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Facile synthesis of rapamycin-peptide conjugates as mTOR and Akt inhibitors†

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A simple and straightforward process for the synthesis of rapamycin peptide conjugates in a regio and chemoselective manner was developed. The methodology comprises the tagging of chemoselective functionalities to rapamycin and peptides which enables the conjugation of free peptides, without protecting the functionality of the side chain amino acids, in high yield and purity. From this methodology, we successfully conjugate free peptides containing up to 15 amino acids. Rapamycin is also conjugated to the peptides known for inhibiting the kinase activity of Akt protein. These conjugates act as dual target inhibitors and inhibit the kinase activity of both mTOR and Akt.

Rapamycin (sirolimus), a 31-membered macrocyclic polyketide produced by *Streptomyces rapamycinicus*, was first isolated in 1975 and was initially used as antifungal agent.[1–3] But later studies revealed its immunosuppressant,[4] neuroprotective/neuroregenerative[5–7] and anti-aging[8] activities. The potential of rapamycin to act as an antiproliferative agent was first recognized by Dr Suren Shegal and was sent for anti-tumor activity screening against the standard 60 human tumor cell lines of NCI.[9,10] After that, a lot of studies on the antiproliferative activity of rapamycin were done.[9,11–13]

Rapamycin is known as a potent inhibitor of the serine/threonine kinase mTOR (the mammalian target of rapamycin). The inhibition of mTOR in turn inhibits cell progression from the G1 to the S phase in most cells, and in some cells it induces p53-independent apoptosis.[14–16] mTOR forms two distinct complexes, named as mTORC1 and mTORC2, as a result of it binding with different regulatory proteins.[17,18] Rapamycin or rapamycin derivatives (rapalogs) are only partial inhibitors of mTOR and inhibit only mTORC1 and not mTORC2, which also plays an important role in cancer progression.[19–22] The inhibition of only mTORC1 by rapamycin and not mTORC2 in some tumors can stimulate PI3K/Akt signaling and antagonize its antitumor efficacy, leading to more aggressive tumor development.[23–25] This was recognized during preclinical studies in which treatment with rapamycin or rapalogs resulted in only minimal inhibition of tumor cell growth due to the increase in Akt signaling and PI3K activity.[26,27] To cope with this situation, several rapamycin or rapalog formulations have been made in which rapamycin was combined or conjugated with a PI3K inhibitor such as LY294002 or wortmannin, and the resulting combinations were shown to inhibit the Akt activation induced by rapamycin, resulting in the synergistic inhibition of tumor growth.[28,29]

Therefore, a number of strategies have been developed for the synthesis of new rapamycin derivatives, mainly involving substitution at the hydroxyl group of the 42-and/or 31 positions of rapamycin.[29–34] Recent efforts have been made to synthesize rapamycin peptide conjugates in which rapamycin is conjugated to peptides which are known for their antiproliferative activity. These rapamycin peptide conjugates were prepared by the reaction of 42-*O*-(4-nitrophenoxycarbonyl) rapamycin with a free amino group on the protected peptides known for their CDK (cyclin dependent kinase) inhibitory activity (Scheme 1a).[35] The resulting rapamycin peptide conjugates have the capacity to target the two protein kinases and act as dual target inhibitors. These rapamycin peptide conjugates were found to be more efficient than rapamycin in inhibiting cell proliferation. In spite of the better bioactivity, this method for the synthesis of rapamycin peptide conjugates has several drawbacks such as the use of only protected peptide fragments, tedious purification, low yield *etc*. Herein, we developed a simple and efficient methodology for the synthesis of rapamycin peptide conjugates. Subsequently, rapamycin was conjugated to known Akt inhibitory peptides to overcome the problem of the simultaneous activation of Akt by rapamycin.

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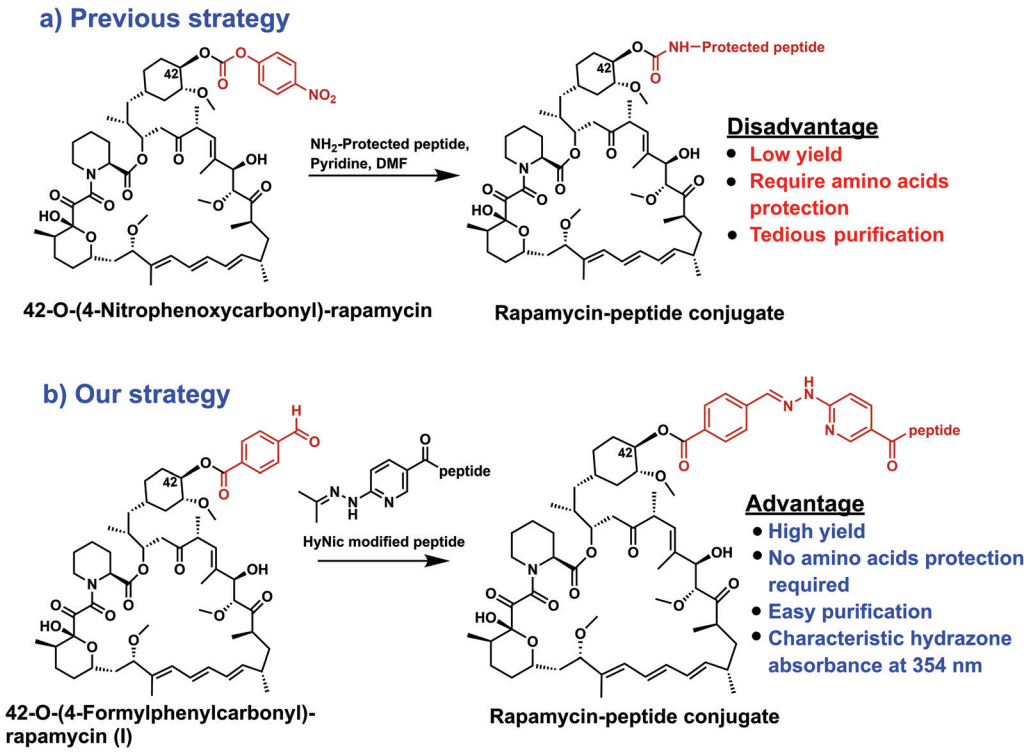
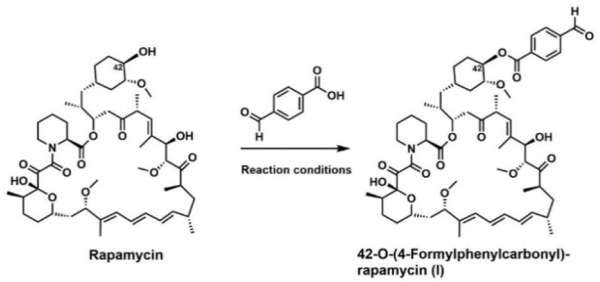


Scheme 1 Strategy for the synthesis of the rapamycin peptide conjugates.

Results and discussion

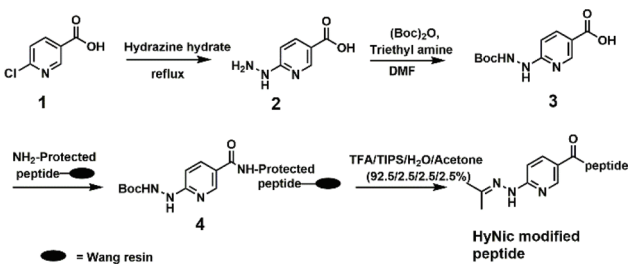
Rapamycin has a complex and delicate structure and the reagents and chemical conditions used for the earlier synthesis of rapamycin peptide conjugates restricts their further development, in spite of the potential shown by the rapamycin peptide conjugates. In view of this, we utilized hydrazone chemistry in line with Solulink bioconjugation technology[36] for the synthesis of rapamycin peptide conjugates, as shown in Scheme 1b. The synthesis required appropriately functionalized fragments, namely the aldehyde bearing component 42-*O*-(4-formylphenylcarbonyl)-rapamycin (I) first and the hydrazinonicotinyl (HyNic) modified peptide second.

The complex structure of rapamycin makes it difficult to introduce aldehyde groups in a selective manner. For this, we took 4-formyl benzoic acid, which was then regioselectively attached to the 42-hydroxyl group of rapamycin by forming an ester bond with the carboxylic group of 4-formyl benzoic acid under mild conditions (Scheme 2). For esterification we used different coupling reagents like HBTU and DCC with or without using DMAP in its catalytic amount. We got the best result with DCC/DMAP and the functionalization of rapamycin was successfully carried out by the regioselective acylation of the 42-hydroxy group of rapamycin with 4-formyl benzoic acid to form the corresponding 42-*O*-(4-formylphenylcarbonyl)-rapamycin in good yield and purity. We next prepared the hydrazinonicotinoyl modified peptide component by employing the solid phase method of peptide synthesis using Wang resin as the solid support (Scheme 3). The Boc-hydrazinonicotinic acid **3**

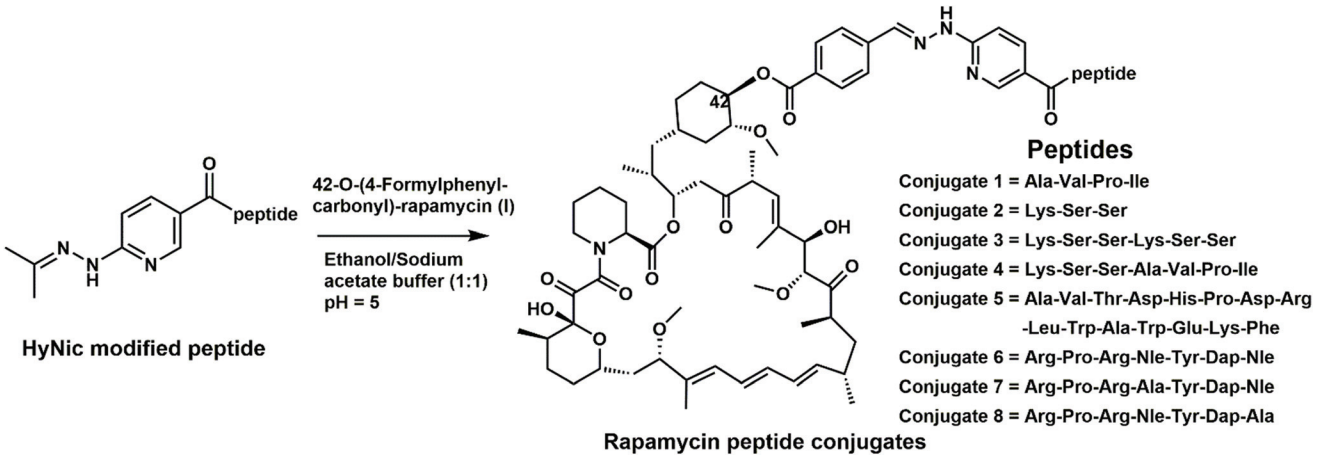


S.No.	Reaction conditions	% Yield of I
1.	DCC, DMAP(cat.), Dichloromethane 12 h	47.79
2.	HBTU, DIPEA, Acetonitrile, 12 h	5.7
3.	HBTU,DIPEA,DMAP(cat), Acetonitrile, 12 h	38.23

Scheme 2 Synthesis of 42-*O*-(4-formylphenylcarbonyl)-rapamycin.



Scheme 3 Synthesis of the hydrazinonicotinyl (HyNic) modified peptides.



Scheme 4 Synthesis of rapamycin peptide conjugates.

used in the synthesis of the hydrazinonicotinyl modified peptides was prepared from 6-chloronicotinic acid **1** by following the reported protocol.[37] The peptide was finally deprotected and cleaved by TFA/TIPS/acetone/water (92.5/2.5/2.5/2.5%) which gave the isopropylidenyl-hydrazinonicotinoyl tagged peptides. The addition of acetone in the peptide cleavage solution resulted in the protection of the hydrazine group as hydrazone, thus preventing hydrazide formation with trifluoroacetic acid.

Both the compounds have been synthesized separately, the peptide rapamycin conjugates were prepared by mixing stoichiometric ratios of these components in a pH 5 sodium acetate buffer and ethanol mixture for a couple of hours (Scheme 4). Initially, tetrapeptide AVPI, which contains only amino acids with no side chain functional groups, was conjugated to give conjugate **1**. Then, more complicated peptides were selected, having amino acids with amino and hydroxy group functionality in their side chains to give conjugates **2–4**. The reaction progress was monitored by HPLC and, after completion, the reaction mixture was subjected to purification by HPLC without additional workup to obtain the rapamycin peptide conjugates in almost quantitative yields. The progress of the formation of the conjugates was easily identified as the bis-aryl hydrazone bond formed during the conjugation reaction is highly chromophoric and has a characteristic absorbance at 354 nm (Fig. S9†). The conjugation chemistry used for the synthesis of the rapamycin conjugates is highly efficient and chemoselective, as demonstrated by the clean reaction despite the highly functional and complex nature of the peptides and rapamycin. One of the major advantages of this methodology is that the conjugation reaction was carried out using free peptides after the removal of the side chain protecting groups of the hydrazinonicotinyl tagged peptides to obtain ready to use conjugates after purification. The reaction conditions were extremely mild and did not require any reagent for conjugation.

Interestingly, after the conjugation of the peptides no apparent loss in the biological activity of rapamycin in the conjugates was observed in cell-based assays (Tables S1 and S2†). This encouraged us to conjugate rapamycin to the peptides known for inhibiting the kinase activity of Akt. Akt overexpression resulted in resistance to chemotherapy and Akt overexpression is also one of the major factors responsible for resistance to cancer therapy by rapamycin or rapalogs.[12,38] For the present study, we chose four Akt inhibitory peptides, Ala–Val–Thr–Asp–His–Pro–Asp–Arg–Leu–Trp–Ala–Trp–Glu–Lys–Phe (**Akt-in**),[39] Arg–Pro–Arg–Nle–Tyr–Dap–Nle,[40] Arg–Pro–Arg–Ala–Tyr–Dap–Nle and Arg–Pro–Arg–Nle–Tyr–Dap–Ala, to give the rapamycin peptide conjugates **5**, **6**, **7** and **8**, respectively. The peptide **Akt-in** is a well known potent inhibitor of the kinase activity of Akt, whereas the other peptides used for the conjugation were already found to be active in our in-house assays as Akt inhibitors. It must be noted that all of these peptide conjugates were prepared in high yield and purity without protecting the highly reactive side chain functionality like guanidino, carboxyl amino or phenolic groups, showing the potential and versatility of the methodology.

The conjugates **5–8** were evaluated for their biological activity for the inhibition of the kinase activity of mTOR and Akt proteins *in vitro*, as well as their ability to inhibit cell proliferation in a cell-based assay. The mTOR kinase inhibition assay was performed using active recombinant mTOR protein (Fig. 1a). mTOR phosphorylates the ribosomal protein S6 kinase (S6K) at Thr389. It is clear from the western blots that the rapamycin peptide conjugates **7** and **8**, like rapamycin, completely inhibited the kinase activity of recombinant mTOR at 10 nM concentration. However, conjugates **5** and **6** did not inhibit the kinase activity of the mTOR protein as much as rapamycin. Different concentrations of conjugates **7** and **8** were then evaluated for their inhibitory activity against the full mTORC1 complex in the human breast cancer MCF-7 cell line (Fig. 1b). The conjugate **7** showed similar inhibition of mTORC1 to rapamycin, whereas conjugate **8** showed better inhibition of mTORC1 as compared to rapamycin at 100 nM concentration. It has been reported that incomplete mTOR inhibition by rapamycin (*i.e.* inhibiting only mTORC1 and not mTORC2) results in an increased level of Akt, which results in the development of an aggressive tumor like situation. It was

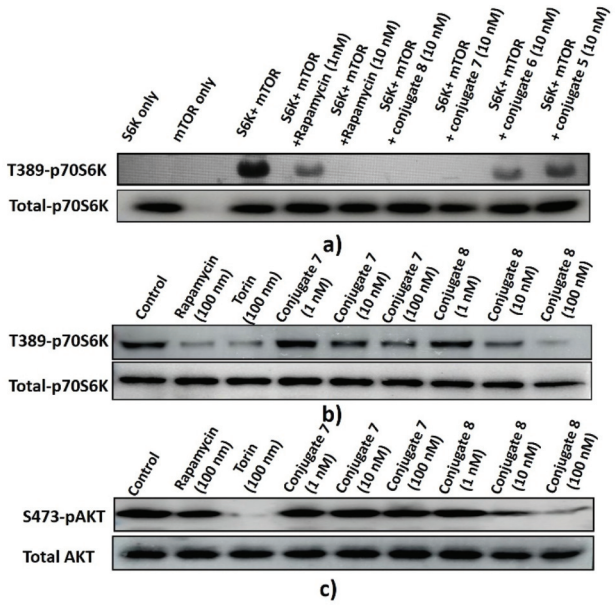


Fig. 1 The *in vitro* inhibition of the kinase activity of the recombinant mTOR protein, mTOR complex 1 and mTOR complex 2 in the MCF-7 breast cancer cell line. Western blots showing the inhibition of the kinase activity of; (a) recombinant mTOR protein; (b) mTOR complex 1 in MCF-7 cells and (c) mTOR complex 2 in mCF-7 cells. Torin is used as a standard inhibitor of the kinase activity of mTORC1 and mTORC2.

therefore considered appropriate to evaluate the ability of the novel conjugates for mTORC2 inhibition. mTORC2 phosphorylates the protein kinase Akt at Ser473 and is insensitive to rapamycin. It is clear from Fig. 1c that conjugate **8**, unlike rapamycin, also significantly inhibits the kinase activity of mTORC2 at 100 nM concentration, suggesting that conjugate **8** acts on dual targets which inhibit the kinase activities of mTORC1 and mTORC2, resulting in less phosphorylation of the S6K and Akt proteins, respectively. Quantitative analysis of protein phosphorylation inhibition was performed by the densitometric analysis of the respective western blots and the results are given in Fig. S15.† A similar trend was observed when we performed these experiments with isolated mTORC1 and mTORC2 complexes (Fig. S14†). To evaluate the potential of these conjugates for inhibiting the kinase activity of the Akt protein more precisely (through binding at the ATP binding site), optimized non-radioactive *in vitro* ELISA based Akt kinase assays were performed and the results are shown in Table 1.[41] The conjugates **6**, **7** and **8** at 10 μM concentration

Table 1 Percentage inhibition of Akt kinase activity at 10 μM concentration

S. no.	Compounds	% Inhibition ± SE
1	A443654	85.44 ± 4.1
2	Conjugate **5**	No inhibition
3	Conjugate **6**	80.20 ± 8.89
4	Conjugate **7**	80.53 ± 7.4
5	Conjugate **8**	62.97 ± 14.68

Table 2 Percentage growth inhibition at 10 μM concentration in breast cancer cell lines

S. no.	Compounds	MCF-7	MDA-MB-231
1	Rapamycin	42.42 ± 4.3	15.73 ± 0.57
2	Conjugate **5**	42.94 ± 4.7	No inhibition
3	Conjugate **6**	32.69 ± 1.5	No inhibition
4	Conjugate **7**	51.1 ± 8.8	10.46 ± 2.1
5	Conjugate **8**	57.19 ± 8.43	No inhibition

inhibited the kinase activity of Akt and were comparable with the standard Akt inhibitor A443654.

These conjugates were then evaluated for their antiproliferative activity in two breast cancer cell lines (MCF-7 and MDA-MB-231), which have different sensitivities towards rapamycin, using the well established SRB (Sulforhodamine B) assay.[42] The MCF-7 cell line is sensitive to rapamycin and MDA-MB-231 is almost rapamycin insensitive.[43] Similarly to rapamycin, no cytotoxicity of these conjugates was observed in the MDA-MB-231 cell line, whereas these conjugates showed cytotoxicity comparable to rapamycin in the MCF-7 cell line and conjugate **8** was found to be most potent one (Table 2).

Conclusions

In conclusion, we have developed an efficient methodology for the regioselective functionalization of rapamycin, which was used for the preparation of rapamycin peptide conjugates in excellent yield and purity under mild reaction conditions in a chemoselective manner. One of the major advantages of this process is that we could directly conjugate free peptides to rapamycin without the need of protecting side chain functional groups of the amino acids which are otherwise very difficult to remove once the peptide is conjugated to rapamycin. We could easily couple short and long peptides containing arginine, lysine or serine amino acids to rapamycin, which is otherwise difficult to conjugate, showing the versatility of the methodology. Furthermore, after making peptide conjugates of rapamycin, no loss of the apparent activity of rapamycin was observed. These conjugates may exhibit the ability to bind dual targets depending upon the pharmacological properties of the other ligand used for conjugation, for example conjugate **8** completely inhibited the kinase activity of the recombinant mTOR protein like rapamycin, and unlike rapamycin it significantly inhibited the kinase activity of Akt as well. This will provide a new insight into the area of research comprising the discovery of new rapamycin conjugates with improved activity and selectivity and that of target based new chemical entities.

Experimental section

General information

The Fmoc protected amino acids and Wang resin (100–200 mesh) used in the solid phase peptide synthesis were

purchased from Novabiochem. Rapamycin was purchased from APExBIO. Flame-dried or oven dried glassware was used to carried out the reactions. All of the reactions were performed with magnetic stirring under nitrogen atmosphere using freshly dried and distilled solvents, unless otherwise noted. Ready made TLC silica gel 60 F_{254} plates (Merck, Dermstadt, Germany) were used for reaction monitoring. The TLC plates were either developed under iodine vapors or visualised directly under UV light at 254 nm. Silica gels of 100–200 and 230–400 mesh were used for column chromatography. High resolution mass spectra were recorded using an Agilent 6520-Q-Tof MS/MS system. MALDI-MS spectra were recorded with an AB Sciex 4800 plus MALDI TOF–TOF Analyzer mass spectrometer. ^{1}H NMR spectra were recorded on a Bruker Av III HD 400 MHz spectrometer operating at 400 MHz at 25 °C using 2–10 mM concentrations in appropriate solvents, using TMS as the internal standard or the solvent signals as secondary standards, and the chemical shifts (δ) are shown in ppm scales. Multiplicities of NMR signals are designated as s (singlet), d (doublet), t (triplet), q (quartet), br (broad), td (triplet of a doublet), dt (doublet of a triplet) and m (multiplet, for unresolved lines).

Synthesis of 6-hydrazinonicotinic acid (2)

Hydrazine hydrate (5 mL) was added to 6-chloronicotinic acid **1** (980 mg, 6.22 mmol) and the reaction mixture was refluxed at 100 °C for 4 h. The reaction mixture was concentrated to dryness to give a solid residue. The residue was dissolved in a minimum amount of water and acidified with hydrochloric acid (35%) up to pH 5 to precipitate the HCl salt of 6-hydrazinonicotinic acid. The precipitate was filtered off and washed with ethanol to yield 800 mg (83.99%) of 6-hydrazinonicotinic acid. ^{1}H NMR (DMSO-d_6, 400 MHz): δ/ppm = 8.53 (d, J = 1.72, 1H, Ar–H), 8.27 (s, 1H, NH), 7.86 (dd, J = 2.28, 8.88, 1H, Ar–H), 6.86 (d, J = 8.8, 1H, Ar–H).

Synthesis of 6-Boc-hydrazinonicotinic acid (3)

To a solution of 6-hydrazinonicotinic acid (900 mg, 5.85 mmol) and triethylamine (0.7 mL, 5.85 mmol) in DMF (6.3 mL), di-*tert*-butyl dicarbonate (1.34 mL, 5.85 mmol) was added. The reaction mixture became homogeneous over 1 h and stirring was continued for 16 h at room temperature. The reaction mixture was concentrated to dryness under reduced pressure to give a brown solid. The residue was dissolved in a minimum amount of ethyl acetate and passed through silica gel using ethyl acetate as the eluent to remove the coloured impurities. The eluate was concentrated to dryness to give 1.3 g (87.75%) of 6-Boc-hydrazinonicotinic acid which was used for the next steps. ^{1}H NMR (DMSO-d_6, 400 MHz): δ/ppm = 8.99 (s, 1H, NH), 8.89 (s, 1H, NH), 8.59 (d, J = 1.8, 1H, Ar–H), 7.97 (dd, J = 1.6, 10.88, 1H, Ar–H), 6.54 (d, J = 8.76, 1H, Ar–H), 1.43 (s, 9H); HRMS (ESI) calculated for $C_{11}H_{16}N_3O_4^+$ = 254.1135, found = 254.1134.

Synthesis of hydrazinonicotinyl (HyNic) modified peptides

The desired peptides were assembled on the Wang resin using standard Fmoc based solid phase peptide synthesis. The Fmoc group of the assembled peptide was deprotected with 20% piperidine in DMF. To this resin a solution of 6-Boc-hydrazinonicotinic acid (3 equiv.) and HBTU (3 equiv.) in DMF was added and the reaction mixture was stirred by purging nitrogen gas followed by the addition of DIPEA (3 equiv. to HBTU), and the reaction mixture was stirred by purging nitrogen gas for 3 h under nitrogen. After the reaction was completed, the resin was then washed with DMF (three times) and DCM (three times) and finally dried under vacuum. The peptide bound resin was then treated with a mixture of TFA : TIPS : acetone : water (92.5 : 2.5 : 2.5 : 2.5%) and the reaction mixture was stirred for 3 h. The resin was removed by filtration and the filtrate was reduced to half by evaporation under reduced pressure at 45 °C. The peptide was precipitated with diethyl ether. The precipitate obtained was filtered and further washed three times with diethyl ether. The precipitate obtained was dried under vacuum to give the crude peptide. Purification with RP-HPLC using a C-18 column gave the desired HyNic modified peptides.

Synthesis of 42-*O*-(4-formylphenylcarbonyl) rapamycin (I)

To a solution of 4-carboxy benzaldehyde (40 mg, 0.2 mmol) in DCM (8 mL) at 0 °C, DCC (41.28 mg, 0.2 mmol) was added. After 15 minutes, rapamycin (200 mg, 0.2 mmol) and DMAP (4 mg, 0.03 mmol) was added to it at 0 °C. The reaction was then stirred overnight at 25 °C. After that, DCU was removed by filtration and washed with DCM. The organic layer was then concentrated *in vacuo*, affording the crude product. The crude residue was then purified on a silica gel column, eluting with 1% MeOH/DCM to give 100 mg (47.79%) of the title compound as a light yellow solid. Characteristic ^{1}H NMR peak ($CDCl_3$, 400 MHz): δ/ppm = 10.11(s, CHO, 1H), 8.20 (d, J = 8.2, Ar–H, 2H) and 7.96 (d, J = 8.52, Ar–H, 2H), the detailed NMR spectra is shown in Fig. S3;† MALDI-MS $(M + Na)^+$ calculated for $C_{59}H_{83}NNaO_{15}^+$ = 1068.56, found 1068.51.

Synthesis of the rapamycin peptide conjugates

To the solution of HyNic modified peptides (5–10 μmol) in 1–2 mL of sodium acetate buffer of pH 5.1 add solution of equivalent amount of 42-*O*-(4-formylphenylcarbonyl) rapamycin in 1–2 mL of ethanol. Reaction was stirred for 3 h at room temperature (monitor by RP-HPLC). After that rapamycin peptide conjugate was purified by RP-HPLC in C-4 column using gradient solvent system of 0.1% TFA in DI water and acetonitrile to give desired rapamycin peptide conjugate in almost quantitative yield. The product is confirmed by mass spectroscopy and the purity of peptide was checked with analytical HPLC.

Author contributions

WH, SS and RA contributed to the designing, conceptualization and synthesis. JM, VS and SB performed the mTOR related experiments. MH and JS performed the Akt kinase assays. SM and DD performed the cell-based assays.

Conflicts of interest

There are no conflicts to declare. An Indian Patent application has been filed for part of this work with patent no. 341387.

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