FT-IR spectroscopy as a tool for identification of apoptosis-induced structural changes in A549 cells treated with PM 701

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Abstract: Fourier transform infrared (FTIR) spectroscopy has been found useful for detecting apoptotic changes induced in human lung cancer cells (A549) that treated with PM 701 (natural product) in tissue culture level. Characteristic bands alterations were identified in the apoptotic cells arising from cellular protein, lipid and DNA, there were specific changes that affected the secondary structure of proteins in the apoptotic lung cells appeared in the FTIR spectra confirmed with the second derivative analysis. To follow the induced changes in the apoptotic A549 cells, different band absorbance ratios were calculated for different times up to 24 hours. The results show that there is a marked decrease in the phosphate absorbance ratio from nucleic acids, the minimum value was observed at five minutes of PM 701 treatment meanwhile there is a sharp increase in the lipid-protein ratio the maximum value was obtained for cells treated at five minutes with PM 701. This study suggests that FTIR spectroscopy could possibly be used to monitor apoptosis in A549 cells and prove the efficiency of our new drug PM 701 in cancer treatment.

Keywords: FTIR spectroscopy; apoptosis; A549 cells; structural changes; quantitative analysis.

1 Introduction

Apoptosis, as a pre-programmed physiological mode of cell death, plays an important role in the pathogenesis and progression of cancer. Understanding of the basic mechanisms that underlie apoptosis will point to potentially new targets of therapeutic treatment of diseases that show an imbalance between cell proliferation and cell loss. As an active process, apoptosis involves biochemical changes on three essential cellular components, DNA, protein and lipid. There are three steps in apoptosis, the initiation phase triggered by a stimulus received by the cell, the effector or decision phase during which the cell commits itself to live or to die and the degradation phase when the cells acquire the morphological and biochemical hallmarks of apoptosis. Common biochemical events include changes in cell membrane potential, altered levels of protein and RNA biosynthesis and degradation of DNA into small fragments. DNA fragmentation, activation of caspases and externalisation of phosphatidyserine are considered as three hallmarks of apoptosis (Willingham, 1999; Coelho et al., 2000). Morphologically, cells undergoing apoptosis display membrane blebbing, cell shrinkage, nuclear condensation, fragmentation and packaging of cellular material into apoptotic bodies. Clearly, it would be desirable to detect apoptosis at an early stage, i.e., before phase that entails the visible changes of apoptosis.

Consequently, a method that can detect all three processes simultaneously would provide a useful addition for the study of apoptosis. Because chemotherapeutic agents exert their in vivo anti-tumour activity by triggering apoptosis in susceptible tumour cells, the in vitro measurement of drug-induced apoptosis should provide a mechanism-based test for the chemosensitivity of the tumour cells isolated from an individual patient and may represent a prognostic marker for the treatment response (Kravtsov et al., 1998; Hannun, 1997). Furthermore, it was demonstrated that IR spectroscopy can distinguish the different phases of cell division when examining purified populations (Boydston-White et al., 1999) and render diagnoses and even prognoses on the outcome of disease (Schulz et al., 1997). The understanding of infrared spectra of individual cells becomes of paramount importance for the interpretation of spectra of tissue samples. Cancer becomes one of the leading cause of death in many countries over the world. Early diagnosis and treatment increases the chances of survival and full recovery (Dukor, 2001). Fourier transform infrared (FTIR) spectroscopy has been used by chemists as a powerful tool to characterise inorganic and organic compounds (Khorsid and Mushref, 2006). It has been applied in biology for studying the
structure and conformation of molecules like proteins (Cooper and Knutson, 1995),
nucleic acids (Mantsch and Chapman, 1996) and lipids (Brandenburg and Seydel, 1998).

Herein, we continue the work done earlier by Khorsid and Mushref (2006) to explore
the potential of PM 701 as anti-cancer agent, which produce apoptosis in human lung
cancer cells (A549) dry samples at the tissue culture level together with the potential of
infrared (FTIR) spectroscopy as a means for the early detection of apoptosis.

2 Materials and methods

• **Media:** The following commercially available media were prepared according to
  published literature, these include: ordinary media, minimal essential medium
  (MEM) (10% FCS); MEM is a rich, multipurpose medium that was used for
cultivation of human lung cancer cells (A549). Phosphate buffer saline (PBS) is a
  phosphate-buffered physiological saline solution. Calcium and magnesium free
  solution trypsin.

• **Examined media:** PM 701 is a natural product, easily available, cheap, sterile and
  non-toxic according to our chemical and microbiological testing. PM 701 added to
  the ordinary media with ratio 1:100.

• **Human lung cancer cells line:** A549 cells, non-small cell carcinoma was obtained
  from cell strain from American Type Cultural Collection (ATCC), available in the
cell bank of Tissue Culture Unit, King Fahd Medical Research Center (KAU,
  Jeddah).

• **In vitro proliferation of cells**
  1. A549 was suspended in culture medium MEM
  2. the cells were dispensed in 3X (six wells plate), $1 \times 10^5$/ml in each well
  3. each group of cells was incubated 24 hours in suitable media and apoptosis was
     induced by incubating with dissolved lyophilised PM 701 at different time
     points.

• **Infrared spectroscopy and statistical analysis:** At various time points, the cells
treated with PM 701 were washed twice (by centrifugation for three minutes at
300g). The cell pellet was kept at $-20^\circ$C before freeze drying, then the dried A549
cells dispersed in potassium bromide (KBr) to form 1% concentration discs. The
FTIR spectra from three separate samples were recorded in absorbance form using
Shimadzu FTIR-8400s spectrophotometer. For each examined sample, the spectrum
was taken as the average of three different measurements. The spectra were obtained
in the wave number range of 400 cm$^{-1}$ to 4,000 cm$^{-1}$ with an average of 20 scans to
increase the signal to noise ratio and at spectral resolution of 4 cm$^{-1}$. All the samples
were baseline corrected and normalised by using IR solution software before any
measurements. The averages of the normalised spectra were used for subsequent
analysis. Other normalisation method such as amide I for the 800 cm$^{-1}$ to 1,800 cm$^{-1}$
region have also been tested and gave negligible changes in the results. To extract
the maximum information from the IR bands, we applied Fourier deconvolution to
all spectra before using difference methods between the spectra under investigations.
Second derivative spectra were tacking in comparison studies. The parameters
studied were nucleic acid ratio, lipid-protein ratio (Mourant et al., 2003; Gaingneaux et al., 2002) and phosphate levels (Ramesh et al., 2003; Gasparri and Muzio, 2003).

3 Results and discussions

Representative IR spectra of A549 cells before and five minutes to 30 minutes and 24 hours after treatment with PM 701 are shown in Figure 1 (samples treated for 15 minute and 2 hours are not shown for clarity reasons). It is observed from the figure that there are dynamic changes in the intensities of the normalised spectra during treatment at various wave numbers. Since the absorbance is dependent on the concentration of various metabolites, alteration of any/all of the metabolites with respect to each other would manifest themselves as changes in the spectra. These several important changes in the IR spectra are related to molecular vibrations of proteins, lipids and DNA. Thus, drug affect A549 cells, cause biochemical changes in A549 cells and induces apoptosis (Khorshid and Mushref, 2006).

Figure 1 Representative IR spectra of A549 cells before and after treatment with PM701 for different times (see online version for colours)

The amide I band (which provides information about the overall protein secondary structure in the cells (Rigas and Wong, 1992; Surewicz and Mantsch, 1988; Liu and Mantsch, 2001) arising from the amide C = O stretching vibration of the peptide groups in all proteins (Figure 1) shifts from at 1,652 cm^{-1} in the control cells to 1,645 cm^{-1} in the cells treated with PM 701 for five minutes and 15 minutes. These amide I frequencies are compatible with the fact that overall protein structure in the control cells consists primarily of α-helix, whereas at the first step of apoptosis apoptotic A549 cells have a relatively high proportion of β-sheet after five minutes of treatment while they have a relatively high proportion of unordered proteins constituents after 24 hours of PM 701.
treatment. Since apoptosis involves the modification of existing proteins, as well as the synthesis of new proteins (Liu et al., 2001), we analysed the changes of protein structure upon PM 701 treatment in more detail. Figure 2 shows the mean spectra of control and PM 701 treated cells in the region of the amide I (1,600 cm$^{-1}$ to 1,700 cm$^{-1}$) and amide II (1,500 cm$^{-1}$ to 1,600 cm$^{-1}$) bands. Spectra are shown as second derivative, a method commonly used for narrowing broad IR bands for better visualisation. It is obvious from the figure that the changes in the secondary structure of the proteins are time dependant. The corresponding absorbance’s bands of amide I and amide II at the examined time of treatment are presented in Table 1. A similar shift in the amide I and amide II bands during apoptosis was reported earlier (Liu et al., 2001).

**Figure 2** Mean spectra for control and PM 701-treated A549 cells for different times up to 24 hours as second derivatives (see online version for colours)

**Table 1** The different absorbance peaks of the amide I and amide II bands as appeared in the second derivatives spectra

<table>
<thead>
<tr>
<th>Time of PM 701 treatment</th>
<th>Amide I absorbance band (cm$^{-1}$)</th>
<th>Amide II absorbance band (cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1,652s 1,684 sh</td>
<td>1,542s 1,516m</td>
</tr>
<tr>
<td>5 min</td>
<td>1,636s 1,656s 1,681 sh</td>
<td>1,542s 1,519 sh 1,572 w,sh</td>
</tr>
<tr>
<td>15 min</td>
<td>1,636s 1,656s 1,681 s.sh</td>
<td>1,542s 1,522 sh 1,572 s.sh</td>
</tr>
<tr>
<td>30 min</td>
<td>1,653s 1,685 sh</td>
<td>1,542 1,515</td>
</tr>
<tr>
<td>2 hrs</td>
<td>1,650 1,681 s.sh</td>
<td>1,544 1,513</td>
</tr>
<tr>
<td>24 hrs</td>
<td>1,660s 1,636 sh 1,681 sh</td>
<td>1,544 1,524 1,570 sh</td>
</tr>
</tbody>
</table>

Note: s = strong; w = weak; m = medium; sh = shoulder.
The effect of apoptosis on DNA can be identified from IR bands associated with vibrations of various structural groups in DNA such as the band at 972 cm\(^{-1}\) (marked as C-C/C-O), a single bond stretching vibration characteristic of the deoxyribose sugar moiety in DNA. The bands at 1,087 cm\(^{-1}\) and 1,238 cm\(^{-1}\) (marked as sPO\(_2\) and as PO\(_2\)) due to vibrations of the phosphodiester groups in DNA. The FTIR spectra in Figure 1 clearly showed decrease in the absorbance of phosphate bands corresponding to nucleic acids. Also, the band at 967 cm\(^{-1}\) accounting for symmetric stretching vibration of the phosphodiester bonds in nucleic acids showed reduction in the intensity after PM 170 treatment and disappeared up to 24 hours of drug treatment.

The absorbance ratios RIII and RIV calculated as the ratio of absorbance at 1,238 cm\(^{-1}\) over the absorbance at 2,925 cm\(^{-1}\) and the ratio of absorbance at 1,087 cm\(^{-1}\) over the absorbance at 2,925 cm\(^{-1}\) respectively are plotted versus the time of treatment in Figure 3. The figure shows that the behaviour of the absorbance ratios RIII and RIV are similarly with all time of treatment. There are dramatic decreases in the values of these ratios at five minutes of treatment. There are observed increase over the minimum value in these ratios in late time of treatment.

Figure 3  The changes in RIII (A1238 cm\(^{-1}\)/A2925 cm\(^{-1}\)), RIV (A1087 cm\(^{-1}\)/A2925 cm\(^{-1}\)) ratios with PM 701 treating time (see online version for colours)

Note: The values are average of three different measurements on each sample.

The absorbance ratio R (amide II/amide I) calculated as the ratio of absorbance at 1,542 cm\(^{-1}\) over the absorbance at 1,652 cm\(^{-1}\) and plotted versus the time of treatment is shown in Figure 4. This ratio has been indicated to represent the variation in nucleic acids (Benedetti et al., 1997). The figure shows a marked decrease in the value of this ratio for the samples treated during the first 15 minutes than that of the control sample. The minimum value was achieved after five minutes of treatment followed by an increase in the value of this ratio at 30 minutes of treatment. The amide II/amide I ratio was dramatically decreases up to 24 hours of treatment.
**Figure 4** The changes in amide II/amide I (A1,542 cm\(^{-1}\)/A1,652 cm\(^{-1}\)) ratio with PM 701 treating time (see online version for colours)

Note: The values are average of three different measurements on each sample.

**Figure 5** IR difference spectrum between control and PM 701-treated A549 cells for 24 hours (see online version for colours)
Figures 5(a) and 5(b) illustrate the difference spectrum between treated samples for five minutes, 24 hours and the control A549 cells, generated by subtracting the mean spectrum of control cells from that of PM 701 treated A549 cells. From the difference spectrum, one can clearly observe that DNA content in the cells treated with PM 701 for 24 hours has significantly decreased. These results are in good agreement with the results obtained earlier by Khorsid and Mushref (2006) who studied the effect of PM 701 as anti-cancer agent in vitro on the same type of cancer A549 cells. Live images of the cells showed that the severe lethal effects of PM701 on cancer cells started immediately after five minutes to six minutes since adding the examined substrate. They continued that the cancer cells incubated in PM 701 showed that the substrate attacks the cell’s nuclei, which is indicated by the appearance of pale ring around the nucleus of the lung cancer cell after 30 minutes of incubation. This leads to the degradation of the cells, which could not be reversed to recover the cells by re-growing the cells in ordinary media again. These results are also consistent with the finding that at the early stage of apoptosis, initiation phase is triggered by a stimulus received by the cell, this can be explained by the first five minutes to 15 minutes of PM 701 treatment where the R ratio is decreasing together with the observed band shift in the amide I and amide II (Liu and Mantsch, 2001; Liu et al., 2001). These results are also in good concistence with the results obtained by the electron microscope (EM)
Figure 6  EM showed the morphological hallmarks features that characterise apoptosis, as shown by chromatin condensation and membrane blebbing in treated cells with PM 701 (arrows) data not published (see online version for colours). (Figure 6) showed the morphological hallmarks features that characterise apoptosis, as shown by chromatin condensation and membrane blebbing in treated cells with PM 701 (arrows) (data not published). The sharp decrease in the value of (I Amide II/I Amide I) ratio at five minutes and 15 minutes of treatment and the marked decrease in RIII and RIV values at the same time of treatment may be due to condensation of chromatin during the early stage of apoptosis, which in turn decreases the path length. This is may referred to dark DNA, one would expect that less photons are transmitted within the strong absorption bands of DNA (which make up the chromatin) by particles of such relatively high optical density. Furthermore, given the number of transitions and the width of the DNA and protein bands, the entire highly condensed chromatin would be virtually opaque (Brian et al., 2005; Jamin et al., 2003). The relatively increase in the RIII and RIV values than the minimum value when the sample treated up to 24 hours with PM 701 may referred to the degradation of DNA. (Jamin et al., 2003) reported a large
increase in the DNA signals of apoptotic cells. In apoptosis, DNA is degraded into oligonucleotides with a few hundreds of base pairs that subsequently diffuse out of the nucleus. This observation suggests that the DNA, although highly condensed in the nucleus, has a low absorption, yet its degradation products in late apoptosis are detected by IR spectroscopy. The decrease in protein concentration was evident with lower intensity for the amide II band.

The lipids in the plasma membrane are composed mainly of phospholipids that determine membrane stability, fluidity and membrane enzymatic activity. Thus, monitoring lipid absorbance in the treated samples is important for detecting apoptosis. Lipid changes in the treated cells can be derived from other IR bands as (figure not shown) the dominant lipid bands in the range 2,800 cm\(^{-1}\) to 3,000 cm\(^{-1}\) originate from CH stretching vibrations of the fatty acyl chains of all cellular lipids (unsaturated lipids show = CH band at 3,012 cm\(^{-1}\), while the band at 1,740 cm\(^{-1}\) originates from lipid ester C = O groups (appeared in the mean spectra as a weak shoulder and as a strong band in the second derivative spectra. The treatment of A549 cells with PM 701 increases the CH\(_2\) content, this is indicated by the marked increase in lipid protein ratio calculated as the ratio of absorbance at 2,854 cm\(^{-1}\) over the absorbance at 1,400 cm\(^{-1}\) CH\(_2/\)CH\(_3\) and illustrated in Figure 7. It is obvious that (all) lipid bands show a tendency to increase in intensity, starting five minutes after the treatment. We also followed the overall cellular lipid changes after PM 701 treatment. The treatment of A549 cells with PM 701 increases the lipid content as most of these lipid bands show positive intensities in the difference spectrum (not shown). On the other hand, the characteristic CH\(_3\) group stretching vibrations at 2,870 cm\(^{-1}\) and 2,954 cm\(^{-1}\) show negative band intensities in the difference spectrum which may reflect structural changes of phospholipids rather than relative changes of lipid in the treated cells (Liu and Mantsch, 2001; Liu et al., 2001). It is noted that the initial value of lipid-protein ratio 2,854 cm\(^{-1}\)/1,400 cm\(^{-1}\) is greatly lower than the final value. This may be due to membrane blebbing and increases content of CH\(_2\). The PM 701 contains proteins (paper in progress), which may contact with A549 cell membrane and may form some sort of an ion channel or pore in the cell membrane thereby, enhancing the ion permeability of the membrane and destroying the cell, i.e., induce apoptosis (Matsuzaki, 1998; Shai, 1999). Zasloff (1987) suggested that the peptide could perturb membrane functions responsible for osmotic balance. The relative observed decrease after five minutes of treatment in (Figure 8) may be due to that pore formation is a transient process observable mainly during the early stage of the peptide-membrane interactions (Matsuzaki, 1998). Matsuzaki (1998) proved that the peptides translocate into the inner monolayers after forming the pores. There was a temporal relationship between the onset of apoptosis induced by PM 701 treatment and a 1.4 fold increase in the lipid-protein ratio (CH\(_2/\)CH\(_3\)). The increase in the methylene \(^1\)H NMR signal intensity could be attributable to changes in plasma membrane fluidity or could also be because of an increase in the amount of fatty acids within the lipid bilayer. Francis et al. (1996) cleared that the dominant methylene \(^1\)H NMR resonance shown in their studies do not appear without a large fraction of apoptotic cells. They suggested that the increase in the methylene in the lipid-protein ratio is associated with the onset of apoptosis and independent of the stimuli used to induce apoptotic cell death.
The dying cell (apoptotic cell) shows two characteristic spectral signatures indicative of death (Jamin et al., 2003). First, the centroids of the protein amide I and II peaks shift to lower frequency, indicating a change in the overall protein conformational states within the cells. Second, the appearance of a peak at around 1,743 cm\(^{-1}\). These observations can now be used as signatures of cell death (Hoi-Ying et al., 2000). Moreover, Liu et al. (1997) found that FTIR spectroscopy could be used for the detection of drug sensitive/resistant leukemic cells.

4 Conclusions

To conclude, we can state that PM 701 can induce apoptosis to A549 cells and FTIR spectroscopy is able to identify overall changes in the apoptotic cells in the form of dry samples. The dominant protein secondary structure shifts from \(\alpha\)-helix structure to unordered, indicating an altered protein profile in the apoptotic cells. In addition, the total cellular lipid content increases starting from the first five minutes up to 24 hours of treatment while the amount of DNA decreases dramatically during treatment with PM70 as observed in difference spectrum. It should be mentioned here that condensation of chromatins in early stage of apoptosis was detected in the first time by IR spectroscopy for dry samples after five minutes of PM 701 treatment and confirmed by the previously published paper (Khorshid and Mushref, 2006).
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