Structural implications of the DFD-in domain in computer-aided molecular design of MAP kinase interacting kinase 2 inhibitors.

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Implicaciones estructurales del dominio DFD-in en el diseño molecular de inhibidores de la proteína MAP kinase interacting kinase 2.

Implicacions estructurals del domini DFD-in en el disseny molecular d'inhibidors de la proteïna MAP kinase interacting kinase 2.

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SUMMARY

Protein translation is a key process on cell development and proliferation that is often deregulated in cancer. MAP kinase interacting kinases 1 and 2 (Mnk1/2) play a pivotal role in regulating the cap-dependent translation through phosphorylation of eIF4E transcription factor. Thus, Mnk1/2 targeting have been proposed as a novel therapeutic strategy that would minimize side-effects in contrast to other therapies. For this reason, there is a growing interest in designing *in silico* new Mnk1/2 inhibitors which demands from reliable structural models. Interestingly, the catalytic domain of Mnk proteins are characterized by a DFD motif instead of the characteristic DFG motif of other kinases. However, Mnk2 structural models described in literature are DFG mutated and do not contain the activation loop. Molecular design techniques have been applied to obtain a structural model of the full wildtype Mnk2 protein including the activation loop. The effect of the loop on the interaction mechanism of well-known ligands has been evaluated. Obtained results suggest that the presence of the activation loop is determinant for the correct prediction of the active site and it is essential for the design of new inhibitors.

Keywords: Drug design; MNK2 inhibition; molecular docking.

RESUMEN

La traducción de proteínas es un proceso clave para el desarrollo y la proliferación celular que se encuentra desregulado en muchos cánceres. Las proteínas MAP kinase interacting kinases 1 and 2 (Mnk1/2) juegan un papel fundamental en la traducción *cap*-dependiente regulando la fosforilación del factor eIF4E y se han postulado como una diana terapéutica de gran interés para intentar minimizar los efectos secundarios de las terapias convencionales. Por este motivo, hay un interés creciente en el diseño *in silico* de nuevos inhibidores de Mnk1/2 que, en consecuencia, requiere de modelos estructurales fiables. El dominio catalítico de las proteínas Mnk presenta un motivo DFD que sustituye el motivo DFG característico de las proteínas quinasas. Sin embargo, en el caso particular de la Mnk2, los modelos estructurales disponibles en la bibliografía presentan la mutación DFG y les falta el *loop* de activación. Mediante técnicas de diseño molecular se ha obtenido un modelo completo de la proteína Mnk2 *wildtype* que incluye el *loop* de activación y se ha evaluado el efecto de éste sobre el mecanismo de interacción de ligandos conocidos. Los resultados obtenidos indican que la presencia del *loop* de activación es determinante para la correcta identificación del centro activo y se considera esencial para el diseño de nuevos inhibidores.

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Palabras clave: Diseño de fármacos; inhibición de MN2; docking molecular.

RESUM

La traducció de proteïnes és un procés clau per al desenvolupament i la proliferació cel·lular que es troba desregulat en molts càncers. Les proteïnes MAP kinase interacting kinases 1 and 2 (Mnk1/2) juguen un paper fonamental en la traducció cap-depenent mediant la fosforilació del factor eIF4E i han esdevingut una diana terapèutica de gran interès per intentar minimitzar els efectes secundaris de teràpies convencionals. Per aquest motiu, hi ha un interès creixent en el disseny *in silico* de nous inhibidors de Mnk1/2 que, en conseqüència, requereix de models estructurals fiables. El domini catalític de les proteïnes Mnk presenta un motiu DFD que substitueix el característic motiu DFG de les proteïnes cinases. No obstant, en el cas particular de la Mnk2, els models estructurals disponibles en la bibliografia presenten la mutació DFG i els manca el *loop* d'activació. Mitjançant tècniques de disseny molecular s'ha obtingut un model complet de la proteïna Mnk2 *wildtype* que inclou el *loop* d'activació i s'ha avaluat l'efecte d'aquest sobre el mecanisme d'interacció de lligands coneguts. Els resultats obtinguts indiquen que la presència del *loop* d'activació és determinant per a la correcta identificació del centre actiu i es considera essencial pel disseny de nous inhibidors.

Paraules clau: Disseny de fàrmacs; inhibició de MNK2; docking molecular.

INTRODUCTION

Overexpression of the components of the translation initiation machinery has been correlated to malignant transformation. The limiting eukaryotic translation initiation factor 4E (eIF4E) was found to be up-regulated in most human cancers[1] and has been related to poor prognosis[2]. Overexpression of eIF4E increases the translation of mRNAs with a structured 5' untranslated region (5' UTR) which are usually translated with low efficiency as they include transcription factors, growth factors, receptors and tyrosine kinases[2-6].

The mitogen-activated protein kinase (MAPK) interacting protein kinases (Mnks 1/2) are serine/threonine kinases that regulate the activity of proteins involved in diverse cellular functions through phosphorylation. Mnk inhibition decreases the levels of phosphorylated eIF4E and attenuates the expression of mRNA involved in cell proliferation becoming a potential strategy for the treatment of many cancers. Interestingly, experimental studies on Mnk1/2 knockout mice conclude that eIF4E phosphorylation is crucial for tumorigenesis but dispensable for normal cell development[7].

Design of new inhibitors to target Mnk proteins has become an interesting starting point for search of novel cancer therapies. In this regard, molecular modelling techniques have risen as an effective strategy to guide the drug design process. Unfortunately, limited data is available on known inhibitors commonly used as reference for drug design (Figure 1). Moreover, these molecules are planar heterocyclic systems that mimic the adenine moiety of ATP in order to act as ATP competitors and, therefore, present a low specificity for Mnks. In view of the lack of ligand-based information, structural information about the receptor becomes mandatory to allow the application of structure-based drug design.

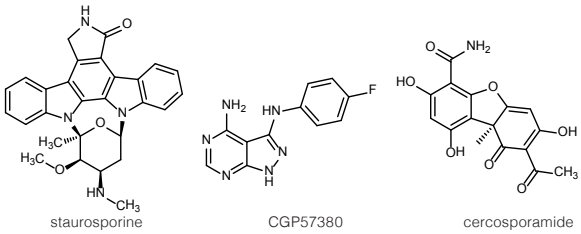

Figure 1. *Molecular structure of known Mnk1/2 inhibitors taken as reference in this study.*

Molecular structure of Mnk proteins has a strong resemblance to other protein kinases; they have a bilobed arrangement in which the N-terminal lobe contains the regulatory α-helix (αC), a twisted sheet of five antiparallel β-strands and a highly flexible glycine-rich loop which plays a key role in the ATP binding. The C-terminal lobe mainly consists on hydrophobic α-helical bundles and contains the elements required for phosphate transfer and for peptide substrate binding, including the activation loop. Interestingly, the ATP binding pocket is an hydrophobic cleft found abutting the hinge region. Both Mnk1/2 present a 80% identity of the active site despite the N-terminal lobe of Mnk1 is inclined 10° leaving a slightly more closed binding site[8]. However, Mnks present two unique features in contrast to tyrosine kinases[4, 11] consisting of (1) an specific insert in the catalytic domain (EAFSE in Mnk2) that seems to promote their activation or to guide the substrate recognition and (2) the replacement of the conserved DFG-motif for a particular DFD-motif (Figure 2).

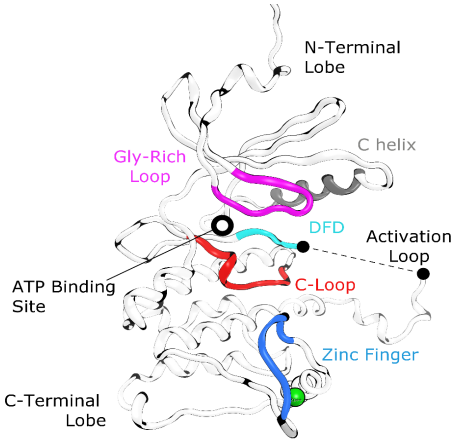

Figure 2. *General structure of the Mnk2 catalytic domain. The dotted line indicates that the activation loop has not been experimentally solved yet.*

DFD-motif is abutting the activation loop, which controls the binding of the ligand due to steric hindrance. In absence of ligands, Mnk2 adopts an inactive conformation (referred as the DFD-out conformation), sticking Phe227 into the ATP binding pocket and blocking the access to the binding site. When activated, the Phe227 leaves the hydrophobic pocket and flips clockwise about 180°. During this process, the salt bridge between Asp226 and Lys234 found on the DFD-out conformation is broken and a new salt bridge between Asp226 and Lys113 is formed (DFD-in conformation) [10].

To the best of our knowledge, the crystal structure of the wildtype (wt) Mnk2 protein presenting the DFD-in active conformation has not been described yet, and it is only available for the DFD-out conformation (PDB ID: 2AC3[9]). Most of the studies reported in literature are leaned on crystallographic structures of D228G Mnk2 mutants[12-15] (PDB ID: 2HW7[9], 2AC5[8]), considering that this mutation affects the conformation of Mnk2, but not the ATP binding and its kinase activity[8]. Noteworthy, none of the crystallographic models mentioned include information about the activation loop, thus it is usually neglected. This would lead to an over-simplistic model, particularly when molecular dynamics simulations suggested that DFD flip is directly associated with conformational changes in the activation loop[10, 16].

In this study we have applied molecular modeling techniques to generate an structural model of Mnk2 DFD-in conformation including the activation loop. Having available the three dimensional structure of Mnk2 protein allows the design of new inhibitors through the application of Structure-Based Drug Design (SBDD) methodologies. Moreover, the effect of the activation loop has been evaluated by predicting the interaction mechanism of well-known Mnk2 inhibitors (i.e. staurosporine, cercosporamide and CGP57380, see Figure 1) by means of molecular docking.

Computational details

Modelling the activation loop of the Mnk2 DFD-in conformation

Structure of the Mnk2 D228G mutant in the active conformation (PDB ID: 2HW7) was downloaded from the PDB and prepared by using Molecular Operating Environment (MOE2014.09[17]). Hydrogen atoms were added, minimized and protonation states were assigned, and crystallographic waters were removed.

This structure was used as reference to generate two additional structural models: (1) a model without the activation loop, obtained by manually removing the residues of the DFD-motif (D226-G228) included in 2HW7 and (2) a second model of the wildtype Mnk2 protein including the activation loop in the active conformation. To generate the latest model, residue G228 was de-mutated to recover the wildtype sequence. The tertiary structure of the activation loop (involving L229-C251 residues) was predicted *de novo* by applying the loop modeler module available in MOE. All calculations were conducted using the Amber12 forcefield. The best loop candidate was selected and energy minimized by molecular dynamics (MD) simulations, using AMBER software[18], to obtain the final model. Amber ff13 forcefield was used for the parameters of standard amino acids. The system was subjected to a first minimization including a 5000-step minimization of TIP3P water molecules followed by a 20000-step energy minimization of the entire system. The system was therefore heated to 300 K in 200 ps using the Langevin thermostat restraining the backbone atoms except the loop atoms using an 8 $kcal{\cdot}mol^{-1}{\cdot}Å^{-2}$ force constant. Pressure equilibration (1 atm) was performed for 1000 ps maintaining the restraints previously described. The SHAKE algorithm[19] was used throughout to restrain the bonds involving hydrogens and the Particle Mesh Ewald[20] method for long range electrostatic, while the short range interactions had a 10 Å cutoff radius. The production phase was extended to 20 ns defining a 2 fs time-step.

Molecular docking

Molecular docking was performed using AutoDock 4 and AutoDock Tools[21]on a 1.80 GHz Intel® Core™ i5-3337U processor with 4 GB RAM. A 126x126x126 Å grid was defined to perform a blind docking. Genetic Algorithms (GA) were used as docking search method, fixing the number of GA runs to 200, defining a population size of 500 and a maximum number of 2500000 evaluations. Evaluation of the results is performed by cluster analysis. Alternatively, MOE 2014.09 software was applied to include a flexible receptor into docking procedure (i.e. induced fit protocol). Docking poses are generated using the triangle matcher placement method (2000 poses) and scored using London ΔG scoring function and GBVI/WSA ΔG for rescoring (100 poses).

RESULTS AND DISCUSSION

Three models were considered for the evaluation of the activation loop effect: (1) the reported 2HW7 PDB structure, which contains the resolved structure for only few atoms of the activation loop and presents the DFG mutation, (2) the wildtype Mnk2 protein with the modelled loop and (3) 2HW7 without loop.

Two docking methods (rigid receptor and induced fit) were compared in order to discuss the relevance of the D228G mutation and the presence of the activation loop when studying the binding mechanism of drug candidates to Mnk2. For this purpose, we docked the reference compounds (i.e. staurosporine, cercosporamide and CGP57380, see figure 1) on the three Mnk2 models.

Molecular docking protocol was validated (RMSD < 0.25 Å) by predicting the binding mechanism of staurosporine and comparing the result to the crystal structure of staurosporine complexed to the Mnk2 D228G mutant structure available in the PDB (2HW7).

Rigid docking

According to the results, the recognition of the active site by the ligands could be conditioned by the presence of the activation loop when applying rigid docking and the protein structure was fixed.

Cluster analysis showed that staurosporine was able to mostly recognize the active site in all models, suggesting that the presence of the loop did not induce a remarkable effect on the location of the ligand. On the contrary, the presence of the activation loop hindered the access to the active site for cercosporamide and CGP57380. The combined effect of activation loop and the DFD motif conformations blocked the active site abutting the C-helix, moving their preferred binding site from the ATP binding site to the helix region at the C-terminal lobe (Figure 3).

From Figure 3 it can be seen that an appreciable percentage of conformations are located in the ATP binding site, regardless of the presence of the activation loop. Residues included in the active site were defined by proximity (4.5 Å) to the crystallized staurosporine-Mnk2 complex available in literature. Nevertheless, conformations located into the active site do not correspond to the best ranked conformations in terms of the scoring function and this could lead to confusing results.

Although staurosporine preferentially bounds to the ATP binding site, the spatial orientation of the most stable binding conformations predicted with and without the activation loop were slightly different (Figure 4). Structural differences could be appreciated when comparing the binding mechanism, obtaining two flipped conformations according to a symmetry axis, with comparable score.

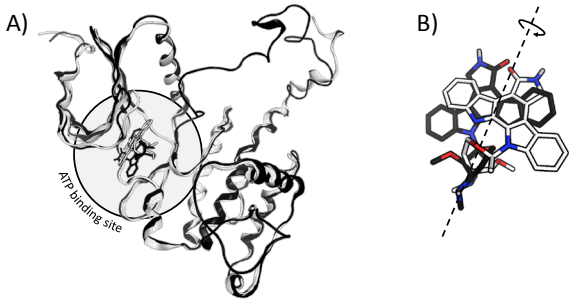

Figure 4. *Comparison between the most stable staurosporine conformations predicted by docking when considering the Mnk2 protein without activation loop (white) and containing a de novo modelled activation loop (black). (A) Position of the best ranked staurosporine conformations within the Mnk2 protein, corresponding to the active site. (B) Comparison between the spatial orientations of both conformations obtained.*

According to these results, the activation loop may have a pivotal role in drug recognition. Moreover, rigid docking did not allowed to obtain a correlation between the active site recognition and the scoring function value. This situation gives rise to unrealistic docking results and we hypothesized the need of including protein flexibility in the study.

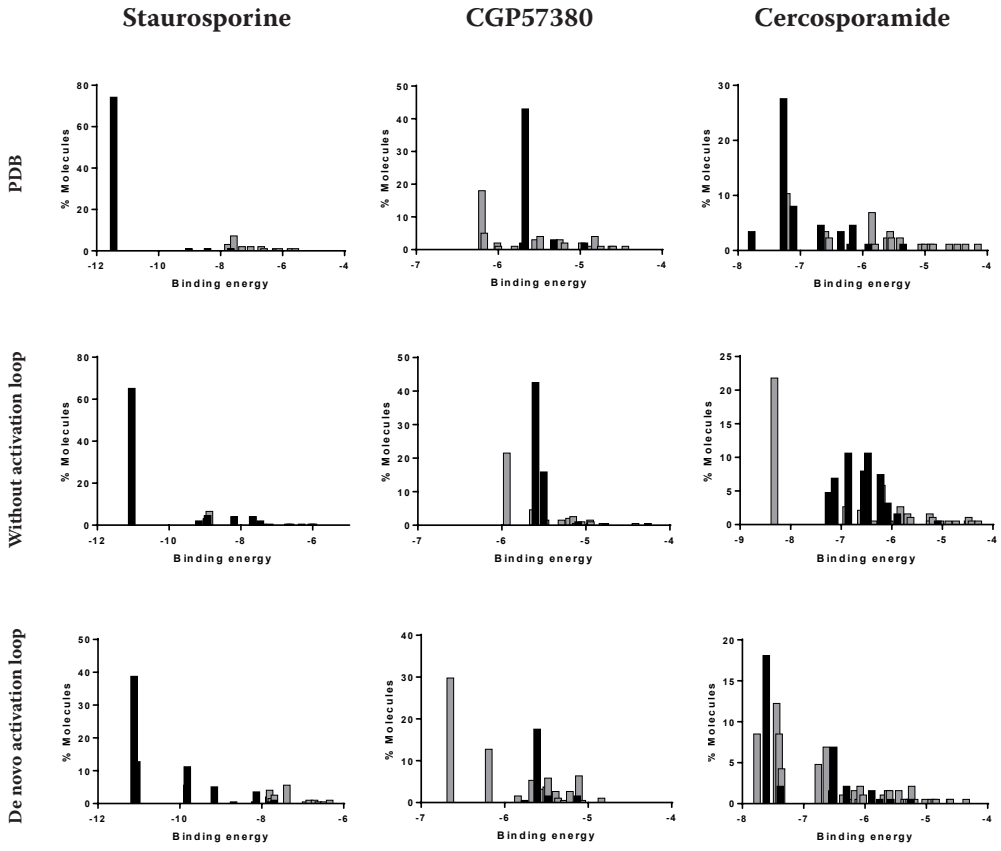

Figure 3. *Cluster analysis from rigid docking. Molecules are divided in two groups depending on where they bind: the active site (black) or other parts of the protein, usually the α-helixes (grey).*

Induced fit docking

Induced fit docking revealed that the flexibility of the activation loop may contribute to guide ligands towards the active site. Interestingly, reference compounds showed a similar behavior in all models when allowing receptor flexibility. Ligands tend to bind to the external part of the β-sheets in absence of the activation loop. When a small part of the loop is present (corresponding to the structure directly obtained from the PDB), more conformations are found in the helixes despite some conformations bind into the active site.

In the presence of the modelled loop a higher percentage of conformations end up in the ATP binding site. The modelled loop guides molecules to the Glycine rich loop and avoids binding to the C-helix. Interestingly, the conformations with the best score are those located in the ATP binding site and conformations with a lower score bind to helixes. The distribution of docking conformations obtained for each model is included in Figure 5.

Cluster analysis revealed that the model including the activation loop *de novo* modeled is the one that allows to lead the higher number of conformations with higher score within the active site (Table 1). The flexibility of the activation loop is determinant in order to obtain these results.

Table 1. *Percentage of molecules bound to the Mnk2 ATP binding site.*

	Staurosporine	CGP57380	Cercosporamide
PDB	28%	20%	12%
Without activation loop	18%	15%	11%
***De novo* activation loop**	33%	36%	22%

In order to make the visualization of these results easier, the three dimensional coordinates of each Mnk2 residue were projected into the H^2 hyperbolic plane. This procedure allows the representation of multidimensional points into a bidimensional space in which the distance between projected points increase exponentially with the radii by changing the metric definition (in this case, using Poincaré's disk model) [22]. Residues were colored according to the protein region and their positions were compared with the location of staurosporine conformations (Figure 6).

As expected, results showed that a higher number of staurosporine conformations are located close to the DFD motive and the ATP binding site. It is a worth of attention the effect of the activation loop on the Mnk2 projection, which modifies the conformation of the protein and induces a change into staurosporine distribution reducing binding affinity towards the β-sheets and the C-helix and concentrating conformations on the active site.

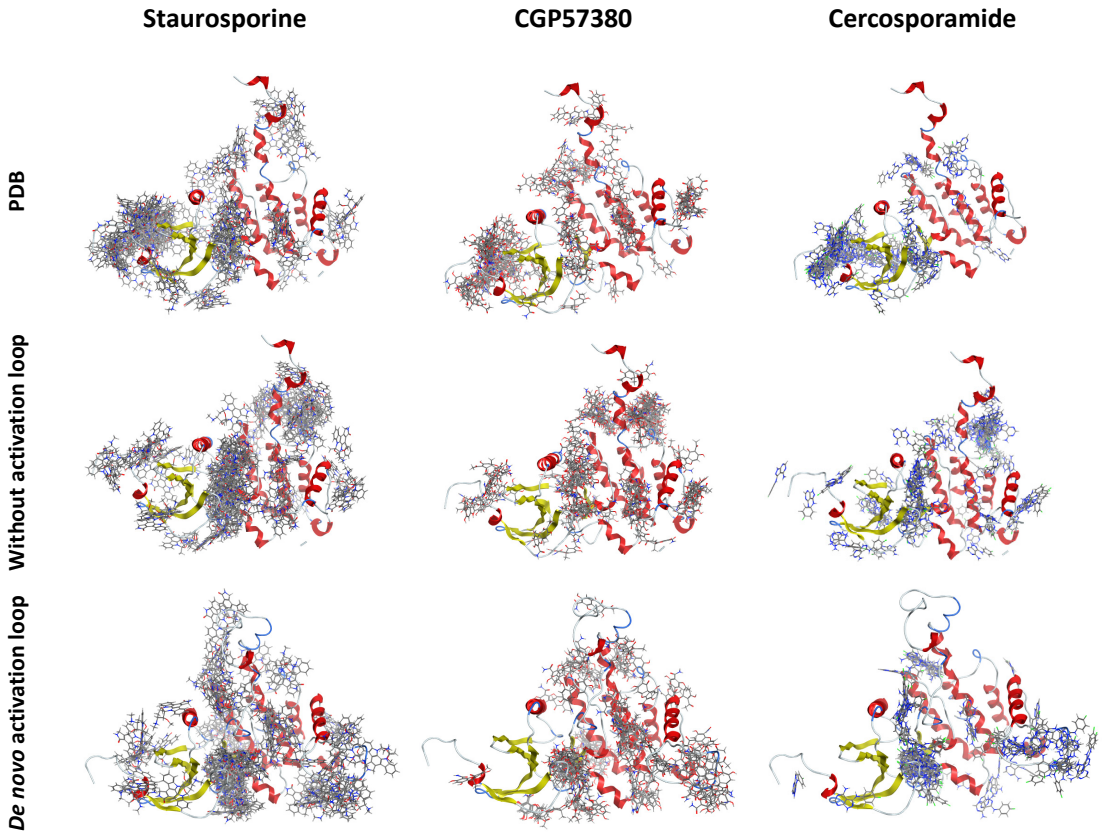

Figure 5. *Graphical representation of the conformations obtained by induced fit docking.*

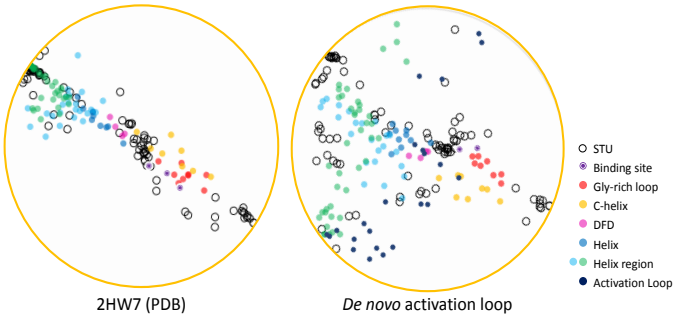

Figure 6. *Hyperbolic projection of Mnk2 residues resulting from flexible docking on PDB (2HW7) and the structure with the modelled loop.*

These results are in disagreement with methodologies commonly reported in literature for designing new Mnk2 inhibitors. Most of research articles directly apply molecular docking on the mutated Mnk2 structure available in the PDB. Our findings suggest that these models may be over simplistic and they could lead to unrealistic results. The presence of the activation loop and considering the flexibility of the receptor may be crucial for the correct description of the Mnk2-ligand complex and both should be considered when applying structure-based drug design techniques.

CONCLUSIONS

The effect of the activation loop on predicting the preferred binding site of Mnk2 inhibitors has been evaluated by molecular docking. The wildtype Mnk2 structure in the DFD-in conformation has been modeled by modifying the structure of Mnk2 D228G mutant available in the PDB. The three dimensional structure of the activation loop has been predicted computationally by *de novo* design methodologies.

The presence of the activation loop was essential to lead ligands towards the active site and receptor flexibility was required in order to obtain reliable results. Thus, obtained results suggest that the presence of the activation loop is determinant for the correct prediction of the active site of known ligands in docking procedures and it should be therefore considered in molecular design of new Mnk2 inhibitors.

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REFERENCES

1. Wheater, M. J.; Johnson, P. W.; Blaydes, J. P. The role of MNK proteins and eIF4E phosphorylation in breast cancer cell proliferation and survival. Cancer Biol. Ther. **2010**, 10, 728–735.
2. Ozretic, P.; Bisio, A.; Inga, A.; Levanat, S. The growing relevance of cap-independent translation initiation in cancer-related genes. Period. Biol. **2012**. 114, 471–478.
3. Diab, S.; Kumarasiri, M.; Yu, M.; Teo, T.; Proud, C.; Milne, R.; Wang, S. MAP Kinase-Interacting Kinases—Emerging Targets against Cancer. Chem. Biol. **2014**. 21, 441–452.
4. Hou, J.; Kam, F.; Proud, C.G.; Wang, S. Targeting Mnks for Cancer Therapy. Oncotarget. **2012**. 3, 118–131.
5. Furic, L.; Rong, L.; Larsson, O.; Koumakpayi, I.H.; Yoshida, K.; Brueschke, A.; Petroulakis, E.; et al. eIF4E phosphorylation promotes tumorigenesis and is associated with prostate cancer progression. Proc. Natl. Acad. Sci. **2010**, 107, 14134–14139.
6. Wendel, H.-G.; Silva, R.L.; Malina, A.; Mills, J.R.; Zhu, H.; Ueda, T.; Watanabe-Fukunaga, R.; et al. Dissecting eIF4E action in tumorigenesis. Genes Dev. **2007**, 21, 3232–3237.
7. Ueda, T.; Sasaki, M.; Elia, A.J.; Chio, I.I.; Hamada, K.; Fukunaga, R.; Mark, T.W. Combined deficiency for MAP kinase-interacting kinase 1 and 2 (Mnk1 and Mnk2) delays tumor development. Proc. Natl. Acad. Sci. U. S. A. **2010**, 107, 32, 13984–13990.
8. Jauch, R.; Jäkel, S.; Netter, C.; Schreiter, K.; Aicher, B.; Jäckle, H.; Wahl, M.C. Crystal Structures of the Mnk2 Kinase Domain Reveal an Inhibitory Conformation and a Zinc Binding Site. Structure. **2005** 13, 1559–1568.
9. Jauch, R.; Cho, M.; Jäckel, S.; Netter, C.; Schreiter, K.; Aicher, B.; Zweckstetter, M.; Jäckle, H.; Wahl, M.C. Mitogen-activated protein kinases interacting kinases are autoinhibited by a reprogrammed activation segment. EMBO J. **2006**, 25, 4020–4032.
10. Hou, J.; Teo, T.; Sykes, M. J.; Wang, S. Insights into the Importance of DFD-Motif and Insertion I1 in Stabilizing the DFD-Out Conformation of Mnk2 Kinase. ACS Med. Chem. Lett. **2013**, 4, 736–741.
11. Cargnello, M.; Roux, P.P. Activation and Function of the MAPKs and Their Substrates, the MAPK-Activated Protein Kinases. Microbiol. Mol. Biol. Rev. **2011**, 75, 50–83.
12. Teo, T.; Yang, Y.; Yu, M.; Basnet, S. K.; Gillam, T.; Hou, J.; Schmid, R.M.; Kumarasiri, M.; Diab, S.; Albrecht, H.; Sykes, M.J.; Wang, S. An integrated approach for discovery of highly potent and selective Mnk inhibitors: Screening, synthesis and SAR analysis. Eur. J. Med. Chem. **2015**, 103, 539–50.
13. Wu, H.; Wu, H. S.; Wang, A.; Weisberg, E.L.; Chen, Y.; Yun, C.; Wang, W.; Liu, Y.; et al. Disco-

very of a BTK/MNK dual inhibitor for lymphoma and leukemia. Leukemia. **2016**. 30, 173–181.
14. Yu, M.; Li, P.; Basnet, S.K.; Kumarasiri, M.; Diab, S.; Teo, T.; Albrecht, H.; Wang, S. Discovery of 4-(dihydropyridinon-3-yl)amino-5-methylthieno[2,3-d]pyrimidine derivatives as potent Mnk inhibitors: synthesis, structure–activity relationship analysis and biological evaluation. Eur. J. Med. Chem.**2015**. 95, 116–126.
15. Oyarzabal, J.; Zarich, N.; Albarran, M.I.; Palacios, I.; Urbano-Cuadrado, M.; Mateos, G.; Raymundo, I.; Rabal, O.; et al. Discovery of Mitogen-Activated Protein Kinase-Interacting Kinase 1 Inhibitors by a Comprehensive Fragment-Oriented Virtual Screening Approach. J. Med. Chem. **2010**, 53, 6618–6628.
16. Kumarasiri, M.; Teo, T.; Wang, S. Dynamical insights of Mnk2 kinase activation by phosphorylation to facilitate inhibitor discovery. Future Med. Chem.**2015**. 7, 91–102.
17. Chemical Computing Group Inc. Molecular Operating Environment (MOE). **2014**, 2014.09.
18. Case, D. A.; Berryman, J. T.; Betz, R. M.; Cai, Q.; Cerutti, D. S., Cheatham, T. E. AMBER 14. University of California, San Francisco, **2014**.
19. Ryckaert, J.P.; Ciccotti, G.; Berendsen, H.J. Numerical integration of the cartesian equations of motion of a system with constraints: molecular dynamics of n-alkanes. J. Comput. Phys.**1997**, 23, 327–341.
20. Darden, T.; York, D.; Pedersen, L. Particle mesh Ewald: An N ·log(N) method for Ewald sums in large systems. J. Chem. Phys. **1993**, 98, 10089–10092.
21. Morris, G. M.; Huey, R.; Lindstorm, W.; Sanner, M.F.; Belew, R.K.; Goodsell, D.S.; Olson, A.J. AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. J. Comput. Chem. **2009**, 30, 2785–2791.
22. Estrada, R.; Nonell, S.; Teixidó, J. Changing the way of viewing QSAR methods: the application of hyperbolic projection in medicinal chemistry. Rev. la Soc. Catalana Química. **2012**, 11, 61–67.