Anticancer Effects of Thymoquinone, Caffeic Acid Phenethyl Ester and Resveratrol on A549 Non-small Cell Lung Cancer Cells Exposed to Benzo(a)pyrene

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Abstract

Background: Phytochemical compounds are emerging as a new generation of anticancer agents with limited toxicity in cancer patients. The purpose of this study was to investigate the potential effects of thymoquinone, caffeic acid phenylester (CAPE) and resveratrol on inflammatory markers, oxidative stress parameters, mRNA expression levels of proteins and survival of lung cancer cells in vitro. Materials and Methods: The A549 cell line was treated with benzo(a)pyrene, benzo(a)pyrene plus caffeic acid phenylester (CAPE), benzo(a)pyrene plus resveratrol (RES), and benzo(a)pyrene plus thymoquinone (TQ). Inflammatory markers, oxidative stress parameters, mRNA expression levels of apoptotic and anti-apoptotic proteins and cell viability were assessed and results were compared among study groups. Results: TQ treatment up-regulated Bax and down-regulated Bcl2 proteins and increased the Bax/Bcl2 ratio. CAPE and TQ also up-regulated Bax expression. RES and TQ down-regulated the expression of Bcl-2. All three agents decreased the expression of cyclin D and increased the expression of p21. However, the most significant up-regulation of p21 expression was observed in TQ treated cells. CAPE, RES and TQ up-regulated TRAIL receptor 1 and 2 expression. RES and TQ down-regulated the expression of NF-kappa B and IKK1. Viability of CAPE, RES and TQ treated cells was found to be significantly decreased when compared with the control group (p=0.004). Conclusions: Our results revealed up-regulation of the key upstream signaling factors, which ultimately cause increase in their regulatory p53 levels affecting the induction of G2/M cell cycle arrest and apoptosis. Overall these results provide mechanistic insights for understanding the molecular basis and utility of the anti-tumor activity of TQ, RES and CAPE.

Keywords: A 549 cell line - adenocarcinoma - benzo(a)pyrene - caffeic acid phenylester - resveratrol - thymoquinone

Introduction

Throughout the world, 1.6 million new cases of lung cancer recorded and 1.4 million people died from the disease, which represented 18% of all cancer deaths in 2008 (Jemal et al., 2011). In 2010, the number of deceased subjects from lung cancer increased to 1.5 million, representing 19% of all cancer deaths in all around the world (Lozano et al., 2012).

Non-small-cell lung cancer (~80%) is the most frequent type of lung cancer and with chemotherapy only limited efficacy and short survival times can be provided (Devesa et al., 2005; Goldstraw et al., 2011). Poor prognosis in non-small-cell lung cancer is partly due to the development of chemotherapy resistance (Rosell et al., 2013; Zhou et al., 2013).

Polycyclic aromatic hydrocarbons (PAH) are environmental and tobacco carcinogens and are suspect agents in the causation of human lung cancer (Eom et al., 2013). PAHs should be activated to reactive genotoxins to cause carcinogenic effects. Benzo(a)pyrene (B(a)P) -a type of PAH- is a potent tobacco carcinogen has been used in previous studies to understand molecular insights of cancer (Pfeifer et al., 2002).

Thymoquinone (TQ) is the predominant bioactive constituent present in black seed oil (Nigella sativa). In previous studies it has been demonstrated that thymoquinone inhibits cell proliferation, decreases cellular viability, induces apoptosis, arrests cell cycle, modulates multiple molecular targets including p53, p73, PTEN, STAT3, PPAR-γ, activation of caspases and generation of ROS in vivo and in vitro conditions of different cancer types (Randhawa et al., 2011). The anti-tumor effects of thymoquinone have also been investigated in tumor xenograft mice models for colon, prostate, pancreatic and lung cancer (Woo et al., 2012).

Caffeic acid phenethyl ester (CAPE); a phenolic compound, is found and isolated from propolis, has wide spectrum effects like antioxidant, anti-inflammatory, antiviral, immune stimulant, carcinostatic...
and anticarcinogenic (Orsolic et al., 2005). CAPE is one of the reported chemoprotective agent to interfere with the intracellular signal that is related to carcinogenesis, the proliferation of cancer cells, apoptosis, and cell migration (Orsolic et al., 2004; 2005).

Resveratrol, trans-resveratrol (trans-3,5,4’-trihydroxystilbene) is a phytoalexin and present in many plants and fruits including red grapes, peanuts and pineapples. Reactive oxygen radicals are cleared with antioxidant activity. Resveratrol inhibits DNA damage and prevents lipid peroxidation of cellular membrane. Resveratrol is expected to be effective in cancer related processes (Chen et al., 2010; Bae et al., 2011; Zhang et al., 2011; Yin et al., 2013).

In this study we aimed to investigate the effects of thymoquinone, CAPE and resveratrol on B(a)P exposed lung cancer cell line and compare the effectiveness with each other.

Materials and Methods

**Major reagents**

The human lung adenocarcinoma cell line A549 was obtained from Dr J Mazella (CNRS, Valbonne, France). DMEM (Dulbecco’s modified Eagle’s medium), fetal bovine serum and benz(α)pyrene [B(a)P] were purchased from Sigma-Aldrich. Thymoquinone was purchased from Spectrum Chemical Manufacturing Corp (USA). Resveratrol was purchased from Cayman Chemical. Caffeic acid phenethyl ester (CAPE) was purchased from ABCR GmbH & Co (Germany).

**Cell culture and treatments**

A549, human lung adenocarcinoma cell line was maintained in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10% fetal bovine serum (FBS), 100units/ml penicillin and 100µg/ml streptomycin, and 2mM glutamine. The cells were incubated in 5%CO2 and humidified at 37°C for growth.

A549 cells were seeded in 75-cm2 culture flasks and grown for 1-2 days before use. When 50% confluence was obtained, the culture medium was changed to fresh medium containing 20µM B(a)P (C20H12; purity>96%) for 48h (Tampioa, 2008). B(a)P was first dissolved in dimethyl sulfoxide (DMSO) and then added to the culture medium with the final concentration of DMSO less than 0.1%. The medium was changed daily to maintain the concentration of B(a)P.

CAPE, thymoquinone and resveratrol treatments

After 24 h incubation of A549 cells with B(a)P, culture medium containing B(a)P was removed, and fresh medium containing 2µg/ml CAPE (BaP+CAPE group) (Chen et al., 2004), 5µM Thymoquinone (BaP+TQ group) (Khan et al., 2012), and 10 µM Resveratrol (BaP+RES group) (Kode et al., 2008) alone were added into the flasks according to the groups. As control, A549 cells were incubated with medium containing vehicle (DMSO) at the same concentration. After 48 h incubation with agents medium was removed and the cells were used to perform the experiments described as follows.

**Protein analyses**

Cells from the all groups were washed twice with ice-cold PBS and then harvested by scraping. The harvested cells were lysed in lysis buffer (100mM NaH2PO4, 1% Triton X-100, 1 M HEPES, and protease inhibitor cocktail 1%). The homogenate was centrifuged at 13,000g at 4°C for 40 min. The supernatants were collected and protein concentrations were determined using Bradford method (Bradford et al., 1976).

**Measurements of cell survival by MTT assay**

The number of viable A549 cells after treatments was evaluated by the MTT (3-[4,5-methylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay. In brief, A549 cells (2x10^6 cells/well) were seeded in a 24-well plate and kept overnight for attachment. The next day the medium was replaced with fresh medium with B(a)P (20 µM) and cells were allowed to grow for 48 h. After completion of incubation, agents were added in each well and after completion of second incubation (24 h), 100µl of MTT (10mg/ml, Sigma USA) was added in each well, followed by 4h incubation at 37°C, then medium was removed and 150µl DMSO was added to each well. The plate was then shaken for 10 min in the dark at room temperature. The absorbance value at 490nm was read using a UV/VIS spectrophotometer (T70, PG Instruments). The percentage of cell viability was calculated as follows: cell viability (%)=OD treatment / OD control ×100%.

**Analyses of cytokine production in cells**

The effect of TQ, CAPE and RES on release of cytokines (IFN-γ, IL-1β, TNF-α) in cell lysate was assessed by ELISA technique. Values were expressed as pg per mg protein.

**Determination of NO production**

Nitric oxide concentrations in culture medium and cell lysate were determined by Griess reaction (Miranda et al., 2001). Samples were deproteinated with 75 mmol zinc sulphate and total nitrite was determined by spectrophotometer at 546 nm after conversion of nitrate to nitrite by vanadium-III-chloride. Results were calculated in µmol per liter in medium and µmol per gram protein in cell lysate.

**Measurement of GSH levels**

GSH level of cell lysates was measured by the method described by Buettel et al. (1963). In this reaction GSH is recycled from GSSG and steadily reduces DTNB to TNB. The increase in OD at 412nm over time (i.e., ΔODt=412nm) is directly proportional to the GSH content in the deproteinized sample (with TCAA 10%). Results were implied as µmol per gram protein.

**MDA measurement in cell lysate**

MDA levels were measured with the method described by Yoshioka et al. (1979). The method is essentially based on the formation of a pink color under the acidic condition upon the reaction of MDA and thiobarbituric acid. Absorbance of pink compound receiving n-butanol was measured spectrophotometrically at 535nm. In the
computation of results, nanomole per gram protein was used as units.

Measurement of total oxidant status in cell lysate

The TAS of the cell lysate was measured using an automated colorimetric measurement method for TAS (Erel, 2005). This method is based on the bleaching of color characteristics of a more stable ABTS [2,2’-azino-bis(3-ethylbenzothiazole-6-sulfonic acid)] radical cation by antioxidants. The assay has excellent precision values, which are lower than 3%. The results were expressed as mmol H\textsubscript{2}O\textsubscript{2} Eq/g protein.

Determination of oxidative stress index

The ratio of TOS to TAS represents the OSI, an indicator of the degree of OS. The OSI value is calculated according to the formula: OSI (arbitrary unit)=TOS (μmol H\textsubscript{2}O\textsubscript{2} Eq/g protein)/TAS (mmol Trolox Eq/g protein)×100 (Esen et al., 2012).

Total RNA isolation and mRNA expression levels of genes by real-time reverse transcription-polymerase chain reaction (RT-PCR)

A549 cells were seeded in 25-cm\textsuperscript{2} culture flasks and grown for 1-2 days before use. Cells were collected and washed with phosphate-buffered saline (PBS) after completion of incubations given above. Total RNA was isolated by RNAeasy kit (QIAGEN) and cDNA was generated with a First Strand cDNA Synthesis kit (Thermo Scientific) at a total volume of 20 μl according to the manufacturer’s instructions. Real-time quantitative PCR was performed in a Strategene Mx3005P QPCR system (USA). Expression levels of target gene were normalized to the housekeeping gene β-actin (β-actin). Gene expression values were then calculated based on the \( \Delta\Delta Ct \) method using the equation: \( RQ = 2^{-\Delta\Delta Ct} \). PCR amplification was performed with Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific). The primer sequences used in PCR reactions and PCR conditions are described in Table 1. Each assay was performed in triplicate and repeated three times.

Statistical analysis

The statistical analyses of our study were performed using SPSS statistical software version 20.0. The variables were investigated using visual (histograms, probability plots) and analytical methods (Shapiro-Wilk test) to determine the normality of distributions. The results were expressed as mean±standard deviation and median value (min-max range). ANOVA was used to compare parameters with normal distribution among study groups. Levene test was used to assess homogeneity of variances. The p value less than 0.05 was accepted as significance level. When an overall significance was observed pairwise post hoc tests were performed using Tukey’s test for homogenous variances and Tamhane T2 test for heterogenous variances. For continuous variables without normal distribution Kruskal-Wallis test for the comparison of parameters among study groups and Mann-Whitney U test was used for the comparison of two groups.

Results

Inflammatory markers and oxidative stress parameters

Inflammatory markers and oxidative stress parameters of study groups are outlined in Table 1. CAPE, TQ and RES treated cells had decreased levels of interferon gama, interleukin 1 beta, glutathione and malondialdehyde when compared with control group (Table 2). Total oxidan status was also lower in CAPE, TQ and RES groups than control group.

mRNA expression levels of genes in A549 cells

Comparison of mRNA expression levels of genes in A549 cells is presented in Table 3. CAPE, RES and TQ upregulated TRAIL receptor 1 and 2 expression. CAPE and TQ upregulated bax expression. RES and TQ downregulated the expression of Bcl-2 (Table 3). RES and TQ downregulated the expression of NF-kappa B and IKK1. All three agents decreased the expression of cyclin D and increased the expression of p21. The highest increase of p 21 expression was observed in TQ group.

Viability of cells was investigated and compared among groups (Figure 1). Viability of TQ, CAPE and RES treated cells were significantly decreased when compared with control group (p<0.004). However there were no significant differences in terms of viability of cells between TQ treated cells and CAPE treated cells.
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dependent kinases. A family of negative cell cycle regulated by interactions between cyclins and cyclin-cell line are the considerable results of the present study. apoptotic proteins and survival of benzo(a)pyrene exposed resveratrol on inflammatory markers, oxidative stress study investigating and comparing the potential roles Discussion

**Table 3. Comparison of mRNA Expression Levels of Genes**

<table>
<thead>
<tr>
<th>Groups</th>
<th>mRNA expression levels of genes (fold increase/ decrease)</th>
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<tbody>
<tr>
<td></td>
<td>BAX</td>
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<tr>
<td>Bi(At)</td>
<td>(0.1)</td>
</tr>
<tr>
<td>CAPE**</td>
<td>(0.2)</td>
</tr>
<tr>
<td>Resb</td>
<td>(0.1)</td>
</tr>
<tr>
<td>TQb</td>
<td>(0.1)</td>
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*According to the control group; **According to the Bi(At) group; B(a)P: Benzo(a)pyrene. CAPE: Caffeic acid phenethyl ester, RES: Resveratrol, TQ: Thymoquinone

**Figure 1. Results of Viability Assay using A 549 Cell Line 24 Hours after Treatment with Caffeic Acid Phenethyl Ester (CAPE), Resveratrol and Thymoquinone.** Cell viability was significantly decreased in treatment groups when compared with control group (p=0.004)
cells (p=0.746), TQ treated cells and RES treated cells (p=0.746) and CAPE treated cells and RES treated cells (p=0.332).

**Discussion**

To our knowledge, the present study is the first study investigating and comparing the potential roles of thymoquinine, caffeic acid phenylester (CAPE) and resveratrol on inflammatory markers, oxidative stress parameters, mRNA expression levels of apoptotic and anti apoptotic proteins and survival of benzo(a)pyrene exposed A 549 cells in vitro. And down regulated NF-kappaB, IKKb and cyclin D1 expression together with the highest increase in p21 expression by TQ in lung adenocarcinoma cell line are the considerable results of the present study. The basic principle in cancer treatment is the stimulation of apoptosis. Cell cycle progression is regulated by interactions between cyclins and cyclin-dependent kinases. A family of negative cell cycle regulators, cyclin-dependent kinase inhibitors, modulates these events, especially those controlling the transition of G1 to S-phase (Chellappan et al., 1998; Chen et al., 1996). p21 is a member of cyclin-dependent kinase inhibitor and plays an important role in growth arrest (Gartel and Tyner, 2002). In addition, there are various reports that reveal the essential role of p21 in growth arrest of cancer cells (Brugarolas et al., 1995). p21 expression has also been demonstrated as a potential prognostic factor in patients with non-small cell lung carcinoma, (Komiya et al., 1997; Niklinkski et al., 2001) and it may represent an important clinical marker of outcome in non-small cell lung carcinoma (Shoji et al., 2002). Benzo(a)pyrene (BP)-induced DNA damage in association with p53 expression in A 549 cell line was first investigated by Ramet al. (Ramet et al., 1995). Activation of BP in A-549 lung carcinoma and MCF-7 breast adenocarcinoma cell lines was resulted to an increase in p53 protein expression indicating that p53 protein is part of the response of the cells to BP-induced DNA damage (Ramet et al., 1995). Kaspin and Baird (1996) and Luch et al. (1999) reported the induction of p53 protein levels together with the increase of p21WAF1 protein levels in MCF-7 cell line after the exposure to BP.

Binkova et al. (2000) also found more than 1.5-fold increase of p53 and p21WAF1 proteins in BP treated human normal diploid lung fibroblasts as compared with controls. The present study also demonstrates 1.2 fold increase of p 21 expression in BP treated A 549 cells.

Malhotra et al. (2011) reported that treatment with curcumin and resveratrol enhanced protein expression of p21, thereby resulting in a significant decrease in tumor incidence and multiplicity in BP-treated mice. Kubota et al. (2003) investigated the effects of resveratrol on proliferation and inducing apoptosis in three lung cancer cell lines (A549, EBC-1, Lu65) and also combined effects of resveratrol and paclitaxel. Although exposure to resveratrol plus paclitaxel did not result in significant synergy, resveratrol significantly enhanced the subsequent antiproliferative effect of paclitaxel and resveratrol prior to paclitaxel induced p21waf1 expression approximately 4-fold in their study. Similar to these studies resveratrol upregulated the expression of p21 in A549 cells of the present study. However the most significant upregulation of p21 expression was observed in TQ. To the best of our knowledge the data of increased p21 gene expression by TQ in lung adenocarcinoma cell line has not been reported in the literature till the present study.

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NF-kappaB controls proliferation by increasing expression of cyclin D1 and apoptosis is increased by inhibition of NF-kappaB and proliferation is decreased by reducing cyclin D1 expression (Connelly et al., 2011). In parallel with the previous studies, mRNA expression levels of NF-kappaB were found to be 1.2 fold decreased and mRNA expression levels of cyclin D1 were found to be 2.5 fold decreased in TQ treated A 549 Cells (Sethi et al., 2008). Down regulated NF-kappaB, IKKβ and cyclin D1 expression together with the highest increase in p21 expression by TQ suggest that TQ may prove useful in overcoming this devastating disease in addition to other chemotherapeutic agents.

In a recent study by Attoub et al. (2013) exposure of cells derived from lung (LNM35), liver (HepG2), colon (HT29), melanoma (MDA-MB-435), and breast (MDA-MB-231 and MCF-7) tumors to increasing TQ concentrations resulted in a significant inhibition of viability through the activation of Akt phosphorylation leading to DNA damage and activation of the mitochondrial-signaling proapoptotic pathway. Bax is an important protein for pore formation in the permeabilization of the mitochondrial outer membrane, which not only couples mitochondria to the activation of caspases but also initiates caspase-independent mitochondria dysfunction. Bax is constantly held in check by the anti-apoptotic activity of the Bcl-2 proteins (Cheng et al., 2001; Li et al., 2013). In the present study although TQ and CAPE treatment up-regulated Bax, RES treatment down-regulated Bax proteins. TQ and RES treatment down-regulated Bcl2 proteins. Thus only TQ treatment up-regulated Bax, and down-regulated Bcl2 proteins. TNF-related apoptosis-inducing ligand (TRAIL), is a cytokine functioning as a ligand that induces apoptosis by binding to the death receptors DR4 (TRAIL-R1) and DR5 (TRAIL-RII). CAPE, RES and TQ treatments enhanced TRAIL induced apoptosis by upregulating TRAIL receptor 1 and 2 expression in the present study. Moreover IKK1 expression was down regulated by RES and TQ. Therefore viability of CAPE, RES and TQ treated cells was found to be significantly decreased when compared with control group.

Shigoeaka et al. (2004) investigated the effects of CAPE on the transforming growth factor-beta (TGF-β)-induced invasive phenotype using A549 lung adenocarcinoma cells as a model system and determined the suppression of TGF- β-enhanced cell motility and TGF-β-induced Akt (protein kinase β) activation as well as a specific inhibitor of phosphatidyl inositol 3-kinase (PI3K)/Akt pathway, LY294002. CAPE treatment has been reported as inhibiting cell proliferation, causing apoptosis and S/ G2 cell cycle arrest, and increasing radiosensitization in A549 cells (Chen et al., 2004; Lin et al., 2012).

Natarjan et al. (1996) determined the blockade of the activation of NF-κappaB by CAPE treatment in the dose- and time-dependent manner. However in the present study CAPE is the only agent increasing NFκB expression when compared to control group and BP exposed group. This result may be due to the different dose of CAPE in our study.

It is claimed that CAPE inhibits the growth of tumor cells using oxidative stress pathways connected to p53-independent pathways and observed to inhibit oxidative processes by decreasing the generation of intracellular hydrogen peroxide (H₂O₂) in A549 cells (Cotgreave and Gerdes, 1998). Chen et al. (2004) also determined the decrease of intracellular GSH and H₂O₂ in CAPE treated A 549 cells than untreated A 549 cells. In the present study GSH level and TOS were found to be reduced in CAPE group when compared with control group. Moreover the most significant decrease in GSH level was observed in thymoquinone group and the most significant decrease in TOS was in resveratrol group. Therefore, the depleting intracellular stores of GSH and decreased TOS by CAPE, resveratrol and thymoquinone can make cells more susceptible to oxidative stress-induced apoptosis which is a target point in cancer treatment (Cotgreave and Gerdes, 1998).

Further studies including the combination of these three agents are needed to determine whether synergistic action of these agents on adenocarcinoma cells are present or not.

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