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Inhibition of Notch signaling pathway using γ-secretase inhibitor delivered by a low dose of Triton-X100 in cultured oral cancer cells

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ABSTRACT

How to effectively delivering therapeutic agents, including γ-secretase inhibitors (GSIs), into live cells, remains a significant challenge. This study assessed the effect of Notch signaling inhibition by examining levels of the Notch1 intracellular domain (N1ICD) in cultured oral cancer cells analyzed with random stitched images (2D) and 3D visualizations using confocal microscopy and quantitative gene analysis. Substantially, we have developed a novel method to assist the delivery of γ-secretase inhibitor, DAPT, into live cells in the presence of an effective minimum concentration of Triton-X100 (0.001%) without damaging cell activity and membrane integrity assessed with cell proliferation assays. The images obtained in this study showed that DAPT alone could not block the γ-secretase inhibitor despite inhibiting cell growth. Further analysis of quantitative gene expressions of Notch signaling canonical pathway to verify the effectiveness of the novel method for delivering inhibitor into live cells, displayed deregulation of Notch1, Delta-like ligand 1 (DLL1) and hairy and enhancer of split 1 (Hes1). Our data suggest that Notch1/Hes1 signaling pathway is deactivated using DAPT with a low dose of Triton-X100 in this cancer cells. And the finding also suggests that Notch1 could be engaged by DLL1 to promote differentiation in oral cancer cells. Using this approach, we demonstrate that Triton-X100 is a promising and effective permeabilization agent to deliver γ-secretase inhibitor DAPT into live oral epithelial cells. This strategy has the potential to implicate in the treatment of cancer diseases.

1. Introduction

Notch signaling cascades undergo in conserved pathways that regulate cellular differentiation, proliferation, and homeostasis [1]. However, the deregulation of this pathway was found to assist the survival of various cancers such as T-cell leukemia, breast cancer, prostate cancer, colorectal cancer and lung cancer as well as central nervous system malignancies [2].

Notch receptors (Notch1–4) are transmembrane receptors harboring on extracellular domains which are responsible for the binding of their specific ligands and on intracellular domains which are involved in transcriptional regulation. Notch receptors can receive signals from neighboring cells that express transmembrane-type ligands such as Delta-like ligands (DLLs) and Jaggeds (JAGs) [3]. Upon ligand binding, Notch receptors undertake two cleavage processes mediated by a member of a disintegrin and metalloproteinase (ADAM) family and gamma-secretase, leading to the release of the Notch intracellular domain (NICD). NICD then translocates into the nucleus forming a complex which is activated by the Mastermind family of coactivators and finally activates Notch target genes such as mammalian hairy and enhancer of split (Hes) and Hey family members [4].

Since the release of NICD activates the transcriptional complex, the strategy of blocking the NICD cleavage emerges as a prominent treatment option to combat cancers [5,6]. Several clinical trials have been launched to test efficacy and safety of Notch inhibitors in tumors [5,6]. Although the availability of potent Notch inhibitors such as alpha, γ-secretase inhibitors (GSIs), peptides, siRNA, antibodies or probodies, Notch-related treatments are currently prevented by side effects, due to the non-specific binding of inhibitors in most tissues [2]. Cancer treatment with GSIs induces diarrhea and suppression of lymphopoiesis [7]. Antibody-based targeting of Notch ligands is associated with induction of vascular tumours in

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mice [8] and a variety of side effects including a headache, hypertension, fatigue, right and left ventricular dysfunction in patients in clinical trials [9]. Therefore, clinically efficient suppression of Notch activity requires more efficient and targeted delivery of these inhibitors.

To overcome this barrier in delivery, cell permeabilization agent such as chemical detergent can be used in conjunction with the GSI inhibitors. Detergents are the most commonly used cell permeabilization agents and have been utilized for the intracellular delivery of biomolecules [10] since at sub-solubilizing concentrations. It efficiently perturbs membrane structure and increases cell permeability [11]. Triton-X100 has been successfully used to reversibly permeabilize live cells for delivery of optical contrast agents [12]. It was reported that for the treatment of cells with appropriate concentrations of Triton-X100, membrane permeabilization is reversible and membrane integrity typically restored after 24 h. The treatment of cells with low concentrations of digitonin was shown to selectively permeabilize the plasma membrane, leaving the nuclear envelope intact [13].

GSI inhibitor, such as the γ-secretase inhibitor, DAPT (N-[N-(3,5-difluorophenacetyl)- L-alanyl]-S-phenylglycine t-butyl ester), has been used on several cancer cell lines [14–16], but rarely investigated on oral squamous cell carcinoma (OSCC) [17]. Oral cancer is one of the ten leading causes of cancer death in developing countries [18], and the survival rate of patients with oral cancer has not improved despite improvements and innovations in diagnostic techniques and treatments [19]. In this regard inhibition of Notch signaling pathway in OSCC could be an effective means to improve treatment. So far no systematic study regarding Notch inhibition therapy has been performed in mammalian cells using the permeabilizing agent in delivering GSIs for cancer. Here we have developed a novel method to actively inhibit Notch activity using γ-secretase inhibitor DAPT delivered by a low dose of Triton-X100 in cultured oral cancer cells.

2. Materials and methods

2.1. Oral cancer cell culture

The cancer cell line (H413) derived from a human oral squamous cell carcinoma (OSCC) [20], displays stratified epithelial cell morphology in culture. H413 cell clonal lines were established using a limit dilution method in our laboratory as described previously [21]. H413 clone-1 cells exhibiting characteristic epithelial morphology and tumor marker CD24 were chosen for this study [21]. The cloned cells were cultured in Joklik modification's minimum essential medium (Sigma-Aldrich), supplemented with penicillin/streptomycin (100 IU/ml, Sigma) and 10% fetal calf serum (FCS, CSL Limited, Victoria, Australia) at 37 °C in 5% CO2. Cultures were collected with trypsin replacement - triple express (Invitrogen, Australia) in PBS and sub-cultured every 3 days.

2.2. Cell proliferation assay

To determine cell proliferation in response to DAPT alone or in combination with Triton-X100, the cell proliferation ELISA, BrdU (colorimetric) assay (Roche) was used. DAPT (Sigma Aldrich) was dissolved in dimethyl sulfoxide (DMSO) in a stock 10 mM. Briefly, oral cancer cells H413-1 (SOCC) were harvested and plated in 96-well flat bottom plates at 20000 cells per well in 100 μl medium. Overnight after plating, each set of triplicate wells of cells was treated with 5, 10 and 20 μM DAPT (Sigma Aldrich) dissolved in DMSO concentration 0.05%, 0.1%, and 0.2% respectively or in combination with Triton X-100 (0.01%, 0.001%, 0.0001%) or medium alone (as a growth control) or DMSO (0.05%, 0.1%, 0.2%) alone for 4 h. Then 10 μl of BrdU labeling solution was added to each well and cultured for 2 h at 37 °C in incubator. After removal of labeling medium, 100 μl/well of Fix Denature solution was added and cultured for 30 min at room temperature. Then flicking off Fix Denature solution thoroughly, 70 μl/well of anti-BrdU-POD working solution was added and cultured for 1.5 h at room temperature. For background controls, wells contained media (100 μl), BrdU (10 μl), Fix Denature solution (100 μl), and anti-BrdU-POD (70 μl) respectively without cells. Remove antibody conjugate by flicking off and rinse wells 3 times with 200 μl/well washing solution (PBS, 1×). After removal of washing solution, added 100 μl substrate solution to each well, then read absorbance at OD 370 nm with a reference of 492 nm (SpectraMax) within 30 min. The experiment was repeated three times. Ten μM of DAPT (0.05% DMSO) has been referred as an optimal working solution [22].

2.3. Immunostaining analysis for Notch1 intracellular domain (N1ICD)

Confluent cancer cells ($2 \times 10^5/cm^2$, H413 clone-1) grown on 8-well chamber slides (Corning, USA) were co-cultured with γ-secretase inhibitor (DAPT, 10 μM) in the presence of Triton-X100 (0.001%), or DAPT alone, or without inhibitor but with or without Triton-X100 (0.001%) for 4 h. Series dilutions of Triton-X100 were performed to determine the minimum effective concentration to deliver DAPT into live cells. Slides washed in PBS then fixed with 4% paraformaldehyde/PBS for 30 min, permeabilized with 0.2% Triton-X100/PBS for 15 min, and blocked with 3% BSA/PBS for 1 h, then probed with Rabbit polyclonal to activated Notch1 antibody (1:100, Abcam, UK) for 1 h at 37 °C which is corresponding to activated Notch1 aa 1755–1767 (intracellular). The epitope is only exposed after gamma secretase cleavage and is not accessible in the uncleaved form. After washing with PBS, Goat anti-Rabbit IgG (H + L) Secondary Antibody, Alexa Fluor 594 (1:100, Life Sciences), was added for 1 h at 37 °C. For negative control, the primary antibody was replaced with isotype control antibody IgG (DAKO). Slides were washed with PBS and mounted with ProLong Gold antifade reagent with DAPI (Molecular Probes, Invitrogen).

Confocal images were captured with an Olympus Fluoview (FV) 1000, equipped with Olympus three lasers: 405 nm, 473 nm and 633 nm, at our microscopy laboratory. Fields were selected at random [objective: Olympus 40X/1.30/0.20 (WD) Oil UPLSAPO] and the cells were brought into focus under bright-field conditions. All fluorescence images, including random stitched 2D images and 3D reconstruction images prepared with confocal acquisition software (FV10-ASW 4.2) were stored and exported as TIF image files.

2.4. RNA isolation and quantitative real-time RT-PCR analysis for Notch family genes

Confluent H413 clone-1 cells were incubated with one of the following conditions for 4 h: co-cultured with or without Triton-X100 (0.001%) as two control groups, or treated with γ-secretase inhibitor (DAPT, 10 μM), in the presence of Triton-X100 (0.001%) or DAPT alone. Cells were harvested in 1 ml of Trizol reagent (Invitrogen) and RNA extracted as per the Trizol protocol. For reverse transcription, the First-Strand cDNAs were synthesized with oligo(dT)12–18 (Invitrogen), 10 mM dNTP (Promega), 5 × first strand buffer, RNaseOUT™ Recombinant RNase Inhibitor (Invitrogen) and SuperScript™ III Reverse Transcriptase (Invitrogen) according to the manufacturer's (Invitrogen) protocol.

Primers for genes encoding Notch family (Additional file 1: Table 1) were designed using Oligo Explorer software (1.1.0) and synthesized by Integrated DNA Technologies (IDT, USA). Real-time RT-PCR analyses were performed by SYBR Green-based assays

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