destroyed the biological activity of endomorphin 1.

4. Several new analogues of endomorphin 2 were prepared by means of chemical modifications of the C-terminal amino acid phenylalanine. The new peptides were evaluated by radioligand binding experiments and by functional (GTP binding) assay.

Free C-terminal amino acid containing analogue of endomorphin 2 (Tyr-Pro-Phe-Phe) displayed low affinity to the μ receptor but the reduction of carboxyl group ($COOH \rightarrow CH_2OH$) resulted in increased affinity for μ binding sites. Preparation of amide derivatives of Tyr-Pro-Phe tripeptide was also achieved but these substances showed low potency.

These studies corroborated that the amide function of the C-terminal amino acid has an essential role in the regulation of binding and agonist and antagonist properties of endomorphin 2.

It was found that endomorphin 2-ol has a low intrinsic activity and on the basis of sodium index and in vitro bioassays it behaves as a partial agonist. In my opinion the sodium index is still high (8) to consider this compound as a full agonist.

How can the candidate explain that E 2-ol is a partial agonist? According to the pharmacological definition a partial agonist has low intrinsic efficacy (activity) so that its dose response curve exhibits a ceiling effect at less than the maximal effect produced by a full agonist. Buprenorphine is the main example of a partial agonist opioid. Increasing the dose of such a drug above its ceiling level does not result in any further increase in response. On the other hand the Na^+ index of buprenorphine is low ~ 1.

The peptide YPF-benzyl-allyl-amide has a low sodium index and this observation is interesting because this substance behaves as an agonist-antagonist peptide. Similar results have been reported in case of N-allyl leucine enkephalin too.

I have to mention some critical remarks but these issues have no influence to the main point of the thesis.

The chemical name ''benzofuromorphinans'' is wrong these compounds are called benzofuranomorphinans.

On page 39 the abbreviations are not explained for the peptides PWF-NH_2 and YPW-OH.

On page 44 the formula for peptide YPF-benzylallylamide is wrong it represents a β-phenylethyl derivative.

The formulas of etorphines (p. 31) does not contain the numbering of carbon atoms. On the other hand the numbering of etorphine used in the text is not generally accepted. (See The Merck Index or Chemical Abstracts Service.) The proper chemical name of etorphine is derived from 6,14 –endoethenomorphinan-7-methanol i.e. the new compounds are epimeric at C-7.

The scientific results and the presentation of the thesis are well documented and the submitted thesis meets the requirements to obtain the Ph. D. degree. The new results

has been published in well recognized journals. In case of a successful defense I can support to judge the Ph. D. degree for D. Biyashev.

Sandor Hosztafi Ph. D. ICN Hungary Ltd. Tiszavasvari

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Referee's opinion

on the Ph.D. Thesis entitled

„Biological activity of structurally modified opioid receptor ligands"

written by **Dauren Biyashev**

The Thesis of Dauren Biyashev both in style and form meets the specified requirements of the Ph.D. program of the University. In my opinion, it satisfies the scientific requirements of getting the degree.

Mr. Biyashev has spent some years in this internationally well known laboratory dedicated to opioid research in Szeged. He published his results in respectable international journals such as BBRC and Peptides and presented them at several international meetings. Some of his results are submitted or are in preparation.

The Thesis consists of 52 pages. The major headings are: Introduction (17 pages), Aim of the study (1 page) Materials and Methods (6 pages), Results and Discussion (28 pages), Conclusions (2 pages). More than 200 references complement the Thesis, more than adequate for a work of the scope. The dissertation is well organized and clearly presented. In the Introduction there are some really excellently well-written parts (for example 1.2.). The methodologies employed are appropriate for the studies undertaken. With the exception of some concerns listed below, the conclusions are supported by the experimental results.

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<u>The aim of the Thesis was:</u>

1. To demonstrate the biochemical characterization of a series of newly synthesized analogues of delta selective antagonists by in vitro competitive radioligand binding experiments.
2. To investigate the biochemical and functional properties of etorphine derivatives.
3. To assess the effects of possible enzymatic degradation of endomorphin-1 on the binding characteristic and in vivo effect.
4. to determine the influence of C-terminal structural modification of endomorphin-2 on receptor binding and the consequent activation of G-proteins.

<u>Some of the major findings of the Thesis:</u>

Ad 1. The binding analysis of NTI and NBI derivatives have shown that some of the new drugs have better characteristics as regards the selectivity and binding activity.

Ad 2. All new etorphine derivatives showed high affinity and potency in the binding assays.

Ad 3. The functional analysis showed that endomorphin-1 derived peptides had low binding potency and did not influence the GTP binding.

Ad 4. The distance between the C-terminal aromatic ring and the peptide backbone of endomorphin-2 has strong effect on the receptor binding and the functional activity of the peptides.

Some major remarks:

1. The introduction gives a very excellent review about the history and the action mechanisms of opioids. 1.4. chapter is also very well written, which deals with the heteromeric opioid receptors. Speaking about it is very elegant but it is unnecessary and out of the scope of the Thesis. What is the relevance of it in this deepness? How could be determined whether the interaction between different opioid receptors are manifested at heteromer receptors, or in one cells but different mu or kappa receptors or at different cells?
2. The binding properties of the different compounds were excellently characterized, although only one study investigated the potential in vivo effects of endomorphin-1 derived peptides. What type of studies do you plan to investigate their *in vivo* effects in the future?
3. What are the new results about the effects of endomorphins on non-opioid receptors?

I missed from the introduction:

What are the main results about etorphine? What are the problems with it?

What are the main results about endomorphins? What are the problems with them?

Minor remarks

General:

In some cases I missed the references.

The data of the tables should not be repeated in the text.

Page 4. Line 1-3 lines

I do not think, that mu and delta opioid receptors are considered to be responsible for analgesic effects at supraspinal level, and delta and kappa at spinal level. I missed the references for this claim. Furthermore, there are several data about the mu receptor at the

spinal level, and a lot of human studies apply mu opioid agonists for pain therapy with good results.

There is no correlation between Table 1.2 and the text. Since the opioid receptor activation influences almost all of the cell activity, it is very funny, that the author mentioned the pituitary hormone regulation.

It is very difficult to discuss the effect of opioids at calcium or potassium channels, because these effects could be mediated by cAMP, PLC, Gβγ. It would have been better to discuss the effect of opioid receptor activation on different channel function at the end of the 1.2.3 section.

Page 3. Last paragraph:

Opioid receptors are present not only in the brain and peripheral tissues, but also in the spinal cord, as is mentioned in other sections. Therefore the term "central nervous system" is better.

Table 1.2. I suggest the title: Main effects mediated by opioid receptors.

Page 6. 1.2.3 section, 2nd paragraph:

Since in the earlier section there is no mention about the possible endogenous ligands for opioid receptors, but later there will be a section about it, I prefer to delete the first sentence:

„A number of different tissues...."

Since opioid could decrease but also increase the intracellular level of calcium, I suggest a sentence about it, to emphasize this fact.

There is no reference here about „...regulate phospholipase C activity."

Page 9. 1st, 2nd and 3rd paragraphs

There are no any references for the claims.

Page 9. 4th paragraph

„.. Later endomorphins were found in human brain and **spinal cord..**

Page 10.

Table 1.3

Page 11. 1st paragraph

There are also a lot of studies, which could reverse the antinociceptive effect of endomorphins with mu opioid antagonists.

„Experiments performed in our group suggest that endomorphin-1 is able to bind to both mu-opioid receptors..." Are there any references about it?

1.3.2.

The title is not correlated with the content of this section. The section deals with approaches for creating endogenous ligands.

Aim of the studies

Page 17. 2nd paragraph

I do not agree with first sentence:

„Mu and delta opioid receptors have enormous importance for both basic research and **clinical use." We do not use opioid receptors in the clinical practice.**

Results and Discussion

Page 24.

Affinity and selectivity

I would prefer a more systematic analysis of the different compound, because I found some repetition about the same compound. See page 25 1st and 2nd paragraphs.

3rd paragraph

I would move the Table 4.1.1 at the end of the first sentence of the next section.

5th paragraph, last sentence:

I would mention that C6 has the lowest selectivity and activity for delta receptor.

Page 25. 1st paragraph.

I would not say, that C1 had a loss in affinity towards all binding sites, because it is the 3rd best new compound at delta receptor.

Furthermore C2 showed decrease in affinity for all receptors compared to the parent drug.

2nd paragraph

I do not understand that introduction of Me at R2 is not favorable for delta selectivity, because in C1 it is not a problem.
I would have more discussion on the C10, because it has the largest selectivity and activity at delta opioid receptor.

Page 29.

In contrast to the suggestion, the presence of a 5-Me group (C1 and C4) induced change in delta affinity.

Page 32.

It would be better to discuss in detail the structure-activity studies of etorphine derivatives.

Page 43. 2nd paragraph

Since, only YPW-OH could inhibit the G-protein activation by E1, therefore it would be better in the following way:
However, due to **its (not their) ability to displace... this fragment might be ....**

Page 44-45.

The author did not mention the benzyl-amide substituted compound.

Last paragraph

In the „The E2 stimulated maximal... „ the mentioned data are shown in Fig. 4.3.2.3, but not in Table.

Based upon the above, the dissertation proved that it clearly and greatly fulfills the criteria of the Ph.D. and I suggest to the Dean of Faculty to accept his Thesis and honor his work with a Ph.D. degree.

Szeged. 2001. 11. 20. Dr. Horváth Gyöngyi, M.D. Ph.D