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The anti-proliferation and pro-apoptosis of hydroxybenzoate calcium complexes in HT-1080 human fibrosarcoma cells

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Summary. *Aim:* Hydroxybenzoic acids are one of the major classes of phenolic compounds and are widely distributed in the phytochemicals of various plants. The aim of this study is to assess the efficacy of 2-, 3- and 4-hydroxybenzoate calcium (HBCa) complexes on HT-1080 cells and examine their anti-proliferative and pro-apoptotic effects in relation to 2-acetylbenzoic acid (2-ABA), or aspirin. *Materials and Methods:* The cytotoxicity of HBCa complexes was analysed in HT-1080 cells by MTT assay. Immunochemical and morphological assessment techniques were conducted to detect apoptosis, while Western blot was used to measure the expression of pro- and anti-apoptotic proteins. *Results:* The current in vitro study showed that HBCa complexes exert their cytotoxic activity in a concentration and chemical structure-dependent manner. 4-HBCa showed more potency than 3-HBCa, 2-ABA and 2-HBCa analogues. HBCa complexes inhibited cell proliferation, inducing apoptosis and cell cycle arrest at G0/G1 in human fibrosarcoma HT-1080. In addition, HBCa complexes modulated anti-apoptotic protein Bcl-2 and the induction of Bax, p53 and caspases-3 in favour of apoptosis. *Conclusion:* These results suggest that the apoptotic effects of the HBCa complexes are driven via the intrinsic apoptotic pathway which is regulated, at least to some extent, by the relative expression of Bcl-2 family proteins. The increased potency of 4-HBCa compared to 2-HBCa, 3-HBCa and 2-ABA provides a rationale for the continued exploration of these analogues as anti-cancer agents.

Key words: hydroxybenzoate calcium, anti-proliferation, apoptosis, HT-1080 fibrosarcoma cell line

«L'attività anti-proliferativa e pro-apoptotica dei complessi di idrossibenzoato di calcio in cellule di fibrosarcoma umano HT-1080»

Riassunto. *Scopo:* Gli acidi idrossibenzoici sono una delle principali classi di composti fenolici e sono ampiamente presenti tra i fitochimici di piante diverse. Lo scopo di questo studio è quello di valutare l'efficacia dei complessi di 2-, 3- e 4- idrossibenzoato di calcio (HBCa) su cellule HT-1080 ed esaminare i loro effetti anti-proliferativi e pro-apoptotici in relazione all'acido 2-acetilbenzoico (2-ABA) o aspirina. *Materiali e Metodi:* La citotossicità dei complessi HBCa è stata analizzata in cellule HT-1080 mediante saggio MTT. Per rilevare l'apoptosi sono state utilizzate tecniche di valutazione immunochimica e morfologica, mentre il Western blot è stato utilizzato per misurare l'espressione di proteine pro- e anti-apoptotiche. *Risultati:* L'attuale studio in vitro ha dimostrato che i complessi HBCa esercitano la loro attività citotossica in maniera dipendente da concentrazione e struttura chimica. 4-HBCa ha mostrato maggiore efficacia degli analoghi 3-HBCa, 2-ABA e 2-HBCa. I complessi HBCa inibiscono la proliferazione cellulare, inducono apoptosi e arrestano il ciclo cellulare in G0/G1 in fibrosarcoma umano HT-1080. Inoltre, i complessi HBCa modulano la proteina anti-apoptotica Bcl-2 e determinano l'induzione di Bax, p53 e caspasi-3 in favore dell'apoptosi. *Conclusione:*

Questi risultati suggeriscono che gli effetti apoptotici dei complessi HBCa sono guidati attraverso la via apoptotica intrinseca che è regolata, almeno in parte, dalla relativa espressione di proteine della famiglia Bcl-2. La maggiore efficacia di 4-HBCa rispetto al 2-HBCa, 3-HBCa e 2-ABA fornisce un fondamento logico per l'esplorazione continua di tali analoghi come agenti anti-cancro.

Parole chiave: calcio idrossibenzoato, anti-proliferazione, apoptosi, linea cellulare di fibrosarcoma HT-1080

Introduction

The commonest drug belongins to the hydroxybenzoic acid (HBA) group of phytochemicals is 2-acetylhydroxybenzoic acid (2-ABA, Figure 1), or aspirin. Pharmacologically, 2-ABA and its precursor, 2-HBA were recognised in 1987 as non-steroidal anti-inflammatory drugs (1). Research has indicated that the additional compounds: 3-HBA, 4-HBA and their metal ion counterparts display various pharmacological properties, including anti-cancer, anti-inflammation and anti-proliferation (1-4). The mechanisms of these biological activities are *via* modulation of the apoptosis related proteins in cancer cells. They modulate the expression of NF-κB and induce apoptosis (4).Although a complicated process, it is characterised by the key biochemical hallmarks and morphological features of activation of caspases, chromatin condensation and membrane blebbing (5-7). Furthermore, these phytochemicals and their analogues can alter the ratio of pro-apoptotic (e.g. Bax) to anti-apoptotic (e.g. Bcl-2) members of the Bcl-2 family; these proteins are the necessary regulators of apoptotic pathways (7-10). 2-Hydroxybenzoate calcium (2-HBCa, Figure 1) upregulates the expression of p21, p53 and Bax while it down regulates Bcl-2 in a concentration-dependent manner (2). 2-HBCa also inhibitd cell proliferation in the HT-1080 fibrosarcoma cell line by approximately 25% as compared to the control.

The presence of the hydroxyl group at the *ortho* (or C2) position in 2-HBA is more acidico than the hydroxyl group at the *meta*- (C3) or *para*- (C4) position (Figure 1). Thus, these compounds possess different chemical and pharmacological properties. Previous research has shown that HBA and their organometallic counterparts express different efficacy in inducing anti-proliferation and pro-apoptotic activits (1-4). In addition, 2-ABA has a side effecs profile on the cardiovascular and gastrointestinal systems, thus, demonstrating the need for development of more specific anti-inflammatory agents. This study focuses the effects of 2-HBCa, 3-HBCa and 4-HBCa (Figure 1) in promoting anti-proliferation and pro-apoptotic activities as compared to 2-ABA. Additionally, the significance of the hydroxyl group position on the aromatic moiety and the way different organometallic complex counterparts influence efficacy are issues that we discussed.

Materials and methods

HT-1080 human fibrosarcoma cell lines (American Type Culture Collection; Rockville, USA) were cultured at 37°C in a humidified atmosphere containing 5% CO_2. Optimal Dulbecco's Minimum Essential Medium (DMEM, Gibco, USA) was supplemented with 10% foetal bovine serum (FBS) and 1% gentamicin-streptomycin (Gibco BRL), 1% L-glutamine, 0.1% Hepes buffer (1M), 0.1% sodium pyruvate (104 lM) and 0.1% ascorbic acid. HT-1080 cells were cultured for 3 days (based on the growth curve of HT-1080 cells), giving approximately 70% confluence, and then further cultured for another 24 hours, an, 72 hours, with different concentrations of HBCa complexes.

Cell proliferation

The cell proliferation of human HT-1080 cells was assessed by the standard MTT assay. Cell viability or cell survival was expressed relative to the control. Briefly, cells were grown to their exponential stage of growth and then seeded into 96-well plates at a density of approximately $4X10^3$ cells per well in 100 μL of complete DMEM medium for 24 hours. HBCa complex treated cells were further allowed to grow for 24, 48, and 72 hours. The concentrations of HBCa complexes were as follows: 0, 0.05, 0.1, 0.2, 0.3 and 0.6 mM. At the end of each incubation period, an MTT

assay was carried out on the cells. The media were removed from each well of the 96-well plate. Then, using a multi-channel pipette, 110 μL of the mixture containing plain media and MTT was added to each well, including the control wells, and incubated for 1 hour. After 1 hour, 100 μl of MTT lysis buffer was added into each well and mixed by pipetting up and down. These cells were incubated for a further hour. Two hours after the addition of MTT, the amount of formazan production was measured spectrophotometrically at 550-600 nm using an ELISA plate reader.

Light microscopy

Six-wetts plates containing coverslips were used to seed HT-1080 cells, each containing approximately 100 x 10^3 cells/ml. Cells were allowed to grow in a standars Eagle Medium and under standard conditions for 24 hours. Cells were treated with 400 and 800 μM of 2-HBCa, 3-HBCa and 4-HBCa for 48 hours. The media was removed and cells were washed with 2 ml of phosphate-buffered saline and left for 2-5 minutes before 2 ml of formalin was added and left for 10 minutes. Cells attached to the coverslips were then stained with Haematoxylin and Eosin for 4 minutes, washed with distilled water, dehydrated using 75%, 95% and 100% alcohol, before being treated with xylene solutions. Finally, the coverslips were individually mounted on glass slides with DPX. Stained cells were viewed and analysed by microscope attached to a computer run by the AnalySIS programme. Cells were counted and expressed as a percentage.

Annexin V-FITC

The effect of HBCa complexes on HT-1080 cells was measured by Annexin V-FITC apoptotic assay (Bender Medsystems, Vienna, Austria). 5x10^5 cells were treated with an increasing concentration of HBCa complexes for up to 72 hours at 37°C in a 25 cm^2 flask. Cells were trypsinised with 2 ml trypsin and the cell concentration was adjusted to 10^6 cells/ml 0.5 ml of the cell suspension was seeded in Eppendorf tubes and 10 μl Media Binding Reagent was added in each tube before 1.25 μl Annexin V-FITC was added. This was followed by incubation of cells at room temperature for 15 min before removal of the medium, gentle re-suspension of cells and labelling with 10 μl propidium iodide. The samples were then immediately analysed by flow cytometry.

Scanning electron microscopy

HT-1080 cells were seeded into 12-well plates containing microscopic slide cover slips at a density of 15x10^3 cells and incubated for 48 hours with 2 mM or 6mM HBCa complexes. Cells were fixed for 1 hour with 0.8% glutaraldehyde, 0.6% osmium tetraoxide, 2mM $CaCl_2$ and 0.2M sucrose in 0.1M cacodylate buffer pH 7.4. HT-1080 cells were then washed several times with PBS buffer and dehydrated using a sequence of alcohol concentrations (30%, 50%, 70%, 90%, each for 5 min and 100% for 10 min twice). The dehydrated HT-1080 cells were then dried to the critical point in Blazers CPD 030 using CO_2, cells were mounted onto 12 mm 'Philips type' aluminium stubs using silver paint, then gold sputter coated in an Edwards S150B sputter coater. Finally, the samples were imaged using a Philips XL20 SEM.

Cell cycle analysis

HT-1080 cells were seeded in T-25 flasks (Coster) at an initial density of 1x10^3 cells per flask. Cells were cultured as described in previous discussions before being treated with 3-HBCa and 4-HBCa complexes for 24 hours. In addition, control experiments were performed in which no salicylate compounds were incorporated in the cultures. At the end of each treatment, the cells were harvested by trypsinization, centrifuged and re-suspended in 200 μl of PBS. Aliquots (2 ml) of ice-cold 70% ethanol were then added and the cells were vortexed prior to being cooled to -20° for 30 min. Subsequently, 100 μl of RNase (Sigma, UK) at a concentration of 1 mg/ml and 100 μl of propidium iodide (Biovision Incorporate, CA, USA) at a concentration of 0.4 mg/ml was added prior to incubation at 37°C for 30 min. Quantitative fluorescent staining and flow cytometric analysis of cellular DNA assessed the relative DNA contents for cell cycle kinetics, using (Becton Dickinson, San Jose, CA, USA) and Cell Quest software (Becton Dickinson).

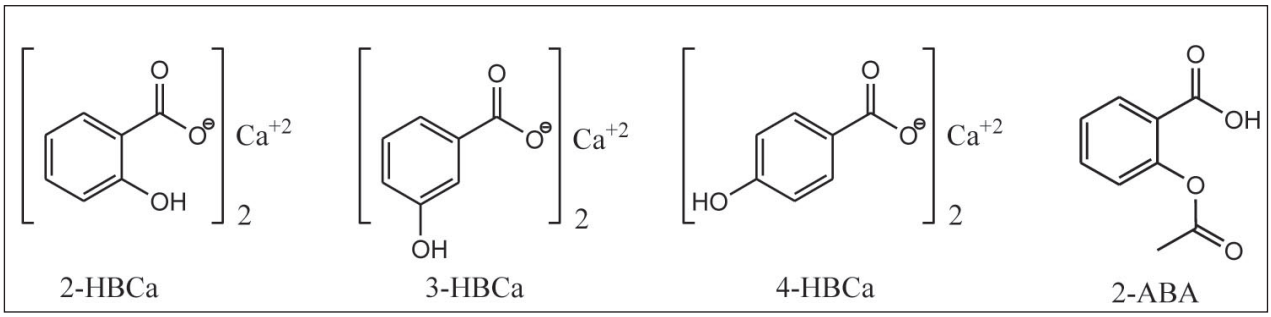


Figure 1. The chemical structures of HBCa complexes and 2-ABA.

Immunoblot analysis

HT-1080 cells were seeded in six-well plates at 3x104 initial density and cultured in DMEM medium. Cells were allowed to grow for 48 hours in the presence of 0.2 or 6 mM of 2-HBCa, 3-HBCa or 4-HBCa under optimal culture conditions, before being washed with cold PBS (10 mM, pH 7.4) to remove any remaining medium. This step was followed by adding 200 ml of 2 x sample buffer (250 mm Tris-HCl pH 6.8, 4% SDS, 0.006% bromophenol blue, 2% β-mercaptoethanol; Pharmacia, Uppsala, Sweden) to each well and the cells were then harvested using a cell scraper. HT-1080 cells were treated with lysate buffer, after which they were transferred to a 1.5 ml Eppendorf tube, heated at 100˚C for 10 min, cooled to room temperature, and centrifuged at 12000 G for 5 min. The supernatant was further centrifuged at 4˚C and 16 000 G for 5 min to obtain a clear solution of protein mixture, which was used to measure the expression of p53, Bcl-2, Bax and caspase-3 by Western blotting. β-actin was used as the internal standard. Total cell lysate protein concentrations were determined according to the method in the literature (11).

Thirty micrograms (22 μl) of the protein sample and 10 μl of molecular marker were loaded to 4–12% bis- tris acrylamide gel in NuPAGE MOPS (3-(Nmorpholino) propanesulphonic acid) SDS running buffer (Invitrogen, Life Technologies, Scotland, UK). After running the gel at 200V for 30–50 min, resolved proteins were transferred to a nitrocellulose membrane (Sigma). Membranes were incubated first with an appropriate primary antibody (p53, Bcl-2, Bax, or β–actin, then with peroxidase conjugated anti-mouse IgG antibody (Sigma). They were then washed and developed using a chemiluminescent reagent (Amersham, Cardiff, UK) and then were exposed to photographic films. The protein bands intensities were scanned and quantified using a densitometer.

Statistical analysis

Data obtained in these experiments were evaluated using equal variance and paired Student's t-test (two-tails). The Pearson correlation coefficient was calculated, along with other statistical analyses, using Graphpad Prism 5.0 software (Graphpad Software Inc., San Diego, CA, USA).

Results

The effects of HBCa complexes on HT-1080 cell proliferation

The effect of 2-HBCa, 3-HBCa and 4-HBCa on the cell proliferation of HT-1080 cells was conducted using the MTT assay. The results demonstrated that HBCa complexes exert a bi-phasic effect on HT-1080 cells; low doses (≤ 0.3 μM) appeas to induce a slight pro-proliferative effect whilst higher doses (≥0.3 mM) had more pronounced anti-proliferative effects (Figure 2). The effect of HBCa complexes in HT-1080 cell proliferation was observed in a dose- and time-dependent manner. Generally, HT-1080 cells showed an overall range of 4% to 16% proliferation increase. In contrast, 0.6 mM HBCa complexes reduced proliferation in the

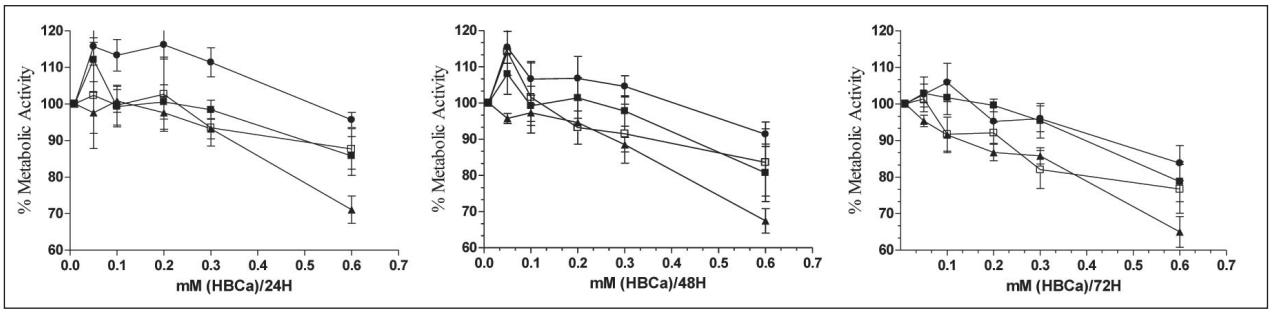


Figure 2. The cell proliferation of HT-1080 cells treated with 2-HBCa (●), 3-HBCa (■),4-HBCa (▲) and 2-ABA (□) at different concentrations (0.01,0.05, 0.1, 0.2, 0.3, 0.6mM) for 24, 48 and 72 hours. The cell proliferation was measured by the MTT method and data were normalised to the control (untreated) samples.

range of 4-29%, 9-32% and 16-53% when HT-1080 cells were treated for 24, 48, and 72 hours respectively. The results indicate that the anti-proliferative effect of HBCa complexes mainly follows the following order: 2-HBCa < 2-ABA < 3-HBCa < 4-HBCa (Figure 2).

In addition, cells stained with Haematoxylin-Eosin showed a reduction in cell density when HT-1080 cells were treated with 0.3 and 0.6 mM HBCa complexes for 48 hours as compared to the control (Figure 3). 4-HBCa was more effective than 3-HBCa and 2-ABA.

The detection and assessment of apoptosis induced by HBCa Complexes

Treatment of HT-1080 cells with 0.6 mM 4-HBCa for 48 hours revealed morphological features of apoptosis. These features include loss of microvilli, condensation of chromatin at the nuclear periphery, membrane blebbing, formation of apoptotic bodies and consequent cell shrinkage (Figure 3). The treatment of HT-1080 cells with 0.3 mM and 0.6 mM 4-HBCa for 48 hours induced apoptotic cell death, which was confirmed by SEM analysis. Figure 4 shows evidence of the apoptotic effects of 4-HBCa. Untreated HT-1080 cells displayed a flat monolayer structure and retained attachment to the surface as well as pseudopodial attachment to each other (Figure 4.2). In contrast, these features were not evident in cells treated with 4-HBCa and instead were replaced by the appearance of apoptotic bodies (Figure 4.2).

Figure 5A shows an example of the annexin-V/ propidium iodide plots for HT-1080 cells treated

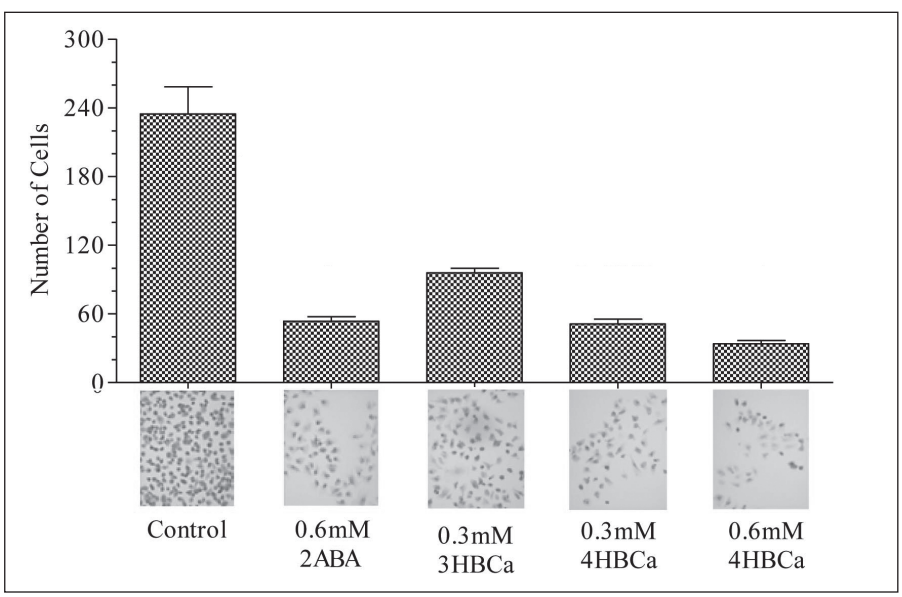


Figure 3. The effects of HBCa complexes on HT-1080 cell density stained with Haematoxylin and Eosin. The graph represents the mean values of the number of cells which were obtained from 4 slides. The pictures underneath represent a sample of each treatment [T-test (2-tailed, p < 95%)].

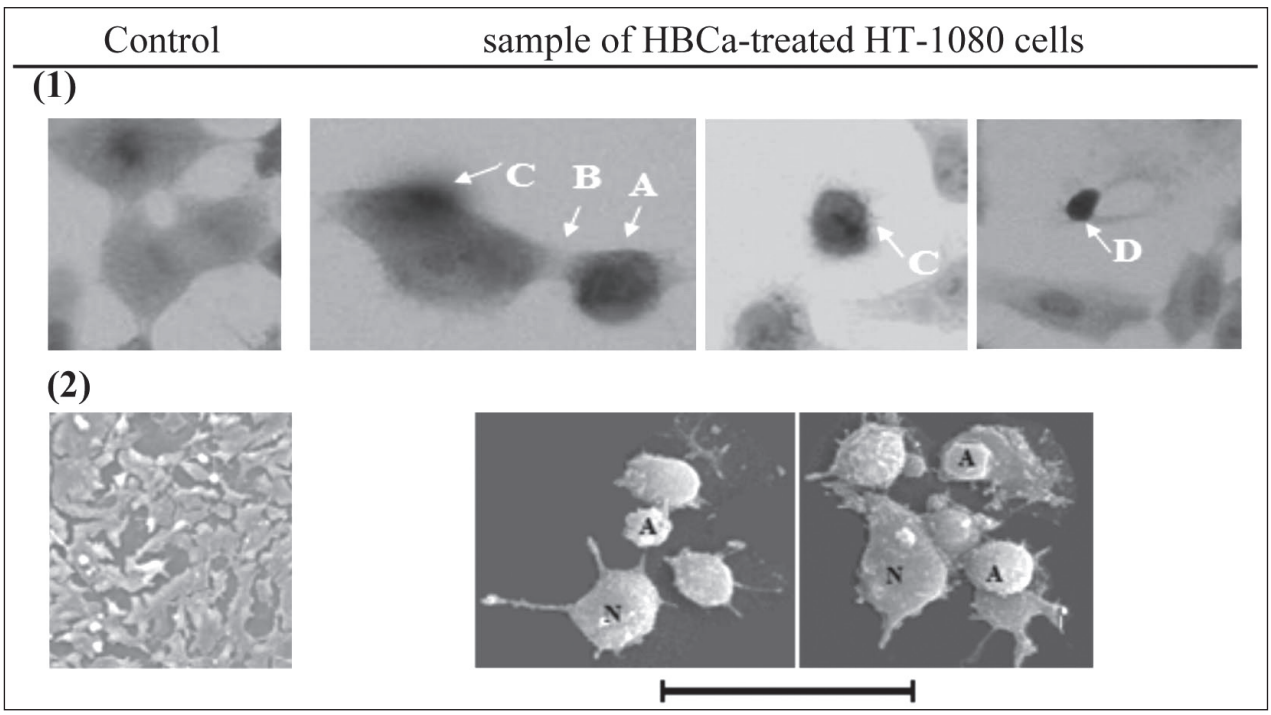


Figure 4. (1) Light micrograph of HT-1080 cells stained by Haematoxylin and Eosin showing different features of apoptosis: A = loss of microvilli; B = chromatin margination; C = apoptotic cell. (2) SEM of HBCa-treated HT-1080 cells whereas (N) refers to normal HT-1080 cells and A an apoptotic cell. Cells were treated with 0.6 mM 4-HBCa for 48 hours. Magnification bars represent 50 μm.

with 0.3mM and 0.6mM, 3-HBCa or 4-HBCa for 48 hours. Both 3-HBCa and 4-HBCa induced apoptosis in a concentration-dependent manner. 3-HBCa induced early and late apoptosis by at least 11.1% at 0.3 mM and 20.6% at 0.6 mM (Figure 5B). The induction of the overall apoptosis increased to 22.1 and 33% when HT-1080 cells were treated with 0.3 and 0.6 mM 4-HBCa respectively (Figure 5B).

The effects of HBCa complexes on the cell cycle distribution of HT-1080 cells

The effect of 3-HBCa and 4-HBCa on HT-1080 cell cycle phases were analysed based on the DNA contents after culturing for 24 hours. Results showed that 3-HBCa and 4-HBCa treatments did not alter the sub-G1 phase. However, a significant increase in the G0/G1 phase occurred following exposure to 3-HBCa and 4-HBCa: 21.4% (p= 0.0299) and 24.7% (p= 0.0054) respectively (Figure 6). Both 3-HBCa and 4-HBCa complexes increased G0/G1 in a concentration-dependent manner. The arrest of HT-1080 cells in G0/G1 resulted in suppression of cell progression to the S-phase. As a result of 3- or 4-HBCa treatment, the DNA content at the S-phase decreased in a concentration-dependent manner. At 0.6 mM concentration, 4-HBCa induced more reduction (from 34.3 to 22.2, approximately 1.54 fold) in DNA at the S-phase than did 3-HBCa (from 34.2 to 30.0, 1.132 fold). These results indicate that 3- and 4-HBCa exert an anti-proliferative effect that is to some extent dependent upon the chemical structure (Figure 6).

The effects of HBCa complexes pro- and anti-apoptotic gene expression

In order to assess the molecular response of HT-1080 cells treated with HBCa complexes for 48 hours, pro- and anti- apoptotic proteins were analysed using Western blot analysis. The expression of β-actin was used as a loading control. Figure 7A shows that HBCa complexes decreased the expression of Bcl-2 in

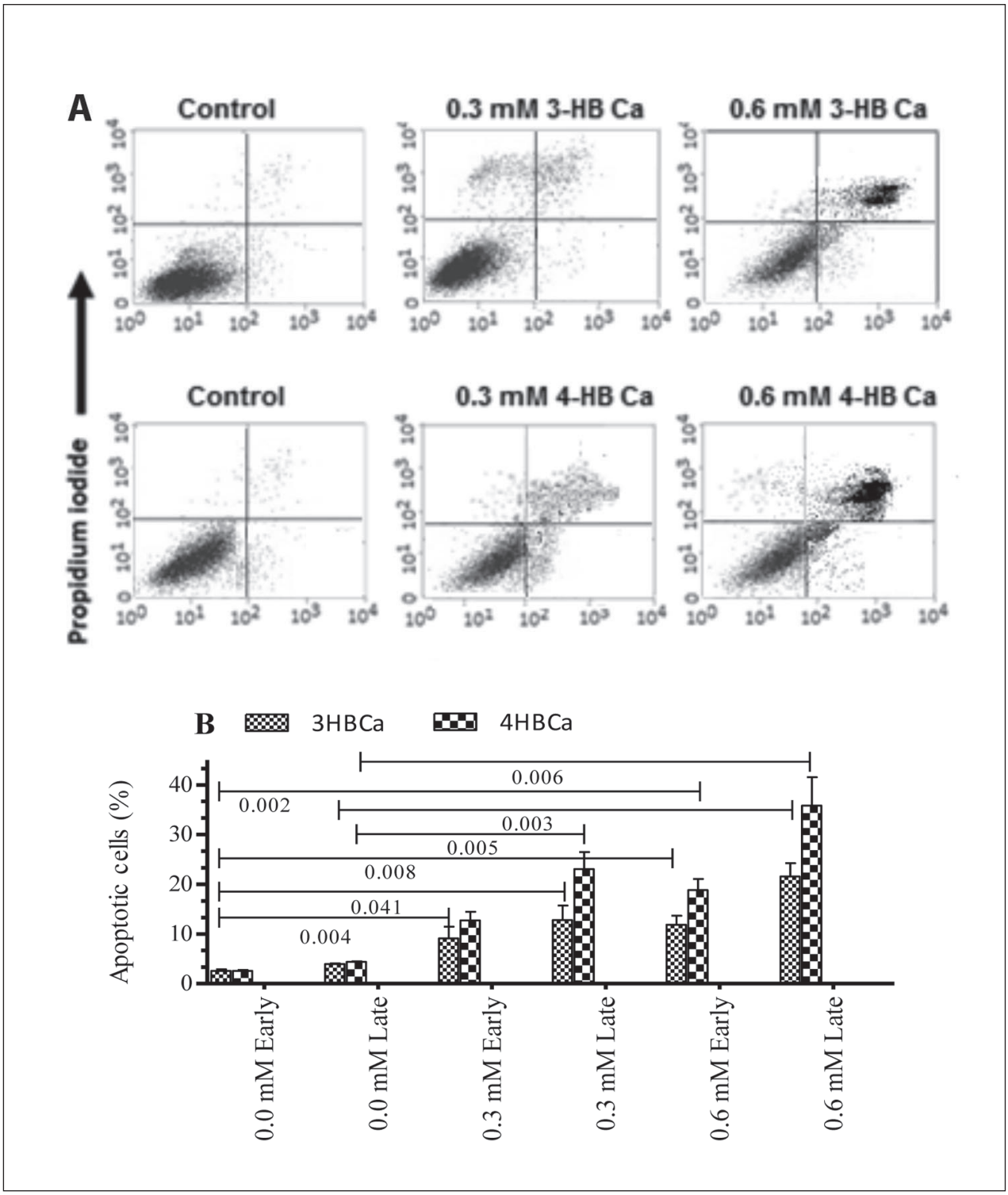


Figure 5. A) Examples of Annexin V/propidium iodide (PI) dot plots. B) The apoptotic effect of 3-HBCa and 4-HBCa on HT-1080 cells by Annexin-V/propidium iodide after treatment for 48 hours under optimal culture conditions. Values are a mean of 2 replicates.

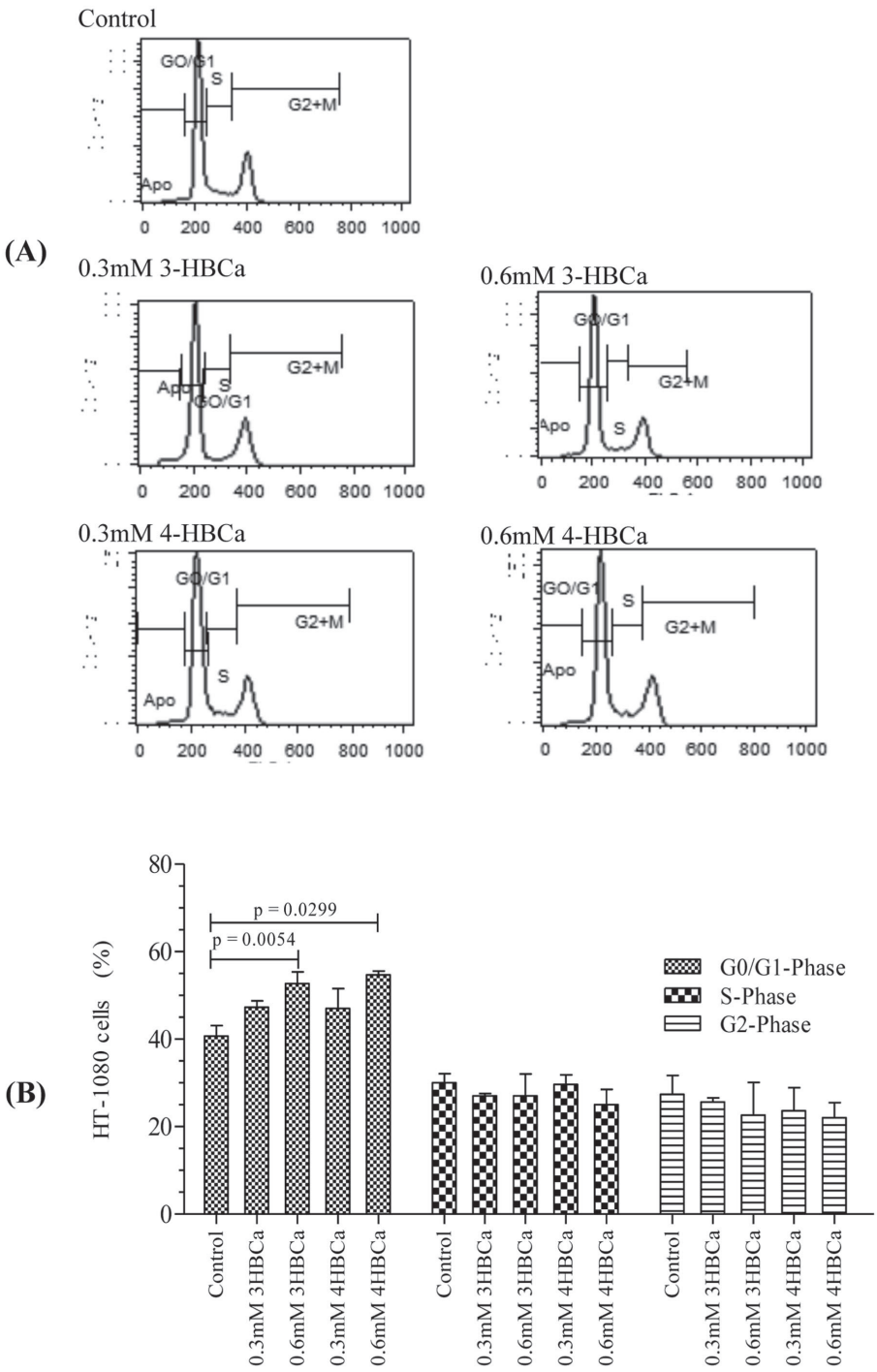


Figure 6. The effect of HBCa complexes on the HT-1080 cell cycle. A- DNA histograms for G0/G1, S and G2 phases measured by flow cytometer. B- Analysis of cell cycle phase distribution. Cells were cultured at optimal conditions and treated separately with 0.3 and 0.6 mM 3-HBCa or 4-HBCa for 24 hours.

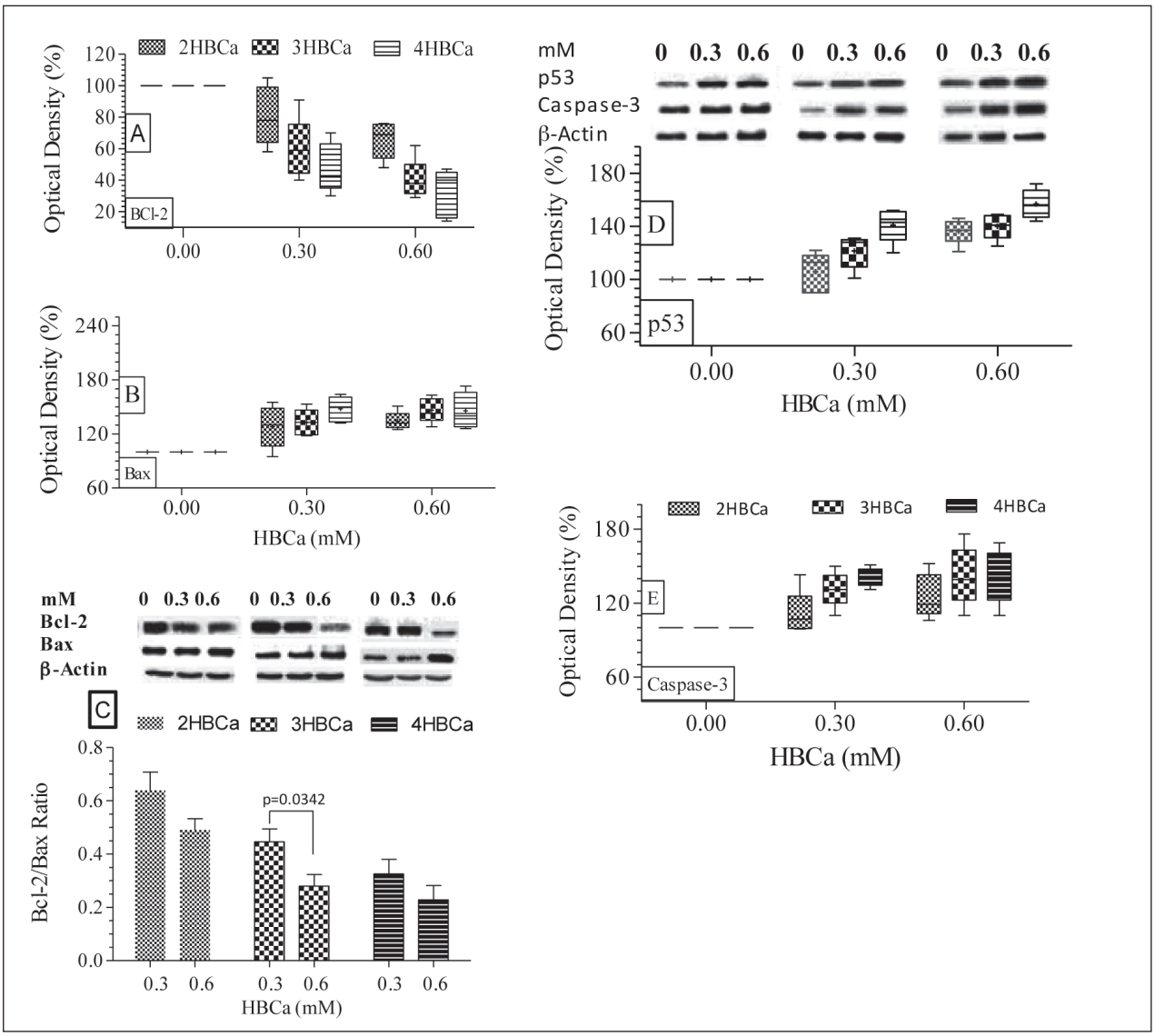


Figure 7. Immunoblotting analysis of (A) BCl-2, (B) Bax, (C) Ratios of Bcl-2/Bax (2-tails t-test: 2-HBCa vs 3-HBCa at 0.3 mM p = 0.0492; at 0.6 mM p = 0.0087; 2-HBCa vs 4-HBCa at 0.3 mM p = 0.0071; at 0.6 mM p = 0048), (D) p53, and (E) caspase-3 protein expression in treated HT-1080 cells cultured with different concentrations of 2-HBCa, 3-HBCa and 4-HBCa for 48 hours. β–actin antibody was used as an internal standard. Immunoblotting of each protein was conducted on three independent lysates. The values for optical density (OD) were calculated by normalising relative optical density values to that of the control. Thus OD for the control is 100% while treatment is either below or above 100%. Normalised values were calculated as ratios of (ODcontrol-ODtreatement/ODcontrol).

HT-1080 cells in a concentration- and hydroxyl position-dependent manner when compared to untreated cells. The magnitude of Bcl-2 down regulation was in the following order: 2-HBCa (1.235 and 1.524 folds) < 3-HBCa (1.66 and 2.5 fols) <4-HBCa (2.083 and 3.125 fols) at 0.3 mM and 0.6 mM respectively (Figure 7A). In contrast, the treatment of HT-1080 cells with 0.3 mM or 0.6 mM HBCa complexes showed a concentration-dependent increase in the expression of Bax (Figure 7B). The increase in the expressed Bax ranged between 1.28-1.48 folds and 1.342 and 1.456 when HT-1080 cells were treated with 0.3 and 0.6 mM respectively. In addition, the quantification of the Bcl-2/Bax ratio by densitometric analysis of Western blots (Figure 7C) showed that a low ratio was characteristic for the sensitivity of HT-1080 cell population to HBCa

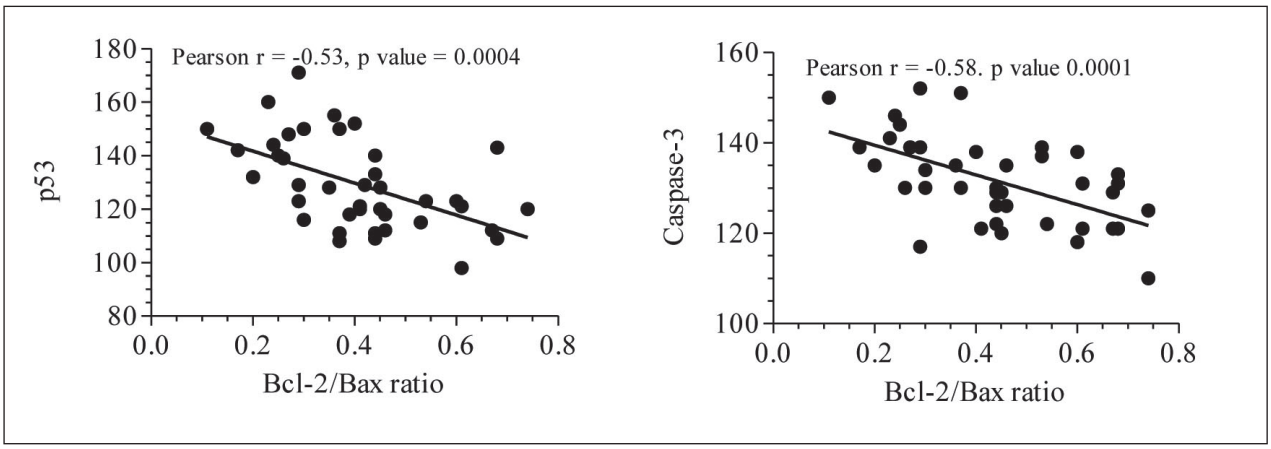


Figure 8. Pearson correlation coefficient pattern between Bcl-2/Bax ratios and p53 and caspase-3 respectively. Data were derived from all three compounds at 0.0, 0.3 and 0.6 mM.

complexes. The Bcl-2/Bax ratios decreased in concentration- and chemical structure- dependent manners in the same order as defined above. 0.6mM HBCa complexes decreased the ratios by 22.74% (2-HBCa), 45.34% (3-HBCa) and 51.67% (4-HBCa) (Figure 7C). Furthermore, the pro-apoptotic protein p53 (Figure 7D) increased in the range of 1.058-1.568 folds and in a concentration-dependent manner. p53 was up-regulated by 1.410 and 1.568 fold when HT-1080 cells were treated with 0.3 mM and 0.6 mM 2-HBCa respectively. The u regulation of p53 induced by 0.3 mM and 0.6 mM 3-HBCa was increased by 1.2 and 1.4-fold respectively. In addition, the highest up regulation of p53 (0.6 mM =1.4, 0.6 mM = 1.6) was observed when HT-1080 cells were treated with 4-HBCa as compared to control samples (Figure 7D). The expression of total caspase-3 was also increased in treated HT-1080 cells compared to controls. In addition, the expression of Bcl-2/Bax ratios negatively correlated with p53 ($r = -0.53$; $p = 0.0004$) and caspase-3 ($r = -0.58$; $p = 0.0001$) (Figure 8).

Discussion

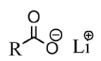
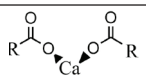
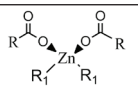
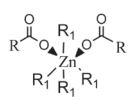
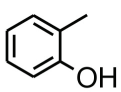
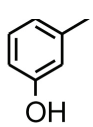
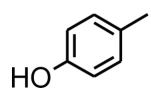
HBAs are novel phytochemicals that exhibit simple chemical structures with two functional groups; the hydroxyl and carboxylic acid. Although 2-, 3- and 4-HBCa complexes have the same molecular formula ($C_{14}H_{10}O_6Ca$) and molecular mass (314.14), they vary in their chemical structures due to the presence of the hydroxyl group at different positions on the benzene ring, i.e. at C2 (*ortho*), C3 (*meta*) or C4 (*para*) positions (Figure 1). Hence, these HBAs possess different chemical and biological properties. The acidity of these molecules, for example, increases in the following order 2-HBA >3-HBA> 4-HBA. The lower acidic properties of 4-HBA make it a more pharmacologically attractive molecule than the common anti-inflammatory 2-HBA. Thus, the aim of this study was to compare the efficacy of 3-HBCa and 4-HBCa with 2-HBCa and 2-ABA, using the human fibrosarcoma cell line HT-1080. The results here indicate that the position of the hydroxyl group on the benzene ring can alter the modulation of HT-1080 cell proliferation, the cell cycle and the expression of pro- and anti- apoptotic proteins in a structure-dependent manner. The results obtained support the fact of induction of apoptosis in HBCa complex-treated HT-1080 cells. 4-HBCa was more cytotoxic than 2-HBCa, 3-HBCa and 2-ABA. The 3- and 4-HBCa complexes arrested HT-1080 cells at G0/G1 which agrees with the similar effect of 2-HBCa and 2-ABA (12-13). The similarity in the biological activities of these compounds could be attributed to the same activation pathways that lead to induction of G0/G1 arrest and apoptosis. Indeed, the evidence indicates that modulation of the cell cycle is closely associated with the response to apoptosis and proliferation. Different proteins have been recognised as playing significant roles in managing the direction of the cell cycle towards apoptosis or proliferation, including p53, RBc-Myc and various Cdks (14-16). These pro-

teins were found to function in proliferative pathways and may also sensitize cells to apoptosis (14, 16).

Apoptosis is often favoured as a cell-killing mechanism for anti-cancer therapies as it is less inflammatory than necrotic cell death (17-19). However, cancer cells often lack the appropriate apoptotic responses that can be mediated by increased expression of anti-apoptotic genes or inactivation of apoptotic pathways, resulting in the promotion of cancer cell survival (20-21). The common signalling pathways that trigger apoptosis include the intrinsic and extrinsic pathways (20-21). The results presented here suggest that HBCa complexes can effectively induce apoptosis in HT-1080 cells *via* the intrinsic pathway. Furthermore, 2- (12), 3- and 4-HBCa complexes induced down-regulation of Bcl-2 and up-regulation of Bax, as well as induction of p53, which plays a pivotal role in regulating apoptosis and the cell cycle (22-23). The loss of p53, for example, enhances survival of cells with DNA damage and aids cancer progression (24-25). The expression of Bcl-2 suppresses apoptosis in response to specific stimuli (26). In contrast, Bax enhances apoptosis *via* the release of cytochrome *c* from the mitochondria, which results in the activation of caspases including caspase-9 and downstream caspases-3 (27). In addition, the HBCa complexes evaluated in this study were able to modulate the ratio of Bcl-2/Bax, thereby increasing the apoptotic potential of the cells. High Bcl-2/Bax ratios are associated with inhibition of apoptosis. Consistent with these results, the HBCa complex-mediated reduction in Bcl-2/Bax ratios was associated with induction of both p53 and caspase-3 expression in HT-1080 cells treated with 0.3 and 0.6 mM HBCa complexes. Similar results were obtained with HBLi and HBZn complexes when the same cell line was used (2, 28-29).

The efficacy of HB metal ion complexes on cancer cells is associated with both type of metal ion (Li+, Ca2+ or Zn2+) and the position (2HB, 3-HB, 4-HB) of hydroxyl group in HB ligand (2HB, 3-HB, 4-HB). These results can be accounted for the structure-activity relationships. Based on our current and previous results, it is indicated that that efficacy of HB metal ions complexes is proportionally increased with more coordination centres: mono-valance < di-valance < transition metal ion (Table 1). Hence, the zinc ion in HBZn complex showed more interaction sites with the target biological molecule for inducing anti-inflammatory and

Table 1. The effect of metal ion, chemical structure and the number of coordination sites of the metal ion on the efficacy of HB complexes.

	Chemical structure	MI* valance	MI* coordination site	Efficacy
HBLi		1	1	Lowest
HBCa		2	2	In between
HBZn	 or 	2	4-6 (30)	highest
R =	2-HB MI* 	or 3-HB MI* 	or 4-HB MI* 	
R1	Different centres for coordination with the target molecule			
Efficacy	Lowest	In between	Highest	

*MI = metal ion

pro-apoptotic activities than HBLi or HBCa (Table 1). In addition, the position of the hydroxyl group at C4 may give a better microenvironment of interactions for regulating anti-proliferation and pro-apoptotic activities (2, 31).In addition, the position of the hydroxyl group at C4 may give a better interaction microenvironment for regulating anti-proliferation and pro-apoptotic activities (2, 31).

Conclusion

These results demonstrate that HBCa complexes exert apoptotic effects in a concentration- and chemical structure-dependent manner. The cytotoxic effects of HBCas follow the order: 2-HBCa <3-HBCa/2-ABA <4-HBCa suggesting that 4-HBCa may be a promising lead compound for the development of novel anticancer therapeutics. Furthermore, the cellular modulation of HT-1080 cells by HBCa complexes showed a clear association between apoptosis induction and the reduction in Bcl-2/Bax ratios, suggesting that this ratio represents a potential molecular marker for predicting response to HBCa complexes. In addition, although HBZn complexes give better efficacy, the results here may encourage further research towards developing new anti-proliferative and pro-apoptotic strategies, including the use of HBCa as an agent.

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