universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

„DOCKING OF PYRAZOLOQUINOLINONES INTO THE BENZODIAZEPINE BINDING SITE OF THE $GABA_A$ ION GATED CHLORIDE CHANNEL & ANALYSIS OF THEIR BINDING CONFORMATION“

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1. BIOLOGICAL & PHARMACOLOGICAL BACKGROUND

1.1. GABA RECEPTORS & SYNAPTIC NEUROTRANSMISSION

GABA receptors

Glutamate, as an essential amino acid, is relevant for transmitting chemical signals and is the major protein in the mammalian brain.

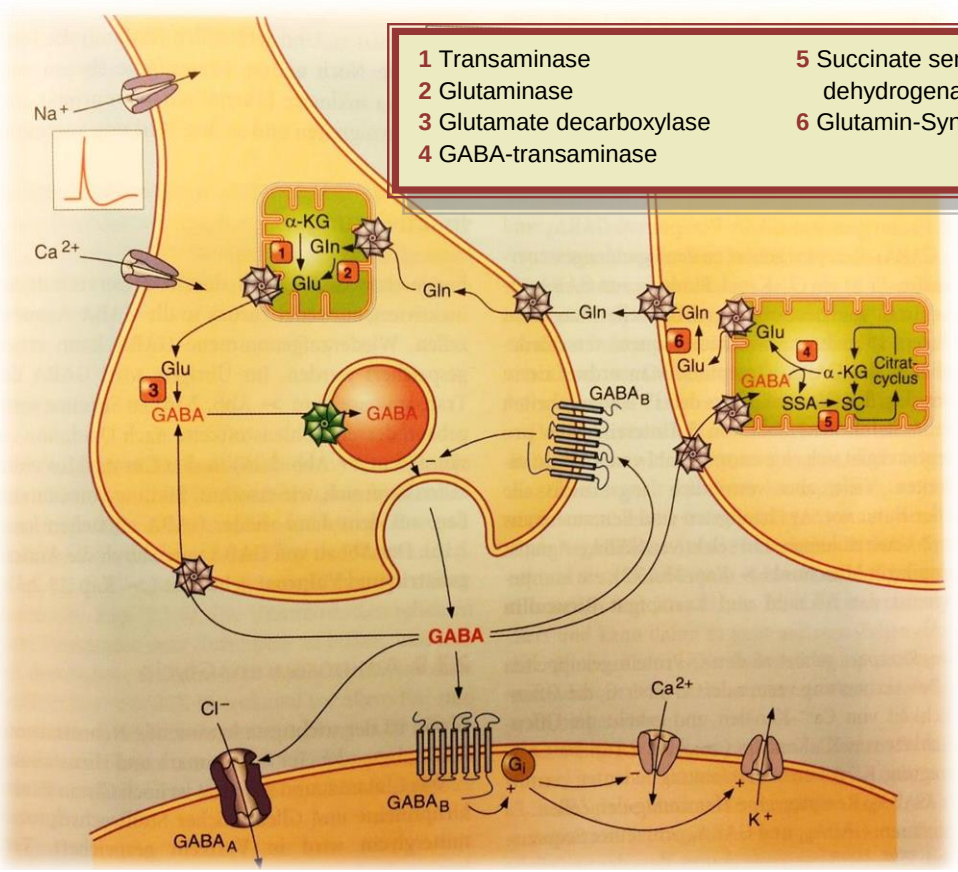
Hence glutamine is integrated in the citric acid cycle and as well as α-Ketoglutarate demonstrates to be the biochemical precursor of GABA. (1)

The creation of GABA (gamma aminobutyric acid) can take place in two ways:

1. **α-Ketoglutarat**: The conversion of α-KG to glutamate within the glutamate neurone is run by the transaminase. Glutamate is then decarboxylated by the glutamate decarboxylase to GABA and can then be implemented in a vesicle and released from the axoplasma. From there it is then transported to the GABA receptor.

2. **Glutamine**: Glutamine is transported into the GABAergic nerve endings by the glutaminase which hydrolyses glutamine to glutamate and is finally decarboxylated to GABA by the glutamate decarboxylase. Then it can be enclosed in a vesicle and released from the axoplasma for transportation to the GABA receptor.

The postsynaptic cell contains two main types of GABA receptors, $GABA_A$ and $GABA_B$ receptors. The $GABA_B$ receptors belong to the G-protein-coupled receptors of the Gi-family and are coupled to the Ca^{2+}- and the K^+-channel. If the $GABA_B$ receptor is activated the opening-possibility for Ca^{2+}-channels is decreased through Gi whereas the opening possibility for K^+-channels is increased. Ca^{2+}-influx leads to an excitation and the K^+-efflux leads to inhibition of the presynaptic release of GABA. Hereby GABA release is downregulated, via presynaptic $GABA_B$ auto receptors.



ill. 1: synaptic transmission through GABA from presynaptic to postsynaptic GABA receptor. (1)

The postsynaptic $GABA_A$ receptor is a membrane bound receptor complex, which acts as a ligand gated chloride ion channel.

It consists principally of α, β, and γ subunits. These subunits belong to the specific family which withholds 19 different putative subunits within the $GABA_A$ receptor. These subunits vary in their amino acid sequences divided into following subunits: α1-6, β1–3, χ1-3, δ, ε, π, η and θ1-3. (2) These can be arranged in different compositions within the receptor complex, but usually appear in counts of pentamers, whereby an enormous amount of possible combinations are given. Most frequently found is the heteropentamer with the subunits α1, β2 and γ2. (4) These have been mostly found in the mammalian central nervous system. (3)

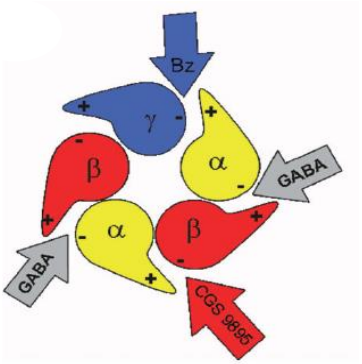
$GABA_A$ receptor subtypes

The $GABA_A$ receptor as a major inhibitory neurotransmitter receptor mainly in the mammalian brain is the target of many clinically important drugs interacting with different specific binding sites within the receptor. (4)

When GABA binds it triggers the chloride ion channel to open leading to a chloride ion flux into the nerve cell and inhibiting thereby the neurotransmission. (5)
This is how GABA triggers numerous neurological effects including convulsion, anxiety and sleep, and also influencing memory and learning processes.

Each different receptor subtype within anatomically distinct regions of the brain is responsible for specific physiological and pathological processes.
The binding site for the neurotransmitter GABA itself is located at the interface of β+α–. (1)

For the predominant pharmacological effect on $GABA_A$ receptors the α-subunit showed to play a determined role. (6)
Whilst the α1βγ2 receptor subtype has a prominent role in seizure, susceptibility and sedation, the α2βγ2 and presumably also the α3βγ2 subtypes are involved in anxiety. The α5βγ2 subtype has an important function in learning processes and memory. These determinations are important to take in consideration when it is aimed to design subtype selective ligands so that specific wished for reactions can be released. (6)



ill. 2: top view from heteropentamer with subunits a1, b2 and g2. Arrows point at the interface of the binding target for the respective ligand. (4)

1.2. BENZODIAZEPINES & ANALOGUES

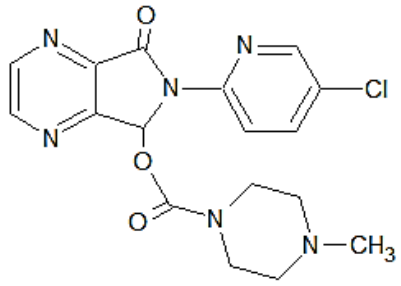
Benzodiazepines are up to now the best characterized drugs that interact with the $GABA_A$ receptor. The term “benzodiazepine” relates to compounds with the chemical structure of Diazepam (usually 1,4-Benzodiazepine). From the initial 1,4-

Benzodiazepine a class of various drugs has derived. At present there are more than a dozen of different Benzodiazepine types in clinical use which all have the same mechanism of acting. (1)

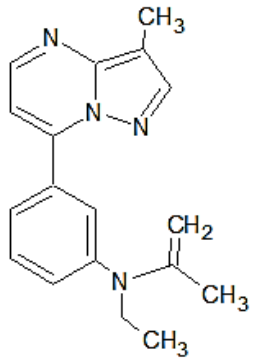
In general the subtypes of drugs that interact with the $GABA_A$ receptor are

- $GABA_A$ antagonists: i.g. Flumazenil
- $GABA_A$ agonists: i.g. Alprazolam, Diazepam, Midazolam, Triazolam etc.
- $GABA_A$ partial agonists: i.g. Bretazenil
- $GABA_A$ inverse agonists: i.g. beta-Carboline DMCM

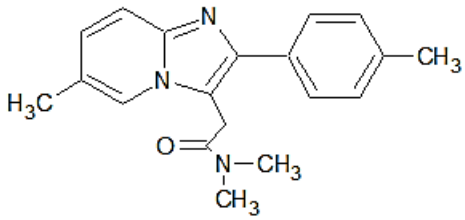
Structural benzodiazepine (BZD) analogues, which also bind to the Benzodiazepine binding site, are the so called “Z-Hypnotics”, which are Zolpidem, Zaleplon and Zopiclon. (1)



ill. 3/a: Zopiclon



ill. 3/b: Zaleplon

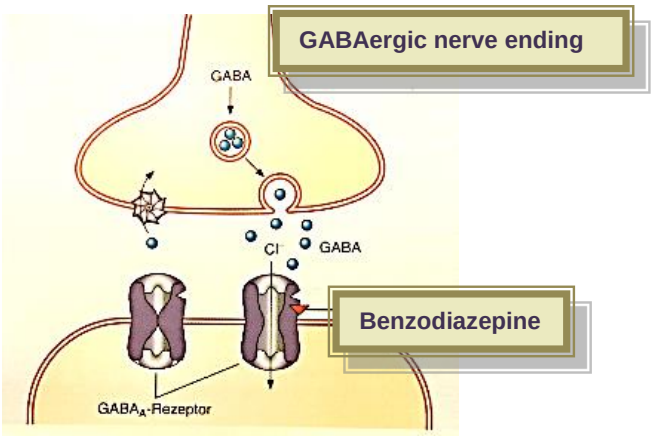


ill. 3/c: Zolpidem

ill. 3: chemical structures of “Z-Hypnotics”, BZD analogues. (1) Drawn in Chem ACD/Chem Sketch (Freeware) 2012.

1.2.1. EFFECT ON RECEPTOR

In general the pharmacological effects of Benzodiazepines are anxiolytic, sedative, muscle relaxing, anticonvulsive and downregulates endocrinologic effects.



ill. 4: The $GABA_A$ receptor is a GABA dependent chloride ion channel. Its activation is increased if BZD is bound to the receptor. BZD alone does not release any effect as long as GABA does not activate the $GABA_A$ receptor. (1)

Activation of the $GABA_A$ receptor is followed by the binding of the neurotransmitter GABA, which is released from the GABAergic nerve endings. When Benzodiazepine binds to the allosteric high affinity benzodiazepine binding site which is located in the extracellular domain at the $\alpha+\gamma$- interface of the $GABA_A$ receptor it modulates GABAergic activity increasing the effect of GABA. (1)

The potency of the effect, determined by the affinity to its benzodiazepine binding site, is dependent on the chemical structure of the specific benzodiazepine. If GABA is bound in the $GABA_A$ receptor, Benzodiazepine causes a left shift in the dose-effect-curve of the signaling. The stronger the left shift of the dose-effect-curve, the stronger the effect. (1) The termination of GABA's effect is triggered by the reuptake of GABA in the GABAergic nerve-endings.

Is GABA not bound, Benzodiazepine cannot release any effect, indicating that Benzodiazepine's effect is dependent on GABA being present.

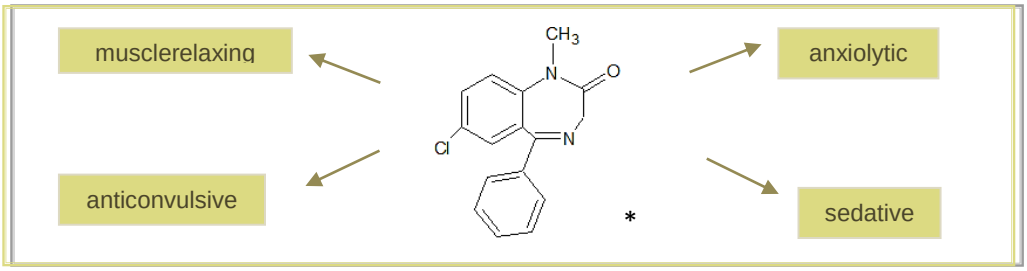
1.2.2. PHARMAKOKINETICS & -DYNAMICS

In general the bioavailability is > 80%. The maximum level after oral intake is usually reached after 1 to 2 hours and the plasma protein binding and its active metabolites are mostly > 80%. Duration of the benzodiazepine's effectiveness is dependent on the speed of its metabolic elimination which is carried out by the enzymes CYP2C19 and CYP3A4 and also via the coupling of glucuronic acid. (1)

1.2.3. INDICATION

The clinical use of benzodiazepines mainly involves the treatment of

- anxiety disorders within schizophrenia or depression and panic attacks,
- sleeping disorders
- epilepsy or cerebral convulsion caused through intoxication (1)



* structure drawn with ACD/ChemSketch (Freeware) 2012.

Furthermore it is used

- in anesthesia as a pre-medication or in combined anesthesia
- within internal medication, for the sedation of specific body parts in order to conduct investigations, such as gastroscopy or colposcopy.
- during heart attacks, for the relief of psychological stress and anxiety (1)

1.3. OBSTACLES OF BENZODIAZEPINES

1.3.1. SIDE EFFECTS

Depending on the initial reason for indicating, the wished for effect as well as the side effects vary. The acute side effects under an oral therapy of Benzodiazepines can mainly be somnolence, fatigue, lack of concentration, attention and alertness, if not part of the wished for outcome. Furthermore it can cause ataxia, akinesia, ataxic disorders, anterograde amnesia, decreased libido and appetite as well as gain of

weight. In older patients it can cause insomnia and tension. After intravenous application or high-dose-intake or when combined with alcohol, it may cause bradycardia and hypoventilation. In rare cases it can even lead to asystolia.

When it is under chronic use, it leads to risk of dependence and tolerance. As an antidote Flumazenil, a Benzodiazepine antagonist, is indicated. (1)

1.4. CGS-COMPOUNDS

In the 1980s a new class of benzodiazepine site ligands, 2-arylpyrazolo[4,3-c]quinolin-3-ones, have been discovered to perform highly affine towards the BZR (benzodiazepine receptor). (7) These are known under the name “CGS compounds”, since the first lead components were CGS 8216, CGS 9896 and CGS 9895.

These compounds behave as null modulators (antagonists) and have low affinity via the $\alpha 2\beta\gamma 2$ interface of the $GABA_A$ receptor.

Since 2011 it has been found out that these CGS-compounds not only act as antagonists by the binding to the benzodiazepine binding site but also are able to cause an increased GABA induced flux by binding to a binding site located at the extracellular $\alpha+$ $\beta-$ interface, called the “CGS binding site” with high affinity. (8),(4)

During the process of closer identification of Pyrazoloquinolinone derivatives, more compounds were detected which aspire a modulatory effect via the $\alpha 1+\beta 3$-binding site, mostly acting as null modulators or with low affinity to the BZD binding site and high affinity towards the CGS binding site. (4)

1.5. THE SUITABLE BINDIG-FIT

It turned out that some positive allosteric modulators or null modulators elicited their effect by solely binding to the $\alpha 1+\beta 3-$ binding site.

Activation of the α+β- interface, and by this triggering benzodiazepine like effects, has so far only been observed under the use of Pyrazoloquinolinones and Pyrazolopyridinones. (9)

Activation of the CGS-BS (α+β- interface) has been proven to show benefits in comparison to the BZD-BS (α+γ- interface) activation by classical benzodiazepines:

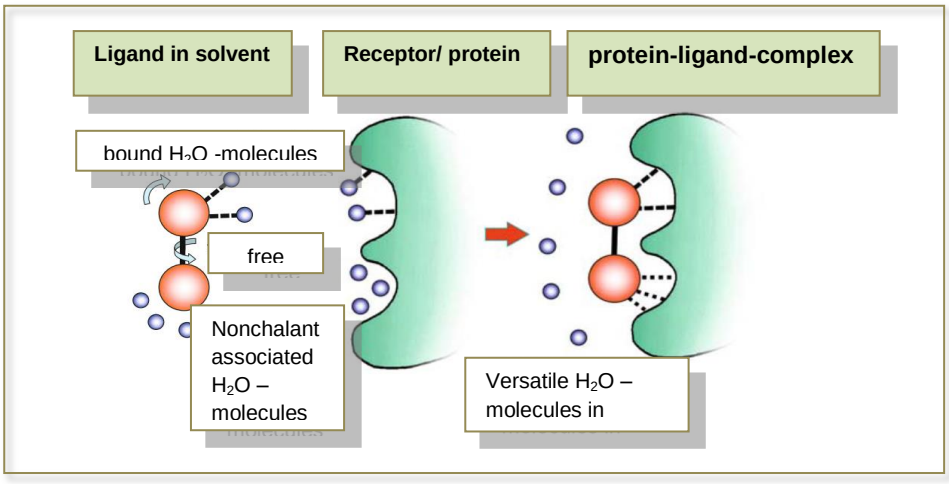
- It is presumed that the binding in the pocket of the α+β– interface may be the reason for fewer side effects than if ligands are bound in the pocket of the α+γ– interface. (4),(10)
- There are no effects in absence of GABA. As a consequence lower risk of toxicity is expected.
- They do not only react with the αβγ receptor subtype but a far broader range of subtype receptors, such as the subtypes αβ, αβγ, αβδ and αβε. Since more receptor subtypes are available for the CGS compounds to potentially react with, it is presumed that less substance is needed in order to preserve a pharmacological effect. This is another reason for assuming that lower risk of toxicity is present under CGS' binding.
- Especially tolerance is under suspicion to be caused by the activation of the g-sheet. This would be abolished with the subtype selective activation of the α+β- interface in the $GABA_A$ receptor. (11)
- Therefore they are more appropriate in the treatment of chronic disease like epilepsy as a prevention of tolerance development could be achieved. (11)

Taking these benefits into consideration, the importance of gaining insight of subtype selective activation of the α+β– interface of the $GABA_A$ receptor, is revealed. (4) Thereby the path to a novel prospectus of therapy may be paved in the course of long term therapy when treating disease such as epilepsy without causing serious side effects typically for classical BZDs.

For the prediction of interactions and steric reactions between synapses in general, construct and positioning play an important role. Also external factors can increase or decrease the transmission of hormones. (12) This becomes relevant in the research of subtype specific activation.

1.5.1. ACTIVATED DRUGS

The fundamental work in developing active pharmaceutical drugs particularly involves considering types and intensity of interactions between protein and ligand. (13)



ill. 5: process of a protein-ligand-complex being made up through the formation of H-bond interactions. (13)

For an active compound to release its effect, it needs to bind to a very specific target. This can either be a protein, such as an enzyme or receptor, or it can be a target in form of RNA and DNA. In order to be able to sufficiently bind, size and form have to match with one another. In this context the image of a lock and key model presents an example for the necessary fit between ligand and protein. This comparison was firstly introduced by Emil Fischer in 1894 to which Paul Ehrlich two decades after this commented, ”Corpora non agunt nisi fixate”, which translated means that the bodies cannot release an effect unless they are bound.
Additionally to an ideal fit to the binding protein’s surface, the ligand’s structural characteristics should suit the protein’s surface character so that beneficial interactions can be released. (13)

The dimension of the protein-ligand interaction is defined by the binding affinity described by the inhibition constant, Ki. A change in Ki is caused by a change in the free enthalpy of the binding. The free enthalpy is a form of characterization of the energy relationship when a complex is being built.
The ability of a protein-ligand-complex being formed is answered in the aspiration of all natural processes wanting to bring order in a disordered condition. For the description of the disordered condition entropy S is used.

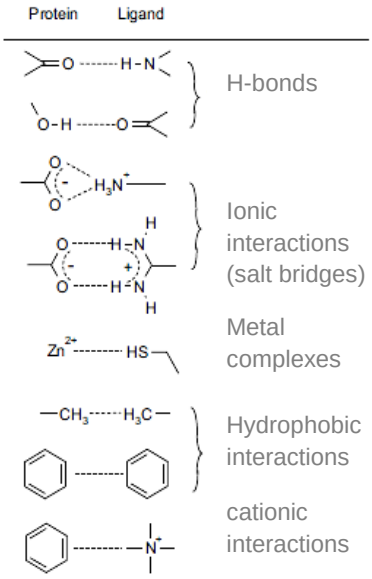
Consequently to describe the process of a protein-ligand complex being generated, both enthalpy and entropy are needed, which are in constant exchange between the binding partners. Taking the energy balance into account, the change in entropy, in terms of the variable energy spread by the degree of freedom in the system, is also considered. (13)

1.5.2. PROTEIN-LIGAND INTERACTIONS

Very often **hydrogen bonds** are seen in protein-ligand complexes. Here the partner that withholds the proton is defined as the H-bond donor. Usual H-bond donors are -NH or -OH groups which interact with H-bond acceptors. To the H-bond acceptors belongs a group of electronegative atoms with a negative charge like O or N and therefore pull protons towards them. Thereby the **electrostatic interaction** rises and ligands are drawn closer towards the protein.

Ionic interactions (salt bridges) represent a kind of electrostatic attraction which take place between controversially charged components. This appears fairly often and moreover indicates a strong electrostatic attraction when the ligands' distance is defined by only 2.7-3.0 Armstrong.

The presence of **metal ions** in proteins like Zn^{2+} or Cu^{2+} etcetera can enable an interaction with a controversially charged group of the ligand. Some groups, like thiol RSH or acidic groups and many other nitrogen heterocycles, can also form complexes. (13)



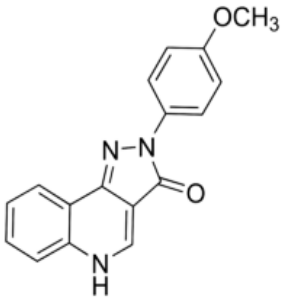
ill. 6: examples of protein-ligand-interactions, with contribution towards a good binding-affinity quality. (13)

Hydrophobic interactions take place when apolar sidechains of the proteins' amino residues and lipophilic groups (e.g. CH-rests, tiophen, furan, halogens etc.) of the ligand are close to one another. This type of interaction is not specifically oriented, which is why exact coordinates of the lipophilic are not important, thus knowing the distinctive region which it is drawn near. (13)

1.5.3. ANALYSIS OF CGS' BINDING MODE

In the journal paper of *L.Savini et al.* **"High Affinity Central Benzodiazepine Receptor Ligands. Part 2: Quantitative Structure-Activity Relationships and Comparative Molecular Field Analysis of Pyrazolo[4,3-c]quinolin-3-ones" (3)** a 3D-QSAR in form of a COMFA with 106 pyrazolo[4,3-c]quinolin-3-ones was proceeded in order to analyze the potential interactions of pyrazoloquinolinones (PQs) in the BZD pocket.

The core structure they used here is the CGS-8216, 2-Phenyl-3H-pyrazolo [4,3-c]quinolin-3-one.



ill. 7: chemical structure of 2—[4'-Methoxy] Phenyl-3H-pyrazolo [4,3-c]quinolin-3-one → CGS-9895 (3)

EXPERIMENTAL METHODS

They tested a dataset of 106 PQs for their ability to displace [3H] flunitrazepam from the benzodiazepine binding site of rat brain membranes in the course of a radio ligand binding measurement (pIC50 values are reported). Also they give an extensive review on a 3D-QSAR of several classes of central BZR-ligands.

For the ***3D-QSAR***, the compounds were divided into four subsets for which equations were derived, assessing electronic and hydrophobic effects, molar refractivity, van

der Waals volume and verloop sterimol parameters illustrating bulkiness and polarizability and describing their character.

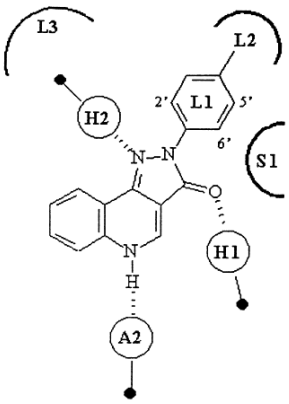
The results of the van der Waals coefficient suggested that binding of a 2-Phenyl-substituted-ring at the L1 and also L2 pocket region of the receptor is favorably filled out by small electron donor substituents at meta (3'/5') and para (4'). This can be seen in the picture illustrated above from the paper of L. Savini (3), which is also in accordance with the model presented in the paper of Richter et al in 2012. (14)

The substituent's **lipophilic character** is presumed to play a very important role in the receptor-ligand interaction, which delivers evidence of the lipophilic character of the receptor region, specifically L2, which is assumed to interact with the para positioned substituent of the 2-phenyl ring.

π–π stacking interaction is suggested at the L1 receptor region and as a consequence aromaticity has to be offered by the substituent here.

Regions of **H2** and **H1** would prefer **electron donating** moieties.

In contrast **A2** indicates wanting to accommodate **electron withdrawing** moieties



ill. 8: pharmacophore model for BZD-receptor. H represents an HB donor site, A an HB acceptor site, L a hydrophobic site and S a sterically inaccessible region. For an inverse agonist activity H2 and A2 do not have to be triggered. (3)

3D-QSAR – CoMFA

The performed 3D-QSAR, presented in this paper included 106 PQ ligands. (3) The delivered steric and electrostatic isocontour maps showed following results:

- STERIC ISOCONTOUR MAP: A steric fit is gained with substitution in position 5’ and 8 and to a lower degree at position 2’ which means that by substituting here, the pocket is well occupied. Also the favour of substitution in position 5, 6, 7 and/ or 8 as well as 4’ lead to a strong drop of affinity. In concerns of position 8 the report is contradicting as it suggests in the beginning of the description through the steric isocontour map that in position 8, substitution is favored. But shortly afterwards it is written that detrimental substitution is seen in position 7 and 8. (3)

- ELECTROSTATIC ISOCONTOUR MAP: In the report it is indicated, that non-favored electrostatic signals are seen in position 4’, 5’, 6’, 6 and 7, causing a drop in affinity. The report is in contrast by once describing the favourability of low electron density and positive charges (nucleophils) in position 4’, leading to a rise in affinity. Later it is implied that electron rich groups near 9(1), 4’ and 1’ (2’) increase the binding affinity and vice versa positive charges in this region decrease affinity. (3)

Hence it is of great interest to get clarification about the uncertain regions, which are especially seen in C4' and C8.

2. COMPUTATIONAL BACKGROUND

2.1 LIGAND BASED DRUG DESIGN

2.1.1 3D-QSAR/ COMFA- Comparative Molecular Field Analysis

- Methodology

2.2 STRUCTURE BASED DRUG DESIGN

2.2.1 GABA Protein Models

- The alignment of the protein models
- Differences between the protein models

2.2.2 Molecular Docking

- Use of Docking
- Docking Program GOLD
- CLUSTERANALYSIS
- PLIF – PROTEIN-LIGAND INTERACTION FINGERPRINTS
- CBM ANALYSIS
- SCORING OF BINDING MODE

2.3 OBSTACLES OF COMPUTATIONAL WORKFLOW

2.3.1 Challenges of Docking procedure

- Flexibility
- Soft potential
- Time issues

2.3.2 Challenges concerning scoring functions

2.3.3 Challenges of clustering docking poses

2. COMPUTATIONAL BACKGROUND

Within an in silico workflow, knowledge of the protein-ligand fit, is vital for creating new ligands towards a specific interaction profile. (15)
Designing ligands that will bind tightly to their target is done by modeling techniques using special computer programs that visualize the compounds' docking poses in the binding-protein and also yield predicted affinity values (Ki) and represent protein-ligand interactions. (16)

➢ RATIONAL DRUG DESIGN

Molecular modeling is one part of various systematic approaches to attain the lead compound as well as to optimize the existing ones by looking into the protein-ligand quantitative structure activity relationship. By this the correlation of the substance's chemical structure and the biological activity can be understood and as a consequence the correlation between lipophilicity, electrostatic, steric factors and biological activity, transport and biological distribution can be put into a significant statistical system. Thereby the construction of a hypothesis is possible and enables realization of where to set the starting point for improving the interactions between protein and ligands or a compound series. Conclusively the ligands structure can be adapted so that high affine subtype selective binders are created. This is done within molecular drug design also known as ***computer aided drug design***. **(13)**

This rational drug discovery is an approach which is in **contrast to the traditional method of drug discovery**, where it is relied on trial and error testing of chemical substances on cultured cells or animals. Contrary to this, rational drug design begins with a hypothesis that modulation of a specific biological target may have therapeutic value. Once a suitable target is identified, the target is normally cloned and expressed, and on this a screening assay is established. This enables the three dimensional structure of the target being determined, which makes the screening of small molecules, as potential drug compounds that bind the target, possible. (13)

Within an in silico approach influences of binding affinity, like polarizability and electrostatic potentials, are calculated. Also knowledge based scoring functions may be used to provide prediction of binding affinity by using methods like linear regression, machine learning, neural nets and other statistical techniques to obtain predictive binding affinity equations. This is possible by translating experimental affinities to computationally derived interaction energies between the molecule and target.

The aim behind this in silico workflow is to predict affinity before synthesizing, so that a lot of time as well as costs can be saved. (13)

Although discovery of an optimal drug still needs several iterations of design, synthesis and testing, the computational method accelerates discovery by reducing the number of iterations required and has often provided novel structures. (13)

For example a novel, potent, and selective amidosulfonamide 5-HT1A agonist for the treatment of anxiety and depression was discovered through an integrated in silico 3D-model-driven discovery, which is possible by increasing a lead compound's binding affinity to its target. (17)

Typically a drug target is a key protein involved in a particular metabolic or signaling pathway that is specific to a disease, pathological condition or to the infectivity or survival of a microbial pathogen. The attempt in drug design is creating drugs that inhibit the key molecule in the dysfunctional pathway which is responsible for causing the disease. This is ensued by them binding to the active region and inhibiting it without affecting any other important "off-target" molecules or anti-targets whereby this could lead to side effects.

Principally it is distinguished between two different types of approaches in drug design, referring either to ligand based or structure based drug design. (15)

2.1. LIGAND BASED DRUG DESIGN

The method of ligand based drug design is based on the knowledge of active compounds with their property to a specific target protein. By the finding of a correlation between their structure and ligand-receptor interaction, novel drugs can

then be elaborated. This was previously only possible by trial and error approaches. Hereby ligands with pertained 3D structure can be used for ligand based drug design by creating a pharmacophore model, using the molecular similarity approach or performing a quantitative structure activity relationship, which leads to progressively designing ligand based drugs. (18)

2.1.1. 3D-QSAR/CoMFA -Comparative Molecular Field Analysis

Comparative molecular field analysis is an in silico approach of correlating structure and activity from a data table containing known molecules. Here columns withhold property values in form of numbers, representing the activity property, and rows contain compounds, which represent the structure property. The aim of CoMFA is predicting the behaviour of new molecules, which is done by creating a form of a linear equation between the structure and activity information, which is called quantitative structure/activity relationship (QSAR) in a three dimensional format. (19)

METHODOLOGY

CoMFA, as a 3D-QSAR technique, summarizes the correlation between biological activity and 3D-shape of a set of molecules. The results are represented in form of steric isocontour maps and electrostatic isocontour maps, where electro negative or – positive moieties are represented. (20)

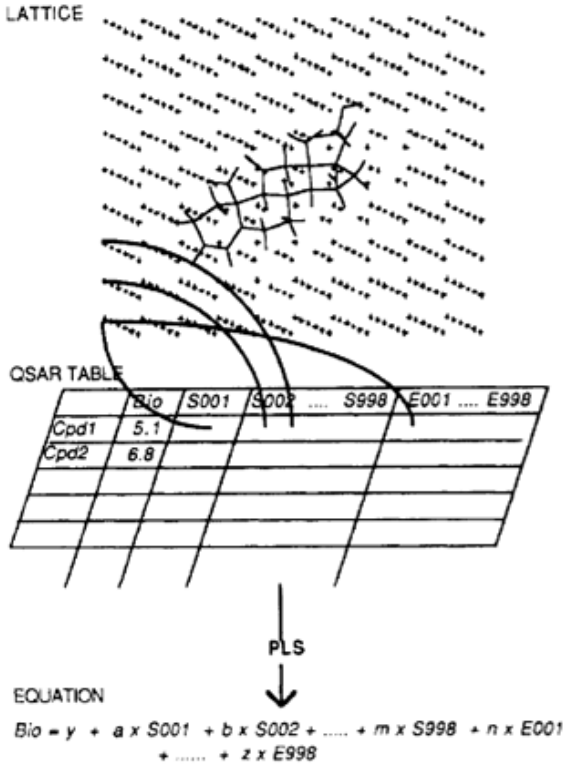
1. First of all a set of molecules has to be selected, which interacts with the same binding target in an equal manner and geometry. This is referred to as the training set.
2. The Calculation and low energy conformations have to be generated for the atomic parts.
3. A pharmacophore hypothesis can be set up, whereby all individual molecules can be superimposed and aligned.
4. A lattice is positioned around the molecules and measures the distances of each atom to each atom in the grid system.
5. The field values in each grid point are calculated by the different atomic probes, such as positively or negatively charged atoms, hydrogen bond donors or acceptor or lipohphilic probes that are situated in the regular 3D lattice.

6. The PLS analysis is the most used method to extract a stable QSAR from this data table. Components run by a PLS analysis, deliver a cross-validated r^2.

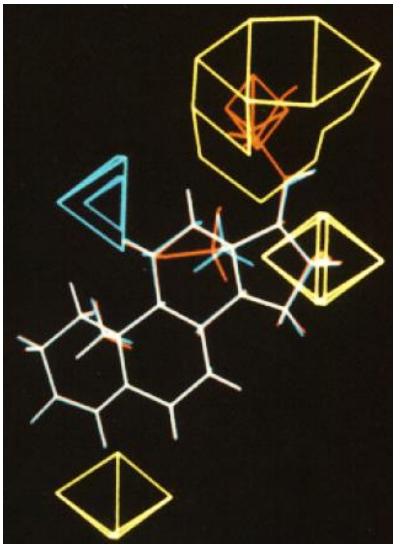
7. A regression equation is created with thousands of coefficients by making use of the PLS, which is used back in the original data space. The QSAR equation includes a coefficient per column in the table to each two lattice points that represents zero. By this it can be put into a 3D-space, of the fixed lattice. (ill. 9a)

8. The results are pictured in form as contour maps which are colour indicating images of the ligands: the electrostatic isocontour maps indicate electropositive or -negative favoured regions and the steric isocontour maps show favoured and nonfavoured steric regions of the ligands. (ill.9b)

9. Summing up these pieces of information, the compound's characteristic features and behaviour is understood. (20)



ill. 9a: chemical structure is placed into a 3D-space of a fixed lattice where each distance between each atom is measured by which a regression equation is created through PLS. (19)



ill. 9b: 3D-QSAR delivered image, which gives a stereoscopic view of the respective molecules' steric features to the target.

Colour indication: blue means high steric interaction/ high binding affinity; yellow and red means low steric interaction/ low binding affinity

This example represents the binding of aldosterone (red molecule) and testosterone (blue molecule) to the testosterone-binding globulin (TBG). Conclusively aldosterone has poor and testosterone high binding affinity to the TBG. (19)

2.2 STRUCTURE BASED DRUG DESIGN

The approach of structur based drug design is based on the knowledge of the three dimensional structure of the biological **target** obtained through X-ray-crystallography or NMR spectroscopy or similar methods. Also it is possible to create a homology model of the target based on the experimental structure of a protein related to it. Most of the published structural data are distributed to subscribers from the Brookhaven Protein Data Bank on-line on http://www.pdb.bnl.edu.
Within structure based drug design obtained structural information of the ligand, mostly deduced by the natural substrate to the target, is therefore used to look for substances with improved binding and drug property. (21)

2.2.1 GABA PROTEIN MODELS

For docking procedures protein models with well-known order of the hydrogen bonds are needed. Specific $GABA_A$ crystal structures have been used a lot for past binding mode evaluations and for characterizing certain compound classes, such as β-Carbolines, 1,4-Benzodiazepines, Triazolopyridazines, Imidazopyridines and more. As a consequence $GABA_A$ crystal structures were already very well investigated. (22)

Among the available protein models usable for docking procedures into the crystal structure of the $GABA_A$ receptor, four different protein models were chosen in the course of this work: The latest published model, a β3 $GABA_A$ homopentamer, with the PDB code **4COF**, was included (23), as well as two models based on the Acetylcholin binding protein, **X77** and **N103**, and another binding protein model based on **GluCl,** the glutamate gated chloride ion channel. The GluCl BP model is an α-homopentamer and is the first anion selective Cys-loop receptor of the Caenorhabditis elegans. (24)

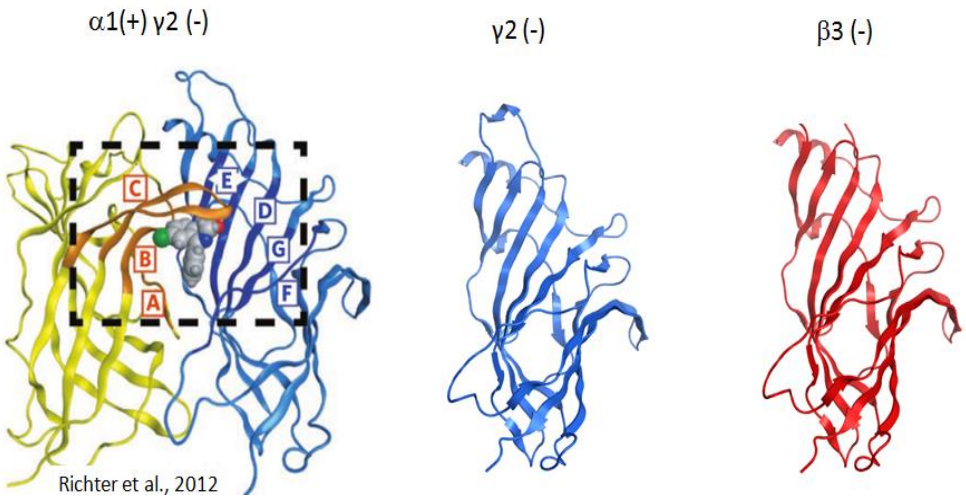
So far atomic models have been determined with a resolution at 4 Armstrong. With the X-ray structure of the human β3-homopentamer $GABA_A$ receptor, it was possible to reach a resolution at 3 Armstrong. This novel $GABA_A$ receptor model shall be able to explain rules of assembly, ligand binding and more. (23)

By using these respective protein models a significant range of the most realistic suggested docking poses should be offered from which further distinctions can be followed up.

➢ THE ALIGNMENT OF THE PROTEIN MODELS

In the binding protein there is a **principle** and **complementary part**. The principle is the part that is necessary to make the binding possible. The complementary can vary subtype specifically and is presented by the subunits α, β, γ or δ. (14)

Principals of the $GABA_A$ crystal structure are represented by loop **A, B, C** and form the **plus** side, whereas the complementary is represented by loop **D, E, G, F** and provide the **minus** side. (14)



ill. 10: On the left side it is shown the ribbon mode view of the GABA protein structure with subunits a1 (+) and g2 (-) representing the BZD Binding Site parallel with the lipid bilayer. Diazepam is bound in it, shown in framed region, and demonstrated in its space filling model. (22) Loop C is coloured in orange demonstrating the flexible binding part allowing adaption to the ligand's most preferred binding mode. The loops C,B,A,E,D,G, F are accordingly marked .On the right side g2 (-) subunit is illustrated next to the b2 (-) subunit demonstrating their exchange when mutated to the CGS binding site. (14)

The **loops E, D, G** lie next to one another and are conserved regions, corresponding to the gamma subunit.

Not conserved regions are for example represented by Tyr209, in **loop C**, and also the region between Gln169 and Lys171 in **loop F**. The beneficial use of flexibility in this area is seen in the possibility of the protein adjusting itself to the ligand's structure so that an efficient fit between protein and ligand can take place.

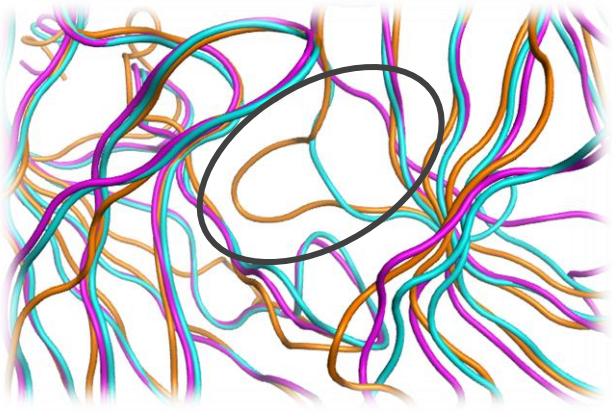
The most relevant amino residues revealed in the alpha subunit are S205, T209, H101 and Y159. The most relevant amino residues revealed in the gamma2 subunit are Y58, F77, A79, Y142 and E189.

It is known that these respective amino residues form the relevant parts of the binding pocket, but it is not known in which rotamer they appear. Depending on the rotamer they take in, the space in the pocket available to the binding ligand, varies. **Tyr159** is an important part of the pocket-forming area in the alpha subunit and corresponds towards the other subunit. Correct placement of Tyr159 in the protein model is therefore of great relevance and has an effect on how the ligand is placed in the pocket, by the gamma subunit interacting with it. When comparing the BP models, X77 and N103, it is seen that Tyr159 in the gamma sheet, takes in different conformations and suggesting different docking pose results.

Altogether the main differences between the docked binding protein models are seen within the region of the gamma sheet which could lead to a slightly dissimilar ligand binding placement.
When hypothesizing on the basis of suggested docking poses, it is important to keep in mind whether the involved amino residues are part of the conservative or the flexible complementary side of the binding pocket (e.g. E189 in loop F)

➢ DIFFERENCES BETWEEN THE PROTEIN MODELS

The Pre-E-Region is called the part of the binding pocket, which is located at the backside of the extracellular binding site, along the conserved amino acid sequence in Loop E between M130 and T142, where the amino residues form the shape of a “hairpin”. The consequence of this being different, is a slight change in the actual binding site. (25)



ill. 11: BPs, X77, GluCl and N103 aligned in MOE. Encircled is the area of the Pre-E-region which is uncertain and might lead to different binding mode suggestions. Orange encoloured is GluCL, turquoise is X77 and pink colour coded is N103.

In the binding pocket of X77 “integrated gaps”

are spread along the conserved parts within loop E, which causes an aerial constriction in the binding site and narrows down the bottom area of the “formed basket. (25)

Compared to the protein’s origin, the ACh-models contain a deletion of three amino acids within the Pre-E-Region, whereas the GluCl model has the same sequence length as the human $GABA_A$ receptor, which is why it is presumed to be the most correct BP (binding protein) model concerning the Pre-E-region. (25)

In the ACh-BP, X77, the deleted amino acids are built in as integrated gaps, thus spread along the region of the conserved parts. In N103, the deleted amino acids are distributed in the sequence as fused gaps, thus meaning that the missing amino acids are inserted all at once next to one another. (25)

Concerning the similarity of the GluCl model to the origin, it was considered the most reliable model till 4COF was published. (25)

2.2.2. MOLECULAR DOCKING

Use of Docking

Molecular Docking is a bioinformatic approach of predicting the binding energy of two molecules. Docking is subdivided into different subtypes, depending on the partners interacting in the binding to one another:

- protein-protein docking
- protein-ligand docking
- docking of DNA and RNA (26)

The docking approach is often used to research among pharmaceutical areas in order to discover novel drug compounds with higher and better binding affinity for a specific pharmacological therapy with a better output of the one of the lead compound. (26)

Through the use of a large dataset of compounds, the binding pose can be virtually tested by placing them in the target molecule in multiple possible conformations.

The most important programs for this available are Schrödinger Suite, GOLD and SYBYL.

	Schrödinger Suite	GOLD v1.6.2.	SYBYL-X 2.1.1.
COMPANY	Schrödinger, LLC	CCDC	Tripos, L.P.
GRAPHICAL INTERFACE	Maestro	HERMES	SYBYL
OPERATING SYSTEM	(Windows), Linux	Windows, Linux	Linux, SGI
MOLECULE MODULE	Yes	No	Yes
LIGAND FLEXIBILITY	Yes	Yes	Yes
PROTEIN FLEXIBILITY	Yes	Yes(limited to 10 sidechains)	No (?)

(26)

DOCKING-PROGRAM GOLD

Behind the name “GOLD” stands the description “**G**enetic **O**ptimisation for **L**igand **D**ocking”. GOLD is a genetic algorithm for docking flexible ligands into a protein binding site. (27)

The program itself is supplied as part of the GOLD suite, including two software components, which are called Hermes and GoldMine.
Hermes visualizer is used to prepare input files for the docking process, visualization of docking results and calculation of the descriptors. It is also used to setup an interactive docking, if for example the defining of the binding site and the setting of constraints is needed.
The other software component GoldMine is a tool for analyzing and post-processing the docking results. (27)

The most commonly used molecular modeling environments to create and edit starting models, used in the docking process, include SYBYL, MOE, Insight II and Discovery Studio.

After the ligands are modeled, they can be docked into the protein binding site via GOLD from prepared input files.

With the preparation, Hermes assists for adding hydrogen atoms, and everything else necessary for defining the correct ionization and tautomer status as well as the flexibility and potential appearance of side clashes of the protein residue.
The GOLD scoring functions are GoldScore, ChemScore, ASP, CHEMPLP and User Defined Scores.
Genetic algorithm parameters help to control the balance between the speed of GOLD and the reliability of its prediction. (27)

VIEWING AND ANALYZING RESULTS FROM GOLD

During the Docking process various output files are created, containing

- the initialized protein and ligand
- the docked ligand/ ligands
  Each ligand is normally docked several times and per given input ligand a set of files is produced, each containing the results of a separate docking attempt. All of these files can be collected to a single file.
- the protein binding site geometry
  During a docking run hydrogen bond geometries are optimized by rotating groups like OH and NH_3 groups. The possibility of keeping specific protein sidechains flexible is given.
- ranked fitness scores for an individual ligand and a set of ligands
- information on the progress of each docking run, where the genetic algorithm is recorded with each docking run of a ligand also comparing various docking solutions presented in a matrix of RMS deviations between the various docked ligand positions.
- Moreover GOLD creates files containing error messages, process information and more

The similarity of the docking poses collected in the clusters is shown in RMSD-values, which is important for objectively identifying different binding modes.
The docked solutions can be visualized with HERMES once the job is completed. By calculating additional descriptors for the docking poses they can be defined by descriptors, which is very beneficial for the use of further analysis of a GoldMine DB.

➢ CLUSTERANALYSIS

A clusteranalysis aims to subdivide a group of entities into more homogeneous subgroups on the basis of similarity among the entity of objects. Once the clusters represent a newly created group, it is possible to use them for further classifications, identification of present groups in a dataset, definition of hypotheses about relationships and for the recognition of patterns to predict the behaviour of objects within other various segmentation workflows and to design new series of compounds. (28)

Thereby a whole pool of docking poses, suggested by the docking program, subdivides into different clusters according to their similarity containing specific main conformations, represented by their “central object”. After the clustering process each cluster contains those poses which are most similar to one another. The main conformation is represented by the “central object”. (28)

By looking into and analyzing the central object poses of the cluster analysis, an overview of the main different conformations out of a huge suggestion on different modes can be gained. Implementing the cluster analysis in order to look into the binding mode of docked compounds it is spoken from the CBM analysis (Cluster binding mode analysis) where all docking poses are clustered into specific cluster classes.

Different clustering methods can be used in the process of the cluster analysis:
In the method of overlapping clusters, each object can be stored in more than one cluster. In the method of non-overlapping clusters, each object occurs only once stored in a specific cluster class. This method of clustering is further subdivided into hierarchical and non-hierarchical clustering methods. (28)

- **Non-hierarchical clustering** (28)
  - **Single pass**
  - **Relocation**
  - **Nearest neighbor**
  - **Others** (Centroid-, Distribution- or Density-Based Clustering)

- **Hierarchical Clustering** (28)

The arrangement of clusters is usually visualized within a dendrogram, where the similarity level and the path of the molecules' fusion is illustrated

- **divisive**: the observations start of in one cluster in a "top-down" manner, where the single cluster is gradually split into more clusters, moving down the hierarchy.
- **agglomerative**: each observation starts in its own cluster and cluster-pairs are merged as they move up the hierarchy, representing the classification in the "bottom-up" manner. The final wished for amount of clusters has to be specified to which amount larger created clusters are to be fused to.

The ward method is a distance measure, which defines the most similar or dissimilar pair within a geometric clustering method. This makes the inclusion of the Euclidean Distance necessary, which defines a cluster centroid by determining distances between points. (28)

XLSTAT (29)

XLSTAT is a software, that operates in combination with Microsoft EXCEL and offers a variety of statistical functions, carrying out evaluations in order to create an interpretable set of information.

Some of the approaches withheld under this software are 3D Plots, advanced data analysis (ADA), conjoint analysis (Conjoint), dose efficacy analysis (Dose), Correlated component regressions (CCR), Partial least square regression (PLS), various clustering analysis (hierarchical as well as agglomerative) and many other functions. In this case XLSTAT was used for carrying out the clustering-analysis.

Under Windows XLSTAT works with Excel versions 97 to Version 2013 and also under Mac OS X XLSTAT can be used with the Version 2011. If it is only used for a limited time it can even be downloaded for free from the internet.

➢ PLIF–PROTEIN LIGAND INTERACTION FINGERPRINTS

PLIF is a tool, integrated in MOE, which creates **P**rotein **L**igand **I**nteraction **F**ingerprints. It generates queries from bound ligands that are given from complexes like docking results. (30)

It summarizes the interactions between the respective ligands and the binding protein. Each row represents the object and the column indicates an interaction that can be potentially followed out between protein and ligand. (30)

Interactions which are recognized and indicated by this tool are hydrogen bonds, ionic interactions and surface contacts. These are translated into a fingerprint scheme represented in form of protein-ligand complex interactions. Thereby interactions among the objects can be compared. (30)

The terms for the translation of the protein-ligand interactions into fingerprints are as follows:

- Contact to the surface is encoded by a small "c".
- Hydrogenbond acceptors are represented by a small "a" if they originate from the backbone and a capital "A" if originating from the sidechain.
- Hydrogenbond donors are represented by a small "d" when originating from the backbone and a capital "D" if originating from the sidechain.
- Each of these letters is called a "bit" and each “bit” is assigned to its specific amino residue from which the interaction derives. (30)

The quality of the hydrogen bond interactions is represented by the indication of either one or two bits. One given letter code means that the quality of the interaction lies between 1% and 10% and everything above 10% is shown by the indication of two bits. (30)

➢ CBM ANALYSIS

In the course of an objectified cluster binding mode analysis, a CBM analysis, the letter codation of PLIF is transferred into an excel sheet where it is possible to assign each interaction to each clustered pose. As each pose is assigned to its cluster, the various appearing interactions between ligand and protein are then summarized for each cluster.

This makes comparison and analysis of each cluster’s central objects (this pose is the representative of the other poses within the cluster) possible. This saves from having to run complex comparisons with the huge amount of single poses and getting lost in a heap of detailed information.

➢ Scoring of Binding Modes

The clustered and PLIF assigned central object poses, are summarized in an Excel sheet, where they can be compared and analyzed in the process of a CBM analysis. Although the CBM analysis represents a hugely simplified method of assessing binding poses, nevertheless a profound scoring method of validating this type of resulted docking poses is to date still lacking. This demonstrates the challenging opportunity for searching after such a statistically grounded scoring method. The necessity of the scoring method is seen in validating the docking pose according to the probability of the suggested pose by integrating bioactivity data into the process of binding mode evaluation.

2.3 OBSTACLES OF THE COMPUTATIONAL WORKFLOW

2.3.1 CHALLENGES OF THE DOCKING PROCEDURE

➢ Flexibility

Getting the flexibility setting of the binding protein right is a tricky task especially as the selection in GOLD is limited to choosing maximum 10 sidechains for flexibility and SYBYL X 2.1.1. is not able to include flexibility at all. (26) Therefore various docking runs have to be preceded until the right set up can be chosen.

➢ Soft Potential

Finding the right selection as well as the right intensity of soft potentials also makes it necessary to conduct docking test runs. If Soft Potentials are falsely set too intense it consults in side clashes being so harsh, that the ligand is bound beyond the actual binding pocket as it is too easily deformed. On the other hand, if the soft potential is not set in a region where in reality plasticity would be present, then this may lead to the ligand's inability in binding, where it should be able to and as a consequence deliver wrong docking results.

- **Time issues**

Sudden publications of novel protein models or similar discoveries being published near the end of a project leads to the previously obtained results having to be adapted to the new results, overworked which can result in great extensions or even the project being discarded.

Not to forget is that the algorithms used in in silico approaches are very complex. The process of a single docking run itself consumes a long running time, until it is completed. Moreover computational workflows may involve license or compatibility problems which also involve time delays.

While time consuming analysis are being carried out, others may already be in the process of releasing the results that oneself is still looking for. Therefore it is necessary to keep in track in the field of researches that focus on up-to-date topics.

2.3.2. CHALLENGES CONCERNING SCORING FUNCTIONS

Finding the right set up concerning scoring functions is very important for delivering a correct outcome. In the choice of the suitable scoring function the needs of the workflow have to be met. For instance the prediction of affinity values there are among others the options **GBVI/WSA dG** and **London dG** available.

GBVI/WSA dG is a force field based Scoring function. The force fields define specific parameters for each type of atom and can describe the potential energy of a molecule and atom in a system. It relates to atoms in covalent bondages, electrostatic forces and van der Waals forces.

Thereby each cut off plane interaction and bond stretch interaction in the force field is given in kcal/mol/A, whilst bond angle interactions as well as stretch-bends in bond angles are listed in kcal/mol/deg and kcal/mol/deg/A. (31)

Another option would be choosing the scoring function **London dG** which is not able to take ionized interactions into account for affinity predictions which would mean that when an ionized data set is being used London dG cannot be used.

2.3.3. CHALLENGES OF CLUSTERING DOCKING POSES

It has to be sought after the right number of clusters so that clusters do not contain single poses, when too many clusters are selected, and clusters do not contain various poses and are inhomogeneous, when an outcome of too few clusters is selected. In general the more clusters are submitted, the more distinctive the poses become. Therefore the finding of the correct cluster count is an incremental approach.

INTRODUCTION A

3 INTRODUCING THE DIPLOMA THESIS

3.1. SCIENTIFIC AIM

3.2. CONCEPT OF THE WORKFLOW

1. INTRODUCING THE DIPLOMA THESIS

3.1. SCIENTIFIC AIM

Diazepam, as a $GABA_A$ receptor agonist, is shown to be very successful in various treatments of illnesses. Nevertheless it involves side effects, such as sedation, ataxia, potentiation with alcohol and most importantly it also increases the risk of tolerance and dependence, when chronically used. (32) This is why the treatment of chronic disorders is queried. Therefore risks need to be weighed against the advantages. Unfortunately the list of alternatives is at present fairly limited which is why disadvantages are often simply tolerated. Thus there is still a need of finding therapeutic drugs, which have reduced side effects but still retain the other beneficial therapeutic effects.

Therefore this thesis aims to evaluate and identify the PQ's functional role within the $GABA_A$ receptor binding pocket, which shall be started with an analysis of the subtype selective affinity and application of an in silico workflow. (33)

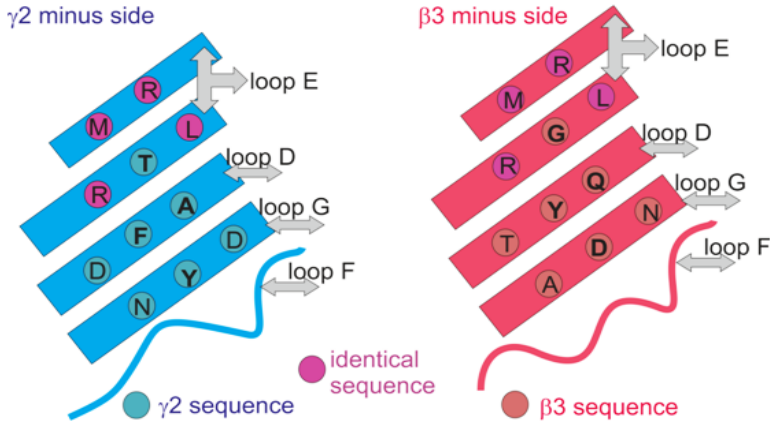
Doing this emphasizes on finding a hypothesis of the Pyrazoloquinolinones' binding mode in the benzodiazepine binding site and understanding the differences of interactions between the two binding sites, BZD and CGS BS. The obtained knowledge of the binding affinity and behaviour between the BZD BS and ligand shall enable to predict the CGS-series' binding conformation within the CGS binding site, by exchanging four specific amino residues, which are known to be different among the two binding sites.

2.2. CONCEPT OF WORKFLOW

The **knowledge of the BZD binding site** is already very consistent and well profound. Adapting obtained information of the familiar BZD binding site to the CGS binding site is more sensible than starting to create completely new homology models of an unknown docking region from scratch.

The difference of these two binding sites is seen in the exchange of the gamma-sheet with the beta sheet, with a distinction in four amino residues as listed in the table below and schematically pictured in the images of the γ2(-) and β3(-) subunits (ill. 12) with their certain relevant aminoresidues within loop E, D, G and F.

loop	γ2	β3
E	T142	G127
D2	F77	Y62
D1	A79	Q64
G	Y58	D43



ill. 12: schematic image of γ2 and β3 sequence with indication of relevant aminoresidues to compare the differences among the subunit – specific loops. (34)

It is suggested that the changes between Y62 and F77 as well as Q64 and A79 are mainly responsible for the selectivity in binding to the BZD or the CGS binding site.

Therefore, by knowing how a ligand is posed in the BZD binding site it is possible to apply this gained knowledge to the binding mode in the CGS binding site by exchanging F77 and A79 with Y62 and Q64. Subsequently the interactions appearing in the CGS-binding site can be predicted. Selectivity to the specific binding site is of great pharmacological importance through which the prevention of side effects is enabled in this case.

For the understanding of the PQs' binding-mode, the experimental data guided docking workflow (14) included performing a **CoMFA,** molecular **docking** and an exhaustive analysis of the suggested poses via two **newly developed individualized scoring-methods.** This enabled an evaluation of the poses in view of fitting the activity landscape of PQs.

➢ CoMFA

The obtained CoMFA results represented the regional favoured and unfavoured electrostatic and steric features for a good protein ligand interaction. 138 CGS-compounds (4), (3), (33) were included in the CoMFA using Sybyl-X 2.1.1.

➢ Molecular docking

For in silico docking the software GOLD v1.6.2 was used and detained into four different protein binding models of the human BDZ site, including the latest published model, 4COF, as well as models based on GluCl and Ach BP (N103, X77), created in the group of Margot Ernst in the Medicinal University of Vienna. After the docking procedure the complexes were energy minimized using MOE and multiple CGS-poses were attained.

➢ Post-docking modification

The idea behind the post-docking modification was to integrate the information of bioactivity data among a series of PQ into the process of binding mode evaluation.
Firstly each of the docking poses was computationally modified to 13 different specifically selected derivatives within the binding pocket itself, followed by an energy minimization of the binding site shaping sidechain atoms. For all ligands the bioactivity data was known by previously conducted experiments, with varied pKi value of 5.44 – 10.046.
Finally this computational modification led to multiple in silico binding complexes per CGS-pose, summing up to 14 receptor complexes per docking pose, which each was represented in an array and demonstrating the potential correct binding mode.

➢ Individualized scoring schemes

Subsequently scores were newly calculated on MOE delivering predicted affinity values for each docking pose and its 13 derivatives. The better the in silico predicted affinity differences correlated with the actual affinity differences between the CGS-compound and the modified structure, the more probable the respective pose was to be the true binding conformation. (3) The assessment of the conformation's actual probability took place by transforming the poses information into two individualized

scoring methods, named the **Penalty scoring method** and the **Simple scoring method.**

The scores of each pose and its 13 complexes are summed up and ranked with the highest ranked pose theoretically representing the most plausible pose in view of fitting the activity landscape of PQs in the binding pocket.

MATERIALS AND METHODS B

1. COLLECTING COMPOUNDS

2. 3D-QSAR – COMPARATIVE MOLECULAR FIELD ANALYSIS

2.1 COMFA SET UP

3. DOCKING

3.1 DOCKING SET UP WITH CGS-8216
3.2 CLUSTERANALYSIS OF DOCKING POSES
3.3 APPLYING PLIF ON DOCKING POSES

4. EVALUATION OF DOCKING POSES

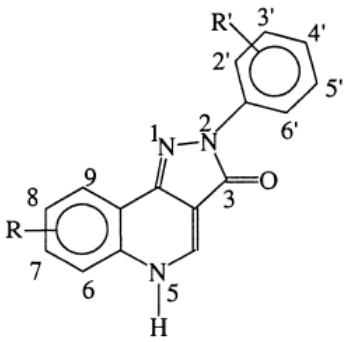
4.1 POST-DOCKING MODIFICATION
4.2 METHODOLOGY OF SCORING BINDING MODE
4.3 INTEGRATION OF INDIVIDUALIZED SCORING METHODS
- 4.3.1 Implementing Penalty Scoring Method
- 4.3.2 Implementing Simple Scoring Method

After a basic research of the already existing evidence and information of the binding pocket in correlation with the CGS compounds the experimental part was ready for being started.

The approach, under which the diploma work proceeded, partly had to be developed progressively, by individualizing the analysis suitable for the data, obtained from the previous workflow. The approached workflow was as follows:

1. COLLECTING COMPOUNDS
2. CoMFA
3. PROTEIN MODELS
4. DOCKING
5. CLUSTERING OF DOCKING-POSES
6. PLIF ASSIGNING
7. POST DOCKING MODIFICATION
8. INDIVIDUALIZED SCORING METHODS OF DOCKING- POSES
9. VALIDATING RESULTS
10. CONCLUSION OF BINDING MODE

1. COLLECTING COMPOUNDS



ill. 13: core structure of pyrazolo[4,3-c]quinolin-3-one. (35)

Firstly a collection of compounds with the core structure of pyrazoloquinolin-3-ones with presented affinity values on the $GABA_A$ Benzodiazepine receptor derived from sufficient experimental measurements had to be conducted.

After a thoroughly withheld research, three journal papers were used as a main source for the diploma work:

1. The paper from L. Savini et al.: ***"High Affinity Central Benzodiazepine Receptor Ligands. Part 2: Quantitative Structure-Activity Relationships and Comparative Molecular Field Analysis of Pyrazolo[4,3-c]quinolin-3-ones"*** (3)

2. The publication from Zdravko Varagic et al.: ***"Identification of novel positive allosteric modulators and null modulators at the GABAA receptor a+b-interface"*** (4)

3. The journal paper from Xiaohui He: ***"Studies of Molecular Pharmacophore/Receptor Models for GABAA/BzR Subtypes: Binding Affinities of Symmetrically Substituted Pyrazolo[4,3-c]quinolin-3-ones at Recombinant axb3y2 Subtypes and Quantitative Structure-Activity Relationship Studies via a Comparative Molecular Field Analysis"*** (33)

1) L. Savini et al. "High Affinity Central Benzodiazepine Receptor Ligands. Part 2: Quantitative Structure-Activity Relationships and Comparative Molecular Field Analysis of Pyrazolo[4,3-c]quinolin-3-ones" (3)

In this paper a COMFA analysis with 106 pyrazolo[4,3-c]quinolin-3-ones is presented. The report's resulted isocontour maps show specific parts of these PQs which were not clearly described. Therefore a CoMFA with a huger dataset than presented in the paper of L. Savini was aimed to run in order to specify their results. The additional

PQs included in this respective COMFA, were taken from the reported data listed in the papers from *Zdravko Varagic et al.* and *Xiaohui He et al.*

2) Zdravko Varagic et al.: ***"Identification of novel positive allosteric modulators and null modulators at the GABAA receptor a+b- interface" (4)***

3) Xiaohui He et al.: ***"Studies of Molecular Pharmacophore/Receptor Models for GABAA/BzR Subtypes: Binding Affinities of Symmetrically Substituted Pyrazolo[4,3-c]quinolin-3-ones at Recombinant axb3y2 Subtypes and Quantitative Structure-Activity Relationship Studies via a Comparative Molecular Field Analysis" (33)***

Eventually 138 diverse PQs were collected and included in the experimental data guided analysis.

2.3 3D-QSAR- COMPARATIVE MOLECULAR FIELD ANALYSIS

2.1. CoMFA SET UP

SOFTWARE: *Tripos Bookshelf, SYBYL – X.2.1.1*

The 3D-QSAR/ CoMFA was run with SYBYL – X.2.1.1, available from Tripos, Inc. Software for research in Life Sciences using the functions of the Topomer CoMFA. In order to get the whole system started, a set of compounds had to be created in form of a datatable in MOE.

Therefore **138 compounds** with the specific PQ core structure were taken from the journal paper of *L.Savini et al.*, *Zdravko Varagic et al.* and *Xiaohui He et al* where measured affinity values were given.
These compounds were drawn in MOE and the affinity values were assigned to each compound. Hence the different affinity values had to be converted to pKi:

- ➔ The compound's affinity values presented in the paper of *Xiaohui He. et al.* were Ki affinity values and therefore easily converted into pKi by using this formula: pKi=-log Ki

- ➔ Also the Ki affinity values given for the compounds in the *Zdravko Varagic et al.* paper were converted into pKi using the same formula as the settings were comparable.

- ➔ The compound's values presented in the paper of *L.Savini et al.* were all IC50 values, which derived from the displacement of [3H]flunitrazepam. Converting the values of pIC50 into pKi in the course of this evaluation was necessary for obtaining equal parameters to be able to compare the compounds from different sets. Moreover, pKi is a normalized value, independent from assay conditions such as flunitrazepam concentration. Transferring these into Ki affinity values was done by applying the Cheng Prusoff equation. This was possible as the settings for the measurement affinity values measured for the

compounds illustrated in the papers of *Zdravko Varagic et al. (4)* and *Xiaohui He et al*. (33) were the same. The Cheng Prusoff equation is as follows:

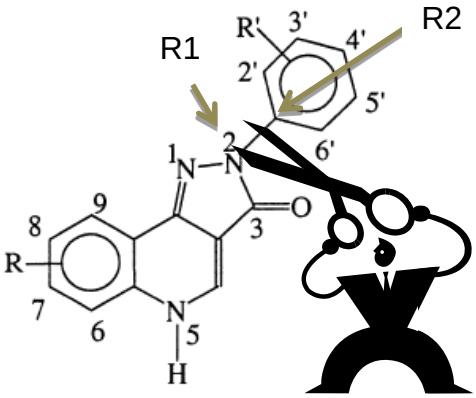
$$K_i = \frac{EC_{50}}{1 + \frac{[ligand]}{K_D}}$$

KD/Km *= Michaeliskonstante*
S *= Substratkonzentration*
Ki *= Enzym-Inhibitor-Komplex*

$$K_i = \frac{x}{1 + \frac{[ligand]}{K_D}}$$

KD / Km= 2.8 nM
S = [ligand] = 2 nM => 1+ (S/KD) = 1.714285714
EC50 = IC50→ pIC50 => -log (IC50)

By applying the **Topomer CoMFA** the core structure was **split into two** subsets. Therefore N2 was defined as R1 and C1' of ring D was defined as R2.



ill. 14: PQ core structure.(35) Arrows point at the elected rests R1 and R2, for splitting the structure into two subsets as a preparatory workflow before conducting the Topomer CoMFA.

The choice of using Topomer CoMFA was based on the rotatability between N2 and C1'. Otherwise the CoMFA results might be influenced if rotation would appear which was hereby eliminated and results could be assessed independently of the torsion angle between R1 and R2.

Since the molecules should not be adapted in their placement, they were **not energy minimized**.

For the calculation **Gasteiger Hückel charges** were chosen, and the **PLS** (partial least squares, which is a regression analysis system) was selected.

The data set containing **138 compounds** for the 3D-QSAR was randomly divided into test and training set. From these all of the 138 components were included in the training and 10 components in the test set with an intercept of 3.82. Biological activity data goes from a minimum of 4.657 to a maximum of 10.42.

3. DOCKING

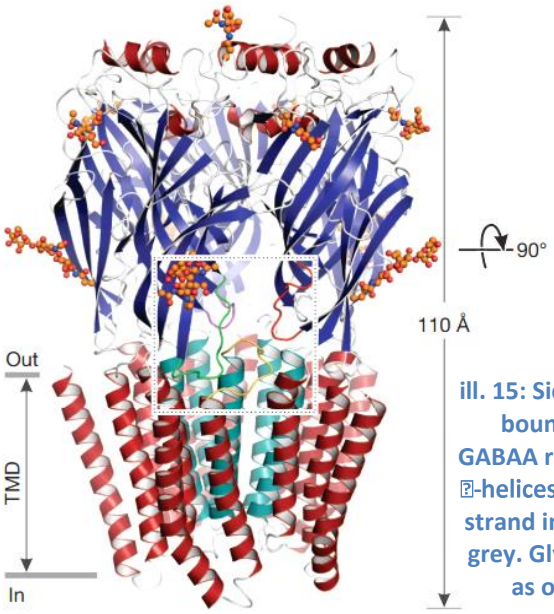
The docking process was solely performed with CGS-8216 into 4 different binding protein models, finally delivering 400 poses => 1 cpd x 100 conformations x 4 BPs = 400 suggested docking poses.

3.1. DOCKING SET UP WITH CGS-8216

CGS-8216 was selected representing the core structure of the PQ series. This was docked **into 4 different binding protein** models, which slightly varied in the flexible sidechain, as described in the chapter of the Introduction “2.2.1. Protein Models”.

Considering the available options, the docking program GOLD was assorted since it allowed keeping ligands and the 10 amino residues in the protein sidechain flexible. For the drawing of compounds the program MOE (**M**olecular **O**perating **E**nvironment) and in order to operate the **program GOLD v1.6.2.** the **software HERMES** was used**.**

For each docked protein model and per docked compound **100 conformations** were suggested, and finally 400 suggested conformations were delivered.



ill. 15: Sideview of membrane bound protein model of GABAA receptor--β3, 4COF. The α-helices is illustrated in red, β-strand in blue and the loops in grey. Glycans are represented as orange strings. (23)

➢ **PROTEINMODELS**

The binding protein models were created by the group of Margot Ernst in the Neuroscience Center of the University of Medicine:

1) N103
2) X77
3) GluCl
4) 4COF

➢ SOFT POTENTIALS

Selecting **Soft potentials** is an option for allowing clashes in specific areas in two different magnitudes, intensity 1 or intensity 2. The greatest importance of flexibility is seen and therefore selected for Ser204, Ser205, Thr206, Gly207, Glu189, Arg185 and Lys184.

For the BP models **X77** and **GluCl**, the amino residues **Arg185** and **Lys184** were not taken into account in this case.

In the BP model X77 Arg185 and Lys184 are located so far away from the actual binding target, that they are most unlikely to interact with the ligand or influence their binding mode. Therefore they are not taken into account for this setup.

In the BP of GluCl Lys184 was also not taken into account as it takes in another pose than in the other BP models and would most probably not cause any interference since it is distantly located to the actual binding target.

Final Soft Potential set up:

- N103: intensity 2: Arg185, Lys184
  intensity 1: Ser204, Ser205, Thr206, Gly207, Glu189
- X77: intensity 1: Ser204, Ser205, Thr206, Gly207
- GluCl: intensity 1: Ser204, Ser205, Thr206, Gly207
- 4COF: intensity 1: Ser204, Ser205, Thr206, Gly207

➢ FLEXIBILITY

Ten amino acids of the following were kept flexible per DOCKING run:

FLEXIBLE SIDECHAINS IN ALL 4 BPs

Flexible sidechains in $\alpha 1$

Loop A: His101

Loop B: Tyr159

Loop C: Ser204, Ser205, Thr206, Gly207, Tyr209

Flexible sidechains in $\gamma 2$

Loop G: Tyr158

Loop D: Phe77

Loop E: Thr142

THE TENTH FLEXIBLE SIDECHAIN

- GluCl: Met130
- N103 & X77: Glu189
- 4COF: Lys155

FLEXIBILITY SET UP:

The four BP models have slight differences within the undefined strand F. Hence flexibility is differently set up in this area. Instead **of Glu189** the amino residue **Lys155** is chosen for flexibility in 4COF, due to Lys155 being orientated more towards the binding area than in the other three BP models.

Flexibility in the BP-models **N103** and **X77** was selected for Glu189 and in **GluCl** for the amino residues Met130.

➢ SCORING FUNCTION

The **Scoring function** used for all docking procedures in Hermes was GOLDscore. This used to be the original scoring function provided with GOLD, and is the scoring function selected by default for versions 5.0. It is specified for predicting ligand binding positions on the basis of H-bond formation and van der Waals energy, metal interaction and ligand torsion strain. (36)

➢ **GA-RUNS:**100 per BP-model

400 suggested conformations per compound were delivered, after four binding proteins were docked:

→1 compound x 100 conformations x 4 BP models = 400 suggested docking poses

After the docking procedure the attained docking poses of CGS-8216 were energy minimized within the binding protein using the MOE built-in tool LigX.

1.6. CLUSTERANALYSIS OF DOCKING POSES

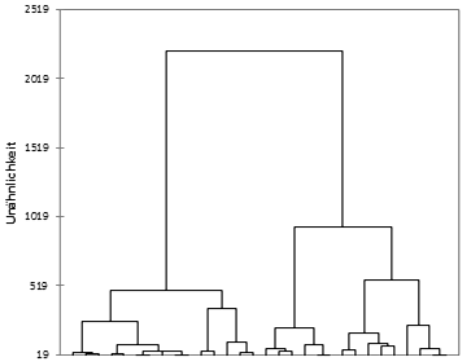
As a next step the clusteranalysis was performed applying to all 400 suggested docking poses obtained from the docking process using the program XLSTAT.

➢ RMSD-MATRIX:

The created RMSD-Matrix contained 400 docking results from the structure, CGS-8216, which was docked into the BP-models X77, N103, GluCl and 4COF.

➢ SELECTED SETTINGS:

- Agglomerative Hierarchical Cluster
- Proximity Metric (distance or similarity metric)
    - distance metric
- Euclidean Distance
- Ward method
- dissimilarities
- Dendrogram
- Central Objects
- It was selected to obtain 30 clusters. This choice was made after multiple test runs with various counts of clusters.



ill. 16: dendrogram obtained with XLSTAT clustering. The branches show the development of 30 obtained clusters processed by the gradual splitting.

3.2. APPLYING PLIF ON DOCKING POSES

After the clustering procedure, each docking pose was assigned to its specific cluster. Then the function PLIF, Protein-Ligand Interactions Fingerprint, was applied. Hereby interactions between the respective binding protein and CGS-8216 in its specific pose were indicated in form of bits. After converting these into a text file via a customized script, this enabled the comparison of the interactions among the different docking poses summarized in an Excel sheet.

4. EVALUATION OF DOCKING POSES

4.1. POST-DOCKING MODIFICATION

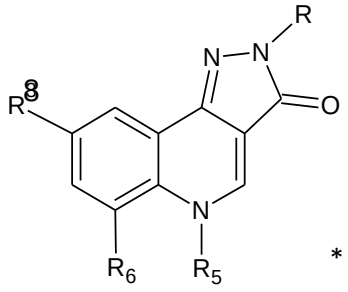
As a next step the suggested PQ binding modes had to be assessed in order to gain confirmation of the pose's plausibility.

Therefore a new method was developed within this diploma work, named the "Post–docking modification". Hereby the bioactivity in form of pKi from a series of PQs, could be integrated into this individualized scoring scheme.

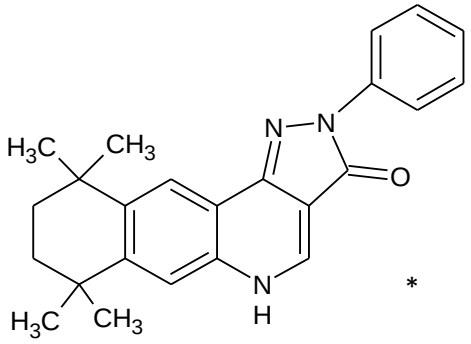
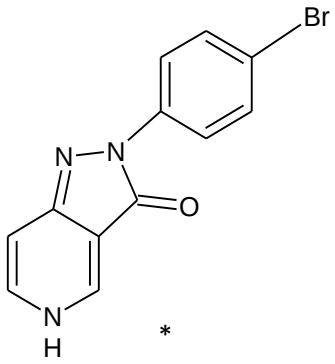
This was done by consulting each of the 400 docking poses and computationally changing these to specially assorted PQ derivatives, which were hoped to offer explanation for an authentic binding mode hypothesis.

These CGS-8216 binding complexes were energy minimized within the binding protein and rebuilt to these following compounds by using the MOE built-in tool LigX:

- **POST-DOCKED MODIFIED COMPLEXES**



Compound	R5	R6	R7	R8	R9	R	pKi
ACTIVES							
1 (CGS-8216)[a]	H	H	H	H	H	Phenyl	9.42
2 (5_9)[b]	H	H	H	H	H	C≡CCH$_3$	8.70
3 (102)[c]	H	H	H	8,9-BENZOFUSED		Phenyl	8.21
4 (103)[c]	H	H	7,8-OCH$_2$0		H	Phenyl	8.59
5 (46)[c]	H	H	H	Phenyl	H	Phenyl	8.42
6 (5_20)[b]	H	H	H	tert. Butyl	H	Phenyl	10.05
7 (68)[c]	H	H	H	OCF3	H	p-Aminophenyl	9.17

INACTIVES							
8 (2.72)[c]	H	H	H	OCF3	H	p-Carboxyphenyl	5.57
9 (1)[c]	H	H	H	H	H	H	6.37
10 (5_29)[b]	H	tert. Butyl	H	H	H	Phenyl	5.52
11 (101)[c]	H	6,7-BENZOFUSED		H	H	Phenyl	6.07
12 (11)[c]	CH_3	H	H	H	H	Phenyl	6.91
13 (XHe-098a)[a]							5.44
14 (XHe-III-56)[a]							5.57

**Structures drawn with ACD/ChemSketch (Freeware) 2012.*
[a]Ref. 4, IC50 values have been calculated to pKi values
[b]Ref. 33, Ki values have been calculated to pKi values
[c]Ref. 3, pIC50 values have been calculated to pKi values

- **POST-DOCK DERIVATIZATION**

The CGS-8216 docking poses were computationally modified to these specially assorted CGS derivatives. The selection was carried out after their potential to deliver information of the pocket's shape and activity behaviour. This was done using the MOE SVL functions sm_Build and oDestroy by which the ligands structure was transformed to the respective 13 derivatives, keeping the CGS-8216 poses coordinates upright. Thereby a suggestion of the CGS-8216 fitting mode, in the specific conformation out of all 400 suggested conformations, is given. Each conformation was put into a PQ-series-pose-array thus delivering a table of 400 arrays, representing each conformation, 14 columns, representing the specific

conformation of CGS-8216 and the 13 CGS derivatives. The derivative had to shape its sidechain atoms into the binding pocket by keeping the conformation of the core structure fixed.
Finally this procedure summed up to 400 x 14 receptor complexes.

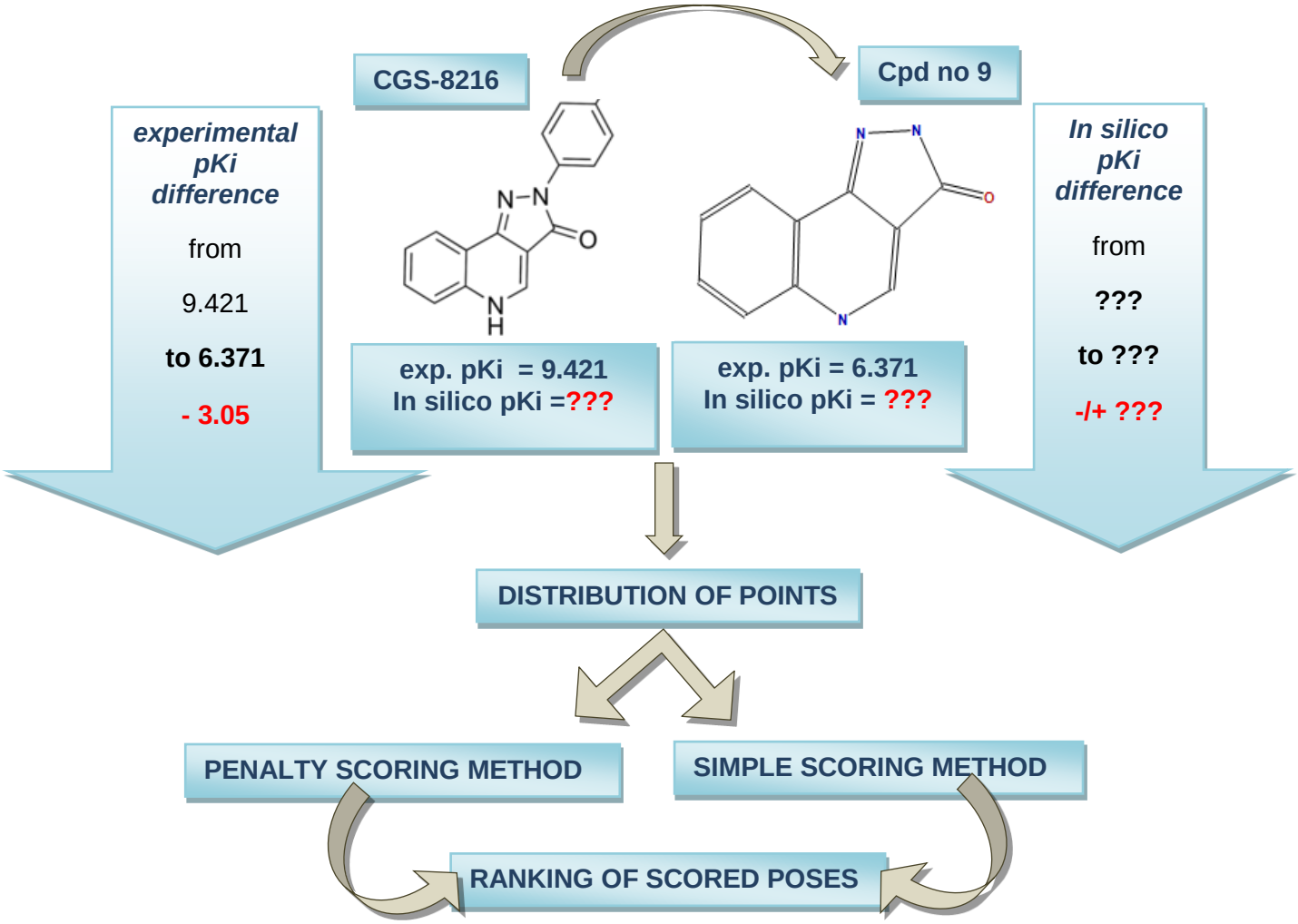
After each computationally created modification, a deep descent energy minimization of the compound was realized whilst the scaffold was kept rigid.
RMSD values were calculated via common scaffolding. Subsequently the scores for these derivatives were newly calculated in MOE using the scoring function **GBVI/WSA dG**, yielding predicted pKi affinity values for each CGS-8216 pose and its 13 modified complexes. The choice of this scoring function is based on the fact that an other optional scoring function, **London dG,** is not able to take ionized interactions e.g. carboxylated compounds into account for predicting affinity. As an ionized compound is among the data set, 8 (2.72), London dG would deliver wrong results.(37)

4.2. METHODOLOGY OF SCORING BINDING MODE

The newly calculated predicted affinity values for each newly modified derivative in its original pose were then scored according to their plausibility.
The higher the correlation between the predicted and the experimental affinity difference between CGS-8216 and the modified compound, the higher the chances of this respective pose demonstrating the true binding mode. Consequently, if the affinity difference between two compounds is predicted correctly, this pose is ranked high.

For better understanding of this scoring methodology an example taken from the diploma work is illustrated below (ill. 17).
In the given example the experimental affinity difference between CGS-8216 and the N2-unsubstituted CGS compound (= cpd no 9, which is a member of the group of inactives) are known to be -3.05 pKi.
The coordinates of each in silico suggested CGS-8216 docking pose, which are clustered, PLIF-assigned and summarized in an Excel sheet, were taken to transform

the ligand structure into the N2-unsubstituted derivative, cpd no 9. Subsequently the computed affinity decrease was calculated for this in silico pair, using GBVI/WSA dG within MOE. The in silico predicted pKi affinity difference was then compared with the experimental pKi value.



ill. 17: illustration for the understanding of the two scoring methodologies.

The assessment of the accuracy of the binding pose by means of this specific correlation was followed up by the two significant individualized scoring methods:

1. **Penalty scoring method**
2. **Simple scoring method**

➢ Penalty scoring method

In the penalty scoring method, the poses' plausibility were scored by distributing plus or minus points, depending on how well or badly the in silico prediction consulted to be:

First the difference of affinity values between the original ligand and the modified structure was calculated for every compound over each pose.

After the compound was divided into the group of actives or inactives, the awarding of points was specified, depending on which group the compound belonged to.

In case of actives, the distribution of points is generally ensued the following way:

divergence of difference of experimental vs in silico pKi	Awarding of points
< -1	- 0.25
> -1; < -0,5	0
> -0.5; < +0.5	+ 1
> +0.5; < +1	+ 0.5
> +1	+ 0.25

In case of inactives, the general distribution of points is ensued the following way:

divergence of difference of experimental vs in silico pKi	Awarding of points
< -1	+ 0.25
> -1; < -0,5	+ 0.5
> -0.5; < +0.5	+ 1
>+0.5; < +1	0
> +1	- 0.25

In the case of the example demonstrated above (ill.17), the predicted pKi affinity value between CGS-8216 and the N2-unsubstituted cpd no 9 should be -3.05 and in order to be ranked high should therefore be fairly close to this value: *Every pose with a prediction between -2.55 and -3.55 was scored with 1 point. Every prediction between -2.55 and -2.05 would be rated with 0 points. If its prediction goes beyond -2.05, a deduction of 0.25 points is carried out, as it is being predicted more active, although it should be more inactive than CGS-8216. Every prediction between -3.55 and -4.05 would be scored with 0.5 points and if predicted with an affinity value more*

negative than -4.05, it is scored with 0.25 points as the predicted value is at least led towards the right activity mode.

➢ SIMPLE SCORING METHOD

The simple scoring method works by distributing either plus or zero points, thus the maximum number of modified structures results to be the maximum amount of points that can be reached per pose.
Again the derivative is divided into the group of actives or inactives, specifying the awarding of points, dependent on the group it is assigned to.

In case of inactives, the distribution of points is ensued the following way:

divergence of difference of experimental vs in silico pKi	Awarding of points
< -0,5	+ 0.5
> -0.5; < +0.5	+ 1
>+0.5	0

In case of actives, the distribution of points is ensued the following way:

divergence of difference of experimental vs in silico pKi	Awarding of points
< -0,5	0
> -0.5; < +0.5	+ 1
>+0.5	+ 0.5

When taking the example from above again (ill.17), the predicted pKi affinity value should be -3.05, for a high ranking: Every prediction between -2.55 and -3.55 is scored with 1 point. Every prediction more positive than -2.55 would be rated with 0 points, because it is being predicted more active, although it should be more inactive than CGS-8216. Every pose predicted more negative than -3.55, would be scored with 0.5 points since the predicted value is at least led towards the right activity mode.

After once applying the penalty scoring method and the other time applying the simple scoring method, the scores of each pose and its 13 complexes, which were attained through the post docking modification, were summed up and the pose arrays

were ranked in descending order according to their score. The highest ranked pose theoretically represents the most plausible pose on the basis of being able to explain the experimentally ascertained affinity increase or decrease, when CGS-8216 is modified into a specific derivative.

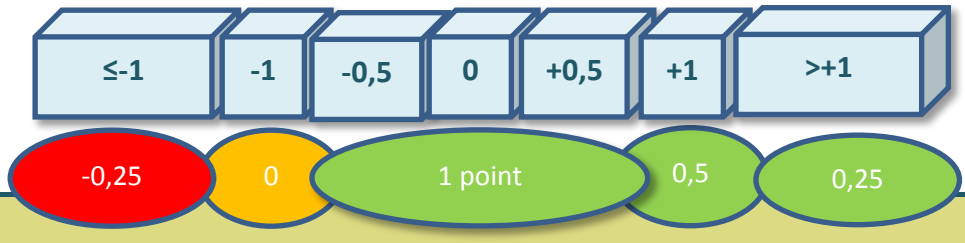
4.3. INTEGRATION OF INDIVIDUALIZED SCORING METHODS

For summing up the scores of each pose and its 13 complexes, the threshold for per derivative were worked out in order to integrate these into a formula-function within an excel spreadsheet, so that the calculation for each pose could then be carried out within it.

4.3.1. IMPLEMENTING PENALTY SCORING METHOD

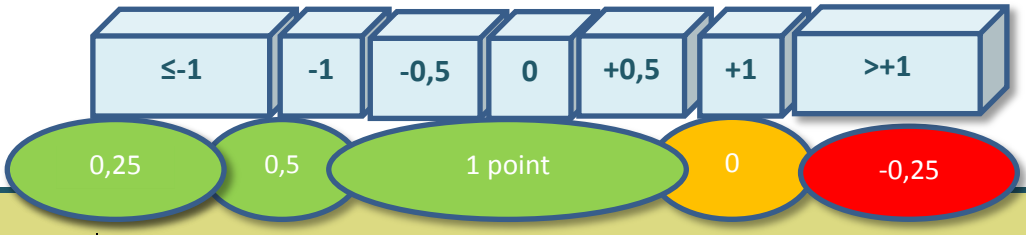
Cpd2 from Ref[3] (pKi=9,4214) is structurally the same as CGS-8216 from Ref[4] (pKi=10,30). However the measured affinity values are slightly apart, as the conditions under which these were measured varied. Therefore, to gain most correct results, all the affinity differences from compounds (3, 4, 5, 7, 9, 11, 12) taken from Ref[3] were calculated, by deducting from the pKi value (9,4214) of Cpd2.

➢ AWARDING OF POINTS FOR ACTIVE CGS DERIVATIVES



	cpd	pKi	exp. Diff.	AWARDING OF POINTS IN PENALTY SCORING METHOD				
0	2	9,4214	-------					
1	CGS8216	10,3	-------	-0,25	0	+1	+0,5	+0,25
2	5_9	8,7	-1,6	≤-2,6	>-2,6; ≤-2,10;	>-2,10; ≤-1,10	>-1,10; ≤-0,6	>-0,6
3	102	8,21	-1,21	≤-2,21	>-2,21; ≤-1,71	>-1,71; ≤-0,71	>-0,71; ≤-0,21	>-0,21
4	103	8,59	-0,83	≤-1,83	>-1,83; ≤-1,33	>-1,33; ≤-0,33	>-0,33; ≤0,17	>0,17
5	46	8,42	-1	≤-2,0	>-2,0; ≤-1,5	>-1,5; ≤-0,5	>-0,5; ≤0,0	>0,0
6	5_20	10,05	-0,25	≤-1,25	>-1,25; ≤-0,75	>-0,75; ≤0,25	>0,25; ≤0,75	>0,75
7	68	9,17	-0,25	≤-1,25	>-1,25; ≤-0,75	>-0,75; ≤0,25	>0,25; ≤0,75	>0,75

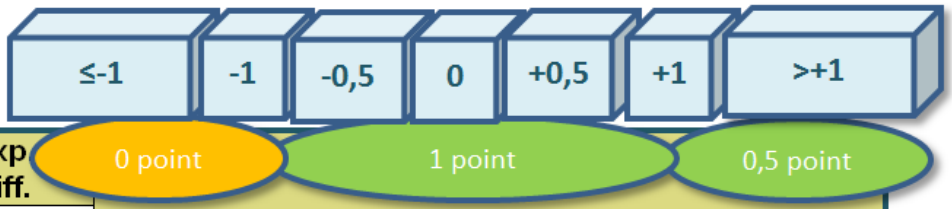
➢ AWARDING OF POINTS FOR INACTIVE CGS DERIVATIVES



				AWARDING OF POINTS IN PENALTY SCORING METHOD				
	cpd	pki	Exp. Diff.	+0,25	+0,5	+1	0	-0,25
8	2_72	5,57	-3,85	≤-4,85	>-4,85; ≤-4,35	>-4,35; ≤-3,35	>-3,35; ≤-2,85	>-2,85
9	1	6,37	-3,05	≤-4,05	>-4,05; ≤-3,55	>-3,55; ≤-2,55	>-2,55; ≤-2,05	>-2,05
10	5_29	5,52	-4,78	≤-5,78	>-5,78; ≤-5,28	>-5,23; ≤-4,23	>-4,23; ≤-3,73	>-3,73
11	101	6,07	-3,35	≤-4,35	>-4,35; ≤-3,85	>-3,85; ≤-2,85	>-2,85; ≤-2,35	>-2,35
12	11	6,91	-2,51	≤-3,51	>-3,51; ≤-3,01	>-3,01; ≤-2,01	>-2,01; ≤-1,51	>-1,51
13	XHe-098a	5,44	-3,98	≤-4,98	>-4,98; ≤-4,48	>-4,48; ≤-3,48	>-3,48; ≤-2,93	>-2,93
14	Xhe-III-56	6	-3,42	≤-4,42	>-4,42; ≤-3,92	>-3,92; ≤-2,92	>-2,92; ≤-2,42	>-2,42

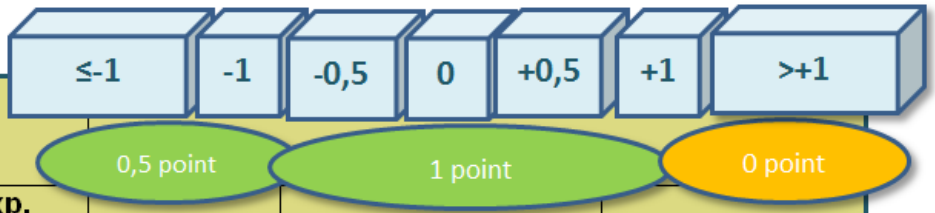
4.3.2. IMPLEMENTING SIMPLE SCORING METHOD

➢ AWARDING OF POINTS FOR ACTIVE CGS DERIVATIVES



	cpd	pKi	exp Diff.	AWARDING OF POINTS SIMPLE SCORING M.		
0	2	9,4214	----------			
1	CGS8216	10,3	----------	0	1	0,5
2	5_9	8,7	-1,6	≤-2,1	>-2,10; ≤-1,1	>-1,1
3	102	8,21	-1,21	≤-1,71	>-1,71; ≤-0,71	>-0,71
4	103	8,59	-0,83	≤-1,33	>-1,33; ≤-0,33	>-0,33
5	46	8,42	-1	≤-1,5	>-1,50; ≤-0,5	>-0,5
6	5_20	10,05	-0,25	≤-0,75	>-0,75; ≤0,25	>0,25
7	68	9,17	-0,25	≤-0,75	>-0,75; ≤0,25	>0,25

➢ AWARDING OF POINTS FOR INACTIVE CGS DERIVATIVES



	Cpd	pki	exp. Diff.	0,5	1	0
8	2_72	5,57	-3,85	≤-4,35	>-4,35; ≤-3,35	>-3,35
9	1	6,37	-3,05	≤-3,55	>-3,55; ≤-2,55	>-2,55
10	5_29	5,52	-4,78	≤-5,28	>-5,28; ≤-4,28	>-4,28
11	101	6,07	-3,35	≤-3,85	>-3,85; ≤-2,85	>-2,85
12	11	6,91	-2,51	≤-3,01	>-3,01; ≤-2,01	>-2,01
13	XHe-098a	5,44	-3,98	≤-4,48	>-4,48; ≤-3,48	>-3,48
14	XHe-III-56	6	-3,42	≤-3,92	>-3,92; ≤-2,92	>-2,92

RESULTS & DISCUSSION C

1. COMFA RESULTS

1.1 DEFINING COMFA OUTCOME

1.1.1 Electrostatic isocontour map

1.1.2 Steric isocontour map

2. DOCKING POSE RESULTS

2.1 CLUSTERED DOCKING POSE RESULTS

2.2 PLIF APPLIED TO DOCKING RESULTS

3. SCORING SCHEME RESULTS

3.1 DEFINING OUTCOME OF SCORING METHOD

3.1.1 Penalty scoring method

3.1.2 Simple scoring method

4. FINAL BINDING MODE FINDING

4.1 BINDING MODE CONFIRMING EXPERIMENTAL REQUESTS

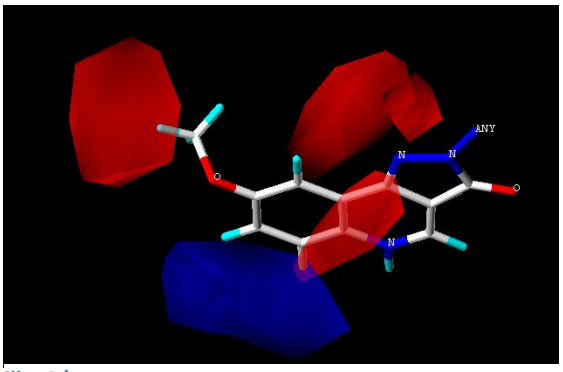
4.2 THE FINAL RESULT

1. CoMFA RESULTS

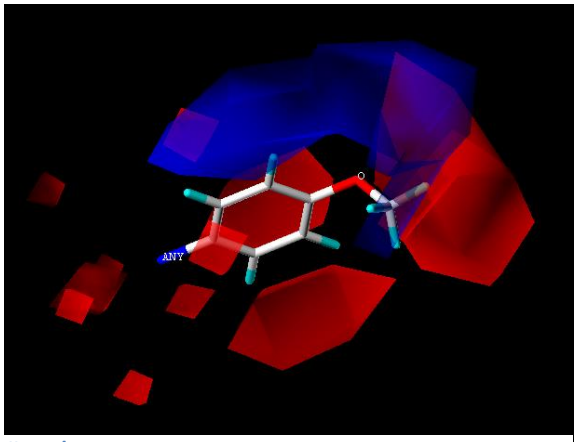
delivered CoMFA results were as follows:

r^2: 0,915 & **q^2:** 0,703 and **r^2 stderr**: 0,38 & **q^2 stderr**: 0,70

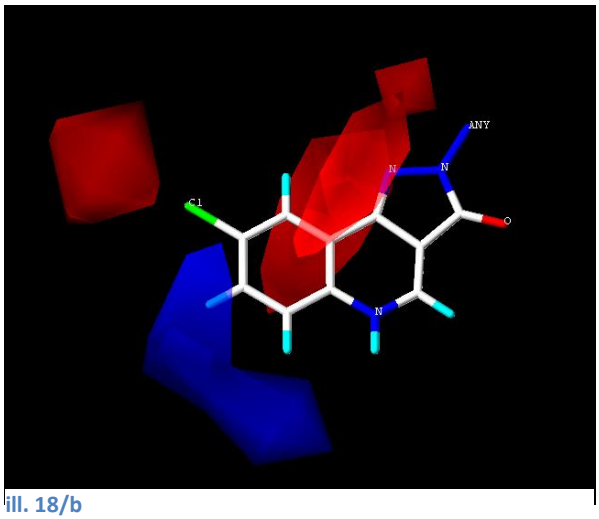
➢ PICTURES OF ELECTROSTATIC ISOCONTOUR MAP



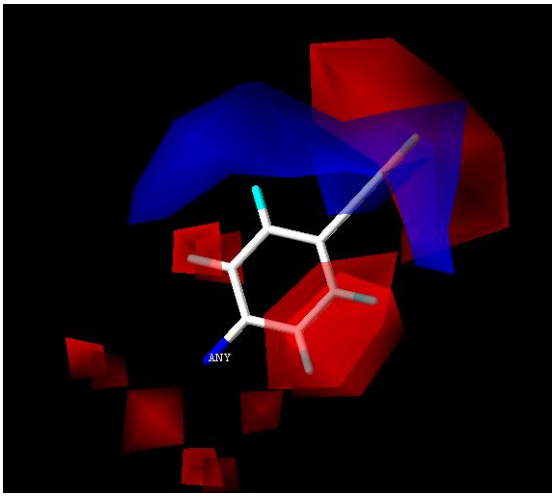
ill. 18/a



ill. 18/c



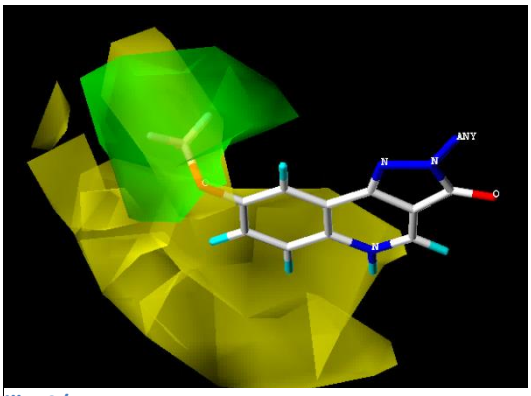
ill. 18/b



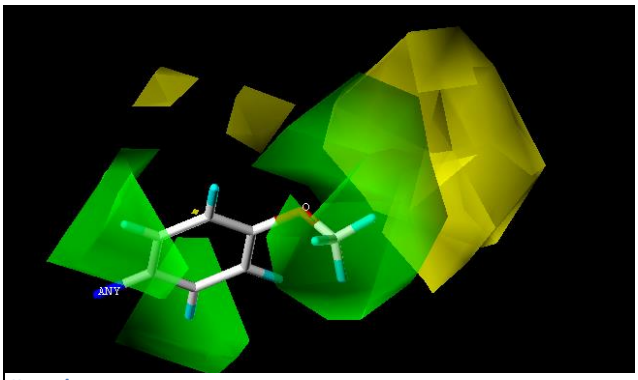
ill. 18/d

ill. 18:electrostatic isocontour maps: red indicates a e⁻ negative favoured region; blue indicates that positive moiety is favoured.

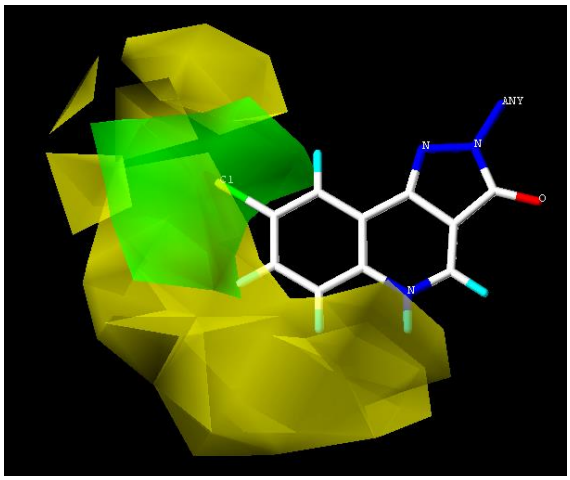
➢ PICTURES OF STERIC ISOCONTOUR MAP



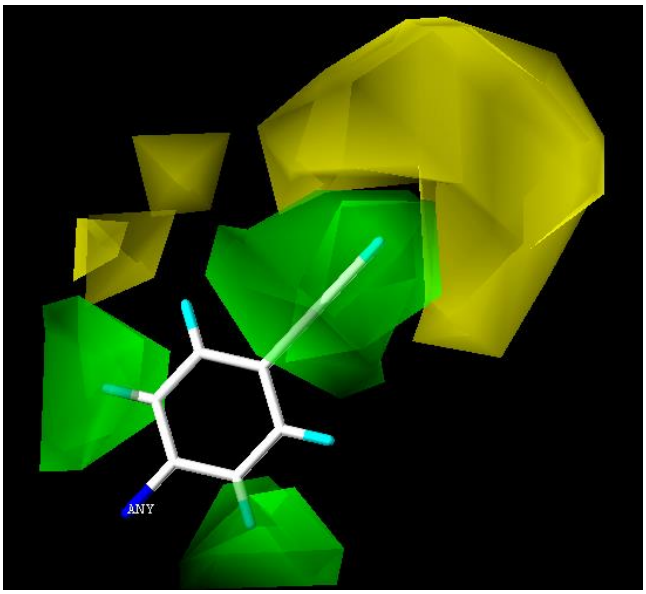
ill. 19/a



ill. 19/c



ill. 19/b



ill. 19/d

ill. 19: steric isocontour maps: green indicates sterically favoured regions; yellow indicates sterically unfavoured regions.

➢ ELECTROSTATIC ISOCONTOUR MAP

The electrostatic isocontour map, indicates that positively charged substitution in the **C4'** within ring D, but in a certain degree electron withdrawing substitution is favoured in the tip of c4' as well as in **C2'/C6'**. This explains the higher affinity when the 2-phenyl ring is substituted with 2-pyrimidin–2'-yl. The red colouring of ring **D** shows importance of its negative moiety for increasing affinity. (see ill.18/c & d)

Around the area of **C8** the red colouring indicates that negatively charged substitution increases the affinity. Also red colouring at **N1** and **N2** is present, indicating their importance in order to keep the affinity high. (see ill. 18/a & b)

➢ STERIC ISOCONTOUR MAP

The steric isocontour maps of the CoMFA indicate that a substitution in **C4'** (see ill. 19/c & d) and **C8** region (see ill. 19/a & b) is preferred to a certain degree, with the most preferred fit, by looking at the affinity results, is presented by the substituent OCF3 at C8 and in C4' by e.g. NH_2 or ethylen. By this the binding pocket is filled out most conveniently and affinity rises.

Electron withdrawing insertion in ring D at **R2'/ R6'**, leads to a slight bulky proportion in this region, and increases the affinity by performing a better fit in the binding pocket. (see ill 19/c & d)

Sterically unfavoured is substitution in **C5**, **C6** and **C9**. C5 unfavoured substitution is due to the loss of the Hydrogen - bond formation of NH. (see ill. 19/a & b)

2. DOCKING POSE RESULTS

The delivered docking poses are clustered and interactions for each pose are characterized by PLIF.

2.1. CLUSTERED DOCKING POSE RESULTS

The cluster analysis delivered 30 clusters each containing minimum 0.4% (1 pose) and maximum 10% (41 poses) out of all 400 docking poses.

The obtained results are mostly homogeneous with some exceptions.
The one cluster containing only one pose was therefore not really representing a cluster at all but an outlier in its own.

Class	amount of poses	percentage
22	41	10%
4	33	8%
27	27	7%
9	23	6%
16	23	6%
1	22	6%
25	22	6%
5	19	5%
8	18	5%
29	16	4%
18	15	4%
3	14	4%
12	14	4%
11	12	3%
26	12	3%
7	10	3%
19	10	3%
28	10	3%
2	9	2%
10	8	2%
6	7	2%
21	6	2%
13	5	1%
14	5	1%
15	5	1%
20	5	1%
30	4	1%
17	2	1%
24	2	1%
23	1	0%

2.2. PLIF APPLIED TO DOCKING RESULTS

The application of the function PLIF delivered 67 bits, representing interactions between CGS-8216 and 17 various amino residues of the binding protein. The amino residues involved in interacting with CGS-8216 are

D56, Y58, H101, R132, T142, S158, Y159, K184, E189, V202, Q203, S204, S205, T206, G207, E208, Y209

The percentage of the interaction's appearance in relation to the sum of all possible interactions is illustrated in the table below

residue	Percentage
S204	22,29
Y58	21,39
T206	16,21
Y209	14,59
S205	8,18
H101	3,16
S158	2,92
D56	2,51
R132	2,27
T142	1,78
Y159	1,54
V202	1,46

3. SCORING SCHEME RESULTS

After applying the scoring schemes, “penalty scoring method” and “simple scoring method” to all of the 400 clustered docking poses, with their associated interactions, the scores were summed up, and then poses were ranked in the order largest to smallest, according to their scoring points. The following best scored poses per scoring scheme were attained, as seen in the tables below.

- Rank of the docking poses after applying the “penalty scoring scheme” is seen in the table below:

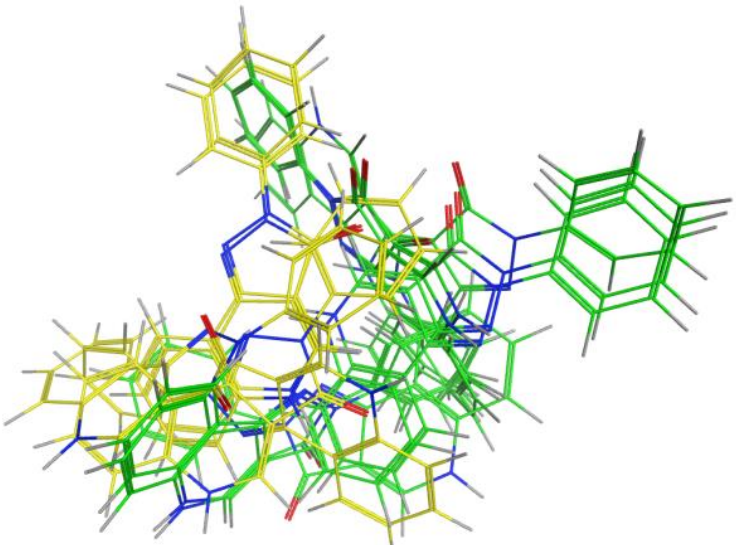
pose_id.	**present in**
1-100	N103
101-200	X77
201-300	GluCl
301-400	4COF

Rank	pose_id	Class	penalty score
1	165	3	9
2	161	3	8,75
2	182	3	8,75
3	8	1	8,25
3	9	1	8,25
4	185	3	7,5
5	174	3	7
6	167	3	6,5
6	173	3	6,5
7	1	1	6,25
7	13	1	6,25
7	150	3	6,25
7	155	3	6,25

BEST RANKED POSES:

- Cluster 3: yellow poses
- Cluster 1: green poses

➔ Altogether inhomogeneous suggestion of poses



ill. 20: Best ranked poses, delivered by the penalty scoring method. Poses from cluster 3 (yellow) and poses from cluster 1 (green) were overlaid and imaged in MOE.

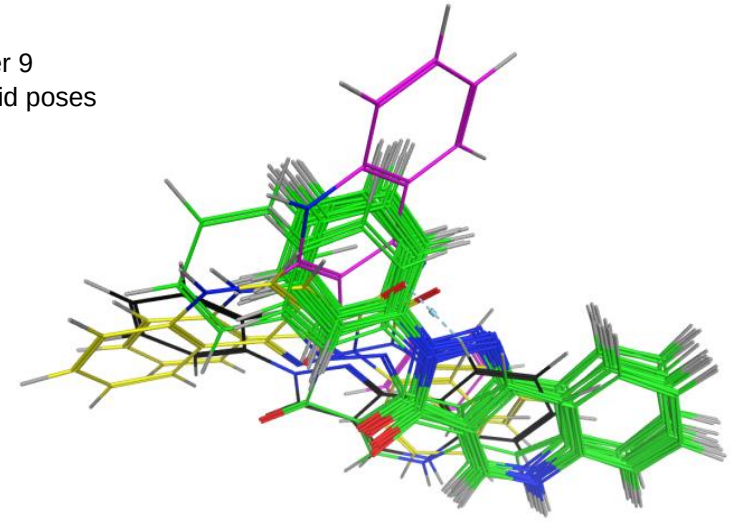
- Rank of the docking poses after applying the “simple scoring scheme” is listed in the table below:

Rank	pose_id	Class	simple_score
1	333	9	7
2	312	9	6,5
2	316	9	6,5
2	317	9	6,5
2	327	9	6,5
2	331	9	6,5
2	343	9	6,5
2	365	9	6,5
2	398	29	6,5
3	311	9	6
3	315	9	6
3	324	9	6
3	325	5	6
3	329	9	6
3	332	9	6
3	334	9	6
3	362	29	6
3	389	22	6
3	391	5	6

BEST RANKED POSES:

- Cluster 9: green poses
- Cluster 29: yellow poses
- Cluster 22: pink poses
- Cluster 5: black pose

➔ homogenous poses in cluster 9 represented as green overlaid poses



ill. 21: Best ranked poses, delivered by the simple scoring method. Poses from the specific clusters were overlaid and respective snapshot was taken in MOE.

➢ PENALTY SCORING METHOD

The highest ranked poses suggested by the penalty scoring scheme are representatives of the cluster class **3** (score = 9) and **1** (score = 8,25). Visually observed these differences are not insignificantly (see ill. 20). Looking into these clusters' interactions the amino residues involved are the following, which are listed in the table below. These are listed in descending order according to their amount of appearance among all the poses present within the respective cluster.

cluster 1	amount
Y58	22
T206	21
S204	20
S158	16
Y209	10
S205	1
Y159	1

cluster 3	Amount
Y58	14
Y209	11
S204	10
T206	7
S158	5
H101	4
S205	1
Y159	1
E189	1

1 & 3	amount
S158	80
Y58	32
S204	32
T206	20
Y209	8
H101	4
S205	1
Y159	1
E189	1

➢ SIMPLE SCORING METHOD

The highest ranked poses that are suggested when applying the simple scoring scheme are mainly poses from cluster number 9, 29, next to cluster number 5 and 22: From nineteen poses fourteen belong to cluster no 9, two belong to cluster no 29, another two belong to cluster no 5 and one belongs to cluster no 22.

When cutting down to the five best ranked poses solely poses from cluster no **9** (score = 5) and **29** (score = 4,53) are suggested. The poses which are seen in cluster 9 are very homogeneous when visualizing their binding mode with MOE (see ill. 21).

In terms of interactions the amino residues, that are involved are listed in the table below, which are sorted in ranked order according to their amount of appearance among all the poses that appear within the respective cluster.

cluster 9	amount
T206	38
S204	33
S158	32
Y209	31
H101	18
V202	16
Y58	15
Y159	10
S205	1

cluster 29	Amount
S204	36
T206	28
Y58	26
S205	18

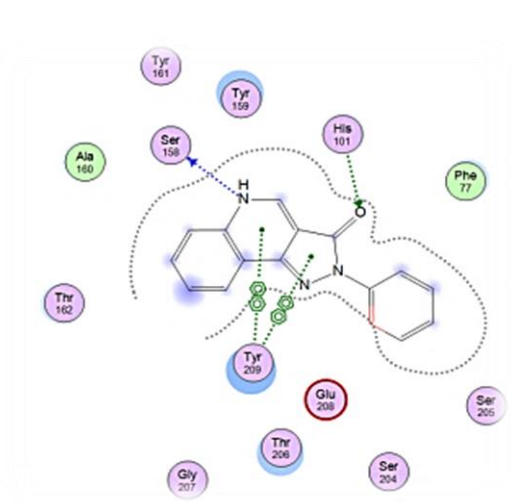
9 & 29	amount
S204	69
T206	59
Y58	31
S158	31
Y209	18
H101	16
V202	15
Y159	10
S205	1

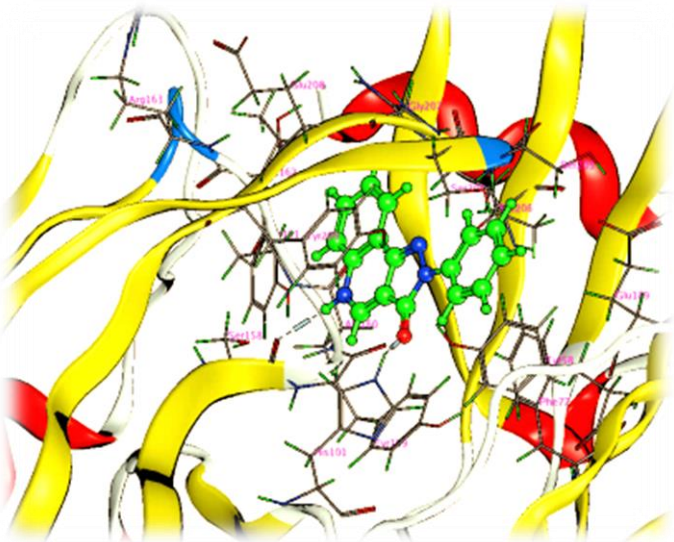
4. FINAL BINDING MODE FINDING

Looking into both scoring methods, six clusters were altogether submitted, which were cluster 1, 3, 5, 9, 22 and cluster number 29. Cluster 1 and 3 suggested by the penalty scoring method were quite essentially different, whereas the best ranked poses of cluster 9 were very similar, which were suggested after applying the simple scoring method. This proves the unity within the simple scoring scheme. Other suggested poses were mostly not in agreement with experimentally obtained data.
Consequently, within the simple scoring scheme as well as within the penalty scoring scheme, binding modes that were submitted were evaluated.
The highest ranked binding poses suggested by the penalty scoring scheme were illustrated as binding mode A and the binding mode suggested by the simple scoring scheme is illustrated as binding mode B.
The assessment of both binding poses suggested by the two scoring schemes takes place with their binding behaviour being in accordance with the CoMFA results and experimentally derived data information.

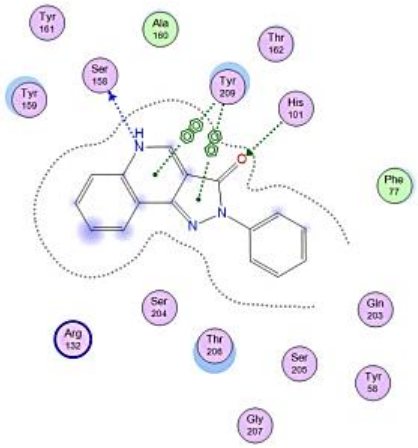
BINDING MODE A

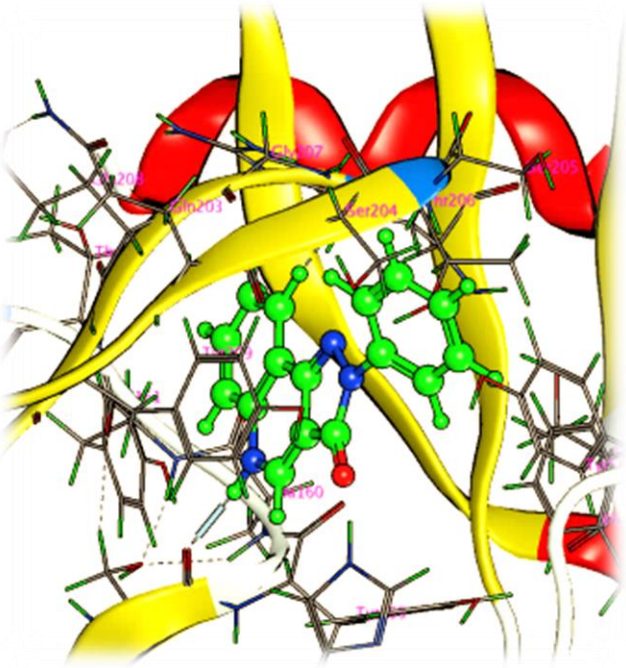
- POSE IDENTITY 165



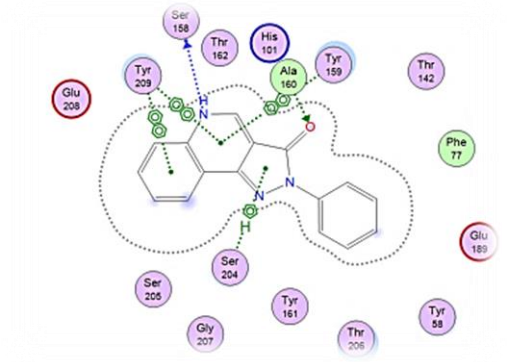


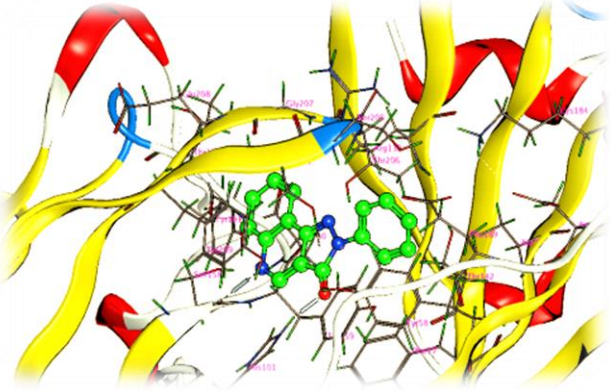
➢ POSE IDENTITY 182



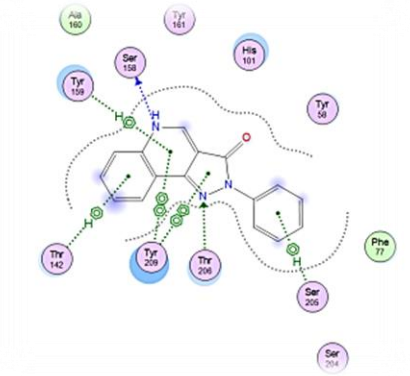


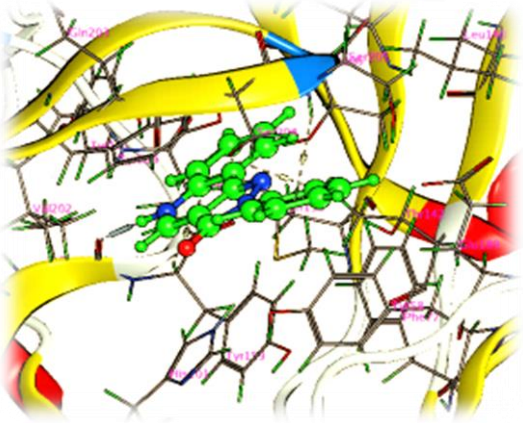
➢ POSE IDENTITY 8





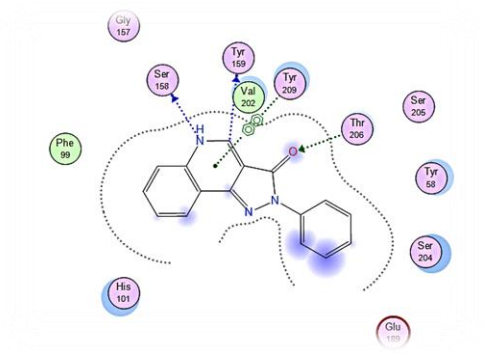
➢ POSE IDENTITY 185

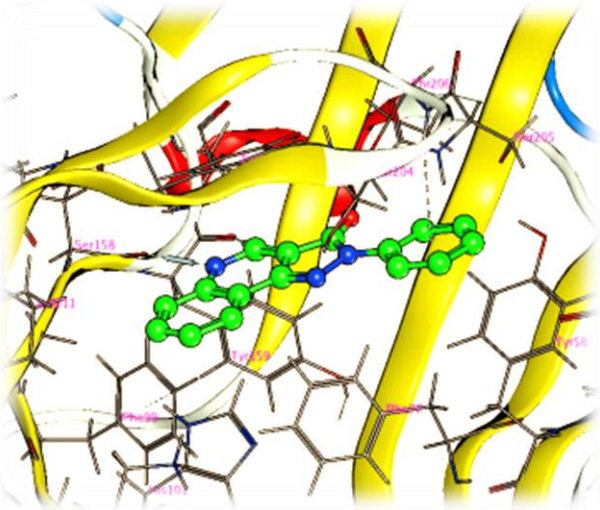




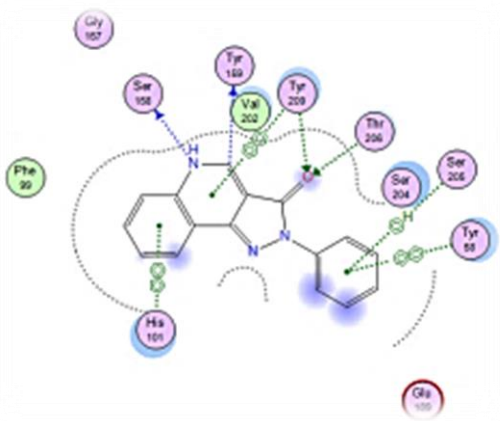
BINDING MODE B

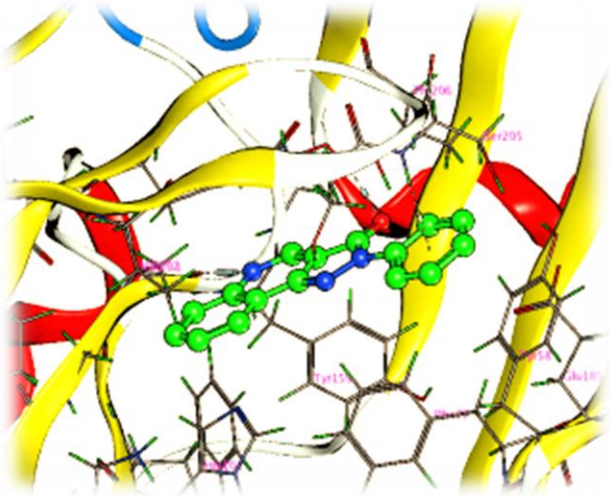
- POSE IDENTITY 333



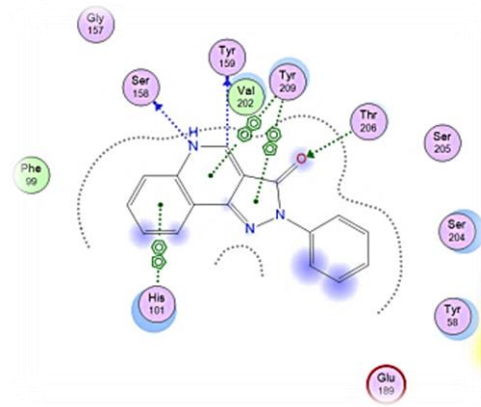


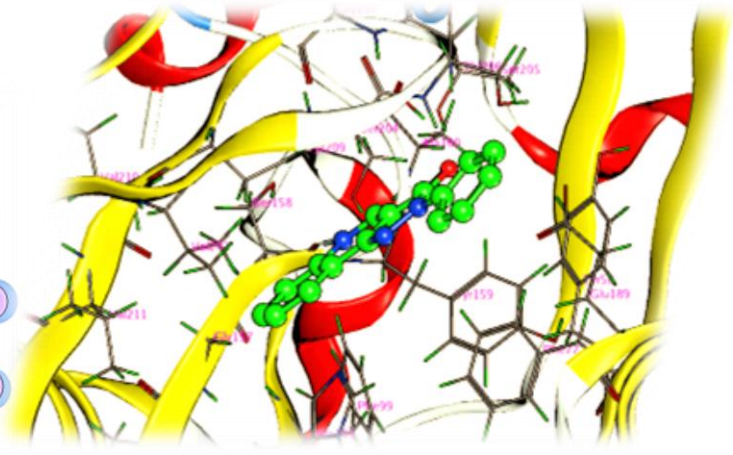
- POSE IDENTITY 316



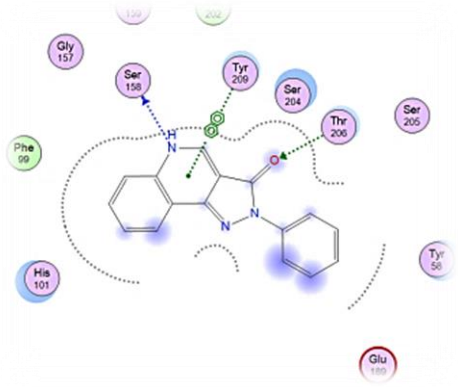


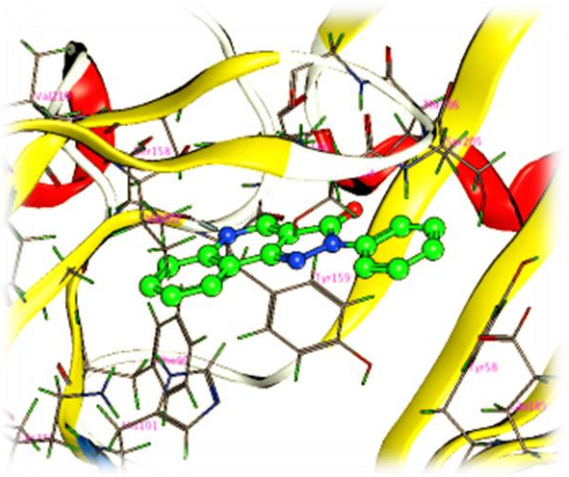
- POSE IDENTITY 317





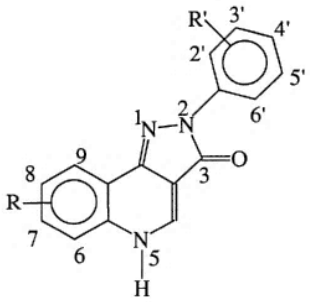
➢ POSE IDENTITY 343

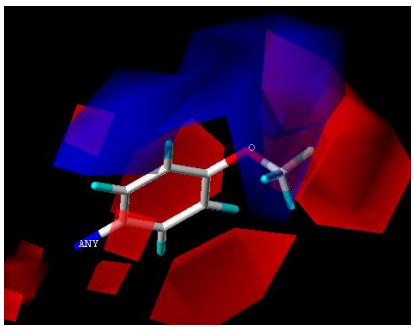
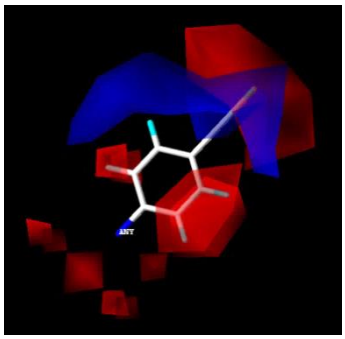
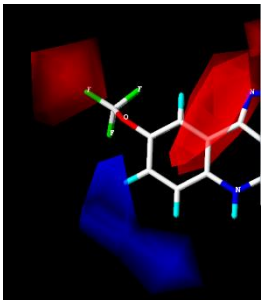
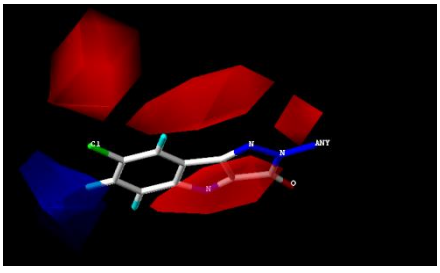


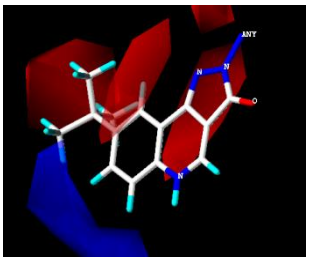
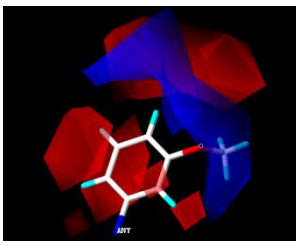
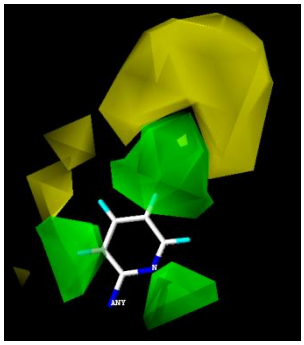
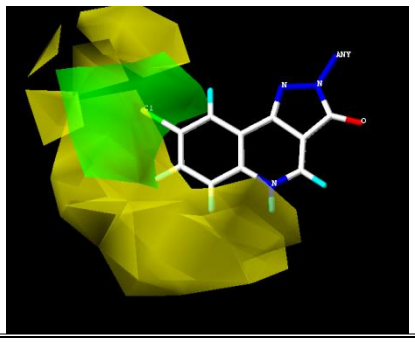


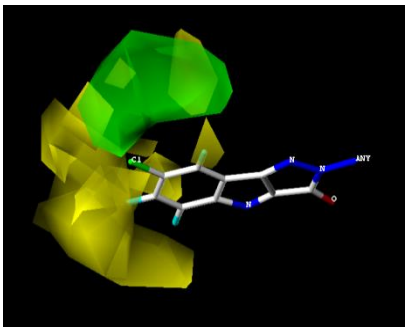
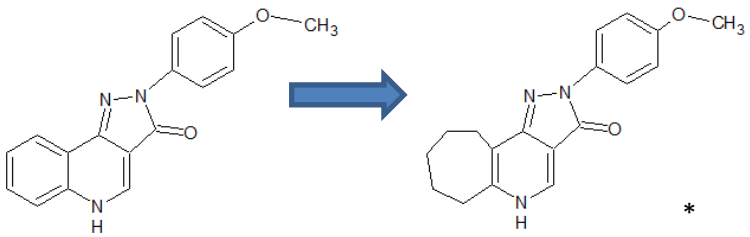
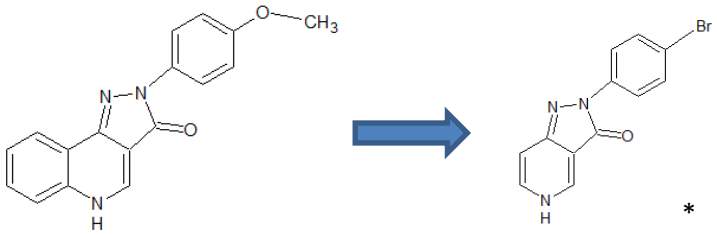
4.1. BINDING MODE CONFIRMING EXPERIMENTAL REQUEST

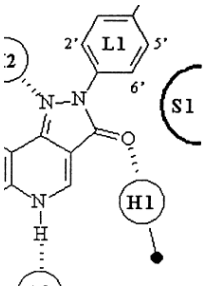
In the course of the end analysis, the two remaining binding poses were examined according to the CoMFA outcome and literature derived information for pinning down the final correct binding conformation.



COMFA REQUEST	Binding mode A	Binding mode B
ELECTROSTATIC FEATURES		
C4'- substitution in ring D: BM has to explain an affinity rise with the 4' region mainly containing a positive charge, but also a negative charge to a certain degree at its tip. 	An affinity rise with a positively charged substitution in 4' region is explained.	BM B also fulfills required feature.
C2'/C6' in ring D: e⁻-withdrawing substitution in ring D at **C2'/C6' increases affinity.** Hence substituting the 2-phenyl ring with 2-pyrimidin–2'-yl increases the binding affinity. 	Yes	Yes
According to the COMFA images, indication of red colour in C8, suggests preference of electron withdrawing substituents. 	BM A fulfills required feature.	BM B fulfills required feature.
Red colouring at **N1** and **N2** indicates its importance for keeping affinity up high. 	Carbonylic interaction from the pyrazoloring.	An interaction is seen between carbonyl of ring C and Y209 of the binding protein.

Red colouring at ring **B** and **D** indicates importance of its electron withdrawing feature. Hence it is suggested that π–π stacking here is important for high affinity.  	π–π-stacking-interaction is present towards ring B and ring D.	π–π-stacking interaction is present towards ring B and ring D.
STERIC FEATURES		
Steric request in C8: Best steric fit is represented with substitution of e.g. **O-C-F_3** or **tert. Butyl**, as these fill out the binding pocket most conveniently and as a consequence show high affinity in experimental measurements. Everything larger than this would cause steric clashes and decrease the affinity.	Yes	Yes
Substitution in **C4':** High affinity is explained in respective BM if C4' is substituted with for example • *Ethylin* • *-NH2* 	BM A explains an affinity rise C4' contains a substitution to a certain degree.	BM B also explains an affinity rise with a limited substitution in C4'.
Electron withdrawing insertion at **C2' and/or C6'** in ring D is preferred, also leading to a beneficial steric fit in the pocket and thereby increases the affinity.	BM A explains a rise in affinity with e^--negative ortho substitution(s) in ring D.	BM B also explains a rise in affinity with e^--withdrawing substitution(s) in C2' and/or C6'.
Preference of unsubstituted status in **C5** is suggested. It is presumed to be important for keeping an HB-formation of NH upright (14). C5-substitution would cause clashes with the binding protein.  Confirmation of **C6**- and	H-bond formation with NH is present. Is confirmed with BM A.	H-bond formation with NH is present → towards Ser158. Is confirmed with BM B. Compared to BM A, more space is available in BM B.

C9-substitution causing a drop of affinity. The assumption is made that steric clashes are easily created in this region, giving a closer idea of the binding pocket's borders.		
 CoMFA indicates sterically unfavoured: substituation in **C5**, **C6** and **C9**.	This is explained as there is little space seen.	In BM B this is also explained as there is also limited space available, although distinguished from BM A more space is offered in this conformation.
EXPERIMENTALLY PROVEN NECESSITY; Confirmed by visual inspection of the Binding Mode		
There is no π–π-stacking interaction present towards ring A. This evidence is based on the experimentally derived measurement of the pKi value staying the same when the CGS-8216 is changed in its ring A to for example CGS-20625, which has a non-aromatic cycloheptan ring.  CGS-8216 with pKi 9,42 → CGS-20625 with pKi 9,30	BM A fulfills requirement.	BM B also fulfills requirement.
Confirmation of drop in affinity by the loss of ring A. Experimental evidence of this fact is given by a measured drop in affinity when CGS8216 is modified to for example XHe-III-56.  CGS-8216 with pKi 9,42 → XHE-III-56 with pKi 5,57	No → In fact ring A in this position is more solvent exposed than in the other binding conformation. This means that for keeping activity upright, a substitution is not important. <u>Conclusion</u>: Ring A does not take in the function of a steric interacting component towards the binding	Yes → Ring A functions as an important part for steric interactions. The loss of ring A, whether it is an aromatic or non-aromatic ring results in an affinity-decrease. Controversially to the other binding pose, this can be explained with this binding conformation. The amino residue His 101 shows a

	pocket.	hydrophobic interaction towards ring A. Besides ring A is more centrally embedded within the pocket area and not as much solvent exposed as seen in the other BM.
interaction between the pyrazolo-carbonyl and the binding protein (3) 	yes	Yes (→ *towards Y209)*
Binding mode pose presented by cluster number	1 & 3 Altogether inhomogeneous poses	9, 29, 22 & 5 First 8 best ranked poses are all from cluster 9 and are very homogeneous, when looking at the overlayed poses within MOE.
most important Interactions of CGS-8216 to the binding protein	**His101, T142, F77** and **Y58**	**His 101, T142, F77** and **Y58** Additional interaction versus cluster 1 & 3: **Y159**

*compounds drawn with ACD/ChemSketch (Freeware) 2012.

4.2. THE FINAL RESULT

The additional visual assessment of the scored docking poses confirmed the requested binding features in most areas, from which binding mode A fulfilled less requirements than binding mode B. Binding mode B could fulfill all of the required binding features. The important divergences between binding mode A and B are as follows:

1) The request of the affinity property increasing when C4' is derivatized with positively charged substitution, cannot be fulfilled with binding mode A whilst binding mode B can explain this manner.

2) Furthermore, ring A of the CGS derivatives are in binding mode B embedded more centrally within the binding pocket which is important for a good binding conformation whilst in binding mode A it is more solvent exposed.

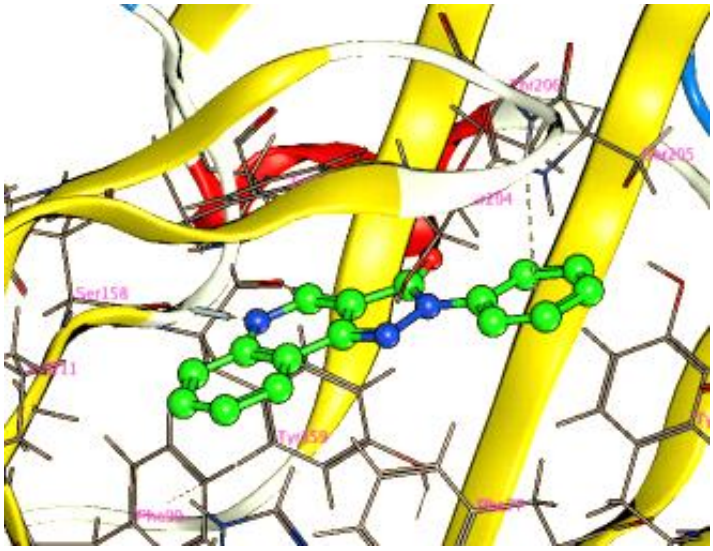
3) Moreover binding mode B includes docking poses from within the BP 4COF, which is the latest binding protein. Therefore it is plausible that this is the most correct one and should therefore withhold the most reliable pose.

4) Another aspect is that the binding mode B is a suggestion obtained after applying the simple scoring method, which withholds a condenser set of information than the penalty scoring method. Through the comparison of the delivered outcome of both scoring methods, the penalty scoring method does not yield a uniform suggestion of binding poses, whereas the simple scoring method proves a unity when looking at the high similarity of the first best ranked poses.

   The awarding of points in the penalty scoring method may be too strict as it deducts points, whereby certain poses, which are originally ranked high, get ranked lower by a few outliers, which cannot explain the pose. Therefore poses which are originally middle ranked get ranked higher as the high ranked poses drop beneath the originally middle-ranked in the ranking list. This is why the simple scoring method is thought to deliver more correct results.

By taking these aspects into account, binding mode B is confirmed as the correct conformation, taken in by CGS compounds!

HIGHEST RANKED POSE, POSE_ID 333, WITH SIMPLE SCORE METHOD:



CONCLUSION& OUTLOOK D

1. SOLVING DIFFICULTIES IN DRUG DESIGN

4 SOLVING DIFFICULTIES IN DRUG DESIGN

In the course of this diploma work the focus was set on tackling two different kinds of difficulties. One difficulty concerns the relevance of researching novel therapeutic approach by selective binding of a specific substance class' and the other referring to the difficulty during computer-targeted discovery for structure activity relationship revelation.

On the one hand the thesis reveals the importance of the compounds binding selectivity to achieve a specific biological and pharmacological outcome.
Depending on which subtype of the $GABA_A$ receptor is activated, physiological and also pathological effects vary. Undesired side effects differ from somnolence, fatigue, lack of concentration through to the development of dependence and tolerance when chronically used.
By identifying these determinations, it is considered that only through the design of subtype selective ligands, specific wished for reactions can be released whilst side effects can be prevented.

In the specific case of this diploma work the attention was set on CGS-compounds, which behave as null modulators via the $\alpha 2\beta\gamma 2$ interface of the $GABA_A$-receptor but also bind to a novel drug binding site, at the $\alpha 1+\beta 3-$ interface thus triggering benzodiazepine like effects, with far less observed side effects. (4)
Taking this into consideration, the aim is to ground a novel outlook of therapy through the detection of the CGS' high affinity subtype selective binding conformation with the final aim of a more successful therapeutic approach.

The other difficulty tackled in the course of the diploma thesis was focused on overcoming hindrances found on various levels of computational workflows.

In order to obtain decent results workflow settings had to be adjusted and therefore kept being adapted to the system, appropriate for the used materials. By this final realization, the outcome's evaluation was possible.

Computational hindrances include faults in the protein modeling as well as limitations in the ligand recognition due to challenging issues in molecular modeling and simulation studies. However, structural plasticity plays a crucial role in drug discovery simulations for the characterization of ligand-receptor complexes. (38)

This is why it is important to find the most adequate set up by keep adjusting and looking into the outcome of a few test runs. A standard set up for perfect docking results or cluster analysis does not exist. It is a process of successively approximating towards an appropriate result, which is why flexibility, soft potentials and docked ligands had to be changed over the first few test runs. Also finding the correct count of clusters took place progressively.

Most importantly is the presented invention of the novel evaluation method for docking pose results, which is finally realized by the "simple scoring method" of "post dock modified" docking poses. This represents a significant systematic approach of detecting the plausibility of a broad set of suggested docking poses.

As a consequence not only the original aim of finding the CGS binding mode was reached but also future inquiries are facilitated since this novel approach of solving in silico difficulties was enlightened.

BIBLIOGRAPHY E

(1) Aktories, K., Förstermann, U., Hofmann, F. & Starke, K. (2013). *Allgemeine und spezielle Pharmakologie und Toxkikologie*. (11. Überarbeitete Auflage). München: Elsevier GmbH, Urban Fischer Verlag,

(2) Jennings, A.S.R. et al. (2006). Imidazo[1,2-*b*][1,2,4]triazines as a2/a3 subtype selective GABAA agonists for the treatment of anxiety. *Bioorganic & Medicinal Chemistry Letters, 16,* 1477–1480.

(3) Savini, L. et al. (2001). High Affinity Central Benzodiazepine Receptor Ligands: Part 2: Quantitative Structure-Activity Relationships and Comparative Molecular Field Analysis of Pyrazolo[4,3-c]quinolin-3-ones. *Bioorganic & Medicinal Chemistry, 9*, 431-444.

(4) Varagic, Z. et al. (2013). Identification of novel positive allosteric modulators and null modulators at the GABAA receptor a+b- interface. *British Journal of Pharmacology*, *169,* 371-381.

(5) Han, D. et al. (2008). A study of the structure–activity relationship of $GABA_A$–benzodiazepine receptor bivalent ligands by conformational analysis with low temperature NMR and X-ray analysis. *Bioorganic & Medicinal Chemistry*, *16,* 8853–8862.

(6) Clayton, T. et al. (2007). An updated unified pharmacophore model of the Benzodiazepine Binding site on g-aminobutyric $acid_A$ receptors: correlation with comparative models. *Current Medicinal Chemistry, 14,* 2755-2775.

(7) Yokoyama, N., Ritter, B. & Neubert, A.D. (1982). 2-Arylpyrazolo [4,3c]quinolin-3-ones: novel agonist, partial agonist and antagonist of benzodiazepines. *J. Med. Chem., 25*, 337-339.

(8) *Ramerstorfer*, J. *et al*. (*2011*) The GABAA receptor a+b– interface: a novel target for subtype selective drugs. *J. Neurosci., 31*, 870–877. Fig 1a

(9) Sieghart, W., Ramerstorfer, J., Sarto-Jackson, I., Varagic, Z. & Ernst, M. (2012) A novel GABA-A receptor pharmacology: drugs interacting with the alpha+beta-interface. *Br J Pharmacol*, *166*, 476-485.

(10) Vinkers, C.H. & Olivier, B. (2012). Mechanisms Underlying Tolerance after Long-Term Benzodiazepine Use: A Future for Subtype- Selective GABAA Receptor Modulators?. *Hindawi Publishing Corporation; Advances in Pharmacological Sciences*, *Volume 2012*, *Article ID 416864*, 19 pages.

(11) Vinkers, C.H., van Oorschot, R., Nielsen, E.Ø., Cook, J.M. & Hansen, H.H. (2012) $GABA_A$ Receptor α Subunits Differentially Contribute to Diazepam Tolerance after Chronic Treatment. *PLoS ONE, 7*(8), e43054.

(12) Ewald, G. (2006). Gehirn, Seele und Computer: Der Mensch im Quantenalter. Darmstadt: WBG (Wissenschaftliche Buchgesellschaft).

(13) Klebe, G. (2009). *Wirkstoffdesign: Entwurf und Wirkung von Arzneistoffen* (2. Aufl.). Heidelberg: Springer.

(14) Richter, L. et al. (2012). Diazepam-bound GABAA receptor models identify new benzodiazepine binding-site ligands. *Nat Chem Biol*, *8* (5), 455-64.

(15) Böhm, H.-J., Klebe, G. & Kubinyi H. (2002). Wirkstoffdesign: Heidelberg: Spektrum Akademischer Verlag GmbH.

(16) Schneider, G. & Baringhaus, K.-H. (2008). Molecular Design: Concepts and Applications with a foreword by Hugo Kubinyi. Weinheim: WILEY-VCH.

(17) Becker, O.M. et al. (2006). An integrated in silico 3D model-driven discovery of a novel, potent, and selective amidosulfonamide 5-HT1A agonist (PRX-00023) for the treatment of anxiety and depression. *J Med Chem*, *49*(11), 3116-35.

(18) Lee, C.-H., Huang, H.-C. & Juan, H.-F. (2011). Reviewing Ligand-Based Rational Drug Design: The Search for an ATP Synthase Inhibitor. *Int. J. Mol. Sci., 12*, 5304-5318.

(19) Cramer, R.D., Patterson, D.E. & Bunce, J.D. (1988). Comparative Molecular Field Analysis (CoMFA).1. Effect of Shape on binding of Steroids to Carrier Proteins*. J. Am Chem. Soc, 110,* 5959-5967.

(20) Pinky, S.V. (2012). Comparatative Molecular Field Analysis (CoMFA). Available under: http://de.slideshare.net/sheetalvincent/comfa-comfa-comparative-molecular-field-analysis [28.10.2014]

(21) Gubernator, K. & Böhm, H.-J. (1998). Structure based ligand design. Weinheim: Wiley-VCH.

(22) Ogris, W. et al. (2004). Affinity of various benzodiazepine site ligands in mice with a point mutation in the GABAA receptor g2 subunit. *Biochemical Pharmacology, 68,* 1621-1629.

(23) Miller, P. S. & A. Aricescu, R. (2014). Crystal structure of a human GABAA receptor. *Nature, 512*(7514), 270-275.

(24) Hibbs, R.E. & Gouaux, E. (2011). Principles of activation and permeation in an anion-selective Cys-loop receptor. *Nature, 474* (7349), 54-60.

(25) Varagic, Z. et al. (2013). Subtype selectivity of a+b- site ligands of $GABA_A$ receptors: identification of the first highly specific positive modulators at a6b2/3g2 receptors. *British Journal of Pharmacology, 169* (2); 384-399.

(26) Jurik, A. (2009). *Flexible Docking Studies of a Series of Ligands in a Homology Model of the Human Serotonin Transporter*. Diplomarbeit, Hauptuniversität, Wien.

(27) GOLD tutorial (2014) available under: file:///C:/Users/Lydia/Documents/Docking%20Gold/goldtutorial.pdf

(28) Van de Waterbeemd, H. (1995). Advanced computer assisted techniques in drug discovery. Weinheim: VCH.

(29) Addinsoft, XLSTAT (2014). Available under: http://www.xlstat.com/de/mein-xlstat.html [7.10.2014]

(30) moe tutorial (2014) available under: http://www.chemcomp.com/MOE-Pharmacophore_Discovery.htm [15.5.2014]

(31) moe tutorial (2014) available under: file:///C:/moe2013/html/moe/pot.htm#Charge

(32) Crawforth, J. (2004). Tricyclic pyridones as functionally selective human GABAA_2/3 receptor-ion channel ligands. *Bioorganic & Medicinal Chemistry Letters, 14*, 1679–1682.

(33) He, X., Huang, Q., Yu, S., Ma, C., McKernan, R. & Cook, J.M. (1999). Studies of Molecular Pharmacophore/Receptor Models for GABAA/BzR Subtypes: Binding Affinities of Symmetrically Substituted Pyrazolo[4,3-c]quinolin-3-ones at Recombinant axb3g2 subtypes and Quantitative Structure-Activity Relationship Studies via a Comparative Molecular Field Analysis. *Drug Design and Discovery, 16,* 77-91.

(34) Puthenkalam, R. (2014). Unpublished dissertation: *Comparing the high affinity benzodiazepine binding site with the homologous "CGS9895" site in $GABA_A$ receptors*, Department of Medical Neurosciences Vienna.

(35) Savini, L. et al. (2011). High Affinity Central Benzodiazepine Receptor Ligands. Part 2: Quantitative Structure-Activity Relationships and Comparative Molecular Field Analysis of Pyrazolo [4,3-c]quinolin-3-ones. *Bioorg. Med. Chem., 9*, 431-444.

(36) CCDC online (2014). Available under: http://www.ccdc.cam.ac.uk/SupportandResources/Support/Pages/SupportSolution.aspx?supportsolutionid=92 [7.4.2014]

(37) moe tutorial (2014) available under: file:///C:/moe2013/html/apps/docking.htm#Example_PostDocking

(38) Spyrakis, F., Bidon-Chanal, A., Barril, X. & Luque, F.J. (2011). Protein Flexibility and Ligand Recognition: Challenges for Molecular Modeling. *Current Topics in Medicinal Chemistry, 11* (2), 192-210.

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2. ABSTRACT IN ENGLISH

The $GABA_A$ receptor is a major inhibitory neurotransmitter receptor and the target of many clinically important drugs.[1]It is a membrane bound heteropentameric receptor complex consisting principally of α, β, and γ subunits, which are arranged in different combinations, mostly found in the central nervous system.[2] When GABA binds it causes the chloride ion channel to open and thereby inhibits neurotransmission and triggers numerous neurological effects including convulsion, anxiety and sleep.[3]

Since 2011 it is known that CGS compounds (Pyrazoloquinolinones), a new class of benzodiazepine Site ligands, additionally to the benzodiazepine binding site also bind to another binding site, 'CGS binding site', located at the $\alpha+$ $\beta-$ interface. Binding of CGS compounds to the CGS-BS is supposed to be responsible for fewer side effects than with the classical benzodiazepines [1], which would mainly be sedation, ataxia, potentiation with alcohol and risk of tolerance and dependence when chronically used.[4]

In order to understand the binding-mode of CGS compounds, we pursued an experimental data guided docking workflow.[5] Docking of the pyrazoloquinolinone, CGS-8216, into a set of four different homology models of $GABA_A$, followed by exhaustive analysis of the docking poses including a new scoring function based on steric hindrance analysis, revealed two potential binding hypotheses. Comparison of the two different binding pocket conformations with the very recent publication of Miller & Aricescu [6] allowed the final prioritization. This will allow designing high affinity CGS compounds with same efficacy but less side effects than with classical benzodiazepines.

[1]Zdravko Varagic et al.; Identification of novel positive allosteric modulators and null modulators at the GABAA receptor
[2]L.Savini. et al.; High Affinity Central Benzodiazepine Receptor Ligands. Part 2: Quantitative Structure-Activity Relationships and Comparative Molecular Field Analysis of Pyrazolo[4,3-c]quinolin-3-ones; *Bioorganic & Medicinal Chemistry*; **9**; 431-444; (2001)
[3]Han Dongmei et al.; A study of the structure–activity relationship of GABAA–benzodiazepine receptor bivalent ligands by conformational analysis with low temperature NMR and X-ray analysis;*Bioorg Med Chem.*; 16(**19**); 8853-8862 (2008)
[4]Crawforth James et al.; Tricyclic pyridones as functionally selective human GABAA alpha 2/3 receptor-ion channel ligands.; *Bioorg Med Chem.*; 14(**7**); 1679-82 (2004)
[5]Richter et al; Diazepam-bound GABAA receptor models identify new benzodiazepine binding-site ligands.; *Nat Chem Biol.;* 8(**5**); 455-64 (2012)
[6]Miller PS, Aricescu AR; Crystal structure of a human GABAA receptor; *Nature*; 0028-**0836**; 1476-4687 (2014)

3. ABSTRACT IN GERMAN

Der $GABA_A$ Rezeptor ist ein bedeutender inhibitorischer Neurotransmitter-Rezeptor, der den Angriffspunkt vieler klinisch relevanter Arzneistoffe darstellt.[7] Es handelt sich hierbei um einen membrangebundenen heteropentameren Rezeptor-Komplex, welcher grundsätzlich aus den Subeinheiten α, β, und γ besteht. Diese kommen in verschiedenen Kompositionen und dabei hauptsächlich im ZNS vor.[8]

Kommt es zur Bindung von GABA, wird der Chlorid-Ionen-Kanal geöffnet und die Neurotransmission wird unterdrückt. Dies hat Einfluss auf eine Vielzahl neurologischer Effekte, wie etwa Konvulsion, Angst- und Schlafzustände. [9]

Seit 2011 ist die neue Klasse der BZD-BS-bindender Liganden, CGS-Liganden (Pyrazoloquinolinone) bekannt, welche zusätzlich zur BZD-BS, die „CGS-Bindestelle“, welche sich an der $\alpha+ \beta-$ Schnittstelle befindet, binden.

Es wird angenommen, dass in der Bindung der CGS-BS der Grund für das Auftreten weniger unerwünschter Nebeneffekte, als unter klassischen Benzodiazepinen, liegt.[1] Häufig auftretende unerwünschte Nebeneffekte sind mitunter Sedierung, Ataxie, Potenzierung mit Alkohol so wie das Risiko einer Toleranz und Abhängigkeit, wenn chronisch angewendet. [10]

Um den Bindemodus von CGS-substanzen zu erläutern, wurde ein Docking-prozedere durchgeführt, in dem experimentell ermittelte Datensätze zu Hand genommen wurden. [11]

Im Zuge dessen wurde das Pyrazoloquinolinon, CGS-8216, in ein Set von 4 verschiedenen Homologie-Modellen des $GABA_A$ Rezeptors gedockt, welches von einer signifikanten Docking-Pose-Analyse gefolgt wurde. Jene basiert auf einer

[7] Zdravko Varagic et al.; Identification of novel positive allosteric modulators and null modulators at the GABAA receptor a+b- interface; *British Journal of Pharmacology*; **169**; 371-381 (2013)
[8] L.Savini. et al.; High Affinity Central Benzodiazepine Receptor Ligands. Part 2: Quantitative Structure-Activity Relationships and Comparative Molecular Field Analysis of Pyrazolo[4,3-c]quinolin-3-ones; *Bioorganic & Medicinal Chemistry*; **9**; 431-444; (2001)
[9] Han Dongmei et al.; A study of the structure–activity relationship of GABAA–benzodiazepine receptor bivalent ligands by conformational analysis with low temperature NMR and X-ray analysis;*Bioorg Med Chem.*; 16(**19**); 8853-8862 (2008)
[10] Crawforth James et al.; Tricyclic pyridones as functionally selective human GABAA alpha 2/3 receptor-ion channel ligands.; *Bioorg Med Chem.*; 14(**7**); 1679-82 (2004)
[11] Richter et al; Diazepam-bound GABAA receptor models identify new benzodiazepine binding-site ligands.; *Nat Chem Biol.;* 8(**5**); 455-64 (2012)

neuartigen Scoring-Funktion, die auf einer Begutachtung sterischer Hindernisse aufbaut.

Im Endresultat werden 2 potentielle Bindungshypothesen offenbart. Der Vergleich beider Bindungsmodi mit dem vor Kurzem veröffentlichten $GABA_A$-Rezeptor-Modell von Miller & Aricescu[12], erlaubte die finale Priorisierung.

Die Offenlegung der Bindungskonformation von CGS-Komponenten in der BZD-BS, soll den Grundstein für den Design von Liganden, die selektiv in der CGS-BS binden, darstellen und mit diesen soll auf weiterer Sicht betrachtet eine neuartige Therapieform mit weniger unerwünschten Nebeneffekten ermöglicht werden.

[12]Miller PS, Aricescu AR; Crystal structure of a human GABAA receptor; *Nature*; 0028-**0836**; 1476-4687 (2014)

4. LIST OF ABBREVIATIONS

GABA	Gamma aminobutyric acid
3D	Three dimensional
3D-QSAR	3D Quantitative structure-activity relationship
PLIF	Protein-ligand interaction fingerprint
CoMFA	Comparative molecular field analysis
α-KG	alpha Ketoglutarat
BZD	Benzodiazepine
CYP2C19/ CYP3A4	Cytochrome P450 2C19/ Cytochrome P450 3A4
BZR	Benzodiazepine receptor
PQ	Pyrazoloquinolinone
BS (BZD-; CGS-BS)	Binding site (Benzodiazepine-; CGS-binding site
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
IC50	Median inhibition concentration
pIC50	The negative logarithm of the IC50 value
Ki	Receptor affinity
pKi	The negative logarithm of the Ki value
CBM analysis	Cluster binding mode analysis
5-HT1A agonist	5-Hydroxytryptophan 1A agonist
PLS	Partial Least Squares
NMR	Nuclear magnetic resonance
GluCl	Glutamate gated chloride ion channel
BP model	Binding protein model
A Ala	Alanine
R Arg	Arginine
D Asp	Aspartic acid
E Glu	Glutamic acid
Q Gln	Glutamine
G Gly	Glycine
H His	Histidine
K Lys	Lysine
M Met	Methionine

F Phe	Phenylalanine
P Pro	Proline
S Ser	Serine
T Thr	Threonine
Y Tyr	Tyrosine
Ach-BP	Acetylcholine binding protein
GOLD	**G**enetic **O**ptimisation for **L**igand **D**ocking (software)
MOE	**M**olecular **O**perating **E**nvironment (software)
RMSD	Root-mean-square deviation
ATP	Adenosine triphosphate
cpd/ cpd no	Compound/ Compound number
MOE SVL function	MOE Scientific Vector language function
ref.	Reference
ill.	Illustration
vs.	Versus
r^2	Coefficient of determination
q^2	Predicted variance
r^2 stderr/ q^2 stderr	Standard error of estimate of r^2/ q^2
BM	Binding mode
Pose-ID	Pose identity

5. CURRICULUM VITAE

PERSÖNLICHE DATEN

Name	Lydia Schlener
Staatsbürgerschaft	Britisch
Religion	evangelisch A.B.
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Geburtsdatum	29. September 1987

AUSBILDUNG

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2002 - 2007	HBLA für Tourismus und Wirtschaftliche Berufe; Bergheidengasse 5-19; 1130 Wien
2006	Reife-, Diplomprüfung zum diplomierten Koch und Kellner (ausgezeichneter Erfolg)
06.2007	Matura (ausgezeichneter Erfolg)
10.2007	Studium der Pharmazie an der Universität Wien
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03.2014	Diplomarbeit am Department pharmazeutische Chemie

BISHERIGE ARBEITSERFAHRUNG

Datum	07.07. – 10.08. 2004 01.08. – 31.08. 2007
Arbeitsplatz	Österreichische Blindenwohlfahrt; Baumgartenstr. 69; 1140 Wien

Datum	08.06. – 30.09. 2005

	03.07. – 20.08 2006
Arbeitsplatz	Bristol Marriott Royal Hotel, College Green, Bristol; BS1 5TA; UK

Datum	01. – 30. 09.2010 - ***Ameisapotheke KG*** ; 1140 Wien
Arbeitsplatz	08. –12. 2011 - ***Living Concept –The Pharmacy***; 1010 Wien
	01. – 28.02. 2013 ***- Alpha-Apotheke***; 1220 Wien
	05.08 – 05.09. 2013 - ***Apotheke der Kaiserstrasse***; 1070 Wien

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Datum	01. – 31.07. 2014
	ab 10. 2014 geringfügige Anstellung
Arbeitsplatz	***Bayer Austria GesmbH***; Medical Affairs; 1160 Wien
Aufgabenbereich	Administrations-, Recherchen-Arbeit, Mitwirken ähnl. Anfragen

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Datum	SS 14 & WS 14/15
Arbeitsplatz	Universität Wien – Tutor am Departement f. Pharm.Chemie

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Datum	08. - 10.09. 2014
	Symposium SFB 35
Ort	Bernhard Gottlieb University Clinic of Dentistry; 1090 Wien
Abstracts & Poster	*“Docking of Pyrazoloquinolinones into the Benzodiazepine Binding Site of the GABAA Ion Gated Chloride Channel and the analysis of their Binding conformation”*