Development of kinase inhibitors and activity-based probes

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4 Biological evaluation of H-89-analogues: searching for selective AKT1 and FLT3 inhibitors

4.1 Introduction

Protein kinase B (PKB/AKT) is an essential factor in the phosphatidylinositol-3-kinase (PI3K)-PKB signaling pathway. This pathway is altered in many human cancers, to inlcude breast, colorectal, ovarian[1] and prostate cancers[2], and aberrant activation of the PI3K-PKB pathway promotes cell growth and survival, invasion, metastasis and angiogenesis in these tumors. PKB/AKT also plays an important role in diabetes[3] and intracellular bacterial infections[4], underscoring its relevance as a target for clinical drug development.

Most cells express three isoforms of PKB/AKT, namely, AKT1, AKT2 and AKT3.[5] Genetic deletion of both AKT1 and AKT2 in mice is lethal, whereas elimination of only AKT1

appeared to have no detrimental effect on the development and health of the thus genetically modified mice.[6] Given the importance of PKB/AKT activation in cancer and other diseases, several studies have been conducted in the past that aimed to develop active and selective PKB/AKT1 inhibitors.[7] Kuijl *et al.*[4] have, for instance, revealed that the isoquinoline derivative, H-89 (**3**), which was originally identified as an inhibitor of protein kinase A (PKA),[8] inhibited AKT1 quite efficiently.[9]

Following the discovery that H-89 inhibits AKT1, a systematic library of H-89 analogues (**1** – **239**)[10] was synthesized with the aim to identify inhibitors selective for AKT1 over both AKT2 and PKA. In this ligand-based drug discovery approach, focused libraries were assembled based on H-89 as a lead structure and by varying the linker in length and distal phenyl ring as well as by modifying the styrene moiety, while keeping the isoquinoline moiety intact (Figure 1). This isoquinoline moiety was kept intact, since co-crystallisation studies of isoquinoline sulfonamides (H-series) complexed with PKA, which shares a close sequence (± 68%) homology in the kinase domain and within the adenine binding site itself only three residues differ between AKT2 and PKA, revealed that a single hydrogen bond is formed between the heteroaromatic nitrogen of the isoquinoline and a backbone amide on the kinase hinge (Val123 in PKA), an interaction that is highly conserved in kinase-inhibitor recognition.[11,12,13] With the aim to establish their selectivity, H-89 and selected members of the focused library that were found to be effective AKT1 inhibitors were tested in a commercial kinase-panel screen (Kinomescan)[14], in which their inhibitory activity at 10 micromolar against 100+ kinases was assessed (see Chapter 2). One of these H-89 analogues that contains a bulky naphthalene group instead of a bromide, namely compound **195** proved to inhibit, next to AKT1, also FMS-like tyrosine kinase 3 (FLT3)[15], which is an interesting drug target in its own right (see Chapter 3). FLT3 is a membrane-bound receptor tyrosine kinase expressed on hematopoietic cells. Upon binding to the FLT3 ligand, the activated receptor triggers both Ras/Raf and PI3K-PKB pathways resulting in cell proliferation and inhibition of apoptosis.[16,17] FLT3 mutations have been found in acute lymphoblastic leukemia (ALL)[18] and acute myeloid leukemia (AML)[19] patients. FLT3 inhibitors are currently in clinical trials for the treatment of ALL and AML patients. Moreover, inhibitors that target at the same time both AKT1 and FLT3 may act in a synergistic fashion, since both AKT1 and FLT3 activate the PI3K-PKB pathway.

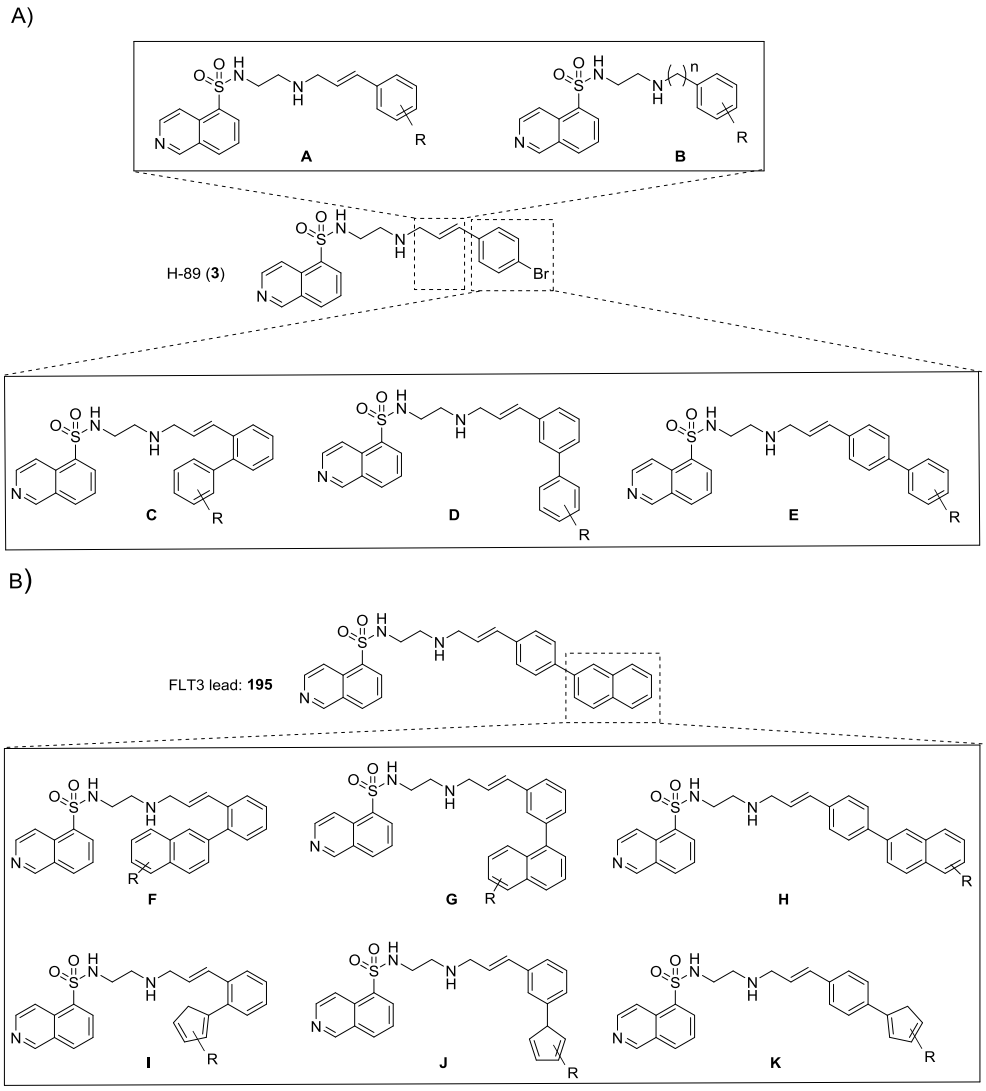


Figure 1. **H-89-based libraries.** A) H-89 (**3**) was modified by varying the linker length (**A**) or at the styrene moiety (**B** – **E**). B) FLT3 lead compound **195** was modified at the bulky naphthalene side (**F** – **H**) or it was displaced by thiophene or pyrrole containing moieties (**I** – **K)**.

Based on the interest in AKT1 inhibitors, FLT3 inhibitors as well as the potential of dual active inhibitors targeting both kinases, a study was initiated in which focused libraries were synthesized based on lead structure **195**. Synthesis details on these compounds are given in the preceding chapter. This chapter describes the inhibitor activities towards PKA, AKT1, AKT2 and FLT3 of the full set of isoquinolinesulfonamide derivatives prepared over the years – both those whose synthesis was described previously and the set of

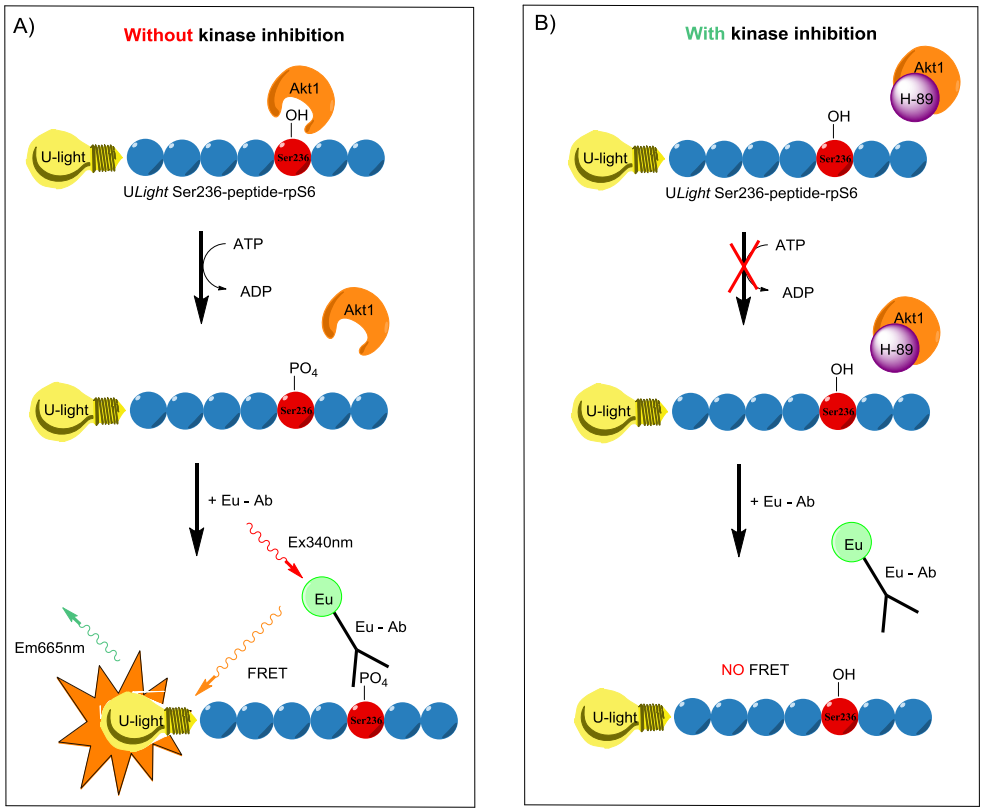


Figure 2. **TR-FRET kinase assay.** A) In presence of ATP, Ser/Thr kinase AKT1 phosphorylates the acceptor dye-labeled substrate, which is a U*Light*-labeled peptide containing residues surrounding Ser235 and Ser236 of human 40S ribosomal protein S6 (U*Light* Ser236 rpS6). The phosphorylated Ser236 will be bound by Eu-chelate donor dye containing rabbit monoclonal antibody. Upon irradiation at 320 nm, a FRET signal will be transferred from the Eu chelate to the U*Light* fluorophore, which, in turn, generates a light signal at 665 nm. B) In case of addition of an AKT1 inhibitor (e.g. H-89, **3**) to AKT1, phosphorylation of U*light*-substrate is (partially) blocked, with (partial) abolishment of the FRET signal as the result.

compounds described in Chapter 2. The inhibitor activities are all determined using a time-resolved fluorescence resonance energy transfer (TR-FRET) kinase activity assay.[20]

This assay is illustrated in Figure 2, in which AKT1 has been taken as the example kinase. A synthetic U*Light*-labeled peptide containing residues surrounding Ser235 and Ser236 of human 40S ribosomal protein S6 (U*Light* Ser236 rpS6) has been used as the acceptor-fluorophore substrate for the Ser/Thr kinases PKA, AKT1 and AKT2. By adding this substrate to a Ser/Thr kinase, the kinase recognizes and in turn phosphorylates amino acid Ser236 in the presence of ATP. Subsequently, the phosphorylated Ser235 will be recognized by the europium chelate donor dye

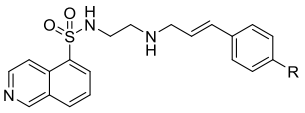
containing rabbit monoclonal antibody. The binding of this antibody to the U*Light*-labeled substrate brings donor and acceptor dyes into close proximity, which leads to energy transfer for the Eu donor to the U*Light* acceptor after irradiation of the kinase reaction at 320 nm. The generated light at 665 nm is measured and the intensity of the light emission is proportional to the level of U*Light*-substrate phosphorylation, which is a measure of kinase activity (Figure 2A).
Figure 2B describes the situation wherein an inhibitor (for instance, H-89) is present. In this situation, AKT1 is not able to phosphorylate Ser236 of the U*Light*-containing peptide, which in turn will not be recognized by the Eu-containing antibody. As a result, no FRET signal will be generated. A similar assay was used to determine FLT3 inhibitory activities. However, in that case, a tyrosine containing U*Light* peptide (U*Light*-TK peptide) and an europium-labeled anti-phospho-tyrosine antibody were used instead.

4.2 Results and discussion

In total, the inhibitor activities of 239 isoquinolinesulfonamides were determined. All structures feature the same isoquinoline moiety (left hand part of the structure of the lead compound, H-89) and vary in the nature of the spacer (length, substitution, saturation) and/or the aryl moiety that makes up the right hand part of the molecules (*para*-bromobenzene in H-89). H-89 (**3** in Figure 1) and its 238 analogues were comparatively investigated with respect to their ability to inhibit the Ser/Thr kinases PKA, AKT1 and AKT2 and the Tyr kinase FLT3. In the first instance, relative inhibition percentages of the four kinases were measured at 2 μM final concentrations of each compound. These numbers are given in Figures 3-11, in which the isoquinoline derivatives are grouped around common themes (linker substitutions, nature and regiochemistry of substituted phenyls, etc.). Following these studies, the most effective AKT1 inhibitors (both in terms of activity and selectivity) were selected and inhibition constants were determined. These numbers are given in Table 1 and the chapter will end with a structure-activity relationship evaluation of the data, with a projection on the use of the most promising compounds as either AKT1 inhibitors or FLT3 inhibitors. All inhibition data were obtained using a TR-FRET kinase activity assay.

Unsubstituted alkene series

The eight analogues of H-89 (**3**), in which the bromide group is substituted with other halogens, alkyl or phenolic groups, were evaluated and compared to **3** in the TR-FRET assay for inhibition of the four kinases PKA, AKT1, AKT2 and FLT3 (Figure 3).



H	F	Br	Cl	CH_3
1 PKA 89 ± 1	**2** PKA 81 ± 1	**3** PKA *53 ± 1*	**4** PKA *68 ± 0*	**5** PKA 79 ± 1
Akt1 76 ± 5	Akt1 *58 ± 1*	Akt1 **34 ± 1**	Akt1 **41 ± 1**	Akt1 *66 ± 1*
Akt2 *71 ± 9*	Akt2 *66 ± 7*	Akt2 **49 ± 8**	Akt2 *62 ± 3*	Akt2 *69 ± 5*
FLT3 76 ± 5	FLT3 *74 ± 6*	FLT3 *58 ± 4*	FLT3 *67 ± 4*	FLT3 *62 ± 5*

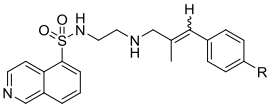
CF_3	O	O	NO_2
6 PKA *73 ± 18*	**7** PKA 89 ± 1	**8** PKA 94 ± 3	**9** PKA *75 ± 2*
Akt1 *57 ± 1*	Akt1 82 ± 1	Akt1 76 ± 0	Akt1 **31 ± 1**
Akt2 *63 ± 5*	Akt2 79 ± 1	Akt2 *74 ± 3*	Akt2 **50 ± 4**
FLT3 100 ± 54	FLT3 91 ± 1	FLT3 **23 ± 0**	FLT3 *54 ± 3*

Figure 3. **Unsubstituted alkene.** Relative remaining activity (%) towards PKA, PKB/AKT1, PKB/AKT2 and FLT3 in an *in vitro* kinase reaction in the absence or presence of 2 μM compound. Relative remaining activity ranges from 0 – 25%, 25 – 50%, 50 – 75%, > 75% are in bold and underlined, bold, italics and normal, respectively. Results are normalized to the activity detected in the absence of any compound, containing DMSO only, from *n = 3* experiments performed. Negative control: absence of ATP. Positive control: 2 μM commercial H-89.

Based on the results of Figure 3, substitution of the bromide group with other halogens or other small groups does not improve the relative activity towards all the four different kinases when compared to H-89 (**3**).
In fact and excepting compound **8** in all cases this substitution led to less potent inhibition. Compound **8** showed to be two times more active against FLT3 than compound **3** and it is slightly more active than its lead compound **195** (Figure 9).

Alkylated alkene series

The relative remaining enzymatic activity towards PKA, AKT1, AKT2 and FLT3 of a set of compounds with methyl (**10** – **24)**, ethyl (**25** – **39**) or isopropyl (**40** – **55**) substitution at the double bond and with replacement of the bromide group were determined (Figure 4 – 6 respectively). In the synthesis of these compounds (see the preceding chapter) it was observed that *E/Z* stereoisomers with respect to the alkene configuration could not always be separated. Accordingly, where possible pure *E* and *Z* isomers were evaluated and otherwise *E/Z* mixtures were put to the test.

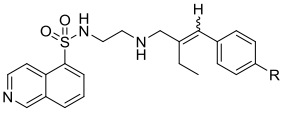


(*E*)	(*Z*)	(*E*)	(*Z*)	(*E/Z*) (5/3)	(*E*)
10 PKA 84 ± 2 Akt1 *70 ± 1* Akt2 78 ± 2 FLT3 79 ± 4	**11** PKA 93 ± 2 Akt1 84 ± 1 Akt2 80 ± 4 FLT3 82 ± 3	**12** PKA 80 ± 3 Akt1 *54 ± 1* Akt2 *71 ± 8* FLT3 75 ± 5	**13** PKA 90 ± 3 Akt1 *65 ± 0* Akt2 79 ± 12 FLT3 *69 ± 3*	**14** PKA 88 ± 2 Akt1 *62 ± 1* Akt2 *73 ± 5* FLT3 *73 ± 4*	**15** PKA *64 ± 2* Akt1 **43 ± 1** Akt2 *62 ± 1* FLT3 *60 ± 7*

(*E*)	(*E*)	(*Z*)	(*E*)	(*E/Z*) (1/4)	(*E*)
16 PKA *72 ± 2* Akt1 **48 ± 2** Akt2 *67 ± 3* FLT3 *74 ± 5*	**17** PKA 84 ± 2 Akt1 *69 ± 1* Akt2 78 ± 4 FLT3 *70 ± 5*	**18** PKA 94 ± 3 Akt1 79 ± 1 Akt2 84 ± 1 FLT3 *71 ± 10*	**19** PKA 93 ± 2 Akt1 *68 ± 3* Akt2 80 ± 4 FLT3 85 ± 5	**20** PKA 96 ± 3 Akt1 *66 ± 5* Akt2 83 ± 2 FLT3 77 ± 13	**21** PKA 89 ± 3 Akt1 81 ± 3 Akt2 86 ± 5 FLT3 77 ± 6

(*E*)	(*Z*)	(*E/Z*) (6/1)
22 PKA 99 ± 1 Akt1 82 ± 1 Akt2 *70 ± 26* FLT3 **40 ± 5**	**23** PKA 79 ± 1 Akt1 **31 ± 1** Akt2 *53 ± 3* FLT3 *55 ± 3*	**24** PKA 77 ± 2 Akt1 **28 ± 1** Akt2 **50 ± 4** FLT3 65 ± 3

Figure 4. **Methylated alkene.** Relative remaining activity (%) towards PKA, PKB/AKT1, PKB/AKT2 and FLT3 in an *in vitro* kinase reaction in the absence or presence of 2 µM compound. Relative remaining activity ranges from 0 – 25%, 25 – 50%, 50 – 75%, > 75% are in bold and underlined, bold, italics and normal, respectively. Results are normalized to the activity detected in the absence of any compound, containing DMSO only, from *n = 3* experiments performed. Negative control: absence of ATP. Positive control: 2 µM commercial H-89.

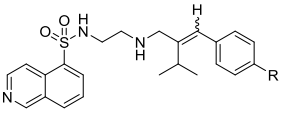


R = H (*E*)	R = H (*E/Z*) (1/5)	R = F (*E*)	R = F (*E/Z*) (1/5)	R = Br (*Z*)	R = Br (*E/Z*) (5/1)
25 PKA 89 ± 2	**26** PKA 90 ± 5	**27** PKA 84 ± 2	**28** PKA 93 ± 3	**29** PKA 84 ± 2	**30** PKA 82 ± 2
Akt1 *69 ± 0*	Akt1 78 ± 0	Akt1 *57 ± 1*	Akt1 *70 ± 2*	Akt1 **48 ± 2**	Akt1 **50 ± 0**
Akt2 81 ± 10	Akt2 79 ± 3	Akt2 *75 ± 1*	Akt2 79 ± 5	Akt2 *66 ± 4*	Akt2 *68 ± 4*
FLT3 76 ± 3	FLT3 80 ± 2	FLT3 *75 ± 8*	FLT3 79 ± 11	FLT3 *71 ± 4*	FLT3 *66 ± 4*

R = Cl (*E*)	R = CH_3 (*E*)	R = CH_3 (*E/Z*) (1/2)	R = CF_3 (*E*)	R = CF_3 (*Z*)	R = O (*E/Z*) (5/1)
31 PKA 88 ± 4	**32** PKA 88 ± 4	**33** PKA 88 ± 1	**34** PKA 89 ± 2	**35** PKA 90 ± 2	**36** PKA 90 ± 3
Akt1 *60 ± 0*	Akt1 *69 ± 2*	Akt1 *65 ± 1*	Akt1 *64 ± 0*	Akt1 *66 ± 0*	Akt1 *75 ± 1*
Akt2 77 ± 1	Akt2 78 ± 3	Akt2 79 ± 3	Akt2 *74 ± 3*	Akt2 79 ± 5	Akt2 83 ± 1
FLT3 *75 ± 5*	FLT3 *71 ± 6*	FLT3 *68 ± 8*	FLT3 *72 ± 5*	FLT3 *70 ± 4*	FLT3 *67 ± 2*

R = O (*E*)	R = O (*Z*)	R = NO_2 (*E/Z*) (3/2)
37 PKA 100 ± 1	**38** PKA 96 ± 2	**39** PKA 99 ± 5
Akt1 83 ± 4	Akt1 **30 ± 2**	Akt1 *58 ± 2*
Akt2 92 ± 8	Akt2 *58 ± 2*	Akt2 *67 ± 9*
FLT3 **39 ± 1**	FLT3 **39 ± 8**	FLT3 77 ± 8

Figure 5. **Ethylated alkene.** Relative remaining activity (%) towards PKA, PKB/AKT1, PKB/AKT2 and FLT3 in an *in vitro* kinase reaction in the absence or presence of 2 µM compound. Relative remaining activity ranges from 0 – 25%, 25 – 50%, 50 – 75%, > 75% are in bold and underlined, bold, italics and normal, respectively. Results are normalized to the activity detected in the absence of any compound, containing DMSO only, from *n* = *3* experiments performed. Negative control: absence of ATP. Positive control: 2 µM commercial H-89.



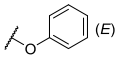
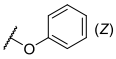
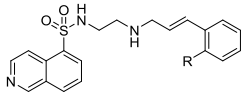
 (*E*)	 (*E/Z*) (1/10)	 (*E*)	 (*E/Z*) (3/2)	 (*E*)	 (*Z*)
40 PKA 89 ± 3	**41** PKA 98 ± 2	**42** PKA 88 ± 1	**43** PKA 93 ± 2	**44** PKA 79 ± 2	**45** PKA 89 ± 1
Akt1 *74 ± 1*	Akt1 89 ± 5	Akt1 *62 ± 0*	Akt1 *69 ± 0*	Akt1 **35 ± 3**	Akt1 *53 ± 2*
Akt2 76 ± 4	Akt2 80 ± 11	Akt2 *70 ± 4*	Akt2 *75 ± 4*	Akt2 *58 ± 1*	Akt2 76 ± 6
FLT3 *74 ± 3*	FLT3 84 ± 10	FLT3 *71 ± 4*	FLT3 *74 ± 4*	FLT3 **<u>15 ± 5</u>**	FLT3 *75 ± 7*
 (*Z*)	 (*E*)	 (*Z*)	 (*E*)	 (*Z*)	 (*E/Z*) (1/4)
46 PKA 88 ± 3	**47** PKA 93 ± 2	**48** PKA 93 ± 1	**49** PKA 93 ± 1	**50** PKA 93 ± 1	**51** PKA 92 ± 2
Akt1 *53 ± 3*	Akt1 *75 ± 1*	Akt1 77 ± 3	Akt1 79 ± 2	Akt1 *70 ± 1*	Akt1 76 ± 1
Akt2 *67 ± 3*	Akt2 77 ± 6	Akt2 *75 ± 5*	Akt2 78 ± 4	Akt2 *73 ± 2*	Akt2 83 ± 11
FLT3 *60 ± 3*	FLT3 *57 ± 1*	FLT3 *74 ± 3*	FLT3 81 ± 8	FLT3 *74 ± 1*	FLT3 *71 ± 0*
 (*E*)	 (*Z*)	 (*E*)	 (*Z*)		
52 PKA 96 ± 1	**53** PKA 95 ± 2	**54** PKA 88 ± 1	**55** PKA 88 ± 1		
Akt1 80 ± 0	Akt1 **48 ± 2**	Akt1 **45 ± 1**	Akt1 **46 ± 1**		
Akt2 77 ± 2	Akt2 *68 ± 6*	Akt2 *68 ± 11*	Akt2 *65 ± 3*		
FLT3 **34 ± 4**	FLT3 **41 ± 3**	FLT3 *67 ± 3*	FLT3 *66 ± 11*		

Figure 6. **Isopropylated alkene.** Relative remaining activity (%) towards PKA, PKB/AKT1, PKB/AKT2 and FLT3 in an *in vitro* kinase reaction in the absence or presence of 2 µM compound. Relative remaining activity ranges from 0 – 25%, 25 – 50%, 50 – 75%, > 75% are in bold and underlined, bold, italics and normal, respectively. Results are normalized to the activity detected in the absence of any compound, containing DMSO only, from *n = 3* experiments performed. Negative control: absence of ATP. Positive control: 2 µM commercial H-89.

The results that are given in Figure 4 – 6 show that addition of an alkyl group to the double bond does not improve the inhibition potency towards PKA, AKT1, AKT2 or FLT3 remarkably. Also the influence of the *E* or *Z* confirmation appeared to be negligible. An exception can be made for compound **44**, which showed to be twice as active against FLT3 as its lead compound **195** and it is remarkable that the *E* compound is five times more active that its corresponding *Z* isomer.

Ortho/*meta*/*para*-phenyl sulphonamide series

To obtain more insight into the size and electronics of the pocket that accommodates the distal phenyl ring of H-89 (**3**), this aryl ring has been systematically modified by introducing various large substituents, including bulky aromatic and/or hetero-aromatic groups at the ortho (**56** – **112**), meta (**113** – **169**) and para position (**170** - **229**) (Figure 7 – 9), while keeping the alkene unsubstituted.

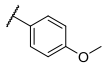


56 PKA 98 ± 1
Akt1 **41 ± 0**
Akt2 **50 ± 7**
FLT3 *73 ± 8*

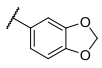
57 PKA 100 ± 2
Akt1 *59 ± 2*
Akt2 *64 ± 7*
FLT3 *72 ± 4*

58 PKA 98 ± 1
Akt1 **40 ± 1**
Akt2 **50 ± 2**
FLT3 76 ± 5

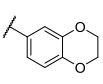
59 PKA 98 ± 2
Akt1 **43 ± 1**
Akt2 **50 ± 5**
FLT3 *74 ± 8*



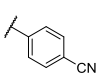
60 PKA 99 ± 3
Akt1 **42 ± 0**
Akt2 **50 ± 5**
FLT3 *60 ± 10*



61 PKA 100 ± 2
Akt1 **35 ± 2**
Akt2 **47 ± 5**
FLT3 *63 ± 1*



62 PKA 97 ± 1
Akt1 **33 ± 1**
Akt2 **47 ± 4**
FLT3 76 ± 5



63 PKA 99 ± 3
Akt1 **41 ± 1**
Akt2 *56 ± 8*
FLT3 *68 ± 2*

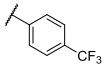
64 PKA 100 ± 2
Akt1 **45 ± 0**
Akt2 *55 ± 2*
FLT3 85 ± 4

65 PKA 94 ± 1
Akt1 **39 ± 1**
Akt2 *52 ± 4*
FLT3 76 ± 5

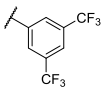
66 PKA 96 ± 3
Akt1 **43 ± 1**
Akt2 **50 ± 4**
FLT3 *62 ± 4*

67 PKA 100 ± 5
Akt1 **46 ± 1**
Akt2 *56 ± 4*
FLT3 83 ± 11

68 PKA 100 ± 2
Akt1 **36 ± 1**
Akt2 **47 ± 4**
FLT3 *74 ± 5*



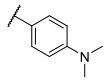
69 PKA 100 ± 4
Akt1 *62 ± 2*
Akt2 *64 ± 4*
FLT3 87 ± 4



70 PKA 100 ± 3
Akt1 93 ± 0
Akt2 91 ± 4
FLT3 92 ± 2

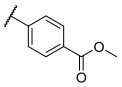
71 PKA 88 ± 23
Akt1 88 ± 0
Akt2 86 ± 2
FLT3 84 ± 2

72 PKA 100 ± 2
Akt1 **45 ± 1**
Akt2 *59 ± 7*
FLT3 89 ± 7

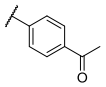


73 PKA 100 ± 3
Akt1 77 ± 0
Akt2 76 ± 3
FLT3 87 ± 8

74 PKA 88 ± 5
Akt1 *59 ± 2*
Akt2 79 ± 7
FLT3 78 ± 1

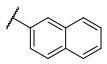


75 PKA 98 ± 9
Akt1 79 ± 1
Akt2 83 ± 8
FLT3 84 ± 2



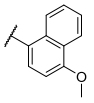
76 PKA 89 ± 19
Akt1 *59 ± 2*
Akt2 *66 ± 7*
FLT3 *74 ± 1*

77 PKA 96 ± 6
Akt1 *65 ± 1*
Akt2 93 ± 38
FLT3 77 ± 0

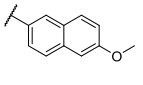


78 PKA 98 ± 5
Akt1 79 ± 1
Akt2 84 ± 5
FLT3 **37 ± 4**

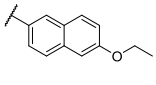
79 PKA 97 ± 3
Akt1 88 ± 1
Akt2 85 ± 9
FLT3 78 ± 3



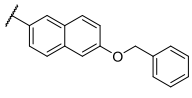
80 PKA 100 ± 1
Akt1 86 ± 1
Akt2 90 ± 13
FLT3 79 ± 5



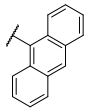
81 PKA 100 ± 1
Akt1 89 ± 1
Akt2 93 ± 14
FLT3 *72 ± 5*



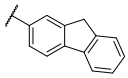
82 PKA 100 ± 3
Akt1 99 ± 1
Akt2 100 ± 13
FLT3 84 ± 10



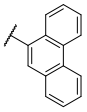
83 PKA 100 ± 3
Akt1 100 ± 1
Akt2 100 ± 13
FLT3 91 ± 3



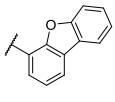
84 PKA 100 ± 1
Akt1 90 ± 1
Akt2 79 ± 33
FLT3 82 ± 3



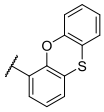
85 PKA 99 ± 9
Akt1 100 ± 1
Akt2 100 ± 12
FLT3 84 ± 3



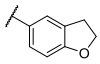
86 PKA 100 ± 1
Akt1 99 ± 2
Akt2 100 ± 19
FLT3 84 ± 2



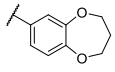
87 PKA 100 ± 1
Akt1 98 ± 2
Ak2 98 ± 8
FLT3 *63 ± 3*



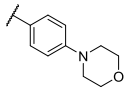
88 PKA 100 ± 1
Akt1 100 ± 1
Akt2 96 ± 4
FLT3 92 ± 1



89 PKA 100 ± 1
Akt1 **45 ± 1**
Akt2 *58 ± 4*
FLT3 *71 ± 8*



90 PKA 100 ± 2
Akt1 **48 ± 3**
Akt2 *58 ± 4*
FLT3 78 ± 3



91 PKA 100 ± 5
Akt1 87 ± 0
Akt2 91 ± 8
FLT3 87 ± 5

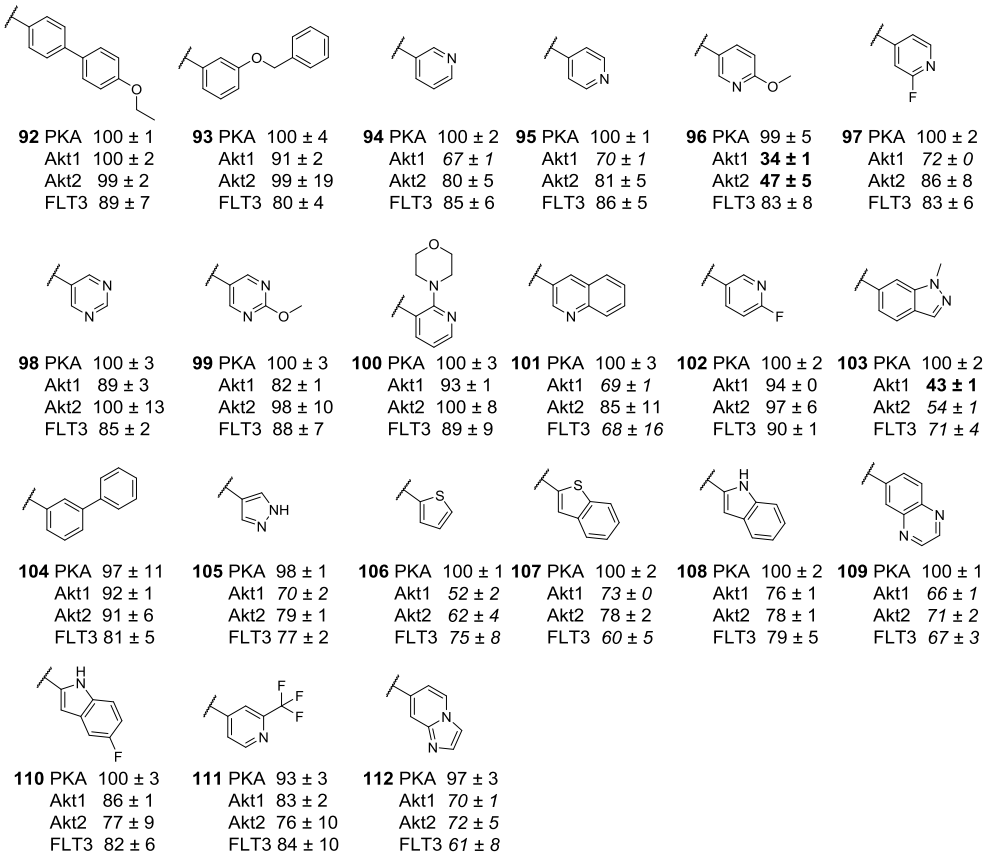
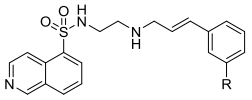


Figure 7. *Ortho*-phenyl sulphonamide inhibitors. Relative remaining activity (%) towards purified PKA, PKB/AKT1, PKB/AKT2 and FLT3 in an *in vitro* kinase reaction in the absence or presence of 2 µM compound. Relative remaining activity ranges from 0 – 25%, 25 – 50%, 50 – 75%, > 75% are in bold and underlined, bold, italics and normal, respectively. Results are normalized to the activity detected in the absence of any compound, containing DMSO only, from *n = 3* experiments performed. Negative control: absence of ATP. Positive control: 2 µM commercial H-89.

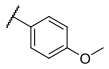


113 PKA 100 ± 3
Akt1 85 ± 1
Akt2 90 ± 6
FLT3 ***55 ± 5***

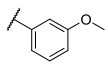
114 PKA 100 ± 2
Akt1 88 ± 0
Akt2 91 ± 9
FLT3 ***57 ± 3***

115 PKA 100 ± 2
Akt1 91 ± 1
Akt2 88 ± 5
FLT3 *65 ± 2*

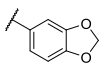
116 PKA 100 ± 0
Akt1 84 ± 3
Akt2 85 ± 6
FLT3 *60 ± 2*



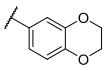
117 PKA 99 ± 2
Akt1 84 ± 1
Akt2 90 ± 10
FLT3 ***44 ± 2***



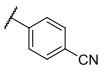
118 PKA 99 ± 2
Akt1 84 ± 1
Akt2 89 ± 10
FLT3 *54 ± 1*



119 PKA 96 ± 10
Akt1 85 ± 0
Akt2 86 ± 4
FLT3 **38 ± 5**



120 PKA 100 ± 1
Akt1 93 ± 1
Akt2 92 ± 5
FLT3 *56 ± 5*



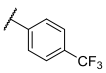
121 PKA 100 ± 1
Akt1 87 ± 1
Akt2 *69 ± 5*
FLT3 *63 ± 4*

122 PKA 100 ± 3
Akt1 95 ± 1
Akt2 95 ± 6
FLT3 *72 ± 8*

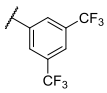
123 PKA 100 ± 5
Akt1 100 ± 2
Akt2 89 ± 10
FLT3 *55 ± 3*

124 PKA 98 ± 5
Akt1 95 ± 1
Akt2 88 ± 9
FLT3 79 ± 10

125 PKA 98 ± 3
Akt1 89 ± 3
Akt2 85 ± 9
FLT3 *58 ± 9*



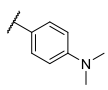
126 PKA 100 ± 2
Akt1 96 ± 1
Akt2 84 ± 8
FLT3 *70 ± 3*



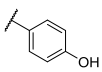
127 PKA 98 ± 5
Akt1 97 ± 1
Akt2 85 ± 8
FLT3 90 ± 5

128 PKA 100 ± 1
Akt1 97 ± 0
Akt2 87 ± 8
FLT3 77 ± 3

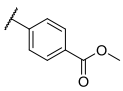
129 PKA 98 ± 3
Akt1 86 ± 0
Akt2 85 ± 4
FLT3 *69 ± 7*



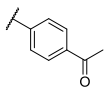
130 PKA 100 ± 3
Akt1 *67 ± 2*
Akt2 *74 ± 4*
FLT3 *64 ± 5*



131 PKA 87 ± 2
Akt1 *67 ± 0*
Akt2 76 ± 4
FLT3 *69 ± 6*

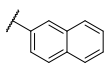


132 PKA 98 ± 3
Akt1 91 ± 2
Akt2 87 ± 4
FLT3 *62 ± 7*



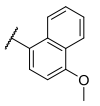
133 PKA 94 ± 13
Akt1 93 ± 2
Akt2 94 ± 5
FLT3 79 ± 2

134 PKA 99 ± 3
Akt1 96 ± 2
Akt2 90 ± 3
FLT3 *53 ± 5*

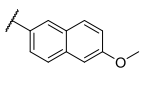


135 PKA 100 ± 2
Akt1 95 ± 0
Akt2 89 ± 3
FLT3 **42 ± 1**

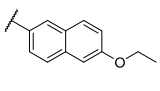
136 PKA 100 ± 1
Akt1 96 ± 0
Akt2 89 ± 8
FLT3 77 ± 7



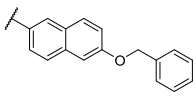
137 PKA 97 ± 9
Akt1 97 ± 0
Akt2 87 ± 8
FLT3 *72 ± 1*



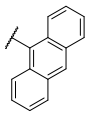
138 PKA 100 ± 0
Akt1 97 ± 2
Akt2 87 ± 13
FLT3 *70 ± 2*



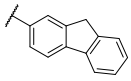
139 PKA 100 ± 1
Akt1 97 ± 2
Akt2 90 ± 9
FLT3 81 ± 6



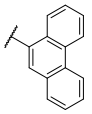
140 PKA 100 ± 2
Akt1 97 ± 1
Akt2 93 ± 11
FLT3 88 ± 4



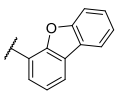
141 PKA 99 ± 1
Akt1 76 ± 1
Akt2 73 ± 9
FLT3 *52 ± 3*



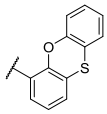
142 PKA 100 ± 1
Akt1 98 ± 1
Akt2 90 ± 10
FLT3 80 ± 4



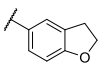
143 PKA 100 ± 1
Akt1 98 ± 0
Akt2 91 ± 8
FLT3 91 ± 5



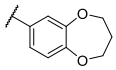
144 PKA 100 ± 0
Akt1 97 ± 1
Akt2 89 ± 8
FLT3 *66 ± 1*



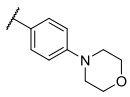
145 PKA 100 ± 2
Akt1 97 ± 1
Akt2 89 ± 6
FLT3 86 ± 7



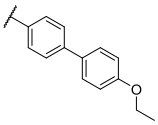
146 PKA 99 ± 5
Akt1 88 ± 1
Akt2 85 ± 4
FLT3 **44 ± 2**



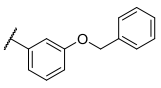
147 PKA 100 ± 3
Akt1 91 ± 1
Akt2 84 ± 4
FLT3 *55 ± 2*



148 PKA 100 ± 4
Akt1 87 ± 1
Akt2 81 ± 2
FLT3 *72 ± 7*



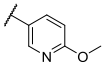
149 PKA 99 ± 7
Akt1 96 ± 2
Akt2 90 ± 2
FLT3 81 ± 3



150 PKA 100 ± 2
Akt1 100 ± 2
Akt2 92 ± 2
FLT3 *58 ± 2*

151 PKA 90 ± 0
Akt1 *66 ± 0*
Akt2 76 ± 8
FLT3 *57 ± 2*

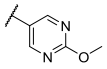
152 PKA 98 ± 1
Akt1 90 ± 1
Akt2 91 ± 6
FLT3 80 ± 2



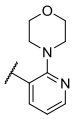
153 PKA 98 ± 2
Akt1 89 ± 3
Akt2 90 ± 6
FLT3 *64 ± 5*

154 PKA 89 ± 2
Akt1 88 ± 1
Akt2 91 ± 6
FLT3 80 ± 7

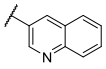
155 PKA 98 ± 4
Akt1 89 ± 0
Akt2 93 ± 9
FLT3 *75 ± 2*



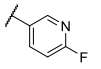
156 PKA 95 ± 3
Akt1 89 ± 1
Akt2 94 ± 5
FLT3 *75 ± 7*



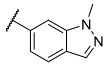
157 PKA 100 ± 3
Akt1 89 ± 1
Akt2 94 ± 10
FLT3 **50 ± 2**



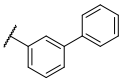
158 PKA 96 ± 12
Akt1 81 ± 2
Akt2 91 ± 9
FLT3 **35 ± 3**



159 PKA 99 ± 3
Akt1 91 ± 3
Akt2 91 ± 7
FLT3 *73 ± 6*



160 PKA 100 ± 3
Akt1 88 ± 3
Akt2 84 ± 5
FLT3 **44 ± 1**



161 PKA 100 ± 4
Akt1 95 ± 1
Akt2 93 ± 7
FLT3 78 ± 2

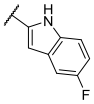
162 PKA 98 ± 4
Akt1 75 ± 1
Akt2 79 ± 2
FLT3 **28 ± 6**

163 PKA 98 ± 3
Akt1 73 ± 1
Akt2 72 ± 4
FLT3 *56 ± 4*

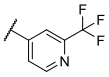
164 PKA 100 ± 4
Akt1 94 ± 3
Akt2 89 ± 4
FLT3 *57 ± 3*

165 PKA 100 ± 2
Akt1 86 ± 3
Akt2 82 ± 4
FLT3 *68 ± 5*

166 PKA 98 ± 1
Akt1 79 ± 0
Akt2 79 ± 4
FLT3 **35 ± 5**



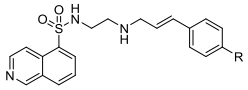
167 PKA 100 ± 1
Akt1 91 ± 1
Akt2 87 ± 3
FLT3 *71 ± 4*



168 PKA 100 ± 2
Akt1 100 ± 0
Akt2 85 ± 13
FLT3 85 ± 1

169 PKA 79 ± 3
Akt1 81 ± 0
Akt2 *75 ± 9*
FLT3 *54 ± 4*

Figure 8. ***Meta*-phenyl sulphonamide inhibitors.** Relative remaining activity (%) towards PKA, PKB/AKT1, PKB/AKT2 and FLT3 in an *in vitro* kinase reaction in the absence or presence of 2 μM compound. Relative remaining activity ranges from 0 – 25%, 25 – 50%, 50 – 75%, > 75% are in bold and underlined, bold, italics and normal, respectively. Results are normalized to the activity detected in the absence of any compound, containing DMSO only, from *n* = *3* experiments performed. Negative control: absence of ATP. Positive control: 2 μM commercial H-89.



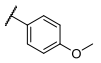
170 PKA 99 ± 2
Akt1 84 ± 1
Akt2 88 ± 0
FLT3 **36 ± 1**

171 PKA 98 ± 0
Akt1 88 ± 1
Akt2 88 ± 3
FLT3 **43 ± 7**

172 PKA 100 ± 2
Akt1 92 ± 1
Akt2 92 ± 5
FLT3 **31 ± 3**

173 PKA 100 ± 2
Akt1 88 ± 1
Akt2 96 ± 6
FLT3 *58 ± 6*

174 PKA 100 ± 3
Akt1 100 ± 4
Akt2 100 ± 3
FLT3 92 ± 6



175 PKA 100 ± 2
Akt1 98 ± 2
Akt2 98 ± 6
FLT3 **45 ± 2**

176 PKA 100 ± 1
Akt1 93 ± 0
Akt2 94 ± 5
FLT3 **43 ± 3**

177 PKA 100 ± 2
Akt1 93 ± 1
Akt2 99 ± 15
FLT3 **40 ± 6**

178 PKA 99 ± 2
Akt1 89 ± 1
Akt2 95 ± 4
FLT3 **41 ± 7**

179 PKA 98 ± 2
Akt1 85 ± 1
Akt2 90 ± 8
FLT3 *56 ± 1*

180 PKA 99 ± 4
Akt1 88 ± 2
Akt2 88 ± 6
FLT3 *51 ± 1*

181 PKA 97 ± 2
Akt1 82 ± 2
Akt2 89 ± 6
FLT3 **34 ± 4**

182 PKA 99 ± 0
Akt1 88 ± 1
Akt2 89 ± 7
FLT3 **47 ± 3**

183 PKA 99 ± 0
Akt1 87 ± 1
Akt2 100 ± 21
FLT3 *62 ± 29*

184 PKA 98 ± 1
Akt1 83 ± 2
Akt2 90 ± 2
FLT3 **36 ± 5**

185 PKA 96 ± 6
Akt1 93 ± 0
Akt2 91 ± 4
FLT3 78 ± 7

186 PKA 99 ± 2
Akt1 94 ± 2
Akt2 87 ± 3
FLT3 100 ± 4

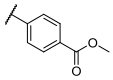
187 PKA 100 ± 2
Akt1 91 ± 1
Akt2 91 ± 4
FLT3 **49 ± 4**

188 PKA 100 ± 0
Akt1 97 ± 2
Akt2 87 ± 4
FLT3 *71 ± 4*

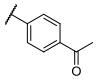
189 PKA 98 ± 1
Akt1 86 ± 0
Akt2 85 ± 4
FLT3 *52 ± 7*

190 PKA 100 ± 1
Akt1 *67 ± 1*
Akt2 *74 ± 4*
FLT3 **35 ± 8**

191 PKA 87 ± 2
Akt1 *67 ± 2*
Akt2 76 ± 5
FLT3 *64 ± 1*



192 PKA 98 ± 4
Akt1 91 ± 5
Akt2 87 ± 5
FLT3 **41 ± 3**



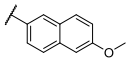
193 PKA 94 ± 2
Akt1 93 ± 3
Akt2 94 ± 5
FLT3 **49 ± 5**

194 PKA 99 ± 1
Akt1 96 ± 3
Akt2 90 ± 5
FLT3 **35 ± 16**

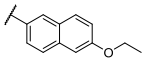
195 PKA 100 ± 3
Akt1 95 ± 5
Akt2 89 ± 14
FLT3 **36 ± 4**

196 PKA 100 ± 4
Akt1 100 ± 1
Akt2 86 ± 10
FLT3 85 ± 13

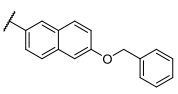
197 PKA 97 ± 8
Akt1 99 ± 4
Akt2 89 ± 10
FLT3 *72 ± 7*



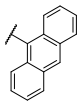
198 PKA 100 ± 7
Akt1 100 ± 3
Akt2 95 ± 15
FLT3 169 ± 15



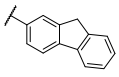
199 PKA 100 ± 5
Akt1 152 ± 3
Akt2 183 ± 20
FLT3 **50 ± 3**



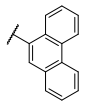
200 PKA 100 ± 3
Akt1 100 ± 2
Akt2 99 ± 12
FLT3 90 ± 6



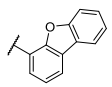
201 PKA 100 ± 4
Akt1 100 ± 3
Akt2 100 ± 10
FLT3 *57 ± 8*



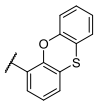
202 PKA 100 ± 4
Akt1 98 ± 1
Akt2 100 ± 16
FLT3 272 ± 49



203 PKA 100 ± 0
Akt1 100 ± 1
Akt2 100 ± 9
FLT3 *74 ± 2*



204 PKA 100 ± 4
Akt1 100 ± 2
Akt2 100 ± 6
FLT3 **28 ± 4**



205 PKA 100 ± 3
Akt1 100 ± 1
Akt2 100 ± 10
FLT3 100 ± 4

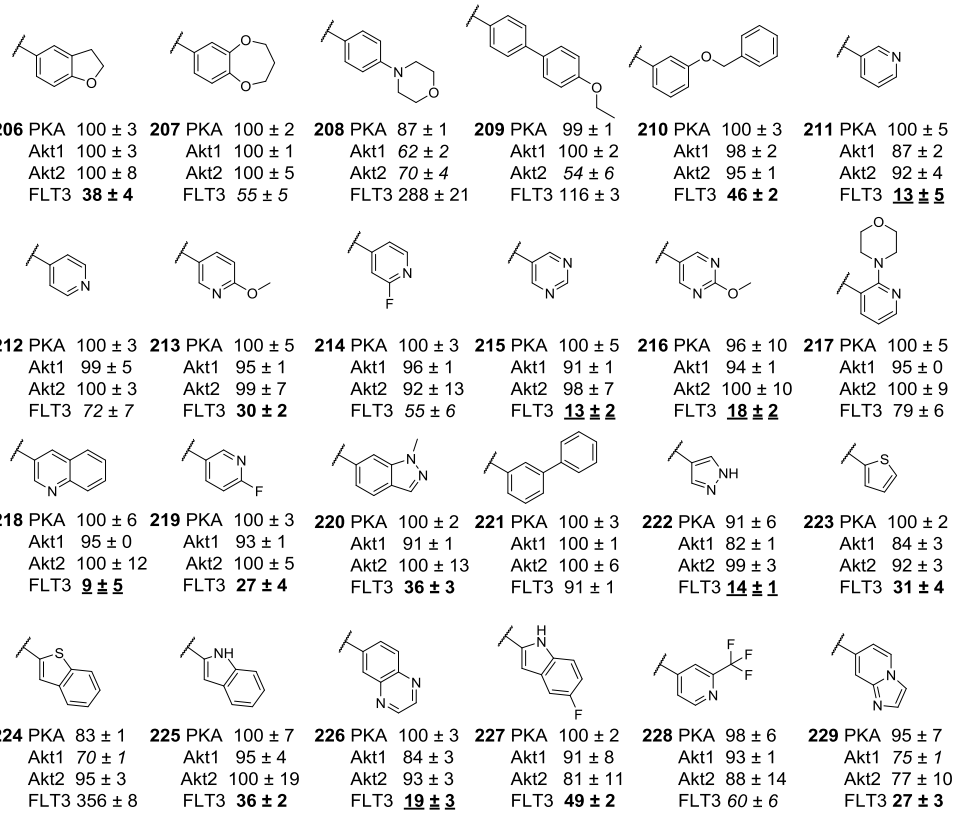


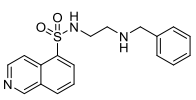
Figure 9. ***Para*-phenyl sulphonamide inhibitors of first (A) and second (B) generation library.** Relative remaining activity (%) towards PKA, PKB/AKT1, PKB/AKT2 and FLT3 in an *in vitro* kinase reaction in the absence or presence of 2 μM compound. Relative remaining activity ranges from 0 – 25%, 25 – 50%, 50 – 75%, > 75% are in bold and underlined, bold, italics and normal, respectively. Results are normalized to the activity detected in the absence of any compound, containing DMSO only, from *n* = *3* experiments performed. Negative control: absence of ATP. Positive control: 2 μM commercial H-89.

It can be seen from Figure 7 - 9 that the compounds having a functional group at the meta position have the least activity against the kinases PKA, AKT1, and AKT2 when compared to its corresponding ortho and para analogues. Analogues having an aromatic group at the ortho position appear to have the most activity for AKT1 and AKT2. Of note, all compounds are poor PKA inhibitors. This indicates that substitution of the bromide in H-89 (**3**) for an aromatic moiety is detrimental for PKA/AKT1/AKT2 inhibition. In addition, bulky aromatic groups at the para position result in active inhibitors against FLT3. Compounds with nitrogen-containing aromatic rings at the para position are amongst the most active FLT3 inhibitors.

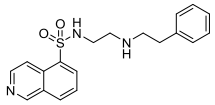
Introduction of functional groups at the ortho position seems to be least favourable for gaining active FLT3 inhibitors. Finally, a few activators for FLT3 has been observed, namely compounds **198**, **202**, **208**, **209** and **224**.

Linker length alteration

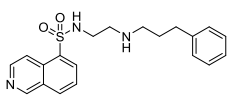
The linker length has been altered and the double bond has been excluded in compounds **230** – **236** (Figure 10). This will give information about the importance of the double bond and the influence of the linker length on the activity.



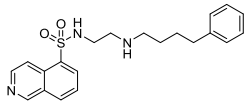
230 PKA 95 ± 1
Akt1 85 ± 1
Akt2 92 ± 16
FLT3 86 ± 7



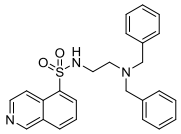
231 PKA 92 ± 5
Akt1 84 ± 0
Akt2 84 ± 4
FLT3 82 ± 4



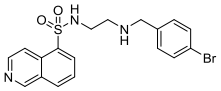
232 PKA 89 ± 10
Akt1 82 ± 1
Akt2 85 ± 3
FLT3 80 ± 4



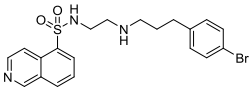
233 PKA 100 ± 1
Akt1 95 ± 2
Akt2 97 ± 4
FLT3 93 ± 8



234 PKA 100 ± 6
Akt1 100 ± 5
Akt2 87 ± 3
FLT3 100 ± 11



235 PKA 86 ± 1
Akt1 76 ± 0
Akt2 82 ± 6
FLT3 78 ± 5



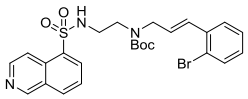
236 PKA 82 ± 1
Akt1 **42 ± 0**
Akt2 *67 ± 13*
FLT3 *58 ± 2*

Figure 10. **Variation in linker length.** Relative remaining activity (%) towards PKA, PKB/AKT1, PKB/AKT2 and FLT3 in an *in vitro* kinase reaction in the absence or presence of 2 μM compound. Relative remaining activity ranges from 0 – 25%, 25 – 50%, 50 – 75%, > 75% are in bold and underlined, bold, italics and normal, respectively. Results are normalized to the activity detected in the absence of any compound, containing DMSO only, from *n = 3* experiments performed. Negative control: absence of ATP. Positive control: 2 μM commercial H-89.

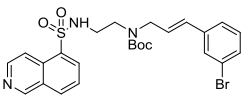
Figure 10 shows that changes in linker length and double bond have drastic influence on the activity towards the four different kinases. The linker length in H-89 (**3**) appears to be optimal and the double bond, which leads to rigidity in the molecule, appears to be important for activity.

Boc protected analogues

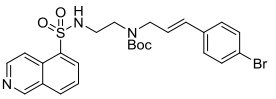
The amine group in compounds **237** – **239** have been protected by a Boc group (Figure **11**). According to the relative activities given in Figure 11, it seems that the amine functionality needs to be unprotected and or the bulky Boc-group seems to give steric hindrance with the active site. Thus a free amine contributes to the inhibition towards PKA, AKT1, AKT2 and FLT3.



237 PKA 98 ± 1
Akt1 93 ± 0
Akt2 90 ± 1
FLT3 97 ± 5



238 PKA 96 ± 1
Akt1 93 ± 2
Akt2 93 ± 4
FLT3 84 ± 5



239 PKA 96 ± 1
Akt1 94 ± 2
Akt2 90 ± 5
FLT3 100 ± 33

Figure 11. **Boc protected isoquinoline sulfonamide analogues.** Relative remaining activity (%) towards PKA, PKB/AKT1, PKB/AKT2 and FLT3 in an *in vitro* kinase reaction in the absence or presence of 2 μM compound. Relative remaining activity ranges from 0 – 25%, 25 – 50%, 50 – 75%, > 75% are in bold and underlined, bold, italics and normal, respectively. Results are normalized to the activity detected in the absence of any compound, containing DMSO only, from *n = 3* experiments performed. Negative control: absence of ATP. Positive control: 2 μM commercial H-89.

K_i and IC_{50} values of selected compounds

An estimation of the activities of the 239 inhibitors has been made by determining the relative activities towards PKA, AKT1, AKT2 and FLT3. However, to compare the activity and the selectivity between these compounds, it is necessary to determine their K_i values. K_i values were only determined for potential active and selective compounds, that is to say, only compounds showing > 50% inhibitory activity towards PKA, AKT1 or AKT2 (Table 1).

Table 1. Inhibition constant (K_i) values in µM of H-89 analogues. The mean K_i values are calculated using the Cheng-Prusoff equation from the IC_{50} values from *n = 3* experiments performed. The conditions are: K_M = 10.35, 207.8 and 0.82 µM for AKT1, AKT2 and PKA respectively and [ATP] was 100 µM.

Compound	AKT1	AKT2	PKA
24	0.06 ± 0.01	1.40 ± 0.26	0.04 ± 0.01
9	0.08 ± 0.01	1.28 ± 0.21	0.05 ± 0.01
38	0.09 ± 0.02	2.17 ± 0.55	0.46 ± 0.30
23	0.10 ± 0.03	1.76 ± 0.48	0.07 ± 0.01
68	0.09 ± 0.01	1.30 ± 0.21	0.28 ± 0.12
62	0.08 ± 0.02	1.19 ± 0.33	> 0.16
3	0.11 ± 0.02	2.25 ± 0.37	0.02 ± 0.00
96	0.07 ± 0.01	0.78 ± 0.16	0.20 ± 0.09
61	0.07 ± 0.02	1.34 ± 0.30	> 0.16
65	0.10 ± 0.01	1.86 ± 0.30	0.17 ± 0.06
56	0.17 ± 0.05	2.63 ± 0.63	0.20 ± 0.09
4	0.17 ± 0.04	3.40 ± 0.87	-
60	0.19 ± 0.03	2.87 ± 0.59	-
58	0.14 ± 0.03	2.16 ± 0.26	-
3 (H-89)	0.09 ± 0.01	1.67 ± 0.26	0.02 ± 0.01
236	0.13 ± 0.02	2.22 ± 0.34	0.02 ± 0.01
59	0.12 ± 0.02	1.74 ± 0.40	-
191	0.10 ± 0.01	1.64 ± 0.33	0.02 ± 0.01
218	0.08 ± 0.01	1.22 ± 0.19	0.01 ± 0.00
66	0.14 ± 0.03	1.76 ± 0.30	-
64	0.13 ± 0.02	2.12 ± 0.41	-
63	0.08 ± 0.02	1.17 ± 0.21	0.18 ± 0.04
103	0.06 ± 0.01	1.13 ± 0.19	0.36 ± 0.20
15	0.08 ± 0.01	2.22 ± 0.36	0.03 ± 0.01
89	0.05 ± 0.01	1.12 ± 0.23	> 0.16
54	0.19 ± 0.05	5.34 ± 0.96	0.10 ± 0.02
72	0.09 ± 0.01	1.51 ± 0.42	0.26 ± 0.14
67	0.11 ± 0.02	2.12 ± 0.35	-
55	0.13 ± 0.02	3.88 ± 0.65	-
29	0.09 ± 0.01	2.63 ± 0.37	-
16	0.09 ± 0.01	2.19 ± 0.23	0.04 ± 0.02
53	0.16 ± 0.02	3.74 ± 0.83	-
30	0.16 ± 0.04	4.42 ± 1.30	
45	0.08 ± 0.01	2.32 ± 0.36	0.04 ± 0.01

106	0.13 ± 0.01	1.64 ± 0.29	-
46	0.09 ± 0.02	2.13 ± 0.39	-
12	0.13 ± 0.01	6.15 ± 4.18	-
101	0.17 ± 0.03	2.76 ± 0.49	-
6	0.12 ± 0.02	1.91 ± 0.26	-
90	0.10 ± 0.01	1.72 ± 0.22	-
34	0.14 ± 0.02	3.11 ± 0.54	-
74	0.35 ± 0.10	7.93 ± 2.52	0.08 ± 0.02
39	0.15 ± 0.03	2.97 ± 1.34	-
2	0.14 ± 0.05	6.41 ± 1.77	-
27	0.33 ± 0.07	6.34 ± 1.43	-
31	0.22 ± 0.06	5.55 ± 1.54	-
45	0.47 ± 0.18	9.25 ± 2.55	-
57	0.41 ± 0.13	7.05 ± 2.49	-
76	0.36 ± 0.10	6.74 ± 2.31	-
190	0.10 ± 0.04	0.97 ± 0.13	0.04 ± 0.01
99	0.55 ± 0.30	-	-
224	0.42 ± 0.07	1.71 ± 0.43	0.05 ± 0.01
13	0.24 ± 0.10	20.42 ± 12.91	-
97	0.69 ± 0.22	12.25 ± 2.63	-
77	0.95 ± 0.27	14.97 ± 7.08	-
69	0.74 ± 0.24	8.47 ± 2.04	-
109	0.38 ± 0.06	5.04 ± 1.46	-
95	0.25 ± 0.04	5.37 ± 1.47	-
94	0.23 ± 0.03	3.03 ± 0.73	-
130	0.73 ± 0.08	6.61 ± 1.56	-
209	0.72 ± 0.18	1.47 ± 0.29	0.05 ± 0.01
199	3.10 ± 2.04	-	-
44	0.10 ± 0.02	1.92 ± 0.39	-

Based on the results shown in Table 1, a selection of most active AKT1 inhibitors has been made (Figure 12). These compounds (**24**, **61**, **89**, **96** and **103**) showed to have a significantly lower K_i value, which varies between 1.3 – 1.8 times more activity, than the lead compound H-89 (**3**).

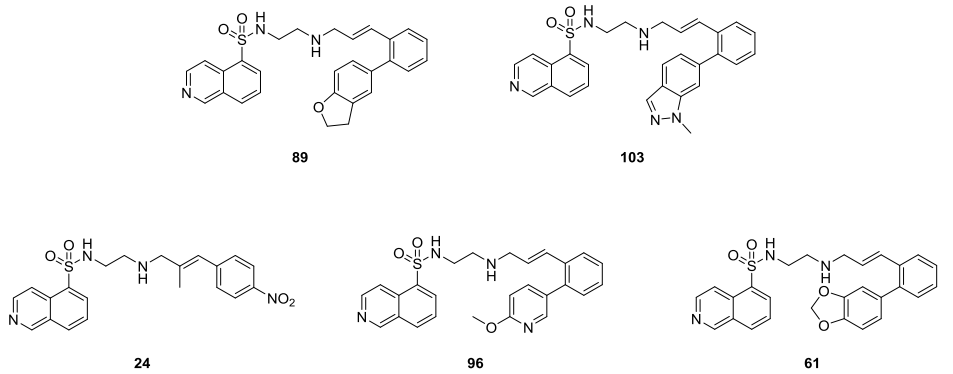


Figure 12. Selection of AKT1 inhibitors, which are more active than lead compound H-89 (**3**) based on their K_i values.

The ortho-phenyl substituted compounds are the most active inhibitors towards AKT1 in this library (Figure 10). It appears that bulky and electron-donating groups are favoured in the active site of AKT1. In addition, methyl-alkene derivatives appear to be more active AKT1 inhibitors compared to their non-substituted (at the alkene) counterparts. In general, the *E* configuration is preferred over the *Z* configuration.

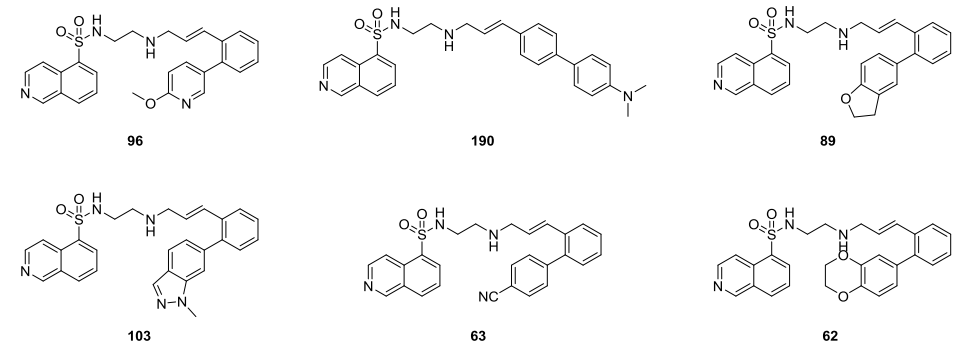


Figure 13. Selection of AKT2 inhibitors, which are more active than lead compound H-89 (**3**) based on their K_i values.

The six compounds (**62**, **63**, **89**, **96**, **103** and **190**) that are shown in Figure 13 are the most active AKT2 inhibitors found in this library. They are all significant more active than H-89 (**3**) showing an increase varying from 1.5 – 2.1 times. Based on these structures it can be seen that bulky, electron negative containing phenyl groups which are on the ortho position are preferred in the active site of AKT2. Compound

190 is the only compound in the top six having an aromatic group on the para position. The high activity is likely due to the dimethylamine group.

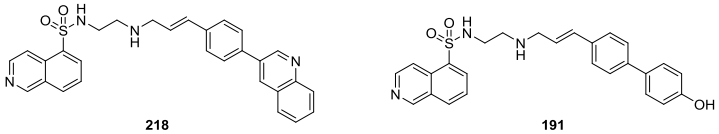


Figure 14. Selection of PKA inhibitors, which are more active than lead compound H-89 (**3**) based on their K_i values.

The lead compound H-89 (**3**) is already a potent PKA inhibitor, and it is therefore not surprising (also given that optimization was conducted towards AKT1, and not PKA) that stronger PKA inhibitors are scarce in the evaluated series of compounds. In fact, only compound **218** (Figure 14) showed a significant lower K_i value than lead compound H-89 (**3**). According to the results presented in Table 1, the most active PKA inhibitors are all containing a functional group at the para position, for instance, compound **191** (Figure 14). This is perhaps not surprising since the lead compound H-89 (**3**), which is a PKA inhibitor, contains a bromide on the para position.

Both AKT1 and AKT2 prefer roughly the same compounds (compounds **89**, **96** and **103**). Since inhibition of AKT2 is lethal in mice, it is important to find active and selective compounds towards AKT1. In Figure 15 the three most selective AKT1 over AKT2 compounds are shown.

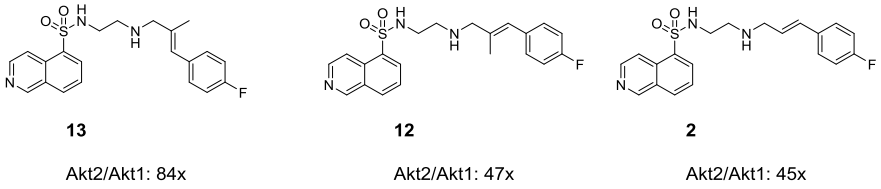


Figure 15. Most selective AKT1 over AKT2 inhibitors based on their K_i values difference.

It appears that a methyl substituent at the linker alkene contributes to AKT1 selectivity over AKT2. Next to this, introduction of a para-halogen at the phenyl ring appears to improve the selectivity of AKT1 over AKT2. Compared to the selectivity of the lead compound H-89 (**3**), which is 18 times more selective for AKT1 over AKT2, these compounds showed to have at least twice as selective as their lead compound. As can be seen in Figure 16, the most selective AKT1 over PKA inhibitors prefer to contain aromatic groups having oxygen or nitrogen groups at an ortho position.

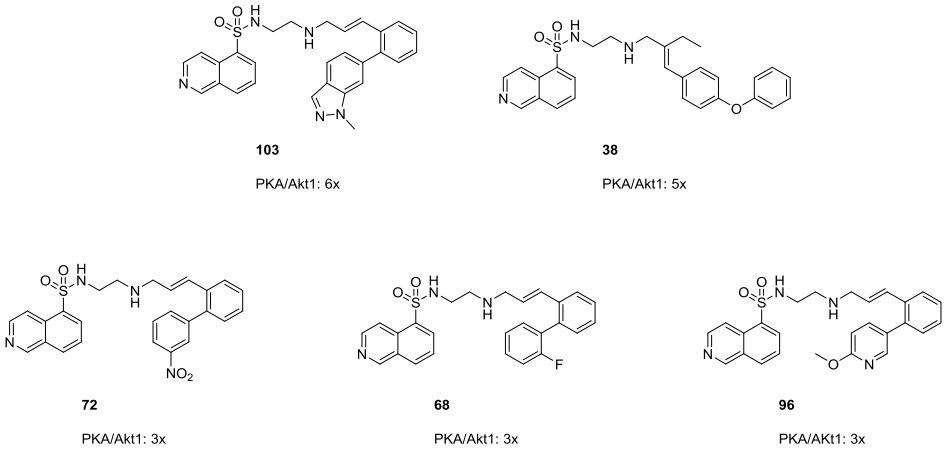


Figure 16. The most selective AKT1 over PKA inhibitors based on their K_i values difference.

Since FLT3 plays a role in the same pathway as AKT1, IC_{50} values of a few compounds showing a lower relative activity value than the FLT3 lead compound **195** and other relevant compounds have been determined (Table 2). This Table shows the importance of the position at which the aryl side is substituted. When comparing compounds **101**, **158** and **218**, which all contain an isoquinoline group at the ortho, meta or para position respectively, it becomes evident that the compound having a *para*-isoquinoline group (**218**) is more than 40 times more active than the ortho and meta analogues (**101** and **158**).

Table 2. IC_{50} values of selected isoquinolinesulfonamide compounds for FLT3.

Compound	IC_{50} (nM)	Compound	IC_{50} (nM)
101	3500 ± 3043	**215**	42.5 ± 15.3
158	715.5 ± 66.1	**216**	202.8 ± 73.6
195	290.7 ± 92.2	**218**	15.4 ± 1.4
211	17.8 ± 7.2	**222**	28.0 ± 10.3

Figure 17 shows the structures of the most active FLT3 compounds based on their IC_{50} values. These compounds are 5x or more active than **195** and it seemed that bulky, nitrogen containing aromatic groups at the para position leads to more inhibition of FLT3.

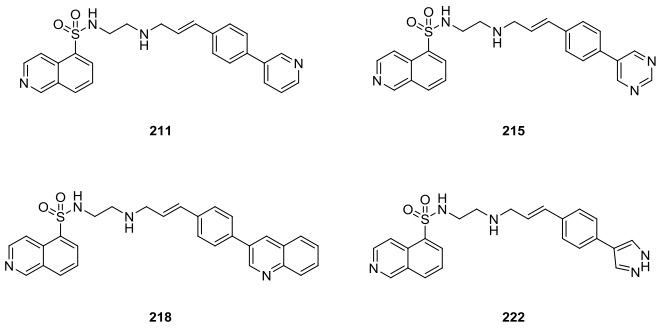


Figure 17. Selection of FLT3 inhibitors, which are more active than lead compound **195** based on their IC_{50} values.

4.3 Conclusion

This chapter describes the biological evaluation of 239 H-89-based compounds **1 – 239**. Evaluation of these compounds reveal that the structure-activity relationship (SAR) of AKT1 inhibition favours a hetero-aromatic group at the ortho position, which gives a more active AKT1 inhibitor and a more selective AKT1 over PKA inhibitor. The most active AKT1 inhibitor in this study is compound **89**, which has a benzofuran group at the ortho position, and the most selective AKT1 over PKA inhibitor is compound **103**, which has a methyl indazole group at the ortho position. Substitution of the double bond with a methyl or isopropyl group gives a more selective AKT1 over AKT2 inhibitor. This finding is applicable on the most selective AKT1 over AKT2 inhibitor **13**, which has a methyl group at the double bond. These results indicate that the ATP binding pocket of AKT1, in contrast with AKT2, does contain an additional cavity that can be occupied by a bulkier apolar group.
Furthermore, the SAR of FLT3 inhibition prefers bulky hetero-aromatic groups at the para position, especially nitrogen-containing aromatic groups are favourable. In this study, the most active FLT3 inhibitor is compound **218.**
In summary, thus far the two kinases AKT1 and FLT3, which act in the same biological pathway, cannot be inhibited by the same compound having this isoquinolinesulfonamide scaffold, since one prefers substitution at the para position and the other the ortho position, respectively. Therefore, new scaffolds need to be developed to cope this.

Experimental

Synthesis

Compounds 1 – 76, 113 – 133, 170 – 193 and 230 – 239: see thesis "Synthetic studies on kinase inhibitors and cyclic peptides: strategies towards new antibiotics", Adriaan W. Tuin, 2008, https://openaccess.leidenuniv.nl/bitstream/handle/1887/13365/Proefschrift%2BAW_Tuin%2Balles.pdf?sequence=7.

Compounds 77 – 112, 134 – 169 and 194 - 229: see Chapter 3.

Biochemistry

Kinase assay: Determination of the relative activity, IC_{50} and K_i of inhibitors 1 – 239 at 2 µM final concentration towards PKA, AKT1 and AKT2

A solution of 2 µM of inhibitor,10 nM U*Light*-rpS6 (pSer235/Ser236) peptide (Perkin-Elmer, human 40S ribosomal protein), 2 nM Eu-labeled anti-phospho-rpS6 antibody (Perkin-Elmer, Europium-labeled rabbit monoclonal antibody) and 100 µM ATP in 50 mM HEPES buffer pH 7.5 was incubated with 0.5 nM/min AKT1, AKT2 or 0.05 nM/min of PKA (SignalChem) for 6 h at RT. During these 6 h of incubation, the intensity of the light emission was measured with intervals of 30 min on a PE Envision reader using the Lance Ultra kinase assay settings (λ_{ex} 320 nm; λ_{em} 665 nm) and a secondary control emission was measured at 615 nm. In control experiments, no ATP was added into the buffer (negative control) or DMSO was added to the reaction instead of an inhibitor (background control). Alternatively, the kinases were incubated with 2 µM commercial H-89 (CalBiochem) (positive control).
To determine the K_M for the kinases, the same assay was performed using 0, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, 200, 500, 1000 µM ATP. K_M values were calculated using GraphPad Prism 5 (GraphPad software, La Jolla, USA).
To determine K_i values for inhibitors **1 - 239**, they were tested at a concentration ranging from 0.05 to 20 µM. Data was analyzed using GraphPad Prism 5 (GraphPad software, La Jolla, USA).
K_i values were calculated via equation 3.1:

$$K_i = IC_{50} / (1 + ([S]/K_M)) \quad (3.1)$$

where K_i is the inhibition constant, IC_{50} is the half maximal inhibitory concentration, S is the concentration of substrate and K_M is the Michaelis-Menten constant, which is the substrate concentration at which the reaction rate is half maximum. All experiments were conducted in triplicate and curves were corrected for background fluorescent of the solvent.

Kinase assays for FLT3

A solution of 2 µM of inhibitors **101, 158, 195, 211, 215, 216, 218** or **222**, 10 nM U*Light*-TK peptide (Perkin-Elmer, phosphorylated tyrosine residues), 2 nM Eu-labeled anti-phospho-tyrosine antibody (Perkin-Elmer, Europium-labeled rabbit monoclonal antibody) and 100 µM ATP in 50 mM HEPES buffer pH 7.5 was incubated with 0.05 nM/min FLT3 (Signalchem) for 6 h at RT. During these 6 h of incubation, the intensity of the light emission was

measured with intervals of 30 min similar to as for AKT1. In control experiments, no ATP was added into the buffer (negative control) or DMSO was added to the reaction instead of an inhibitor (background control). Alternatively, the kinases were incubated with 2 μM H-89 (CalBiochem) (positive control). To determine the IC_{50} values, inhibitors were tested at a concentration range from 0.3 to 10000 nM. All experiments were conducted in triplicate. Data was analyzed using GraphPad Prism 5 and curves were corrected for background fluorescent of the solvent.

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