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Resistance to FLT3 inhibitors involves different molecular mechanisms and reduces new DNA synthesis

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ABSTRACT

Acute myeloid leukaemia (AML) is a hard to treat blood cancer. Mutations in FLT3 are common among the genetic aberrations that characterise the cancer. Patients initially react to FLT3 inhibitors but drug resistance is a hinder to successful therapy. To better understand the mechanisms leading to drug resistance, we generated four AML cell lines resistant to the inhibitors gilteritinib or FF-10101, and explored their resistance mechanisms. We further tested whether the novel inhibitor Chen-9u could be used to limit cell growth. The results showed that each of the four resistant cell lines became resistant through a different mechanism. Resistant cells showed decreased FLT3 and increased NRAS pathway activity and reduced DNA synthesis due to decrease in CDK4 activity. Resistance mechanisms included resistance mutations in FLT3 (C695F and N701K), and a novel mutation in NRAS (G12C). In a fourth line, resistance might have developed through a MYCN mutation. Cell growth was inhibited by Chen-9u and resistant clones could not be obtained with this inhibitor. The results highlight opportunities and limitations. On the one hand, resistant cells were produced due to different mechanisms, showing the versatility of tumour cells. Furthermore, resistance developed to the most advanced inhibitors, one of which is covalent and the other non-covalent but highly specific. On the other hand, it is shown that DNA synthesis is reduced, which means that resistance has evolutionary consequences. Finally, the novel drug-resistant cell lines may serve as useful models for better understanding of the cellular events associated with inherent and acquired drug resistance.

1. Introduction

FMS-like tyrosine kinase 3 (FLT3) is a member of the class III receptor tyrosine kinase family. Activating mutations in FLT3 are observed in ~30% of acute myeloid leukaemia (AML) cases [1]. AML with activating FLT3 mutations is referred to as $FLT3^{+}$-AML. Three FLT3 inhibitors are available in the global market. Midostaurin is a multi-kinase inhibitor that inhibits a mutated variant of FLT3 where the protein became activated due to internal tandem duplications (FLT3/ITD) [2]. Quizartinib is a highly specific FLT3/ITD inhibitor [3,4]. Gilteritinib is also a specific FLT inhibitor that inhibits not only FLT3/ITD but also FLT3 that carries kinase domain (KD) activating mutations and even wild type (wt) FLT3 [5–7]. It is the most widely use FLT3 inhibitor in Sweden and many other countries.

Unfortunately, resistance to targeted therapy leads to relapse on all FLT3 inhibitors in the market today. Resistance mutations, i.e. mutations to the drug target that lead to a reduction in the drug's efficacy are a common mechanism [8–12]. Of the approved inhibitors, gilteritinib has the most favourable resistance profile when in comes to resistance mutations, but the gatekeeper mutation F691L confers resistance to all three inhibitors and is often observed at relapse following gilteritinib therapy [13]. Pexidartinib is a FLT3 inhibitor that overcomes this mutation, but many other mutations limit its efficacy in AML [14,15]. More recently, an irreversible (covalent) FLT3 inhibitor was developed, FF-10101. FF-10101 has been shown to have potent activity against nearly all mutations associated with clinical resistance to FLT3 tyrosine kinase inhibitors (TKIs) except for the crenolanib-resistant Y693C mutation [16] (crenolanib inhibits FLT3 but is not currently approved for AML). It also synergises with the hypomethylating drug azacitidine, in contrast to other FLT3 inhibitors [17]. In vitro studies suggest that mutations in Cys^{695} confer resistance to FF-10101 as this residue covalently binds the drug [18,19]. Other modes of resistance have also been described against various FLT3 inhibitors [20], including mutations that activate secondary pathways such as RAS, activation of cytokines, and overexpression of its natural ligand [21]. The precise role of each mechanism is less well known, and it remains to be seen if more effective FLT3 inhibitors could achieve

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longer periods of progression free survival without relapse.

Understanding of the molecular mechanisms behind drug resistance to FLT3 inhibitors is of utmost importance in the quest to design better therapies [22,23]. This is especially true since FLT3 activating mutations are so prevalent among AML patients. Unfortunately, data from patients is scarce. Moreover, the rapid progress of the disease makes it difficult to follow on the development of resistance mutations in real time. Our earlier studies on blood cancers revealed that cell studies could be predictive for resistance [15,24]. Thus, in this study we took an experimental approach and set to develop and characterise AML cell lines that express FLT3/ITD and are resistant to the newest inhibitors, namely gilterinib and FF-10101. We have further tested Chen-9u, a novel FLT3 inhibitor with high potency also against FLT3/F691L [25,26]. Chen-9u is a pre-clinical inhibitor that has shown high efficacy and low toxicity in cellular and animal studies. In the original work reporting its development, the growth IC50 values in Ba/F3 cells for FLT3/wt and FLT3/F691L were reported as 0.9 and 13.0 nM, respectively [25]. These values were lower then those reported for quizartinib in the same work (4.9 and >1000 nM). The inhibitor's small size and unique binding mode make it highly effective for FLT3 mutants. Specifically, it does not interact with Phe^{691}, which is a mutated in patients that are exposed to other FLT3 inhibitors. In terms of activity, it was shown to lead to inhibition of phosphorylation of FLT3 and proteins that play a role in its downstream signalling (ERK and STAT5). [25].

2. Materials and methods

2.1. Reagents

Cell culture medium (RPMI 1640, Iscove's Modified Dulbecco's Medium -IMDM), Pierce Protease Inhibitor Mini Tablets, antibiotics (Penicillin-Streptomycin) and fetal bovine serum (FBS) were purchased from Fisher Scientific, Sweden. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) was purchased from Promega. Cell Extraction Buffer was bought from Sigma-Aldrich.

Primary antibody to NRAS (ab77392) was purchased from Abcam. The phospho-FLT3 (p-FLT3), β-actin and secondary antibody Anti-rabbit IgG (H+L), $F(ab')_2$ Fragment (Alexa Fluor 555 Conjugate) were purchased from Cell Signaling Technology. Secondary antibody Goat anti-Rabbit IgG (H+L) Secondary Antibody, HRP conjugate, was purchased from Thermo Fisher Scientific.

2.2. FLT3 inhibitors

FF-10101 was purchased from Chemtronica AB (Sweden). Gilteritinib was purchased from Cayman Chemical. Chen-9u was a generous gift from Prof. Lijuan Chen, Department of Hematology and Research Laboratory of Hematology, West China Hospital of Sichuan University, Chengdu, China. Each inhibitor was dissolved in dimethyl sulfoxide (DMSO). Aliquots were stored at −20 ℃ and thawed immediately before use.

2.3. Cell lines cell proliferation assay

MOLM-14 and MV4-11 were a generous gift from Prof. Stefan Fröhling, National Center for Tumor Diseases, Germany. MV4-11 cells were cultured in IMDM supplemented with 10% (vol/vol) FBS and 1% (vol/vol) antibiotics (Penicillin-Streptomycin). MOLM-14 cells were cultured in RPMI-1640 medium supplemented with 10% (vol/vol) FBS and 1% (vol/vol) antibiotics (Penicillin-Streptomycin). All cells were cultured in a humidified incubator at 37 ℃ with 5% CO_2 and maintained at a density of 0.5×10^6 to 1.5×10^6 cells/mL by splitting the cultures every 2 to 3 days.

Cell viability was assessed using the CellTiter 96 AQ_{ueous} One Solution® Cell Proliferation Assay (Promega, Sweden) in accordance with the manufacturer's instructions. Briefly, exponentially growing cells were plated in each well of a 96-well plate with 100 μL of complete medium containing the appropriate concentration of the drug in triplicate. Cells were allowed to expand for 48 h. Cell viability was determined by measuring fluorescent signals at 490 nm. The concentration of inhibitor corresponding to a 50% inhibition of cell proliferation (IC50) values were calculated using Prism 8.0 (GraphPad Software). Cell counting was performed using trypan blue exclusion on a LUNA-2 cell counter (Logos Biosystems).

2.4. Establishment of human leukaemia subline resistant to drug-induced growth inhibition

Resistant sub-lines were developed by growing AML cells in escalating concentrations of FF-10101 or gilteritinib, see Fig. 1. Briefly, exponentially growing AML cells (parental cells, PT) were collected and exposed continuously to gradually increasing concentrations of gilteritinib (from 10 nM to 100 nM) or FF-10101 (from 2 to 100 nM). Cells treated with DMSO (0.1% (vol/vol)) were set as control. The medium (with drugs) was replenished every four days. Cell numbers were counted every two days. Development of fully resistant cells took approximately six months, after which IC50 concentrations were re-assessed in each resistant cell line. The resistant cells were labelled as MOLM-14/Gilt, MV4-11/Gilt, MOLM-14/FF and MV4-11/FF. The degree of resistance was calculated by dividing the IC50 value of the resistant cells by that of the PT.

2.5. Cell cycle analysis

Cells were collected and pelleted by centrifugation at 1000 rpm for 5 min and suspended in 1 mL phosphate-buffered saline (PBS). They were subsequently fixed in 90% cold (4 ℃) ethanol and incubated at room temperature for 30 min. Cells were pelleted and resuspended in 1 mL PBS containing propidium iodide (25 μg/mL) and DNase-free RNase A (100 μg/mL). Following incubation at 37 ℃ for 30 min, cell cycle distributions of the parental and drug resistant cells were analysed using FACS.

2.6. Western blot analysis and immunofluorescence microscopy

To prepare extracts, cells were washed once with cold PBS and lysed in 1 × Cell Extraction Buffer (Sigma-Aldrich) as described by the manufacturer. Bound antibody complexes were detected and visualised using Amersham™ ECL system (GE Healthcare) according to the manufacturer's instructions. Densitometric analysis was carried out using FIJI software.

Immunofluorescence staining and measurement of p-FLT3 was performed as our previous work, with minor modifications. Briefly, cells needed in the experiment were fixed by 4% formaldehyde for 30 min at 37 ℃ and rinsed briefly two times with PBS to remove traces of the fixative. The cells were then smeared on gelatin-coated slides gently with the side of a pipette tip. When the liquid had been evaporated, cells were washed two times with washing buffer (0.1% BSA in PBS). The cells were thereafter blocked in PBS containing 10% normal donkey serum and 0.3% Triton X-100 for 45 min to 1 h at room temperature followed by permeabilisation with PBS containing 0.5% Tween-20 for 10 min at room temperature. After removing the blocking buffer, cells were incubated with diluted (1:1000) primary antibody p-FLT3 at 4 ℃ overnight. This was followed by two washes with washing buffer. The preparation continued with the cells incubated with diluted (1:2000) fluorescein-labelled secondary antibody anti-rabbit IgG (H+L), $F(ab')_2$ Fragment (Alexa Fluor 555 Conjugate, Cell Signaling Technology) in TBST containing 5% low fat milk powder for 1 h at room temperature protected from light. Finally, the cells were rinsed two times with washing buffer, and then counter-stained with 0.1 μg/mL DAPI for nucleic acid staining in the dark. Prior to incubation, the cells were washed two times with PBS, then covered with cover slides by mounting medium.

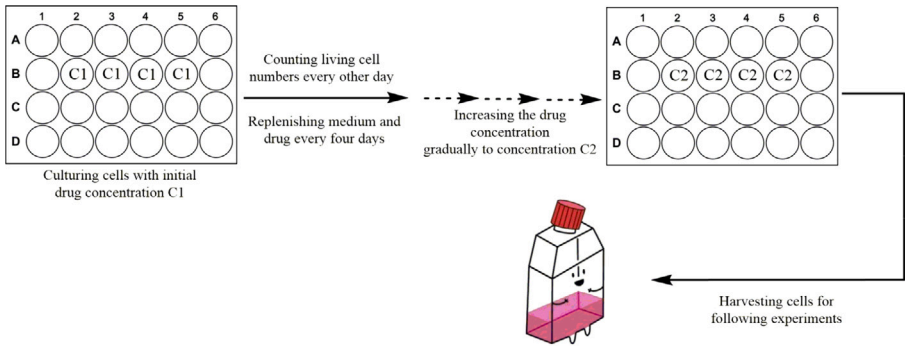

Fig. 1. Establishment of drug-resistant cell sub-lines.

Table 1
IC50 values (nM) for the three inhibitors tested on the various cell lines used in this study. Drug resistant cell lines are indicated by the name of the drug that was used for treating them. For example, MV4-11/Gilt = MV4-11 cells that are resistant to gilteritinib.

	MOLM-14	MOLM-14/ FF	MOLM-14/ Gilt	MV4-11	MV4-11/ FF	MV4-11/ Gilt
FF-10101	1.7 ± 0.2	320 ± 25	8.7 ± 0.9	2.1 ± 0.3	610 ± 240	48 ± 12
Gilteritinib	17 ± 1.7	50 ± 0.8	160 ± 40	28 ± 6.6	33 ± 6.3	280 ± 72
Chen-9u	0.5 ± 0.1	1.5 ± 0.3	1.4 ± 0.1	0.7 ± 0.1	1.0 ± 0.1	1.8 ± 0.1

2.7. DNA sequencing analysis

Genomic DNA was extracted using proteinase K and phenol from the cells mentioned above. Sequencing was performed by Genome Sequencer Illumina NovaSeq. The sequences were analysed for expression of relevant genes with the help of Geneyx Analysis Platform.

2.8. Sequence analysis

The observed mutations in FLT3 were analysed to examine if these were novel (new functionality) or if the changes could be observed in other residues, following [27]. Briefly, the kinase domain of FLT3 was downloaded and matched to the Swissprot database using DeltaBlast [28] which employs a domain search, limiting the number of hits to 2000. Hits with >95% similarity to the FLT3 sequence were not retained; all other 1996 sequences were downloaded from Swissprot. These sequences were further filtered by the EMBOSS `skipredundant` tool to remove sequences that were >95% similar to each other. 1324 sequences were retained and aligned by mafft [29] using the default setup. Finally, the `get_seq_pos_variation.pl` Perl script was used to analyse the variations at selected positions. The `get_seq_pos_variation.pl` and the complete protocol are available in https://github.com/Ranger1976/MSA_variance_tools.

2.9. Statistical analysis

ANOVA test was used for comparison between multiple groups when applicable. The analysis was performed in GraphPad Prism version 8.

3. Results

3.1. AML cells developed cross-resistance to gilteritinib and FF-10101

To be able to study drug resistant cells we first aimed at generating resistant cell lines. To this aim, drug-sensitive AML cell lines were used as parental lines to generate a series of drug resistant cell lines through repeated cycles of drug exposure to drugs (Fig. 1). To examine the effect the drugs on continuous growth we started by incubating the cells with gilteritinib or FF-10101 for four days at IC50 and IC90 concentrations. We found that AML parental cells continued growing under IC50 drug treatment but at a lower growth rate than in the absence of drug. At IC90 concentrations the drugs effectively suppressed cell growth, which was not regained after two additional days (Fig. 2A and B).

To examine whether resistance can be established, we started by growing the cells for four days each time together with concentrations of drugs that matched their IC50. After four days cells were washed, diluted, and incubated with fresh medium and drugs. This procedure was repeated 11 to 14 times to examine any changes in the growth rates. Indeed, the cells could be grown at IC50 concentrations for many weeks with their growth initially (first 1–5 4 day cycles) slow but thereafter much faster although in general not reaching the same cell density as in untreated cells (Fig. 2C–F).

Having seen an indication of resistance we set up to grow drug resistant cells by the method depicted in Fig. 1. The lines derived through incubation with FF-10101 (MOLM-14/FF and MV4-11/FF) and gilteritinib (MOLM-14/Gilt and MV4-11/Gilt) exhibited significant resistance to their corresponding drugs compared with the parental cell lines (Table 1). When cross resistance was investigated, we found that both the FF-10101 and gilteritinib-derived resistant cells exhibited resistance to both agents, though not to the same degree as to the drug that was used in growing them.

3.2. The novel inhibitor Chen-9u does not lead to resistance and inhibits the growth of cross-resistant cells

The fact that the cells developed resistance to both gilteritinib and FF-10101 is alarming. Gilteritinib is the most widely used specific FLT3 inhibitor in clinical use today, whereas FF-10101 was suggested to inhibit cells that display gilteritinib resistance due to various mechanisms [19]. Having observed cross-resistance to these two agents, we set to examine whether Chen-9u, a pan-FLT3 inhibitor [26] that is under development, leads to resistance and whether resistance to this inhibitor might also develop.

Interestingly, growing AML cells with Chen-9u at a concentration that matched its IC50 did not lead to resistance even after multiple cycles of growth (Figure S1). This indicates that resistance to this inhibitor is less likely to occur. Furthermore, the inhibitor remains potent against the four strains of drug-resistant cells (Table 1), with IC50 values that, although above those obtained for the parental lines, are below 2 nM. In the parental cell lines, Chen-9u was shown to be more potent than both FF-10101 and Gileritinib. Previously, it was shown to have IC50 values lower than quizartinib as well [25].

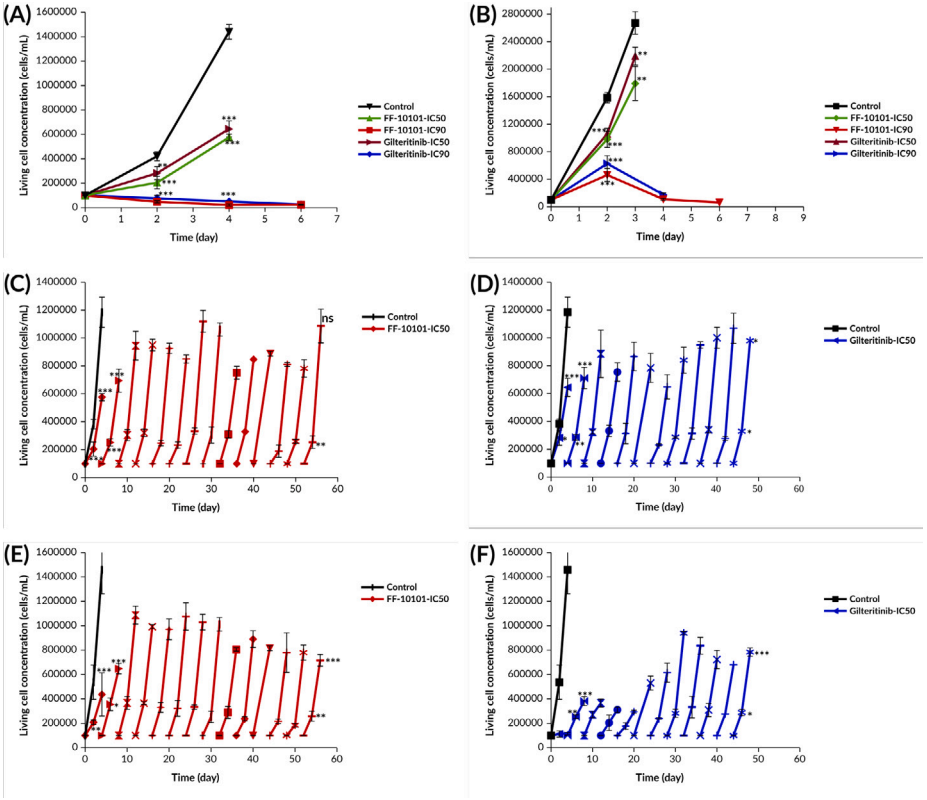

Fig. 2. MOLM-14 (A) and MV4-11 (B) parental cells were treated with different concentrations of drugs, and at days after the treatment as indicated, and living cells were counted. Growth curves of MOLM-14 (C, D) and MV4-11 (E, F) in the presence of FF-10101 (C, E) or gilteritinib (D, F) at the concentration of IC50 respectively. ANOVA test - one asterisk indicates $p \leq 0.05$ between experiment and control groups on day 2 and day 4, respectively, two asterisks indicate $p \leq 0.01$, three asterisks indicate $p \leq 0.001$ while ns means $p > 0.05$.

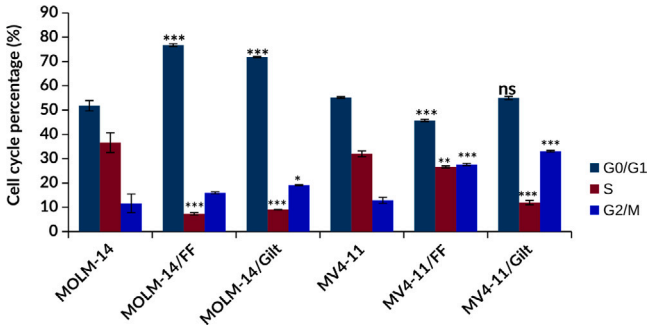

Fig. 3. Comparison of cell cycle of parent cell lines and drug-resistant cell lines (MOLM-14/FF, MOLM-14/Gilt, MV4-11/FF and MV4-11/Gilt) with propidium iodine staining. Representative histogram analyses were performed for the distribution of cell cycle phases. ANOVA test - one asterisk indicates $p \leq 0.05$ between the phase of PT and drug-resistant cell line, respectively, two asterisks indicate $p \leq 0.01$, three asterisks indicate $p \leq 0.001$ while ns means $p > 0.05$.

3.3. Drug-resistant cells exhibited a different cell cycle from the parental cells

To examine the features of the resistant cells, we first performed cell cycle analysis by propidium iodine staining, which can reveal three distinct cell populations: namely those that are in the G0/G1, S and G2/M phases of the cell cycle. As shown in Fig. 3 and Figure S2, most cells were in the G0/G1 phase. Yet, a large fraction of the AML cells were in the S phase, whereas the drug resistant cells had a lower tendency to be in the S phase. This indicates that the cells spent less of their lifetimes synthesising new DNA, which can explain the somewhat lower growth rates that was observed when the cells were incubated with the drugs at the IC50 concentration (Fig. 2).

3.4. Resistant cells showed increased expression of NRAS and decreased expression of CDK4 and phoshorylated FLT3

The proliferation of the resistant cells on the one hand, and changes to the cell cycle on the other hand, prompted us to explore whether the expression of several key proteins differed between the cell lines. NRAS activation is a known factor driving drug resistance. Here, it was observed in all treated cell lines (Fig. 4A). Given that DNA synthesis was reduced in the treated cells (Fig. 2), we examined also the expression of CDK4 since it regulates the entry of cells to the S phase [30]. This analysis reveals that the expression of CDK4 was reduced in the drug- resistant cells, particularly those treated by FF-10101. (Fig. 4B). Examination of phosphorylated FLT3 (p-FLT3) on the cell surface revealed a decrease but no elimination of FLT3 phosphorylation (Fig. 4C–F), consistent with partial inhibition of the protein (FLT3 auto-phosphorylates).

3.5. DNA sequencing of drug resistant AML cells revealed different strategies for resistance

In an effort to understand the cause of resistance to gilteritinib and FF-10101, DNA was extracted from the resistant cells and screened for novel mutations that did not exist in the parental MOLM-14 and MV4-11 cell lines. The characteristic FLT3/ITD mutations were maintained in all cells, and secondary mutations were identified in the resistant cells.

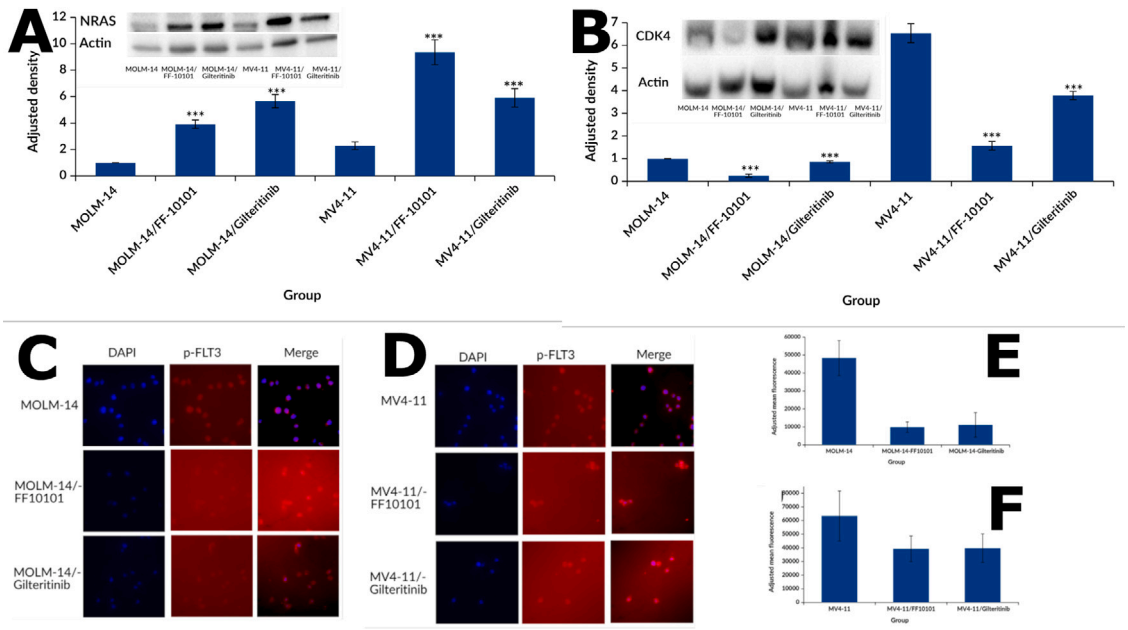

Fig. 4. (A and B). NRAS (A) and CDK4 (B) protein expression in the parental and resistant cells. Total proteins were isolated from parent and corresponding drug-resistant sub-lines and subjected to SDS-PAGE gel electrophoresis and transfer by Western blot. Protein expression was examined between parent and resistant cell lines. Adjusted density values were calculated by dividing the relative density of each sample lane by the relative density of the loading-control for the same lane, using the ImageJ software. ANOVA test - one asterisk indicates $p \leq 0.05$ between parental cell line and drug-resistant cell line, two asterisks indicate $p \leq 0.01$, three asterisks indicate $p \leq 0.001$ while ns means $p > 0.05$. (C–F). p-FLT3 immunostaining in MOLM-14 (C) and MV4-11 (D). DAPI staining is shown in blue. Photographs were taken by confocal microscopy. A Quantification of the fluorescence of p-FLT3 was carried out by the Fiji software. (E) shows the results for MOLM-14, and (F) represents the result for MV4-11.

3.5.1. *FLT3/N701K in MOLM-14/gilteritinib*

The cause for resistance to gilteritinib in MOLM-14 cells was found to be a secondary FLT3 mutation, N701K. This mutation was shown to promote resistance to gilteritinib in Ba/F3 cells that express FLT3 and FLT3/ITD [31]. This is although residue Asn^{701} was not shown to interact with gilteritinib in a comprehensive modelling study [12]. Recently, the presence of the N701K mutation was verified in patients treated by gilteritinib [32], and it was suggested that quizartinib still inhibits FLT3/N701K. Here we show that the mutation also conferred some resistance also to FF-10101 (Table 1, with over 4-fold increase in the IC50).

3.5.2. *MYCN/D31P in MV4-11/gilteritinib*

In MV4-11 cells, we identified a novel mutation, MYCN/D31P. MYCN (also known as NMYC) is a transcription factor, and multiple other mutations of the gene have been associated with cancer (data from the COSMIC database [33]). Its N-terminal part interacts with Aurora-A kinase, which stabilises the protein and prevents its degradation [34]. We hypothesise that the D31P mutation stabilises the N-terminal part, through interaction with Aurora-A kinase or with another factor. We could not identify any other mutation or mechanism that could explain the observed resistance to both gilteritinib and FF-10101.

3.5.3. *NRAS/G12C*

Resistance to FF-10101 showed a clear-cut profile. In MOLM-14 cells, resistance was acquired by an activating mutation in NRAS, G12C. G12C mutations are well characterised in RAS proteins, especially in KRAS but also in NRAS. In a study of AML patients who relapsed on gilteritinib, it has been shown that 32% of the patients had NRAS mutations [35]. The authors of the study have grown MOLM-14 cells with quizartinib, and showed that the NRAS/G12C was observed in the resistant cells. In addition, they showed also that the same mutation has been shown to confer resistance against the FLT3 inhibitor quizartinib and reduce apoptosis when the cells were treated by gilteritinib (ibid.). Later, it was found that also FF-10101 exhibited reduced potency against MOLM-14 cells that were made resistant to quizartinib due to an NRAS/G12C mutation [19]. Here we show that this mutation is acquired also upon treatment with FF-10101, not only quizartinib. When it occurred, it led to marked resistance against FF-10101 (over 180-fold increase in IC50) and a more modest resistance to gilteritinib (about 3-fold increase in IC50, to 52 nM).

3.5.4. *FLT3/C695F*

MV4-11 cells acquired resistance to FF-10101 through a C695F mutation. As the inhibitor binds covalently to Cys^{695}, the mutation impairs this binding. It also affects gilteritinib, but not nearly as much. This is the first report of a C695F mutation but other mutations at the same residue (C695R/S/Y) were shown to lead to FF-10101 resistance in Ba/F3 cells that were made to depend on FLT3 [19]. There is a difference between such cells and AML cell lines though. The Ba/F3 cells were transformed in a way that made them dependent of FLT3/ITD for survival, thus creating a strong evolutionary pressure to mutate the FLT3 gene, whereas we used AML cell lines that could develop resistance in other forms as well. That it is exactly the C695F mutation that was discovered may suggest that it has overall beneficial properties when it comes to cell growth in the presence of FF-10101.

4. Discussion

Cell lines are a highly useful tool for the development of efficient cancer therapies and understanding of drug resistance. While patient cells can sometimes be used to test drugs or combinations, such cells are vulnerable ex vivo and cannot be used to study drug resistance. For this reason, our main aim in this study has been to develop and study cells lines that are resistant to the most advanced $FLT3^{+}$ inhibitors. We started by utilising two cell lines that carry FLT3/ITD mutations. Previously, we studied the same type of cells with different FLT3 inhibitors and observed a rapid increase in cell growth rate [15]. This, however, did not immediately translate to a marked increase in the IC50 for the drugs in the case of gilteritinib and FF-10101. Growth of the resistant cell lines has proven to be a lengthy process which took

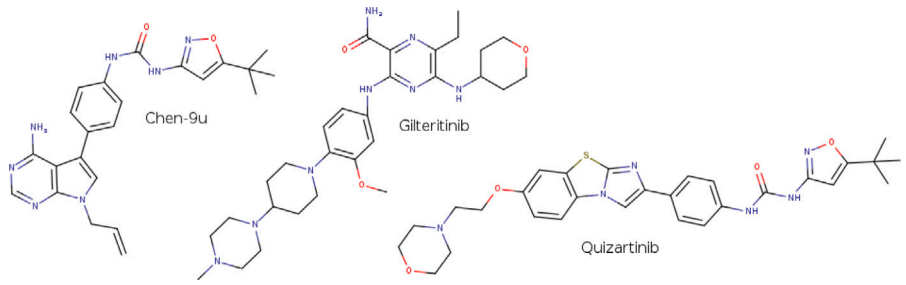

Fig. 5. The structures of the FLT3 inhibitors Chen-9u, gilteritinib and quizartinib.

about six months per line. The same was shown for another type of leukaemia cells [36].

Mechanisms that do not depend on mutations have been attributed to resistance in many other scenarios. One such mechanism is overexpression of the FLT3 ligand, but this is expected to be a contributing factor if wild type FLT3 is expressed instead of FLT3/ITD which was not the case here. Another mechanism is overexpression of efflux pumps, but neither FLT3 nor FF-10101 are known as substrates for such pumps. Thus, mutations and epigenetic resistance mechanisms are the likely culprits in this specific instance. We identified mutations that are specific for each cell line and these appear to be the most likely explanation for emergence of a drug resistant phenotype.

It is well known that resistant cell lines often revert back to a drug sensitive phenotype when the drug is not present anymore [37]. Following on the cell cycle it was shown here that all resistant cells are found less often in the S phase (Fig. 3). It is apparent that developing resistance comes at an evolutionary cost to the cells. Given that all the cells here showed some degree of cross-resistance (Table 1) it is apparent that switching between gilteritinib and FF-10101 is unlikely to be a useful approach. The slower growth of the resistant cells might indicate that a so-called 'drug holiday', where the patient is left untreated for a short period might be beneficial. However, as some residual resistant cells are likely to persist, such approach to treatment is not advisable [38]. A novel inhibitor tested here, Chen-9u, has shown to be adequate to inhibit cell growth of resistant cells; the cells showed no or only moderate resistance to this inhibitor (with IC50 < 2 nM in all cases). Chen-9u is structurally similar to quizartinib (Fig. 5) and achieves higher affinity to the protein than quizartinib and gilteritinib as it maintains strong interactions with FLT3 despite its smaller size. The other two inhibitors include additional groups for better solubility (piperize and morpholine) that do not bind to the protein. Chen-9u does not contain such groups and binds with higher potency owing to its strategically positioned 4-Amino-7H-pyrrolo[2,3-d]pyrimidine group, that affords good solubility as well. We were not able to grow cells that were resistant to Chen-9u. Overall, a pan-resistant inhibitor, if such can be developed appears to be a viable approach to postpone the emergence of drug resistance. Combination therapies are also useful in this respect [39]. However, mechanisms such as RAS mutations can lead to resistance against different therapies [40,41]. Moreover, there is a need for caution with combination therapies owing to increased risk for toxicities and because such therapies may be antagonistic.

Two mutations in FLT3 were identified in this study of which one (C695F) has not been described before. Resistance mutations can be completely novel, i.e. a new sequence that was not threaded in the evolution, but in most cases the new residue can be seen in the corresponding position in other related proteins [27]. The distinction between 'novel mutations' (never seen before in similar proteins) and mutations that are not novel is important from an evolutionary perspective as in the latter case the mutation might hint at new functionality. Upon examination of proteins homologous to FLT3 we identified both Cys and Phe in the position corresponding to Cys^{695}. Position 695 seems to be highly variable and hence it is likely that the protein retains its activity upon mutation to a similar level. Likewise, Asn and Lys are

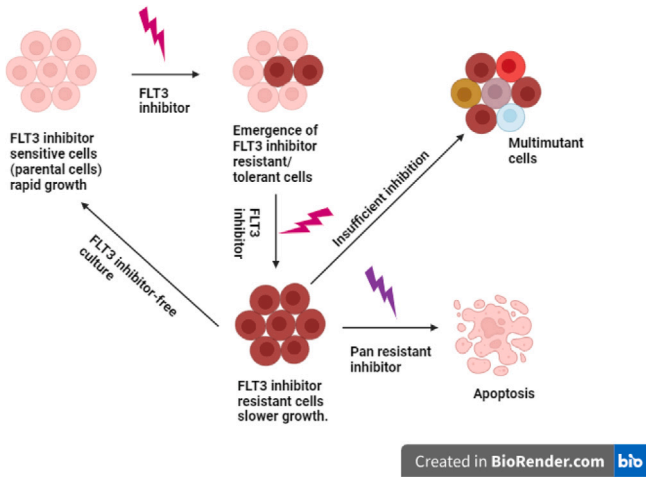

Fig. 6. A pan-resistant inhibitor appears to be a good strategy to avoid drug resistance. Tumour cells normally grow rapidly. FLT3 inhibitors kill the cells, while also making them grow more slowly due to limitation on synthesis of new DNA. If therapy is taken out, the cells revert to the parental phenotype. A low concentration of drugs (insufficient inhibition) allow the cells to grow while at the same time they are under evolutionary stress to develop new mutations. Rapid elimination of the cells by a pan-resistance inhibitor may this afford longer survival.

equally likely in the multiple sequence alignment of proteins related to FLT3 in the position that matches residue Asn^{701} and the position is highly variable. Thus, it is not likely that the mutations bring any kind of new functionality to FLT3. Rather, they promote resistance while maintaining the activity of the protein.

Considering this study and the current state of knowledge, it is suggested that using an inhibitor with high efficiency and low sensitivity is the best approach to delay the onset of drug resistance in $FLT3^{+}$-AML (Fig. 6). With the current efforts by multiple labs and the interest of pharmaceutical companies we remain hopeful that such approach will eventually be used. The current inhibitors have excellent affinity but drug resistance remains a significant hurdle.

5. Conclusions

$FLT3^{+}$-leukaemia is treated by chemotherapy together with FLT3 inhibitors for induction and consolidation cycles, and FLT3i (sometimes with an hypomethylating agent) for maintenance [42]. The acquisition of resistance is a major problem in cancer therapy [8,43], and in particular limits the effect of FLT3 inhibitors [11]. There are currently no good options for patients that relapse and they are recommended clinical trials as the first option [42]. Some other options include FLT3 inhibitors combined with other drugs (ibid.). We developed in vitro drug resistance models using MOLM-14 and MV4-11 cell lines, acquiring four new cell lines with distinct genomic changes. The cells are resistant to gilteritinib and FF-10101 despite a different mode of binding. They include mutations that have hitherto, to the best of

our knowledge, not been observed (MYCN/D31P and FLT3/C695F). Resistance mutants are shown to be less often in the S phase, which is likely partially due to lesser expression of CDK4. The NRAS pathway is activated in all mutants and is mutated in one case. A high degree of cross-resistance is observed between gilteritinib and FF-10101, suggesting that the use of the latter might be limited in case of resistance to the former. Encouragingly, the Chen-9u inhibitor is able to inhibit the growth of resistant cells and no resistance has been observed against it by use of the same protocol. This suggests the possibility to obtain inhibitors with a narrow resistance profile to treat FLT3^{+}-AML. The NRAS/G12C mutation limited cell growth and is thus less likely to become fixed in a sub-clonal population prior to treatment with a FLT3-inhibitor. It remains to be seen what are the effects of the other mutations on the growth rate.

CRediT authorship contribution statement

Jingmei Yang: Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Ran Friedman:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

This file contains growth curves of MOLM-14 and MV4-11 in the presence of compound Chen-9u and the CDK4 expressions in different groups and a figure that shows propidium iodine staining.

Supplementary material related to this article can be found online at https://doi.org/10.1016/j.bbrep.2024.101894.

Data availability

All raw data have been deposited in the https://www.Figshare.com under DOI number: 10.6084/m9.figshare.22269859. Other data and reagents are available upon reasonable request.

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