Probing Real-Time Response to Multitargeted Tyrosine Kinase Inhibitor 4-N-(3′-Bromo-Phenyl) Amino-6, 7-Dimethoxyquinazoline in Single Living Cells Using Biofunctionalized Quantum Dots

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Abstract

Recently, the quinazolinederivative, 4-N-(3′-bromo-phenyl) amino-6, 7-dimethoxyquinazoline (PD153035), has been reported not only to inhibit the epidermal growth factor receptor (EGFR) tyrosine kinase but also to bind to DNA double helical structures by intercalation. However, several important pharmacology issues such as whether PD153035 is a specific and reversible inhibitor of the EGFR tyrosine kinase should be addressed in more detail. In this study, we propose a nanotechnology-based approach to monitoring the real-time EGF-EGFR complex trafficking process and its relationship to cytoskeleton, as well as spatio-temporal cellular response to PD153035 at the single-cell level. We utilize the biofunctionalized quantum dots (QDs) conjugated with EGF to monitor the cellular distribution of QD-EGF-EGFR complexes, which can provide a more direct access to probing the spatio-temporal distribution of EGF-EGFR complex in the absence and presence of PD153035. We found that QD-EGF-EGFR complexes undergo retrograde transport before receptor-mediated internalization. In addition, QD-EGF-EGFR complexes colocalize with actin filaments, especially in filopodia regions. Furthermore, the cellular distribution of fluorescing QDs was strongly localized inside the cell after washing PD153035 for time period longer than 15 minutes. This observation demonstrated that PD153035 could be removed from the intracellular kinase domain, namely, PD153035 is a reversible EGFR inhibitor. We anticipate these approaches based on the platform at single-cell level could be applied to build a quick screening method for detection and treatment evaluation of many types of cancer expressed high levels of EGFR.

Keywords: Biofunctionalized quantum dots; Single living cell; Epidermal growth factor receptor; Tyrosine kinase inhibitor; PD153035; Non-small cell lung cancer

Introduction

The activation of epidermal growth factor receptor (EGFR) controls the intracellular signal transduction pathways that regulate cell proliferation, apoptosis, angiogenesis, adhesion, motility and invasion [1,2]. Nowadays, the EGFR, which is expressed or highly expressed in colorectal cancer, gliomas, head and neck carcinomas, and non-small-cell lung cancer (NSCLC), is recognized as a particularly promising therapeutic target for anticancer therapies [3-8]. Presently, different strategies for EGFR inhibition have been reported such as monoclonal antibodies, recombinant EGF vaccine, antisense oligonucleotides, and small-molecule EGFR tyrosine kinase inhibitors (EGFR-TKIs) [9].

Over the past years, inhibition of protein tyrosine kinase activity by ATP-site-directed compounds has become attractive target for rational drug design [10]. The quinazoline derivatives such as Iressa and Tarceva have been widely used for targeted therapy [reviewed in [11]]; see also [12,13]; Iressa and Tarceva currently constitute the second and third line treatment in NSCLC [13,14]. Numerous in vitro studies revealed that Iressa and Tarceva inhibit the EGFR tyrosine kinase by 50%, i.e., IC50 at concentrations of 27–33 nM [15,16] and 20 nM [17,18] respectively.

Recently, another quinazoline-type compound, 4-N-(3′-bromo-phenyl)amino-6,7-dimethoxyquinazoline (PD153035), has been reported to be not only a highly selective reversible inhibitor of EGFR (IC50=29 pM) [19,20] but also a DNA intercalator [21-23]. However, on the one hand, the DNA binding affinity constant of PD153035 cannot be determined using bulk fluorescence titration experiments due to

that changes in fluorescence emission are very weak [21]. On the other hand, it also requires advanced approaches to monitoring the real-time cellular responses to PD153035. Nowadays, due to the ongoing advances in various nanomaterials and nanotechnology, these advances motivate us to examine and address the aforementioned pharmacological issues about multitargeted tyrosine kinase inhibitor PD153035 in more detail.

In our previous study, we have presented single-molecule approach using optical tweezers to determine the binding mode and binding affinity constant of PD153035 to DNA for the first time; we found that PD153035, although this drug exhibits a noticeable increment in contour length of DNA molecules at 1 mM sodium cacodylate, have weak DNA-binding ability at physiological salt concentrations (100 mM for monovalent salt) [24].

Currently, quantum dot (QD) conjugates have been utilized for prolonged real-time visualization of the signaling mechanisms in living cells [25]. In this study, we will propose a nanotechnology-based approach to probing real-time response to PD153035 at the...
single-cell level. To this end, we have developed a platform in which the temperature-controlled microfluidic flowcell is incorporated into single-molecule fluorescence microscopy systems (Figure 1). In addition, we utilize the biofunctionalized quantum dots conjugated with EGF (QD-EGF) to monitor the cellular distribution of EGF, which can provide a more direct access to probing the spatio-temporal distribution of EGF-EGFR complex and the corresponding transport pathway. Furthermore, this approach provides direct evidence from single-cell analysis to demonstrate that PD153035 inhibits downstream signaling events and is a reversible inhibitor of the EGFR tyrosine kinase.

Materials and Methods

Cell culture and drug treatments

A human epidermoid carcinoma A431 cell line (Bioresource Collection and Research Center), which possesses 2×10^5 EGFR per cell [20], was cultured up to ~60% confluence in DMEM medium containing 10% heat-inactivated FBS. Cultures of A431 cells were maintained at 37°C in a humid atmosphere of 5% CO₂. A431 cells were trypsinized, counted and resuspended at the appropriate densities (≈1×10^5 cells/mL) in fresh culture medium. The dissociated A431 cells were then introduced into the inlet port of the flow chamber using an automated syringe pump at a constant volumetric flow rate of 0.5μl/sec. During the experiments, the temperature inside the flow chamber is 37°C. To promote adhesion, A431 cells were seeded onto collagen1-coated glass coverslip assembled onto the bottom of the flow chamber. To disrupt actin filaments, the A431 cells were incubated with medium containing 1 µM PD53035 for 2 hours at 37ºC. Note that the EGFR tyrosine kinase activity, the A431 cells were incubated with 4 nM QD-EGF at 37°C for 30 minutes. For two-dimensional (2-D) random walk, the 2-D mean square displacement (MSD) increases linearly with time according to MSD=4D∆t, where D and ∆t are diffusion coefficient and time lag, respectively [26-28]. An image-based particle-tracking microrheology is presented to probe the mechanical properties of living A431 cells in the absence and presence of tyrosine kinase inhibitor PD153035. We apply Fourier-based cross-correlation processing [29] to determine the lateral displacement of the vesicles within the living cells. Hence, the diffusion coefficient in the intracellular microenvironment could be obtained from the linear slope of MSD plot.

Results and Discussion

Dynamic visualization of single cell adhesion

Quality of cell adhesion and growth on collagen I coated glass coverslips was the most important ingredient to assure the proposed single-cell platform as a means to examine cellular behavior in response to drug treatment. We first visualized the morphology of the cell adhesion and growth on glass coverslips. We found that it required 1 h for stable cell adhesion, as shown in Figures 2(A) through 2(I). The filopodial structures and granular vesicles are visible after 60 minutes of the dissociated A431 cells introduced into the microfluidic flow cell. On the other hand, from multiple bright field images taken at different depths of focus, the corresponding thickness of the A431 cells, in general, is about 11 µm.

Exploring the QD-EGF trafficking pathway at single-cell level

In this study, QDs conjugated to EGF were utilized to monitor the cellular distribution of EGF-EGFR complexes. First, we investigate whether the QD-EGF entered cells via either a Fickian diffusion transport process or receptor-mediated internalization. To eliminate the possibility of receptor-mediated internalization, A431 cells were treated with 4 nM QD-EGF at 4°C for 30 minutes. We then acquired the fluorescence images of QD-EGF-EGFR complexes along the z-axis (the optical axis) corresponding to sequential optical sections spaced 1 µm apart via repeated fine-tuning of the objective lens along the z axis. As it can be seen, QD accumulation with a large amount was observed along the periphery of the cell membrane but not in the cytoplasm. This finding suggested that Fickian diffusion transport process had less effect on the QD-EGF trafficking pathway.

In contrast, Figure 4 showed that there existed retrograde transport of QD-EGF-EGFR complexes along filopodia to the cell body after incubation with 4 nM QD-EGF at 37°C. In addition, wave-like propagation of QD-EGF-EGFR complexes was approaching the cell body at the time scale of 15 minutes, where the arrow indicated the

Figure 1: Single-molecule detection platform, including temperature control system and flow delivery system, was integrated into an inverted fluorescence microscope (upper left). The lower right figure showed the schematic of streptavidin-conjugated quantum dots with biotinylated ligand EGF, where binding of EGF to the extracellular domain of EGFR leads to receptor dimerization and tyrosine autophosphorylation of receptor tyrosine kinases.
determined to be 3.5 nm/s (supplementary information). Movie 1 (20 frames per second), showing the wave-like propagation of QD-EGF is published as supplementary information on the web site.

Figure 5 showed the bright field cell image (left) and the simultaneous fluorescence image (right) of the QD in living A431 cell after 90 minutes of QD-EGF stimulation. As it can be seen in Figure 5(B) the cellular distribution of fluorescence signals of QDs was strongly localized inside the cell, namely, the internalization of the QD-EGF was occurred after longer incubation time periods. Movies 2 and 3 (10 frames per second), showing the bright field cell image and the corresponding intracellular trafficking of QDs are published as supplementary information on the web site. Based on the above investigations of the QD-EGF trafficking pathway, the present finding revealed that QD-EGF-EGFR complexes undergo retrograde transport before endocytosis.

Relationship between EGF-EGFR complex and cytoskeleton

First, we investigated the relationship between QD-EGF trafficking pathway and actin filaments, where F-actin was visualized using Alexa Fluor® 635 phalloidin (Invitrogen Cat. no. A34054). Here A431 living cells were incubated with 44 nM QD-EGF for 30 minitues at 37ºC, followed by further incubation with 4 nM QD-EGF for 30 minutes at 37ºC. Finally, cells were fixed in 4% PFA and immunostained with Alexa Fluor® 635 phalloidin for 5 minutes at 37ºC. We then compared bright field image of A431 cell, fluorescence image of QD-EGF (red pseudo-color), and fluorescence image of actin filaments; note that direction of retrograde transport process, as illustrated in Figure 4(B). Note that this time scale is consistent with the internalization of the receptor [30]. Here, the transport speed of wave-like propagation was
the colocalization between QD-EGF and actin filaments, especially in filopodia regions, as illustrated in the merged image.

The relationship between cellular localization of QD-EGF-EGFR complexes and actin filament distributions in A431 cell was further investigated using cytochalasin D, which can induce depolymerization of actin filaments. A431 living cells were exposed to 4 \( \mu \text{M} \) cytochalasin D for 30 minutes at 37\(^\circ\)C, followed by further incubation with 4 nM QD-EGF for 30 minutes at 37\(^\circ\)C. Finally, cells were fixed in 4\% PFA and immunostained with Alexa Fluor® 635 phalloidin. Figures 7(A) and 7(B) showed that active contractions of filopodia were occurred due to depolymerization of actin filaments (Figure 7(D)). In addition, QD-EGF-EGFR complexes accumulated outside the cell body, as illustrated in Figure 7(C). Furthermore, cellular localization of QD-EGF-EGFR complexes seemed to be highly correlated with the the morphology of filopodial structures before depolymerization by cytochalasin D. Hence, the retrograde transport was strongly inhibited when actin filaments were depolymerized.

**Reversal of PD153035-induced inhibition of EGFR tyrosine kinase activity**

The main focus of this study is to provide a direct evidence to prove that PD153035 is a specific and reversible inhibitor of the EGFR tyrosine kinase. A431 living cells were exposed to 1 \( \mu \text{M} \) PD153035 for 2 hours at 37\(^\circ\)C, followed by further incubation with 4 nM QD-EGF in the presence of 1 \( \mu \text{M} \) PD153035 for 30 minutes at 37\(^\circ\)C. Finally, the medium containing 4 nM QD-EGF in the presence of 1 \( \mu \text{M} \) PD153035 was removed and replaced by fresh medium in the absence of PD153035. The result showed that QD-EGF-EGFR complexes accumulated outside the cell body in the presence of 1 \( \mu \text{M} \) PD153035, as illustrated in Figures 8(D) through 8(F). However, after PD153035 was removed by washing of the cells with drug-free medium, the cellular distribution of fluorescing QDs was strongly localized inside the cell after washing of the cells for time period longer than 15 minutes, as illustrated in Figures 8(G) through 8(I). This observation demonstrated that PD153035 is a reversible EGFR inhibitor, namely, PD153035 could be removed from the intracellular kinase domain.

On the other hand, we are interested in validating whether PD153035 had influence on intracellular microrheology. To this end, these images were taken in the same focal plane for direct comparison (Figures 6(A) through 6(C)). Figure 6(D) showed the merged image indicated the overlay of QD-EGF signals and actin filaments. As it can be seen fluorescence signals of QD-EGF were discrete; however, while comparing fluorescence signals of actin filaments, there existed
we applied particle-tracking microrheology of living cells to determine the lateral displacement of the vesicles within the living cells in the absence and presence of PD153035; therefore, both the mean square displacement (MSD) and the diffusion coefficient (D) can be obtained. Note that for quantitative analysis of complete trajectories, only those vesicles that move roughly within the focal plane are analyzed. Figure 9 showed the bright-field cell image (left), the simultaneous single vesicle image (middle) of the QD in living A431 cell, and the corresponding MSD plot (right) in the absence and presence of PD153035. We found that values of diffusion coefficient were nearly identical, which suggested that PD153035 played a relatively minor role in the intracellular microrheology.

In the present work, we applied nanobiotechnology to address pharmacologic inhibition of EGFR kinase activity with PD153035 at single cell level. This approach was suitable for qualitative understanding and quantitative analysis of various aspects of cellular responses to EGFR inhibitors and their relevance to cancer therapy at single-cell level. The present measurements and analyses revealed the following. (i) EGF-EGFR complexes undergo retrograde transport before endocytosis. (ii) the retrograde transport of EGF-EGFR complexes is directly correlated with actin filaments. (iii) PD153035 is a reversible EGFR inhibitor and can be removed from the intracellular kinase domain, and (iv) PD153035 played a relatively minor role in the intracellular microrheology. We are currently applying the proposed approach, together with our high-resolution optical tweezers system to determine stiffness of cellular membrane and chemotactic responses to EGF in the absence and presence of EGFR tyrosine kinase inhibitors. All of these results will be reported elsewhere in the near future.

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