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Microwave Assisted Peptide Synthesis of Some Rationally Designed Cell Penetrating Peptides from C-Kit Receptor

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IN the interest of public health, cell-penetrating peptides (CPPs) have proven to be important way for the highly efficient intracellular delivery of bioactive cargos like peptides, proteins, and oligonucliotides. It is feasible to combine one or multiple CPPs with bioactive cargos either by direct chemical conjugation or, more rarely, as non-covalent complexes. The term bioportides was introduced to describe monomeric CPPs that are intrinsically bioactive. One of the consistent problems that may compromise the in vivo applications of CPPs and bioportides, is the stimulation of mast cell (MC) secretion. Therefore, in this study two decapeptides, S6 and S6P, has been designed from the c-kit protein, a mast cells receptor, and synthesized using the technology of microwave assisted solid phase peptide synthesis. In an attempt to find bioportides from the c-Kit protein, and studying their structure- activity relationship, the present work was carried out, investigating the effect of the presence of the phosphotyrosine moiety as an active site in the synthesized peptides.

Keywords: Microwave assisted solid phase peptide synthesis, Cell-penetrating peptide, Bioportides, C-Kit receptor.

Introduction

Cell penetrating peptides (CPPs), or protein transduction domains, is a technology has been used to overcome the lipophilic barrier of the cellular membranes to afford an efficient delivery of bioactive molecules[1–4]. The first examples of CPPs that were clearly effective included penetratin (RQIKIWFQNRRMKWKK [5], corresponding to the third helix of the Antennapedia homeodomain, and Tat (GRKKRRQRRRPPQ [6,7], corresponding to the basic domain of HIV-1 Tat protein. A recent genre of CPP, designated as bioportides, distinct from conventionally inert vectors, has been described. [8,9] these proteomimetic CPPs combine the dual properties of cellular penetration and biological activity and often derive from cationic helical domains of key signaling proteins and commonly act as biological modulators of protein-protein interactions (PPIs) [8–11].

The majority of recently developed CPPs are relatively short [12-25](amino acids) polycationic sequences. Some of these derive from known helical domains of transcription factors which facilitate membrane translocation, including the common delivery vectors Tat [6] and Penetratin[5]. Other CPP sequences, including the Transportans [12], can be modeled as an amphipathic helix. Polyarginine-containing CPP sequences have also been identified in a range of other human proteins[13].

Thus, it is possible to speculate that one or multiple polycationic sequences may enable functionally-diverse charged proteins [14]. to traverse biological membranes and so fulfill their

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biological role(s).

Many practical limitations associated with the development of peptide and peptide-like drugs, including both CPP vectors and bioportide technologies, particularly the utilization of peptides in a clinical setting due to rapid proteolysis and the need to employ undesirable routes of delivery, have been widely documented [15,16] yet a consistent problem may compromise the in vivo applications of CPPs and bioportides that is the stimulation of mast cell (MC) secretion. This potential limitation is a consequence of the exposure of circulating MCs to peptides that are potentially potent secretagogues [17].

As key mediators of inflammatory and allergic responses, MCs are sensitive to some polycationic and amphiphilic peptides. Besides, this mode of action is commonly receptor-independent and may arise in part from the direct activation of intracellular heterotrimeric G proteins [18,19].

C-Kit receptor is a protein found on the surface of many different types of cells including hematopoietic cells, germ cells, mast cells, melanoma cells and the gastrointestinal tract cajal cells. It may also be found in higher than normal amounts, or in a changed form, on some types of cancer cells, including gastrointestinal stromal tumors and melanoma [20,21] measuring the amount of c-kit in tumor tissue may help diagnose cancer and plan treatment. C-kit is a type of tumor marker and also called CD117 and stem cell factor receptor.

The c-kit receptor (976 amino acids (aa)) is consists of; an extra-cellular domain of 5immunoglobulin-like folds (519 aa), a trans-membrane domain (23 aa), and an intracellular tail (433 aa) composed of a juxta-membrane domain and a tyrosine kinase domain [22] (Fig. 1). The extracellular domain of c-kit activate downstream signal transduction and then regulate a variety of cells biological behavior, such as normal cells proliferation and differentiation, tumor occurrence, development, migration and recurrence [23–25].

In this study, we chose a peptide sequence

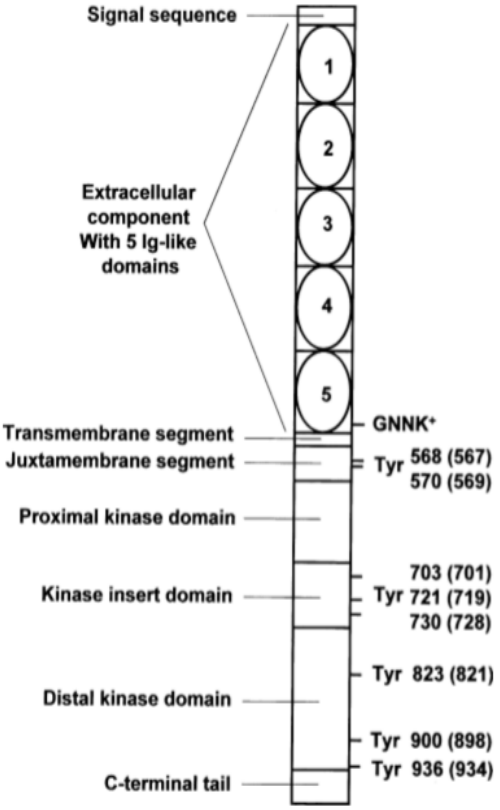


Fig. 1. [47]Tyrosine phosphorylation sites and organization of Kit. The relative length of the domains is to scale. The human (mouse) numbering system is displayed. G refers to glycine; N, asparagine; K, lysine; and Ig, immunoglobulin.

containing a tyrosine phosphorylation site S6 (NYVYIDPTQL) from the whole c-kit protein [577-586] to be synthesized of normal tyrosine and its analogue S6P (NpYVpYIDPTQL), in which pY is a phosphotyrosine, using microwave technology for solid phase peptide synthesis. Microwave irradiation significantly reduces the time of synthesis, and also improves the quality and the yield of the peptides produced [26,27].

Since its introduction by Merrifield's in 1963 Solid-phase peptide synthesis (SPPS) has become the primary route for synthetic peptides which are essential for research in many fields. SPPS involves a solid support on which the peptide becomes elongated and excess reagents and by-products being withdrawn through simple filtration after every reaction step (incorporating α-amino and deprotection) [28,29]. In the last decades Significant advances have been made in SPPS; however, this methodology has involved difficulties regarding synthesis in some cases concerning difficult peptide sequences that have severe inefficiencies regarding acylation and deprotection reactions. Depending on the sequence of amino acid (aa) residues, Peptide sequences of much hydrophobic amino acids, bulky side chains or bulky protecting groups are more prone to such inefficiencies like peptide chain deletion or truncation which produces from peptide-resin and/or secondary structure (mostly β-sheet) aggregation causing steric hindrance [30–32]. These associations produce low peptide-resin complex solvation and low reagent accessibility to reaction sites. The combination of Microwave (MW) radiation with Solid-phase synthesis strategy has been used for disrupting these associations in addition to accelerating synthesis even with long peptide sequences [33,34].

Microwave assisted Fmoc-SPPS has now become the method of choice to produce peptides, It permitted the development of automation [35], also it facilitates the development of greener methods that are organic solvent-free and environmentally friendly method for peptide synthesis [36,37].

In an attempt to find bioportides from the c-Kit protein, and studying their structure- activity relationship, the present work was carried out, investigating the effect of the presence of the phosphotyrosine moiety as an active site in the synthesized peptides.

Materials and Methods

Peptides Selection

Most of the phosphorylation sites that occur upon ligand stimulated activation of c-Kit reside either in the juxtamembrane region, the kinase insert, or the COOH-terminal tail. S6 (NYVYIDPTQL) peptide sequence (567-576) includes tyrosine 568 and tyrosine 570 active sites, so it has been selected as a possible bioportide and its analog S6P where both Tyrosine 568 and 570 amino acids have been replaced with phosphotyrosines to examine the effect of the phosphate group on the translocation of the peptide and its ability to mimic the parent protein.

Microwave Enhanced Peptide Synthesis

Peptides sequences were synthesized using a Discover SPS Microwave Peptide Synthesizer (CEM Microwave Technology Ltd, Buckingham, UK, [38]) with a 0.1 mmol scale on Rink amide methylbenzhydrylamine (MBHA) resin (Novabiochem, Beeston, UK), and an N-α-Fmoc protection strategy with O-(6-Chloro-1-hydrocibenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU; AGTC Bioproducts, Hessle, UK) activation.

First step were the deprotection of the rink amide MBHA resin in Microwave Peptide Synthesizer, where 7 ml of 20% vol/vol piperidine in DMF (Dimethylformamide) was added to 0.169gm (0.1 mmol) of resin, then put in Microwave Synthesizer at 50 W/75 °C for 3min (Kaiser test showed +ve result), the resin then was filtered off and washed several times with DMF and DCM (Dichloromethane).

Coupling of the amino acids (Scheme 1):

1- Coupling of the first amino acid (Fmoc-Leu) was performed twice to ensure complete loading on the rink resin, in each, 4 fold of Fmoc-Leu (0.45 mmol, 0.159gm) was dissolved in a mixture of HCTU(0.1mmol) and diisopropylethylamine (DIPEA, 160µl) in 4 ml DMF then put in the CEM Microwave Peptide Synthesizer for 10-20 min at 25 W/75 °C. The coupling result ensured by qualitative Kaiser test, then deprotection step, where 7 ml mixture of 20% vol/vol piperidine in DMF was added to the Fmoc-Leu- rink amide resin and subjected to microwave in the Peptide Synthesizer at 50 W/75 °C for 3min.

2- The following coupling reactions were accomplished with about 4-fold molar excess of Fmoc-protected AA (0.45 mmol) with HCTU (0.1mmol) and diisopropylethylamine (DIPEA, 160µl), in 4 ml DMF for 10-20 min (till Kaiser test showed +ve results) at 25 W/75 °C in the Microwave Peptide Synthesizer.

3- Each complete coupling step followed by deprotection step with 7 ml of 20% vol/ vol piperidine in DMF for 3 min at 50 W/75°C.To reduce aspartimide formation, [33] 7ml of 5 % wt/vol piperazine in 0.1 M 1-hydroxybenzotriazole hydrate (HOBt) was employed as the deprotection solution for the Fmoc-Asp(otBu) amino acid and protected amino acids after [Fmoc-Ile, Fmoc-Tyr(tBu)/ Fmoc-Tyr(PO(OBzl)OH), Fmoc-Val, and Fmoc-Asn(Trt)], for 3min at 50 W/75 °C in the Microwave Peptide Synthesizer.

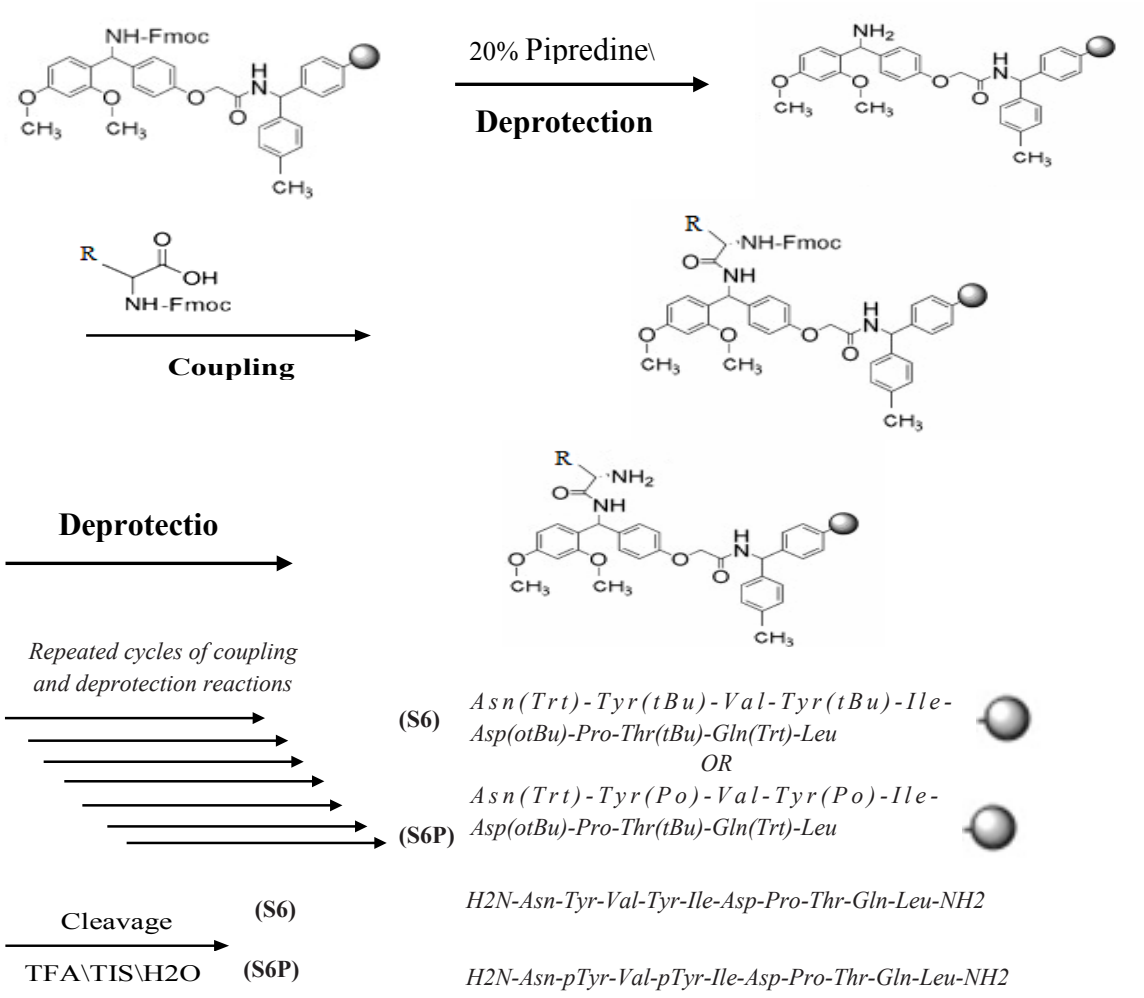
4- To ensure the incorporation, the coupling and deprotection steps were confirmed by a routinely employed qualitative ninhydrin test (Kaiser Test, [39].

5- All coupling reagents and amino acids were dissolved in DMF. After each coupling and deprotection step, all resins are washed with DMF (~5 ml) and DCM then thoroughly drained three times.

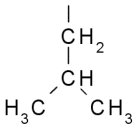
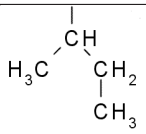
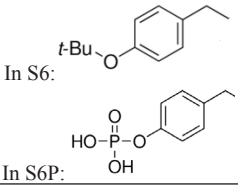
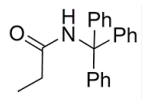
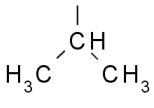
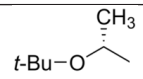
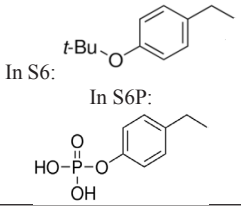
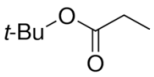
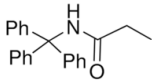
Coupling with Fluorescent:

Peptides, to be used in confocal live cell imaging, were synthesized by amino-terminal acylation of peptides sequences with 6-Carboxy-tetramethylrhodamine (TAMRA) (Novabiochem, Beeston, UK) as previously reported [40,41].

About 2 fold molar excess of TAMRA (50 µmol, 25mg) with HCTU (0.1mmol, 0.5ml) and 45µl and DIPEA were added to 25µmol peptide resin and shacked at Room Temperature for 5hrs then washed with DMF, DMC and methanol 3 times each and once with ethylether for complete dryness.



Scheme 1. synthetic route to peptides S6 and S6p, Where R is R1 ⟶ R10:

R1		In Leucine (Leu, L)	R6		In Isolucine (Ile, I)
			R7	 In S6: In S6P:	In S6: Tyrosine(tBu) (Tyr, Y) In S6P: Phosphotyrosine
R2		In Glutamine(Trt) (Gln, Q)	R8		In Valine (Val, V)
R3		In Threonine(tBu) (Thr, T)	R9	 In S6: In S6P:	In S6: Tyrosine(tBu) (Tyr, Y) In S6P: Phosphotyrosine
R4		In Proline (Pro. P)			
R5		In Aspartic acid(otBu) j(Asp, D)	R10		In Asparagine(Trt) (Asn, N)

Cleavage of Peptides from resin:

The synthesized peptides were Cleaved from the resin and all the side-chain protecting groups were removed in one step (Scheme 1), carried out using 4ml TFA/100µl H_2O/100µl triisopropylsilane (TIS) with agitation at R.T for 2-3 hours followed by filtration of the TFA mixture through a glass wool into a tube then blowing of N_2 gas over TFA until volume reduced to about 0.5ml. Ice cold ether was added to the precipitated peptide and peptide pellet in centrifuge 2500 RPM for 2min then washed twice with ether.

Culture of RBL-2H3 cells

RBL-2H3 cells were maintained in DMEM (Dulbecco's modified Eagle's medium) containing L-glutamine (0.1 mg ml−1) supplemented with fetal bovine serum (10% v/v), penicillin (100 U ml−1) and streptomycin (100 µg ml−1) in a humidified atmosphere of 5% CO_2 at 37 °C[42].

Secretory Efficacies of Synthesized Peptides

RBL-2H3 cells were cultured in 24-well plates. To determine the secretory efficacies of selected peptides, the lysosomal enzyme β-hexosaminidase was assayed in samples of cell medium following exogenous application of peptides to RBL-2H3 cells in HAMS F12 medium [42,43]. 5 µl aliquots of medium were transferred into 96 well plates and incubated with ρ-nitrophenyl-N-acetyl-β-D-glucosamide (20 µl of 1 mM in 0.1 M sodium citrate buffer, pH 4.5) for 1 h at 37 °C. Na_2CO_3/$NaHCO_3$ buffer (200 µl of 0.1 M, pH 10.5) was then added and β-hexosaminidase activity determined by colourimetric analysis at 405 nm.

Cell viability assays

To evaluate the potential cytoxicity of synthesized peptides, cellular viability was measured using the 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) conversion assay [40,41] in RBL-2H3 cells that were cultured in 96-well plates and treated with peptides (0.1–30 µM) for 24–72 h at 37 °C. Following the removal of stimulation medium, cells were incubated with MTT (0.5 mg/ml) for 3 h at 37 °C. Medium was aspirated and the insoluble formazan product solubilized with DMSO (Dimethyl sulfoxide). MTT conversion was determined by colorimetric analysis at 540 nm.

Live Cell Scanning Confocal Microscopy

RBL-2H3 cells were transferred to 35 mm sterile glass base dishes (IWAKI, Japan) and grown to ~75% confluence. Immediately prior to the addition of TAMRA-labeled peptides (5 µM, to a maximum volume of 2 ml), cells were washed with and transferred into HAMS F12 without phenol red medium. During the period of exposure to peptides, cell layers were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

Immediately prior to observation, cells were washed gently with phenol red free HAMS F12 (8×) and analyzed with a Carl Zeiss LSM510Meta confocal microscope equipped with a live cell imaging chamber.

Results and Discussion

Two peptide chains were synthesized using the Microwave assisted Fmoc-solid phase peptide synthesis (MSPPS) method:

S6 (H_2N) Asn- Tyr- Val- Tyr- Ile-Asp- Pro-Thr-Gln -Leu($CONH_2$). And S6P (H_2N)Asn-Tyr(PO_3H_2)-Val-Tyr(PO_3H_2)-Ile-Asp-Pro-Thr-Gln -Leu($CONH_2$). S6 sequence is a decapeptide from C-kit protein receptor [from aa 577 to aa 586] and the analogue S6P where tyrosine amino acids were replaced with phosphotyrosines.

Phosphotyrosine is a phosphorylated amino acid that occurs in a number of proteins. Tyrosine phosphorylation and dephosphorylation controls many cellular functions [44].

Rink amide methylbenzhydrylamine (MBHA) resin has been used to obtain a C-terminal amidated peptide sequences to increase the metabolic stability of the synthesized peptides as well as their ability to resist enzymatic degradation by aminopetidases, exopeptidases, and synthetases. The synthesized peptide ends are uncharged, so they more closely mimic the native protein. Also, this increases their ability to enter cells. Amidation not only enhances the activity of peptides, but also prolongs their shelf life [45].

The essential advantages of the microwave assisted solid phase peptide synthesis are that the reduction of coupling and deprotection required time, the decreasing of the racemization and the excellent purity of the crude peptide [26,34]. Discover SPS Microwave Peptide Synthesizer has been used for peptides synthesis in which the time required for all coupling steps was within 10 to 20 min, and for all the deprotection steps was 3min each.

All the synthesized peptides were purified to apparent homogeneity by semi-preparative scale high performance liquid chromatography (HPLC) [9,41] (Fig. 2), and their predicted masses (average M+H+) were confirmed to an accuracy of ±1.0 by matrix-assisted laser desorption ionization (MALDI) time of flight mass spectrometry operated in positive ion mode using α-cyano-4-hydroxycinnamic acid (Sigma) as a matrix [41] (Fig. 3).

Peaks obtained from the HPLC analysis of the synthesized crude peptides suggested the absence of diastereomeric compounds. Thus, there is no significant racemization during the MW assisted coupling reactions. The overall yield calculated from the amino group content of the used resin was about 75%.

Biological Evaluation:

Mastoparan [MP][46][21], A mast cell degranulating polycationic peptide in wasp venoms. MP stimulates the exocytosis from MCs in a receptor-independent fashion and trigger a localized inflammatory response initiated by the degranulation of MCs [43]. Copaired with MP, Both the synthesized peptides, S6 and S6P, has no influence upon β-hexosaminidase release from RBL-2H3 cells even at a concentration of 30 μM (Fig. 4).

The Phosphotyrosine moiety in S6P sequence enhances the cytotoxic potency of the synthesized peptide with a small extent, while S6 sequence was inactive.

The results indicate that both peptides [S6 and its Phosphotyrosine analogue (S6P)] is a part of protein receptor already expressed by mast cells, and that may explain why they don't stimulate the mast cells degranulation as shown in the secretion assay results (Fig. 4). Also, however, neither peptides shown a clear apoptotic effect on RBL-2H3 cells (Fig. 5), S6P- The phosphotyrosine analogue- still has a considerable cell penetrating activity as shown in Fig.6 by confocal microscope. From the studies described herein, however, many cationic CPPs have exocytotic effect on MCs, both peptides S6 and S6P have no influence upon β-hexosaminidase release from RBL-2H3 cells even at a concentration of 30 μM. In addition, the Phosphotyrosine analogue (S6P) appeared to be very promising CPP that could be employed to deliver cargoes into MCs without promoting exocytosis i.e in a relatively inert fashion.

Conclusion

The present study identifies and characterizes a peptide selected as bioportide from the stem cell receptor protein (ckit) , S6, and its analog, S6P. The peptide S6 was unable to translocate into the RBL-2H3 cells, while the analog, S6P, with

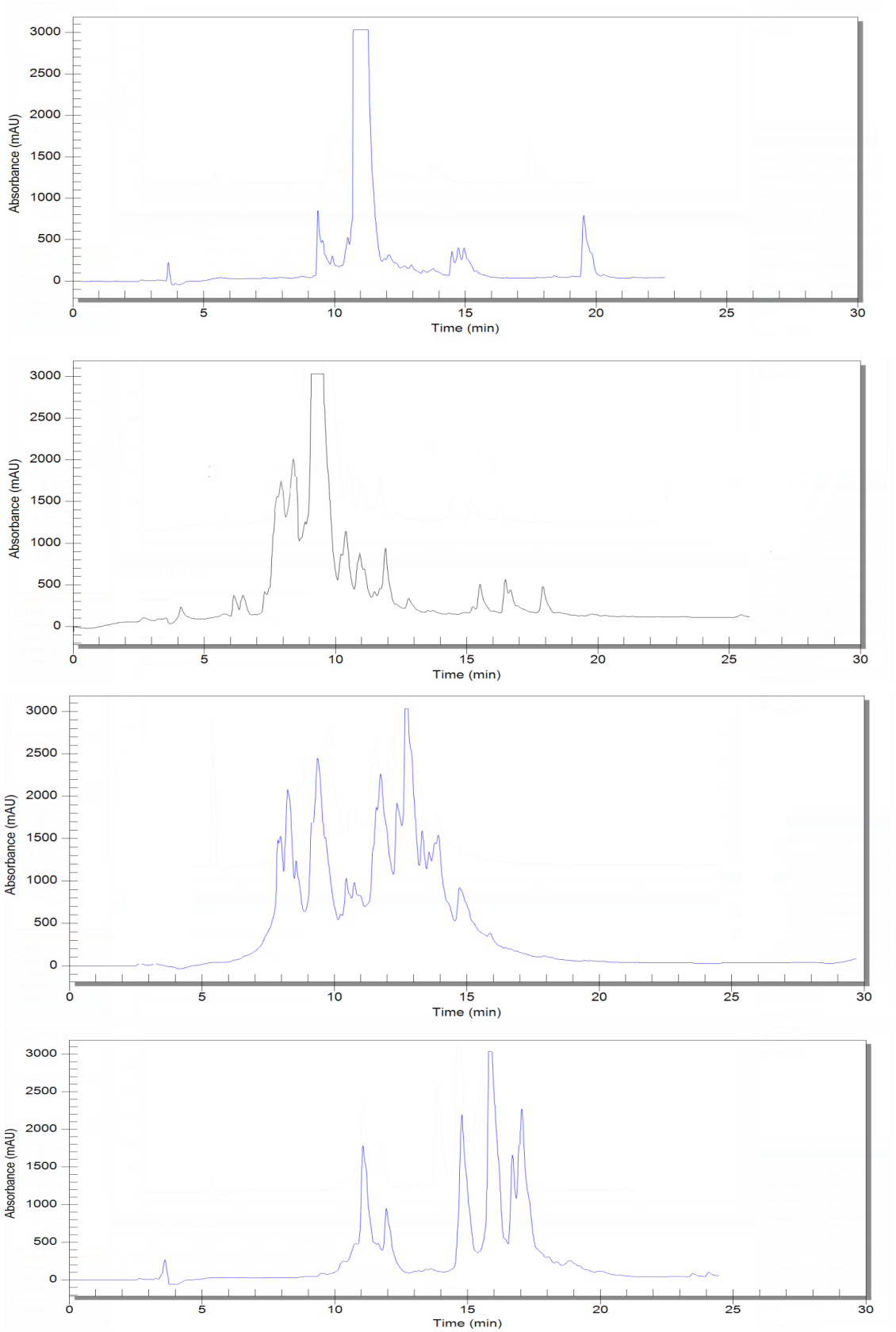


Fig. 2. HPLC charts for the synthesized peptides; a) represents S6, b) represents S6P, c) represents TAMRA-S6 and d) represents TAMRA-S6P.

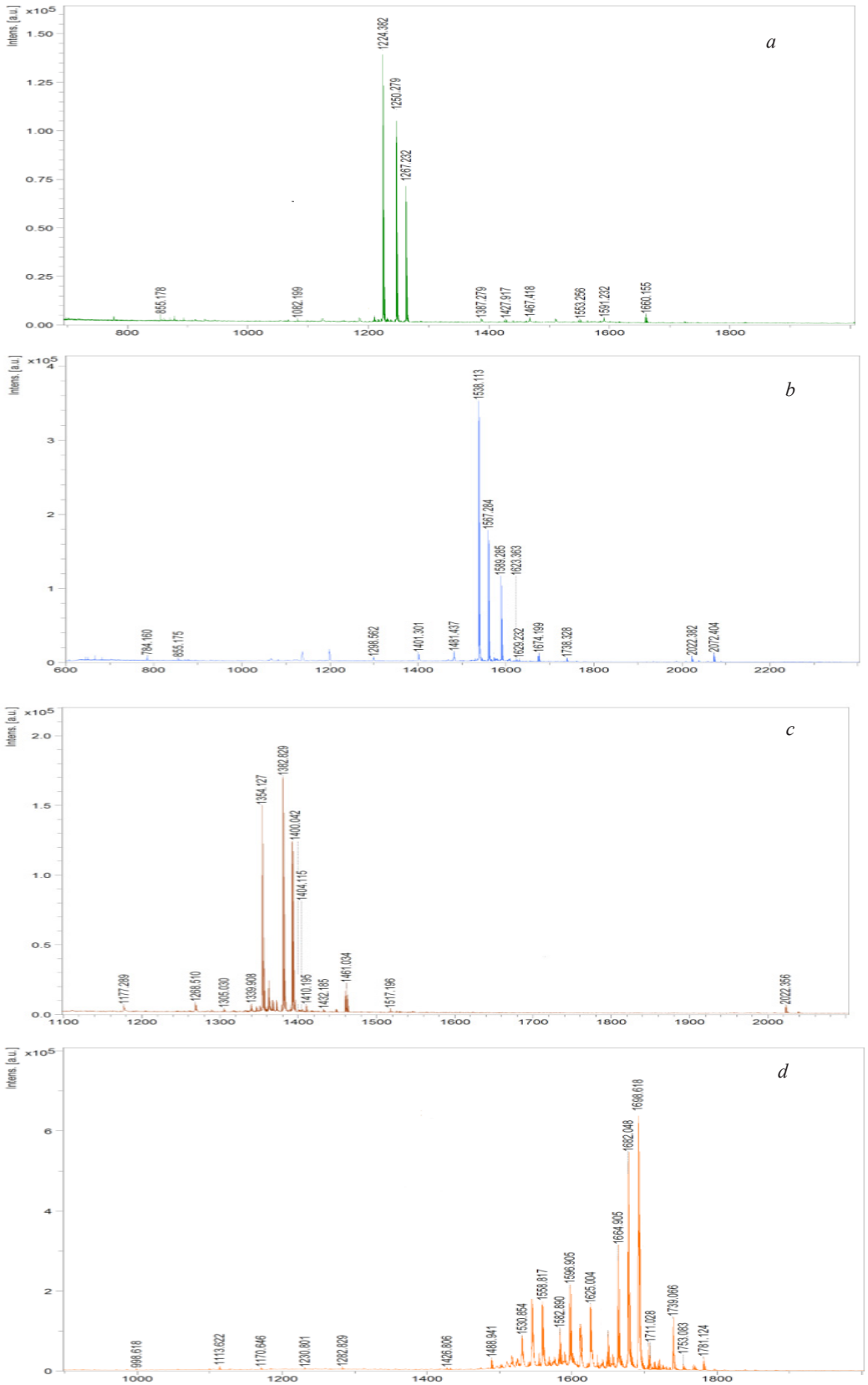


Fig. 3. Mass spectra for the synthesized peptides; a) represents S6 (M.wt.: 1225.37), b) represents S6P (M.wt.: 1383.82), c) represents TAMRA-S6 (M.wt.: 1539.74), and d) represents TAMRA-S6P (M.wt.: 1699.18).

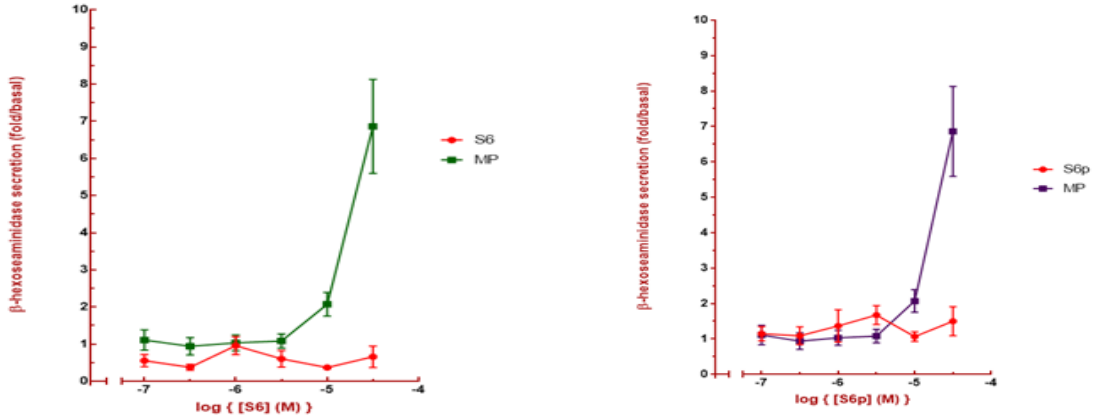


Fig. 4. Comparative concentration-dependent secretory activities of C-Kit-derived peptides and Mastoparan(MP)43, a) represents S6 and b) represents S6P. RBL-2H3 cells were treated with increasing concentrations of peptides for 15 min at the concentrations indicated. Data points are mean ± SEM from 2 experiments performed in triplicate.

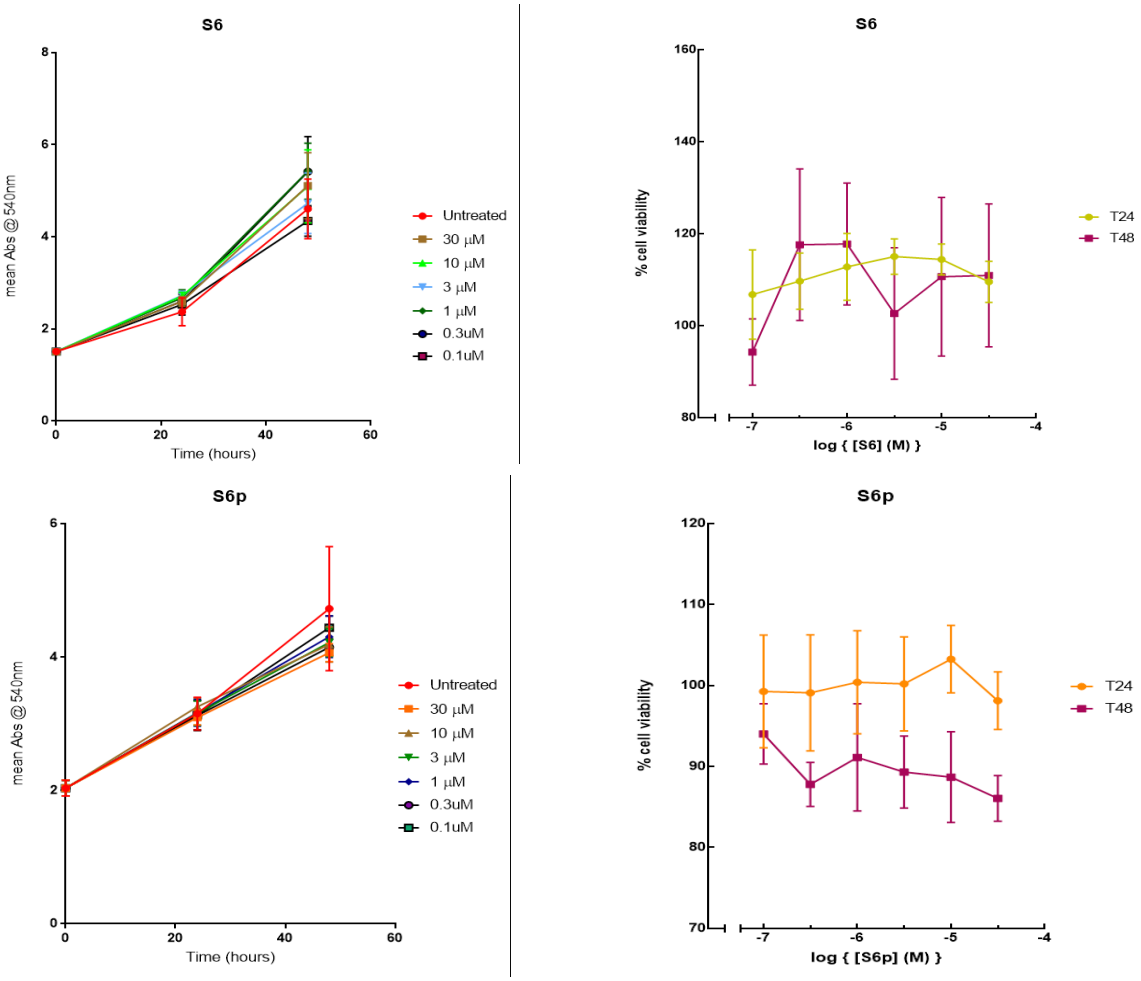


Fig. 5. The Toxicity of S6 and S6P, RBL-2H3 cells were exposed to increasing concentrations of peptides (0.001–30 mM) for 24 hr and 48hr. Cell viability was measured by MTT conversion and expressed as a percentage of those cells treated with vehicle (medium) alone. Data points are mean ± SEM from three experiments performed in triplicate.

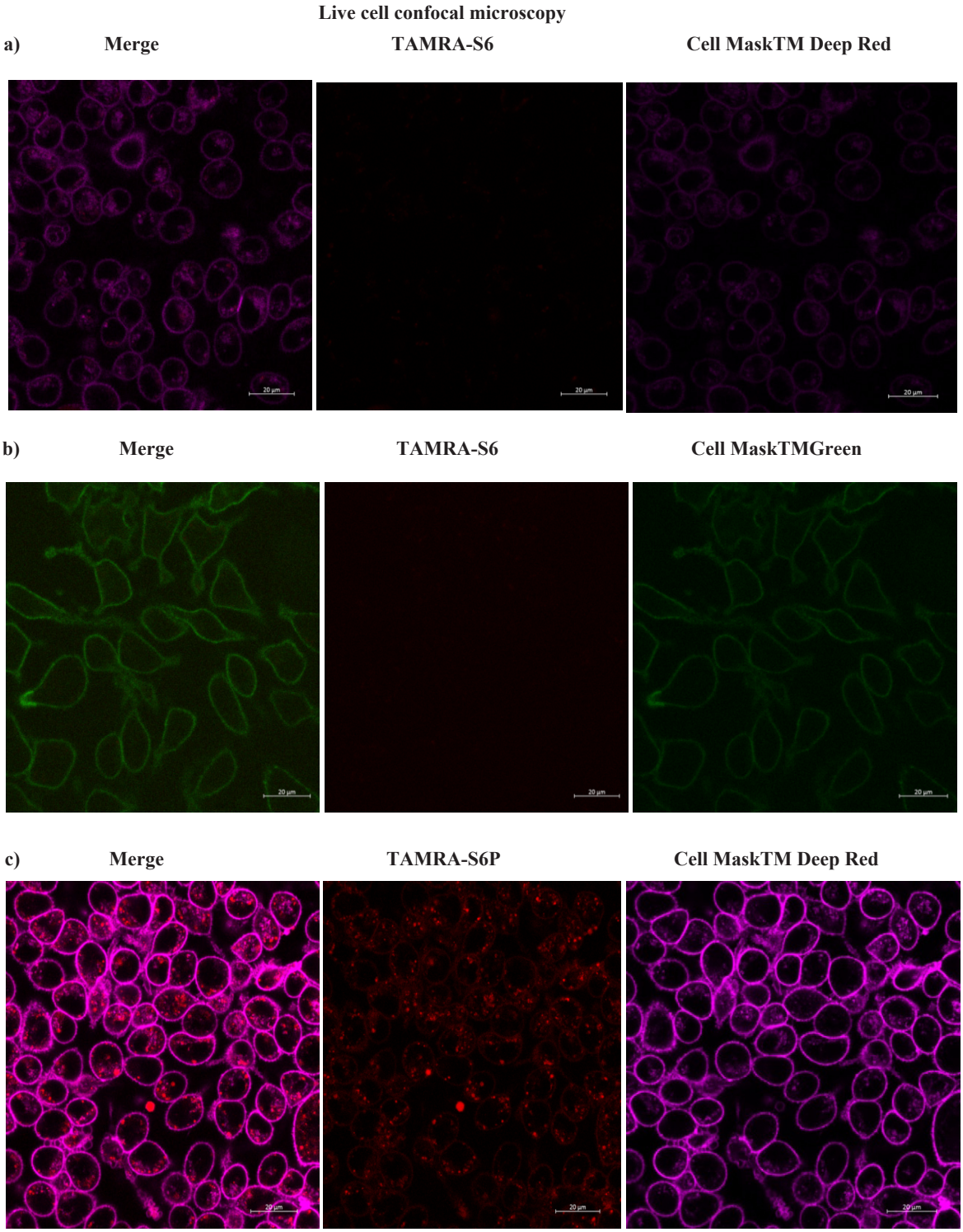


Fig. 6. Intracellular distributions of TAMRA–S6 and TAMRA–S6P in RBL-2H3 cells. Cells were incubated with TAMRA-peptides (5 mM) for 55 min, followed by Cell MaskTM Deep Red (3.75 mg/ml) treatment for 5 min so as to visualize secretory lysosomal structures. Cells were washed and re-suspended in DMEM w/o phenol red for confocal image capture using a live cell imaging chamber. Visualization by live confocal cell imaging demonstrated that TAMRA–S6P accumulated within secretory granules as designated by red colocalization (c). Incontrast (a,b), TAMRA–S6 has any intracellular distribution, [(b) RBL-2H3 cells incubated with TAMRA–S6 and treated with cell mask™ Green].

the phosphotyrosine moiety was able to enhance apoptosis in the RBL-2H3 cells following its efficient translocation. The conformational changes caused by phosphotyrosine moiety in sequence S6P may enhance the cell penetrating properties of the peptide and regulates its catalytic activity. Also, it may recruit some proteins inside cells that have structurally conserved domains that recognize and bind to phosphomotifs. The ability of phosphoproteins to recruit other proteins is critical for signal transduction, in which downstream effector proteins are recruited to phosphorylated signaling proteins.

Acknowledgment

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استخدام تقنية الميكروويف في تشييد بعض الببتيدات المصممه لتكون مخترقه للخلايا من المستقبل C-Kit

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أثبتت الببتيدات المخترقه للخلايا**(CPP)** في مجال الصحة العامة أنها طريقة ذات فعالية عاليه لتوصيل المواد البيولوجيه داخل الخلايا مثل الببتيدات والبروتينات وأوليغونبكليوتيدات. ومن الممكن ايضا الجمع بين واحد أو أكثر من هذه الببتيدات مع المواد النشطة بيولوجيا إما عن طريق الاقتران الكيميائي المباشر، أو غيرها ، كمجموعات غير تساهمية. كما تم تعريف مصطلح **bioportides** لوصف الـ **CPPs** الأحادية التي لها نشاط بيولوجي جوهري. وكواحدة من المشاكل التي قد تعوق بعض تطبيقات ال **CPPs** وال **bioportides** فى الجسم الحي هى تحفيز إفراز الخلايا البدينة**(Mast Cell)**. لذلك ، في هذه الدراسة، تم تصميم اثنين من الببتيدات، **S6** و **S6P**، من بروتين **c-kit** وهومستقبل الخلايا البدينة، ويتم تصنيعها باستخدام تقنية تشييد الببتيدات باستخدام السطح الصلب بمساعدة الميكروويف. في محاولة للعثور على **bioportides** من بروتين **c-Kit**، ودراسة العلاقة بين البنية الجزيئيه والنشاط ، تم تنفيذ العمل الحالي، والتحقق من تأثير وجود شق الفوسفوتيروسين كموقع نشط في الببتيدات المشيده.