



Published in final edited form as:

Bioorg Med Chem Lett. 2023 May 15; 88: 129288. doi:10.1016/j.bmcl.2023.129288.

Identification of A Novel Spirocyclic Nek2 Inhibitor Using High Throughput Virtual Screening

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Abstract

NIMA Related Kinase 2 (Nek2) kinase is an attractive target for the development of therapeutic agents for several types of highly invasive cancers. Despite this, no small molecule inhibitor has advanced to the late clinical stages thus far. In this work, we have identified a novel spirocyclic inhibitor (**V8**) of Nek2 kinase, utilizing a high-throughput virtual screening (HTVS) approach. Using recombinant Nek2 enzyme assays, we show that **V8** can inhibit Nek2 kinase activity ($IC_{50} = 2.4 \pm 0.2 \mu M$) by binding to the enzyme's ATP pocket. The inhibition is selective, reversible and is not time dependent. To understand the key chemotype features responsible for Nek2 inhibition, a detailed structure-activity relationships (SAR) was performed. Using molecular models of the energy-minimized structures of Nek2-inhibitory complexes, we identify key hydrogen-bonding

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Declaration of Competing Interest

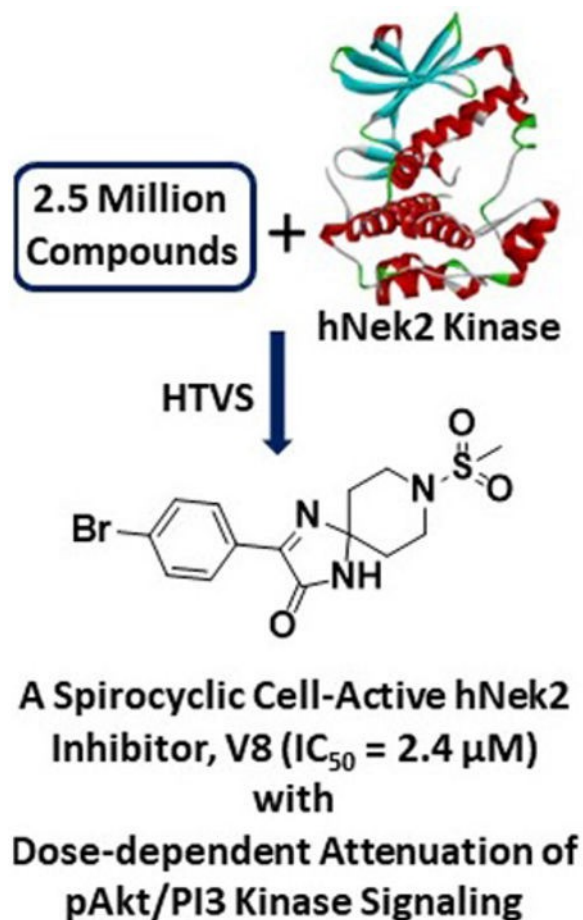
T.T.T. is a co-founder of Hysplex, Inc. with interests in PARP inhibitor development. Other authors declare no known competing financial interests or personal relationships.

Appendix A. Supplementary data

Supplementary data to this article can be found online.

interactions, including two from the hinge-binding region, likely responsible for the observed affinity. Finally using cell-based studies, we show that **V8** attenuates (a) pAkt/PI3 Kinase signaling in a dose-dependent manner, and (b) proliferative and migratory phenotypes of highly aggressive human MDA-MB-231 breast and A549 lung cancer cell lines. Thus, **V8** is an important novel lead compound for the development of highly potent and selective Nek2 inhibitory agents.

Graphical Abstract



Keywords

Nek2 kinase; Nek2 inhibitor; Nek2 hinge pharmacophore; Nek2 HTVS; Cytotoxicity of Nek2 inhibitor; Nek2 and Akt/PI3K Signaling

Mitosis is a fundamental cellular process that is carefully orchestrated by an exquisite group of kinases working to coordinate a sequence of complex events in a highly organized manner.^{1, 2} A controlled mitotic process is essential for maintaining cellular homeostasis during normal survival and growth processes. Aberrant mitosis as a result of dysregulated kinase signaling can be catastrophic, resulting in multinucleated daughter cells with abnormal chromosomal contents and more than two centrosomes. If cellular apoptotic machinery is nonfunctional in cells containing mitotic errors, cells will divide

‘asymmetrically’ in the next round of cell cycle, creating aneuploidic cells. A body of strong evidence suggest that aneuploidy and abnormal number of centrosomes can promote cancer development.³⁻⁵ One of the prominent mitotic kinases is Never in mitosis (NIMA)-related kinase 2 (Nek2) that plays critical roles in several mitotic events, including centrosomal disjunction, microtubule anchoring and stabilization, and spindle assembly checkpoint signaling.⁶ Overexpression of Nek2 kinase stimulates centrosome amplification (CA) and chromosomal instability (CIN), promoting the onset of tumorigenesis.⁷⁻⁹

Several recent studies further indicate that higher levels of Nek2 kinase can promote drug resistance, and accelerate tumor growth.^{8, 10-13} Results published from our laboratory further established that overabundant Nek2 can activate PI3K/Akt signaling and promote metastasis *in vivo*.¹⁴ The active role of Nek2 kinase in metastasis has been corroborated by other subsequent studies.^{12, 15} This finding is highly important since at least two thirds of deaths from solid tissue cancers are due to migration of primary tumor cells to secondary sites.^{16, 17} Furthermore, expression analysis in cancer cells from various origins, including the ones from highly invasive tumors, revealed overexpression of Nek2 kinase.¹⁸⁻²² This kinase is thus considered an attractive therapeutic target for anti-cancer drug development.²³ Indeed, inhibitory perturbation of Nek2 kinase signaling by RNAi-based agents, active-site targeted small molecules, and protein-protein interaction (PPI) inhibitory agents of Nek2-Hec1 protein interface has been shown to exert strong anti-tumor effects and reverse drug resistance (Figure 1).^{13, 24-28} Despite this, no small molecule Nek2 inhibitors have advanced to late stage clinical trials, primarily due to lack of cellular activity, low potency, and kinase nonspecificity. Novel chemotypes are thus needed to develop them into potent, selective and cell-permeable Nek2 inhibitory agents as potential anti-cancer agents. In this work, we have implemented a High-Throughput Virtual Screening (HTVS) protocol on a library of 2.5 million compounds, and identified a novel spirocyclic compound, **V8**, that inhibits Nek2 kinase activity *in-vitro*. The binding of **V8** is selective, fast and reversible, and ATP-site directed. Additionally, to provide further support that a spirocyclic motif is well-tolerated within the Nek2 active site, we explore structure-activity relationships (SAR) using a commercially available **V8**-analog compound library. To further probe that a 3-ring system, as in **V8**, was essential for affinity, a set of 3 bicyclic derivatives was synthesized and biochemically evaluated. We also utilized the energy-minimized inhibitory complexes of Nek2, created by computational modeling, to investigate the key structural features responsible for Nek2 inhibition at the molecular level. Finally, using western-blot analysis, we showed that **V8** is cell-active and inhibits pAkt/PI3K signaling in live human A549 lung cancer cells in a dose-dependent manner. Consistently, it also reduces cell-proliferative and migratory phenotypes in both human MDA-MB-231 breast and A549 cancer cell lines.

To acquire a novel inhibitory chemotype for Nek2 kinase, we performed a HTVS of a 2.5 million compound library (ChemDiv Inc., USA and Hit2Lead Inc., USA) that was readily available for experimental evaluation. Schrodinger Maestro version 12 was used for the docking protocols that included sequential implementation of HTVS, SP, and XP modes for enhanced computational efficiency (See Supporting Information, Section I). Because each of the three docking modes follows a unique algorithm, identifying an appropriate Nek2 crystal structure from the existing PDB database was essential for accurate docking

prediction. This was accomplished by screening the twenty-eight available Nek2 structures in the PDB database for each docking mode and identifying the one where strongest linear negative correlation existed between the docking score and the reported IC₅₀ values. These efforts led to the selection of three crystal structures: 2XKF²⁹ (for HTVS mode), 4AFE³⁰ (for SP mode) and 2JAV³¹ (for XP Mode). The HTVS procedures were then implemented as depicted in Figure 2. A total of 1600 chemical structures were obtained after the XP mode of docking. A general physicochemical evaluation of the structures in combination with visual inspection, based on their drug-likeness, resulted in a set of 63 compounds. These were then subjected to further evaluation, based on novelty of the chemotypes, especially in the context of reported kinase inhibitors.

We finalized a list of eight compounds, obtained them from commercial vendors (ChemDiv Inc., USA and Hit2Lead Inc., USA) and evaluated them for biochemical inhibition against the active recombinant hNek2 kinase (SignalChem Inc., Canada). A previously utilized, commercially available ADP-Glo Kinase assay for small molecule inhibitory screen was used (for ADP-Glo assay protocol, see the Supporting Information, Section II).^{32, 33} This initial screen identified five Nek2 inhibitory compounds. Interestingly, three of these compounds activated Nek2 kinase, although to a different degree; this may warrant further biochemical investigation and was not currently pursued. The lead spirocyclic inhibitory compound, **V8**, was then subjected to a concentration-dependent inhibitory experiment. The data was plotted and fitted to a standard concentration-response curve using KaleidaGraph software (Version 4.5), yielding the IC₅₀ value of $2.4 \pm 0.2 \mu\text{M}$ (Figure 3A and Table 1). To confirm that **V8** was binding in the ATP-binding pocket of Nek2 kinase, [ATP] concentration was increased by 3-fold to $150 \mu\text{M}$ in the assays. As expected, the concentration-response curve shifted to the right, with the new IC₅₀ at $17.5 \pm 0.2 \mu\text{M}$ (Figure 3A and Table 1), thereby indicating that **V8** was a competitive inhibitor for ATP binding to the Nek2 active site pocket. We next evaluated the selectivity of inhibition. Given that obtaining selectivity for Nek2 inhibitors against other cell cycle kinases have proven challenging, we assessed **V8**'s activity against CDK1, Plk1, and Aurora A kinases.^{31, 34} Furthermore, we included Nek7, another member of NIMA family of kinase with functional overlap with Nek2 kinase in mitotic processes involving separation of centrosomes and appropriate arrangement of the microtubule-based mitotic spindle.³⁵ We also included EGFR enzyme, a receptor tyrosine kinase that has been shown to share inhibitor preferences with Nek2 kinase in the ATP binding site.¹⁴ A concentration-response curve was thus generated for each enzyme and the data fitted to obtain the IC₅₀ values (Figure 3B and Table 1). Analysis of the data revealed that **V8** was at least 9- to 13-fold selective towards Nek2 kinase over the other tested kinases. This suggested that **V8** is a preferred Nek2 inhibitory scaffold. To further corroborate our finding that (a) **V8** was indeed a valid lead inhibitory compound, and (b) a phenyl-substituted spirocyclic scaffold was generally well tolerated, we evaluated the inhibitory efficacies of fourteen structurally homologous compounds (Table 2; **V8-1** to **V8-14**). This study was anticipated to provide important information for the future during the scaffold optimization process. The inhibitory assessments of **V8** analogs revealed that potency was only modestly affected, IC₅₀ values increasing from 1.3-fold to 6-fold when compared to original hit **V8**, with substitutions at both the aromatic ring and methylsulfone group (see Table 2). To rationalize comparable

potency of **V8-1** to **V8-14** analogs to that of **V8**, we performed XP-Glide docking using Nek2 crystal structure (PDB ID: 2JAV).³¹ The XP-Glide scores for these analogs were in the range of -8.4 to -6.5 kcal/mol (see Table 2); this is consistent with the inhibition profile observed for **V8-1** to **V8-14** compounds. While replacement of the 4-bromine atom with a bulky 4-*tert*-butyl moiety on the phenyl ring decreased the inhibitory potency nearly 4-fold, smaller aliphatic substitutions, such as the 4-methyl group in **V8-2** and the 3,4-dimethyl group in **V8-3**, were less detrimental. In addition, cyclic substitutions on the sulfonyl group as in **V8-4** and **V8-9** and **V8-11** to **V8-14** were also somewhat well tolerated. Unsubstituted arylsulfone derivatives (**V8-5** to **V8-9**) generally showed comparable Nek2 inhibitory potency. Isosteric replacement of the sulfone group in **V8-6** with a carbonyl group led to **V8-10** with retention of potency. The **V8-6**, **V8-7**, and **V8-8** analogs showed a preference for strong electron-withdrawing group, fluoro, compared to the moderate electron-withdrawing chlorine and bromine atoms. Aliphatic substituents on the *para*-position of the phenylsulfone moiety (**V8-12**) are generally tolerated compared to 3,4-disubstituted (**V8-14**) or 2,4,6-trisubstituted (**V8-13**) analogs. To understand the importance of bromophenyl ring on the planar imidazolinone ring in Nek2 inhibition, we synthesized three compounds, **V0-1**, **V0-2**, and **V0-3**, and assessed their inhibition profile against Nek2 kinase (see Table 2). **V0-1** that lacks the bromophenyl ring showed >13 -fold loss of activity, compared to **V8**. A similar loss in activity was also noted for **V0-2** that lacked both bromophenyl ring and the methylsulfone group. The methylated derivative of the spiro-piperidine ring -NH- as in **V0-3** also led to a significant loss of potency. Substantial loss of Nek2 inhibitory activity by truncated analogs, **V0-1**, **V0-2**, and **V0-3**, are further corroborated with poor Glide scores (-6.2 to -3.8 kcal/mol). The SAR thus observed with this series of compounds indicates that phenyl-substituted spirocyclic **V8** scaffold may well be suited for further optimization to obtain potent and selective Nek2 inhibitors using structure-based design approach.

To understand the key interactions observed in the most potent analog **V8**, the moderately active **V8-14**, and the least active **V0-3** with the active site residues of Nek2 enzyme, the docked and energy minimized inhibitory complexes were analyzed at the molecular levels (Figure 4A-C). This analysis revealed that **V8** retained two key hinge-binding interactions: (i) The backbone -NH- from Cys89 formed a hydrogen bonding interaction with the carbonyl oxygen (C=O) of **V8**, and (ii) The backbone carbonyl oxygen (C=O) from Glu87 formed a hydrogen bond interaction with -NH- group of **V8**. Hinge-binding interactions are critically important and are present in majority of ATP-binding site directed Type I protein kinase inhibitors.³⁶ Other notable interaction exists where the sulfonyl oxygen (S=O) in **V8** forms an electrostatic interaction with the backbone -NH- of Asp159. Furthermore, the bromophenyl ring forms aromatic-aromatic and hydrophobic interactions respectively with the side chains of Tyr88 and Ile14. Finally, the methyl of the methanesulfonyl group snugly fits in a pocket lined by the T-helix residues such as Leu162, Ala163, and Leu166 (Figure 4A). These non-covalent interactions seen in the energy-minimized inhibitory complex of **V8**-Nek2 kinase support the experimentally observed inhibitory potency. Although moderately active **V8-14** analog preserved two hydrogen bonds with the hinge region and electrostatic interaction of the sulfone group with the backbone -NH- of Asp159 residue, its 3,4-dimethyl substituted phenyl ring formed unfavorable steric clash with the side chains of T-helix residues, Leu162, Ala163, and Leu166 (Figure 4B), thus explaining its moderate

activity. Similarly, although the least active **V0-3** showed two hydrogen bonds with the hinge residues in addition to the cation- π interaction between the piperidine ring -N- and Phe148, it lacked the key sulfone group and a *para*-substituted phenyl ring and could not fit into the T-helix to form favorable interactions with the side chain residues of the helix (Figure 4C). Collectively, it is thus inferred that a halogen substituted phenyl ring on imidazolinone scaffold and a spiropiperidine ring with methanesulfonyl moiety are key structural features responsible for Nek2 inhibition.

We have shown that **V8** can bind Nek2 kinase *in-silico* and inhibit its activity in *in-vitro* kinase assay. We next determined the efficacy of **V8** in a biological context in living cells. Our hypothesis was that if **V8** was a cell-active inhibitor, it is anticipated to downregulate Nek2-mediated signaling pathways. Using *Drosophila melanogaster* as an overexpression model system, we previously reported that Nek2 overabundance enhanced cell proliferative signaling via Akt/PI3 kinase pathway *in-vivo*.¹⁴ This finding was also corroborated by Zhou et al.¹⁰ We therefore first investigated if treatment of human A549 lung cancer cell line with **V8** would mitigate Akt/PI3 kinase signaling. Since phosphorylation of Akt at Ser-473 activates Akt/PI3 kinase signaling pathway, we investigated the effect of **V8** on the cellular levels of pAkt (Ser-473).³⁷ Western-blot analysis of **V8**-treated A549 cells (24 h) resulted in reduced levels of pAkt (Ser-473) in a dose-dependent manner (Figure 5). DMSO, and rac-cct 250863 - a commercially available potent inhibitor of Nek2 ($IC_{50} = 73$ nM) served as -ve and +ve controls respectively.³⁰ Since **V8** successfully downregulated pAkt/PI3 kinase signaling, we thus anticipated that cellular inhibition of Nek2 kinase by **V8** would also lead to enhanced cytotoxicity and reduced cell migration.^{8, 12, 14} To demonstrate this, highly aggressive human MDA-MB-231 breast cancer and A549 cell lines were chosen.²⁵ To assess cytotoxicity, cell viability was determined (see Supporting Information, Section III) using MTT assay.³⁸ **V8**-treated cells exhibited reduced cell viability in both human MDA-MB-231 breast cancer ($IC_{50} = 18.1 \pm 0.2$ μ M) and human A549 lung cancer ($IC_{50} = 22.7 \pm 0.2$ μ M) cell lines (Figure 6A). Further, to assess the effect of **V8** on cell's migratory behavior, wound-healing assay (see Supporting Information, Section IV) was performed on human A549 lung cancer cell line.³⁸ **V8**-treated A549 cells migrated slower than the vehicle control (Figure 6B); this is consistent with Nek2's role in promoting cell migration.^{39, 40}

In summary, using HTVS protocol we have identified a novel spirocyclic lead compound, **V8**, that inhibits the activity of human Nek2 ($IC_{50} = 2.4 \pm 0.2$ μ M). The mode of inhibition is competitive with respect to ATP, reversible, and selective. A modest SAR using structurally-similar **V8** analog library validates the inhibitory profile of **V8** and suggests that it may be readily amenable to the development of highly potent, selective, and cell-active Nek2 inhibitors. The synthesized truncated 2-ring analogs of **V8** (**V0-1**, **V0-2**, and **V0-3**) exhibit significantly lower affinity for Nek2 enzyme, further corroborating the observation that the 3-ring spirocyclic system in **V8** is required for effective inhibition. Molecular modeling studies involving the energy-minimized structure of Nek2 inhibitory complexes provide support for the observed inhibition profile. Finally, we show that **V8** inhibits pAkt/PI3K signaling pathway and has favorable anti-proliferative and anti-migratory phenotypes in two highly aggressive human MDA-MB-231 breast and human A549 lung cancer cell

lines. Future studies involving an expanded and systematic SAR should identify Nek2 inhibitors that are highly potent and selective with desirable cellular profile.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

Research reported in this publication was financially supported by the National Institutes of Health (NIH) under award number 1R15CA243109 to SKP. JYC was supported by NIH SC2 GM130470. SKP also gratefully acknowledges the support of Chemistry and Biochemistry Department and the Office of Dean of Math and Natural Sciences (MNS) of Queens College for their continued support.

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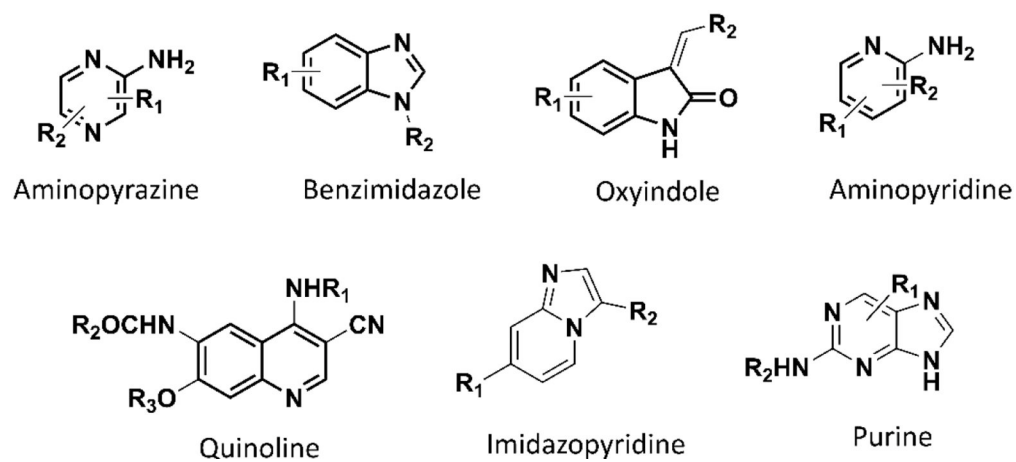


Figure 1.
The chemotypes utilized in the development of the reported small molecule inhibitors of Nek2 kinase activity.^{14, 29-31, 34, 41-45}

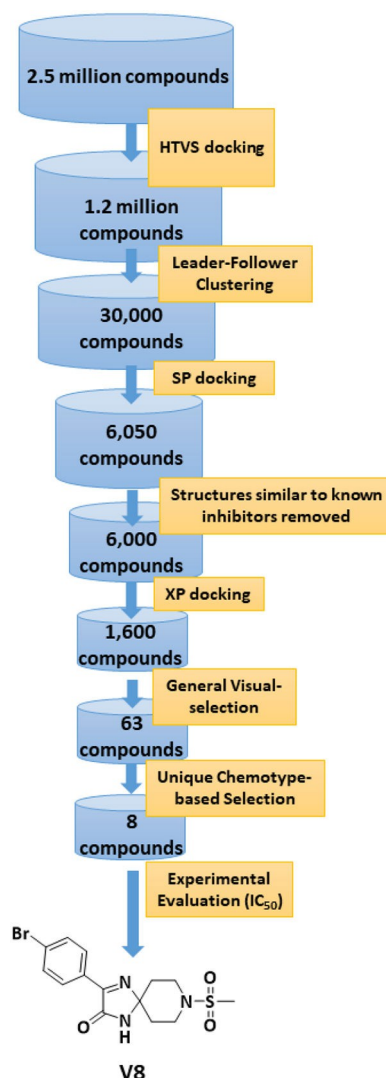


Figure 2. HTVS workflow implemented in the discovery of spirocyclic inhibitory Nek2 kinase chemotype, **V8**.

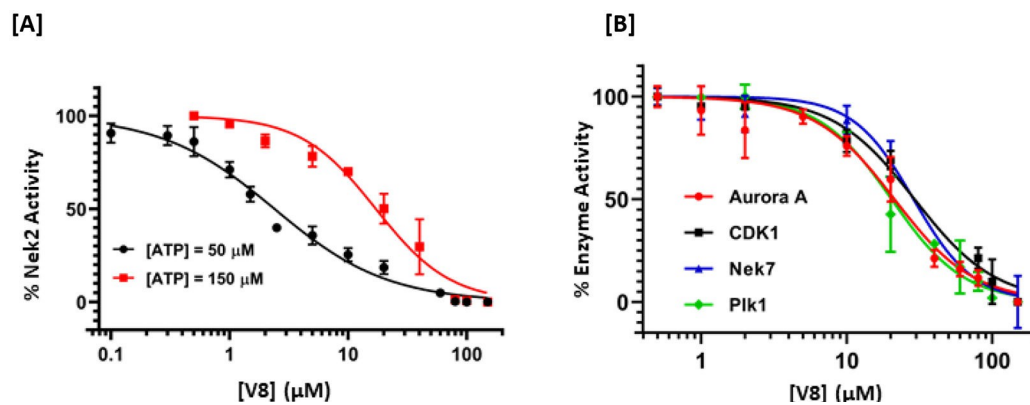
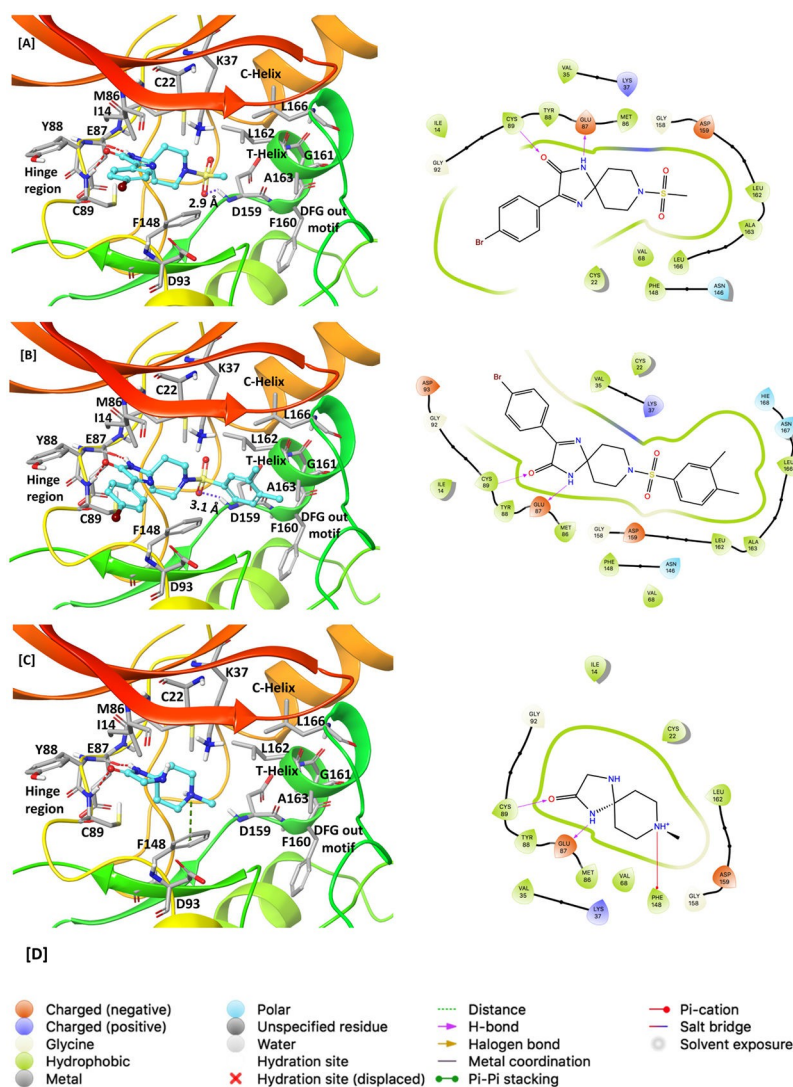


Figure 3.

Activity-based dose-response curves of **V8** against Nek2 kinase [A], and other related mitotic kinases [B], Plk1, Nek7, CDK1, and Aurora A. The points are experimental and the line joining them are the fit to obtain the IC₅₀ values.

**Figure 4.**

Comparison of molecular interactions of compounds **V8** ($IC_{50} = 2.4 \pm 0.2 \mu M$), **V8-14** ($IC_{50} = 12.3 \pm 1.2 \mu M$) and **V0-3** ($IC_{50} = 58.1 \pm 1.0 \mu M$) with the Nek2 active site. [A] Active-site view of the energy minimized complex of inhibitor **V8** docked into the ATP-binding pocket of Nek2 kinase (left) and its 2D-interaction map (right). Two key hinge-binding interactions are evident: The carbonyl oxygen (C=O) of **V8** formed a hydrogen-bonding interaction with amide backbone NH from Cys89, and NH of **V8** formed a hydrogen-bonding interaction with the backbone carbonyl oxygen (C=O) of Glu87 residue. In addition, the sulfonyl oxygen (S=O) of **V8** formed an electrostatic interaction with the backbone NH from Asp159 residue. In 3D-interaction maps (left): Dashed red lines indicate hydrogen bonds, the dashed purple line indicates an electrostatic interaction, and the dashed green line indicates a cation- π interaction; ligands are shown as ball and stick model whereas active site residues are shown in stick model. [B] 3D- (left) and 2D- (right) interaction map of the **V8-14** with the active site of Nek2 kinase [C] 3D- (left) and 2D- (right) interaction map of the **V0-3** with the active site of Nek2 kinase. [D] Legends for 2D-interaction maps.

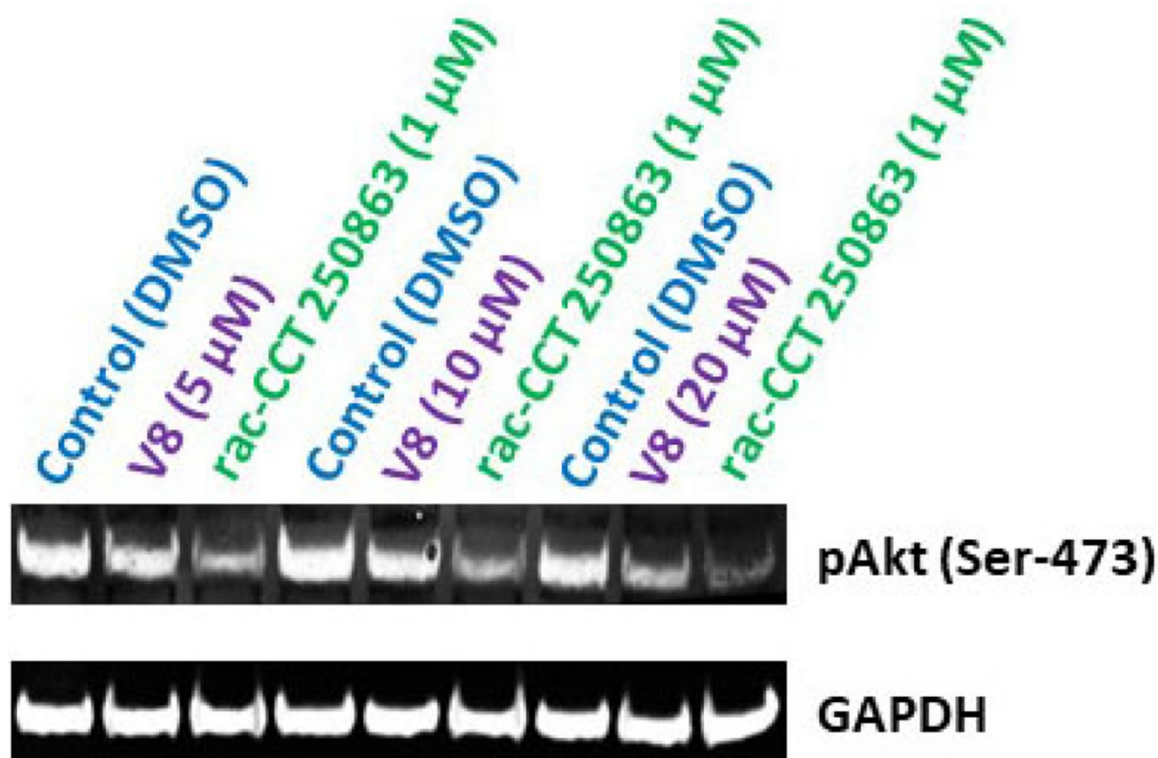
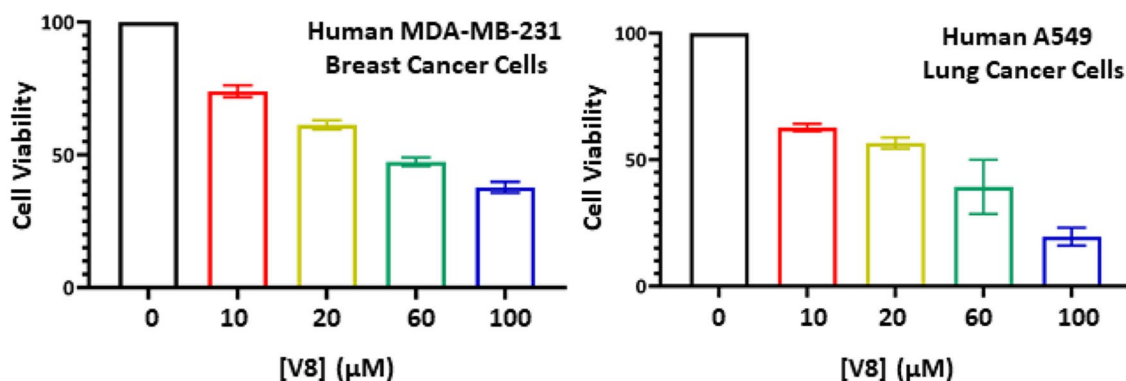


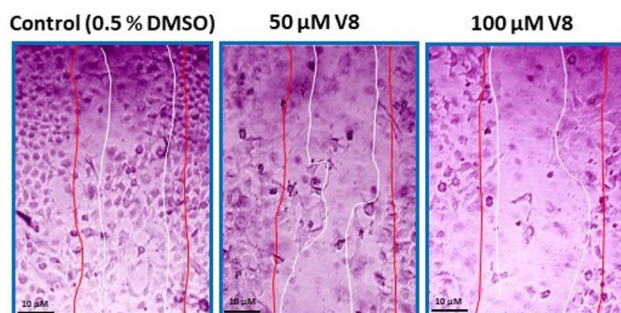
Figure 5.

Cellular effect of Nek2 inhibitor, **V8**, on pAkt levels in the human lung A549 cancer cell line. Western-blot analysis of A549 cells treated with **V8**. DMSO and a highly potent commercially available Nek2 inhibitor, **rac-cct 250863**, served as -ve and +ve control respectively. A dose-dependent level of pAkt is observed upon treatment of cells with **V8**, compared to the control, DMSO. GAPDH served as a loading control for protein normalization.

[A]



[B]

**Figure 6.**

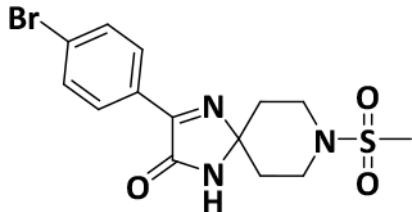
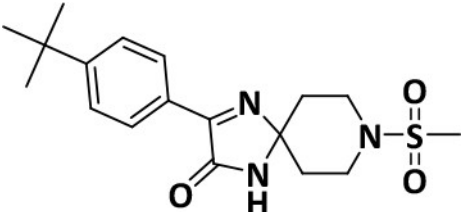
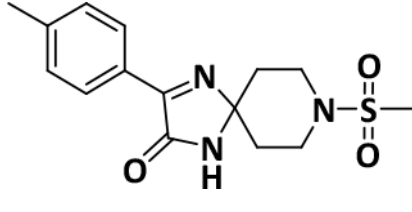
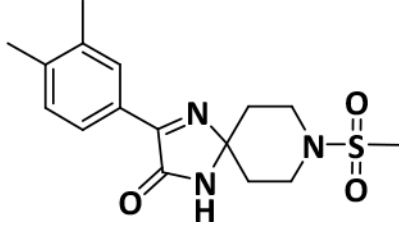
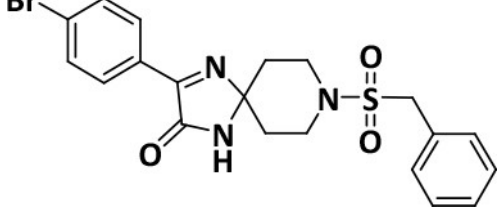
[A] Dose-dependent Effect of Nek2 inhibitor, **V8**, on cell viability in human MDA-MB-231 breast cancer (EC_{50} : $18.1 \pm 0.2 \mu\text{M}$) and human A549 lung cancer (EC_{50} : $22.7 \pm 0.2 \mu\text{M}$) cell lines. [B] Assessment of migratory behavior of human A549 lung cancer cells treated with **V8**. The pair of red lines represents the visual boundary at the initial scratch time ($t = 0$) while the pair of white lines represent the visual boundary at 24 hr post-treatment. Dose-dependent inhibition of cell migration is observed in **V8**-treated A549 cells.

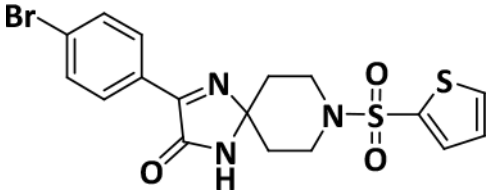
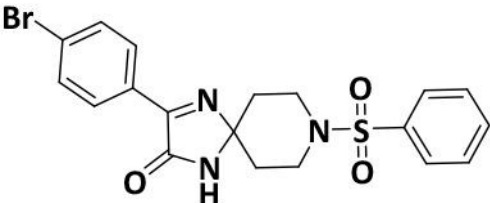
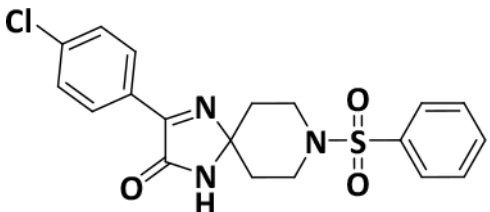
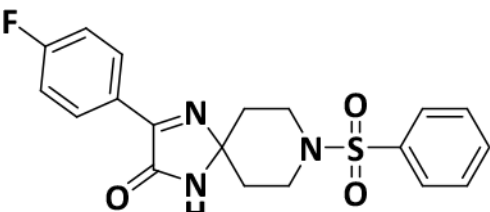
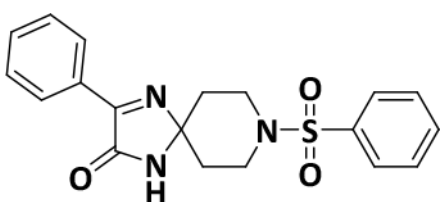
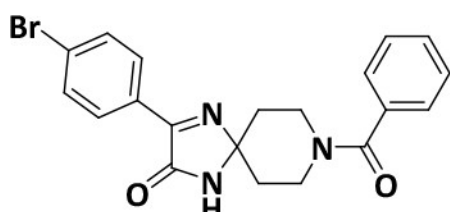
Table 1.Experimental IC₅₀ values of V8 against Nek2 kinase and other related mitotic kinases.

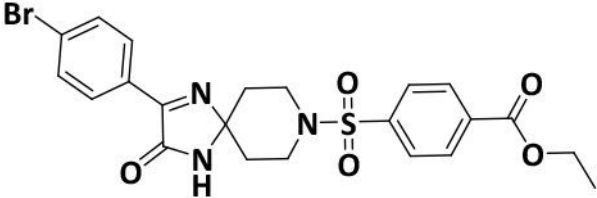
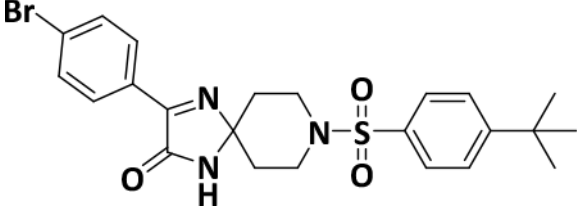
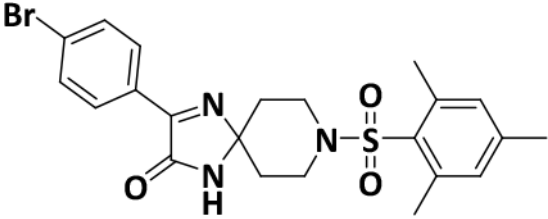
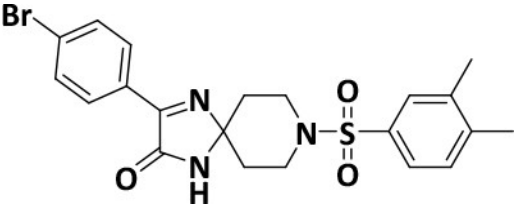
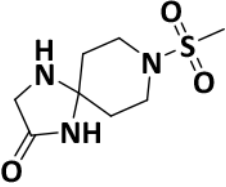
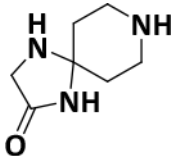
Enzymes	IC ₅₀ (μM) [ATP = 50 μM]
Nek2	2.4 ± 0.2
	*17.1 ± 0.2
Aurora A	21.3 ± 0.8
CDK1	29.3 ± 0.2
Nek7	28.8 ± 0.1
Plk1	20.4 ± 0.2

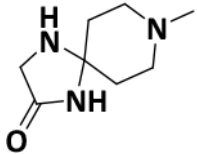
* At [ATP] = 150 μM

Table 2.Experimental IC₅₀ values and XP-Glide scores for V8 and V8-analogue compounds against hNek2 kinase.

Compound I.D.	Chemical Structure	IC ₅₀ (μ M)	XP-Glide Score (kcal/mol)
V8		2.4 $\pm$ 0.2	-7.9
V8-1		8.7 $\pm$ 0.2	-7.8
V8-2		4.9 $\pm$ 0.6	-7.9
V8-3		3.2 $\pm$ 0.1	-8.1
V8-4		6.8 $\pm$ 0.1	-8.0

Compound I.D.	Chemical Structure	IC ₅₀ (μM)	XP-Glide Score (kcal/mol)
V8-5		5.6 ± 0.1	-7.7
V8-6		9.3 ± 0.1	-7.9
V8-7		6.0 ± 0.1	-8.0
V8-8		4.5 ± 1.0	-8.4
V8-9		8.1 ± 1.0	-7.9
V8-10		9.2 ± 0.7	-6.5

Compound I.D.	Chemical Structure	IC ₅₀ (μM)	XP-Glide Score (kcal/mol)
V8-11		14.6 ± 1.0	-8.0
V8-12		5.0 ± 1.0	-7.3
V8-13		9.8 ± 1.3	-6.6
V8-14		12.3 ± 1.2	-7.2
V0-1		32.1 ± 0.6	-5.4
V0-2		32.5 ± 0.7	-6.2

Compound I.D.	Chemical Structure	IC ₅₀ (μM)	XP-Glide Score (kcal/mol)
V0-3		58.1 ± 1.0	-3.8