HHS Public Access

Author manuscript

ACS Chem Biol. Author manuscript; available in PMC 2018 September 10.

Published in final edited form as:

ACS Chem Biol. 2018 June 15; 13(6): 1551–1559. doi:10.1021/acschembio.8b00154.

Selective inhibition of the myeloid Src-family kinase Fgr potently suppresses AML cell growth *in vitro* and *in vivo*

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Abstract

Acute myelogenous leukemia (AML) is the most common hematologic malignancy in adults, and is often associated with constitutive tyrosine kinase signaling. These pathways involve the non-receptor tyrosine kinases Fes, Syk and the three Src-family kinases expressed in myeloid cells (Fgr, Hck, and Lyn). In this study, we report remarkable anti-AML efficacy of an N-phenylbenzamide kinase inhibitor, TL02–59. This compound potently suppressed the proliferation of bone marrow samples from twenty of twenty-six AML patients, with a striking correlation between inhibitor sensitivity and expression levels of the myeloid Src family kinases Fgr, Hck, and Lyn. No correlation was observed with Flt3 expression or mutational status, with the four most sensitive patient samples wild-type for Flt3. Kinome-wide target specificity profiling coupled with in vitro kinase assays demonstrated a narrow overall target specificity profile for TL02–59, with picomolar potency against the myeloid Src-family member Fgr. In a mouse xenograft model of AML, oral administration of TL02–59 for three weeks at 10 mg/kg completely eliminated leukemic cells from the spleen and peripheral blood while significantly reducing bone marrow engraftment. These results identify Fgr as a previously unrecognized kinase inhibitor target in AML, and TL02–59 as a possible lead compound for clinical development in AML cases that overexpress this kinase independent of Flt3 mutations.

Keywords

acute myelogenous leukemia; AML; Src-family kinases; Fgr; Flt3

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ASSOCIATED CONTENT

Supporting Information. Supporting Information is available free of charge on the ACS Publications website at http://pubs.acs.org. This information includes Supplementary Figures S1 and S2, Tables S1–S4, and detailed descriptions of materials and methods including inhibitor analog synthesis and analytical data.

Acute myelogenous leukemia (AML) is a devastating hematologic cancer with limited treatment options. While diverse genetic changes are associated with AML, upregulation of tyrosine kinase signaling pathways is a common feature that offers opportunities for targeted therapy. One important example involves activating mutations in the Flt3 receptor tyrosine kinase, which are observed in about one-third of AML cases[1,2]. Two types of Flt3 mutations commonly occur in AML: internal tandem duplications (ITDs) and tyrosine kinase domain point mutations[3,4]. Multiple small molecule Flt3 kinase inhibitors have been tested for clinical management of AML. Broad-spectrum kinase inhibitors that target Flt3, such as midostaurin, lestaurtinib, and sorafenib, have limited efficacy due to lack of sustained Flt3 inhibition and off-target associated toxicity[5]. Newer inhibitors with enhanced specificity for the Flt3 kinase domain, including quizartinib and crenolanib, have demonstrated efficacy as single agents[6,7]. However, inhibitor resistance, largely though acquired mutations in the Flt3 kinase domain, has limited their clinical utility[8]. Compounds that inhibit common Flt3 resistance mutants have also been developed and are in clinical trials, such as FF-10101[9] and PLX3397[10].

Non-receptor tyrosine kinases have also been implicated in AML pathogenesis, including Fes, Syk and the three Src-family kinases expressed in myeloid cells (Fgr, Hck, and Lyn). Both Fes and Syk are constitutively active in Flt3-ITD$^+$ AML cell lines and patient samples, where RNAi-mediated knockdown and kinase inhibition suppress AML cell growth[11–14]. Hck is highly overexpressed in gene expression profiles of myeloid leukemia stem cells[15], and an ATP-site inhibitor of Hck reduced the growth of primary AML cells in engrafted immunocompromised mice[16], although the selectivity of the inhibitor for Hck has not been established. Lyn is active in a majority of clinical AML isolates and has been linked to the activation of Stat5 by Flt3-ITD[17–19]. Fgr is also highly expressed in Flt3-ITD$^+$ AML samples and siRNA knockdown significantly reduced primary AML cell growth[20]. However, whether or not selective inhibition of Fgr kinase activity is of clinical value in AML is unknown. Taken together, these studies suggest that targeted inhibition of non-receptor tyrosine kinases may be a viable therapeutic strategy for AML patients in which these kinases are over-expressed and active.

In this study, we describe a unique non-receptor tyrosine kinase inhibitor, TL02–59, with pM potency against the AML-associated Src-family kinase, Fgr, both in vitro and in cell-based kinase assays. TL02–59 inhibited the growth and induced apoptosis of AML cell lines expressing this kinase with single-digit nM potency. More importantly, TL02–59 induced growth arrest in primary AML bone marrow samples, with Fgr expression correlating most strongly with TL02–59 sensitivity. Primary AML bone marrow cell responses to TL02–59 were independent of Flt3 expression and mutational status, with the four most sensitive patient samples wild-type for Flt3. Finally, oral administration of TL02–59 completely eliminated AML cells from the spleen and peripheral blood in a mouse model of AML, while dramatically suppressing bone marrow involvement. These results demonstrate that selective targeting of Fgr may provide clinical benefit in the subset of AML patients that over-express this Src-family member.

RESULTS AND DISCUSSION

TL02–59 is a potent inhibitor of AML cell lines expressing constitutively active tyrosine kinases.

AML pathogenesis is associated with over-expression and constitutive activation of several non-receptor tyrosine kinases, including Syk, Fes, and the myeloid Src-family members Hck, Lyn and Fgr (see Introduction). While previous studies have suggested that that inhibition of these kinases may be of benefit in the context of Flt3-ITD$^+$ AML, the broader impact of AML-associated non-receptor tyrosine kinase inhibition has not been directly addressed due to the lack of selective ATP-site inhibitors. To begin to pursue this question, we evaluated a set of ten N-phenylbenzamide kinase inhibitors for growth inhibitory activity towards the Flt3-ITD$^+$ AML cell line, MV4–11, which also expresses active Fes[13,14], Syk[11,12], and all three myeloid Src-family members[19]. Remarkably, three of these compounds (TL02–59, TL8–133, and TL8–187) inhibited MV4–11 AML cell growth with an IC_{50} value less than 1 nM (Fig. 1A). Subsequent mechanistic studies focused on TL02–59, because of its favorable pharmacokinetic properties.

To determine whether TL02–59 was selective for AML cells expressing active tyrosine kinases, we examined its inhibitory potency against a two other commonly used AML cell lines, MOLM-14 and THP-1. MOLM-14 cells are Flt3-ITD$^+$ and express a similar complement of active non-receptor protein tyrosine kinases as MV4–11 cells. TL02–59 also potently induced growth arrest in MOLM-14 cells, with an IC_{50} value of 6.6 nM (Fig. 1B). In contrast, TL02–59 treatment had no effect on THP-1 AML cell proliferation at concentrations as high as 1 μM, with slight growth suppression evident at 3 μM (Fig. 1B and data not shown). THP-1 cells are transformed by tyrosine kinase-independent mechanisms that involve an *MLL-AF9* translocation [21,22] as well as a point mutation in the *NRAS* locus [23]. TL02–59 also selectively induced apoptosis in MV4–11 and MOLM-14 cells, without affecting THP-1 cells (Fig. 1C), and was a more potent inducer of apoptosis than tandutinib, a Flt3 inhibitor previously tested in clinical trials against Flt3-ITD$^+$ AML[24].

KINOMEscan analysis and primary AML cell kinase expression profiling implicate myeloid non-receptor tyrosine kinases as dominant TL02–59 targets.

To define the broadest possible range of kinase targets for TL02–59, we performed KINOMEscan analysis, a kinome-wide competition assay for ATP-site inhibitor specificity[25]. We profiled TL02–59 against 456 kinases that cover all eight kinase subfamilies at the relatively high screening concentration of 1 μM relative to its potency against AML cell lines (~ 1 nM; Fig. 1). TL02–59 displayed remarkable selectivity, interacting with only twenty-three of the 456 kinases tested for a selectivity (S) score of 0.07 (complete KINOMEscan data are shown in Table S1). Most of the putative targets are members of the tyrosine kinase family, and include the AML-associated kinases Flt3, c-Kit, Fes, and the myeloid Src-family members Hck and Lyn. Additional Src-family members, including Lck, Blk, and c-Src, as well as Abl (*ABL1*) and Arg (*ABL2*) also scored a positive hits. Several Ser/Thr kinases were also identified, including members of the STE (Taok2, Taok3/Jik, Slk, Stk10/Lok, MAP4K2) and CMGC (p38α, Jnk2) families. While p38α and Jnk have been linked to AML in previous studies[26,27], a role for the remaining Ser/Thr

kinases in AML is less clear. By contrast, the broad-spectrum kinase inhibitor midostaurin (Rydapt), a derivative of staurosporine which was recently FDA-approved for FLT3-ITD$^+$ AML in combination with cytarabine, interacts with 166 out of 386 unique kinases in the KINOMEscan assay, for an S score of 0.43.[28] This lack of selectivity is likely to contribute to non-selective cytotoxicity due to off-target kinase inhibition.

To define the subset of potential target kinases for TL02–59 identified by KINOMEscan that are relevant to AML, we next determined the expression profile of all TL02–59 KINOMEscan hits in a set of bone marrow specimens from twenty-six AML patients (clinical characteristics and cytogenetic profiles are presented in Table S2). PCR of genomic DNA and sequence analysis showed that thirteen AML samples carried an ITD in one *FLT3* allele, with the remainder wild type (Table S3). Using quantitative real-time RT-PCR (qPCR), we then profiled the relative expression of the 23 TL02–59 target kinases from the KINOMEscan analysis plus four additional non-receptor tyrosine kinases close to the specificity cut-off of 1.0 (Src-family members Fgr, Fyn, and Yes plus Syk). Relative expression was calculated as the base 2 antilog of the ΔC_t value for each kinase relative to GAPDH. Each value was then normalized to the mean expression value for all TL02–59 target kinases in the sample to create a relative expression profile for each patient (Fig. 2). Using this approach, we found that 15 of the 27 target kinases for TL02–59 identified by KINOMEscan analysis showed relative expression values greater than 2 in at least four of the Flt3-ITD$^+$ bone marrow samples (Fig. 2A). Remarkably, a very similar pattern of kinase gene expression was observed in patient AML samples wild type for Flt3, with the same group of 15 kinases showing relative expression values greater than 2 in at least four samples (Fig. 2B). The most highly expressed kinase in the Flt3-ITD$^+$ samples is the Src-family kinase Fgr, which was observed in 8 of 13 samples. A similar Fgr expression pattern was mirrored in the AML samples wild type for Flt3, where Fgr was among the most highly expressed protein kinases. Lyn, another Src family member, showed average expression levels of greater that two in all twenty-six samples examined. The putative TL02–59 target kinases identified by KINOMEscan analysis and expressed in primary AML bone marrow samples are listed in Table 1.

We next evaluated whether the TL02–59 target kinase expression pattern in our cohort of primary AML bone marrow samples is representative of AML cases more generally. To do this, TL02–59 target kinase gene expression values from AML specimens (determined by RNAseq analysis) with wild type (n = 111) or ITD (n = 34) forms of *FLT3* were download from the TCGA database[29] and normalized to the average expression across all 27 target kinases for TL02–59 within each sample as per our qPCR data. The average of the normalized expression values for each kinase from the TCGA database was then plotted against the average value obtained for each kinase by qPCR in our AML cohort for the Flt3-ITD and Flt3-WT populations (Fig. 3A). A significant linear correlation was observed between the two data sets ($p < 0.0001$), indicating that the relative TL02–59 target kinase expression pattern observed in our primary AML bone marrow samples is mirrored in this larger population of AML specimens.

AML bone marrow cell sensitivity to TL02–59 correlates with expression of Fgr and other AML-associated non-receptor tyrosine kinase but not Flt3.

We next explored the sensitivity of primary AML bone marrow samples to growth arrest in response to TL02–59 treatment. Primary AML bone marrow samples were cultured on mitotically inactive Hs27 feeder fibroblasts in the presence of the myeloid cytokines IL-3, IL-6, and SCF according to the method of Klco, et al.[30] This approach allowed the primary cells to survive *ex vivo* and expand in number for about one week. Cultures were then established in the presence of a range of TL02–59 concentrations, and IC_{50} values for growth inhibition were determined using the Cell Titer Blue cell viability assay. The AML bone marrow samples showed a remarkable range of responses to this kinase inhibitor, ranging from 77 nM to greater than 3,000 nM (Table S3). Relative expression levels of the three myeloid Src-family kinases (Fgr, Hck and Lyn) as well as Syk showed a significant inverse correlation with the IC_{50} values for TL02–59 across the 26 AML samples (Fig. 3B). Fes expression also showed a similar trend with inhibitor sensitivity that did not reach statistical significance, which may reflect the relatively small patient sample size. Notably, the four AML bone marrow samples most responsive to TL02–59 were wild-type for Flt3, indicating that its mechanism of action is unlikely due to direct Flt3 inhibition. Kinase expression profiles for the AML samples most (cases 454 and 451) and least (cases 104 and 505) sensitive to TL02–59 are highlighted in Figure 2.

Kinase assays identify the myeloid Src-family member Fgr as the primary target for TL02–59.

Kinase expression profiling and correlation analyses using primary AML bone marrow cells presented in the previous sections strongly implicate the Src-family kinases Fgr, Hck and Lyn as well as Syk as the most likely targets for TL02–59 action in AML cells. While each of these kinases was identified as a TL02–59 target in the KINOMEscan analysis, this ligand displacement assay does not necessarily predict inhibitory activity. Therefore, we performed in vitro kinase assays with recombinant Syk, Fgr, Hck and Lyn, as well as four additional kinases that scored as hits in the KINOMEscan analysis. This latter group included the Flt3-ITD kinase domain, Fes, p38α as well as Taok3, which has not been previously linked to AML. All kinase assays were performed over a wide range of TL02–59 concentrations, with the ATP concentration set to the respective K_m for each kinase. The resulting IC_{50} values are shown in Table 1. Of all of the kinases tested, Fgr was by far the most sensitive to TL02–59, with an IC_{50} value of just 31 pM. Lyn was also potently inhibited, with an IC_{50} value of 100 pM. All of the other kinases showed IC_{50} values in the three-digit nM range, including Flt3-ITD (440 nM), Fes (290 nM), Syk (470 nM), and Hck (160 nM). Similar results were obtained with the Ser/Thr kinases p38α (126 nM) and Taok3 (509 nM). The difference in potency between Fgr and Lyn vs. each of the other AML-associated kinases is more than 1000-fold, suggesting that the primary targets for TL02–59 action in AML cells are these Src-family members. These in vitro findings are consistent with growth suppression of primary AML bone marrow cells by this compound, where the most sensitive samples express the highest levels of Fgr and Lyn.

To determine whether the target specificity profile for TL02–59 observed in vitro was also observed in cells, we used the human myeloid leukemia cell line TF-1 as a model system.

These cells express low or undetectable levels of Flt3, Fgr, Hck, and Lyn, allowing us to introduce each kinase individually via retroviral transduction and thereby avoid the possible complication of cross-talk between the kinases. TF-1 cell populations expressing each AML-associated tyrosine kinase were then treated with TL02–59 over a wide range of concentrations (0.1 to 1000 nM). The kinases were immunoprecipitated, followed by immunoblotting with antibodies to the activation loop phosphotyrosine (pTyr416 in the case of Hck, Fgr, and Lyn), or with antiphosphotyrosine antibodies in the case of Flt3, as a measure of kinase activity in the cells. TL02–59 treatment potently inhibited Fgr autophosphorylation in TF-1 cells, with paritial inhibition observed at 0.1–1 nM and complete inhibition above 10 nM (Figure 4). In contrast, Hck, Lyn and Flt3 were inhibited in the 100 to 1000 nM range.

To provide independent validation of Fgr as an inhibitor target in MV4–11 AML cells, which are extraordinarily sensitive to TL02–59 in terms of growth inhibition (IC_{50} of 0.78 nM; Figure 1), we knocked down Fgr expression using lentiviral shRNAs. Each of two independent shRNA sequences suppressed MV4–11 proliferation compared to non-targeted controls, with the degree of inhibition consistent with the degree of reduction in Fgr transcript levels (Figure S1).

TL02–59 is an orally active inhibitor of AML in vivo.

Studies presented above demonstrate that TL02–59 potently inhibits the growth of AML-derived cell lines and primary bone marrow samples in vitro, through inhibition of Fgr and possibly other Src-family kinases. Pharmacokinetic studies demonstrated that TL02–59 is orally bioavailable and displays a plasma half-life of about 6 hours in mice, making it an attractive candidate for in vivo evaluation (Table S4). To test the efficacy of TL02–59 in vivo, we used an MV4–11 AML cell xenograft model based on NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ (NSG) immunocompromised mice. A recent study demonstrated that tail vein injection of NSG mice with MV4–11 cells leads to rapid engraftment of the bone marrow and spleen, providing a rigorous model to test TL02–59 efficacy in vivo[7].

Cohorts of NSG mice were injected with MV4–11 cells through the tail vein, and allowed to engraft for two weeks. Groups of eight mice were then treated once daily by oral gavage with TL02–59 at 1 and 10 mg/kg, with the clinically used Flt3 inhibitor sorafenib (10 mg/kg), or vehicle alone for three weeks. The presence of MV4–11 cells in the bone marrow, spleen, and peripheral blood was then assessed by flow cytometry using antibodies specific for human $CD45^+$ and $CD33^+$ (Figs. 5A and B). Three weeks of TL02–59 treatment at 1 mg/kg reduced bone marrow engraftment of MV4–11 cells by 20% and spleen engraftment by half while suppressing levels of leukemia cells in the peripheral blood by 70%. At 10 mg/kg, TL02–59 treatment completely eradicated leukemic cells from the spleen and peripheral blood while reducing bone marrow engraftment by 60%. On the other hand, sorafenib (10 mg/kg) had no effect on either bone marrow or spleen engraftment of MV4–11 cells, although a significant reduction of leukemic cell counts in the peripheral blood was observed. Thin sections from the bone marrow and spleen of all five groups were then stained with antibodies to human CD45 and representative images are presented in Figure 5C. Bone marrow and spleen sections from untreated mice show heavy infiltration of MV4–

11 cells. Treatment with TL02–59 at the higher dose completely eliminated MV4–11 cells from the spleen and restored the normal bone marrow architecture, although some MV4–11 cells are still evident. In contrast, spleen and bone marrow sections from animals treated with sorafenib at 10 mg/kg still stained strongly for MV4–11 cells in a manner indistinguishable from the vehicle controls. Note that while sorafenib is a nM inhibitor of Flt3-ITD kinase activity in vitro, it does not inhibit Fgr or other myeloid Src-family kinases (IC_{50} > 100 μM; data not shown).

Summary and conclusions.

Here we report the identification of a unique N-phenylbenzamide tyrosine kinase inhibitor, TL02–59, with potent growth inhibitory activity against AML both in vitro and in vivo. Parallel KINOMEscan analysis, in vitro kinase assays, target kinase gene expression profiling and efficacy studies in AML bone marrow samples identified the Src-family kinase Fgr as the primary target for this inhibitor. In the patient-derived AML bone marrow cells, we observed a wide range of inhibitor responses that were not linked to Flt3 expression levels or mutations. In fact, the four most sensitive primary AML bone marrow samples were wild-type for Flt3, suggesting that TL02–59 may be efficacious against AML patients with diverse cytogenetic backgrounds provided they overexpress Fgr. Indeed, three of the four most sensitive patient-derived samples showed complex cytogenetic changes, including translocations and chromosomal duplications (see Table S2). While many Flt3 inhibitors have been tested in clinical trials, none has produced durable responses as monotherapy for AML. One important example is quizartinib (also known as AC220), a very potent and selective Flt3 inhibitor that was among the first to show efficacy as a single agent against Flt3-ITD$^+$ AML[5,31]. Quizartinib often induces rapid remission in Flt3-ITD$^+$ AML, but acquired drug resistance, commonly in the form of Flt3 kinase domain mutations, has limited its effectiveness[4,5,32–34]. Unlike TL02–59, we found that quizartinib does not inhibit Fgr or other AML-associated kinases, including Syk, Fes, or Hck in vitro; the same is true for other Flt3 inhibitors tested in the clinic, including sorafenib, sunitinib, and tandutinib (data not shown). Clinical compounds based on TL02–59, which selectively target Fgr and other myeloid Src-family kinases such as Hck and Lyn, may complement the action of quizartinib to suppress acquired resistance when used in combination for therapy of Flt3-ITD$^+$ AML. In addition, such compounds may have general utility in AML cases where Fgr is over-expressed. Based on our analysis of kinase gene expression profiles in AML samples from the TCGA database, this may represent one-third or more of AML cases. Furthermore, Fgr is uniquely over-expressed across more than 30 distinct tumor types (Figure S2).

METHODS

Sources of cell lines, antibodies, inhibitors, and other reagents used in this study, as well as additional methodological details, are provided in the Supporting Information.

Kinase Immunoprecipitation and Western Blotting.

Myeloid cells were cultured in the presence of kinase inhibitors or DMSO for 16 hours prior to lysis as described[35]. Kinase proteins were immunoprecipitated, separated by SDS-PAGE,

transferred to PVDF membranes and probed with primary antibodies followed by alkaline phosphatase-linked secondary antibodies.

In vitro kinase assays.

Recombinant protein kinase assays were performed using the FRET-based Z'-LYTE Kinase Assay according to the manufacturer's instructions (ThermoFisher Scientific).

RNA Isolation, cDNA preparation, qPCR.

Real-time quantitative PCR assays were performed on total RNA using SYBR Green detection and gene-specific QuantiTect primers (Qiagen).

Mouse AML Xenograft Model.

NOD.Cg-Prkdc^{scid}*Il2rg*^{tm1Wjl}/SzJ (NSG) mice were obtained from Jackson Laboratories and injected with human MV4–11 AML cells (10^7) suspended in PBS via the tail vein. After 14 days of engraftment, groups of eight mice received daily treatments of vehicle, TL02–59, or sorafenib by oral gavage for 21 days. The presence of human cells was assayed by flow cytometry and immunohistochemistry of fixed tissues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

This work was supported by National Institutes Health Grants CA185702 to T.E.S. and K01 OD01843 to S.T.S. R.K.P. was supported by the Pharmacology and Chemical Biology Training Program Grant NIH T32 GM08424. We also acknowledge the UPMC Hillman Cancer Center Tissue and Research Pathology/Health Sciences Tissue Bank, a shared resource supported in part by NIH award P30 CA047904.

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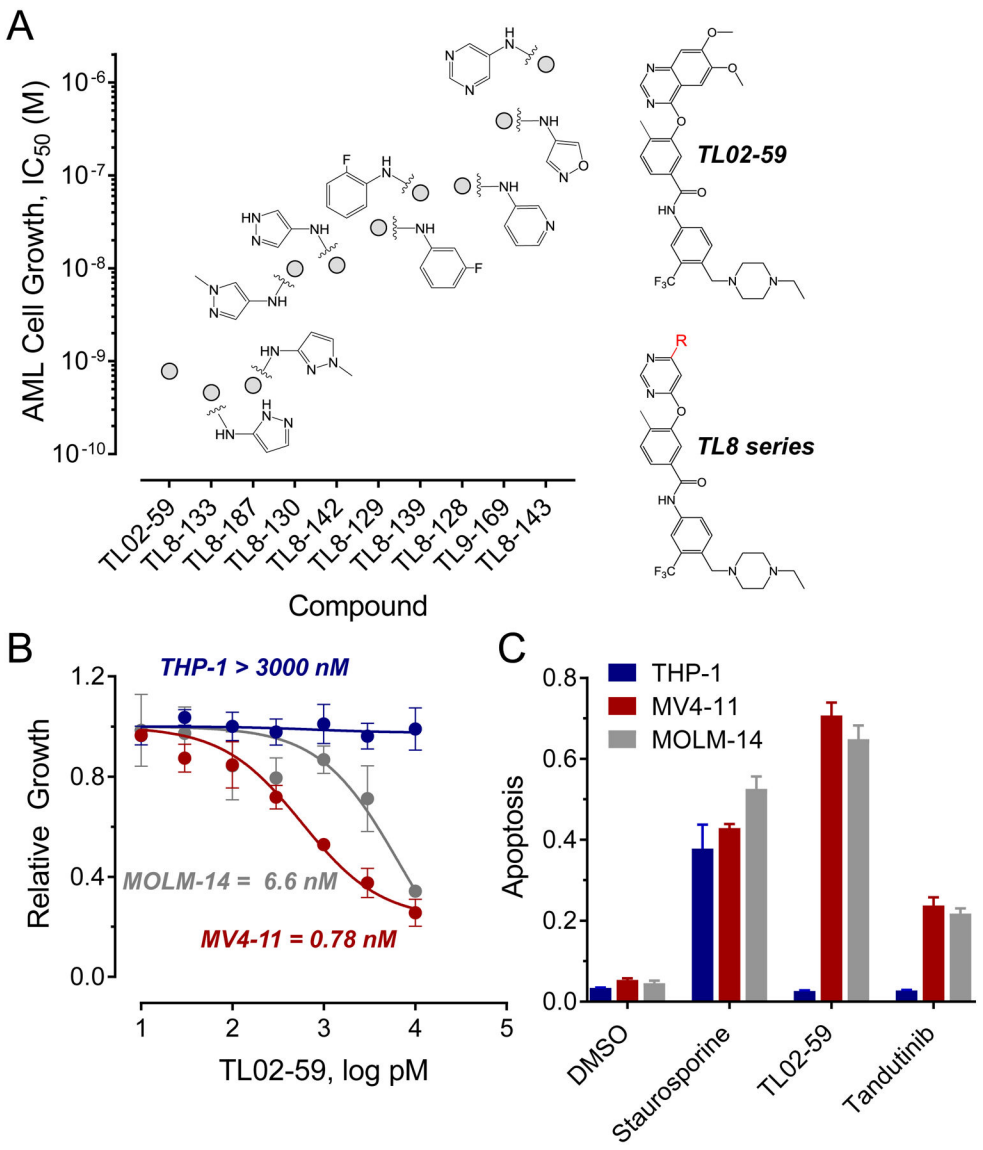

Figure 1.

Identification of TL02–59 as a potent inhibitor of AML cell growth. A) TL02–59 and nine N-phenylbenzamide analogs were evaluated for their growth inhibitory activity in the FLT3-ITD$^+$ AML cell line, MV4–11, using the CellTiter Blue assay (Promega). IC_{50} values for each compound are shown, with the R-group unique to each analog plotted next to the corresponding data point. The structures of TL02–59 and the related TL8 scaffold are shown on the right. B) Proliferation of MV4–11, MOLM-14 and THP-1 AML cells in the presence of TL02–59 was measured using the CellTiter-Blue assay over a range of concentrations for 72 hours. For THP-1 cells, additional concentrations up to 3.0 μM did not significantly inhibit growth (not shown). C) MV4–11, MOLM-14 and THP-1 AML cells were incubated with tandutinib (100 nM), TL02–59 (100 nM), staurosporine (1 μM) or DMSO for 72 hours. Apoptosis and cell viability were independently measured and results are presented as mean ratios of caspase activity to cell viability from three independent experiments ± SD.

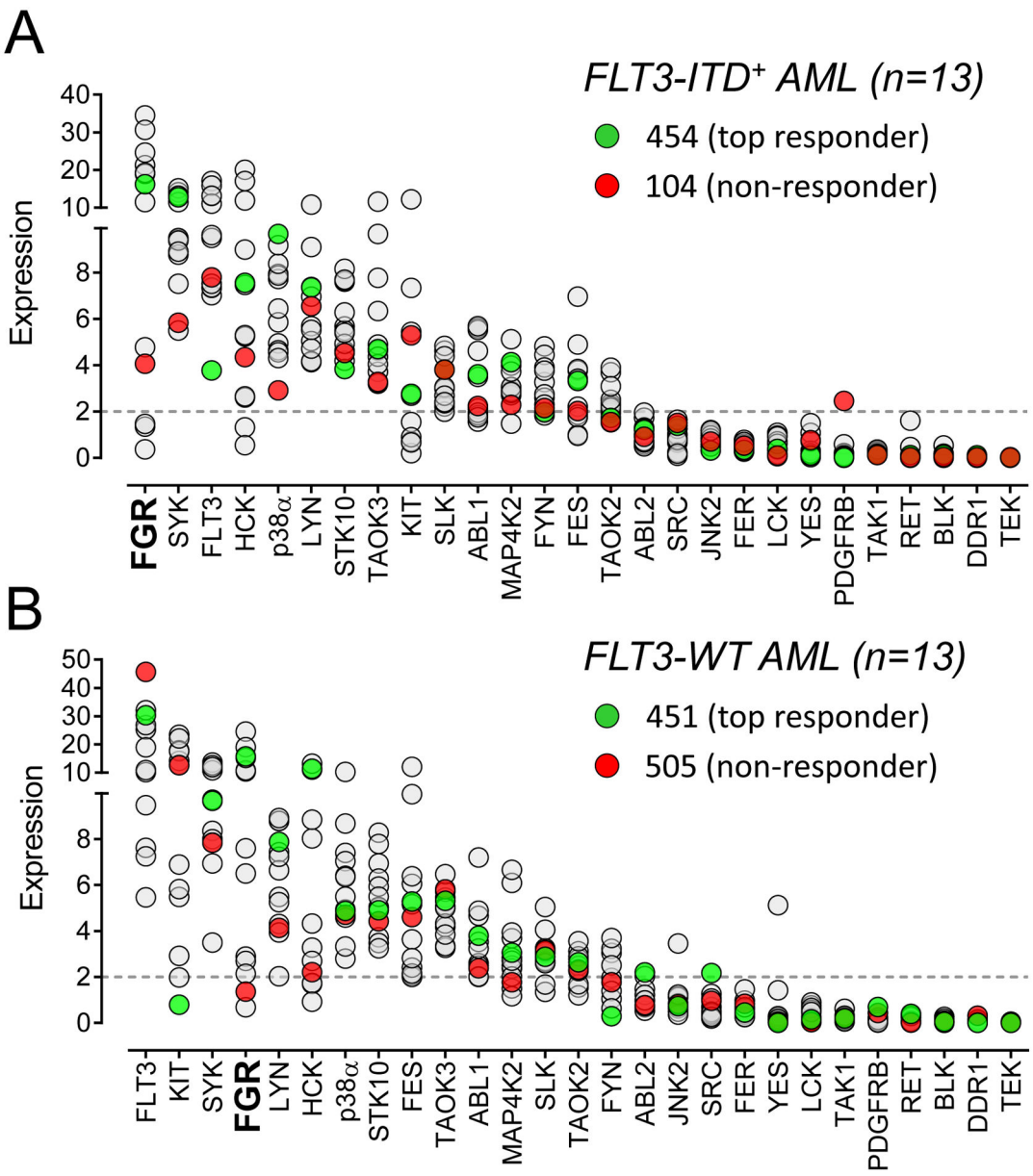

Figure 2.

Expression of TL02–59 target kinases in primary AML bone marrow samples. Expression of the 27 putative TL02–59 target kinases identified by KINOMEscan analysis was determined in 26 primary AML bone marrow samples by qPCR. Relative expression values were calculated as the base 2 antilog of the qPCR ΔC_t values relative to GAPDH for each kinase. These values were then plotted as a distribution relative to the mean value for all 27 kinases analyzed in each patient sample. All determinations were made on at least two independent RNA samples from each patient. Each patient is represented by a dot. The Flt3 genotype of each sample was determined by PCR and sequencing (Table S2) and results are grouped as Flt3-ITD$^+$ (panel A) or Flt3 wild-type (Flt3-WT; panel B). Expression profiles for the AML samples most sensitive to TL02–59 are highlighted in green (top responder; cases 454 and

451) while the least responsive cases are highlighted in red (non-responder; cases 104 and 505).

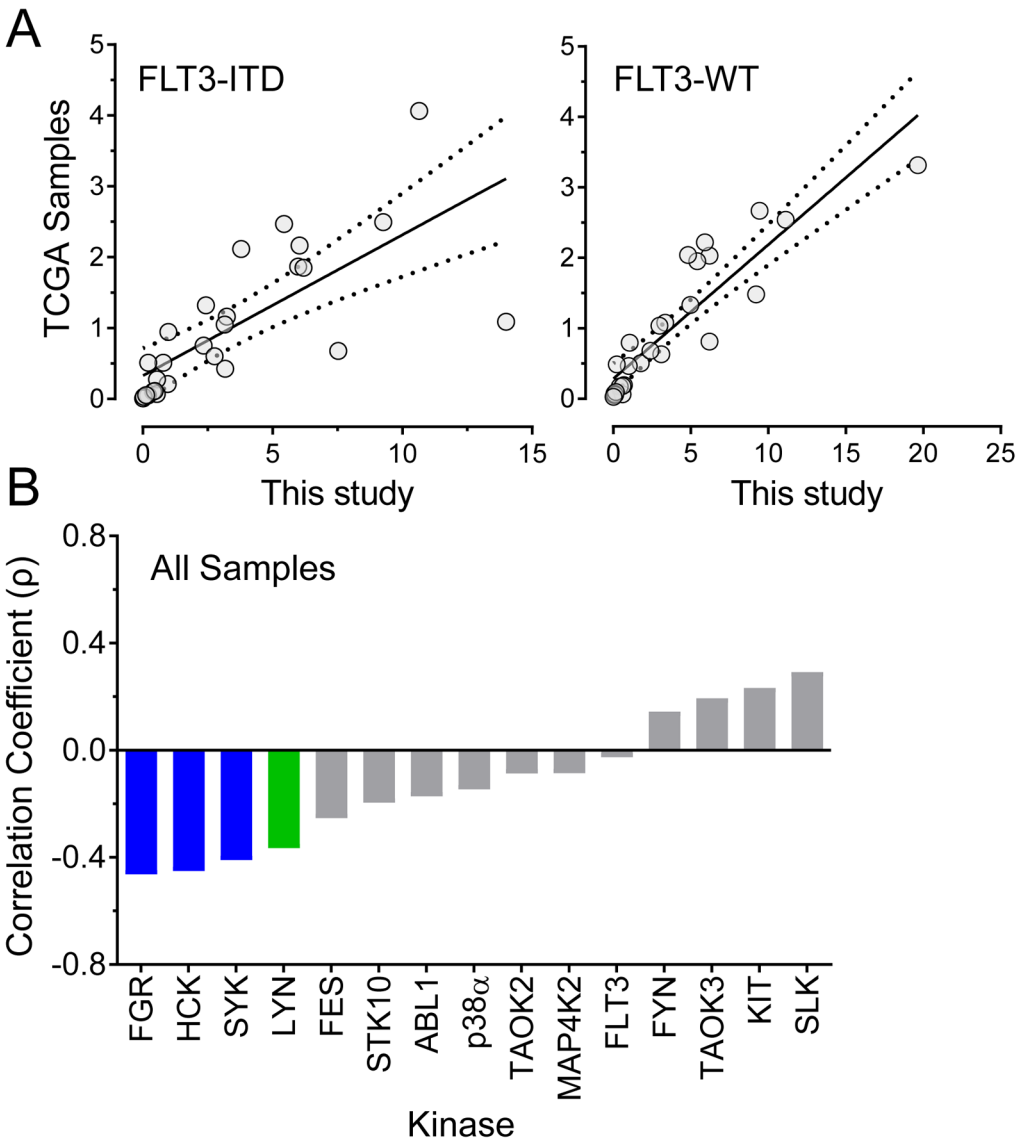

Figure 3.

A) Comparison of TL02–59 target kinase expression in primary AML samples from the TCGA database vs. primary AML bone marrow samples from this study. TL02–59 target kinase RNAseq expression data were downloaded as FPKM values from the TCGA database[36] and normalized to the average expression across all 27 kinases within each sample. Average values for each kinase from the TCGA database (Flt3-ITD: n = 34; Flt3-WT: n =111) were plotted against the average values obtained for each kinase in this study for Flt3-ITD (n = 13; *left*) and Flt3-WT (n = 12; *right*) primary AML bone marrow samples by qPCR. Data were analyzed by linear regression and the best-fit line and 95% confidence intervals are shown; $p < 0.0001$ in both cases. B) Myeloid-restricted Src-family kinases and Syk correlate with TL02–59 sensitivity in patient AML bone marrow samples. Relative TL02–59 target kinase expression values from Figure 2 were correlated with TL02–59 IC_{50} values determined for each of the primary AML bone marrow samples (Table S3).

Spearman's correlations were calculated for each kinase across all of the AML samples, and the resulting correlation coefficients are plotted in ascending order. Blue bars represent kinases whose expression shows a significant negative correlation with TL02–59 IC_{50} values with $p < 0.05$; green bar (Lyn) represents $p < 0.1$. Grey bars represent kinases that did not show statistical significance.

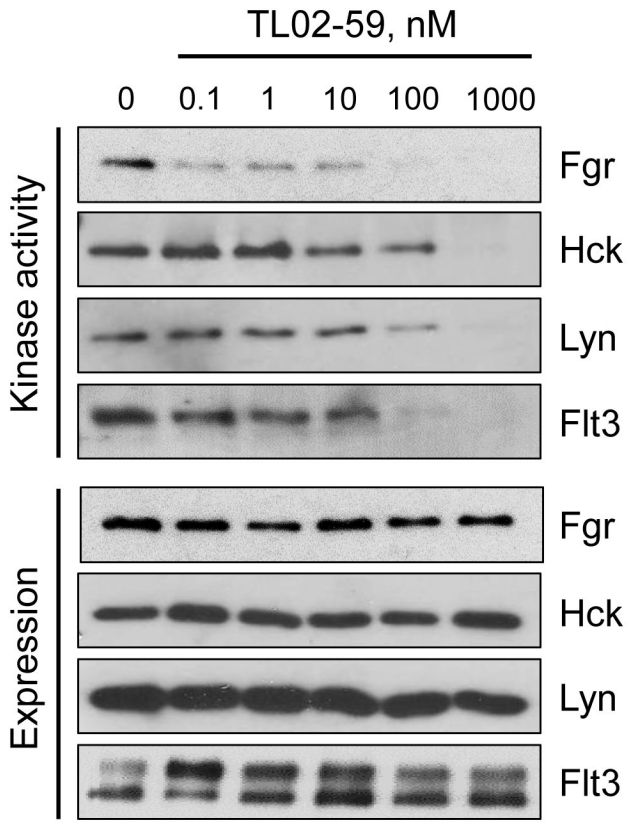

Figure 4.

TL02–59 selectively inhibits Fgr autophosphorylation in myeloid cells. TF-1 myeloid cells were transduced with recombinant retroviruses carrying Fgr, Lyn, Hck, and Flt3-ITD. Cells were treated with TL02–59 for 6 h over the range of concentrations shown, followed by immunoprecipitation of each kinase from clarified cell protein extracts. The immunoprecipitates were resolved by SDS-PAGE, followed by immunoblotting with antibodies to the Src-family kinase activation loop tyrosine (pTyr416) or antiphosphotyrosine in the case of Flt3-ITD (*upper panels*). The presence of kinase protein in each lane was verified by immunoblotting with kinase-specific antibodies (*lower panels*). This experiment was repeated three times with equivalent results.

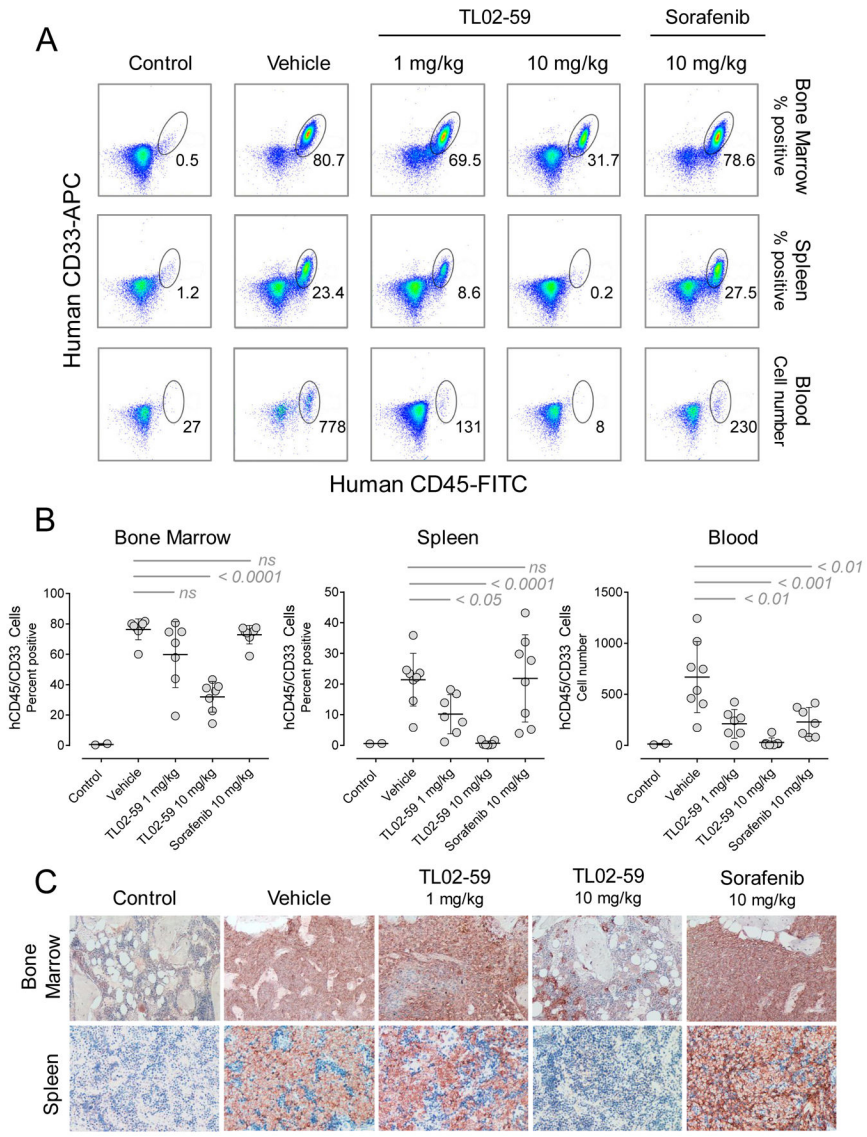

Figure 5.

TL02–59 reduces MV4–11 cell engraftment in an AML mouse model. Human Flt3-ITD$^+$ MV4–11 AML cells were injected into the tail vein of immunocompromised (NSG) mice and allowed to engraft for two weeks. Groups of eight mice were then treated daily by oral gavage with TL02–59 (1 and 10 mg/kg), sorafenib (10 mg/kg), or vehicle. Two mice were not engrafted and received no treatment for use as baseline controls. Following three weeks of treatment, the animals were sacrificed and the presence of MV4–11 AML cells in the bone marrow, spleen and peripheral blood were assayed by flow cytometry. A) Representative flow cytometry diagrams for a single mouse within each treatment group. For the bone marrow and spleen, the inset number represents the percentage of human CD45$^+$/CD33$^+$ MV4–11 leukemia cells present. For whole blood, the number of MV4–11 cells present per 25 μL of blood is shown. B) Summary of all flow cytometry results, where each dot represents a single mouse. Horizontal bars indicate the mean value in each group +/− SD. Statistical significance was determined by pairwise Student's t-test, with p values

indicated for significantly different groups; *ns*, not significant. C) Representative thin sections of spleen and bone marrow from all five treatment groups. MV4–11 AML cells were visualized in the sections by immunohistochemistry with an antibody specific for human CD45.

Table 1.

In vitro kinase assays.

Putative TL02–59 kinase inhibitor targets identified in the KINOMEscan assay and expressed in primary AML blasts are listed. KINOMEscan values represent percent of residual kinase binding to the immobilized probe at a TL02–59 concentration of 1.0 μM (e.g. a value of zero equals 100% probe displacement while a value of 100 equals no binding of TL02–59 to the target kinase). The complete KINOMEscan dataset is provided in Supplementary Table S1. Select target kinases were also tested for inhibitor sensitivity in the Z'LYTE in vitro kinase assay with the ATP concentration set to the K_m value for each kinase. IC_{50} values (nM) were calculated by nonlinear regression analysis of the resulting dose-response curves using GraphPad Prism. For Flt3-ITD and Kit, the recombinant kinase domain was used in the assay; *n.d.*, not determined.

Kinase	KINOMEscan *Residual probe bound*	*In vitro* Kinase Assay *IC50, nM*
Src-family kinases		
Fgr	2.3	0.03
Lyn	1.0	0.10
Hck	0.3	160
Other non-receptor tyrosine kinases		
Syk	1.2	470
Fes	0.8	290
Abl	0.2	*n.d.*
Receptor tyrosine kinases		
Flt3-WT	0.6	633
Flt3-ITD	5.2	440
Kit	0.8	*n.d.*
Ser/Thr Kinases		
p38α	0	126
Stk10	0	*n.d.*
Taok2	0.1	*n.d.*
MAP4K2	0.3	*n.d.*
Taok3	0.3	509
Slk	0.8	*n.d.*