Quizartinib (AC220) is a potent second generation class III tyrosine kinase inhibitor that displays a distinct inhibition profile against mutant-FLT3, -PDGFRA and -KIT isoforms

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Abstract

Background: Activating mutations of class III receptor tyrosine kinases (RTK) FLT3, PDGFR and KIT are associated with multiple human neoplasms including hematologic malignancies, for example: systemic mast cell disorders (KIT), non-CML myeloproliferative neoplasms (PDGFR) and subsets of acute leukemias (FLT3 and KIT). First generation tyrosine kinase inhibitors (TKI) are rapidly being integrated into routine cancer care. However, the expanding spectrum of TK-mutations, bioavailability issues and the emerging problem of primary or secondary TKI-therapy resistance have lead to the search for novel second generation TKIs to improve target potency and to overcome resistant clones. Quizartinib was recently demonstrated to be a selective FLT3 inhibitor with excellent pharmacokinetics and promising in vivo activity in a phase II study for FLT3 ITD + AML patients. In vitro kinase assays have suggested that in addition to FLT3, quizartinib also targets related class III RTK isoforms.

Methods: Various FLT3 or KIT leukemia cell lines and native blasts were used to determine the antiproliferative and proapoptotic efficacy of quizartinib. To better compare differences between the mutant kinase isoforms, we generated an isogenic BaF3 cell line expressing different FLT3, KIT or BCR/ABL isoforms. Using immunoblotting, we examined the effects of quizartinib on activation of mutant KIT or FLT3 isoforms.

Results: Kinase inhibition of (mutant) KIT, PDGFR and FLT3 isoforms by quizartinib leads to potent inhibition of cellular proliferation and induction of apoptosis in in vitro leukemia models as well as in native leukemia blasts treated ex vivo. However, the sensitivity patterns vary widely depending on the underlying (mutant)-kinase isoform, with some isoforms being relatively insensitive to this agent (e.g. FLT3 D835V and KIT codon D816 mutations). Evaluation of sensitivities in an isogenic cellular background confirms a direct association with the underlying mutant-TK isoform – which is further validated by immunoblotting experiments demonstrating kinase inhibition consistent with the cellular sensitivity/resistance to quizartinib.

Conclusion: Quizartinib is a potent second-generation class III receptor TK-inhibitor – but specific, mutation restricted spectrum of activity may require mutation screening prior to therapy.

Keywords: AC220, Quizartinib, Leukemia, KIT, FLT3, PDGFR
Background
Gain-of-function mutations of the FLT3, KIT and PDGFR class III receptor tyrosine kinases (RTK) play important roles as oncogenesis-driving events in several hematologic malignancies. For example, FLT3 mutations are predominantly found in AML associating with a poor prognosis [1-4], but are also reported in (pediatric) acute lymphoblastic leukemia (ALL) [5]. KIT mutations occur in the vast majority of systemic mastocytosis (SM) [6] and subsets of acute leukemia, including core-binding factor (CBF) [7] and pediatric [8] AML. Certain FLT3 and KIT mutations correlate with inferior outcome in adult AML [4,9,10].

PDGFR mutations are frequently found in myeloproliferative disorders, such as Philadelphia chromosome-negative chronic myeloid leukemia (CML), where PDGFR alpha or beta fuses with another gene allowing autoactivation of the tyrosine kinase. Several fusion partners have been described, including FIP1L1 leading to the FIP1L1-PDGFRA fusion gene. This translocation has been associated with hypereosinophilic syndromes and mastocytosis with eosinophilia [11-13].

Numerous tyrosine kinase inhibitors have been developed to target class III RTKs (see also Discussion). These TKIs have a variable spectrum of activity against different class III RTKs and against various mutant isoforms of these kinases. To date, translation from bench to bedside has resulted in only modest or short-lived effectiveness of these inhibitors in most entities [14-23] and only a few agents have achieved FDA approval for selected indications such as CML and HES. With the exception of Ph+ALL, no TKIs have been approved for treatment of acute leukemia so far.

Quizartinib is a novel second generation class III receptor tyrosine kinase inhibitor with superior pharmacological properties and an excellent pharmacokinetic profile compared to other agents. Quizartinib was demonstrated to have high efficacy and tolerability in tumor xenograft models that express a FLT3 ITD mutant kinase [24,25].

A previous study used recombinant enzyme in in vitro kinase assays to identify that quizartinib targets related class III RTKs, such as wildtype and gain-of-function mutant KIT and PDGFR isoforms [24].

Using several cell based assays, we now show, that quizartinib treatment of leukemic cells leads to inhibition of mutant KIT, PDGFR and FLT3 isoforms - with resultant inhibition of cellular proliferation and induction of apoptosis. These effects are seen in vitro as well as ex vivo (primary leukemic blasts). Importantly, potent antitumor activity was seen against distinct (mutated) kinase isoforms, including FIP1L1-PDGFRA, and FLT3 ITD, FLT3 TKD1 and FLT3 TKD2 mutations. Whereas some mutant-KIT and -FLT3 isoforms were sensitive to quizartinib treatment, some mutations such as FLT3 D835V and the most prevalent KIT gain-of-function mutation detected in CBF AML, KIT D816V, was relatively insensitive with regard to quizartinib treatment.

Quizartinib is currently under clinical investigation in FLT3 ITD and wildtype AML. Our data suggests that quizartinib may be an attractive agent for clinical investigation in other settings as outlined here. This would not include the group of mutant-KIT CBF AML that have KIT D816V mutations. However, patients with CBF AML with KIT D816Y or exon 11 mutations or patients with solid tumors associated with KIT and PDGFR mutations, such as GIST might benefit from this agent. Clinical mutation analysis could help identify individuals that are the most likely to respond to quizartinib.

Results
Quizartinib inhibits cellular proliferation of mutant-FLT3, -KIT or –PDGFR leukemia cell lines in a dose dependent manner
Quizartinib was previously reported to be a potent inhibitor of wildtype FLT3 and FLT3 ITD kinases [24]. Structural considerations suggest quizartinib could inhibit other members of the class III RTK family that are frequently mutated in leukemia or myeloproliferative disorders (i.e. KIT and PDGFR). These findings prompted us to evaluate quizartinib sensitivity in a variety of leukemia cell line models harboring RTK mutations.

The human mast cell leukemia cell lines HMC1.1 (KIT V560G) and HMC1.2 (KIT V560G + D816V), the murine mast cell line p815 (harboring a KIT D816Y mutation analogous to the human D816Y mutation), the eosinophilic leukemia cell line EOL-1 (FIP1L1-PDGFRA), the CBF AML cell line Kasumi-1 (N822K), the myeloid leukemia cell line MOLM14 (heterozygous FLT3 ITD), M-07e (growth factor dependent wildtype KIT), the APL cell line HL60 (growth factor independent, wildtype FLT3 and KIT), the lymphoblastic leukemia cell line Jurkat (no known activated RTK) and the CML blast crisis cell line K562 (BCR/ABL1) were treated with quizartinib in a dose-dependent manner for 48 hours and the cellular antiproliferative capacity was measured using an XTT-based assay.

The proliferation of cell lines with FLT3 ITD (MV4;11, MOLM14), FIP1L1-PDGFRA (EOL-1), ligand-stimulated wildtype KIT (M0-7e), or certain KIT mutations (KIT exon 11 V560G, HMC1.1; Kasumi KIT exon 17 N822K) was strongly inhibited by quizartinib (Figure 1). In contrast, the proliferation of a cell line with a KIT exon 11 V560G and KIT exon 17 D816V mutation on the same allele (HMC1.2) was basically insensitive to quizartinib. Noteworthy, the murine cell line p815, harboring an alternative (tyrosine) substitution at the same codon (KIT
D814Y, which corresponds to D816Y in human KIT), retained intermediate sensitivity to quizartinib.

As experimental controls, we also tested quizartinib against several cell lines lacking an activated type III RTK (K562 [BCR-ABL1], Jurkat [no known tyrosine kinase oncogene], and HL60 [no known tyrosine kinase oncogene, but expressed wild-type FLT3]; quizartinib had no meaningful anti-proliferative effects against these cell lines (IC50 > 10,000 nM; Table 1).

DMSO alone, used in the highest concentration in any of the quizartinib dosing experiments, had no significant antiproliferative effect (MOLM14 cell line, Table 1). To further show the specificity of the anti-proliferative effect of quizartinib, we tested the effects of this drug against M-07e cultured in GM-CSF rather than SCF: Whereas the SCF-stimulated M-07e were sensitive to quizartinib (IC50 77 nM), GM-CSF stimulated M-07e were completely resistant (quizartinib IC50 > 10,000 nM).

Notably, the sensitivity patterns observed for different mutant RTK isoforms were not totally consistent with IC50s previously published in a kinase assay reported by Zarrinkar and colleagues [24] (e.g. IC50 KIT D816V 150 nM versus >1500 nM in our assays). There are several possible explanations for this disparity. First, the results by Zarrinkar et al. were performed using drug binding to recombinant kinases as opposed to enzymatic inhibition of full-length cellular kinases. Second, while we did not change FBS levels prior to quizartinib administration (10% for leukemia cell lines, 20% for native leukemia blasts) – the earlier report by Zarrinkar et al. [24] used cells that were pre-sensitized by exposure to reduced serum levels (0.5% FBS) 12 hours prior to therapy.

Serum deprivation is a commonly used method to reduce serum-drug interactions – but also has profound cellular effects including accumulation and synchronization of cells in the G1/G0 [26]. Moreover, given the high protein binding of quizartinib (99% protein bound), it is not surprising that changes in serum concentration would affect drug potency in cell-based models.

For example, we repeated our experiments with the KIT D816V-positive cell line HMC1.2, but this time cultured the cells in serum-reduced media overnight prior to quizartinib treatment the next day. This change in experimental conditions profoundly altered the anti-proliferative effect of quizartinib, as the IC50 for serum-deprived cells was ~260 nM compared to the serum replete conditions (IC50 ~1700 nM). The potency of the serum-deprived cells is in the range of the predicted IC50 for the D816V mutation reported by Zarrinkar et al. [24] (Table 1).

This effect was further validated using an isogenic cell model as well as native FLT3 ITD positive leukemia blasts as described below. (Please, refer to Table 2 and Additional file 1: Figure S1 for similar experiments using native cells and to Table 3 for serum-deprivation experiments using Ba/B3 FLT3 ITD and KIT D816V cells).

Quizartinib induces apoptosis in in vitro leukemia cell lines

The extraordinary antiproliferative effect seen in some cell models tested, was also accompanied by microscopically condensed pyknotic cells that accumulated over time. This observation suggests that quizartinib may induce apoptosis via inhibition of (mutant) FLT3, KIT or PDGFRα.

Using an annexin V-based immunofluorescence assay, we were able to demonstrate potent dose-dependent induction of apoptosis in several leukemia cell lines:

In analogy to the demonstrated antiproliferative effects, evaluation of quizartinib in several cell lines lacking an activated type III RTK (K562, Jurkat and HL60) did not reveal any significant proapoptotic effects. In contrast, cell lines harboring FLT3 ITD (MV4;11,
MOLM14), FIP1L1-PDGFRα (EOL-1), SCF (but not GM-CSF)-stimulated wild-type KIT (M0-7e), or certain KIT mutations (KIT exon 11 V560G, HMC1.1; Kasumi KIT exon 17 N822K) potently underwent apoptosis upon quizartinib exposure with IC50s in the lower nanomolar ranges (Table 2). Notably, IC50s were similar or somewhat higher compared to the antiproliferative effects achieved in these cell lines (compare with Table 1).

HMC1.2, the sister cell line of HMC1.1 harboring an additional KIT D816V mutation, revealed a complete loss of sensitivity towards quizartinib in all tested doses (Figure 2; IC50s are provided with Table 2). This finding suggests that the distinct mutant-KIT isoform directly orchestrates sensitivity towards quizartinib. In this context it is noteworthy, that the KIT D814Y-positive (D816Y in human KIT) murine cell line p815 was still capable to induce apoptosis with an IC50 in the hundreds nanomolar range (Figure 2).

**Comparison of quizartinib sensitivity towards different leukemia-driving KIT and FLT3 mutations in an isogenic cellular background**

Quizartinib potently inhibits cellular proliferation and induces apoptosis in leukemia cell lines that are dependent on FLT3, KIT or PDGFRα activity. However, the potency of quizartinib differs widely between the tested cell lines – from complete insensitivity to doses in the low nanomolar range.

The divergent inhibitory effects may be due to differential sensitivity profiles of different (mutant) RTK isoforms (compare findings about HMC1.1 versus HMC1.2 in Table 1).
cells) – but may also have been obscured by additional genomic abnormalities contributing to leukemogenesis and resistance to therapeutics.

To exclude cell line-specific off-target biology interfering with the effects of kinase-inhibition, we tested leukemia-driving RTK mutations in an isogenic cellular background: Various human (mutant) FLT3 or KIT isoforms were stably transfected in the IL3-dependent murine pro B-cell line Ba/F3. Activation of the transfected mutant isoforms was demonstrated by selecting for cells with IL-3 growth factor-independent proliferation. However, BaF3 cells expressing wildtype KIT or FLT3 isoforms required the addition of the corresponding ligand, (KIT (SCF) or FLT3L)).

We were able to directly cross-check the clinically most relevant RTK mutations in acute leukemia (i.e.

| Cell line | Target | IC50 (nM) |
|-----------|--------|-----------|
| HMC1.1    | KIT V560G | 31        |
| HMC1.2    | KIT V560G/D816V | not reached* |
| p815      | KIT D814Y (murine) | 341 |
| Kasumi-1  | KIT N822K | 67        |
| M-O7e + SCF | KIT-activated | 78        |
| M-O7e + GM-CSF | unspecific stimulation | not reached* |
| EOL-1     | FIP1L1-PDGFRα | < 1 |
| K562      | BCR/ABL | not reached* |
| HL60      | N/A | not reached* |
| Jurkat    | N/A | not reached* |
| MV4;11    | FLT3 ITD (hemizygous) | 2        |
| MOLM14    | FLT3 ITD | 3        |
| GIST822   | KIT K642E | 109 |
| GIST48    | KIT V560D/D820A | not reached* |
| Pat.368   | FLT3 amplification | 2998 |
| Pat.601   | FLT3 ITD | 876 |
| Pat.695, 20% FBS | FLT3 ITD | 2335 |
| Pat.695, 0.5% FBS | FLT3 ITD | 25 |
| Pat.139, 20% FBS | FLT3 ITD, Relaps | 760 |
| Pat.139, 0.5% FBS | FLT3 ITD, Relaps | 10 |
| Pat.507   | CBF AML (KIT WT) | 1275 |
| Pat.317   | CBF AML (KIT D816Y) | 1294 |
| Pat.521   | CBF AML (KIT WT) | 2018 |
| Pat.305   | CBF AML (KIT WT) | 2954 |
| Pat.511   | CBF AML (KIT WT) | 4272 |
| Pat.281   | CBF AML (KIT WT) | 5758 |
| Pat.279   | CBF AML (KIT WT) / FLT3 amplification? (subclone) | 6607 |
| Pat.523   | CBF AML (KIT WT) | 7175 |
| Pat.361   | CBF AML (KIT D816V) | 8443 |
| Pat.239   | CBF AML (KIT D816V) | not reached* |

* tested up to 10 000 nM.

The table summarizes estimated IC50 values obtained by non-linear regression analysis for the cytotoxic activity of quizartinib in leukemia cell lines and primary native leukemia blasts. Native patient blasts were cultured in 20% FBS,” to “Cell lines were cultured in 10% FBS; native patient blasts were cultured in 20% FBS. To address methodology aspects towards sensitivity profiles two native FLT3 ITD + patient samples (Pat. 695 with newly diagnosed AML, Pat. 139 with relapsed AML) were co-treated with reduced serum (0.5% FBS).

In addition IC50 estimates for the proapoptotic effect of quizartinib in the imatinib-sensitive GIST solid tumor cell line GIST882, harboring a K642E mutation, and the imatinib-insensitive cell line GIST48, harboring a V560D mutation in addition to a D820A mutation in the tyrosine kinase domain, are provided, revealing sensitivity profiles similar to imatinib.

Table 2 Non-linear regression analysis of IC50s (Apoptosis/Viability)
FLT3-ITD, KIT D816V/Y, BCR/ABL1) transfected into an isogenic Ba/F3 background against a panel of leukemic cell lines harboring a corresponding RTK mutation. Comparison of inhibition of cellular proliferation after quizartinib treatment revealed strong correlation between naturally occurring and engineered cell lines expressing identical mutant kinases (Figure 3A): Ba/F3 cells stably transfected with a vector encoding for a FLT3 ITD were equally highly sensitive to quizartinib compared to the human FLT3 ITD positive leukemia cell line MOLM14. Vice versa, transfection of a KIT D816V mutation retained Ba/F3 cells highly resistant towards quizartinib, which is consistent with findings in the human mast cell leukemia cell line HMC1.2 as discussed earlier. Interestingly, replacing the valine substitution with tyrosine at codon 816 (D816Y) rendered Ba/F3 cells to relative sensitivity to quizartinib – so was the KIT D814Y-positive cell line p815.

This observation is not unique to quizartinib – but is in line with previous data for other KIT tyrosine kinase inhibitors, such as dasatinib [27]. In this context, a recent study suggested structural reasons that underlay drug sensitivity of different mutant-KIT kinases using sunitinib and imatinib mesylate [28].

Surprisingly, transfection of BCR/ABL1 into Ba/F3 cells not only did not halt proliferation of cells – but did confer a proliferation advantage for quizartinib treated cells in a dose-dependent manner. This observation deserves further exploration with regard to molecular mechanisms.

Together, these findings suggest a direct mutant-specific tyrosine kinase-mediated effect of quizartinib towards modulation of cellular proliferation. Table 3 provides additional information of sensitivity patterns, with regard to inhibition of proliferation as well as induction of apoptosis, for several mutant-FLT3, -KIT and BCR/ABL1 isoforms transfected into an isogenic Ba/F3 cellular background: Of note, transfection of a FLT3 D835V kinase domain mutation, which is homologous to D816V in KIT, reveals restricted sensitivity towards quizartinib – which is in line with a recent study by Smith and colleagues demonstrating a conformational clash preventing proper binding of quizartinib to the FLT3 binding pocket [29]. Importantly, our data further show that alternative substitution of aspartic acid with a tyrosine residue (D835Y) renders cells to sensitivity, which underlines our findings for KIT D816Y as discussed above.

### Table 3 Estimated IC50s for the proapoptotic and antiproliferative effects of quizartinib in an isogenic cell model of Ba/F3 cells transfected with various mutant TKs

| Isoform   | Mutation locus | IC50 (nM) Ba/F3 transfectants | IC50 (nM) leukemia cell lines |
|-----------|----------------|-------------------------------|------------------------------|
|           |                | Inhibition of proliferation   | Induction of apoptosis     |
|           |                | not reached (MOLM14)          | n.r.* (MOLM14)             |
| BCR/ABL   | fusion         | n.r.*                         | n.r.*                       |
| FLT3 KIT  | WT N/A         | 49                            | 11                           |
| FLT3 ITD  | 10% FBS       | juxtamembrane domain 9        | 5                            |
| FLT3 ITD  | 0.5%FBS       | juxtamembrane domain <1       | N/D                          |
| FLT3 K663Q| tyrosine kinase domain I | 14  | 23                           |
| FLT D835V | tyrosine kinase domain II | 172 | 888                          |
| FLT D835Y | tyrosine kinase domain II | 84  | 24                           |
| KIT WT    | N/A            | 474                           | n.r.*                       |
| KIT D816F | tyrosine kinase domain II | 2871 | 6254                          |
| KIT D816V | 10% FBS        | tyrosine kinase domain II 3074 | 8982                        |
| KIT D816V | 0.5% FBS       | tyrosine kinase domain II 633 | N/A                          |
| KIT D816V | 0.5% FBS       | tyrosine kinase domain II 366 | 611                          |
| parental  | N/A            | n.r.*                         | n.r.*                       |
| parental + DMSO | N/A | n.r.* | n.r.* |

*(not reached with tested doses up to 10 000 nM).

Sensitivity of quizartinib is distinct to and differs widely in between different tyrosine kinase isoforms transfected into an isogenic Ba/F3 cellular background. Estimated IC50s were computed using non-linear regression analysis of an average mean of at least 3 experiments for each cell line.

If applicable, IC50s of leukemic cell lines harboring a similar mutation are provided (rows on the right).

Influence of serum-deprivation on sensitivity profiles of quizartinib was tested in two cell strains (Ba/F3 FLT3 ITD or KIT D816V): Cells were cultured in media with a reduced serum concentration (0.5% FBS) and treated with quizartinib the next day.

Solvent-associated non-specific cytotoxicity was excluded using the parental Ba/F3 cell strain treated with DMSO in the highest concentration used for the quizartinib dose experiments.
Inhibition of cellular proliferation associates with distinct inhibition of phosphorylation of the target receptor tyrosine kinase in an isogenic cell model

In our cell biology experiments, the sensitivity of the tested cell lines to quizartinib was linked to inhibition of (mutant) class III RTK. The isogenic cell models confirm IC50s obtained for the leukemia cell lines harboring similar mutations, further suggesting a direct interaction of tyrosine kinase inhibition and the observed antiproliferative and proapoptotic effects in the tested cell lines (rather than off-target effects).

To address this question at the protein level, we additionally performed immunoblotting experiments for Ba/F3 cell lines transfected with mutant KIT or FLT3 kinases and treated with quizartinib for 90 minutes. Indeed, sensitivity towards quizartinib, as indicated by loss of RTK autophosphorylation, proved to be kinase-specific and was in agreement with the functional assays using the same cell lines. Notably, the KIT D816V mutation, exchanging aspartic acid for valine at codon 816 and thus rendering the kinase autophosphorylated, does not show significant reduction of phosphorylation levels – while tyrosine or phenylalanine substitutions at the same codon (D816Y or D816F), similarly leading to autoactivity of the kinase, proved to be sensitive towards quizartinib treatment with loss of autophosphorylation in the nanomolar ranges (Figure 3B). Interestingly, D816Y was thereby dephosphorylated at the glycosylated membrane-bound (∼145 KDa), as well as the intracellular isoform (∼125 KDa) – whereas sensitivity of D816F was virtually restricted to the glycosylated isoform.

These results are in line with the viability assays provided in Figure 3A and Table 3, and again argue against nonspecific off-target – but for TK-mediated effects. Moreover, it underlines that TKI sensitivity patterns are not just tyrosine kinase, kinase-domain or codon specific – but may even depend on the type of amino acid substitution at a given codon.
In vitro inhibition of cellular proliferation by quizartinib translates into ex vivo antiproliferative effects in native leukemia blasts

We further evaluated the antiproliferative effects of quizartinib using native blasts isolated from patients with newly diagnosed FLT3- or KIT-activated AML (additional patient characteristics are provided in Additional file 2: Table S1 with the online version of the article).

Notably, quizartinib was able to inhibit proliferation of ex vivo CBF AML blasts and FLT3 ITD positive blasts in the upper nanomolar or lower micromolar ranges (Table 1).

CBF AML is associated with high CD117 (i.e. KIT) expression and/or autoactivating mutations within the KIT gene [7]. KIT mutation screening of exons 8, 9, 11, 13 and 17 was performed. No autoactivating mutation in our patient cohort used for antiproliferation assays was detected, suggesting a paracrine activation of KIT in the quizartinib-responsive patient cohort as demonstrated earlier for ~50% of FLT3/KIT wildtype patients [30].

In FLT3-associated leukemia patients, the antiproliferative effect of quizartinib was inconsistent – with refractory as well as sensitive cases identified. For example, one case sensitive to quizartinib treatment was from a patient with an MLLT3-MLL rearrangement (patient #368). We were not able to detect any FLT3 or KIT mutations in this patient – although, karyotyping revealed trisomy of chromosome 13 (FLT3 genomic location), which potentially contributed to treatment response via FLT3 amplification. Potent inhibition of amplified FLT3 wildtype gene via quizartinib was recently shown in an in vitro leukemia cell model using the SEM-K2 ALL cell line by Gunawardane and colleagues [31]. Another of our cases demonstrating sensitivity towards quizartinib harbored a FLT3 ITD, but interestingly, two more cases with a FLT3 ITD were refractory to quizartinib (patient #176 and #602).

FLT3 ITD sequencing revealed that the internal tandem duplication was located in the beta1 sheet of the first tyrosine kinase domain in both resistant cases. This particular class of mutant kinase is resistant to FLT3 inhibition by midostaurin (PKC412) and is associated with a poor clinical outcome [32-34]. Our data suggests a similar sensitivity profile for quizartinib against FLT3 ITD-beta1 mutant kinases.
Quizartinib induces apoptosis in ex vivo native leukemia cells

We next tested isolated native blasts derived from patients with newly diagnosed AML to confirm the proapoptotic effect observed for quizartinib in in vitro leukemia and isogenic mutant-TK models (IC50s for all patients are provided with Table 2; patient characteristics are available as supplementary material in Additional file 2: Table S1).

One sample, taken from a bone marrow aspirate of a patient with de novo AML, was identified to harbor a FLT3 ITD mutation in the juxtamembrane domain of the gene (patient #601). IC50 was in the higher nanomolar range – which is considerably higher compared to the in vitro FLT3 ITD models. The cause of this discrepancy is unknown, but is commonly observed in ex vivo blasts compared to in vitro models [35,36]. In addition to the above comments about the effect of serum concentration on sensitivity to quizartinib, other genomic abnormalities acquired in the context of complex cytogenetic AML may have contributed to the observed effects in cultured ex vivo blasts.

In a second patient sample, obtained from the bone marrow aspirate of an elderly patient with MLL-associated AML – including trisomy of chromosome 13 (leading to overrepresentation of the FLT3 gene), quizartinib treatment induced apoptosis in these cells with an IC50 ~3000 nM. Due to the high bioavailability of quizartinib, ex vivo IC50s in the lower micromolar ranges may translate into antileukemic activity in vivo – but this observation needs clinical validation.

A subset of native leukemia samples analyzed presented with a high proportion of dead/apoptotic cells in the untreated control samples. This obscured conventional analysis via annexin/PI staining of proapoptotic effects induced by quizartinib treatment. Nevertheless, we were able to assess reduction of the proportion of viable cells in a cohort of CBF AML patients 48 hours after quizartinib treatment compared to treatment-naive cells (Figure 4 for representative analysis of one patient). Table 2 provides a summary of IC50s of all tested samples.

The cytotoxic effect of quizartinib varied widely. Analysis of the KIT mutation status revealed a wildtype gene for KIT in most patients – some of these were sensitive to quizartinib therapy, arguing for paracrine activation of KIT or FLT3 [30].

Of interest, similar to our cell line results, two patients with a KIT D816V mutation revealed relative insensitivity towards quizartinib. However, as predicted by our in vitro models (see also Figure 3), one patient with CBF AML harboring the less common KIT D816Y mutation demonstrated sensitivity with an IC50 for reduction of viable cells of approximately 1300 nM. Notably, this lies in the same range as seen for the proapoptotic effect of ex vivo blasts of a newly diagnosed treatment-naive patient (patient #601) harboring a FLT3 ITD (JM) mutation. Additional patient characteristics are provided in Additional file 2: Table S1 with the online version of the article.

In general, the reported IC50s in our study for native leukemia cells lie in contrast to a previous report by Kampa-Schittenhelm et al. Molecular Cancer 2013, 12:19

http://www.molecular-cancer.com/content/12/1/19

Figure 4 Quizartinib reduces the proportion of viable cells in samples of native leukemia cells. Patient samples were cultured in DMEM containing 20% FBS and treated with different concentrations of quizartinib for 48 hours. (A) The plot of one patient is shown. The mononuclear cell fraction was gated in a FSC/SSC scatter and viability was confirmed using an annexin V/propidium iodide (PI) stain demonstrating negativity for annexin V and PI. (B) Relative reduction of the proportion of viable cells in response to quizartinib was assessed by flow cytometry. A dose-effect plot of all tested concentrations of quizartinib is shown on the right. Estimated IC50s by non-linear regression analysis of all tested patient samples are provided in Table 2.
Zarrinkar and colleagues [24], which have suggested IC50s for FLT3 ITD-positive native blasts in the lower nanomolar ranges.

Several issues need to be discussed in this context. Besides individual cell-context specific additional effects (such as additional mutations rendering signal transduction pathways or drug sensitivities), which may have obscured TK-targeted effects of quizartinib, a couple of methodology-related aspects need to be addressed: As discussed earlier, we did not use serum reduced conditions in our assays — but serum-rich media containing 10% FBS for cell lines and 20% FBS for experiments involving native blasts. Moreover, it has been reported that blasts obtained from patients with relapsed FLT3 ITD-positive leukemia may show higher sensitivities towards tyrosine kinase inhibitors due to a higher addiction to FLT3 gain-of-function signal transduction of leukemia blasts in the relapse setting compared to de novo AML samples [37]. Notably, the Zarrinkar study evaluated the efficacy towards quizartinib in relapsed patients while our work included only specimens from newly diagnosed patients. We addressed these issues and treated a newly diagnosed patient with AML as well as a patient with relapsed AML, both harboring a FLT3 ITD mutation, with quizartinib in a dose-dependent manner. Both samples were cultured in serum-repleted (20% FBS) as well as serum-reduced (0.5% FBS) conditions. In line with our theory, the average concentration to induce apoptosis was markedly reduced with IC50s in the low nanomolar range in samples cultured in serum-reduced conditions. Even more, sensitivity towards quizartinib was increased in the relapsed leukemia patient. Exemplary AnnexinV-based density plots illustrating the influence of culture conditions with regard to the achievable proapoptotic effects (~22% versus 80% dead/apoptotic cells upon exposure to 10 nM quizartinib when cultured in 20%, resp. 0.5% FBS) are provided as Additional file 1: Figure S1; IC50s are provided with Table 2.

Antitumor activity of quizartinib in mutant-KIT solid tumor cell lines

Besides acute leukemia, KIT mutations are found in a large proportion of gastrointestinal stromal tumors (GIST) [38], in subsets of seminomas [39] and melanoma [40]. PDGFR mutations are further reported in myeloproliferative disorders and GIST as well [38].

KIT or PDGFRα tyrosine kinase inhibition is the only known medical treatment option for advanced GIST. Due to the excellent bioavailability properties of quizartinib, higher plasma concentrations are achieved compared to other inhibitors with a similar sensitivity profile. This may be advantageous in particular for the treatment of solid tumor lesions.

We treated an imatinib-sensitive GIST cell line (GIST822) harboring a KIT exon 13 mutation (K642E) and a second cell line, GIST48, harboring an imatinib-sensitive V560D mutation plus a secondary imatinib-insensitive activation loop mutation (D820A) with varying concentrations of quizartinib. Due to a much slower in vitro cell doubling time of the GIST cell lines compared to leukemic cell lines, GIST cells were treated for 7 days. Figure 5 demonstrates a potent proapoptotic effect of quizartinib targeting the KIT K642E mutation in the GIST822 cell line, whereas the imatinib-insensitive cell line GIST48 did not display any significant signs of induction of apoptosis following quizartinib treatment. Calculated IC50s are provided in Table 2.

Discussion

Tyrosine kinase inhibitors are rapidly entering into the clinic. These agents are subject of intensive clinical investigation for the treatment of acute leukemia. For example the following inhibitors are under investigation for mutant ABL1, FLT3 or KIT-associated subtypes (clinicaltrials.gov): sorafenib (e.g. AML: NCT00127646, NCT00373373), sunitinib (e.g. AML: NCT00783653), dasatinib (e.g. CBF-AML: NCT00850382; SM: NCT00979160; Ph + ALL: NCT00103701, NCT00940524), imatinib (e.g. AML: NCT00707408, NCT00744081; activated RTKs/ various tumors: NCT00171912), lesartininib (e.g. AML: NCT00030186, NCT00079482), tandutinib (e.g. AML: NCT0064584, NCT00274248), masitinib (e.g. SM: NCT00814073 and nilotinib (e.g. AML: NCT01222143; SM/HES: NCT00109707).

Quizartinib (formerly AC220), a N-(5-tert-butyl-isoxazol-3-yl)-N’-[4-[7-(2-morpholin-4-yl-ethoxy)imidazo [2,1-b] [1,3] benzothiazol 2-yl]phenyl]urea dihydrochloride, is a novel class III tyrosine kinase inhibitor with promising in vitro as well as in vivo activity against FLT3 wildtype and mutant isoforms [24]. Compared to other tyrosine kinase inhibitors in evaluation for the treatment of acute leukemia subtypes, quizartinib provides superior bioavailability with longer and higher plasma concentrations achieved in vivo [25] thereby targeting and suppressing the activated kinases more effectively.

Early data from a phase II trial of quizartinib in refractory or relapsed FLT3 ITD+ patients revealed an acceptable safety profile. Interim analysis of 62 patients (29 female, 33 male) was previously presented (Cortes, EHA annual meeting 2011, #1019): Common drug-related adverse events for evaluable patients were nausea, QTc prolongation, vomiting, fatigue, dysgeusia, anorexia, febrile neutropenia, diarrhea, and dyspepsia. Drug-related severe adverse events in >15% of patients were febrile neutropenia and asymptomatic Grade 3 QTc prolongation. 85% of patients were evaluable for efficacy: The
composite CR (CRc = CR + CRp + CRi) rate was 43% (1 CRp, 22 CRi) and PR rate was 28%. Of note, of the patients refractory to any prior therapy, 56% had CRc and 22% had PR in response to quizartinib treatment. 26% of patients were bridged to allogenic transplantation concepts.

We now report that quizartinib not only targets FLT3 wildtype and ITD mutant kinases [24] – but also potently inhibits cellular proliferation and induces apoptosis of cells expressing a broad range of other clinically relevant class III (mutant) RTK isoforms associated with various diseases, including pediatric and adult leukemia (FLT3 and KIT), GIST, seminoma and melanoma (KIT), as well as myeloproliferative neoplasms associated with eosinophilia (PDGFRA). Quizartinib-responsive mutations were thereby detected in the juxtamembrane (ITDs), but also the tyrosine kinase domains (TKD I - K663Q, TKD II - D835Y) of FLT3, as well as in the KIT JM- (V560G) and TK-domains (D816Y). However, as with all tyrosine kinase inhibitors, quizartinib has a distinct potency profile against different autoactivating mutant RTK isoforms - and some mutations in the TKD of FLT3 (D835V, B1-sheet ITDs) and KIT (D816V) proved to be resistant towards quizartinib. Resisnticity of FLT3 ITD mutations located in the beta-1 sheet of the first TKD has previously been shown for other TKI [32-34] as well. In contrast to the BCR-ABL1 fusion transcript demonstrating resistance towards quizartinib, the FIP1L1-PDGFRα fusion mutation revealed extraordinary sensitivity. In CBF AML, which is frequently dependent upon KIT-mediated (gain-of-function) signal transduction, quizartinib demonstrated varying antiproliferative and cytotoxic efficacy in in vitro and ex vivo leukemia cells – in some cases within the low nanomolar range of FLT3 ITD (JM) positive samples. However, the most prevalent KIT mutation in CBF AML, substituting aspartic acid at codon 816 (KIT D816V), was demonstrated to be basically insensitive towards quizartinib in in vitro and ex vivo leukemia cell lines, primary myeloblasts, and in an isogenic Ba/F3 cell model – while substitution of a tyrosine residue (D816Y) retained some sensitivity to quizartinib. Therefore tyrosine kinase genotyping may become a prerequisite for clinical use of this agent.

Moreover, based on our data, we speculate that quizartinib may be a promising agent in solid tumors associated with KIT mutations, such as GIST or melanoma: In addition to a favorable activity against KIT mutant kinases expressed in GIST (and other mutant-KIT neoplasms), the excellent pharmacokinetics with unprecedented achievable plasma concentrations may be advantageous to target bulky solid tumor lesions that have impaired drug uptake. Thus, our data opens new avenues for clinical investigation and further testing of the efficacy of quizartinib in these settings is warranted.

It has to be noted, that IC50s in our studies were coherent in between all cell models used – but higher compared to a previous report [24]. The phenomenon was seen throughout the assays and is therefore most likely due to methodology reasons as we have illustrated with several experimental data:

While individual cell-context specific additional effects (such as additional mutations) can not be fully excluded to have obscured sensitivity profiles in some cell models,
methodological differences most likely will account for most of the discrepancies observed: In contrast to previous studies using serum-depleted culture conditions (0.5% FBS), we used serum-repleted medium in all assays (10% FBS for cell lines, 20% for native blasts).

Even more, data from Zarrinkar and colleagues were based on treating refractory/relapsed AML samples [24] – in contrast, samples tested in our assays were isolated from patients with newly diagnosed disease. However, it is believed that refractory/relapsed patient samples have higher sensitivities towards TK-inhibition due to a higher addiction to the respective oncogenic (mutant) tyrosine kinase [37].

To underline our theories, we have provided substantial experimental data using serum-deprived versus serum-rich culture conditions in cell lines and native blasts derived from newly diagnosed as well as relapsed patients to treat with quizartinib in a dose dependent manner. High correlation of serum-levels and achievable IC50s was confirmed in all cell models. In addition, treating newly diagnosed versus relapsed FLT3 ITD-positive leukemia native patient samples, a higher sensitivity profile for relapsed AML was verified. Thus, the data will need to be interpreted in this context.

Conclusion
To summarize, our findings suggest that quizartinib is a promising agent for treatment of several hematologic and solid human neoplasms. However, due to the quizartinib-specific mutation restricted spectrum of activity, tyrosine kinase mutation screening may be required to identify patients most likely to respond to quizartinib therapy.

Methods
Cell lines
The CML blast crisis cell line K562 was a generous gift of Dr. Lopez, Oregon Health and Science University, Portland, OR. The FLT3 ITD positive AML cell line MOLM14 (heterozygous for an ITD of DFREYE at amino acid position 593-598 [2]) was acquired through the Fujisaki Cell Center (Okayama, Japan). The acute myeloid leukemia cell line HL60, the KIT N822K positive CBF AML cell line Kasumi1 [41] and the FLT3 ITD positive AML cell line MV4-11 were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). MV4-11 cells are hemizygous for an ITD of amino acids VDFREYEYDH at position 592-601 [42]. The interleukin 3 (IL-3)–dependent murine pro-B cell line Ba/F3, the FIP1L1-PDGFRα positive cell line EOL-1 [43], from a patient with eosinophilic leukemia following hypereosinophilic syndrome [44], and the p815 mast cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA). The murine KIT D814Y mutant isoform expressed by the p815 cell line [45] is homologous to the human KIT D816Y mutation. The human hematopoietic growth factor–dependent M-07e cell line was kindly provided by Dr. Hal Broxmeyer (Department of Microbiology and Immunology, Walther Oncology Center, Indiana University School of Medicine, Indianapolis, IN). The human HMC-1.1 mast cell line, expressing a KIT juxtamembrane domain mutant isoform (V560G), was provided by Dr. Butterfield (Division of Allergic Diseases, Department of Internal Medicine, Mayo Clinic, Rochester, MN). A spontaneously occurring subclone of the HMC-1.1 cell line, HMC-1.2, which has an additional mutation in the activation loop (D816V) [46], was kindly provided by Dr. Akin (Laboratory of Allergic Diseases, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD). All cell lines were cultured in RPMI media containing 10% FBS.

M-07e cells were cultured using recombinant human granulocyte-macrophage colony stimulating factor (GM-CSF, R&D, Minneapolis, MN) as a growth supplement as previously described [47]. Negativity for mycoplasma contamination was confirmed using the pluriportent PCR Mycoplasma test Kit (AppliChem, Darmstadt, Germany). Cell lines harboring a mutant KIT, FLT3 or BCR-ABL1 were sequence confirmed.

The gastrointestinal stromal tumor cell lines GIST822, harboring a KIT exon 13 mutation (K642E), and GIST48, harboring an imatinib-sensitive V560D mutation plus a secondary imatinib-insensitive activation loop mutation (D820A) were generously provided by Dr. Kopp (University of Tübingen) [48,49].

Site-directed mutagenesis and generation of a Ba/F3 cell line expressing KIT or FLT3 isoforms
Site-directed mutagenesis and generation of Ba/F3 cell lines stably expressing mutant KIT D816V, D816Y, D816F, FLT3 ITD, D835Y, D835Y, K663Q and FLT3 wildtype were performed as previously described [50-52].

KIT Wildtype cDNA cloned into a pJP1563 plasmid vector was obtained from the DNASU Plasmid Repository at the Biodense Institute of the Arizona State University (ASU). Lipofection transfection into the parental Ba/F3 cell line was performed to stably express KIT Wildtype by double selection for neomycin (pCMVneo plasmid), blasticidin (pJP1563 plasmid) or gentamicin (G418; all other plasmids) resistance and IL-3-independent growth. The Ba/F3 KIT Wildtype cell line was cultured using recombinant human stem cell factor (SCF/KITLigand, R&D, Minneapolis, MN) as a growth supplement.

Antibodies and reagents
The small-molecule compounds quizartinib was obtained from Ambit Biosystems and dissolved in DMSO to create 10 mmol/L stock solutions and stored at –20°C.
Anti-KIT and anti-FLT3 rabbit polyclonal antibodies were used at a 1:5,000 to 1:1,000 dilution. Antiphosphotyrosine p-KIT antibodies (Tyr568/570 and Tyr703), p-FLT3 antibodies (Tyr 589/591) and a pan-antiphosphotyrosine antibody (clone PY20) were administered at dilutions of 1:100 to 1:2,000 (all from Cell Signaling Technology).

Infrared dye-conjugated secondary goat anti-rabbit or anti-mouse antibodies to use in a LI-COR® imaging detection system were prepared according to standard protocols (LI-COR Biosciences, Lincoln, NE).

Isolation of bone marrow and peripheral blood mononuclear cells
Bone marrow aspirate and peripheral blood samples from patients with AML were collected in 5000 U heparin after informed consent and approval of the ethics committee of the University of Tübingen or Ulm. Mononuclear cells were isolated by Ficoll Hypaque density gradient fractionation [35]. Additional acute myeloid leukemia samples were generously provided by the German-Austrian AML Study Group (AMLsG) leukemia biobank (patient characteristics summarized in Additional file 2: Table S1). Native ex vivo blasts were cultured in DMEM media containing 20% FBS.

Immunoblotting
Cell pellets were lysed with 100 to 150 μL of protein lysis buffer (50 mmol/L Tris, 150 mmol/L NaCl, 1% NP40, 0.25% deoxycholate with added inhibitors aprotinin, AEBSF, leupeptin, pepstatin, sodium orthovanadate, and sodium pyruvate, respectively phosphatase inhibitor cocktails “2” and “1” or “3” (Sigma, St. Louis, MO). Protein from cell lysates (75 to 200 μg protein) was used for whole cell protein analysis after denaturing by Western immunoblot assays using a BioRad Criterion system (protein separation by SDS-PAGE in 3-8% or 10% polyacrylamide gels followed by electroblotting onto nitrocellulose membranes). Nonspecific binding was blocked by incubating the blots in nonfat dry milk or BSA. Primary antibodies were incubated for one hour or over night, followed by several washes of Tris-buffered saline (TBS) containing 0.005% Tween 20. The appropriate secondary antibody was applied for 30 min, followed by several washes. Antibody-reactive proteins were detected using a LI-COR Odyssey® fluorescence optical system (LI-COR Biosciences, Lincoln, NE).

Apoptosis and cell viability assays
Induction of apoptosis upon quizartinib treatment was assayed in dilution series (0 – 5000 nM) and translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane as an early indicator of apoptosis was analyzed using an Annexin V-based assay (Immunotech, Marseilles, France) and a FACScalibur® flow cytometer loaded with CellQuest® analysis software (BD, Heidelberg, Germany) [27,47].

A proportion of ex vivo leukemia blasts were not available for induction of apoptosis assays using Annexin V/PI staining due to a higher percentage of apoptotic cells in the untreated negative control population. Nevertheless, viability assays were assessed using FSC/SSC-flow cytometry experiments with a gate on the living cell population. Reduction of viable cells in the presence of quizartinib was measured 48 hours post quizartinib treatment.

Proliferation assays
Cells were added to 96-well plates at densities of 50 000 cells per well. Quizartinib was added in dilution series (0 – 5000 nM) and proliferation was measured at 48 hours using an 2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT)–based assay (Sigma, MO) [27,47].

Polymerase Chain Reaction (PCR) and Sequencing
Genomic DNA was isolated using a DNeasy® DNA purification kit (Qiagen, Hilden, Germany). FLT3 mutation status was assessed by routine standard PCR techniques. KIT mutation status of exon 8, 9, 11, 13 and 17 was analyzed by PCR followed by bidirectional sequencing. The primer sets are as follows: KIT exon 8, sense: GCT GAG GTT TTC CAG CAC TC; KIT exon 8, antisense: AAT TGC AGT CCT TCC CCT CT; KIT exon 9, sense: AGCCAGGGTCTTTTGTTC; KIT exon 9, antisense: CAGAGCTTAAACATCCCTTTA; KIT exon 11, sense: CCTTTGCTGATTGGTTTCGT; KIT exon 11, antisense: AAACAAAGGAAGCCACTGGA; KIT exon 13, sense: GTTCTCTGTATGTCATGCG; KIT exon 13, antisense: CAGTTTATAATCTAGCATGG; KIT exon 17, sense: GGTTTTCTTTTCTCCCTCA ACC; KIT exon 17, antisense: GGATTTACATTATGAAAGTCACAGG.

Data analysis
Inhibition of proliferation or the proportion of apoptotic/viable cells was assessed in dilution bar diagrams. Non-linear 4-parameter median-effect regression analysis was performed to compute IC50s using Prism® (GraphPad Software, Inc., LaJolla, CA) or MasterPlex® software (Hitachi Solutions, Tokyo, Japan).

Additional files

Additional file 1: Figure S1. Native FLT3 ITD positive patient blasts were treated with quizartinib in a dose-dependent manner and cultured in serum reduced (0.5%) versus serum repleted (20%) conditions. Cells were incubated for 48 hours and induction of apoptosis was measured using an annexin V-based assay. Density plots for quizartinib at 10 nM are shown - estimated IC50s are provided with Table 2.

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Additional file 2: Table S1. Supplementary information on patient characteristics is available at the website of MOLECULAR CANCER (see “Additional file 2: Table S1/Additional file 1: Figure S1”).

Abbreviations
AML: Acute myeloid leukemia; ALL: Acute lymphoid leukemia; CML: Chronic myeloid leukemia; FIP1L1: FIP1-like 1; FLT3: FMS-like tyrosine kinase 3; GIST: Gastrointestinal stromal tumor; HES: Hypereosinophilic syndrome; IC50: Concentration sufficient to achieve a 50% inhibition; IL3: Interleukin 3; ITD: Internal tandem duplication; KIT: v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog; PDGFRA: Platelet-derived growth factor receptor alpha; RTK: Class III receptor tyrosine kinases; SM: Systemic mastocytosis; TKD1/2: Tyrosine kinase domain 1 resp. 2; TKI: Tyrosine kinase inhibitor; XTT: 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilid-sodium salt.

Competing interests
The authors (s) declare that they have no competing interest.

Authors’ contributions
Dr. KMKS conceived of the design of the study, carried out experiments, analyzed and interpreted data and drafted the manuscript. FA: substantially participated in design and acquisition of experiments, analyzed data and helped drafting the manuscript. Dr. HD conceived of the study, analyzed and interpreted data and critically revised the manuscript. Dr. KO participated in the acquisition of data, analyzed and interpreted data and critically reviewed manuscript. Dr. MCH designed experiments, analyzed and interpreted data and critically revised the manuscript. Dr. MMS conceived of the design of the study, carried out experiments, and supervised the project. Dr. KMKS conceived of the design of the study, carried out experiments, and critically revised the manuscript. Dr. KD participated in the design of the study, helped drafting the manuscript. Dr. HD conceived of the study, analyzed and critically revised the manuscript. Dr. KMKS conceived of the design of the study, carried out experiments, and critically revised the manuscript. Dr. HD conceived of the study, analyzed and critically revised the manuscript. All authors read and approved the final manuscript.

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