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PRECLINICAL STUDIES

Caffeic acid phenethyl ester exerts apoptotic and oxidative stress on human multiple myeloma cells

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Summary

Caffeic acid phenethyl ester (CAPE) is a phenolic compound initially identified in bee glue. CAPE is reported to exhibit antitumor activity in many cancer models. However, the effect of CAPE on multiple myeloma (MM) is not well studied. We investigated the anti-myeloma effect of CAPE, and the data showed that CAPE inhibited the growth of human MM cells in a dose (1 ~ 30 μM) and time (24 ~72 h) dependent manner without altering the viability of normal human peripheral blood B cells. Stress and toxicity pathway analysis demonstrated that CAPE, in a dose- and time-related fashion, induced the expression of apoptotic and oxidative stress-response genes including growth arrest and DNA-damage inducible, alpha and gamma (GADD45A and GADD45G) and heme oxygenase-1. Apoptosis of MM cells by CAPE was further confirmed through flow cytometric analysis with up to 50% apoptotic cells induced by 50 μM CAPE within 24 h. Western blot analysis revealed the CAPE-induced activation of apoptosis executioner enzyme caspase-3, and corresponding cleavage of its downstream target poly(ADP-ribose)polymerase (PARP). The oxidative stress caused by CAPE cytotoxicity in MM cells was evaluated through measurement of reactive oxygen species (ROS) level, antioxidant intervention and glutathione depletion. The intracellular ROS level was not elevated by CAPE, but the pretreatment of antioxidant (N-acetyl cysteine) and glutathione synthesis inhibitor (buthionine sulfoximine) suggested that CAPE may cause oxidative stress by decrease of intracellular antioxidant level rather than over production of ROS. These data suggest that CAPE promotes apoptosis through oxidative stress in human multiple myeloma cells.

Keywords Caffeic acid phenethyl ester · Multiple myeloma · Cytotoxicity · Apoptosis · Oxidative stress

Introduction

Multiple myeloma (MM) is a plasma cell cancer characterized by accumulation of malignant cells preferentially in the bone marrow. It is associated with severe bone disease and remains incurable even with high dose chemotherapy. About 10 ~ 15% of hematologic malignancies and 20% of human death related to blood and bone marrow cancers are the consequences of MM [1]. The understanding of MM pathophysiology has improved considerably in recent years. It is known that B cell differentiation occurs in early antigen-independent and late antigen-dependent stages, resulting in the generation of plasma cells and memory B cells [2]. The pathogenesis of MM involves a high degree of immunoglobulin heavy chain gene hypermutation [3], chromosomal abnormalities [4], and interactions between MM cells and the bone marrow (BM) microenvironment [5, 6]. The BM microenvironment contributes significantly to MM tumor survival and progression. BM microenvironment is composed of extracellular matrix proteins including fibronectin, collagen, laminin and osteopontin and cell components such as immune cells, endothelial cells, erythrocytes, bone cells (osteoclasts and osteoblasts) and bone marrow stromal cells (BMSCs) [7, 8].

The interactions between MM cells and BM microenvironment, especially the binding of MM cells to BMSCs, lead to an increased expression of cytokines (interleukin −1 and 6) and growth factors (insulin-like growth factor and vascular endothelial growth factor). The release of these

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cytokines and growth factors enhances the growth and survival of the MM cells through cell adhesion-mediated drug resistance [9, 10]. A number of intracellular signaling pathways in MM cells are activated due to MM cell and BMSC interaction including Ras-Raf-MAPK kinase /extracellular signal-regulated kinase (MEK/Erk), Janus kinase/signal transducers and activators of transcription 3 (JAK/STAT3), phosphatidylinositol 3-kinase/Akt (PI3K/Akt), and nuclear factor-kappaB (NF-κB) [8, 11–13]. Improved understanding of myeloma biology has led to the development of drugs targeting intracellular survival pathways and interactions between myeloma cells and BM microenvironment. Such drugs include thalidomide and bortezomib [14]. However, chronic relapse and treatment resistance are often observed [15]. This disease is still far from being cured, therefore new therapeutic advances are of great importance and worthy of investigation.

Over the past several decades, considerable progress has been made in the development of various anticancer drugs and strategies. One such strategy is the application of naturally occurring substances to intervene in the pathogenesis of cancer with reduced drug toxicity toward normal cells [16]. Caffeic acid phenethyl ester (CAPE) is one of the predominant bioactive components identified in honeybee propolis, which is held accountable for the anticancer property of bee glue [17]. This polyphenolic phytochemical has been reported to exhibit numerous bioactive properties including antioxidant [18], anti-inflammatory [19], and anticancer/tumor [20] activities. CAPE was also found to inhibit activation of NF-κB, the major transcriptional modulator of inflammation and tumor cell proliferation [21, 22]. Anticancer effects of CAPE have been reported in different types of malignant cells including human breast cancer and prostate cancer cells [23, 24]. The mechanism of CAPE anticancer action is found possibly through induction of apoptosis and other cancer progression pathways involving various protein kinases, growth factors, transcription factors, cell cycle proteins and cell adhesion molecules [25]. Reports about the effects of CAPE on multiple myeloma, however, are limited, and the mechanism of CAPE action remains largely undefined.

The purpose of the present investigation was to explore the potential of CAPE in multiple myeloma treatment by evaluating the growth-inhibitory effect of CAPE on MM cells and elucidating the mechanism of CAPE action. In this study, we examined the cytotoxicity of CAPE in various MM cell lines. To obtain insight into potential mechanism of CAPE, we assessed the expression of genes associated with cellular stress and toxicity in MM cells following CAPE treatment. Here, we report that caffeic acid phenethyl ester inhibits growth of multiple myeloma cells through induction of apoptosis and possible involvement of oxidative stress.

Materials and methods

Chemicals

CAPE and buthionine sulfoximine (BSO) were purchased from Cayman Chemicals (Ann Arbor, MI, USA). N-acetylcysteine (NAC) was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

Myeloma cell line RPMI 8226, NCI-H929, and U266 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). RPMI 8226 cells were cultured using RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). NCI-H929 cells were maintained in RPMI 1640 medium supplemented with 10% FBS and 0.05 mM 2-mercaptoethanol. U266 cells were cultured using RPMI 1640 medium supplemented with 15% FBS. Human peripheral blood B (HPBB) cells were purchased from Cell Applications (San Diego, CA, USA) and cultured according to manufacturer's instruction. Cells were maintained at 37 °C in the presence of 5% CO_2 and 95% air in a humidified incubator.

Cell viability and toxicity assay

Cell viability was assessed using the Presto Blue™ assay kit following manufacturer's protocol (Life Technologies, Carlsbad, CA, USA). Briefly, at the time of experiments, MM cells were counted and subcultivated in 48-well multiplates (Corning Incorporated, Corning, NY, USA) at a concentration of 40,000 cells/well. Cells were then exposed to 1, 5, 10, 20, and 30 μM of CAPE or 0.1% DMSO (vehicle control) for 24, 48, or 72 h incubation. At the end of each treatment, cells were incubated with culture medium containing 10% Presto Blue reagent for additional one hour. Fluorescence was measured at 545 nm excitation and 590 nm emissions using a Synergy HT plate reader (BioTek Instruments Inc., Winooski, VM, USA).

Pathway-focused and real-time PCR-based array analysis

Cultured RPMI 8226 (MM 8226) cells were treated with 10 μM CAPE, 20 μM CAPE or vehicle control (0.1% DMSO) for 6 h, and 20 μM CAPE or vehicle control for 24 h, respectively. After pharmacological exposure, treated cells were frozen and submitted for gene expression profiling using the Human Stress & Toxicity PathwayFinder™, RT^2Profiler™ PCR Array (Qiagen Inc., Valencia, CA, USA). This array contains gene-specific primer sets for 84 stress- and toxicity-related genes involved in different stress

and toxicity pathways and 5 housekeeping genes, whose Ct values were used as a normalization factor. Expression values were averaged across three independent array experiments. A cutoff of 2 fold in terms of fold changes between CAPE-treated and control groups was selected. A p value less than 0.05 were used to identify statistically significant up- and down-regulated genes.

Flow cytometric analysis

Induction of treated MM 8226 cell death (early and late apoptosis) was examined using an Annexin V-FITC (Annexin V) / 7-Amino-Actinomycin (7-AAD) dual staining of cells followed by flow cytometric analysis. Cultured MM 8226 cells (1.0×10^6 cells/well) were plated in 6-well multiplates and treated with 1, 5, 10, 25, 50 μM CAPE or 0.1% DMSO (vehicle control) for 24 h. After treatment, cell staining was performed using a FITC Annexin V Apoptosis Detection Kit following the manufacturer's protocol and flow cytometry done on the BD Accuri™ C6 Flow Cytometer (BD Biosciences, San Jose, CA, USA).

Caspase 3 enzyme activation assay

Caspase-3 activity was measured using the EnzChek Caspase-3 Assay Kit II (Molecular Probes, Invitrogen, ThermoFisher Scientific Inc., MA, USA). Briefly, cultured MM 8226 cells (1.0×10^6 cells/well) were treated with 1, 5, 10, 25 μM CAPE or 0.1% DMSO (vehicle control) for 24 h. After treatment, treated cells were harvested and washed in phosphate-buffered saline (PBS). Cell pellets were treated with cell lysis buffer and substrate Z-DEVD-R110 working solution according to the manufacturer's protocol. Fluorescence was measured at 485 nm excitation and 530 nm emissions using a Synergy HT plate reader (BioTek Instruments Inc., Winooski, VM, USA). Caspase-3 activity was expressed as arbitrary units of fluorescence.

Western blotting

MM 8226 cells were treated with various concentrations of CAPE and were allowed to incubate for 24 h. Protein from treated cells was collected using complete 1X RIPA lysis buffer (Thermo-Fischer Scientific Inc., MA, USA). Protein concentration was estimated by using the Pierce™ BCA assay kit (Thermo-Fischer Scientific). Equal amount of protein (30 μg) collected from each treatment was loaded and run on 10-well 4–20% Mini-PROTEAN® TGX™ precast gel (Bio-Rad, Hercules, CA, USA) at 100 V for one and a half hours. Protein was then transferred from the gel to an Immuno-Blot PVDF membrane (Bio-Rad), and the membrane was washed 3 times for 5 min with PBST (1X PBS plus 0.1% Tween20) then blocked for an hour at room temperature using blocking buffer (Li-COR Biosciences, Lincoln, NE, USA). The blot was incubated with primary antibodies for Caspase 3, PARP, and β-actin (Abcam, Cambridge, MA, USA) at a ratio of 1:5000 overnight at 4 °C. After been washed for three times with PBST, the blot was incubated with Li-COR IRDye® secondary antibodies at a ratio of 1:10000 for one hour at room temperature. After additional washes for five minutes three times, the membrane was imaged with the Li-COR Odyssey CLX imaging system. Protein bands were detected using the 700 and 800 channels. Intensity of target protein bands was quantified using Image J software (NIH, Bethesda, MA, USA).

Determination of intracellular level of reactive oxygen species

Intracellular level of reactive oxygen species (ROS) was determined when exposing CAPE treated MM 8226 cells to the fluorescent probe CM-H_2DCFDA (Thermo-Fischer Scientific Inc., MA, USA) as described previously [26]. Briefly, cultured MM 8226 cells were rinsed twice with pre-warmed working buffer (Hank's balanced salt solution with 10 mM HEPES) and incubated with CM-H_2DCFDA at 8 μM for 45 min at 37 °C in the dark. MM 8226 cells were then washed twice with pre-warmed working buffer and treated with CAPE (1, 10, 25, and 50 μM) or 0.1% DMSO (vehicle control) dissolved in working buffer for 24 h. Fluorescence was measured at 480 nm excitation and 520 nm emissions using a Synergy HT plate reader (BioTek Instruments Inc., Winooski, VM, USA). The fluorescent signals were normalized to the vehicle control group, and relative fluorescence was taken to calculate the percentage of ROS production compared to control.

Antioxidant and glutathione intervention assay

MM 8226 Cells were incubated 1 h with N-acetyl cysteine (NAC, 0.1 ~ 1 mM), a known antioxidant, or 24 h with buthionine sulfoximine (BSO, 0.1 ~ 1 mM), a known glutathione synthesis inhibitor. After NAC pretreatment, MM 8226 cells were incubated with 30 μM CAPE for 24, 48, or 72 h. After BSO pretreatment, MM 8226 cells were incubated with 30 μM CAPE for 48 h. Cell viability was determined using Presto Blue™ assay kit as described above.

Statistical analysis

Statistical analysis was conducted using IBM SPSS Statistics® version 22 (IBM, Chicago, IL, USA), and the results were presented as mean ± standard deviation. Differences between and within groups were analyzed using one-way analysis of variance, and, if found significant, the analyses were followed by post hoc tests of Tukey (equal variances assumed) or Games-Howell (equal variances not assumed) for inter-group comparisons. A difference of P value ≤0.05 was considered statistically significant.

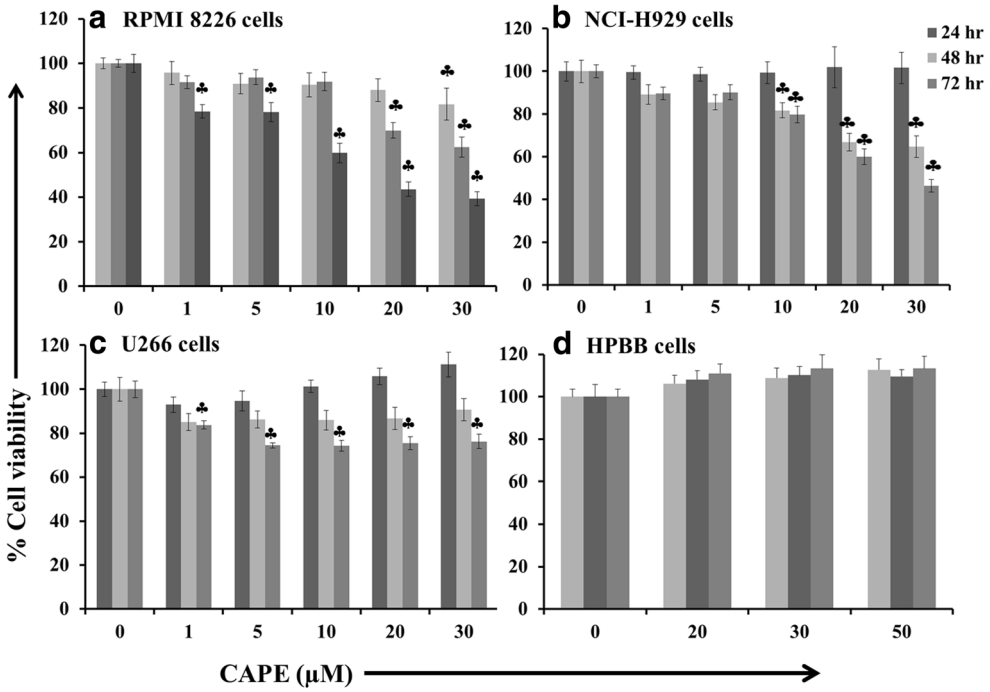


Fig. 1 Cytotoxic effects of CAPE in multiple myeloma RPMI 8226 (**a**), NCI-H929 (**b**), and U266 (**c**) cells. CAPE inhibited growth of MM cells in a dose and time – dependent manner within 72 h. ♣: $p < 0.05$ versus control (0 μM CAPE). CAPE did not reduce viability of normal human peripheral blood B cells (**d**) up to 50 μM within 72 h

Results

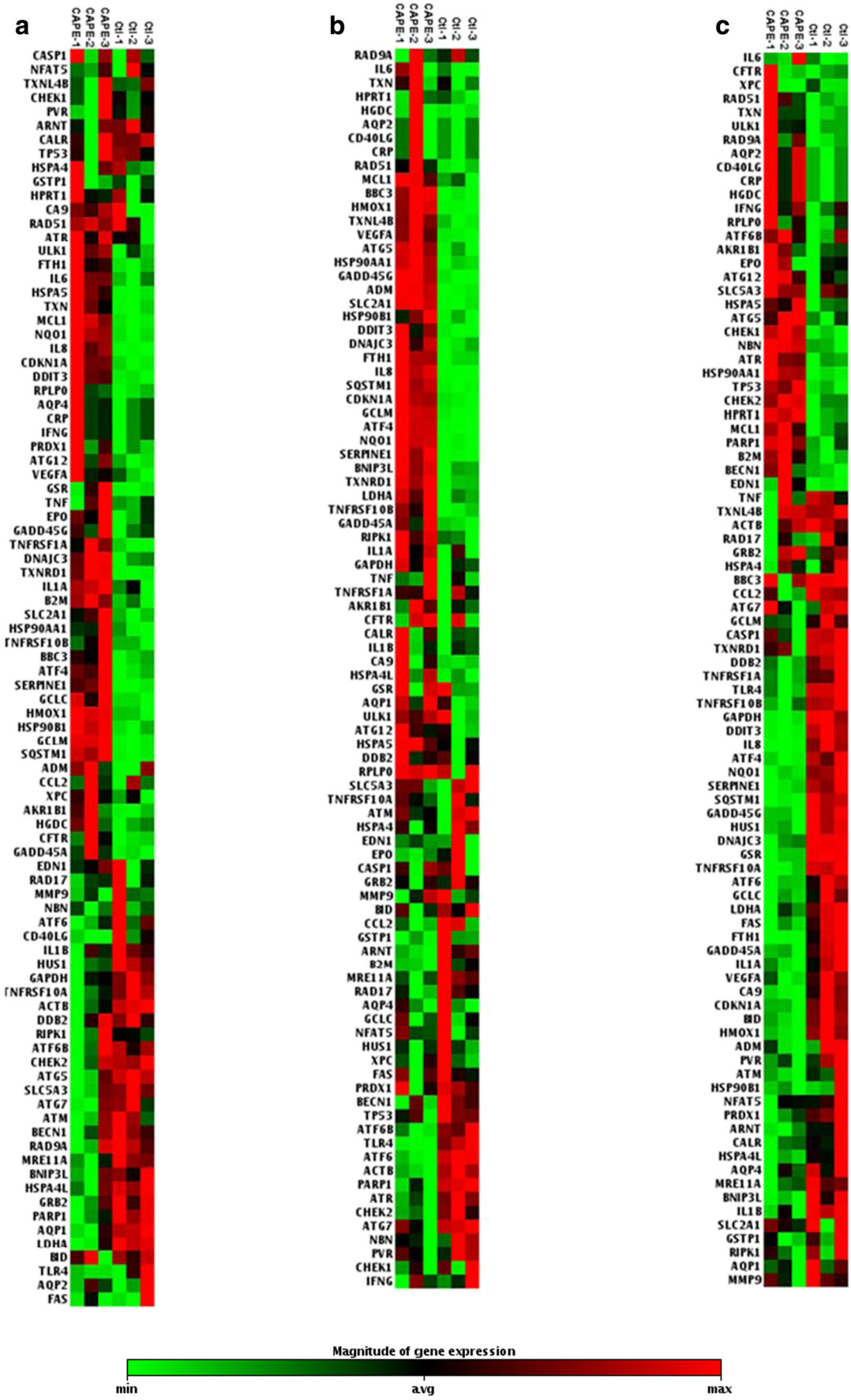
CAPE inhibits growth of multiple myeloma cells

To evaluate the effect of CAPE on MM cells, we treated three human MM cell lines (RPMI 8226, NCI-H929, and U266) with various concentrations of CAPE (0 ~ 30 μM) and determined cell viability after 24, 48, and 72 h incubation. The data show that CAPE significantly inhibited MM cell growth in a dose- and time-dependent manner (Fig. 1). When MM 8226 cells were treated with CAPE at 30 μM, the highest dose tested, significant decrease of cell viability was observed at 24 h from 20% up to 60% at 72 h (Fig. 1a). After 72 h incubation, viability of MM 8226 cells dropped to 80% even at 1 μM, the lowest dose tested. Viability of NCI-H929 cells did not change after 24 h treatment of CAPE compared to control, which suggests that this MM cell line can tolerate CAPE toxicity within 24 h (Fig. 1b). However, inhibitory effect of CAPE on NCI-H929 cell growth was observed after 48 and 72 h incubation. Cell viability was significantly decreased starting from 48 h treatment of CAPE at 10 μM. After 72 h treatment of CAPE at 30 μM, viability of NCI-H929 cells dropped to 45%. Interestingly, U266 cells were more resistant to CAPE cytotoxicity (Fig. 1c). Viability of these MM cells was significantly decreased only after 72 h incubation of CAPE and ranged from around 85% (CAPE at 1 μM) to 75% (5 ~ 30 μM). Although these MM cell lines are from donors of different gender and age, CAPE was able to reduce the viability of all three of them to different extent. To examine the effect of CAPE on the normal counterpart of these malignant plasma cells, we treated human peripheral blood B cells with CAPE up to 50 μM and assessed cell viability at the same time and dose points as those for MM cell treatment. No significant difference in cell viability was found up to 72 h incubation at the tested doses of CAPE compared to control (Fig. 1d). Taken together, these results suggest that CAPE exhibits common anti-myeloma effect by reducing viability of myeloma RPMI 8226, NCI-H929, and U266 cell lines in a dose and time dependent manner but is non-toxic to normal human blood B cells at the tested doses.

CAPE triggers upregulation of growth arrest, apoptotic and oxidative stress-response genes in MM 8226 cells

To explore the mechanism of CAPE anti-myeloma effort at molecular level, we examined regulation of genes responsible for cellular stress and toxicity in CAPE-treated MM 8226 cells through pathway-focused PCR arrays. We checked the early expression of stress-response genes after 6 h exposure to 10 and 20 μM CAPE, doses of CAPE at which MM 8226 cell viability was not affected up to 24 h treatment (Fig. 1a). A clustering heat map of pathway-focused genes was illustrated in Fig. 2. These selected genes belong to various human stress and toxicity pathways including oxidative stress, hypoxia signaling, osmotic stress, cell death, inflammatory response, DNA damage & repair, and unfolded protein response. A list

Fig. 2 Clustering heatmaps of all pathway-focused genes whose expression was up- and down-regulated by 10 μM CAPE in 6 h (**a**), 20 μM CAPE in 6 h (**b**), and 24 h (**c**) versus 0.1% DMSO as vehicle control (Ctl), respectively, in MM 8226 cells ▶


a
CAPE-1
CAPE-2
CAPE-3
Ctl-1
Ctl-2
Ctl-3
CASP1
NFAT5
TXNL4B
CHEK1
PVR
ARNT
CALR
TP53
HSPA4
GSTP1
HPRT1
CA9
RAD51
ATR
ULK1
FTH1
IL6
HSPA5
TXN
MCL1
NQO1
IL8
CDKN1A
DDIT3
RPLP0
AQP4
CRP
IFNG
PRDX1
ATG12
VEGFA
GSR
TNF
EPO
GADD45G
TNFRSF1A
DNAJC3
TXNRD1
IL1A
B2M
SLC2A1
HSP90AA1
TNFRSF10B
BBC3
ATF4
SERPINE1
GCLC
HMOX1
HSP90B1
GCLM
SQSTM1
ADM
CCL2
XPC
AKR1B1
HGDC
CFTR
GADD45A
EDN1
RAD17
MMP9
NBN
ATF6
CD40LG
IL1B
HUS1
GAPDH
TNFRSF10A
ACTB
DDB2
RIPK1
ATF6B
CHEK2
ATG5
SLC5A3
ATG7
ATM
BECN1
RAD9A
MRE11A
BNIP3L
HSPA4L
GRB2
PARP1
AQP1
LDHA
BID
TLR4
AQP2
FAS
b
CAPE-1
CAPE-2
CAPE-3
Ctl-1
Ctl-2
Ctl-3
RAD9A
IL6
TXN
HPRT1
HGDC
AQP2
CD40LG
CRP
RAD51
MCL1
BBC3
HMOX1
TXNL4B
VEGFA
ATG5
HSP90AA1
GADD45G
ADM
SLC2A1
HSP90B1
DDIT3
DNAJC3
FTH1
IL8
SQSTM1
CDKN1A
GCLM
ATF4
NQO1
SERPINE1
BNIP3L
TXNRD1
LDHA
TNFRSF10B
GADD45A
RIPK1
IL1A
GAPDH
TNF
TNFRSF1A
AKR1B1
CFTR
CALR
IL1B
CA9
HSPA4L
GSR
AQP1
ULK1
ATG12
HSPA5
DDB2
RPLP0
SLC5A3
TNFRSF10A
ATM
HSPA4
EDN1
EPO
CASP1
GRB2
MMP9
BID
CCL2
GSTP1
ARNT
B2M
MRE11A
RAD17
AQP4
GCLC
NFAT5
HUS1
XPC
FAS
PRDX1
BECN1
TP53
ATF6B
TLR4
ATF6
ACTB
PARP1
ATR
CHEK2
ATG7
NBN
PVR
CHEK1
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ATG5
CHEK1
NBN
ATR
HSP90AA1
TP53
CHEK2
HPRT1
MCL1
PARP1
B2M
BECN1
EDN1
TNF
TXNL4B
ACTB
RAD17
GRB2
HSPA4
BBC3
CCL2
ATG7
GCLM
CASP1
TXNRD1
DDB2
TNFRSF1A
TLR4
TNFRSF10B
GAPDH
DDIT3
IL8
ATF4
NQO1
SERPINE1
SQSTM1
GADD45G
HUS1
DNAJC3
GSR
TNFRSF10A
ATF6
GCLC
LDHA
FAS
FTH1
GADD45A
IL1A
VEGFA
CA9
CDKN1A
BID
HMOX1
ADM
PVR
ATM
HSP90B1
NFAT5
PRDX1
ARNT
CALR
HSPA4L
AQP4
MRE11A
BNIP3L
IL1B
SLC2A1
GSTP1
RIPK1
AQP1
MMP9
Magnitude of gene expression
min
avg
max

of genes that were significantly altered by at least two fold in either one or two dose group(s) was extracted from the heat map and summarized in Tables 1 and 2. Among them, upregulation of cell cycle regulator and stress-responsive tumor suppressor gene CDKN1A, DNA-damage response gene DDIT3 and pro-apoptotic gene BBC3 were observed at both doses after 6 h exposure to CAPE in a dose-dependent manner (Table 1). In addition, the upregulation of growth arrest and DNA-damage response genes GADD45A and GADD45G were induced after incubation with 20 μM CAPE, which further strengthens the initiation of DNA damage signaling and apoptotic pathway. To examine the temporal change in gene expression, MM 8226 cells were treated with 20 μM CAPE for 24 h. The upregulation of the abovementioned genes (6 h treatment of 20 μM CAPE) was decreased after 24 h (Table 2). Interestingly, a group of genes in response to oxidative stress was significantly induced in a dose-dependent manner within 6 h of exposure to 10 and 20 μM CAPE (Table 1). These genes include HMOX-1, NQO-1, SQSTM-1, TXNRD-1, FTH-1, and GCLM. Similarly, upregulation of those genes was decreased after 24 h incubation compared to those after 6 h incubation (Table 2). Decrease in up-regulation of these genes at 24 h suggests that transcriptional activation of early (6 h) initiated gene expression in response to CAPE treatment was attenuated. Taken together, these data suggest that CAPE treatment triggers oxidative stress response and DNA damage signaling and apoptotic pathways, which may provide an explanation for CAPE effects on MM cells.

CAPE induces apoptosis of MM 8226 cells through activation of caspase-3

CAPE was reported as a potent inducer of apoptosis in many tumor cell lines [25]. Our previous data (Tables 1 and 2) showed upregulation of genes involved in apoptotic and DNA damage signaling pathway following CAPE treatment. To further substantiate that MM cell death is due to CAPE-induced apoptosis, we performed a flow cytometric analysis on MM 8226 cells pretreated with CAPE using Annexin V and 7-AAD double staining. In order to observe the possible apoptogenic effect of CAPE within 24 h, we increased the dose of CAPE up to 50 μM. The results showed an induction of apoptosis in MM 8226 cells upon CAPE treatment (Fig. 3). The number of early and late apoptotic cells was increased up to 6 and 12-fold, respectively, after 50 μM CAPE treatment compared to control group (Fig. 3a). The percentages of apoptotic cells increased and were close to 50% of total number of cells following CAPE treatment (Fig. 3b). Of all the key factors involved in programmed cell death, caspase-3 is the downstream executioner for both intrinsic and extrinsic apoptotic pathways [27]. To explore the mechanism of apoptosis induced by CAPE, we assessed the activation of caspase-3 in MM 8226 cells following CAPE treatment. Activity of caspase-3 was found increased in a dose-dependent manner after 24 h incubation with CAPE (Fig. 4a). Decrease of pro caspase-3 protein expression was observed corresponding

Table 1 Differential upregulation of genes involved in various stress and toxicity pathways in MM 8226 cells challenged with 10 and 20 μM CAPE for 6 h

Gene symbol	Gene name	Fold Change 10 μM	*p* Value	20 μM	*p* Value
DNA Damage Signaling/Apoptosis					
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21)	2.91	0.00123	3.20	0.000105
DDIT3	DNA-damage-inducible transcript 3	2.44	0.002225	6.85	0.010171
BBC3	BCL2 binding component 3 (PUMA)	1.83	0.019052	4.29	0.000167
GADD45A	Growth arrest and DNA-damage-inducible, alpha			2.18	0.019383
GADD45G	Growth arrest and DNA-damage-inducible, gamma			6.71	0.00006
Oxidative Stress					
HMOX-1	Heme Oxygenase (Decycling) 1	3.40	0.000022	5.63	0.000142
NQO-1	NAD(P)H dehydrogenase (quinone 1)	2.79	0.000198	2.43	0.000007
SQSTM-1	Sequestosome 1	2.22	0.000071	3.72	0.000074
TXNRD-1	Thioredoxin reductase 1	3.65	0.000764	2.19	0.00023
FTH-1	Ferritin, heavy polypeptide 1	1.42	0.011308	3.36	0.000158
GCLM	Glutamate-cysteine ligase, modifier subunit	2.69	0.000001	3.09	0.00001
GCLC	Glutamate-cysteine ligase, catalytic subunit	2.75	0.004565		

Fold change is expressed relative to the respective untreated controls. The PCR expression array analysis was conducted in three independent repeats. Upregulation of genes is considered statistically significant if p value ≤0.5

Table 2 Differential upregulation of genes involved in various stress and toxicity pathways in MM 8226 cells challenged with 20 μM CAPE for 6 and 24 h

Gene symbol	Gene name	Fold Change 6 h	*p* Value	24 h	*p* Value
DNA Damage Signaling / Apoptosis					
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21)	3.20	0.000105	1.55	0.001822
DDIT3	DNA-damage-inducible transcript 3	6.85	0.010171	3.69	0.000045
BBC3	BCL2 binding component 3 (PUMA)	4.29	0.000167		
GADD45A	Growth arrest and DNA-damage-inducible, alpha	2.18	0.019383	1.70	0.004533
GADD45G	Growth arrest and DNA-damage-inducible, gamma	6.71	0.00006	1.80	0.000163
Oxidative Stress					
HMOX-1	Heme Oxygenase (Decycling) 1	5.63	0.000142	2.13	0.000647
NQO-1	NAD(P)H dehydrogenase (quinone 1)	2.43	0.000007	1.82	0.000435
SQSTM-1	Sequestosome 1	3.72	0.000074	1.83	0.000035
TXNRD-1	Thioredoxin reductase 1	2.19	0.00023		
FTH-1	Ferritin, heavy polypeptide 1	3.36	0.000158	2.41	0.004725
GCLM	Glutamate-cysteine ligase, modifier subunit	3.09	0.00001	1.17	0.038405
GCLC	Glutamate-cysteine ligase, catalytic subunit			2.24	0.017411

Fold change is expressed relative to the respective untreated controls. The PCR expression array analysis was conducted in three independent repeats. Upregulation of genes is considered statistically significant if p value ≤0.5

to an increase of active caspase-3 in a dose-dependent manner (Fig. 4b). In addition, poly(ADP-ribose)polymerase (PARP), a caspase-3 downstream target known for repair of DNA damage, was found to be degraded with decrease of full-length PARP protein expression in a dose-dependent manner (Fig. 4c). These results suggest that CAPE induces apoptosis of MM 8226 cells through caspase-3 mediated apoptotic pathway.

CAPE exerts cytotoxicity in MM 8226 cells through oxidative stress

CAPE induction of genes responsive to oxidative stress suggests that cellular oxidative stress may contribute to the cytotoxic effect of CAPE in MM 8226 cells. To explore the role of oxidative stress behind CAPE action, we first determined the intracellular production of ROS in the presence and absence of CAPE. Our data showed that intracellular ROS level in MM 8226 cells was significantly decreased in the presence of CAPE up to 50 μM for 24 h (Fig. 5a). We then examined the effects of antioxidant intervention and glutathione depletion on CAPE cytotoxicity. One-hour pretreatment of NAC (0.1 ~ 1 mM), a known intracellular antioxidant and glutathione precursor, intervened the inhibitory effect of 30 μM CAPE on MM 8226 cell growth (Fig.5b). Viability of MM 8226 cells was recovered in a dose-dependent manner at all time points tested (24, 48, and 72 h). On the other hand, 24 h pretreatment of BSO (0.1 ~ 1 mM), a known glutathione synthesis inhibitor, sensitized MM 8226 cells to CAPE cytotoxicity following additional 24 h incubation (Fig. 5c). With co-treatment of BSO, cell viability dropped more than 20% compared to 30 μM CAPE alone. Interestingly, BSO from 0.1 to 1 mM resulted in similar cell viability of around 50%, indicating BSO as low as 0.1 mM was sufficient to exert its effect. These data, taken together, indicate that CAPE exerts cytotoxicity in MM 8226 cells through oxidative stress by glutathione depletion but not ROS overproduction.

Discussion

Multiple myeloma is a B cell neoplasm featured by proliferation and accumulation of malignant plasma cells in the bone marrow. It is associated with severe bone disease and remains incurable even with high dose of chemotherapy [28]. Therefore, there is a great need to identify new therapeutic strategies that inhibit the progression of multiple myeloma disease without significant drug resistance and eventually increase quality of life and survival of multiple myeloma patients.

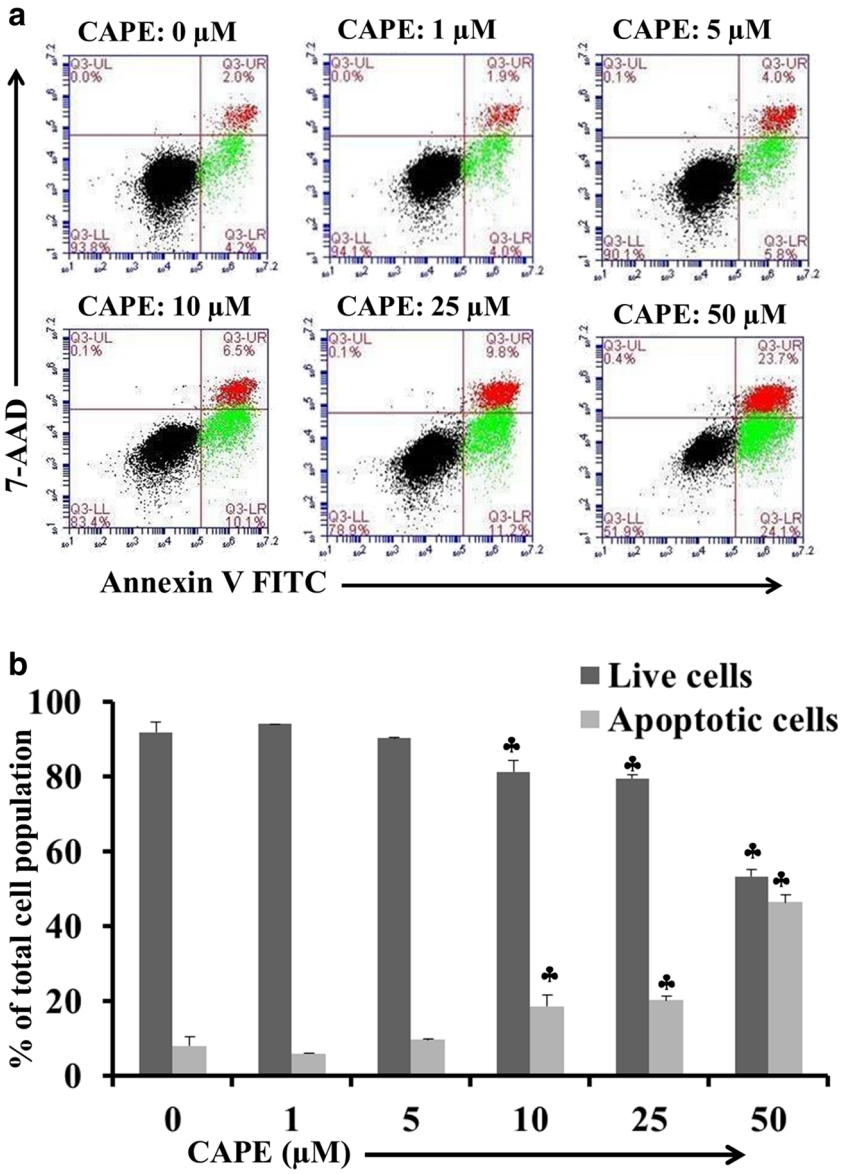

Fig. 3 Apoptosis of MM 8226 cells induced by CAPE within 24 h. Annexin V FITC-A vs 7-AAD-A plots from the gated cells show the populations corresponding to viable and non-apoptotic (Annexin V^- 7-AAD^-, black dots), early (Annexin V^+ 7-AAD^-, green dots), and late (Annexin V^+ 7-AAD^+, red dots) apoptotic cells (**a**). Percentages of live versus apoptotic cells obtained from flow cytometric analysis are compared below (**b**). ♣: $p < 0.05$ versus control (0 μM CAPE)

In this study, we evaluated the potential anti-myeloma effect of CAPE, a natural phenolic compound identified from bee glue. We found that CAPE inhibited the growth of human MM cell line RPMI 8226 in a dose and time-dependent manner. Similar inhibitory effect by CAPE but to different extent was observed on the growth of two other MM cell lines NCI-H929 and U266, confirming the common activity of CAPE in reduction of MM cell viability. No inhibitory effect of CAPE on the growth of human peripheral blood B cells was observed, suggesting that the CAPE doses applied to MM cells is not toxic to normal plasma cells. To explore the molecular mechanism of CAPE-induced cytotoxicity in MM cells, we evaluated the potential cytotoxic effect of CAPE through a panel of genes responsible for cellular stress and toxicity in MM 8226 cells. These genes are selected to represent various pathways including oxidative stress, hypoxia signaling, osmotic stress, cell death, inflammatory response, DNA damage signaling, and heat shock proteins/unfolded protein response. Our data showed that CAPE significantly altered expression of genes from DNA damage signaling and oxidative stress pathways in a dose and time dependent manner (Tables 1 and 2). After 6 h incubation with 10 and 20 μM CAPE, expression of several genes responsible for DNA damage, growth arrest and apoptosis increased toward higher concentrations of CAPE tested. This result suggests that CAPE may trigger DNA damage-induced cell death by apoptosis, which led us to verify if apoptosis of MM cells is actually induced by CAPE through various approaches. In addition, seven genes responding to oxidative stress were significantly

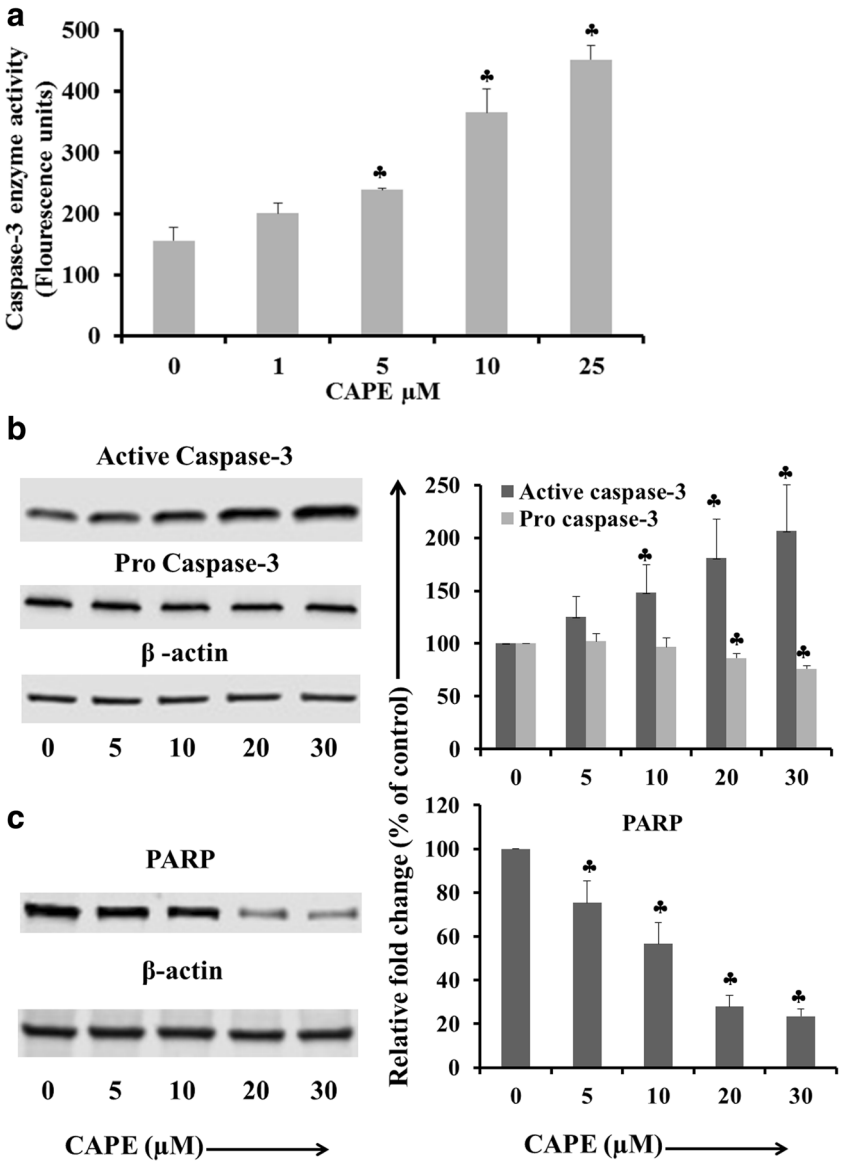

Fig. 4 CAPE-induced apoptosis was mediated through caspase-3 pathway evidenced by dose-dependent activation of caspase-3 enzyme activity (**a**), increased protein expression of active caspase-3 and corresponding decreased protein expression of pro caspase-3 (**b**), and decreased protein expression of PARP, a caspase-3 downstream gene (**c**). ♣: $p < 0.05$ versus control (0 μM CAPE)

upregulated. This suggests that oxidative stress may be involved in the effect of CAPE on MM cells, which directed our follow-up investigation on the role of oxidative stress. After 24 h incubation with 20 μM CAPE, expression of those genes from both categories significantly decreased, indicating that DNA damage, apoptosis and oxidative stress pathways responded to CAPE action in a time-dependent manner. Understanding the time effect in toxicological studies may shed a light on the mechanism of drug action. We recently reported potent cytoprotectant-induced gene expression profiling at different time point within 24 h, which reveals key players responsible for different phase of drug response [29]. Similarly, we observed that upregulation of genes responsible for oxidative stress was increased up to 6 h and down by end of 24 h. In addition, it is unquestionable that initiation of gene expression by a stressor, a compound like CAPE, is sequentially followed by cell signaling and corresponding protein synthesis. It is not surprising to see a later decreased expression of those early upregulated genes especially after a single dose of the tested compound since alteration of gene expression and corresponding protein expression usually follows a bell-shape curve [30].

Modes of cell death include apoptosis, necrosis, and autophagy [31]. Apoptosis, or programmed cell death, is a fundamental and complex process that allows killing and removal of unwanted cells by the host organism during the processes of development, normal homeostasis, and disease states. The anti-cancer effect of CAPE was recently reported through induction of caspase or p53-mediated apoptosis in breast cancer, lung cancer, and melanomas [32, 33]. Here,

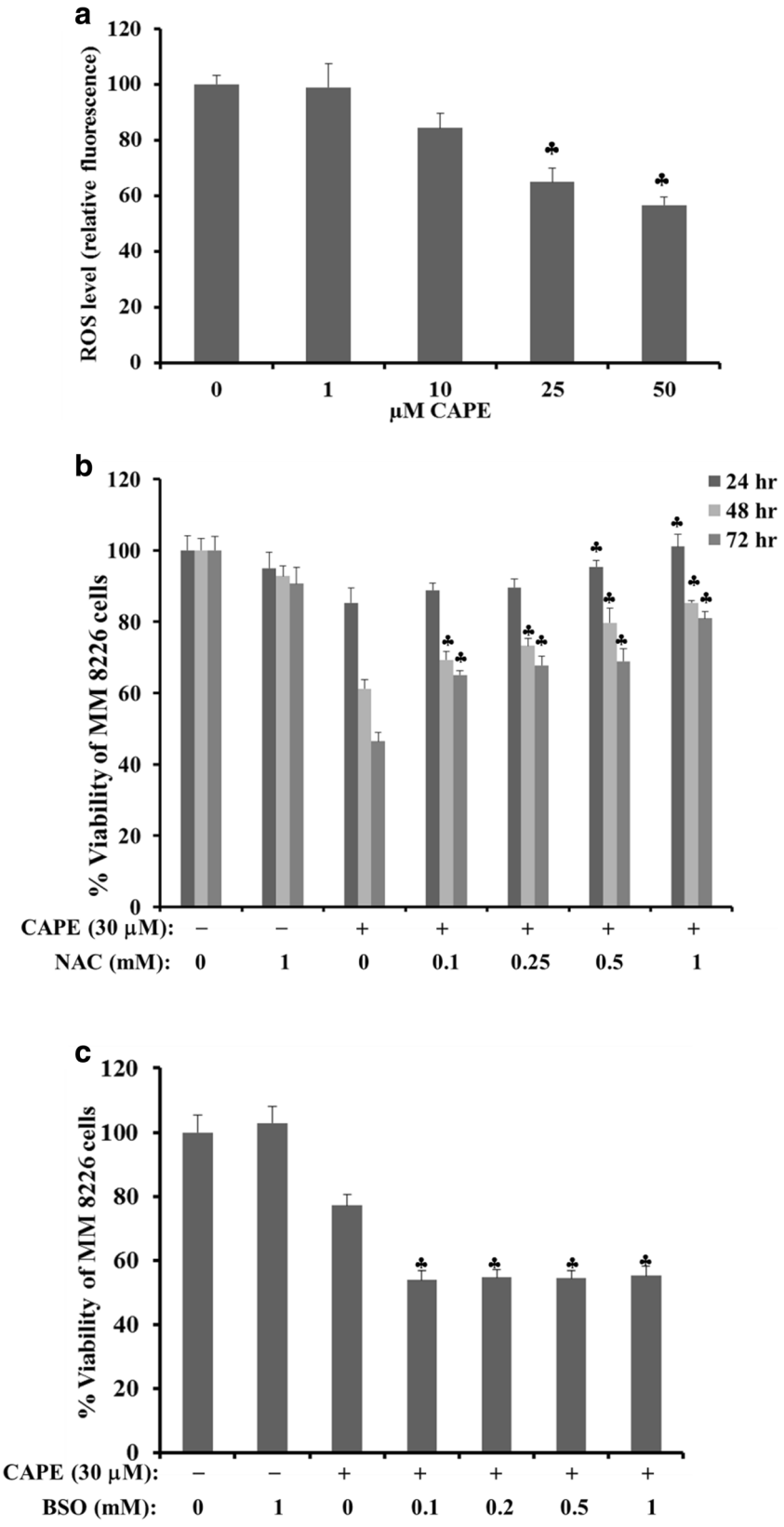

Fig. 5 Effects of ROS production, antioxidant intervention and glutathione depletion on cytotoxicity of CAPE in MM 8226 cells. Intracellular ROS level in MM 8226 cells was significantly decreased after 24 h incubation of CAPE in a dose– dependent manner (**a**). ♣: $p < 0.05$ versus control (0 μM CAPE). One-hour pretreatment of antioxidant NAC ameliorated cytotoxicity of 30 μM CAPE in MM 8226 cells in a dose and time – dependent manner within 72 h (**b**). Pretreatment of BSO for 24 h enhanced cytotoxicity of 30 μM CAPE following additional 24 h incubation in MM 8226 cells (**c**). ♣: $p < 0.05$ versus control (30 μM CAPE only)

our results show that MM 8226 cells underwent apoptosis dose–dependently after 24 h exposure of CAPE by flow cytometric analysis. In addition, CAPE was found activating caspase-3 enzyme, which correlated well with increase

of active form along with corresponding decrease of pro form of this key executioner of apoptosis. CAPE cleavage of PARP, a nuclear substrate of caspase 3 [34], further confirmed what we discovered earlier from stress and toxicity pathway analysis.

Oxidative stress is a condition resulting from an imbalance between intracellular ROS and antioxidant defense system. ROS is known to be induced to a level that triggers apoptosis of cancer cells by chemotherapy agents [35, 36]. Although our previous work found that CAPE protects human endothelial cells from cellular oxidative stress induced by menadione [26], more evidence has indicated that polyphenols such as epigallocatechin gallate, resveratrol, and curcumin can act as pro-oxidants and induce oxidative stress in cancer cells [37]. Pro-oxidant activity along with the difference in redox state between cancer and normal cells may provide an explanation for the selective cytotoxicity of polyphenolic compounds. Compounds with pro-oxidant activity are usually considered inducers of oxidative stress through either over production of ROS or inhibition of intracellular antioxidants [38]. Measurement of intracellular ROS level indicates that CAPE lowered the production of ROS in MM 8226 cells, which is consistent with what we previously reported in normal human endothelial cells [26]. On the other hand, alteration of the level of major intracellular antioxidant glutathione may provide an explanation to CAPE cytotoxicity in MM cells. This is evidenced by a dose- and time-dependent increase of CAPE-treated MM 8226 cell viability when pretreating the cells with glutathione precursor NAC and additional inhibition of CAPE-treated MM 8226 cell growth with pretreatment of glutathione synthesis inhibitor BSO. Taken together, these studies suggest that oxidative stress, particularly induced by glutathione intervention, may play an important role in the anti-myeloma effect of CAPE on MM 8226 cells.

In conclusion, our data support that CAPE inhibits the growth of malignant blood cells including RPMI 8226, NCI –H929 and U266 but not its normal counterpart at the tested doses. The apoptotic effect of CAPE mainly involves the induction of caspase 3 and generation of oxidative stress through glutathione intervention. Results of these studies suggest CAPE as a potential anti-myeloma drug candidate.

Author contributions Conceived and designed the experiments: XW. Performed the experiments: EM, HP, ML.YB, and MK. Analyzed the data: EM, HP, ML, RS, and XW. Critically reviewed the paper: RS. Wrote the paper: XW.

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Compliance with ethical standards

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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