Bachelor's thesis

NTNU
Norwegian University of Science and Technology

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# Abstract

Protein kinases are involved in many of the fundamental reactions in cells, and further implicated in several diseases. Hence, they have become drug targets with major attention during the last decades, where the goal is to regulate abnormal cellular activity. One of these kinases are Colony-Stimulating Factor-1 Receptor (CSF1R), where an abnormal CSF1R signaling is linked to many diseases, one of which is tenosynovial giant cell tumor (TGCT). By inhibiting CSF1R in patients suffering from complicated cases of TGCT, studies have shown that the size of the tumors decreases. This thesis has investigated and compared, based on published literature, two CSF1R inhibitors, PLX3397 and DCC-3014, both of which are linked to TGCT. PLX3397 is an approved drug by the FDA, whereas DCC-3014 is a promising drug currently undergoing clinical trials. Based on presumed interactions and currently available data regarding pharmaceutical properties, it seems like DCC-3014 is better suited to be used as a treatment for TGCT not amenable to surgery. However, it is to early to make a conclusion since data from the phase III clinical trial (NCT05059262) is not yet published nor finished.

# Sammendrag

Proteinkinaser er involvert i flere av de grunnleggende reaksjonene i celler, og er videre sett i sammenheng med flere sykdommer. De siste tiårene har de derfor fått mye oppmerksomhet i legemiddelutviklingen, hvor målet er å regulere unormal cellulær aktivitet. En av disse kinasene er kolonistimulerende faktor 1-reseptor (CSF1R), hvor unormal CSF1R signalisering er knyttet til mange sykdommer, hvorav en er tenosynovial kjempecelletumor (TGCT). Ved å inhibere CSF1R hos pasienter som lider av kompliserte tilfeller av TGCT, har studier vist at størrelsen på svulstene reduseres. Denne oppgaven har undersøkt og sammenlignet, basert på publisert litteratur, to CSF1R-inhibitorer, PLX3397 og DCC-3014, som begge er knyttet til TGCT. PLX3397 er et godkjent legemiddel av FDA, mens DCC-3014 er et lovende legemiddel som foreløpig undersøkes i kliniske studier. Basert på antatte interaksjoner og tilgjengelige data tilhørende farmasøytiske egenskaper, ser det ut til at DCC-3014 er bedre egnet til å brukes som behandling for TGCT som ikke egnet for kirurgi. Det er dog for tidlig å konkludere, da data fra den kliniske studien som er i fase III (NCT05059262) ikke enda er publisert eller fullført.

# Contents

# Abbreviations

List of all abbreviations in alphabetical order:

- **ΔG** Binding Free Energy, Gibbs Free Energy
- **ADME** Absorption, distribution, metabolism and excretion assay
- **ATP** Adenosine Triphosphate
- $\mathbf{C_{max}}$ Maximum observed concentration
- **CSD** Cambridge Structural Database
- **CSF1** Colony-Stimulating Factor-1
- **CSF1R** Colony-Stimulating Factor-1 Receptor
- **C-terminal** Carboxyterminal tail
- **DCC-3014** Vimseltinib
- **DFG** Amino acid sequence of Asp-Phe-Gly
- **FDA** Food and Drug Administration
- $\mathbf{IC_{50}}$ Half-maximal inhibitory concentration
- **IL-34** Interleukin-34
- **JMD** Juxtamembrane domain
- **KD1** Kinase domain 1
- **KD2** Kinase domain 2
- **PDB** Protein Data Bank
- **PLIP** Protein-Ligand Interaction Profiler
- **PLX3397** Pexidartinib, TURALIO$^{TM}$
- **RTK** III Class III receptor tyrosine kinase
- **TGCT** Tenosynovial giant cell tumor
- $\mathbf{T_{max}}$ Time of maximum observed concentration

# 1 | Introduction

The human genome is coding for about 518 protein kinases, where their task is to catalyze chemical reactions that involves transferring a $\gamma$-phosphate from adenosine triphosphate (ATP) to its protein substrates.[1–3] Colony-Stimulating Factor-1 Receptor (CSFIR) is one of these kinases, and belong to the class III receptor tyrosine kinase (RTK III). CSF1R is normally expressed in myeloid lineage cells, which depend on CSF1R signaling for survival, differentiation, and proliferation.[4–6] Like most of the enzymes in RTK III, CSF1R is a transmembrane protein, thus it consists of an extracellular and intracellular domain which are connected through a single transmembrane segment.[4,5] Colony-Stimulating Factor-1 (CSF1) and Interleukin-34 (IL-34) are the ligands that binds the extracellular domain of CSF1R and induces dimerization.[7] The intracellular domain consist of a juxtamembrane domain (JMD), a kinase domain divided into two parts (KD1 and KD2) by a hydrophilic insert sequence, and a carboxyterminal tail (C-terminal).[4,7] Whereas KD1 anchor the binding of ATP, KD2 mediates the substrate binding and is mainly responsible for its catalytic activity.[4] When the dimerization occurs, the kinase domain of CSF1R is phosphorylated, and transfer it into its activated form.[8] This leads to multiple downstream reactions inside the cell that are crucial for normal cell function. Overexpression of CSF1 or elevated activity of CSF1R leads to abnormal CSF1R signaling, that is observed in several diseases.[4,6,8] Hence, research regarding development of novel small-molecule CSF1R inhibitors and CSF1-antibodies, and expanding their potential indications, has garnered a lot of attention the last decade.[4] The structure of CSF1R with ligands and the following downstream reactions after activation, as well as some of the inhibitors, are illustrated in Figure 1.0.1.

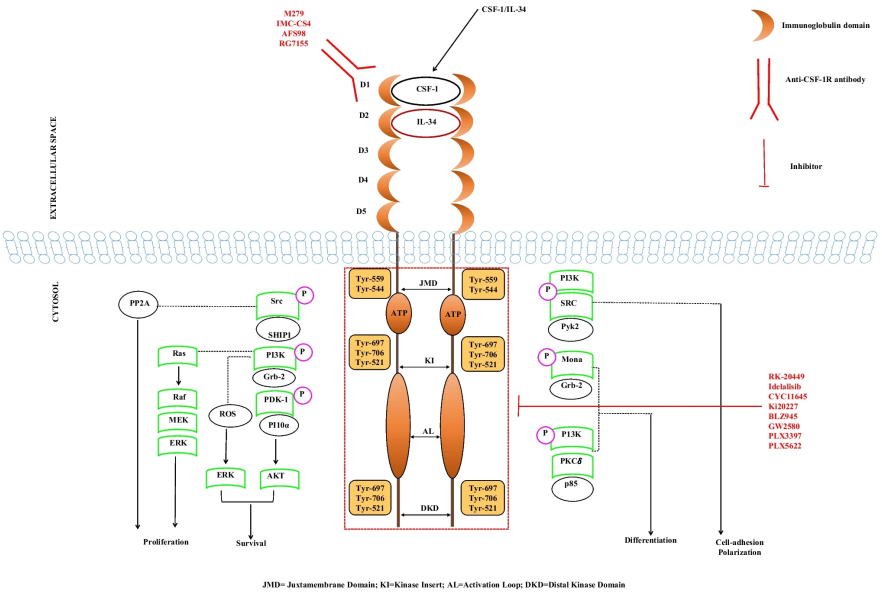



**Figure 1.0.1:** Illustration of CSF1R, the cascade effects in the cells after activation and its inhibitors. Reprinted from Yadav *et al.* with permission.[5]

The first small-molecule inhibitor of CSF1R approved by the Food and Drug Administration (FDA) in 2019 was Plexidartinib (PLX3397, TURALIO$^{TM}$).[4,9–11]It was approved for treatment of tenosynovial giant cell tumor (TGCT), that is a rare benign tumor that appears in different tissue in joints.[4,6,9,12] TGCT is caused by a genetic translocation that results in an overexpression of CSF1.[6] This disease often leads to poor quality of life, since patients with TGCT often experience swelling and pain in the joints with a limited range of motion.[9,12] The main treatment for TGCT is surgical excision, often multiple surgeries are required which leads to a reduced quality of life and increased morbidity.[9,12] In rare cases the tumor can be located in such a way that surgical removal is not an option. Historically, the only treatment options for these patients were joint replacement or amputation.[9] Hence it has been investigated to find treatment options for TGCT, especially CSF1R inhibitor that compensates the overexpression of CSF1. Although PLX3397 is a selective CSF1R inhibitor, it also inhibits other closely related kinases.[9,11] Furthermore, PLX3397 also have an increased risk for liver toxicity,[4,6,11] which necessitates development of new CSF1R inhibitors.

Today there are several small-molecule CSF1R inhibitors in clinical trials for potential treatments and immunotherapies.[4,6,11] One of these are Vimseltinib (DCC-3014), that currently are in a phase III trial as a treatment for cases of TGCT where the tumor is unresectable (NCT05059262).[4,6] This thesis will examine DCC-3014 and PLX3397 based on the published literature with respect to the following research question: "How does the structural differences in PLX3397 and DCC-3014 affect binding to CSF1R and their properties as drugs?".

# 2 | Theory

## 2.1 Structure of Binding Site at Protein Kinases

Kinases, including CSF1R, are dynamic proteins, thus the conformation and 3D structure can change slightly. Indeed, it seems like most kinases have a conformational heterogeneity, ranging between an active and an inactive conformation. This is justified by the crystallographic structures of several kinases obtained from the Protein Data Bank (PDB).[2] The amino acid sequence Asp-Phe-Gly (DFG) motif is a part the flexible activation loop, found in the C-terminal, and have an important regulatory role during catalysis and substrate binding.[2,4,11,13] This DFG motif exist in two conformations, called DFG-in and DFG-out, that depends on the conformation of the kinase.[2,13] When the kinase is in the activated state, the aspartic acid of DFG will point towards the ATP binding site, whereas phenylalanine points outwards. Hence the DFG motif is in its DFG-in conformation.[2,7,13] Further the aspartic acid will also coordinate two $Mg^{2+}$ ions, which are highly important to the catalytic effect. However, when the kinase is in the inactivated conformation this causes the DFG motif to flip around 180° relative to the activated state, hence the aspartic acid and phenylalanine switch places.[2,7,11,13] This causes the aspartic acid to be around 5 Å away from the ATP binding site, thus leading the kinase into an catalytically inactive state, called DFG-out.[2,7,13] Most importantly the DFG-out conformation makes a new allosteric and hydrophobic pocket adjacent to the ATP binding site accessible, whilst the phenylalanine is blocking the ATP binding site.[2,11] Specifically for CSF1R, this is initiated when Trp550, found inside the hydrophobic pocket, interacts by forming a hydrogen bond with the side chain of Asp796, one of the residues in the DFG motif.[7,13] This pocket is a popular target for drugs designed to inhibit kinases, and further stabilize the DFG-out conformation and an inactivated state of the kinase. Furthermore, CSF1R also have a conserved salt bridge between Glu633 and Lys616, that is as an essential part for catalytic activity, which also are a target for drugs.[11,13] Additionally, CSF1R has the combination of Met637 and Gly795, which are only found in 10 other human kinases. If a drug interacts with these residues, it is presumed that it acquires a significant selectivity towards CSF1R.[11] Azhar *et al.* [13] proposed, based on the findings in the in silico study, that Met637 and Leu588 are specificity markers for CSF1R, and are often targeted by inhibitors.[13]

## 2.2 PLX3397 – Pexidartinib

The small-molecule CSF1R inhibitor developed by Plexxikon, pexidartinib (PLX3397, TURALIO$^{TM}$), is the first treatment for TGCT approved by the Food and Drug Administration (FDA) in US.[4,9,14] PLX3397 has the following IUPAC name 5-((5-chloro-1*H*-pyrrolo[2,3-*b*]pyridin-3-yl)methyl)-*N*-((6-(trifluoromethyl)pyridin-3-yl)methyl)pyridin-2-amine, and its chemical structure is shown in Figure 2.2.1.

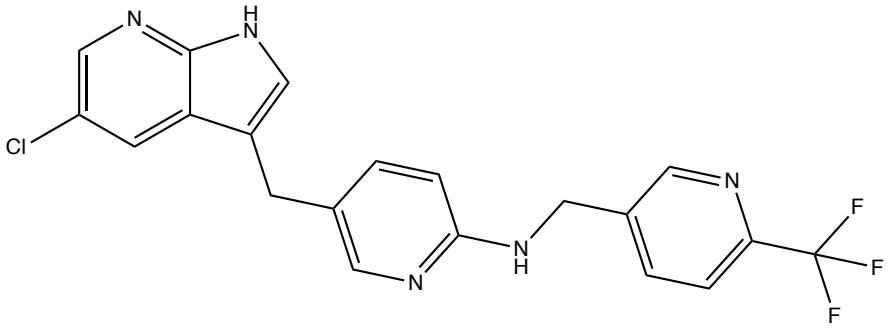



**Figure 2.2.1:** The chemical structure of PLX3397.

PLX3397 is orally administered, and is designed to interact with residues of JMD when CSF1R is in an autoinhibited conformation. Thus PLX3397 aims to further stabilize CSF1R in the inactive state.[4,15] Figure 2.2.2 shows the surface of the crystallographic model of the kinase domain of CSF1R with PLX3397 bound inside the hydrophobic pocket. Even though PLX3397 is a selective kinase inhibitor, it also potently inhibits other kinases in the RTK III family.[4] Thus the suppression of CSF1R may be a bit limited by the off-target activity.[11] Unfortunately, PLX3397 have a high risk of serious hepatotoxicity, which are the main reason PLX3397 are not been approved by European Medicines Agency.[4] It has also been in several clinical trials the last years, for monotherapy and with combination of other drugs for different diseases, like melanoma and lung cancer (Trials: NCT02452424, 01499043, 01349036, 01826448, 01090570).[4] However, corporate decisions and insufficient results are the reasons why some of these trials have been withdrawn or terminated.[4]

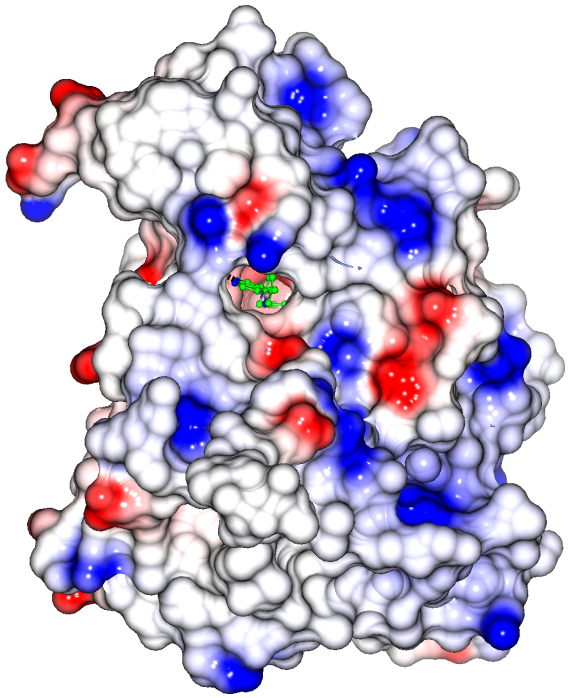


**Figure 2.2.2:** Surface of the kinase domain of CSF1R with PLX3397 bound inside.

## 2.3 DCC-3014 – Vimseltinib

Vimseltinib (DCC-3014) is an orally active selective CSF1R inhibitor[6,13] produced by Deciphera Pharmaceuticals.[4] DCC-3014 have the chemical structure shown in Figure 2.3.1, with the following IUPAC name 3-methyl-5-[6-methyl-5-[2-(1-methylpyrazol-4-yl)pyridin-4-yl]oxypyridin-2-yl]-2-(propan-2-ylamino)pyrimidin-4-one.

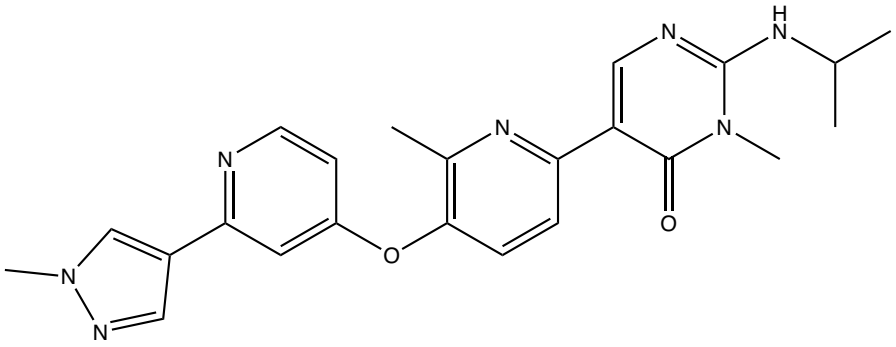



**Figure 2.3.1:** The chemical structure of DCC-3014.

Caldwell *et al.* [6] developed DCC-3014 by using structure-based drug design and traditional medicinal chemistry approaches for Deciphera Pharmaceuticals, where PLX3397 was used as the reference drug. It is, like PLX3397, designed to target JMD in the inactive conformation and stabilizing the DFG motif in its DFG-out conformation. In Figure 2.3.2 the surface of the crystallographic structure of kinase domain of CSF1R with DCC-3014 bound inside the hydrophobic pocket are shown. Currently DCC-3014 is in several clinical trials for TGCT, advanced tumors and sarcomas, both as monotherapy and in combination with avelumab (Trials: NCT0306946469, 04242238, 05059262).[4] Early disclosed data from the trials shows that DCC-3014 is a well-tolerated oral drug that has promising pharmaceutical properties, in addition to good results from assays regarding selectivity and binding.[16]

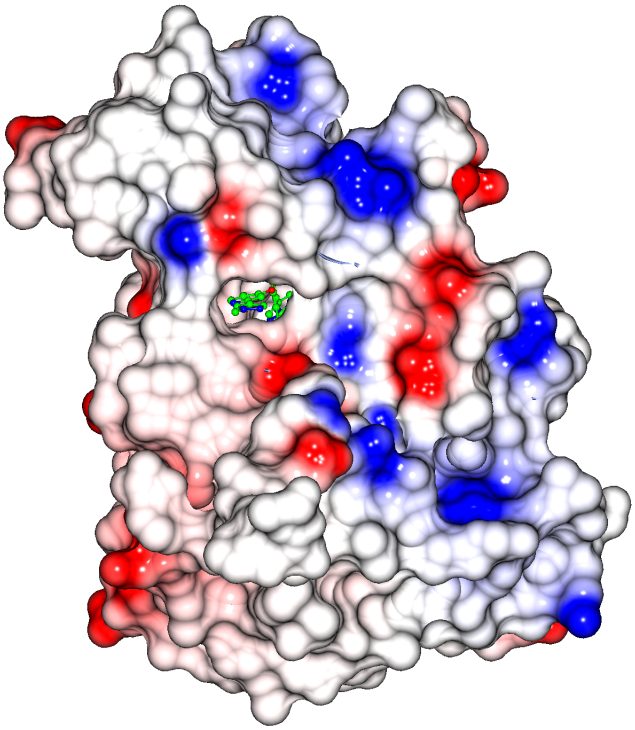


**Figure 2.3.2:** Surface of the kinase domain of CSF1R with DCC-3014 bound inside.

## 2.4 Molecular Interactions in Protein-Ligand Complexes

Drugs made today are mostly antagonists, beside drugs against infections and corrections of deficiencies, where its purpose is to obstruct the agonist to bind the binding site.[17] This way the drug could moderate or abolish an over-response related to the disease. Therefore, it is important to understand how molecules interact and are recognized in biological systems to develop and optimize these drugs. Usually, biological systems depend on specific attractive interactions between two partner molecules for recognition.[18] In structure-based drug design, these partner molecules typically are proteins and ligands. Here, the goal is to identify and optimize such interactions between these molecules. Crystallographic structures with associated affinity data, for example collected from PDB or CSD, can provide information about the interaction geometries and approximate affinity contributions, that could be useful in the optimization process.[18] Below are some of the most important considerations and interactions in protein-ligand complexes of CSF1R with PLX3397 and DCC-3014.

### 2.4.1 Entropic and Enthalpic Consideration of Binding

In a protein-ligand complex there would be several attractive interactions between the molecules, as mentioned above. These interactions are mostly characterised as noncovalent, hence they must be associated with a negative binding free energy ($\Delta G$) to occur.[18] $\Delta G$ is defined as the sum of an enthalpic term ($\Delta H$) and an entropic term ($T - \Delta S$), which both are highly dependent on several environmental properties.[18] Two important concepts regarding $\Delta G$, are the flexibility and desolvation effect.

Protein-ligand complexes are flexible entities, where binding can have an impact on the entropy and enthalpy of the system. A phenomenon often linked to this effect is the entropy-enthalpy

compensation, where a favorable binding enthalpy often is compensated with an unfavorable system entropy.[18] When a ligand has a tightly and strongly directed interaction with the protein the complex becomes more rigid, which leads to a less favorable entropy.[18] On the other hand, tightening caused by additional interactions often lead to shorter interaction distances, which are more enthalpically favorable.[18] The amount of entropy and enthalpy compensation will vary in different systems, where more flexible domains may prefer a flexible ligand than highly ordered ones.[18] Furthermore, some studies have shown that the optimal binding in protein-ligand complex occurs when the ligand occupies around 55% of the volume in the binding site.[18] In literature this is referred to as the "55% filling rule", and further implies the importance of a flexible ligand.[18,19]

Proteins, ligands, and other organic molecules will be surrounded by a solvation shell of structured water to solvate them in the bulk water phase.[18,20,21] Thus, in order to establish new interactions between the protein and ligand, the solvation shell of both molecules needs to be partially removed and rearranged.[19,21] This phenomenon is known as the desolvation effect. The formation of a protein-ligand complex creates a new and smaller surface area that is covered by a solvation shell, compared with the two molecules not interacting. In other words, the hydrophobic area is minimized, and less water is needed to form the solvation shell, which is called the hydrophobic effect. Thus the amount of free water is maximized, which often is entropic favorable to the system.[18,20–22] However, this energetic effect depends on the system and which residues that approaches the solvation shell.[18,21,22] Hence, the regions with most gain of binding energy could be found by probing the hydration state of the unbound protein binding site, and should be used as a standard element in structure-based drug design.[18]

### 2.4.2 Hydrogen Bond

Hydrogen bonds play an important role in biological systems, and are the most important specific interaction in the recognition process.[17–19] To form a hydrogen bond between two atoms, it must be an proton donor and acceptor with the correct orientation and distance.[17,18] The most common proton donors are OH- and NH-groups, where OH is a better donor than NH. This is partial due to the elevated electronegativity of oxygen, compared to nitrogen, which makes the hydrogen more acidic. Conversely, the most common proton acceptors are nitrogen, oxygen and sulfur, where the strength depends highly on which groups nitrogen, oxygen, and sulfur are bound to, as well as environmental factors, like polarity and pH.[17,18] However in protein-ligand complexes the most common donor and acceptors are the NH-group and carbonyl group found in the peptide backbone of the protein, respectively. In Figure 2.4.1 a simplified illustration on a hydrogen bond is shown. In some cases CH, often bound to nitrogen or oxygen in aromatic heterocycles, can also act as a proton donor.[17,18] This interactions are often referred to as weak hydrogen bonds, since they are considerable weaker than OH and NH proton donors.

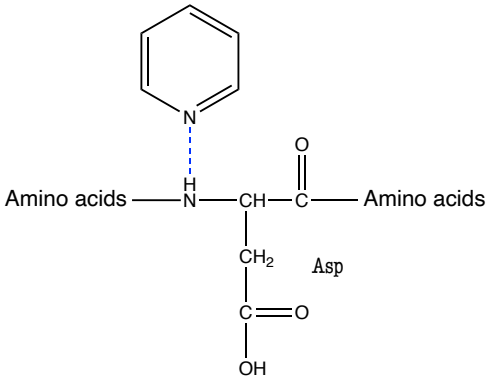



**Figure 2.4.1:** Simplified illustration of a hydrogen bond, dashed bond in blue, where NH in the backbone of Asp is the proton donor and the acceptor is nitrogen of the pyridine ring.

Furthermore, the geometries of hydrogen bonds also follow strict rules.[18] The length of a hydrogen bond is approximately 3 Å long, but this depends on the donors and acceptors. Bissantz *et al.*[18] found that OH as donor usually give shorter hydrogen bonds, this is based on data collected from both Cambridge Structural Database (CSD) and Protein Data Bank (PDB).[18] Furthermore, hydrogen bonds also have distinct angular preferences.[18] Most hydrogen bonds are formed along the direction of the lone pair of the proton acceptor. However, sulfonyl groups does not follow that rule, and tend to prefer to form hydrogen bonds along the S=O axis.[18] Even though hydrogen bonds convey specificity to the recognition process, it does not always contribute with negative $\Delta G$.[18,23] Hence, during a structure-based drug design it is important to identify which proton donors and acceptors which need to be satisfied, rather than how many hydrogen bonds that can be formed.[18]

### 2.4.3 Hydrophobic Interaction

The size of the hydrophobic surface in the ligand binding site highly correlate with the binding affinity, and are considered as one of the best structural parameters for this estimation.[18] Drug molecules consist mainly of hydrophobic atoms, hence hydrophobic interactions are a major source in protein-ligand complexes.[19] According to Teague and Davis [19] hydrophobic interactions contribute with a minimum of 3.2-fold increase in binding per methyl group.[19] Furthermore, a proposed method for increasing the affinity and selectivity for receptors, can be by exploitation of specific hydrophobic interactions. However, hydrophobic interactions, despite their importance in binding, are nonspecific.[19]

### 2.4.4 $\pi$-$\pi$ Interaction

Noncovalent interactions in aromatic rings have an important role in protein-ligand recognition, which also is used in structure-based drug design.[24,25] These interactions can be divided into three groups called $\pi$-$\pi$, cation-$\pi$ or anion-$\pi$ interactions, but this thesis will only consider $\pi$-$\pi$ interactions. In proteins, such interactions are ubiquitous and mainly formed with amino acids containing an arylic side group, like Phe, Tyr, Trp or His.[18] When these residues are found near the binding site, they also tend to expose the aromatic side chain out in the binding site, which make them accessible to form $\pi$-$\pi$ interaction with the ligand.[18] This is confirmed by the vast majority of the crystallographic structures of protein-ligand complexes, which reveals bonding interaction involving the receptors aromatic side chain and ligands either aromatic

or heteroaromatic rings.[24] Due to the shape and electronic properties of aromatic rings, $\pi$-$\pi$ interactions have two preferred geometries: T-shaped edge-to-face and parallel-displaced stacking.[18,24,25] These structures are shown in Figure 2.4.2. In the parallel-displaced orientation the interplanar distance is approximately 3.5 Å, with a displacement of the rings about 1.6-1.8 Å.[24] Whereas, the edge-to-face arrangement, with the hydrogen atom pointing perpendicular in the center of the other ring, have a distance close to 5 Å between the ring centers.[24] However, the parallel-displaced geometry is found more frequently in proteins, but this depends highly on the $\pi$-system.[18,24,25] Studies have found that the strongest binding occur with one electron rich and one electron poor aromatic unit.[24]

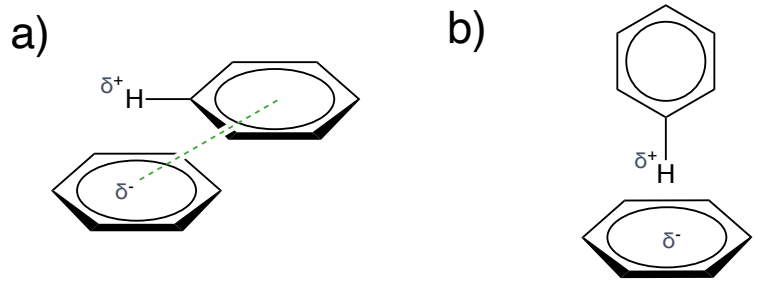



**Figure 2.4.2:** Illustration of the geometries of $\pi$-$\pi$ interactions, a) parallel-displaced and b) T-shaped edge-to-face, of two bezene rings. Adapted from Meyer *et al.* [24].

### 2.4.5 Van der Waals Force

Van der Waals is a weak interaction between two uncharged molecules, which is due to transient electric dipoles that weakly attract each other.[20] When the dipoles are close in space, repulsion forces also occur. The point where the attraction is maximized is called the van der Waals contact, and this depends on the van der Waals radii to the given atoms.[20] These interactions are ubiquitous but subtle in protein-ligand complexes, where the interaction is non directional and not frequently associated in gain of specificity.[18]

# 3 | Discussion

## 3.1 Binding to CSF1R

In this thesis, data regarding interactions in or near the binding site to CSF1R has been collected from several studies.[4,6,11,13] Further the crystallographic structure to DCC-3014 and PLX3397 within CSF1R from the X-ray structures 7MFC and 4R7H respectively, have been analyzed using the programs Protein-Ligand Interaction Profiler (PLIP)[26] and CCP4MG. The presumed most important amino acids, in CSF1R and their roles, are summarized in Table 3.1.1. The table also provides information about possible interactions between the given residues and DCC-3014 or PLX3397.

**Table 3.1.1:** Important residues, and their associated functions, of CSF1R, with information about if they interact with DCC-3014 or PLX3397. This table is adapted, with modification, from Azhar *et al.* [13].[13]

| Interacting amino acid residues at binding site | Kinase functions | Interacts with | |
|---|---|---|---|
| | | DCC-3014 | PLX3397 |
| Cys666 | ATP binding site and hinge region residue | Yes[6,11,13,26] | Yes[4,26] |
| Tyr 546, Trp 550 | Regulates JMD to maintain an autoinhibitory state | No, Yes[13] | No, Yes[15,26] |
| Asp 796, Phe797 | Parts of the DFG motif, regulates the activation loop to maintain an autoinhibitory conformation | Yes,[6,11,26] Yes[6,11,26] | Yes,[4,26] Yes[4,26] |
| Gly795 | Prior to DFG motif, creates a glycine selectivity pocket | Yes[6,11,13] | Yes[4] |
| Met637, Leu588 | Residues of CSF1R specificity | Yes,[6,11,13] Yes[26] | No, Yes[4] |
| Lys616, Glu633 | Forms a salt bridge, that regulates activation | Yes,[6,11,26] No | Yes,[4] Yes[26] |
| Leu640, Ile646, Leu769, Leu785, Val596, Cys774, Ile794, Ala614 | Forms a hydrophobic pocket | Ala614,[26] Leu640,[26] Leu785[26] | Leu785,[26] Val596[26] |

In Figure 3.1.2 & 3.1.1, the interactions formed in the protein-ligand complexes by the kinase domain of CSF1R with DCC-3014 and PLX3397 are illustrated in 2D, respectively. By using the program CCP4MG, models of these complexes, collected form PDB, are shown 3D in Figure 3.1.3. Based on the hydrophobic pocket illustrated in Figure 3.1.3, it seems like both inhibitors approximately are fulfilling the 55% filling rule.

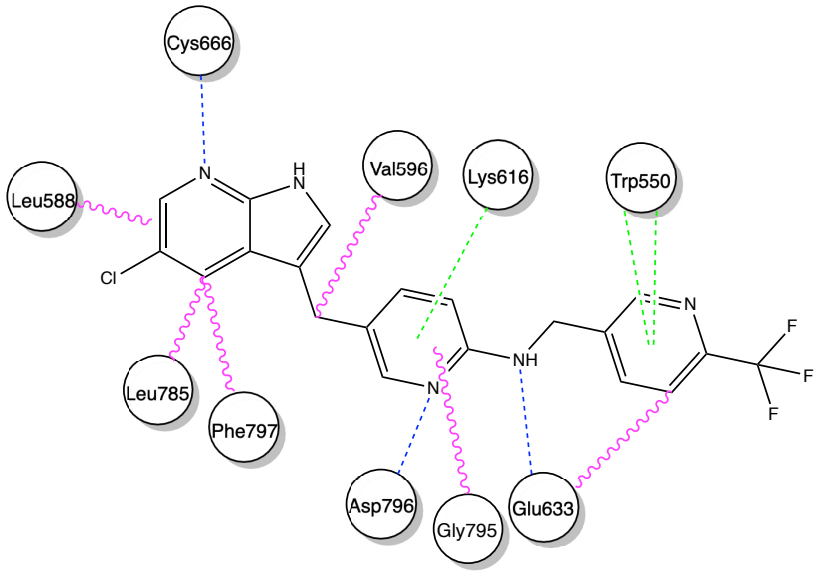



**Figure 3.1.1:** Simplified illustration of the interactions between CSF1R and PLX3397 in 2D. The pink wavy bonds represent hydrophobic interactions, blue dashed bonds symbolize hydrogen bonds, and green dashed bonds are $\pi$-interactions.

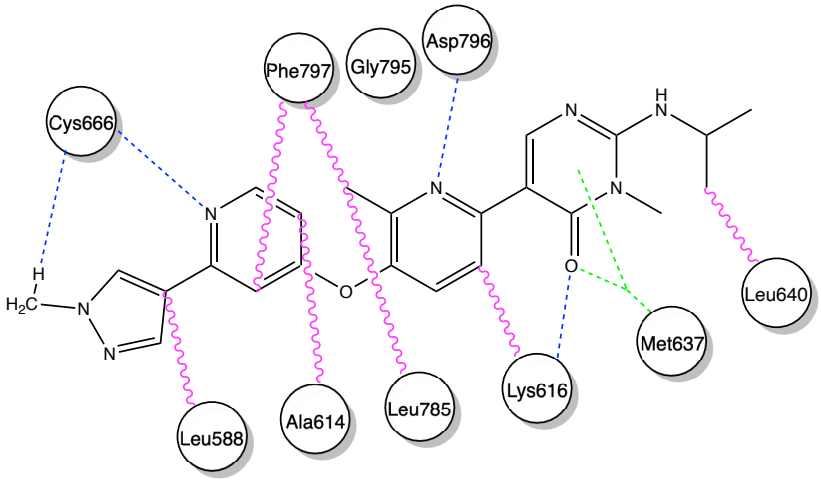
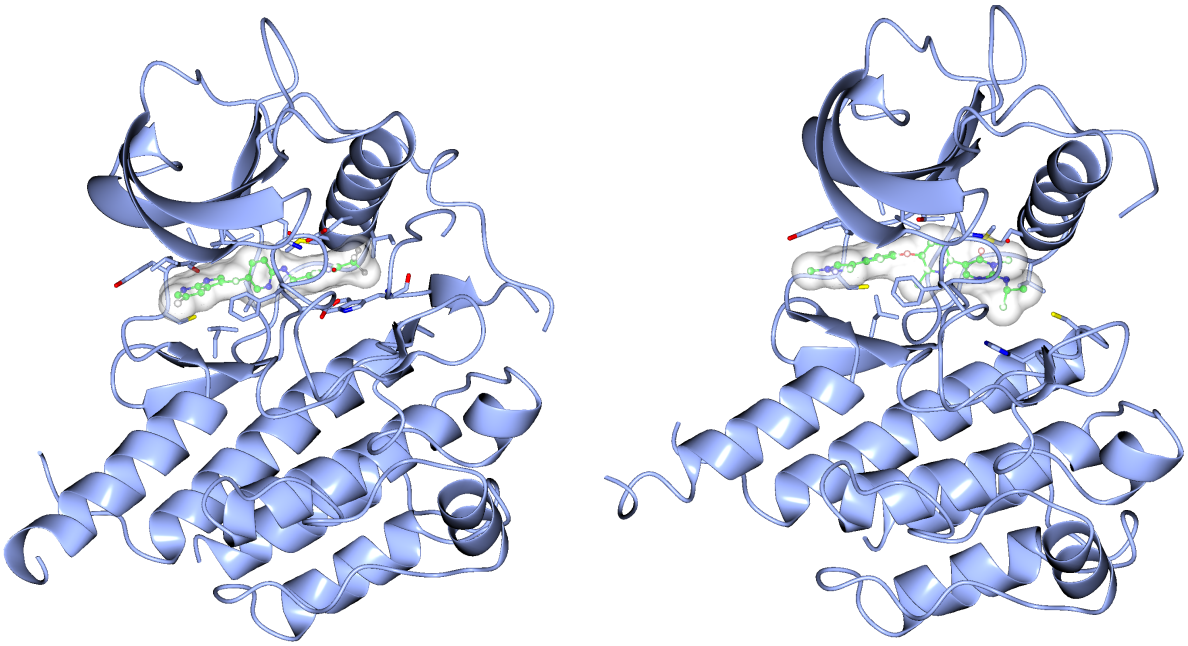


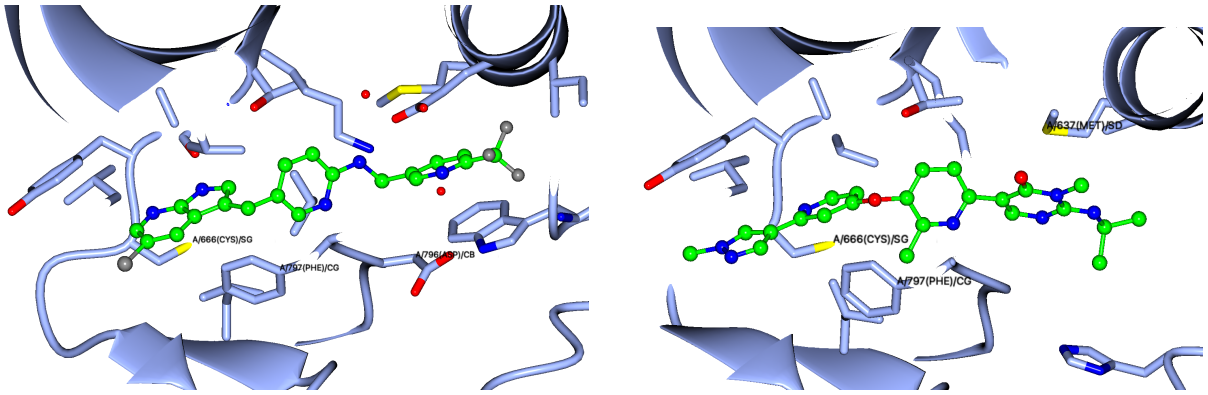

**Figure 3.1.2:** Simplified illustration of the interactions between CSF1R and DCC-3014 in 2D. The pink wavy bonds represent hydrophobic interactions, blue dashed bonds symbolize hydrogen bonds, and green dashed bonds are $\pi$-interactions.



**(a)** **(b)**

**Figure 3.1.3:** Crystallographic structure of the kinase domain of CSF1R with the inhibitors, a) PLX3397 and b) DCC-3014, inside the hydrophobic pocket. The structures are collected form PDB, 4R7H and 7MFC.

It can be assumed that differences in how deeply inhibitors is bound to the hydrophobic pocket has an affect on interactions and $\Delta G$ associated with the protein-ligand complex. Therefore, it is desirable to estimate how deeply different inhibitors bind during comparison. Since Cys666 is close to the opening of the binding pocket, and presumable forms hydrogen bond with both PLX3397 and DCC-3014, it can be used as a parameter to presume how deep the two ligands bind. According to the prediction made of PLIP, the proton acceptor in PLX3397 is assumed to be the nitrogen at the pyridine of the pyrrolopyridine ring, this forms a bond distance at approximately 2,8 Å.[26] On the other hand, DCC-3014 is assumed to form a hydrogen bond to Cys666 with the nitrogen in the disubstituted pyridine ring, as the proton acceptor, with a distance of 3,2 Å.[26] Thus, the pyrazole ring of DCC-3014, which are larger than half of the chlorine substituted pyridine ring at PLX3397, is pointing outwards of the binding pocket. However, Caldwell *et al.* [6] and Smith *et al.* [11] predict that DCC-3014 also forms a hydrogen bond to the carbonyl group in the backbone to Cys666 with the methyl group to the pyrazole ring, in addition to the hydrogen bond described above.[6,11] This weak interaction suggests that DCC-3014 is not necessarily bound as far out of the binding site, as first assumed. Despite the weak hydrogen bond formed between DCC-3014 and Cys666, Figure 3.1.4 shows that PLX3397 binds CSF1R with slightly more of its structure inside the binding pocket, than DCC-3014 does.[26]




**(a)** **(b)**

**Figure 3.1.4:** Inhibitors, a) PLX3397 and b) DCC-3014, inside the hydrophobic pocket of CSF1R. Amino acid residues Cys666, Phe797 and Asp796 (only a)) are marked.

The crystallographic data also provide information about the direction these two ligands interacts within the binding site. PLX3397 will have the trifluoromethyl group pointing inwards in the protein, with the pyrrolopyridine near the opening of the binding site. Whereas DCC-3014 binds with the isopropylamine group at the pyrimidinone ring inwards the protein, and the pyrazole ring towards the opening of the binding site.[26] They both have quite similar shape in 2D, they are both long without many substituents branching out of the scaffold. Since DCC-3014 is developed by structure-based drug design with PLX3397 as the reference drug, this could explain the similarities in 2D.[6] However, in 3D DCC-3014 appear to prefer a more bent shape and have some more substituents than PLX3397, as shown in Figure 3.1.4. The methyl group at the central pyridine is believed to occupy the space near Gly795, the residue preceding the DFG motif. Other related kinases, like KIT, FLT3, PDGFRA, PDGFRB, have a bulkier cystein group prior to the DFG motif, where CSF1R creates a "glysine selectivity pocket".[4,11] Hence, by occupying this space DCC-3014 contributes with specificity towards CSF1R, since the methyl group would be sterically hindered by cysteine of the other kinases.[11] Based on the data published by Wen *et al.* [4], it can be assumed that PLX3397 also interacts with Gly795, by van der Waals interaction.[4] However, they have not indicated which part of PLX3397 that interacts with Gly, nor if some part of its structure occupies and take advantage of the pocket close to Gly795.

Other CSF1R specific residues are Met637 and Leu588. PLIP presumes that DCC-3014 and PLX3397 forms hydrophobic interaction with Leu588. Smith *et al.* [11] claims that DCC-3014 interacts with the sulfur atom in Met637 by an orbital overlap to the pyrimidione ring.[11] It is not clear if sulfur interacts with the carbonyl group or the $\pi$-system of the pyrimidione ring. Thus, it is assumed that DCC-3014 interacts with both Met637 and Gly795 which make DCC-3014 highly selective to CSF1R. This is due to the seldom combination of these residues together in kinases.[11] PLX3397 does not seem to interact with Met637, and this could be one of the factors that causes it to also inhibit other kinases. Additionally, the salt bridge created by Glu633 and Lys616, believed to be of catalytic importance, is targeted by both ligands.[4,6,11,13] By forming a hydrogen bond between the carbonyl group at pyrimidinone ring with Lys616, and with a hydrophobic interaction with the central pyridine ring, DCC-3014 breaks this conserved salt bridge.[4,11] On the other hand, the central pyridine group at PLX3397 forms a $\pi$-interaction with Lys616.[4] In addition, PLX3397 also forms a hydrogen bond from the amine group with Glu633,

where both could be the proton acceptor and donor.[26] By breaking this salt bridge, the inhibitors is assumed to disrupt CSF1Rs catalytic activity.

Further, both PLX3397 and DCC-3014 is designed to stabilize the DFG motif in its inactive DFG-out conformation.[4,6,15] According to PLIP, Smith *et al.* [11], and Wen *et al.* [4], it can be assumed that both PLX3397 and DCC-3014 interact with the DFG motif in the same way. The NH in Asp796 forms hydrogen bond to the nitrogen atom in the central pyridine ring with a distance at approximately 3 Å, which stabilizes it in the DFG-out position.[11,26] In addition, Phe797 forms van der Waals interaction with the methyl group on the central pyridine ring and with the adjacent pyridine in DCC-3014.[11,26] This will stabilize the DFG-out conformation even more. Similarly and with the same effect, the pyrrolopyridine ring in PLX3397 interacts with Phe797 by hydrophobic interaction.[26] Moreover, both ligands interacts with Trp550, which also stabilizes the DFG-out conformation.[13,26] PLIP predicts that the pyridine ring bound to trifluoromethyl group in PLX3397 forms $\pi$-$\pi$ interactions with both rings in Trp550.[15,26] Whereas the interaction with DCC-3014 and Trp550 is not further described.[13] As a result, both PLX3397 and DCC-3014 seems to stabilize CSF1R in the inactive conformation.

A hydrophobic pocket is formed by several amino acid residues, as listed in Table 3.1.1. According to PLIP, DCC-3014 and PLX3397 form hydrophobic interactions with four of these residues, Ala614, Leu640, Leu785 and Val596.[26] Overall, these interactions between CSF1R and both DCC-3014 and PLX3397 are mainly stabilizing the DFG motif in its DFG-out inactive conformation. Azhar *et al.* [13] reported that DCC-3014 had the lowest $\Delta G$, of the seven kinases they studied in silico, with a value of -10.64 kcal/mol.[13] To conclude, both inhibitors break the salt bridge, which is assumed to interrupt the catalytic activity.[4] Further, DCC-3014 interacts with both Met637 and Gly795, which is assumed to give high selectivity for CSF1R compared to other kinases in RTK III. In comparison, PLX3397 does not as clearly interact with these two residues, which could be the main reason for why it also inhibits other related kinases. Nevertheless, PLX3397 seems to potently bind CSF1R by other interactions.

Notwithstanding the benefit of crystallographic structures, mainly used to predict the interactions described above, this modeling also has limitations. Firstly, there is a lack of an integrated crystallographic structure of CSF1R, which includes the extracellular, intracellular and transmembrane segment with its conformations preferences in the cell environment.[4] It can be assumed that the actual kinase domain is slightly different from the crystallographic structure without the extracellular and transmembrane segment. Hence, this can lead to deviation between the results of cellular and enzyme assay. Furthermore, as Bissantz *et al.* [18] stated, protein-ligand complexes are not characterized by a single structure in reality, but rather an ensemble of structures.[18] Where changes in the degrees of freedom during the binding event, for both the protein and the ligand, could have large impact on the systems $\Delta G$. Lastly, it is also important to consider which energetic contribution new interaction give the system, during the development of novel inhibitors.[18,23] As mentioned, more interactions does not always imply a more potent inhibitor, due to a possible entropy penalty.

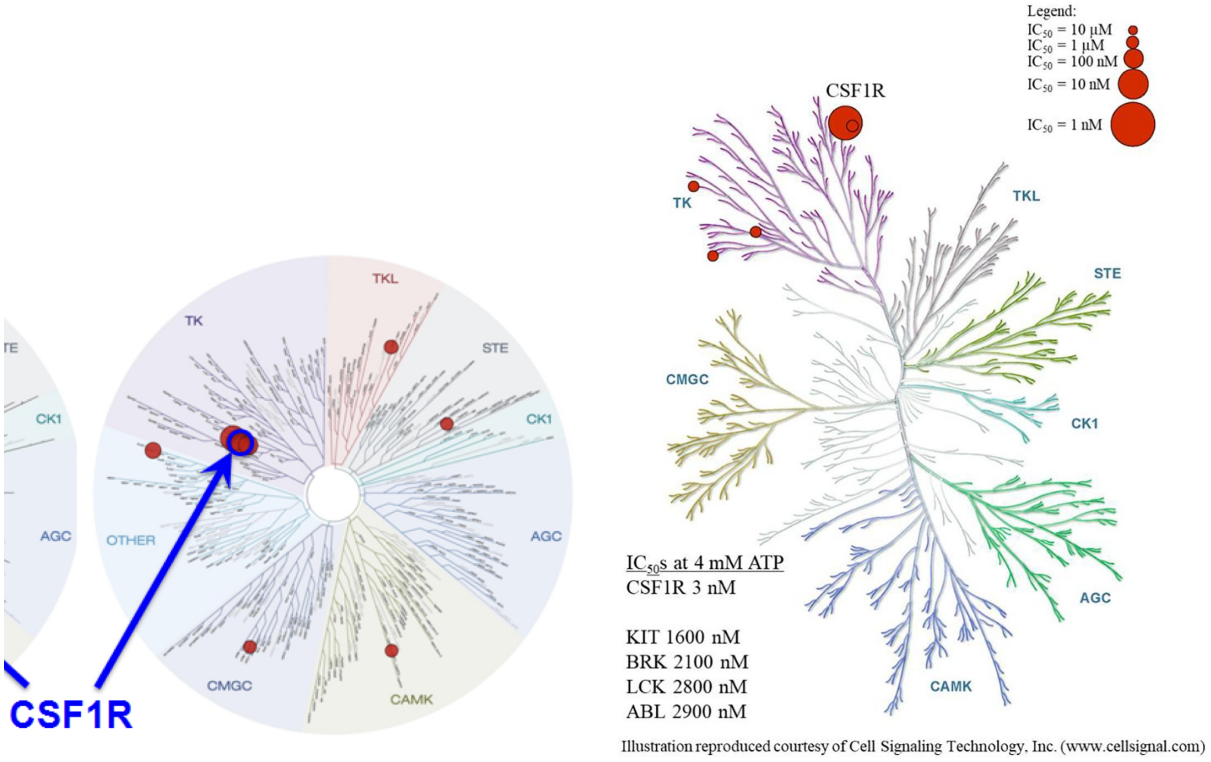
## 3.2 Pharmaceutical properties

PLX3397 and DCC-3014 are both orally distributed CSF1R inhibitors. Hence, they, like other small molecule drugs, are assumed to be absorbed through passive permeability in the small

intestinal.[27] However, the absorption that occurs in the small intestinal only lasts 3-4 hours. Therefore, high permeability is usually desirable since this gives high absorption and rapid onset of the effect.[27] Hence, a low time ($T_{max}$) at which the drug reaches its maximum drug concentration ($C_{max}$) is advantageous. Below, some of the most important assays and properties belonging to DCC-3014 and PLX3397 as drugs are discussed.

### 3.2.1 Kinase and Cellular Assay with Selectivity

Even though both DCC-3014 and PLX3397 are CSF1R selective inhibitors, data from kinase selectivity profiles, Figure 3.2.1, indicates that DCC-3014 is significantly more selective against CSF1R than PLX3397.[4,6,15] PLX3397 also potently inhibits KIT, FLT3, PDGFRA, and PDGFRB.[4,6]




**(a)** **(b)**

**Figure 3.2.1:** a) Kinase selectivity profile of DCC-3014. Reprinted from Caldwell *et al.*. b) Kinase selectivity profile of PLX3397. Reproduced with permission from Tap *et al.* [15], Copyright Massachusetts Medical Society.[15]

Caldewell *et al.* [6] performed a screening of several compounds, including DCC-3014 and PLX3397, for CSF1R, KIT, PDGFRA, PDGFRB, FLT3 kinase activity.[6] The results shows that the half-maximal inhibitory concentration ($IC_{50}$) for DCC-3014 are 3.7 nM, >3300 nM, 436 nM, 2300 nM and 476 nM for CSF1R, FLT3, PDGFRA, PDGFRB, and KIT, respectively. Whereas PLX3397 got 1.7 nM, 7.1 nM, 9.6 nM, 36 nM and 1.6 nM as $IC_{50}$ for CSF1R, FLT3, PDGFRA, PDGFRB, and KIT, respectively. Thus, DCC-3014 is >500-fold selective to CSF1R versus the other RTK III kinases, whereas PLX3397 are more potent than DCC-3014, but also inhibits these other kinases with 5-fold selectivity.[11] Based on the kinase selectivity profiles, Figure 3.2.1, it can be seen that PLX3397 also inhibit kinases form different kinase families, whereas DCC-3014

only inhibits kinases in RTK III. This also correlates with the findings above that DCC-3014 tends to interact more strongly with CSF1R specific residues, than PLX3397.[4,6,11,13,26]

M-NFS-60 is a murine leukemia cell line that is depended upon CSF1 for differentiation and proliferation, and is often used in cellular assay for novel CSF1R inhibitors.[6,11] According to results form Caldwell *et al.* [6] and Smith *et al.* [11], DCC-3014 inhibits M-NFS-60 more potently than PLX3397,[6,11] with $IC_{50}$-values at 18 nM and 33 nM respectively.[6] This deviation from the kinase assay, will most likely be due to several factors. Furthermore, Smith *et al.* [11] found that DCC-3014 is relatively unaffected by high levels of CSF1 on the inhibition of M-NFS-60 cells.[11] High concentration of CSF1 can often reduce the potency of the kinase inhibitors, since the extracellular ligand induces dimerization and activation of the kinase.[11] PLX3397 was however more affected on high CSF1 values, presumably because of the preferred conformation of CSF1R during binding, which are less accessible when CSF1R are dimerizated.[11] Further, DCC-3014 is also insensitive to high cellular concentrations of ATP.[11] These are important properties for drugs made to inhibit CSF1R and tumor growth in TGCT.[11]

Smith *et al.* [11] also performed a whole blood assay, to examine DCC-3014 inhibition of CSF1R in human primary monocytes.[11] The results show that DCC-3014 binds human plasma protein at approximately 96.5%, and that it inhibits CSF1R signaling in monocytes with an average $IC_{50}$ at 403 nM.[11] These findings correlate well with the results of Caldwell *et al.* [6].[6] On the other hand, PLX3397 was a weaker inhibitor in the whole blood assay with $IC_{50}$ at 2900 nM, which are likely reflecting PLX3397 high human plasma protein binding at >99%.[11]

### 3.2.2 Pharmacokinetics and in vitro ADME

To determine the suitability of drugs for human oral dosing, normally absorption, distribution, metabolism and excretion (ADME) assays, as well as other assays examine the pharmaceutical properties, are performed.[11] Based on the findings of Caldwell *et al.* [6] & Smith *et al.* [11] it can be argued that DCC-3014 has favorable drug-like properties, including good stability in liver and high aqueous solubility, and high oral bioavailability.[6,11] Since PLX3397 is a FDA-approved drug, similar properties as described for DCC-3014 is expected. Further, DCC-3014 does not inhibit the hERG channel, implying low risk for cardiovascular disorders.[6]

Pharmacokinetic assay with rats has been performed for both PLX3397[15] and DCC-3014.[6] At an oral dose of 10 mg/kg of DCC-3014, $C_{max}$ was determined to 3735 nM with $T_{max}$ 2.67 hours, bioavailability at 76% with a low systemic plasma clearance at 0.03 L/h/kg.[6] The latter is presumably due to the high liver stability. According to Tap *et al.* [15], $C_{max}$ for PLX3397 was determined to 4253 nM, with a bioavailability at 43%, and systemic plasma clearance at 0.164 L/h/kg in rat with an oral dose of 30 mg/kg.[15] Again, DCC-3014 has better results than PLX3397.

### 3.2.3 Clinical Trials on Humans Suffering from TGCT

Results from clinical trials of PLX3397 reveal that it successfully inhibits CSF1R, thus reducing tumor growth by up to 50%.[15] Hence, PLX3397 is assumed to be a valuable drug for patients with TGCT not amenable to improvement with surgery, even though there were not seen any big differences regarding pain.[9] PLX3397 is associated with a rare and serious hepatotoxicity, thus it is only available through a Risk Evaluation and Mitigation Strategy Program.[4,6,9–11] It

is not completely known in the literature what causes this toxicity, but one common theory is pointing towards some of the metabolites of PLX3397.[11] Another possibility could be linked to the CSF1R inhibition in Kupfler cells, specialized liver macrophages.[4] If the latter is the case, this could be a severe problem regarding drugs that aim to inhibit CSF1R.

At the 2022 CTOS Congress, data from phase II expansion of DCC-3014 in TGCT were presented by Blay et al.[16] They stated that DCC-3014 is well tolerated in patients suffering form TGCT, not amenable to surgery, at a dose of 30 mg given two times a week.[16] This could imply that DCC-3014 does not tend to have the same hepatotoxicity as PLX3397. However, there were only five patients out of 140 that suffered from these adverse events in all of the trials with PLX3397.[11] Further findings have revealed that DCC-3014 also have promising antitumor activity, where disease progression is not observed in any of the patients. More importantly, patients reported a meaningful symptomatic benefit with use of DCC-3014, both with respect to pain and stiffness.[16] Hence, DCC-3014 is now under a phase III MOTION trial for treatment of TGCT (NCT05059262).[10,16]

# 4 | Conclusion

When PLX3397 was approved by FDA as a treatment for patients with TGCT not amenable to surgery in 2019, this further encouraged development of novel CSF1R inhibitors.[4] Even though it potently inhibits CSF1R and shows great results in patients with TGCT, it also inhibits other kinases, including KIT, FLT3, PDGFRA, and PDGFRB. Unfortunately, PLX3397 has a risk for hepatotoxicity, and is not available in Europe.[11] DCC-3014 was designed to be highly selective to CSF1R, and it shows signs to be a promising CSF1R inhibitor. Thus, it appears like the structure-based design of DCC-3014 from PLX3397, has led to a more potent and specific inhibitor of CSF1R with favorable pharmaceutical properties. It shows repeatedly improved parameters compared with PLX3397, both during modeling interactions in the hydrophobic pocket of CSF1R, and more importantly for pharmaceutical properties. According to the phase II expansion of DCC-3014 reported by Blay *et al.* [16], it seems like DCC-3014 is well tolerated by patients.[16] However, it is too early to conclude about the successfulness of DCC-3014, since the phase III clinical trial for TGCT (NCT05059262) is not yet completed. Considering the enormous potential kinase inhibitors have as drugs,[28] it can be assumed that research in this field will not stop any time soon.

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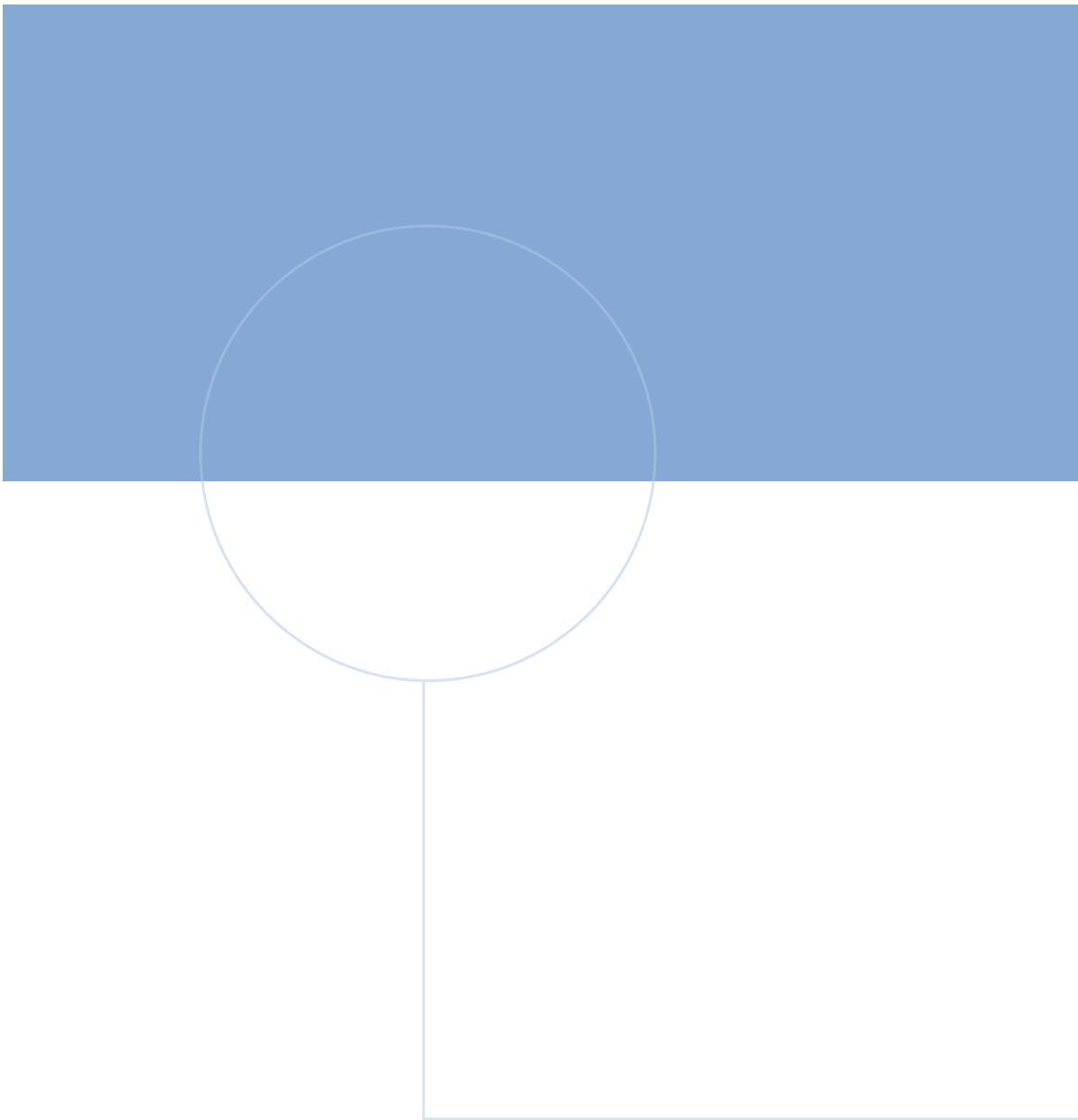

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