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Identification of an Inhibitory Mechanism of Luteolin on Insulin-like Growth Factor-1 Ligand-receptor Interaction

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Abstract

Using single-molecule force measurement and single-molecule fluorescence imaging, we demonstrated that luteolin is able to perform an inhibitory effect on IGF-1 ligand-receptor binding, the initial step of IGF-1 signaling. Furthermore, this inhibition mechanism was also confirmed by flow cytometry and molecular docking. Our study demonstrates the novel molecular mechanism of luteolin as an IGF-1 signaling inhibitor in cancer therapy.

Keywords

Insulin-like growth factor 1 receptor; Luteolin; Single-molecule force measurement; Single-molecule fluorescence imaging

Insulin-like growth factor-1 (IGF-1) signaling plays a pivotal role in a wide variety of biological processes, such as cell growth, differentiation, apoptosis, and biological aging process.^[1,2] Aberration of IGF-1 signaling has been implicated in the pathogenesis of many diseases including cancer. For example, IGF-1R overexpression promotes growth of existing cancer cells.^[3-6] Developing IGF-1 signaling inhibitors not only facilitates the regulation of biological processes for cell biology study, but also becomes an attractive way to develop new therapeutic reagents and anticancer drugs.^[7]

IGF-1 signaling is initiated by the ligand interaction with IGF-1 receptor (IGF-1R), which triggers IGF-1R to form a homodimer signaling complex on cell membrane. This leads to the phosphorylation of IGF-1R and then the phosphorylation of intracellular insulin receptor substrate 1 (IRS-1), to propagate the signal transduction.^[8,9] Several antisense oligonucleotides, antibodies and small chemical molecules have been developed for IGF-1 signaling inhibition. Currently, the most widely used antagonists are large molecules, including oligonucleotides that target IGF-1R mRNA to reduce IGF-1R expression, and antibodies that disrupt IGF-1 ligand binding to the receptor complex.^[10-13] Tremendous effort has been made to develop small-molecule inhibitors which have the advantages in reproducible chemical synthesis, simple delivery and deep tissue penetration, and long-term stability. To date, nearly all the small-molecule inhibitors are functioned by binding the ATP-binding site of the IGF-1R kinase domains to prevent IGF-1R phosphorylation and

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activation.^[14-16] Chemical compounds targeting other steps in IGF-1 signaling pathway have not been reported.

Recent reports have shown that luteolin (3',4',5,7-Tetrahydroxy flavone), a natural predominant flavonoid with many pharmacological activities, has the ability to reduce IGF-1R and IRS-1 phosphorylation in the presence of IGF-1.^[17] The results also demonstrated its potential in cancer treatment as an IGF-1 signaling antagonist. The structure of luteolin is absolutely different from the current small-molecule ATP competitive compounds for IGF-1 antagonism, e.g. BMS-536924,^[15] and the molecular mechanism on the inhibition effect remains unclear. In this work, we applied two single-molecule techniques, single-molecule force spectroscopy and single-molecule fluorescence microscopy, to investigate its inhibition mechanism, especially whether it is a new class of small molecule inhibitors to disrupt IGF-1 ligand-receptor binding and receptor activation. Both single-molecule force spectroscopy and single-molecule fluorescence microscopy are emerging techniques to probe the initial cell signaling events on the live-cell membrane, which are difficult to study by the conventional biochemical assays with cell lysates and ensemble averaging.^[18] It is the first time that the mechanisms of the small-molecule inhibitors are revealed by single-molecule techniques.

We firstly used single-molecule fluorescence microscopy to examine the effect of luteolin on the formation of ligand induced IGF-1R dimerization, which is a prerequisite for IGF-1R phosphorylation and signal propagation.^[19] As shown in the typical TIRFM image of resting MCF7 cells (Figure 1A and SI Movie 1), IGF-1R-GFP molecules appeared as well-dispersed diffraction-limited fluorescent spots (5×5 pixels, 800×800 nm). The fluorescence intensities of these spots maintained mostly for less than 4.5 s and then suddenly dropped to the background level, indicating that they are single IGF-1R molecules. The fluorescence intensity distribution of the spots exhibited a sum of two Gaussian distributions (Figure S1A). While the first population of IGF-1R monomers had a peak intensity close to that of single GFP molecules on the glass coverslips (Figure S1C), the second population of IGF-1R dimers had a peak value about twice of the first one. With 303 counted spots, 89.8% were monomers and 10.2% were dimers. After IGF-1 stimulation, the population of monomers decreased to 58.3% and that of dimers increased to 41.7% (Figure 1B). This is in agreement with the previous report that IGF-1 stimulation results in IGF-1 receptor dimerization for activation.^[19]

Then we treated the cells with 100 μ M luteolin for 1h before fluorescence imaging. The fluorescence intensity distribution of the spots from the resting MCF7 cells was very similar to that obtained in the cells without luteolin treatment (with 332 counted spots, monomers 90.1% and dimers 9.9%) (Figure S1B). However, after IGF-1 stimulation, the fluorescence intensity distribution (with 337 spots counted monomers 78.6% and dimers 21.4%) obtained from luteolin-treated cells (Figure 1C) was significantly different from that obtained with the untreated cells. The population of IGF-1R dimers decreased from 41.7% to 21.4% for luteolin-treated cells. This indicated that luteolin inhibited ligand-induced IGF-1R dimerization.

We then counted the photobleaching steps of individual fluorescent IGF-1R-GFP molecules under different conditions to confirm the above results from intensity distribution analysis (Figure 1D). Bleaching-step counting is a new approach to determine the subunit number of membrane-bound proteins tagged with GFP.^[20] From the photobleaching plots (Figure S2A-B), we obtained the similar results that the population of two-step photobleaching spots, which represent dimers, decreased from 39.7±2.6% to 21.9±1.3% after luteolin treatment for the stimulated cells.

Furthermore, we did the same experiments with BMS-536924 instead of luteolin for comparison. BMS-536924 is a typical small-molecule inhibitor of IGF-1 signaling which has the known function of binding to IGF-1R kinase domains.^[15] By analyzing both fluorescence intensity distributions and photobleaching steps, we did not find that BMS-536924 treatment caused a significant difference in IGF-1R dimer/monomer population before and after ligand stimulation (Figure S3A-B, Figure 1D). This suggested that luteolin had a different inhibition mechanism on IGF-1 signal transduction from those of previously reported chemical compounds.

Since ligand-induced IGF-1R dimerization is the consequence of IGF-1 ligand-receptor interaction,^[8] we expected that luteolin could affect IGF-1/IGF-1R binding. To test this hypothesis, we performed IGF-1/IGF-1R binding force measurements with living cells in the absence and presence of luteolin by single-molecule force spectroscopy. Previous reports had used atomic force microscopy (AFM) to investigate the binding strength for several pairs of ligand and its membrane receptor at the single-molecule level.^[21, 22] To directly probe the binding force of IGF-1 to its high-affinity receptor IGF-1R, the AFM tip was modified with IGF-1 and moved to the MCF7 cells which expressed IGF-1R.^[17] The rupture forces were detected when the tip and cell were brought into and out of contact. Blocking of the force with IGF-1R extracellular domain (IGF-1R-ECD) was carried out to confirm the measured forces were the specific ones between IGF-1 and IGF-1R.

As shown in Figure 2, the force distribution histogram displayed a single maximum by a Gaussian fit, indicating that the single-molecule forces were measured. The low frequency of the adhesion events during the measurement also ensured that the force was mediated by a single ligand-receptor pair (< 25% adhesion events ensured > 90% probability of single-molecule force measurement).^[23] For the cells without luteolin treatment, the averaged histogram peak value of the binding forces from three independent experiments was 70.2 ± 3.1 pN (Figure 2B). While a lower binding force of IGF-1 with IGF-1R on the cell membrane was observed (49.6 ± 2.9 pN) for the luteolin-treated cells (Figure 2C). Furthermore, there is a significant difference in the binding probabilities under two conditions. Luteolin treatment caused a lower binding probability, which decreased from $22.4\% \pm 2.6\%$ for untreated cells to $10.3\% \pm 1.2\%$ for treated cells (Figure 2D). In addition, flow cytometry was performed using biotinylated IGF-1 and avidin-FITC. We found that the fluorescence signal decreased in the presence of luteolin (Figure 2E).

The discovery that luteolin affects the IGF-1/IGF-1R binding indicates that the inhibition effect may be related to the disruption of the binding site between the ligand-receptor pair. This possibility was supported by the molecular docking study. Based upon the crystal structure of the extracellular domain of IGF-1R in complex with IGF-1, we predicted the binding model of luteolin with IGF-1/IGF-1R complex.^[24-26] Analysis of this binding model suggests that four binding sites (PHE1, THR516, GLY520 and ASP533) are the key interactions between luteolin and IGF-1/IGF-1R complex (Figure 3). Furthermore, previous reports have shown that these four binding sites are also critical for IGF-1 ligand-receptor binding.^[24-26] Most surprisingly, we found that luteolin affected not only the binding probability but also the binding force. This suggests that the inhibitory mechanism of luteolin seems to be complex so as not to be simply thought as a competitive inhibition. One possibility is that luteolin might change the conformational dynamics of IGF-1R, which causes the decrease in the binding force. Previous report has shown that protein dynamics, which can be modulated by small molecules, is one of the key determinants in protein-protein binding.^[27] Therefore our docking study, to some extent, demonstrates that luteolin has the potential to lower the binding between IGF-1 and IGF-1R, which provides a better understanding for the luteolin inhibition on IGF-1 signaling.

In summary, we have employed single-molecule force spectroscopy and fluorescence microscopy to study the inhibition mechanism of luteolin on IGF-1 signaling in living cells. Our results demonstrated that luteolin reduced the binding of IGF-1 to its specific receptor IGF-1R, thus inhibited the receptor dimerization and activation for the signaling complex formation and the subsequent IRS-1 phosphorylation for the downstream signal transduction. Therefore, luteolin and its analogs have the potential to be developed as a new class of small-molecule inhibitors of IGF-1 signaling. Our study also provides a new approach of applying single-molecule techniques to identify the chemical compounds for cell signaling inhibition.

Experimental Section

Cell Culture and Transfection

MCF7 cells were cultured in DMEM (Gibco) supplemented with 10% FBS (HyClone) at 37°C in 5% CO₂. The DNA fragment encoding full-length IGF-1R was subcloned into the HindIII and BamHI sites of pEGFP-N1 (Clontech), yielding the IGF-1R-GFP expression plasmid. Transfection of IGF-1R-GFP plasmid was performed using lipofectamine2000 (invitrogen). Cells growing in a 35-mm glass-bottom dish (MatTek) were transfected with 0.4 µg/mL plasmids in the phenol red-free DMEM. To achieve a low-level protein expression, cells were incubated with the plasmid for 4 h, washed, and then imaged in the phenol red-free DMEM under the fluorescence microscopy. For luteolin or BMS-536924 treatment, the transfected cells which were ready for single-molecule imaging were incubated with 100 µM luteolin and 100 µM BMS-536924 (Sigma) in the serum-free DMEM for 1 h at 37°C before fluorescence imaging. For IGF-1 stimulation experiment, the transfected cells were incubated with 50 ng/mL IGF-1 (R&D) in the serum-free DMEM for 15 min at 37°C.

Single-Molecule Fluorescence Imaging

Single-molecule fluorescence imaging was performed with the objective-type total internal reflection fluorescence microscopy using an inverted microscope (IX 81, Olympus, Japan), a total internal reflective fluorescence illuminator, a 100X/1.45NA Plan Apochromat TIR objective (Olympus, Japan) and a 14-bit back-illuminated electron-multiplying charge-coupled device (EMCCD) (Andor iXon DU-897 BV). The microscope was equipped with a CO₂ incubation system (INU-ZIL-F1, TOKAI HIT) and live cell imaging was performed at 37°C in 5% CO₂. GFP was excited at 488-nm by an argon laser (Melles Griot, Carlsbad, CA) with the power of 5 mW measured after the laser passing through the objective in the epi-fluorescence mode. The collected fluorescent signals were passed through two filters, BA510IF and HQ 525/50 (Chroma Technology), before directed to the EMCCD. The gain of EMCCD was set at 300. Only the central quarter of the chip (256×256 pixels) was used for imaging analysis to ensure homogeneous illumination. Movies of 200 frames were acquired for each sample at a frame rate of 10 Hz using MetaMorph software (Molecular Device). For the photobleaching-step counting study, before the single-molecule fluorescence imaging, the cells were washed with cold PBS (4°C) twice and fixed in cold 4% paraformaldehyde/PBS solution for 10 min. For the control experiment of single GFP molecule imaged on coverslip, GFP protein purified from *E. coli* was firstly dissolved in the high salt buffer (600 mM NaCl, 150 mM PBS buffer, pH 7.4) to prevent the dimer formation and then immobilized on the coverslips through biotin coupled GFP antibody (Clontech) as previously reported.^[28]

Image Analysis

For analysis of single-molecule fluorescence intensity in a movie acquired from living cells, the background fluorescence was first subtracted from each frame using the rolling ball

method in Image J software (National Institute of Health). Then the first frame of each movie was used for fluorescent spot (regions of interest) selection. The image was thresholded (four times of the mean intensity of an area with no fluorescent spots), then filtered again with a user-defined program in Matlab. To analyze the photobleaching steps, regions of interest for bleaching analysis were selected according to the method previously reported.^[20] The background fluorescence was subtracted from the movie acquired from the fixed cells using the rolling ball method in Image J software.

AFM Force Measurement

Preparations of chemical modification of AFM tips (type: NP, from Veeco, Santa Barbara, CA, USA) and extracellular domain of IGF-1R (IGF-1R-ECD) were carried out as previously reported.^[21,29] The force measurements of the IGF-1-modified AFM tip on the living MCF7 cells were carried out on a PicoSPM II with PicoScan 3000 controller and a large scanner (Molecular Imaging, Tempe, AZ). The AFM scanner was mounted on an inverted fluorescence microscopy (Olympus IX81, Japan). The loading rate of Force measurements was 1.0×10^4 pN/s. The force curves measured in living MCF7 cells were recorded and analyzed by PicoScan 5 software (Molecular Imaging, Tempe, AZ). All forces were measured with contact mode at room temperature.

Flow Cytometry

Flow cytometry was used to measure the binding of IGF-1 to IGF-1R. IGF-1 was labeled using the Fluorokine IGF-1-biotin kit (R&D Systems) according to manufacturer's instructions. MCF7 cells were incubated without or with luteolin (100 μ M) or negative control reagent, in the presence of biotinylated IGF-1 for one hour.

Theoretic study with molecular docking

Molecular docking study was carried out for the extracellular domain of IGF-1R/IGF-1 complex and luteolin using ZDock method (Discover Studio 2.5), followed by docking luteolin with Surflex-Dock. The IGF-1R/IGF-1 coordinates were downloaded from the Protein Data Bank. ArgusLab4.0.1 and UFF force field were used to prepare the small molecule structure. The Surflex-Dock used an empirical scoring function and a patented search engine to dock luteolin into the IGF-1/IGF-1R complex to identify the most stable and favorable orientations. The image in the manuscript was created from SYBYL 7.3 with the intercalation site.^[30]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Measurements of IGF-1R monomers and dimers. (A) A typical single-molecule image of IGF-1R-GFP on the MCF7 cell membrane. The spots enclosed with green circles represented the signals from individual IGF-1R-GFP molecules. Scale bar: 4 μ m. (B and C) Distributions of the fluorescence intensity of individual IGF-1R-GFP spots from the stimulated cells without (B) and with (C) 100 μ M luteolin treatment. For IGF-1 stimulation experiment, the transfected cells were incubated with 50 ng/mL IGF-1 (R&D) in the serum-free DMEM for 15 min at 37°C. The solid curves show the fitting of the Gaussian function with the arrowheads indicating the peak positions. The two peaks represented monomers and dimers, respectively, and the numbers in the parentheses are the fractions. (D) Frequency of one- and two-step bleaching events under various conditions. Data is expressed as mean $\pm$ SD. * P <0.05.

Figure 2.

Measurements of the binding between IGF-1 and IGF-1R in living cells. (A) Representative force curves obtained with IGF-1-modified AFM tips on MCF7 cells in the absence (1) or presence (2) of luteolin or after blocking the binding with extracellular domain of IGF-1R (IGF-1RECD) (3). (B and C) Histograms of binding forces of IGF-1/IGF-1R with untreated cells (B) and the cells treated with 100 μ M luteolin (C) (grey bars, experimental data; solid line, theoretical Gaussian distribution curves). (D) The binding probability of IGF-1 and IGF-1R when the cells are treated without or with 100 μ M luteolin or the blocking reagent. Data is expressed as mean $\pm$ SD. * $P < 0.05$. (E) Fluorescence intensity by flow cytometry

using biotinylated IGF-1 and avidin-FITC. The cells were treated without or with luteolin (100 μ M) or negative control reagent.

Figure 3.
Predicted binding model of the extracellular domain of IGF-1R/IGF-1 complexed with luteolin (sticks) and four binding sites (PHE1, THR516, GLY520 and ASP533).