Bioorganic & Medicinal Chemistry Letters 22 (2012) 7634–7640

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Modification of a promiscuous inhibitor shifts the inhibition from γ-secretase to FLT-3

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ARTICLE INFO

Article history:
Received 14 August 2012
Revised 28 September 2012
Accepted 1 October 2012
Available online 11 October 2012

Keywords:
Acute myeloid leukemia
FLT-3
Kinase inhibitor
γ-Secretase

ABSTRACT

The inhibition of FLT-3 activity is an interesting target for the treatment of acute myeloid leukemia (AML). The serendipitous identification of FLT-3 inhibitors from a CK1/γ-secretase programme provided compounds with dual inhibitory activity. We analyzed the structure–activity relationship of these inhibitors and derivatized them to arrive at compounds with reduced impact on γ-secretase activity and enhanced FLT-3 inhibition.

Acute myeloid leukemia (AML) is an aggressive haematological malignancy with long-term survival rates of 25–70% in patients younger than 60 years and only 5–15% in older patients.[1,2] Activating mutations of FLT-3 (FMS-like tyrosine kinase-3) are abundant molecular abnormalities found in AML.[3] FLT-3 is essential for the normal function of stem cells and the immune system and is primarily expressed in immature hematopoietic cells.[4] It contains an extracellular ligand binding domain, a transmembrane domain, and an intracellular juxtamembrane domain followed by the tyrosine kinase domain, which is interrupted by a kinase insert region.[5,6] Internal-tandem duplications (ITDs) and tyrosine kinase domain (TKD) point mutations are the two major classes of activating FLT-3 mutations identified in AML patients.[7] FLT-3/ITD mutations were estimated to occur in ~23% of de novo AML.[8] Several ATP-competitive FLT-3 inhibitors have been developed for a targeted therapy of this disease.[9] The FLT-3 inhibitors are derived from different structural classes and some of them displayed high potential in preclinical and clinical trials, Scheme 1.[5–10]

The published data suggest that FLT-3 is an attractive therapeutic target for the development of kinase inhibitors for AML and other associated diseases.[11]

Working on the structure–activity relationship of the indolinone scaffold of dual CK1/γ-secretase inhibitors in the context of Alzheimer's disease we additionally profiled the compounds for kinase selectivity. Serendipitously FLT-3 was part of the kinase panel and two substances displayed significant inhibition (98% and 89% at 10 mM) of this tyrosine kinase, Scheme 2.[11]

Regarding this and the fact that FLT-3 is not known to exert effects on the amyloid precursor protein metabolism we focused on optimizing the FLT-3 inhibitory activity of **1** and reducing its modulation of γ-secretase activity. Primary objective of our research was to decrease the effect on γ-secretase and to improve meanwhile the inhibitory activity against FLT-3. The structure of **1** guided the variation of the structure–activity relationship (SAR) study.

The Knoevenagel condensation of indolinones and aldehydes provides a rapid and efficient method to generate a chemical diversity. The Koevenagel products were obtained from microwave irradiated reaction mixtures within 30 min, which was followed by rapid purification. In the first step alkyl or aryl benzyl halides were coupled with 4-hydroxybenzaldhydes **3** under basic conditions to obtain a series of elongated benzaldehydes **4a–n** for the subsequent Knoevenagel condensation.[11,12] A further nucleophilic substitution of the chlorides **4k,l** resulted in the tertiary amines **5a**,**b** potential intermediate iminium salts were hydrolysed in the aqueous work up.[11] The ether **7** was formed in a microwave heated reactor by substitution of an aromatic fluoride **6** with a 4-(4-methyl-1*H*-imidazole-1-yl)phenol.[13] The 4-fluoro-benzaldehyde **8** was substituted to its corresponding sulfonyl **9** and imidazol benzaldehyde **10**, Scheme 3.[13,14] The phenylimidazoles **7** and **10** were prepared to evaluate this motif, which is frequently employed on γ-secretase

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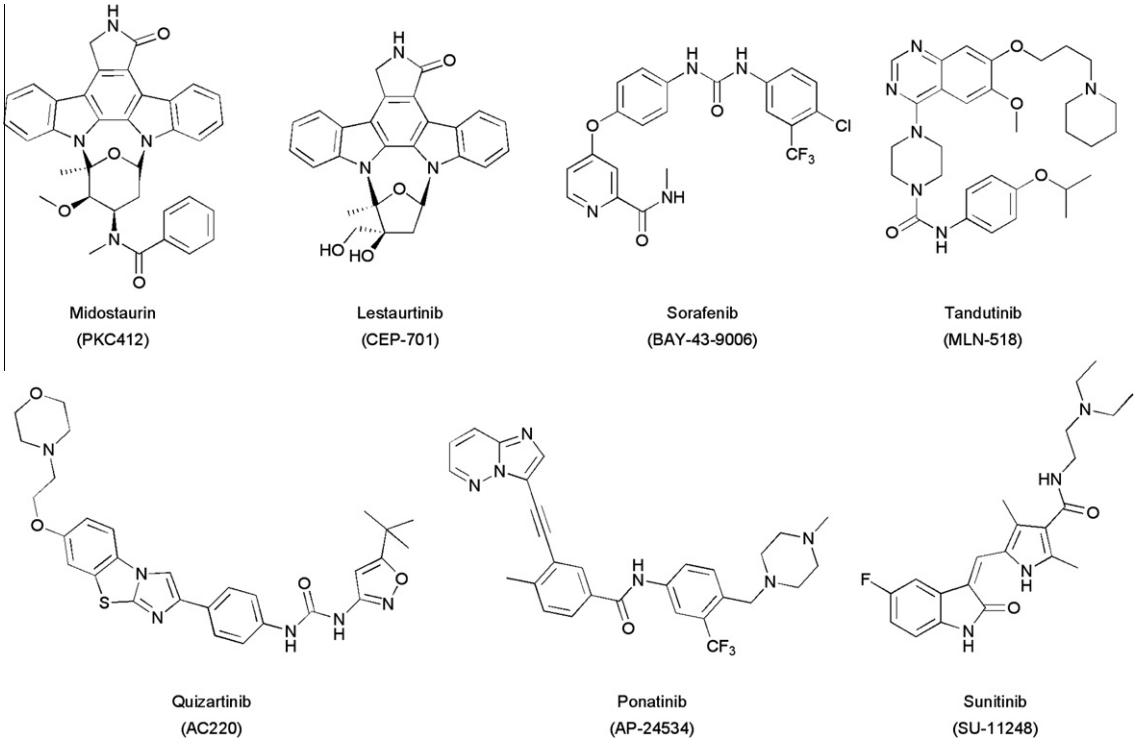
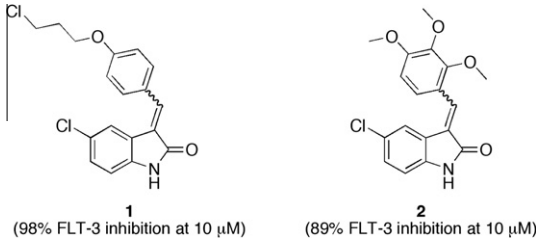
http://dx.doi.org/10.1016/j.bmcl.2012.10.016



Scheme 1. FLT-3 inhibitors evaluated in preclinical or clinical trials.[5–7,9,10]



Scheme 2. Screening hits of potential FLT-3 inhibibitors in a kinase panel of 43 kinases.[11]

modulators.[15] The opportunity to obtain fluorescent derivatives was addressed by two derivatives: (A) a two step synthesis resulted in the azobenzene derivative **12**, the 4-aminobenzaldehyde **11** was converted to its diazonium derivative via diazotation and the final azobenzene **12** was formed by an azo coupling reaction.[16] (B) The 9-methyl-9*H*-carbazole **13** was converted to the bulky aldehyde **14** in a two step procedure under Vilsmeier–Haack conditions, Scheme 3.[17]

Commercial aldehydes and the previously synthesized benzaldehydes (Scheme 3) were coupled with the indolinones **15a,b** under microwave supported Knoevenagel conditions, Scheme 4.[11] The resulting products **16a–x** were obtained in yields up to 97%, Table 1. This class of compounds was shown to isomerize within 2 days in methanol, thus no attempts were made to separate the isomers.[11] A further reduction of compound **16e** resulted in 77% yield of product **16y**, Scheme 5a. Finally, the product **16i** was derivatized to obtain the tetrazole **16z** in a moderate yield of 62%, Scheme 5b.[18]

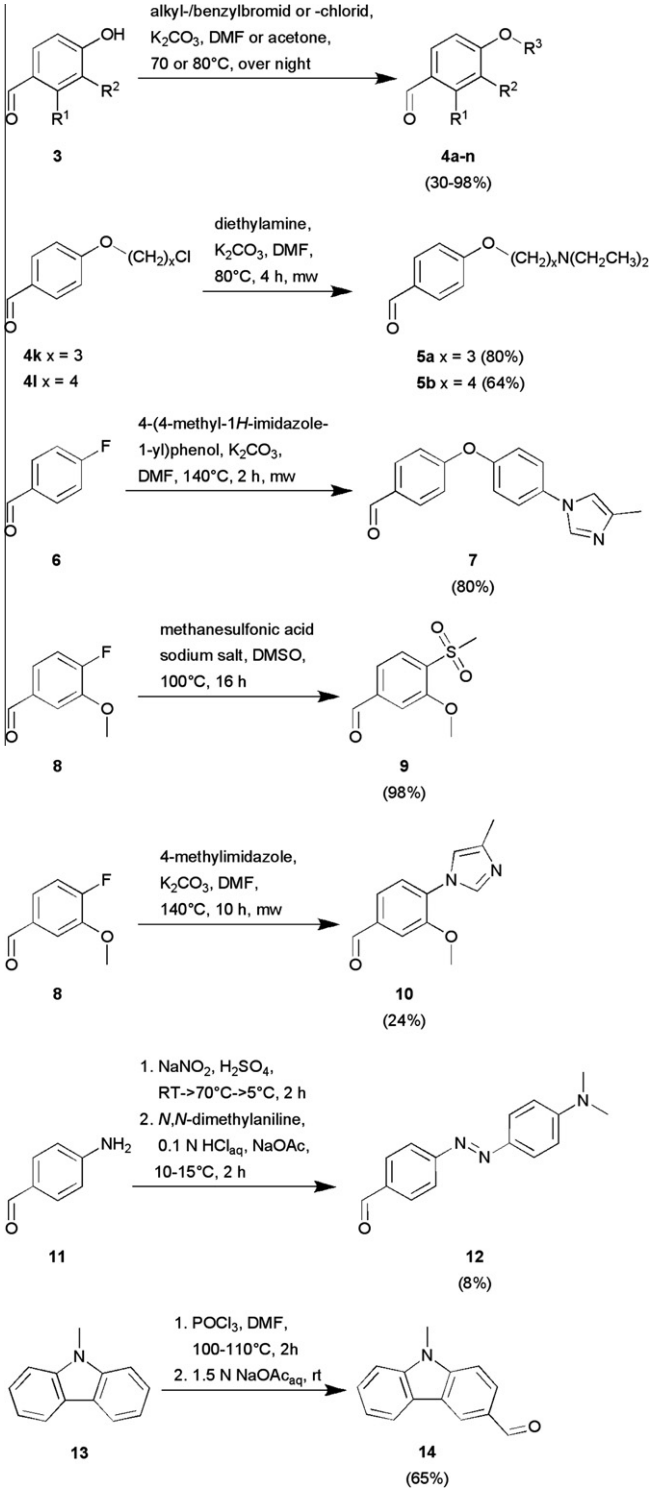
Human recombinant FLT-3 was used in the FLT-3 in vitro kinase assay to ascertain the inhibitory activity. The concentration of the phosphorylated substrate peptide phospho-Ulight-CAGAGAIETDK EYYTVKD (Starting unphosphorylated peptide concentration: 100 nM) was determined after 90 min incubation time at room temperature by the LANCE detection method.[24] All experiments were carried out as technical replicates, the average of two such replicates is listed in Table 1.

The results are expressed as a percent of control (Staurosporine) specific activity ((measured specific activity/control specific activity) × 100) obtained in the presence of the test compounds (10 μM), Table 1. The previously described liquid phase electrochemiluminescence (LPECL) assay was used to measure the Aβ42 isoform to evaluate the compounds for their potency in inhibiting γ-secretase activity, Table 1.[23]

To confirm the FLT-3 inhibition-ratio observed at 10 μM concentration we determined the IC_{50} values of compounds **16e,j,l,n**. The four compounds showed potent inhibition of FLT-3 activity: IC_{50} = 4.1 nM for **16e**, IC_{50} = 14.0 nM for **16j**, IC_{50} = 29.0 nM for **16l** and IC_{50} = 17.0 nM for **16n**, which exceeded the IC_{50} in the Aβ42 generation assay by 3 powers of 10. These activities indicated that we have differentiated FLT-3 inhibition from γ-secretase inhibition.

The most potent γ-secretase inhibitors **16s,v** feature a phenylimidazole, which is frequently encountered in γ-secretase modulators. However, both compounds display reduced FLT-3 inhibition. As indolinones are well known as kinase inhibitors[25,26] we determined the broader selectivity of our most active FLT-3 inhibitor **16e**. The selectivity of compound **16e** was evaluated at a concentration of 1 μM against 50 human protein kinases, Figure 1. Most of the 50 kinases in this panel showed a residual activity higher than 80%, whereas FLT-3 displayed a residual activity of only 14.7%. The only kinases, which were also significantly inhibited by this compound, were the Ser/Thr kinase HGK (MAP4K4) and the Tyr kinase JAK3.

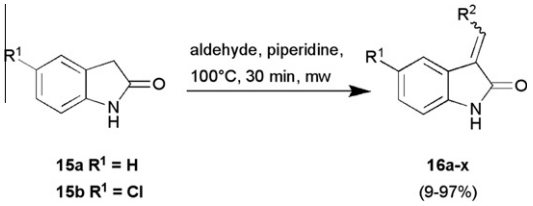
In addition to the H4 APP#9 cell-based toxicity assay, we evaluated the compounds in a zebrafish embryo phenotype assay, which enabled toxicity determination in whole organisms.[27] The embryos were collected and maintained in E2 medium at 28 °C. Compound **16e** was added 4–5 hpf (hours post fertilization) and the phenotypes were compared after 48 hpf. Compound **16e** causes



Scheme 3. Aldehyde syntheses (R^1 = H, OMe; R^2 = H, OMe; R^3 = alkyl/benzyl moieties).[11–14,16,17]

a development delay at 20 μM, Figure 2. Compared to the control, zebrafish embryos treated with **16e** were still covered by the chorion. Nevertheless, they did not reveal other abnormalities. The zebrafish embryo assay did not reveal lethality and peculiarities at a concentration below 20 μM of **16e**.

The docking studies (Molegro Virtual Docker 5) of the FLT-3 crystal structure (PDB: 1RJB) and compounds **16e** and **16n**



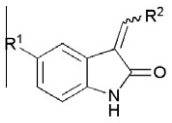
Scheme 4. Synthesis of indolinone derivatives under microwave conditions (R^2 = phenyl moieties).[11,19–22]

revealed potential inhibitor-enzyme interactions, Figure 3.[8] In both cases the docking result indicates four potential interactions. The indolinone motif interacts edge-to-face with the Phe830 of the DFG motif. In addition, the indolinone fits to the FLT-3 hinge region by face-to-face interplay with Phe691 and the hydrogen bonding with Lys644.[8] The phenyl group of the inhibitors interacts with Met665.[28] In addition to these interactions, the rigidity of the bridge between the indolinone and the phenyl moiety may contribute to potent FLT-3 inhibition. Therefore, we synthesized compounds **16e** and **16y**, which differ in orientation and rotational flexibility of the aryl substituent. The FLT-3 in vitro assay results of compounds **16e** (FLT-3 inhibition-ratio = 100%) and **16y** (FLT-3 inhibition-ratio = 47%) revealed a twofold increased FLT-3 inhibition-ratio for **16e** and exemplifying the influence of rigidity. A FLT-3 inhibition comparison of all compounds revealed that an electron donor motif is needed at the end of the elongated, variable alkyl chain. For example compound **16c**, which lacks this motif, showed a decreased inhibition activity compared to **16d,i**. This may be explained by a polar area formed by Glu573 and Gln577, which is in close vicinity. A comparison of the FLT-3 inhibition results and the docking studies offers the possible suggestion that the benzylidene indolinone moiety may occupy the entrance of the ATP-binding site (compound **16a,b,k**) and the elongated alkyl chain ranging from C_2 (**16e**) to C_4 (**16h**) could act as a flexible 'anchor' in the inner side of the ATP-binding pocket, for example, **16d–j**. Furthermore, a comparison of the FLT-3 inhibition results of Table 1 revealed that bulky residues at the phenyl moiety lead to a decreased inhibitory activity.

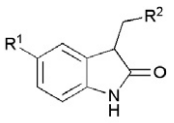
The inhibitory activity of the synthesized compounds in the γ-secretase assay cannot be correlated to the FLT-3 inhibition-ratio, Figure 4. In the case of compounds **16i** (FLT-3 inhibition-ratio = 96%; IC_{50} (Aβ42) > 40 μM) and **16n** (FLT-3 inhibition-ratio = 87%; IC_{50} (Aβ42) > 160 μM) we expected the same result, but surprisingly, they were not active against the γ-secretase, Table 1 and Fig. 4. In comparison with other compounds, for example, **16d,l** and **16p,r**, the nitrile and alkyl chain combination is important for this selectivity.

Conclusion: we have identified several potent FLT-3 inhibitors based on the scaffold of the screening hit **1**, which display neglible activity on γ-secretase inhibition. The most active compound **16e** did not display significant toxicity in H4 APP#9 cells and the zebrafish embryo phenotype assay. This lack of apparent toxicity may be due to lack of permeation, yet permeation is indicated by the activity in the Aβ42 generation assay. The combination of FLT-3 in vitro results and docking studies revealed likely enzyme-inhibitor interactions with the amino acids Lys644, Met665, Phe691 and Phe830. Further improvement of these FLT-3 inhibitors will focus on the reduction of the log*P* to enhance to pharmacokinetic properties. Our most active FLT-3 inhibitor **16e** exhibits a *C*log*P* value of 5.28, Table 1, which implies impaired solubility.

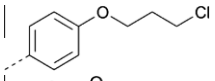
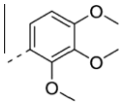
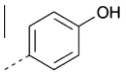
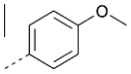
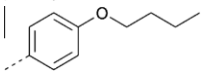
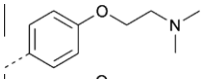
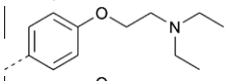
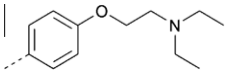
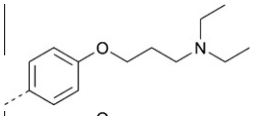
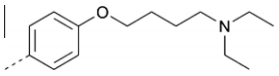
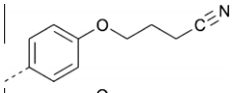
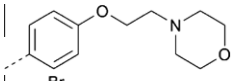
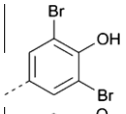
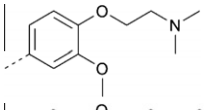
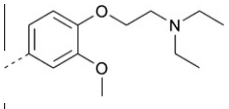
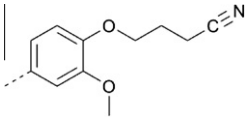
Table 1
Synthesized indolinones, their chemical properties and in vitro assay results[23,24]



1-2, 16a-x, 16z

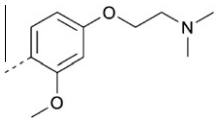
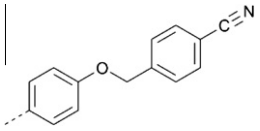
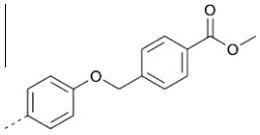
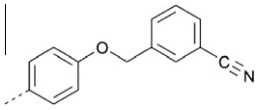
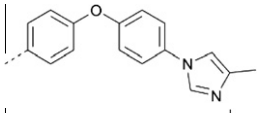
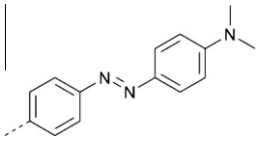
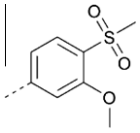
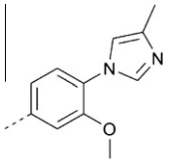
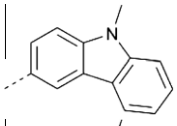
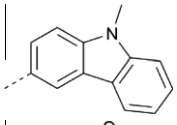
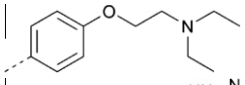
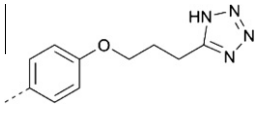


16y

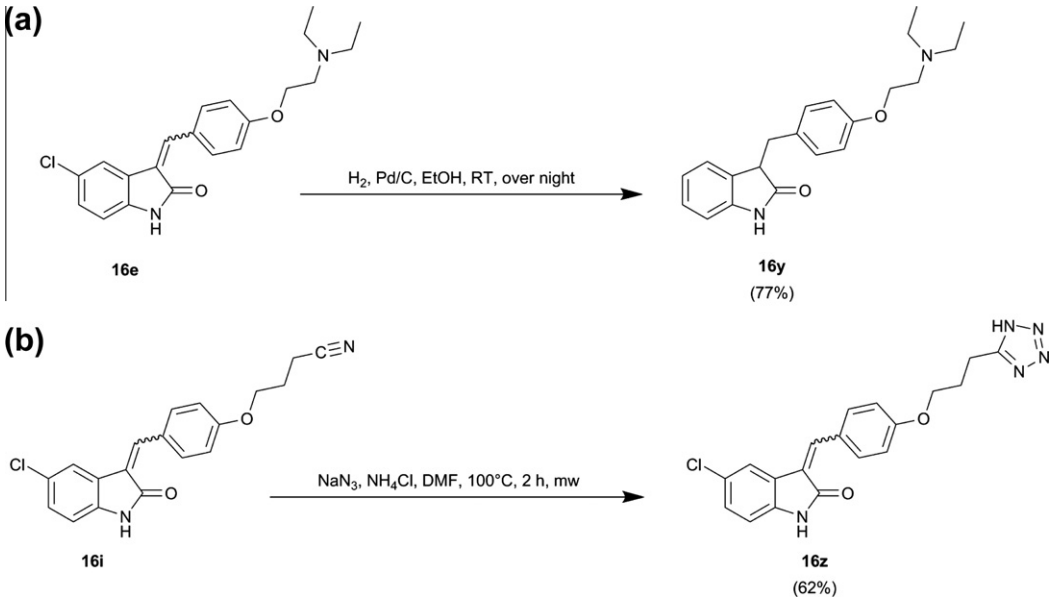
Compound	R^1	R^2	FLT-3 inhibition -ratio[a] (%)	IC_{50} of A_{42} LPECL assay[b] (μM)	Toxicity (μM)[c]	Yield (%)	Clog*P*[d]
Staurosporine[e]	—	—	100	n.t.[f]	n.t.[f]	—	4.19
Sunitinib[e]	—	—	100	n.t.[f]	n.t.[f]	—	3.00
1	Cl		98	4.7	Y (40)	—	5.04
2	Cl		89	—	—	—	3.50
16a	Cl		94	47.6	N (40)	28	3.53
16b	Cl		85	>40	N (40)	88	4.12
16c	Cl		79	>40	N (40)	83	5.71
16d	Cl		99	7.6	N (40)	65	4.22
16e	Cl		100	17.7	Y (40)	55	5.28
16f	H		97	—	—	95	4.31
16g	Cl		99	—	—	80	5.63
16h	Cl		99	—	—	64	5.56
16i	Cl		96	>40	Y (40)	90	3.94
16j	Cl		96	11.3	N (40)	84	4.24
16k	Cl		97	15.0	N (40)	97	5.10
16l	Cl		98	11.5	Y (40)	31	3.96
16m	Cl		99	11.5	Y (40)	42	5.02
16n	Cl		87	>160	N (160)	67	3.68

(*continued on next page*)

Table 1 (*continued*)

Compound	R^1	R^2	FLT-3 inhibition -ratio[a] (%)	IC_{50} of A_{42} LPECL assay[b] (μM)	Toxicity (μM)[c]	Yield (%)	Clog *P*[d]
16o	Cl		100	10.0	N (40)	71	4.31
16p	Cl		75	27.3	N (40)	93	5.32
16q	Cl		58	38.8	N (40)	70	5.86
16r	Cl		77	>40	N (40)	48	5.32
16s	Cl		83	5.4	Y (10)	41	5.88
16t	H		77	>80	N (80)	9	5.55
16u	Cl		48	7.3	Y (20)	85	2.49
16v	Cl		74	6.7	Y (80)	55	4.25
16w	Cl		69	19.8	N (80)	17	6.04
16x	H		78	27.6	Y (80)	15	5.07
16y	H		47	—	—	77	3.87
16z	Cl		83	17.7	Y (40)	62	3.76

[a] Percent of control (Staurosporine) specific activity ((measured specific activity/control specific activity) × 100); activity at a concentration of 10 μM.
[b] Aβ liquid phase electrochemiluminescence (LPECL) assay, H4 APP#9 cells.
[c] Determined in H4-cells, Y = Yes, N = No.
[d] Calculated by ChemDraw Ultra (9.0.1).
[e] Control.
[f] n.t. = Not tested.



Scheme 5. Post-Knoevenagel modifications: (a) Reduction with H_2 and Pd/C; (b) Tetrazole synthesis under microwave conditions.[18]

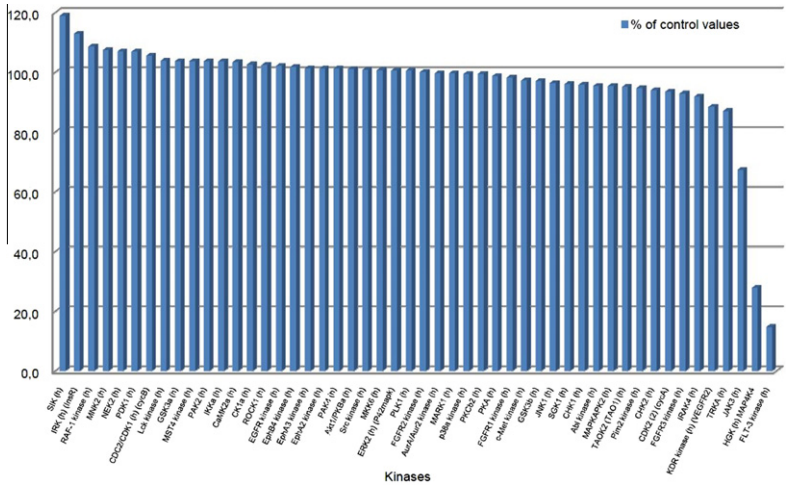


Figure 1. Screening of compound **16e** against a panel of human protein kinases. Each bar represents the activity of one individual protein kinase (determination method: percent of control (Staurosporine) specific activity [(measured specific activity/control specific activity) × 100]). Compound **16e** was tested at a concentration of 1 μM against 50 protein kinases. See the Supplementary data for more details.[24]

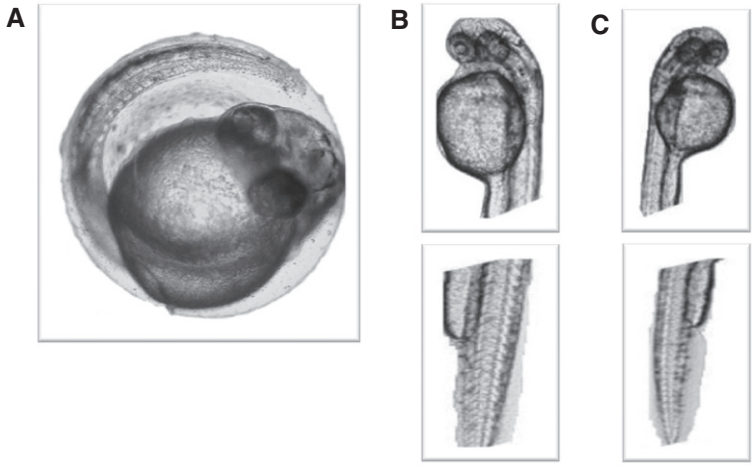


Figure 2. Exposure of zebrafish embryos to (A) 20 μM of **16e**, (B) 5 μM of **16e**, (C) control. The embryos were collected and maintained in E2 medium at 28 °C. Compound **16e** was added 4–5 hpf (hours post fertilization) and the phenotypes were compared after 48 hpf.

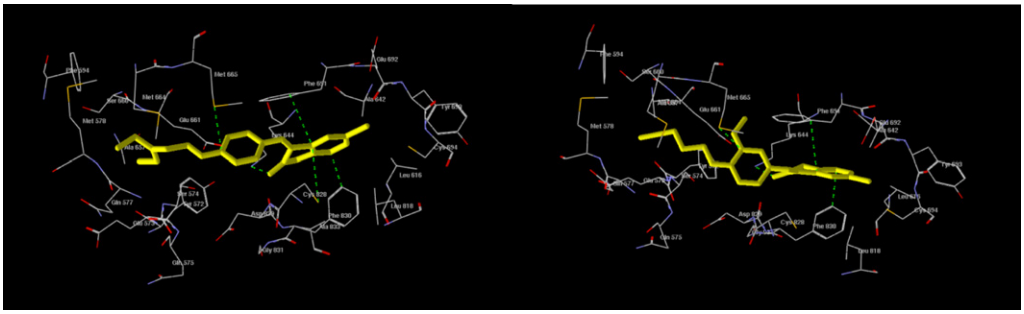


Figure 3. Docking of compound **16e** (left) and **16n** (right) into the PDB crystal structure 1RJB of FLT-3; important interactions are highlighted; Software: Molegro Virtual Docker 5.

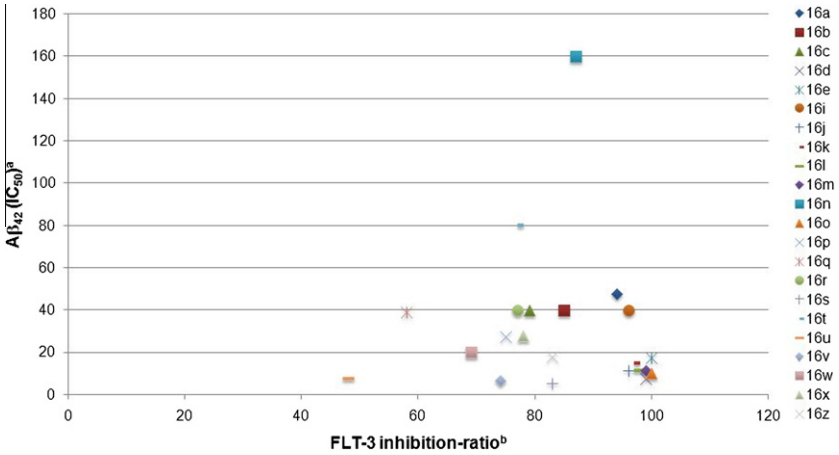


Figure 4. Correlation plot of FLT-3 activity inhibition and Aβ42 inhibition (IC_{50}); [a]values at 40 μM are determined as >40, see Table 1; [b]determination method: percent of control (Staurosporine) specific activity [(measured specific activity/control specific activity) × 100]).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.10.016.

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