Melatonin induces apoptosis through biomolecular changes, in SK-LU-1 human lung adenocarcinoma cells

P. Plamee*, N. Weerapreeyakul*†, K. Thumanu‡, W. Tanthanuch‡ and S. Barusrux*§

*Melatonin Research Group, Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, 40002, Thailand, †Center for Research and Development of Herbal Health Product (CRD-HHP), Khon Kaen University, Khon Kaen, 40002, Thailand, ‡Synchrotron Light Research Institute (Public Organization), Nakhon Ratchasima, 30000, Thailand and §Centre for Research and Development of Medical Diagnostic Laboratories (CMDL), Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, 40002, Thailand

Received 19 May 2014; revision accepted 14 August 2014

Abstract

Objectives: Anti-cancer effects of melatonin (*N*-acetyl-5-methoxytryptamine, an indole-amine), have been widely reported, however, little has been known, regarding its mechanism(s) of action in lung cancer. Thus, we investigated its induction of apoptosis through biomolecular changes (lipid, protein and nucleic acid/DNA) in the SK-LU-1 human lung cancer cell line.

Materials and methods: We used Fourier transform infrared (FTIR) microspectroscopy, and conventional methods, to confirm changes in lipid (annexin V/PI staining for membrane alteration), protein (caspase-3/7 protein activity) and DNA (DAPI staining for DNA fragmentation).

Results: We observed from FTIR data that melatonin increased lipid content and reduced intensity of nucleic acid/DNA, confirmed by annexin V/PI and DAPI respectively. Secondary protein structure at 1656 cm^{-1} (α -helix) was reduced and peak position of β -sheet structure (1637 cm^{-1}) was shifted to lower frequency. Alteration in apoptotic proteins was demonstrated *via* caspase-3/7 activity induction.

Conclusions: High melatonin concentration exerted anti-cancer effects by changing biomolecular structure of lipids, nucleic acids and proteins, supporting its enhancement of apoptotic induction.

Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine, an indole-amine), is produced primarily by the pineal gland – then

secreted into blood circulation (1). Its well-known function is to regulate circadian rhythms (2) and it has been used in treatment of insomnia, jetlag, migraine and headaches (3–5). Several studies have reported immune system modulation (6,7), antioxidant (8,9), anti-inflammatory (10) and anti-cancer (7,11) characteristics for it, and *in vitro* anti-cancer effects on various cancer cells with possible mechanisms including induction of apoptosis (12–16).

Most chemotherapeutic agents induce cancer cell apoptosis [autonomous cell disassembly that avoids environmental inflammation (17)]; essential mediators of it including a subset of caspases (caspase-2, -3, -6, -7, -8, -9 and -10). Activation of caspases results in chromatin cleavage, DNA fragmentation, nuclear shrinkage, detachment of the intact plasma membrane from neighbouring structures and exposure of its phosphatidylserine (18). These biomolecular changes serve as markers for tracking induction of apoptotic cell death.

When infrared energy is absorbed by material it causes vibration of chemical bonds. Functional groups of molecules tend to absorb infrared radiation of the same wavelength range, depending on structure of the molecule (19). Fourier transform infrared (FTIR) microspectroscopy is the method used to detect biochemical changes in cell samples with no need for use of reagents or complicated sample handling (20,21). Recently, it has been used to study apoptosis, cell cycle staging, differentiation and proliferation of cells of a variety of lines and tissues (20,22,23). FTIR has also been adapted to differentiate between human brain tumours and normal brain tissue (24). Conventional techniques for discrimination of cell death – including annexin V, TdT-mediated deoxyuridine triphosphate nick end labelling and caspase activity – however, tend to be expensive, complicated and time-consuming (25). A simple diagnostic method that does not involve sample processing nor

Correspondence: S. Barusrux, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen 40002, Thailand. Tel./Fax: +66 43 202089; E-mail: sahapat@kku.ac.th

destruction, and can monitor biochemical changes during cell death has been needed. As cell biomolecular components may change and be detected before and during cell death, FTIR can be applied to investigate them and structures of cells (22,23).

Lung cancer is the most common malignancy that leads to death (26); in the order of 60% of them are non-small cell lung adenocarcinomas (NSCLC) (27). Cisplatin is the first-line treatment for NSCLC in the majority of countries (28) but incompletely controlled remission (27) and low survival rate (16%) for all stages (26) are a persistent challenge in its treatment. Thus, effective strategies for improving its outcome are needed (28).

Up to now, little has been known regarding mechanism(s) of action of melatonin in NSCLC; this work aims, therefore, to study its apoptotic induction through biomolecular changes in the cisplatin-sensitive NSCLC (SK-LU-1) cell line. The study was designed to test single measure FTIR microspectroscopy as a technique to track overall biochemical changes during cell death. Changes in cell composition induced by melatonin were focused on lipid, protein and nucleic acid contents and conventional methods were used to confirm changes observed.

Materials and methods

Cell line and reagents

Human lung adenocarcinoma cisplatin-sensitive cell line (SK-LU-1) was purchased from Cell Lines Service – CLS (Eppelheim, Germany). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Invitrogen Life Technologies, Barcelona, Spain) supplemented with 10% foetal bovine serum (FBS) and 100 U/ml penicillin and 100 µg/ml streptomycin, maintained at 37 °C in a 5% CO₂ atmosphere. Melatonin (GMP) was from Huanggang Saikang Pharmaceutical Co. Ltd., Hubei, China (purity confirmed at >99.4% by DSC and HPLC). Cisplatin was manufactured by Boryung Pharmaceutical (Kyunggi-do, Korea) and MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) was purchased from Amresco LLC (Solon, OH, USA). Media and reagents used in cell culture experiments were of commercial biological grade. Melatonin was freshly prepared by dissolving it in dimethyl sulphoxide (DMSO, Lab-Scan, Analytical Science, Dublin, Ireland) to reach appropriate final physiological to pharmacological concentrations (1 pM to 10 mM) and incubated for 24 h. Cisplatin 120 µM (IC₅₀) was used as positive control throughout the study. Control-untreated cells were incubated in culture medium only. Vehicle

control-treated cells received less than 2.5% v/v DMSO (in culture medium), which caused less than 10% cytotoxicity compared to control-untreated cells.

Cell viability detection by MTT assay

MTT was used for rapid determination of chemosensitivity of the living cancer cells by their converting MTT to formazan crystals; we employed the method of Denizot, with minor modifications (29). Cells were seeded at 5×10^3 in 96-well plates and left for 24 h. They were then treated with melatonin at specified concentrations. MTT solution (20 µl = 5 mg/ml) was added 21 h after treatments. MTT solution was removed and formazan was lysed with DMSO, then measured using a microplate reader (Sunrise, Tecan Group, Männedorf, Switzerland), at 555 nm (650 nm reference wavelength). Finally, %cytotoxicity was calculated in comparison to untreated or DMSO control group [$\% \text{cytotoxicity} = \{(\text{OD}_{\text{vehicle control}} - \text{OD}_{\text{sample}}) / \text{OD}_{\text{vehicle control}}\} \times 100$].

FTIR microspectroscopy for biomolecule changes

Fourier transform infrared was used to assess apoptotic cell death induced by melatonin; alteration of proteins, lipids and nucleic acids was examined following treatment with it. Treated cells were washed in 0.9% NaCl and dropped on low-e slides (MirrIR; Kevley Technologies, Chesterland, OH, USA), these were then rinsed in distilled water (30). Fixed cells were dried and kept in desiccators until used. Measurements were performed at the IR End Station, Synchrotron Light Research Institute (Public Organization), Nakhon Ratchasima, Thailand. IR data acquisition was accomplished using a Bruker Hyperion 2000 microscope (Bruker Optics Inc., Ettlingen, Germany), equipped with a nitrogen-cooled MCT (HgCdTe) detector with a 36×IR objective lens coupled to a Bruker Vertex 70 spectrophotometer. IR spectra were obtained from the reflection mode by collecting 64 scans, at 4 cm⁻¹ resolution, over a measurement range of 4000–600 cm⁻¹. Spectral acquisition and instrument control were performed using OPUS 7.0 (Bruker Optics Ltd, Ettlingen, Germany) and Unscrambler 9.7 software (CAMO, Norway). We averaged original FTIR spectra of melatonin-treated SK-LU-1 cells and untreated cells. To explore mechanisms of apoptotic induction, FTIR data from the standard chemotherapeutic drug were also evaluated. Spectra of each condition were used for further analysis including (a) SK-LU-1 treated with 1, 5 and 10 mM melatonin ($n = 140, 110$ and 112 , respectively), (b) cisplatin-treated cells ($n = 70$) and (c) untreated cells ($n = 135$). Average original FTIR spectra

of melatonin-treated SK-LU-1 cells and untreated cells are presented in Fig. 1. At high melatonin concentrations (1–10 mM) versus untreated cells, biomolecular changes were clearly observed in the lung cancer cell line treated with melatonin. Infrared spectra of biomolecules provided novel information on the lipids, proteins and nucleic acids investigated.

Detection of membrane alteration of apoptotic cells by annexin V/PI staining

Membrane phospholipid phosphatidylserines (PS) became exposed during the apoptotic process. PS is translocated from inner to outer leaflets of membranes; this then can bind to annexin V. Necrotic cells with internal and external membrane disruption is stained with propidium iodide (PI) (31) – as used in the annexin V-FITC apoptosis detection kit (cat no. 88-8005-74; eBioscience, San Diego, CA, USA). After completing melatonin treatment, cells were washed in phosphate buffer saline (PBS) and suspended in 1x binding buffer (HEPES-buffered saline supplemented with 25 mM CaCl_2). Then, in a darkened room, 5 μl annexin V-FITC and 5 μl PI were added (15 min for each dye) at room temperature. Once the incubation period was complete, cells were analysed by flow cytometry (BD FACSCanto

II, BD Biosciences, San Jose, CA, USA) within 1 h of staining.

Detection of caspase-3/7 protein activity

Caspase-3/7 activity was measured using a colorimetric method following the manufacturer's instructions (Caspase-glo 3/7 assay kit (cat no. G8090; Promega, Madison, WI, USA). Briefly, 2×10^4 SK-LU-1 cells were seeded in 96-well plates and left for 24 h, and then treated with melatonin. Next, lysate of SK-LU-1 cells was mixed with equilibrated Caspase-glo 3/7 reagents for 1 h at room temperature. Luminescence was measured using an AB-2270 luminometer (Bio-instrument ATTO, Tokyo, Japan).

DNA fragmentation/DAPI fluorescence staining

Apoptotic bodies were labelled with DAPI staining – intact nuclei being stained and observed by 4'-6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), as per Yu *et al.* with modifications (32). After treatment with melatonin, cells were washed once in PBS, fixed in methanol for 10 min, then stained with 1 $\mu\text{g/ml}$ DAPI for 30 min. Results were accompanied by complementary nuclear

Figure 1. Original spectra for Fourier transform infrared analysis of SK-LU-1 treated with melatonin at 1, 5 and 10 mM concentrations compared to cisplatin and untreated cells. Spectra from each group measured with 64 scans co-added for each individual spectrum. Untreated cells ($n = 135$), 1 mM melatonin ($n = 140$), 5 mM melatonin ($n = 110$), 10 mM melatonin ($n = 112$) and cisplatin ($n = 70$).

morphological observations, gathered using fluorescence microscopy.

Statistical analysis

Means ($\pm$ SD) of treatment groups were calculated against untreated or DMSO control group. One-way ANOVA (SPSS; SPSS Inc., Chicago, IL, USA) was used to compare melatonin- and cisplatin-treated groups with untreated or DMSO control group. Statistical significance within the 95% confidence interval was set at $P < 0.05$.

To distinguish chemical components of the samples, principal component analysis (PCA) (Unscrambler 9.7 software – CAMO, Oslo, Norway) was used to analyse individual spectra of each study group. A second derivative spectrum was performed to identify individual peaks among the complex spectra, more easily. Spectra were processed using the second derivative and the vector was normalized using the Savitzky–Golay method (third polynomial, nine smoothing points), and then normalized with extended multiplicative signal correction (EMSC) in the spectral regions from 1800 to 750 cm^{-1} . The latter normalizes for effects of differing sample thicknesses. The second derivative helps resolve neighbouring peaks and sharpens spectral features. Results were interpreted by focusing on second derivative spectra with a negative peak. Peaks in raw spectra become negative peaks on either side of the second derivative spectra. Spectra can, therefore, be used to explain differences between control and treated groups based on absolute absorbance intensity. EMSC is a transformation method used to compensate for multiplicative, additive scatter effect in data and can account for physical or chemical phenomena affecting spectra. Baseline corrections are used to adjust spectral to minimum point or by making a linear correction based on defined variables.

Principal component analysis has proven to be useful in the analysis of biospectroscopic data, providing two types of information: (a) visualization of clustering of similar spectra within data sets in score plots and (b) identification of variables (i.e. spectral bands representing various molecular groups within samples) in loading plots, thereby, explaining clustering observed in score plots. Score plots describe data structure in terms of sample patterns and more generally show sample differences or similarities. Loading plots describe data structure in terms of variable contributions and correlations. Results were interpreted by taking second derivative spectra showing the negative peak. Positive and negative loading plots had opposite correlation with negative and positive score plots respectively.

Using OPUS software, a comparative analysis was performed to determine peak integral area of normalized second derivative spectra. Amide I band (~ 1720 to $\sim 1500 \text{ cm}^{-1}$) was analysed for discrimination of its components, by curve-fitting. Correlation between integrated peak area of the biomolecules after treatment with melatonin and mode of cell death were assessed using Pearson's rho (SPSS).

Results

Melatonin increased direct cytotoxicity of SK-LU-1 cells

Melatonin from 1 μM to 10 mM was significantly cytotoxic to the SK-LU-1 cell line (7.3 ± 2.7 to $77.1 \pm 4.7\%$ compared to control, $p < 0.05$); in contrast, lower concentrations were not toxic to the lung cancer cell line (Fig. 2). Cisplatin (120 μM) caused $48.2 \pm 0.3\%$ cytotoxicity and was used as positive control throughout the current study.

Lipid changes

Lipid changes detected by FTIR microspectroscopy. Cell lipid regions are characterized by fatty acid vibrations of symmetrical and asymmetrical stretching of CH_2 - and CH_3 - groups. Fatty acid esterase was assigned to the 1800–1700 cm^{-1} spectral range, owing to stretching of the C=O contained in lipid head groups (33,34). C–H peaks of the lipid region at 2852, 2922 and 2958 cm^{-1} increased during treatment with both melatonin and cisplatin (Fig. 3a). C=O stretching of lipids at 1737 cm^{-1} also increased after treatment with melatonin, in a dose-dependent manner (Fig. 3a). Integral of absorbance between 1750 and 1715 cm^{-1} of lipid ester group (Fig. 3b) and 3000–2800 cm^{-1} of lipid contents (Fig. 3c) were observed. Lipid contents were significantly higher in SK-LU-1 cells treated with 5 and 10 mM melatonin.

Increase in lipid membrane alteration in melatonin-treated SK-LU-1 cells. Alteration in lipid detected by FTIR microspectroscopy in SK-LU-1 apoptotic cell death was confirmed using annexin V and PI dye staining, after melatonin treatment. Annexin V and PI were used to stain and thus separate apoptotic from necrotic cell death. Four populations of cells were identified by flow cytometry: (Q1) necrotic cells (PI only stained cells), (Q2) late apoptotic cells (both annexin V-FITC and PI-stained cells), (Q3) viable cells (low annexin V-FITC and PI-stained cells) and (Q4) early apoptotic cells – as indicated by high annexin V-FITC and low PI fluorescence-stained cells (Fig. 4). Apoptotic cells

Figure 2. Dose-response study on the cytotoxicity of melatonin on SK-LU-1 cells. Cell viability assessed by MTT reduction assay after 24 h treatment with melatonin (1 pM–10 mM) or cisplatin (120 μM). Experiments performed at least three times. * $P < 0.05$ versus untreated cells.

increased in number, in a dose-dependent manner with melatonin treatment, higher the dose (2.5–10 mM), greater the apoptotic effect (27.1 ± 4.7 to $44.1 \pm 1.9\%$). Melatonin at 2.5–10 mM enhanced early and late apoptosis (12.9 ± 2.7 to $26.5 \pm 1.3\%$ and 4.2 ± 0.5 to $23.7 \pm 6.2\%$, respectively). Simultaneously, high concentrations of melatonin (5 and 10 mM) increased SK-LU-1 necrotic cells (19.1 ± 3.3 and $6.8 \pm 1.6\%$, respectively). Treatment with 10 mM melatonin resulted in fewer necrotic cells than with 5 mM. This result reveals that 10 mM melatonin caused loss of intact cell membranes, leading to cell debris which cannot be detected by flow analysis. The same trends – for apoptosis or necrosis – were found at 1 and 2 mM melatonin treatment compared to untreated cells. Positive control (cisplatin 120 μM) resulted in 31.9% apoptotic cells.

Protein changes

Protein changes detected by FTIR microspectroscopy. Protein region was assigned to amide I and amide II regions (Fig. 5a). Two major band envelopes of amide I and amide II of the protein arise from specific stretching and bending vibrations of the protein backbone. Amide I (~ 1700 to 1580 cm^{-1}) band arises predominantly from C=O stretching vibration (80%) of the amide C=O group and C–N stretching vibration (20%). Amide II (~ 1540 to 1500 cm^{-1}) vibration frequency occurs from N–H bending vibration (60%) coupled with C–N stretching (40%) (33).

Intensity of α -helix secondary protein structures at $\sim 1656\text{ cm}^{-1}$ was reduced after treatment with melatonin; peak area at 1637 cm^{-1} of β -sheet protein increased and peak position was shifted to a lower frequency (1623 cm^{-1}) (Fig. 5a). Secondary structures of protein absorption in the 1700 – 1500 cm^{-1} spectral range were

also observed. Second derivative spectra of the amide I band of each treatment revealed significantly different patterns. Amide I band reflects a secondary structure of protein, containing four components (34): (a) at $\sim 1615\text{ cm}^{-1}$, $\sim 1629\text{ cm}^{-1}$ and $\sim 1642\text{ cm}^{-1}$ assigned to the β -sheet; (b) at $\sim 1656\text{ cm}^{-1}$ associated with presence of an α -helix secondary structure; (c) at $\sim 1671\text{ cm}^{-1}$ assigned to a β -turn in the peptide secondary structure; and (d) at $\sim 1685\text{ cm}^{-1}$ associated with an anti-parallel β -sheet.

Integral of absorbance between 1700 and 1600 cm^{-1} was used as an estimate of total amide I. Amide I contents were reduced by treatment with melatonin, but increased by treatment with cisplatin ($P < 0.05$, Fig. 5b). Overall structural change of amide I protein did not, however, explain the mechanism of how melatonin concentration affected SK-LU-1 cells. We, therefore, performed further curve-fitting analyses (Table 1) to ascertain relative content (percentage) of secondary structures (α -helix, β -sheet and β -turn) of protein.

Amide I contents were reduced by treatment with melatonin ($P < 0.05$, Fig. 5b), but these increased after treatment with cisplatin (Fig. 5b). It is, however, quite difficult to compare data from melatonin at 5 mM and cisplatin, from overall changes in amide I protein (1700 – 1600 cm^{-1}) data. Curve-fitting was, therefore, performed around the amide I band to try to discern details of the secondary structure of the proteins (i.e. α -helix, β -sheet and of β -turn) (Table 1).

Relative integral area of the β -sheet of melatonin-treated cells was significantly elevated compared to untreated cells; in contrast, relative integral area of the α -helix declined. Integral areas of the β -sheet and α -helix were significantly changed in melatonin-treated cells (10 mM) versus the untreated ones ($P < 0.05$, Table 1).

Figure 3. Average second-derivative spectra for primary spectra and relative integral area (%) from Fourier transform infrared analysis of SK-LU-1 treated with melatonin at 1, 5 and 10 mM compared to cisplatin and untreated cells. (a) Average second-derivative spectra after 9 points of smoothing and normalized with extended multiplicative signal correction over the range of lipid (3000–2800 cm^{-1}). Alteration of biomolecular structures and amount observed by peak position and height. Relative integral area (%) of (b) lipid ester group (1750–1715 cm^{-1}) and (c) lipid region (3000–2800 cm^{-1}) calculated in normalized second-derivative infrared spectra using OPUS software. * $P < 0.05$ or # $P < 0.001$.

Increase in caspase-3/7 protein activation in melatonin-treated SK-LU-1 cells. Previously, melatonin has been shown to induce apoptosis in HepG₂ hepatocellular carcinoma, HL-60 human myeloid and MCF-7 breast adenocarcinoma cell lines *via* caspase activation (16,35–37). Alteration in protein, detected by FTIR microspectroscopy in SK-LU-1 apoptotic cell death, was confirmed by determination of caspase enzyme activity. Melatonin induction of apoptosis through caspase-3/7 in SK-LU-1 cells, was determined with a variety (1–10 mM) of concentrations; at 5 and 10 mM it increased caspase-3/7 protein activity (2.1 ± 0.3 and 5.1 ± 0.7

ratio compared to control, $P < 0.001$) (Fig. 6). Positive controls (treated with 120 μM cisplatin) also had elevated caspase-3/7 activity (6.0 ± 1.1 ratio compared to control). These results confirm that high concentrations (5 and 10 mM) of melatonin induced SK-LU-1 cell apoptosis *via* executioner caspase-3/7 protein activation.

DNA changes

Nucleic acid changes detected by FTIR microspectroscopy. Vibration of carbohydrates and phosphates associated with nucleic acids, characterizes the nucleic acid

Figure 4. Annexin V- and PI-stained cells present after detection by flow analysis. Flow cytogram and mode of cell death after treatment with melatonin or cisplatin (120 μ M). (a) Untreated group, (b) melatonin-treated group at 1 mM, (c) 2 mM, (d) 2.5 mM, (e) 5 mM and (f) 10 mM. Data represent percentage of living and mode of cell death (g). Experiments performed in triplicate. * $P < 0.05$, # $P < 0.001$ versus untreated cells.

region. We investigated spectra of asymmetrical and symmetrical phosphodiester vibrations of nucleic acids (1240 cm^{-1} and 1085 cm^{-1}) and their RNA ribose phosphate main chain and C–C bonds ($970\text{--}966\text{ cm}^{-1}$) (38–40). Major peak areas at 1086 cm^{-1} phosphate stretching modes were reduced by melatonin (Fig. 7a); this originated from nucleic acid phosphodiester groups. The PO_2^- group of nucleic acids at $\sim 1240\text{ cm}^{-1}$ wavelength was shifted to a lower value (1238 and 1234 cm^{-1}) after treatment with melatonin at 5 and 10 mM respectively (Fig. 7a).

The prominent peak of cell nucleic acids at 968 cm^{-1} was similar; whether cells were treated with melatonin or not (untreated). Integral of absorbance between 1200 and 900 cm^{-1} was used as an estimate of total nucleic acid content, which decreased after being

treated with 5 and 10 mM melatonin ($P < 0.05$, Fig. 7b).

Increase in DNA fragmentation in melatonin-treated SK-LU-1 cells. Alteration in DNA, detected by FTIR microspectroscopy in SK-LU-1 apoptotic cell death, was confirmed using DAPI dye staining after melatonin treatment. DAPI is commonly used to reveal apoptotic bodies as it distinguishes between nuclear DNA condensation or fragmented cells, and normal cells. Apoptotic bodies induced by melatonin were detected and counted (Fig. 8). Melatonin at concentrations of 5 and 10 mM significantly induced apoptosis of SK-LU-1 cells (43.9 ± 5.4 and $66.8 \pm 5.0\%$) compared to control, $P < 0.05$. At concentrations of 1, 2 and 2.5 mM melatonin, apoptotic bodies were not significantly greater in

Figure 5. Average second-derivative spectra for primary spectra and relative integral area (%) from Fourier transform infrared analysis of SK-LU-1 treated with melatonin at 1, 5 and 10 mM compared to cisplatin and untreated cells. (a) Average second-derivative spectra after 9 points of smoothing and normalized with extended multiplicative signal correction over the range of protein region (1700–1500 cm⁻¹). Alteration of biomolecular structures and amount observed by peak position and height. Relative integral area (%) of (b) amide I region (1700–1600 cm⁻¹) calculated in normalized second-derivative infrared spectra using OPUS software. * $P < 0.05$ or # $P < 0.001$.

Table 1. Spectral data and vibrational assignments of amide I proteins bands of untreated SK-LU-1 cells compared to melatonin- and cisplatin-treated cells

Assignment	Untreated		Melatonin 1 mM		Melatonin 5 mM		Melatonin 10 mM		Cisplatin	
	Position	%RIA	Position	%RIA	Position	%RIA	Position	%RIA	Position	%RIA
Anti-parallel β -sheet	1685.8	6.5	1686.8	4.7	1684.2	7.7	1683.7	8.0	1683.4	6.6
β -turn	1671.7	16.7	1672.4	18.6	1669.9	15.0	1669.1	14.1	1669.1	14.1
α -helix	1656.4	32.3	1656.3	35.2	1655.4	28.2	1654.9	24.0 ^a	1654.0	29.4
β -sheet	1615.8–1642.2	44.5	1616.1–1641.9	41.4	1611.9–1640.3	49.1	1609.5–1639.7	53.9 ^a	1608.4–1638.7	49.9

RIA, relative integral peak area.

^aAll treatments were different to untreated cells $P < 0.05$.

number than in the untreated group (5.8 ± 1.1 to $11.6 \pm 3.4\%$). Cisplatin at $120 \mu\text{M}$ also increased numbers of apoptotic bodies by $66.8 \pm 5.0\%$ compared to control. These results indicate that at higher concentrations, melatonin induced SK-LU-1 cell apoptosis, whereas lower concentrations inhibited cancer cell proliferation.

Distinguishing effects of melatonin on SK-LU-1 cells using PCA by FTIR microspectroscopy

Distributions of each data set were analysed by PCA from second derivative spectra and presented as first and

second principal components (PCs) (Fig. 9a). Two-dimensional score plots – for PC1 and PC2 comprising PCA modelling – were separated into groups of SK-LU-1: (a) treated with melatonin (b) treated with cisplatin and (c) untreated cells (Fig. 9a). From PC1, untreated SK-LU-1 cells and 1 mM melatonin-treated cells were separated from the 5 and 10 mM melatonin-treated cells and cisplatin, for a total of 56%. From PC2, 5 and 10 mM melatonin treatments were separated from the cisplatin-treated group, for a total of 27%. Regions having a major effect on discrimination of each clustering (from PCA score plots) are represented in PCA loading plots (Fig. 9b).

Figure 6. Activity of caspase-3/7 in SK-LU-1 cells evaluated by caspase-Glo 3/7 assay. Caspase-3/7 activity assessed after 24 h treatment with melatonin (1–10 mM) or cisplatin (120 μ M). Experiments performed in duplicate. [#] $P < 0.001$ versus untreated cells.

Melatonin-treated cells (1 mM) were categorized in the same group as untreated SK-LU-1 cells, suggesting that the lower concentration did not induce biomolecular changes. This clustering is perhaps the result of positive loading plots of amide I from proteins at 1658 cm^{-1} (assigned to the α -helix structure), the highest loading for PC1 that discriminated negative score of spectra of untreated SK-LU-1 cells from positive score of spectra of melatonin treatment at 5 and 10 mM, and cisplatin-treated cells (Fig. 9a,b). These data indicate that the respective α -helix protein structural peak (1658 cm^{-1})

of untreated cells was higher than that of spectra of melatonin-treated cells.

Significant separation was observed after 5 and 10 mM treatment of both melatonin and cisplatin, probably because these spectra had the highest negative value for PC1 loading at 1624 cm^{-1} (β -sheet protein structure), 2958, 2922 and 2852 cm^{-1} (C–H stretching of lipids) (Fig. 9b). These findings reflect a significant respective increase in lipid and β -sheet protein content after melatonin and cisplatin treatment versus untreated cells.

Moreover, spectra of the nucleic acids region (phosphodiester groups and C–O/C–C stretching of deoxyribose–ribose vibrations from the nucleic acids at ~1089 to ~966 cm^{-1}) had a significant positive value for PC1 loading, suggesting that the untreated group had higher nucleic content than did cells treated with high concentration melatonin (Fig. 9a,b).

The cisplatin-treated group was 27% separated from 5 and 10 mM melatonin treatment according to PC2 (Fig. 9a). The region most affecting discrimination of this group was the amide I band protein structure at 1649 cm^{-1} , which was heavily loaded for PC2 and helped to differentiate negative score of cisplatin-treated cells from positive score of spectra of 5 and 10 mM melatonin treatment. The implication is that SK-LU-1 cells responded to cisplatin and melatonin differently, inducing different protein expression patterns. Consequently, 5 and 10 mM melatonin-treated groups had the highest

Figure 7. Average second-derivative spectra for primary spectra and relative integral area (%) from Fourier transform infrared analysis of SK-LU-1 treated with melatonin at 1, 5 and 10 mM compared to cisplatin and untreated cells. (a) Average second-derivative spectra after 9 points of smoothing and normalized with extended multiplicative signal correction over the range of nucleic acid region (1300–900 cm^{-1}). Alteration of biomolecular structures and amount observed by peak position and height. Relative integral area (%) of (b) nucleic acid region (1200–900 cm^{-1}) calculated in normalized second-derivative infrared spectra using OPUS software. ^{*} $P < 0.05$ or [#] $P < 0.001$.

Figure 8. Detection of apoptotic bodies by DAPI staining and counting them under inverted fluorescence microscopy (40 $\times$ magnification). (a) Untreated group (b) melatonin-treated group at 1 mM, (c) 2 mM, (d) 2.5 mM, (e) 5 mM and (f) 10 mM. Data represent a percentage of apoptotic bodies compared to whole population (g) of melatonin- or cisplatin (120 μ M)-treated groups. Triplicate sampling performed for each group. * $P < 0.05$ versus untreated cells.

negative value for PC2 loading at 1662 cm^{-1} (β -sheet protein structure), 2958, 2923 and 2852 cm^{-1} (C–H stretching of lipids) (Fig. 9b).

Based on PCA analysis, intensity of FTIR bands of SK-LU-1 cells treated with melatonin and cisplatin were differentiated from untreated cells by biochemical changes in their lipids, proteins and nucleic acids. Increasing β -sheet intensity and reducing amount of α -helix structure suggest alteration in secondary structure of the protein band. These changes may be associated with pro-apoptotic proteins which induce apoptosis (41).

Correlation between biomolecular changes and mode of cell death

Correlation of the mode of SK-LU-1 cell death and integrated peak area of the biomolecules after treatment with melatonin are presented in Table 2. There was correlation between late apoptosis and nucleic acid and fatty acid ester content ($P < 0.05$), whereas early apoptosis was associated with amide I content ($P < 0.05$). These

results suggest that apoptotic cell death was related to reducing nucleic acids and increasing lipids.

Discussion

This piece of our recent research demonstrated anti-lung cancer activity of melatonin in a dose-dependent manner; SK-LU-1 cell growth was inhibited by 1 and 2 mM of melatonin. Meanwhile, cytotoxic effect of melatonin ranged between doses 2.5 and 10 mM. In a previous study, activity of melatonin was detected in A549 cells – a non-small cell lung cancer and cisplatin non-sensitive cell line. No significant alterations in A549 cell number or apoptotic cell death occurred after treatment with 0.1 and 1 mM melatonin. Melatonin, however, synergized with doxorubicin and increased doxorubicin-induced cytotoxicity to A549 cells (42). 1 mM concentration of melatonin inhibited A549 cell growth, by an anti-proliferative mechanism, without alteration of antioxidant capacity of the cells (43). Anti-cancer effect of melatonin on the HepG₂ hepatocellular carcinoma cell line was assessed at concentrations between 100 μ M and

Figure 9. Principal component analysis score plots (a) and loading plots (b) from 2nd derivative spectra of SK-LU-1 treated with melatonin at 1, 5 and 10 mM concentrations compared to cisplatin and untreated cells. Biochemical changes from each group classified as per their PC1 versus PC2 score plot. PC1 and PC2 explained 56% and 27% of the total variance, respectively. Spectra derived using second-derivative processing with the entire biochemical cell fingerprint region (1800–900 cm^{-1}) and (3000–2800 cm^{-1}).

10 mM. Both a time- and dose-dependent cytotoxic response to melatonin were observed (35). The current study, therefore, focused on apoptotic induction using pharmacological levels of melatonin (1–10 mM) on the SK-LU-1 cells. Monitoring changes in biomolecular contents of cells was performed to ascertain apoptotic induction effects of melatonin on SK-LU-1 cells.

Significantly, greater apoptosis occurred at high concentrations of melatonin (than lower ones), as observed using conventional methods – annexin V, DAPI staining and caspase activity. These techniques, however, tend to be complicated and time-consuming (25). A simple diagnostic method that does not involve sample processing nor destruction, that can monitor several biochemical changes during cell death, has been needed. As cell bio-

Table 2. Correlation coefficient for modes of cell death and relative integrated area (%) of biomolecules from melatonin-treated SK-LU-1 cells

Correlation coefficient for mode of cell death and biomolecules	Apoptosis	Early apoptosis	Late apoptosis	Necrosis
Nucleic acid	−0.984	−0.829	−1.000 ^a	−0.421
Amide I	0.908	1.000 ^a	0.836	−0.172
Lipid ester	0.974	0.800	0.997 ^a	0.466
Lipid	0.781	0.460	0.865	0.804

^aCorrelation significant at 0.05 level (two-tailed).

molecular components and structural changes can be detected before and during cell death by FTIR (22,23), we therefore, applied this technique for detection of cell

alteration after SK-LU-1 cells were treated and had undergone apoptosis.

Our study supports use of the FTIR technique for detection of biomolecular changes in cancer cells during apoptotic induction; this was demonstrated by using SK-LU-1 cells after being treated with melatonin. Infra-red spectra of macromolecules can provide information on cells (the fingerprint region) such as pattern of lipids, proteins and nucleic acids (33,38,39). Our work demonstrates that spectral changes from FTIR data can be used to evaluate biochemical changes due to treatment and effects of medication on cells, at the biomolecular level, which can then be used to classify mode of cell death.

Our results are in agreement with a previous report on the erythroleukaemic K562 cell line treated with imatinib mesylate; the lipid ester band integral area (1723 cm^{-1} and 1756 cm^{-1}) in apoptotic cells was greater than that of viable cells (44). Liu and Mantsch reported an increase in cell lipid, based on the observation of a lipid ester band at 1740 cm^{-1} and between 2800 and 3000 cm^{-1} of C–H stretching in a T-lymphoblastic cell line (CEM) treated with etoposide (45). Studies on human leukaemia cell lines support the trend showing that apoptotic induction correlates with accrual of lipid content (20,46,47). Presence of lipid esters can indicate several different kinds of metabolic alteration (21,48) including the apoptotic process. Many specific markers can be observed during apoptosis, from cell changes such as membrane fluidity, ionic charge, membrane proteins and lipid structure. A well-known marker of apoptosis is the presence of phosphatidylserine on the outer leaflet of plasma membranes (49). Accordingly, the increased of peak FTIR spectra – between 3000 cm^{-1} and 2800 cm^{-1} from the plasma membrane phosphatidylserine – is called the ‘marker band of the apoptosis’ (46). By comparison, membrane changes in necrotic cells – due to swelling of organelles and loss of plasma membrane integrity – result in reduction of lipid membrane. Lipid spectra alone are, however, inadequate for discrimination between apoptosis and necrosis (20).

Relative integral area of β -sheet and α -helix of melatonin-treated SK-LU-1 cells was respectively elevated and reduced compared to untreated cells. This result may be due to (a) protein α -helix structural shift to β -pleated sheet in apoptotic cells as previously reported in melphalan-treated U937 leukaemic (47) and boric acid-treated SK-MEL28 cells (30); (b) anti-proliferative effect so that signs of apoptosis would be associated with broadened amide I peak, and shift to a lower wavelength (from 1655 to 1649 cm^{-1}) as reported in boric acid-treated SK-MEL28 cells (30); and (c) changing confor-

mation of caspases detected as changes in parallel β -sheets (41).

It is well recognised that caspases play an important role in the apoptotic process (18). Caspases-3/7 – proteolytic enzymes involved in apoptotic activity was increased in melatonin-treated SK-LU-1 cells. Previous studies of melatonin on various cell lines such as of human leukaemia, colon cancer, breast cancer, hepatocellular carcinoma and pancreatic carcinoma, support the observation of apoptotic induction of melatonin by caspase activation (12,14–16,35,50). Conformation of caspases, as visualized by X-ray spectroscopy, mostly consists of parallel β -pleated sheets (41). Accordingly, our observation of shifting peak position of β -sheets confirms the conformation change of caspases, and increasing β -sheet peaks seems to confirm the induction of apoptosis through caspases activity.

Nucleic acid content of SK-LU-1 cells was shown to be reduced by melatonin treatment, in the current study. These findings support previous reports which indicate that nucleic acid content declines after apoptosis yet is induced by standard chemotherapeutic drugs, in human leukaemia cell lines CEM and U937 (20,45,47). Nucleic acid/protein ratio observed by FTIR data was significantly reduced after SK-MEL28 human skin melanoma cells were treated with boric acid (30). Apoptotic induction by melatonin was by caspase-3/7 activation resulting in DNA fragmentation as observed by DAPI staining. DNA condensation of apoptosis leads to DNA ‘radiation opacity’ and consequently less IR absorption (non-Beer-Lambert absorption); this phenomenon was observed more often in apoptosis than necrosis. Here, DNA opacity was found to be inversely correlated to integral area of spectra at 1200 – 900 cm^{-1} and number of apoptotic cells; also observed more often in apoptosis than necrosis (20,51).

Fourier transform infrared microspectroscopy proved to be a useful technique for assessing effects of melatonin on differentiating the mode of cell death. Apoptosis correlated to reduced nucleic acids and increase in lipids, supporting previous studies on human leukaemia cell lines (20,46,47), which proposed the use of FTIR for detection of apoptotic cell death.

In summary, we found that melatonin exerted anti-cancer activity on our lung cancer cells by direct cytotoxicity as well as by induction of apoptosis by caspase activation. These were investigated by observing changes in the biomolecular structure of lipids, nucleic acids and proteins, by FTIR microspectroscopy. FTIR spectra revealed reduction in nucleic acid and α -helix protein structures, augmentation of lipid content and change in conformation of β -sheet protein structure, that seemed attributable to the apoptotic process. This work

introduces FTIR microspectroscopy as an alternative for observing overall biochemical changes during apoptosis, with a single measurement. The precise mechanism of anti-cancer action by melatonin requires further investigation before conducting trials on its use in cancer patients.

Acknowledgements

We are grateful for financial support from Khon Kaen University through the Incubation Researcher Project, Integrated Multidisciplinary Research Cluster under the National Research University Project of Thailand (NRU-KKU), the Melatonin Research Group (MRG). The authors thank Mr. Bryan Roderick Hamman and Mrs. Janice Loewen-Hamman for assisting in English-language presentation of the manuscript.

Conflict of interest

The authors declare that there are no conflicts of interest.

References

- Stehle JH, Saade A, Rawashdeh O, Ackermann K, Jilg A, Sebesteny T *et al.* (2011) A survey of molecular details in the human pineal gland in the light of phylogeny, structure, function and chronobiological diseases. *J. Pineal Res.* **51**, 17–43.
- Reiter RJ, Rosales-Corral S, Coto-Montes A, Boga JA, Tan DX, Davis JM *et al.* (2011) The photoperiod, circadian regulation and chronodisruption: the requisite interplay between the suprachiasmatic nuclei and the pineal and gut melatonin. *J. Physiol. Pharmacol.* **62**, 269–274.
- Hardeland R, Madrid JA, Tan DX, Reiter RJ (2012) Melatonin, the circadian multioscillator system and health: the need for detailed analyses of peripheral melatonin signaling. *J. Pineal Res.* **52**, 139–166.
- Reiter RJ (2003) Melatonin: clinical relevance. *Best Pract. Res. Clin. Endocrinol. Metab.* **17**, 273–285.
- Wilhelmsen M, Amirian I, Reiter RJ, Rosenberg J, Gogenur I (2011) Analgesic effects of melatonin: a review of current evidence from experimental and clinical studies. *J. Pineal Res.* **51**, 270–277.
- Liu F, Ng TB, Fung MC (2001) Pineal indoles stimulate the gene expression of immunomodulating cytokines. *J. Neural. Transm.* **108**, 397–405.
- Plaimée P, Khamphio M, Weerapreeyakul N, Barusrux S, Johns NP (2014) Immunomodulatory effect of melatonin in SK-LU-1 human lung adenocarcinoma cells co-cultured with peripheral blood mononuclear cells. *Cell Prolif.* **47**, 406–415. doi: 10.1111/cpr.12119.
- Korkmaz A, Reiter RJ, Topal T, Manchester LC, Oter S, Tan DX (2009) Melatonin: an established antioxidant worthy of use in clinical trials. *Mol. Med.* **15**, 43–50.
- El-Sokkary GH (2008) Melatonin and vitamin C administration ameliorate diazepam-induced oxidative stress and cell proliferation in the liver of rats. *Cell Prolif.* **41**, 168–176.
- Mauriz JL, Collado PS, Veneroso C, Reiter RJ, Gonzalez-Gallego J (2012) A review of the molecular aspects of melatonin's anti-inflammatory actions: recent insights and new perspectives. *J. Pineal Res.* **54**, 1–14.
- Cutando A, Lopez-Valverde A, Arias-Santiago S, Vicente J, Diego RG (2012) Role of melatonin in cancer treatment. *Anticancer Res.* **32**, 2747–2753.
- Sanchez-Hidalgo M, Lee M, de la Lastra CA, Guerrero JM, Packham G (2012) Melatonin inhibits cell proliferation and induces caspase activation and apoptosis in human malignant lymphoid cell lines. *J. Pineal Res.* **53**, 366–373.
- Xu C, Wu A, Zhu H, Fang H, Xu L, Ye J *et al.* (2013) Melatonin is involved in the apoptosis and necrosis of pancreatic cancer cell line SW-1990 via modulating of Bcl-2/Bax balance. *Biomed. Pharmacother.* **67**, 133–139.
- Wang J, Guo W, Chen W, Yu W, Tian Y, Fu L *et al.* (2012) Melatonin potentiates the antiproliferative and pro-apoptotic effects of ursolic acid in colon cancer cells by modulating multiple signaling pathways. *J. Pineal Res.* **54**, 406–416.
- Wang J, Xiao X, Zhang Y, Shi D, Chen W, Fu L *et al.* (2012) Simultaneous modulation of COX-2, p300, Akt, and Apaf-1 signaling by melatonin to inhibit proliferation and induce apoptosis in breast cancer cells. *J. Pineal Res.* **53**, 77–90.
- Carbajo-Pescador S, Steinmetz C, Kashyap A, Lorenz S, Mauriz JL, Heise M *et al.* (2013) Melatonin induces transcriptional regulation of Bim by FoxO3a in HepG2 cells. *Br. J. Cancer* **108**, 442–449.
- Lowe SW, Lin AW (2000) Apoptosis in cancer. *Carcinogenesis* **21**, 485–495.
- Fink SL, Cookson BT (2005) Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells. *Infect. Immun.* **73**, 1907–1916.
- Smith BC (1996) Fundamentals of Fourier Transform Infrared Spectroscopy. Boca Raton, FL: CRC Press, Inc. 202pp.
- Zelig U, Kapelushnik J, Moreh R, Mordechai S, Nathan I (2009) Diagnosis of cell death by means of infrared spectroscopy. *Biophys. J.* **97**, 2107–2114.
- Mourant JR, Yamada YR, Carpenter S, Dominique LR, Freyer JP (2003) FTIR spectroscopy demonstrates biochemical differences in mammalian cell cultures at different growth stages. *Biophys. J.* **85**, 1938–1947.
- Berthomieu C, Hienerwadel R (2009) Fourier transform infrared (FTIR) spectroscopy. *Photosynth. Res.* **101**, 157–170.
- Bellisola G, Sorio C (2012) Infrared spectroscopy and microscopy in cancer research and diagnosis. *Am. J. Cancer Res.* **2**, 1–21.
- Krafft C, Sobottka SB, Schackert G, Salzer R (2004) Analysis of human brain tissue, brain tumors and tumor cells by infrared spectroscopic mapping. *Analyst* **129**, 921–925.
- Sgonc R, Gruber J (1998) Apoptosis detection: an overview. *Exp. Gerontol.* **33**, 525–533.
- Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D (2011) Global cancer statistics. *CA Cancer J. Clin.* **61**, 69–90.
- Ashworth A, Rodrigues G, Boldt G, Palma D (2013) Is there an oligometastatic state in non-small cell lung cancer? A systematic review of the literature. *Lung Cancer* **20**, 379–376.
- Stahel R, Peters S, Baas P, Brambilla E, Cappuzzo F, De Ruyscher D *et al.* (2013) Strategies for improving outcomes in NSCLC: a look to the future. *Lung Cancer* **8**, 390–395.
- Yeap SK, Alitheen NB, Ali AM, Omar AR, Raha AR, Suraini AA *et al.* (2007) Effect of Rhabdophora korthalsii methanol extract on human peripheral blood mononuclear cell (PBMC) proliferation and cytolytic activity toward HepG2. *J. Ethnopharmacol.* **114**, 406–411.

- 30 Acerbo AS, Miller LM (2009) Assessment of the chemical changes induced in human melanoma cells by boric acid treatment using infrared imaging. *Analyst* **134**, 1669–1674.
- 31 Bishayee K, Ghosh S, Mukherjee A, Sadhukhan R, Mondal J, Khuda-Bukhsh AR (2013) Quercetin induces cytochrome-c release and ROS accumulation to promote apoptosis and arrest the cell cycle in G2/M, in cervical carcinoma: signal cascade and drug-DNA interaction. *Cell Prolif.* **46**, 153–163.
- 32 Yu R, Mandlekar S, Harvey KJ, Ucker DS, Kong AN (1998) Chemopreventive isothiocyanates induce apoptosis and caspase-3-like protease activity. *Cancer Res.* **58**, 402–408.
- 33 Movasaghi Z, Rehman S, ur Rehman DI (2008) Fourier transform infrared (FTIR) spectroscopy of biological tissues. *Appl. Spectrosc. Rev.* **43**, 134–179.
- 34 Petitbois C, Délris G (2006) Chemical mapping of tumor progression by FT-IR imaging: towards molecular histopathology. *Trends Biotechnol.* **24**, 455–462.
- 35 Martin-Renedo J, Mauriz JL, Jorquera F, Ruiz-Andres O, Gonzalez P, Gonzalez-Gallego J (2008) Melatonin induces cell cycle arrest and apoptosis in hepatocarcinoma HepG2 cell line. *J. Pineal Res.* **45**, 532–540.
- 36 Bejarano I, Redondo PC, Espino J, Rosado JA, Paredes SD, Barriga C *et al.* (2009) Melatonin induces mitochondrial-mediated apoptosis in human myeloid HL-60 cells. *J. Pineal Res.* **46**, 392–400.
- 37 Cucina A, Proietti S, D'Anselmi F, Coluccia P, Dinicola S, Frati L *et al.* (2009) Evidence for a biphasic apoptotic pathway induced by melatonin in MCF-7 breast cancer cells. *J. Pineal Res.* **46**, 172–180.
- 38 Lasch P, Boese M, Pacifico A, Diem M (2002) FT-IR spectroscopic investigations of single cells on the subcellular level. *Vib. Spectrosc.* **28**, 147–157.
- 39 Norris KP (1959) Infra-red spectroscopy and its application to microbiology. *J. Hyg. (Lond)* **57**, 326–345.
- 40 Ye D, Tanthanuch W, Thumanu K, Sangmalee A, Parnpai R, Heraud P (2012) Discrimination of functional hepatocytes derived from mesenchymal stem cells using FTIR microspectroscopy. *Analyst* **137**, 4774–4784.
- 41 Lavrik IN, Golks A, Krammer PH (2005) Caspases: pharmacological manipulation of cell death. *J. Clin. Invest.* **115**, 2665–2672.
- 42 Fic M, Podhorska-Okolow M, Dziegiel P, Gebarowska E, Wysocka T, Drag-Zalesinska M *et al.* (2007) Effect of melatonin on cytotoxicity of doxorubicin toward selected cell lines (human keratinocytes, lung cancer cell line A-549, laryngeal cancer cell line Hep-2). *In Vivo* **21**, 513–518.
- 43 Sanchez-Sanchez AM, Martin V, Garcia-Santos G, Rodriguez-Blanco J, Casado-Zapico S, Suarez-Garnacho S *et al.* (2011) Intracellular redox state as determinant for melatonin antiproliferative vs cytotoxic effects in cancer cells. *Free Radic. Res.* **45**, 1333–1341.
- 44 Bellisola G, Della Peruta M, Vezzalini M, Moratti E, Vaccari L, Birarda G *et al.* (2010) Tracking infrared signatures of drugs in cancer cells by Fourier transform microspectroscopy. *Analyst* **135**, 3077–3086.
- 45 Liu KZ, Mantsch HH (2001) Apoptosis-induced structural changes in leukemia cells identified by IR spectroscopy. *J. Mol. Struct.* **565–566**, 299–304.
- 46 Gaudenzi S, Pozzi D, Toro P, Silvestri I, Morrone S, Castellano AC (2004) Cell apoptosis specific marker found by Fourier transform infrared spectroscopy. *Spectroscopy* **18**, 415–422.
- 47 Machana S, Weerapreeyakul N, Barusrux S, Thumanu K, Tanthanuch W (2012) FTIR microspectroscopy discriminates anticancer action on human leukemic cells by extracts of *Pinus kesiya*; *Cratogeomys formosus* ssp. *pruniflorum* and melphalan. *Talanta* **93**, 371–382.
- 48 Holman HY, Martin MC, Blakely EA, Bjornstad K, McKinney WR (2000) IR spectroscopic characteristics of cell cycle and cell death probed by synchrotron radiation based Fourier transform IR spectromicroscopy. *Biopolymers* **57**, 329–335.
- 49 Koopman G, Reutelingsperger CP, Kuijten GA, Keehnen RM, Pals ST, van Oers MH (1994) Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood* **84**, 1415–1420.
- 50 Leja-Szpak A, Jaworek J, Pierzchalski P, Reiter RJ (2010) Melatonin induces pro-apoptotic signaling pathway in human pancreatic carcinoma cells (PANC-1). *J. Pineal Res.* **49**, 248–255.
- 51 Gasparri F, Muzio M (2003) Monitoring of apoptosis of HL60 cells by Fourier-transform infrared spectroscopy. *Biochem. J.* **369**, 239–248.