Peptides 101 (2018) 150–156

Contents lists available at ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

Screening of quorum sensing peptides for biological effects in neuronal cells

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ARTICLE INFO

Keywords:
Quorum sensing peptides
Screening
Biological effects
Neuronal cells
Microbiome

ABSTRACT

Quorum sensing peptides (QSP) are an important class of bacterial peptides which can have an effect on human host cells. These peptides are used by bacteria to communicate with each other. Some QSP are able to cross the blood-brain barrier and reach the brain parenchyma. However, nothing is known about the effects of these peptides in the brain. Therefore, 85 quorum sensing peptides were screened on six different neuronal cell lines using MTT toxicity, neurite differentiation, cytokine production and morphology as biological outcomes. This primary screening resulted in 22 peptides with effects observed on neuronal cell lines, indicating a possible role in the gut-brain axis. Four peptides (Q138, Q143, Q180 and Q212) showed induction of neurite outgrowth while two peptides (Q162 and Q208) inhibited NGF-induced neurite outgrowth in PC12 cells. Eight peptides (Q25, Q135, Q137, Q146, Q151, Q165, Q208 and Q298) induced neurite outgrowth in human SH-SY5Y neuroblastoma cells. Two peptides (Q13 and Q52) were toxic for SH-SY5Y cells and one (Q123) for BV-2 microglia cells based on the MTT assay. Six peptides had an effect on BV-2 microglia, Q180, Q184 and Q191 were able to induce IL-6 expression and Q164, Q192 and Q208 induced NO production. Finally, Q75 and Q147 treated C8D1A astrocytes demonstrated a higher fraction of round cells. Overall, these in vitro screening study results indicate for the first time possible effects of QSP on neuronal cells.

1. Introduction

Quorum sensing is a system used by bacteria to communicate with each other. This way, gene expression is regulated in response to the cell density. A variety of cell functions are controlled by this communication system *e.g.* virulence, biofilm formation, competence or sporulation [1]. Different types of quorum sensing molecules are used: Gram-negative bacteria mostly use N-acyl homoserine lactones while gram-positive bacteria produce i.a. oligopeptides for their communication called quorum sensing peptides (QSP) [2]. Other quorum sensing molecules such as, furan borate derivatives and other miscellaneous molecules exist as well [3–5]. These QSP are produced as large pro-peptides, secreted by ATP-binding cassette transporters, whilst hydrolyzed to the active QSP and interact with membrane located receptors (mainly histidine kinases) for signal transduction or directly with cytoplasmic sensors (e.g. RNPP) after being incorporated by permeases [6]. The QSP are chemically and microbiologically described in the Quorumpeps® database [7]. Recently, it has been discovered that these peptides are also able to influence mammalian host cells by promoting tumor cell invasion and angiogenesis in vitro, implying that these QSP are working via epigenetic mechanisms to stimulate cancer stem cell migration and normal stem cell differentiation for angiogenesis of vascular endothelial cells [8,9]. In general, it has been hypothesized that the colon microbiome influences colon cancer stem cells in the ascending and descending colon to become either treatable or non-treatable colon cancers [10]. Moreover, various peptides including certain QSP are able to permeate the intestinal mucosa, reach the systemic circulation and penetrate the blood-brain barrier, thereby reaching the brain parenchyma [11]. Hence, QSP are potential mediators of the gut-brain axis influenced by the microbiome.

The gut-brain axis is a bidirectional communication system, enabling gut microbes to communicate with the brain and vice versa. These communication signals traverse via neural, endocrine, immune and metabolic pathways. It has been implicated that the vagus nerve may play a communicative role in this axis as the risk to develop Parkinson's Disease is reduced after vagotomy [12] and the effects of

Abbreviations: QSP, Quorum sensing peptide; NGF, Nerve growth factor; DMSO, Dimethylsulfoxide; FBS, Fetal bovine serum; NEAA, Non-essential amino acids; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RA, Retinoic acid; NO, Nitric oxide; TMB, tetramethylbenzidine; SSMD, strictly standardized mean difference; MAD, mean absolute deviation

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https://doi.org/10.1016/j.peptides.2018.01.013
Received 8 December 2017; Received in revised form 17 January 2018; Accepted 17 January 2018

Lactobacillus rhamnosus ingestion are absent in vagotomized mice [13]. It remains unclear how this mechanism of signal transmission works [14]. Clinical observations indicate that the gut microbiome is correlated to the development of psychiatric- and neurodegenerative disorders [15,16]. It has been observed that the microbiota composition is changed in human patients of Parkinson's Disease [17], Alzheimer's Disease [18], multiple sclerosis [19], autism [20,21] and depression [22]. There are indications that modulation of the gut microbiota composition can have beneficial effects on disease outcome, e.g. treatment with probiotics alleviates anxiety-like behavior and improves core symptoms of autism spectrum disorder [23,24]. However, these strong clinically observed correlations are not yet well understood from a causality point of view: while the possibility of a reciprocal relationship between the host disease and its microbiota cannot be ruled out, the effectors and mechanisms are still largely unknown. Our hypothesis is that QSP are one of the causal factors.

Therefore, we investigated the biological effects of 85 chemically diverse QSP on different neuronal cell types (PC12, NB41A3, BV-2, C8D1A, GT1-7 and SH-SY5Y) to investigate their potential role in the gut-brain axis. These immortalized and cancer cell lines are mixtures of abnormal stem cells and partially differentiated stem cells. The PC12 cell line is derived from a rat pheochromocytoma and shows a dopaminergic neuron like phenotype after stimulation with nerve growth factor (NGF). Therefore, it is often used in neurotoxicity and neurodegenerative studies [25,26]. NB41A3 is a mouse neuroblastoma cell line and often used as a neuron model for neurite outgrowth [27,28]. The BV-2 microglia cell line is a suitable alternative for primary glia [29]. These cells are often used to investigate neuro-inflammation [30]. C8D1A is a murine astrocyte cell line and is used to study the effects on astrocytes and are incorporated in *in vitro* models of the blood-brain barrier (BBB) [31]. The GT1-7 cell line is an immortalized hypothalamic mouse cell line which is often used as an *in vitro* model of neurons in neuropeptide research [32,33]. SH-SY5Y is a human neuroblastoma cell line which has the potential to be used as an *in vitro* neuron model for neurodegenerative and neurodevelopmental disorders [34,35]. Investigated responses included toxicity, neurite differentiations, cytokine production and morphological changes as measures for biological activity.

2. Materials and methods

2.1. Peptides

All peptides were purchased at GL Biochem (Shanghai, China) and labelled by their Quorumpeps® ID number [7]. Peptides were dissolved in their solvent as specified in supplementary Table 1, e.g. water or water + dimethylsulfoxide (DMSO).

2.2. Cell lines

PC12 cells (ATCC, CRL-1721.1, Sedex, France) and NB41A3 cells (ATCC, CCL-147, Sedex, France) were grown in F12 K medium, supplemented with 15% V/V horse serum (HS), 5% V/V fetal bovine serum (FBS) and 100 units/ml penicillin and 100 µg/mL streptomycin (Pen/Strep). BV-2 cells were a kind gift of Prof. Minelli (Perugia, Italy) and were cultured in RPMI 1640 medium, supplemented with 10% V/V FBS and Pen/Strep. GT1-7 cells were kindly provided by Prof. Mellon (La Jolla, California, USA) and cultured in DMEM, supplemented with 10% V/V FBS and Pen/Strep. C8D1A cells (ATCC, CRL-2451, Sedex, France) were cultured in DMEM + 10% V/V FBS + Pen/Strep. SH-SY5Y cells (Sigma-Aldrich, Saint-Louis, USA) were grown in F12:MEM (1:1 V/V) medium, supplemented with 15% V/V FBS, 1% V/V Non-essential amino acids (NEAA), 2 m $_L$-Glutamine and Pen/Strep. All culture media and additives were obtained from Gibco (Thermofisher, Waltham, USA). All cell culture material and plastic disposals were obtained from Greiner (Vilvoorde, Belgium).

2.3. Toxicity

Toxicity of QSP was assessed in all cell lines using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells were seeded in a 96-well plate (1×10^4 cells/well for PC12 and NB41A3; 4×10^4 cells/well for BV-2; 2.5×10^4 for C8D1A; 5×10^4 cells/well for GT1-7 and SH-SY5Y). 24 h post-seeding, cells were treated with peptide (100 nM) and incubated for 24 h (BV-2, C8D1A, SH-SY5Y and GT1-7) or 48 h (PC12 and NB41A3). Control cells were treated with medium. Next, 20 µL of MTT reagent was added to each well and incubated for 1.5 h (BV-2) or 3 h (SH-SY5Y, GT1-7, C8D1A, PC12 and NB41A3) at 37 °C. After incubation, cell medium was removed and 150 µL DMSO was added. Absorbance was measured at 570 nm using a microplate reader (Thermofisher, Waltham, USA). The percentage of viable cells was calculated using the absorbance of peptide treated cells divided by the absorbance of medium treated cells.

2.4. Evaluation of neurite outgrowth

The effect of QSP on neurite outgrowth was assessed in PC12 and SH-SY5Y cells. PC12 cells (5×10^3 cells/well) were seeded in 48-well plates and incubated for 24 h after which they were treated with peptide (100 nM) or NGF (100 ng/mL). After 6 days of incubation, neurite outgrowth was assessed under a phase-contrast microscope equipped with a camera (Olympus CKX53, Tokyo, Japan). Pictures were taken in the center of the well. Cells were also co-treated with 100 nM peptide and 100 ng/mL of NGF to assess inhibition of NGF induced neurite outgrowth.

SH-SY5Y cells (2.5×10^4 cells/well) were seeded in 48-well plates in serum deprived medium and incubated for 24 h after which they were treated with peptide (100 nM) or retinoic acid (10 µM). After 7 days of incubation, neurite outgrowth was assessed under the phase-contrast microscope. Co-treatment of QSP and retinoic acid was performed to assess inhibition of neurite outgrowth. All experiments were performed in duplicate and evaluated by two independent blinded investigators. Differentiation is also assessed using an MTT assay as differentiation causes inhibition of proliferation. Cells (5.0×10^3 cells/well) were seeded in a 96-well plate in serum deprived medium. After 24 h, cells were treated with QSP (100 nM) or RA (10 µM) after which the MTT assay is performed, as described in 2.3., after 7 days of incubation.

2.5. TNFα and IL-6 determination

TNFα and IL-6 levels were determined in cell free supernatants of BV-2 cells after QSP treatment. BV-2 cells (2×10^5 cells/well) were seeded in 24-well plates and treated with 100 nM of peptide for 24 h. ELISA was performed according to the supplier's protocol (eBioscience, Vienna, Austria). Briefly, after incubation with biotinylated detection antibody, avidin-HRP conjugate and subsequently chromogenic tetramethylbenzidine (TMB) substrate were added. Absorbance was measured at 450 nm and 570 nm using the Multiskan Ascent 354 (Thermofisher, Waltham, USA). Concentrations were determined using the standard curve generated using known concentrations of TNFα and IL-6. Concentrations are expressed relative of placebo treated cells.

2.6. Nitric oxide determination

Nitric oxide (NO) concentrations in cell free supernatants of BV-2 cells were determined using Griess reagent (Sigma-Aldrich, Saint-Louis, USA). BV-2 cells (2×10^5 cells/well) were seeded in 24-well plates and treated with 100 nM of peptide for 24 h. Aliquots of 100 µL of supernatants were mixed with an equal volume of Griess reagent and absorbance was measured at 492 nm after 15 min. Nitrite concentrations were calculated using a standard curve generated by known concentrations of $NaNO_2$ (Janssen Chimica, Beerse, Belgium).