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OXYMATRINE (AN INHIBITOR OF HSPA8) REDUCES BINDING OF VGF DERIVED BIOACTIVE PEPTIDE TLQP-21 TO THE SURFACE OF LIVE SH-SY5Y CELLS

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Abstract: Use of active site-targeting inhibitor is an effective approach leading to pharmacological inventions. Heat shock cognate protein A8 (HSPA8) has been found as a receptor of VGF derived bioactive peptide TLQP-21 in SH-SY5Y cells. So, it was of much interest to carry out this study in order to observe whether Oxymatrine (OMTR), an inhibitor of HSPA8, inhibits the binding of TLQP-21 to the surface of intact, live SH-SY5Y cells. The results confirmed, as expected, that OMTR reduces the binding of TLQP-21 to the surface of intact, live SH-SY5Y cells, with a strong conclusion that the binding of TLQP-21 to the surface of SH-SY5Y cell model was through HSPA8. The inhibition efficacy of OMTR potentiates its application in drug targeting.

Keywords: VGF, TLQP-21, SH-SY5Y, OMTR, HSPA8

Introduction

VGF (a non-acronymic name) gene was originally identified as a nerve growth factor (NGF) responsive gene and should not be confused with VEGF (vascular endothelial growth factor) (Akhter, 2015). NGF33.1, a nervous system-specific mRNA was cloned by treatment of PC12 cells with NGF. After elucidating the nucleic acid as well as amino acid sequences of the NGF33.1 cDNA clone, Levi *et al.* (1985) designated this clone corresponding to the NGF-inducible mRNA as VGF. The term 'VGF' derived from the selection of this clone from plate **V** of the nerve **G**rowth **F**actor induced PC12 cell cDNA library (Levi *et al.*, 1985; Possenti *et al.*, 1989).

TLQP-21 is a 21 residue peptide named after its three amino terminal amino acid residues---threonine (thr) - leucine (leu)–glutamine (gln) – proline (pro), spans residues 556-576 of the precursor sequence. The molecular weight of TLQP-21 human is 2490.88 Da, formula: $C_{107}H_{170}N_{40}O_{26}$ (Levi *et al.*, 2004; Akhter, 2015). Out of several bioactive peptides derived from VGF, TLQP-21 is of great importance because of its multi-physiological roles. TLQP-21 was found provoking energy consumption and increased resting energy expenditure (Possenti *et al.*, 2012; Jethwa *et al.*, 2007; Bartolomucci *et al.* 2006). TLQP-21 also plays a key role in stress (Razzoli *et al.*, 2012, Bartolomucci *et al.*, 2011), diet induced obesity (Possenti *et al.*, 2012), choronic pain modulation/nociception (Fairbanks *et al.*, 2014;

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Chen *et al*., 2013; Rizzi *et al*., 2008, Ayub, 2012), anorexia (Bartolomucci *et al*., 2006; Jethwa *et al*., 2007), gastric contractility (Severini et.al., 2009; Bartolomucci *et al*., 2008), blood pressure/hypertension regulation (Fargali *et al*., 2014), glucose-stimulated insulin secretion (GSIS) (Stephens *et al*., 2012), reproduction (Aguilar *et al*., 2013; Pinilla *et al*., 2011), neuroprotective agent (Severini *et al*., 2008, diabetes (Stephens *et al*., 2012. All these observations suggest that TLQP-21 will render a very interesting pharmacological target in many aspects (Akhter, 2015).

HSPA8 (71 kDa), a constitutively expressed protein, is a fascinating member of the HSP70 family. HSPA8 expressing on the cell surface performed as a cellular receptor (Sagara *et al*., 1998; Page *et al*, 2009; Chakraborty *et al*., 2015; Akhter, 2015). Over 70 candidate compounds were screened for HSPA8 inhibitor. Among the compounds examined, Oxymatrine (OMTR, matrine oxide, matrine N-oxide, matrine 1-oxide: one of many quinolizidine alkaloid compounds), molecular weight (MW) 264.31 (Fig. 1), an alkaloid extracted from *Sophera flavescens* (Ling *et al*., 2007) (popularly known as KuShen plant, Fig. 2), showed significant activity to down regulate the expression of HSPA8 in HepG2 liver cells without showing any toxicity to the cells. Additionally, Western blotting confirmed the reducing effect at the protein level, showing reduction of HSPA8 protein in the OMTR treated cells (Wang *et al*., 2010).

Considering all these, it was of interest to investigate the effect of OMTR on SH-SY5Y cells; whether it reduces binding of TLQP-21 to the surface of intact, live SH-SY5Y cells in flow cytometry, popularly known as Fluorescence-activated Cell Sorting (FACS) analysis.

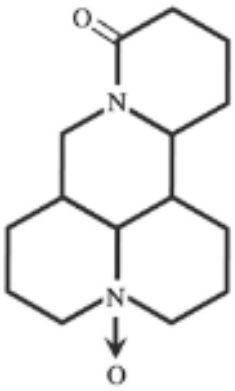

Fig. 1: Chemical structure of OMTR

Fig. 2: *Sophora flavescens*: A Chinese herb, popularly known as Ku Shen: a typical traditional Chinese medicine plant (Cai *et al*., 1997)

Materials and Methods

Oxymatrine (OMTR), inhibitor of HSPA8 protein expression, with purity 99% was purchased from the Selleck Chemicals, USA.

SH-SY5Y cells (European Collection of Cell Cultures, ECACC; catalog number-94030304) are thrice cloned (SK-N-SH → SH-SY → SH-SY5 → SH-SY5Y) subline of the

neuroblastoma cell line SK-N-SH and were grown at 37°C in a 5% CO_2-humidified incubator on a culture medium composed of 1:1 Earle's Balanced Salt Solution (EBSS) (Sigma Aldrich) and F12 HAM (Sigma Aldrich) supplemented with 15% fetal bovine serum (FBS) (Gibco), 1% Glutamine (Gln)(Sigma Aldrich), 1% Non-Essential Amino Acids (NEAA) (Sigma Aldrich), and 1% Penicilin-Streptomicin (P/S) (Invitrogen) in Falcon Petri dishes, 100×20 mm (Life Sciences).

OMTR was solubilised in sterilized milliQ water at concentration of 25 mg/ml (94.5 mM). Growing SH-SY5Y cells in Falcon Petri dishes, 100×20 mm (Life Sciences), treated overnight with solution of OMTR at final concentration of 0.4 mg/ml (1.51 mM) or not treated, were washed with Phosphate-buffered saline (PBS) at room temperature. Then for detachment from the bottom of the Petri dish, cells were incubated with 1.5 ml of 0.05% Trypsin/Ethylene diamine tetraacetic acid (EDTA) solution (1× Gibco, Life Technologies) at 37°C with 5% CO_2 for 2-5 minutes and finally cell detachment was empirically assessed by visual inspection. Subsequently, 1.5 ml of SH-SY5Y culture medium was added and mixed gently by pipetting. Cells were taken in a Falcon tube and spun down at 1500 rpm for 10 minutes. Cells were resuspended in PBS containing 1% bovine serum albumin (BSA) at a concentration of 0.5-2 × 10^6 cells/ml, as determined by counting using a FACS Calibur™ (Becton Dickinson; San Jose).

TLQP-21 (28, 10 or 5 µl of a 0.1 mM solution in filtered PBS, corresponding to 2.8, 1.0 and 0.5 mmoles of TLQP-21) was added to resuspended cells, already treated or not treated with OMTR, and the mixture was incubated at 4 °C for 30 minutes. The cells were then washed twice with PBS supplemented with 1% BSA (wash buffer), followed by centrifugation at 300×g for 7 minutes. After the second wash, the supernatant was discarded gently, followed by vortexing to resuspend the pellet in test tube. Fluorescein-conjugated avidin (Thermo Scientific) was added at 10 or 20 µg/ml as per instructions of the supplier, and the mixture incubated at 4°C for 30 minutes in the dark. Then after washing two times as before, the cells were measured with a FACSCalibur™ (Becton Dickinson; San Jose). Gates were set by forward and side scatter. For analysis, cells were calculated using Cell Quest Software (Becton Dickinson). Forcontrol, cells were treated with fluorescein-conjugated avidin (Thermo Scientific) only (Akhter, 2015).

SH-SY5Y cells treated with OMTR (0.4 mg/ml; 1.51 mM) were collected, as described above. Aliquots of the cells were taken and lysed in lysis buffer containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2 mM ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 1 mM Dithiothreitol (DTT),1mM sodium orthovanadate, 1% Triton X-100, 10% Glycerol, 2 µM leupeptin, 400 µM Phenylmethylsulfonyl fluoride (PMSF), 50 µM ß-glycerophosphate, 100 µg/ml trasylol. The cells were scrapped on ice for 10 minutes and were incubated on ice for 30 minutes with periodic vortexing at each 5 minutes interval, followed by centrifugation for 20 minutes at 14000×g at 4°C. The supernatant was collected for further use. Protein concentration was quantified using the bicinchoninic acid (BCA) protein assay kit (Pierce Chemical).

Results

Incubation of SH-SY5Y cells with TLQP-21, followed by FACS analysis showed that OMTR reduces the binding of TLQP-21 to the surface of SH-SY5Y cells. Surface binding of TLQP-21 by the cells treated with OMTR were 2.81% (control, Fig. 3A), 57.37% (Fig. 3C), 53.61% (Fig. 3E) and 53.16% (Fig. 3G); while surface binding of TLQP-21 by the cells not treated with OMTR were 2.28% (control, Fig.3B), 57.36% (Fig. 3D), 59.33% (Fig. 3F) and 58.73%(Fig. 3H) when the SH-SY5Y cells were incubated with 0.1 mM TLQP-21 with 5,10 and 28 µl, respectively. In this case, fluorescein-conjugated avidin was added at a concentration of 10 µg/ml.

In addition, using fluorescein-conjugated avidin at double concentration, 20 µg/ml ; surface binding of TLQP-21 by the cells treated with OMTR were 1.98% (control, Fig.3I), 30.78% (Fig. 3K), 32.64% (Fig. 3M) and 50.36% (Fig. 3O); whereas, surface binding of TLQP-21 by the cells not treated with OMTR were 3.51% (control, Fig. 3J), 32.13% (Fig. 3L), 40.75% (Fig. 3N) and 61.16% (Fig. 3P) when the SH-SY5Y cells were incubated with 0.1 mM TLQP-21 with 5,10 and 28 µl, respectively.

SH-SY5Y cells treated with OMTR showed a significant reduction of binding of the peptide, particularly at the optimum conditions (16.66%): This is, when the cells were incubated with 5 µl of the peptide (corresponding to 0.5 mmole) followed by the addition of fluorescein-conjugated avidin added at a concentration of 20 µg/ml (Fig. 4).

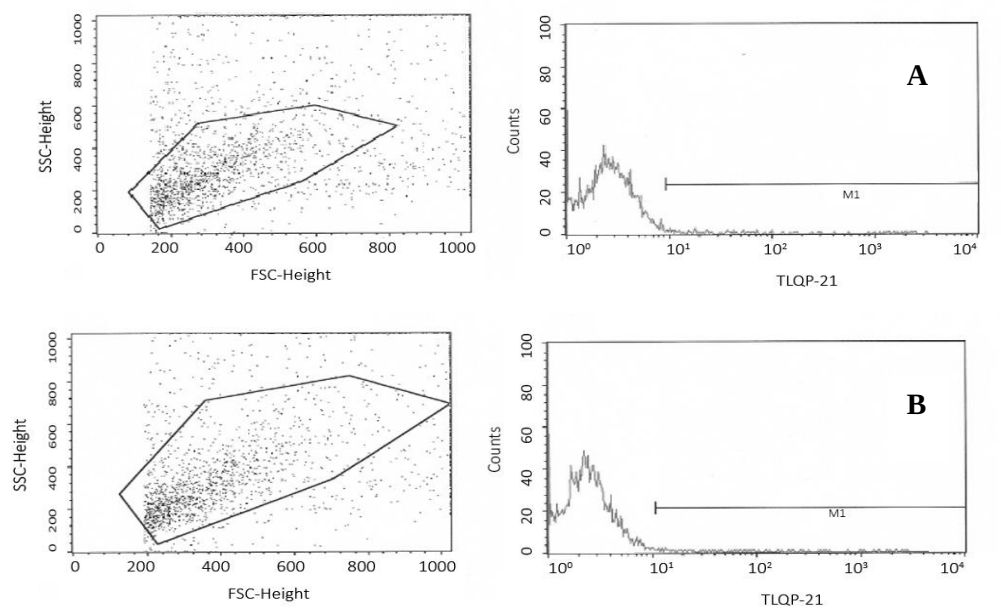

Fig. 3(A-B): Expression of HSPA8 protein in SH-SY5Y cells reduced in flow cytometry assays (24 hour treatment), resulting in decreased binding of TLQP-21 to the surface of live SH-SY5Y cells. Incubation of SH-SY5Y cells withoutTLQP-21, staining with avidin, fluorescein conjugated, at a concentration of 10µg/ml showed surface binding of TLQP-21 as 2.81% (control, with inhibitor, A) and 2.28 % (control, without inhibitor, B)

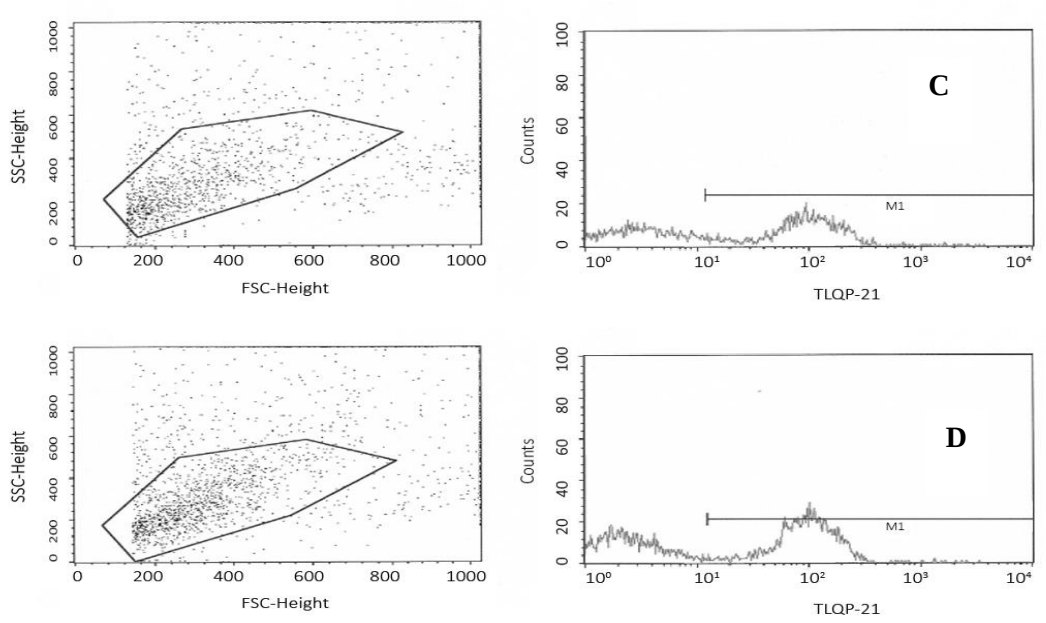

Fig. 3(C-D): Expression of HSPA8 protein in SH-SY5Y cells reduced in flow cytometry assays (24 hour treatment), resulting in decreased binding of TLQP-21 to the surface of live SH-SY5Y cells. Incubation of SH-SY5Y cells with 28 µl of 0.1mM TLQP-21 followed by staining with avidin, fluorescein conjugated (10µg/ml) showed surface binding of TLQP-21 as 57.37% (with inhibitor, C), and 57.36% (without inhibitor, D)

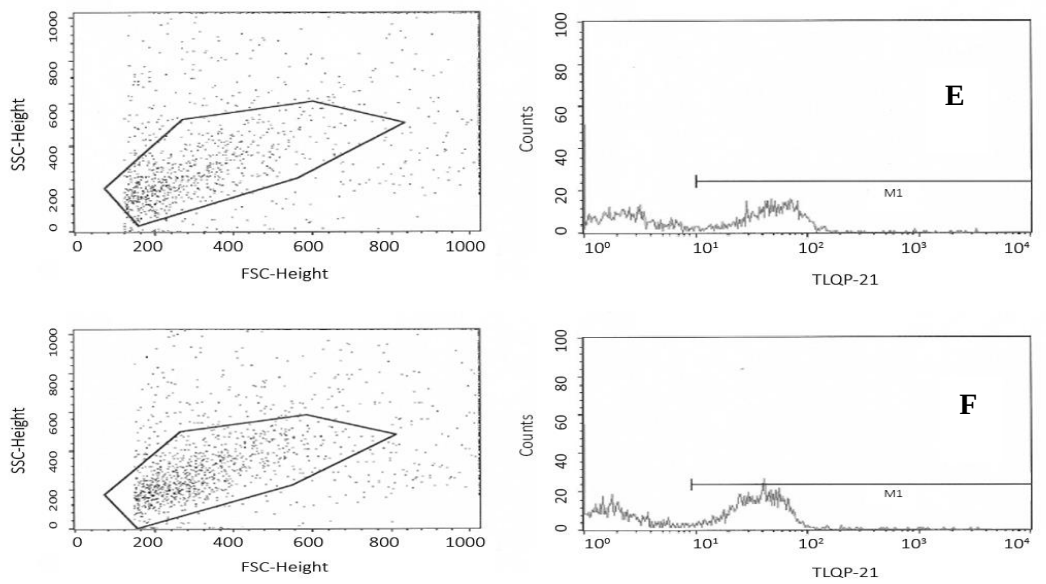

Fig. 3(E-F): Expression of HSPA8 protein in SH-SY5Y cells reduced in flow cytometry assays (24 hour treatment), resulting in decreased binding of TLQP-21 to the surface of live SH-SY5Y cells. Incubation of SH-SY5Y cells with 10 µl of 0.1 mM TLQP-21 followed by staining with avidin, fluorescein conjugated (10 µg/ml) showed surface binding of TLQP-21 as 53.61% (with inhibitor, E), and 59.33% (without inhibitor, F)

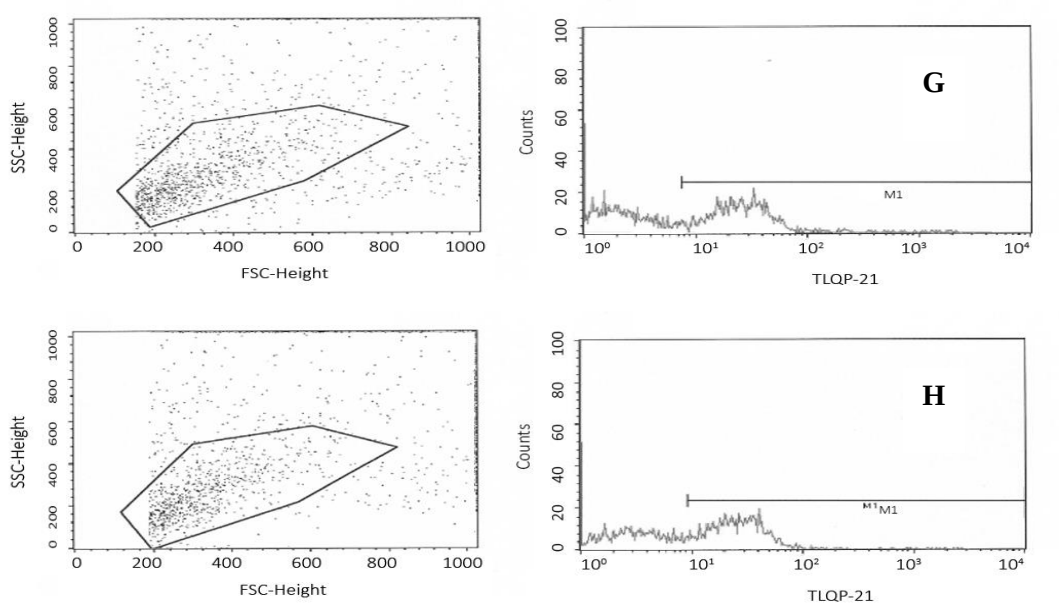

Fig. 3(G-H): Expression of HSPA8 protein in SH-SY5Y cells reduced in flow cytometry assays (24 hour treatment), resulting in decreased binding of TLQP-21 to the surface of live SH-SY5Y cells. Incubation of SH-SY5Y cells with 5 µl of 0.1mM TLQP-21 followed by staining with avidin, fluorescein conjugated (10µg/ml) showed surface binding of TLQP-21 as 53.16% (with inhibitor, G), and 58.73% (without inhibitor, H)

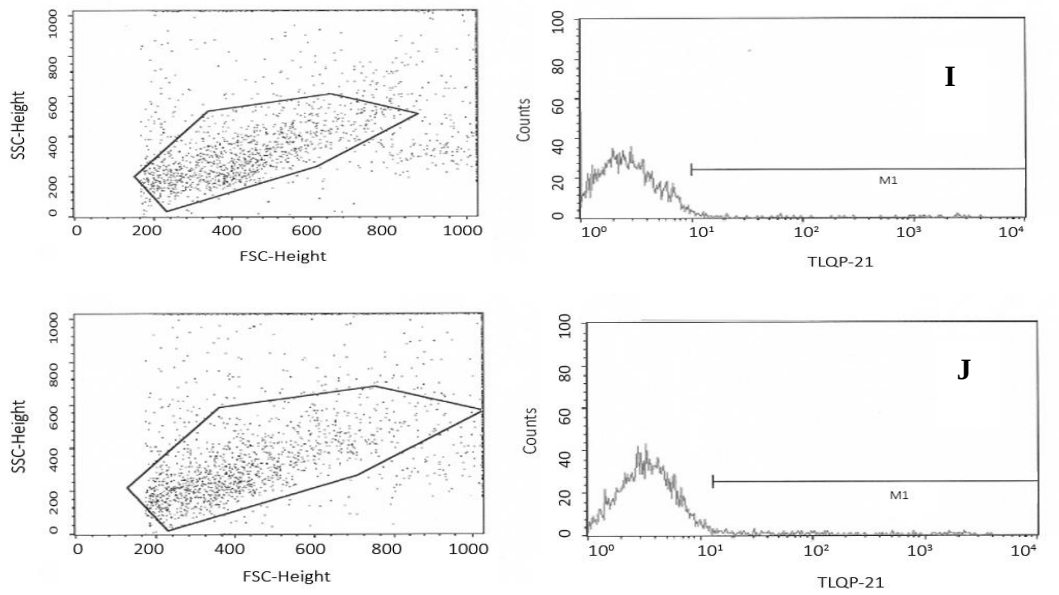

Fig. 3(I-J): Expression of HSPA8 protein in SH-SY5Y cells reduced in flow cytometry assays (24 hour treatment), resulting in decreased binding of TLQP-21 to the surface of live SH-SY5Y cells. Incubation of SH-SY5Y cells without TLQP-21, staining with avidin, fluorescein conjugated at a concentration of 20µg/ml showed as 1.98% (control, with inhibitor, I) and 3.51% (control, without inhibitor, J)

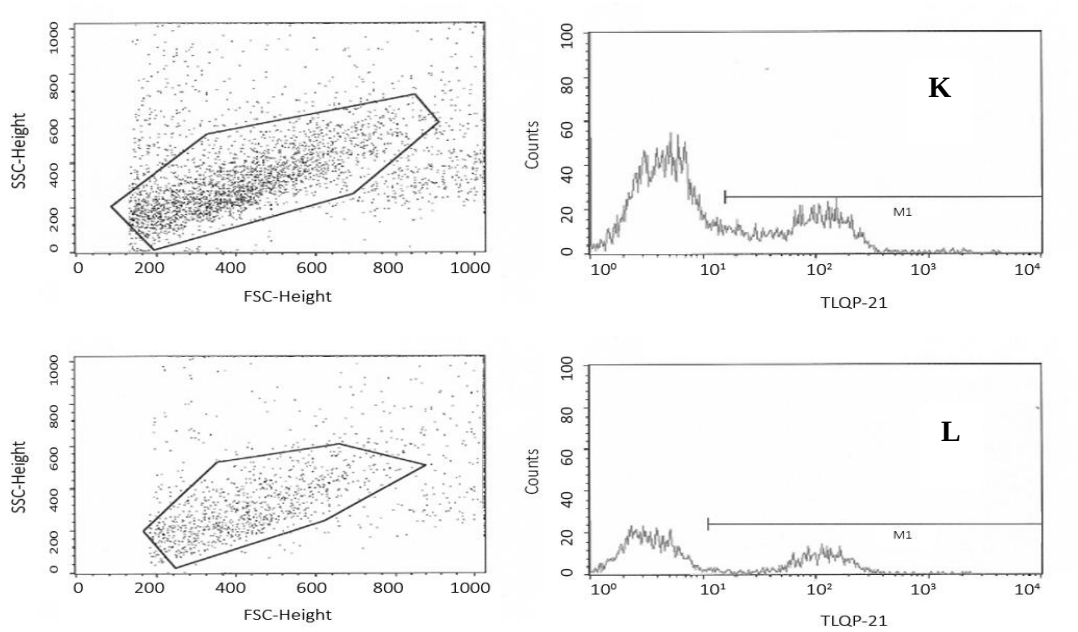

Fig. 3(K-L): Expression of HSPA8 protein in SH-SY5Y cells reduced in flow cytometry assays (24 hour treatment), resulting in decreased binding of TLQP-21 to the surface of live SH-SY5Y cells. Incubation of SH-SY5Y cells with 28 µl of 0.1mM TLQP-21 followed by staining with avidin, fluorescein conjugated (20µg/ml) showed surface binding of TLQP-21 as 30.78% (with inhibitor, K), and 32.13% (without inhibitor, L)

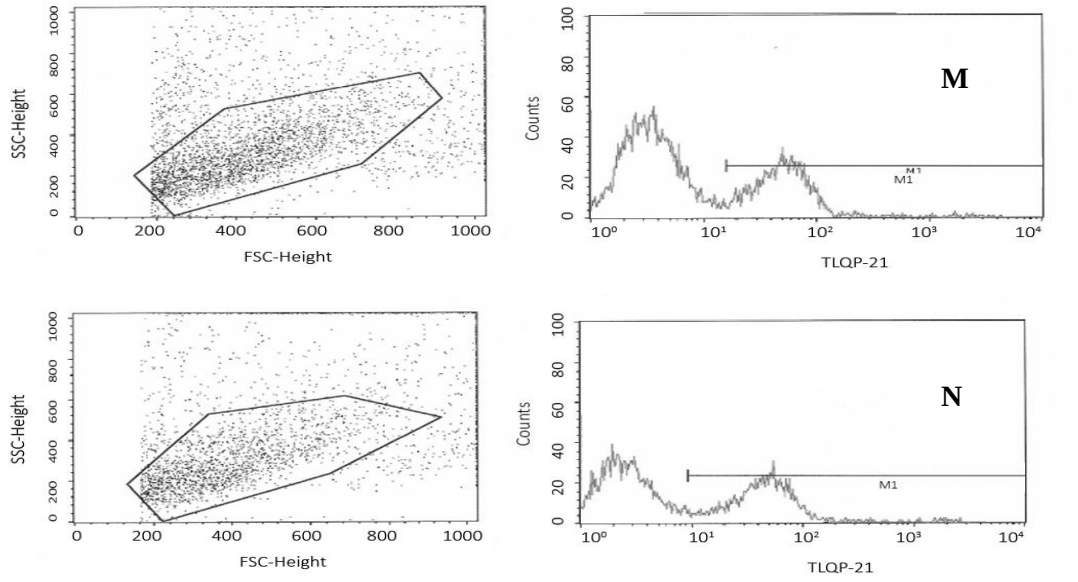

Fig. 3(M-N): Expression of HSPA8 protein in SH-SY5Y cells reduced in flow cytometry assays (24 hour treatment), resulting in decreased binding of TLQP-21 to the surface of live SH-SY5Y cells. Incubation of SH-SY5Y cells with 10 µl of 0.1mM TLQP-21 followed by staining with avidin, fluorescein conjugated (20 µg/ml) showed surface binding of TLQP-21 as 32.64% (with inhibitor, M), and 40.75% (without inhibitor, N)

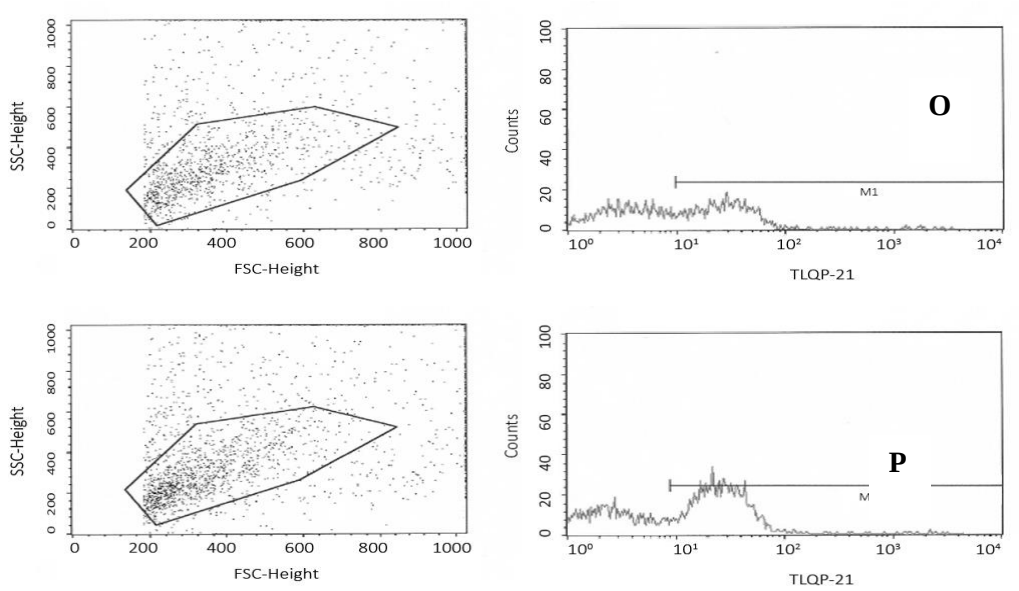

Fig. 3(O-P): Expression of HSPA8 protein in SH-SY5Y cells reduced in flow cytometry assays (24 hour treatment),resulting in decreased binding of TLQP-21 to the surface of live SH-SY5Y cells. Incubation of SH-SY5Y cells with 5 µl of 0.1mM TLQP-21 followed by staining with avidin, fluorescein conjugated (20 µg/ml) showed surface binding of TLQP-21 as 50.36% (with inhibitor, O), and 61.16% (without inhibitor, P)

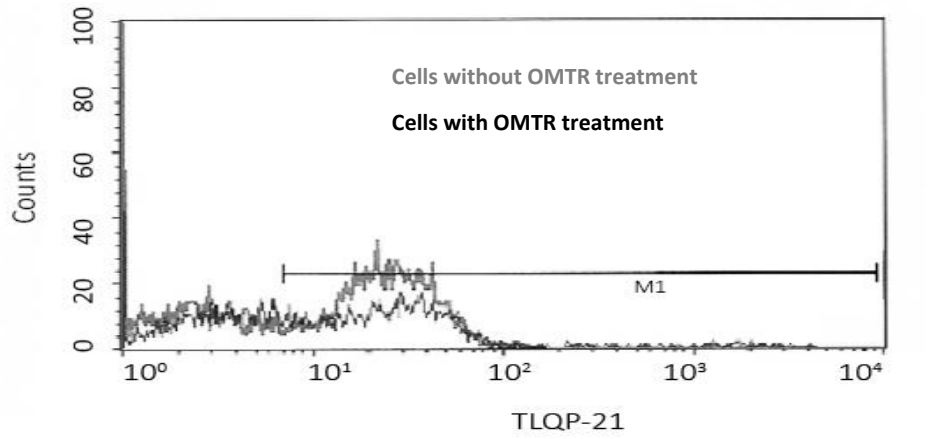

Fig. 4: Representative frequency polygon showing decreased binding of TLQP-21 to the surface of intact, live SH-SY5Y cells treated with OMTR vs. control (without OMTR treatment) in flow cytometry

Discussion

In this study, FACS analysis has been used to confirm that OMTR reduces the binding of TLQP-21 to the surface of intact, live SH-SY5Y cells. Previously, in SH-SY5Y cell line, immunochemical techniques established that OMTR treatment for 60 hours downregulated

expression of HSPA8 protein level in comparision to control, though 24 hours treatment showed no effect on expression of HSPA8 protein (Akhter, 2015), in consistent with the findings of Wang *et al.*, 2010. In another cell line HepG2, OMTR showed the downregulation of HSPA8 at the protein level confirmed by Western blotting. A flow-cytometric assay also exhibited the same results, showing a subsequent reduction of HSPA8 protein in the OMTR-treated cells. And this downregulatory effect was reversible (Wang *et al.*, 2010; 2011).

Among the VGF derived peptides, TLQP-21 is the most studied beneficial bioactive compound and of great importance because of growing evidences of its biologically significant effects (Akhter, 2015, Bartolomucci *et al.*, 2011), as already mentioned. TLQP-21 and its receptors could be drug target in the following biological activities: reproduction (Hahm *et al.*, 2002; 1999), feeding, energy balance, obesity and lypolysis (Jethwa *et al.*, 2007; Bartolomucci *et al.*, 2006, 2008, 2011, Possenti *et al.*, 2012, Hannedouche *et al.*, 2013*),* nociception (Fairbanks *et al.*, 2014; Chen *et al.*, 2013, Rizzi *et al.*, 2008), and diabetes (Stephens *et al.*, 2012). Considering all these significances of the peptide TLQP-21, the results reported in this study were of great interest to us, because HSPA8 and its inhibitor OMTR were already reported as drug target (Muller *et al.*, 2008; Zimmer *et al.*, 2013; Stricher *et al.*, 2013; Wang *et al.*, 2010; 2011). HSPA8, receptor of TLQP-21 and its inhibitor OMTR could be a drug target in the physiological activities, as noted above. Use of OMTR as inhibitor could open new avenues to explore the molecular mechanisms of the physiological actions of TLQP-21 and of pharmacological modulation thereof.

Of note, OMTR treatment had no lethal effect on cells both in short (84 hours) and long (8 months) term therapy. Moreover, it is of great interests that the down regulatory effect of OMTR was detected only in HSPA8 but not in other heat shock protein (HSP) members like HSP90, HSPA5, HSPA9 and HSPA4 (Wang *et al.*, 2010). So, most likely that reduced binding of TLQP-21 to the surface of SH-SY5Ycells is due to the down regulation of HSPA8, the receptor of TLQP-21.

Conclusion

TLQP-21 binds to HSPA8 on the surface of SH-SY5Y cells. But when OMTR, an inhibitor of the expression of HSPA8 was used, binding of TLQP-21 to the surface of cells decreased, strongly suggesting that TLQP-21 binding was through HSPA8. Further studies are required to find out the mode of action of OMTR in HSPA8 down regulation and to observe the change in protein expressions in OMTR treated vs. Control (without OMTR treatment) cells using 2D proteomic analysis, that will ultimately unveil or at least give clue about the mechanism(s) of inhibition of TLQP-21 binding to HSPA8 by OMTR treatment.

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