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Label-free cell phenotypic profiling and pathway deconvolution of neurotensin receptor-1

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ABSTRACT

Neurotensin (NT), an endogenous peptide found in the central nervous system and in peripheral tissues, contributes to the pathophysiology of neurodegenerative and psychiatric diseases, cancer, inflammation, and immunomodulatory disease. NT exerts its physiological effects predominantly through its cognate high-affinity neurotensin receptor-1 (NTS1). NTS1 emerges as a druggable target; however, there are limited numbers of NTS1 active compounds reported to date. Here we reported a label-free cell phenotypic profiling model for screening NTS1 ligands and differentiating their biased agonism. Resonant waveguide grating enabled dynamic mass redistribution (DMR) assay was first optimized against cell confluency and then used to characterize the endogenous NTS1 in HT-29 cell using known agonists and antagonists. Pathway modulators were also used to deconvolute the signaling pathways of endogenous NTS1. Results showed that the NTS1 DMR assay is robust for screening and can differentiate biased agonism; and the activation of NTS1 in HT-29 triggers multiple pathways including G_q signaling and epidermal growth factor receptor transactivation. This study highlighted the power of label-free DMR assay to characterize receptor signaling and pharmacology of distinct classes of ligands for NTS1, G protein-coupled receptors in general.

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1. Introduction

Neurotensin is an endogenous tridecapeptide found in the central nervous system (CNS) and in peripheral tissues, and exerts a wide range of physiological effects primarily through its high-affinity neurotensin receptor subtype 1 (NTS1). NTS1, a seven putative transmembrane G protein-coupled receptor (GPCR), is highly expressed in the CNS and peripheral nervous system [1], and has been implicated in several important diseases including neurodegenerative and psychiatric diseases [1], cardiovascular diseases [2], and opioid-independent analgesic effects [3]. NTS1 is also expressed in several human neoplastic tissues, and has stimulatory proliferation, survival, pro-migratory and pro-invasive effects for multiple malignancies [4–7]. SR48692, a NTS1 antagonist, was found to significantly suppress tumor growth in xenograft models of colon and small cell lung cancer cells [8], and breast and non-small cell lung carcinomas (NSCLC) [9].

NTS1 is known to mediate multiple signaling pathways, as revealed using various cell models including murine neuroblastoma N1E-115 cells [10–12], human colon adenocarcinoma HT-29 cells [13], murine macrophage Raw264.7 cells [14], and over-expressed Chinese hamster ovary (CHO) cells [15,16]. Several signaling pathways after NT stimulation are potentially involved in proliferation, survival, migration, and invasion processes of cancer cells. For example, NT activated NTS1 in KM20 cells, resulted in Ca^{2+}- and mitogen-activated protein kinase (MAPK) signaling pathways [17], while the activation of NTS1 in N1E115 cells resulted in cGMP [18,19] and phospholipase C (PLC) activation [20,21]. In human prostate cancer cell line PC3, the activation of NTS1 by NT transactivates epidermal growth factor receptor (EGFR), an important receptor in cancer progression [22]. In human pancreatic cancer MIAPaCa-2 cells, both the ERK and Jun N-terminal kinase (JNK) pathway were activated [23–25]. In macrophages, JAK-STAT pathway was clearly involved in the neurotensin-activated NTS1

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[26]. These results suggest that NTS1 is capable of mediating multiple signaling pathways in a cellular background sensitive manner.

Here, we described a label-free cell phenotypic profiling method to characterize ligand pharmacology and signaling of endogenous NTS1 in HT-29 cells. This method uses resonant waveguide grating (RWG) biosensor system to non-invasively monitor ligand-induced dynamic redistribution of cellular mass in living cells, which enables holistic cell phenotypic measurement of ligand pharmacology and allows for systematic deconvolution of receptor signaling when combined with chemical biology approaches [27].

2. Materials and method

2.1. Compounds and reagents

NT (Glp-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu), NT_{8-13}, JMV449 and GF109203X were obtained from Tocris Bioscience Co. (St. Louis, MO, USA). NT_{1-8} was purchased from GL Biochem Ltd. (Shanghai). PD149163, SR48692, SR142948A, U73122, erlotinib, cyclopiazonic acid, Y27632, Tofacitinib and LY294002 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hank's balanced salt solution (HBSS), Hepes, fetal bovine serum (FBS) and McCoy's 5A medium were obtained from Sigma Chemical Co. (St. Louis, MO).

2.2. Cell culture

Human colorectal adenocarcinoma HT-29 cells were obtained from the Type Culture Collection of the Chinese Academy of Science (Shanghai, China). HT-29 cells were cultured in McCoy's 5A medium supplemented with 10% FBS, 50 μg/mL penicillin and 100 μg/mL streptomycin at 37 °C under air/5% CO_2. The cells were passed with trypsin/ethylene-diaminetetraacetic acid when approaching 90% confluence to provide new maintenance culture on T-75 flasks and experimental culture on the biosensor microplates. For dynamic mass redistribution (DMR) assays, the cell seeding density was optimized when the culture duration in biosensor microplates was fixed to be 20 h.

2.3. Dynamic mass redistribution assay

DMR assays were carried out on an Epic® BT system (Corning, NY, USA). Unless specific mentioned, cells were directly seeded in Epic® 384 biosensor microplate and cultured overnight to form a confluent monolayer in the cell culture medium. The cells were then manually washed twice, and maintained with the assay buffer (1 × HBSS buffer, 10 mM Hepes, pH 7.2) for 1 h before measurement.

For DMR agonism profiling, a 2-min baseline was first established, followed by adding compounds using the multi-channel pipette and recording the compound-triggered DMR signals for about 1 h. For DMR antagonism or pathway deconvolution assays, cells were initially treated with either an antagonist, or a pathway modulator for 1 h, respectively. Afterwards, the baseline was reestablished, followed by adding the known NTS1 agonists at a fixed dose (typically at its EC_{80} or EC_{100}) and monitoring the cellular responses induced by the agonists for 1 h. NT dissolved in the assay buffer with 0.1% dimethyl sulfoxide (DMSO) and 0.1% bovine serum albumin (BSA). All DMR signals were background-corrected.

2.4. Data analysis

All data were analyzed by using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA). All EC_{50} or IC_{50} were calculated based on the maximum amplitudes of DMR signals at 45 min after agonist stimulation unless otherwise stated. The EC_{50} values were obtained by fitting the dose DMR response curves with nonlinear regression. The Z' factor was calculated using the formula of [1 − (3 × CV of the positive control + 3 × CV of the negative control)/(the mean of the positive control − the mean of the negative control)].

Table 1
The EC_{50} value of NTSR agonists in HT-29 cells at two different time points.

Agonist	EC_{50} (n = 4)	
	4 min	45 min
Neurotensin (nM)	0.63 ± 0.04	0.30 ± 0.02
$Neurotensin_{1-8}$ (nM)	/	/
$Neurotensin_{8-13}$ (nM)	3.85 ± 1.13	4.02 ± 1.27
PD149163(μM)	2.36 ± 0.07	1.56 ± 0.15
JMV449 (nM)	0.64 ± 0.06	1.25 ± 0.25

3. Results

3.1. NT triggered a cell confluency sensitive DMR signal in HT-29

We first examined the sensitivity of NT-triggered DMR in HT29 to cell confluency. Since cell confluency is difficult to be accurately measured, we fixed the cell culture duration to 20 h and varied the cell seeding density ranging from 5000 to 40,000 cells per well. Results showed that NT at 32 nM triggered a cell seeding density dependent DMR (Fig. 1A). The DMR maximum amplitude increased initially as the seeding density increased, until it reached a plateau when the cell seeding density exceed 20,000 cells per well. This was consistent with light microscopic imaging, showing that when the seeding density exceeds 20,000 cells per well overnight culture of HT-29 all led to >95% confluency. Thus, all following assays were performed in HT-29 cells with the seeding density at 3×10^4 cells per well.

3.2. NTS1 agonists display distinct time-dependent potency

Next, we characterized several known NTS1 agonists-triggered DMR using DMR agonism assay. Result showed that NT triggered a robust DMR consisting of three phases: an initial fast increased DMR event (peak-DMR, P-DMR), a slow-decayed DMR event (valley-DMR, V-DMR) and a stable increased DMR (stable-DMR, S-DMR) (Fig. 1B). NT_{8-13}, JMV449, and PD149163 all triggered dose dependent DMR with similar characteristics (exemplified in Fig. 1C). In contrast, the inactive fragment NT_{1-8} triggered little DMR, as expected. PD149163 and JMV449 are two NT_{8-13} analogues. These results are consistent with previous findings [28].

Based on the DMR characteristics, two time points (4 min and 45 min) were used to fit the dose responses of the four NTS1 agonists examined (Fig. 1D and E, Table 1). Results showed that as the post-stimulation time increased, the EC_{50} values of both NT and PD149163 slightly decreased, while the EC_{50} value of JMV449 slightly increased, but NT_{8-13} largely unchanged. One possibility is that distinct signal pathways contribute to the early and late event of the NTS1 DMR signals (see below), and different agonists may have different biased activities for these events [29]. This possibility is worthy of further investigation.

3.3. NTS1 DMR assay is robust

Next, we assess the appropriateness of the NTS1 DMR assay for screening and pathway deconvolution study. Three parameters, including signal to noise ratio (S/N), Z' factor and coefficient of variation (CV), were evaluated using the DMR response from 2 nM NT (EC_{100}) and 0.1 nM NT (EC_{20}), respectively (Table 2). Results showed an averaged response of 6.67 ± 8:75 pmfor the negative controls

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