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Research paper

Discovery and development of extreme selective inhibitors of the ITD and D835Y mutant FLT3 kinases

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ABSTRACT

Aberrant activation of FMS-like tyrosine receptor kinase 3 (FLT3) is implicated in the pathogenesis of acute myeloid leukemia (AML) in 20–30% of patients. In this study we identified a highly selective (phenylethenyl)quinazoline compound family as novel potent inhibitors of the FLT3-ITD and FLT3-D835Y kinases. Their prominent effects were confirmed by biochemical and cellular proliferation assays followed by mice xenograft studies. Our modelling experiments and the chemical structures of the compounds predict the possibility of covalent inhibition. The most effective compounds triggered apoptosis in FLT3-ITD AML cells but had either weak or no effect in FLT3-independent leukemic and non-leukemic cell lines. Our results strongly suggest that our compounds may become therapeutics in relapsing and refractory AML disease harboring various ITD and tyrosine kinase domain mutations, by their ability to overcome drug resistance.

1. Introduction

The FMS-like tyrosine receptor kinase 3 is a cell surface receptor belonging to the third class of tyrosine kinase family receptors [1]. In the human body FLT3 is expressed by immature blood cells to the highest extent and plays a pivotal role in the differentiation and survival of hematopoietic stem cells in the bone marrow [2–4]. Like other receptor kinases, it consists of three main structural elements: the extracellular, transmembrane and intracellular domains. The intracellular part contains the juxtamembrane domain and the two kinase domains which are separated by the small kinase insert domain [5,6]. The ATP-binding site of the kinase is located between the N- and C-terminal lobes surrounded by the common structural elements (hinge region, DFG motif, activation loop and αC-helix; Fig. 1A). The juxtamembrane domain plays an important role in the autoinhibition of FLT3 and mutations often occur in this part of the protein [7].

Under physiological conditions the protein can be found in the cell membrane in a monomeric form. The binding of the FLT3 ligand (FL or FLT3L) leads to its activation, causing conformation changes (dimerization) and phosphorylation of the intracellular part [8]. The activated FLT3 kinase turns on various signal transduction pathways (Fig. 1B) to convey the information to the nucleus, which pathways control transcription, translation, differentiation and apoptosis. The two most important pathways are the PI3K/Akt and RAS/RAF/MEK/ERK [9].

Due to its function, overexpression and/or mutation of the FLT3

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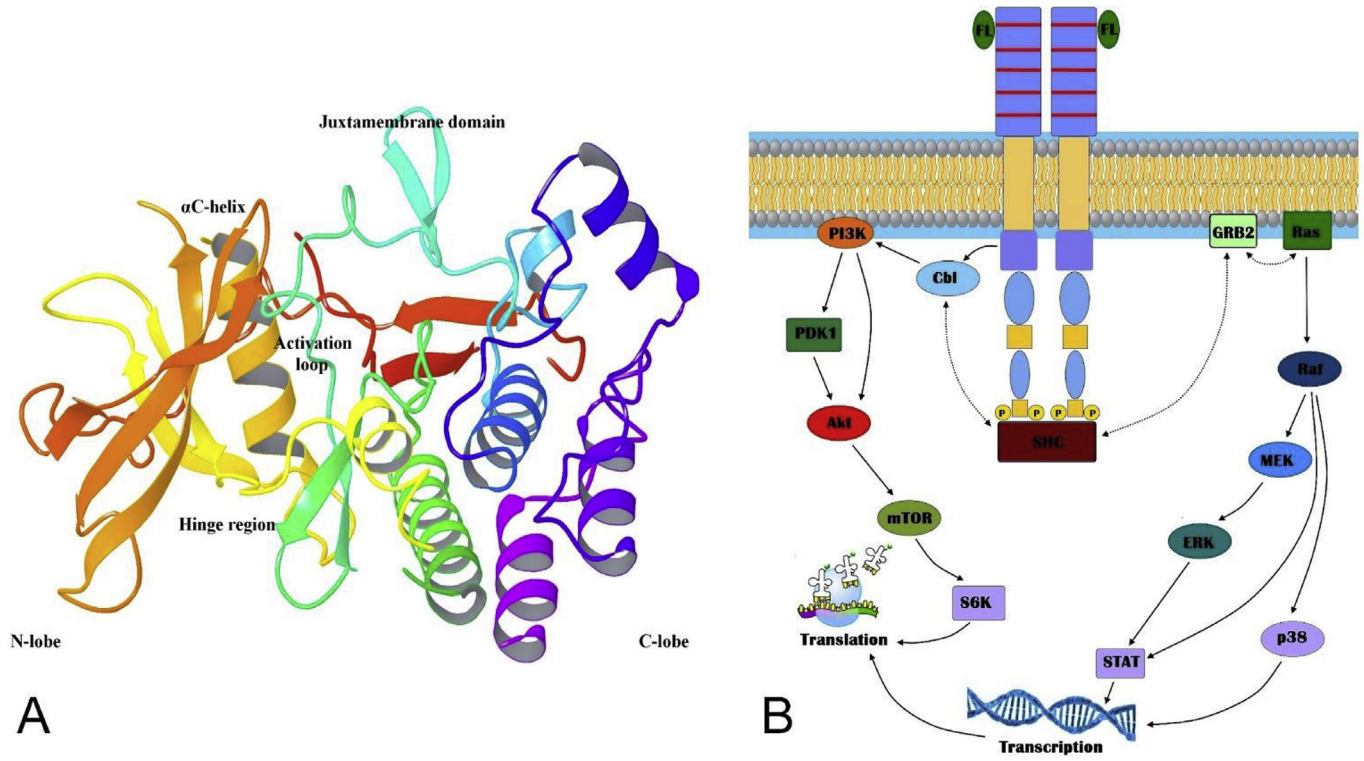


Fig. 1. (A) Structure of the FLT3 kinase (PDB ID: 1RJB); (B) the FLT3 pathway based on Ref. [9].

kinase are one of the most commonly observed phenomena in malignant tumors of the hematopoietic system and they are potential risk factors in AML patients [10–12]. The most frequent activating mutation of FLT3 (~23% in AML) is the internal tandem duplication (ITD) which extends the length of the juxtamembrane domain and interrupts the autoinhibition mechanism of the kinase [13,14]. The length of the ITD mutation varies from patient to patient but it is usually 3–400 base pairs [15]. The second most common mutation (~7% in AML) of FLT3 is the D835Y point mutation, which alters the amino acid sequence of the activation loop causing enhanced protein activation [16,17]. FLT3-activating mutations critically regulate leukemic transformation by accelerating proliferation and suppressing apoptosis and the FLT3-ITD mutations are known to confer poor prognosis, while the prognostic impact of D835Y mutation is controversial. Moreover FLT3 and its mutations were identified as the most important driver genes not only in AML, but in ALL and CML as well [18,19]. These findings highlight FLT3 as a highly attractive target for drug development.

Most of the known FLT3 inhibitors are multikinase inhibitors, which demonstrated promising efficiency in clinical trials but they have unfavourable side effects and/or poor pharmacokinetic profiles [20].

Although the quinazoline-based tandutinib (Fig. 2) inhibits FLT3 in the nanomolar range ($IC_{50} = 220$ nM), it also binds to other receptor kinases (PDGFRß, FGFR, VEGFR, c-Kit) in the same concentration range ($IC_{50} = 170–200$ nM) [21]. Despite its poor pharmacokinetic properties, tandutinib was investigated in clinical trials in combination therapy for other cancer types as well [22–24].

Compound KW-2449 (Fig. 2) was developed as an FLT3 kinase inhibitor ($IC_{50} = 6.6$ nM) but further profiling revealed that the compound is active also against many other kinase enzymes [25]. Because of its poor pharmacokinetic profile KW-2449 showed weaker effect in clinical trials than expected and under physiological conditions the inhibition of FLT3 was insignificant [26]. Sunitinib, an oxindole based multikinase inhibitor, got the approval of the U.S. Food and Drug Administration (FDA) against renal cell carcinoma and gastrointestinal stromal tumor in 2006 [27]. According to selectivity studies sunitinib inhibits approximately 150 kinases including FLT3 (wt FLT3 $IC_{50} = 250$ nM, FLT3-ITD $IC_{50} = 50$ nM, FLT3-D835Y $IC_{50} = 30$ nM) and showed remarkable results in phase I trials [28–30]. In recent years sunitinib has also been tested in combination with standard chemotherapy in patients with FLT3-mutated AML [31].

The natural product staurosporine was the initial lead compound for many semi-synthesized inhibitors including midostaurin and lestaurtinib (Fig. 2). Both compounds inhibit a broad spectrum of kinases [32,33]. In preclinical experiments midostaurin showed an IC_{50} value of 30 nM on FLT3-ITD enzyme and induced apoptosis in high proportions of FLT3-mutant cells [34]. Midostaurin results were promising and it has been approved by the FDA for the treatment of FLT3 mutation-positive adult AML patients in 2017 April [35–37]. Lestaurtinib (developed by Teva/Cephalon) showed not only stronger inhibitory effect on FLT3 kinase ($IC_{50} = 2–3$ nM) but also improved cytotoxicity on cell lines expressing wild type and mutant FLT3 [38]. Results of lestaurtinib suggest a well-tolerated compound with mild side-effects, and it is still in clinical development [39,40].

Although the styryl-pyrimidine derivative ENMD-2076 (Fig. 2) was originally published as an Aurora inhibitor ($IC_{50} = 14$ nM), additional targets have been identified with kinase selectivity profiling. ENMD-2076 inhibits RET, Src, VEGFR2, PDGFRα, FMS with the same efficacy ($IC_{50} = 10–60$ nM), but it is a ten-fold better FLT3 inhibitor ($IC_{50} = 1.86$ nM) [41].

Quizartinib (Fig. 2), developed by Ambit Biosciences Corporation, is an inhibitor which was originally designed against FLT3. Notably, quizartinib displayed better selectivity over kinases than the previously described inhibitors. The IC_{50} of quizartinib is 1.1 nM

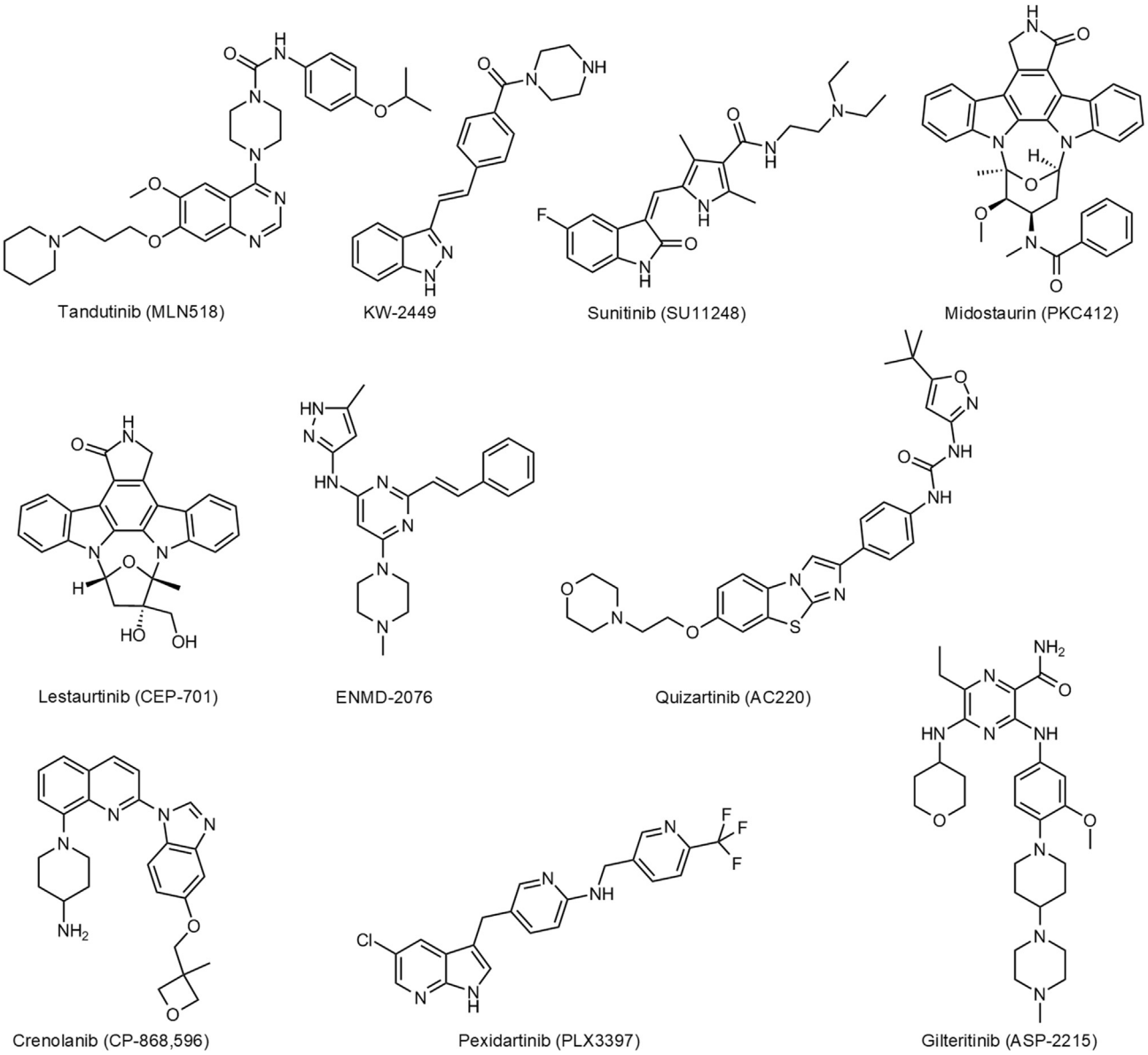


Fig. 2. Chemical structures of known FLT3 inhibitors have been investigated in clinical trials.

on FLT3-ITD and 4.2 nM on the wild type kinase [42]. In clinical trials the compound showed favorable and very encouraging effect, especially in the case of FLT3-ITD patients [43]. However, acquired drug resistance reduces the applicability of the compound. It was previously published that quizartinib-treated FLT3-ITD patients developed D835Y and F691L point mutations in the activation loop [44] and in the gatekeeper residue respectively, which decreased affinity of the compound. Quizartinib is a type II kinase inhibitor which binds to the DFG-out inactive conformation of the protein. The D835Y point mutation destabilizes the inactive state and quizartinib is not able to bind to FLT3 [45]. Although other FLT3 inhibitors like midostaurin and lestaurtinib are type I inhibitors and can bind to the active conformation, they are staurosporine analogues with low selectivity. Thus, there is a high demand for a selective FLT3-ITD/FLT3-D835Y inhibitor.

PLX3397 (Fig. 2), a triple kinase inhibitor of CSFR1R (enzymatic $IC_{50} = 13$ nM), c-KIT (enzymatic $IC_{50} = 27$ nM) and FLT3-ITD (enzymatic $IC_{50} = 11$ nM) inhibits FLT3 signaling in FLT3-ITD mutant cells (e.g. biochemical $IC_{50} = 18$ nM in MV4-11 cells) [46]. PLX3397 is a novel FLT3 inhibitor that overrides F691L. Based upon its encouraging preclinical activity, a phase 1/2 safety and efficacy study of orally administered PLX3397 was initiated in adults with relapsed or refractory FLT3-ITD acute myeloid leukemia (AML) [47].

The investigational type I inhibitor, crenolanib (Fig. 2), is a highly selective and potent FLT3 tyrosine kinase inhibitor (TKI) with activity against the FLT3-ITD mutants as well as against the FLT3-D835Y point mutants that are resistant to quizartinib; however, it shows a loss of potency against the gatekeeper mutation F691L and shows moderate activity against c-KIT [48,49]. Correlative data from an ongoing clinical trial demonstrate that crenolanib can achieve sufficient plasma level to inhibit both FLT3-ITD and resistance-conferring FLT3-D835 mutants in AML patients, *in vivo* [50].

The development of drug resistance during treatment of hematologic malignancies has been a challenging issue for TKIs [19,51,52]. Various factors have been identified in the background of resistance against FLT3 inhibitors including point mutations, plasma inhibitory activity (PIA), protective effect on bone marrow

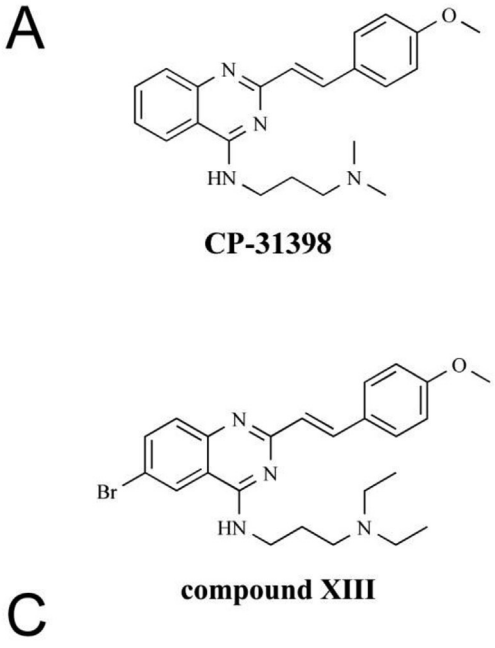
stromal cells and high levels of FLT3 ligand (FL). Point mutations within the kinase domain of FLT3-ITD especially at positions N676, F691 and D835 lead to substantial resistance to quizartinib and midostaurin [19,53]. Additional mutations causing quizartinib-resistance have been described using *in vitro* models [54].

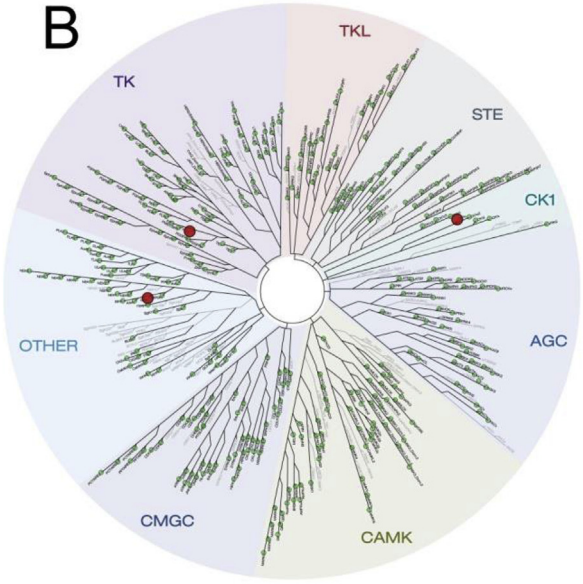
Myelosuppression can be a challenging clinical parameter to evaluate in any trial that involves AML patients. Most AML patients enrolled on early phase studies are already myelosuppressed from the burden of their disease. Nonetheless, suppression of bone marrow function to some degree is almost always a feature of certain TKIs, which have activity against c-KIT [49], as c-KIT is essential for normal erythropoiesis and megakaryocyte function [55]. For this reason it is important to determine a therapeutic index between the targeted receptor and c-KIT for TKIs used to treat hematologic malignancies in order to maintain normal hematopoiesis and improve the outcomes of treatments [56]. Based on these observations there is an unmet need for next-generation FLT3 inhibitors that overcome drug resistance and can reduce myelosuppression.

Here we report the development of novel and selective FLT3-ITD and FLT3-D835Y inhibitors having high affinity *in vitro* for the quizartinib-resistant FLT3-ITD/D835Y double mutant enzyme. Another unique advantage of our compounds is that they have no or negligible activity against c-KIT. Thus, we believe that **compound III** is a promising drug candidate with strong therapeutic potential to overcome drug resistance and to prolong disease-free survival of AML patients.

2. Results and discussion

The Nested Chemical Library™ [57] of Vichem was used to discover hit compounds against the FLT3 kinase. The phenylethenylpyrimidine compound ENMD-2076 (Fig. 2) was described as a low nanomolar FLT3 inhibitor [41]. The kinase inhibitory profile of a similar quinazoline-based compound (CP-31398, Fig. 3A) had not been investigated before. Previously it was published as a p53 activator molecule [58]. Since structural similarity can be found between ENMD-2076 and CP-31398 we measured the FLT3-ITD inhibitory activity of CP-31398, which was found to be 3.81 ± 0.87 (IC_{50}). Therefore, we chose our CP-31398-related phenylethenylquinazoline derivative **compound XIII** (Fig. 3A) for target identification and selectivity profiling study. The kinase inhibitory





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Kinase	Group	Family	Displacement (%)
FLT3	TK	PDGFR	81
FLT3(D835H)	TK	PDGFR	97
FLT3(D835Y)	TK	PDGFR	95
FLT3(ITD)	TK	PDGFR	96
FLT3(K663Q)	TK	PDGFR	80
FLT3(N841I)	TK	PDGFR	95
CSNK1A1	CK1	CK1	77
RIOK1	Atypical	RIO	63
GAK	Other	NAK	65

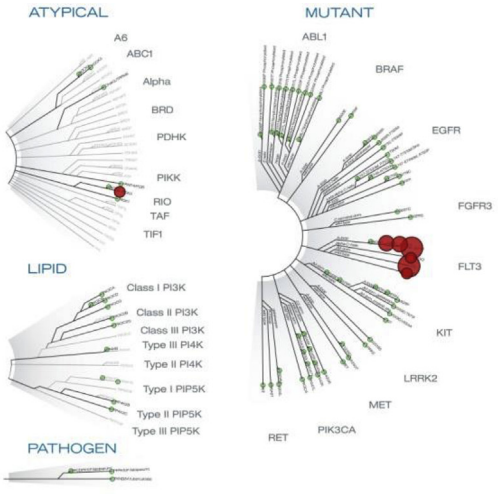


Fig. 3. (A) Structure of CP-31398 and **compound XIII** (B) Representation of quantitative measurement of the interaction between **compound XIII** and 451 kinases including clinically relevant mutants, lipid, atypical and pathogen kinases. (C) Displacement values of **compound XIII** at a concentration of 5 μM.

profile of **compound XIII** was determined by the KINOMEscan® binding assay (DiscoveRx Corporation) and the FLT3 kinase was identified as the primary target (wt FLT3 = 81% displacement) of the molecule (Fig. 3C). Moreover, in the case of mutant FLT3 kinases we found >90% displacement: FLT3-D835H = 97%, FLT3-ITD = 96%, FLT3-D835Y = 95%, FLT3-N842I = 95%, at 5 μM concentration. Only lower affinity was detected against a few kinases (CSNK1A1 77%, GAK 65% and RIOK1 63%), but it is important to note that the hit compound did not bind either to the members of the third class of the receptor tyrosine kinase family (c-Kit, CSFR, PDGFR), nor to any other receptor kinases. This is a very promising result considering the fact that some marketed kinase inhibitors hit more than 30% of the whole kinome [59].

2.1. Chemistry

Using the synthetic route (Fig. 4) developed for the preparation of CP-31398 (see Supplementary data) we synthesized additional 122 (phenylethenyl)quinazoline derivatives in order to establish a detailed structure-activity relationship.

First, the 2-methylquinazolone intermediates were prepared in good overall yields using the appropriate anthranilic acids, acetic anhydride and concentrated ammonium hydroxide solution. These intermediates were converted into phenylethenylquinazolones with various benzaldehydes *via* aldol-type condensation on the 2-methyl group. Using a microwave reactor not only increased the yield of these reactions, but also shortened the reaction time to 1.5–2 h. Next, these phenylethenylquinazolones were mixed with phosphoryl chloride and stirred at 90 °C in the presence of catalytic amount of dimethylformamide. The formed 4-chloro-phenylethenylquinazolines are unstable: therefore all derivatives were freshly synthesized and used in the last step without any analytical confirmation.

In the end, 4-amino-phenylethenylquinazolines were prepared from the imidoyl chlorides *via* nucleophilic aromatic substitution (S_NAr). The instability of the 4-chloro derivatives resulted in a mixture of the 4-hydroxyl- and 4-alkylamino derivatives, even under dry reaction conditions. Therefore the crude products were purified by column chromatography to separate the desired (phenylethenyl)quinazoline compounds with 10–60% yield.

2.2. Biochemical properties and selectivity of the compounds

In the first instance all compounds were tested with fluorescence polarization based IMAP™ (Immobilized Metal Assay for Phosphochemicals) kinase assay which was suitable for MTS/HTS screening at one concentration (10 μM), then only the best compounds were selected for the further IC_{50} measurements. The best FLT3 inhibitors synthesized are summarized in Table 1.

Based on the results of the biochemical assays we concluded that the best molecules are low-nanomolar inhibitors of the FLT3-ITD and FLT3-D835Y kinases, moreover, they showed selectivity over the wild-type FLT3 (Table 1). The significance of this finding is that the compounds also have a verified high inhibitory effect on D835Y mutant protein. Although the D835Y point mutation occurs less frequently (~7%) in AML, this mutation has been identified to play a role in acquired resistance against FLT3 inhibitors. Therefore, we also analyzed the inhibitory effect of tandutinib and quizartinib on the D835Y mutant FLT3. As expected, quizartinib showed an activity of only 1.33 ± 0.28 μM (IC_{50}) against the D835Y mutant enzyme which confirmed the previously described discovery that the mutation destabilizes the inactive (DFG-out) conformation and obstructs the binding of quizartinib [43]. Our lead molecules (indicated in grey in Table 1) are highly effective on FLT3-D835Y in the sub-micromolar range, not to mention the improved selectivity of the compound family over quizartinib [59].

2.3. Structure-activity relationship (SAR) study

The large number of the synthesized molecules gave us the opportunity to establish a detailed structure-activity relationship, based on the FLT3-ITD biochemical assay results and molecular docking experiments. The measured IC_{50} values for all prepared compounds are shown in the Supplementary data, Table 1. The quinazoline scaffold (Fig. 4) is essential for the FLT3 inhibitory effect and the core ring can be unsubstituted or monosubstituted (R^1), while the disubstituted (R^1, R^2) compounds were ineffective. Incorporation of a halogen atom (R^1) at the 6th position did not alter the effect substantially. However, the fluorinated derivatives had lower activities than the molecules containing bromine or chlorine at this position. Compounds having substituents in the 7th and 8th positions were ineffective, possibly due to reasons described in the molecular modelling paragraph. The benzo[*g*] quinazoline-based molecules condensed with benzaldehydes were also ineffective. The R^3 group can be substituted with a phenyl or a thiophene ring. In the case of the unsubstituted quinazolines, the substituted phenyl derivatives were effective but the thiophene analogues did not show any activity. However, in case of the tricyclic compounds, the thiophene derivatives were active while the substituted phenyl derivatives were completely inactive. The R^3 group has to be a monosubstituted aromatic ring for the strongest inhibition. Derivatives containing a 3,4-disubstituted phenyl

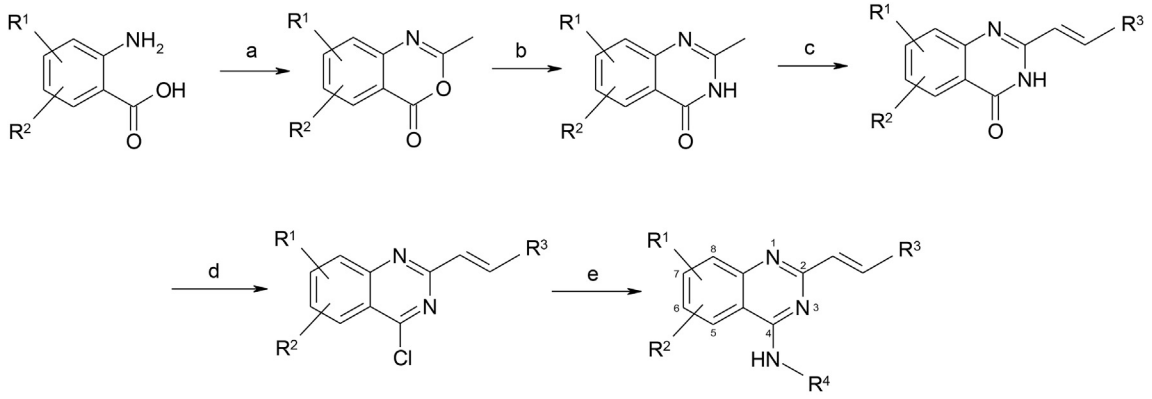
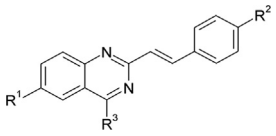


Fig. 4. General synthetic route of the phenylethenylquinazoline derivatives. Reagents and conditions: (a) acetic anhydride, reflux, 4 h; (b) 28% NH_4OH, RT 12 h; (c) benzaldehydes, 190 °C, microwave reactor, 1.5–2 h; (d) phosphorous oxychloride, DMF, 90 °C, 12 h; (e) primary amines, DIPEA, dioxane, 80 °C, 12 h (see details in **Supplementary data**).

Table 1
Structure-activity relationship and enzymatic data of the phenylethenylquinazolines. Biochemical kinase assay for FLT3-WT, FLT3-ITD, FLT3-D835Y was performed with IMAP-FP method at various concentrations of the compounds, and their IC_{50} values were calculated. All data are represented as means ± SD of three independent experiments. Grey coloring indicate the lead compounds.



Compound	R^1	R^2	R^3	FLT3 inhibition (IC_{50} μM)		
				FLT3-WT	FLT3-ITD	FLT3-D835Y
Tandutinib				0.29 ± 0.00	0.27 ± 0.04	9.22 ± 2.73
Quizartinib				0.17 ± 0.02	0.03 ± 0.00	1.33 ± 0.28
I	H	4-F	*N,N*-dimethylethane-1,2-diamine	7.02 ± 1.60	0.76 ± 0.02	0.56 ± 0.18
II	H	4-methylsulfanyl	*N,N*-dimethylethane-1,2-diamine	6.78 ± 0.88	0.48 ± 0.04	0.34 ± 0.15
III	H	4-isopropyl	*N,N*-dimethylethane-1,2-diamine	3.50 ± 0.50	0.20 ± 0.02	0.12 ± 0.04
IV	H	4-methylsulfonyl	*N,N*-dimethylethane-1,2-diamine	2.40 ± 0.26	0.33 ± 0.01	0.15 ± 0.02
V	Cl	4-F	*N,N*-dimethylpropane-1,3-diamine	24.9 ± 5.35	0.91 ± 0.56	0.79 ± 0.01
VI	Cl	4-methylsulfanyl	*N,N*-dimethylethane-1,2-diamine	3.40 ± 0.69	0.62 ± 0.28	0.10 ± 0.01
VII	Cl	4-isopropyl	*N,N*-dimethylethane-1,2-diamine	3.62 ± 0.57	0.41 ± 0.10	0.08 ± 0.03
VIII	Cl	4-methylsulfonyl	*N,N*-dimethylpropane-1,3-diamine	11.37 ± 0.02	0.87 ± 0.33	0.20 ± 0.02
IX	Cl	4-methylsulfonyl	*N,N*-dimethylethane-1,2-diamine	3.66 ± 0.69	0.24 ± 0.11	0.03 ± 0.00
X	Cl	4-methylsulfonyl	*N,N*-diethylethane-1,2-diamine	6.13 ± 1.06	0.44 ± 0.32	0.07 ± 0.00
XI	F	4-isopropyl	*N,N*-dimethylethane-1,2-diamine	7.08 ± 1.16	1.36 ± 0.41	0.19 ± 0.07
XII	F	4-isopropyl	*N,N*-diethylethane-1,2-diamine	18.92 ± 5.43	1.67 ± 1.57	0.24 ± 0.01
XIII	Br	4-methoxy	*N,N*-diethylpropane-1,3-diamine	>12.50	1.95 ± 0.70	1.07 ± 0.00

groups showed reduced effect and the 3,4,5-trisubstituted phenyl containing molecules were completely ineffective. The para substitution of the R^3 phenyl ring was the most preferred; substituents in the meta position greatly reduced the inhibitory effect. These observations are explained well by the inspection of the size and shape of the binding site that shows unfavourable properties for the binding of analogues bearing substituents other than the 4th position. The best substituents were the isopropyl, methylsulfonyl and methylsulfanyl functional groups. The R^4 group has to be a side chain that contains a tertiary amino group. The shorter chains (*N,N*-dimethylethane-1,2-diamine, *N,N*-dimethylpropane-1,3-diamine) were much better than the longer and larger (*N,N*-diethylpropane-1,3-diamine, N^1,N^1-diethylpentane-1,4-diamine, morpholine, *N*-methylpiperazine or pyrrolidine) ones. The presence of the side-chain is essential for the activity because compounds without it had no inhibitory effect (see Supplementary data, Table 2).

2.4. Antiproliferative activity in leukemia cells

After the selective and potent inhibitory effect of the compounds was demonstrated in different biochemical assays (FLT3-WT, FLT3-ITD and FLT3-D835Y), the cell proliferation inhibitory activity was tested in a panel of leukemic and non-leukemic cells. The compounds inhibited the cellular proliferation of the FLT3-ITD bearing leukemic (MV4-11 FLT3-dependent) cell line with an IC_{50} ranging from 0.01 to 2.34 μM. FLT3-ITD mutation was identified as a driver mutation in this cell line [29] and the cells acquired growth advantage due to the ITD mutation. FLT3 signal independent leukemic cell lines (U937 [60] and K562 [61]) and other non-leukemic cell lines were either weakly or not inhibited at all by the compounds (Table 2). Overall, these compounds are potent and highly selective inhibitors of the proliferation of FLT3-driven cells.

Table 2
Inhibition of cell proliferation by phenylethenylquinazolines on a panel of human cell lines. Cell proliferation was measured with CellTiter-Glo Assay. All data are represented as means ± SD of three independent experiments.

	Ihibitory effect on human cell lines (IC_{50} μM)			
	Leukemia			Bone Marrow Stroma
	MV4-11	K-562	U-937	HS-5
Tandutinib	0.06 ± 0.05	9.21 ± 0.39	19.80 ± 6.50	11.37 ± 0.42
Quizartinib	0.00 ± 0.00	10.33 ± 1.03	8.55 ± 1.60	2.98 ± 0.01
I	0.32 ± 0.01	5.81 ± 1.43	10.90 ± 1.08	6.37 ± 0.20
II	0.25 ± 0.06	5.21 ± 0.25	6.63 ± 1.61	4.00 ± 0.15
III	0.03 ± 0.00	2.49 ± 0.34	3.53 ± 0.10	2.55 ± 0.64
IV	0.05 ± 0.01	5.54 ± 1.00	11.32 ± 0.89	8.14 ± 0.19
V	0.43 ± 0.16	2.04 ± 0.19	5.35 ± 1.06	3.03 ± 0.02
VI	0.38 ± 0.46	3.00 ± 0.15	4.95 ± 0.24	3.30 ± 0.07
VII	0.03 ± 0.01	2.87 ± 0.13	3.56 ± 0.28	3.03 ± 0.07
VIII	0.10 ± 0.09	4.84 ± 1.37	9.02 ± 1.08	4.03 ± 0.25
IX	0.01 ± 0.01	6.94 ± 0.38	12.29 ± 0.12	4.76 ± 1.00
X	0.04 ± 0.01	2.59 ± 0.63	4.95 ± 1.18	4.09 ± 0.03
XI	0.06 ± 0.06	4.03 ± 0.02	4.10 ± 0.50	3.28 ± 0.00
XII	0.24 ± 0.13	2.12 ± 0.15	4.11 ± 0.56	3.36 ± 0.03
XIII	2.34 ± 1.32	4.03 ± 0.23	6.36 ± 2.85	2.07 ± 0.06

Table 3
Determination of kinetic solubility and passive, transcellular permeability (P_e ($*10^{-6}$ cm/s)) values (mean ± SD; n = 3) of the phenylethenylquinazoline compounds.

Compound	Solubility [μM]		P_e [$*10^{-6}$ cm/s]
	pH = 7.4	pH = 2.0	
I	10	120	9.61 ± 1.45
II	116	119	5.86 ± 1.13
III	108	120	5.85 ± 0.98
IV	119	120	1.42 ± 0.15
V	56	113	6.29 ± 0.70
VI	8	94	3.49 ± 0.56
VII	12	110	0.73 ± 0.52
VIII	23	111	0.61 ± 0.20
IX	8	112	0.36 ± 0.16
X	12	115	0.56 ± 0.01
XI	5	119	3.38 ± 0.00
XII	36	120	1.74 ± 0.04
XIII	96	96	5.35 ± 0.05
caffeine			5.84 ± 0.11
amiloride			0.05 ± 0.00

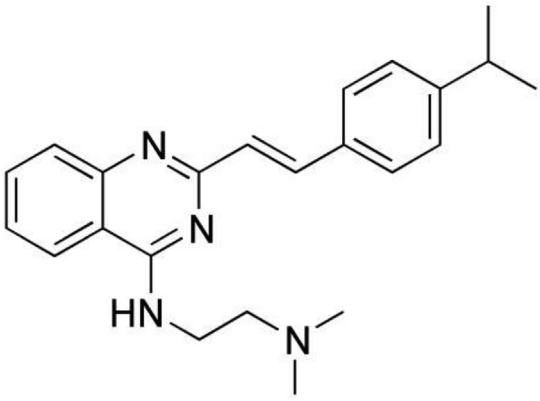
Because some of our molecules demonstrated strong inhibition both in biochemical and in cellular assays, the selectivity of these lead compounds had to be verified. The 4-isopropyl derivative **compound III** demonstrated good solubility and membrane penetration values (Table 3), therefore, this molecule was chosen for the selectivity validation by KINOME*scan*™ binding assay.

The remarkable selectivity profile of **compound III** (Fig. 5) did not change due to the structural modifications (compared to **compound XIII** - Fig. 3A and B) and even improved the FLT3 binding affinity. The results also confirmed the high binding affinity for the double mutant FLT3-ITD/D835Y quizartinib resistant enzyme (Fig. 5).

Next, we determined the mechanism of the inhibition. Most of the quinazoline-based kinase inhibitors are ATP-competitive, therefore, we hypothesized that our compounds are ATP-competitive as well. In order to prove this hypothesis, the IC_{50} values of the lead compounds were measured at three different ATP concentrations: at the K_mATP concentration (5.1 μM for the FLT3-ITD enzyme), and at a lower (0.51 μM) and at one higher (51 μM) concentration. According to the Cheng–Prusoff equation [62], the increasing ATP concentration will increase the IC_{50} value of the compounds if the they are ATP-competitive inhibitors.

The results (Fig. 6) demonstrated that our compounds have higher IC_{50} at higher ATP concentration so compounds III, VII and IX are ATP-competitive.

Due to the high homology of the ATP binding sites, the ATP-competitive inhibitors are generally not selective for their target proteins; therefore there is no particular explanation for the extreme selectivity of the compounds. We suppose that the cause of the selectivity may be attributable to favorable combination of non-covalent and covalent binding to FLT3. Covalent kinase inhibitors can target a nucleophilic group at a special position in the



Compound III

Kinase	Group	Family	Displacement (%)
FLT3(ITD/D835V)	TK	PDGFR	100
FLT3(ITD)	TK	PDGFR	100
FLT3(N841I)	TK	PDGFR	100
FLT3(R834Q)	TK	PDGFR	100
FLT3	TK	PDGFR	98
FLT3(D835V)	TK	PDGFR	98
FLT3(D835H)	TK	PDGFR	95
FLT3(K663Q)	TK	PDGFR	93
FLT3(D835Y)	TK	PDGFR	90
MEK5	STE	MAPKK	82
TRKA	TK	TRK	76
KIT(D816V)	TK	PDGFR	69
INSR	TK	Insulin receptor	68
PDGFRB	TK	PDGFR	68

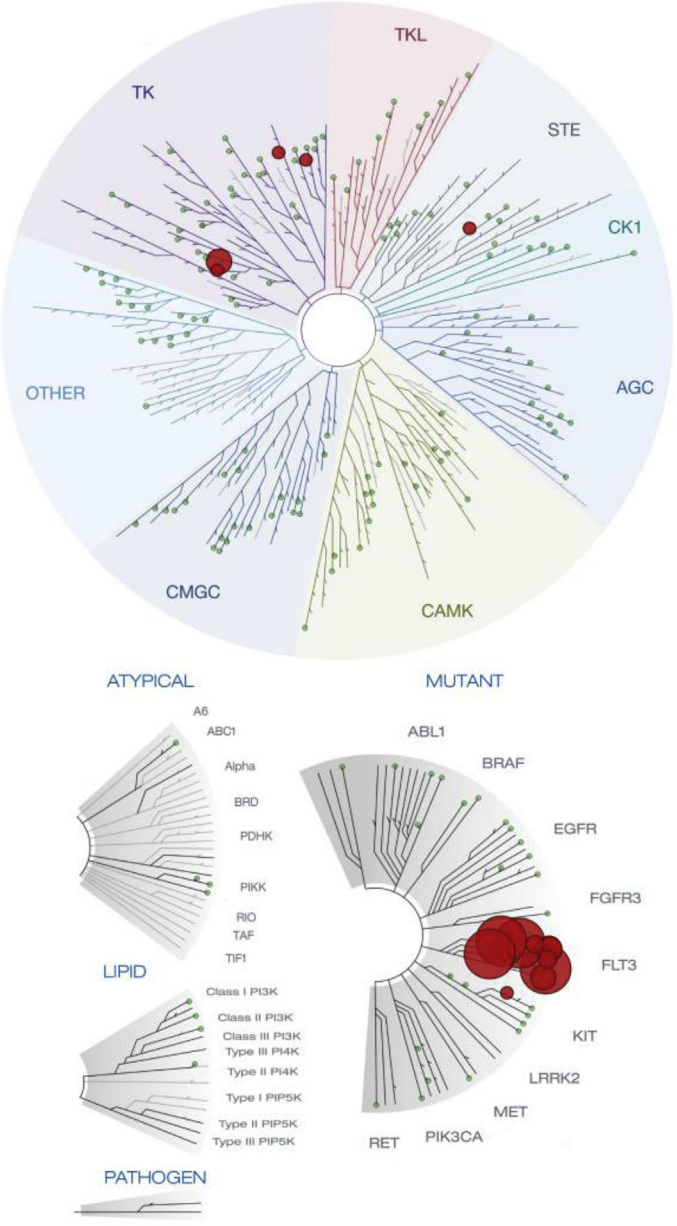


Fig. 5. Representation of quantitative measurement of the interaction between **compound III** and 451 kinases including clinically relevant mutants, lipid, atypical and pathogen kinases.

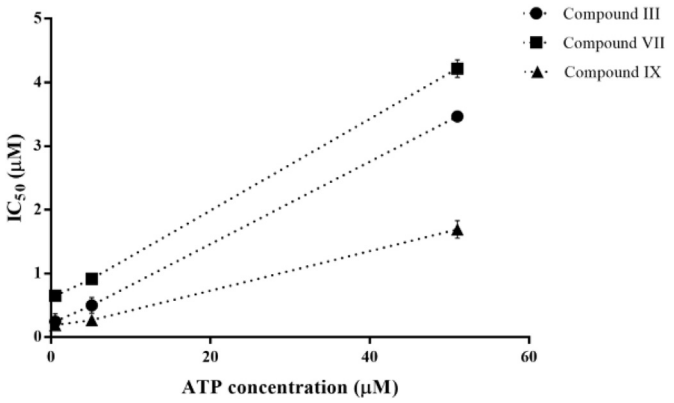


Fig. 6. Assessing the binding mode of **compound III**, **compound VII** and **compound IX** to recombinant FLT3-ITD enzyme. All data are represented as means ± SD of two independent experiments.

binding site (e.g. cystein residue). Other kinases or family members will not react, because they do not have the targeted nucleophilic residue in the appropriate position [63]. It is important to note that, besides FLT3, only a handful of kinases have an adequately positioned cysteine residue in the hinge region for the covalent reaction to occur. The Aurora kinase isoforms do not contain a similarly positioned cysteine either [64], which may also explain why our compounds have no such inhibitory activity on Aurora described for ENMD-2076, despite structural similarities.

Our developed FLT3 inhibitors contain a double bond between the two aromatic rings and this ethenyl group can act as a Michael acceptor. To prove this hypothesis we used 2-mercaptoethanol (ME) and *N*-acetylcysteine (AcCys) as a Michael donor and performed chemical experiments to model this reaction. The addition product ($[M+H]^+$ 439.3) was detected first after 24 h of incubation with ME (ratio 1:1) at room temperature (Supplementary data, Fig. 1). By using excessive amount of ME (ratio 1:50), the addition product appeared after 60 min (Supplementary data, Fig. 2). These measurements were repeated with AcCys as well and resulted in very similar results. In case of equivalent quantity (inhibitor and AcCys 1:1), the addition product ($[M+H]^+$ 524.3) could be detected after 18 h (Supplementary data, Fig. 3). By using AcCys in excess, 30 min incubation was enough for the reaction to occur (Supplementary data, Fig. 4).

Based on these measurements, our compounds may likewise form covalent bonds with the ATP binding site of the FLT3 kinase and this could partly answer the extreme selectivity. According to a recent report on kinase inhibitors, they can show ATP-competitive kinetics while binding covalently to the active site [65].

2.5. *In silico modelling of the covalent binding mode*

Molecular modelling was applied to further investigate the binding mode of the compounds. Reversible glide docking was conducted on all of the derivatives. Covalent docking was performed on the ligands using a custom made covalent docking mechanism protocol [66].

Our theory is that a covalent reaction occurs between the ethenyl group and Cys694 of the hinge region after initial binding of the molecule. Such mode of action was reported by Nijmeijer, Engelhardt et al. of a hH4 partial agonist compound with a 2-ethenylpyrimidine structure, that is able to form covalent adducts with thiol containing molecules glutathione and cysteine ethyl ester [67]. No similar reaction was observed in the case of the 2-methylpyrimidine derivative, suggesting a covalent mechanism of the ethenyl compound. Alkenyl and alkynyl substituted heteroarene compounds and their ability to form covalent bonds with cysteines were described in a recent overview of covalent warheads by Matthias Gehringer and Stefan A. Laufer [68].

Our glide docking models indicate a minimal distance of 3 Å between the sulfhydryl group of Cys694 and the ethenyl carbon of our compounds. The reversible ligand-enzyme complexes show π-π stacking interactions of the phenyl ring with Phe830 and, to a lesser extent, Phe691. Either a hydrogen bond or an ionic bond is also observed between the protonated amino group of the side chain and the carboxylate group of Asp698.

The supposed covalent reaction takes place on the β-carbon of the ethenyl group, analogous to α,β-unsaturated carbonyl compounds [69] and acrylamide moiety-containing covalent inhibitors [63]. Covalent docking of the compounds indicate a uniform binding mode of the active derivatives (Fig. 7), with minimal displacement relative to the reversible docking model. Interactions described above with Phe830 and Asp698 are also present in the covalent models, while a small movement of the molecules outwards the pocket, towards the solvent is observed. Docking scores also confirm the favorable energy balance of the covalent reaction (Supplementary Data, Table 3). Models of inactive compounds bearing substituents in the 8th position of the quinazoline ring show a substantial displacement compared to the actives. The also inactive 6,7-dimethoxy substituted analogues are represented as protonated both on the amino side chain and on the pyrimidine nitrogen at pH 7.4 simulations, possibly disrupting the active binding conformation. Benzo[g]quinazoline derivatives mostly overlap with their quinazoline analogues, although steric clashes between the core and the hinge region prevent them from tighter binding and properly interacting with the phenylalanines nearby through their phenyl or thienyl rings. This is in accordance with their reduced inhibitory activity. The models also suggest that only molecules with para positioned substituents have adequate space inside the binding site when covalently docked; analogues with

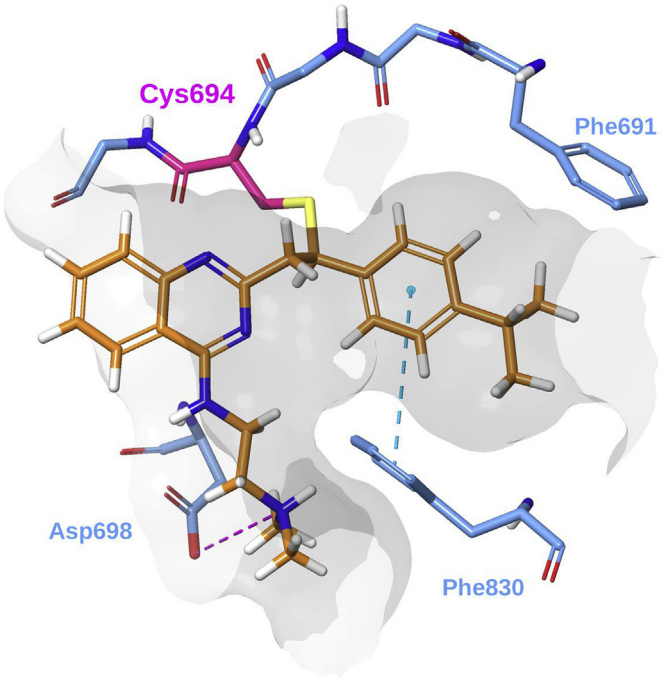


Fig. 7. Covalent binding mode of **compound III** (orange) to Cys694 (magenta) of the hinge region. Interactions with Asp698 and Phe830 are represented by the dashed lines. The inner surface of the receptor binding site is the area shown in grey.

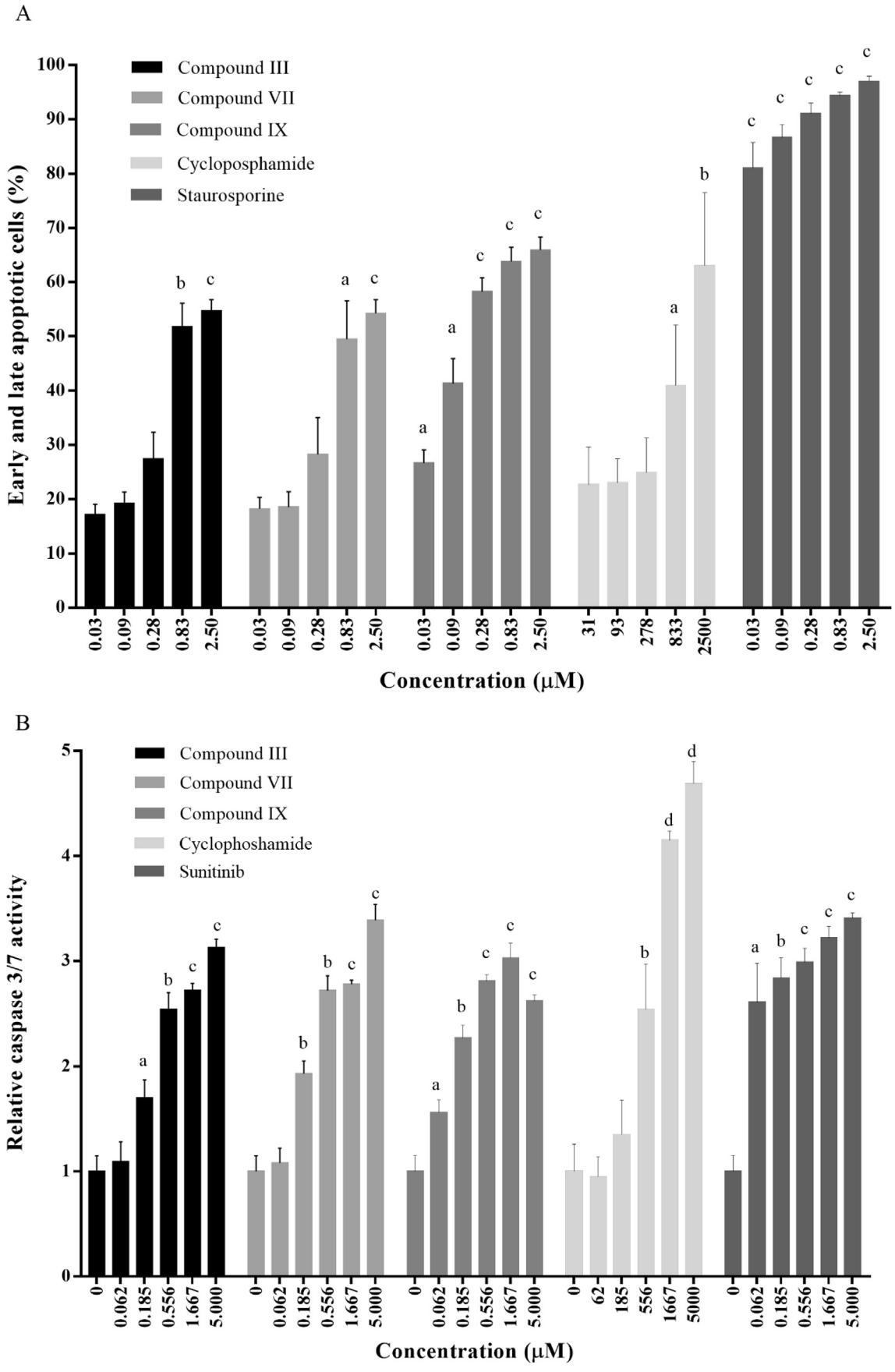


Fig. 8. (**A**) The percentages of early and late apoptotic cells stained with Annexin V-FITC and PI analyzed by flow cytometry. MV4-11 cells were treated with the indicated concentrations of the compounds for 24 h. Concentrations are in μM. Data are displayed as means of n = 3 + SEM. Variance analysis was performed prior identifying the unpaired *t*-test as appropriate to calculate significance between treated and untreated cells [a] $p \leq 0.05$; [b] $p \leq 0.005$; [c] $p \leq 0.0005$. (**B**) Caspase-3/7 activities are shown after 24 h of treatment with increasing concentrations of compounds in MV4-11 cells. Concentrations are in μM. Data are displayed as means of n = 3, +SEM. Variance analysis was performed prior choosing the unpaired *t*-test to calculate significance values compared to basal caspase activity [a] $p \leq 0.05$; [b] $p \leq 0.01$; [c] $p \leq 0.001$; [d] $p \leq 0.0001$. According to the analysis of raw data by Shapiro-Wilk test, we can assume that samples have normal distributions in both experiments ($P > 0.05$).

multiple phenyl ring substituents do not fit well in the pocket, which explains their inactivity.

2.6. Compounds induce apoptosis in FLT3-ITD expressing MV4-11 cells

With the reproducible anti-proliferative effect observed for **compound III**, **compound VII** and **compound IX** against the MV4-11 cell line, their mechanism of action was investigated by flow cytometry. Apoptosis and necrosis were demonstrated by staining the cells with Annexin V and propidium iodide. The cells were treated with increasing concentrations of **compound III**, **compound VII** and **compound IX** to demonstrate the dose-dependent effect on the induced apoptosis.

The majority of the untreated cells (DMSO was used as control) was viable (82.6 ± 3.6%) and showed neither Annexin V-FITC nor PI positivity. As expected a dose-dependent increase was observed in early ($AN^+ PI^-$) and late apoptotic cells ($AN^+ PI^+$) (Fig. 8A) following treatment with **compound III**, **compound VII** and **compound IX**.

Caspase-3 is a key downstream effector in the apoptosis pathway therefore Caspase-Glo® 3/7 assay was used for measuring caspase 3/7 activity. Using the assay as a determinant, a significant elevation of caspase activity was detected after exposure to as little as 0.185 μM **compound III**, **VII** and **IX** in the FLT3-ITD mutant MV4-11 cells (Fig. 8B). This effect was as pronounced as the effect of cyclophosphamide used at higher concentrations and verified the early stage of apoptosis induced by the compounds in the MV4-11 cell line in a dose-dependent manner.

Together, the data indicate that **compound III**, **compound VII** and **compound IX** effectively induce apoptosis.

Table 4
Pharmacokinetic parameters of **compound III** after i.v. and oral administrations.

	Unit	iv (3 mg/kg)	oral (10 mg/kg)
AUC	ng/mLh	1663 ± 96	264 ± 18
$T_{1/2}$	min	50.6 ± 1.8	ND
CL_obs	L/h/kg	0.60 ± 0.04	ND
Vss_obs	L/kg	4.26 ± 0.62	ND
C_{max}	ng/mL	399 ± 56	16.6 ± 1.9
T_{max}	h	0.017	2
F%			16

C_{max} maximum concentration.
T_{max} time of maximum concentration.
AUC area under the curve from the time of dosing to the last measurable concentration.
CL_obs total clearance.
Vss_obs volume of distribution at steady state.
$T_{1/2}$ half-life.
F% bioavailability.
ND could not be calculated.

2.7. Determination of ADME properties

The solubility of the compounds was measured at two pH values using HPLC (Table 3). The compounds showed good solubility at pH 7.4 but some of them were considerably more soluble at acidic pH of 2.0, which is not surprising due to the secondary and tertiary amino groups in their structures. Some molecules and one of the lead compounds (**III**) showed acceptable solubility (108 μM) at physiological pH. These results suggest that special compound formulation is not required for early preclinical development.

Most of the tested phenylethenylquinazoline compounds demonstrated good penetration values (Table 3). According to our results (P_e values = 0.36–9.61*10^{-6} cm/s), we can conclude that some of these phenylethenylquinazoline derivatives can cross the cell membranes by passive diffusion.

2.8. Pharmacokinetic parameters and in vivo studies of compound III

Compound III has a large volume of distribution (Vss_obs = 4.26 L/kg) and a plasma half-life (T ½) of 50.6 min (Table 4). Although oral bioavailability (F (%) = 16) was relatively poor, suggesting high first pass metabolism and/or poor gastrointestinal absorption, **compound III** reached sufficiently high plasma concentrations (16.6 ng/ml ± 1.9) after a single oral administration to exert an effect in a xenograft model.

SCID mice were subcutaneously inoculated with MV4-11 cells.

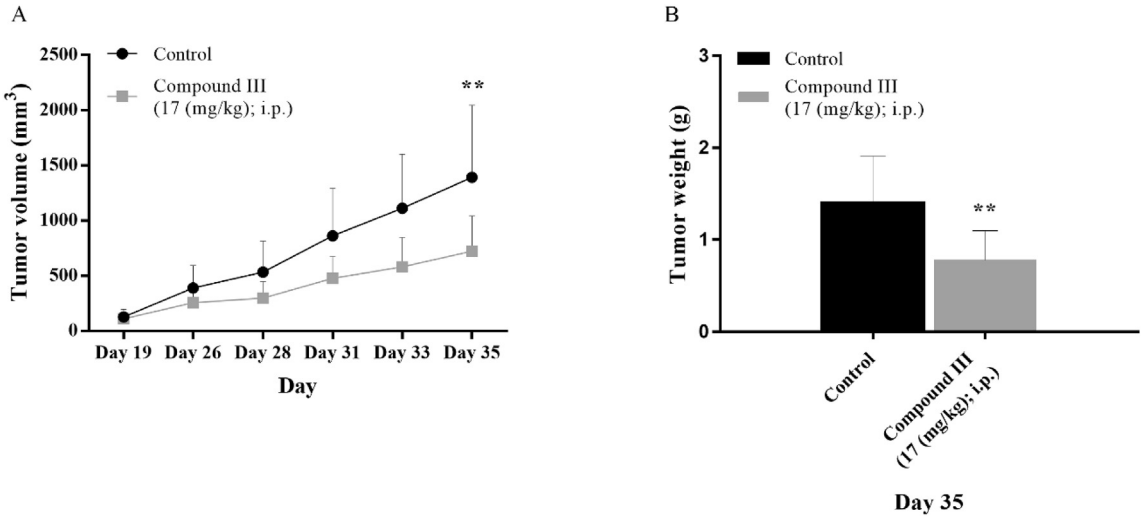


Fig. 9. (A) *In vivo* antitumor activity of **compound III** against FLT3-ITD-driven leukemia tumor growth in SCID mice. Decreasing rate of tumor growth was observed during **compound III**-treatment (volume = width^2 x length x π/6); means ± SD; n = 11, *p < 0.05). Mann-Whitney *U*-test was used to compare tumor volumes between the vehicle-treated group and the treatment group. On day 35, the median of the tumor volume in the control group was 1306.952 mm^3, while in the treated group the median of the tumor volume was 613.819 mm^3; U = 14; Z = −3.053; p = 0.002 (2-tailed); the effect size (r = 0.6509) is strong. **(B)** Tumor weight on day 35 (means ± SD; n = 11; *p < 0.05). Mann-Whitney *U*-test was made to compare tumor weights between the vehicle-treated group and the treatment group. The median of the tumor weight in the control group was 1.367 g and 0.687 g was in the treatment group; U = 15; Z = −2.988; p = 0.002 (1-tailed); the effect size (r = 0.637) is strong.

Table 5
Determination of body weight during **compound III**-treatment. Mann-Whitney *U*-test was used to compare body weight after tumor inoculation on Day 19 between the vehicle-treated group and the treatment group. On day 19 the median of the (weight_D19) in the control group was 30.400 g, while in the treated group the median of (weight_D19) was 31.700; U = 51.5; Z = −0.591; p = 0.554 (2-tailed); the effect size (r = 0.053). Mann-Whitney *U*-test was used to compare body weight after tumor inoculation on Day 35 without tumor weight (weight_D35) between the vehicle-treated group and the treatment group. On day 35 the median of the (weight_D35) in the control group was 31.428 g while in the treated group the median of the (weight_D35) was 30.231; U = 31; Z = −1.937; p = 0.053 (2-tailed); the effect size (r = 0.011) is. Thus, it can be stated that there is no significant difference in body (weight_D19) and (weight_D35). This result indicates that the administration of **compound III** was well-tolerated at the dose we employed.

n° animal	Control				Treated			
	Animal weight after tumor inoculation on Day 19 [g]	Animal weight after tumor inoculation on Day 35 [g]	Tumor weight after tumor inoculation on Day 35 [g]	Animal weight after tumor inoculation on Day 35 without tumor weight [g]	Animal weight after tumor inoculation on Day 19 [g]	Animal weight after tumor inoculation on Day 35 [g]	Tumor weight after tumor inoculation on Day 35 [g]	Animal weight after tumor inoculaton on Day 35 without tumor weight [g]
1	29.80	32.90	1.33	31.13	30.60	29.60	0.45	29.15
2	33.50	34.50	0.66	34.16	31.80	31.50	0.80	30.70
3	30.40	31.50	1.03	31.43	32.50	33.30	1.43	31.87
4	30.40	32.30	1.38	31.78	32.30	31.80	0.68	31.11
5	31.30	31.40	1.06	32.36	27.40	28.40	0.56	27.84
6	32.80	34.60	1.54	34.34	31.10	29.50	0.64	28.86
7	26.20	28.20	1.45	27.65	33.50	33.00	0.58	32.42
8	29.80	31.50	1.15	30.95	26.30	26.50	0.69	25.81
9	33.20	36.80	2.62	35.82	31.70	31.60	1.01	30.59
10	27.90	29.70	1.38	29.28	32.10	31.50	1.27	30.23
11	28.40	30.70	1.83	30.23	29.40	29.50	0.50	29.01

19 days after the inoculation, **compound III** was administered intraperitoneally at a dose of 17 mg/kg on every other day for 3 weeks. By the end of this period, the MV4-11 tumors have reached an average volume of 1393.13 mm^3. Compared to the vehicle control, the treatment with **compound III** reduced the tumor volume and weight significantly, by 48% and 49% in average, respectively (Fig. 9). During the treatment period no additional body weight loss and no death was observed (data shown in Table 5). These results demonstrated that **compound III** effectively reduced the tumor size and was well-tolerated at the dose we employed.

3. Conclusions

In summary, a novel series of phenylethenylquinazoline derivatives were designed and synthesized by general and affordable methods. The exploration and optimization of the different substituents positioned on the quinazoline scaffold resulted in the discovery of a new, highly selective FLT3 inhibitor compound family. To our current knowledge these are selective FLT3 inhibitors, which showed an impressive *in vitro* efficacy on single mutant enzymes (FLT3-ITD, FLT3-D835Y) and also had a high affinity for the quizartinib-resistant double mutant (FLT3-ITD/D835Y) enzyme. We demonstrated with chemical reactions and *in silico* modelling experiments that the compounds can form a covalent bond with the thiol side chains of cysteines and we assume that this mechanism is present when binding to the FLT3 kinase as well. This phenomenon can probably contribute to the high degree of selectivity. Besides these observations we detected an ATP-competitive characteristic for **compound III**.

In addition, the selectivity and specificity were demonstrated in cellular assays and in xenograft leukemia model. **Compound III** showed effective antitumor activity *in vivo*, by significantly reducing tumor volume and weight after treatment on every other day for 3 weeks. In conclusion, these molecules should be evaluated in further drug discovery and development studies to treat FLT3-ITD bearing, quizartinib-resistant AML.

Author contributions

FB designed and synthesized the compounds. AS set and performed the FLT3 biochemical assays, cellular and solubility experiments. ZŐ discovered the high selectivity to FLT3 of an early hit compound and accomplished the flow cytometric and caspase 3/7 assay measurements on MV4-11 cells. ZN measured the solubility of the molecules. JD performed cellular experiments. CsSzK supervised the *in vitro* FLT3 kinase assays and ESz did the instrumental analysis in the PK studies. GSZ, LD and PH performed the PK studies on mice. MTC and JT managed and performed the *in vivo* xenograft model. RG helped in the chemistry and designed the figures for the paper. MK accomplished the *in silico* molecular modelling experiments and covalent docking. LŐ initiated, supervised and directed the project and prepared the paper with FB, AS and ZŐ.

Declaration of competing interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmech.2019.111710.

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