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# Growth inhibitory, apoptotic and anti-inflammatory activities displayed by a novel modified triterpenoid, cyano enone of methyl boswellates

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Triterpenoids are pentacyclic secondary metabolites present in many terrestrial plants. Natural triterpenoids have been reported to exhibit anti-inflammatory and anti-carcinogenic activities. Here, we show that modifications of ring A of boswellic acid (2 cyano, 3 enone) resulted in a highly active growth inhibitory, anti-inflammatory, pro-differentiative and anti-tumour triterpenoid compound called cyano enone of methyl boswellates (CEMB). This compound showed cytotoxic activity on a number of cancer cell lines with IC<sub>50</sub> ranging from 0.2 to 0.6 μM. CEMB inhibits DNA synthesis and induces apoptosis in A549 cell line at 0.25 μM and 1 μM concentrations, respectively. CEMB induces adipogenic differentiation in 3T3-L1 cells at a concentration of 0.1 μM. Finally, administration of CEMB intra-tumourally significantly inhibited the growth of C6 glioma tumour xenograft in immuno-compromised mice. Collectively, these results suggest that CEMB is a very potent anti-tumour compound.

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## 1. Introduction

The discovery that 2-cyano-3,12-dioxoolean-1,9(11)-diene-28-oic acid (CDDO) (Honda *et al.* 1998), synthesized from naturally abundant oleanolic acid, displayed diverse biological activities ranging from suppression of iNOS and COX2, cellular proliferation of malignant and premalignant cells, to induce differentiation of malignant and nonmalignant cells (Suh *et al.* 1999), has renewed interest in the pentacyclic triterpenoids and natural products. CDDO and its derivatives are highly active in suppressing cellular proliferation

of human leukaemia (Suh *et al.* 1999; Konopleva *et al.* 2002; Place *et al.* 2003; Konopleva *et al.* 2004), breast cancer (Honda *et al.* 1999; Lapillonne *et al.* 2003; Konopleva *et al.* 2006) and several other cancer cells. In addition, 2-cyano-3,12-dioxooleana-1,9-dien-28-imidazole (CDDO-Im) exhibits strong inhibitory activity against production of NO, induced by IFN-γ in mouse macrophages (IC<sub>50</sub>=0.1 nM) (Honda *et al.* 1998; Suh *et al.* 1999). CDDO was also found to induce monocytic differentiation of human myeloid leukaemia cells and adipogenic differentiation of mouse 3T3-L1 fibroblasts

**Keywords.** Cancer therapeutics; cytotoxicity; differentiation; natural products

Abbreviations used: AMC, 7-amino-4-methylcoumerin; BrdU, 5-bromo-2'-deoxyuridine; CDDO, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid; CEMB, cyano enone of methyl boswellates; COX-2, cyclooxygenase-2; DAPI, 4',6-diamidino-2-phenylindole; DMEM, Dulbecco's modified eagle's medium; DMSO, dimethyl sulphoxide; DPBS, Dulbecco's phosphate buffer saline; FBS, fetal bovine serum; IC<sub>50</sub>, 50% inhibitory concentration; IFN IFN-γ, interferon interferon-γ; iNOS, induced nitric oxide synthase; MTT, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide; NED, *N*-(1-naphthyl) ethyl-enediamine; TGF-β, transforming growth factor factor-β

(Suh *et al.* 1999; Wang *et al.* 2000). It has been shown that CDDO and CDDO-Im inhibit inflammatory response and tumour growth *in vivo* (Place *et al.* 2003). Notably, CDDO-Im is approximately 10-fold more potent than CDDO in inhibiting cancer cell proliferation and in inducing differentiation in leukaemia cells (Kim *et al.* 2002). Various derivatives of CDDO also showed potential apoptotic activity on many types of cancer cell lines. For instance, CDDO induces apoptosis of acute myelogenous leukaemia (Konopleva *et al.* 2004), osteosarcoma (Ito *et al.* 2001) and skin cancer (Hail *et al.* 2004). The methyl ester of CDDO, CDDO-Me, triggers apoptosis in lung carcinoma (Kim *et al.* 2002) and prostate cancer cells (Hyer *et al.* 2008) whereas its imidazole ester, CDDO-Im, induces apoptosis in pancreatic (Samudio *et al.* 2005) and ovarian cancer (Petronelli *et al.* 2009). Despite the multifunctional activities of synthetic triterpenoids, the molecular mechanisms that mediate the effects are not fully understood except that CDDO and its derivatives have been shown to induce apoptosis either by activating intrinsic mitochondria-dependent or extrinsic-death-receptor-mediated pathways, depending on the cell types (Ito *et al.* 2000, 2001; Samudio *et al.* 2006; Brookes *et al.* 2007). In addition, it has recently been shown that CDDO-Me activates endoplasmic reticulum stress (ER stress) and thereby triggers the DR5-mediated apoptotic pathway (Zou *et al.* 2008). CDDO and derivative compounds are being used in phase I/II clinical trials as novel cancer therapeutic agents (Liby *et al.* 2007b; Hyer *et al.* 2008). All these studies suggest the potential of modified triterpenoids in anti-inflammatory and anti-carcinogenic applications.

Although, CDDO and its derivatives show great promise as therapeutic molecules, it is important to explore other triterpenoids for their efficacy in these actions, to provide alternatives to CDDO. A recent publication suggests that modified betulinic acid also shows activities similar to those

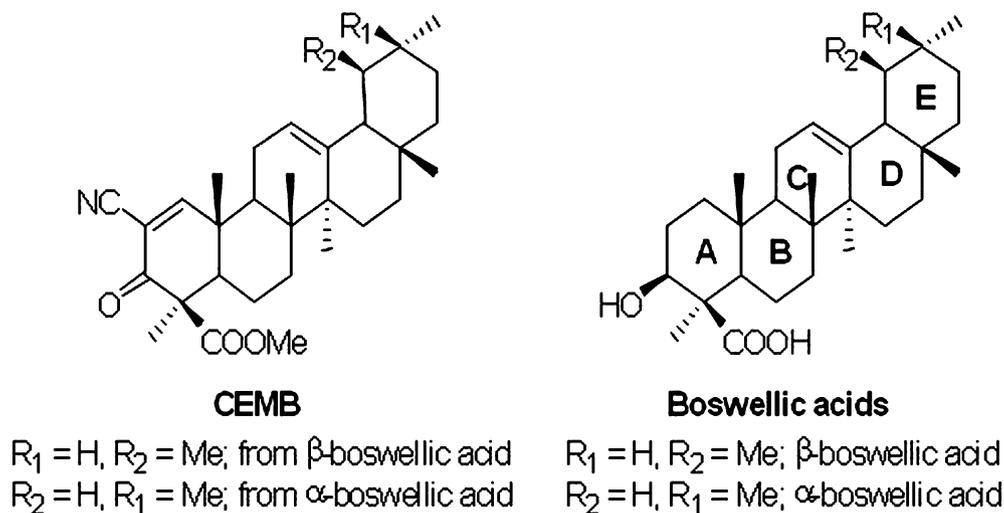
reported for CDDO (Liby *et al.* 2007a). However, it is necessary to explore other triterpenoids because of uncertainties in their metabolic turnover and other potential side effects that may be dependent on the molecular species. To this end, we obtained several triterpenoids available from Indian plants and made chemical modifications that are similar to CDDO. The details of their synthesis, characterization and preliminary activities of these triterpenoids such as inhibition of IFN- $\gamma$ -induced NO production and cytotoxicity have been reported earlier (Subba Rao *et al.* 2008).

Here we report that cyano-enone modified methyl boswallates (CEMB) (figure 1) (1) is a potent inhibitor of NO production induced by IFN- $\gamma$  in elicited mouse primary macrophages, (2) is an inhibitor of DNA synthesis, (3) is an inducer of caspase 8 and caspase 3 activation and thereby apoptosis in A549 cells, (4) is an inducer of adipogenic differentiation in 3T3-L1 cells and (5) has anti-tumour activity on tumour xenografts in mouse model. Our results suggest the potential of CEMB as a chemotherapeutic agent.

## 2. Materials and methods

### 2.1 Cell cultures and reagents

Dimethyl sulphoxide (DMSO), 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), phosphoric acid, *N*-(1-naphthyl) ethyl-enediamine (NED) and sulfanilamide were purchased from Sigma. Dulbecco's modified eagle's medium (DMEM), DMEM-F12, RPMI, Dulbecco's phosphate buffer saline (DPBS), certified fetal bovine Serum, penicillin-streptomycin, amphotericin B and trypsin were purchased from GIBCO. Caspase 8 and Caspase 3 Activity Assay Kits were from Sigma (Product code: CASP-8-F and CASP-3-F). 5-Bromo-2'-deoxyuridine (BrdU), anti-BrdU



**Figure 1.** Structure of CEMB. The structure of synthetic triterpenoids CEMB and the parent compound of CEMB, boswellic acid.

antibody (ab-3) and Texas red-conjugated goat antimouse antibody were purchased from Oncogene. Human cancer cell lines A549, A431, HT1080, HepG2, HeLa, HCT116, MCF-7, T47D, T84, CaCO2, JEG, PC3, mouse 3T3-L1, Rat C6 glioma cells, and human keratinocyte cell line HaCaT were routinely grown in DMEM with 10% FBS. Jurkat, HL-60, THP-1 and K562 cell lines were grown in RPMI with 10% FBS. Penicillin-streptomycin and amphotericin B were added in the culture to prevent microbial growth. CEMB was synthesized from boswellic acid as described previously. CEMB was dissolved in DMSO at 10 mM concentration and stored at  $-20^{\circ}\text{C}$ . The dilutions were made in culture medium just before the treatments.

### 2.2 Nitric oxide assay

Two-month-old C57black6 mice were injected intraperitoneally with 4% thioglycolate route. On the third day, the mice were sacrificed by cervical dislocation, and 6 ml of 0.32 M sucrose solution was injected in to the peritoneal region, and the macrophages were collected. Cells were centrifuged, washed with DPBS and plated ( $1.5 \times 10^5$  cells/well) in a 96-well plate. The cells were treated with IFN- $\gamma$  (10 U/ml) in the presence or absence of CEMB and incubated for 3 days. NO was rapidly oxidized to nitrite in the culture medium (RPMI+10% FBS), and determination of nitrite concentration was used as a measure of NO production. Equal volumes of cell supernatants and Griess reagent (Ding *et al.* 1990) were added to individual wells of a 96-well plate and the absorbance was measured at 590 nm in an ELISA reader. Nitrite concentration was estimated using a sodium nitrite standard curve. Nitrite concentration was normalized with total protein determined by the Bradford method. The experiments were performed multiple times and the result presented here are from three independent experiments. Graph Pad Prism software was used to assess the inhibitory concentration ( $\text{IC}_{50}$ ) of the compound.

### 2.3 Cytotoxicity assay using MTT reagent

The cells were plated in 96-well tissue culture clusters (seeded at densities ranging from  $5 \times 10^3$  to  $8 \times 10^3$  cells/well) depending on the cell line and incubated at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$  atmosphere. After attachment of the cells (usually taking 3–4 h), different concentrations of the compound were added and incubated for 72 h. MTT solution (20  $\mu\text{l}$  of 5 mg/ml) was added to each well and the incubation continued for an additional 3 h. The dark blue formazan crystals formed within the healthy cells were solubilized with DMSO, the plates read in ELISA plate reader (7520 Microplate Reader, Cambridge Technologies Inc.) at 550 nm and the absorbance was correlated with the cell number. Experiments were performed in triplicates and the values are the average of three ( $n=3$ )

independent experiments. The inhibitory concentration ( $\text{IC}_{50}$ ) of the compound was assessed by Graph Pad Prism software.

### 2.4 Cell proliferation assay using BrdU incorporation

BrdU incorporation assay was carried out as described earlier (Wajapeyee and Somasundaram 2003). In brief, cells were plated at a density of  $1.2 \times 10^4$  cells/well in a 96-well plate. After 24 h, cells were treated with the compound for another 24 h and BrdU (20  $\mu\text{M}$ ) was added to the culture medium 4 h prior to the termination. The cells were washed with phosphate buffered saline (DPBS) and fixed with 70% ethanol for 5 min. The cells were washed again with DPBS and incubated with 2 N HCl+Triton $\times 100$  to denature the chromosomal DNA. The cells were washed thrice to completely remove HCl, followed by incubation again with 1% BSA for blocking nonspecific binding. BrdU incorporation was determined by treating with anti-BrdU antibody and Texas red conjugated goat-antimouse IgG.

### 2.5 DNA fragmentation assay

Low-molecular-weight genomic DNA was extracted as described previously (Yawata *et al.* 1998). In brief, approximately  $1 \times 10^6$  cells were plated and treated with 1  $\mu\text{M}$  (A549 cells) and 0.5  $\mu\text{M}$  (HL60 cells) of CEMB for various treatment hours. All the cells (including floating cells) were harvested by trypsinization and washed with DPBS. Cells were lysed with the lysis buffer, and treated 40 mg/ml RNase A and proteinase K at  $37^{\circ}\text{C}$  for 1 h. DNA was precipitated using isopropanol, and subjected on to 1.0% agarose gels. The gels were stained with 1  $\mu\text{g/ml}$  ethidium bromide.

### 2.6 Caspase activity assay

Caspase assays were performed using fluorometric kits (Sigma-Aldrich, St. Louis, USA). The cells were treated with CEMB (1  $\mu\text{M}$ ) for 3, 6, 12 and 24 h and lysed with the lysis buffer (50 mM HEPES, pH 7.4, with 5 mM CHAPS and 5 mM DTT). Later, 100  $\mu\text{l}$  of the lysate was incubated with the caspase 3 or caspase 8 peptide substrates (Ac-DEVD-AMC and Ac-IETD-AMC, respectively) for 1 h as described in the manufacturer's protocol (Caspase 8 and Caspase 3 Assay Kits, Fluorometric, SIGMA). Caspase inhibitors were also used to confirm the result. The caspase activity was represented as quantity of fluorescent 7-amino-4-methylcoumerin (AMC) released/mg protein/h.

### 2.7 Oil red O staining

Induction of differentiation in 3T3-L1 cells was performed as described earlier (Wang *et al.* 2000). In brief, 3T3-L1

fibroblasts cells were seeded in 6-well plate at a cell density of 0.4 million cells/well and cultured for around 4 days until it reached confluence. The culture medium was replaced with fresh medium once in 2 days. Cells were treated with CEMB at various concentrations starting from 0.05  $\mu\text{M}$  to 0.25  $\mu\text{M}$ . The standard method to induce adipogenesis (0.5 mM IBMX+0.5  $\mu\text{M}$  dexamethasone + 1.7  $\mu\text{M}$  insulin) was also included in the experiment to compare the efficiency of CEMB-induced differentiation. To stain the triglycerides, cells were washed twice with PBS and fixed in 3.7% formaldehyde for 1 h followed by oil red O staining as described previously (Hansen *et al.* 1999).

### 2.8 Tumour xenograft study

Female nude mice at approximately 2 months age and 20–25 g weight were used for tumour xenograft studies. C6 rat glioma cells were injected subcutaneously ( $3 \times 10^6$  cells/mouse) over the left flank of mice (day 0). After 4 days, treatment with CEMB was initiated with CEMB in DMSO, castor oil and PBS mixed in the ratio of 1:1:8 in 0.1 ml solution and injected at the site of the tumours. An equivalent number of mice were injected with vehicle control (DMSO, castor oil and PBS in the same proportions). The treatment was carried out twice daily (every 12 h) for the first 2 days and then daily (every 24 h) for 3 more days. On day 14, the mice were sacrificed and tumours was excised and weighed. A portion of the tumour was fixed in 10% buffered formalin for histological analysis.

## 3. Results

### 3.1 CEMB inhibits NO production in primary mouse macrophages

To test whether CEMB inhibits NO production, mouse primary macrophages were plated and NO production was induced by IFN- $\gamma$ , in the presence or absence of CEMB. Natural boswellic acid has been reported to possess anti-inflammatory activity at a concentration of 40  $\mu\text{M}$  (Gayathri *et al.* 2007). In our study, 20  $\mu\text{M}$  of boswellic acid was used as the highest concentration, and at this concentration, naturally occurring boswellic acid did not inhibit NO production (data not shown). However, appropriate modifications in the A ring resulted in CEMB showed potent inhibition of NO production ( $\text{IC}_{50}=57.3$  nM) in a concentration-dependent manner starting from 1, 0.1, 0.01 and 0.001  $\mu\text{M}$  (figure 2). DMSO was used as a negative control, whereas CDDO and CDDO-Me were used as positive controls. We observed that CDDO and CDDO-Me were more potent in inhibiting NO production ( $\text{IC}_{50} \leq 1$  nM) than CEMB.

### 3.2 Cytotoxic effect of CEMB in tumour cell lines

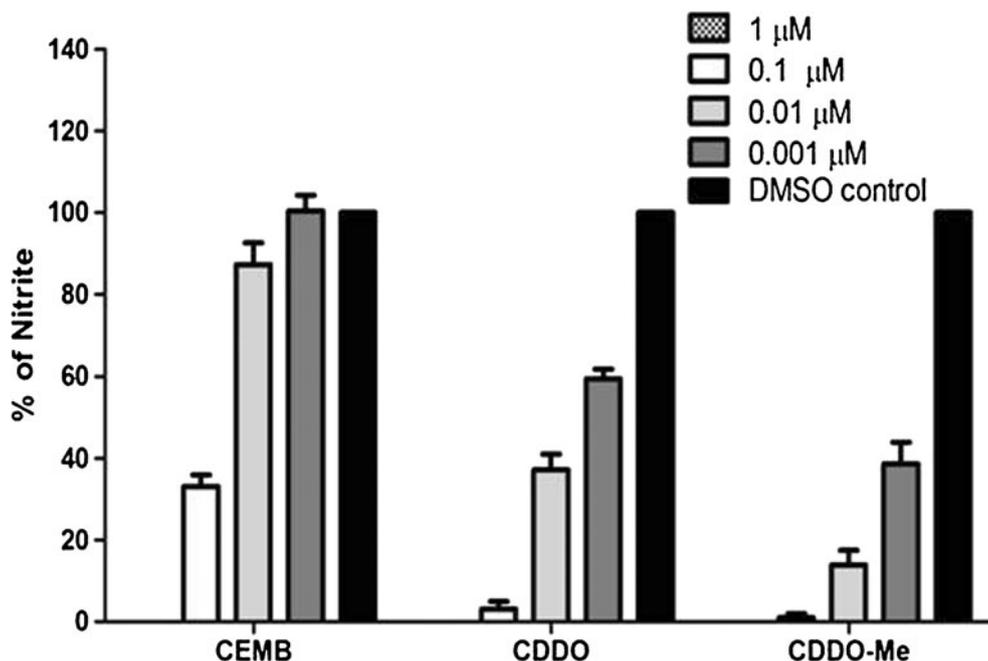
To investigate whether CEMB inhibits cancer cells growth, various tumour cell lines were treated with different concentrations of CEMB and evaluated using MTT assay. The CEMB treatment resulted in reduced concentration of formazan crystals, indicating either a growth inhibitory or cytotoxic effect of CEMB. The compound was active in all cancer cell lines we tested with  $\text{IC}_{50}$  ranging from 0.10  $\mu\text{M}$  (HL60) to 0.7  $\mu\text{M}$  (MCF7) (table 1). Lung and breast carcinoma cells ( $\text{IC}_{50} \geq 0.7$   $\mu\text{M}$ ) were comparatively less sensitive to the compound than the leukaemia cells ( $\text{IC}_{50} \leq 0.25$   $\mu\text{M}$ ). The percentage of cells showing cytotoxicity at 1  $\mu\text{M}$  concentration was in the range of 97% (Raji cells-Bcl-2 overexpressed) to 56.8% (JEG cells). These results indicate that the compound CEMB has a potent cytotoxic/growth inhibitory activity.

### 3.3 CEMB inhibits DNA synthesis preferentially in carcinoma cells

By MTT assay, CEMB was found to be a potent cytotoxic/growth inhibitory molecule. In order to test whether CEMB is specific against cancer cells, A549 (human lung carcinoma) and HaCaT (human keratinocyte) cells were treated with CEMB at various concentrations. BrdU incorporation assay was done as described in materials and methods. As shown in figure 3, CEMB inhibited BrdU incorporation in both the cell lines at higher concentrations. However, at lower concentration, we observed differential sensitivity between A549 and HaCaT cells (compared at 0.1  $\mu\text{M}$  and 0.25  $\mu\text{M}$ ). This differential response could be due to cell type differences. Alternatively, this could be due to higher sensitivity of tumour cells to CEMB than the normal cells. An extensive validation of this study in various cell lines of tumour and normal origin is required to confirm the finding.

### 3.4 CEMB induces DNA fragmentation

CEMB showed cytotoxic activity on various cancer cell lines and it also affects DNA synthesis, at least in A549 cells. In order to delineate the mechanism of cell death mediated by CEMB, we performed DNA fragmentation assay, which is characteristic for apoptosis. A549 (24, 48, and 72 h treatments) and HL60 (12, 24 and 48 h treatments) cells were treated with 1  $\mu\text{M}$  and 0.5  $\mu\text{M}$  of CEMB, respectively. Low-molecular-weight DNA from these cells was resolved in 1.0% agarose gels. Figure 4A shows DNA ladder formation in A549 cells at indicated time points at a concentration of 1  $\mu\text{M}$ . Figure 4B shows DNA ladder formation after 48 h of treatment in HL60 cells at 0.5  $\mu\text{M}$  concentration. These data suggest that CEMB is a potent inducer of apoptosis in A549 and HL60 cells.

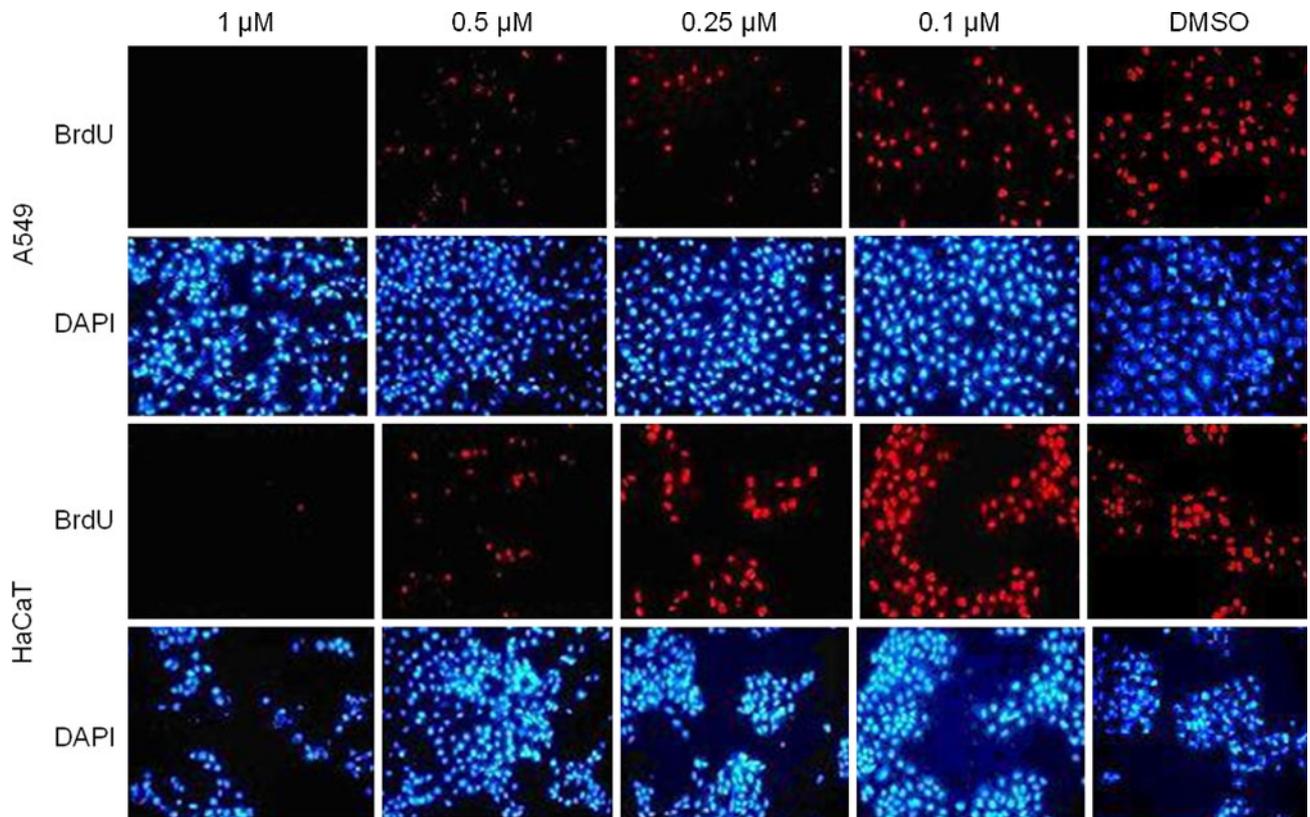


**Figure 2.** Nitric oxide assay on mouse primary macrophages using Griess reagent. Mice were injected with 4% thioglycolate solution and sacrificed on the third day. Macrophages were collected with 0.32 M of ice cold sucrose solution. The collected cells were plated at a density of  $1.5 \times 10^5$  cells/well in a 96-well plate. After 3–4 h, the cells were treated with IFN- $\gamma$  (10 U/ml) in presence or absence of CEMB with various concentrations for 72 h. Griess assay was done as described in materials and methods. The experiment was performed at least three times and the result presented here is the mean  $\pm$  SEM of three independent experiments.

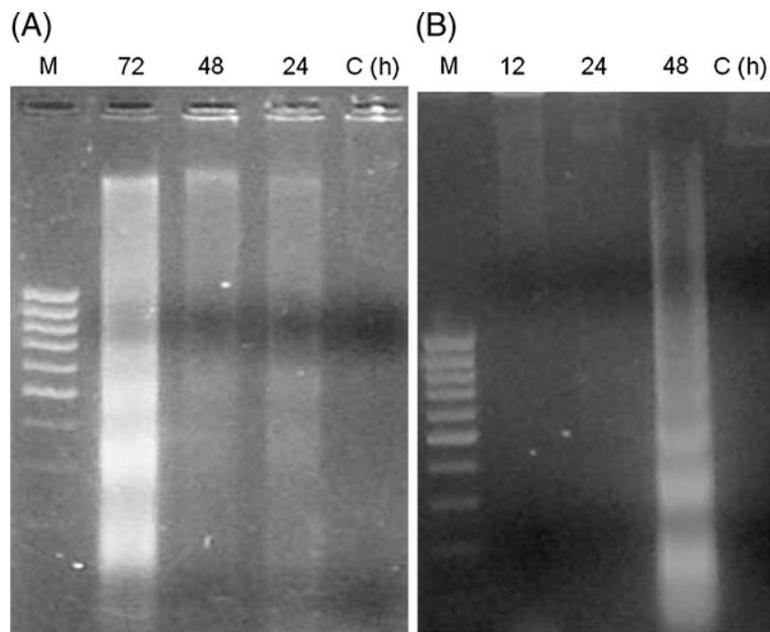
**Table 1.** Cytotoxic effect of CEMB on cancer cell lines

No.	Cell line	Description	% of cytotoxicity at 1 $\mu$ M conc.	IC <sub>50</sub> ( $\mu$ M)
1	A549	Lung carcinoma	77.4 $\pm$ 11	0.51
2	PC3	Prostate carcinoma	71.7 $\pm$ 7	0.51
3	K562	Leukemia	58.6 $\pm$ 7	0.24
4	Jurkat	Leukemia	83.7 $\pm$ 1	0.16
5	THP-1	Leukemia	72.5 $\pm$ 6	0.25
6	HeLa	Cervical carcinoma	78.5 $\pm$ 3	0.27
7	CaCo2	Colon carcinoma	71.5 $\pm$ 9	0.35
8	T84	Colon carcinoma	65.5 $\pm$ 0.5	0.29
9	MCF-7	Breast carcinoma	61.4 $\pm$ 11	0.70
10	HepG2	Hepato carcinoma	86.6 $\pm$ 3	0.39
11	T47D	Breast carcinoma	79.1 $\pm$ 5	0.55
12	JEG	Chorio carcinoma	56.8 $\pm$ 16	0.23
13	HCT116	Colon carcinoma	68.4 $\pm$ 1	0.31
14	HT1080	Fibrosarcoma	60.8 $\pm$ 5	0.34
15	C6	Rat glioma	82.8 $\pm$ 1	0.34
16	Jurkat (C8 deficient)	Leukemia	79.1 $\pm$ 5	0.24
17	Raji (Bcl-2 over expressed)	Lymphoma	96.4 $\pm$ 0.4	0.12
18	HL60	Leukemia	95.0 $\pm$ 0.6	0.10

Cells were plated ( $5 \times 10^3$ – $8 \times 10^3$ ) cells/well in a 96-well cluster plate. After 3–4 h, the cells were treated with various concentrations of CEMB. MTT assay was done after 72 h of incubation. The results are presented as the mean  $\pm$  SEM of three independent experiments. IC<sub>50</sub> values were calculated using Graph Pad Prism software.



**Figure 3.** BrdU incorporation assay. A549 and HaCaT cells were plated in a 96-well plate and treated with CEMB for 24 h. BrdU (20  $\mu$ M) was added after 20 h of treatment for a period of 4 h. BrdU staining was done using anti-BrdU antibody as described in materials and methods. The experiment was performed twice.

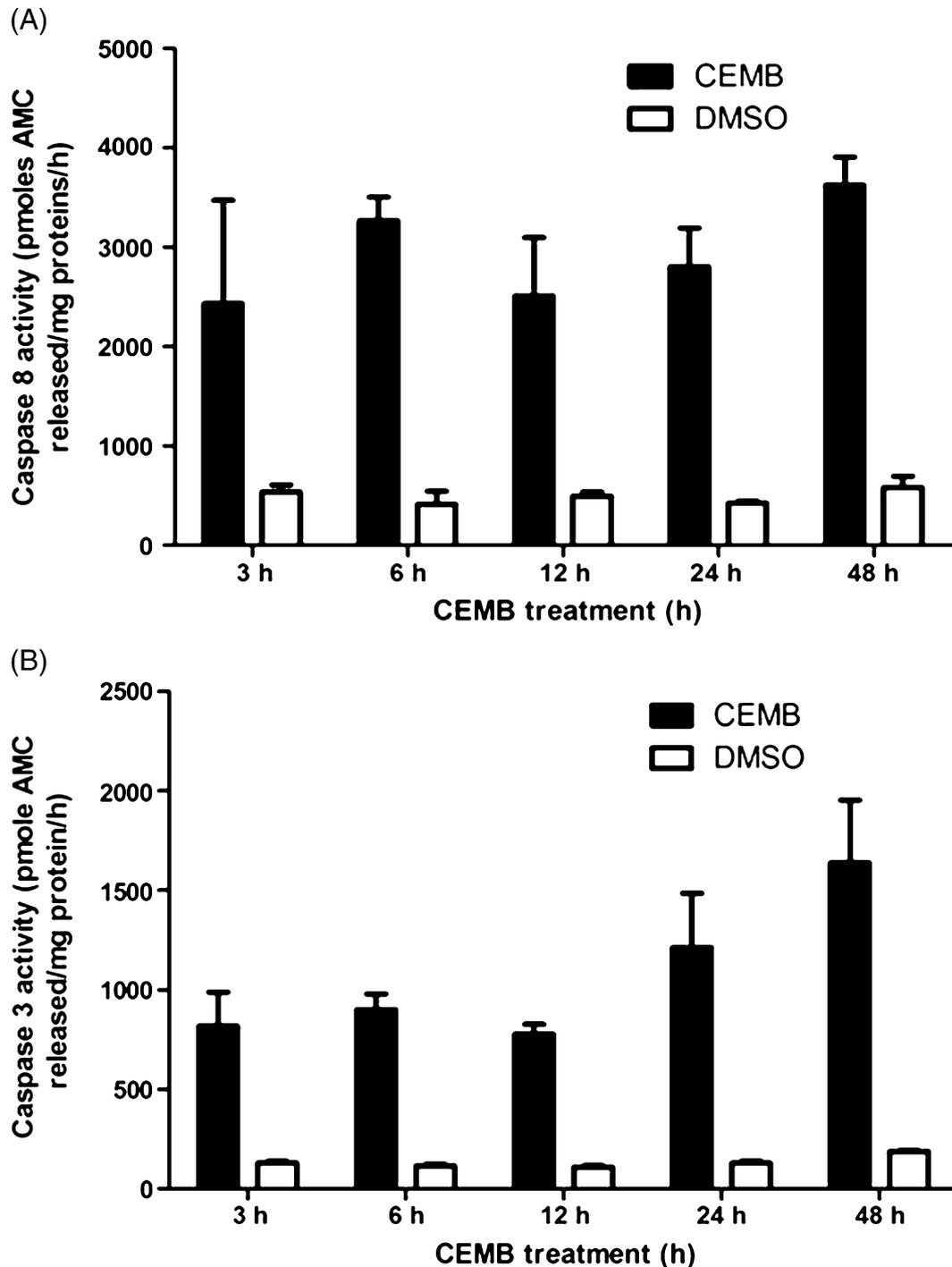


**Figure 4.** DNA fragmentation of A549 (A) and HL60 (B) exposed to CEMB. (A) A549 cells were treated with CEMB at 1  $\mu$ M concentration for 24, 48 and 72 h. (B) HL60 cells were incubated with 0.5  $\mu$ M of CEMB for 12, 24 and 48 h. DNA laddering formation was viewed on ethidium bromide-stained gel. M, molecular weight marker; C, DMSO control.

## 3.5 CEMB activates caspase 8 and caspase 3

Because CEMB induces cell death by apoptosis, we tested to check whether caspase 3 and caspase 8 were

involved in the apoptotic process. A549 cells were treated with 1  $\mu$ M of CEMB for various durations (3, 6, 12 and 24 h). As shown in figure 5, both caspase 8 (figure 5A) and caspase 3 (figure 5B) enzymes are



**Figure 5.** Activation of caspase 8 (A) and caspase 3 (B) exposed to CEMB. A549 cells were treated with CEMB (1  $\mu$ M) for increasing time points. Caspase activity assay was carried out using appropriate fluorogenic-caspase substrates, measured in a fluorimeter. The results presented are the mean  $\pm$  SEM of two independent experiments.

activated in A549 cells at all time points we tested, starting as early as 3 h. A maximal activity of 8.5- and 6.2-fold for caspase 8 and 3, respectively, was observed after 48 h of treatment. DMSO was used as a negative control for each time points. This establishes CEMB as a potent inducer of apoptosis by activating of caspase 8 and caspase 3 in A549 cells.

### 3.6 Induction of differentiation in 3T3-L1 cells by CEMB

Natural triterpenoids and synthetic triterpenoids have been shown to induce differentiation in malignant and nonmalignant cells. To determine whether CEMB is capable of inducing differentiation, we tested CEMB on 3T3-L1 fibroblast cell line, which is known to induce adipogenesis. To test this, cells were plated and allowed to reach confluence. After 48 h, cells were treated with 50 nM and 100 nM of CEMB for 48 h as described in materials and methods and stained with oil red O dye. DMSO was used as a negative control. 3-Isobutyl-1-methylxanthine (IBMX) was used as a positive control for differentiation of 3T3-L1 cells. As shown in figure 6, treatment of 3T3-L1 cells with CEMB-induced adipogenic differentiation is seen by the increased number of oil droplets (stained with oil red O) in both 50 nM and 100 nM treatments.

### 3.7 CEMB suppresses tumour growth in xenografts mouse model

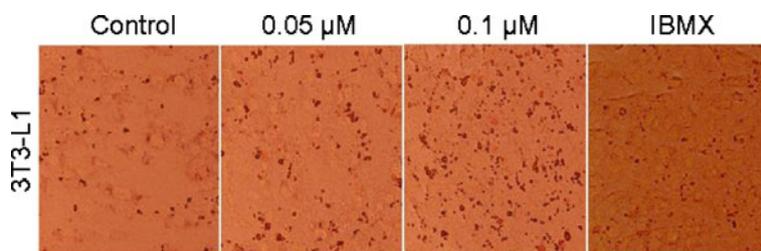
Our *in vitro* studies showed that CEMB is a potent apoptotic agent for most tumour cell lines. To study the activity of CEMB *in vivo*, subcutaneous C6 rat glioma tumours were induced in nude mice as described in the methods section. CEMB treated tumours showed a reduction in both size (volume) and weight (figure 7A). In the group of mice treated with 100 µg/day CEMB, the tumour weight was  $0.23 \pm 0.09$  g, whereas treatment with 200 µg/day showed tumour weights of  $0.1 \pm 0.03$  g (figure 7B), compared with  $0.38 \pm 0.10$  g with vehicle control. Histological analysis of tumours showed that CEMB-induced cell death was

apoptotic and necrotic in the tumour tissue. Thus, CEMB displays *in vivo* anti-tumour activity against C6 glioma, at least under these experimental conditions.

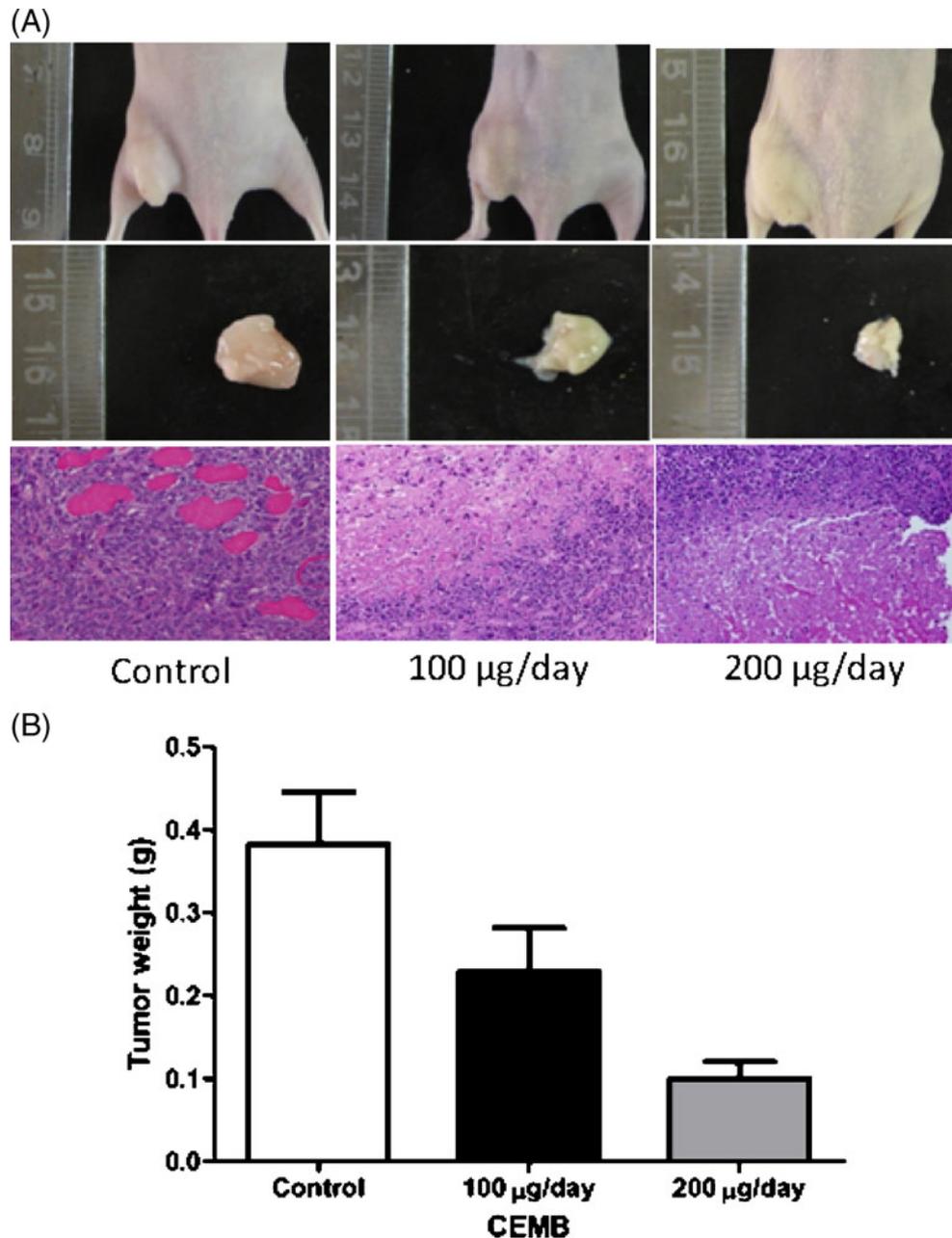
## 4. Discussion

The results presented here, reveal that modification to the A ring of pentacyclic triterpenoid  $\alpha$  and  $\beta$  boswellic acids (extracted from *Boswellia serrata*) potentiates the anti-inflammatory and anti-carcinogenic properties of the compound. As the separation of the  $\beta$  and  $\alpha$  (60:40) isomers of boswellic acid is a tedious process, we performed the synthetic scheme on the mixture to generate the CEMB (Subba Rao *et al.* 2008). In our study, we screened the compound CEMB on a number of cancer cell lines from different tissue origins. We found that CEMB is a potent cytotoxic agent ( $IC_{50} \sim 0.15\text{--}0.7$  µM) by the MTT assay on several cell lines. We have also shown that CEMB inhibits DNA synthesis in lung carcinoma cell line A549 and this effect is comparatively less in an untransformed cell line such as HaCaT, particularly at 0.25 and 0.1 µM concentrations. This compound is also shown to possess anti-inflammatory property as it inhibits NO production ( $IC_{50} \sim 0.05$  µM) induced by IFN- $\gamma$ . A strong link between cancer and inflammation is well known in lung, colon, bladder, cervical, pancreas and stomach cancers (Aggarwal *et al.* 2006; Lu *et al.* 2006). NO is a multifactorial molecule in tumorigenesis, known to participate in carcinogenesis by inducing DNA damage, supporting tumour progression and suppression of immune response (Lala and Orucevic 1998). NO also plays an important role in angiogenesis, invasion and metastatic processes (Palmer *et al.* 1988; Radomski *et al.* 1990). Therefore, inhibiting NO production could facilitate cancer therapy. 3T3-L1 mouse fibroblasts cells show induction of adipogenic differentiation in the presence of differentiation inducers (IBMX, dexamethasone and insulin). In our study, using CEMB we have shown that in absence of these inducers, CEMB (0.05–0.1 µM) alone could induce differentiation.

In the present work, we have shown that CEMB induces cell death via apoptosis. Specifically, we have demonstrated that CEMB activates the death-receptor-mediated caspase



**Figure 6.** CEMB induces differentiation. 3T3-L1 cells were treated with CEMB and the intracellular triglycerides accumulation was stained using Oil Red O. IBMX, standard method to induce differentiation; control, neither with differentiation cocktail nor CEMB.



**Figure 7.** *In vivo* effect of CEMB on xenograft mouse model. (A) Nude mice were subcutaneously injected with C6 rat glioma ( $3 \times 10^6$ ) cells /mouse over the left flank of mice (day 0). After 4 days, CEMB in DMSO, castor oil and PBS was mixed in the ratio of 1:1:8 in 0.1 ml solution and injected at the site of tumours. Equal number of nude mice were injected with DMSO, castor oil and PBS (1:1:8) as vehicle control. The treatment was carried out twice a day (every 12 h) for the first 2 days and then daily (every 24 h) for 3 more days. On day 14, the mice were sacrificed and tumours were excised and weighed. (B) Tumour weights of each group computed as mean of three tumours weights from three different nude mice  $\pm$  SEM.

8 apoptotic pathway, leading to caspase 3 activation followed by DNA breakage. We have tested CEMB on caspase 8 deficient Jurkat cells and Bcl-2-overexpressed Raji cells. In MTT assay, CEMB was surprisingly sensitive even to the Jurkat cells that are caspase 8 deficient, indicating that CEMB can also trigger caspases-independent cell death

mechanisms. Bcl-2 has been extensively studied for chemoresistance as it is able to suppress chemotherapy-induced apoptosis (Korsmeyer 1992; Yang and Korsmeyer 1996; Reed 1997). Interestingly, we observed that CEMB was effective even in Bcl-2-overexpressed cells, suggesting that CEMB is capable of inducing cell death in cells resistant to

apoptosis. The actual mechanism of cell death in caspase-8-deficient and Bcl-2-overexpressed cells remains to be established. This is an important observation and extensive testing is warranted to confirm the effectiveness of this compound on drug-resistant tumour cells. Furthermore, we have tested the *in vivo* effect of CEMB on a xenograft tumour model in immuno-compromised mice. Our data show that CEMB significantly reduced the tumour size in a dose-dependent manner, suggesting the potential of this as an anti-cancer compound.

In summary, we conclude that CEMB is a novel synthetic triterpenoid compound showing potent cytotoxic, growth inhibitory, anti-inflammatory and pro-differentiative activities. CEMB induced cell death is via apoptosis in A549 cells, mediated by caspase 8. CEMB could effectively reduce the tumour size in xenograft mouse model. Thus we advocate further studies to explore the development of CEMB as an anti-tumour therapeutic agent.

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