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Diversity oriented approach to triazole based peptidomimetics as mammalian sterile 20 kinase inhibitors†

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Protein kinases are important drug targets due to their involvement in several pathological conditions. Here, we demonstrate a strategy to develop small molecule inhibitors by using peptidomimetic compounds as a target for serine/threonine kinases. In this regard, we employ a unique approach to modify peptides by using a copper catalyzed 1,3-dipolar cycloaddition reaction between various azides and peptides containing an alkyne moiety to create a triazole based peptidomimetic library. Interestingly, these compounds have yielded competent kinase inhibitors, exhibiting an IC_{50} value of 1.2 μM for the pro-apoptotic mammalian sterile 20 1 (MST1) kinase, which is an important drug target in cardiomyopathy. Further, kinase profiling studies were performed with a panel of kinases both within the sterile 20 kinase family and also with PI-3K. Results revealed that compound **7** is a competent MST1 kinase inhibitor, which exhibits a high degree of selectivity and specificity for MST1 and does not inhibit the other tested kinases. In addition, docking studies shed light on the binding mode of these compounds. Hence, this knowledge can serve as a starting point for further development of triazole based peptidomimetics for designing selective serine/threonine kinase inhibitors.

Introduction

Kinases are important drug targets due to their role in cellular signaling.[1] Misregulation of the signaling cascade can lead to several diseases including cancer of different types, and many a times kinase inhibitors are employed as a potential cure for these diseases.[2] Over the years a plethora of small molecule kinase inhibitors have been developed, many of which exploit the conserved ATP binding signature present in these proteins for the development of potent inhibitors.[2] In several instances staurosporine, a generic kinase inhibitor, which mimics ATP binding, has been used as a starting scaffold to search the chemical space for optimal kinase inhibitors.[3–5] In other cases allosteric binding sites and non-catalytic sites have been identified to develop selective inhibitors for a particular kinase.[2,6,7] The problem faced by current research in the field of kinase inhibitors is to develop inhibitors that meet a balance between selectivity and potency without being toxic to the host.[8–10] Many of these small molecule inhibitors are promiscuous and inhibit several kinases. Nevertheless with proper screening and exploration of the chemical space, several such inhibitors have been successfully developed over the years and some of them like Gleevac, a drug for BCR-Abl-driven chronic myelogenous leukemia cancers, is now been made available as a pharmaceutical drug.[2,11]

In this work, we use triazole based peptidomimetics to develop MST1 kinase inhibitors. To date, the only known inhibitors of MST1 are staurosporine based organoruthenium compounds.[4] MST1 is a pro-apoptotic kinase and has two characterized physiological substrates: fork head box protein O (FoxO) transcription factor and histone H2B.[12–14] The MST1–FoxO signaling pathway is an important therapeutic target as misregulation of FoxO has been implicated in various diseases like cancer and diabetes mellitus.[15,16] On the other hand, H2B phosphorylation by MST1 is the first step towards initiating apoptosis.[12,17] Over expression of MST1 under environmental stress results in uncontrolled cell death which leads to ischemia. For example, over expression of MST1 in cardiac tissue has been reported to result in a condition known as dilated cardiac myopathy.[18] Hence recently, in an effort to control the damage caused by uncontrolled expression of MST1, various therapies like usage of wild type embryonic stem cells into MST1 transgenic blastocysts have been performed to control the onset of cardiomyopathy.[19,20]

In order to develop efficient MST1 kinase inhibitors, instead of only using the ATP binding site as a starting scaffold, we use a mix of the ATP and the peptide binding site as a target for

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† Electronic supplementary (ESI) information available: Spectroscopic data, ^{1}H NMR and ^{13}C NMR spectra for all the new compounds, Table S1 and Fig. S1. See DOI: 10.1039/c3ra44318c

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kinase inhibition. Several attempts have been made to develop peptide based inhibitors, as many kinases employ peptides as substrates and phosphorylate other amino acids like serine, threonine and tyrosine.[2,21] In particular, the triazole based peptidomimetics derivatives offer an interesting structural motif and have been widely used as inhibitors of various classes of enzymes ranging from protein phosphatases[22,23] to proteases[24] and apoptotic caspases.[25,26] The triazole scaffold has also been employed to generate high nanomolar kinase inhibitors for the Abelson (Abl) tyrosine kinase by combinatorially screening libraries by clicking an ADP–alkyne to a selection of azides and then further refining the hits.[27] Click chemistry approaches have in addition been employed to generate inhibitors in the range of 10–20 μM inhibitors for plasmodium falciparum protein kinase 7 (PfPK7).[28] As a first step towards the development of a dual ATP and peptide directing class of hybrid compounds as MST1 kinase inhibitors, we have synthesized a library of molecules using click chemistry[29–33] consisting of both peptide and triazole moieties (1,4-triazole-based peptidomimetics) which we have subsequently screened to identify potent inhibitors.[25,34–37] In addition, to establish selectivity of the identified inhibitor, a small scale profiling with a panel of kinases was also performed. Together these studies open doors for the future development of triazole based peptidomimetic compounds as effective serine/threonine kinase inhibitors.

Results and discussion

Scaffold design chemistry

To realize our synthetic strategy towards triazole based peptidomimetics, initially we tested our idea with simple mono-alkyne based peptides. In this regard, different mono-alkyne building blocks prepared in our group have been considered.[38] Various azides were prepared from the corresponding amines by using literature procedures.[39] Initially mono-alkyne **1a** was reacted with a simple phenyl azide to optimize the reaction conditions for Cu catalyzed cycloaddition (Scheme 1). Addition product **2** (Table S1†) was obtained in best yield when 0.2 equivalents of sodium ascorbate were used in the presence of 0.1 equivalents of $Cu(OAc)_2$. The formation of compound **2** was confirmed by complementary spectral data (IR, ^1H & ^{13}C NMR and HRMS). Along similar lines, different mono-alkynes were reacted with various azides to give the corresponding mono-triazole based peptidomimetics **2–9** (ESI Table S1†).

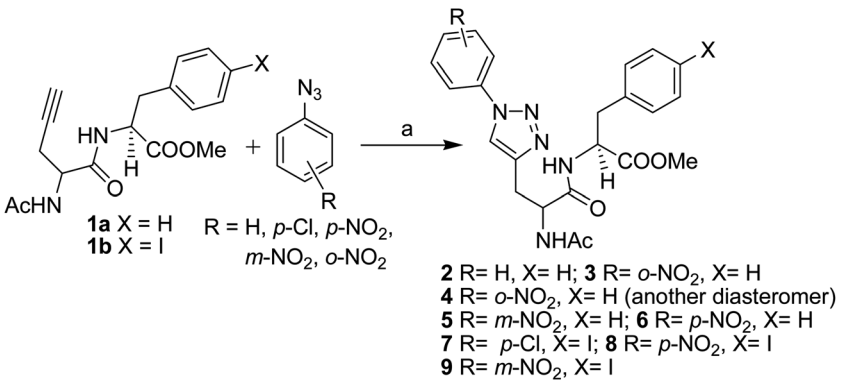
Having demonstrated the copper catalyzed cycloaddition reaction involving various mono-alkynes with different azides, the same protocol has been extended with dipeptides under similar conditions to generate di-triazole based peptidomimetics. Towards this goal, synthesis of the corresponding di-alkyne peptide was undertaken according to our earlier procedure.[40] After successful synthesis of a di-alkyne based peptide, attention was turned towards the final objective of modifying the peptide *via* a copper catalyzed cycloaddition reaction. The cycloaddition of di-peptide **10** was performed with various azides successively using the conditions developed in the case of mono-alkyne peptides to generate modified analogues (Scheme 2). Typically 66–99% yields of the addition product were obtained and the results are summarized in ESI Table S1.†

Later on, we extended this methodology to di-alkyne based tri-peptide **17**. In this regard, hydrolysis of the dipeptide **10** with 2 N NaOH solution gave the acid **16** derivative, which was used in the next step without further purification. Later, the acid was condensed with H-(L)-Ala-OMe·HCl in the presence of DCC–HOBt. Filtration of the crude product through a silica gel column with a ethyl acetate–petroleum ether gradient mixture, furnished the tripeptide **17** (Scheme 3). Having prepared the tripeptide **17** in good yield, then it was reacted with various azides in the presence of $Cu(OAc)_2$ and sodium ascorbate (Scheme 4) to deliver the di-triazole based peptidomimetics **18–20** (ESI Table S1†).

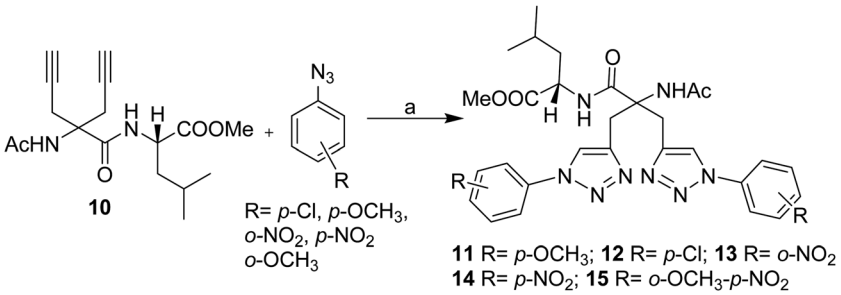
It is noteworthy to mention that no racemization of peptides was observed during the cycloaddition sequence of the various alkyne-based peptides with different azides in the presence of $Cu(OAc)_2$ and sodium ascorbate. Also, in all cases, the reaction was clean with good overall yield of the final products.

Rational for scaffold design

As described above to find a suitable inhibitor for MST1 kinase a hybrid library of various compounds consisting of mono- and di-triazole based peptides were screened. The synthesized di-peptide based unnatural amino acids consisting of



Scheme 1 Reagents and conditions: (a) $Cu(OAc)_2$, sodium ascorbate, *t*-BuOH–H_2O (1 : 1), rt.



Scheme 2 Reagents and conditions: (a) $Cu(OAc)_2$, sodium ascorbate, *t*-BuOH–H_2O (1 : 1), rt.

mono-triazole based compounds were designed as analogues of a fusion peptide system consisting of a substituted tyrosine like residue followed by an unnatural proline–tyrosine hybrid mimic of known amino acids. Thus the modified peptides mostly contained groups which are bulky and hydrophobic in nature and many of them had a benzene ring with additional substitutions such as a +R group (halogen, methoxy) and a −R group (nitro). The di-triazole, di-peptide series of compounds was more bulky and was designed as a peptide system with the first amino acid always being a leucine and the second being the di-triazole based unnatural amino acid that was subject to functionalization as shown in Scheme 2. Alanine and leucine are chosen for simplicity as they are the simplest chiral amino acids. The di-triazole based peptides consisted of two branched mono-triazole based proline–tyrosine hybrid like units tethered together. The di-triazole peptides were designed with an intension that they may provide a better binding site for the substrate. The tri-peptide based peptidomimetic compounds were structural extensions of the di-triazole, di-peptide series of compounds and consisted of an alanine residue N-terminal to the pre-existing di-peptide system. All the peptidomimetics were designed keeping in mind that the triazole moiety is the key unit, as triazoles are known to readily bind with various biological targets through hydrogen bonding and dipole–dipole interactions.

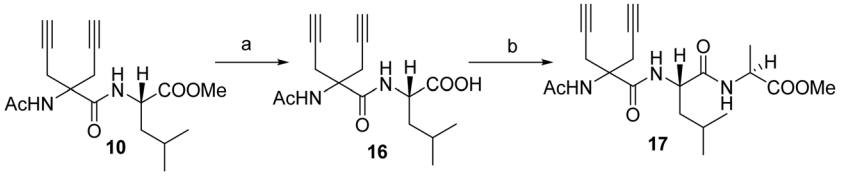
Biological activity

The radioactive enzyme assay was performed using 100 nM of MST1 kinase, 100 μM of ATP and histone H2B as the substrate, whose phosphorylation was monitored by SDS page electrophoresis.[4,12] An initial screening for all the 22 modified peptides was performed at 100 μM concentration.

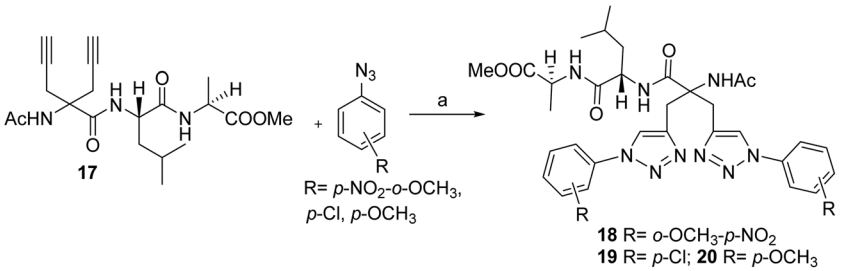
A list of various triazole based peptides tested for activity, along with their chemical structure are listed in ESI Table S1.† Among the compounds tested, six modified peptides inhibit enzyme activity greater than 50% at 100 μM concentration. The results are summarized in Fig. 1A as a histogram bar graph with percentage activity plotted against compound number.

Close examination of the structures showing kinase inhibition revealed that the presence of a nitrobenzene or a chlorobenzene substitution at the triazole end of the mono-triazole peptidomimetic enhanced their ability to inhibit MST1 kinase, with the *para* substitution of the phenyl ring exhibiting enhanced inhibitory effect as compared to the *meta* substituted derivative. A similar trend was also observed in the di-substituted triazoles, with both *para* and *meta* substituted nitro or halogenated benzene modifications being identified as potential inhibitors in the first round of screening. Only one of the tri-peptides showed significant inhibition. This tripeptide also had a *para*-chloro substitution at the triazole end.

Among the six compounds which showed initial inhibitory effect at 100 μM, only compound numbers **7**, **8** and **9** demonstrate greater than 50% inhibition at 10 μM concentration. However, further testing at 1 μM showed that only compound **7** exhibits substantial inhibition at 1 μM (Fig. S1†). Due to the low inhibition potential demonstrated by **8** and **9** at 1 μM, these compounds were not deemed fit for further IC_{50} calculations (Fig. S1†). All of these compounds, **7**, **8** and **9** which were found to be potential inhibitors are di-peptide based compounds that contain a mono-triazole moiety. Hence, it appears that di-peptide mono-triazole based inhibitors are better than larger mimics and branching of the alpha carbon to create di-triazoles decreases the inhibitory potential of this class of compounds. The data reveals that too many triazole substitutions or an increase in peptide length of the compound makes the substrate bulky and discourages its approach to the binding pocket of MST1 kinase. Furthermore, compound **7** with a *para*-chlorobenzene substituent, demonstrates good inhibition



Scheme 3 Reagents and conditions: (a) 2 N NaOH, MeOH, rt, 12 h, 98%; (b) H-(L)-Ala-OMe·HCl, DCC, HOBt, THF, NMM, DMF, rt, 24 h, 70%.



Scheme 4 Reagents and conditions: (a) $Cu(OAc)_2$, Na-ascorbate, *t*-BuOH–H_2O (1 : 1), rt.

around 50% even at 1 μM concentration. Subsequent IC_{50} studies were performed with compound 7 using a dose response curve and the results are shown in Fig. 1B. Compound 7 exhibits an IC_{50} value of 1.2 μM and is currently the best known inhibitor among these compounds. As reported in literature, MST1 kinases prefers peptide substrates with recognition sequence WYNTMKRR,[14,41] therefore it is possible that the triazole based modified di-peptide apart from occupying the ATP binding site protrudes into the hydrophobic space occupied by the first two amino acids WY in the recognition sequence of MST1. Thus, this prevents the native substrate from binding efficiently and results in enzyme inhibition.

Kinase profiling studies

To ascertain selectivity of the inhibitor, the most competent inhibitor, compound 7 was subject to a small scale kinase profiling study. Three serine/threonine kinases from the sterile 20 kinases were screened as representative kinases from the family for profiling. In addition, to check for general selectivity, the fourth serine/threonine kinase, phosphoinositide 3-kinase (PI-3K) was also screened, as it is an evolutionary divergent kinase and is not related to the Ste20 family. The three Ste20 kinases selected for study play an important role in various biological pathways like cell proliferation, apoptosis and stress responses. For example one of the Ste20 enzymes tested was

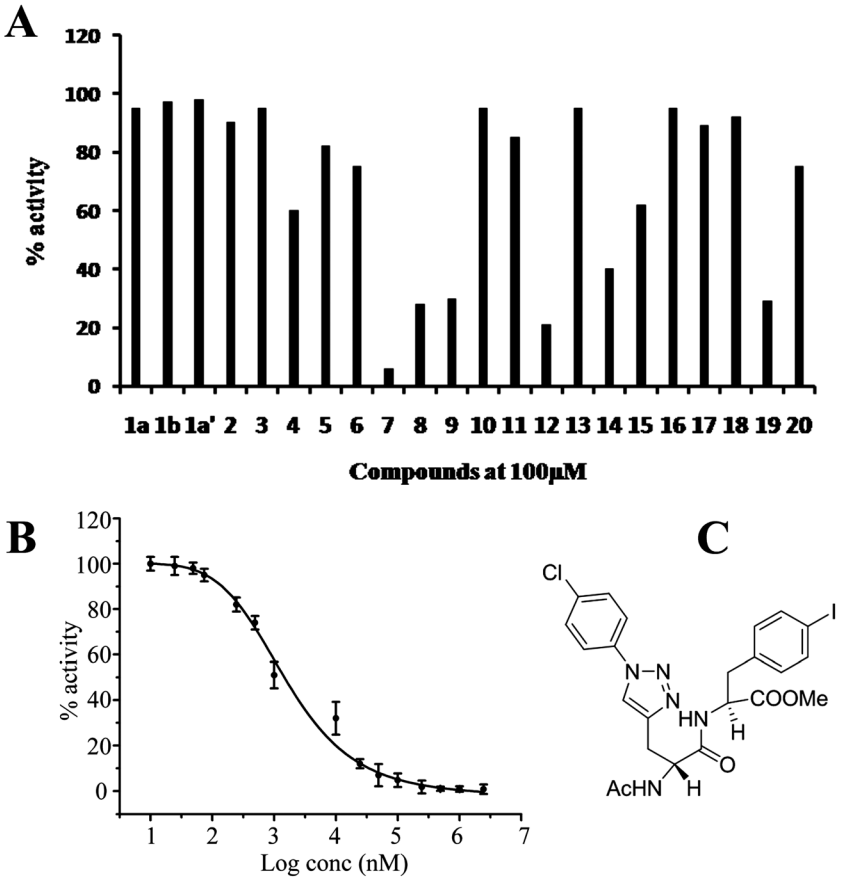


Fig. 1 (A) Screening of mono- and di-triazole based peptidomimetics against MST1 kinase. The histogram was plotted with % activity of MST1 towards H2B phosphorylation on the *y*-axis and compound number on the *X*-axis. (B) The IC_{50} curve for compound **7** against MST1 kinase. (C) Structure of compound **7**.

Hippo kinase (named after the "hippopotamus" like phenotype in animals), which regulates the pathway controlling organ size in animals and is also known to be a tumor suppressor.[42,43] The other two kinases screened from the Ste20 family were Ste20 like oxidative stress response (SOK)[44] kinase and TAO2 kinase.[45] Results shown in Fig. 2 demonstrate that compound 7 inhibits MST1 with a high degree of selectivity. SOK kinase only strongly inhibits effectively at an inhibitor concentration of 1 mM and has roughly 50% inhibition at 100 μM. However, by 10 μM concentration it exhibits no inhibition. In the case of TAO2 and Hippo kinase the selectivity is even more pronounced, no inhibition is seen even at very high concentrations. Therefore, we conclude that compound 7 is very selective towards MST1 and shows close to 100 fold selectivity within the Ste20 family members. To demonstrate that the inhibitor is selective towards unrelated kinases, an inhibition profile of compound 7 was also performed with PI-3K which is not related to the Ste20 kinase family. The result as depicted in Fig. 2 shows that compound 7 inhibits PI-3K only at very high inhibitor concentrations of 1 mM. Hence, compound 7 is more than 1000 fold selective towards MST1 as compared to PI-3K. This study creates a solid platform to initiate future work in the peptidomimetic space to improve the inhibition potency of MST1 kinase and also to design and use these compounds as inhibitors for other kinases. The aim is to create hybrid inhibitors which will block not only the ATP binding site but will also partially occupy the peptide binding site, thus providing handles to tune kinase specificity at both the sites.

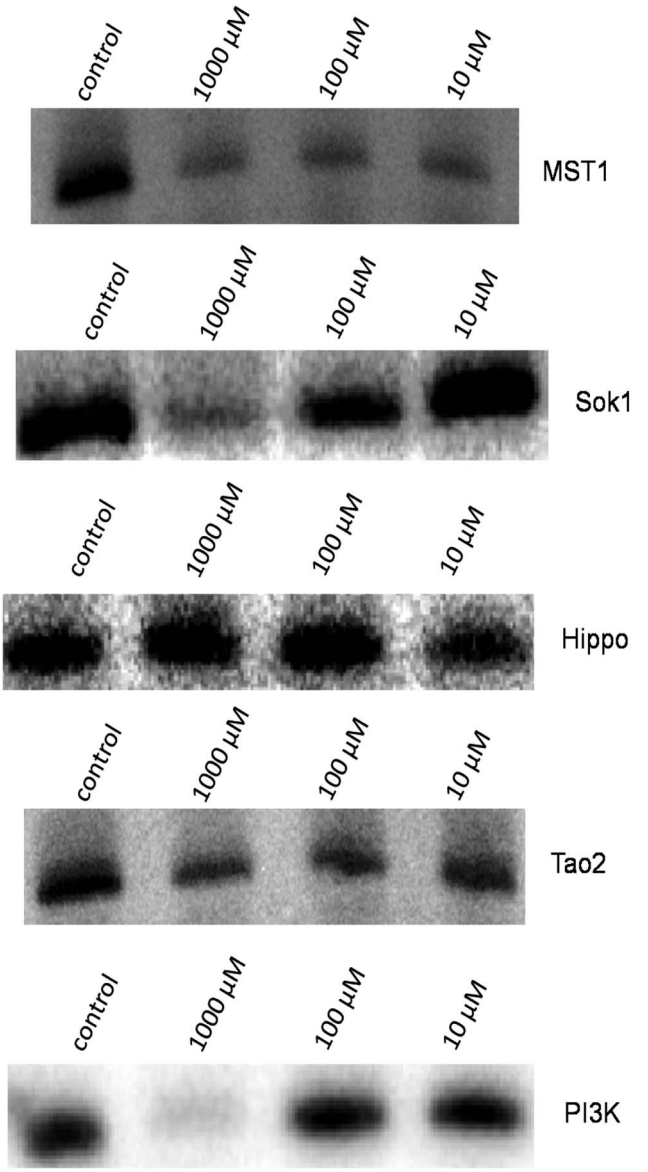


Fig. 2 Kinase profiling with compound **7**. Screening of compound **7** against different protein kinases belonging to the Ste20 family (MST1, Sok1, Hippo, and Tao2) and other kinase like PI-3K. The phosphorylation of the substrate H2B/Foxo by different kinases was shown in the absence (control) and in the presence of different concentrations of compound **7**.

Docking studies

To understand the mode of binding of compound 7 with MST1 kinase, computational docking using AUTODOCK version 4.2 (ref. 46) with the available crystal structure of human MST1 (PDB code 3COM) was performed. The importance of various structural groups that enhance kinase inhibition and to help in the design of future inhibitors, the structure was carefully studied. Docking results show that the compound occupies the active site cavity and its binding is stabilized by several hydrogen bonding and hydrophobic interactions as shown in Fig. 3A and B. The probable interactions of the compound 7 with the MST1 kinase active site residues are also shown as a ChemDraw picture (Fig. 3C). The model is able to explain the inhibition profile observed in Fig. 1A. The preference of monotriazole substitution can be attributed to the limited space for accommodating another triazole substitution, as this substitution will result in a clash with the outer wall of the enzyme pocket lined with residues Val86 and Leu156. Similarly, the bulky tripeptide moiety is too large and cannot be properly accommodated in the MST1 kinase binding pocket. The model predicts that an extension of the structure from a di to a tripeptidomimetics in the current configuration will result in a steric clash, thus disabling the compound from binding. This is reflected in the tripeptide showing weak inhibitory activity.

Inhibition assay results had demonstrated that although both nitro and halogenated substitution of the phenyl substitute triazole ring were important to confer kinase inhibition. Presence of chlorine is important for inhibition, since similar peptides where the chlorine is replaced by a nitro group showed decreased inhibition properties. Docking reveals that the active site pocket of the enzyme, occupied by the chlorine atom is capable of stabilizing the conformation by interaction of the electronegative halogen with various amino acids. The chlorine atom of the ligand makes hydrogen bonding contacts with the NH^1 atom of Arg115 and the O^{γ} atom of Ser111 respectively. Additionally in the model, it also seems to interact with the NH^2 moiety of Arg115, the O atom of Gly153 and the $O^{\delta 2}$ atom of Asp112. Thus the *para*-chloro substituted benzene ring is favored in this position as opposed to a more bulky nitro group, which is not only hard pressed for space but also possesses

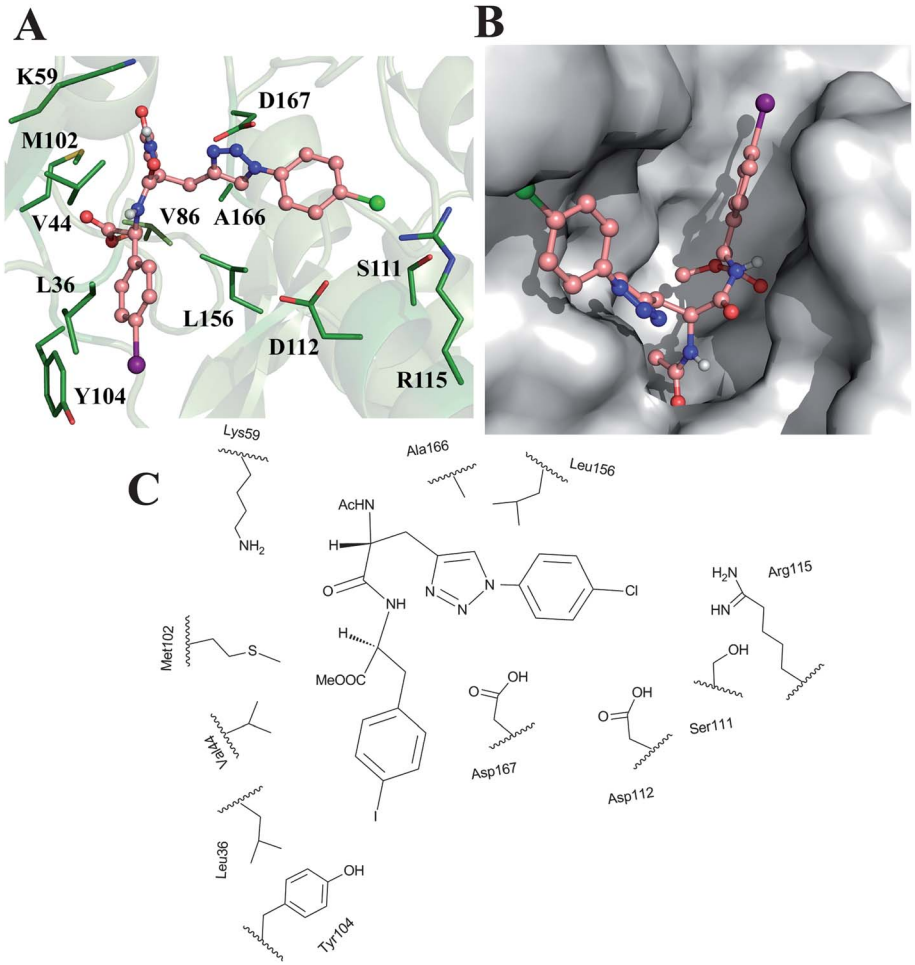


Fig. 3 Docking studies of compound **7** with MST1 1-311 (PDB code: 3COM). (A) Active site interactions of compound **7** in the binding pocket of MST1 kinase. (B) Surface representation of MST1 kinase active site with bound inhibitor. Figures were made using PyMol.[49] (C) ChemDraw picture showing the probable interactions of compound **7** with the MST1 kinase active site residues.

opposite stereo electronic properties and is electron deficient as compared to the halogen moiety.

There are some other groups like the N^{AS} atom of the ligand which is in close vicinity of the $O^{\delta 1}$ atom of Asp 167, the $N^{\delta 2}$ atom of the ligand with the $O^{\delta 2}$ atom of Asp 167 and the O^{AC} atom of the ligand with the N^{2} atom Lys59 that may stabilize the ligand in the binding pocket of MST1. In the current orientation, the ligand can also be stabilized by a lot of hydrophobic interactions with residues like Ala 166, Met102, Val44, Tyr104, Leu36, Leu156 and Val86 forming a hydrophobic pocket.

Conclusion

Here, we have demonstrated an exceptionally simple and efficient method for the synthesis of modified triazole-based peptidomimetics using a 1,3-dipolar cycloaddition reaction without the racemization. The synthetic methodology demonstrated here may open a new door for the synthesis of biologically active compounds. This is the first instance of serine/threonine kinases where C-α, α blocked triazole based peptidomimetics have been shown as low micro molar inhibitors. The advantage of using this class of compounds as potential kinase inhibitors is that on one hand we can design novel scaffolds using the peptide like substrate binding properties and on the other hand we can make them unique by tweaking their steric and electronic properties to suit the system of interest thereby creating inhibitors that are specific to a particular kinase. Therefore, this study has generated new possibilities for the design of future potent and selective peptidomimetics as kinase inhibitors.

Experimental section

General procedure for synthesis of mono-triazole based peptidomimetics

The mono alkyne precursor (1 mmol) was dissolved in *t*-BuOH–H_2O (3 : 3 mL) and the azide (1.1 mmol), $Cu(OAc)_2$ (0.1 mmol) and sodium ascorbate (0.2 mmol) were added. The resulting mixture was stirred at rt, until TLC indicated completion of the reaction. The mixture was diluted with ethyl acetate and washed with aq. NH_4OH (0.2%) and brine. The aqueous phases were extracted with ethyl acetate (2 × 10 mL). The combined organic extracts were dried over Na_2SO_4 and concentrated *in vacuo*. The

crude product was purified by flash chromatography on silica gel. Details of synthesis and corresponding characterization details are complied in the ESI.†

General procedure for synthesis of di-triazole based peptidomimetics

The alkyne precursor (1 mmol) was dissolved in *t*-BuOH–H_2O (3 : 3 mL) and the azide (2.2 mmol), $Cu(OAc)_2$ (0.2 mmol) and sodium ascorbate (0.4 mmol) were added. The resulting mixture was stirred at rt, until TLC indicated completion of the reaction. The mixture was diluted with ethyl acetate and washed with aq. NH_4OH (0.2%) and brine. The aqueous phases were extracted with ethyl acetate (2 × 10 mL). The combined organic extracts were dried over Na_2SO_4 and concentrated *in vacuo*. The crude product was purified by flash chromatography on silica gel. Details of synthesis and corresponding characterization details are complied in the ESI.†

Cloning expression and protein purification

Human MST1 kinase gene was cloned in the pachis-Tev baculoviral transfer vector.[12] The recombinant protein was expressed in Sf9 cells. All procedures on the protein including growth and purification were performed as described previously.[12] TAO2 kinase was also cloned in the above mentioned baculoviral transfer vector and was purified as mentioned previously.[45] Sok kinase and Hippo kinase were cloned in pET28a bacterial expression vectors and were expressed as 6X-His tag fusion protein in *E. coli* BL21 (DE3) cells and was purified using Qiagen Ni-NTA resin. The bound protein was eluted with 100 mM imidazole. The proteins were further purified by size exclusion chromatography using a superdex 200 column in 20 mM HEPES (pH 7.5), 100 mM NaCl. The protein fractions from the column are pooled together and concentrated to 5 mg mL^{-1}, flash frozen in liquid N_2 and stored at −80 °C until use. Histone H2B was purchased from New England Biolabs. FoxO was cloned in pETDuet-1 vector containing the gene for yeast SMT3, a ubiquitin-like protein of the SUMO family.[47,48] All procedures on the FoxO protein including growth and purification were performed as described previously.[12]

Kinase assay

In order to find the potential inhibitor for MST1 kinase enzyme, a library of small peptides were screened against the phosphorylation of histone H2B by MST1 kinase. The protein kinase assay was performed by measuring the incorporation of radio labelled γ-^{32}P into the protein histone H2B.[4] The initial screen of various compounds was performed first by incubating 100 nM MST full length enzyme in kinase buffer (50 mM Tris pH 7.5, 0.1 mM $MgCl_2$, 0.1 mM DTT) with 100 μM of the peptidomimetics for 30 min. Then the substrate histone H2B was added at a final concentration of 4 μM and the mixture was further incubated for 5 min. The reaction was started by adding 2 μCi of labelled γ-^{32}P ATP, 100 μM ATP was used in each reaction. After 30 min incubation, the reaction was stopped by adding 6 μL of 10× SDS loading dye and the samples were boiled for 5 min at 100 °C. 20 μL of the reaction volume was loaded in each well of SDS gel. The gel was transferred to the cassette for 1 h exposure and the signal was measured using a phosphoimager. The IC_{50} measurements for the best inhibitor was performed by keeping the enzyme and substrate concentration constant and varying the inhibitor concentration from 1 mM to 50 nM. The IC_{50} curve was plotted using origin 8.0 software and the sigmoidal curve fitting by logistic method.

Kinase profiling with compound **7** was done with four different kinases, Sok kinase, Hippo kinase, PI-3 kinase and TAO2 protein. The procedure for kinase assay is the same as mentioned above. Except TAO2, for the remaining kinases the activity assay was performed with H2B as a substrate and for TAO2, FoxO was used as the substrate.

Docking studies

To study the interactions of the peptidomimetics with MST1 kinase, docking studies were performed with compound **7** and MST1 kinase 1-311 (PDB 3COM) using Auto dock version 4.2 software and the docking calculations were performed using Lamarckian Genetic Algorithm, with a maximum of 2 500 000 energy evaluations. The grid box was selected in the active site region with the box dimensions 39.201, 57.077, 51.462 from the centre along *X*, *Y* and *Z* axes. Around 250 runs were performed and the best run was selected which showed a docking energy of −6.64 kcal mol^{-1}.

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