Communication

Enhancing Anti-Proliferative Activity and Selectivity of a FLT-3 Inhibitor by PROTAC Conversion

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J. Am. Chem. Soc., **Just Accepted Manuscript** • DOI: 10.1021/jacs.8b10320 • Publication Date (Web): 14 Nov 2018

Enhancing Anti-Proliferative Activity and Selectivity of a FLT-3 Inhibitor by PROTAC Conversion

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Supporting Information Placeholder

ABSTRACT: The receptor tyrosine kinase FLT-3 is frequently mutated in acute myeloid leukemia, however current small molecule inhibitors suffer from limited efficacy in the clinic. Conversion of a FLT-3 inhibitor (quizartinib) into a proteolysis targeting chimera (PROTAC) results in a compound which induces degradation of FLT-3 ITD mutant~~protein degradation~~ at low nanomolar concentrations. Furthermore, the PROTAC is capable of inhibiting cell growth more potently than the warhead alone whil~~est~~ inhibiting fewer off-target kinases. This enhanced anti-proliferative activity occurs, despite a slight reduction in the PROTAC's kinase inhibitory activity, via an increased level of apoptosis induction~~,~~ suggesting non-kinase roles for the FLT-3 ITD protein. Additionally, the PROTAC is capable of inducing FLT-3 ITD degradation *in vivo*. These results suggest that degradation of FLT-3 ITD may provide a useful method for therapeutic intervention.~~.~~

Targeted protein degradation is emerging as a powerful new tool to combat disease.[1] At the forefront of targeted protein degradation technologies are Proteolysis Targeting Chimeras (PROTACs).[2] This class of heterobifunctional small molecules, developed in our lab, function via recruitment of the cellular protein degradation machinery to a target of interest. More specifically, recruitment of an E3 ligase complex by a PROTAC to a disease-causing protein results in the ubiquitination and subsequent proteasomal degradation of that protein target[2a]. In addition to being irreversible, this also results in eradication of all functional activities of the protein rather than the inhibition of a single one. This approach has been used to degrade multiple protein targets using those E3 ligases proven amenable for use in this technology.[3] Crucial to the development of a successful PROTAC is the careful selection of the protein target. Receptor Tyrosine Kinases (RTKs) represent ideal targets for the PROTAC technology as they possess both well-documented scaffolding roles as well as their cognate kinase activities.[4]

An important example of a disease relevant RTK is the FMS-Like Tyrosine kinase 3 (FLT-3), which is expressed in the majority of Acute Myeloid Leukemia (AML) cases (41/44 tested).[5] AML is the most common form of acute leukemia,[6] and 20-30% of newly diagnosed AML patients feature FLT-3 mutations that are associated with poor prognoses.[7] The most common mutation is an internal tandem duplication (ITD) in the juxta-membrane domain, which has been validated as the driving mutation and ~~a~~ thus a therapeutic target for AML treatment.[8] Several FLT-3 kinase inhibitors have been assessed in clinical trials but demonstrated only limited benefit.[9] It appears that maximum, sustained inhibition of FLT3 ITD signaling is essential to achieve an effective clinical response,[9-10] and achieving such a complete inhibition can be challenging with small molecule kinase inhibitors: it often necessitates the use of high doses, potentially resulting in undesirable off-target effects. In our eyes, this makes FLT-3 ITD an ideal target for a targeted protein degradation approach since degradation of the FLT-3 ITD protein will necessarily result in sustained, maximum inhibition. Additionally, previous studies have shown that siRNA induced knockdown of FLT-3 ITD in AML cells sensitized them to apoptosis[11] and that FLT-3 ITD can act as a scaffold for Pim kinases resulting in FLT-3 ITD inhibitor resistance.[12]

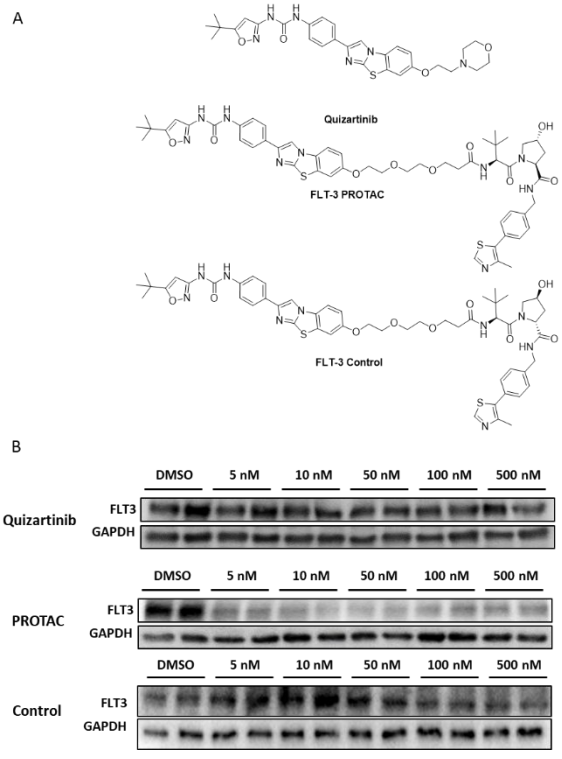


Figure 1. PROTAC-induced degradation of FLT-3 ITD. A – Structures of compounds used in this study. B – Western blot

showing the FLT-3 protein levels in MV4-11 cells treated with the indicated concentration of each compound.

Having recently demonstrated that RTKs are amenable to targeted protein degradation via PROTACs,[4a] we sought to extend this approach to target FLT-3 ITD. Employing the clinical candidate quizartinib (AC220[13]) as a recruiting element and replacing the solubilizing morpholine group with our VHL ligand[14] via an optimized linker, we were able to develop a FLT-3 PROTAC (Figure 1A) that potently induces FLT-3 ITD protein degradation in MV4-11 cells (Figure 1B and Figure S2) and MOLM-14 cells (Figure S1) at low nanomolar concentrations. Inversion of the stereocenters in the hydroxyproline VHL binding element results in an diastereomer of equal FLT-3 binding affinity and physicochemical properties but unable to induce degradation, thus providing an excellent control molecule (Figure 1A).[2c, 3a, 4a, 14a] Indeed, the binding of the FLT-3 control appears to stabilize the FLT-3 protein as has been previously reported for other FLT-3 ligands.[3h, 15] This mechanism of resistance to FLT-3 ITD inhibitors may be the reason for the limited benefit in clinical trials, further emphasizing the benefits of a FLT-3 PROTAC.

With this optimized compound in hand, we sought to characterize the consequence of PROTAC conversion on the activity and selectivity of our compound as a kinase inhibitor, compared to the parent compound, quizartinib. We performed kinase activity assays to confirm that the PROTAC can indeed still inhibit both FLT-3 ITD as well as wild type FLT-3. The PROTAC has slightly reduced ability to inhibit both FLT-3 ITD (43 ± 3.2 nM) (Figure 2A) and FLT-3 WT (36 ± 3 nM) (Figure 2B) compared to quizartinib (10 ± 0.3 nM and 7.4 ± 0.3 nM respectively) but retains low nanomolar activity in both cases. Quizartinib is a moderately selective kinase inhibitor with a limited number of targets at 1 μM as shown in Figure 2D. Profiling our PROTAC in a similar fashion, significantly fewer hits were observed. Conversion of quizartinib into a PROTAC retained FLT-3 binding but significantly reduced binding affinity for the other members of the kinase super-family that had beenwere inhibited (Figure 2C). Furthermore, we investigated the effect of the PROTAC on the degradation of other kinases to which it binds and found no reduction in protein levels (Figure S3). This is consistent with previous observations, from our lab and others, that replacement of supposed solubilizing groups during PROTAC conversion can have profound effects on compound promiscuity.[3c, 3h]

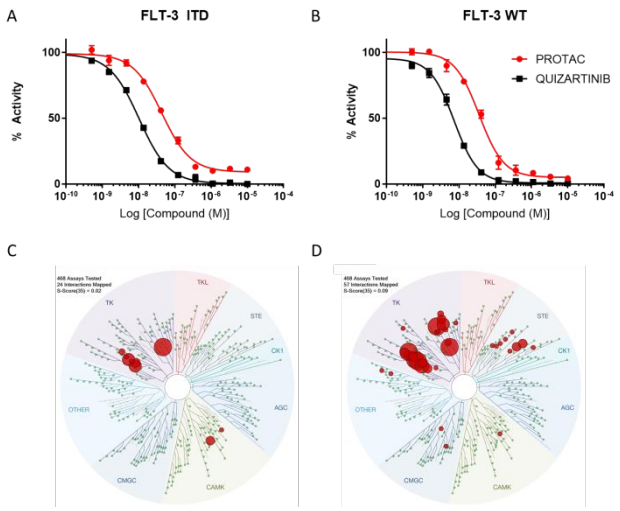


Figure 2. *In Vitro* Profiling of the FLT-3 PROTAC A – *In vitro* kinase inhibition assay for FLT-3 ITD. B – *In vitro* kinase inhibition assay for FLT-3 wt. C – KinomeScan™ data for PROTAC at 1 μM. D – KinomeScan™ data for quizartinib at 1 μM.

Evaluating our compounds in cellular proliferation assays revealed the distinct advantage of degradation over inhibition with the PROTAC not only performing significantly better than the control compound but also better than the clinical candidate quizartinib in both MV4-11 cells (Figure 3A) and MOLM-14 cells (See S2). Indeed, the FLT-3 PROTAC is >3.5 fold more potent than quizartinib, with a sub-nanomolar IC_{50} (0.6 ± 0.08 nM). Comparison of the control (IC_{50} = 2.27 ± 0.2 nM) to quizartinib (IC_{50} = 1.87 ± 0.1 nM) in this proliferation assay shows a slight reduction in potency for the former, which is expected from the reduced inhibitory activity in the *in vitro* assay following PROTAC conversion. This indicates that degradation of FLT-3 is crucial for the enhanced anti-proliferative effect. This is in contrast to the previously reported FLT-3 PROTACs which, although also derived from quizartinib, failed to give an advantage over the warhead alone; perhaps this may be due to decreased cell permeability or their less potent induction of degradation.[3h] In some cases, degradation has proven a viable approach to combat resistance mechanisms against inhibitors[3e, 3f, 16] so we tested the FLT-3 PROTAC against cell lines expressing two of the most common FLT-3 kinase domain mutations, as well as the ITD, which impart resistance to quizartinib, D835Y and F691L.[13a, 17] In both mutants the PROTAC displayed a similar response to quizartinib but all compounds showed a similar right-shift in the dose response curve relative to those for FLT-3 ITD WT, attributable to reduced binding affinity of the mutants for the recruiting element (Figure S2S4). Given the potent activity of these compounds in cell lines bearing a FLT-3 ITD mutation, we also assessed the anti-proliferative activity against a WT FLT-3 AML cell line (OCI-AML-3) and a CML cell line (K562) to ensure against general toxicity (Figure S3S5). Quizartinib has moderate antiproliferative activity in OCI-AML-3 cells (IC_{50} – 773 ± 74 nM) but in this case the PROTAC is comparatively less potent (IC_{50} >2800 nM). This may be due to more restricted kinase engagement/enhanced selectivity of the PROTAC compared to quizartinib (Figure 2C/D); however, the results suggest that only in the case of FLT-3 ITD driven cells is degradation better than inhibition. The PROTAC showed no anti-proliferative activity up to 5 μM in K562 cells revealing a >8000-fold selectivity window against non-AML cells.

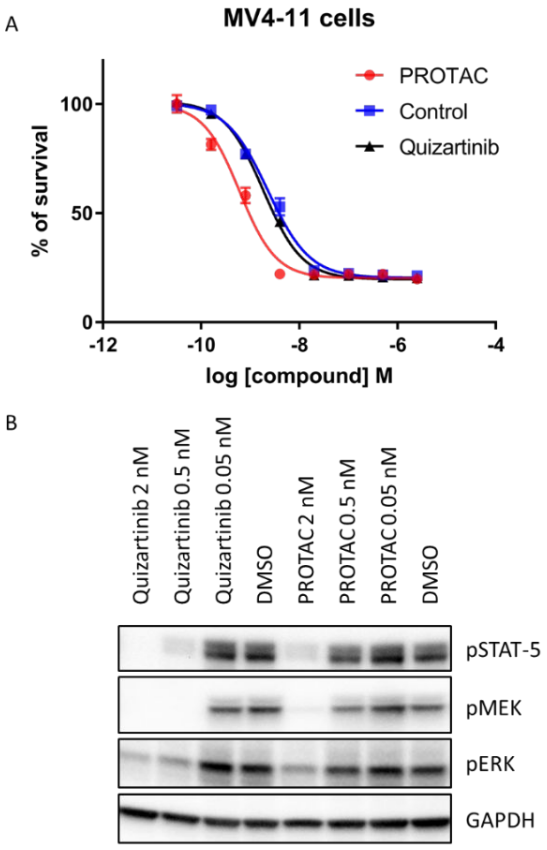


Figure 3. Cellular Evaluation of FLT-3 PROTACs. A – Cell Proliferation assay in MV4-11 cells. B – Analysis of downstream signaling by immunoblotting.

Given the enhanced anti-proliferative activity associated with FLT-3 ITD degradation, we sought to assess the effect of our PROTAC on signaling downstream of the kinase. MV4-11 cells were treated with either quizartinib or FLT-3 PROTAC in serum free media for 24 hours before lysis and immunoblotting for phosphorylation events downstream of FLT-3 (Figure 3B). Curiously, the PROTAC seems to be slightly less able to inhibit phosphorylation of STAT-5, MEK and ERK than quizartinib in MV4-11 cells despite its enhanced anti-proliferative effects (Figure 3A).

Seeking to further understand what may be providing the apparent disconnect between signaling and proliferation, the levels of apoptosis in MV4-11 cells treated with PROTAC, control and quizartinib were assessed by CaspaseGlo assay. At concentrations as low as 0.1 nM, a significant level of apoptosis induction (i.e. caspase activation) can be observed with PROTAC but not with quizartinib or the inactive control compound (Figure 4). Indeed, at all doses of PROTAC below 10 nM, caspase activation was significantly higher ~~better~~ than that following inhibition with either quizartinib or the inactive control. The greater potency of the PROTAC to cause caspase 3 activation (cleavage) was also confirmed by western blot (Figure S4). This suggests that full FLT-3 ITD-driven AML cell proliferation may depend on the presence of the protein itself and not just the increased activity of the kinase domain. This could partially explain the limited response of traditional inhibitors in the clinic. Further studies are required to elucidate the FLT-3 ITD kinase-independent functions and the corresponding pathway(s) through which degradation of FLT-3 ITD induces apoptosis.

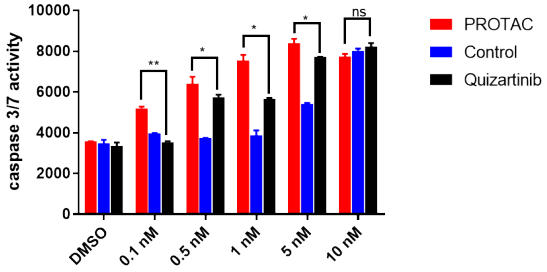
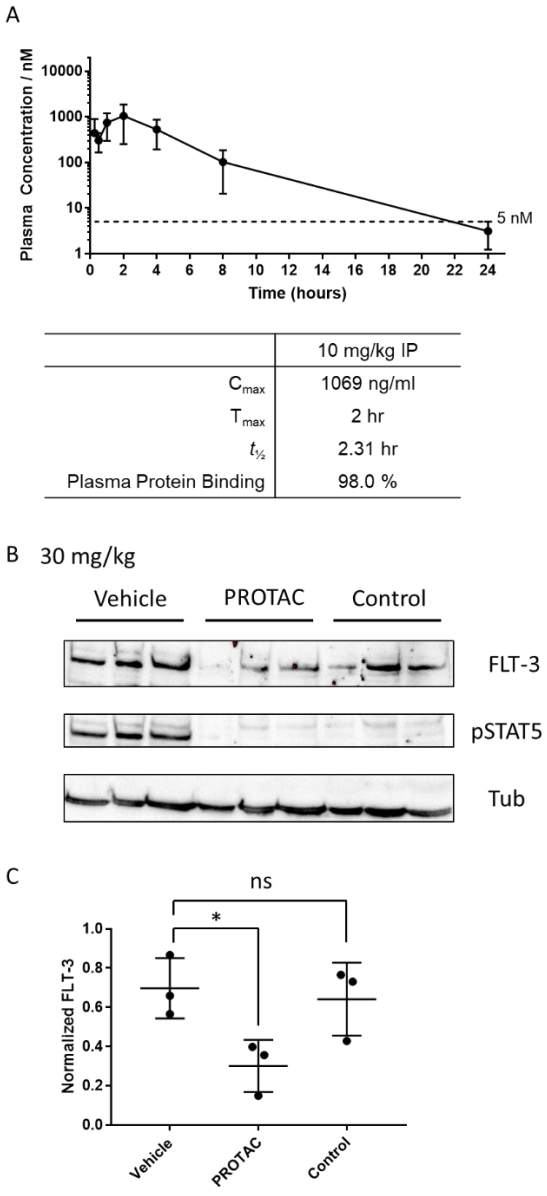


Figure 4. Apoptosis Induction in MV4-11 Cells. Caspase 3/7 activation in MV4-11 cells treated with PROTAC, control or quizartinib measured by CaspaseGlo assay

Finally, we sought to explore the *in vivo* activity of our compounds. Initial assessment of the pharmacokinetic properties of the FLT-3 PROTAC revealed acceptable pharmacological properties: a single 10 mg/kg IP injection followed by plasma level quantitation over the following 24 hours revealed a maximum concentration of just over 1 µM at 2 hours post injection and a half-life of just over 2.3 hours (n=3) (Figure 5A). Importantly, the plasma concentration remains above 5 nM, a concentration at which significant degradation is observed *in cellulo*, for approximately 22 hours post-injection. Based on these favorable pharmacokinetic properties, the PROTAC and the control compound were progressed into a small-scale pharmacodynamics study using a previously reported model system in which 9 athymic mice were injected subcutaneously with MV4-11 cells and tumors allowed to develop.[18] Once the tumors had reached approximately 200 mm^3, the mice were randomly assigned into three treatment groups; vehicle, PROTAC or control. The mice were treated once every 24 hours for 3 days with 30 mg/kg compound (to ensure sustained plasma levels >5 nM over 24 hours) or vehicle equivalent before euthanasia 5 hours following the final dose. No adverse effects were observed during this period. Tumor samples were collected and probed for FLT-3 levels as well as phospho-STAT-5 (Figure 5B). Under this treatment regime, both PROTAC and diastereomeric control are capable of inhibiting downstream signaling of FLT-3 ITD but, as expected, only the PROTAC is capable of inducing the degradation of FLT-3. Quantitation by densitometry and averaging of western blots reveals a significant decrease (approximately 60%) in total FLT-3 protein (normalized to tubulin as a loading control) in tumors from mice treated with PROTAC but no significant change in control treated animals (Figure 5C).



	10 mg/kg IP
C_{max}	1069 ng/ml
T_{max}	2 hr
$t_{½}$	2.31 hr
Plasma Protein Binding	98.0 %

Figure 5. In vivo evaluation of PROTACs. A – PK profile for PROTAC in mouse. B – Immunoblotting of MV4-11 xenograft tissue after 3 days of treatment with 30 mg/kg IP treatment. C – Quantification of FLT-3 protein levels in B.

In conclusion, we have shown that conversion of quizartinib into a VHL-recruiting PROTAC results in an *in vivo* active compound with a more restricted kinase interactome and that is a more potent inhibitor of FLT-3 ITD driven proliferation than quizartinib itself despite a moderate loss of inhibitory activity, both *in vitro* and *in cellulo*. The increased efficacy of growth inhibition can be attributed to the loss of the FLT-3 ITD protein itself, which appears to accelerate the induction of apoptosis. This study demonstrates that conversion of a kinase inhibitor into a PROTAC can not only yield a more efficacious compound but also a compound with enhanced selectivity, further revealing the power of this approach. Conceptually, this demonstrates a shift from occupancy-driven pharmacology to event-driven pharmacology.[19] Ongoing studies in our laboratory will focus on the elucidation of the mechanism of apoptosis induction and assessment of *in vivo* efficacy compared to quizartinib.

ASSOCIATED CONTENT

Supporting Information

Supplemental Figures 1-4, Scheme S1, experimental procedures, and details on the synthesis of compounds discussed above are included in the supporting information. (PDF) The Supporting Information is available free of charge on the ACS Publications website.

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Notes

C.M.C. is founder of, consultant to and shareholder in Arvinas Inc., which partially supports research in his lab. X.C. is an employee of Arvinas Inc.

ACKNOWLEDGMENT

G.M.B is a Fellow of The Leukemia & Lymphoma Society. J.S. acknowledges the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2016R1A6A3A03012989). J.H. gratefully acknowledges the US National Cancer Institute for their support (R50CA211252). C.M.C. gratefully acknowledges the US National Institutes of Health for their support (R35CA197589). We thank members of the Crews Laboratory for useful discussions. The MOLM-14 cell line was a gift from David Scadden, Harvard University. The MOLM-14 mutant FLT-3 ITD cell lines were a gift from Neil Shah, University of California, San Francisco.

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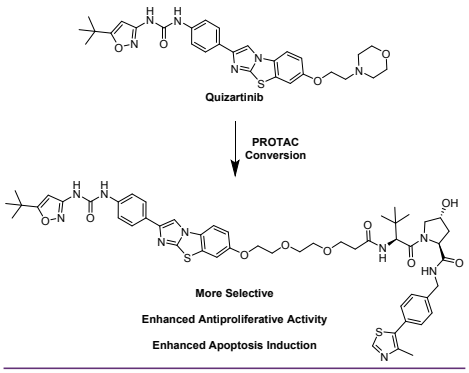
TOC Graphic:


Quizartinib
PROTAC
Conversion
More Selective
Enhanced Antiproliferative Activity
Enhanced Apoptosis Induction