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# Quantitative Analysis with Atomic Force Microscopy of Cisplatin-induced Morphological Changes in HeLa and Ishikawa Cells

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**Abstract**

Because the cell membrane is an important regulator of cell function, its morphological changes are important markers of cell apoptosis. These changes can differ for each cell type, and depend on the treatment conditions, including the drug, doses, and treatment time. To quantify morphological changes, HeLa and Ishikawa cells were investigated with atomic force microscopy. Both cells were treated with cisplatin (1 mM) for 24 hours. The viability and proliferation of the cells were analyzed with methylthiazol tetrazolium method. The proliferation rates of both cells treated with cisplatin decreased more than 50%. The morphological changes induced by cisplatin were dependent on the cell type, and the results were determined quantitatively. The surface of HeLa cells became rougher with cisplatin treatment, whereas cisplatin-treated Ishikawa cells were smoother than untreated cells. In both cases, cell height was decreased with cisplatin treatment. These results suggest that atomic force microscopy can be used to analyze anticancer drug activity in cancer cells.
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**Key words:** atomic force microscopy, cisplatin, HeLa cell, Ishikawa cell

## Introduction

Cervical cancer is the second most frequent cancer among women worldwide and the most frequent cancer among women in Asia, Africa, and South America[1]. In addition, it is a main cause of death in women in developing countries[2]. Reported 5-year survival rates are 63% to 70% and 16% to 25% for stage IIB and IVA disease, respectively[3]. Endometrial cancer is the most common gynecologic malignancy and accounts for 6% of all cancers in women. Anticancer drugs based on platinum have commonly been used to treat gynaecologic cancers. Cisplatin (*cis*-diamminedichloroplatinum) is a primary drug in the treatment of advanced cancer of the

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cervix, endometrium, and ovary. Cisplatin interacts with DNA by binding to it and then causing the production of intrastructural cross links and the formation of DNA adducts[4]. Intrastrand cisplatin adducts can cause changes in DNA conformation that may affect DNA replication[5].

The cell membrane constitutes a barrier between the cell and the external environment. The functions of the intact cell membrane are to to act as an exchange interface between the intracellular and extracellular environments to maintain the dynamic balance of the intracellular environment from external variation, and to, provide a relatively stable intracellular environment for the normal physiological viability of the cell. The morphological changes that occur in a cell are important markers of cell apoptosis[6]. Recently, an analysis of changes in cancer cell morphology and cytoskeletal elements induced by external stimuli generated intense interest in the field of cancer chemotherapeutics[7].

Atomic force microscopy (AFM) has become an important medical and biological tool for non-invasive imaging of cells and biomaterials since its invention by Binnig *et al.*[8]. AFM can be used to examine the properties of biomaterials, such as the surface topography, structure, and micromechanical properties. First, AFM is a very high-resolution type of scanning probe microscopy, with a demonstrated resolution in the sub-nanometer range, which is more than 1,000 times the optical diffraction limit. Therefore, AFM is an extremely powerful tool for observing the surface or structure of biomaterials from cells to protein molecules. Second, AFM is a non-invasive technique with the ability to image under different conditions, for example in air and in physiological solutions. These abilities make it an ideal technique for examining the dynamics of living cells. Third, AFM can also be used to study the micromechanical properties of biomaterials from force-curve measurement. The force curve is a plot of the applied force to the cantilever as the AFM tip moves (approaching and retracting). The micromechanical properties, such as stiffness, elasticity, and adhesive force, can be determined from the relationship between the applied force and tip movement. Because of this superior ability, AFM has frequently been used in molecular, cellular, and medical biology research[9]. Several reports on cancer cells examined with AFM have been published, including reports on cancer cell morphology[10] and elasticity[11], and their relations to cell function[12].

Morphological changes in cancer cells mediated by anticancer agents may potentially be valuable for evaluating the anticancer activity of these agents[13]. The changes in cell morphology are also helpful for understanding the pathophysiology of cancer[14]. Therefore, the combination of morphological analysis and molecular analysis could be used for cancer diagnosis and therapeutics[15]. However, there have been only a few studies of the morphological changes of gynecologic cancer cell membranes after cisplatin treatment. Therefore, in the present study, changes in the morphology of cancer cells after cisplatin treatment were investigated with AFM and analyzed quantitatively. Two types of cancer cells, human cervical carcinoma (HeLa cells), and human endometrial adenocarcinoma (Ishikawa cells), were used because they are the most common cancer cells in women.

## Material and Methods

### Cell Culture and Drug Treatments

HeLa cells were derived from human cervical cancer cells (KCLB-10002; Korean Cell Line Bank, Seoul, Korea), maintained in RPMI 1640 (WelGENE Inc., Daegu, Korea) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St.Louis, MO, USA). Ishikawa cells (European Collection of Animal Cell Cultures, Salisbury, Wiltshire, UK) were derived from a well-differentiated human endometrial epithelial adenocarcinomas cell line and grown in Minimum Essential Medium (Gibco, Auckland, New Zealand) containing 5% fetal bovine serum (Sigma-Aldrich), antibiotics (100 U/mL 122 of penicillin and 100 μg/mL of streptomycin) and 2 mM glutamate. Both cell types were cultured on plastic substrata (SPL Life Science, Inc., Pocheon, Korea) in a humidified 5% $CO_2$ atmosphere at 37-℃. The cells ($2 \times 10^5$) were seeded in dishes. Cisplatin (P4394, Sigma-Aldrich) diluted in dimethyl sulfoxide (DMSO) was applied at 37-℃ for 4 different times.

### Cell Fixation

For AFM measurement, cancer cells were cultured in a 30 × 30 cm dish (SPL Life Sciences, Inc.). The cells were rinsed twice in phosphate-buffered saline solution for 5 minutes before being fixed by immersion in 2.5% gluteraldehyde. The cells were fixed for 30 minutes and then rinsed in PBS and stored at room temperature under dark conditions.

### Light Microscopic Imaging

Photomicrographs were obtained with an Olympus optical microscope ckx41 digital camera to observe changes in cell morphology. The cells were photographed before and after treatment with cisplatin.

### Methylthiazoltetrazolium Assay

Cell viability and proliferation were analyzed with the methylthiazoltetrazolium (MTT) mitochondrial reaction. This colorimetric assay is based on the ability of live cells to reduce yellow MTT reagent (Sigma-Aldrich) to a purple formazan product. Cells ($2 \times 10^5$) were seeded in 12- well- plates (SPL life Sciences, Inc.) at different times (0, 12, 24, and 48 hours). Then, the cells were treated with different doses of cisplatin (P4392, Sigma-Aldrich: 10 μM, 100 μM, 1 mM, 10 mM). A total of 100 μL of MTT solution was added to each well at 37℃ and 5% $CO_2$. After incubation, MTT was aspirated and 100 μL of dimethyl sulfoxide (DMSO) was added to each well. Subsequently, an enzyme-linked immunosorbent assay reader was used to measure the optical densities of plates at 540 nm.

### Atomic Force Microscopy

The effects of cisplastin on the properties of cancer cells were investigated with the AFM NANOStation II (Surface Imaging Systems GmbH, Herzogenrath, Germany), which consists of an AFM scanner and an optical microscope (Epiplan 500x, Carl Zeiss Microimaging, Jena, Germany). The AFM images were measured with the reflex-coated gold cantilever in contact mode (Budget-Sensors, Sofia, Bulgaria). The material property and dimension of the probe used in the contact mode were: resonance frequency, 13 ± 4 kHz: force constant, 0.2 N/m (0.07 to 0.4 N/m): cantilever length, 450 ± 10 μm: cantilever width, 38 ± 5 μm: cantilever thickness, 2 ± 1 μm: tip radius, < 10 nm: and tip height, 17 ± 2 μm. The images were obtained at a resolution of 512 × 512 pixels and a scan speed of 0.1 line/second. The scan area were dependent on the size of the cancer cell. Image processing and data analysis were performed with image-processing software (Scanning Probe Image Processor version 4.1, Image Metrology, Hørsholm, Denmark). The surface roughness of cancer cells was determined in a small area of 3 × 3 $\mu m^2$. The roughness was measured at more than 20 sites for each cell and mean values were determined.

### Statistics

Statistical analysis with two-tailed Student's *t*-tests was performed to compare mean values of surface roughness of cancer cells. A p-value < 0.05 was considered to indicate statistical significances.

## Results

### Cell Viability

In untreated Ishikawa and HeLa cells, the viability rates increased with time from 100% to more than 170% but decreased in the cisplatin- treated cells (**Fig. 1 (a) and (b)**). The proliferation rates of cisplatin-treated HeLa cells were 11% to 29% of baseline. These rates were 18% to 28% in the treated Ishikawa cells.

### Light Microscopic Imaging

In *in vitro* studies, changes in the general morphology of tumor cells under the effects of drugs are commonly used as a basis for assessing drug effects. On light microscopic examination of HeLa and Ishikawa cells, the nuclei were clearly visible (**Fig. 2**). The shape of the membrane, however, differed according to the treatment. In both types of untreated cells (**Figs. 2 (a) and (c)**), the membranes were nearly circular, but changed into irregular polygons following cisplatin treatment (**Figs. 2 (b) and (d)**).

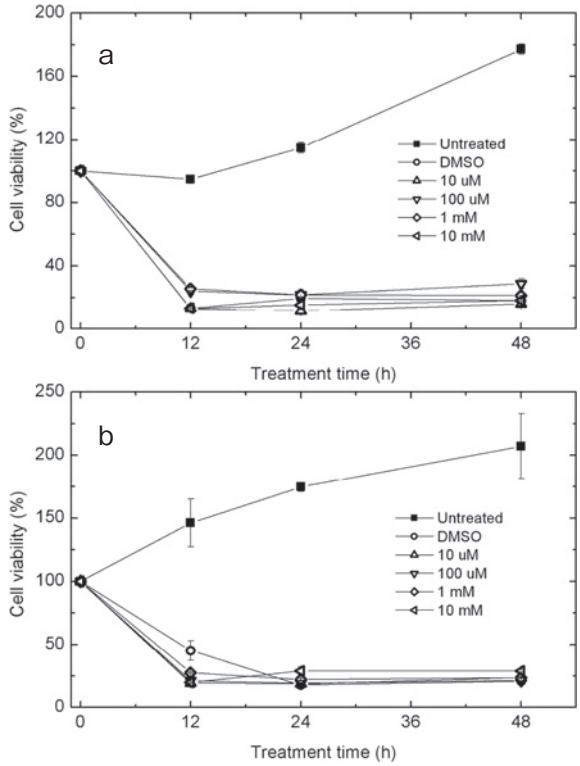


Fig. 1 The viabilities of HeLa (a) and Ishikawa (b) cells. The cells were untreated or treated with DMSO, or various concentrations of cisplatin (10 μM, 100 μM, 1 mM, 10 mM) for various lengths of time (0, 12, 24, and 48 hours) and analyzed with the MTT assay.

**AFM Images and Morphological Changes**

Untreated HeLa cells were circular and had a diameter of 27 μm (**Fig. 3**). In the center of the cell the nucleus was clear and brightly colored. The greatest height of cells was 2.72 μm. After treatment with cisplatin HeLa cells showed physical changes: the cells became oval, and the surface became extremely rough. Because the nuclei of the treated cells had exploded, as indicated by the large area of the bright region, the height of treated cells was less (2.29 μm) than that of untreated cells. Untreated Ishikawa cells (**Fig. 4**) had a distinct boundary, smooth surface, and centrally located nucleus. After treatment with cisplastin, the cells showed an uneven boundary and a rougher surface. In particular, the height of Ishikawa cells was 5.16 μm before treatment but decreased to 2.83 μm after treatment.

For both HeLa and Ishikawa cells, the height of cells not treated with cisplatin was greater than that of cells treated with cisplatin (**Fig. 5**). The change in height was dependent on the type of cell. The height of HeLa cells decreased by 3 μm, but the height of

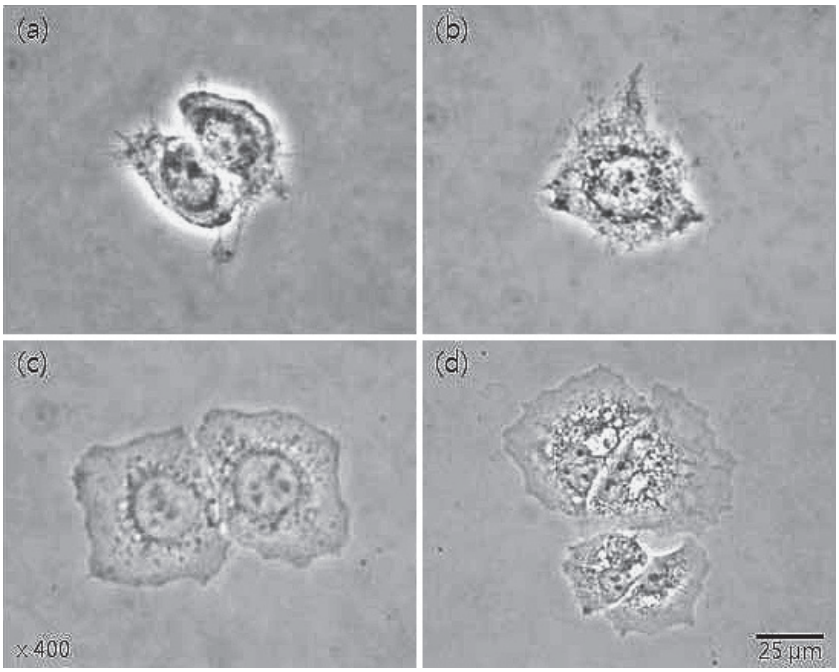


Fig. 2 Light microscopic images of cancer cells. (a) and (b) are the images of untreated and cisplatin-treated (1 mM) HeLa cells, respectively, and (c) and (d) are the images of untreated and treated Ishikawa cells for 24 hours, respectively.

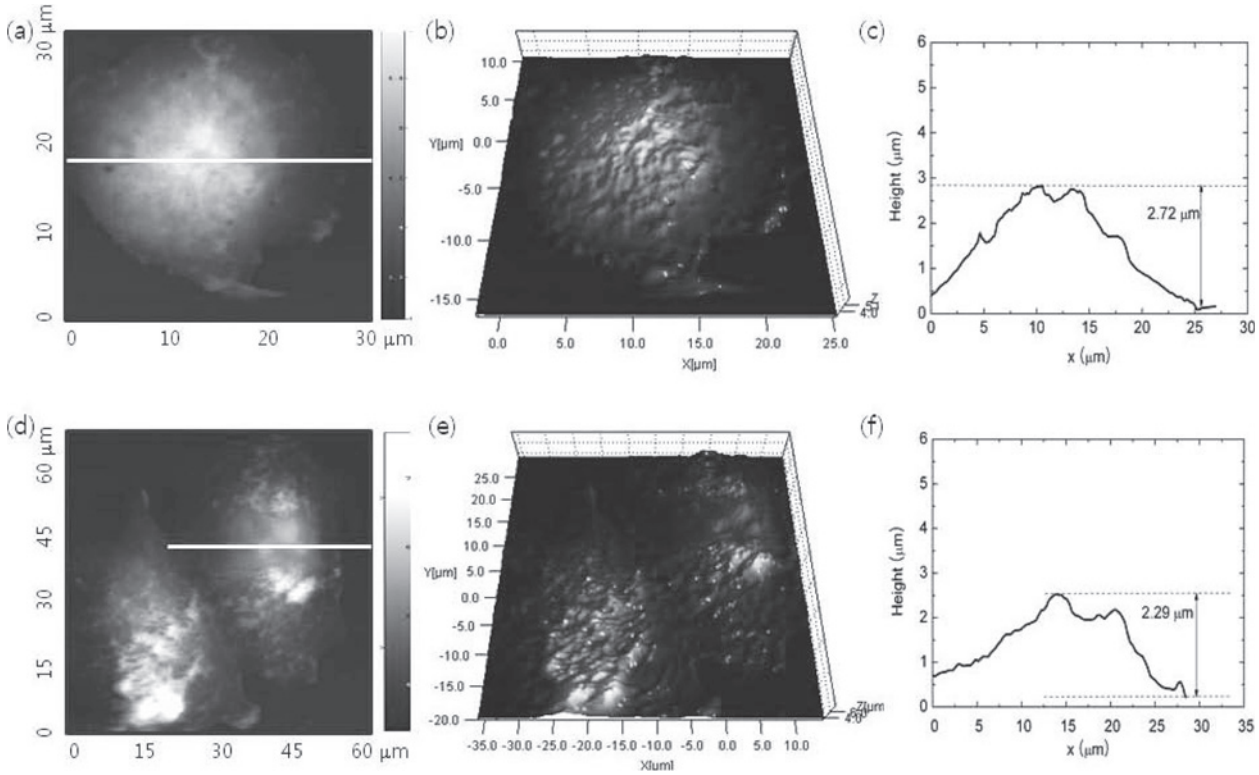


Fig. 3 Representative AFM images of untreated HeLa cells (upper row) and HeLa cells treated with cisplatin (lower row) for 24 hours. Figures (a) and (d) are topographic images (in which light and dark colors correspond to the higher and lower topography, respectively), (b) and (e) are 3-dimensional images of the entire cell, and (c) and (f) are line profiles measured in the first column ; the white line shows the position taken by the line profile.

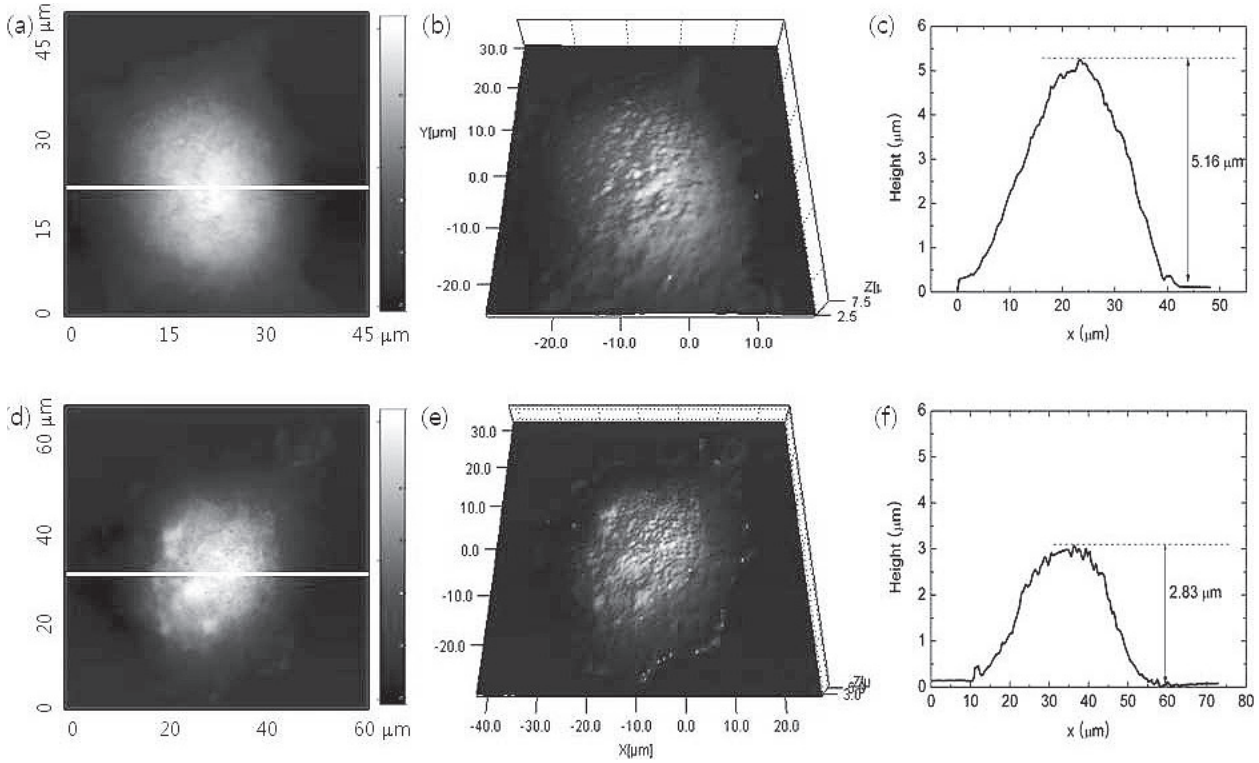


Fig. 4 Representative AFM images of untreated Ishikawa cells (upper row) and Ishikawa cells treated with cisplatin (lower row) for 24 hours. Figures (a) and (d) are topographic images (in which light and dark colors correspond to the higher and lower topography, respectively), (b) and (e) are 3-dimensional images of the entire cell, and (c) and (f) are line profiles measured in the first column; the white line shows the position taken by the kine profile.

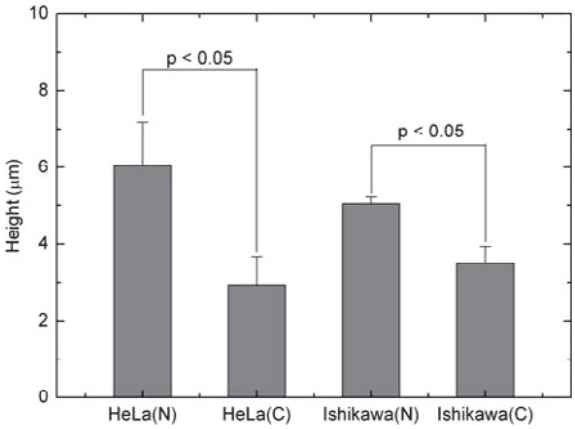


Fig. 5 Comparison of height between untreated (N) and cisplatin-treated (C) HeLa and Ishikawa cells. The results are mean values for 10 cells in each case.

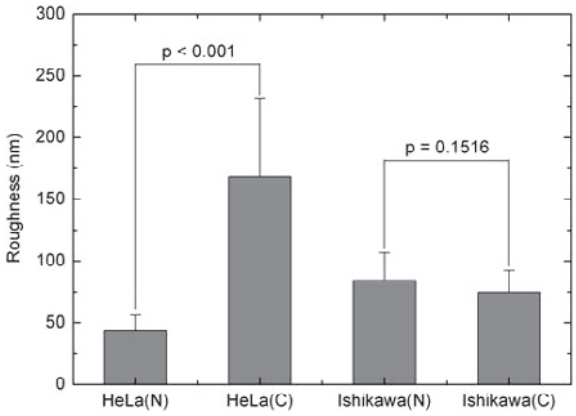


Fig. 6 Comparison of the surface roughness between the untreated (N) and cisplatin-treated (C) HeLa and Ishikawa cells.

Ishikawa cells decreased by only 1.5 μm.

The roughness value of cisplatin-treated HeLa cells (168.4 nm) was significantly greater ($p<0.05$) than that of untreated HeLa cells (43.3 nm) (**Fig. 6**). Ishikawa cells, in contrast, showed the opposite result: untreated Ishikawa cells (84 nm) were rougher than treated cells (74 nm). However, the difference was not significant.

## Discussion

Cervical and endometrial cancers are the most common gynecologic cancers. Therefore, the development of chemotherapeutic agents against these cancers is important for reducing their incidence and mortality[1,17]. Cisplatin is a widely used anticancer agent in gynecologic cancers; it is believed to damage cellular DNA by forming different types of bifunctional adducts with DNA and to thereby induce apoptosis[18]. Cisplatin induces 2 different modes of cell death[19]: necrosis and apoptosis. Necrosis is characterized by a cytosolic swelling and early loss of plasma-membrane integrity. In contrast, early features of cells undergoing apoptosis include cell shrinkage, chromatin condensation, and DNA fragmentation.

The cell membrane is an important regulator of cell function and plays a key role in the physiological processes of cells, such as signal transduction, cellular transport, energy conversion, cell-surface recognition, cell survival, and differentiation[20]. Changes in cell membrane structure can therefore directly influence the normal functions of cells[21]. Several studies of the apoptotic changes that occur on cancer-cell surfaces have been reported. Kerr *et al.*[22] have reported that the onset of apoptosis can be characterized by shrinkage of the cell membrane, condensation of its cytoplasm, and detachment of the cell from the surrounding tissue. Cai *et al.*[14] have demonstrated that the anticancer drug artesunate inhibits cell viability and induces changes in the height and roughness of Jurkat (acute lymphoid leukemia) cells. Venkatesan *et al.*[7] have reported that HCT15 colon cancer are shrunk by the anticancer drugs AEE788 and celecoxib.

In the present study, the morphological changes induced by cisplatin in HeLa and Ishikawa cells were clearly observed with AFM imaging. In both cell types, cisplatin damaged membranes, as indicated by an uneven boundary and a rougher surface. Cell height decreased following treatment. As expected, the changes depended on the type of cancer cell, and the difference was determined quantitatively. Therefore, as demonstrated in several studies[7,14,22]. AFM can be used to analyze the properties of cancer cells and to evaluate the activity of anticancer drugs. Importantly, changes in the morphological properties of cells can be determined qualitatively and quantitatively.

Even though AFM has several advantages for

studying cellular systems, there are limitations to imaging live cells. AFM scans the topography of the sample by detecting differences in height; thus, the AFM tip is brought close to the sample surface. When the sample is a dead cell, the approaching tip has no affect on the sample. However, if the sample is a living cell, the tip interacts with the cell and can change its shape or position. Therefore, measuring living cells is extremely difficult. This is the reason we fixed the cells.

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