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The impact of cell membrane organization on the binding efficacy of the epidermal growth factor and elisidepsin

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1. INTRODUCTION

1.1. Membrane microdomains, lipid rafts

Microdomains are those parts of the membrane that are characterized by distinct physical and chemical qualities. There are two approaches to investigate them: treatment with non-ionic detergents and analysis of the residual fraction or examination with microscopy. The latter was successful in finding small and transient structures.

Lipid rafts are the most important subgroup of membrane microdomains. They are small (10-200 nm), heterogeneous, dynamic, sterol- and sphingolipid-rich domains that compartmentalize the cellular processes. Smaller rafts can get stabilized by protein-protein or protein-lipid interactions in order to provide greater surface for certain processes. The main components of lipid rafts are cholesterol and (glyco-) sphingolipids and they are able to recruit cell surface proteins or those that are attached to the cytofacial leaflet. On the other hand, they keep certain proteins apart as well, so they are considered cell signaling platforms. EGFR, a protein examined in the current thesis is also found in lipid rafts, especially its phosphorylated form.

1.2. The EGF receptor family

The family of EGF/ErbB receptors is considered the prototype of receptor tyrosine-kinases. Its members are: EGFR/ErbB1/HER1, ErbB2/HER2, ErbB3/HER3 and ErbB4/HER4. They play an important role in intracellular cell signaling processes, differentiation, apoptosis and in physiological and pathological cell division.

They consist of three major structural elements: an extracellular domain (ECD) that binds the ligand, an α-helical transmembrane domain and an intracellular region that contains the kinase domain (KD) and the C-terminal region with the tyrosine autophosphorylation sites.

1.3. Homodimerization of EGFR

EGF receptors tend to form homo-and heteroassociations resulting in the diversity of signal transduction. Ligand induced dimerization and activation became the dogma of the activation of EGFR. It states that EGF binds to domains I and III of the ECD in the same time, then due to the conformational changes the dimerization arm is released that is able to bind to an other activated EGFR nearby.

However, novel evidence emerged suggesting that pre-assembled dimers can be found in the membrane in the absence of ligand. Formation of such dimers is usually initiated by the kinase domains. Functionally active, asymmetric KD dimers are formed by an activator and a receiver kinase, while symmetric, functionally inactive KD dimers consist of two kinases in the same conformation.

1.4. The cooperativity of EGF binding

In protein associations with multiple ligand binding domains a phenomenon called cooperativity might be observed, so that the binding of molecules is not independent but influenced by interactions among the binding sites. Positive cooperativity is suggested if the binding of the first ligand facilitates the binding of the second one, while negative cooperativity occurs if the binding of the second ligand is inhibited.

The Hill model is frequently applied to fit concentration-dependent ligand-binding assays. The Hill coefficient allows to reveal the nature of cooperativity: a coefficient higher than 1 suggests positive cooperativity. However, the Hill model fails to describe the molecular details of ligand binding events, a feature that should be of importance in the EGFR system.

As for the EGFR, negative cooperativity became the widely accepted theory, explaining the heterogeneity observed in EGF binding. Negative cooperativity was also supported by structural studies of the ECD in

drosophila. In human EGFR, negative cooperativity was only observed if ligand bound to the full-length receptor. Besides these studies, several other ones can be found suggesting positive cooperativity. These contradictions support the idea that EGF binding is influenced by many interactions, conformational changes of receptor domains (e.g. the kinase) and the presence of other structures (e.g. cytoskeleton). For these reasons we refer to the binding parameters obtained by the Hill model as apparent cooperativity and affinity.

1.5. Antibodies and kinase inhibitors binding to EGFR

Anti-EGFR monoclonal antibodies are able to bind to a certain region of the ECD with high affinity. Once they bind they inhibit the ligand binding of the receptor and initiate internalization, downregulation and anti-tumour cytotoxic response. Cetuximab (Erbitux®) is the most successfully applied anti-EGFR antibody so far, that is a mouse/human chimeric IgG used in the therapy of colorectal and head-neck carcinomas.

Tyrosine kinase inhibitors constitute an other subgroup of anti-EGFR therapeutic agents. Kinase inhibitors are small, membrane-permeable molecules that interact with the kinase region of the receptor abrogating its enzymatic activity. Kinase inhibitors can be classified according to the conformation of the kinase they can bind to. Type-I inhibitors are ATP analogues that bind to the active conformation of the kinase (e.g. gefitinib/Iressa® or erlotinib /Tarceva®). Type-II inhibitors bind to the inactive conformation of the kinase (e.g. lapatinib/Tykerb® or neratinib).

1.6. Elisidepsin and its supposed mechanism of action

Elisidepsin is a cyclic depsipeptide that seemed useful in the treatment of several tumour types in vitro. Its mechanism of action differs from most chemotherapeutic drugs in some aspects. According to the literature, elisidepsin leads to the disruption of lysosomal membranes, nucleus-

fragmentation and necrosis. Necrosis is beneficial as the risk of developing resistence against the drug is lower, however, it can lead to inflammation.

In sensitivity studies, fatty acid-2-hydroxilase enzyme came into the focus, as the presence of 2-hydroxy fatty acids resulted in increased sensitivity against the drug, while inhibiting the enzyme led to decreased sensitivity.

This finding suggests that lipid rafts might play a role in the mechanism of action of elisidepsin, as 2-hydroxy fatty acids are essential in developing and stabilizing lipid rafts.

2. OBJECTIVES

In my dissertation I examined the cell membrane's organizing function in connection with two molecules (a growth factor and a drug).

- We investigated the ligand binding properties of EGFR: kinetics, affinity, cooperativity and conformation-dependence.
- We wanted to identify some circumstances that might influence the cooperativity and affinity of EGF binding (e.g. polimerization of cytoskeleton or glycosylation of the receptor).
- The effect of the kinase domain's conformational changes was also studied.
- To interpret the obtained results at the molecular level we aimed to develop a model that describes not only the details of the interactions behind EGF binding but can also predict the presence and concentration of each receptor species depending on stimulation by the ligand.
- We also aimed to study the mechanism of action of elisidepsin in the aspect of oxygen-depletion, a circumstance that often occurs in tumours.

3. MATERIALS AND METHODS

3.1 Cell lines, treatments and stimulation with EGF

We used two cell lines during the investigation of EGF binding: A431, a human epidermoid carcinoma cell line and F1-4, that is a subline of CHO (chinese hamster ovary) stably transfected with GFP-conjugated EGFR. For experiments examining elisidepsin we used the following cell lines: A431, CHO, HaCaT (human keratinocyte), HeLa (human cervical cancer), MCF7, MDA-MB-453, SKBR-3 (human breast cancer cell lines).

Cells were cultured at 37°C and in the presence of 5% CO_2 in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum (FCS) and 50 μg/ml gentamycin.

To alter the actin content of cells, latrunculin-B and jasplakinolide toxines were used, as the former depolimerizes F-actin while the latter enhances its polimerization. Latrunculin-B was applied at a concentration of 2 μM in HBSS solution at 37°C for 10 mintues. Jasplakinolide was added to cells at a concentration of 1 μM in HBSS solution at 37°C for 30 mintues.

Tunicamycin, an agent inhibiting N-glycosylation of proteins was applied at a concentration of 1 μg/ml in DMEM medium supplemented as written above. In the presence of the toxin cells were cultured for 24 h at 37°C.

EGF stimulation was carried out on cells that were cultured in DMEM supplemented with 0.1% of FCS and 50 μg/ml gentamycin for 12 h. Then EGF was added at a concentration of 130 nM in HBSS at 37°C or on ice depending on the type of experiment. Incubation with EGF was 15 min long.

3.2 Labelling cells with fluorescent EGF and intensity measurements with flow cytometry

For EGF binding experiments tetramethylrhodamine (TAMRA) conjugated EGF was used as a ligand. It was reconstituted at a concentration of 10 μM. A two-fold dilution series of TAMRA-EGF was prepared with each

vial containing 500 μl TAMRA-EGF dissolved in phosphate-buffered saline supplemented with 0.5 mg/ml BSA. The solutions were kept on ice before adding 20 μl of a cold cell suspension containing 100,000 cells. Cells were incubated in the presence of TAMRA-EGF for 1 hour on ice with shaking. The fluorescence intensity of the samples was measured on a FACS Aria III flow cytometer. TAMRA was excited at 561 nm, and its emission was detected through a 595 nm band-pass filter. GFP was excited at 488 nm. The temperature of the sample holder was adjusted to 4°C. At least 20,000 cells/sample were analyzed by the instrument.

3.3 Elisidepsin treatment and cell culturing in oxygen-depleted environment

While we examined the binding of elisidepsin to the cell membrane we used the OregonGreen488 fluorophore conjugated and unlabeled version of the drug at 1:4 ratio. The final concentration of the drug was 0.5 μM for confocal microscopic and 2 μM for flow cytometric observations.

Hypoxic environment was established with a Billups-Rothenberg modular hypoxic chamber: cells grown on 8-well Ibidi slides or flasks were placed in the chamber and they were flushed by a gas mixture at a rate of 25 L/min for 4 min. The gas mixture consisted of 1% O_2, 5% CO_2 and 94% N_2. The chamber was then closed and placed in an incubator and cells were further cultured at 37°C for 4 days.

4. RESULTS

4.1. Gylcosylation of EGFR, and the cytoskeletal network influence EGF binding

We investigated if the membrane environment or the anchoring of EGFR to actin could alter the receptor's function. F1-4 cells were treated with latrunculin-B, a toxin that depolimerizes F-actin. Treated cells were characterized by reduced ligand binding cooperativity and affinity compared to non-treated cells. As a positive control we used jasplakinolide to enhance the polimerization of F-actin. Results of Hill fits are as follows: $n_{control}$=1.24; $K_{d\ control}$=4 nM; $n_{lat\text{-}B}$=1.03; $K_{d\ lat\text{-}B}$=8.2 nM; n_{jaspl}=1.13; $K_{d\ jaspl}$=4.3 nM.

The two agents' effects were supported by confocal microscopic experiments: cells grown on 8-well Ibidi chambers were treated with latrunculin-B and jasplakinolid, then they were permeabilized and the actin filaments were labelled by TRITC-phalloidin. According to our observations, latrunculin-B effectively depolimerized F-actin, while jasplakinolid did not have a remarkable effect on the amount and organization of microfilaments.

It also remained a question whether the glycosylation of EGFR has an effect on the cooperativity and affinity of EGF binding.

According to our observations, gained by the Hill fits, both ligand binding cooperativity and affinity decreased upon the treatment by tunicamycin ($n_{control}$=1.24; $K_{d\ control}$=4 nM; $n_{tunicamycin}$=0.77; $K_{d\ tunicamycin}$=15 nM). However, one must keep in mind that as tunicamycin is an aspecific inhibitor, other proteins' inadequate glycosylation might also contribute to this observation.

The decrease of the molecular weight of EGFR caused by the treatment has been supported by western blots.

4.2. Conformational changes of the kinase domain of EGFR influence EGF binding

Afterwards we aimed to study the effect of kinase inhibitors on the ligand binding and affinity of EGFR, not only in the context of kinase conformation but the level of receptor expression as well. Erlotinib- and lapatinib- treated cells were labelled with a dilution series of fluorescent EGF in the continued presence of the appropriate inhibitor. During the data analysis the population of cells was separated into 3 subpopulations: low, medium and high expressers of the receptor and EGF binding was analyzed independently in these gates.

We could observe the following ligand binding parameters by the Hill fits of the whole cell population: $n_{control}$=1.26; $K_{d\ control}$=3.9 nM; $n_{erlotinib}$=0.95; $K_{d\ erlotinib}$=1.9 nM; $n_{lapatinib}$=1.2; $K_{d\ lapatinib}$=4.9 nM. It can be concluded that erloinib treatment resulted in decreased ligand binding cooperativity and higher affinity, while lapatinib did not alter cooperativity but decreased the ligand binding affinity.

4.3. A new, molecular model of EGF binding

Our experiments allowed us to conclude that the possible conformations of the kinase substantially influence the ligand binding properties of the receptor. It is also known that kinase domains are able to create associations as well. In order to clarify the relationship between the conformations of the extra- and intracellular domain we established a new model that is based on the following assumptions:

- The open and closed conformations of the ECD are in equilibrium. Although ligandless ECDs may exhibit other conformations as well, the model does not involve those ones in order to keep the number of parameters low.

- Dimerization of the receptor can only occur if the ECD is in the open conformation.
- The two possible conformations of the kinase are the active and the inactive one. Although several other conformations have been published, those ones are not covered by the model to minimize the number of receptor species appearing.
- The conformations of the intra- and extracellular domains are independent of each other.
- By having two open ECDs, the kinase domains can develop symmetric and asymmetric dimers, as it is suggested by electron microscopic studies. In symmetric KD dimers the kinases are both inactive, while in asymmetric dimers active kinases are present.
- Dimers consisting of symmetric or asymmetric KD dimers bind EGF with different affinity.
- Ligand can only bind to the open conformation of the ECD.

The established model involves 12 molecular species whose equilibrium states are described by 11 equations and 9 constants. The 11 equations can be written as a quadratic equation set containing 13 unknowns (the concentration of the 12 receptor species and the amount of EGF bound to cells). The equation set was solved in Mathematica and the set of roots in which all 13 concentrations were positive was selected as the meaningful solution.

As control, erlotinib- and lapatinib- treated cells were divided into 3 subpopulations according to the expression level of EGFR (3 subpopulations and the whole cell population were included), 12 experimental conditions were fitted by the model. The sum of squared deviations between the fitted equations and the experimental data was minimized by an algorithm, which was global in two respects. Just one parameter (K_1 characterizing the conformational equilibrium between the active and inactive KDs) was allowed to have three

different values for the control, erlotinib- and lapatinib-treated samples. Secondly, all 12 data sets were fitted simultaneously with parameters K_2-K_9. In this way, the 11 free parameters in the model were globally fitted to 156 data points from the 12 data sets. The Global Search algorithm of Matlab was used for finding the global minimum of the norm. In order to define the confidence interval of the fitted parameters the optimization procedure was repeated 100-times.

4.4. Describing the cooperativity of EGF binding with the new model

According to the fittings by the new model, the inactive conformation of the kinase domain is favored in the absence of EGF under all experimental conditions. This equilibrium is shifted by the inhibitors by stabilizing the active or the inactive conformation of the kinase. It can also be noted that the ECD's closed conformation is favored in the absence of ligand and monomers with inactive kinase tend to dimerize with lower propensity than the active ones.

The ligand binding cooperativity and affinity of the induced dimers are strongly influenced by the conformation of the KD. While dimers consisting of asymmetric KD dimers (erlotinib treatment) exhibit subnanomolar affinity for the first ligand, the second EGF binds with a ~30-times lower affinity due to significant negative cooperativity. Dimers with symmetric kinase dimers (lapatinib treatmennt) have an extremely low affinity for the first ligand, but a subnanomolar binding constant is found for the second EGF, suggesting positive cooperativity.

4.5. The kinase- and temperature dependence of the clustering of EGFR

The value of parameters fitted to the equations not only gives us the amount of EGF bound to cells, but the prevalence of each receptor species and their dependence on the expression and concentration.

It was observed that the presence of EGF does not substantially alter the ratio of monomers and dimers, and that considerable amount of ligand-independent, pre-assembled dimers can be found on cells. The amount of dimers with symmetric kinases seems independent of the expression level and it decreases only if the ligand is present in a high concentration. These predictions of the model seem controversial, as the ligand-induced dimerization and activation mechanism (the theory, being widely accepted in terms of the functions of EGFR) suggests otherwise, so one would expect higher dimerization propensity- at least in control cells. One possible explanation of this phenomenon is the fact that all EGF binding assays were carried out on ice in order to decrease internalization. To resolve this contradiction, we performed homo-FRET experiments to study the clustering of the receptor and its temperature dependence.

The principle of homo-FRET is that the donor and the acceptor fluorophores are identical and energy is shared between them. The only read-out parameter of the interaction is the altered fluorescence anisotropy. If the density of fluorophores decreases, the probability of homo-FRET interaction also decreases and anisotropy becomes higher. Anisotropies were evaluated in the cell membrane and were plotted as a function of residual GFP fluorescence. We could observe that EGF stimulation on ice only resulted in the decrease of anisotropy in the case of erlotinib treatment that suggests clustering of EGFR. In contrast, control and lapatinib-treated samples do not show changes in the anisotropy whether they were or were not stimulated by EGF on ice. These findings support the predictions of our model.

At 37°C we could observe EGF-induced clustering under all experimental conditions, according to low anisotropy values.

4.6. Binding of elisidepsin to cells depends on the oxygen level

Anti-cancer drugs often tend to be less effective in the oxygen-deprived central parts of tumors. Many of the frequently applied cell lines were investigated by my colleagues concerning their sensitivity to elisidepsin under normoxic and hypoxic conditions. They defined the half-maximal inhibitory concentrations (IC_{50}) of elisidepsin and divided cell lines into two groups: cell lines (A431, CHO, HaCaT, HeLa) sensitive to elisidepsin according to higher IC_{50} values. They developed higher insensitivity under oxygen-deprivation. The other strain (MCF-7, MDA-MB-543, SKBR-3) seemed sensitive to elisidepsin under both normoxic and hypoxic conditions.

First we aimed to investigate the binding of fluorescent elisidepsin to two cell lines by confocal microscopy: A431, as according to the IC_{50} values it is expected that hypoxia might decrease the binding of the drug and SKBR-3 as a control, because change of binding is not predicted in this case.

Elisidepsin substantially bound to A431 cells cultured in normoxia and it appeared intracellulary as well. Oxygen-deprived version of this cell line did not exhibit such high fluorescence. In contrast, the drug considerably bound to SKBR-3 cells regardless of the presence or absence of oxygen during the cell culturing phase.

By flow cytometric experiments we further wanted to prove the aforementioned loss of sensitivity against the drug due to hypoxia. We cultured cells under normoxic and 5-day hypoxic conditions and measured the extent of binding immediately after adding elisidepsin. Time of measurements was also saved for further data analysis. By a method, developed by our workgroup we could measure the amount of membrane-bound elisidepsin. Based on the results we can now conclude that the decreased binding of the drug is the reason of insensitivity observed in A431, CHO, HaCaT and HeLa cell lines.

5. DISCUSSION

5.1 The ligand binding of the epidermal growth factor receptor

During my PhD work I devised experimental systems that allowed us to examine the ligand binding properties of the epidermal growth factor receptor. Our main point of interest was to study the cooperativity and affinity of EGF binding and their dependence on the membrane environment and several other features related to the receptor. To define the nature of cooperativity, the Hill model is frequently used that is adequate for completely cooperative systems. In those cases, the Hill coefficient equals to the number of ligand binding sites and the K_d characterizes the affinity of binding. However, in the EGFR system molecular details behind the binding events and conformational changes of the receptor subunits must be of importance and it is impossible to take them into consideration with the Hill model. For these reasons we refer to data obtained by Hill fits as apparent cooperativity and affinity.

Firstly, we studied the possible role of the cytoskeleton by applying two agents that alter the actin structure: latrunculin-B depolymerises F-actin while jasplakinolid supports actin polymerization. Examining the ligand binding of treated cells and fitting the experimental results with the Hill model, we could see that both the cooperativity and affinity of EGF binding decreased in the case of latrunculin-B treatment, while jasplakinolid did not have such an effect. To examine visually the outcome of toxin application on cells, TRITC-phalloidin was used to stain the actin filaments and by confocal microscopic measurements it became clear that jasplakinolide did not change the amount of microfilaments in the cytoplasm, or more importantly, in the proximity of the plasma membrane. It can explain why jasplakinolid did not substantially alter the parameters of ligand binding. Latrunculin-B, in contrast, considerably reduced the actin content of cells.

It also remained a question if the proper glycosylation of the receptor might have importance in the nature of ligand binding of EGFR. Tunicamycin, an N-glycosylation inhibitor was applied that caused negative apparent cooperativity and low ligand binding affinity. The decrease of the molecular weight of EGFR upon tunicamycin treatment was also supported by western blots. However, one must keep in mind that as tunicamycin is an aspecific inhibitor, other proteins' inadequate glycosylation might also contribute to the detected changes in ligand binding.

Based on the findings described previously, we conclude that the receptor's anchoring to the cytoskeleton („confinement") and its proper glycosylation are needed to achieve positively cooperative ligand binding.

We also aimed to study the role of the intracellular kinase domain in EGF binding. By performing a treatment with the appropriate molecule it becomes possible to stabilize the active or the inactive conformation of the kinase. We applied two reversible inhibitors, erlotinib and lapatinib, with the former stabilizing the active, and the latter stabilizing the inactive conformation of the kinase. Having the kinase in its active conformation resulted in lower apparent cooperativity and higher ligand binding affinity, compared to non-treated cells. Lapatinib treatment, however, did not cause an effect on apparent cooperativity, but reduced the ligand binding affinity.

Based on the results of experiments examining the kinase domain, it seemed necessary to describe the distinct manners of dimerization and ligand binding events in detail. To achieve this, we came up with a new, molecular model. The model allows the kinase and the extracellular domain to exhibit independent conformations and the formation of different types of dimers. The model also covers the distinct ligand binding steps of the receptor species. However, several simplifications have also been introduced in the model in order not to increase the number of variables unreasonably.

The model seemed useful to fit our experimental results with. According to the results, the inactive conformation of the kinase and the closed conformation of the ECD is favoured in the absence of ligand. Differences in the dimerization tendencies were also detected: formation of asymmetric KD dimers (achieved by stabilizing the active conformation of the kinase) is characterized by considerably higher affinity than formation of the symmetric ones (occurs in the case of stabilizing the inactive structure of the kinase).

The affinity of binding the first molecule of EGF also takes place with different affinity depending on the kinase: symmetric KD dimers bind it with higher affinity than asymmetric ones. Asymmetric KD dimers bind the first molecule of EGF with high affinity and the binding of the second one occurs with substantially lower affinity, a typical feature of negative cooperativity. Symmetric KD dimers are characterized by positive cooperativity as the first molecule of ligand binds with lower affinity than the second one.

By using the model, it becomes possible to determine the amount of the 12 receptor species, taking the expression level and the ligand's concentration into consideration.

Presence of dimers consisting of symmetric KD dimers is remarkably high even if the concentration of EGF is low. Also, the amount of dimers does not seem to increase at high receptor expression level and concentration of EGF, a controversy we aimed to resolve by microscopic homo-FRET experiments. Clustering of EGFR was investigated on ice or at 37°C, with or without stimulating cells by EGF. We came to the conclusion that adding EGF to the system on ice did not cause lower homo-FRET efficiency in control and lapatinib-pretreated cells but stimulation at 37°C resulted in the clustering of the receptor. In the case of erlotinib treatment, we recognized that clustering is initiated by the ligand even if it was applied on ice- an observation supporting the predictions of our model.

To sum up, our results could resolve parts of the contradictions regarding the cooperativity of the EGFR system, as we could demonstrate that positive and negative apparent cooperativity is highly influenced by the conformation of the kinase domain and several other factors. We believe that activation of the EGFR system takes place only in the presence of abundant concentration of the ligand, avoiding aspecific effect to lead to cell responses. However, if the kinase is stabilized in its active conformation by a suitable inhibitor, dimerization and activation of the receptor occurs even in the presence of low concentration of the ligand. Taking these into consideration, we suggest that inhibitors stabilizing the kinase in its inactive form (lapatinib-like molecules) could be safer and more potent to apply in medicine, at least from a theoretical point of view.

5.2 The oxygen dependence of the binding of elisidepsin

Elisidepsin is a recently discovered cytostatic agent showing anti-tumour effects. Its mechanism of action is not completely described yet, but 2-hydroxy-fatty acids in lipid rafts and oxygen concentration are suggested to contribute to it. Earlier my colleagues demonstrated that many cell lines are relatively insensitive against the drug, and oxygen-depletion makes them even more resistant. Other examined cell lines were characterized by higher sensitivity, regardless of the level of oxygen.

To determine visually the binding of elisidepsin to cells, we set up confocal microscopic observations on A431 and SKBR-3 cell lines. Cells grown in hypoxic environment for 4 days and those grown in normoxic conditions were treated with a mixture of the fluorescent and unlabelled type of the drug at a ratio of 1:4. On the microscopic pictures we observed high fluorescence in the case of A431 cells grown in normoxia but we did not do so

in the case of oxygen-deprivation. SKBR-3 cells, in contrast, bound elisidepsin effectively, regardless of the oxygen level they were cultured in.

To study the binding of elisidepsin to the cell membrane we performed flow cytometric experiments as well. It was demonstrated that cells that became resistant against the drug due to hypoxic culturing conditions, bind elisidepsin more weakly- an observation clarifying the reason of low sensitivity by examining high number of cells.

According to our results and that of others, elisidepsin is a promising drug-candidate. Due to its encouraging anti-tumour effect and therapy index Phase-II examinations were initiated.

6. SUMMARY

In my PhD work, I examined the binding of two different proteins to the cell membrane. The function of the epidermal growth factor receptor and elisidepsin is influenced by the membrane's ability to produce protein and lipid associations.

Associations with multiple ligand binding sites usually do not bind ligands independent of each other, but some kind of cooperativity exists between the ligand binding domains. During my research I succeeded in identifying some conditions that play a role in the nature of cooperativity of the EGFR system: in control case cooperativity of EGF binding was positive, but it turned to negative due to the lack of anchoring to the membrane and inadequate glycosylation.

The effect of the conformational changes of the intracellular kinase domain was also investigated by a molecular model of ours. Equilibrium EGF binding was examined to cells with active or inactive conformation of the kinase, stabilized by kinase inhibitors. Dimers consisting of active, asymmetric KD dimers bind EGF in a negatively cooperative manner, while dimers with inactive, symmetric KDs were characterized by positive EGF binding cooperativity.

Elisidepsin turned out to bind to the membrane in an oxygen concentration-dependent manner: the loss of sensitivity observed in most examined cell lines is caused by decreased binding to cells- supported by various methods.

7. PUBLICATIONS

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List of publications related to the dissertation

1. **Hajdu, T.**, Váradi, T., Rebenku, I., Kovács, T., Szöllősi, J., Nagy, P.: Comprehensive Model for Epidermal Growth Factor Receptor Ligand Binding Involving Conformational States of the Extracellular and the Kinase Domains.
   Front. Cell. Dev. Biol. 8, 1-53, 2020.
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   IF: 5.201 (2019)

2. Király, A., Váradi, T., **Hajdu, T.**, Rühl, R., Galmarini, C. M., Szöllősi, J., Nagy, P.: Hypoxia reduces the efficiency of elisidepsin by inhibiting hydroxylation and altering the structure of lipid rafts.
   Mar. Drugs. 11 (12), 4858-4875, 2013.
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List of other publications

3. Kovács, T., Batta, G., **Hajdu, T.**, Nagyné Szabó, Á. T., Váradi, T., Zákány, F., Csomós, I., Szöllősi, J., Nagy, P.: The Dipole Potential Modifies the Clustering and Ligand Binding Affinity of ErbB Proteins and Their Signaling Efficiency.
Sci. Rep. 6, 35850, 2016.
DOI: http://dx.doi.org/10.1038/srep35850
IF: 4.259

Total IF of journals (all publications): 12,972
Total IF of journals (publications related to the dissertation): 8,713

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

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