Defence Life Science Journal, Vol. 1, No. 1, June 2016, pp. 57-62, DOI : 10.14429/dlsj.1.10062

RESEARCH ARTICLE

Bivalent KDP Peptide to Enhance Neurite Growth for Traumatic Brain Injury

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ABSTRACT

A silent epidemic of modern world and largely neglected field in drug development is traumatic brain injury. There is no treatment available for the patients suffering from brain trauma. Peptide is a naturally occurring biological alternative that could represent a new generation of future medications. Authors have designed and modified the C-terminal amino acids of KDI peptide responsible for neurite growth. The inclusion of a Cys (C), Gly (G), Cys and Pro (P) amino acids in the sequence leads to the possibility of better binding with the target receptor 3T1M as compared to the known KDI sequence for neurite growth. Studies on human SH-SY5Y cells for neurite outgrowth demonstrates that the CGCKDP could plausibly be useful in neuroregeneration in neurodegenerative disorders by neurite growth formation.

Keywords: Traumatic brain injury, KDI Peptide, posttraumatic stress disorde, central nervous system, peripheral nervous system, neurite growth

1. INTRODUCTION

The common problems in military service are traumatic brain injury (TBI) and posttraumatic stress disorder (PTSD). There is an indication that the individual having the history of traumatic brain injury (TBI) are at greater risk for developing neurodegenerative diseases like the Alzheimer's type dementia[1–4]. However, mostly research has focused on the risk associated with the brain injuries, and repeated mild injuries[5,6]. In military wars, the extensive use of improvised explosive devices reportedly produced up to 23% confirmed cases of TBI in one group combat team of nearly 4000 soldiers[7].

The common neurophathological consequences of TBI is neurite growth and degeneration, which includes both dendritic and axonal damage[8]. Neurite are important in forming functional synapses that mediate neuronal signalling. The injured axons in the peripheral nervous system (PNS) often regenerate, however the injury is usually permanent in the central nervous system (CNS). The key challenges in neuroscience to regenerate the CNS. Laminin has been identified as an active mediator of neuronal development, response to trauma and linked to the regeneration of the CNS[9-11]. Laminin1 (α1β1γ1) is a cross shaped glycoprotein composed of the three short arms and one long arm. The important role of laminin is in the developmental process which includes migration and development of neuronal pathways[12]. In injured neuronal pathways, the major factors hinders in regeneration of central axons are proteins, released from the damaged myelin sheets and formation of the glial scar[13]. The decapeptide (RDIAEIIKDI) is responsible for the neurite outgrowth function of γ1 laminin. The decapeptide of γ1 laminin induces potassium currents in primary cultured cerebellar neurons. The smallest peptide is capable of inducing the same properties and supporting neurite outgrowth is the KDI tripeptide[14,15].

The role of KDI tripeptide in promoting the survival of CNS neurons has been recently shown in Parkinson's disease[14]. The KDI tripeptide was the first compound to be used to protect the dopaminergic neurons of the rat substantia nigra (SN) against death induced by high doses 6-hydroxydopamine (OHDA)[14] to such an extent that over 30% of the dopaminergic neurons of the injected SN remained viable[14].

Bivalent approach has been applied to conjugate two KDP peptide molecule through glutamate linker which is finally conjugated to DO3A to yield the desired imaging agent. The proposed design is aimed to obtain a higher affinity of the molecule towards the target receptors[16]. The peptide linker was synthesised on rink amide resin using the Fmoc strategy. The synthesised bis-KDP-peptide has been characterised by the mass spectrometry and HPLC. In addition, the peptide was radiolabled with a ^{99m}Tc for real time monitoring of its blood clearance kinetics and bio-distribution to see the brain uptake of the bis-peptide. Finally we proceeded with the *in vivo* evaluation in Neuro-2A cell line and neurite outgrowth activity in human SH-SY5Y cells.

Received 29 December 2015, Revised 09 April 2016
Accepted 16 April 2016

2. RESULTS AND DISCUSSION

2.1 Design and Synthesis

2.1.1 Molecular Docking Studies

The molecular docking results in general elaborates all the necessary interaction between peptides and human galectin-3, aromatic-aromatic interactions, lipophilic-lipophilic interactions, hydrogen bond interactions, hydrophobic region in terms of glide XP score. The proline amino acids introduced in place of leucine in the know active KDI sequence, considering the fact that proline residue is a vital factor for the binding of N-methyl-D-aspartate (NMDA) receptor and the neuroprotective effects of tripeptide Gly-Pro-Glu (GPE)[17]. Moreover the proline-rich protein 1A is define and promotes axotomised neurons and outgrowth respectively. The pose was selected on the basis of Glide score and the interactions formed between the ligands and active site amino acids. Modified KDP has got maximum Glide Gscore with -5.92 and -8.61 were comparable with KDI's docking score -5.43, respectively (Table 1).

Table 1. Gscore and various thermodynamic parameters of ligand receptor (3T1M) complex.

Ligand	XP Gscore	Glide evdw	Glide ecoul	Glide energy	XP Hbond
CGC-bis-KDP	-5.92	-34.62	-22.10	-56.73	-3.27
DOTA-bis-KDP	-8.61	-38.15	-28.77	-66.92	-4.17
KDI	-5.43	-14.14	-21.99	-36.13	-3.25

The crucial ligand-1 and 3TIM interaction could be defined by ligands interacting through hydrogen bonding to Asn143, Arg144, Ser237, Asp148 and resides in pocket of residues formed by Asn143, Asn153, His158, Asn160, Arg162, Lys176, Trp181, Glu184, and Gly235. These important anchoring residues were also found to be residing in the vicinity of ligand-2 including hydrogen bonding with Asn160, Trp181 Lys176 Glu184, and Asp239 in addition to contacts in ligand 1. The reference peptides and designed ligand 1 and 2 were showing the remarkable resemblance in the docking interactions as they have their interaction with the common amino acid residues in the ligand binding site as shown in Table 2. Hence, our designed ligands are efficient, novel designed peptides towards human galectin-3 with satisfactory results in the form of final docking score.

Table 2. Amino acid residues of importance in receptor 3T1M.

Ligand	H-bond forming Amino acid residues
CGC-bis-KDP (1)	Asn143, Arg144, Ser237, Asp148
DOTA-bis-KDP (2)	Asn143, Arg144, Ser237, Asp148, Asn160, Trp181, Lys176, Glu184, Asp239
KDI	Arg144, Asp148, His158, Asn160, Lys176, Trp181, Gly235
KDP	Asp148, Lys176, Trp181, Gly235

2.1.2 Synthesis and Characterisation of Peptide-ligand

Our attempt was to synthesize a bisconjugated peptide and peptide macrocycle as SPECT, PET and MRI contrast agent for diagnosis and as a neurite growth enhancer related to neurodegenerative disorder. 'Bivalent approach' has been used to employ two peptide molecule across the spacer length of glutamate amino acid in ligand -1 (Fig. 1). In this study the bis-peptide was prepared on rink amide resin using solid phase peptide synthesis in good yield and cys-gly-cys amino acids was used for ^{99m}Tc labelling (SPECT imaging) with high efficiency. While in case of second ligand the trisubstituted cyclen was synthesised by reacting tert. butylbromoacetate with cyclen preferentially at three nitrogen atoms as reported in literature[18].

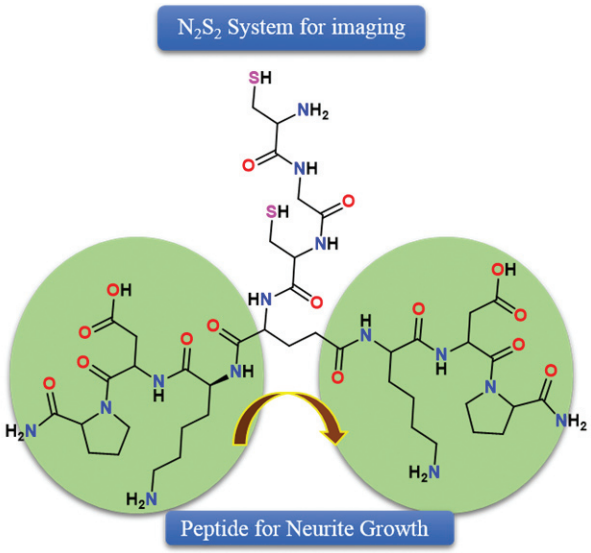


Figure 1. Ligand-1 Synthesised by solid phase peptide strategy.

By applying this strategy bis-KDP peptide was easily attached to DOTA. The tertiary butyl groups of DO3A were deprotected using TFA to obtain the compound which on further complexation with $GdCl_3$/Ga^{3+} gave peptide based ligand for MRI/PET application respectively (Fig. 2). The formation of both the ligands were confirmed by mass spectrometry.

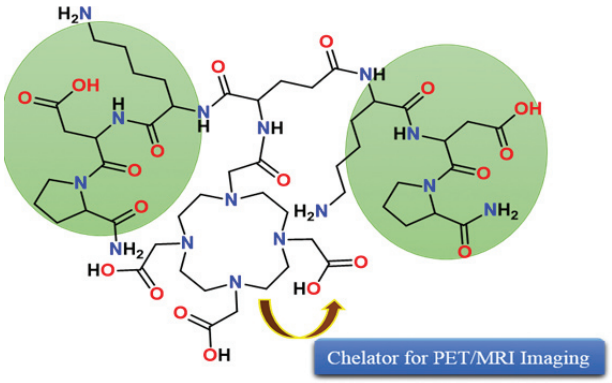


Figure 2. Ligand-2 Synthesised by solid phase peptide strategy.

2.2 Cytotoxic Studies

2.2.1 MTT Assay

The quantification of a cell proliferation and viability forms the basis for numerous *in vitro* assays of a cell population's response to external factors[19]. The yellow tetrazolium MTT's (3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) reduction is a way to examine cell proliferation[20]. MTT is reduced metabolically to generate reducing equivalents such as NADH and NADPH into intracellular purple formazan which can be solubilised and quantified spectrophotometrically[21,22]. The peptide ligand was evaluated for the ability to induce cytotoxicity on Neuro-2A cell line using methylthiazole tetrazolium (MTT) assay. The cells exposed to peptide- conjugate showed concentration dependent cell death (Fig. 3).

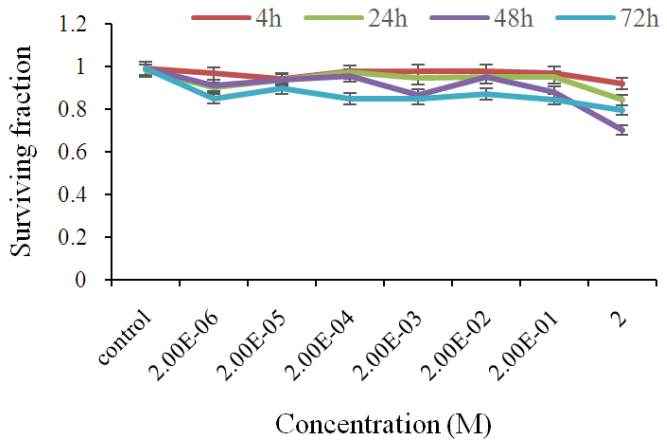
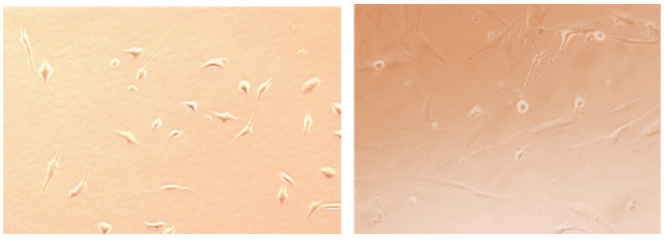


Figure 3. The mitochondrial activity (MTT) assay for cytotoxicity of unlabeled peptide-ligand in Neuro2A cell line.

2.3 Neurite Growth Assay

Retinoic acid (RA) is known to be a trophic factor for inducing neurite outgrowth through retinoic acid and the retinoid X receptors by alteration in gene expression[23]. No significant changes in neurite growth were observed in the number or length of neurites in the control condition on comparing with the ones treated with KDP peptide ligand treated neuron at any time point. The preliminary imaging in human SH-SY5Y showed peptide was able to initiate neurite growth formation (Fig. 4).

Furthermore the labeled peptide-compounds were highly stable *in vitro* and *in vivo* conditions. The stability in serum indicated that ^{99m}Tc remained bound to the complex up to 24 h. The blood kinetic activity shows a biphasic clearance and accounts for high *in vivo* stability of the radiolabeled conjugate. The biodistribution pattern of peptide ligand carried out in normal BALB/C mice displays moderate uptake in the brain at 5 min post injection. The results from the growth survival assay support the quantitative neurite growth assay. Further evident by high binding affinity in Neuro2A cell line demonstrating its capability of interacting with neuronal cell which could be translated in animal models for treatment of brain trauma in future.



(a) (b)

Figure 4. Bright field images of control (a), peptide-ligand (b) in human SH-SY5Y cells depicting neurite growth.

3. EXPERIMENTAL

3.1 Materials and Methods

All reagents and solvents were used in purest grade as available commercially without further purification. All amino acids, HOBT, DIC were purchased from Fluka. ^{99m}Tc was procured from Regional centre for Radiopharmaceuticals (Northern Region), Board of Radiation and Isotope Technology (BRIT), Institute of Nuclear medicine and Allied Sciences (INMAS), DRDO, India. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA). Column chromatography was carried out using silica MN60 (60-120 mesh) and neutral alumina (70-290 or ~150 mesh)), TLC sheets coated with silica gel 60, F254 (Merck).

3.2 Instrumentation

The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance II 400 MHz. Chemical shifts are reported relative to TMS. Mass spectra ESI-MS (Electrospray Ionisation-Mass Spectrometry) was performed on Agilent 6310 ion trap LCMS system from Germany with ion trap detection in the positive and negative mode. Radioactive samples were counted using Capintech automated well type counter. The scintigraphic imaging and biodistribution studies were done using a planar gamma camera Symbia true point from Siemens. The scintigraphs were obtained using a rectangular large field of view gamma camera (Symbia true point dual head) with a low energy all-purpose collimator.

3.3 Computational Methodology

Molecular modeling studies were carried out using an advanced molecular docking program GLIDE, version 9.2 (Schrodinger, Inc, USA). Firstly a complete systematic search of the conformational, orientational and positional space of the docked ligand were taken. Then it uses scoring to eliminate unwanted conformations followed by energy optimisation. For this study, PDB entry 3T1M (X-ray crystal structure of human galectin-3; www.rcsb.org), were used. The resolution was 1.55 A°. Protein preparation was performed for exclusion of needless heteroatom and solvent. The crystal ligand and protein were adjusted and minimised up to 0.30 A° RMSD. The extra precision (XP) mode of GLIDE were used to increase the accuracy to this study. The 'Glide grid boundaries' was defined by a 17.04 AX°9.31 A°X0.58 A°. Van der Waals radii were applied without scaling factors. For rest of the parameter default settings were used.

3.4 Synthesis of KDP Tripeptide

The resin (200 mg) was swelled in DCM 4 mL for 120 min and drained. The Fmoc group was deprotected using 20 % (v/v) solution of piperidine in DMF. A mixture of 4 equiv. of the Fmoc-Pro(otbu)-OH (relative to resin capacity), 4 equiv. of HOBT and DIC (relative to amino acid) and 2 mL of dry DMF was added to the resin and the mixture was stirred for 2 h. The mixture was again washed with DMF (3×25 mL), MeOH (3×25 mL) and DCM (3×25 mL), and dried in vacuo. Subsequently, a mixture of 4 equiv. of the Fmoc-Asp(otbu)-OH (relative to resin capacity), 4equiv. of HOBT and DIC (relative to amino acid) and 2 mL of dry DMF was added to the resin and the mixture was stirred for 2 h. The mixture was washed again with DMF (3×25 mL), MeOH (3×25 mL) and DCM (3×25 mL), and dried in vacuo. Finally Fmoc-Lys(Boc)-OH was coupled to NH_2-Asp-Pro(OtBu)-CONH-Rink amide with above mentioned protocol. The mixture was washed again with DMF (3×25 mL), MeOH (3×25 mL) and DCM (3×25 mL), and dried in vacuo. The tripeptide was obtained as a white solid after subjecting the resin first to Fmoc group deprotection by using 20 % (v/v) solution of piperidine in DMF and later to TFA cocktail (H_2O: TIS: EDA :: 2: 2: 1 and TFA) cleavage. The peptide was synthesised in high yield (> 70 %). The synthesised KDP peptide was characterised by mass spectrometry: KDP; *m/z* = found 579.9 [M^+]; calculated = 579.64. The progress of the amino acid coupling and deprotection of the Fmoc group was determined by colour change of ninhydrin in the Kaiser test.

3.5 Synthesis of Tri-tert-butyl 2,2',2''- (1,4,7,10-tetraazacyclododecane-1,4,7-triyl) Triacetate

A solution of 1,4,7,10-tetraazacyclododecane (1 eq., 1 g, 5.80 mmol) was stirred with of sodium bicarbonate (3 eq., 1.46 g, 17.38 mmol) in acetonitrile at 0 °C in an inert atmosphere. Thereafter, a solution of tert. butylbromoacetate (3 eq., 3.39 g, 17.38 mmol) in 40-50 mL acetonitrile was added dropwise to the reaction mixture after 20 minutes. Progress of the reaction was checked by TLC plates in chloroform and methanol (9.5:0.5 v/v). The reaction mixture was allowed to stir for 36 h and then solvent was evaporated to dryness under reduced pressure. The reaction mixture was extracted with chloroform/water and combined organic extracts were dried over anhydrous sodium sulphate, evaporated under reduced pressure to obtain the white powder as product and purified by silica gel chromatography (1.5% methanol in chloroform with a yield of 1.294 g). ^{1}H NMR (400 MHz, CDCl3, Me4Si): 3.38 (s, 4H, 2×CH_2), 3.29 (s, 2H, CH_2), 3.10 (brs, 4H, 2×CH_2), 2.93-2.88 (m, 12H, 6×CH_2), 1.45 (s, 27H, C(CH_3)$_3$). ^{13}C NMR (100 MHz, $CDCl_3$, Me_4Si): 172.86, 170.50, 169.60, 81.96, 81.79, 81.63, 58.13, 55.62, 51.28, 51.21, 49.14, 48.75, 47.48, 28.19, 28.16, 27.85, 27.77, 27.47; ESI-MS: *m/z* $C_{26}H_{50}N_4O_6$ Found: 515.40 [M+H]$^+$ calculated=514.70.

3.6 Synthesis of Cys-Gly-Cys-glutamic(KDP)$_2$ Ligand-1

The Fmoc group conjugated to Fmocglutamic(KDP)$_2$ bound to the resin was deprotected using 20 % (v/v) solution of piperidine in DMF. A mixture of 4 equiv. of the Fmoc-Cys(S-trityl)-OH (relative to resin capacity), 10 equiv. of HBTU and DIC (relative to amino acid) and 4 mL of dry DMF was added to the resin and the mixture was stirred for 5 h to obtain Fmoc-Cys-glutamic(KDP)$_2$. The mixture was again washed with DMF (3×25 mL), MeOH (3×25 mL) and DCM (3×25 mL), and dried in vacuo. The Fmoc group of Fmoc-Cys(S-trityl)-glutamic(KDP)$_2$ was deprotected using 20 % (v/v) solution of piperidine in DMF. Subsequently, a mixture of 4 equiv. of the Fmoc-Gly-OH (relative to resin capacity), 10 equiv. of HBTU and DIC (relative to amino acid) and 4 mL of dry DMF was added to the resin and the mixture was stirred for 5 h to obtain Fmoc-Gly-Cys(S-trityl)-glutamic(KDP)$_2$. The mixture was again washed with DMF (3×25 mL), MeOH (3×25 mL) and DCM (3×25 mL), and dried in vacuo. The Fmoc group of Fmoc-Gly-Cys(S-trityl)-glutamic(KDP)$_2$ was deprotected using 20 % (v/v) solution of piperidine in DMF. Finally, Fmoc-Cys(S-trityl)-OH was coupled to NH_2-Cys(S-trityl)-Gly-Cys(S-trityl) glutamic(KDP)$_2$ amide with above mentioned protocol. The mixture was washed again with DMF (3×25 mL), MeOH (3×25 mL) and DCM (3×25 mL), and dried in vacuo. The tripeptide conjugated with glutamic(KDP)$_2$ was obtained as a white solid after subjecting the resin first to Fmoc group deportection by using 20 % (v/v) solution of piperidine in DMF and later to TFA cocktail (H_2O: TIS: EDA :: 2: 2: 1 and TFA) cleavage. The synthesised KDP peptide was characterised by mass spectrometry: 1089.4816 [M/2]; calculated = 1089.25.

3.7 Synthesis of Tri-tert-butyl 2,2',2''-(1,4,7,10-tetraazacyclododecane-1,4,7-triyl)Triacetate Conjugated Glutamic(KDP)$_2$ Chloroacetylated Glutamic(KDP)$_2$ Ligand-2

In a sealed and inert assembly of a three necked flask took the required amount of resin bound chloroacetylated glutamic(KDP)$_2$, in acetonitrile, 18 equiv. of potassium carbonate were added in an inert atmosphere and the temperature of the reaction vessel was raised to 70 °C. After the desired temperature was obtained, 5 equiv. of trisubstituted cyclen dissolved in acetonitrile was added. The reaction mixture was allowed to stir for 18-20 h . The resin was again washed with ACN (3×25 mL), H_2O (3×25 mL), MeOH (3×25 mL) and DCM (3×25 mL), and dried in vacuo. The DO3A conjugated chloroacetylated glutamic(KDP)$_2$ was subjected to TFA cocktail (H_2O: TIS: EDA :: 2: 2: 1 and TFA) cleavage. The peptide was synthesised in high yield (> 60 %). The synthesised DO3A conjugated chloroacetylated glutamic(KDP)$_2$ peptide was characterised by mass spectrometry: *m/z* = found [M^+]; calculated. ESI-MS: *m/z* $C_{33}H_{61}N_{11}O_7$ Found: 1211.62 [M-H]$^-$, calculated = 1212.31.

3.8 Cell Culture and Cytotoxicity

Monolayer cultures of Neuro2A cells with uniform density of 4000 cells/well, 24 h before treatment were taken. Neuro2A cells were treated with different

concentrations (nM-mM range) of drugs for 4 h, 24 h, 48 h and 72 h. After the treatment including control and drug treated cells were incubated with MTT to have the last concentration of 50 µg/mL for 2 h at 37 °C. Afterwards the medium was removed. The cells were processed and 150 µL of DMSO was used to dissolve the formazan crystals. Optical density was measured on cell extracts in DMSO at 595 nm. Percentage metabolic viability was calculated using the optical density.

3.9 Quantitative Neurite Outgrowth Assay

RA was used as a positive control for generation of neurite growth. Nine field of views (FOVs) were taken in triplicate from different cells containing RA and Peptide ligand. At least 500 cells were counted. At least one neurite with a length of more than 10 µ was taken as standard. 0.1 % DMSO was used as a negative control in control experiments.

4. CONCLUSIONS

We present a modular approach, *in vitro* and *in vivo* studies for the development of peptide-ligand for neurite growth. What is needed a simple approach that attract, and develops, scientifically recognised aspects for their use in the treatment of neurological disease and trauma. The advantage of the KDP based peptide ligand over designed drugs is that occurs naturally in our CNS, therefore in the incident of injury or neurological disease is unlikely to have harmful side effects. The designed peptide-ligand may provide a new peptide sequence as a neurite growth enhancer for viability of neurons in neurodegenerative diseases.

ACKNOWLEDGEMENTS

We thank Director A.K. Singh, Institute of Nuclear Medicine and Allied Sciences. The work was carried out under Defence Research and Development Organisation Project no. INM 311 (3.1).

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Corrigendum (2021)

Defence Life Science Journal, Vol. 1, No. 1, June 2016, pp. 57-62, DOI : 10.14429/dlsj.1.10062, 2016, DESIDOC

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In original manuscript the following Figure has been depicted and named by the author.

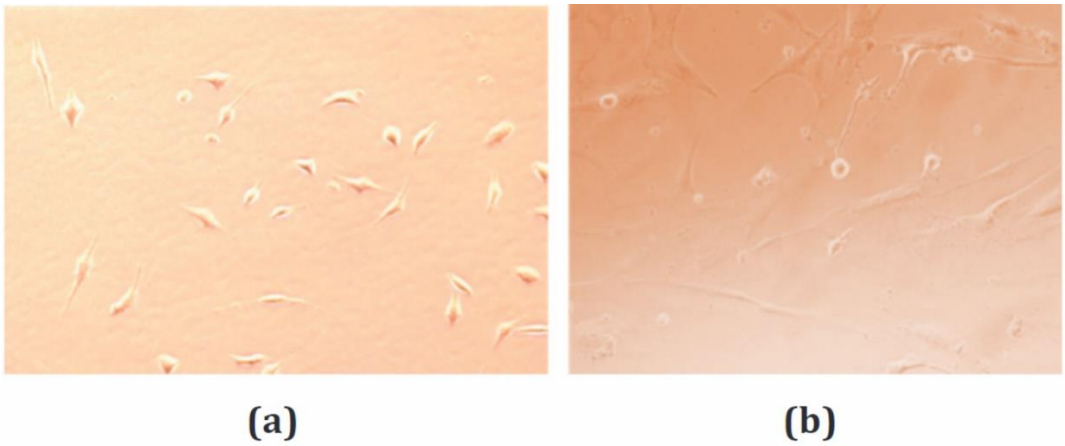
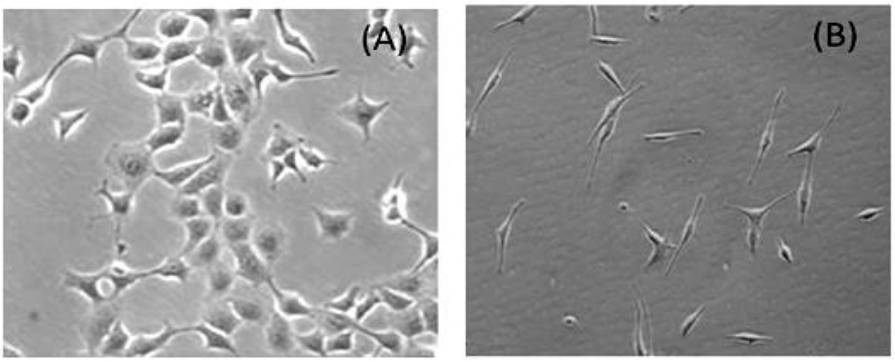


Figure 4. Bright filed images of control (a), peptide-ligand (b) in human SH-SY5Y cells depicting neurite growth

The Corrigendum to Figure 4 (A) (B) needs replacement. The unintentional error of image occurs due to the synchronized work on the other KDP ligand. The correct images are attached. The correction does not affect the result or any outcome of the article.

Correct Images:



Correct Caption:

Figure 4 (A) Bright field images of undifferentiated control human SH-SY5Y cells, (B) KDP peptide-ligand treated differentiated human SH-SY5Y cells depicting neurite growth.

Signature of Authors

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Date: 26/11/2021