An Investigation of A549 Human Lung Cancer Cells Treated with Colchicine Using Fourier Transform Infrared Spectra

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Abstract

In this study, we used Fourier Transform Infrared (FT-IR) spectroscopy with second-derivative analysis techniques to obtain Infrared (IR) spectra of A549 cells and confirm the peak positions and assignments of the IR spectra to characterize intracellular protein secondary structure of A549 human lung cancer cells treated with colchicine. Treatment with colchinine reduced the lipid content decreased in A549 cells, with peak shifts occurring at 2962, 2916, 2875, and 2847 cm^{-1}. After treatment with colchicine, the peak positions of α-helix of amide I (1650 cm^{-1}) and amide II (1540 cm^{-1}) in A549 cells were shifted to high frequency. The differences between A549 cells that underwent mitosis were significant, especially with regard to the conformational changes of the proteins. We also observed that increasing concentrations of colchicine led to clear differences in nucleic acid PO_2^- stretching (1087 and 1240 cm^{-1}). These results suggest colchicine dose-dependently induced conformational changes of a-helical secondary structures in A549 cells. However, the band at 965 cm^{-1} did not show distinct changes, indicating no marked changes of this DNA stretching vibration. Therefore, these changes of the protein structures may be related to the mode through which the agents interact with the cell, as has been reported previously.

Keywords: A549 human lung cancer cells, colchicine, second-derivative analysis, protein secondary structure, FT-IR

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Introduction

Colchicine is a naturally occurring secondary metabolite alkaloid, originally extracted from plants of the genus *Colchicum*. It was used only to treat gout but is currently being investigated as an anti-cancer drug. It inhibits microtubule polymerization by binding to tubulin dimers and prevents the assembly of microtubules. It has been used as a model antimitotic drug and frequently used as an antimitotic agent in cancer research involving cell cultures[1,2]. Though its use has been limited because of its toxicity, colchicine can still be used as a reference compound for the exploration of anti-cancer drug mechanisms.

In recent years, spectral methods have been used in the evaluation of cancer, and some attempts have been made to utilize them as a diagnostic tool[3,5]. Infrared (IR) spectroscopy is becoming an increasingly powerful tool for the study of the composition and structure of cellular components within intact tissues[6,7]. Fourier-transform infrared (FT-IR) spectroscopy has also been used as an extension of current histopathological techniques used to characterize various forms of cancer, multiple sclerosis, and Alzheimer's disease[8-11]. FT-IR spectroscopy as a non-destructive approach to examination of biological materials has provided interesting and useful information on the secondary conformation of intact protein in biological tissue[12,13]. The advantages of FT-IR over other spectroscopy techniques are that it requires fewer samples and less time. The availability of modern computers has enabled the rapid and powerful FT-IR mathematical data analysis and processing. These vibrations of the intensity and frequency of IR spectra are sensitive to the interactions and the environment of biomolecules. The IR spectra give complementary information on the molecular vibrations of biological polymers and provide a characteristic fingerprint of the cell constituents, so the global effect of a therapeutic agent can be distinguished from those of treated cell. The A549 lung cancer cell line consists of carcinomic human alvelar basal epithelial cells and derived through culture of lung adenocarcinoma tissue. This study uses FT-IR spectroscopy to investigate the intracellular interaction between colchicine and A549 cells to understand the conformational changes of A549 human lung cancer cells induced by colchicine.

Materials and Methods

I. *Chemicals and Culture Medium*

Colchcine, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and trypan blue were obtained from Sigma Chemical Co. (St. Louis, MO, USA). RPMI-1640 medium, fetal bovine serum (FBS), penicillin and streptomycin were purchased from GIBCO BRL (Grand Island, NY, USA). Other chemicals were analytical grade.

II. *Cell Line and Cell Culture*

The A549 human lung carcinoma epithelial cell line, a type II epithelial-like cell line obtained from the American Type Culture Collection

(ATCC) (Rockville, MD, USA), was cultured following standard methodology and grown on tissue culture dishes in a humidified incubator at 37°C in a 5% CO_2 atmosphere. They were maintained in a complete medium consisting of RPMI 1640 medium, supplemented with 10% heat-inactivated FBS, penicillin (100 units/ml)/ streptomycin (100 µg/ml) and 1% *L*-glutamine.

III. *MTT Cytotoxicity Test*

Cytotoxicity effect was assessed using MTT assay[14]. A549 cells were seeded at a density of 5×10^3 cells/well in a 96-well culture plates, and treated with colchicine or culture medium alone for 48 hours. Colchicines were dissolved in DMSO (final test concentration were 0.01, 0.05, 0.1, 0.25, 0.5, 1, 5, 10, and 50 µM), and the final concentration of DMSO in the culture medium was kept below 0.1% in the treated cells and control cells. Briefly, we added 10 µl of MTT solution (5 mg/ml) with 50 µl of cells suspension in HBSS into a 96-well plate which we incubated at 37°C in the dark for 2 hours. Treatment of living cells with MTT produces a dark blue formazan product, whereas no such staining is observed in dead cells. And the formazan product was dissolved by adding 150 µl DMSO and then the absorbance was measured on an ELISA reader at a wavelength of 570 nm. The IC_{50} value of colchicine on cells was calculated by MTT assay. Values are expressed as means ± S.D. of six independent experiments.

IV. Immunofluorescence microscopy

A549 cells (5×10^4 cells/well) plated on 4-well chamber slides were treated without or with 1 µM colchicine for 12 hours. After treatment, cells were fixed in 4% formaldehyde in PBS for 15 min, permeabilized with 0.3% Triton X-100 in PBS for 1 hour with blocking of nonspecific binding sites using 2% BSA. Fixed cells were then incubated with antihuman α-tubulin antibody (1:100 dilution) or phospho-histone H3 (1:100 dilution) overnight and then exposed to the secondary antibody (FITC-conjugated goat anti-mouse IgG at 1:100 dilution), followed by DNA staining with PI. Photomicro-graphs were obtained using a Leica TCS SP2 Confocal Spectral Microscope (Chen et al., 2005).

V. Infrared Spectral Analysis

A549 cells (1×10^5 cells/well) were treated without or with 0.025, 0.05, 0.075 and 0.1 µM colchicine for 24 hours. They were harvested by gently scraping them off the tissue culture plates in 10 ml saline solution. After treatment, cells were washed once with PBS, and then fixed with 70% ice-cold ethanol and re-suspended in PBS before infrared spectrum measurement. The infrared spectrum of A549 human lung cancer cells was obtained using a Perkin-Elmer Spectrum One FT-IR spectrometer (Perkin Elmer Ltd., Buckinghamshire, UK) equipped with a liquid nitrogen-cooled mercury-cadmium-telluride (MCT) detector and a zinc selenide (ZnSe) attenuated total reflectance (ATR) prism. For each spectrum, 128 scans were performed with 20 µl cell suspension solution on ATR prism at a spectral resolution of 4 cm^{-1}, with a normal wave number ranging from 4000 to 700 cm^{-1} in triplicate. Spectra were baseline

corrected, normalized on the whole spectral window and offset corrected. By comparing the second derivative spectra used to confirm the peak positions and assignments of the IR spectra, we identified the most significant regions. All spectral data were analyzed using Windows software supplied by Perkin-Elmer and second derivative spectra were then obtained using a five-point, two-degree polynomial function, which eliminated the need for further resolution enhancement by Fourier self-deconvolution. The spectra were then smoothed with an 11-point, two-degree polynomial function to reduce signal noise before further data analysis. The derivatized and smoothed spectra were individually integrated, and the second derivative band area was calculated using the integration function.

The experiments were performed in quadruplicate and the pellets were stored at -80°C until analysis. Repetition of experiments using fresh or previously frozen samples revealed that IR spectra of these cells were not affected by storage at -80°C.

VI. Statistical analyses

Values are expressed as the mean ± S.D. of three independent experiments. Student's t tests were used to assess the significance of the differences, with "p" values of less than 0.05 considered significant.

Table 1. Characteristic infrared bands of biomolecule

Designation	Approximate frequency (cm^{-1})		Description
Lipids	2800-3000	2847	Symmetric lipid CH_2
		2916	Asymmetric lipid CH_2
		2875	Symmetric CH_3-stretching
		2960	Aymmetric CH_3-stretching
Proteins	1400-1800	1650 (1600-1690)	Amide I
		1541 (1480-1575)	Amide II
DNA	800-1400	965	Deoxyribose & phosphate
		1087	Symmetric PO_2^-
		1240	Asymmetric PO_2^-

Results

1. *Cytotoxicity effect of colchicine on A549 cells*

In order to analyze the cytotoxic effect of colchicine on A549 cells, we initially conducted the MTT assay for 48-hour screening to determine the IC_{50} values (the concentration of 50% cell proliferation-inhibitory effect) with different concentrations of colchicine. Control cells were incubated in medium supplemented with 0.1% DMSO, as a drug vehicle. As shown in Fig.1, colchicine dose-dependently inhibited

the proliferation of A549 cells at concentrations ranging from 0 to 0.5 μM. Treatment with colchicine in A549 cells for 48 hours resulted in an IC_{50} value of 0.084 μM (Fig.1). These results suggest that colchicine had a significant anti-proliferative effect on the cells. Actin filaments or microtubule in treated-A549 (1 μM) cells were investigated using confocal laser scanning microscopy and image analysis to help investigate cellular changes upon exposure to colchicine. As seen in Fig.2, A549 cell shape changed after disruption of actin filaments or microtubules.

2. *IR Spectroscopic Analysis*

Infrared spectra of colchicine-treated and untreated A549 human lung cancer cell lines indicated colchicine had intracellular effects. Fig.3, Fig.4 and Fig.5 illustrate more subtle differences in the second derivative spectra. They illustrate the changes in bands corresponding to lipids (3000-2800 cm^{-1}), proteins (1800-1400 cm^{-1}), and DNA (1400-800 cm^{-1}), respectively. While the second derivative spectra in the 1800-1400 cm^{-1} range showed a significant change in lipid, amide I, and amide II regions in Fig.4. The second derivative spectrum further shows a band at 1734 cm^{-1} indicating that lipids increased as the concentration of colchicine increased. This finding was corroborated by the second derivative spectrum in the 3000-2800 cm^{-1} range depicted in Fig.3, which shows negative bands at 2847 and 2916 cm^{-1} corresponding to lipid CH_2 symmetric and asymmetric stretching modes[15]. These bands were shifted to low frequency relative to the absorptions at 2851 and 2917 cm^{-1} in the spectrum after the cells were treated with 100 nM colchicines; however, this observed spectral difference affected by colchicine was not significant. After derivatization, the maximum wave number shifted slightly because the relative intensities of the CH band changed. The band near 2875 cm^{-1} was mainly due to the symmetric CH_3-stretching mode of methyl chains in membrane lipids, and the band near 2960 cm^{-1} was due to the asymmetric CH_3-stretching mode of the methyl end groups of membrane lipids as well as the methyl, side groups in cellular proteins[15]. These symmetric and asymmetric CH_3- stretching bands are relative to the absorptions at 2874 and 2956 cm^{-1} in the spectrum, respectively, after the cells were treated with 100 nM colchicine.

The second derivative spectrum in the 1700-1400 cm^{-1} region contained amide I (1650 cm^{-1}) and amide II (1541 cm^{-1}), both typical features of protein. The amide I band is often employed in the study of protein structures, since the amide I band in spectra is the most suitable probe for differentiating among different secondary structures of proteins and polypeptides[12]. Fig.4 shows the amide I peak (1649 cm^{-1}) of A549 cell was not significantly affected by treatment with 100 nM colchicine. The α-helix peak reached 1650 cm^{-1} in amide I. The movement of the amide II absorption peak confirmed this observation. The amide II peak of the A549 cells treated with 100 nM colchicine moved slightly from 1540 cm^{-1} to 1544 cm^{-1}, corresponding to structural

changes in α-helix[12]. These results suggest that the α-helical structures in A549 cells were slightly changed by increasing concentrations of colchicine.

The bands in the spectrum at 965 cm^{-1}, 1087 cm^{-1} and 1240 cm^{-1} are well-known DNA bands[8]. The band near 965 cm^{-1} is thought to originate from a C-C/C-O stretching vibration involving the characteristic deoxyribose and phosphate moiety of the DNA backbone[8]. The band near 1087 cm^{-1} and the band centered around 1240 cm^{-1} originate, respectively, from the symmetric PO_2^- and asymmetric PO_2^- stretching vibrations of the phosphate groups that are part of the DNA backbone[16]. In all the tumor-cell spectra that have been studied, the most significant change comes from the symmetric PO_2^- and asymmetric PO_2^- of the phosphate groups[17]. The intensity of the symmetric PO_2^- band is reported to be related to the cell DNA content[17]. The asymmetric PO_2^- of the phosphate group in the cells also often changes when treated with various agents[18]. After treating the cells with various concentrations of colchicine, we found no distinct changes in the band at 965 cm^{-1}, indicating no marked changes of this stretching vibration (Fig.4). The second derivative spectrum of A549 cells were negatively correlated with intensity in the 1087 and 1240 cm^{-1} absorptions and increasing concentrations of colchicine.

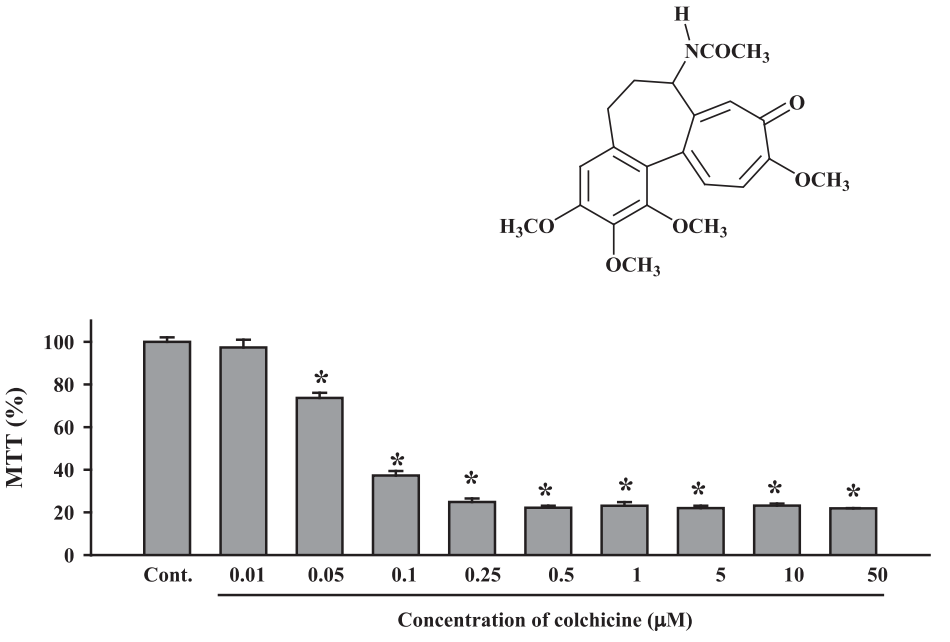


Fig.1 Colchicine inhibition of growth in human lung cancer A549 cells. A549 cells (5×10^3 cells/well in a 96-well plate) were treated without or with the indicated concentrations of colchicine for 48 hours. After treatment, the relative cell proliferation was measured by scanning with an ELISA reader with a 570 nm filter and the IC_{50} (the concentration of 50% cell proliferation-inhibitory effect) value of colchicine on cells was calculated by MTT assay (IC_{50} value was approximately 0.084 μM). Data are expressed as means ± S.D. of six independent experiments. * $p<0.001$ compared with the control.

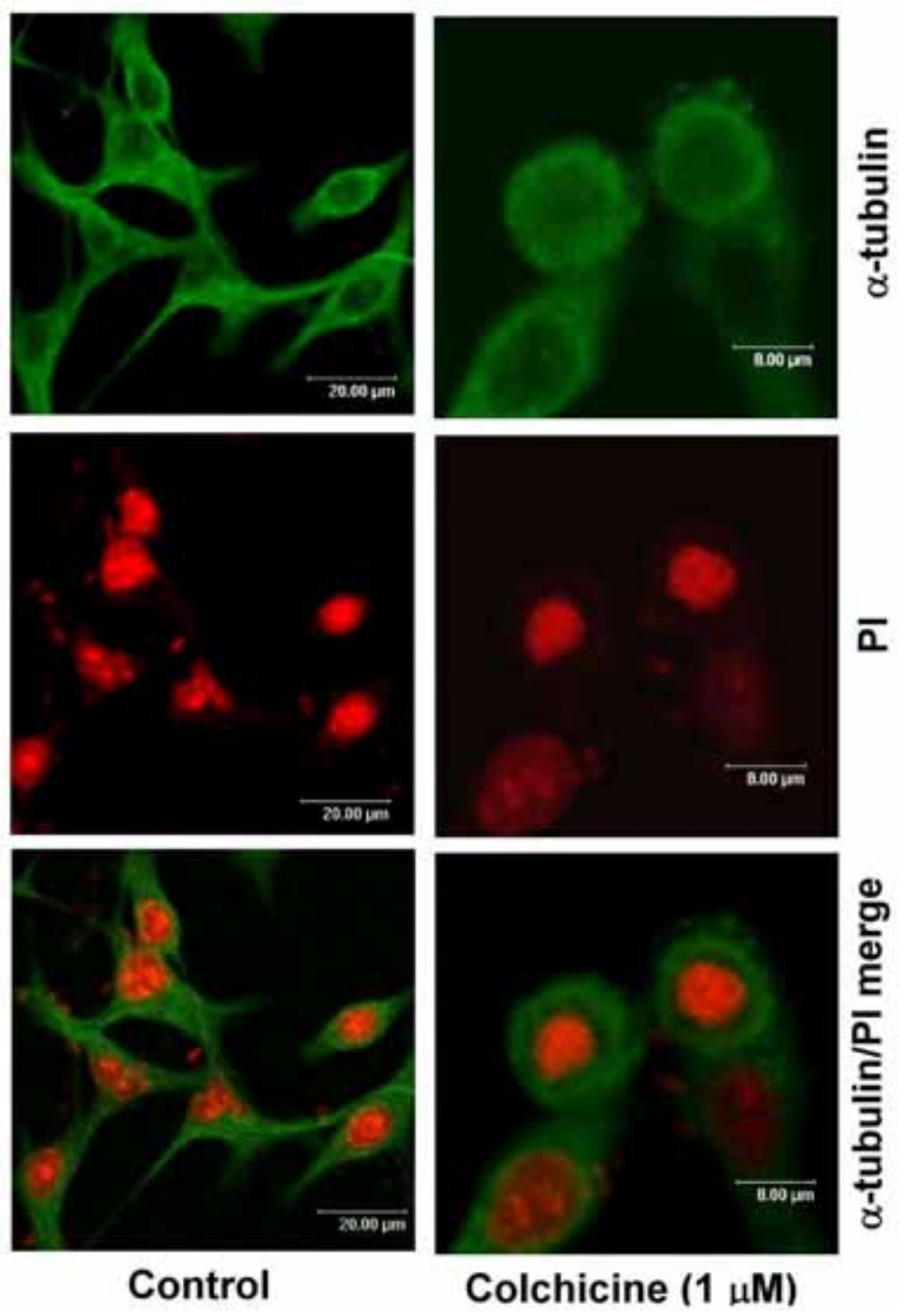


Fig.2 Effects of colchicine on microtubules of A549 human lung cancer cells. Confocal microscopy of tubulin immunofluorescence staining [α-tubulin, (green), Nucleus, (red)] reveals that colchicine (1 μM for 12 hours) caused A549 cells mitotic arrests.

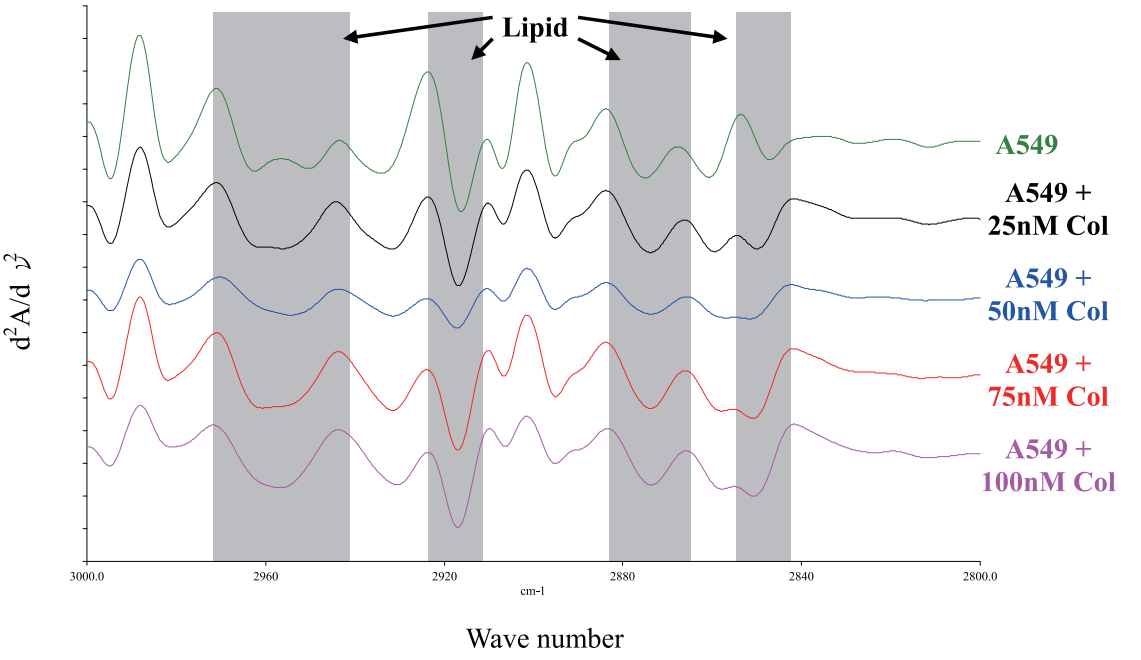


Fig.3 Second derivative FT-IR spectra of colchicine (Col)-treated A549 cells highlighted in the 3000-2800 cm^{-1} range regions. Cells were treated with 25 nM, 50 nM, 75 nM, and 100 nM colchicines for 24 hours and the effects on lipids were observed.

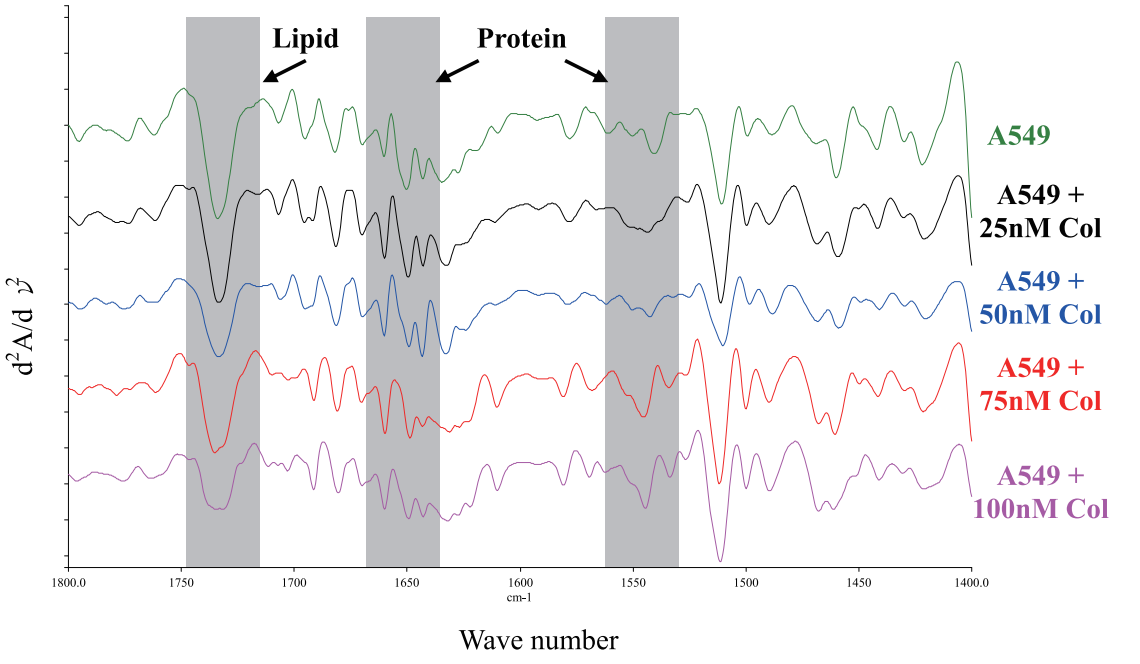


Fig.4 Second derivative FT-IR spectra of colchicine (Col)-treated A549 cells highlighted in the 1800-1400 cm^{-1} range regions. Cells were treated with 25 nM, 50 nM, 75 nM, and 100 nM colchicines for 24 hours and the treatment effects on lipids and proteins were observed.

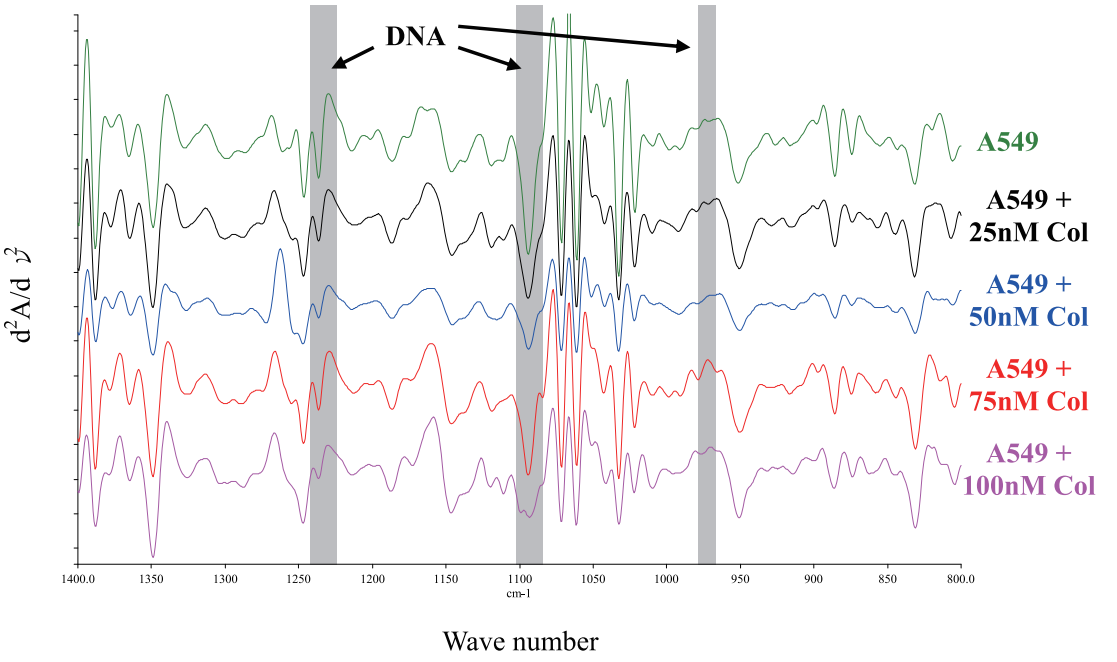


Fig.5 Second derivative FT-IR spectra of colchicine (Col)-treated A549 cells highlighted in the 1400-800 cm^{-1} range regions. Cells were treated with 25 nM, 50 nM, 75 nM, and 100 nM colchicines for 24 hours and treatment effects on nucleic acid were observed.

Discussion

Effect of colchicine on A549 cells

Colchicine interacts with tubulin and perturbs the assembly dynamics of microtubules. Prior to FTIR spectroscopic measurements, it is crucial to estimate the proliferation-inhibitory effect of colchicine on A549 cells. Cells were incubated with colchicine concentrations ranging from 0.01 to 50 μM for 48 hours. As shown in Fig.1, the drug concentrations at 1μM or higher inhibited A549 cell growth by approximately 20%. Colchicine inhibited the proliferation of A549 cells in a concentration-dependent manner under drug concentrations ranging from 0 to 0.5 μM. Moreover, the shape of the A549 cell changed after the disruption of actin filaments or microtubules that occurred after exposure to 1μM colchicine (Fig.2).

IR spectroscopy of colchicine-treated A549 cells

In recent years, FT-IR has become one of the most well established experimental techniques used to analyze the secondary structure of biomolecules. As shown in Fig.1, these colchicine concentrations, which ranged from 0 to 1.0 μM, inhibited the proliferation of A549 cells in a concentration-dependent manner. These experimental FT-IR spectral results showed several of the changes that occurred when A549 cells were treated with

various concentrations of colchicine (0.025, 0.05, 0.075, 0.1 μM and control). They revealed that only cell proliferation was affected, not cell viability. These IR spectra findings show the distinct spectral regions that correspond to lipids (3000-2800 cm^{-1}), proteins (1800-1400 cm^{-1}), and nucleic acid (1400-1800 cm^{-1}) described in Table 1.The lipid content decreased when A549 cells were treated with colchicine, as indicated by the change of peak shifts at 2962, 2916, 2875, and 2847 cm^{-1}. The A549 cells treated with colchicine were significant different, especially with regard to conformational changes of the proteins. Table 1 illustrates the characteristic infrared bands of peptide linkage and the differences in spectral regions of protein secondary linkage. The most sensitive and prominent spectral region to protein backbone is the amide I (1700-1600 cm^{-1}). After treatment with colchicine, the peak positions of α-helix of amide I and amide II in A549 cells were slightly shifted to higher frequencies, but these band shifts were too small to be significant. The bands in the spectrum at 965 cm^{-1}, 1087 cm^{-1} and 1240 cm^{-1} were DNA bands. Obvious differences were occurred in nucleic acid PO_2^- stretching (1087 and 1240 cm^{-1}) absorptions as concentrations of colchicine increased. The binding mode might play an important role in how the process agents affect the cells. However, the band at 965 cm^{-1} did not show distinct changes, indicating no marked changes of this DNA stretching vibration (Fig.4). The band near 965 cm^{-1} originated from a C-C/C-O stretching vibration involving the characteristic deoxyribose and phosphate moiety of the DNA backbone. This finding is consistent with those of Panayiotidis (2006) [19], who observed no changes in global DNA methylation status in cells synchronized at either phase of the cell cycle.

Most changes in the cells that underwent conformational change induced by colchicine were identical, as indicated by the changes of the lipids, proteins and DNA measured in the present study. It is encouraging to compare the results with the findings of Andreu in 1982 who indicated that the conformation of the tubulin-colchicine complex appears to be nonidentical with that of the unliganded protein[20].

In conclusion, our study indicates that these proliferative A549 cells possess similar properties, although they undergo proliferation along different routes. The investigative techniques used in this study showed clear and reproducible differences between the complex spectra of this interaction of A549 cells and colchicine, suggesting that infrared spectroscopy in conjunction with spectral processing technology may be used to reveal subtle spectral differences occurring following induction of conformational changes and to interpret their biochemical origins.

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利用傅立葉轉換紅外線顯微光譜儀探討瞭解秋水仙素與A549人類肺癌細胞間之細胞內交互作用

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摘 要

為了瞭解秋水仙素 (colchicine) 與A549人類肺癌細胞間之細胞內交互作用的情形，我們利用了傅立葉轉換紅外線 (FTIR) 顯微光譜儀結合二次微分分析技術獲得A549細胞的IR圖譜與確認IR圖譜的波峰位置及波峰解析。當A549細胞經由秋水仙素作用後，其脂質的含量隨即減少，而此可由位在脂質吸收區之2962 cm^{-1}，2916 cm^{-1}，2875 cm^{-1}及2847 cm^{-1}的波峰位置改變而證實之。A549細胞經由秋水仙素作用後，A549細胞的醯胺鍵I (1650 cm^{-1}) 及醯胺鍵II (1540 cm^{-1}) 之α-helix波峰位置被位移至高頻率吸收區。隨著秋水仙素濃度的增加，核苷酸PO_2^-伸縮作用吸收峰位置1087 cm^{-1}及1240 cm^{-1}亦可被觀察到明顯的不同改變。A549細胞進行細胞有絲分裂作用間的差異是很明顯的，特別是在細胞蛋白質構型的改變。這些結果指出A549細胞的α-helix結構的構型變化在分裂的細胞蛋白質中隨著秋水仙素濃度增加而些微地被誘導。而本研究中A549細胞的細胞蛋白質結構變化情形與先前的藥物與細胞間交互作用模式之研究報告相類似。

關鍵詞：人類肺癌細胞 (A549)、秋水仙素、二次微分分析、蛋白質二次結構、傅立葉轉換紅外線

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