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Rational Design and In-Silico Studies of Novel Potential Covalent and Non-Covalent Fms-like Tyrosine Kinase 3 Inhibitors. Structure-Based Drug Design Approach in Targeted Cancer Drug Discovery †

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Abstract

In the last years, as Acute Myeloid Leukemia rise as one of the deadliest leukemia, a modest number of tyrosine-kinase inhibitors has been introduced to counter this burden. Nevertheless, tyrosine-kinase domain (TKD) and Internal Tandem Duplication (ITD) mutations are limiting therapies outcomes. In this study we developed a novel series of compounds more likely to target both ITD and TKD mutation to overcome resistance. We investigated a novelty covalent bond in the region of the pocket where the F691L mutation occurs and a stronger acceptor-donor interaction in the hinge. Preliminary in-silico studies have shown promising results compared to approved ligands.

Keywords: Acute Myeloid Leukemia; Feline McDonough Sarcoma-like tyrosine-kinase 3; FLT-3; targeted therapy; pyrazine; structure-based drug design; Quizartinib; Molecular Docking; Molecular Dynamics

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1. Introduction

Acute Myeloid Leukemia (AML) represent only the 1.1% of all cancer diseases in the United States, on the contrary though, has one of the highest estimated mortality rates for the year 2025 where near 50% of affected patient will face unfavourable outcome [1]. While Feline McDonough Sarcoma-like tyrosine kinase 3 (FLT3) was identified as a primary deregulated target, Quizartinib was intended to specifically inhibit the inactive conformation of the kinase, nevertheless is facing tolerance due to point mutation in the Kinase Domain and Internal Tandem Duplication mutations, accountable for the modified length of the juxta-membrane domain [2]. Considering the relevance and the poor outcome of this disease, especially for those patients carrying FLT3-ITD and TKD mutations, in this study we used an in-silico approach to build a novel series of compounds with different heterocyclic-based rings, more likely to form stronger acceptor-donor interaction than Quizartinib with a Cys694 residue in the hinge. A novelty covalent bond in the region of the pocket where the F691L mutation occurs has been investigated. So far data harvested, showed encouraging results in both the wild type (WT) and the F691L mutant. Pyrazine-based compounds covalently bound with cyano-moiety, showed an unreported

pose in the binding pocket, enhanced by a stabilizing interaction with Asp829 and a 90° inclined plane. Hence theoretically, the series seems to be unaffected by F691L mutation. We are seeking to harvest more data from Molecular Dynamics simulation, to evaluate the stability of the complexes and analyse the binding-unbinding processes, meanwhile the synthesis and the in-vitro testing will be used for biologically validation of the series.

2. Acute Myeloid Leukemia and FLT3 Expression

Among subtypes of leukemia, Acute Myeloid Leukemia (AML) rise as one of the deadliest encountered with approximately 50% of the affected patient facing high early mortality rate and low overall survival (OS) [3]. This disease is classified as a cancerous due to the unrestrained duplication of poorly developed myeloid cells that spread from the hematopoietic marrow to other tissues [4]. Feline McDonough Sarcoma-like tyrosine-kinase 3 (FLT3) is by far the most expressed and deregulated protein in AML. It is included in the type III family of tyrosine-kinases (TK), a class of transmembrane receptors that comprise three main substructures: an extracellular front, a catalytic domain where phosphorylation takes place and a juxtamembrane segment (JM), crucial for the autoinhibition process. From the physiological perspective, FLT3 has two main conformations, when the receptor lays in dormient state the JM segment prevents the activation. As soon as the ligands from the extracellular domain binds, receptor dimerize, and spatial properties are modified and shifted to sustain activation by loosening the inhibitory influence of the JM domain on the FLT3 via phosphorylation, allowing ATP binding and leading to the intracellular pathway that, in pathological cases, is accountable for the uncontrolled proliferation of the myeloid cell lines in the bone marrow [5]. Intracellularly, FLT3 activation leads and rely on the RAS-RAF, JAK-STAT and PI3K-AKT pathways, accountable for the transcriptional deregulation in the nucleus.

The abnormal activation of the FLT3 kinase usually enhance its physiological roles and highlights duplication, transmigration, preservation of cells, development of novel blood vessels and regulation of the cell cycle [6].

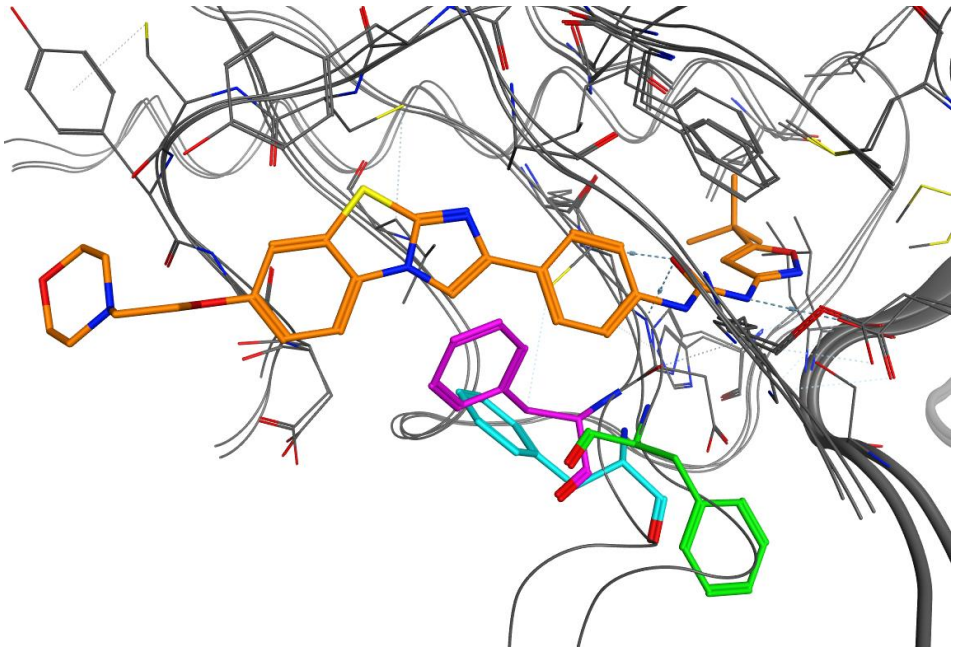


Figure 1. Superposition of FLT3 conformations in active and autoinhibited conformations. Magenta (PDB: 1RJB) and Cyan (PDB:4XUF): DFG-out conformation, Phe830 is pointing into the active site, prevents ATP binding creating a nearby pocket exploited by type II inhibitors. Green (PDB: 6JQR):

DFG-in conformation, Phe830 pointing outside the binding site to allocate ATP. Orange (PDB:4XUF): Quizartinib co-crystallized pose in the binding site [7].

2.1. Internal Tandem Duplication Mutations

Even tough their prognostic significance remains unclear, genomic mutation seems to lavish a central role, especially in FLT3 kinase and seems to be related to lower overall survival especially in those cases where the ratio between mutant to wild-type loom in favour of the mutant. Internal Tandem Duplication (ITD) were discovered in 1996 by Nakao M. et al. [8] and usually occurs in nearly 25% of all diagnosed patient, consisting in a specific insertion in the chromosome where a sequence of DNA is copied and linked directly after the native DNA [9,10]. In so doing the final protein structure will be affected in the lenghth of specific domains, hence the folding and its behaviour will differ. By far the most affected domains are the JM and the TK domains where the different lengths are toutght to be responsible for the constitituve kinase activation, leading to a poorer outcome and a more aggressive diseases. Longer miutated JM segment increase domain's mobility weakening its inhibitory influence on the receptor [9]. In recent years, new drugs have been devoloped and approved to target FLT3 kinase in AML treatment, they are divided in type I inhibitors (targeting FLT3 active conformation) and type II inhibitor (targeting autoinhibited FLT3). Both classes though can target FLT3-ITD mutant without reporting significanly changes in therapy and achieving OS improvement [11].

2.2. Tyrosin-Kinase Domain Mutations

The second most relevant mutation that weaken FLT3 inihibitors-based therapies are the TKD mutations. They are also called on-target resistances, to diversify them from the off-target resistances that occurs in the intracellular pathways signalling system. On-target point mutations belong to the class of the acquired resistances, known to proliferate after prolonged therapies with the same agent. Point mutation targeting FLT3 kinase leads to a shift into an active confromation due to a change in the constitution of the activation loop, by doing so the domain opens up catalizing the ATP binding and hence the activity of the kinase. The dynamical changes at the equilibrium stage regarding the active kinase brings serious concerns when it comes to compare the WT and the mutants. It has been reported that receptors with D835F and Y842H mutations showed an increased amount of configurations when the activation equilibrium is reached, compare to the WT. Thereby, the receptor decreases its chances to be found in the autoinhibited conformation, building up resistance to type II inhibitors. Among all point mutations, F691L is the one that showed ubiquitous tollerance to all approved inhibitors [9,11,12]. In the case of Quizartinib, the F691L mutation really affect its therapeutical outcomes, as a type II inhibitor it binds to the autoinhibited conformation of the kinase preventing the activation. According to docking studies, Quizartinib keeps most of its interaction even in the F691L mutant, interestingly though, more flipped pose were reported and the π-π T-shaped stacking was missing, crucial interaction on which Quizartinib's binding relies on. On the other hand, mutations in the activation-loop like D835F and Y842H have no influence on the drug when its already bound, hence the tolerance might comes from the inability to reach the binding pocket, due to a facilitation to an active conformation [9].

Table 1. Quizartinib's re-docking results on the FLT3-WT and F691L mutant (PDB:4XUF). High RMSD values represent flipped poses inside the pocket, discordant with the crystal structure.

Poses	**FLT3-WT**			**FLT3-F691L**		
	Score	**RMSD**	**E_conf**	**Score**	**RMSD**	**E_conf**
entry 1	−11.1995	1.5639	10.7694	−9.9307	2.9367	14.2753

entry 2	−10.9474	0.7640	12.7669	−9.4001	2.3845	21.5236
entry 3	−10.4476	2.1197	13.8941	−9.2922	15.0881	20.2260
entry 4	−9.8095	1.9405	21.2559	−9.2523	14.6181	21.0484
entry5	−9.3098	15.1669	19.1510	−9.2082	14.6914	18.6701

3. Materials and Methods

3.1. Hits and Database Preparation

Compounds were drawn in ChemDraw 23.1.2 software and then imported in MOE 2024.0601. A new database was created and all the compounds were prepared for docking by washing them at pH set to 7 and protonation state set as Dominant to mimick physiological conditions. Hydrogens and Lone Pairs were adjusted follwed by an energy minimization step using Amber:EHT as force field; R-field 1:80 and Cutoff (8,10). Gradient of the minimization stage set to 0.1 RMS kcal/mol/A^2.

3.2. Protein Preparation; FLT3-WT and FLT3-F691L

Target of the "Crystal structure of the FLT3 kinase domain bound to the inhibitor quizartinib (AC220)" with PDB:4XUF was downloaded from the RCSB Protein Data Bank, opened in MOE and prepared for docking analysis via QuickPrep function. Breaks in the sequence were capped, Protonate3D feature was used for adding hydrogens to the structure, water molecules further than 4.5 A from ligand and receptor deleted, theter was set to 10 in strength for both ligand and receptors due to the high flexibility of the domain. Atoms further than 8 a from the ligand were fixed and hydrogens close to the binding site were not. Refine step was set to an RMS Gradient of 0.1 kcal/mol/A^2 and process ran till completion. FLT3-F691L structure was prepared using the "Protein Builder" feature by substituiting the Phe691 in Leucine followed by minimizing step. The mutant was then prepared using QuickPrep function.

3.3. Re-Docking of the Crystalized Ligand and Non-Covalent Series Docking Settings

Quizartinib was re-docked after target preparation to check if there was any discrepancy between the crystal structure and the prepared complex. Docking panel was left untouched from the standard settings. Triangle matcher with London dG score and Rigid Receptor with GBVI/WSA dG scoring funciont used as Placement and Refinement stages respectively. 30 placement poses for the searching stage, 5 poses screened for refinement stage. Conformation of the ligand fixed as "Rotate Bonds". Equal setting were used for the non-covalent series docking studies

3.4. Covalent Docking

Irreversible binding was achieved by using the covalent feature present in MOE docking panel. The reaction was drawn in ChemDraw and then saved as .rxn file. Once the file was uploaded, the sulfur atom on the Cys828 was selected as reactive site. Rigid receptor was used and 30 poses each ligand configured as placement. 5 poses retained for refinement stage and ranked based on GBVI/WSA dG scoring function.

4. Results and Discussion

Two novel series of compounds have been designed to specifically target the FLT3 kinase. A detailed evaluation on both pocket features and interactions established by Quizartinib had been taken into consideration and isosteric replacement of targeted moieties were assessed. The novel series aim at the same pocket accessible in the autoinhibited conformation of the target, the novelty focused on a stronger interaction in the hinge with the Cys694 residue, feeble in FLT3-Quizartinib complex. In both series, an amide bond

was introduced to attain a more accessible synthesis and enhance the flexibility of the compounds. In the non-covalent series, the morpholine-ethyl bridge was preserved to stabilize the ligand in the binding site and create additional interaction at the entrance of the pocket while the ether function has been replaced with alkylated secondary amine. Noteworthy conformation of the morpholine-alkyne linkage showed poses were the chained bended near the activation loop, suggesting additional dynamics studies to mark the influence of this conformation on the kinase activation. The thiazolidine ring was replaced with five and six membered rings, capable of more powerful interaction with Cys694. The purpose of keeping the phenyl ring was to determine if the F691L mutant develops resistance even with a stronger hinge linkage. The isosteric replacement of the urea linker and the *tert*But-isooxazole ring was achieved with the amino-benzoimidazole core. This substitution respects the interactions with the Glu661 and the benzene ring is allocated in the same hydrophobic pocket as the branched *tert*-butyl residue from Quizartinib. Regarding the covalent series, a novelty covalent bond has been investigated with Cys828, in the region of the pocket where the F691L point mutation occurs. The introduction of the pyrazine ring, a six member heterocycle ring widespread in the pharmaceutical field, especially in the development of tyrosine-kinase inhibitors known for its adaptability and peculiar structural features in place of the Quizartinib's phenyl ring, showed an interesting binding pose recurrent throughout the series [13]. Pyrazine ring doesn't rely on the T-shaped π-π stacking interaction established with Phe691 and Phe830 residues, the ring lays in a 91° inclined plane when compounds bound, and poses are kept in place by an acceptor-donor interaction between pyrazine's nitrogen in position 1 and Asp829.

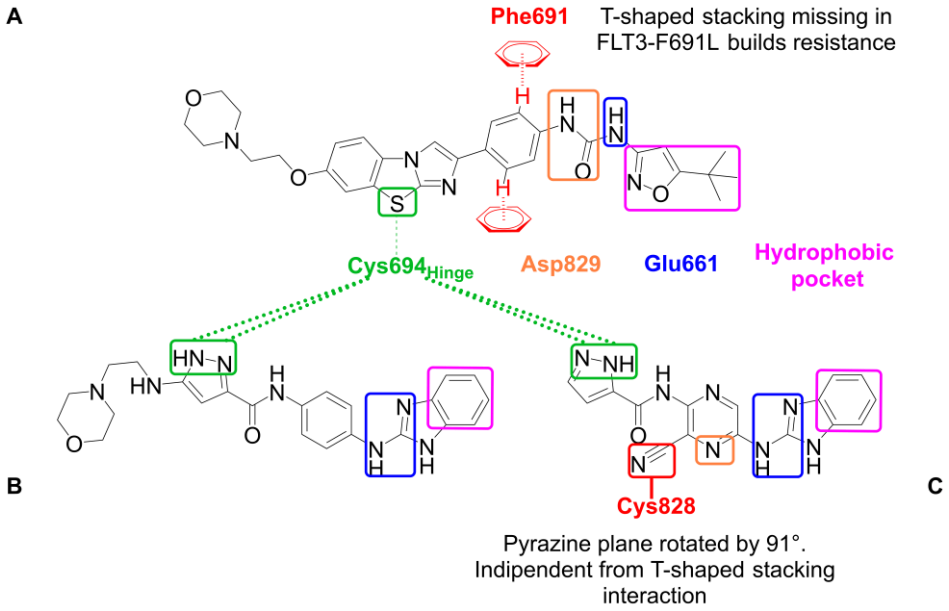


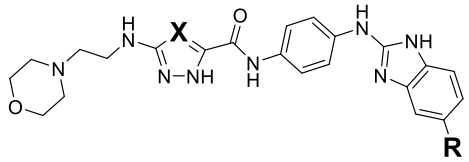
Figure 2. Comparison between Quizartinib's main interactions (**A**) and the novel series. Rational and SAR analysis of non-covalent (**B**) and covalent series (**C**). Highlighted stronger acceptor-donor hinge interaction (in green) and the respective contacts mimicking those of Quizartinib. Carbonitrile reactive site for covalent binding (in red).

Docking results performed on both the WT and the F691L receptors showed consistent results, something that doesn't occurs with Quizartinib, where the docking scores dropped in the FLT3-F691L mutant. High reported RMSD correspond to a clear sign of a flipped pose, unusual behaviour considering the binding mode reported in the crystal. It is interesting to note that the most flipped poses happened in the FLT3-F691L structure, which suggests that Quizartinib has a decreased chance of binding with the correct

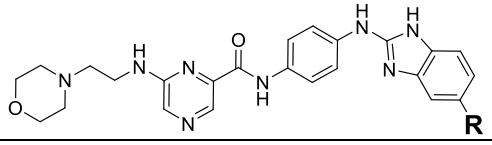
conformation. Introducing a stronger connection in the hinge in both novel series decreased considerably the number of flipped poses, hinting a specific and steady conformation in the binding pocket.

As reported in Table 2, the non-covalent series exhibited comparable results to Quizartinib redocking while maintaining recurrent results even in the FLT3-F691L mutant. In almost every refined poses, all the crucial interaction were respected, suggesting a recurrent binding conformation throughout the series. Greater conformational energies than Quizartinib were also reported along with comparable placement energies.

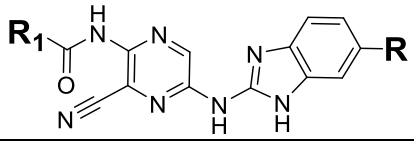
Table 2. Docking results performed on the WT and F691L mutant.



Residues		FLT3-WT			FLT3-F691L		
X	**R**	**Score**	**E_conf**	**E_place**	**Score**	**E_conf**	**E_place**
N	H	−8.91	−98.73	−103.01	−8.64	−95.28	−79.99
N	tertBu	−10.22	−110.06	−108.01	−9.90	−108.17	−72.86
C	H	−9.31	2.66	−129.80	−8.70	−1.78	−95.70
C	tertBu	−10.27	−16.35	−43.91	−9.93	−14.70	−116.12



Residues	FLT3-WT			FLT3-F691L		
R	**Score**	**E_conf**	**E_place**	**Score**	**E_conf**	**E_place**
H	−9.43	−21.91	−129.23	−8.94	−21.88	−106.04
tertBu	−10.07	−48.04	−82.49	−9.66	−53.40	−120.29



Residues		FLT3-WT			FLT3-F691L		
R	**R_1**	**Score**	**E_conf**	**E_place**	**Score**	**E_conf**	**E_place**
Pyrazole	H	−6.83	−81.98	−3.74	−6.45	−81.72	−5.13
1,2,3-triazole	H	−6.43	−116.36	−6.90	−6.57	−117.20	−7.75
1,2,4-Triazole	H	−6.31	−140.02	−7.36	−6.51	−144.56	−7.06
2-Pyridine [1]	H	−6.97	−92.02	−6.40	−7.03	−96.86	−7.06
3-Pyridine	H	−6.70	−134.33	−8.15	−6.76	−135.90	−6.38
4-Pyridine	H	−6.77	−135.40	2.20	−6.88	−145.59	−8.59
Pyrazole	tertBu	−8.02	−97.90	−10.42	−7.80	−90.97	−7.77

[1] No hinge interaction reported across the proposed poses.

5. Conclusions

Currently the first irreversible drug was developed to target the FLT3 kinase in the Cys695 residue displaying a broad efficacy across all mutations associated with the use of type II inhibitors [14]. To our knowledge no studies on the FLT3 kinase have ever targeted the Cys828 residue covalently. We are looking forward to running additional in-silico studies based on Molecular Dynamics simulation. Time aspect in the simulation can give additional insights data on the stability of the complexes. Synthesis is currently in progress and detailed information on in-vitro testing will be made available at later time.

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Abbreviations

The following abbreviations are used in this manuscript:

AML	Acute Myeloid Leukemia
FLT3	Feline McDonough Sarcoma-like Tyrosine Kinase 3
FLT3-ITD	Feline McDonough Sarcoma-like Tyrosine Kinase 3 Internal Tandem Duplication mutant
FLT3-F691L	Feline McDonough Sarcoma-like Tyrosine Kinase 3 F691L mutant
JM	Juxtamembrane
TK	Tyrosine-Kinase
TKD	Tyrosine Kinase Domain
ITD	Internal Tandem Duplication
OS	Overall Survival

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