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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*

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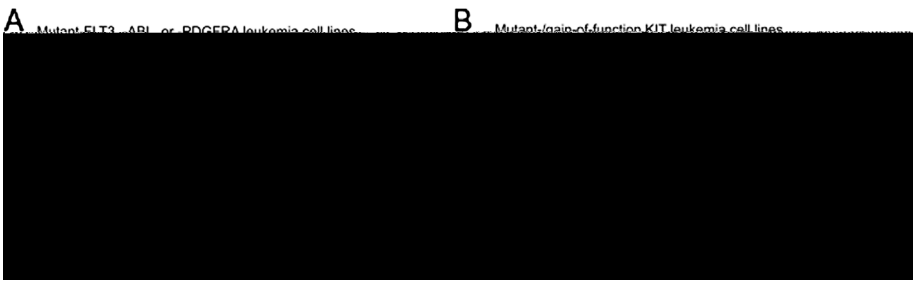

Figure 1 Quizartinib inhibits cellular proliferation of *KIT*-, *FLT3*- or PDGFR-dependent leukemia cells. Dilution series of quizartinib for several cell lines harboring mutant-*FLT3*, *ABL1* or *PDGFRA* (**1A**) or mutant-*KIT* isoforms (**1B**) were performed and cellular proliferation was assessed using an XTT-based assay. Sensitivity towards quizartinib varied widely between the tested cell lines. IC50s and mutation isoforms for all tested cell lines are listed in Table 1.

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 *PDGFRA*.

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,

Table 1 Non-linear regression analysis of IC50s (Antiproliferation)

Cell line	Target	IC50 (nM)
		Inhibition of proliferation
HMC1.1	*KIT* V560G	14
HMC1.2	*KIT* V560G/D816V	1727
HMC1.2, 0.5% FBS	*KIT* V560G/D816V	263
p815	*KIT* D814Y (murine)	445
Kasumi-1	*KIT* N822K	36
M-07e + SCF	*KIT*-activated	77
M-07e + GM-CSF	*GM-CSF* signaling	not reached*
EOL-1	*FIP1L1-PDGFRA*	1
K562	*BCR/ABL*	not reached*
HL60	unknown	not reached*
Jurkat	unknown	not reached*
MV4;11	*FLT3* ITD (hemizygous)	< 1
MOLM14	*FLT3* ITD	< 1
MOLM14 + DMSO	*FLT3* ITD	not reached*
Pat.221	CBF AML (*KIT* WT)	675
Pat.279	CBF AML (*KIT* WT) / *FLT3* amplification (subclone)?	3434
Pat.299	CBF AML (*KIT* WT)	7248
Pat.305	CBF AML (*KIT* WT)	7079
Pat.375	CBF AML (*KIT* N/A)	503
Pat.379	CBF AML (*KIT* WT)	806
Pat.368	*FLT3* amplification ?	2700
Pat.601	*FLT3* ITD	1153
Pat.176	*FLT3* ITD (Beta1)	not reached*
Pat.602	*FLT3* ITD (Beta1)	not reached*

** tested up to 10 000 nM.*
The table summarizes estimated IC50 values obtained by non-linear regression analysis for the antiproliferative activity of quizartinib in leukemia cell lines and primary native leukemia blasts.
The cell line HMC1.2 was additionally pre-treated with reduced serum (0.5% FBS) to address the influence of methodology aspects on sensitivity profiles. To exclude solvent-associated non-specific cytotoxicity, the MOLM-14 cell line was treated with DMSO using the highest concentration for the quizartinib dose experiments.

MOLM14), *FIP1L1-PDGFRA* (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 *PDGFRA* 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

Table 2 Non-linear regression analysis of IC50s (Apoptosis/Viability)

Cell line	Target	IC50 (nM)
		Induction of apoptosis
HMC1.1	*KIT* V560G	31
HMC1.2	*KIT* V560G/D816V	not reached*
p815	*KIT* D814Y (murine)	341
Kasumi-1	*KIT* N822K	67
M-07e + SCF	*KIT*-activated	78
M-07e + GM-CSF	unspecific stimulation	not reached*
EOL-1	*FIP1L1-PDGFRA*	< 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
		Reduction of viable cells
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.

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 *FLT3* (*FLT3*L)).

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

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)	IC50 (nM)	IC50 (nM)	IC50 (nM)
		Ba/F3 transfectants		*leukemia cell lines*	
		Inhibition of proliferation	*Induction of apotosis*	*Inhibition of proliferation*	*Induction of apotosis*
BCR/ABL	fusion	n.r.*	n.r.*	not reached *(K562)*	n.r.* *(K562)*
FLT3 WT	N/A	49	11		
FLT3 ITD, 10% FBS	juxtamembrane domain	9	5	<1 *(MOLM14)*	3 *(MOLM14)*
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		
FLT3 D835Y	tyrosine kinase domain II	84	24		
KIT WT	N/A	474	n.r.*	77 *(M0-7e)*	210 *(MO-7e)*
KIT D816F	tyrosine kinase domain II	2871	6254		
KIT D816V, 10% FBS	tyrosine kinase domain II	3074	8982	1727 *(HMC1.2)*	n.r.* *(HMC1.2)*
KIT D816V, 0.5% FBS	tyrosine kinase domain II	633	N/A		
KIT D816Y, 0.5% FBS	tyrosine kinase domain II	366	611	*445 (p815)*	341 *(p815)*
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.

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.

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, proof 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.

Figure 3 Cellular effects of quizartinib are tyrosine kinase-mediated (A) Quizartinib displays distinct antiproliferative effects of genetically altered Ba/F3 cells in dependence of the tyrosine kinase isoform transfected. The sensitivity of inhibition of proliferation is thereby similar to the sensitivity achieved in natural leukemia cell lines harboring a similar mutation. Estimated IC50s are provided in Table 3 along with IC50s for the proapoptotic effects in the same cellular context. (**B**) The observed cellular effects are directly linked to the potency of inhibition of phosphorylation of mutant-*KIT* and *FLT3* isoforms. Whole cell lysates of Ba/F3 cells transfected with different human mutant-*KIT* or -*FLT3* isoforms were immunoblotted using a pan-phosphotyrosine antibody or a total-*KIT* or -*FLT3* antibody. Pretreatment of cells with quizartinib revealed isoform-specific inhibition of phosphorylation. Notably, inhibition of phosphorylation of the D816V mutation was significantly reduced compared to the D816Y and D816F isoforms.

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

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 d*e 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 PDGFRA 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: NCT00217646, 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), lestaurtinib (e.g. AML: NCT00030186, NCT00079482), tandutinib (e.g. AML: NCT00064584, 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 i*n 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

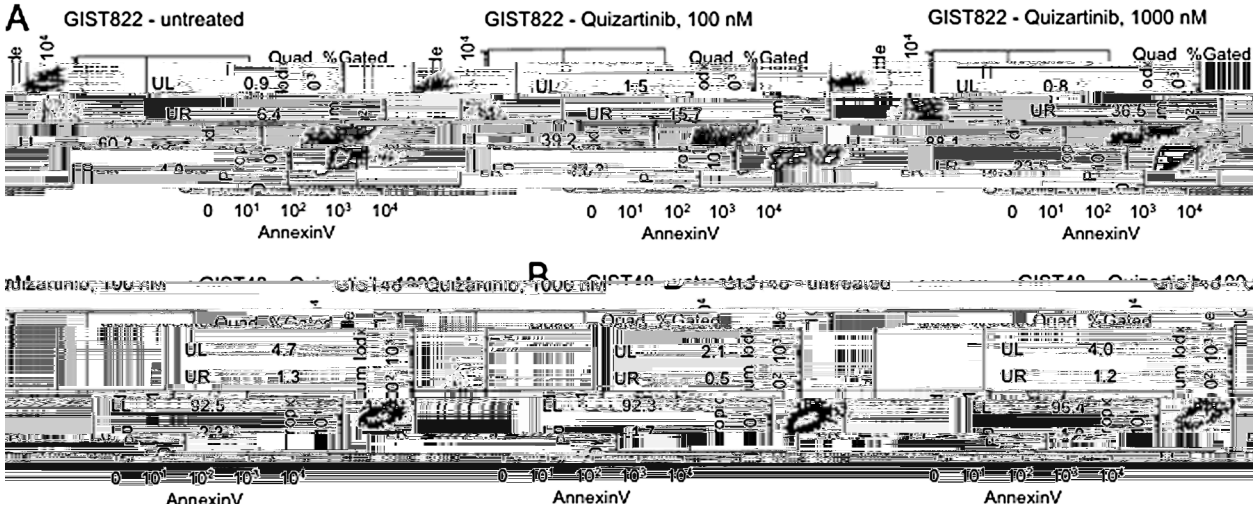

Figure 5 Quizartinib selectively induces apoptosis in gastrointestinal stromal tumor (GIST) cells. The imatinib-sensitive cell line GIST822, harboring a KIT K642E mutation (**A**) and the imatinib-insensitive cell line GIST48, harboring a KIT V560D plus an additional D820A mutation, were treated with quizartinib for 7 days and assayed for induction of apoptosis. Quadrant density dot plots are provided showing the apoptotic cell fraction as indicated by annexin V positivity (LR quadrant: early apoptosis) and/or positive propidium iodide stain (UR: late apoptosis, UL: dead cells) of quizartinib-treated versus treatment-naive cells. Estimated IC50s by non-linear dose regression analysis are provided with Table 2.

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. Resistancy 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-PDGFRA* 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 valine for 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 MV4-11were 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-PDGFRA 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 pluripotent 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 Biodesign 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/*KIT*Ligand, 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', 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-sulfophenyl]-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: AGCCAGGGCTTTTGTTTTCT; KIT exon 9, antisense: CAGAGCCTAAACATCCCCTTA; KIT exon 11, sense: CCTTTGCTGATTGGTTTCGT; KIT exon 11, antisense: AAACAAAGGAAGCCACTGGA; KIT exon 13, sense: GTTCCTGTATGGTACTGCATGCG; KIT exon 13, antisense: CAGTTTATAATCTAGCATTG CC; KIT exon 17, sense: GGTTTTCTTTTCTCCTCCA ACC; KIT exon 17, antisense: GGATTTACATTATGA AAGTCACAGG.

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. Densitiy plots for quizartinib at 10 nM are shown - estimated IC50s are provided with Table 2.

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 author(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. KD participated in the acquisition of data, analyzed and interpreted data and critically revised manuscript. Dr. MCH designed experiments, analyzed and interpreted data and critically revised manuscript. Dr. MMS conceived of the design of the study, analyzed and interpreted data and drafted the manuscript. All authors read and approved the final manuscript.

Authors' information
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