Effects of curcumin in combination with cyclophosphamide on canine mammary tumour cell lines

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ABSTRACT: In recent years, significant emphasis has been placed on combination chemotherapy in cancer using cytotoxic agents and plant derived-bioactive substances that are capable of selectively arresting cell growth and inducing apoptosis in tumour cells. The present study was undertaken to evaluate the possibility that the combination of curcumin and cyclophosphamide could show synergistic anti-proliferative effects towards CMT-U27 and CMT-U309 canine mammary cancer cells and, if so, to clarify the mechanism involved. The anti-proliferative activities of curcumin, cyclophosphamide and a combined treatment on CMT cells were determined using the MTT and LDH assays. The concentration of drug required for 50% inhibition of cell viability (IC_{50}) and combination index (*CI*) values were calculated from log dose-response curves of fixed-combinations of curcumin and cyclophosphamide generated from MTT assays. Apoptosis was detected using a DNA fragmentation assay and Annexin-V/propidium iodide staining followed by flow cytometry. Cell cycle analyses were also performed using flow cytometry. The expression of the apoptosis-related proteins Bax and Bcl-2 was determined by immunocytochemical staining. MTT and LDH assays showed that curcumin and cyclophosphamide induced a dose- and a time-dependent decrease in cell viability. Isobole analysis revealed that the substances exhibited a synergistic interaction when IC_{50} and 1/2 IC_{50} concentrations of curcumin and cyclophosphamide were added concurrently to the cultures. This synergy was characterised by a significant increase in the percentage of early and late apoptotic CMT-U27 and CMT-U309 cells. However, internucleosomal fragmentation of DNA was not observed in the DNA fragmentation assay. Cells treated with curcumin and cyclophosphamide arrested at the G_2/M and S phases of the cell cycle, respectively. In combined treatments cells were arrested in both phases of the cell cycle. Furthermore, immunocytochemical stainings demonstrated that the curcumin induced apoptosis in CMT cells by the modulation of Bcl-2/Bax protein expression, as the expression of Bcl-2 was decreased and that of Bax increased. This effect was more pronounced in combination treatments. In conclusion, our study shows that a combination of curcumin and cyclophosphamide shows synergistic growth inhibitory activity on CMT cells via induction of apoptosis and cell cycle arrest accompanied by modulation of Bcl-2/Bax protein expression. This finding provides a molecular basis for the development of natural compounds as novel anticancer agents and will allow lowering the dose of cytotoxic agents, which will in turn lead to more specific and less toxic therapies for mammary cancer in dogs.

Keywords: curcumin; cyclophosphamide; apoptosis; combination index; canine mammary cancer

Canine mammary tumours are the most common neoplasms in dogs and approximately 50% of these tumours are malignant (Dore et al. 2003). Surgical resection is the treatment of choice for mammary gland neoplasia and radiotherapy and chemotherapy are used as an adjuvant therapy to surgery (Sorenmo et al. 2013). Although chemotherapy could be effective against systemic or metastatic disease of unknown origin, it has not been fully effective in combatting high incidence cancers

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characterised by low survival rates. Further, it is accompanied by various side effects. Drug toxicity and the development of resistance to chemotherapeutic agents represent major problems in cancer treatment that limit the dose of chemotherapeutic agents (Simon et al. 2009). The development of alternative treatments which act by enhancing the effect of chemotherapeutic drugs to allow the use of lower concentrations of the drugs may alleviate these side effects (Menendez et al. 2001). Therefore, new therapeutic approaches are needed in which cytotoxic agents are combined with novel chemotherapeutic agents, allowing a lower of the dose of the cytotoxic agent and enhancing the efficacy of treatment. One promising approach involves the administration of plant bioactive compounds that possess cancer-preventative activity and/or growth inhibitory activity against cancer cells. Plant bioactive compounds are normally cheaper and cause fewer side effects with respect to chemotherapy (Al-Qubaisi et al. 2011). Thus, the search for drugs (or compounds) extracted from plants as potential cytotoxic agents for cancer is an important line of research in the search for novel anti-cancer candidates (Elhawary et al. 2013).

In recent years, curcumin, a plant derived-bioactive substance that is capable of selectively arresting cell growth and inducing apoptosis in tumour cells has received considerable attention in cancer chemopreventive approaches (Son et al. 2004). Curcumin (1,7-Bis(4-hydroxy-3-methoxyphenyl) hepta-1,6-diene-3,5-dione) diferuloyl methane), a polyphenol extracted from rhizomes of *Curcuma* species, has in fact been shown to possess powerful antioxidant and anti-inflammatory properties (Walters et al. 2008; Ravindran et al. 2009). Extensive investigations in recent years have confirmed that curcumin has potent anti-proliferative activity *in vitro* against many different types of cancer cells, including breast, colon and prostate (Sharma et al. 2005; Shishodia et al. 2007). In addition, the chemotherapeutic efficacy of curcumin is being evaluated in Phase I and II clinical trials for a variety of tumours (Cheng et al. 2001; Dhillon et al. 2008). Moreover, numerous reports point to the benefits of administering curcumin alongside chemotherapeutic drugs (Zaidi et al. 2011). For example, combination therapy of curcumin with cisplatin resulted in a synergistic anti-tumour activity while combination therapy with doxorubicin resulted in additive or sub-additive effects (Notarbartolo et al. 2005). Also, dasatinib and curcumin demonstrated synergistic interactions in chemo-resistant colon cancer cells (Nautiyal et al. 2011).

Strategies for cancer treatment using combined therapies or agents with distinct molecular mechanisms show greater potential for higher efficacy resulting in superior survival (Singh et al. 2011). Recent studies have revealed that the anti-tumour activities of curcumin involve the regulation of various signalling pathways in breast cancer cells (Ramachandran and You 1999; Choudhuri et al. 2002); therefore, cytotoxic therapies combined with this compound may exert enhanced anti-tumour effects through synergistic actions and may also decrease systemic toxicity. Thus, the present study was designed to investigate the *in vitro* effects of curcumin alone and in combination with the chemotherapeutic agent cyclophosphamide, a drug which is frequently used for the treatment of mammary cancer in dogs (Sorenmo 2003). To explore the mechanism by which curcumin inhibits the development of canine mammary cancer, we studied its effects on the proliferation, apoptosis and cell cycle progression of the cultured canine mammary tumour cell lines CMT-U27 and CMT-U309. In order to further elucidate the molecular mechanism responsible for the curcumin-induced apoptosis in CMT cells, the expression of certain apoptosis-related genes such as Bcl-2 and Bax was analysed. The promising data reported in this study may lay the basis for a novel anti-neoplastic regimen of therapy for mammary cancer in dogs.

MATERIAL AND METHODS

CMT-U27 and CMT-U309 canine mammary tumour cells. CMT-U27 canine mammary carcinoma and CMT-U309 canine mammary spindle-cell tumour cell lines were generously provided by Dr. Eva Hellmen (Department of Anatomy and Physiology, Uppsala University, Sweden). CMT-U27 and CMT-U309 canine mammary tumour cells were grown in Dulbecco's modified Eagle's medium-F12 (DMEM-F12) containing 10% foetal bovine serum (FBS), 2mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin and 2.5 µg/ml amphotericin B. The cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2. Cells were sub-cultured as they reached 80–90% confluence and adherent cells were detached by incubation with Trypsin/EDTA solution. Cell number was determined using a 0.2% trypan blue dye with the

Cedex XS cell counter system (Innovatis, Roche, Germany).

Chemicals. Curcumin and most other chemicals were obtained from Sigma (St. Louis, MO, USA). Cyclophosphamide monohydrate (purity 97%) was obtained from Acros Organics (Belgium). A stock solution (20mM) of curcumin was made with 100% dimethyl sulfoxide (DMSO) and cyclophosphamide was dissolved in DMEM-F12. Prior to the experiment, curcumin was diluted in the culture medium to final concentrations of 2.5, 5, 10, 25, 50, 100 and 200μM and cyclophosphamide to concentrations of 0.5, 1, 2.5, 5, 10, 25 and 50mM, respectively. Polyclonal anti-Bcl-2 (sc-492), polyclonal anti-Bax (sc-493) antibodies, a secondary antibody kit and 3,3'-diaminobenzidine (DAB) (sc-2018) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

MTT assay. Cell viability was assessed using a commercial cell proliferation MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltrazolium bromide] kit (Roche Applied Science, Germany) according to the manufacturer's instructions. CMT-U27 and CMT-U309 canine mammary tumour cells were seeded at a density of 1×10^4 cells/well in 96-well plates (Jet Biofil, Canada) and then allowed to settle and attach overnight. At time 0, medium was replaced with fresh complete medium containing different concentrations of curcumin, cyclophosphamide or combinations thereof. The cells were then incubated for 24, 48 and 72 h. At each time point, 10 μl of MTT solution [5 mg/ml in phosphate buffered saline (PBS)] were added to each well. The plates were incubated for 4 h in a humidified atmosphere at 37 °C with 5% CO_2. The purple water insoluble formazan salt was then dissolved with 10% SDS in 0.01M HCl and the plates were incubated overnight in a cell culture incubator. The optical densities of the wells were measured at 595 nm using a Multi-Mode microplate reader (FilterMax F5, Molecular Devices, USA). All samples were assayed in triplicate, and the mean for each experiment was calculated. The effect of each compound on viability was assessed as percentage cell viability; vehicle-treated cells were taken as 100% viable. The mean of triplicate experiments for each dose was used to calculate the concentration of drug required for 50% inhibition of cell viability (IC_{50}) as determined using the Biosoft CalcuSyn program. Concerns have been raised that high doses of curcumin are not achievable systemically due to poor absorption, rapid metabolism and systemic elimination (Anand et al. 2007). Therefore, in combination experiments we investigated the effects of a combination of curcumin with cyclophosphamide at concentrations lower than the IC_{50} (1/2 IC_{50} and 1/4 IC_{50} and 1/8 IC_{50}) doses of both drugs with fixed-drug combination ratios (1 : 1) in order to increase efficacy and reduce toxicity and chemotherapeutic resistance. In addition, changes in cell morphology commonly used as indicators of cell survival were determined microscopically (Olympus CKX41).

Drug interaction effect. The multiple drug effect analysis of Chou and Talalay (1984), which is based on the median-effect principle, was used to examine the nature of the interaction between curcumin and cyclophosphamide. The details of this methodology have been published (Chou and Talalay 1984). Determination of the synergistic versus additive versus antagonistic cytotoxic effects of the combined treatment of cells with curcumin and cyclophosphamide were assessed using the combination index (*CI*) where $CI < 1$, $CI = 1$ and $CI > 1$ indicate synergistic, additive and antagonistic effects, respectively. Based on the classic isobologram, the *CI* was calculated as:

$$CI = [(D)_1/(D_x)_1] + [(D)_2/(D_x)_2]$$

were:

$(D_x)_1$, $(D_x)_2$ = the concentrations of curcumin and cyclophosphamide that induced a 50% inhibition of cell proliferation

$(D)_1$, $(D)_2$ = the concentrations of curcumin and cyclophosphamide in combination that also inhibited cell growth by 50% (isoeffective as compared with the single drugs alone). The *CI* index was calculated using Biosoft CalcuSyn software (Biosoft, Cambridge, UK)

LDH assay. The effects of curcumin and cyclophosphamide on membrane permeability in CMT-U27 and CMT-U309 canine mammary tumour cell lines were determined using the Cytotoxicity Detection Kit (Roche Applied Science, Germany), which is based on the detection of lactate dehydrogenase (LDH) released from dead cells as a result of cytotoxicity. The cells were seeded at a density of 1×10^4 cells/well in 96-well plates (Jet Biofil, Canada) and then allowed to settle and attach overnight. At time 0, medium was replaced with fresh complete medium containing different concentrations of curcumin, cyclophosphamide or combinations thereof as indicated for the MTT

assay. At each time point, cell-free culture supernatants (100 µl/well) from curcumin- and cyclophosphamide-treated CMT-U27 and CMT-U309 cells were collected and transferred to 96-well plates. A reaction mixture (100 µl/well) containing iodotetrazolium chloride and sodium lactate and diaphorase/NAD^+ mixture was added, followed by incubation for 30 min. The formazan dye was quantified by measuring the absorbance at 450 nm. Triton X-100-treated cells (1% Triton X-100) that give the maximum loss of LDH were used as a positive control and taken as 100% dead.

DNA fragmentation assay. Apoptosis was evaluated in an enzyme-linked immunosorbent assay (ELISA) using the Cell Death Detection ELISA Plus Kit (Roche Applied Science, Germany), in accordance with the instruction manual. The relative amounts of mono- and oligonucleosomes generated from the apoptotic cells were quantified using monoclonal antibodies directed against DNA and histones and an ELISA. Briefly, CMT-U27 and CMT-U309 canine mammary tumour cells were seeded at a density of 1×10^4 cells/well in 96-well plates and then allowed to settle and attach overnight. At time 0, medium was replaced with fresh complete medium containing different concentrations of curcumin, cyclophosphamide or combinations thereof as for MTT assay. At each time point, the supernatant was removed and the cell pellet was lysed in lysis buffer. The cytoplasmic fractions of the untreated control, and curcumin- and/or cyclophosphamide-treated cells and a combination of both treated cell lines were transferred onto a streptavidin-coated plate and incubated for two hours at room temperature with a mixture of peroxidase-conjugated, anti-DNA and biotin-labeled anti-histone. The plate was washed thoroughly, incubated with 2,2'-azino-di-[3-ethylbenzthiazoline sulphonate] diammonium salt (ABTS), and the absorbance of the wells was measured at 405 nm with a reference wavelength at 490 nm using a Multi-Mode microplate reader (FilterMax F5, Molecular Devices, USA). Samples were measured in triplicate and a positive control DNA-histone-complex was provided with the kit.

Apoptosis assay. Flow cytometric analysis of phosphatidylserine exposure was quantitatively detected on BD FACSCalibur using the Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (BD Bioscience, San Jose, CA, USA) according to the manufacturer's instructions. The assay is based on the binding of Annexin V to the phosphatidylserine that is translocated from the inner membrane leaflet to the outer layer in cells undergoing apoptosis (van Engeland et al. 1998). Live and apoptotic cells are impermeant to the red-fluorescent propidium iodide (PI) nucleic acid binding dye, but in necrotic cells it binds tightly to nucleic acid resulting in red fluorescence. Apoptotic cells stained with Annexin show green fluorescence, dead cells show red and green fluorescence and viable cells show little or no fluorescence (Liu et al. 2013). The cells were seeded at 1×10^5/ml in 24-well flat bottom plates (Jet Biofil, Canada) and then allowed to settle and attach overnight. At time 0, medium was replaced with fresh complete medium containing different concentrations of curcumin, cyclophosphamide or combinations thereof as for the MTT assay. After 72 h, cells were trypsinised, washed twice with ice cold PBS and then resuspended in binding buffer (0.1M Hepes/NaOH (pH 7.4), 1.4M NaCl, 25mM $CaCl_2$), supplemented with 5 µl of Annexin V-FITC and 5 µl of PI. The cell suspension was gently vortexed and incubated for 15 min at room temperature in the dark. Following the incubation, 400 µl of binding buffer were added to each tube and samples were analysed within 1 h on a FACScan flow cytometer (BD Biosciences) using the standard optics for detecting FL1 (FITC) and FL2 (PI). Data were analysed using CellQuest WinMDI software (BD Bioscience, San Jose, CA, USA). Determination of the synergistic versus additive versus antagonistic apoptotic effects of the combined treatment of cells with curcumin and cyclophosphamide were quantified using the cooperativity index (cooperativity index = sum of apoptosis of the single-agent treatment/apoptosis of the combined treatment). A cooperativity index < 1, $= 1$ or > 1 was indicative of synergism, additivity or antagonism respectively (ten Cate et al. 2007).

Cell cycle analysis. Cell cycle distribution was detected using the CycleTEST PLUS DNA Reagent kit (Becton Dickinson, USA) staining solution according to the manufacturer's instructions. In brief, CMT-U27 and CMT-U309 cells were seeded and allowed to settle and attach overnight. At time 0, medium was replaced with fresh complete medium containing different concentrations of curcumin, cyclophosphamide or combinations thereof as indicated. After 72 h 1×10^6 cells from each sample were collected and washed with buffer solution three times, then pelleted by centrifugation at $400 \times g$. The cell pellets were incubated with 250 µl solution A (trypsin in a spermine tetrahy-

drochloride detergent buffer) at room temperature for 10 min, 200 µl solution B (trypsin inhibitor and ribonuclease A in citrate stabilising buffer with spermine tetrahydrochloride) at room temperature for 10 min and 200 µl solution C (propidium iodide and spermine tetrahydrochloride in citrate stabilising buffer) in the dark at 4 °C for 10 min. The suspensions were analysed using a FACScan flow cytometer at an excitation wavelength of 488 nm. Data were analysed using ModFit LT software (BD Bioscience, San Jose, CA, USA).

Immunocytochemistry. In order to determine protein expression of the apoptosis markers Bcl-2 and Bax in CMT-U27 and CMT-U309 cells, sterilised coverslips were placed on the bottom of a 24-well plate and the cells were seeded at a density of 1×10^5 cells/ml and allowed to settle and attach overnight. At time 0, medium was replaced with fresh complete medium containing different concentrations of curcumin, cyclophosphamide or combinations thereof as indicated. At each time point, the cells were rinsed with PBS, and fixed with cold methanol for 10 min. After fixation, the cells were washed with PBS; pH 7.4, 0.1M for 5 min and the endogenous peroxidase activity was inactivated by incubation with 0.3% H_2O_2 in methanol for 10 min. Then, the cells were washed three times with PBS and incubated with protein blocking agent for 10 min to block non-specific immunolabelling. Subsequently, cells were incubated with polyclonal anti-Bcl-2 (sc-492) and polyclonal anti-Bax (sc-493) antibodies at a dilution of 1 : 200 at room temperature for 90 min. After extensive washing in PBS, the cover slips were incubated with a secondary antibody mixture (sc-2018) containing biotinylated secondary antibody and avidin-peroxidase for 20 min at room temperature. Finally, cells were rinsed with PBS and incubated with DAB complexes according to the manufacturer's protocol before counterstaining with Mayer's haematoxylin and mounting onto glass slides. The cells were observed with a light microscope (Olympus BX50). The intensity of immunolabelling was assessed by examination of 10 representative high-power fields (× 400). Positive cells were identified by distinct brown cytoplasmic staining. The number of immunoreactive cells was assessed semi-quantitatively. For staining density, specimens were classified as negative, + (< 10% positive cells), ++ (10–50% positive cells), and +++ (> 50% positive cells). Results were considered inconclusive when there were insufficient neoplastic cells available for analysis (Zuccari et al. 2004).

Statistical analysis. Data were analysed using one-way ANOVA (analysis of variance) followed by Tukey's test (Statistical Product and Service Solutions 15.0 program, Chicago, IL, USA). Data were expressed as mean ± SEM. *P*-values of less than 0.05 were considered to be statistically significant. Correlation analyses between MTT and LDH tests were performed using Pearson's correlation.

RESULTS

The anti-proliferative effects of curcumin and cyclophosphamide and their combination on CMT-U27 and CMT-U309 cells

The effects of curcumin and cyclophosphamide on CMT cell proliferation were determined by MTT assay after 24, 48, and 72 h of treatment. As shown in Figure 1A, 1B, 1C and 1D curcumin and cyclophosphamide treatment significantly inhibited the growth of both cell lines in a dose-and time-dependent manner, which was associated with distinct morphological changes shown in Figure 2A and 2B. The concentration of compound required for a 50% inhibition of cell viability (IC_{50}) was obtained by extrapolation from an inhibition curve. In the presence of curcumin alone, the respective IC_{50} values of CMT-U27 cells were 151.49, 69.24 and 68.39 µM and the IC_{50} values of CMT-U309 cells were 65.23, 56.11 and 49.45µM at 24, 48 and 72 h, respectively. In the presence of cyclophosphamide alone, the IC_{50} values of CMT-U27 cells were 27.93, 15.06 and 10.36mM and the IC_{50} values of CMT-U309 cells were 16.57, 14.99 and 9.51mM at 24, 48 and 72 h, respectively. These results indicate that curcumin and cyclophosphamide exert anti-proliferative effects on CMT cells. Notably, the inhibitory effects of curcumin on these two cancer cell lines were of greater magnitude to those of cyclophosphamide, one of the most commonly used chemotherapeutic drugs for canine mammary tumours (Sorenmo 2003). To investigate the interaction between these drugs combination experiments were performed in order to determine whether a combination of curcumin with cyclophosphamide had synergistic, additive or antagonistic effects. The time point at which the suppression of proliferation was most effective (72 h) and fixed-drug combination ratios of (1 : 1) IC_{50} values for both

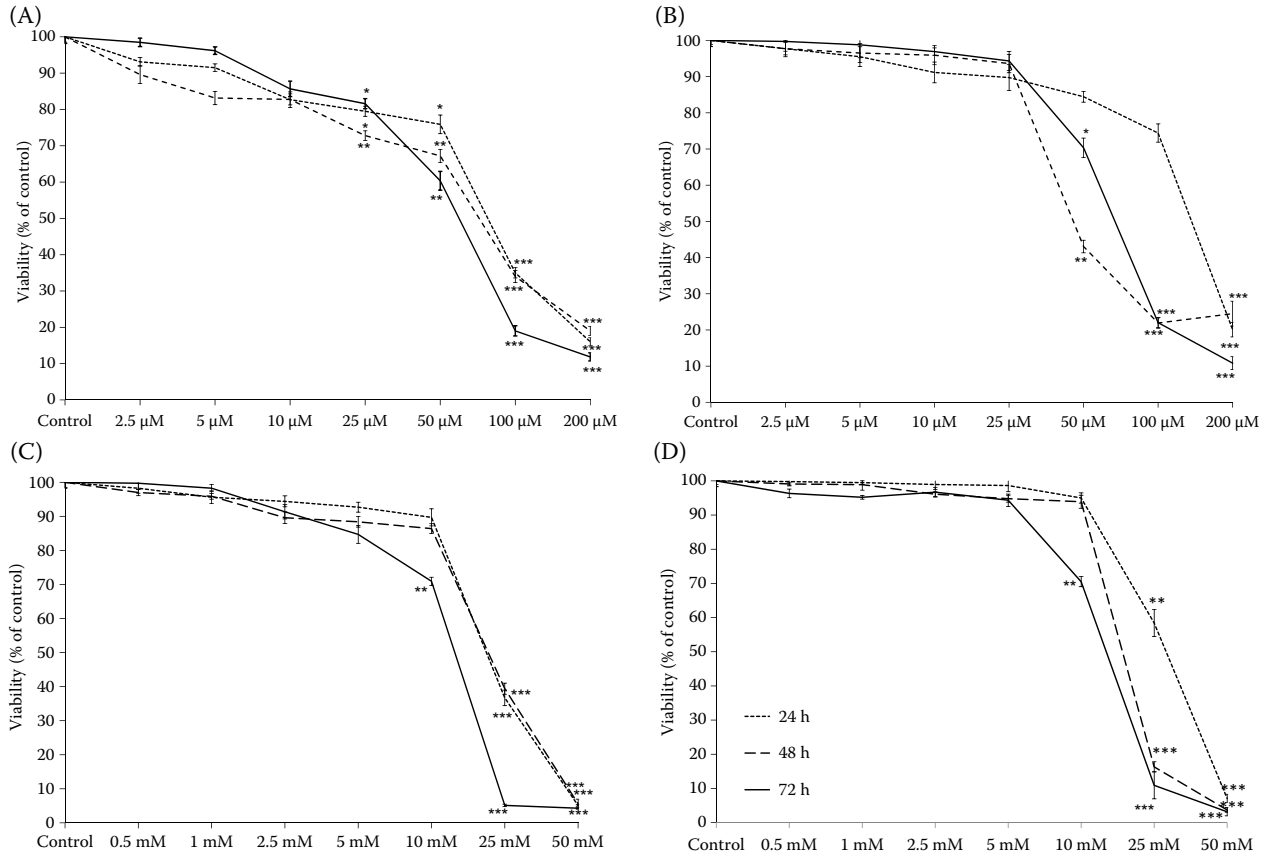

Figure 1. Effects of curcumin treatment in CMT-U309 (**A**) and CMT-U27 cells (**B**) and cyclophosphamide treatment in CMT-U309 (**C**) and CMT-U27 cells (**D**). Data are expressed as mean percentage of cell viabilities ± standard error (SE) from three individual experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to control

drugs were used in subsequent experiments. Both curcumin and cyclophosphamide were added concurrently at a fixed ratio of doses that corresponded to 1/8, 1/4, 1/2 and one times the individual IC_{50}.

Synergistic effects of a combination of curcumin and cyclophosphamide on the proliferation of CMT-U27 and CMT-U309 cells

To assess the drug interaction effect of curcumin and cyclophosphamide together, CMT-U27 and CMT-U309 cells were exposed to different concentrations of both drugs alone and in combination for 72 h. There was a significant synergistic effect in both cell lines when the IC_{50} and 1/2 IC_{50} concentrations of both drugs were combined. The IC_{50} of curcumin and IC_{50} of cyclophosphamide elicited decreases of 40.6%, 49.3%, 34.8% and 43.7% respectively, in CMT-U27 and CMT-U309 cells, but the combination resulted in a 89.7% decrease in CMT-U27, and 90.6% decrease in CMT-U309 cell proliferation (Table 1). Calculation of the *CI* values revealed that the synergistic effect ($CI < 1$) was strongest at a combination of 1/2 IC_{50} and IC_{50} in both cell lines. A 1/4 IC_{50} combination resulted in additivity ($CI = 1$) in CMT-U27 cells and 1/8 and 1/4 IC_{50} combinations resulted in antagonism ($CI > 1$) in CMT-U309 cells (Table 2).

Increased cell membrane permeability and lactate dehydrogenase release after curcumin and cyclophosphamide combination treatments

In order to better characterise the cytotoxic effects observed, we measured the LDH leakage as an indicator of membrane dysfunction. LDH levels were increased by 17.42%, 19.28%, 43.41% and 88.47% in the media of CMT-U27 cells, and reached 14.81%, 51.36%, 52.25% and 64.12% of the positive control in CMT-U309 cells following exposure to 10, 25, 50 and 100μM of curcumin, respectively (Figure 3A and 3B). The cytotoxic effect was more pronounced in combination treatments,

Table 1. Effects of combined curcumin and cyclophosphamide treatment on CMT-U309 and CMT-U27 cells

Drugs	CMT-U27		CMT-U309	
	concentration of drugs	% viability (% of control)	concentration of drugs	% viability (% of control)
Control		100 ± 0.74		100 ± 1.88
IC_{50} CUR	68.39μM	59.4 ± 1.05***	49.45μM	65.2 ± 1.03***
1/2 IC_{50} CUR	34.20μM	81.1 ± 1.08**	24.73μM	72.6 ± 1.33**
1/4 IC_{50} CUR	17.10μM	91.2 ± 1.66	12.36μM	93.7 ± 1.95
1/8 IC_{50} CUR	8.55μM	94.5 ± 2.00	6.18μM	97.2 ± 1.32
IC_{50} CYP	10.36mM	50.7 ± 0.70***	9.51mM	56.3 ± 0.62***
1/2 IC_{50} CYP	5.18mM	72.0 ± 0.61***	4.75mM	69.9 ± 1.01***
1/4 IC_{50} CYP	2.59mM	90.0 ± 1.95*	2.37mM	78.2 ± 0.98**
1/8 IC_{50} CYP	1.30mM	98.3 ± 0.98	1.13mM	97.5 ± 1.65
IC_{50} CUR + IC_{50} CYP	68.39μM + 10.36mM	10.3 ± 0.41***	49.45μM + 9.51mM	9.4 ± 0.35***
1/2 IC_{50} CUR +1/2 IC_{50} CYP	34.20μM + 5.18mM	14.4 ± 0.58***	24.73μM + 4.75mM	35.0 ± 0.59***
1/4 IC_{50} CUR + 1/4 IC_{50} CYP	17.10μM + 2.59mM	79.2 ± 1.11***	12.36μM + 2.37mM	89.2 ± 1.90*
1/8 IC_{50} CUR + 1/8 IC_{50} CYP	8.55μM + 1.30mM	94.1 ± 0.78	6.18μM + 1.13mM	98.5 ± 0.66

Data are expressed as mean percentage of cell viabilities ± standard error (SE) from three individual experiments; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to control

CUR = curcumin, CYP = cyclophosphamide

and significant effects were observed in IC_{50} and 1/2 IC_{50} combinations when compared with control CMT-U27 and CMT-U309 cells (Table 3). A significant ($P < 0.001$) negative correlation (correlation coefficient, $r = –0.962$) was found between the MTT and LDH test.

Cyclophosphamide induces DNA fragmentation in CMT-U27 and CMT-U309 cells

To examine the possible synergistic effects of curcumin and cyclophosphamide and their combination, on induction of DNA fragmentation as a

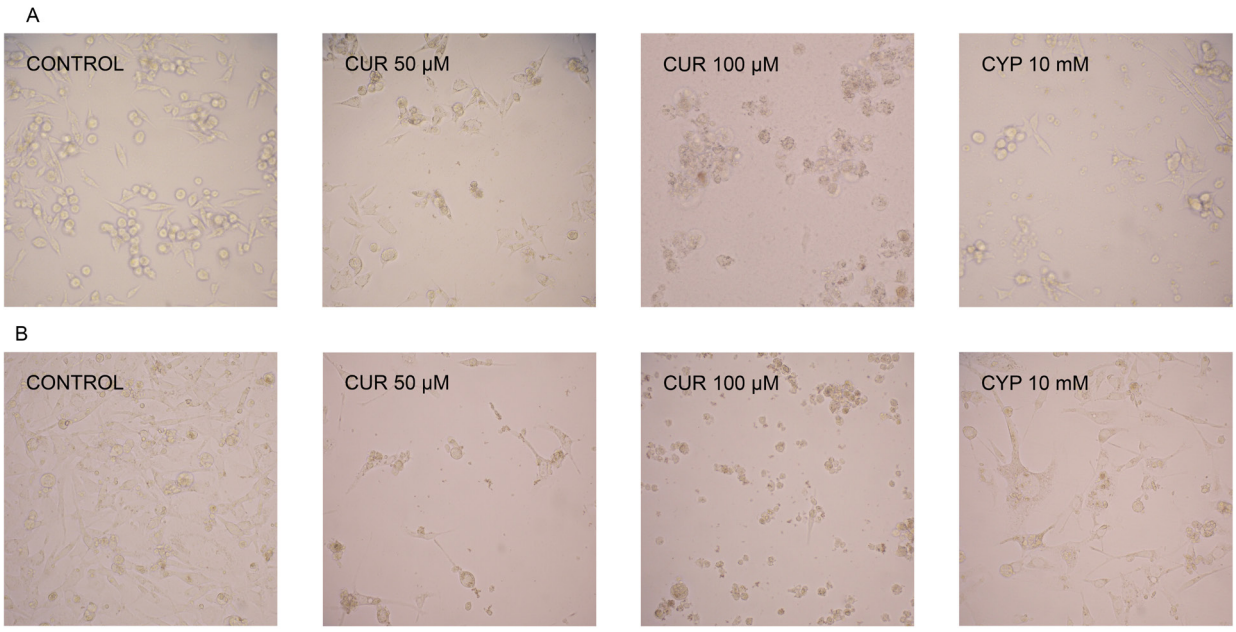

Figure 2. Light microscopy findings. Typical morphologic changes in CMT-U27 cells (**A**) and CMT-U309 cells (**B**) after curcumin and cyclophosphamide treatment for 72 h; 50μM of curcumin reduced cell numbers and 100μM of curcumin resulted in nearly complete cell detachment and nuclear condensation. Viable cells were rarely observed after treatment with 100μM of curcumin and 10mM cyclophosphamide

Table 2. Combination index values for CMT-U27 and CMT-U309 cells exposed to scheduled/concurrent combinations of curcumin and cyclophosphamide

Drugs	Concentration of drugs	CI value	Interpretation
CMT-U27			
IC_{50} CUR + IC_{50} CYP	68.39μM + 10.36mM	0.47	synergism
1/2 IC_{50} CUR + 1/2 IC_{50} CYP	34.20μM + 5.18mM	0.29	synergism
1/4 IC_{50} CUR + 1/4 IC_{50} CYP	17.10μM + 2.59mM	1.05	additive
1/8 IC_{50} CUR + 1/8 IC_{50} CYP	8.55μM + 1.30mM	1.41	antagonism
CMT-U309			
IC_{50} CUR + IC_{50} CYP	49.45μM + 9.51mM	0.42	synergism
1/2 IC_{50} CUR + 1/2 IC_{50} CYP	24.73μM + 4.75mM	0.61	synergism
1/4 IC_{50} CUR + 1/4 IC_{50} CYP	12.36μM + 2.37mM	1.83	antagonism
1/8 IC_{50} CUR + 1/8 IC_{50} CYP	6.18μM + 1.13mM	3.55	antagonism

CI = combination index, CUR = curcumin, CYP = cyclophosphamide

marker of apoptosis, we quantified the levels of mono-oligonucleosome fragments. The results showed that when both cell lines were exposed to a high concentration of cyclophosphamide (25mM), there was a significant increase ($P < 0.05$) in DNA fragmentation. Curcumin-treated cells did not exhibit any significant increase in fragmentation when compared with control (Table 4), and likewise, no significant DNA fragmentation was observed in combination groups.

Induction of apoptosis in CMT-U27 and CMT-U309 cells after treatment with curcumin and cyclophosphamide and their combination

To further elucidate the anti-cancer mechanism of curcumin and cyclophosphamide and their combination in CMT-U27 and CMT-U309 cells, we performed apoptosis assays. After treating the cells with various concentrations of curcumin and

Table 3. Effects of combined curcumin and cyclophosphamide combination treatment on CMT-U309 and CMT-U27 cells

Drugs	CMT-U27		CMT-U309	
	concentration of drugs	% cytotoxicity (% of Triton X-100)	concentration of drugs	% cytotoxicity (% of Triton X-100)
Control		13.74 ± 0.30		15.74 ± 0.25
IC_{50} CUR	68.39μM	60.4 ± 0.82***	49.45μM	58.82 ± 1.17***
1/2 IC_{50} CUR	34.20μM	35.35 ± 1.23**	24.73μM	39.46 ± 0.82**
1/4 IC_{50} CUR	17.10μM	22.0 ± 1.14*	12.36μM	31.86 ± 0.70**
1/8 IC_{50} CUR	8.55μM	18.46 ± 0.65	6.18μM	20.14 ± 0.10
IC_{50} CYP	10.36mM	66.13 ± 0.68***	9.51mM	62.5 ± 1.10***
1/2 IC_{50} CYP	5.18mM	24.84 ± 1.15*	4.75mM	38.23 ± 0.22**
1/4 IC_{50} CYP	2.59mM	23.8 ± 1.21*	2.37mM	33.82 ± 0.47**
1/8 IC_{50} CYP	1.30mM	17.01 ± 0.50	1.13mM	23.03 ± 0.82
IC_{50} CUR + IC_{50} CYP	68.39μM + 10.36mM	86.6 ± 0.86***	49.45μM + 9.51mM	75.73 ± 1.23***
1/2 IC_{50} CUR + 1/2 IC_{50} CYP	34.20μM + 5.18mM	75.96 ± 0.78***	24.73μM + 4.75mM	54.24 ± 0.79***
1/4 IC_{50} CUR + 1/4 IC_{50} CYP	17.10μM + 2.59mM	32.25 ± 0.99**	12.36μM + 2.37mM	26.47 ± 0.59*
1/8 IC_{50} CUR + 1/8 IC_{50} CYP	8.55μM + 1.30mM	14.69 ± 0.40	6.18μM + 1.13mM	15.19 ± 0.72

Data are expressed as mean percentage of cytotoxicity (% of Triton X-100) ± standard error (SE) from three individual experiments; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to control

CUR = curcumin, CYP = cyclophosphamide

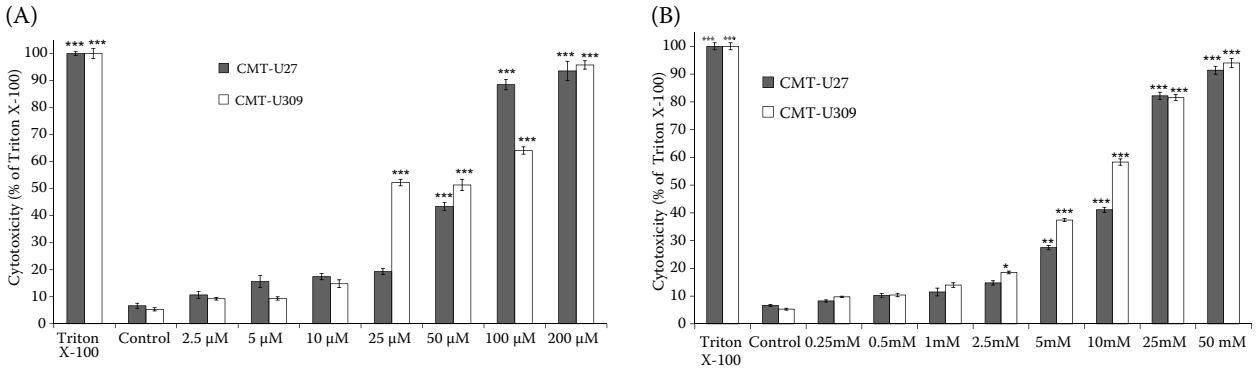

Figure 3. Effects of curcumin treatment on CMT-U27 and CMT-U309 cells (**A**) and cyclophosphamide treatment on CMT-U27 and CMT-U309 cells (**B**). Data are expressed as mean percentage of cytotoxicity (% of Triton X-100) ± standard error (SE) from three individual experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to control

Table 4. Effects of curcumin and cyclophosphamide and their combination on DNA fragmentation CMT-U27 and CMT-U309 cells

Drugs	CMT-U27		CMT-U309	
	concentration of drugs	OD value	concentration of drugs	OD value
	control	1.12 ± 0.04	control	1.06 ± 0.05
	positive control	3.20 ± 0.08**	positive control	2.99 ± 0.04**
CUR	2.5μM	0.94 ± 0.07	2.5μM	1.01 ± 0.08
CUR	5μM	0.98 ± 0.03	5μM	0.90 ± 0.09
CUR	10μM	1.03 ± 0.07	10μM	0.97 ± 0.07
CUR	25μM	1.04 ± 0.04	25μM	1.00 ± 0.08
CUR	50μM	1.09 ± 0.08	50μM	1.00 ± 0.06
CUR	100μM	1.18 ± 0.05	100μM	1.18 ± 0.09
CYP	1mM	1.00 ± 0.06	1mM	1.00 ± 0.01
CYP	2.5mM	1.16 ± 0.06	2.5mM	1.07 ± 0.01
CYP	5mM	1.04 ± 0.04	5mM	1.10 ± 0.06
CYP	10mM	1.34 ± 0.08	10mM	1.34 ± 0.01
CYP	25mM	2.16 ± 0.06*	25mM	1.80 ± 0.03*
IC_{50} CUR	68.39μM	1.20 ± 0.10	49.45μM	0.95 ± 0.05
1/2 IC_{50} CUR	34.20μM	1.15 ± 0.12	24.73μM	0.97 ± 0.08
1/4 IC_{50} CUR	17.10μM	1.02 ± 0.01	12.36μM	1.14 ± 0.08
1/8 IC_{50} CUR	8.55μM	1.12 ± 0.12	6.18μM	1.07 ± 0.08
IC_{50} CYP	10.36mM	1.06 ± 0.02	9.51mM	1.19 ± 0.10
1/2 IC_{50} CYP	5.18mM	1.16 ± 0.09	4.75mM	1.15 ± 0.05
1/4 IC_{50} CYP	2.59mM	1.07 ± 0.02	2.37mM	1.08 ± 0.09
1/8 IC_{50} CYP	1.30mM	1.26 ± 0.13	1.13mM	1.05 ± 0.08
IC_{50} CUR + IC_{50} CYP	68.39μM + 10.36mM	1.27 ± 0.03	49.45μM + 9.51mM	1.11 ± 0.08
1/2 IC_{50} CUR + 1/2 IC_{50} CYP	34.20μM + 5.18mM	1.06 ± 0.11	24.73μM + 4.75mM	1.01 ± 0.14
1/4 IC_{50} CUR + 1/4IC_{50} CYP	17.10μM + 2.59mM	0.99 ± 0.09	12.36μM + 2.37mM	1.07 ± 0.08
1/8 IC_{50} CUR + 1/8 IC_{50} CYP	8.55μM + 1.30mM	1.05 ± 0.06	6.18μM + 1.13mM	0.98 ± 0.10

OD values as measured by ELISA are shown ± standard error (SE) from three individual experiments; $^{*}P < 0.05$, $^{**}P < 0.01$ compared to control

CUR = curcumin, CYP = cyclophosphamide

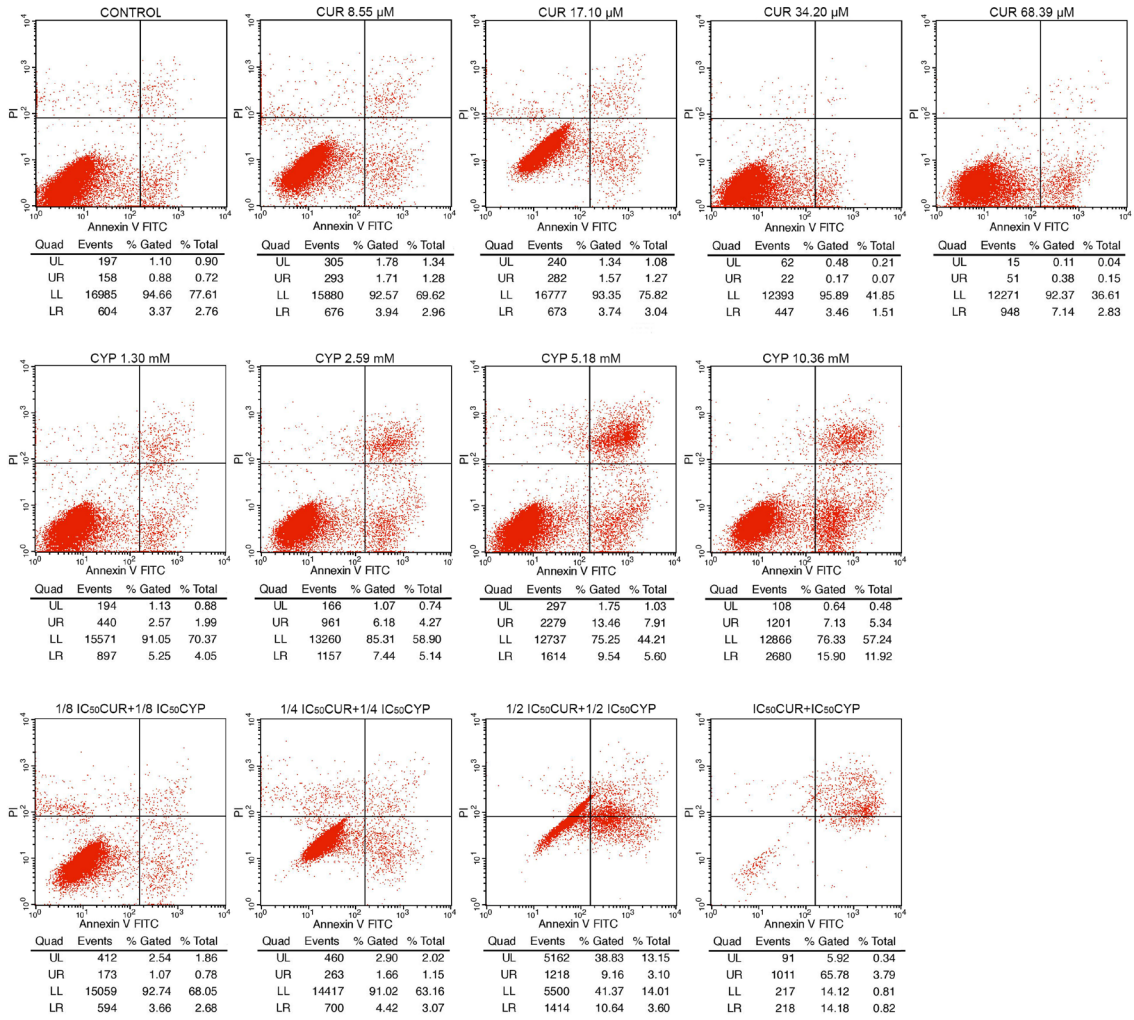

Figure 4. Flow-cytometric analysis of CMT-U27 cells after staining with Annexin-V FITC and propidium iodide (PI). Dual parameter dot plot of FITC-fluorescence (x-axis) vs. PI (y-axis) shows logarithmic intensity. Quadrants: $FITC^-/PI^-$ lower left (LL), viable cells; $FITC^+/PI^-$ lower right (LR), early apoptotic cells; $FITC^+/PI^+$ upper right (UR), late apoptotic cells; $FITC^-/PI^+$ upper left (UL), necrotic cells. Representative data from three independent experiments are shown

cyclophosphamide, the percentage of apoptotic cells was assessed by Annexin V-FITC and PI staining, followed by flow cytometric analysis (Figures 4 and 5). The dot plot of Annexin V-FITC fluorescence versus PI fluorescence indicated a significant increase in the percentage of apoptotic cells that were treated with increasing concentrations of curcumin and cyclophosphamide. It was observed that at concentrations of 10–100µM curcumin and 2.5–25mM cyclophosphamide, there was a marked dose-dependent increase in the sum of the percentages of the cells in early and late stages of apoptosis in both cell lines (Table 5). Combination of curcumin with cyclophosphamide resulted in much stronger apoptotic death compared to each agent alone in both cell lines. The use of 1/8 and 1/4 IC_{50} combinations of both drugs only marginally increased apoptosis in either cell line compared to either drug used alone. In contrast, combined IC_{50} of curcumin and IC_{50} of cyclophosphamide in CMT-U27 cells and combined 1/2 IC_{50} of curcumin and 1/2 IC_{50} of cyclophosphamide in CMT-U309 cells caused a substantial and synergistic induction of apoptosis compared with that resulting from the individual drug treatments (cooperativity index = 0.41 in CMT-U27 and 1.09 in CMT-U309) and elicited a remarkable increase in late apoptotic cells in CMT-U27 and CMT-U309 cell lines. Indeed, 26.99% and 59.32% of cells underwent apoptosis in the presence of a combination of 1/2 IC_{50} of curcumin and 1/2 IC_{50} of cyclophosphamide in CMT-U27 and CMT-U309 cells, respectively (Table 6). An increase in apoptosis was also seen in both cell lines treated with a combination of the IC_{50} of curcumin and IC_{50} of cyclophosphamide.

Table 5. Apoptosis in CMT-U27 and CMT-U309 cells in the presence of curcumin and cyclophosphamide

Drugs	Concentration of drugs	Necrotic	Late apoptotic	Early apoptotic	Viable
CMT-U27					
Control		1.37 ± 0.41	2.49 ± 0.42	4.85 ± 1.20	91.29 ± 1.20
CUR	10μM	2.61 ± 0.52	2.48 ± 0.62	6.34 ± 2.82	88.57 ± 2.56
CUR	25μM	3.07 ± 0.63	5.64 ± 0.81	7.47 ± 1.82	83.83 ± 1.45
CUR	50μM	2.05 ± 0.32	7.84 ± 0.44	11.44 ± 2.61	78.66 ± 2.42
CUR	100μM	1.68 ± 1.09	88.50 ± 2.88***	6.74 ± 2.11	3.08 ± 0.62***
CYP	2.5mM	3.36 ± 0.58	5.24 ± 0.70	5.30 ± 2.12	86.10 ± 1.48
CYP	5mM	4.32 ± 0.65	6.47 ± 0.57	6.09 ± 0.66	83.13 ± 0.85
CYP	10mM	2.54 ± 1.07	16.51 ± 2.68	9.66 ± 3.83	71.29 ± 1.82**
CYP	25mM	7.45 ± 1.32	46.61 ± 3.29***	29.19 ± 3.56*	16.75 ± 1.17***
CMT-U309					
Control		0.70 ± 0.29	2.91 ± 0.57	7.29 ± 0.65	89.10 ± 0.88
CUR	10μM	2.40 ± 0.69	4.49 ± 0.52	11.82 ± 0.98	81.29 ± 1.26
CUR	25μM	9.53 ± 1.31	10.92 ± 1.55	24.87 ± 0.93*	54.68 ± 1.33**
CUR	50μM	8.45 ± 1.75	26.01 ± 1.04**	34.71 ± 2.95**	31.10 ± 2.56***
CUR	100μM	6.11 ± 1.25	44.64 ± 2.76***	40.12 ± 3.04**	9.13 ± 1.52***
CYP	2.5mM	3.46 ± 0.92	3.79 ± 1.21	17.34 ± 1.45	75.41 ± 2.83
CYP	5mM	0.10 ± 0.03	1.63 ± 0.19	26.25 ± 1.79**	72.01 ± 1.83*
CYP	10mM	3.58 ± 1.03	2.78 ± 0.34	32.54 ± 1.15**	59.10 ± 0.49**
CYP	25mM	0.52 ± 0.10	2.53 ± 0.23	88.07 ± 1.97***	8.88 ± 1.66***

Data are expressed as means ± SE from three independent experiments; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to control
CUR = curcumin, CYP = cyclophosphamide

Combination of curcumin and cyclophosphamide causes CMT-U27 and CMT-U309 cells to accumulate in the S and G_2/M phases of the cell cycle

To determine whether curcumin and cyclophosphamide or a combination of both elicited alterations in cell cycle progression, cell cycle analysis was performed in CMT cells. Curcumin, at concentrations of 50 and 100μM, elicited a significant increase in the number of cells in the G_2/M phase, compared to the control group (Table 7). This was accompanied by a significant decrease in the percentage of cells in the G_0/G_1 phase, suggesting that the inhibitory effects of curcumin on the proliferation of CMT cells involved a block during the G_2/M phase. Table 7 shows the percentages of cells in G_0/G_1, S and G_2/M phases of the cell cycle. Cyclophosphamide treatment (10 and 25mM) resulted in a significant accumulation of cells in S phase, which was accompanied by a significant decrease in G_0/G_1 phase cells. Treatment with IC_{50} and 1/2 IC_{50} combinations of drugs resulted in the accumulation of cells in both S and G_2/M phases in both cell lines. The IC_{50} and 1/2 IC_{50} combined treatment increased the proportion of cells in S phase modestly in CMT-U309 cells (Figure 6).

Increased expression of Bax and decreased expression of Bcl-2 in CMT-U27 and CMT-U309 cells in response to curcumin and cyclophosphamide treatment

To determine the apoptotic pathway activated by curcumin, we examined the apoptosis- related targets, Bcl-2 and Bax in CMT-U27 and CMT-U309 cells. Immunocytochemical staining revealed that increasing concentrations of curcumin and cyclophosphamide inhibited the expression of the anti-apoptotic protein Bcl-2 and induced the expression of the pro-apoptotic protein Bax (Figures 7 and 8). In combination groups, these effects were more pronounced (Table 8). These data suggest that Bcl-2 family members play a major role in curcumin-induced apoptosis in canine mammary cancer cells.

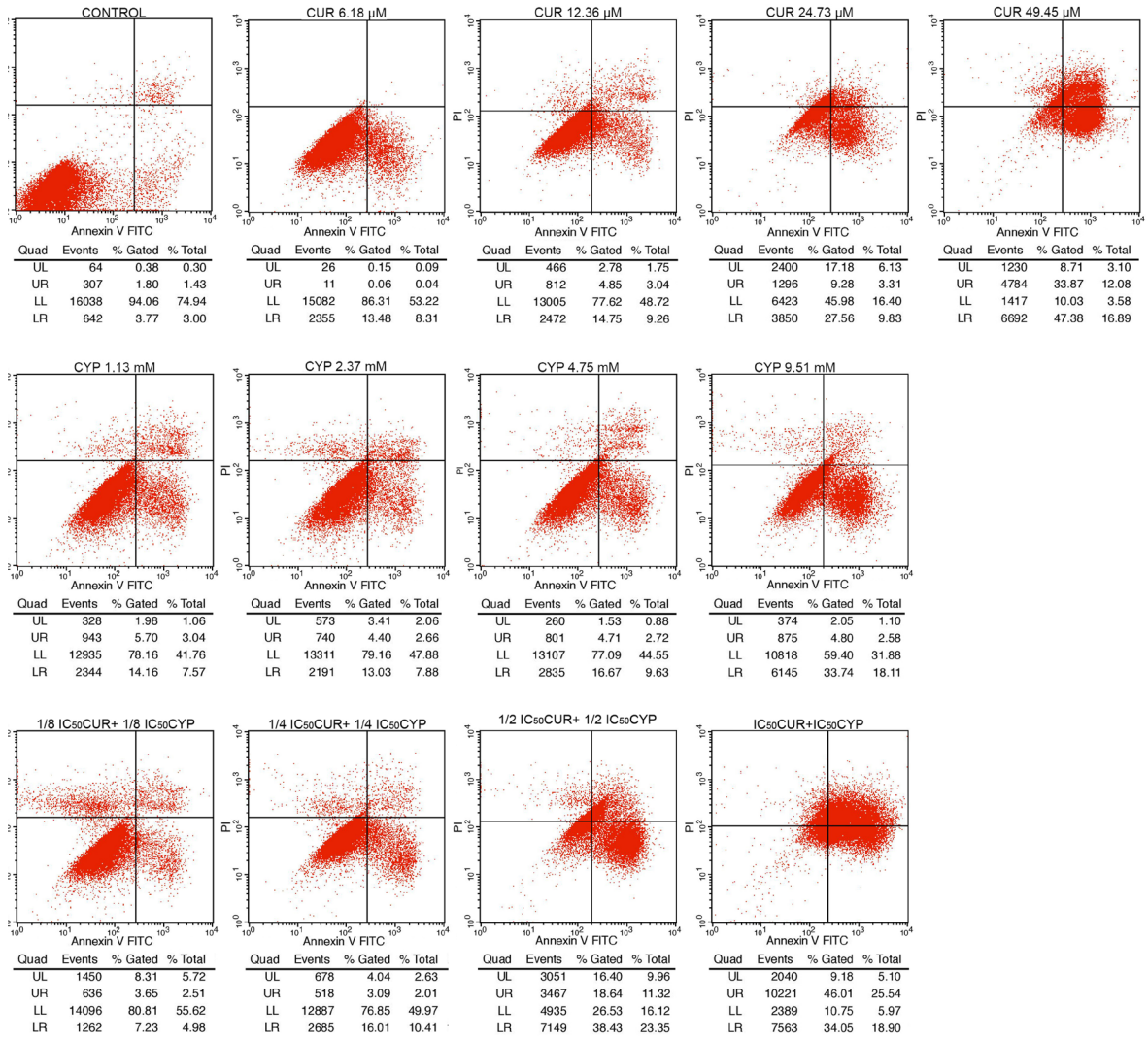

Figure 5. Flow-cytometric analysis of CMT-U309 cells after staining with Annexin-V FITC and propidium iodide (PI). Dual parameter dot plot of FITC-fluorescence (x-axis) vs. PI (y-axis) shows logarithmic intensity. Quadrants: $FITC^-/PI^-$ lower left (LL), viable cells; $FITC^+/PI^-$ lower right (LR), early apoptotic cells; $FITC^+/PI^+$ upper right (UR), late apoptotic cells; $FITC^-/PI^+$ upper left (UL), necrotic cells. Representative data from three independent experiments are shown

DISCUSSION

Combination chemotherapy of cancer using cytotoxic agents and dietary phytochemicals with different mechanisms of action may ameliorate the side effects associated with conventional chemotherapy (Tyagi et al. 2004). Further, combining different anti-cancer agents that induce or enhance apoptosis seems to be a promising strategy for overcoming drug resistance in cancerous cells (Hirsch and Lippman 2005; Atmaca et al. 2009). Curcumin is currently the subject of early phase clinical trials as a potential chemopreventive agent and has shown potent activity against some cancers (Wu et al. 2010; Zaidi et al. 2011; Yang et al. 2012). In this regard, here we assessed whether curcumin synergizes with the chemotherapeutic drug cyclophosphamide in inducing proliferation arrest and apoptosis in CMT-U27 and CMT-U309 canine mammary cancer cell lines. We found that curcumin and cyclophosphamide exerted anti-proliferative effects in both cell lines in a dose-and a time-dependent manner. IC_{50} values in CMT-U309 and CMT-U27 cell lines were found to be 49.45 and 68.39µM, respectively. CMT-U309 cells thus appeared to be more sensitive to curcumin and CMT-U309 viability was consistently lower than that of CMT-U27 at all curcumin concentrations

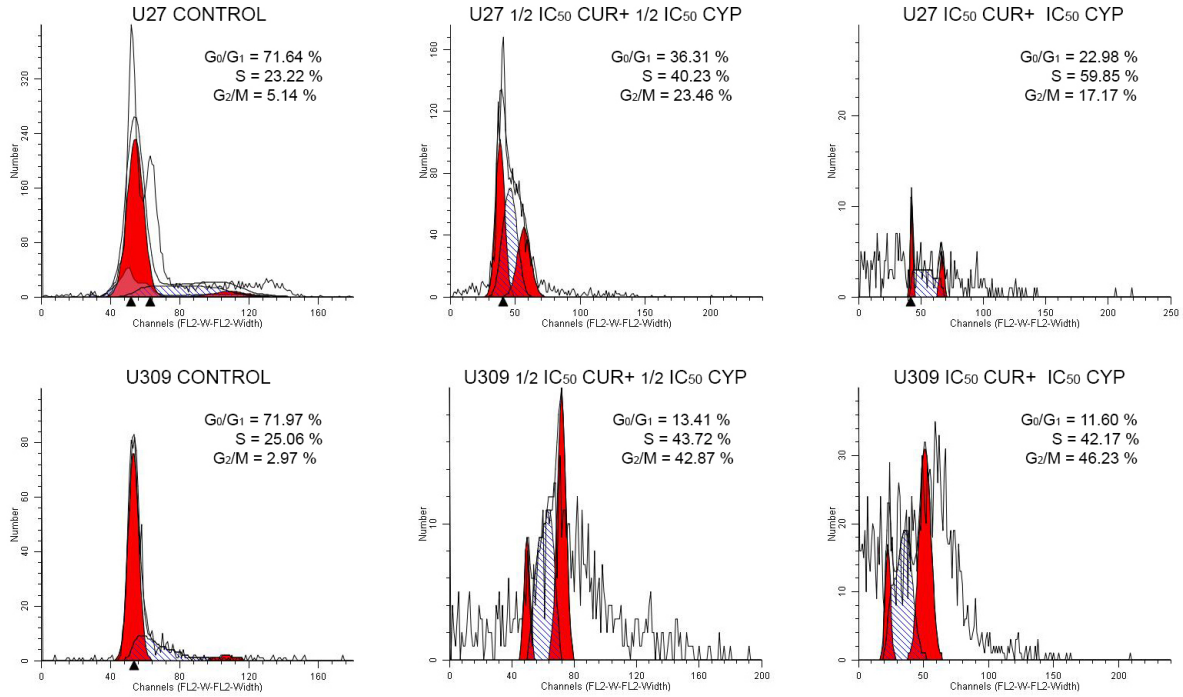

Figure 6. DNA histograms of CMT-U27 and CMT-U309 cells in the presence of different curcumin and cyclophosphamide combinations. Representative data from three independent experiments are shown

tested. It was reported that 50μM curcumin is similarly potent as the antineoplastic agent etoposide against human breast tumour cells (Martin-Cordero et al. 2003); thus, our inhibitory concentrations are within previously reported values (Roy et al. 2002). Since curcumin is described as a non-toxic anticancer compound, it could be useful in combination with antineoplastic agents so as to enhance their efficacy and reduce toxic effects (Yunos et al. 2011). Previous *in vitro* experiments revealed lower IC_{50} concentrations of curcumin (10–39.1μM) which are clinically relevant, and an enhancement of the inhibitory effect of curcumin in the presence of alkylating agents and anthracyclines was noted (Notarbartolo et al. 2005; Qian et al. 2011). These differences may be a factor of the cell lines studied, the rapidity of cell growth *in vitro*, and the methods used for analysis. The detection of LDH leakage is based on the loss of NADH due to its oxidation to NAD, resulting in the conversion of pyruvate to lactate. Enzymatic activity is measured externally, as it leaks from dead cells which lose their membrane integrity (Al-Qubaisi et al. 2011). The reverse correlation between the decreased cell viability and increased LDH activity that we observed suggests that curcumin can alter the membrane properties of CMT cells. Although the exact mechanisms underlying the anti-proliferative effects of curcumin are not clear, many recent reports suggest that its anticancer activity may be mediated by an apoptotic effect (Khar et al. 1999; Choudhuri et al. 2005). DNA fragmentation is considered to be one of the defining hallmarks of apoptosis and is one of the first biochemical phenomena described for this type of cell death. DNA fragmentation is catalysed by two major apoptotic nucleases, termed DNA fragmentation factor (DFF) or caspase-activated DNase (CAD) and endonuclease G (Magalska et al. 2006). Apoptotic chromatin condensation temporally correlates with DNA fragmentation and its efficiency frequently depends on the degree of internucleosomal cleavage (Widlak et al. 2002). However, the cell death induced with curcumin in CMT cells was not characterised by oligonucleosomal DNA fragmentation that is typical for apoptosis. Such a phenomenon was also observed in human Jurkat cells where it was reported that curcumin induces the caspase-3-dependent apoptotic pathway but inhibits DFF40/caspase-activated DNase endonuclease (Sikora et al. 2006). This can be explained by existence of additional cell death pathways in which DFF/CAD might not be the main executor of DNA fragmentation. We assume that curcumin can inhibit DFF or CAD, thus preventing DNA fragmentation, but not affecting CMT cell

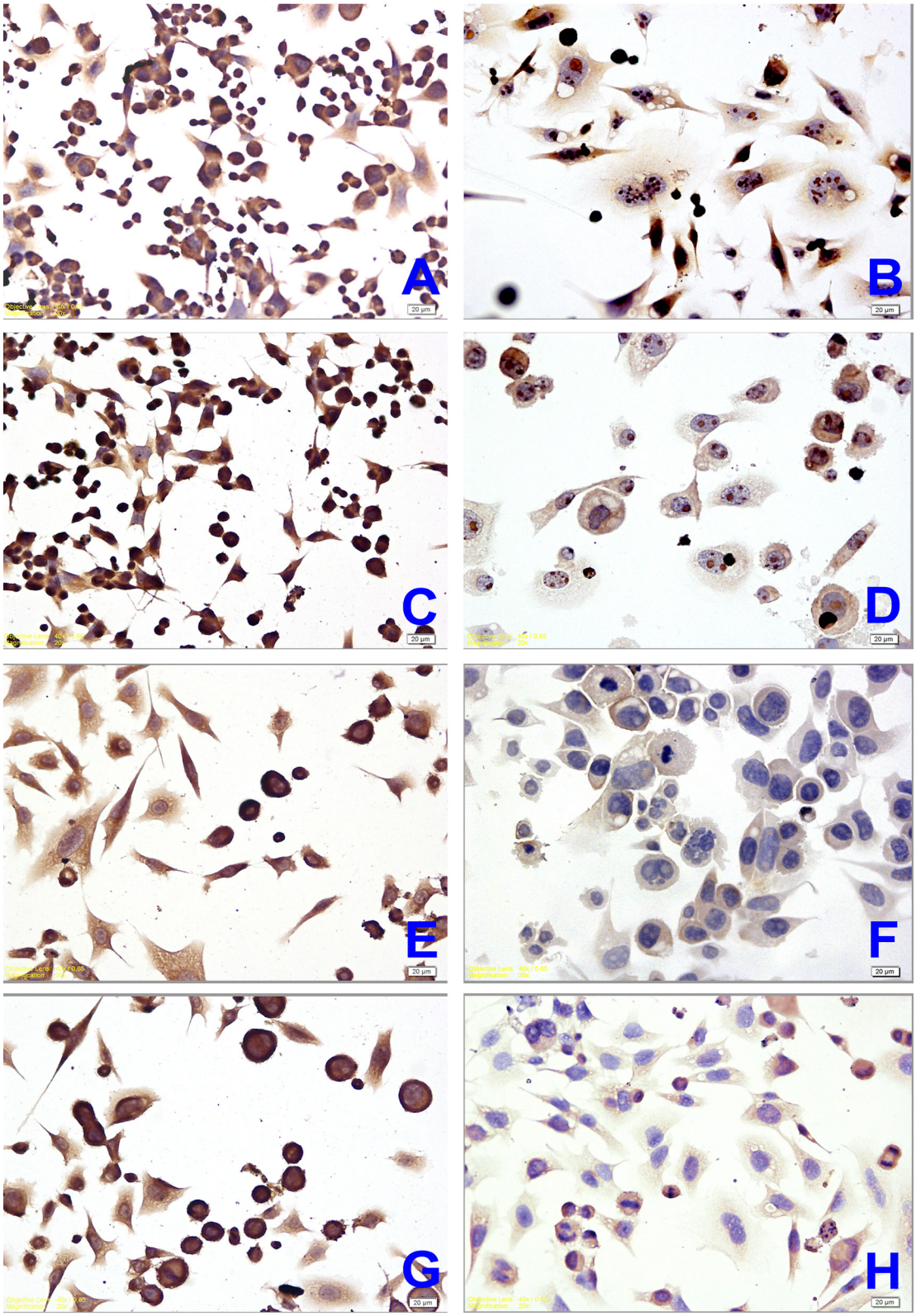

Figure 7. Immunocytochemical expression of Bax and Bcl-2 in CMT U27 cell line; bar = 20 μm **A**. Bax positive control, moderate immunopositivity; **B**. Bcl-2 positive control moderate immunopositivity; **C**. Curcumin 10μM Bax strong immunopositivity; **D**. Curcumin 10μM Bcl-2 moderate immunopositivity; **E**. Curcumin 50μM Bax very strong immunopositivity; **F**. Curcumin 50μM Bcl-2 slight immunopositivity; **G**. Cyclophosphamide 10 000μM Bax very strong immunopositivity; **H**. Cyclophosphamide 10 000μM Bcl-2 slight immunopositivity

Table 6. Apoptosis of CMT-U27 and CMT-U309 cells in the presence of curcumin and cyclophosphamide combinations

Drugs	Concentration of drugs	Late apoptotic	Early apoptotic	Viable
CMT-U27				
Control		2.71 ± 1.23	3.36 ± 0.65	92.37 ± 2.02
IC_{50} CUR	68.39μM	3.58 ± 1.72	7.77 ± 1.28	85.67 ± 3.35
1/2 IC_{50} CUR	34.20μM	1.60 ± 0.72	5.95 ± 1.50	90.75 ± 2.64
1/4 IC_{50} CUR	17.10μM	5.25 ± 2.33	5.03 ± 0.83	85.99 ± 3.89
1/8 IC_{50} CUR	8.55μM	2.54 ± 0.42	6.42 ± 1.51	89.14 ± 2.32
IC_{50} CYP	10.36mM	9.00 ± 1.79	14.22 ± 1.46	73.86 ± 2.88
1/2 IC_{50} CYP	5.18mM	13.39 ± 0.50	8.65 ± 0.76	74.61 ± 2.41
1/4 IC_{50} CYP	2.59mM	6.77 ± 0.31	6.74 ± 1.22	82.73 ± 1.31
1/8 IC_{50} CYP	1.30mM	4.09 ± 1.06	5.41 ± 0.72	86.33 ± 2.24
IC_{50} CUR + IC_{50} CYP	68.39μM + 10.36mM	69.21 ± 2.03***	14.25 ± 1.25	10.41 ± 2.13**
1/2 IC_{50} CUR +1/2 IC_{50} CYP	34.20μM + 5.18mM	14.93 ± 3.73	12.06 ± 1.02	41.67 ± 1.94*
1/4 IC_{50} CUR + 1/4 IC_{50} CYP	17.10μM + 2.59mM	5.69 ± 2.05	6.19 ± 1.18	84.91 ± 3.16
1/8 IC_{50} CUR + 1/8 IC_{50} CYP	8.55μM + 1.30mM	2.91 ± 1.49	3.81 ± 1.02	90.43 ± 2.50
CMT-U309				
Control		1.34 ± 0.23	8.43 ± 3.09	89.40 ± 3.25
IC_{50} CUR	49.45μM	30.31 ± 3.00**	46.37 ± 4.30*	10.61 ±2.12***
1/2 IC_{50} CUR	24.73μM	15.26 ± 3.17	28.82 ± 3.70	45.86 ± 3.13**
1/4 IC_{50} CUR	12.36μM	5.02 ± 0.83	14.82 ± 2.64	77.34 ±3.10
1/8 IC_{50} CUR	6.18μM	3.00 ± 1.58	9.40 ± 2.64	85.80 ± 3.11
IC_{50} CYP	9.51mM	4.44 ± 1.38	34.31 ±2.41*	58.55 ± 3.70*
1/2 IC_{50} CYP	4.75mM	3.78 ± 1.30	17.01 ± 1.60	76.76 ± 2.07
1/4 IC_{50} CYP	2.37mM	4.45 ± 1.02	13.40 ± 2.22	79.66 ± 2.76
1/8 IC_{50} CYP	1.13mM	5.42 ± 0.60	14.58 ± 2.58	78.16 ± 2.48
IC_{50} CUR + IC_{50} CYP	49.45μM + 9.51mM	42.20 ± 1.41***	33.26 ± 2.40*	12.76 ± 2.77***
1/2 IC_{50} CUR + 1/2 IC_{50} CYP	24.73μM + 4.75mM	19.90 ± 0.90**	39.42 ± 1.41**	27.04 ± 3.16***
1/4 IC_{50} CUR + 1/4 IC_{50} CYP	12.36μM + 2.37mM	4.67 ± 2.07	15.52 ± 1.50	75.78 ± 2.84
1/8 IC_{50} CUR + 1/8 IC_{50} CYP	6.18μM + 1.13mM	5.36 ± 1.12	7.80 ± 0.98	80.39 ± 1.03

Data are expressed as means ± SE from three independent experiments; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to control
CUR = curcumin, CYP = cyclophosphamide

death itself. As oligonucleosomal DNA degradation is considered a main hallmark of apoptotic cell death the lack of this sort of degradation can be improperly interpreted as an absence of apoptosis. Thus, we analysed apoptosis in more detailed by focusing on the quantitative distribution of cells using Annexin V staining with flow cytometry. Our FACS analysis showed that curcumin could indeed induce apoptosis in CMT cells as determined by the increased percentages of early and late apoptotic cells. However, after combination treatment the early apoptotic cell percentage was increased in CMT-U309 cells while the late apoptotic cell percentage was increased in CMT-U27 cells. Apoptosis can be triggered by intracellular apoptosis signalling pathways. The intrinsic pathway involves an increase in outer mitochondrial membrane permeability that leads to a loss of mitochondrial function and results in apoptosis (Limtrakul 2007). The apoptosis induced by curcumin in CMT cells is considered to be mediated by the intrinsic pathway related to mitochondrial dysfunction. We observed that curcumin significantly inhibited cell proliferation via the induction of G_2/M cell cycle arrest in both cell lines. Curcumin-induced G_2/M cell cycle arrest has been described to lead to apoptosis, although in cells overexpressing cyclin D1, this arrest does not occur, allowing the cells to pass

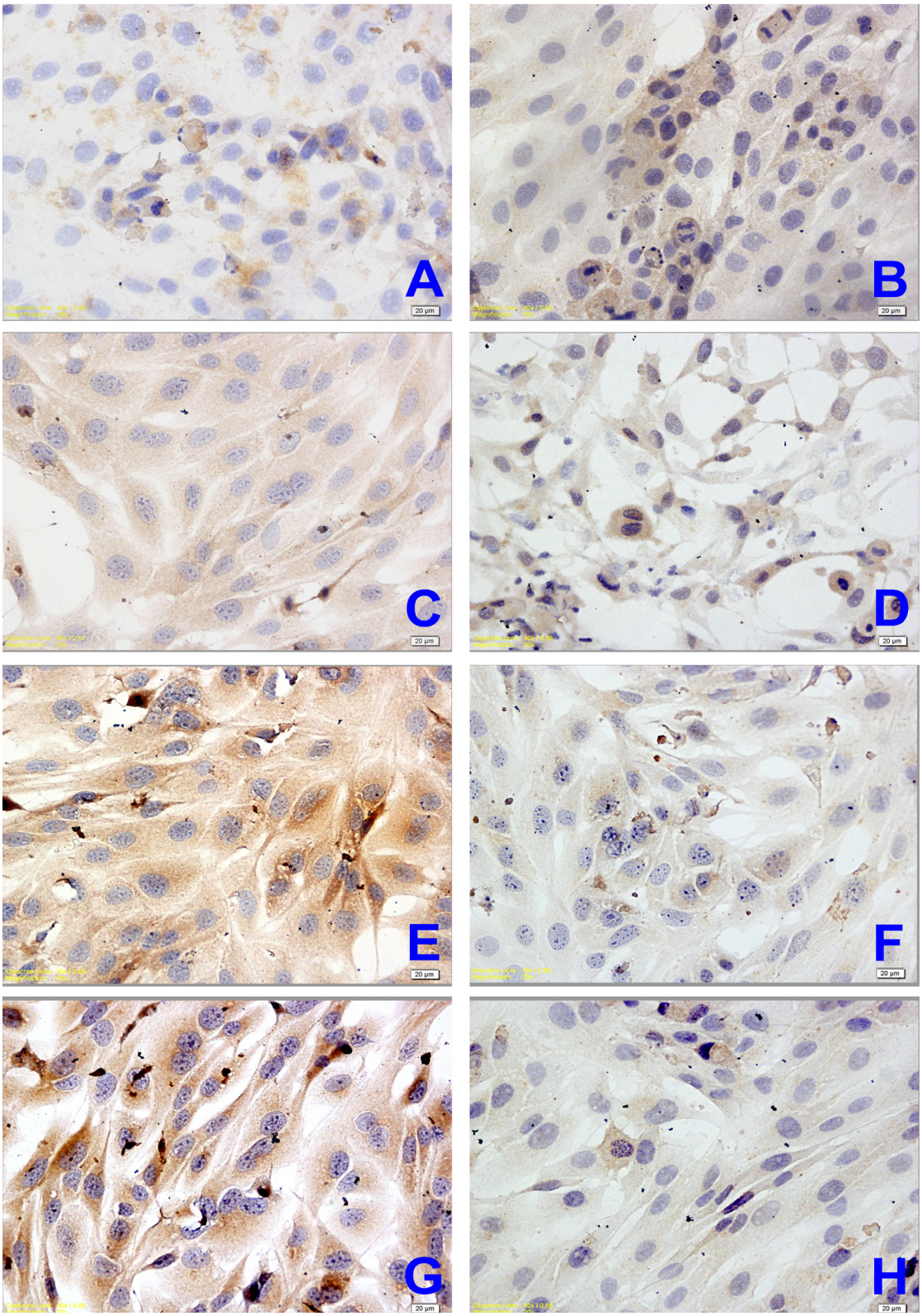

Figure 8. Immunocytochemical expression of Bax and Bcl-2 in CMT U309 cell line; bar = 20 µm **A**. Bax positive control, slight immunopositivity; **B**. Bcl-2 positive control, moderate immunopositivity; **C**. Curcumin 10µM Bax slight immunopositivity; **D**. Curcumin 10µM Bcl-2 immunopositivity; **E**. Curcumin 50µM Bax moderate immunopositivity; **F**. Curcumin 50µM Bcl-2 slight immunopositivity; **G**. Cyclophosphamide 10000µM Bax strong immunopositivity; **H**. Cyclophosphamide 10000µM Bcl-2 slight immunopositivity

Table 7. Cell cycle distribution in CMT-U27 and CMT-U309 cells in the presence of curcumin and cyclophosphamide and their combination

Drugs	Concentration of drugs	G_0/G_1	S	G_2/M
CMT-U27				
Control		69.70 ± 2.23	23.85 ± 2.38	6.41 ± 0.74
CUR	50μM	5.30 ± 1.61***	40.46 ± 1.16	54.24 ± 0.46***
CUR	100μM	10.53 ± 0.97***	61.90 ± 1.54**	27.56 ± 0.58**
CYP	10mM	38.77 ± 1.05**	43.54 ± 2.49*	17.68 ± 1.44*
CYP	25mM	15.31 ± 1.14***	76.88 ± 2.02***	7.80 ± 0.92
IC_{50} CUR + IC_{50} CYP	68.39μM + 10.36mM	22.72 ± 2.31***	60.06 ± 1.55**	17.21 ± 0.76*
1/2 IC_{50} CUR + 1/2 IC_{50} CYP	34.20μM + 5.18mM	36.36 ± 1.64**	39.87 ± 2.39	23.75 ± 0.76**
CMT-U309				
Control		71.48 ± 1.74	25.21 ± 1.31	3.00 ± 0.67
CUR	50μM	2.75 ± 0.90***	21.23 ± 1.73	76.01 ± 0.83***
CUR	100μM	7.12 ± 1.46***	39.56 ± 1.68	53.32 ± 2.52***
CYP	10mM	16.65 ± 1.44***	58.14 ± 2.30**	25.19 ± 0.88**
CYP	25mM	6.10 ± 1.05***	87.75 ± 1.89***	6.14 ± 0.85
IC_{50} CUR + IC_{50} CYP	49.45μM + 9.51mM	11.39 ± 1.02***	42.52 ± 2.77	46.08 ± 1.75***
1/2 IC_{50} CUR + 1/2 IC_{50} CYP	24.73μM + 4.75mM	13.50 ± 1.18***	43.59 ± 2.90	42.90 ± 1.71***

Data are expressed as means ± SE from three independent experiments; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to control
CUR = curcumin, CYP = cyclophosphamide

into G_2/M and undergo apoptosis (Zheng et al. 2004; Choudhuri et al. 2005). Both the magnitude and the temporal sequence of this effect indicate that it is, at least to a large degree, responsible for the reduction in cell number that we observed. In combination treatments, in addition to a G_2/M cell cycle arrest, we also observed arrest in the S phase of the cell cycle. This indicates that curcumin prevents proper DNA replication, and thereby inhibits tumour growth. The changes cell cycle distribution indicated that curcumin in combination with cyclophosphamide may have a synergistic apoptotic effect on the two cell lines. In order to further elucidate the molecular mechanism responsible for the curcumin-induced apoptosis in CMT cells, the expression of some apoptosis-related genes such as Bcl-2 and Bax was analysed. The relative expression of Bax and Bcl-2 has been reported to play a major role in determining whether cells will undergo apoptosis under conditions that promote cell death (Cui et al. 2010). Increased expression of Bax can induce apoptosis, while Bcl-2 protects cells from apoptosis (Cory and Adams 2002). Bcl-2 predominantly localises on the outer mitochondrial membrane and exerts its anti-apoptotic effects via stabilisation of the mitochondrial membrane, thus inhibiting opening of the permeability transition pore and the release of mitochondrial cytochrome c (Egan et al. 1999). By contrast, Bax is predominantly localised in the cytosol and upon activation, translocates to the mitochondria and asserts a pro-apoptotic effect by interacting with membrane pore proteins leading to the release of cytochrome c (Priault et al. 1999). We hypothesised that curcumin-induced apoptosis in CMT cell lines may be mediated by changes in the expression of Bcl-2 and Bax. Our immunocytochemical staining showed that Bcl-2 expression is more pronounced in CMT-U27 cells than CMT-U309 cells and that curcumin downregulated Bcl-2 and upregulated Bax in a dose-dependent manner. Consequently, we suggest that Bcl-2 and Bax are critical regulators of curcumin-induced apoptosis in CMT cell lines. In conclusion, we have demonstrated that curcumin induces apoptosis and cell cycle arrest in canine mammary tumour cells which is accompanied by a modulation of Bcl-2/Bax protein expression. In addition, combined treatment with cyclophosphamide and curcumin had synergistic effects on the inhibition of proliferation in CMT-U27 and CMT-U309 cells via induction of apoptosis and cell cycle arrest. These findings provide a molecular basis for the development of natural compounds as novel anti-cancer agents for canine mammary cancer. Further in-depth investigations

Table 8. Effects of curcumin, cyclophosphamide and their combination on immunocytochemical expression of Bax and Bcl-2 in CMT U309 and CMT-U27 cell line

Drugs	Concentration of drugs	Immunopositivity	
		Bax	Bcl-2
CMT-U27			
Control		++	+++
CUR	10μM	++	++
CUR	25μM	+++	++
CYP	10mM	+++	++
IC_{50} CUR	68.39μM	+++	+
1/2 IC_{50} CUR	34.20μM	++	++
1/4 IC_{50} CUR	17.10μM	++	++
IC_{50} CYP	10.36mM	+++	++
1/2 IC_{50} CYP	5.18mM	++	++
1/4 IC_{50} CYP	2.59mM	++	++
IC_{50} CUR + IC_{50} CYP	68.39μM + 10.36mM	+++	+
1/2 IC_{50} CUR + 1/2 IC_{50} CYP	34.20μM + 5.18mM	+++	+
1/4 IC_{50} CUR + 1/4 IC_{50} CYP	17.10μM + 2.59mM	++	++
CMT-U309			
Control		+	++
CUR	10μM	+	++
CUR	25μM	+	++
CYP	10mM	++	+
IC_{50} CUR	49.45μM	++	+
1/2 IC_{50} CUR	24.73μM	+	+
1/4 IC_{50} CUR	12.36μM	+	++
IC_{50} CYP	9.51mM	++	++
1/2 IC_{50} CYP	4.75mM	+	+
1/4 IC_{50} CYP	2.37mM	++	+
IC_{50} CUR + IC_{50} CYP	49.45μM + 9.51mM	++	+
1/2 IC_{50} CUR + 1/2 IC_{50} CYP	24.73μM + 4.75mM	++	+
1/4 IC_{50} CUR + 1/4 IC_{50} CYP	12.36μM + 2.37mM	++	++

Results were categorised as negative, + (< 10% positive cells), + + (10–50% positive cells), and +++ (> 50% positive cells)
CUR = curcumin, CYP = cyclophosphamide

should be performed in xenograft models to confirm the efficacy of this promising new treatment for canine mammary cancer.

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