Specificity of Chemotactic Ligand –Receptor Interaction

– Chemotaxis and Drug-Targeting –

Short Ph.D. Thesis

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INTRODUCTION

Chemotaxis is one of the most ancestral and essential physiological responses of cells. It developed at prokaryotic and early eukaryotic levels of phylogeny but the two distinct levels possess diverse signalling mechanisms. Interaction of a well described receptor–intracellular signalling system (e.g. Asp- and Thr-receptors and the ChA–ChB–ChY– ChZ network) and several relatively simple ligands (amino acids, dipeptides, saccharides) are present in bacteria. In contrast, still poorly described, different membrane associated receptor complexes and their characterised intracellular signalling networks are present in eukaryotic cells, e.g. chemokine or formyl-peptide receptors.

Although itself the receptor-mediated responsiveness of unicellular and multicellular organisms is essential, chemotactic responsiveness represents more than detection of harmful or beneficial ligands (e.g. toxins and nutrients). Large number of physiological processes are based on the chemotactic ability of the cells, e.g. leukocyte recruitment is a central component of the inflammatory process or reproduction is inconceivable without chemoattraction of the sperm cells. The spreading of tumour cells also based on the directed migration. The biological and pathological significance all of these processes including some still obscure relations of the problem, motivated us to analyze and characterize the chemotactic signalization.

In the present work the ligand–receptor interaction of chemotactic signalling process was studied, in the focus of the chemotactic ligand and in the different levels of phylogeny.

OBJECTIVES

At the first period of our work the chemotactic ability of amino acids and peptide type ligands were evaluated on a unicellular ciliated model, *Tetrahymena pyriformis*.

Our aims were:

- To describe potential relationships of physico-chemical properties of amino acids and the chemotactic responses elicited;
- To learn the possible structural – functional correlation in the case of a peptide library containing the (W)SXWS motif of extracellular domain of hemopoetic cytokine receptors;
- To apply chemotactic selection and describe durability of ligand-receptor relationships in the subpopulations gained;
- To characterize the potential drug-targeting ability of chemotaxis on the unicellular model.

In the second period of our work we investigated in higher ranked model, monocytes that:

- Whether the structural-functional relationships gained on the unicellular model are universal;
- To design drug containing peptide conjugates for chemotactic drug-targeting and analyse their cell physiological effects.

METHODS

Peptides and other chemotactic ligands

The tested peptides were synthesized following Fmoc/^{t}Bu and Boc/Bzl strategy by the Research Group of Peptide Chemistry, Hungarian Academy of Sciences.

The L-amino acids and the formyl-peptides were obtained from Sigma Chemical Co., St Louis, USA and Reanal, Budapest, Hungary.

Model cells

In the first part the unicellular eukaryotic ciliated protozoa, *Tetrahymena pyriformis GL* was our model. Although it represents the lower level of the phylogeny, the chemotaxis is a basic cell physiological response of these cells. Tetrahymena shows more significant similarities (e.g. membrane receptors and second messengers) to higher ranked vertebrate models. The application of this model system offers also practical advantages, due its simple culturing and their short generation time (150 min). For this reason the experiments are quickly prepared, a large number of parallels and umpteenth generation (e.g. 70^{th}) can be studied.

The phylogenic aspects of the chemotaxis were analysed using monocytic and fibroblast cell lines (J774 and MRC5). The model experiments of the chemotactic drug-targeting were performed on human monocytes THP-1.

Assay of chemotaxis

Chemotactic responsiveness of **Tetrahymena cells** was evaluated in a two-chamber capillary assay as modified by Kőhidai et al. In this setup, Tips of an eight-channel-micropipette containing the test substance served as the innert chamber. The cells were placed into microtitration plate as outer chamber. After incubation (20 min) the samples were fixed in 4% formaldehyde. The number of cells was determined oculometrically in Neubauer haemocytometer. The average of ten replica assays of each ligand is presented in the figures.

Chemotaxis of **cell-lines** was measured in NeuroProbe chamber. The upper chamber of the system was filled with the suspension of cells, the lower wells of microtitration plate contained concentrations of the tested conjugates. PC filters (5μm) separated the two chambers. Compounds were dissolved in culturing medium and fresh culture medium was applied as control. After 3 hour incubation the number of positive responder cells was evaluated by detection of mt-dehydrogenase content of cells (Staining with 3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide - MTT assay). The developed of formazan crystals were solubilized by DMSO. The samples were measured in ELISA reader at 540 and 620 nm.

Chemotactic selection

This technique deals with the chemotactic capacity of different signal molecules to form subpopulations of mixed cultures of cells. In this case the chemotaxis assay described above was applied, but after incubations the positive responder cells were transferred to fresh medium. After one week cultivation the selected cultures were assayed again in following combinations: responses of cultures selected with ligand

(X) or control substance (C) were tested in relation to identical ligand (X/X or C/X) or to the control (X/C or C/C). The most effective chemoattractant concentrations of identical ligand were applied both in the selection and in the repeated chemotaxis assays.
The evaluation is based on the coefficient chemotactic selection, an index of which is calculated from the chemotactic responsiveness of the four groups.

Cell physiological properties

The ability of the conjugates using for the chemotactic drug-targeting were specifically characterised by measuring their cell physiological effects:

(i) The role of formyl-peptide receptor (FPR) in the chemotactic response induced by the CDT molecules were analysed by pre-treatments with Con-A and Lens lectins.

(ii) Significance of phosphatydyl inositol pathway was tested by inhibitors, wortmannin and LY 294002.

(iii) The internalization of the fluorescently labelled peptide conjugates was analysed by FACS and confocal microscope.

(iv) The cytotoxic effects of the conjugates containing methotrexate as drug were evaluated by MTT assay.

Computer assisted conformational analysis

Conformational analysis of the SXWS peptides was carried out by performing Monte Carlo Multiple Minimum (MCMM) searches as implemented in MacroModel. 30000 MCMM steps were carried out in each molecule. In drawing conclusions, structures of all low energy conformers were considered. Calculations were carried out using the AMBER force field. Solvent-effects were modelled by the GB/SA algorithm (using water as solvent).

Statistical analysis

Statistical evaluation was performed by ANOVA of Origin 7.0.

RESULTS

Tetrahymena modell

1. In the first part of the experiments reference data of amino acids were obtained by chemotaxis assays. The main purpose of these works was to collect data about chemotactic potency of these relatively simple ligands as the literature of these molecules is rather diverse concerning their chemotactic ability.

   Our results show that, however, the tendency of chemotactic responses elicited by amino acids classified into same chemical groups, there are significant differences also.
   While there was a good correlation between the chemoattractant/repellent character and pKa values or hydropathy indexes of the ligands, the physico-chemical characteristics, (distribution of changes) of amino acids were assumed to be responsible for the above mentioned phenomenon via modification of receptor-ligand interactions.
   An other result of these experiments was the description of a good correlation between the value of solvent exposed areas (SEA) of the ligands (and the side chains) and chemotactic ability of the molecule. Small SEA values furnish the ligand with attractant moiety, while increasing the SEA value results a repellent ligand.

2. Experiments described above were completed by a work which collected data about dynamics of receptors proved to transmit chemoattractant signals. In these experiments chemotactic selection with amino acids was used, to set subpopulations. Further culturing these cells provided us the possibility to analyse whether incubation with the selector ligand in chemotaxis could work on an enhanced level or not.

Data of literature point to that chemotactic selection is a dedicated technique to distinguish „long-term" and „short-term" responsiveness based on two different functional types of receptors. Other data gained by RAPD analysis show that different types of the chemotactic ligands (e.g. formyl peptides, endothelin-1, insulin) are able to select subpopulations characteristically diverse in their genetical markers, too. On the basis of these data we can well distinguish signalling mechanisms using genetically determined (and transmitted from generation to generation) receptors („long-term"). The ad hoc induced receptors at the appearance of the ligand are distinguished from the above mentioned ones as they are composed only upon induction with the ligand, there is no genetical background („short- term").

In Tetrahymena model system only three amino acids (Ile, His, Thr) had a „long-term" selector –signalling character, while a big group of amino acids elicits chemotactic responses via „short term" receptors of Tetrahymena membrane, which conforms to the data of literature describing a plastic surface membrane in this protozoon.

3. Comprehensive analysis of data of literature and computer data bases we could draw a parallel between the chemotactic activity of individual amino acids tested in Tetrahymena and the sequence of appearance of these amino acids in the primordial soup.

   Our calculations and the comprehensive study of data supports the previous data about the essential significance of chemotactic responsiveness in molecular phylogeny. It shows that – upon consensus sequences of databases - amino acids appeared first are working as chemoattractant ones, while the last molecules proved to be chemorepellent in the ciliate model.

The correlation described above supports theory of Lenhoff, which draws the origin of receptors from the simplest, food type molecule recognizing ones. We suggest that chemotaxis receptors and amino acid type ligands embodied the next step in molecular phylogeny, in parallel selection of chemotaxis receptors and ligands.

4. In the next experiments, following the study of chemically simple ligands, SXWS/WSXWS peptides, parts of extracellular domains of cytokine receptors were investigated. The main points of the study of amino acids were followed during characterization of SXWS type ligands and significant differences were recorded. Extremely high chemoattractant moieties were described in SEWS and STWS. Comparison of chemotactic characters and physico-chemical parameters showed that some indexes i.e. hydropathy index are not unequivocally predictive factors of chemoattractant moiety. Diversities from the trends described previously in amino acids could be explained by that the increased size and other significant characters can modify the chemoattractant character. In contrast, SEA values of the SXWS ligands and mass/SEA indexes proved to be decisive. These factors calculated seem to be predictive over amino acid type ligands in SXWS and formyl-peptide sequences, too. This character has not only theoretical but pathological significance as the tetrapeptides (SXWS) investigated are well conserved components of the extracellular domains of the cytokine receptors. These components are working not only as chemoattractant ones, but they can influence the chemotactic quality of surfaces by their slight modifications in the sequence.

5. We have to mention the overlapping effects observed in amino acid (X) and its identical SXWS ligand pairs. Results show that between the two groups of ligands there is a high level (84%) overlapping effect in chemotaxis. Evaluation of the phenomenon on the bases of cell biology suggests that the 2nd member (X) of the SXWS sequence is a special, highly expressed part, which could interact with the chemotaxis receptor alone in a similar way to the identical amino acid.

   To analyse the reality of the above described hypothesis a computer based structure modelling was introduced on the group of SXWS ligands. Structures gained support our hypothesis, the only structural difference of SXWS sequences was detected in the 'X' position.

   Significance of our theory and the supporting data is that it made conceivable that - even in longer sequences – only one amino acid size part is the interacting component with the binding site.

6. A special type of the above mentioned sensibility was found in WSXWS ligands, which differ from SXWS in Trp substitution on the N-terminal. The modification of the ligand results an altered chemotactic potential in 78% of the tested sequences, in 2/3 of them there is a significant decrease. The slight modification of the ligands affect the selection potential as a significant loss was detected also in this parameter, only WSPWS ligand could work by the "long-term" mechanism of signalling.

7. Another result of evaluation of the experimental data gained on different groups of ligands was the description of phenomenon "chemotactic range fitting". In this case the basis of evaluation is not the absolute chemotactic potential of the ligands, but the amplitude of activity of chemotactic responder cells to a ligand. This amplitude has a good

"fitting" to the chemotactic character: both chemoattractant amino acids and SXWS peptides proved to have a wide, while chemorepellent ones narrow ranges of identical chemotactic activities of responder cells. In contrast, and in a good correlation with other data, the fitting of the ranges of WSXWS peptides was not so underlined, which results point to that a wide or narrow range of the functioning receptors could be assumed in the backgrounds of the phenomenon.

8. Formyl-peptides were tested in the next group. However, there are several synthetic peptides available for analysis, fMLF, the most frequent form of the nature proved to have the highest and classical dose-response chemotattractant moiety in Tetrahymena. Investigations of formyl-peptides and the composing amino acids showed also that the presence of the formyl residue itself does not determine the chemoattractant character of molecules and chemotactic character of the identical composing amino acids is not an additive one. It is more conceivable that some optimal (intermedier) physico-chemical data are responsible in determination of the chemoattractant character.

9. The first part of experiments was closed with investigations of tuftsin and its derivatives. Studies of the concentration range dependence and chemotactic selection showed good correlation between the structure of ligands, their chemical characters and the chemotactic potentials. Description the correlation between the low values of SEA and the chemoattractant character of the ligand underlined that this factor (SEA) might be a predictive index in the future to characterize both small and larger chemotactic ligands. The "long-term" signalling of TKPKG

confirmed our hypothesis that this family could be used as carrier molecules in further model-experiments.

Chemotactic drug targeting – Model cells from higher level of phylogeny

In the next part of our work our purpose was to develop a new drug targeting technique and to perform the basic model experiments required for introduction of the novel technique into the further period of evaluation. The main experimental objectives were to validate the measuring techniques and to evaluate groups of newly synthesized complexes containing the drug.
The above mentioned experiments and the synthesis of the new ligands were a result of a step by step analysis and modification of peptide sub-libraries. In the initial period several experiments, previously completed in Tetrahymena model, were repeated in monocyte and fibroblast cell lines.
Results of the pilot experiments indicated that there is a significant difference of chemotactic responsiveness to tuftsin derivatives in monocytes and fibroblasts and that oligotuftsins have an adequate chemoattractant moiety.

1. The first step was to determine and to formulate itself chemotactic drug targeting (CDT) and plan the structural requirements of the peptides. The main components of these complexes are: carrier, chemoattractant ligand and the drug. The purpose was to synthesize such complex which combine more prosperous biological characteristics. Tuftsin was chosen because of its phagocytosis inducer moiety, which could increase the internalization of the complex. Two formyl-peptides (fMLF, fNLF) were used as they proved to have clement chemotactic character, while the ’GFLGC’ spacer sequence was used to link the drug as its sensitivity to

lysosomal enzymes provides fast intracellular effect of the drug. Finally, methotrexate was chosen as drug as very frequently used anti-metabolite in treatment of leukaemic diseases, in this way to monitor its effect is adequate to a leukaemic monocyte cell line THP-1.

2. Evaluation of the novel formyl-peptide-T_{20} conjugates was done at first in Tetrahymena. The results show that the carrier molecule can influence significantly the biological effects of the formyl-peptide detected in native condition. Chemical modification of the carrier (succinyl or formyl residues on the side chains of lysines) could also influence the chemotactic character. Exact reasons of the above described phenomenon are not known, however, on the basis of previous experiments we suppose that modifications of the complex and the accompanying changes in the distribution of charges, as well as differences in sizes of the derivatives might explain the observed changes.

3. In the next part of the experiments effects of drug (Mtx) containing forms were analysed. As more stereochemical forms of the ligands were available for study we could detect a high sensitivity of the ciliate model towards even slight modifications of the complex.

4. Investigations of the chemotactic drug targeting were continued in the above already mentioned human leukaemic model – THP-1 monocyte cell line. Results gained with different groups of ligands in this model and comparison of the results to the data of Tetrahymena are shown in Table 1.

	Conc	THP-1	*Tetrahymena pyriformis*
f-MLF	10^{-8}	++	+++
f-NleLF	10^{-8}	+	+
f-MMM	10^{-8}	0	0
T_{20}	10^{-7}	0	+
T_{20}- fMLF	10^{-7}	+++	0
T_{20}- fNleLF	10^{-7}	++	+++
T_{20}- fMMM	10^{-7}	+	-
succinyl-T_{20}- fMLF	10^{-7}	0	+
formyl-T20-fMLF	10^{-7}	0	+
spacer-T_{20}- fMLF	10^{-7}	++	+++
Mtx_{γ}-T_{20}-fMLF	10^{-6}	+++++	-
Mtx_{γ}-T_{20}-fNleLF	10^{-6}	+++++	-
Mtx_{α}-T_{20}-fNleLF	10^{-6}	+++++	--

Table 1: *Effect of CDT-ligands and conjugates on the chemotactic responsiveness of THP-1 monocyte and Tetrahymena model cells.*

As it is shown in the Table besides the concordant responses elicited, significant diversities were also detected. In the case of formyl-peptides responsiveness of the two relative distinct cell types overlap in its sign and amplitude. In contrast, in conjugates only two (T_{20}- fNleLF, spacer-T20-fMLF) complexes are working in similar way in the two models, while the most significant diversities were detected in the Mtx containing conjugates, THP-1 cells were highly responder, Tetrahymena recognized the complex as a strong repellent one.

Explanation of the above described results rose the requirement of further experiments on the signalling and cell-physiological effects of the tested complexes. In the series of these assays four block of tests were done to clarify: (i) the role of carbohydrate components of the FPR in the effect of conjugates, (ii) to check the role of PI3K, an enzyme which is in the focus of

the intracellular signalling of chemotaxis in general, in this case; (iii) to analyse the internalization and compartmentalization of the complex; and (iv) to test the target cell-physiological effect, cytotoxicity of the drug component methotrexate.

5. As formyl peptides were used as targeting agents in the conjugates it was self-evident to block the FPR by special ligands. As we could not obtain specific antibodies and blocking sequences of the receptor an other way blocking via carbohydrate components of the FPR was chosen. For this purpose α-Man and α-Glu specific lectins (Con-A and Lens) were applied. According to our results effects of the formyl-peptides and the conjugates was blocked by interaction of the α-Man and α-Glu residues of the receptors, which suggest that these saccharide components have a significant role in the FPR signalling.

6. Investigations on the internalization of the labelled derivatives showed that conjugates enter both model cells, however, the time dependency of the internalization was different. In Tetrahymena a rather fast (1 min.) process was recorded which conforms to its ciliate character. It is reassuring that good dynamics were found even in the THP-1 monocytes, and that the confocal study verified the presence of molecules in the vesicular compartment of the cells which shows that there are good chances to target the complex into the compartment of release of the drug.

7. It was also important to analyse whether PI3-kinase, the key enzyme of intracellular chemotactic signalling is triggered or not by the conjugates tested. For this purpose two PI3K inhibitors were applied (wortmannin

and LY294002), both are considered as classic blockers of the enzyme system. Our data confirmed the previous results, as a significant inhibition was registered in drug-free conjugates and complete ones as well. These results verified that our novel chemotactic conjugates over their good chemotactic behaviours are inducers of the classic intracellular signalization pathways.

8. Assays of cytotoxicity induced by the Mtx containing conjugates demonstrated that the therapeutic effect of the drug is retained in both Tetrahymena and THP-1 models. However, the time dependence of action detected in the two cell types was diverse, in Tetrahymena a more rapid effect was recorded, while in THP-1 cells the maximal cytotoxic effect was elicited in 48-72 hours. These data also support our original requirement towards the optimal CDT-ligand, as these novel conjugates possess not only good chemotactic moiety, but their cytotoxic effects are also retained in higher ranked (tumour) cell and it is very important considering their further application in therapy. The time-dependence of cytotoxic effect detected were concordant to the literature, which also underlines that these Mtx containing conjugates are good candidates for further in vivo experiments.

CONCLUSIONS

- We characterized the chemotactic ability of amino acid and oligopeptide type chemotactic ligands and we described their predictive physico-chemical parameters

- We clarified the molecular structural background of the overlapping chemotactic effects of SXWS ligands and their identical amino acids ('X').

- We described phenomenon chemotactic range-fitting as a new aspect in characterization of chemotactic ligands.

- We described the theory of chemotactic drug targeting and structural requirements of effective signalling by formyl-peptide type ligands.

- We designed molecules for CDT and verified their suitability on the ciliate and on the higher ranked monocyte model, as well.

- We analysed chemotaxis and other cell-physiological responses elicited by conjugates containing methotrexate.

PUBLICATIONS

Articles related to the PhD thesis:

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Other publications on this field:

1. **Láng O,** Birinyi J, Bai K, Mező G, Hudecz F, Kőhidai L: Suitability of peptide conjugates containing formyl-peptide residue for chemotactic drug-targeting (CDT). *FEBS Lett.* 272(S1) 527, 2005.
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2. Illyés, E., Bősze, Sz., **Láng**, **O**., Sebestyén, F., Kőhidai, L., Hudecz, F.: A new class of chemotactic peptides containing EWS motif: A mini-review. *Chimica Oggi.* 55-61, 2002.

3. Kőhidai, L., Bősze, Sz., Soós, P., Illyés, E., **Láng, O**., Mák, M., Sebestyén, F., Hudecz, F.: Chemotactic activity of oligopeptides containing EWS motif on *Tetrahymena pyriformis*. The Effect of Amidation of the C-Terminal Residue. *Cell Biochem. Funct.* 21:113-120, 2003.

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5. Hauser, P., Jakab, Z., **Lang, O.**, Kondas, O., Muller, J., Schuler, D., Bognar, L., Garami, M.: Incidence and survival of central nervous system involvement in childhood malignancies: Hungarian experience. *J Pediatr Hematol Oncol.* (3) 125-128, 2005.