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Synthesis, molecular docking and anticancer studies of peptides and *iso*-peptides

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ABSTRACT

Chiral peptides and *iso*-peptides were synthesized in excellent yield by using benzotriazole mediated solution phase synthesis. Benzotriazole acted both as activating and leaving group, eliminating frequent use of protection and subsequent deprotection. The procedure was based on the hypothesis that epimerization should be suppressed in solution due to a faster coupling rate than SPPS. All the synthesized peptides complied with Lipinski's Ro5 except for the rotatable bonds. Inhibition of cell proliferation of cancer cell lines is one of the most commonly used methods to study the effectiveness of any anticancer agents. Synthesized peptides and *iso*-peptides were tested against three cancer cell lines (MCF-7, MDA-MB 231) to determine their anti-proliferative potential. NFkB was also determined. Molecular docking studies were also carried out to complement the experimental results.

A diverse arsenal of peptide based drugs have been developed for the treatment of cancer, viral infections, pain management, and other diseases.[1,2] The ubiquity of biologically active peptides and peptide derivatives has attracted attention of the synthetic community. In this context the 1923 Nobel Prize was awarded to Banting and Macleod for the discovery and extraction of insulin.[2] du Vigneaud, a Nobel laureate in chemistry, presented the first total solution phase synthesis of a naturally occurring bioactive octapeptide, oxytocin.[3–5] Peptides are now routinely prepared by chemical synthesis in solution or solid phase and are being used as therapeutic agents such as leuprolide acetate (Lupron™), octreodide acetate (Sandostatin™) and goserelin acetate (Zoladex™).[2,5]

Peptides are intrinsically able to interact with biological systems and are therefore potent therapeutics,[6–8] but their conformational flexibility, low ability to cross physiological barriers and metabolic instability, represent major hurdles for the successful development of peptide-based drugs.[9]

Advantages for purely synthetic peptides include, large scale preparation, incorporation of unnatural amino acid residues to improve their absorption–distribution–metabolism profile, limitless sequence variations and well-defined homogeneity.[10–12] New and improved strategies lead to more efficient synthesis of complex peptide targets, opening avenues to both new drug candidates and a deeper understanding of the intimate relation between sequence, conformation and properties.

Despite recent progress and the arsenal of reagents available, peptide synthesis remains challenging.

Benzotriazole emerged as a powerful synthetic tool in 1987.[13–16] Since then tremendous progress has been achieved in this field.[17] This solution phase benzotriazole mediated peptide coupling avoids both epimerization and hydrolysis due to fast coupling rate.

Peptides containing *iso*-peptide bonds are called *iso*-peptides. *iso*-Peptides are useful for the synthesis of large peptides and proteins. The *iso*-peptide method led to the efficient preparation and purification of large peptides, which are known to aggregate in solution. Significantly, the combination of both techniques, peptide and *iso*-peptide synthesis has advanced the frontiers of synthetic peptide chemistry. '*O*-Acyl *iso*-peptides' are more hydrophilic and easier to purify by HPLC than the corresponding native peptides.[18]

This study reports the synthesis of chiral peptides and *iso*-peptides by benzotriazole chemistry and their anticancer activity. Molecular docking studies against kinases completes the study.

N-(Pg-Aminoacyl)-benzotriazoles **3a–d** were readily prepared from commercial *N*-protected-amino acids **1a–e** following

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established procedures.[19,20] *N*-Protected dipeptides **5a–h** were synthesized in 87–90% yields by peptide coupling reactions of *N*-(Pg-aminoacyl)benzotriazoles **3a–b** with free amino acid in the presence of triethylamine in aqueous acetonitrile at 20 °C (Scheme 1, Table 1).

Protected dipeptides **5a–b** were treated with benzotriazole **2** in the presence of thionyl chloride in DCM at −20 °C for 5 h to obtain *N*-(Pg-dipeptidioyl)benzotriazoles **6a–b** in good yields (Scheme 2, Table 2).

Isotripeptides **7a–e** were synthesized by peptide coupling reactions between protected dipeptides **5c–f** and *N*-(Pg-aminoacyl)benzotriazoles **3c–d** in dry acetone in the presence of two equivalents of triethylamine for one hour at −10 °C in 70–82% yields (Scheme 3, Table 3).

Deprotection of the Cbz group of **7a–c** with C/Pd in ethanol under hydrogen gave unprotected isotripeptides **8a–c** in good yields (Scheme 4, Table 4).

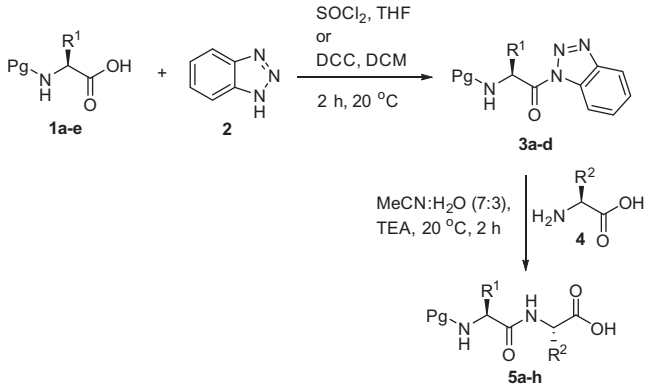
Peptides **5d**, **7d** and **8a** showed QR1 greater than 2 (Table SI 1). The cytotoxic potential of the synthesized peptides toward hormone responsive breast cancer cell line MCF-7 ATCC (HTB-22), and estrogen receptor negative breast cancer cell line MDA-MB-231 (ATCC, HTB-26) was determined and the results are shown in Table SI 1.

Inhibition of cell proliferation of cancer cell lines is one of the most commonly used methods to study the effectiveness of any anticancer agents. In the present study, three cancer cell lines (MCF-7, MDA-MB 231) were used to determine antiproliferative potential of selected samples. Both MDA-MB-231 and MCF-7 are cytotoxicity assays performed on breast cancer cell line. All assays were carried out at 20 μg/mL. In case of NFkB **5d**, and **6a** showed better inhibition.

Nuclear factor kappa-B (NFkB) is an inducible transcription factor that plays an important role in the regulation of apoptosis, cell differentiation, and cell migration. NFkB is commonly involved as a regulator of genes that control cell proliferation and cell survival. Many different types of human tumors have miss-regulated NFkB that is constitutively active. Thus, inhibition of NFkB signaling has a potential application for the prevention or treatment of cancer.[21,22] As NFkB is an important regulator in cell fate decisions, such as programmed cell death, proliferation control, and cell invasion, it is critical in tumor-genesis. Inhibition of NFkB signaling has potential applications for the prevention or treatment of cancer.[22,23]

In the quinoline reductase (QRI) assay,[24,25] after the initial testing of 22 samples, 3 samples were selected to determine CD values (concentration required to double the QRI activity). These samples showed either an induction ratio (IR) > 2 or cytotoxic activity with cell survival ⩽50% at 20 μg/mL.[24–26] In this study, 22 peptides showed various levels of inhibition of NFkB. Among them, the most potent **5d** and **6a** had ⩽50% inhibition at 20 μg/mL. Results are tabulated in Table SI 2.

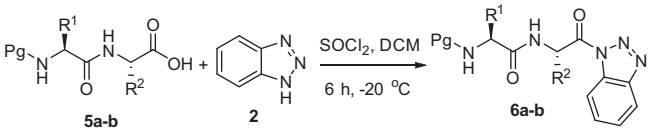
According to Lipinski's Ro5,[27] most drug like molecules have molecular weight ⩽500, logarithm of the octanol/water partition coefficient (log*P*) ⩽ 5, total polar surface area (TPSA) < 140 Å^2, number of hydrogen bond donors (HBD) ⩽ 5 and hydrogen bond acceptor (HBA) ⩽ 10.[27] Further modifications in the Ro5 were made by Veber et al. who suggested the number of rotatable bond (NOR) of a drug like molecule must be fewer or equal to 10.[28] Molecules violating more than one of these rules may have less bioavailability. These molecular descriptors were calculated for all the synthesized peptides, *iso* peptides and benzotriazolides by the ligand property calculation function of MOE (Table SI 3) and all of them were found to obey Lipinski's Ro5 cut-off limits, with the exception of number of rotatable bonds, revealing potent druglike compounds.



Scheme 1. Synthesis of dipeptides **5a–h**.

Table 1
Preparation of dipeptides **5a–h**

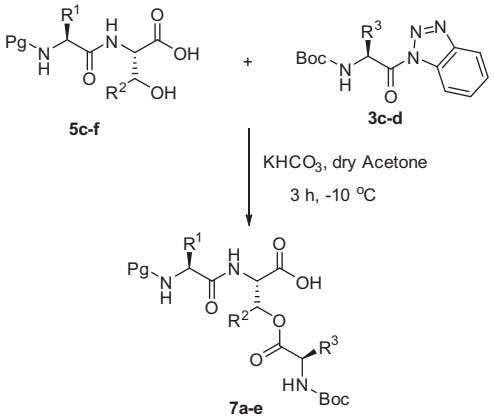
Entry	Compound **5**	Yield (%)	Mp (°C)	Lit mp (°C)
1	Cbz-L-Phe-Bt, **3a**	98	150–152	151–152[19]
2	Cbz-L-Ala-Bt, **3b**	95	109–110	114–115[19]
3	Boc-Gly-Bt, **3c**	94	69–70	68–69[20]
4	Boc-L-Ala-Bt, **3d**	94	84–86	85–86[20]
5	Cbz-L-Phe-Gly-OH, **5a**	89	164–165	163–165[20]
6	15-Cbz-L-Ala-L-Phe-OH, **5b**	88	142–144	141–143[20]
7	Cbz-L-Phe-L-Ser-OH, **5c**	90	140–141	140–141[19]
8	Cbz-L-Phe-L-Thr-OH, **5d**	89	146–148	
9	Cbz-L-Ala-L-Ser-OH, **5e**	80	192–194	192-194[19]
10	Cbz-L-Phe-D-Ser-OH, **5f**	87	191–193	
11	Cbz-L-Ala-D-Ser-OH, **5g**	89	200–202	
12	Cbz-L-Ala-L-Thr-OH, **5h**	88	203–204	



Scheme 2. Synthesis of *N*-(Pg-dipeptidioyl)benzotriazoles **6a–b**.

Table 2
Preparation of *N*-(Pg-dipeptidioyl)benzotriazoles **6a–b**

Entry	Compound **5**	Yield (%)	Mp (°C)	Lit mp (°C)
1	Cbz-L-Phe-Gly-Bt, **6a**	85	166–167	165–167[16]
2	Cbz-L-Ala-L-Phe-Bt, **6b**	86	148–149	148–149[19]



Scheme 3. Synthesis of *iso*-peptides **7a–e**.

Table 3
Preparation of *iso*peptides **7a–e**

Entry	Compound **5**	Yield (%)	Mp (°C)
1	Cbz-L-Phe-L-Ser(Boc-Gly)-OH, **7a**	80	86–87
2	Cbz-L-Phe-L-Thr(Boc-Gly)-OH, **7b**	82	65–66
3	Cbz-L-Phe-D-Ser(Boc-Gly)-OH, **7c**	80	67–68
4	Cbz-L-Ala-L-Ser(Boc-L-Ala)-OH, **7d**	70	92–94
5	Cbz-L-Ala-L-Ser(Boc-Gly)-OH, **7e**	79	90–91

Having studied chemo-preventive activities of peptides, molecular docking studies were carried out in search of a rationale for the lab results.

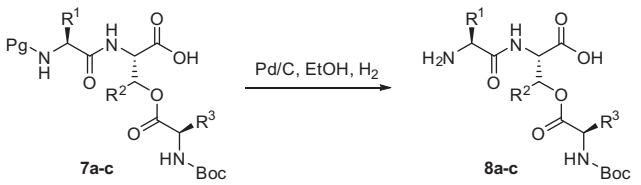
The receptor was cleaned of water molecules, 3D of the protonated and energy minimized by MMFF94[29] by making using MOE suite. The crystal structure of Kinases α and β were obtained from protein data bank (PDB code: 3BRT and 3BRV rec). Both IKKα, (PDB code: 3BRT) and IKKβ, (PDB code: 3BRV) comprise of four chains each as shown in Figure SI 2a and b. Ribbon and surface structure of both the IKKα, (PDB code: 3BRT) and IKKβ, (PDB code: 3BRV) are shown in Figure SI 1.

Since the 3BRT (1KKα) is devoid of a co-crystalized ligand so active site was indicated with the help of site finder module of MOE software by the creation of an alpha center followed by dummies as shown in Figure SI 2a and b. The active site isolated by selecting the longest of the four chains turned to be a deep cleft lined with key residues such as Gly730, Gly732, Phe734, Gly86, Glu89, Lys90, and Leu93. Alpha spheres were created inside the cleft in the form of a compact cluster, similarly dummy atoms were created at the site of alpha sphere to carry out docking on the dummy atoms as the receptor was devoid of its co-crystallized ligand.

3D structures of ligand molecules following the stereochemistry of the peptides by using builder module of the MOE program were drawn and energy minimized. Ligands were 3D protonated. Ligand preparation was by a comprehensive collection of tools, including 3D protonation and energy minimization by using MMFF94 with 0.001 iteration criterion.[29] This exercise generated lower energy, stable and accurate minimized 3D model with correct chiralities.

Detailed results of molecular docking are exhibited in Table 4. All the docked ligands clustered inside the pocket where the dummies were created as shown in Figure 1.

Figures 1 and SI 3 show overlaying of ligands inside active site, Figure SI 3 also exhibits the position of active site in heliacal structure of 3BRT. The dipeptide **5d** with binding energy (London dG −9.0400 kcal/mol) established three deterministic hydrogen bonds with Met734 and Lys90 inside the active site which might be a reason for its good activity. Moreover it also exhibited hydrophobic and polar interaction with key residues. Peptide **6a** also showed a couple of polar interactions and hydrophobic interactions with key residues inside the pocket and showed a binding energy (London dG −8.8484 kcal/mol) Both the active peptides **5d** and **6a** exhibited good binding score, mentioned in Table SI 5 of the Supporting information. Multiple polar and nonpolar interaction coupled with good binding energy could be the possible reason for peptide **5d** and **6a** to exhibit activity against 1KKα (Fig. 2 and Fig. 3).



Scheme 4. Synthesis of unprotected isotripeptides **8a–c**.

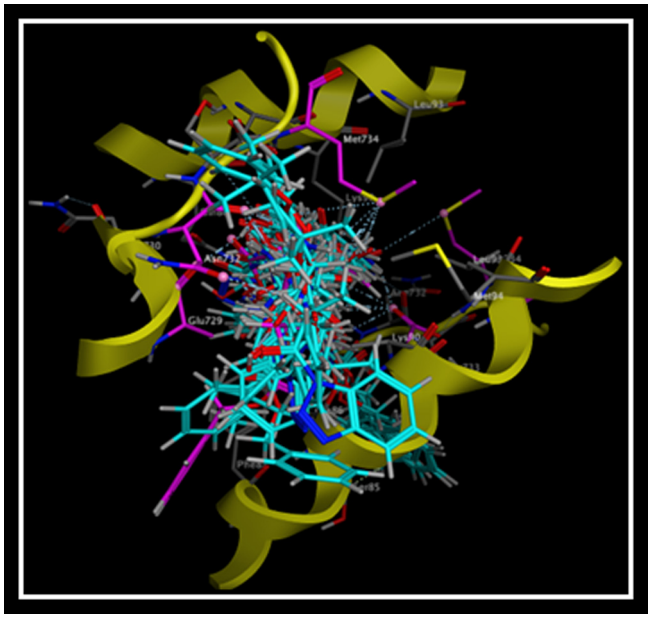


Figure 1. Overlaying of ligands inside cleft of 3BRT.

The protein is a 4-helix bundle of NEMO and IKKβ domains each consisting of two chains B, D and A, C respectively as shown in Figure SI 1. NEMO density extends from residues 49–109 in chain B and from 49–109 in chain D. The IKK peptide density extends from residues 705–743 in chain A and from residues 701–744 in chain C. N and C termini of NEMO chains B and D form dimerization. Three regions are assigned within the IKKβ peptide, designated as helical (705–731), linker (732–736), and the NEMO Binding Domain (NBD) (737–742). Residues 85–101 of dimeric NEMO forms a flat slit paving the way to two broad and extensive IKK binding pockets; each pocket being occupied by the IKK peptide linker and the NBD. The IKK peptide forms intermolecular hydrogen-bond interactions (Ser85:Q730 and Glu89:S733) with NEMO in the NEMO's specificity pocket. Three large IKK side chains inside the NEMO pocket which form consolidated intermolecular hydrophobic interactions (Leu93:F734, Phe92:T735, Met94: F734, Phe97:W739, Ala100:W741, and Arg101:W741) are responsible for formation of NEMO–IKKβ complex.

The active site isolated by selecting the longest chain of the entire four chains turnout to be a deep cleft lined with the key residues such as Gly730, Gly732, Phe734, Gly86, Glu89, Lys90, and Leu93. Alpha spheres were created inside the cleft in the form of a compact cluster, similarly dummy atoms were created at the site of alpha sphere to carry out docking around the dummy atoms as receptor was devoid of having its co-crystallized ligand. Both Figures SI 4a and b show the alpha center and dummies at the pocket of 3BRV (1KKβ) respectively.

All the ligand cluster compactly at the same position where dummies were created by site finder module of MOE suite in active site cleft of 3BRV (1KKβ), however ligand **8a** was found to be off set compared to all other ligands, some of its part seemed to be off lying and this could be a basis for its high binding energy which

Table 4
Preparation of unprotected isotripeptides **8a–c**

Entry	Compound **5**	Yield (%)	Mp (°C)
1	H-L-Phe-L-Ser(Boc-Gly)-OH, **8a**	85	170 (dec.)
2	H-L-Phe-L-Thr(Boc-Gly)-OH, **8b**	87	203–204
3	H-L-Ala-L-Ser(Boc-Gly)-OH, **8c**	85	189–190

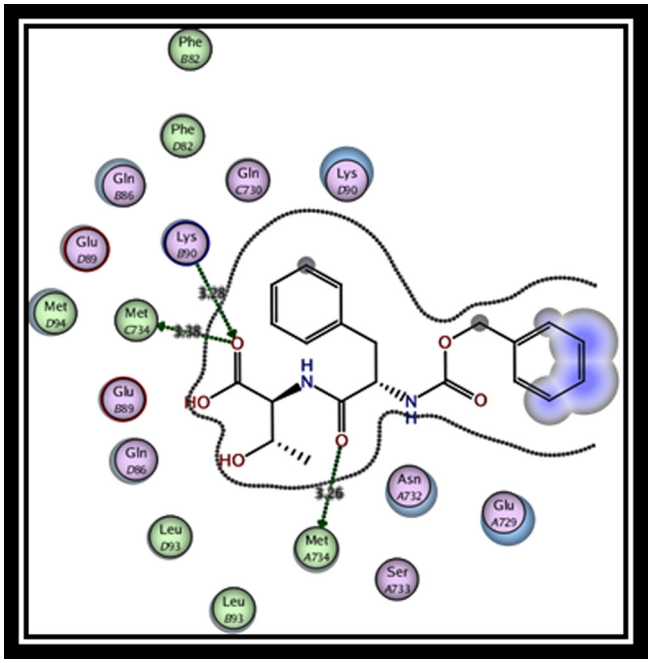


Figure 2. 2D interactions of **5d** with residues in active site (3BRT), dotted lines represents hydrogen bonding, purple in case of 3D and black in case of 2D interaction.

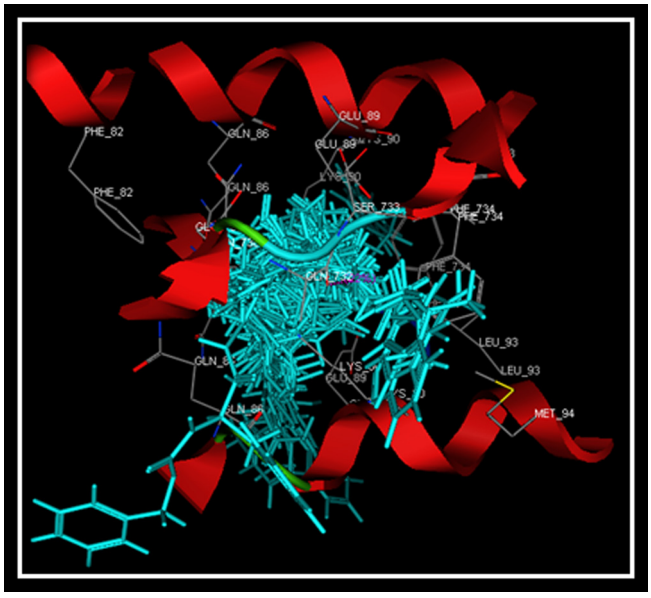


Figure 3. 3BRV (1KKβ), superimposition of ligands (cyan) inside active site cleft.

is −1.774 kcal/mol and quite high London dG (4.7566 kcal/mol). In addition to this ligand, both tri-peptides **7d** and **7e** also showed little higher binding score but they positioned inside the cleft during docking however not squashed as compared to other ligands. It is interesting to note that these compounds were found inactive in wet lab investigations against chemo-preventive assays hence theoretical results complement the experimental data.

Binding mode of ligands inside the active site cleft of 3BRV (1KKβ) was analyzed after docking simulation calculations was investigated. The top ranked docking pose being lowest energy conformation was selected for further analysis. Tremendous strong hydrogen bonding interactions were noticed between the NH groups of ligand **5d** and amino acid residues such as Gly730, Ser 85 and Gly 86 as evidenced from Figures 4 and 5.

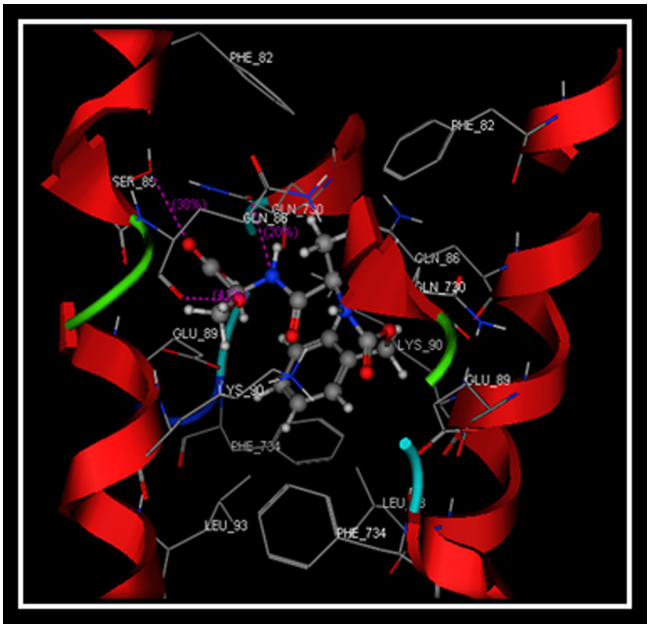


Figure 4. Docking pose of **5d** inside active site of 3BRV. 2D interactions of **5d** (dotted lines represents hydrogen bonding).

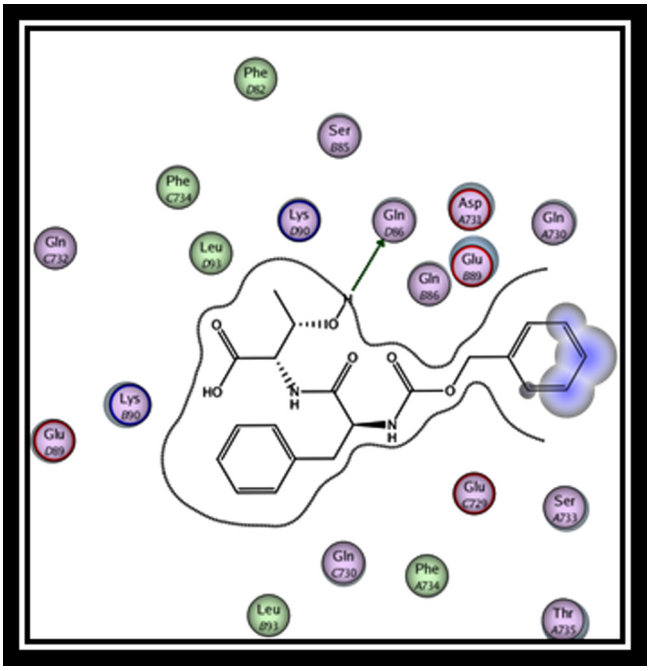


Figure 5. Docking pose of **5d** inside active site of 3BRV. 2D interactions of **5d** (dotted lines represents hydrogen bonding).

All these interactions were indication of strong binding of ligand **10** inside the active side cleft and in turn could be reason for its activity. Ligand **5d** showed low binding energy such as −8.6 kcal/mol (London dG), polar interactions and hydrogen bonding with key residues as shown in Figure SI 4, which might be the possible reason of its activity. Peptide **6a** established hydrogen bonding and also polar interaction with key residues which proclaimed to be possible reason for its activity. Table SI 5 depicts the detailed analysis of molecular docking of active triazoles with 3BRV.

Figure SI 5 shows the position of active site in the helical structure of 1kkβ and it also shows that all docked ligands clustered inside the pocket. Figures 4 and 5 exhibited the 3D and 2D interaction of **5d** with key residues in active site inside the active site.

These results demonstrated the in silico molecular docking studies of peptides with 1KKα and 1KKβ suggested that peptides possess the potential to disturb hydrophobic and H-bond interactions thereby affecting the stability of attachment of 1KKα and 1KKβ chains, and may be effective for other cancer cell lines.

In conclusion, we have synthesized various protected and unprotected dipeptides and *iso*-peptides using benzotriazole chemistry. Some of the dipeptides and *iso*-peptides show promising preliminary anticancer properties. The obtained results were justified by computational studies. These compounds could be used as precursor for developing potential anticancer agents.

Acknowledgments

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Supplementary data

Supplementary data (biological data, synthetic procedure, analysis data, anticancer and molecular docking studies) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.05.020.

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