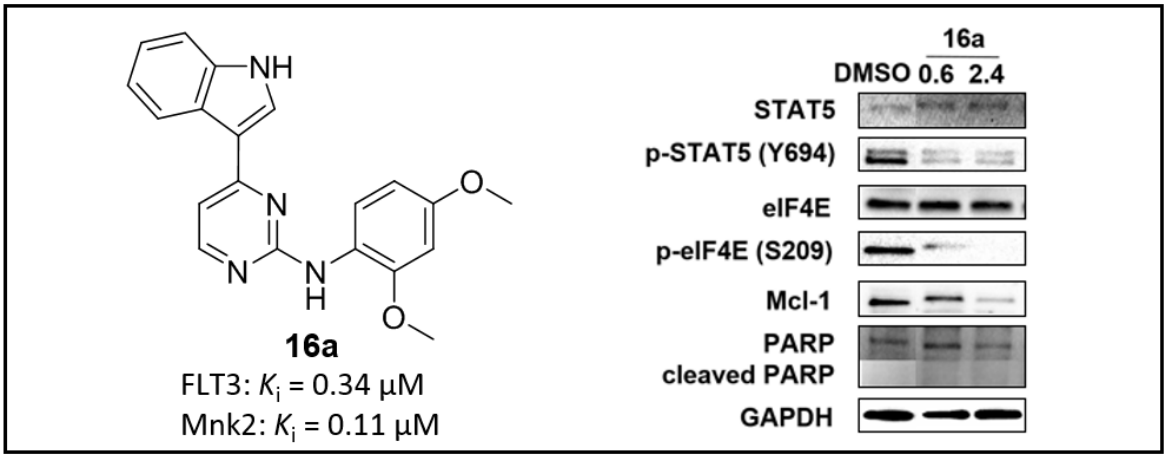
Graphical abstract



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Dual Inhibition of Mnk2 and FLT3 for Potential Treatment of Acute Myeloid Leukaemia

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Keywords

Mnk inhibitor; FLT3 inhibitor; dual inhibitor; AML; drug discovery; anti-leukaemic agent

Abbreviations

AML, acute myeloid leukaemia; CDK, cyclin-dependent kinase; eIF, eukaryotic initiation factor; Erk, extracellular signal-regulated kinase; FLT3, Fms-like tyrosine kinase; GI_{50}, concentration with 50% growth inhibition; IMAP, immobilised metal ion affinity particle; ITD, internal tandem duplication; Jak, Janus kinase; K_i, inhibition constant; $K_{M(ATP)}$, Michaelis-Menten's constant for ATP; MAPK, mitogen-activated protein kinase; Mnk, MAPK-interacting kinase; mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol 3-kinase; PIGF, placental growth factor; SFK, Src family kinase; STAT5, signal transducer and activator of transcription 5; TKD, tyrosine kinase domain; VEGF, vascular endothelial growth factor.

Abstract

The discovery of novel anti-AML therapeutic agents is urgently needed, but the complex heterogeneity of the disease has so far hampered the development of a curative treatment. FLT3 inhibitors have shown therapeutic potential in clinical trials; but a monotherapy regimen has been associated with resistance mediated by the activation of parallel signalling circuitry, including MAPK and mTOR. Therefore, inhibiting a nexus of the two signalling pathways along with inhibition of FLT3 might be advantageous. Herein, we propose that a dual inhibition of FLT3 and Mnk would provide a better clinical option for AML patients compared to targeting FLT3 alone. Thus, a series of *N*-phenyl-4-(thiazol-5-yl)pyrimidin-2-amines and 4-(indol-3-yl)-*N*-phenylpyrimidin-2-amines were prepared. Potent Mnk2 inhibitors, FLT3 inhibitors, and dual inhibitors of Mnk2 and FLT3 were identified and their anti-proliferative activities assessed against MV4-11 AML cell lines. Dual inhibition of FLT3 and Mnk2 caused the increased apoptotic cell death of MV4-11 cells compared to inhibition of FLT3 or Mnk2 alone.

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1. Introduction

Longstanding standard treatment of acute myeloid leukaemia (AML), consisting of cytarabine and an anthracycline, remains unsatisfactory. Cure rates are dismal being only 5-15% for older patients,[1] urging the discovery for more effective therapies targeting the genetic drivers of leukaemogenesis.

Fms-like tyrosine kinase 3 (FLT3) is a transmembrane receptor that controls the proliferation and differentiation of haematopoietic cells.[2] FLT3 is overexpressed on leukaemic blasts of AML patients and the *FLT3* gene is mutated in approximately one third of them.[1, 3, 4] The most common mutations are ITD in the juxtamembrane domain of FLT3 (20%-25%) and point mutations within the TKD (5%-10%), which lead to a loss of the autoinhibitory function of FLT3. Therefore, FLT3 and its downstream effectors, including PI3K/Akt, MAPK/Erks and STAT5 become constitutively active, thereby enhancing the aberrant growth of blasts and inhibiting apoptosis.[4] Hence, FLT3 has emerged as a promising therapeutic target for treating AML, leading to the discovery of several small molecule inhibitors of FLT3, *e.g.* lestaurtinib, midostaurin and quizartinib.[5] However, phase I/II clinical studies of these inhibitors as monotherapy in patients afflicted with AML have shown a partial and transient clinical response.[1] Drug resistance was developed shortly after commencing treatment. Despite inhibition of FLT3, downstream STAT5 and MAPK pathways remained consistently active in some FLT3-mutated AML patients, suggesting compensatory mechanisms, *e.g.* FLT3-independent stimulation of STAT5 and MAPK pathways.[6] Other mechanisms of resistance were unravelled, including an atypical TKD mutation that conferred resistance to FLT3 inhibitors by inducing a substantial upregulation of the anti-apoptotic protein Mcl-1.[7] Data derived from clinical trials suggest that inhibition of FLT3 is unlikely to be beneficial if used alone, and that combination therapy of FLT3 inhibitors with other agents targeting the apoptotic machinery could override drug resistance and be of better clinical value to AML patients.

Deregulation of translation is omnipresent in AML and mediates leukaemic transformation.[8] Being a limiting factor in translation initiation, the eukaryotic initiation factor (eIF) 4E has joined the fray as an anti-AML therapeutic avenue. eIF4E funnels two *bona fide* oncogenic pathways, the Ras/Raf/Erks and PI3K/Akt/mTOR, both of which are commonly deregulated in AML.[9, 10] The transformational activity of eIF4E stems from its role in enhancing the translation and export of transcripts needed only for tumourigenesis, while other house-

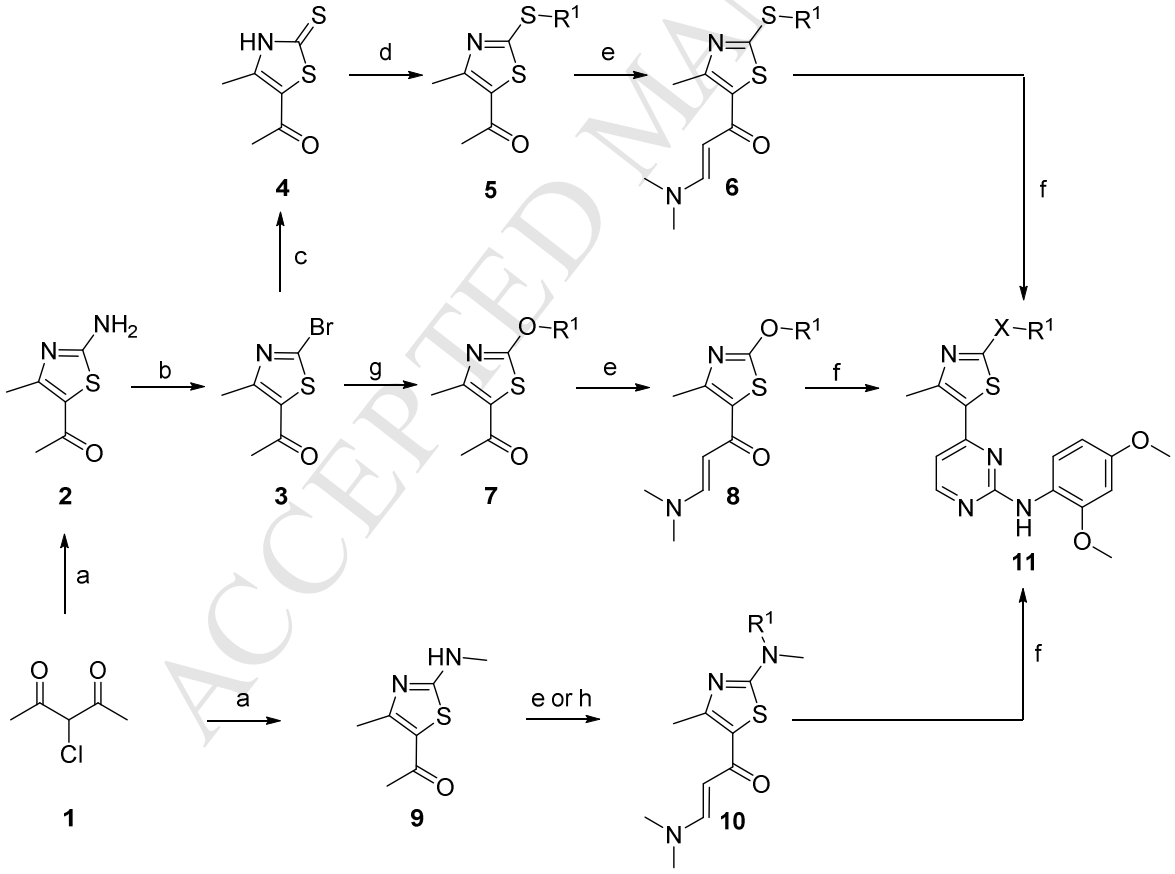
keeping transcripts remain unaffected.[11] Stalling the activity of eIF4E downregulates Mcl-1 in AML cells, which culminates in apoptotic cell demise.[12, 13] Phosphorylation of eIF4E at its Ser209 by MAPK-interacting kinases (Mnk1 and Mnk2) is essential for its oncogenic activity; overexpression of wild-type eIF4E was shown to markedly enhance Myc-mediated lymphomagenesis in mice, compared to those that received $eIF4E^{S209A}$ mutated grafts.[14] Furthermore, Mnk inhibitors significantly reduce the proliferation of AML cells *in vitro* and *in vivo*. We have previously demonstrated that Mnk inhibitors downregulate phosphorylated eIF4E, induce G_1 phase cell cycle arrest and apoptosis in MV4-11 AML cells.[15] In mouse xenografts, a significant reduction of MV4-11 cell proliferation was observed upon treatment with cercosporamide, as monotherapy or in combination with cytarabine[16], and with merestinib.[17] Therefore, Mnk inhibitors hold great promise as anti-AML agents.

Herein, we suggest that the concomitant blockade of Mnks and FLT3 would have an additive effect against AML cells. As eIF4E is a focal point of the MAPK and mTOR pathways, suppressing its translational function might interrupt mechanisms responsible for the development of resistance following FLT3 inhibition. Our endeavour to develop dual inhibitors of Mnk and FLT3 has focused on the *N*-phenylpyrimidin-2-amine scaffold. The derivatives of the latter have demonstrated potent Mnk2 inhibitory activity,[18] and some of them exerted a residual inhibition of FLT3. To gauge improvements in potency and selectivity towards both kinases, two frameworks were designed based on the *N*-(2,4-dimethoxyphenyl)-pyrimidin-2-amine scaffold, given its enhanced selectivity towards Mnks over cyclin-dependent kinases (CDKs).[18] The first scaffold retained the thiazole at C4 position of the pyrimidine while incorporating various substitutions at C2 of the thiazole (*i.e.* compounds **11**). A premise for this design strategy was that previous modifications with bulkier alkylamines had dampened inhibitory potency against CDKs.[23] Thus, it was hypothesised that the introduction of bulkier groups at C2 of the thiazole might marginalise CDK inhibitory activity, but favour the interactions with Mnk2 and FLT3. SAR analysis of the first series revealed the favourable outcomes of grafting nitrogen at the C2 position of the thiazole. Therefore, in the second scaffold, and in order to evaluate the effect of fused heterocyclic systems, thiazole ring at C4 of pyrimidine was replaced by an indole (i.e. compounds **16**) where its 1-NH might be memetic of the C2-NH of thiazole.

2. Results

2.1. Chemistry and Structure-Activity Relationships

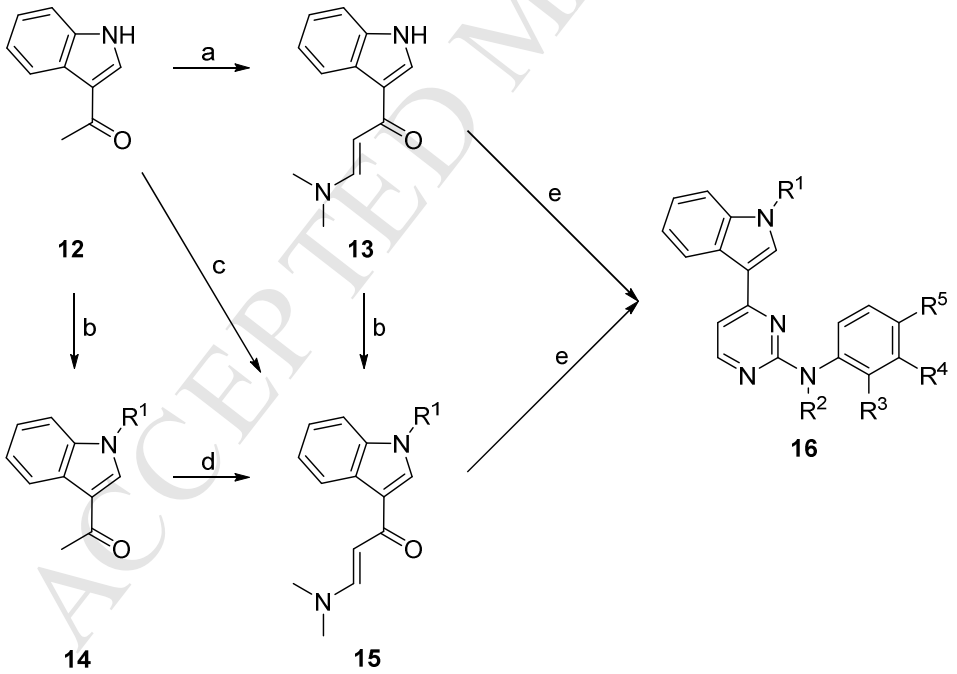
Derivatives of *N*-(2,4-dimethoxyphenyl)-4-(4-methylthiazol-5-yl)pyrimidin-2-amine **11a-11q** were obtained following condensation reactions between each of enaminones **6**, **8** and **10** and 1-(2,4-dimethoxyphenyl)guanidine (**Scheme 1**). A five step synthesis to enaminones **6** included: (i) cyclisation reaction of 3-chloropentane-2,4-dione **1** and thiourea to afford 1-(2-amino-4-methylthiazol-5-yl)ethan-1-one **2**; (ii) bromination of the latter to yield **3**; (iii) treatment of **3** with thiourea to give its 2-thioxo derivative **4**;[20] (iv) alkylation of **4** in the presence of alkyl halides to produce **5**; (v) treatment of **6** with DMF-DMA. The synthesis of enaminones **8** began with a nucleophilic substitution of the bromine of **3** with an alkoxy group to give **10,** followed by treatment with DMF-DMA. Analogues **10a-10b** were prepared as described previously.[21]



Scheme 1: Synthesis of *N*-(2,4-dimethoxyphenyl)-4-(4-methylthiazol-5-yl)pyrimidin-2-amine. *Reagents and conditions*: (a) thiourea or 1-methylthiourea, pyridine, MeOH, rt, 4 h, 73-79%; (b) $NaNO_2$, 32% HBr, H_2O, 0 ºC, 2 h, 77%; (c) thiourea, EtOH, reflux, 4 h, 86%; (d)

alkyl halide, K_2CO_3, DMF, rt, 1-24 h, 43-96%; (e) DMF-DMA, microwave 200-300 W, 150 ºC, 30 min, 22-88%; (f) 1-(2,4-dimethoxyphenyl)guanidine, NaOH, MeCN, microwave 200-300 W, 160 ºC, 30 min, 8-40%, (g) MeONa, anhydrous MeOH, 50 °C, 2 h, 62% for **8a**, EtONa, anhydrous EtOH, 50 °C, 2 h, 33% for **8b**, NaH, butan-1-ol, rt, 16 h, 93% for **8c**, EtONa, *i*-PrOH, 50 °C, 4 h, 34% for **8d**, EtONa, 2-methoxyethan-1-ol, 50 °C, 4 h, 33% for **8e**; (h) DMF-DMA, reflux, 36 h, 26% for **10a**.

Preparation of 4-(indol-3-yl)-*N*-phenylpyrimidin-2-amines, *i.e.* **16a-16n**, mirrored the aforementioned synthetic procedure, where the key intermediates **13** and **15** served as synthons for the construction of the pyrimidines (**Scheme 2**). Treatment of 1-(1*H*-indol-3-yl)ethan-1-one **12** with Bredereck's reagent or DMF-DMA afforded the corresponding **13** or **15a**. The synthesis of **15b**-1**5g** was accessible through two ways. The first involved the alkylation of **13** with the desired alkyl halides; thus **15b** and **15c** were obtained by treating **13** with EtI and PrI, respectively. Due to the costly Bredereck's reagent, alternative route was adopted for **15d**-**15g** *via* alkylation of **12** with alkyl halides to afford **14a**-**14d**, followed by their treatments with DMF-DMA.



Scheme 2: Synthesis of 4-(indol-3-yl)-*N*-phenylpyrimidin-2-amines. *Reagents and conditions*: (a) Bredereck's reagent, reflux, 7 h, 71%; (b) alkyl halide, K_2CO_3, DMF, 50 ºC, 12-24 h, 35-82%; (c) DMF-DMA, reflux, o/n, 76% for 1**5a**; (d) DMF-DMA, microwave 200-300 W, 150 ºC, 30 min, 30-70%; (e) corresponding phenylguanidine , NaOH, MeCN, microwave 200-300 W, 160 ºC, 1 h, 7-35%.

The compound screening cascade has begun with 72 h cell viability assays to determine the activity in MV4-11 AML cell line. FLT3-driven MV4-11 cell line was selected for two reasons: (i) compounds with high anti-proliferative activity against MV4-11 cells would likely be potent FLT3 inhibitors, and (ii) Mnk inhibitors were also most cytotoxic in MV4-11 cells.[18, 22] All compounds were evaluated for their inhibition of Mnks using our IMAP-based assay for biochemical pre-screening, followed by dose-response assessment using the ADP-Glo™ assay[23] for those which achieved > 40% inhibition of Mnks at 10 µM. On the other hand, only the compounds that exerted a cellular anti-proliferative activity with GI_{50} < 1 µM were subjected to enzymatic assays to assess their inhibition of FLT3; a similar approach was adopted by Li *et al.* in their program to identify FLT3 inhibitors.[24] Compounds inhibiting either Mnks or FLT3 were further evaluated for their inhibition of CDK2 and CDK9 (**Table S1**), as *N*-phenylpyrimidin-2-amine derivatives were previously explored as CDK inhibitors.

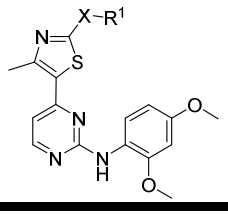
In the context of the *N*-(2,4-dimethoxyphenyl)-4-(4-methylthiazol-5-yl)pyrimidin-2-amine system, an array of substitutions at thiazole-C2 were initially assessed for their impacts on Mnks and FLT3 inhibition; results are summarised in **Table 1**. Compounds bearing alkyl functionalities at R^1 were generally inactive towards Mnks and FLT3, except **11m** and **11q**. For instance, **11a-11c** and **11k-11l,** reduced the activity of Mnks by less than 60%; considered a low inhibitory effect. Among the five compounds, only **11a** exerted a potent anti-proliferative effect with a GI_{50} of 0.53 µM and was subsequently evaluated for its inhibitory activity against FLT3. Nevertheless, its K_i value against FLT3 was > 10 µM, suggesting its off-target cytotoxicity. Derivatives **11b**-**11c** and **11k**-**11l** also displayed weak inhibition of FLT3, as attested by their moderate anti-proliferative effect against MV4-11 cells (GI_{50} = 1.40-32.4 µM). Surprisingly, **11m** (R^1 = Bu) resulted in an improved inhibition of Mnk2 (K_i = 0.56 µM); probably due to favourable hydrophobic interactions with the Mnk2 protein. Analogues **11d** and **11n** (R^1 = *i*-Pr) or **11e** and **11o** (R^1 = CH_2CH_2OMe) exhibited intermediate inhibitory activity against Mnk2 with K_i values in the range of 0.86-1.70 µM. 2-(Alkylthio)thiazole **11e** seemed to be a better inhibitor of FLT3 compared to its 2-alkoxythiazole counterpart **11o**. Derivatives **11e** (R^1 = CH_2CH_2OMe) and **11f** (R^1 = $CH_2CH_2NMe_2$) inhibited FLT3 potently with K_i = 0.064 and 0.007 µM, respectively. **11f** was a potent Mnk2 inhibitor with K_i = 0.23 µM and exhibited a strong anti-proliferative effect with GI_{50} = 0.09 µM. On the other hand, **11g**, where R^1 = $CH_2(CH_2)_2N(Me)_2$, showed five- and four-fold reduced activity against Mnk2 (K_i = 1.15 µM) and FLT3 (K_i = 0.03 µM) respectively compared to **11f**, which was translated into a lower anti-proliferative effect (GI_{50}

= 0.37 μM). Introduction of R^1 = 3-CH_2-pyridine at R^1, *i.e.* **11h**, restored Mnk2 inhibition (K_i = 0.40 μM) but was two-fold less effective against FLT3. Nevertheless, **11h** retained the cellular potency (GI_{50} = 0.27 μM). Incorporation of bulkier groups at R^1 resulting in **11i**, where R^1 = CH_2CH_2-piperazine, and **11j**, where R^1 = CH_2CH_2-morpholine, has dampened the inhibitory effect for Mnk2 by three- and six-fold respectively, compared to **11h**. Analogues **11i** and **11j** were potent inhibitors of FLT3 with K_i = 0.10 and 0.093 μM, respectively, and exhibited potent anti-proliferative activity (GI_{50} = 0.50 and 0.59 μM, respectively). Overall, these compounds exhibited a great selectivity for Mnk2 over Mnk1; only **11n** showing a moderate activity against Mnk1 with a K_i of 1.23 μM.

N-Methylthiazol-2-amine containing analogues, *i.e.* **11p** (R^1 = H) and **11q** (R^1 = Me), were also potent inhibitors of Mnk2 (K_i = 0.11 and 0.20 μM, respectively) and exhibited a nanomolar inhibition against FLT3, with **11q** (K_i = 0.005 μM) being six-fold more potent than **11p** (K_i = 0.032 μM). **11p** and **11q** showed a moderate inhibition of Mnk1 with a K_i = 1.47 and 0.86 μM, respectively, and exerted a potent anti-proliferative activity with respective GI_{50} values of 0.30 and 0.17 μM.

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Table 1: Structure and biological activities of compounds **11**.



Cmpd	X	R^1	Kinase inhibition[a]					72 h cytotoxicity GI_{50} (μM)[b], MV4-11
			Activity remaining (%) at 10 μM		K_i (μM)			
			Mnk1	Mnk2	Mnk1	Mnk2	FLT3	
11a	S	Me	94	46	-	-	> 10	0.53 ± 0.12
11b	S	Et	95	82	-	-	-	32.4 ± 7.28
11c	S	Pr	92	93	-	-	-	2.24 ± 1.08
11d	S	*i*-Pr	55	9	-	1.30	-	5.67 ± 1.37
11e	S	CH_2CH_2OMe	86	39	-	1.70	0.064	0.81 ± 0.23
11f	S	$CH_2CH_2N(Me)_2$	35	7	5.92	0.23	0.007	0.09 ± 0.01
11g	S	$CH_2CH_2CH_2N(Me)_2$	60	16	-	1.15	0.030	0.37 ± 0.05
11h	S	3-CH_2-pyridine	62	14	-	0.40	0.060	0.27 ± 0.02
11i	S	CH_2CH_2piperazine	69	25	-	1.33	0.10	0.50 ± 0.10
11j	S	CH_2CH_2morpholine	87	22	-	2.50	0.093	0.59 ± 0.27
11k	O	Me	80	56	-	-	-	1.40 ± 0.66
11l	O	Et	96	47	-	-	-	3.89 ± 0.40
11m	O	Bu	92	6	-	0.56	-	7.86 ± 0.40
11n	O	*i*-Pr	28	3	1.23	0.98	-	8.69 ± 1.90
11o	O	CH_2CH_2OMe	47	24	-	0.86	-	1.98 ± 0.32
11p	NMe	H	30	7	1.47	0.11	0.032	0.30 ± 0.06
11q	NMe	Me	19	16	0.86	0.20	0.005	0.17 ± 0.07

For Mnk1 and Mnk2, the percentages of remaining activity and K_i values were obtained by in-house IMAP and ATP-Glo™ assays, respectively. K_i values against FLT3 were obtained from Reaction Biology Corp. The percentages of remaining activity are mean values of two replicates. [b] Cytotoxicity was determined by 72 h resazurin assay. The data given are mean values derived from at least two replicates ± SD.

The 4-(indol-3-yl)-*N*-phenylpyrimidin-2-amines, *i.e.* **16a**, **16b**, **16d**-**16h**, exhibited a similar range of activity for Mnk2 (K_i = 0.18-0.39 μM) irrespective of the R^1 substitutions (**Table 2**). Nevertheless, some derivatives have reinstated inhibition against Mnk1, *e.g.* **16b** (R^1 = Me) and **16f** (R^1 = *i*-Pr) had respective K_i values of 0.94 and 0.71 μM. With regard to cellular activity, **16a** exhibited a GI_{50} < 1 μM, thus its K_i value against FLT3 was determined to be 0.34 μM; others were less effective anti-proliferative agents (*i.e.* GI_{50} ~ 1.74-6.65 μM). *N*-

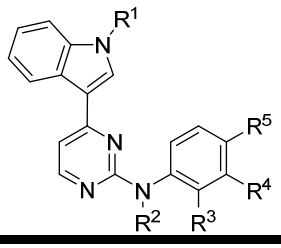
Methylation at C2 of the pyrimidine of **16b**, as in **16c**, abolished the activity for Mnks (K_i values > 10 μM), suggesting that the hydrogen donor ability of the 2C-NH of pyrimidine is vital for interacting to Mnk proteins.

Our previous results have shown that substitutions on the phenyl ring of 5-(2-(phenylamino)pyrimidin-4-yl)thiazol-2(3*H*)-ones were critical for potency towards Mnk2 and selectivity over CDKs; *ortho*-substituents, including methyl, ethyl, methoxy and difluoromethoxy, were the most preferred, whereas the concurrent presence of methoxy groups on the *ortho*- and *para*-positions of the phenyl ring resulted in greater selectivity over CDKs. [18] Herein, the repercussions of various substitutions at the aniline system were examined in the context of 4-(indol-3-yl)pyrimidin-2-amines. Derivatives **16j**-**16l** lost their inhibitory effects towards Mnk2 and Mnk1, and remained virtually ineffective against FLT3. Analogue **16m** (R^1 = CH_2CH_2OMe, R^3 = $OCHF_2$) has curtailed the inhibition of Mnk2 by eight-fold compared to **16i**, and abolished Mnk1 inhibition, whereas **16n**, with R^1= H and R^3 = $OCHF_2$, was ineffective against both Mnks. Derivative **16o** (R^1 = H, R^3 = OH) reinstated Mnk1 inhibition (K_i = 0.89 μM) and was a better inhibitor of Mnk2 (K_i = 0.12 μM) and FLT3 (K_i = 0.063 μM), compared to **16n**.

As some derivatives from the chemical series have been known to target CDK9 and CDK2, the selectivity of inhibitors were screened in CDK2 and CDK9 kinase assays (**Table S1**). None of them inhibited CDK9, but **11f**, **11i**, **11n**, **11q** and **16f** showed > 70% inhibition of CDK2 at 10 μM.

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Table 2: Structure and biological activities of compounds **16**.



Cmpd	R^1	R^2	R^3	R^4	R^5	Kinase inhibition[a] Activity remaining (%) at 10 µM		Kinase inhibition[a] K_i (µM)			72 h cytotoxicity, GI_{50} (µM)[b], MV4-11
						Mnk1	Mnk2	Mnk1	Mnk2	FLT3	
16a	H	H	OMe	H	OMe	30	3	4.77	0.11	0.34	0.60 ± 0.10
16b	Me	H	OMe	H	OMe	8	20	0.94	0.61	-	3.41 ± 0.69
16c	Me	Me	OMe	H	OMe	102	98	-	-	-	2.00 ± 0.37
16d	Et	H	OMe	H	OMe	91	19	-	0.43	-	3.59 ± 0.61
16e	Pr	H	OMe	H	OMe	34	19	1.77	0.43	-	3.62 ± 0.57
16f	*i*-Pr	H	OMe	H	OMe	7	2	0.71	0.22	-	3.25 ± 0.03
16g	CyPn	H	OMe	H	OMe	48	6	-	0.39	-	1.74 ± 0.02
16h	CH_2CH_2 morpholine	H	OMe	H	OMe	44	5	-	0.30	-	6.65 ± 1.68
16i	CH_2CH_2OMe	H	OMe	H	OMe	21	4	1.31	0.18	-	3.79 ± 0.59
16j	CH_2CH_2OMe	H	OMe	H	OEt	40	77	-	-	-	3.72 ± 0.31
16k	CH_2CH_2OMe	H	OMe	F	OMe	54	59	-	-	-	3.89 ± 0.39
16l	CH_2CH_2OMe	H	OCF_3	H	OMe	62	45	-	7.70	-	4.89 ± 0.25
16m	CH_2CH_2OMe	H	$OCHF_2$	H	H	60	30	-	1.57	-	3.42 ± 0.25
16n	H	H	$OCHF_2$	H	H	96	96	-	-	-	4.77 ± 0.70
16o	H	H	OH	H	H	6	1	0.89	0.12	0.063	0.57 ± 0.04

For Mnk1 and Mnk2, the percentages of remaining activity and K_i values were obtained by in-house IMAP and ATP-GloTM assays, respectively. K_i values against FLT3 were obtained from Reaction Biology Corp. The percentages of remaining activity are mean values of two replicates. [b] Cytotoxicity was determined by 72 h resazurin assay. The data given are mean values derived from at least two replicates ± SD.

2.2. Biochemical Competition Assays

Despite that Mnk1 and Mnk2 share 78% sequence similarity in their catalytic domains and 90% in their ATP binding sites,[12, 25, 26] most of our compounds were Mnk2 isoform specific inhibitors. Hence, the difference in the binding modes of the inhibitors to Mnk1 and Mnk2 proteins was scrutinised using biochemical competition assays [23]. Being dual inhibitors of Mnk1 and Mnk2, **11q**, **16f**, **16i** and **16o** were subjected to kinase assays in the presence of

increasing concentrations of ATP or 4E-related peptides, and IC_{50} ratios (ratios of IC_{50}s between the highest and lowest ATP concentrations) were calculated (**Figure 1**). Compounds retaining a ratio > 5 were considered to be competitive. Competition assays of **16a** were performed for Mnk2 only, as it does not inhibit Mnk1 potently.

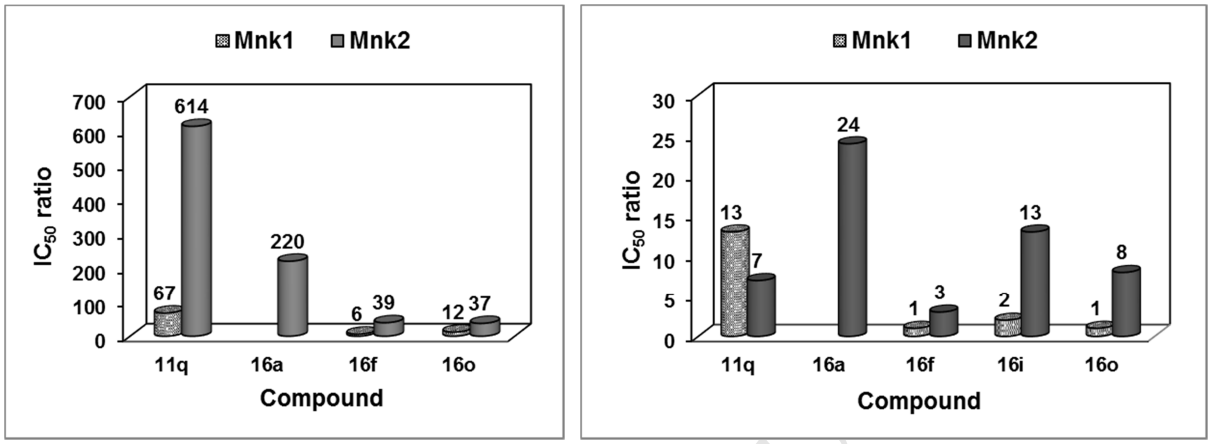


Figure 1: Competition studies on **11q**, **16a**, **16f**, **16i** and **16o** using the ADP-Glo™ assay. (A) ATP competition assays were performed in the presence of ATP at 0.15, 1 and 6 mM for Mnk1 and at 0.05, 0.2 and 2 mM for Mnk2. (B) Substrate competition assays were performed in the presence of three different concentrations of eIF4E-derived peptide: 0.15, 0.6 and 3 mM for Mnk1 and for Mnk2. IC_{50} ratios represent the ratios of IC_{50}s between the highest and lowest ATP concentrations. Only the Mnk2 competition data are presented for **16a**; it was not a potent inhibitor of Mnk1.

Mnk1 ATP competition assays showed that IC_{50} values increased by 67-fold, six-fold and 12-fold for **11q**, **16f** and **16o**, respectively, when the ATP concentration was increased by 40-fold. It is remarkable that the ATP-competitive effect of **11q**, the only compound bearing a thiazole at pyrimidine-C4, was more significant than those of **16f** and **16o**. Results from Mnk2 ATP competition assays were aligned with those obtained with the Mnk1 protein. All compounds were ATP-competitive inhibitors of Mnk2. Compound **11q** remained the most competitive with its inhibitory activity decreasing 614-times with a 40-fold increase in ATP concentration. IC_{50} ratios of **16a**, **16f** and **16o** were 220, 39 and 37, respectively.

Substrate competition assays were performed in the presence of eIF4E-derived peptide at three different concentrations (0.15, 0.6 and 3 mM) for Mnk1 and Mnk2. Derivative **11q** was the only inhibitor to show substrate competition for Mnk1 (IC_{50} ratio = 13), and the IC_{50}s of other compounds, *i.e.* **16f**, **16i** and **16o**, remained almost unaffected by the increase in the

substrate concentration. On the other hand, most of the compounds showed a substrate-competitive effect towards Mnk2; the only exception was **16f** that had an IC_{50} ratio = 3.

2.3. Cellular Mechanistic Studies

The compounds could be divided into four main categories: selective FLT3 inhibitors, selective Mnk inhibitors, dual inhibitors of Mnks and FLT3, and non-inhibitors of FLT3 and Mnks. The difference in their kinase profiles was exploited to examine the importance of dual inhibition of Mnk2 and FLT3 compared to the sole inhibition of either one of them. **11j** was selected as a FLT3 inhibitor, **16a** as a dual inhibitor and **16h** as a selective Mnk2 inhibitor (**Figure 2**). They were nominated for two reasons: (i) the sole and the dual inhibitor should exert a similar range of inhibition of each kinase in order to compare their effects on cancer cells. Otherwise, it would be unclear whether the observed effects stem from the potent inhibition of one kinase or from the simultaneous inhibition of both kinases; (ii) the selected compounds exhibited an appreciable selectivity over CDK2.

The designated compounds were screened against a kinase panel that includes B-raf, MAPKs, p38α, PI3K, Akt, and mTOR, being integral components of the examined signalling pathways (**Figure 2**). All kinases retained activity in the presence of the three compounds of greater than 40%, except for FLT1 which was 80% and 92% inhibited with 10 μM of **11j** and **16a**, respectively.

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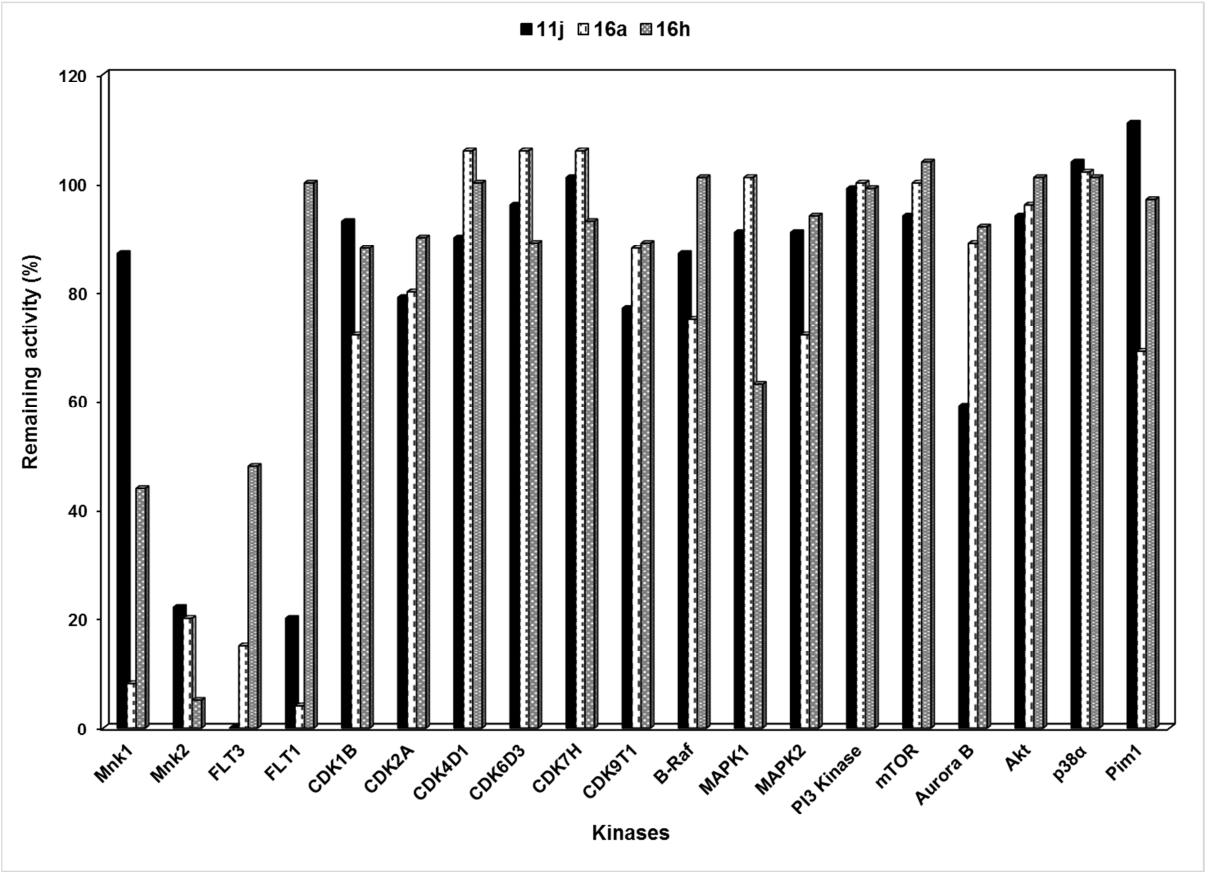


Figure 2: Selectivity of **11j**, **16a** and **16h** against a panel of kinases. Compounds were tested at the concentration of 10 μM.

In order to gauge the outcomes of simultaneous inhibition of FLT3 and Mnk2 on progression of the cell cycle, MV4-cells were exposed to **11j** and **16a** for 48 h, and analysed by flow cytometry (**Figure 3**). Both compounds arrested the cells in the G1 phase of the cell cycle in a dose-dependent manner. This was companied by an increase in the sub-G1 cells, suggesting cell death. The treatment with **11j** resulted in an increase in G1 and sub-G1 cells to 70, 75 and 79% at the drug concentrations of two, four and eight times its GI_{50}, respectively, compared to 64% in untreated cells. The same treatment with **16a** caused greater alterations of cell distribution with 73, 74 and 82% DNA content in the G1/sub-G1, respectively.

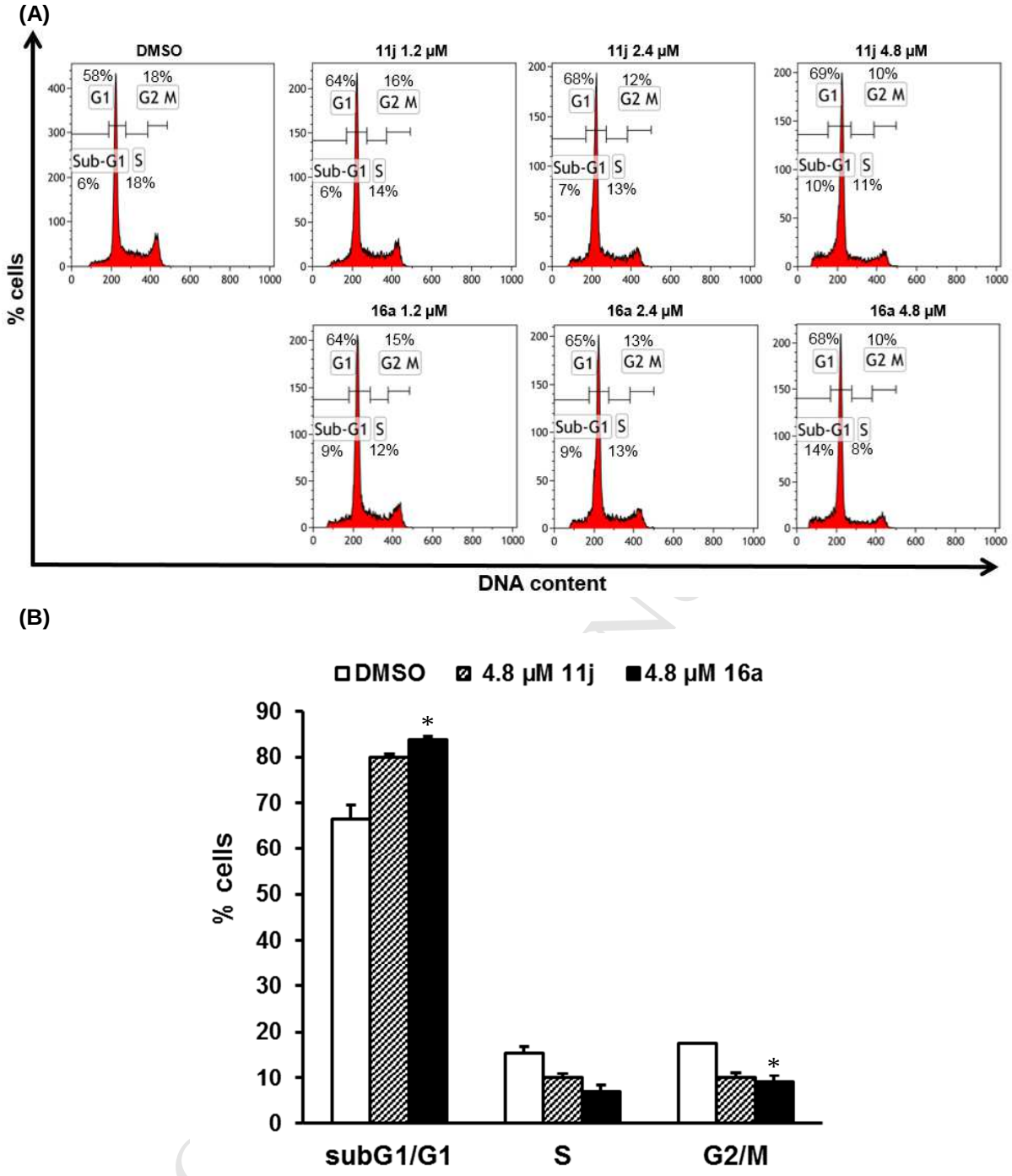
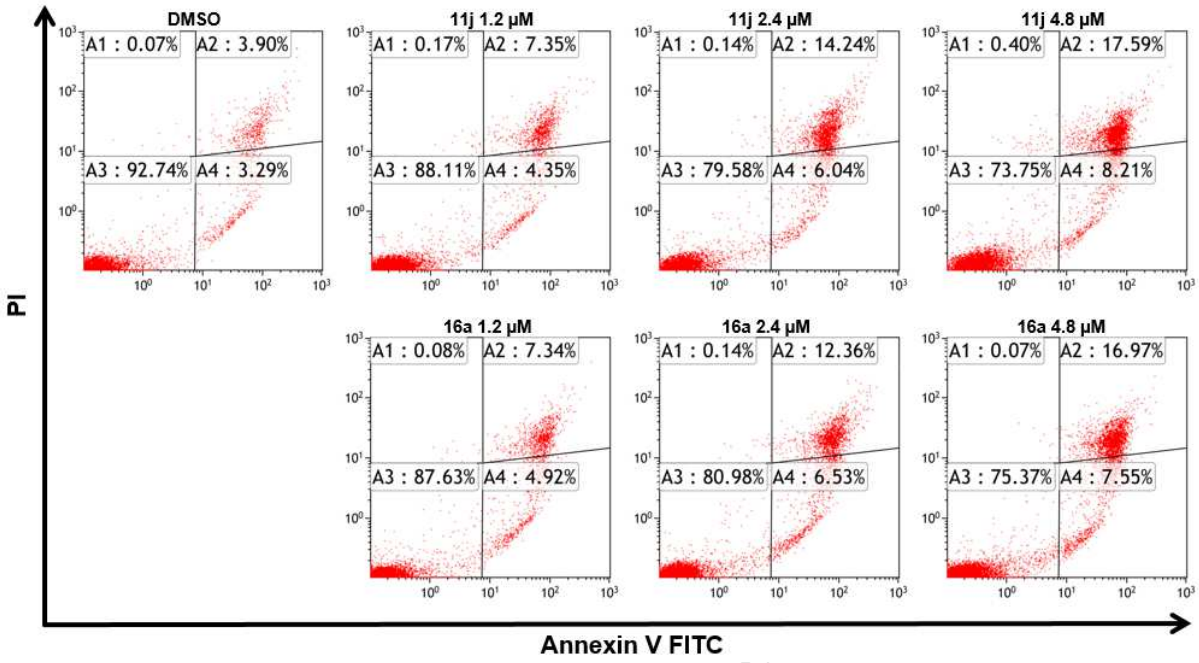


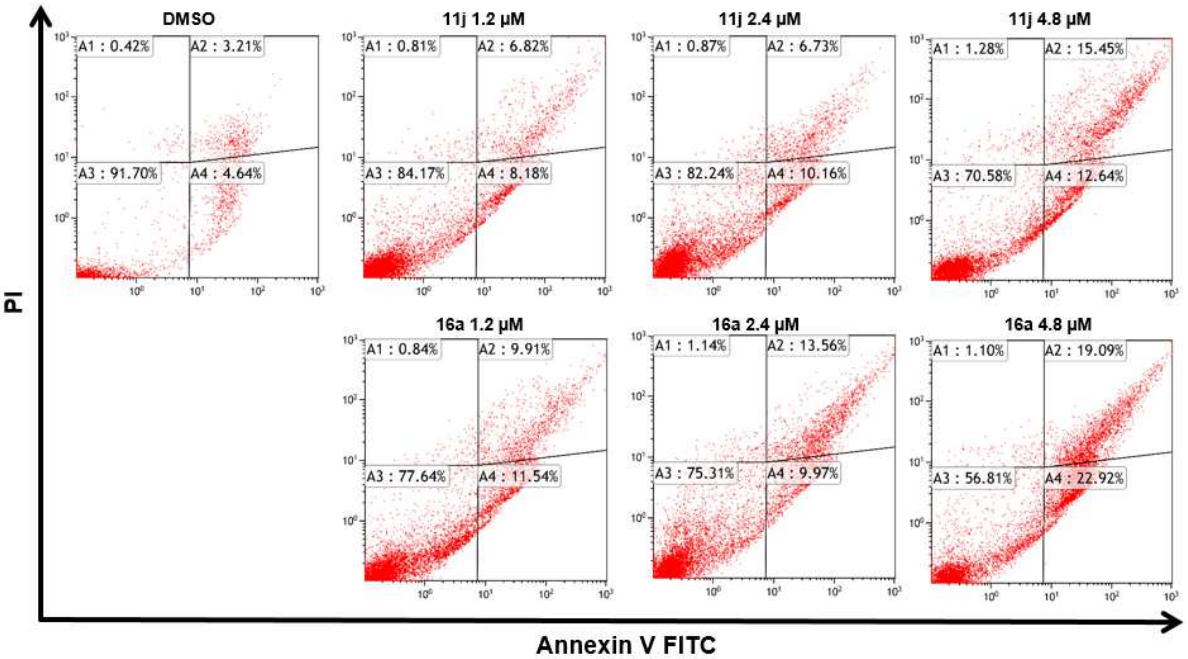
Figure 3: (**A**) Cell cycle analysis of MV4-11 cells after treatment with **11j** and **16a** for 48 h. The diagrams selected from the results of at least two independent repeats. (**B**) Quantitative analysis of cell cycle distribution: * indicates $p \leq 0.05$ when compared to the DMSO-treated cells.

Furthermore, the apoptotic effects of **11j** and **16a** were assessed using Annexin V/PI assay on 48 h (**Figure 4A**) and 72 h (**Figure 4B**) post treatment of the MV4-11 cells. Both **11j** and **16a** were capable of inducing apoptotic cell death, ranging from 12% to 25% at the drug

concentrations shown in a time-dependent manner. After 72 h of exposure to MV4-11 cells, **16a** increased 14% apoptotic cells when compared to **11j (Figure 4C)**.



(B)



(C)

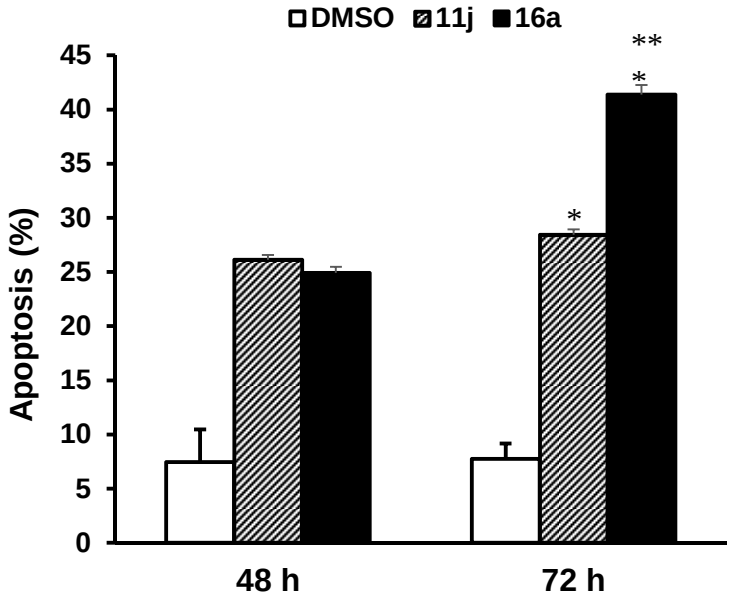


Figure 4: Apoptosis of MV4-11 cells induced by exposure to **11j** and **16a** for 48 h (**A**) and 72 h (**B**) by annexin V/PI assay. The figures were selected from the results of at least two independent experiments. (**C**) Quantitative analysis of cells undergoing apoptosis, defined as the sum of early apoptotic (annexin V^+/PI^-) and late apoptotic (annexin V^+/PI^+). * indicates $p \leq 0.05$ compared to the DMSO-treated cells; ** indicates $p \leq 0.05$ when compared to the **11j**-treated cells.

To investigate the cellular mechanism of action, western blotting analysis was performed on MV4-11 cells following exposure to **11j**, **16a** and **16h** for 1 h and 24 h (**Figures 5**). In the 1 h post-treated cells, **11j** reduced the level of phosphorylated STAT5 at the tyrosine 694, *i.e.* p-STAT5(Y694), a well-validated downstream effector of FLT3, in a dose-dependent manner (**Figure 5A**). **11j** showed little effect on Erk, p38 and eIF4E, thereby confirming its cellular selectivity for FLT3.On the other hand, **16a** was capable of reducing the levels of p-STAT5 (Y694) and p-eIF4E (S209), evidencing its dual inhibition of FLT3 and Mnk2. **16a** did not affect the level of p-Erk(T202/Y204) but decreased the p-p38(T180/Y182). **16h** inhibited cellular Mnk2, *i.e.* p-eIF4E(S209), as well p-STAT5(Y694) despite not showing any inhibition against FLT3 in kinase assay, likely *via* an off-target effect due to the very high concentrations used in the study (*i.e.* 6.5 and 26 μM).

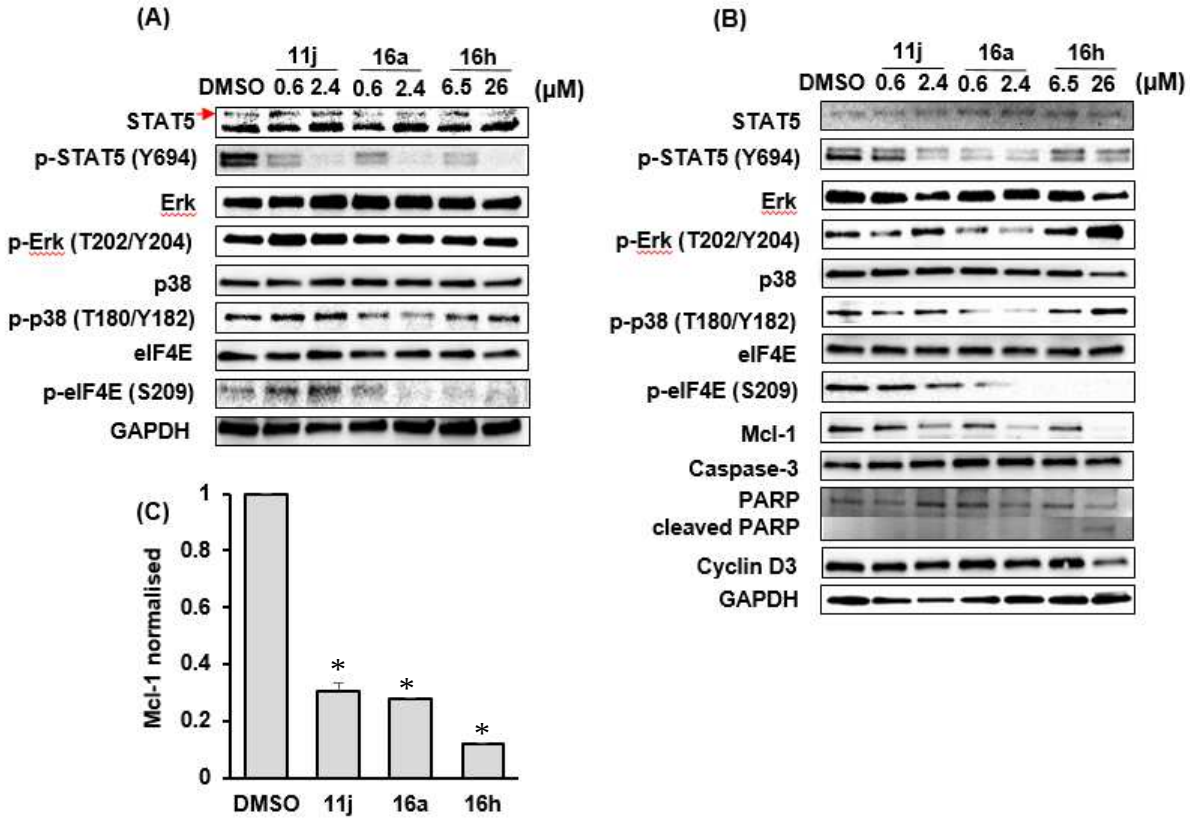


Figure 5: Western blot analysis of MV4-11 cells treated with **11j**, **16a** and **16h** respectively for a period of 1 h (**A**) and 24 h (**B**). GADPH antibody was used as an internal control. DMSO diluent was used as a control in each experiment. The blots present the results of at least two independent experiment. (**C**) The protein level of Mcl-1 in MV4-11 cells 24 h-post treatment with **11j**, **16a** and **16h** at 2.4 µM, 2.4 µM and 26 µM, respectively; $*p \leq 0.05$ compared to the DMSO-treated cells.

Western blots of MV4-11 cells exposed to these compounds for 24 h revealed similar targeting profiles (**Figure 5B**). While **11j** inhibited FLT3 by down-regulating the p-STAT5 (Y694), **16a** reduced phosphorylated STAT5, Erk, p38 and eIF4E. **16h** showed pronounced inhibition of eIF4E phosphorylation, but an upregulation of p-Erk was detected at higher concentrations of **16h**.

We next examined the induction of apoptosis in MV4-11 (**Figure 5B**). A dose-dependent downregulation of pro-survival Mcl-1 protein was observed for all three compounds; dual Mnk2/FLT3 inhibitor **16a** reduced levels of Mcl-1 (also **Figure 5C**) and PARP more than the FLT3 inhibitor **11j**, suggesting an additive effect of **16a**. Likewise, **16h** reduced Mcl-1 at 26 µM. Caspase 3 in this cell line was not affected by all these inhibitors. In agreement with previous studies stating that Mnk inhibitors disturb the transition of cells from the G1 to S

phase through downregulation of cyclin-D3,[15, 27] the levels of cyclin D3 were reduced by **16h**, but were not affected by **16a** and **11j**.

3. Discussion and Conclusions

Having the lowest survival rate of all adult leukaemias, AML remains a worldwide burden. Hence, new anti-AML pharmacological agents are urgently needed. Midostaurin has recently received a breakthrough therapy designation for newly-diagnosed FLT3-mutated AMLs, thereby validating FLT3 as an anti-AML therapeutic target. However, the emerging resistance to monotherapy with FLT3 inhibitors in clinics has fuelled a deviation from the paradigm of targeting an individual genetic alteration to a focus on multiple oncogenic drivers. Indeed, several combination therapies have been evaluated, including dual FLT3 and CDK4 inhibitors, concomitant blockade of Akt and FLT3, simultaneous inhibition of FLT3 and Erk, along with a combination of FLT3 inhibitors with chemotherapy.[28, 29, 30] Herein, the development of dual inhibitors of FLT3 and Mnks as an anti-AML therapeutic option is presented.

The development of dual inhibitors of FLT3 and Mnks was based on the *N*-(2,4-dimethoxyphenyl)-pyrimidin-2-amine scaffold, with compounds bearing either a thiazole (**11**) or an indole system (**16**) at C4 position of pyrimidine. Compounds **11p**-**11q** were the most potent Mnk inhibitors. The hydrogen donor at the 2-aminothiazole of **11p** did not contribute to its superior inhibitory activity since its N-methylated analogue, **11q**, remained effective in inhibiting Mnks. Plausible explanations for the greater potency of **11q** compared to **11a** and **11k** could be either that the binding pose of **11q** to Mnk proteins could be directing the substitution at C2 of thiazole towards a negatively charged residue, allowing favourable electrostatic interactions of **11p** or **11q** with Mnk proteins, or the reduced planarity of **11a** and **11k**. Noteworthy, the 2-(alkylthio)thiazole containing analogues were generally less potent against Mnk2 compared to their 2-alkoxythiazole counterparts, presumably due to the larger size of sulfur atom. Derivatives **11p**-**11q** were also potent inhibitors of FLT3, as were **11e**-**11j** that bore polar thiol substitutions at C2 of the thiazole. The 2-alkoxythiazole containing derivatives, **11k**-**11o**, virtually failed to inhibit FLT3.

Replacing the thiazole with an indole ring at the C4 position of pyrimidine had a detrimental effect on FLT3 inhibition. On the contrary, the indole system was well accommodated in Mnk2, and various substitutions at R^1 of **16** were well tolerated for Mnk2 inhibition. Results

from the kinase competition assays suggest that the functionality grafted at R^1 is slightly altering the binding modes of the compounds. Data of **16a** and **16f** suggest that the hydrogen donor ability of 1*H*-indole, as in **16a**, favours the interaction with the ATP binding site, but overall, is superfluous for enhancing the binding affinity of the inhibitors to Mnk2. Also, **16f** (R^1 = *i*-Pr) was six-fold less ATP competitor than **16a** and did not show competition with the substrate. It remains unclear whether this is due to the hydrophobic nature of the isopropyl group, especially that **16i** ($R^1 = CH_2CH_2OCH_3$) restored a substrate-competitive effect. The substitutions incorporated on the aniline system have also affected the binding pose of the compounds to Mnk2. Despite **16a** and **16o** showing similar K_i values, the ATP- and the substrate-competitive effects of **16a** were more pronounced.

For Mnk1, the data derived from SAR analysis and competition assays suggest that inhibition of Mnk1 depends mainly on the system incorporated at pyridimine-C4, with thiazol-2-amines and indoles being the most favourable. Notably, **11q** was ATP- and substrate-competitive inhibitor of Mnk1 whereas indole derivatives were ATP-competitive only, which could imply that the binding mode of **11q** differs from that of analogues **16**. This hypothesis is borne out by that fact that **11q** was a six-fold and 11-fold superior ATP-competitor than **16o** and **16f**, respectively. In contrast to Mnk2, introducing bulkier group at R^1 of **16**, as in **16g** and **16h**, reduced Mnk1 inhibition. A comparison of the competitive effects on Mnk1 and Mnk2 suggests that **16i** and **16o** are binding differently to these two kinases (**Figure 1B**). It seems that these two compounds are engaging with residues lining the ATP-binding site in both kinases, but interacting with residues of the substrate-binding site only in the Mnk2 protein.

Overall, derivatives **11** were more potent inhibitors for FLT3 over Mnks, with the opposite being true for **16**. The cytotoxic effect against MV4-11 cells rather associated with the inhibition of FLT3, which was expected, given that MV4-11 cells are known to have high expression level of FLT3, and thus high sensitivity to FLT3 inhibition. A concomitant inhibition of Mnks and FLT3 was not always translated into a better growth inhibition of MV4-11 cells. This result is conceivable given that a micromolar inhibition of Mnks was not always reflected on the cellular proliferation. A selective inhibition against Mnks in MV4-11 cells has mostly resulted in GI_{50}s greater than 1 µM. For instance, despite being a potent inhibitor of Mnk2 (K_i = 0.22 µM) and Mnk1 (K_i = 0.71 µM), **16f** caused the moderate growth inhibition with GI_{50} = 3.25 µM

Three compounds, **11j** as a FLT3 inhibitor, **16a** as a dual inhibitor and **16h** as a Mnk2 inhibitor, were screened against a panel of kinases followed by elucidation of cellular mechanisms of action. Compounds were devoid of targeting other kinases, except **11j** and **16a** which inhibited FLT1 moderately (80% and 91% at 10 μM). **11j** and **16a** caused the accumulation of the G1 and sub-G1 cells, in line with previous studies showing that FLT3 inhibitors, *e.g.* midostaurin, and Mnk inhibitors, *e.g.* CGP57380 and MnkI-19, arrested cells in the G1 phase and induced cell death at the sub-G1 phase of the cell cycle.[13, 15, 18, 31] While the effect of **16a** on cell cycle progression was only marginally superior to that of **11j**, the anti-apoptotic effect of the former was significantly greater than that of **11j**. The additive anti-AML effect of **16a**, a dual inhibitor of Mnk2 and FLT3, is remarkable given that its K_i of 0.34 μM indicated about a three-fold lesser inhibition of FLT3 than **11j** (K_i = 0.093 μM). It is supposed that the lower potency of **16a** to inhibit FLT3 was compensated for by the concurrent suppression of Mnk2 activity (K_i = 0.11 μM), causing **16a** to show more effective anti-cancer activity.

Cellular mechanistic studies confirmed that **11j** targeted FLT3 with minimal effect on Mnk2, while **16a** inhibited both FLT3 and Mnk2. **16h** suppressed eIF4E phosphorylation, confirming cellular inhibition of Mnks. It also reduced slightly the level of p-STAT5, likely due to an off-target effect of **16h**. STAT5 is a key signalling molecule and can be phosphorylated by various kinases, including Janus kinase 2 (JAK2), Bcr-Abl, and Src family kinases (SFKs).[32, 33, 34, 35] This off-target effect could also explain the significant reduction of Mcl-1 and cleavage of PARP by the high concentrations of **16h**, *i.e.* 26 μM. Previous studies have demonstrated that JAK2 inhibitors block STAT3 activation, thereby inducing apoptosis through downregulation of Mcl-1 and cleavage of PARP.[36, 37] Interestingly, **16h** upregulated p-Erk; such an effect was previously reported upon exposure of K562 cells to CGP57380.[27] Likewise, the level of p-Erk was induced upon treatment of MV4-11 cells with MnkI-19.[15] It was suggested that inhibition of Mnks triggers a negative-feedback loop that activates Erks. Notably, such an effect was not observed when inhibiting Mnk2 and FLT3 simultaneously with **16a**.

Compound **16a** reduced the phosphorylation of p38. Studies have shown that phosphorylation of p38 in human monocytes and in HUVECs was activated upon stimulation of FLT1 by a vascular endothelial growth factor (VEGF) or a placental growth factor (PIGF), suggesting that inhibition of FLT1 by **16a** is responsible for diminished levels of phosphorylated p38.[38, 39, 40] Nevertheless, a recent study has demonstrated that silencing of FLT1 by shRNA in LN-

208 glioma cells augmented the levels of phosphorylation of p38 and Erks,[41] indicating a FLT1-dependent activation of p38 by VEGF and PIGF. Thus, it remains unclear whether the downregulation of phosphorylated p38 is due to targeting FLT1 or to the dual inhibition of FLT3 and Mnk2, especially given that **11j** did not affect the levels of p-p38 despite targeting FLT1. Most importantly, a dose-dependent downregulation of Mcl-1 was observed upon treatment of MV4-11 cells with the inhibitors with the effect of **16a** being more pronounced than that of **11j**, suggesting the additive effect of inhibiting Mnk2 and FLT3.

The treatment of AML has remained unaltered for the last 25 years, and there is a pressing need for innovative therapies for patients. Herein, we present the development of dual FLT3 and Mnk2 inhibitors as a new approach that may hold promise as an anti-AML strategy. The combination has shown an additive effect, most probably through induction of apoptosis and downregulation of the pro-survival Mcl-1 protein which has been implicated in the resistance to FLT3 inhibitor monotherapy. Such a combination can be valuable for treatment of patients with AML, especially those unable to endure the side-effects of chemotherapeutic agents.

4. Experimental Section

4.1. Chemistry

Derivatives **11** and **16** were synthesised as previously described[21] *via* condensation reaction between enaminones **6**, **8**, **10**, **13** or **15** and the corresponding guanidine. The structural characterisation of these compounds is provided as supplemental information.

4.2. Kinase assays

The immobilised metal ion affinity particle (IMAP) time-resolved fluorescence resonance energy transfer (TR-FRET) screening kit was obtained from Molecular Devices (CA, USA). The ADP-Glo™ kit was obtained from Promega Corporation (Madison, USA). Screening was performed using recombinant GST-Mnk1/2 and His-Mnk2, purchased from Life Technologies and Merck Millipore, respectively. A specially designed eIF4E-derived peptide substrate comprising the thirteen C-terminal amino acids of human eIF4E (D_{202}TATKSGSTTKNR_{214}) was obtained from Mimotopes Pty. Ltd. (Victoria, Australia). With the IMAP technology, the peptide eIF4E202-214 was N-terminally linked with tetramethylrhodamine (TAMRA). Testing was performed as described previously.[23]

Residual activities for CDK2, CDK9, and for the tested kinase panel were measured by radiometric assay using Eurofins Scientific service and a concentration of ATP equal to the $K_{m(ATP)}$ for each kinase. Inhibition of FLT3 was determined by Reaction Biology Corp., using a concentration of ATP equal to the $K_{m(ATP)}$ of each kinase. Half-maximal inhibition (IC_{50}) values were calculated from 10-point dose–response curves and apparent inhibition constants (K_i) were calculated from the IC_{50} values and appropriate $K_{m(ATP)}$ values.

4.3. Cell Culture

MV4-11 leukaemic cells were kindly provided by Prof Richard D'Andrea from the University of South Australia, and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) under 5% CO_2 at 37 ^oC.

4.4. Cell Viability Assays

Resazurin (Sigma) assays were performed with the MV4-11 cell line according to the manufacturer's instructions. Cells were seeded at 5×10^3/well into 96-well plates and incubated overnight at 37 ^oC, 5% CO_2. Tested compounds were diluted from a 10 mM stock solution to prepare a five-fold dilution series in cell medium, added to cells (in triplicate), and incubated for 72 h at 37 ^oC, 5% CO_2. Resazurin was made up as a stock of 0.1 mg/mL in cell medium, 20 µL added per well and incubated in the dark at 37 ^oC, 5% CO_2 for 4 h. The plate was left at room temperature for 10-15 min and the fluorescent product quantified using an EnVision multi-label plate reader (PerkinElmer, Buckinghamshire, UK) with a 570 nm (excitation)/585 nm (emission) filter set. Compound concentrations required to inhibit 50% of cell growth (GI_{50}) were calculated from non-linear regression analysis using Graphpad Prism.

4.5. Cell Cycle Analysis and Detection of Apoptosis

MV4-11 (1×10^5) cells were seeded and incubated overnight at 37 ^oC, 5% CO_2. After treatment with each inhibitor, cells were centrifuged ($300 \times g$) for 5 min. Cell pellets were collected, redispersed in and fixed with 70% EtOH on ice for 15 min, followed by centrifugation ($300 \times g$) for 5 min. The collected pellets were incubated with staining solution (50 µg/mL PI, 0.1 mg/mL RNase A, 0.05% Triton X-100) at 37 ^oC for 1 h and analysed with a Gallios flow cytometer (Beckman Coulter). Detection of apoptosis was performed on 1×10^5 cells as described by Annexin-V FITC/PI commercial kit (Becton Dickenson). Samples were analysed with fluorescence-activated cell sorting (FACS) within 1 h of staining. Data

from the cell cycle and apoptosis detection were analysed using Kaluza v1.2 (Beckman Coulter).

4.6. Western Blots

Western blotting was performed as described previously [42]. Antibodies were used to the following proteins: GADPH, eIF4E, phosphorylated-eIF4E (p-eIF4E(S209)), STAT5, phosphorylated-STAT5 (p-STAT5(Y694)), Erk, phosphorylated-Erk (p-Erk (T202/Y204), p38, phosphorylated p38 (p-p38 (T180/Y182)), Mcl-1, caspase 3, PARP, cleaved PARP and cyclin D3 (Cell Signalling Technologies, Danvers, Massachusetts). Both anti-mouse and anti-rabbit immunoglobulin G horseradish peroxidise-conjugated antibodies were obtained from Dako. Enhanced Chemiluminescence reagents were obtained from GE Life Sciences (Buckinghamshire, UK).

5. Acknowledgements

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Highlights

- Discovery of *N*-phenyl-4-(thiazol-5-yl)pyrimidin-2-amine and 4-(indol-3-yl)-*N*-phenylpyrimidin-2-amine derivatives as dual inhibitors of Mnks and FLT3.
- Binding mode of the compounds to Mnks were assessed using competition assays.
- Confirmation of cellular inhibition of Mnks and FLT3 by western blotting.
- Inhibition of Mnk2 and FLT3 downregulated Mcl-1 protein and enhanced anti-AML activity compared to inhibition FLT3 or Mnk2 alone.

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