The effect of hydroxybenzoate calcium compounds in inducing cell death in epithelial breast cancer cells

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Abstract: Hydroxybenzoate (HB) compounds have shown their significance in inducing apoptosis in primary chronic lymphocytic leukemia (CLL) and cancer cell lines, including HT-1080. The current study focuses on assessing the effects of 2-, 3- and 4-hydroxybenzoate calcium (HBCa) compounds on MCF-10A, MDA-MB231 and MCF-7 epithelial breast cell lines. The HBCa-treated cells were examined using annexin V, to measure apoptosis in the three epithelial breast cell lines, after 48 h of treatment. The results indicated that 0.5 and 2.5 mmol/L of HBCa induced cell death in a dose-dependent manner. The induction of cell death in normal MCF-10A cells was found to be significantly less (p = 0.0003–0.0068), in comparison to the malignant cell lines (MDA-MB231 and MCF-7). HBCa compounds were also found to cause cell cycle arrest in the epithelial breast cells at G1/G0. Furthermore, HBCa compounds induced the upregulation of apoptotic proteins (p53, p21, Bax and caspase-3), as well as the downregulation of the anti-apoptotic protein Bcl-2, which may suggest that apoptosis is induced via the intrinsic pathway.

Keywords: hydroxybenzoate calcium; cell death; breast cancer cells

Citation: Merghani NM, Hazzaa AA, Mahdi EJG, et al. The effect of hydroxybenzoate calcium compounds in inducing cell death in epithelial breast cancer cells. Adv Mod Oncol Res 2015; 1(2); 122–131; http://dx.doi.org/10.18282/amor.v1.i2.35.

Received: 4th September 2015; Accepted: 15th September 2015; Published Online: 2nd December 2015

Breast cancer is a major global health issue that mainly affects women of all age groups. It is the most common cancer worldwide, with more cases in developing countries than in developed countries[1]. Breast cancer is the second leading cause of cancer death after lung cancer in developed countries (198,000 cases, 15.4%). Incidence rates continue to increase globally except in a few high-income countries. The estimated breast cancer incidents of less developed cases in 2012 were 883,000 (52.8%) and 788,000 more developed cancer cases, with mortality rates of 324,000 (62.1%) and 198,000 (37.9%), respectively[2]. In contrast, more than 60% of breast cancer patients survive in developed countries. Lower survival rates occur in developing countries due to the lack of early detection schemes and diagnosis[3]. Statistics related to breast cancer cases have attracted the attention of various researchers to effectively treat the cancer with chemotherapy.

As part of this effort, a large number of compounds have been assessed for their anticancer potential in dif-
different cancer cells\textsuperscript{[4-7]}. The assessed compounds include hydroxybenzoate (HB), 2-acytlenbenzoic acid (2-ABA) or aspirin, and its precursor 2-hydroxybenzoic acid (2-HBA). These compounds showed apoptotic effects at high doses (1–10 mmol/L), which highlight concern regarding their side effects\textsuperscript{[8,9]}. Our previous studies have shown the induction of apoptosis at lower doses in different cancer cell lines and primary chronic lymphocytic leukaemia (CLL)\textsuperscript{[4,10–12]}. For instance, the morphological and immunological evidence for apoptosis were obtained when 4-hydroxybenzoate zinc (HBZn) or 4-hydroxybenzoate calcium (HBCa) compounds were evaluated\textsuperscript{[11–13]}. The mechanism of these compounds is to induce apoptosis via the intrinsic pathway involving the upregulation of the expression of p53, Bax and caspase-9\textsuperscript{[4,10,12]}. In parallel, these compounds downregulated Bcl-2, an anti-apoptotic protein that is able to suppress cytochrome c release and subsequently caspase-3. The regulation of apoptosis-related proteins in the intrinsic pathway is an important indicator for assessing anticancer compounds’ activities. Similar results were obtained in MCF-7 and MDA-MB231 human breast cancer cells when tamoxifen was used\textsuperscript{[14]}.

Hydroxybenzoic acid (HBA) and its metal-bearing analogs are simple compounds with different chemical properties. For example, 2-HBA has a higher acidic content when compared to 3-HBA and 4-HBA. 4HBZn is more potent while 4-HBA is less acidic than the corresponding 2-HBZn and 3-HBZn compounds\textsuperscript{[12]}. The current research examines the cytotoxic effects of three types of HBCa compounds; 2-HBCa, 3-HBCa and 4-HBCa in two epithelial breast cancer cells, MDA-MB231 and MCF-7, and the normal breast cell line MCF-10A, as shown in Figure 1.

![Chemical structures of the three HBCa complexes](image)

\textbf{Figure 1 Chemical structures of the three HBCa complexes}

**Materials and methods**

\textbf{Cell lines:} The mammary epithelial cell lines of both normal (MCF-10A) and cancer (MCF-7 and MDA-MB-231) were obtained from the American Type Culture Collection (ATCC, USA).

\textbf{Chemicals:} 2-, 3-, and 4-HBCa were prepared from 2-, 3- and 4-HBA (Sigma-Aldrich, UK) and calcium carbonate (Sigma-Aldrich, UK), respectively through acid-base reaction.

\textbf{Cell culture}

MCF-10A, MDA-MB-231 and MCF-7 cells (ATCC, USA) were cultured in a RPMI-1640 medium. It contained GlutaMAX, 25 mmol/L HEPES buffer (Sigma-Aldrich, UK), 10% fetal bovine serum (FBS) (Sigma-Aldrich, UK) and 1% penicillin (10,000 U/mL; Sigma-Aldrich, UK), respectively through acid-base reaction.

MCF-10A cells were cultured at 37°C in a humidified atmosphere containing 5% CO\textsubscript{2}.

\textbf{Detection of cell viability by MTT cell proliferation assay}

Breast cells were seeded in a 96-well plate at a density of \(2 \times 10^5\) cell/well in 90 µL optimized medium. The cells were allowed to settle for 24 h before treated with individual dose of HBCa, i.e., 0, 0.1, 0.3, 0.5, 1.0, 2.0 and 5.0 mmol/L. The treated cells were allowed to grow for 48 h. At the end of the incubation period and dose point, 110 µL of 0.22 µm filter-sterilized, 5 mg/mL 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, UK) was added at a temperature of 37°C. The 96-well plate was kept in the dark for 2 h before the medium containing MTT was removed. 100 µL dimethyl sulfoxide (DMSO) obtained from Ajax Finechem Pty Ltd, Australia, was added to dissolve the formazan crystals. The 96-well plates were shaken for 15 min in the dark to dissolve the formazan crystals. The optical density (OD) of each treatment was measured at 570 nm using LabSystems Multiskan EX Version 3.0 (Thermo Fisher Scientific, USA).
Labsystems, Helsinki, Finland). Each experiment was performed in four replicates. The optical densities were normalized according to the control.

Detection of apoptosis by flow cytometry

Vybrant® Apoptosis Assay Kit #2 (Molecular Probes™, Invitrogen™ Life Technologies, USA) was used to detect apoptosis in HBCa-treated breast cells. Breast cells (5 × 10⁵ cell/mL) were cultured to approximately 70% confluence in T-100 tissue culture flasks under optimized media and standard culture conditions. The medium was replaced with medium containing 0.0, 0.5 or 2.5 mmol/L individual HBCa and allowed to culture for 48 h. The cells were then trypsinized, centrifuged and washed in phosphate-buffered saline (PBS). The cells (1 × 10⁶ cells/mL) were suspended in 1 × annexin V binding buffer (10 mmol/L Hepes adjusted to pH 7.4, 140 mmol/L NaCl and 2.5 mmol/L CaCl₂). Then, 5 µL of Alexa Fluor® 488 Annexin V and 1 µL of the 100 µg/mL propidium iodide (PI) solution were added to each 100 µL of cell suspension and incubated for 15 min at room temperature. Next, 400 µL of 1 × annexin V binding buffer was added and mixed gently followed by rapid mixing. The stained cells were then analyzed by flow cytometry. The percentage of apoptotic cells was determined using a FACSCalibur flow cytometer and Cell Quest Pro software (Becton Dickinson Biosciences, New Jersey, USA).

Assessment of cell cycle through flow cytometry

Cells (5 × 10⁵ cell/mL) were seeded in T-100 flasks (Nunc, Denmark) in 10 mL of fresh optimal medium and allowed to grow to approximately 60%–70% confluence before the cells were subjected to serum starvation for 24 h. The cells were then cultured in optimal medium containing 0.0, 0.5 or 2.5 mmol/L of individual HBCa compounds and incubated for 48 h prior to harvesting and washing with PBS. The harvested cells were centrifuged (300 × g for 5 min) and resuspended in 1 mL of PBS before fixation with 3 mL cold 100% methanol. The fixed cells were then centrifuged (300 × g for 10 min), washed with PBS and re-centrifuged (4000 rpm for 10 min). 1 mL of hypotonic DNA staining buffer (Sigma-Aldrich, UK) containing PI at 0.01% (w/v), ribonuclease A (RNase A) at 0.002% (w/v), 0.3% (v/v) Triton X-100 and 0.1% (w/v) sodium citrate (pH 7.8) were added to the pellet. The cells were resuspended and incubated at 4°C for 30 min.

Relative DNA contents was assessed as a function of PI labeling according to the method documented by Crissman and Steinkamp[15] which using the Cell-Quest™ Pro software to acquire and analyze data obtained from the flow cytometer (Becton, Dickinson and Company, San Jose, CA, USA).

Assessment of protein expression by Western blot

Breast cells (5 × 10⁵ cell/mL) were seeded in T-100 flask, cultured and treated with HBCa complexes (0, 0.5, 2.5 mmol/L) for 48 h, as described in the previous section. The medium was removed and the cells were washed with cold PBS to remove the medium. Subsequently, RIPA buffer (150 mmol/L sodium chloride (NaCl), 2 mmol/L ethylenediaminetetraacetic acid (EDTA), 50 mmol/L Tris-HCl, pH 7.5) and lysis buffer (1% deoxycholic acid and 1% NP-40) were added. Protease inhibitor cocktail tablets (Bio-Rad Laboratories, USA) were also added. The cell lysates were then centrifuged at 12,000 × g for 15 min. The supernatant was then centrifuged at 4°C at 16,000 × g for 5 min to obtain a clear solution of the protein mixture. The protein mixture was used to measure the expression of p53, p21, Bcl-2, Bax and caspase-3 by Western blotting using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal antibody control. Total cell lysate protein doses were determined by assay dye (Bio-Rad Laboratories, USA) and absorption was measured at 595 nm. 60 µg of the extracted protein and GAPDH internal antibody control on sodium dodecyl sulfate (SDS) were loaded to 4%-12% Bis-tris acrylamide gel in 3-(N-Morpholino) propanesulfonic acid (NuPAGE MOPS) running buffer (Invitrogen™ Life Technologies, Scotland, UK). After running the gel at 75 V for 3 h at room temperature, the resolved proteins were transferred onto a nitrocellulose membrane (Sigma-Aldrich, UK). The membranes were first incubated with an appropriate primary antibody (p53, Bcl-2, Bax, or GAPDH as a loading control and internal standard), followed by peroxidase conjugated anti-mouse IgG antibody (Sigma-Aldrich, UK). The membranes were washed and developed using a chemiluminescent reagent (Amersham, UK) prior to exposure to photographic films. The protein bands’ intensities were scanned and quantified using a densitometer.

Statistical analysis

Data obtained in these experiments represented an average of different replicates which were evaluated using equal variance and paired with Student’s t-test along with other statistical analyses using GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA).
Results

Response of breast epithelial cell lines to HBCa treatments

The response of breast cells towards various treatments was studied using MTT assay (Figure 2). HBCa compounds showed different effects on cell proliferation after 48 h treatment. However, different doses of HBCa demonstrated that cell proliferation decreased as the dose of HBCa increased in a dose-dependent manner (Figure 2). In addition, the apoptotic response of normal MCF-10A cells and cancer cells (MDA-MB231 and MCF-7) to HBCa compounds were assessed in vitro after 48 h using annexin V/propidium iodide assay (Figure 3A). A clear dose-dependent response was observed for 2-, 3- and 4-HBCa compounds to induce total cell death, particularly in MDA-MB231 and MCF-7 breast epithelial cancer cells (Figure 3B). HBCa-treated MCF-10A, MDA-MB231 and MCF-7 cells showed a higher level of late apoptosis and necrosis of cells (60%–84%) than early apoptosis of cells (18%–40%). The highest total cell death (17.03%) occurred when MCF-7 cells were treated with 2.5 mmol/L 2-HBCa for 48 h (Figure 3B). The 2.5 mmol/L HBCa treatments inhibited cell growth in other cells in a range between 9.10%–10.64%. In addition, statistical analyses indicated that HBCa-treated normal MCF-10A breast cells did not show significant differences ($p = 0.1161–0.7216$) compared to the control, except when treated with 2.5 mmol/L 3-HBCa ($p = 0.0016$). However, the treatment of MDA-MB231 breast cancer cells ($p = 0.0003–0.0068$) and MCF-7 ($p = 0.0001–0.0005$) with 0.5 or 2.5 mmol/L HBCa compounds had significantly increased total cell death compared to the control samples (Figure 3C). Similar results were also obtained with the treated MCF-7 cells ($p = 0.001–0.01$), except when cells were treated with 0.5 mmol/L 2-HBCa ($p = 0.2174$) as shown in Figure 1. The HBCa compounds increased cell death significantly in MDA-MB231 ($p = 0.0001–0.0459$) and MCF-7 ($p = 0.0001–0.0051$) cancer cells in comparison to the normal MCF-10A treated cells. MCF-10A cells were between 3 to 6 times less sensitive to the apoptotic effects of the HBCa compounds when compared with the cancer cell lines.

The effects of HBCa on cell cycle

The regulation of the cell cycle in the HBCa-treated breast epithelial cells (MCF-10A, MDA-MB231 and MCF-7) was analyzed after 48 h. Flow cytometry was used to quantify the DNA content within the cells which indicated that the distribution of different phases of the cell cycle were dependent on both the breast cell line and the HBCa compounds (Figure 4A). The treatment of the normal cell line (MCF-10A) resulted in an accumulation of cells in the G0/G1 phase in a dose-dependent manner compared to the control. The DNA contents increased from 8% to 31.47%. 2-HBCa was more effective in arresting MCF-10A at G0/G1 phase than the 3- and 4-HBCa compounds. However, the effects of different HBCa doses did not show significant differences ($p =$

\[ \text{Figure 2} \text{ Dose-dependent effects of HBCa on the viability of normal (MCF-10A) and cancer (MDA-MB231 and MCF-7) breast epithelial cells as measured by MTT assay. The cells were treated for 48 h and cultured at standard growth conditions. Data is shown as mean ± standard error of mean (SEM) of four values} \]
0.05) in arresting the cell cycle at G0/G1 (Figure 4B). The DNA contents in MCF-10A cells decreased in parallel in the S and G2/M phases, in comparison to the corresponding untreated cells. Cell cycle arrests in breast cancer cells (MDA-MB 231 and MCF-7) showed less response to HBCa in comparison to the control sample and normal breast cells (MCF-10A). The cell cycle in MDA-MB231 cells was arrested at G0/G1 by approximately 2.82% to 15.22% and between 0.19% and 12.06% in MCF-7 cells when treated with HBCa compounds.

Figure 4B

Figure 3 The treatment of normal (MCF-10A) and cancer (MDA-MB231) breast epithelial cells with HBCa for 48 h. (A) Examples of annexin V/propidium iodide dot plots illustrating the effect of HBCa compounds in MCF-7 breast cancer cells. (B) The percentage of breast cell death in normal (MCF-10A) and cancer (MDA-MB231 and MCF-7) cells when they were incubated with 0.5 mmol/L and 2.5 mmol/L HBCa for 48 h. (C) Summary of the statistical analysis of different HBCa compounds’ treatments

<table>
<thead>
<tr>
<th>Cell line/treatment</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-10</td>
<td>Control vs. 0.5 or 2.5 mmol/L 2-HBC, 3-HBC or 4-HBC</td>
</tr>
<tr>
<td>MDA-MB231</td>
<td>Control vs. 0.5 or 2.5 mmol/L 2-HBC, 3-HBC or 4-HBC</td>
</tr>
<tr>
<td>MCF-10</td>
<td>MCF-10A vs. MDA-MB231</td>
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<tr>
<td>MDA-MB231</td>
<td>MCF-10A vs. MDA-MB231</td>
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<tr>
<td>MCF-10</td>
<td>MDA-MB231 vs. MCF-7</td>
</tr>
<tr>
<td>MCF-10</td>
<td>MDA-MB231 vs. MCF-7</td>
</tr>
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Figure 5 showed the effects of 0.5 mmol/L of 2-, 3- and 4-HBCa compounds on the expression of apoptosis-related proteins on MCF-10A, MDA-MB231 and MCF-7 breast cells. These compounds induced the upregulation of the proapoptotic proteins p53, p21, Bax and caspase-3 in MCF-10A, MDA-MB231 and MCF-7 cells after 48 h.
In contrast, the same treatments induced the downregulation of anti-apoptotic Bcl-2. Treatment of normal breast epithelial cells (MCF-10A) with 2-, 3- and 4-HBCa showed downregulated Bcl-2 less than the control by 8%–43%, while the expression of Bax increased in comparison to the control by 42%–92%.

<table>
<thead>
<tr>
<th>A</th>
<th>Control</th>
<th>0.5 mmol/L 2-HBCa</th>
<th>2-HBCa 2.5 mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Counts</td>
<td>0.5 mmol/L 3-HBCa</td>
<td>2.5 mmol/L 3-HBCa</td>
<td>0.5 mmol/L 4-HBCa</td>
</tr>
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</table>

**Figure 4** Effects of HBCa compounds on the cell cycle progression in normal (MCF-10A) and cancer (MDA-MB231) breast cells. (A) Examples of cell cycle analyses of MDA-MB231 cells under different treatments with HBCa compounds for 48 h, illustrating the distribution of the DNA levels at different phases. (B) The percentage of DNA modulation due to treatment with the HBCa compound.
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Figure 5 Molecular expressions of pro- and anti-apoptotic proteins in 0.5 mmol/L HBCa-treated MCF-10A, MDA-MB231 and MCF-7 cells. (A) Western blot analyses of p53, p21, BCl-2, Bax and caspase-3 after treatment with different HBCa compounds for 48 h. (B) Percentage of protein expression was expressed in percentage based on the control sample. Expression level of individual protein was calculated by first converting optical density (OD), OD (%) = (OD of treated sample/OD of control sample) × 100. The expression levels of individual protein were calculated using the following equation (% OD of protein in treated cells − % OD of protein in control) to give positive or negative values. Positive values indicate upregulation, while negative values indicate down-regulation of protein expression.

The 3-HBCa results showed a similar level of Bax and Bcl-2 expressions (Figure 5A). Similar results were obtained when the breast cancer cells (MDA-MB231 or MCF-7) were treated with 2.5 mmol/L HBCa (Figure 5B). Furthermore, the treatment of MCF-7 cells at 0.5 mmol/L was more effective than 2- and 3-HBCa in manipulating the expression of both Bcl-2 and Bax. The reduction of the expressed Bcl-2 in MCF-7 cells was between 25% and 70%, while the increase in Bax expression was between 73% and 97% (Figure 5B).

In addition, the apoptotic effects of HBCa compounds on breast epithelial cells indicated that the level of expressions of p53, p21 and caspase-3 were dependent on the type of cells. 3-HBCa was more effective than 2- and 4-HBCa. 4-HBCa was more effective in the breast cancer cells (MDA-MB231 and MCF-7) (Figures 5B and C). In MDA-MB231, the expression of p53, p21 and caspase-3 were 140%, 86% and 102%, respectively. Furthermore, 4-HBCa upregulated apoptosis-related proteins in MCF-7 by 146%, 168% and 158%, respectively. However, when the breast cells were treated with 2.5 mmol/L HBCa compounds, the expressions of the pro- and anti-apoptotic cells were lowered within 60%–100%. In addition, the treatment of breast epithelial cells with 2.5 mmol/L HBCa induced irregular expression of the pro- and anti-apoptotic proteins.
Discussion

Breast cancer is one of the most commonly diagnosed malignancies in women. Various strategies have been adopted to combat this disease. Chemotherapy is one of the main therapeutic strategies and it has attracted the interest of many researchers aiming to develop effective anticancer compounds. Various compounds including natural and synthetic have been studied. Among these HB compounds include the most common drugs acetylsalicylic acid (ASA) and its precursor 2-HBA, or also known as salicylic acid. Although ASA and 2-HBA exert some side effects, their derivatives exhibit less acidity and possess more apoptotic potential. For example, earlier work has highlighted the potential for 4-HBZn to induce apoptosis, particularly in primary CLL and other cancer cells\[4,16\]. HBCa compounds have also shown their apoptotic potential in human fibrosarcoma HT-1080 cells\[10\]. Metal ions including calcium (Ca\(^{2+}\)), zinc (Zn\(^{2+}\)) and platinum (Pt\(^{2+}\)) showed improved efficacy to various organic compounds against cancer cells. For example, the incorporation of Zn\(^{2+}\) or Ca\(^{2+}\) ions improved the apoptotic potentials of HBA compounds\[4,10,16\]. In addition, platinum-based anticancer drugs have proven to exert highly effective therapeutic potentials in various cancer types\[17\]. Thus, the incorporation of metal ions enhances the anticancer activity of organic compounds and may also be considered as a novel strategy for further development of anticancer drugs\[18-20\]. Our previous results\[12,13,16\] have encouraged us to investigate the apoptotic effects of HBCa compound on human’s normal (MCF-10A) and breast epithelial cancer cell lines (MDA-MB231 and MCF-7) breast epithelial cells. The current study revealed that the treatment of these epithelial cells with HBCa compounds for 48 h has significantly induced cell death in a dose-dependent manner, as assayed by annexin V. The three HBCa compounds showed variable effectiveness in the normal breast epithelial cells and two breast cancer cell lines. The results may suggest the importance of metal-based compounds in the development of a new anticancer drug. It is clear that HBCa caused a notably higher proportion of cell death in breast cancer cells (MDA-MB231 and MCF-7) than in normal breast cells (MCF-10A). The results may encourage further studies in order to explore the anticancer function and potential of HBCa and its corresponding zinc analogues.

Chemotherapeutically, apoptosis is a novel strategy to kill cancer cells without affecting the neighboring normal cells\[21\]. Although physiological and drug-induced apoptosis lead to cell death, both are different in regards to initiating and proceeding apoptosis\[22\]. In both cases, apoptosis inhibits cell growth or proliferation which is associated with modulation of cell cycle check points. Our results showed that HBCa-treated MCF-10A, MDA-MB231 and MCF-7 cells arrested the cell cycle at G1/G0 after 48 h. Normal and cancer cells showed different responses to the increasing doses of HBCa compounds. Lower doses were more effective than higher doses when arresting normal MDA-MB231 cells at the G0/G1 phase. Similar results were obtained when pro-and anti-apoptotic proteins were investigated in HBCa-treated breast cells. The response of cancer cells to different HBCa doses was dependent on the type of cancer cell line and HBCa compound. These results may suggest different cytotoxic mechanisms of the two doses (0.5 and 2.5 mmol/L). Perhaps, 2.5 mmol/L HBCa might cause cytotoxicity to the breast cells, causing cells to undergo cell death. Although, assessing the morphology remains the most significant way to differentiate between apoptosis and necrosis. The results obtained from annexin V and the cell cycle may explain why 2.5 mmol/L HBCa was less effective apoptotically than 0.5 mmol/L HBCa. 2.5 mmol/L HBCa showed a higher level of late apoptosis and necrotic cells. These results may also explain the cytotoxic effect of higher doses of HBCa compounds. This issue highlights the association between the level of doses used and the type of cell death occurring: apoptosis or necrosis. The processes of aponecrosis can occur independently, sequentially and simultaneously, partly depending upon the type and level of stimuli, including anticancer drugs\[23-25\]. Apoptosis occurs via ligand binding and protein cross-linking with FAS (intrinsic pathway) or tumor necrosis factor (TNF) (extrinsic pathway) receptors. Other cells have a default death pathway that must be blocked by a survival factor such as a hormone or growth factor\[25\]. However, it should be noted that due to the lack of phagocytic cells in in vitro culture conditions, apoptosis fragments lyse and induce secondary necrosis or post-apoptotic necrosis in a process similar to necrosis\[20\]. It may be possible that the higher level of HBCa compounds contributed to the intracellular Ca\(^{2+}\) overload, causing post-apoptotic necrosis. Necrosis or death channel in the cytoplasmic membrane can be a result of colloid osmotic forces and entry of cations that cause swelling and ultimately rupturing\[27\]. This effect causes the permeability transition pores to open in the mitochondrial inner membrane in response to the stimuli of intracellular Ca\(^{2+}\) ions leading to necrosis\[28\]. This is also partly due to the inhibition of adenosine triphosphate (ATP) production by glycolysis or oxi-
dative phosphorylation\textsuperscript{27,29}. In addition, the necrotic process is regulated by oxidative stress and p53 when the anticancer compound 2-phenylethynesulfonamide was used\textsuperscript{30}. Reactive oxygen species (ROS) is often highly expressed in cancer cells as a by-product of oxidative phosphorylation\textsuperscript{31}. The higher doses of HBCa may cause a further increase in the level of ROS, either by increasing the level of the oxidative stress or detoxification, in order to regulate cellular processes including cell survival. In both cases, the increased level of ROS production leads to the diversion of apoptosis into necrosis signaling. In this regard, a previous research reported the lack of antioxidants in the breast cancer cells\textsuperscript{32}.

Literature has demonstrated cross-links between apoptosis and the cell cycle through the molecular modulation of different proteins, including Cdk5, p53, Bax and Bcl-2\textsuperscript{33-37}. Thus, the ideal approach to assess the apoptotic effects of HBCa compounds is to measure the expression of both pro- and anti-apoptotic-related proteins. The use of Western blots clearly indicated that 0.5 mmol/L HBCa showed upregulation of p53, p21, Bax and caspase-3, while the expression of the anti-apoptotic proteins showed that Bcl-2 was downregulated. These results may suggest that the HBCa-treated epithelial breast cells underwent apoptosis via the intrinsic pathway. This pathway involves the activation of caspase-9 and caspase-3 in response to anticancer chemotherapy, which is closely associated with the increase of the outer mitochondrial membrane permeability, regulated by the Bcl-2 family\textsuperscript{38}. It mainly involves caspase-3, which specifically activates the endonuclease CAD and degrades chromosomal DNA within the nuclei, as well as causing chromatin condensation\textsuperscript{28}. Previously, we showed that 4-HBZn induced apoptosis via the intrinsic pathway in CLL which is partially abrogated by the caspase-9 inhibitor (Z-LEHD.FMK)\textsuperscript{4}. In conclusion, the apoptotic effects of 2-, 3- and 4-HBCa compounds were significantly lesser in the control samples and normal (MCF-10A) breast epithelial cells (MCF-10A) than the corresponding cancer cells (MAD-Mb231 and MCF-7). These compounds induced cell cycle arrest at G0/G1. HBCa compounds also upregulated pro-apoptotic proteins, p53, p21, Bax and caspase-3 and downregulated the anti-apoptotic protein Bcl-2. These results may suggest the involvement of the intrinsic apoptotic mechanism.

Conclusion

The treatment of breast epithelial cells MCF-10A, MDA-MB231 and MCF-7 with 2-, 3-, and 4-HBCa for 48 h induced more apoptosis at 0.5 mmol/L but more secondary necrosis at 2.5 mmol/L. The cytotoxic effect of the higher dose of HBCa could be explained as these compounds may default the apoptotic pathway, leading cell death into necrosis. Therefore, further studies will be required to investigate the stimulation of cell death in breast cancer cells.

Author contributions

Nada M Merghani performed the experiments and contributed to the analysis of the data. The experimental work was supervised by Amal Al-Hazzaa. Eamon JG Mahdi contributed to review and writing of the manuscript, Abigail J Manning contributed to the review and preparation of the manuscript while Chris J Pepper contributed to the design of the study and review of the manuscript. Andea GS Buggins contributed to the design of the study and reviewed of the manuscript. Jassem G Mahdi designed and supervised the overall study and prepared the manuscript.

Conflict of interest

The authors declared no potential conflict of interest with respect to the research, authorship, and/or publication of this article.

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