Morphological modulation of human fibrosarcoma HT-1080 cells by hydroxybenzoate compounds during apoptosis


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Abstract: Hydroxybenzoate (HB) compounds have shown to modulate the morphology in human fibrosarcoma HT-1080 cells. The changes in HT-1080 cells showed marker signs of apoptosis, which included the condensation of nucleus, cell round, blebbing and the formation of apoptotic bodies. The different stages of apoptosis were assessed microscopically using different staining and immunohistochemical techniques, as well as scanning electron microscopy. In addition, HB compounds increased the expression of caspase-3, which is closely associated with the development of the modulation in HT-1080 cells that are undergoing the programmed cell death. Both acetyl salicylic acid (ASA) and HBZn compounds were dose and treatment duration dependent.

Keywords: hydroxybenzoate compounds; apoptosis; fibrosarcoma HT-1080 cells


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Large efforts of research are continuously plunged to fighting cancer. Our work concentrates on aspirin-based compounds, which are characterized by simple and pharmacologically effective compounds[1]. Among these compounds are simple hydroxybenzoicacids (HBA), that carry hydroxyl group at ortho (or 2), meta (or 3), or para (or 4) positions (Figure 1). The presence of the hydroxyl group at these positions gives these compounds different chemical and pharmacological properties. The acidity of HBA, for example, increases in the following order 2-HBA [1] > 3-HBA [2] > 4-HBA [3]. Pharmacologically, 2-HBA [1], or salicylic acid has side effects on the gastric mucosa of the stomach. In addition, 2-acetoxybenzoicacid (2-ABA [4]), or aspirin and its precursor 2-HBA [1] inhibits cancer cell growth by apoptosis, but at high doses (5 mmol/L)[2]. Pharmacologically, a high dose of salicylates is not compatible with the therapeutic dose (0.01–0.1 mmol/L) that can be achieved to selectively inhibit COX-2 transcription in fibroblasts and endothelial cells[3]. This raises a question concerning the possibility of modifying the chemical structure of 2-HBA [1] with the aim to reduce the dose and improve the apoptotic activities of these compounds. Our experience in this respect showed that 2-Hydroxybenzoate zinc (2-HBZn [5]) exhibited significantly, more cytotoxicity more than 5-fold compared with 2-ABA [4], with mean LD50 values of 210 and 1100 µmol/L respectively[4]. In addition, results showed that the efficacy of salicylic acid compounds were dependent on the chemical structure and the type of
metal ion (Figure 1). The presence of the zinc ion in 2HB [1] induced a higher level of apoptosis at lower doses [1]. The development of effective pro-apoptotic compounds has proved to be the correct strategy to inhibit cancer cells’ growth. The mechanism of HBZn compounds in inducing apoptosis may go through the mitochondria or the intrinsic pathway [4-6]. This pathway involves the activation of caspases-9 and caspases-3 in response to anticancer chemotherapy, which is closely associated with the increase of outer mitochondrial membrane permeability regulated by the BCl-2 family [7].

![Chemical structures of HB compounds with their apoptotic potentials](image)

**Figure 1**

Apoptosis has been recognized as being critical for determining the response to cancer treatment. Inducing apoptosis by anticancer drugs is a crucial strategy used clinically for chemotherapy. Understanding the molecular and morphological changes of cells undergoing apoptosis per se, provides a novel opportunity for a more rational approach to develop molecular-targeted therapies to combat cancer [7]. In this respect, this article focuses on the morphology of apoptotic human fibrosarcoma HT-1080 cells induced by HBZn compounds, using different morphological techniques.

### Materials and methods

#### Cell culture

2-, 3- and 4-HBZn were prepared in our laboratory by acid-base reactions of the corresponding starting materials; 2-, 3- and 4-HBA (Sigma, UK) respectively. Compounds were purified by crystallization from a water-ethanol 40:60 mixture. Stock solutions of HBZn compounds were prepared at different concentrations in Dulbecco’s modified Eagle’s medium (DMEM). The human fibrosarcoma cell line HT-1080 was cultured at different cell densities in DMEM medium containing: 10% foetal calf-serum (FCS, Gibco, UK), L-glutamine (Gibco, UK), 4% gentamicin solution (Gibco, UK), 1% L-glutamine (Gibco, UK), 0.1% hepes buffer (Gibco, UK), 0.1% sodium pyruvate (Gibco, UK), 0.1% ascorbic acid powder (Sigma-Aldrich, UK) and 1 mL/100 mL gentamycin solution. Cells were incubated at 37°C in a humidified atmosphere and 5% CO2. The growth curve was first performed to identify the exponential phase for drug treatment.

#### Determination of apoptosis by caspase-3

HT-1080 cells (7 × 10^4 cell/mL) were plated in T-100 plates, cultured in DMEM medium, under standard conditions for 24 h before treated with HB compounds (0, 0.3 mmol/L) for 48 h. HT-1080 cells were then subsequently harvested by centrifugation and incubated for a further 1 h at 37°C in the presence of the PhiPhiLuxTM G1D2 substrate (Calbiochem, Nottingham, UK). The substrate contains 2 fluorophores, separated by a quenching linker sequence that is specifically cleaved by active caspase-3. Once cleaved, the resulting product is fluorescence green and can be quantified using flow cytometry. For all experiments, 10,000 events were measured and were analyzed using a flow cytometer.

#### Cell preparation before staining

HT1080 cells were seeded into 6 well plates with cover glass at a density of 4 × 10^5 per well unless otherwise stated. Cell culturing and drug treatments were carried out as indicated previously. Control wells were treated with water while other HT-1080 cells were treated with HBZn and ASA of different doses unless otherwise stated. After HB treatment for 24 and 48 h, cells on the cover glass were washed in each well with 2 mL of phosphate buffer saline (PBS) and incubated with PBS for 10 minutes at room temperature before fixing with 2 mL of 4% (v/v) formaldehyde in PBS for 10 min. Once the fixation was completed, different staining procedures were applied.

#### Nuclear morphology-DAPI stain

The attached cells were stained for 20 min with 2.5 µg/mL DAPI (4,6-diamidino-2-phenylindole) solution and each cover glass was mounted in Vectashield mounting medium for fluorescence (Vector Laboratories, Inc, Burlingame, USA). Slides were examined using a fluorescence microscope at 460 nm maximum wave-length of M = Zn, 2-HBZn

[1] 2-HBA

[2] 3-HBA

[3] 4-HBA

[4] 2-ABA


[6] M = Zn, 3-HBZn

[7] M = Zn, 4-HBZn

[8] M = Ca, 2-HBCa

[9] M = Ca, 3-HBCa

[10] M = Ca, 4-HBCa
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length. (Leitz Laborlux 12; Leica, Milton Keynes, UK). Five random fields, each of 100 cells, were counted per slide and the percentage of apoptotic cells was assessed based on nuclear morphology. Representative photographic images were taken using a photometrics FS camera (MCA Leitz; Neoplan system, Tucson, AZ, USA) and CoolSnap image analysis software V1.2 (Rofer Scientific, UK).

**Haematoxylin and Eosin twenty**

The fixed samples were stained with Mayer’s Haematoxylin for 5 min followed by 3 washes in tap water and once with distilled water. The cells were then stained with Eosin for 1 min followed by another 3 washes in tap water and then once with distilled water. Finally, the samples were air dried and mounted in dibutylphthalate polystyrene xylene (DPX). The slides were viewed under a light microscope (Leitz Laborlux 12; Leica, Milton Keynes, UK) and images recorded through the use of Analysis image analysis software V2.3 (Rofer Scientific, UK).

**Methyl-green/pyronin staining for DNA/RNA**

The samples were incubated for 45 min in methyl-green/pyronin staining solution at room temperature. The cover glasses were removed from the wells then washed by dipping for 3–4 s in distilled water at 1°C. The cover glasses were air dried on a blotting paper and mounted with DPX. The cover glasses were viewed under a light microscope (Leitz Laborlux 12; Leica, Milton Keynes, UK) and images recorded through the use of Analysis image analysis software V2.3 (Rofer Scientific, UK).

Methyl green/pyronin staining was adapted from the method quoted by Bancroft and Stevens (1977) attributed to Elias (1969). In summary, 750 mg methyl green was dissolved in 150 mL of acetate buffer (4.4 g sodium acetate dissolved in 160 mL distilled water, pH adjusted to 4.9). The solution was then washed with chloroform (150 mL x 4) to remove methyl violet impurities, until the chloroform layer became translucent. 200 mg of pyronin Y was dissolved in 100 mL of methyl green, and then methyl green/pyronin Y mixture was filtered before use within 5 d.

**Scanning electron microscopy**

HT-1080 cells were seeded in a 12-well plate containing microscopic slide cover slips at the density of 15 x 10^3 cells, incubated for 48 h with the designated HB compound treatment before fixation. The samples were treated for 1 h with the fixative (0.8 % glutaraldehyde, 0.6% osmiumtetroxide, 2 mmol/L CaCl₂, and 0.2 mol/L sucrose in 0.1 mol/L sodium cacodylate buffer pH 7.4), washed several times in PBS buffer and dehydrated with different concentrations of alcohol (30%, 50%, 70%, 90%, each for 5 min, and 100% for 10 min twice). The dehydrated samples were then dried to the critical point in a Blazers CPD030 using CO₂. Cell samples were mounted onto 12 mmol/L Philiptype aluminums tubs using silver paint and then gold sputter coated in an Edwards S150B sputter-coater. Finally, the samples were imaged using a Philips XL20SE Munderarious, magnifications ranging from 100X up to 2000X.

**Statistical analysis**

Data obtained in these experiments represents an average of different replicates, which were evaluated using equal variance and paired Student’s t-test. Along with other statistical analyses, Graphpad Prism5.0 software (Graphpad Software Inc., San Diego, CA, USA) was mainly used.

**Results**

**Caspase-3 activation by HB compounds**

Figure 2 shows the labeling pattern of the activated caspase-3 in HT-1080 cells in response to HB compounds when cells were treated for 48 h. The double dose of ASA compared to HBZn compounds gave a approximately half the increased value of caspase-3 compared to HBZn compounds. However, the three HBZn compounds showed no differences in the activation of caspase-3. The values of caspase-3 ranged between 79.7 and 82.5.

**Morphology of treated HT-1080 cells with HBZn**

Light microscopy revealed that treatment of HT-1080 human fibrosarcoma cells with 2-, 3- and 4-HBZn displayed the same morphological changes. Untreated HT-1080 cells showed characteristics of adherence and growth in the monolayer. Cells clearly showed pseudopodia attachments between cells (Figure 3). Low dose treatments with HBZn (0.05 mmol/L) or ASA treatment showed similar results but some cells gained some morphological changes, including rounding and tapering off the nucleus. HT-1080 cells were more confluent over time when treated with a higher dose of HBZn (0.4 mmol/L).

A clear picture of the morphological changes was seen when HBZn-treated HT-1080 cells were stained with
Figure 2 The effects of HB compounds on the activation of caspase-3 activation in HT-1080 cells

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>0.6 mmol/L ASA</th>
<th>0.3 mmol/L 2-HBZn</th>
<th>0.3 mmol/L 3-HBZn</th>
<th>0.3 mmol/L 4-HBZn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase-3 (%)</td>
<td>Control</td>
<td>12.7</td>
<td>38.5</td>
<td>78.7</td>
<td>80.5</td>
</tr>
<tr>
<td></td>
<td>0.6 mmol/L ASA</td>
<td></td>
<td>85.9</td>
<td></td>
<td></td>
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</tbody>
</table>

Figure 3 Light microscopy of treated HT-1080 fibrosarcoma cells under A) 0.05 mmol/L HBZn for 24 h, B) 0.05 mmol/L HBZn for 48 h, C) 0.4 mmol/L HBZn for 24 h, D) 0.4 mmol/L HBZn for 48 h, E) 0.4 mmol/L HBZn for 24 h, White arrows point to lighter specs, which may possibly be the cytoplasmic vacuoles. Black arrows show plasma membrane protrusions (×400).

DAPI, methyl green-pyronin and hematoxylin-eosin (Figure 4). The treatment of HT-1080 cells with HBZn for 48 h, then stained with DAPI (stain DNA, as blue fluorescence), showed different stages of early apoptotic phase (Figure 4A-E). Figures 4B and 4C show the karyolysis stage, or nuclear fading while Figure 4D shows the nucleus more condensed, reflecting the second stage of the early apoptosis phase, or pyknosis stage. In addition, Figure 4E clearly show the characteristics of the third stage of an early apoptosis phase, or karyorrhexis stage. Figure 4E shows the fragmentation of HBZn- treated HT-1080 cells nuclei, which results from the pyknosis nuclei membrane rupture. Using a green filter also showed the two stages of the nucleus at the early stage of apoptosis, pyknosis and karyorrhexis (Figure 4F).

It is important to note that the majority of HT-1080 cells untreated, or treated with 0.4 mmol/L ASA for 24 h remained attached to the surface of the growth cover slide but with no signs of surface blebbing (Figure 4G). However, treatment with higher dose ASA (0.8 mmol/L) for a longer time (48 h) resulted in more morphological changes, including cell shrinkage, nucleus condensation and blebbing (Figure 4H-I).

The second staining techniques used in the current study are methyl-green (to stain DNA) and pyronin (to stain RNA) staining. The overall results to the effect of ASA and HBZn compounds showed similar morphological changes to the previous 2 staining techniques obtained by DAPI staining (Figure 4A-I). The nuclei of HT-1080 cells were deeply stained with methyl green and looked intact with little or no rupturing. They also looked more condensed. This may indicate that cell death is not associated with the late stage of necrosis, since the plasma membrane remains intact. The other observations indicated that cells appeared densely stained with both methyl-green and pyronin. Although there was no sign of blebbing (Figure 4M), treated HT-1080 cells may indicate that they were in early stages of apoptosis.

Further evidence of the apoptotic effects of HBZn was obtained when treated HT-1080 cells were stained with Hematoxylin and eosin stain. Treated HT-1080 cells with 0.05 and 0.4 mmol/L HBZn and 0.4 mmol/L ASA or 0.8 mmol/L for both 24 and 48 h, appeared as a round or oval mass with dark eosinophilic cytoplasm and dense purple nuclear chromatin fragments. The nuclei of treated HT-1080 cells were clearly stained with the red-black Haematoxylin while the cytoplasm stained light-red with Eosin. Cells remained attached to the surface of the slide even after washing and when treated with 0.4 mmol/L HBZn compounds. This confirms the fact that the apoptotic cells still retain their pseudopodial attachments. The majority of cell plasma membranes have remained intact with little evidence of membrane rupture and little or no swelling. However, these cells when compared to those treated with 0.4 mmol/L HBZn compounds, showed signs of plasma membrane irregularities similar to what
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Figure 4 HT-1080 cells after staining with DAPI (A–I), methyl green pyronin (J, L–M) and Hematoxylin and eosine (K–P) stains after treatment with HBZn or ASA for 24 h or 48 h. A) Control (× 300); B) 0.05 mmol/L HBZn (karyolysis stage, × 300); C) 0.2 mmol/L HBZn (karyolysis stage, × 300); D) 0.3 mmol/L HBZn (pyknotic stage, × 300); E) 0.4 mmol/L HBZn (karyorrhexis stage, × 300); F) 0.4 mmol/L HBZn (karyorrhexis stage, × 300); DAPI staining with green filter (white arrows = nucleus, yellow arrows = cytoplasmic vacuoles), G) 0.4 mmol/L ASA, 24 h (× 300); H) 0.4 mmol/L ASA, 48 h (blue arrows refer to blebbing, × 300); I) 0.8 mmol/L ASA, 24 h (blue arrows refer to blebbing, × 300); J) 0.8 mmol/L ASA, 48 h (blue arrows refer to blebbing); K) 0.8 mmol/L ASA, 48 h (blue arrows refer to blebbing); L) Control, cells with nuclei containing marginated chromatin (blue arrow) (× 300); M) 0.4 mmol/L HBZn, 24 h, cells with a single small nucleus with distinctly dense homogeneously staining chromatin (blue arrow) (× 200); N) 0.4 mmol/L HBZn, 24 h (∗ = cytoplasmic vacuoles, × 400); O) Control; P) 0.4 mmol/L HBZn, 24 h (× 200)

was observed with DAPI and non-stain light microscope. In contrast, untreated or treated HT-1080 cells with low dose (0.05 mmol/L HBZn) generally showed no sign of surface blebbing or rounding up (Figure 4K and 4P).

The above results showed a similarity between the qualitative morphological changes in treated HT-1080 cells, since apoptosis generally involves the same pathway that leads to the main morphological changes, nucleus changes, blebbing and fragmentation. However, quantitative results showed different results in the number of HT-1080 cells that underwent apoptosis. Figure 5 shows the number of apoptotic cells after different treatments, which were derived from the stained HT-1080 cells after staining with methyl-green and pyronin. Results indicated that the treated number of apoptotic HT-1080 cells were increased by dose and treatment duration (Figure 5). The level of apoptotic cells increased

Figure 5 Apoptotic indices as derived by methyl-green and pyronin staining after treatment with 0.4 and 0.8 mmol/L ASA or 0.4 mmol/L HBZn compounds for 48 h incubation period. Data represents the mean and SE of mean for 5 separate slide counts
significantly when HT-1080 cells were treated with ASA or HBZn compared to the control ($p = 0.0029 – 0.0001$). However, no significant differences occurred between 2-, 3- and 4-HBZn (Figure 5).

**Morphology of apoptotic bodies**

*Figure 6* shows the morphological modulation of HT-1080 cells in their ultra-structure after treatment with HBZn and ASA. The use of scanning electron microscope (SEM) in assessing apoptosis is mainly associated with specific markers of changes, including smoothing of cell surface (*Figure 6B*) due to loss of microvillus and blebbing (*Figure 6C*). The other apoptotic marker is the formation of apoptotic bodies (*Figure 6D*). The morphological changes are dose- and incubation period-dependent. In addition, changes depend on the growth stage of treated HT-1080 cells.

**Immunohistological assessment of HB compounds**

*Figure 7* shows the immunohistological assessment of the effect of HB compounds on the induction of apoptosis using annexin V. The interaction of annexin V with the flagged out phospholipid phosphatidylserine found on the surface of the plasma membrane of apoptotic cell gives green florescence (*Figure 7*). Results in *Figure 6* clearly indicate that the induction of apoptosis in HT-1080 cells was dose and treatment duration-dependent. ASA showed the lowest number of apoptotic HT-1080 cells (24.9%). In contrast, 0.4 HBZn compounds showed the highest level of apoptotic induction in 48 h. Furthermore, 2-, 3- and 4-HBZn compounds showed no significant differences in inducing apoptosis.

![Figure 6](image1.jpg)

*Figure 6* Scanning electron microscopy of treated HT-1080 cells with HBZn or ASA for 24 and 48 h. A) Control; B) 0.1 mmol/L HBZn; C) 0.2 mmol/L HBZn; D) 0.4 mmol/L HBZn. Both C) and D) show more apoptotic bodies in a concentration-based manner.

![Figure 7](image2.jpg)

*Figure 7* Detection of apoptosis by labeling annexin V in human fibrosarcoma HT-1080 cells that were treated with ASA and HBZn compounds. The higher dose of HBZn and longer period of treatment increase showed more labeled cells.
Discussions

Apoptosis is associated with distinct sets of biochemical and morphological alteration of a cell’s molecular and component structures[7-14]. The early stage of apoptosis involves nuclear chromatin degradation, DNA condensation into a basophilic mass and rupture of the pyknotic nucleic membrane, which is followed by the nucleus membrane[13]. It also involves flag out of phosphatidylyserine which can be detected by annexin V[15]. The main morphological changes that take place in cell morphology are cell shrinking, blebbing and formation of apoptotic bodies[13]. The membrane of the apoptotic cells is intact before it undergoes fragmentation into apoptotic bodies in the last phase of the apoptotic process[16]. The morphological changes in the apoptotic cell are triggered by the various cytological and biochemical activations of certain enzymes, including caspases that cause organized hydrolysis of the nucleus and cytoplasm. Caspases, per se, play a crucial role in the appearance of the morphological changes. However, in vitro, the removal of caspase-3 did not cause a total elimination of blebbing[17,18]. Nevertheless, this implies that structural events are indeed the consequence of caspase activation. Although blebbing appears to require caspase-3 activity for its initiation, once initiated, it can continue even in the presence of caspase inhibitors. This implies that caspases activate downstream mechanisms, which mediate the appearance of blebs. In the current research, the effects of different HB compounds were used to assess their apoptotic potentials in the human fibrosarcoma HT-1080 cell line. In this study, caspase-3 and different techniques were used to assess the morphological efficacy of HB compounds on HT-1080 cells[19]. The morphological modulation in HT-1080 cells was assessed, using normal, fluorescence and scanning electron microscopes. Different staining and immunohistochemical techniques were included to assess the apoptotic morphological changes of HB compound-treated HT-1080 cells. These staining techniques showed different results that clearly illustrated the signs of apoptosis, which are mentioned above in this section. In this respect, light microscopy is the simplest technique that is used to detect cell morphological changes of treated HT-1080 cells with or without staining. Different dyes were used in this current study to assess the morphological changes and confirm the apoptotic potential of HB compounds in HT-1080 cells. These included DAPI (for nucleus), haematoxylin (for nucleus) and eosin (for cytoplasm and filers). In addition, methyl-green (for DNA) and pyronin (for RNA) staining was next utilized in the morphological analysis. Although this method is far more sensitive to initial set-up conditions than that of DAPI and haematoxylin and eosin, the method has the advantage of being able to categorically identify early apoptotic cells as well as distinguishing those cells that are necrotic and viable. This technique is used to stain nucleic acids, whereas methyl green stains DNA (or pyknotic nuclei) while pyronin stains mRNA. Current results indicated that apoptotic HT-1080 nuclei were denser and appeared heavily stained with methyl green, emphasizing the marginating chromatin and pyknotic nuclei. Dense pyronin staining in the cytoplasm indicated the presence of RNA in apoptotic cells, typical of an apoptotic cell continuing RNA and subsequent protein synthesis, which is required for progressive dismantling of the cell. These results coincide with other results obtained from other techniques in this study, including annexin V and scanning electron microscopy. The morphological changes caused by HB compounds are also in line with the measurement of mainly caspase-3. HB compounds showed an increase in the level of caspase-3 in HT-1080 cells whereas, HBZn compounds were more effective to induce apoptosis compared to ASA.

Conclusion

The morphological modulation of HB compound-treated HT-1080 cells confirms that these cells underwent apoptosis. Different morphological techniques confirmed the development of apoptosis in HT-1080 cells by HB compounds. HBZn compounds were more effective than ASA at a lower dose.

Conflict of interest

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

References


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