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PEGylation of an osteoclast inhibitory peptide: Suitable candidate for the treatment of osteoporosis

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ABSTRACT

Osteoporosis is a condition of bone loss due to excessive osteoclastic activity. Several protein factors, such as receptor activator of nuclear factor kappa-B (RANK), receptor activator of nuclear factor kappa-B ligand (RANKL), osteoprotegerin (OPG), have been identified that are important in the pathogenesis of osteoporosis. RANKL binds to RANK and activates the NF-κB pathway by interaction of its cytoplasmic domain with an intracellular adapter protein, TNF receptor associated factors 6 (TRAF 6). This interaction can be inhibited by cell-permeable peptides that prevent RANK-TRAF 6 interaction. However, similar to the peptides/proteins used in clinical setting, the effective application of this TRAF 6 Inhibitory peptide as a therapeutic agent is marred by several limitations for instance short half-life, rapid renal clearance and immunogenicity. In the present study, we have developed PEGylated TRAF 6 Inhibitory peptide by conjugating TRAF 6 Inhibitory peptide to linear PEG backbone that exhibits longer bioavailability in plasma in the animal model. Besides, it has an enhanced uptake at its site of action, i.e., bone marrow.

1. Introduction

Osteoporosis is a disease characterized by reduction in bone strength and increase in susceptibility to fractures. The reduction in bone strength is a function of reduced bone mass and abnormal bone quality, including microscopic architecture of the bone, bone turnover, damage accumulation, and mineralization (JAMA, 2001). Imbalances between osteoclast and osteoblast activities arise from a variety of hormonal changes or inflammatory growth factors which can cause skeletal abnormalities, such as osteoporosis (decreased bone mass) or osteopetrosis (increased bone mass), due to higher or lower osteoclast activity. It has been shown that sex steroids, estrogen and androgen exert bone protective functions, at least in part, by regulating the development of cells in bone marrow, as well as the rate of programmed cell death of mature cells. At menopause in women (or after castration in men) the reduction of sex hormone levels promotes increased production of the above-mentioned cytokines, which trigger differentiation of a common macrophage/osteoclast precursor towards the osteoclast lineage (Hughes et al., 1996; Jilka et al., 1992; Weinstein et al., 1997). The discovery of RANK/RANKL/OPG system as the mediator of the osteoclastogenesis opens up several therapeutic approach for treatment of different bone ailments (Khosla, 2001; McClung, 2007).

One of the therapeutic approaches is to intervene the interaction of RANKL with its receptor, RANK, which is expressed on the surface of pre osteoblast cells along with interaction of RANK and its association with its cytoplasmic adaptor molecules, TRAFs. RANK has no intrinsic protein kinase activity to mediate signaling after RANKL binds to it (Boyce and Xing, 2007; Darnay and Aggarwal, 1999). The important step in TNFR downstream signaling is the binding of TRAFs to the cytoplasmic domain of RANK. TRAFs 2, 5, and 6 bind to RANK, but of these only TRAF 6 appears to have an essential function in osteoclast development (Boyce and Xing, 2007). The TRAF 6 knockout mice develop osteopetrosis which indicates that TRAF 6 is the most important adaptor molecule of RANK (Lomaga et al., 1999).

Several small molecules like peptides have been reported in the past having inhibitory property of RANKL mediated osteoclast formation (Ye et al., 2002). However, these peptides have their limitation while in use as a therapeutic agent. The peptides consisting of a short chain of amino acids usually have extremely short *in vivo* half-lives due to fast renal clearance (for peptides <5 kDa) (Maeda

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et al., 1992; Sato et al., 2006). Moreover, the therapeutic peptides are degraded by various proteases during circulation in blood and eliminated early from the body by reticuloendothelial system (RES) mainly in liver and spleen (Parveen and Sahoo, 2006). Thus, the low metabolic stability is a major concern for the peptides used as therapeutic agents. To address such issues, various biochemical and drug delivery approaches have received wide acceptance for safeguarding these therapeutic peptides (Sato et al., 2006; Veronese and Morpurgo, 1999).

PEGylation is a process by which the modification of a protein, peptide or non-peptide molecule is carried out by linking it to one or more polyethylene glycol (PEG) chains (Veronese and Pasut, 2005). PEG is an amphiphilic polymer that is non-toxic, non-immunogenic and biocompatible for human use (Parveen and Sahoo, 2006). The conjugation of PEG at specific site of peptide by covalent attachment enhances the pharmacological properties of the therapeutic peptide. In addition, PEGylation increases the ability of the conjugated peptide to resist both proteolysis & systemic clearance without significantly reducing their membrane permeability/bioactivities (Veronese and Pasut, 2005; Werle and Bernkop-Schnurch, 2006).

Hence, we report the PEGylation of TRAF 6 Inhibitory peptide by utilizing the lysine residues of the peptide for conjugation with activated PEG-NHS. The conjugation of TRAF6 Inhibitory peptide with PEG may be useful as long-acting alternative for an efficient pharmacological utilization of this therapeutic agent.

2. Materials and methods

2.1. Materials

L-glutamate (119955000) was procured from Acros organics. Chloroform (12305), disodium hydrogen orthophosphate (15825), potassium dihydrogen orthophosphate (13405), sodium chloride (15198) and potassium chloride (1649161) were purchased from Fisher Scientific. Dulbecco's Modified Eagle Medium (DMEM) (12800-017) was procured from Invitrogen. Fetal bovine serum (RM1112-500ML) and Trypan blue were procured from Himedia. Coomassie Brilliant Blue G 250 (0240109), acrylamide (0148300) were obtained from Sicso Research Lab. Dimethyl sulfoxide (DMSO) (D5879-100 ML), sodium dodecyl sulfate (L4390), penicillin and streptomycin (060M0811), N,N-Methylenebisacrylamide (M7279), Trypsin-EDTA (T4049-500ML) were procured from Sigma Aldrich. FACS buffer (342003) was procured from BD biosciences. FITC (SP-1502) was purchased from Vector Laboratories, USA. Sodium bicarbonate (194553) was procured from MP Biomedicals, Sephadex G10 (17-0010-01) was procured from GE Life Sciences, activated PEG-NHS (MW ≈ 5 kDa) was obtained from NOF CORPORATION (Japan). Dialysis membrane (MWCO: molecular weight cut-off = 3.5 kDa) was purchased from Spectra/Por® 6 (Spectrum Laboratories, Inc., CA, USA).

2.1.1. TRAF6 Inhibitory peptide

The TRAF6 Inhibitory peptide contains RKIPTEDEY, a sequence from cytoplasmic domain of mouse RANK. The peptide binds to RANK, blocking TRAF6 and RANK interaction and thereby inhibiting osteoclast differentiation. The TRAF6 inhibitory peptide also contains a protein transduction (PTD) sequence (DRQIKIWFQNRRMKWKK) derived from antennapedia, which renders the peptide cell permeable. The peptide used in this study having the sequence DRQIKIWFQNRRMKWKK*RKIPTEDEY* was synthesized by IMGENEX Corporation, San Diego, CA.

2.1.2. Cell line

RAW (Mouse leukemic monocyte macrophage cell line) cells were maintained in DMEM (Dulbecco's Modified Eagle Medium) media containing 10% FBS, 100 mM L-glutamate and supplemented with penicillin and streptomycin. Cells were incubated at 5% CO_2 and 90% humidity in a CO_2 incubator (Hera Cell, Thermo Scientific, Waltham, MA). Prior to use the cells were trypsinized with Trypsin-EDTA.

2.1.3. Experimental animals

C57BL/6J female mice were procured from National Centre for Laboratory Animal (NCLA), National Institute of Nutrition, Hyderabad and were kept in separate cages in the animal house. The mice were fed with NCLA supplied pellet feed. They were acclimatized in the animal room at least for two weeks before carrying out the experiment. The tap water was supplied *ad libitum*.

2.2. Synthesis of FITC-TRAF6 Inhibitory peptide

1 mg of FITC dissolved in 50 μl DMSO was added drop-wise to the 2 ml of TRAF6 Inhibitory peptide solution prepared in phosphate buffer (100 mM, pH 7) (5 mg/ml) and incubated for 3 h at room temperature on a shaker (Wadegati, Labequip, India). The FITC-peptide was separated from un-conjugated peptides as well as free FITC by passing through a Sephadex G10 matrix column (Econo-Column® chromatography columns, Bio-Rad, Hercules, CA) of dimension 1.0 cm × 30 cm. The fractions of 1 ml sample size were collected and optical density was measured in two different wavelengths, 280 nm (λ_{max} for TRAF6 Inhibitory peptide) and 495 nm (λ_{max} for FITC) in Bio-Rad Spectrophotometer (Smart Spec, Bio-Rad, Hercules, CA) simultaneously. The fractions showing higher reading at both the wavelength were pooled and lyophilized using a lyophilizer (LABCONCO Corporation, Kansas City, MO) to obtain the powdered form of the FITC-peptide.

2.3. Synthesis of PEGylated TRAF6 Inhibitory peptide

200 μl of TRAF6 Inhibitory peptide dissolved in sodium borate buffer (0.05 M, pH 8.5) (1 mg/ml) was added to activated PEG (PEG_{5000}-NHS) and was stirred under constant magnetic stirring for 2 h at room temperature. The activated PEG derivative, PEG_{5000}-NHS was used at 8-fold molar excess to each lysine in TRAF6 Inhibitory peptide (consist of 5 lysines). The PEGylated peptide thus obtained was subjected to dialysis using dialysis bag (Spectra/Por® 6, Molecular weight cut-off = 3.5 kDa) against sodium borate buffer (0.05 M, pH 8.5) in a 2 l beaker for a period of 6 h with frequent change of dialysate in every 2 h to remove the unconjugated TRAF6 Inhibitory peptide. The resultant solution was then lyophilized using a lyophilizer (LABCONCO Corporation, Kansas City, MO) for 72 h to obtain a white powder form of PEGylated TRAF6 Inhibitory peptide conjugate.

Similarly, the PEGylation of FITC-TRAF6 Inhibitory peptide was done in the process mentioned above for synthesis of PEGylated TRAF6 Inhibitory peptide.

2.4. Characterization of the PEGylated TRAF6 Inhibitory peptide

2.4.1. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

PEGylated TRAF6 inhibitory peptide was characterized by SDS-PAGE. The PEGylated TRAF6 inhibitory peptide and native TRAF6 Inhibitory peptide corresponding to 400 μg each were electrophoresed on 14% separating and 4% stacking gel using the Mini Protean® II electrophoretic apparatus (Bio-Rad, Hercules, CA). Gel electrophoresis was conducted at 100 V in Laemmli buffer, pH 8.3 calibrated with Protein marker (New England Biolabs Inc., Hitchin, UK) with broad range from 3.4 to 212 kDa. Later, the gel was stained with 0.1% (w/v) Coomassie Brilliant Blue R250 for the identification of protein bands on the gel.

2.4.2. MALDI-TOF MS analysis

Molecular weight of PEGylated TRAF6 Inhibitory peptide was obtained from MALDI-TOF mass spectrometry. Analysis of TRAF6 Inhibitory peptide and PEGylated TRAF6 Inhibitory peptide was conducted in a linear mode & data for 2 ns pulses of the 337 nm Nitrogen laser were averaged for each spectrum. Linear, positive ion TOF detection was performed using an acceleration voltage of 20 kV & laser intensity of approximately 10% greater than threshold. The grid voltage & guide wire voltage were chosen for each spectrum to achieve the optimal signal to noise. Spectra are obtained by summing over 128 laser shots to obtain comparable conditions & smoothed with a 19-point Savitzky–Golay filter. The samples for analysis are mixed with sinapinic acid in 70% acetonitrile in water containing 0.1% TFA as final concentration at a sample-to-matrix ratio of 1:1 (v/v) & dried on the sample holder.

2.5. Cytotoxicity assay

The cytotoxicity effect of PEGylated TRAF6 Inhibitory peptide was assayed colorimetrically by Trypan blue dye exclusion assay, as described previously (Terauchi et al., 2007). RAW cells were plated at a density of 1×10^5 cells/well in a 24 well flat bottom plate (NUNC, Denmark) and kept overnight in appropriate growth medium at 37 °C in a CO_2 incubator. Then, different concentrations of PEGylated TRAF6 Inhibitory peptide and 100 μM of native TRAF6 Inhibitory peptide were added in triplicate to the cells and incubated for 24 h in growth medium at 37 °C. After the stipulated time, the cells were washed twice with chilled PBS (0.1 M, pH 7.4) followed by trypsinization and then centrifuged at 1200 rpm for 5 min using SIGMA 1-15K (Munich, Germany). Viability of the pelleted cells was then estimated by Trypan blue dye exclusion assay. In brief, the cell pellets were resuspended in 0.5 ml of PBS (0.1 M, pH 7.4). Then 1 part of 0.4% Trypan blue and 1 part cell suspensions were mixed and incubated for 1 min at room temperature. Both unstained (viable cells) and stained cells (nonviable) cells were counted using a haemacytometer under an inverted phase contrast microscope (Leica Q Win, UK).

2.6. In vivo bioavailability study

In vivo bioavailability study of PEGylated TRAF6 Inhibitory peptide and native TRAF6 Inhibitory peptide were carried out in C57BL/6 mice (Gaur et al., 2000). For this study, FITC-TRAF6 Inhibitory peptide and FITC-PEGylated TRAF6 Inhibitory peptide were used. The mice were divided into two groups. Group I were injected with FITC-TRAF6 Inhibitory peptide and group II were injected with FITC-PEGylated TRAF6 Inhibitory peptide in their tail vein with a dose of 1.5 mg (peptide)/mice (having an approximate body weight of 25 g) at different time periods (i.e., 0.5 h, 2 h, 12 h and 24 h) ($n = 3$; 12 mice/group). Post injection, blood sample was collected at stipulated time periods by retro-orbital bleeding of the mice and centrifuged at 12,000 rpm for 1 min at 4 °C using SIGMA 1-15K (Munich, Germany) to collect the serum. Later, the serum was analyzed by monitoring the fluorescence intensity of FITC having an excitation at 488 nm and emission at 519 nm using a flourimeter (Synergy™ HT, BioTek Instruments Inc., Winooski, VT). The fluorescence intensity was determined by correlating with the standard curve of FITC.

2.7. In vivo biodistribution study

In vivo biodistribution study of FITC-PEGylated TRAF6 Inhibitory peptide and FITC-TRAF6 Inhibitory peptide in various organs was carried out in C57BL/6 mice (Gaur et al., 2000). In addition, the body weights of the mice were measured during the period of investigation. Post injection at different time intervals (0.5 h, 2 h, 12 h and 24 h) after the blood collection, organs such as liver, kidney and bone marrow were collected after sacrificing the mice. The collected organs were thoroughly washed with PBS (0.1 M, pH 7.4) and were homogenized in lysate buffer consisting of 0.005% Triton X in PBS (0.1 M, pH 7.4), according to the protocol described (Gaur et al., 2000). The homogenates were kept at 4 °C for 30 min and then centrifuged at 12,000 rpm for 30 min using SIGMA 1-15K (Munich, Germany). The supernatants were then analyzed by monitoring the fluorescence intensity of FITC having an excitation at 488 nm and emission at 519 nm by using a flourimeter (Synergy™ HT, BioTek Instruments Inc., Winooski, VT). The fluorescence intensity was determined by correlating with the standard curve of FITC.

2.8. In vivo uptake study in bone marrow cells by flow cytometry

Studies have shown TRAF 6 interaction with RANK occurs in the cells of the bone marrow during the pre-osteoclast formation (Khosla, 2001). In this mileu, the uptake of PEGylated TRAF 6 Inhibitory peptide in the bone marrow cells is vital for its potential action. Hence, the cellular uptake of the PEGylated TRAF 6 inhibitory peptide in comparison to native TRAF 6 inhibitory peptide was studied in the bone marrow of mice. In this regard, the bone marrow obtained during the biodistribution experiment of FITC-TRAF 6 inhibitory peptide or FITC-PEGylated TRAF 6 inhibitory peptide for 24 h time period was used for this study. The RBCs from the bone marrow were collected by flushing PBS (0.1 M, pH 7.4) into the marrow. After that, the cells were washed with PBS (0.1 M, pH 7.4) by centrifugation at 1200 rpm for 5 min using SIGMA 1-15K (Munich, Germany) followed by lysis using RBC lysis buffer. And finally the cells were washed with PBS (0.1 M, pH 7.4) before analysis in FACS. The cells were dispensed in FACS buffer and were analyzed by BD FACSCalibur using FL1-H channels. The data were analyzed by CELLQUEST PRO™ (BD Bioscience).

2.9. Statistical analysis

Student's *t*-test was used to conduct statistical analyses. Data are expressed as means ± standard deviation and values of (*) $p < 0.5$ were taken to be indicative of significant differences and (**) $p < 0.05$ were considered very significant differences.

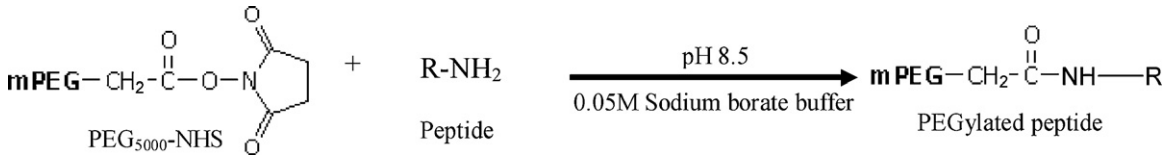
3. Results

3.1. PEGylated TRAF6 Inhibitory peptide

TRAF6 Inhibitory peptide (DRQI*K*IW*F*QNRRM*K*W*KK*R*K*IPTEDEY) contains 6 primary amines that can theoretically react with PEG_{5000}-NHS including the α-amine of phenylalanine and ε-amino groups of 5 lysine side chains. PEGylated TRAF6 Inhibitory peptide was formed as a result of the reaction between an *N*-hydroxysuccinimide ester derivative of PEG molecule and the free ε-amino group present on lysine of TRAF6 Inhibitory peptide forming an amide bond. The reaction mechanism is illustrated in Scheme 1.

3.2. SDS-PAGE analysis

To characterize the PEGylated TRAF 6 Inhibitory peptide, SDS-PAGE was carried out. As shown in the Fig. 1, three bands were visible in lane 1 loaded with PEGylated TRAF 6 Inhibitory peptide signifying mono-, di- and tri-PEGylated TRAF 6 Inhibitory peptide whereas a single distinct band was observed in lane 2 corresponding to native TRAF 6 Inhibitory peptide. The SDS-denatured PEGylated TRAF 6 Inhibitory peptides migrated slowly because of PEG's large hydrodynamic volume (3 mol of water form an adduct with each ethylene oxide subunit of PEG) (Kurfurst, 1992). A strong



Scheme 1. Schematic display of the Schiff base reaction (ε-amino group of lysine react with the reactive group, N-Hydroxy succinimide of PEG) involved in PEGylation of TRAF 6 Inhibitory peptide.

band at ~55 kDa suggests that the maximum mono PEGylated TRAF 6 Inhibitory peptides were formed during conjugation.

3.3. MALDI-TOF MS analysis

MALDI TOF MS analysis was carried out for both native TRAF 6 Inhibitory peptide (Fig. 2a) and PEGylated TRAF 6 Inhibitory peptide (Fig. 2b). Fig. 2(a) showed a sharp peak at m/z 3491 whereas the minor small peaks at m/z 6461 and 10481 signified the aggregates of the peptides. Further, a series of signal peaks was observed at m/z 9406.37, 14649.19 and 20209.32 for PEGylated TRAF 6 Inhibitory peptide (Fig. 2b). The average mass of mono-PEGylated TRAF 6 Inhibitory peptide was observed to be m/z 9406.37. The difference in the masses of native TRAF 6 Inhibitory peptide (3491 Da) and mono-PEGylated TRAF 6 Inhibitory peptide was ~5917 Da, which is almost consistent to the mass of mPEG-NHS. Besides, the peaks at m/z 14649.19 and 20209.32 during PEGylated TRAF 6 Inhibitory peptide analysis in MALDI-TOF MS reveal the di- and tri-PEGylated forms of TRAF 6 Inhibitory peptide. Interestingly, at a higher PEGylation degree, fewer signal peaks in MALDI were observed, indicating less heterogeneity.

3.4. Cytotoxicity of the PEGylated TRAF6 inhibitory peptide

The cytotoxicity of the PEGylated TRAF6 inhibitory peptide was carried out in RAW cell line by Trypan blue dye exclusion method (Fig. 3). The PEGylated TRAF 6 Inhibitory peptide at all concentration was found to be non-toxic to RAW cells with minimal number of dead cells. In addition, the treatment of native peptide at a higher concentration of 100 μM also exhibited non-toxicity (data not shown). Thus, the results clearly indicate that the PEGylated TRAF 6 Inhibitory peptide is non-toxic considering the concentrations tested in the cells.

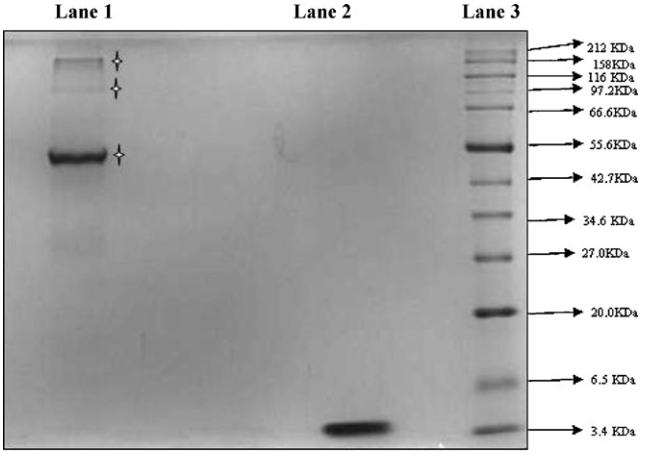


Fig. 1. SDS-PAGE analysis. SDS-PAGE specifically stained for protein with Coomassie blue. Lanes: 1, PEGylated peptide; 2, TRAF 6 Inhibitory peptide; 3, molecular weight marker proteins.

3.5. In vivo bioavailability of the TRAF 6 Inhibitory peptide and PEGylated TRAF 6 Inhibitory peptide in mice

The bioavailability of peptides in serum was studied using spectrofluorometer (Fig. 4). After intravenous injection, the blood collected in four different time intervals, i.e., 30 min, 2 h, 12 h, and 24 h was analyzed for the presence of native TRAF 6 inhibitory peptide and PEGylated TRAF 6 Inhibitory peptide. As depicted from Fig. 4, at initial time period (after 30 min of injection) the PEGylated TRAF 6 Inhibitory peptide exhibited a ~2-fold higher bioavailability than the native TRAF6 inhibitory peptide. Also, as per Table 1, PEGylated TRAF 6 Inhibitory peptide had higher C_{max} than the native TRAF6 inhibitory peptide. Interestingly, the PEGylated TRAF 6 Inhibitory peptide continued to show higher bioavailability in comparison to native TRAF 6 Inhibitory peptide in all time periods. By the end of 24 h PEGyleted TRAF 6 Inhibitory peptide remained ~4 times more than the native TRAF 6 Inhibitory peptide. In addition, the pharmacokinetic parameters and bioavailability were calculated with Kinetica software as listed in Table 1. The table clearly shows half-life ($T_{1/2}$) of PEGylated TRAF 6 Inhibitory peptide at ~9 h whereas native TRAF 6 Inhibitory peptide showed half-life ($T_{1/2}$) at 5 h.

3.6. In vivo biodistribution of the TRAF 6 Inhibitory peptide and PEGylated TRAF 6 Inhibitory peptide in mice tissue

Organ uptake was evaluated for kidney, liver and bone marrow at different time periods after injection of FITC-TRAF 6 Inhibitory peptide and FITC-PEGylated TRAF 6 Inhibitory peptide in C57BL/6 mice, as shown in Fig. 5(a)–(c). The study revealed the presence of PEGylated TRAF 6 Inhibitory peptide in much more quantity in comparison to native TRAF 6 Inhibitory peptide in bone marrow, liver and kidney up to 24 h. The modification of TRAF 6 Inhibitory peptide by PEGylation clearly showed a 2-fold higher uptake in the mice bone marrow till 12 h post incubation. Additionally, PEGylated TRAF 6 Inhibitory peptide has 3-fold higher uptake in bone marrow post 24 h of its administration.

However, PEGylated TRAF 6 Inhibitory peptide in liver and kidney did not exhibit significantly higher uptake in comparison to the native peptide till 24 h. Hence, it can be assumed that the PEGylated TRAF 6 Inhibitory peptides get cleared from these tissues after 24 h post injection.

Table 1
Tabular representation of the pharmacokinetic parameter in serum of C57BL/6 mice after intravenous administration of FITC-PEGylated TRAF 6 Inhibitory peptide and FITC-TRAF 6 Inhibitory peptide in the tail vein with a dose of 1.5 mg/mouse (peptide) ($n = 3$).

Parameters	TRAF 6 Inhibitory peptide (Native)	PEGylated peptide
$T_{1/2}$ (h)	5.08	8.99
AUC_{tot} (μg/μl·h)	0.130925	0.256897
AUC_{Extra} {μg/μl·(h)^2}	0.005872	0.044119
AUC_{last}	0.125053	0.224397
T_{max} (h)	0.5	0.5
C_{max} (ng/ml)	26,840	50,600
MRT (h)	7.51	11.80

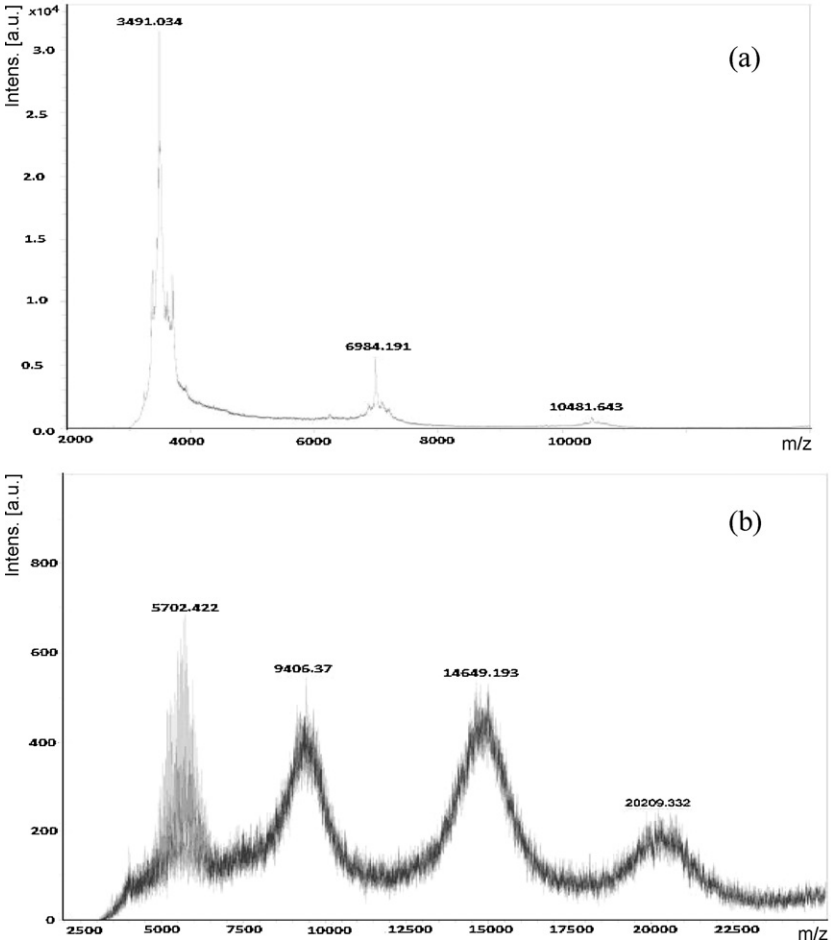


Fig. 2. MALDI TOF MS analysis. MALDI TOF mass spectrum of (a) TRAF 6 Inhibitory peptide and (b) PEGylated TRAF 6 Inhibitory peptide.

3.7. In vivo uptake of the TRAF6 inhibitory peptide and PEGylated TRAF6 inhibitory peptide in mice bone marrow cells

The uptake of the PEGylated TRAF 6 Inhibitory peptide by the bone marrow cells is a crucial determinant for its therapeutic action. As shown in Fig. 6, the PEGylated TRAF 6 Inhibitory peptide tagged with FITC has an absorption maximum at 494 nm and was recorded by FACS in FL1 channel on a FACS Calibur™ (BD Biosciences). In bone marrow, the FITC-PEGylated TRAF 6 Inhibitory peptide showed a slight increase in its uptake compared to FITC-TRAF 6 Inhibitory peptide even after 24 h. Hence, it can be presumed that the higher biodistribution lead to successful higher uptake of PEGylated TRAF 6 Inhibitory peptide in the bone marrow even up to 24 h.

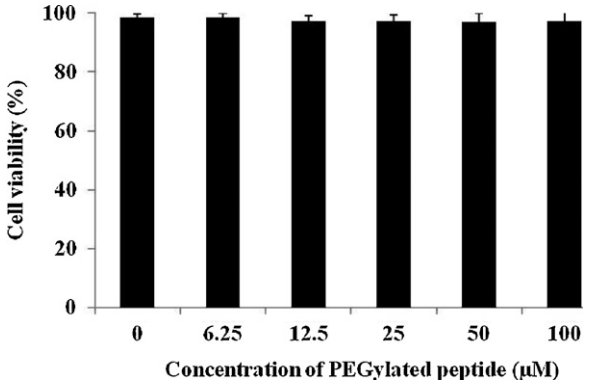


Fig. 3. Bar graph representation of cytotoxic effect of PEGylated TRAF 6 Inhibitory peptide in RAW cell line by Trypan blue assay. The cells were cultured in presence or absence of PEGylated peptide at different concentrations and peptide alone. The percentage of viable cells was counted by Trypan blue exclusion method after 24 h of incubation at 5% CO_2. Data presented are mean ± standard deviation, $n = 3$.

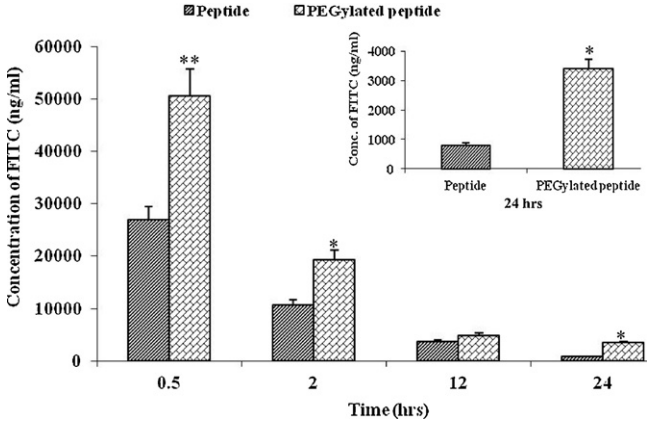


Fig. 4. *In vivo* bioavailability of native TRAF 6 Inhibitory peptide and PEGylated TRAF 6 Inhibitory peptide in serum. FITC-PEGylated TRAF 6 Inhibitory peptide and FITC-TRAF 6 Inhibitory peptide were injected intravenously in tail vein with a dose of 1.5 mg (peptide)/mouse ($n = 3$). Post injection blood sample was collected at different time intervals, processed and subjected to flourimetric analysis. Detailed experimental procedure is mentioned in Section 2. Data presented are mean ± standard deviation, $n = 3$. (**) $p < 0.05$ and (*) $p < 0.5$ FITC-TRAF 6 Inhibitory peptide *versus* FITC-PEGylated TRAF 6 Inhibitory peptide.

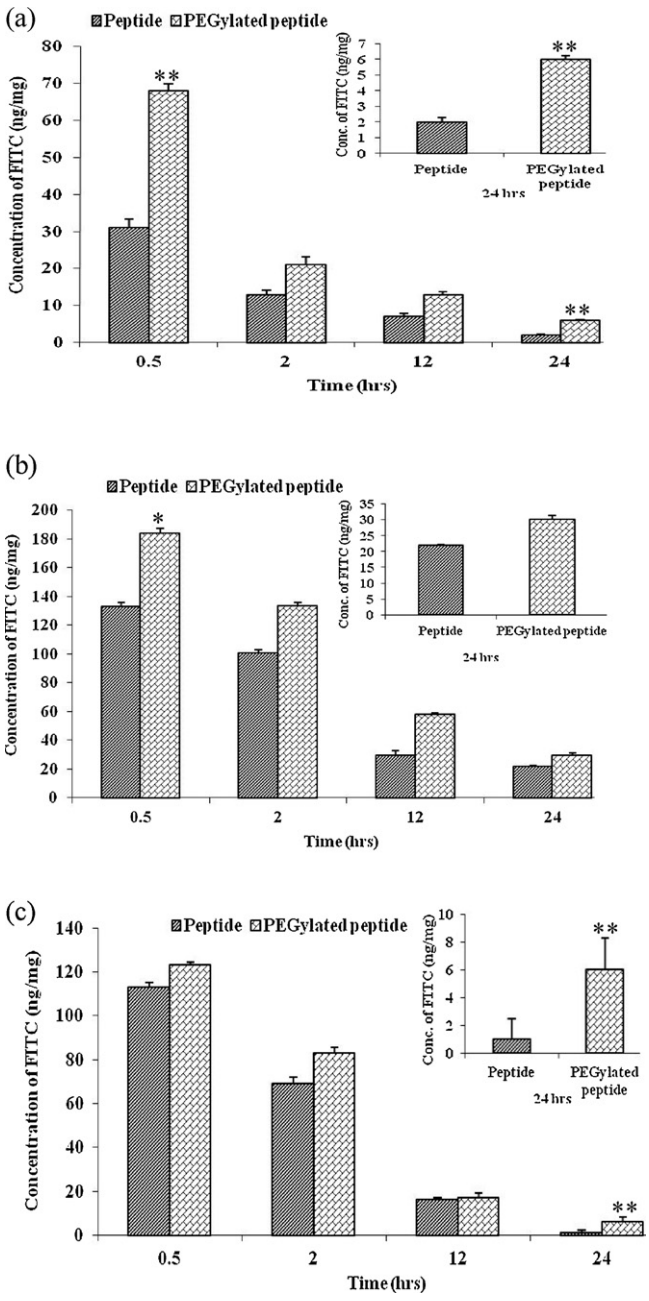


Fig. 5. *In vivo* biodistribution of native TRAF 6 Inhibitory peptide and PEGylated TRAF 6 Inhibitory peptide in (a) bone marrow, (b) liver and (c) kidney. FITC-PEGylated TRAF 6 Inhibitory peptide and FITC-TRAF 6 Inhibitory peptide were injected intravenously in the tail vein with a dose of 1.5 mg (peptide)/mouse ($n = 3$). Post injection organs were collected at different time intervals and processed for flourimetric analysis. Detailed experimental procedure is mentioned in Section 2. Data presented are mean ± standard deviation, $n = 3$. (**) $p < 0.05$ and (*) $p < 0.5$ FITC-TRAF 6 Inhibitory peptide *versus* FITC-PEGylated TRAF 6 Inhibitory peptide.

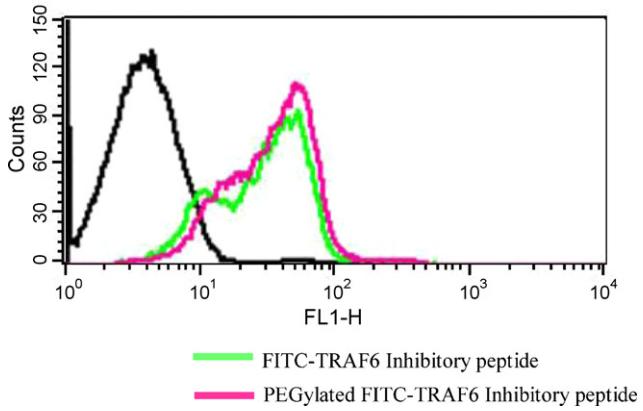


Fig. 6. *In vivo* uptake study in bone marrow cells. FITC-PEGylated TRAF 6 Inhibitory peptide and FITC-TRAF 6 Inhibitory peptide were injected intravenously in the tail vein with a dose of 1.5 mg/mouse (peptide) ($n = 3$). Post injection bone marrow was collected after 24 h that were further processed for flow cytometry analysis.

4. Discussion

The discovery of RANK/RANKL/OPG pathway for osteoclast development paved the path for the identification of drug targets for the treatment of osteoporosis (Khosla, 2001). OPG which is the natural regulator of osteoclast development, when over expressed results osteopetrosis and inhibits the osteoclast differentiation from its precursor cells *in vitro* by inhibiting the interaction of RANK-RANKL (Simonet et al., 1997). The activation of osteoclast, the bone absorbing cell is principally signaled through receptor activator of nuclear factor-κB (RANK) which in interaction with its cytoplasmic adaptor molecule TRAF activates the signaling cascade. Out of all the six TRAF molecules, TRAF 6 is found to be the most critical in carrying out the RANK mediated NF-κB activation and formation of a proper osteoclast cells (Darnay et al., 2007). RANK mutants that specifically lack TRAF 6-binding sites are unable to restore osteoclastogenic potential in RANK$^{-/-}$-derived hematopoietic precursors (Armstrong et al., 2002). TRAF6-knockout mice exhibit severe osteopetrosis which is caused by impaired osteoclast function (Lomaga et al., 1999).

Several approaches have been made in the past for the inhibition of RANK and TRAF 6 interaction; one of them is the TRAF 6 decoy peptide. Ye et al. deduced a decoy peptide which inhibits osteoclast formation *in vitro* (Ye et al., 2002). Latter on Poblenz et al. demonstrated that selective TRAF 6 decoy peptide which is smaller in size in comparison to the earlier reported peptide can effectively inhibit the osteoclast formation (Poblenz et al., 2007). Here, we have selected the smallest TRAF 6 decoy peptide **RKIPTEDEY** for enhancing its bioavailability for potential use in clinical settings.

The peptides which are very small in size are considered to be a poor drug candidate. One of the important drawbacks is their low bioavailability and short half-life because of their rapid degradation by proteolytic enzymes of the digestive system and blood plasma as well as rapid removal from the circulation by the liver and kidney (Vlieghe et al., 2010). The half-life of the peptide can be increased by several procedures including modification of the peptides, glycosylation, fatty acid addition to peptides, entrapment in nanoparticle and conjugation to PEG, etc. (Lien and Lowman, 2003). PEGylation is considered one of the most successful techniques developed so far to enhance the therapeutic potential of peptides and proteins. They belong to the class of synthetic polymers that usually have low polydispersity and tend to increase the molecular size of the peptide conjugated to it thereby, preventing renal clearance (Sato et al., 2006; Veronese, 2001; Veronese and Pasut, 2005).

In this study, the TRAF 6 Inhibitory peptide has a **RKIPTEDEY** sequence along with the antennapedia homodomain peptide sequence. It is 26 amino acid long and has a molecular mass of nearly 3.5 kDa. The conjugation of TRAF 6 Inhibitory peptide with linear PEG5000 occurs by the amide bond formation at the ε-amino group of lysine residues present in the peptide sequence. The formation of the PEGylated TRAF 6 Inhibitory peptide is evident from the SDS-PAGE analysis (Fig. 1). As shown in the figure, three different forms of PEGylated TRAF 6 Inhibitory peptide conjugates are formed in SDS-PAGE due to the conjugation property of the PEG molecules to the lysine molecules. When the peptide with more than one lysine gets conjugated to the PEG then a heterogeneous

mixture of products is formed and the addition of number of PEG molecule to a single peptide increases its stability as well as pharmacological properties particularly molecular weight (Sato et al., 2006; Veronese, 2009). However, the PEGylated TRAF 6 Inhibitory peptide does not show its actual molecular weight in aqueous solution due to the increase in hydrodynamic volumes of PEG leading to lower mobility in SDS-PAGE and higher molecular weight display in comparison to the protein molecular marker during electrophoresis. So it is difficult to calculate the exact molecular weight of the PEGylated peptide by SDS-PAGE electrophoresis (Kurfurst, 1992; Veronese, 2001). Thus, characterization of the PEGylated TRAF 6 Inhibitory peptide was further carried out by MALDI-TOF MS to determine exact number of PEG conjugated to the TRAF 6 Inhibitory peptide, as shown in Fig. 2. The MALDI-TOF MS exhibited corresponding peaks of PEGylated TRAF 6 Inhibitory peptide according to their molecular weight. Also, it shows that maximum PEGylated TRAF 6 Inhibitory peptides formed after PEGylation are either mono or di-PEGylated.

Studies report PEG related toxicity is rare for marketed PEGylated biological products (Parveen and Sahoo, 2006). In line with the perspective, the PEGylated TRAF 6 Inhibitory peptide did not show any cytotoxic effect on RAW cells at different concentrations which indicate that the PEGylated TRAF 6 Inhibitory peptide can be used for the biological application (Fig. 3). In addition, PEGylation is currently used as an improved method for both increasing the bioavailability as well as bioactivity of the peptides and several other therapeutic proteins including single chain antibodies and recombinant proteins (Delgado et al., 1996; Eno-Amooquaye et al., 1996; Katre et al., 1987). Hence, the assessment of bioavailability and biodistribution pattern of PEGylated TRAF 6 Inhibitory peptide is a vital parameter for its efficacy over native TRAF 6 Inhibitory peptide. In our study, the PEGylated TRAF 6 Inhibitory peptide showed increased bioavailability in serum than native TRAF 6 Inhibitory peptide at all time points studied, as evident from Fig. 4. In addition, the PEGylated TRAF 6 Inhibitory peptide exhibited a half-life ($T_{1/2}$) at ~9 h whereas native peptide showed half-life ($T_{1/2}$) at 5 h (Table 1). This signified the longer bioavailability of the PEGylated TRAF6 Inhibitory peptide in plasma. Besides, studies usually indicate the uptake of PEGylated products in RES organs such as liver and kidney partially influenced by molecular target and partially by physicochemical characteristics such as charge or molecular shape, in addition to molecular size. With this backdrop, our study showed the presence of PEGylated TRAF 6 Inhibitory peptide in liver and kidney till 24 h, as shown in Fig. 5. Besides, the PEGylated TRAF 6 Inhibitory peptide tends to act in the bone marrow cells. Our study demonstrated an enhanced presence of the PEGylated TRAF 6 Inhibitory peptide in the bone marrow till 24 h and also at the same time exhibited an increased cellular uptake in the bone marrow cells in comparison to the native TRAF 6 Inhibitory peptide (Figs. 5 and 6).

5. Conclusion

The PEGylated TRAF 6 Inhibitory peptide showed prolonged *in vivo* stability compared to the native TRAF 6 Inhibitory peptide. Therefore, further investigations are warranted to examine the effect of PEGylation on the biological activity of the TRAF 6 Inhibitory peptide. Therefore, PEGylated TRAF 6 Inhibitory peptide possesses a clean advantage over the native TRAF 6 Inhibitory peptide as a superior therapeutic agent for the treatment of osteoporosis.

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