Title
A method for overcoming plasma protein inhibition of tyrosine kinase inhibitors

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Abstract

FMS-like tyrosine kinase 3 (FLT3) is the most frequently-mutated gene in acute myeloid leukemia and a target for tyrosine kinase inhibitors (TKI). FLT3 TKI have yielded limited improvements to clinical outcomes. One reason for this is TKI inhibition by endogenous factors.

We characterized plasma protein binding of FLT3 TKI, specifically staurosporine-derivatives (STS-TKI) by alpha-1-acid glycoprotein (AGP); simulating its effects upon drug efficacy. Human AGP inhibits the anti-proliferative activity of STS-TKI in FLT3-ITD-dependent cells, with IC\textsubscript{50} shifts higher than clinically achievable. This is not seen with non-human plasma.

Mifepristone co-treatment, with its higher AGP affinity, improves TKI activity despite AGP, yielding IC\textsubscript{50}s predicted to be clinically effective. In a mouse model of AGP drug inhibition, mifepristone restores midostaurin activity. This suggests combinatorial methods for overcoming plasma protein inhibition of existing TKIs for leukemia as well as providing a platform for investigating the drug-protein interaction space for developing more potent small-molecule agents.

Statement of Significance

Our data provide a mechanism for the failure of some previous TKI clinical trials. The ability of mifepristone to disinhibit TKIs suggests an approach by which the combination of TKIs with already-approved and well-tolerated drugs may restore TKI activity.

Introduction

FMS-like tyrosine kinase 3 (FLT3) is the most frequently mutated gene in acute myeloid leukemia (AML). Approximately one third of adult cases of AML and a lower fraction of pediatric AML cases express a FLT3 mutation.\(1-3\) Wild-type FLT3 is expressed within early progenitor populations, and may also be expressed at a lower level within the hematopoietic stem cell (HSC) compartment. It continues to be expressed during differentiation, decreasing with terminal differentiation, although it does continue through dendritic cell development.\(4, 5\) In disease, FLT3 mutations lead to constitutive kinase activity, activating multiple signaling pathways including STAT5, AKT and MAPK/ERK.\(6, 7\) These in turn lead to the downstream effects that are the hallmarks of FLT3-mutant AML: differentiation blockade, inhibition of apoptosis, and a proliferative advantage.

FLT3 mutations occur in cooperation with mutations in many other genes, most frequently DNMT3A and NPM1, and are often the last step leading to transformation to AML.\(2\) The most common mutation is the internal tandem duplication (ITD), an in-frame duplication of a small sequence of variable length within the FLT3 juxtamembrane domain.
This leads to loss of autoregulation and subsequent constitutive FLT3/ITD activity. As a high-risk feature, it is associated with increased rates of refractory disease and relapse, and decreased overall survival. In studies, patients with FLT3/ITD AML have a 15-25% event-free survival in response to standard AML chemotherapy protocols compared with 40-60% in patients without. As a result, FLT3-mutated AML has not benefited from many of the advances seen in outcomes for other leukemias. The addition of bone marrow transplant with its short and long-term toxicities is necessary to improve outcome. Yet, in contrast to their chemoresistance, AML blasts with FLT3 mutations are preferentially sensitive to tyrosine kinase inhibitors (TKI) against FLT3, making FLT3 an attractive target for drug development.

Despite the promise of FLT3-directed therapy, FLT3 TKI clinical trials have had limited success. Although several FLT3 TKI have been approved for clinical use, it is clear that more effective targeting is needed. Multiple factors contributed to these outcomes: FLT3 resistance mutations, bone marrow stromal-derived survival factors, overexpression of FLT3 ligand, and activation of alternative “bypass” pathways. The study of these has led to FLT3 TKI with ever-increasing efficacy. However, the role of patient-specific factors in modulating treatment efficacy remains relatively unexplored in the context of FLT3 AML research.

Prior in vitro and clinical trial data indicate that the lack of FLT3-specific clinical impact by TKI therapy may be due, in part, to inadequate activity against FLT3 due to inhibition of these agents by patient-specific factors such as plasma protein binding, stromal protection and altered pharmacokinetics. Indeed, we hypothesize that suppression of FLT3 TKI-mediated inhibition through plasma protein binding is a leading cause of limited clinical impact. Thus, we have undertaken a study to determine the impact of plasma protein binding on FLT3 TKI activity, focusing on the staurosporine family of which the recently-approved drug midostaurin is a member. Furthermore, we report here a method for overcoming plasma protein inhibition to restore the promise of molecularly targeted therapy in FLT3 mutated AML as well as for other TKI whose activity is diminished by protein binding.

Results

Human plasma and plasma proteins reduce TKI efficacy

In the original plasma inhibitory assay (PIA), FLT3/ITD-expressing cells are co-incubated with plasma samples from patients receiving a FLT3 TKI, and the extent of FLT3 inhibitory activity present in their plasma measured by Western blotting analysis of FLT3 auto-phosphorylation. This is compared to total plasma drug levels to determine the relative efficacy of a drug in vivo compared to its activity in vitro and thus impute the effects of host factors on drug efficacy beyond simple metabolism. To model these effects in the pre-clinical setting, we modified the PIA assay. Whereas the PIA was designed to assess in vivo drug activity with regards to a specific target (e.g.: inhibition of FLT3
Overcoming plasma protein inhibition of TKI auto-phosphorylation by TKI), the modified PIA is similarly cell-based, but measures the clinically relevant biological endpoints of cytotoxicity and proliferation inhibition. In the modified assay, FLT3/ITD cell lines are cultured in the presence of human plasma or purified human plasma proteins and treated with a range of concentrations of the drug of interest. The IC\textsubscript{50} measured using the colorimetric MTT method is compared to treatment under typical \textit{in vitro} culture conditions of 10% fetal bovine serum (Figure 1A). Like the original PIA, Western blot analysis can be performed on cells treated in this manner to study specific pathways of interest (Figure 1B). Thus, it is possible to simulate the \textit{in vivo} effect of plasma proteins upon drug activity, allowing one to investigate the effect of endogenous proteins in their native state upon drug dose-response relationships in a controlled manner without the confounding variables of patient-specific drug bioavailability and metabolism. Utilizing the general endpoint of cytotoxicity, the modified PIA is readily adaptable to other disease types and agents where plasma protein binding has been implicated in affecting therapeutic efficacy.

We treated MOLM-14 cells (FLT3/ITD heterozygous, KMT2A-rearranged myeloid leukemia cell line) for 48 hours with midostaurin, lestaurtinib or TTT-3002 (a novel FLT3 TKI) across a range of physiologically relevant concentrations in the presence of either 50% human plasma from healthy donors or standard 10% fetal bovine serum culture conditions (Figure 1A). (10, 11) For all three TKI, the addition of human plasma significantly increased the IC\textsubscript{50} of each drug, and for midostaurin and lestaurtinib, IC\textsubscript{50}s were no longer reached in the range of the conditions tested.

To demonstrate that this effect is through reduced FLT3 inhibition, we treated MOLM-14 cells with each TKI at concentrations greater than the IC\textsubscript{50} for inhibition of FLT3 autophosphorylation (10 nM and 100 nM) and assessed FLT3 autophosphorylation and downstream signaling in the presence or absence of human plasma (Figure 1B). For TTT-3002, the presence of human plasma lead to a significant decrease in inhibition of FLT3 phosphorylation, and decreased downstream inhibition of phosphorylation/activation of STAT5, AKT1 and ERK1/2. Although this effect was overcome by increasing the concentration of TTT-3002, for midostaurin and lestaurtinib, there was no appreciable FLT3 TKI activity in the presence of plasma even with a ten-fold increase in drug. Previous work and clinical trials with FLT3 TKI have suggested that alpha-1-acid glycoprotein (AGP), an acute phase acid glycoprotein responsible for scavenging basic drugs and steroids, plays a role in binding FLT3 TKI.(11-17) We repeated these experiments in the presence of physiologically equivalent concentrations of purified AGP (1 mg/mL) instead of whole human plasma, and comparable inhibition of anti-FLT3 activity was again observed (Figure 1B).

We repeated the modified PIA, assaying for apoptosis after 24 hours, and found that the presence of human plasma abrogates TKI-induced apoptosis (Supplemental Figure 1A) and associated PARP cleavage (Figure 1C). Again, the use of physiologic concentrations of human AGP in place of whole plasma is sufficient to reproduce this effect. These results were also seen in the TKI-mediated effects on cell cycle analysis (Supplemental Figure 1B). The addition of
human AGP alone does not affect FLT3 signaling, baseline apoptosis, or cell cycle distribution. These data indicate that human AGP plays a significant role in the observed plasma inhibition of TKI activity, presumably sequestering the drugs away from their cellular target. Furthermore, when these experiments are repeated using bovine plasma or bovine AGP, there is little effect on the activity of FLT3 TKI (Figure 1D,1E). These results, along with the lower expression of AGP in mice and cow, likely explain why these effects are not observed in pre-clinical in vitro and in vivo murine studies.(18-20)

FLT3 TKI are differentially affected by human plasma and purified human plasma proteins

We tested a panel of FLT3 TKI for activity in the modified PIA. All TKI tested demonstrate increases in IC\textsubscript{50} in the presence of 50% human plasma (Figure 2A). These range from minimal (9-fold) inhibition for TTT-3002 to moderate inhibition (37-fold) for quizartinib to greater than 100-fold inhibition for lestaurtinib, midostaurin, and sorafenib. When tested using purified human AGP at physiologic concentrations (1 mg/mL), quizartinib and sorafenib activity were not significant inhibited, whereas TTT-3002 demonstrated comparable inhibition to that observed with human plasma (Figure 2B). Complete inhibition of lestaurtinib and midostaurin activities was observed, even at sub-physiologic concentrations. Examination of structures show that the TKI affected significantly by AGP (lestaurtinib, midostaurin, TTT-3002) are all derivatives of staurosporine, in contrast to those unaffected by AGP (Figure 2C). In comparison, gilteritinib, the FDA-approved non-staurosporine TKI is minimally affected by the presence of plasma proteins (Supplemental Figure 1C).

Plasma AGP concentration is the primary determinant of plasma protein inhibition of staurosporine-derived TKI

To characterize the impact of human plasma on the staurosporine-derived TKI, we examined the relationship between fold-change in the IC\textsubscript{50} of TTT-3002 and AGP concentration. When the modified PIA was performed for TTT-3002 using purified human AGP across a range of concentrations, a relationship was seen between AGP concentration and the associated TTT-3002 dose-response curves (Figure 3A). Measuring IC\textsubscript{50} at each AGP concentration and comparing it to that observed under standard culture conditions (IC\textsubscript{50} fold-change), a direct linear relationship was noted between IC\textsubscript{50} fold-change and AGP concentration (Figure 3B). A similar relationship was noted for lestaurtinib, wherein, appreciable activity is only seen at AGP concentrations less than one-tenth of physiologic human levels (Supplemental Figure 2).

As these results suggest that the major determinant of plasma inhibition of staurosporine TKI is AGP, we studied the relationship of plasma AGP concentration to plasma inhibition. Plasma samples from 19 human donors were collected, AGP concentration was measured (124 ± 66 mg/dL), and TTT-3002 inhibition by the plasma was tested using the modified PIA. There is a linear correlation between plasma AGP concentration in the samples and the resulting IC\textsubscript{50} fold-change (Figure 3C, solid line) which correlates closely to the results observed using purified AGP (Figure 3C, dashed line). Furthermore, univariate ($R^2$) and multivariate analyses ($P$) of total protein concentration ($R^2 = 0.07$,
Overcoming plasma protein inhibition of TKI

P = 0.38), albumin concentration ($R^2 = 0.02, P = 0.91$), platelet count ($R^2 = 0.04, P = 0.65$), hemoglobin concentration ($R^2 = 0.03, P = 0.25$) did not demonstrate any additional factors with a significant correlation with IC$_{50}$ fold-change, although concomitant medications were not accounted for and may represent a confounding hidden variable.

The modified PIA measures TKI-AGP affinity

We examined the relationship of IC$_{50}$ fold-change and plasma protein concentration (see Supplemental Discussion for derivation), and demonstrated:

$$\Delta = K_A (P_0 - P_A) + 1$$

Where $\Delta$ is the IC$_{50}$ fold-change, $K_A$ is the drug-protein association constant, $P_0$ is the total protein concentration and $P_A$ is the drug-bound protein concentration. In the absence of other drug-protein interactions, fold-change is linearly related to drug-free protein concentration. By using this relationship, comparing IC$_{50}$ fold change to plasma AGP (Figure 3A) yields biochemical data about the interaction of TKI with their associated plasma protein. When the analysis takes into account drug-bound protein, these curves can be used to directly measure $K_A$. To that end, we performed the modified PIA using purified AGP against the staurosporine-derived FLT3 TKI, using MOLM-14 cells (Figure 4A). For comparison, we also assayed the anti-proliferative effects of staurosporine upon HL-60 cells (FLT3 wild-type AML).(21) Different TKI show variable sensitivity to AGP inhibition ($P < 2 \times 10^{-16}$) with TTT-3002 being the least impacted (30-fold inhibition at physiologic concentrations) midostaurin showing intermediate inhibition (300-fold inhibition) and lestaurtinib showing the highest (>1000-fold inhibition predicted). In comparison, staurosporine demonstrated no appreciable activity in the presence of AGP until saturating concentrations of drug were used. Using regression analysis on the linear areas of these curves, we calculated binding constants for each drug (Figure 4B). Of note, although midostaurin shows only intermediate levels of inhibition, when correcting for lower potency for FLT3 inhibition compared to lestaurtinib, the two drugs are predicted to have approximately the same in vivo activity (Supplemental Figure 3).

To validate this approach as a quantitative assay, we also performed traditional competitive fluorescence displacement assays using 8-anilinonaphthalene1-sulfonic acid (ANS), a polyaromatic compound that only fluoresces when bound to AGP.(22) By measuring ANS fluorescence across a range of concentrations with fixed AGP, in the presence or absence of a competing drug of interest and then performing Scatchard analysis, we were able to use the Cheng-Prusoff approximation to measure drug-AGP affinity (Figure 4C). These results were comparable to the results of the cell-based approach.

Finally, to demonstrate the clinical relevance of these findings, we collected samples from a second series of newly-diagnosed adult leukemia patients (Supplemental Table 1), and examined the effect of their plasma upon midostaurin activity. As expected, the modified PIA demonstrated significant loss in potency when co-incubated in 12.5%
human plasma (at higher concentrations, appreciable anti-FLT3 activity could not be quantitatively measured, unlike TTT-3002 which is not as severely shifted). This shift in IC$_{50}$ followed a linear relationship with plasma AGP (Figure 4D, only univariate analysis was performed on this group). For the lowest level AGP samples (patient 9, 1.67 mg/mL), even 12.5% plasma was sufficient to increase the IC$_{50}$ 30-fold, and for about half of the patient samples, there was a hundred-fold or greater shift in IC$_{50}$ from nanomolar to the micromolar range. In 100% plasma, this would be expected to yield an IC$_{50}$ of 10-20 µM, with IC$_{90-95}$ levels (i.e., the pharmacologic goal) five-fold higher still. The patient plasma demonstrated a shift of 220-fold (±39) per mg/mL AGP, which is similar to exogenous human AGP (300-fold per mg/mL, $P = 0.07$). Of note, this new diagnosis cohort had a two-and-a-half-fold greater AGP level compared with the initial cohort (Figure 3C, 270±154 mg/dL versus 117±64 mg/dL, $P = 9 	imes 10^{-4}$).

Overcoming plasma protein inhibition with mifepristone

The above assumes the absence of competitive drug-protein interactions. When we include a competitive binder of AGP (I), we find the fold-change/protein concentration relationship can be formulated as:

$$\Delta = \frac{K_A}{2K_I} \left( \beta - \gamma + \sqrt{(\beta - \gamma)^2 + 4\beta} \right) + 1$$

Where:

$$\gamma = K_I I_0 + 1$$

$$\beta = K_I \tau$$

The derivation and a discussion of the significance of these relationships are described in the supplemental discussion. This relationship suggest that an competitor binding with comparable affinity to AGP will have a significant impact upon fold-change, reducing the overall protein inhibition of the drug (Figure 5A).

We undertook a proof-of-principle demonstration of disinhibiting FLT3 TKI using unrelated drugs to displace TKI. Of the known AGP-bound drugs, the steroid-derived, estrogen receptor antagonist, mifepristone has one of the highest association constants reported.\(^{(22, 23)}\) With a $K_A$ of 7-8 µM$^{-1}$, it is predicted to bind to AGP with higher affinity than TTT-3002 and comparable affinity to lestaurtinib and midostaurin. Competitive fluorescence displacement confirms mifepristone displaces ANS from AGP at concentrations 1.5 to 2-fold lower than midostaurin and lestaurtinib (Figure 5B). We performed the modified PIA using TTT-3002 and purified AGP in the presence of increasing concentrations of mifepristone, we found that the dose-response curve shifted back to that seen in the absence of AGP (Figure 5C). Mifepristone had no impact upon the TTT-3002 dose-response curves in the absence of AGP. Furthermore, when we titrated mifepristone in the presence or absence of AGP and TKI, we found that growth inhibition of MOLM-14 cells by TKI in the presence of AGP was returned to that seen in the absence of AGP at concentrations of mifepristone comparable to
those achieved in clinical trials (Figure 5D). The maximal effect occurs at concentrations where mifepristone alone has no appreciable effect on cell growth. Similar effects were not seen using other known AGP-binding agents with lower affinities (Supplemental Figure 4). These effects (Figure 5E) agree within experimental limits to the model described above (Figure 5A).

When MOLM-14 cells are treated with TTT-3002 in the presence of inhibiting concentrations of AGP, FLT3 inhibition is lost and de-repression of downstream signaling is observed. Mifepristone alone has no effect upon FLT3 autophosphorylation or downstream signaling. However, the addition of mifepristone at increasing concentrations results in a dose-dependent restoration of FLT3 inhibition by TTT-3002 with loss of FLT3-dependent downstream signaling (Figure 5F). This provides further support that mifepristone is acting through competitive displacement of TKI from AGP, not off-target effects.

We hypothesized that among the various FDA-approved drugs and supplements, there exists a subset of agents that, like mifepristone, can bind AGP and displace bound TKI, thus restoring anti-FLT3 activity. These agents might be used as combinatorial agents in restoring clinical activity of the staurosporine derivatives or serve as lead compounds for the development of such agents. Therefore, we screened the Johns Hopkins Drug Library (JHDL), a collection of 2,560 different agents approved for human or veterinary use. FLT3/ITD-expressing Ba/F3 cells were co-cultured in cytotoxic concentrations of midostaurin (100 nM) with inhibiting levels (0.5 mg/mL) of human AGP. To these we added the various agents of the JHDL at 20 µM and measured cell growth by colorimetric means. To confirm that any cytotoxicity was FLT3-specific, and not a consequence of off-target activity by the JHDL compounds themselves, we performed parallel platings in the presence or absence of recombinant murine IL-3. The IL-3 (upon which the parental cells are dependent) will rescue the cells from FLT3-specific cytotoxicity but not from off-target cytotoxicity. The majority of compounds had no impact upon cell growth or were equally cytotoxic in the presence or absence of IL-3 (Figure 6A). However, we identified 219 different agents that showed less cytotoxicity in the presence of IL-3. Although approximately 30 compounds were still significantly cytotoxic in the presence of IL-3 (Figure 6A, area 2), the cytotoxicity of many of the compounds was mostly IL-3-reversible (area 1). This subset of compounds may act by displacing midostaurin from AGP and restoring its anti-FLT3 activity.

To further screen these agents, we performed competitive fluorescence displacement with the JHDL compounds. We measured the relative fluorescence intensity of AGP-bound ANS in the presence of the different agents, normalizing to intensity in the absence of competing drug. When IL-3-independent growth inhibition (1 minus the ratio of growth in the absence and presence of IL-3) is compared to ANS displacement (Figure 6B), several classes of agents are identified. The majority of agents (n = 2343) have neither FLT3-specific growth inhibitory nor AGP-binding activities, while a small
Overcoming plasma protein inhibition of TKI subset \((n = 88)\) bind to AGP but are unable to restore anti-FLT3 activity. Interestingly, a small group of agents \((n = 91, upper \text{ right} \text{ quadrant})\) do not appear to bind to AGP (at least not competitively with ANS), but nonetheless induce cytotoxicity that is IL-3-reversible. It is possible that these agents have intrinsic anti-FLT3 activity, or that they are synergistic with midostaurin such that the low levels of FLT3 inhibition present are now sufficient to induce cytotoxicity by either targeting alternative pathways or directly targeting downstream signaling pathways. In addition, a group of 38 agents (indicated by the dashed ellipse, upper left quadrant) are able to both bind to AGP and restore anti-FLT3 activity. There are six-fold more agents in this fourth group than would be expected based upon the number of agents found in the other groups \((38 \text{ identified vs. } 6.3 \text{ expected}, P = 2.73 \times 10^{-23}, \text{ Fisher’s exact test})\). An initial validation of the 10 lead compounds demonstrated at least one, trihexyphenidyl hydrochloride, that is able to improve midostaurin activity in the presence of inhibiting AGP concentrations \((\text{Figure 6C})\) to an extent similar to the effects seen with mifepristone \((\text{Figure 5C})\). Testing of other candidates is in progress.

We performed an \textit{in vivo} proof-of-principle of competitive disinhibition of TKI in mice \((\text{Figure 7A})\). Murine AGP has very limited homology to human AGP, is expressed at levels five- to ten-fold lower than humans, and work in rats has demonstrated physiologically significant differences between human and rodent AGP. Therefore, we created a mouse model for testing the effects of human AGP \textit{in vivo}. Mice injected with human AGP at 0.3 g/kg intraperitoneally every 48 hours demonstrate serum AGP levels ranging from 0.5 – 3 mg/mL \((\text{Figure 7B})\), with a terminal half-life of approximately 17 hours. Consistent with previous work in rats, mice do not demonstrate any appreciable toxicities from this administration over the two weeks they were observed. We transplanted sublethally-irradiated BALB/c mice with Ba/F3-FLT3/ITD-Luc cells (expressing both FLT3/ITD and a luciferase reporter). After 5 days, luciferin bioluminescence confirmed adequate engraftment \((\text{Figure 7C})\). The mice were then treated with orally gavaged midostaurin or vehicle for four days. At the same time, a subset of the midostaurin-treated mice were also conditioned with intraperitoneal human AGP, or AGP plus oral mifepristone. The remaining were injected with PBS vehicle. Twenty-four hours after the final treatment (day 9), the mice were again imaged \((\text{Figure 7C})\). All mock-treated mice demonstrated progression of the FLT3/ITD-expressing cells whereas the midostaurin alone treated mice showed regression of the cells over the treatment course. However, treated mice that receive intraperitoneal AGP demonstrate progression of the cells comparable to that seen in the control animals. In contrast, when the midostaurin/AGP dually-treated mice were also gavaged mifepristone daily during treatment, the mice again demonstrate regression of the transplanted cells comparable to those of the midostaurin-alone treated mice.
Discussion

Since the discovery of FLT3 and its associated activating mutations, it has remained a consistent target for drug development.(29) Two approaches, rational drug design and library-based screening, have been used for the discovery of new anti-FLT3 agents, with subsequent generations demonstrating increasing potency in vitro. These agents tend to be either staurosporine derivatives such as midostaurin and lestaurtinib or are complex poly-aromatic compounds such as gilteritinib, quizartinib and sorafenib. Staurosporines tend to be very potent, possibly due to additional off-target inhibition of targets such as PKC. They are also active against the FLT3 kinase domain mutations that have been associated with resistance to some of the FLT3 TKI that have no activity against such mutations. However, they have significant dose-limiting toxicities, especially gastrointestinal, likely due to the same off-target effects. By contrast, some non-staurosporine agents, with more selective anti-FLT3 activity, often demonstrate higher in vitro IC₅₀s (i.e. reduced potency) and can be inhibited by more resistance-associated point mutations, but tend to be less dose-limited.(30) Yet, despite this wide selection of diverse inhibitors spanning two decades, clinical trials have often fallen short of the expectations of pre-clinical work. In addition to prior concerns for resistance mutations, we believe that the results presented here highlight an underappreciated pitfall in the development of drugs for FLT3 AML that is likely contributing to their clinical underperformance.

One particularly informative FLT3 TKI clinical trial was that of lestaurtinib for relapsed FLT3 mutant AML.(31-33) There, the addition of lestaurtinib to standard induction therapy failed improve response rates or overall survival. This was despite in vitro potency, and patients achieving plasma drug levels nearly 1000-fold higher than necessary for in vitro cytotoxicity. When patient plasma samples were directly tested for the ability to inhibit FLT3 using the original PIA, most samples failed to demonstrate sufficient levels (>90%) of inhibitory activity. However, for those patients whose samples did achieve high levels of FLT3 inhibitory activity, there was an increase in attaining a complete remission. FLT3-inhibitory activity did not always correlate with plasma drug levels.(9) The dissociation of drug activity from level (pharmacodynamics from pharmacokinetics) was the earliest suggestion that FLT3 TKI therapy is greatly modulated by drug availability within the plasma compartment. The results presented here provide a mechanistic explanation whereby the predicted IC₅₀ is significantly higher than most of the levels seen in patients despite excellent total drug levels.

Concerns regarding the effect of host factors on FLT3 TKI therapy have also been raised in response to the recent trial that led to the FDA approval of midostaurin. Midostaurin added to standard induction therapy yielded a modest, but significant improvement in overall survival for patients regardless of FLT3 mutational type or burden.(34) Yet these different FLT3 mutant disease subtypes have been demonstrated to be clinically and biologically very distinct entities, not equally dependent upon FLT3.(1, 3, 35-38) Relapse data suggests that in some patients there is nonetheless
selective pressure from anti-FLT3 activity. However, our data indicate that for many patients, midostaurin would have adequately cytotoxic anti-FLT3 activity only at concentrations significantly higher than drug levels typically achieved in clinical trials. This suggests that its potent \textit{in vitro} anti-FLT3 activity is significantly attenuated if not largely lost \textit{in vivo}, and survival benefits may be through non-FLT3 effects, especially in non-ITD disease. This is consistent with findings that midostaurin inhibits a multitude of targets in the kinome and may even have general anti-leukemic effects.

The drug-binding effects demonstrated in this report are not limited to AML. At least two other trials have experienced significant complications due to the drug-binding effects of AGP. Trials with 7-hydroxy-staurosporine for refractory neoplasms demonstrated disappointing results despite promising pre-clinical results. Pharmacologic work identified drug-binding to AGP as having been a complicating factor not predicted in pre-clinical models. A separate trial using the non-staurosporine TKI, filanesib for relapsed and refractory multiple myeloma met with similar results. The filanesib trial demonstrated a clear association between plasma AGP concentration and response, with the few responders having the lowest AGP concentrations of the cohort. Further complicating the clinical picture, natural AGP variants can have even greater effects upon drug activity. Finally, as an acute phase reactant, AGP concentration is highly variable in disease states such as AML. Indeed, our new-diagnosis validation cohort demonstrated markedly higher AGP levels compared to the initial cohort comprised of control and post-induction samples. Although this study did not specifically examine the relationship between AGP concentration and disease state, it is intriguing to think that AGP could be used as a biomarker to select either specific TKI or combinatorial approaches for patients.

These effects are not new; FDA approval involves assaying protein binding, and the literature has many similar “re-discoveries” of the problem of plasma protein binding. As drug development becomes more specialized, the health and economic consequences of ignoring drug binding effects during the pre-clinical stage of drug development and until they are observed in the clinical setting can be significant. The work presented here indicates that significant species-specific differences in homology and expression levels of plasma proteins masks these effects in tissue culture and animal models. Even mouse models designed to study AGP binding utilize non-human constructs and could thus fail to predict binding and subsequent failure of the drugs in clinical trials. Our data show that bovine AGP, even when adjusted to human-comparable levels, produces an approximate five-fold IC\textsubscript{50} increase for lestaurtinib, the most tightly bound of the staurosporines, compared to the 500-fold shift produced by human AGP. This mirrors prior work that demonstrated a protective effect of human AGP in reperfusion injury that is not found with mouse AGP. Furthermore, cow and mouse plasma contain significantly lower AGP concentrations (2-10 fold lower) compared with human plasma. These findings indicate the need for new models to study drug-protein binding which faithfully recapitulate
human-specific effects. To our knowledge, this is the first time such a model has been demonstrated for drug testing and represents an exciting new platform for drug development. In the meantime, it is critical for investigators and drug developers to be aware of the limitations of current models and implement testing such as the modified PIA to avoid these failures.

Using a second drug to increase the free-drug concentration of target drugs to levels that are able to more fully inhibit the intended target is one way to potentially increase efficacy. Additionally, by indirectly increasing drug potency, it may be possible to reduce drug dose. This may lead to reduced gastrointestinal exposure and potentially reduce one of the most significant dose-limiting toxicities observed in clinical trials. Thus, it may be possible to decrease drug dose while nonetheless improving efficacy. Although testing and validation continues, our screen has demonstrated that other agents exist with properties similar to mifepristone, and such agents could either form the basis of future combinatorial clinical trials or serve as lead compounds to new, biologically inactive, plasma protein “decoys”. Indeed, even if none of these agents prove to be feasible for clinical use, this new method of screening agents and probing the drug-protein interaction space should provide a wealth of information regarding drug structure-protein interaction to inform the development of new decoys and future drug development (i.e. chemical motifs best avoided during drug development). It is encouraging that mifepristone, a drug that is highly specific for the estrogen receptor, with few off-target effects, has shown promise both in vitro and in vivo. This suggests that this FDA-approved drug may be a candidate itself for use in an early-phase trial to develop this combinatorial approach for overcoming plasma protein binding. Through better awareness of drug-protein interactions, newer models for studying the effects, and the use of combinatorial therapeutic approaches, it may be possible to finally realize the potential of targeted therapies, for FLT3 mutant AML and for other diseases where molecularly target therapies have failed to achieve their full promise.

Methods

Patient plasma

Unless otherwise specified, pooled plasma from healthy donors was used for all experiments. Patient samples were collected from patients seen in the pediatric oncology clinic at Johns Hopkins Hospital between November 2015 and March 2016. Patients were enrolled in an IRB-approved, institutional banking protocol with written informed patient consent in accordance with the Declaration of Helsinki. Whole blood was collected by venipuncture in sodium heparin-treated vacutainer tubes (BD). Within 2 hours of collection, cellular components are separated from the plasma by centrifugation twice at 2,000 x g for 10 minutes, stored until use at -80°C, and clarified immediately prior to use by
Overcoming plasma protein inhibition of TKI centrifugation at ≥15,000 x g for 3 minutes. Plasma AGP concentrations are measured by radial immunodiffusion assay (Kent Labs) according to the manufacturer’s protocols.

**Reagents**

AGP purified from human or bovine plasma (Sigma) was resuspended in unsupplemented RPMI 1640 (Gibco) at 10 mg/mL for working stocks. Recombinant human serum albumin (Sigma) was resuspended in RPMI 1640 at 100 mg/mL. Lyophilized bovine plasma (Sigma) was reconstituted in sterile PBS (Gibco). TTT-3002 was a generous gift of TauTaTis, Inc. (San Diego, CA). Lestaurtinib, midostaurin, sorafenib and quizartinib were purchased from LC Laboratories. Each FLT3 TKI was dissolved at 10 mM in 100% sterile-filtered dimethylsulfoxide (DMSO, Sigma). Miltefrone (Sigma) was dissolved at 100 mM in 100% DMSO. Trihexyphenidyl hydrochloride (Selleckchem) was dissolved at 100 mM in methanol. For cell-based assays, working stocks of 10 µM were prepared for each drug in RPMI 1640 supplemented with 0.1% DMSO and 0.2% bovine serum albumin. For spectrophotometric studies, working stocks of 10 µM were prepared using PBS without sera or albumin. 8-anilinonaphthalene1-sulfonic acid (ANS, Sigma) was dissolved in DMSO at 200 mM, and then diluted to 400 µM with PBS (0.2% DMSO, final). Western analysis was performed using the FLT3 S-18 antibody (Santa Cruz), and for other proteins as indicated (Cell Signaling Technology).

**Cell lines**

Unless otherwise stated, MOLM-14 cells (DSMZ Cat# ACC-777, RRID:CVCL_7916) and Ba/F3 (DSMZ, Cat# ACC-300, RRID:CVCL_0161) are grown in RPMI 1640 media, supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gemini), with antibiotics. The Ba/F3-FLT3/ITD cell line is a Ba/F3 cell line into which the neomycin-selectable pBABE vector with FLT3/ITD has been stably incorporated, as previously described.(30, 52) Ba/F3-FLT3/ITD-Luc cell line was the BaF3 FLT3/ITD cells transfected with the L3-3GFP plasmid containing genes for luciferase and green fluorescent protein (GFP).(52) Parental Ba/F3 cells are cultured with 1 ng/mL recombinant mouse IL-3, whereas Ba/F3-FLT3/ITD and Ba/F3-FLT3/ITD-Luc cells are maintained without supplemental cytokines. Cells are maintained at 4 x 10^5 – 2 x 10^6 cells per milliliter and cultured in humidified, 37°C incubators with 5% carbon dioxide. All cell line work was performed using mycoplasma-free (confirmed by PCR) stocks, and used within 1 month of thawing (approximately 12 passages).

**Modified Plasma Inhibition Assay**

Cells in logarithmic growth are resuspended in either human plasma or serum-free RPMI 1640 media supplemented with the plasma protein of interest and seeded in 96-well format at 50 µL per well. Cell density is 2 x 10^5 cells per well for MOLM-14 and Ba/F3-FLT3/ITD cells. To each well 50 µL of 2x drug dilution in RPMI 1640 media with 10% serum was added. Each condition is plated in quadruplicate. The cells are then incubated at 37°C. After 44 hours of treatment, 10 µL of 5 mg/mL thiazolyl blue tetrazolium bromide (MTT, Sigma) in PBS, sterile-filtered, is added to each well, and the plates
are incubated for an additional 4 hours at 37°C. 100 µL of 10% sodium dodecyl sulfate (JT Baker) in 10 mM hydrochloric acid (Fisher) is then added to each well, and the plates are incubated at 37°C overnight. Optical absorbance at 570 nm is measured via plate reader (iMark, Bio-Rad), averaging across technical replicates. 50% inhibition of proliferation (IC_{50}) values were calculated by linear regression analysis relative to cells cultured without drug(s) in the corresponding presence or absence of plasma or plasma proteins.

**Fluorescence displacement titration**

Drug affinity is measured by displacement of ANS from AGP using the previously described fluorescent spectrophotometric method.(22) The drug of interest, dissolved in serum-free PBS, is combined with AGP and ANS for final concentrations of 1 µM drug, 0.5 mg/mL AGP and ANS at a range of 90 nM to 50 µM. Drugs and protein are allowed to equilibrate in the dark at room temperature for 2 hours. Fluorescence is measured (emission 470 nm, excitation 400 nm) with a SpectraMax M3 Multi-Mode Microplate reader (Molecular Devices). Maximal fluorescence is determined by measuring ANS fluorescence in the presence of 100 µM human serum albumin (Sigma) without drug. Bound ANS is calculated as the product of total ANS concentration times the fraction of maximal fluorescence observed. The association constant (K_a) of ANS with AGP in the absence of drug is calculated by Scatchard analysis, and the binding constants determined by the Cheng-Prusoff approximation.

**Animal Studies**

BALB/c (IMSR Cat# JAX:000651, RRID:IMSR_JAX:000651) mice were sub-lethally X-ray irradiated with 300 cGy (CIXD irradiator, Xstrahl) and injected with 5 x 10^5 Ba/F3-FLT3/ITD-Luc cells via tail vein. Intraperitoneal injections were performed using PBS vehicle with human AGP (Sigma) at 20 mg/mL. Midostaurin was suspended in 30% (w/v) Cremophor EL, 30% (w/v) PEG 400, 10% ethanol, and 10% glucose (all Sigma), and instilled once daily by oral gavage (10, 11). These drug doses have previously been demonstrated to be effective in mice. Mifepristone (Sigma) was dissolved in 50% ethanol and 50% (2-hydroxypropyl)-β-cyclodextrin (Sigma) and gavaged every 48 hours. Mice were imaged by intraperitoneal injection of luciferin (3 mg) and visualizing on an IVIS Spectrum imager (Caliper LifeSciences) using Living Image software for analysis on day 5 (to monitor engraftment) and on day 9 (to assess drug effect). Animal husbandry and procedures were conducted in accordance with the policy of the Johns Hopkins University School of Medicine Animal Care and Use Committee.

**Author Contributions**

D.J.Y. and D.S. initiated the project, designed the experiments, analyzed the data, and wrote the manuscript. D.J.Y., B.N., L.L., and T.H. performed the experiments. J.O.L. provided reagents, assisted in experimental design and interpretation.
M.J.L. provided patient care and supplied patient samples and respective clinical data. All authors reviewed the manuscript.

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References
Overcoming plasma protein inhibition of TKI


Overcoming plasma protein inhibition of TKI


Figures

Figure 1: FLT3 inhibitors are differentially inhibited by human plasma and AGP. Co-incubation with human plasma or human AGP inhibits FLT3 TKI activity. (A) Proliferation of MOLM-14 cells after 48 hours of TKI exposure in the presence or absence of 50% human plasma was assessed by colorimetric methods (MTT). (B) MOLM-14 cells were exposed to low (10 nM) or high (100 nM) concentrations of TKI as well as media (10% FBS), 50% human plasma or human AGP and analyzed by phospho-Western blotting for FLT3 phosphorylation and downstream signaling. (C) Western analysis for cleaved PARP after 24 hours of TKI exposure under standard culture conditions (10% FBS) or in the presence of either 100% human plasma or purified human AGP. (D) Proliferation and (E) Western analysis experiments were repeated in the presence of 50% FBS, bovine plasma, or purified bovine AGP and compared to comparable results with the corresponding human fractions.

Figure 2: FLT3 inhibitors show variable inhibition by human plasma in both AGP-dependent and independent fashions. MOLM-14 cells were exposed to increasing concentrations of the indicated FLT3 TKI under standard culture conditions (10% FBS) or in the presence of either (A) 50% human plasma or (B) various concentrations of purified human AGP (0.25 mg/mL, 0.5 mg/mL, 1.0 mg/mL), and proliferation was measured by MTT after 48 hours. (C) Structures of the various FLT3 TKI used.

Figure 3: AGP demonstrates linear, dose-dependent inhibition of FLT3 TKI. (A) MOLM-14 cells were co-cultured in a range of concentrations of AGP (31 µg/mL – 2 mg/mL) and treated with increasing doses of the FLT3 TKI, TTT-3002. Proliferation was measured by MTT after 48 hours. (B) The IC50 for TTT-3002 was plotted as a function of AGP concentration. (C) Plasma from 19 human patients was assayed for the ability to inhibit TTT-3002 using the modified PIA against MOLM-14 cells. The fold-change in the IC50 (relative to cells treated under standard 10% FBS culture conditions) was calculated and plotted as a function of plasma AGP concentration as measured by radial immunodiffusion assay. Linear regression, best-fit is plotted (solid line) with the curve generated in (B) also plotted for comparison (dashed line). By convention, patient AGP concentration is reported as mg/dL.
**Figure 4:** Human AGP binds staurosporine-derived FLT3 TKI with variable affinities. (A) MOLM-14 cells were co-cultured in a range of concentrations of human AGP and treated with various staurosporine-derived FLT3 TKI. The fold-change in the IC$_{50}$ (relative to cells treated under standard 10% FBS culture conditions) was calculated and plotted as a function of free AGP concentration (corrected for bound drug: IC$_{50}$ with AGP – IC$_{50}$ with 10% FBS). (B) AGP binding constants for each drug were calculated from the linear regions of each curve (solid line); see text and supplemental materials for mathematical treatment. (C) Scatchard plots for fluorescence displacement assays examining competitive displacement of ANS from AGP by the indicated drugs. (D) Plasma from 10 new-diagnosis AML patients was assayed for the ability to inhibit midostaurin using the modified PIA against MOLM-14 cells. The fold-change of IC$_{50}$ in 12.5% plasma was calculated and plotted as a function of plasma AGP concentration (corrected for bound drug) as measured by ELISA. This was compared with the shift predicted by exogenous human AGP in (A) and (B). Patient characteristics can be found in Supplemental Table 1.

**Figure 5:** Inhibition of drug-protein binding is dependent upon protein and protein binding inhibitor characteristics and concentrations and can be abrogated by the addition of competitor compounds. (A) Key aspects of the fold-change growth inhibition curves generated using an idealized drug ($K_A = 10$ µM$^{-1}$) and a binding inhibitor ($K_I = 10$ µM$^{-1}$, $I_0 = 10$ µM). Growth inhibition curves are shown for both the presence (orange) and absence (blue) of a binding inhibitor. Shown are the curves and equations for the asymptotes at low (green) and high (red) protein concentrations, the apparent free-protein concentration (purple) at which the curve has the greatest rate of increasing slope, and the maximal shift (bracket) in the curve caused by the presence of a binding inhibitor. (B) Competitive fluorescence displacement was performed in the presence of increasing concentrations of AGP-binding drugs (Lestaurtinib, Midostaurin, Mifespristone). ANS only exhibits fluorescence when bound to AGP. (C) Dose-response curves using the modified PIA in the presence of increasing doses of mifepristone, a potential binding inhibitor, show a return of the curves towards those seen under standard culture conditions (10% FBS) despite the presence of inhibitory levels of AGP. (D) Co-incubation of MOLM-14 cells with mifepristone restores TTT-3002 activity (5 nM) in the presence of AGP in a mifepristone dose-dependent manner. (E) The effect, as modeled in (A) was experimentally measured in MOLM-14 cells as the fold-change in IC$_{50}$ of lestaurtinib for cells co-cultured with varying concentrations of mifepristone (15 µM, 10 µM, 5 µM) and AGP (2-fold dilutions from 1 mg/mL to 3.9 µg/mL, where 1 mg/mL ≈ 20 µM AGP). (F) MOLM-14 cells were treated with TTT-3002 (2nM) in the presence or absence of inhibitory levels of AGP and the effect of co-incubation with mifepristone upon FLT3/ITD signaling inhibition was measured through phospho-Western analysis.
Figure 6: Identification of compounds that can restore TKI activity despite inhibitory levels of AGP. (A) Ba/F3-FLT3/ITD cells were exposed to cytotoxic concentrations of midostaurin (100 nM) in the presence of inhibitory levels of AGP (0.5 mg/mL) and co-cultured with a library of 2,800 FDA-approved drugs with or without 1 ng/mL of IL-3. After 48 hours in culture, cell growth was assessed by the MTT colorimetric assay and growth in IL-3 was plotted against growth in its absence. Candidate compounds are those that show cytotoxicity that can be completely (area 1) or partially (area 2) rescued by IL-3. (B) Compounds were also measured for their ability to displace AGP-bound ANS, and IL-3-rescuable growth inhibition was plotted as a function of ANS binding to AGP. Compounds that both displace ANS from AGP and restore IL-3-rescuable cytotoxicity are enclosed in the red dashed region. (C) Validation of one candidate compound, trihexyphenidyl hydrochloride. Co-treatment with increasing doses (indicated in legend) result in increasing TKI activity despite the presence of inhibitory (1 mg/mL) AGP. Its position on plots figures A and B is indicated in the inserts.

Figure 7: Human AGP inhibits midostaurin efficacy and can be rescued by mifepristone in vivo. (A) Experimental scheme. (B) Human AGP was injected intraperitoneally at 0 and 48 hours and the plasma concentration was measured at various timepoints via human-specific ELISA. Green line indicates the average of 4 independent mice (gray lines). (C) Sub-lethally irradiated BALB/c mice were engrafted with 5 x 10^5 Ba/F3-FLT3/ITD-luc cells. Bioluminescent imaging on day 5 confirmed engraftment. After engraftment, recipients received human AGP or vehicle by IP injection, and either midostaurin or vehicle by oral gavage for four days. A sub-group of hAGP/midostaurin mice also received mifepristone by oral gavage. Response was assessed on day 9 by repeat bioluminescent imaging. Shown is a representative example of duplicate runs. Average radiance is plotted with significant differences indicated (** < 0.005, * < 0.01, α = 0.05, bars indicate standard deviation).
Figure 1

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Figure 2
Figure 3

TKI dose response shift due to AGP (TTT-3002 with increasing AGP)

TKI potency as a function of patient plasma AGP

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Figure 5

A. Drug-inhibitor competition for protein binding

$\Delta \tau = \frac{K_A}{K_I + 1}$

Maximum $\Delta \tau = I_0$

B. Competitive Fluorescence Displacement

C. Disinhibition of TTT-3002 by Mifepristone

D. Disinhibition of TTT-3002 by Mifepristone

E. Titration of lestauninib disinhibition

$\Delta = 8 \mu M$

F. Western Blot Analysis

pFLT3

FLT3

pSTAT5

pAKT

pERK1/2

ERK1/2

Tubulin
A

Restoration of FLT3-specific cytotoxicity despite AGP binding
(Cytotoxicity with IL-3 versus without IL-3)

1. Growth inhibition rescued by IL-3
2. Both non-specific and resuable inhibition

Ba/F3-FLT3/ITD proliferation with IL-3
(OD_{50} normalized to no-drug controls)

Ba/F3-FLT3/ITD proliferation without IL-3
(OD_{50} normalized to no-drug controls)

B

FLT3-specific cytotoxicity versus AGP binding
(MTT versus ANS fluorescence displacement)

Enrichment of AGP-bound, IL-3 rescued drugs
P = 2.73 x 10^{-23}

IL-3 independent Growth Inhibition
1 - ratio of Proliferation without IL-3 to with IL-3

C

Candidate: Trihexyphenidyl hydrochloride

Figure 6
Figure 7
A method for overcoming plasma protein inhibition of tyrosine kinase inhibitors

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