Proceedings of the International Conference on Modern Medicine and Global Health (ICMMGH 2023)
DOI: 10.54254/2753-8818/6/20230233

# The role and efficacy of PROTAC in FLT3-mutated AML treatment

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**Abstract.** Small molecule Proteolysis Targeting Chimera (PROTAC) is an effective therapy for patients with FLT3-ITD acute myeloid leukemia (AML). By activating the ubiquitination system, PROTAC can generate protein and kinase degradation upon FLT3 to perform outcomes of antiproliferative activities. The focus of this study revolves around the investigation of the efficacy of VHL and CRBN-based PROTAC on FLT3 degradation compared to conventional immunotherapy agents such as quizartinib. By assessing the performances of PROTAC on MOLM-14 and MV4-11 cells with other therapies, comparing both adverse effects and benefits could demonstrate crucial approaches to applying PROTAC for AML treatments. The VHL-recruiting PROTAC based on the modification of quizartinib has shown promising effects where FLT3 within MV4-11 injected athymic mice had experienced around a 60% of decrease. The CRBN-based degrader TL12-186, on the other hand, had also demonstrated antiproliferative outcomes where 14 out of 7559 proteins of the MOLM-14 cell have been successfully degraded, showing a more than 25% decrease. Even though there seem to be some improvements in the VHL-recruiting PROTAC compared to traditional immunotherapy agents like quizartinib, CRBN-mediated PROTAC has shown a relatively less significant result. Critiquing in a variety of aspects, quizartinib has demonstrated better performance in cell permeability, low nanomolecular concentration, and degradation. The significance of this study provides an overview of existing PROTAC technology that shows effects on the treatment of FLT3-mutated AML. Further studies may be conducted on the foundation of this study to demonstrate the enhancements of each modified PROTAC compared to existing therapies.

**Keywords:** PROTACs, FLT3 mutation, AML, quizartinib, TL12-186.

## 1. Introduction

### *1.1. Background*

Acute Myeloid Leukemia (AML) is a lethal myeloid lineage disease that derives from the abnormal proliferation of the blood marrow through mutations in the DNA. The uncontrolled production of cells produces high levels of undifferentiated cells, resulting in the development of white blood cells named myeloblasts. Once the mutation happens, bone marrow failure syndrome takes place in which the proliferation of myeloblasts outnumbers the amount of healthy hematopoietic cells, hindering the normal function of the blood cells [1]. AML is caused by a variety of gene mutations. Among these, FLT3-ITD is one of the most frequent mutations, appearing in ~30% of all AML patients. The FLT3 is

DOI: 10.54254/2753-8818/6/20230233

a receptor tyrosine kinase that is bound onto the hematopoietic cells in regulating the maturation and function of the cell [2]. The mutation of the FLT3 takes place either on the internal tandem duplications (ITD) or as a point mutation on the tyrosine kinase domain (TKD). While available treatments of FLT3 inhibitors are applicable such as gilteritinib and quizartinib, limitations upon the fast proliferation rate of the mutation have made treatments relatively ineffective. Proteolysis Targeting Chimera (PROTAC), on the other hand, overcomes the shortcomings and becomes a possible alternative approach to AML treatments. The technology focuses on using protein modulation in which the targeted protein is degraded by proteasomes by using small bifunctional PROTAC molecules in activating the ubiquitination system [3].

### *1.2. Literature review*

With the recognition of a low 5-year survival rate of 28.3% between 2009 to 2015, AML is in a condition of an unmet medical need [1]. Traditional therapeutic modalities of AML treatment that are currently in application include chemotherapy and allogeneic transplantation. However, not all patients meet the requirement for specific treatments.

Age restrictions and donor matchings are present issues that intervene with patients' access to treatment; hence, increasing the mortality rate. In a clinical study conducted to discuss the effectiveness of treatments on the overall survival rate of 872 adults under 60 years old with AML, results showed that 471 of 872 patients (54%) had died [4]. Out of those who survived, the median survival rate was only 30.2 months, and around ½ of the patients that achieved complete remission experienced a relapse. After the discovery of PROTAC in 2001 by the research group conducted by Craig Crews and Kathleen Sakamoto, it has been applied in various medical fields including drug target identification in drug discovery as well as cancer treatments. By 2018, Crews convened another research group to discover the relationship between PROTAC and FLT3 inhibitors to enhance the anti-proliferation of leukemia cells [5]. While it is known that PROTAC can activate the ubiquitination system, inducing protein degradation and anti-proliferative activities, the technology could be capable of degrading the FLT3-ITD mutant proteins to inhibit cell growth.

Based on PROTACs mechanism and function, there is great significance of this technology in serving as a treatment for the FLT3-ITD-based AML mutations. By combining the PROTAC with the FLT3 inhibitors, the results may lead to advancements in therapies not only for AML but also for various cancerous and proliferative diseases. Developing on the foundation of the degradative characteristics of PROTAC, it may be possible to achieve a fast and efficient method with high selectivity and affinity of obstructing the abnormal growth of mutated and denatured cells. This study would focus on discussing the impact and effects of PROTAC treatment upon AML mutations through performing case analysis and literature review. It would introduce the mechanisms of PROTAC upon FLT3-ITD protein degradation and analyze its effect on the treatment of AML. The study would be divided into two sections where it would discuss the efficacy of VHL-PROTAC modification of quizartinib and CRBN-mediated PROTAC.

## 2. Evaluating VHL and CRBN-based PROTAC

### *2.1. VHL-based PROTAC modification on quizartinib*

The idea of PROTACs was first proposed in 1999, but it wasn't until 2001 that the technology was brought into application. PROTAC was initially developed by the research group conducted by Kathleen Sakamoto, Craig Crews, and Ray Deshaies on using bifunctional PROTAC molecules in degrading the MetAP-2 protein. The first generation of the PROTAC was designed based on peptide fragments, and the research group found out that when injecting these PROTAC molecules into the intact cells, the molecules would eventually bind onto the targeted protein with specificity and degrade the protein. The experiment on the HIF-1α peptide fragment was a milestone in PROTAC development as recruiting Von Hippel-Lindau tumor-suppressing protein (VHL) E3 ligase suggested that the technology carried the future potential to be applied without microinjection [6]. To develop on

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the foundation of the first generation PROTAC, the small-molecule PROTAC was proposed later in 2008 by Craig Crews and Nathanael S. Gray. The first small-molecule PROTAC was designed to consist of a structure made from the combination of a selective androgen receptor modulator (SARM) as well as an MDM2 ligand called nutlin [7]. The SARM-nutlin PROTAC was initially tested upon the ubiquitination and degradation of the androgen receptor, and its results suggested the successful development of PROTAC drugability. The PROTACs serve as heterobifunctional conjugate which binds the targeted protein of interest (POI) with an E3 ubiquitin ligase to activate the ubiquitin-proteasome system (UPS). The ubiquitin-activating enzymes (E1) would transfer the ubiquitin to the ubiquitin-conjugating enzyme (E2) using ATP to remove and attach the ubiquitin onto different compounds like E3. With the presence of E3 on the target protein ligand, the ubiquitin molecules would be transferred to attach to the targeted protein in the form of a small chain to serve as a tag for the 26S proteasome [8]. Regulatory particles of the 26S proteasome will recognize the ubiquitin and perform protein degradation. Once degrading finishes, the ubiquitin molecule detaches and returns to the cytoplasm for re-utilization. This discovery of the small-molecule PROTAC led to the maturity of the technology for modern-day drug discovery.

With the development of PROTAC in recent years, it has been able to target a variety of illnesses and diseases including AML. The FLT3 mutations play a pivotal role in AML pathology as it disrupts the FLT3 function to allow clonal replications of immature blood cells to proliferate. FLT3 inhibitors have been the first approach in treating FLT3-mutated AML, but resistance and limitations for the fast mutation rate of the gene have been a great downplay of the treatment. PROTAC, on the other hand, is a possible novel treatment over traditional small molecule kinase inhibitors as it allows low drug exposure, high cellular efficacy, and long duration. In 2018, Crew's research team conducted a study on degrading FLT3-ITD protein using PROTAC technology made up of conjugates based on the foundation of the FLT3 inhibitor quizartinib, serving as a recruiting element. By linking onto a cereblon (CRBN) and replacing the morpholine group with VHL ligands, the first FLT3-recruiting PROTAC has been developed [2].

*2.1.1. Analysis.* VHL-mediated PROTAC was used for protein degradation of MV4-11 and MOLM-14 cells, and high binding affinity at low nanomolecular concentrations emphasizes its benefit in clinical trials. Through the degradation of FLT3-ITD mutated proteins, significant antiproliferative activity within the cell was displayed. Apoptosis accelerates and it inhibits the rapid mutation of immature AML hematopoietic cells. However, the impact of this new therapeutical method compared to previous treatments is unknown, and experiments would be needed to conclude whether there is an improvement. To find a solution to this issue, Crew's research team created three independent variables including the conventional inhibitor quizartinib, FLT3 PROTAC, as well as an FLT3-control resulting from the diastereomer of the FLT3 PROTAC through stereocenter inversions in the VHL sections. The FLT3 control can perform similar levels of FLT3 binding affinity and chemical properties to FLT3 PROTAC, but it is incapable to induce degradation. By performing the experiment in vitro within both MV4-11 and MOLM-14 cells, data has shown that PROTAC-induced degradation has shown to be a significantly better clinical candidate than the unmodulated quizartinib and the control. At the same survival rate, VHL-mediated PROTAC applied lower drug exposure within the cell. Assessing the drug's safety and efficacy, PROTAC shows potential in future clinical trials as it reduces the likelihood of cytotoxicity and drug resistance. However, there are some aspects in which the quizartinib has shown better therapeutic performances than PROTAC. While PROTAC and quizartinib can both inhibit FLT3-ITD and FLT3 wild-type molecules, quizartinib is able to achieve this at a lower nanomolar activity. PROTAC reduced the ability of FLT-3 ITD at 43± 3.2 nM, while quizartinib reached this at 10± 0.3 nM. Similar to the FLT3 WT model, PROTAC was 36±3 nM, while quizartinib was only 7.4±0.3 nM. Other studies have shown that the conversion of quizartinib into PROTAC has focused on FLT-3 binding, but the binding affinity for other elements in the kinase superfamily is absent. PROTAC degradation on kinases other than FLT3 has shown no diminishing in protein levels, suggesting that the replacement of supposed solubilizing groups may impact the

DOI: 10.54254/2753-8818/6/20230233

selectivity of compounds [6]. PROTAC may have more enhanced selectivity compared to quizartinib, but the degradation of quizartinib in FLT3-ITD cells is occasionally more efficient than inhibition.

In addition to in vitro experiments, Crew's research team also managed activities in vivo within mice to determine and evaluate the functions of PROTAC. The results revealed well pharmacokinetic properties of FLT3 PROTAC where there is significant degradation after 22 hours post-injection of the drug. The plasma concentration maintained a level of 5 nM after a day of drug exposure, and this suggested that PROTAC has significant impacts on the organism. To further examine the pharmacodynamics, the study employed nine athymic mice to inject MV4-11 cells to allow a growing environment for the tumors to proliferate and form an in vivo experiment. The mice were randomly organized into two treatment groups of PROTAC and controlled in which they are treated once every 24 hours to maintain a plasma concentration above 5 nM. The tumor samples are collected for both treatment groups after the experiment. Results show that while both treatments allowed the inhibition of FLT3-ITD downstream signaling, only PROTAC induced the degradation of proteins to occur to constrain the proliferation of the tumor cells. Those mice who were treated with PROTAC has shown a 60% decrease in the FLT3 protein, while the controlled group had no significant changes. This led to the overall conclusion that quizartinib VHL-recruiting PROTAC can increase the efficacy of growth inhibition through accelerating apoptosis. PROTAC not only allowed enhanced selectivity, but it also revealed efficacious data providing new insights into AML treatment and mutant protein degradation that makes it a potential therapeutic treatment for clinical uses.

*2.1.2. Suggestions.* To go beyond the scope of this study to evaluate PROTAC's role in FLT3-mutated AML, the therapeutic method could be tested in clinical trials on patients. Instead of performing experiments in vitro and in vivo, approval for clinical trials authorizes the drug to be directly tested on humans. Different results and efficacy may emerge through the experiments, and it might produce new insights into the development of PROTAC in targeting mutations and hematological diseases like AML. The samples of mice may exhibit relevant information about PROTAC, but it would be ideal to be capable of testing within human pharmacodynamics and pharmacokinetics to go further into the study. Furthermore, another suggestion for future studies would be to perform observations of PROTAC modulation in comparison with other FLT3 inhibitors and to develop more models of PROTAC based on the foundations of these inhibitors. The VHL-recruiting PROTAC was initially modeled by quizartinib, and it indicated the possibility of modifying inhibitors to induce the degradation of specific proteins. It would be a breakthrough if the apoptosis induction mechanism could be elucidated so that more inhibitor based PROTAC could be designed in targeting FLT3 mutations. Other than quizartinib, first and second-generation FLT3 inhibitors like gilteritinib, midostaurin, sorafenib, and sunitinib could all be feasible modified treatments.

### *2.2. CRBN-based PROTAC kinase degrader*

The modification of quizartinib into PROTAC is one of the possible therapeutic approaches toward FLT3-mutated AML cases. However, the first generation of PROTAC was unreliable and demonstrated poor cell permeability due to the E3 ligase binders. While the human genome encodes for more than 600 E3 ligases, only certain types can be operated within the development of PROTAC. For instance, VHL, CRBN, IAPs, and MDM2 are E3 ligases that can degrade target proteins by the chimeric small molecules [3]. The IAPs and MDM2 ligases were involved in creating the second generation to enhance the chemical properties of PROTAC. Yet, the results were minimal and deficient to demonstrate its advantages compared to present methods available in the market. Most importantly, these FDA-approved PROTAC FLT3 kinase inhibitors including but not limited to quizartinib, gilteritinib, and sorafenib has poor clinical responses. The transient effects are followed by high relapse rates as well as drug resistance toward the treatment [9]. Given the contribution of disease progression and the low survival rate of patients, the need for new methods is urgent for this unmet medical condition. The CRBN-mediated PROTAC serves as a breaking point for the treatment of AML. As a possible alternative ligase of PROTAC, CRBN is an immunomodulatory drug (IMiD) that

DOI: 10.54254/2753-8818/6/20230233

could degrade specific proteins and substrates with significant impacts [10]. Beyond inhibiting cell growth, there was also evidence that showed apoptosis induction. It allowed the suppression of the proliferation of tumors, declining the tumor weight and other malignant proteins. This demonstrated a substantial foundation for further study in CRBN-mediated PROTAC degradation to be applied to cancer therapeutic treatments.

In 2017, Nathanael S. Gray assembled a research team to investigate the degradation effect of CRBN-based PROTAC kinase degrader. The study concentrated on developing a methodology that would explore the susceptibility of kinases upon designed degraders. The team elaborated a multi-kinase degrader using a diaminopyrimidine scaffold and discovered approachable degradable targets using the tandem mass tag system. Among the 28 targets filtered, some of them came from the AML cell lines. The kinase of FLT3 relevant in producing the FLT3-ITD protein is one of the targets. With a specific target to aim upon, the team designed target-selective degraders to comprehend certain factors that influence susceptibility. By installing a polyethylene glycol (PEG) linker with an immunomodulatory agent, pomalidomide, they assembled the PROTAC kinase degrader TL12-186 with a CRBN-recruiting IMiD analog [11]. Since MOLM-14 is a human leukemia cell line, the group scrutinized the degradation activity of TL12-186 on MOLM-14 to suggest its efficacy on leukemia like AML [12].

*2.2.1. Analysis.* The experiment focused on comparing the TL12-186 treatment with the non-CRBN-binding TL13-187 PROTAC kinase degrader with both samples having a concentration of 100nM. Since 100nM is where there is a significant difference in the observation of anti-proliferative activities between the wild type and CRBN-based degraders, the treatment concentration would be chosen at this level. The kinase degraders were applied to MOLM-14 cells, and a 4-hour treatment time was set to capture the degradation responses. By comparing the outcomes, the team arrived at a conclusion regarding the efficacy of CRBN-based degraders upon FLT3-mutated AML cells. In the CRBN-based degrader of TL12-186, there was a significant decrease in the identified proteins where 14 out of 7559 MOLM-14 cells protein has been lowered by more than 25% [11]. As a multikinase degrader, out of the 13 degradable proteins of TL12-186, 12 of them were kinases with which FLT3 served as one of them. While the TL13-187 has demonstrated more potential in inhibiting cellular proliferation since low cell permeability for TL12-186 has occurred, only the TL12-186 degrader has reflected pharmacological effects upon the MOLM-14 cells. There has been a significant effect that influences the overall survival of the leukemia cells, laying the foundation to design an FLT3-specific degrader to precisely target FLT3 to serve as a treatment for AML. The advantage of kinase degradation over kinase inhibition is that it can deliver long-term effects on the patient. It decreases the turnover rate for the proteins, and it also allows prevention showing resistance towards the mechanism of this technology [13]. Compared to other therapeutic methodologies, CRBN-based PROTAC kinase degraders can achieve similar effects at a lower intracellular concentration than conventional inhibitors, lowering the possibility for cytotoxicity and fatal consequences.

Other than comparing TL12-186 with similar PROTAC kinase degraders, the study also evaluated the differences between the traditional treatment quizartinib. While the CRBN-based degrader TL12-186 does perform plausible effects on the degradation of proteins and kinases of MOLM-14 cells, it also exhibits multiple downsides that downplay its effect. The TL12-186 shows time-dependent responses in which the FLT3 degradation would take place as time progresses. With the treatment needing 4-8 hours to react on FLT3, TL12-186 degrades faster on other kinases like AUKRA which requires a minimum of 2 hours post-treatment to show significant responses. Compared to the quizartinib, CRBN-based PROTAC is less selective in targeting FLT3. This suggests its disadvantages in serving as an FLT3-ITD mutated AML treatment since there are more selective kinase inhibitors present that allow sufficient degradation. TL12-186 is a potential methodology, but improvements could be made based on its current development. Quizartinib presented more significant anti-proliferative properties than TL12-186 between the difference in IC50. IC50 is the measurement of concentration needed for 50% of inhibition, and quizartinib has shown lower values than TL12-186.

DOI: 10.54254/2753-8818/6/20230233

The FLT3 degradation by the TL12-186, on the other hand, has minimal benefits on sensitivity, cell permeability, and drug concentration compared to quizartinib.

*2.2.2. Suggestions.* The case study presents significant detail analyzing and comparing the efficacy of CRBN-PROTAC TL12-186 with the quizartinib as well as non-CRBN-PROTAC TL13-187, but the experiment is limited in vitro. The demonstration of MOLM-14 cells may be a confounding factor that hinders the performance and efficacy of the TL13-187 degradation activity. Animal testing, such as in mice, would be a possible pathway for conducting future studies investigating the relationship of TL12-186 on the treatment of FLT3-mutated AML. By creating a microenvironment within the mice fostered with malignant AML cells, the degradation of the degraders could simulate similar reactions within human patients, generating more reliable responses. In addition, as the study focuses on comparing the effectiveness of TL12-186, it has neglected other CRBN-mediated PROTACs like TL13-117 or TL13-149. Possible studies could incorporate more FLT3 degraders to use as a comparison for studying the efficacy of CRBN-mediated PROTAC on treatments for AML. This would help identify whether CRBN-based PROTAC will be an appropriate therapeutic method to develop because it takes into consideration a variety of degraders other than just TL12-186. A more holistic view is provided by analyzing the generic characteristic of CRBN-PROTAC.

## 3. Conclusion

The growing unmet medical need for FLT3-mutated AML patients has led to the development and expansion of the landscape of AML treatment. From traditional immunotherapy using agents like quizartinib to PROTAC technology, this has driven many effective anti-proliferative therapies to be under investigation. VHL-recruiting PROTAC modified based on quizartinib has been an effective approach towards AML treatment in degrading FLT3-mutated proteins and kinases. Although the therapy is currently in the experimental stage, it has shown characteristics that make it a promising degrader. It is worth noting PROTAC has demonstrated a 60% decrease in the FLT3 protein within the MV4-11-injected athymic mice, whereas the control group had no significant change in FLT3 level. High binding affinity, as well as low nanomolar concentration levels, made it a notable technology. Other forms of PROTAC have also been recognized to serve as possible candidates for treating FLT3-mutated AML including the CRBN-mediated PROTAC. The CRBN-based degrader TL12-186 had significantly lowered more than 25% of proteins within the MOLM-14 cell, and 12 of them had been kinases including FLT3. Although numerous responses of the degradation activity for TL12-186 are not ideal compared to existing treatments, TL12-186 is still potent in FLT3 degrading for requiring low intracellular concentration. Its demonstration of the low turnover rate of proteins and prevention in mechanism resistance could be a breakthrough in developing improvements for a suitable therapy of AML. The crosstalk between existing AML treatments and innovative PROTAC technology spurred assessments of their potential to integrate immunologic responses with AML patients in clinical trials. Selectivity and reaction time has been a great downplay towards CRBN-based PROTAC, and future research could focus on enhancing the anti-proliferative function. Meanwhile, low cell permeability and poor clinical responses are some disadvantages that need to be researched to understand the possible drawback of this technology. Risk stratification and relapse diagnosis are still not tested as both the VHL-recruiting and CRBN-based PROTAC experiments are strictly limited to within laboratories. To go beyond the scope of this study, further studies are needed to confirm the mechanism of PROTAC upon FLT3 degradation. A larger sample of clinical trials would also be required to guarantee the safety and efficacy of PROTAC as an immunotherapy on human patients of AML.

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