Sub-millimolar concentration of the novel phenol-based compound, 2-hydroxy benzoate zinc, induces apoptosis in human HT-1080 fibrosarcoma cells

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Received 26 February 2009; revision accepted 8 May 2009

Abstract

Objectives: To examine the effect of a novel phenolic-based compound, 2-hydroxy benzoate zinc (2HBZ), and acetylsalicylic acid (ASA) on human HT-1080 fibrosarcoma cells.

Materials and methods: MTT assay was used to assess cell proliferation while different methods were used to detect apoptosis morphologically and immunohistochemically in Human HT-1080 fibrosarcoma cells. Apoptosis was determined by Annexine-V labelling, and caspase-3 activation. In addition, western blot was used to analyse p21, p53 and Bax and flow cytometry was used to analyse the cell cycle.

Results: 2HBZ exhibited a more than 5-fold increase in cytotoxic potency when compared with ASA with mean LD50 values of 210 and 1100 μM respectively ($P < 0.0001$). The cytotoxic effects of 2HBZ were both time- and dose-dependent with marked apoptosis being evident only after 24 h at concentrations as low as 200 μM . In contrast, ASA-induced apoptosis was observed only at concentrations in excess of 1000 μM at the same time point. Both 2HBZ and ASA induced caspase-3 activation in the cells, which confirmed that their cytotoxic effects were the result of apoptotic cell death. These findings were further confirmed by immunomorphological studies for the detection of apoptosis including haematoxylineosin, methyl green/pyronin Y staining and scanning electron microscopy. In addition, 2HBZ caused a marked increase in p21, p53 and Bax protein expressions and these effects were associated with an increase in G1

and G2 arrest of the cell cycle and a reduction in S-phase.

Conclusions: These results demonstrate that the novel phenolic compound 2HBZ is a potent apoptosis-inducing agent in HT-1080 cells and warrants further investigation as a potential chemotherapeutic agent in primary cancer cell models.

Introduction

Phenolic compounds are common natural products found abundantly in plants and have been shown to exhibit multiple biological activities, including anti-cancer potential (1–5). The most common phenolic compound is salicylic acid, precursor of acetylsalicylic acid (ASA) commonly known as aspirin. This compound has had a long and interesting history of development (6). ASA belongs to the class of agents known as non-steroidal anti-inflammatory drugs (NSAID) that exhibit multiple therapeutic properties such as anti-pyretic and anti-inflammatory activities, and anti-cancer properties displayed in a number of human cell lines. In this regard, a novel calcium salicylate has been shown recently to induce apoptosis in the human fibrosarcoma cell line HT-1080 (5).

Despite a great deal of research on anti-cancer potential of phenolic compounds such as ASA, the molecular mechanisms through which these agents induce apoptosis are not well understood. ASA-induced apoptosis has been shown to impair function of both mitochondria and endoplasmic reticulum and subsequently activate downstream pro-apoptotic proteins, which leads to activation of caspases -3, -8 and -9 (7–9). ASA has also been shown to inhibit proliferation of a number of cell lines through induction of cell cycle inhibitor, p21 (10,11). The inhibitory effect of p21, which is largely dependent on presence of p53, is commonly recruited in G₁ phase of the cell cycle (12,13). However, induction of p21 is not always necessarily p53-dependent (14) and hence distinct cell

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Figure 1. Chemical structures of ASA and 2HBZ.

cycle arrest is not required for a cell to enter apoptosis (15). The apparent broad spectrum of apoptotic pathways induced by ASA may be caused, at least in part, by the relatively high concentrations used (for example, 2×10^3 – $50 \times 10^3 \mu\text{M}$). In this article, we report anti-apoptotic activity of a novel phenolic-based analogue, 2-hydroxy benzoate zinc (2HBZ), that has a chemical structure closely related to ASA (Fig. 1) but with cytotoxic activity at sub-millimolar concentrations.

Materials and methods

Cell growth and culture

HT-1080 cells were cultured in optimal Dulbecco's Minimum Essential Medium (DMEM) supplemented with 10% foetal calf serum, gentamicin, 1% L-glutamine, 0.1% Hepes buffer (1 M), 0.1% sodium pyruvate ($10^4 \mu\text{M}$) and 0.1% ascorbic acid. Cells were cultured for 3 days (to approximately 70% confluence) in optimal medium and for a further 24 h in serum-free DMEM medium (cells demonstrated increase in G1 phase of approximately 36%) before they were treated with 2HBZ for 24 h, at concentrations between 50 and $400 \mu\text{M}$. In addition, control cells comprised those to which no drug was added, in all assays outlined below. 2HBZ was prepared from 2-hydroxybenzoic acid and zinc sulphate. Liquid chromatographic analysis showed that 2HBZ was more than 95% pure.

Cell proliferation assay

The cells ($2 \times 10^5/\text{ml}$) were plated in 96-well plates and treated with 2HBZ or ASA (50 – $400 \mu\text{M}$) for 3, 6, 12, 24, 48 and 72 h. Cells were then treated with 10% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent for 1 h and subsequently evaluated colorimetrically at 575 nm using the Labsystems Multiskan MS Version 3.0 (Helsinki, Finland). Three separate experiments were performed and each assay was conducted in triplicate.

Morphological detection of apoptosis

To investigate the apoptotic effect of 2HBZ and ASA, HT-1080 cells (1.25×10^3 – $2 \times 10^3/\text{well}$) were treated with $400 \mu\text{M}$ for 24 h in six-well plates with coverslips. Cells were washed with 2 ml phosphate-buffered saline (PBS) and incubated for 10 min at room temperature before fixation with formaldehyde (2 ml, 4% in PBS) for 10 min at room temperature. Fixed cells were washed again in PBS and treated with Mayer's haematoxylin for 1 min at room temperature. Stained cells were washed three times in tap water and once in distilled water. Preparations were counterstained with 1% eosin for 3–5 min and washed again in water. In addition, cells were treated with methyl green/pyronin Y dye for 45 min at room temperature. Coverslips coated with cells were dipped in iced deionized water for 3–4 s. Then they were then air-dried and mounted using DPX. The cells were subsequently viewed using a Leitz light microscope, and were recorded using Analysis Image; Analysis software V2.3. (FEMLAB, Germany.)

Detection of apoptotic bodies

Cells were treated with 2HBZ (0.1, 0.2, $400 \mu\text{M}$) for 24 h, then were then fixed in 0.8% glutaraldehyde, 0.6% osmium tetroxide, $2 \times 10^3 \mu\text{M}$ CaCl_2 and 0.2 M sucrose in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 h, washed several times in PBS and dehydrated with successive concentration alcohol washes (30%, 50%, 70%, 90%, each for 5 min, and 100% for 10 min twice). Dehydrated cells were then critical point dried in a Blazers CPD 030 using CO_2 . Cell samples were mounted on to 12 mm aluminium stubs using silver paint, then gold sputter coated in an Edwards S150B sputter coater. Treated cells were assessed for morphological signs of apoptosis using a Phillips XL20 scanning electron microscope (SEM) at a variety of magnifications.

Detection of apoptosis by annexin-V labelling

To validate morphological evidence for apoptosis induced by 2HBZ and ASA, HT-1080 cells were

assessed by immunohistochemical and flow cytometric methods using annexin-V labelling. Cells ($1.25 \times 10^5/\text{ml}$) in six-well plates were treated with 400 μM 2HBZ or ASA for 24 and 48 h. Treated cells were subsequently harvested by centrifugation (200 g , 5 min), homogenized, resuspended in 1x binding buffer twice and finally resuspended again in 50 μl of the same buffer. Cells were treated with fluorescein isothiocyanate (FITC)-labelled annexin-V (2 μl) for 15 min and then with propidium iodide (2.5 μl) for 5 min in the dark. Slides were then examined using a fluorescence microscope. Five random fields, each of 100 cells, were counted per slide and the percentage of apoptotic cells was calculated based on number of cells that showed nuclear morphology changes (16). Representative photographic images were taken using a photometrics FS camera and analysed using the Cool-Snap image analysis software V1.2.

Flow cytometric analysis of apoptosis using annexin-V/propidium iodide labelling was also conducted to quantify apoptotic potential of 2HBZ and ASA. Data generated from these assays were used to calculate LD_{50} values for each agent (concentration of compound required to induce 50% apoptosis). Cells ($1 \times 10^4/\text{T-25 flask}$) were treated with 2HBZ (50, 100, 200, 400, 800 and 1600 μM) or ASA (50, 100, 200, 400, 800, 1600 and 3200 μM) for up to 48 h and then were harvested by centrifugation. Cells in 100 μl of binding buffer were treated with FITC-labelled annexin-V, (Dako cytometry, Ely, UK) and then with propidium iodide (Dako cytometry) each for 10 min in the dark, before being analysed by flow cytometry using CellQuest software on a FACSCalibur flow cytometer (Becton Dickinson, Oxford, UK). Apoptosis was also quantified using changes in forward light scatter (FSC) and side light scatter (SSC) characteristics of HT-1080 cells as described previously (17–19).

Detection of apoptosis by caspase-3 activation

Cells ($1 \times 10^4/\text{T-25 flask}$) were treated with 400 μM 2HBZ or 3200 μM ASA for 24 h, 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 two fluorophores separated by a quenching linker sequence that is specifically cleaved by active caspase-3. Once cleaved, resulting products fluoresce green and can be quantified using flow cytometry. For all experiments, 10 000 events were measured and were analysed using a FACSCalibur flow cytometer (Becton Dickinson).

Western blot analysis

Cells in six-well plates at an initial cell density of 3×10^4 were treated with 400 μM 2HBZ for 24 h. They were then washed in PBS and were subsequently extracted in 200 μl of 2x sample buffer [250 mM Tris-HCl pH 6.8, 4% SDS, 0.006% bromophenol blue, 2% β -mercaptoethanol (Pharmacia, Uppsala, Sweden), glycerol (Sigma, Surrey, UK)]. Cells were harvested by scraper, transferred to 1.5 ml Eppendorf tubes, heated at 100 °C for 10 min, cooled to room temperature and harvested by centrifugation (120 g for 5 min). The supernatant was further centrifuged at 4 °C at 16 000 g for 5 min and a clear solution of protein mixture was obtained. Total protein concentrations were determined according to the method of Karlsson *et al.* (20). Twenty-five micrograms (20 μl) of protein samples and 10 μl of molecular marker were loaded on to 4–12% bis-tris acrylamide gel in NuPAGE MOPS [3-(N-morpholino) propanesulphonic acid] SDS running buffer (Invitrogen, Life Technologies, Scotland, UK). After running the gel at 200 V for 30–50 min, resolved proteins were transferred on to a nitrocellulose membrane (Sigma). Membranes were incubated first with an appropriate primary antibody (p21, p53 or Bax), then with peroxidase-conjugated anti-mouse IgG antibody (Sigma). They were then washed and developed using a chemiluminescent reagent (Amersham, Cardiff, UK) and were exposed to photographic films.

Flow cytometric analysis of the cell cycle

HT-1080 cells in T-25 flask at initial density of 1×10^4 were treated with 400 μM 2HBZ or ASA for 2, 6, 12, or 24 h. In addition, control experiments were performed in which no benzoate was incorporated in the cultures. Subsequently, cells were harvested by centrifugation and resuspended in 200 μl of PBS. Aliquots (2 ml) of ice-cold 70% ethanol were then added and cells were vortexed prior to being cooled to –20 °C for 30 min. RNase measuring 100 μl (Sigma) at concentration of 1 mg/ml and propidium iodide measuring 100 μl (Biovision Inc., California, USA) at a concentration of 400 $\mu\text{g}/\text{ml}$ were then added prior to incubation at 37 °C for 30 min. Cells were analysed using a FACSCalibur flow cytometer (Becton Dickinson) and percentages of cells in different phases of the cell cycle were quantified using Cylchred version 1.0.2 software (Terry Hoy, University Hospital of Wales, UK).

Statistical analyses

Data obtained in these experiments were evaluated using equal variance and paired Student's *t*-test, and correlation coefficients were calculated from least squares linear

Figure 2. Comparison of *in vitro* cytotoxicity of 2HBZ and ASA on HT-1080 cells (a) time- and dose-response curves plotted using data derived from MTT viability assays. 2HBZ was significantly more cytotoxic than ASA ($P = 0.0001$). (b) Sigmoidal dose-response curves plotted using data derived from annexin-V/propidium iodide assays. 2HBZ was significantly more cytotoxic than ASA with a mean LD_{50} value of 210 μM ($\pm 24.7 \mu\text{M}$) compared to 1100 μM ($\pm 20.3 \mu\text{M}$) for ASA.

regression plots. LD_{50} values were calculated from line of best-fit analysis of the dose-response curves. All statistical analyses were performed using Graphpad Prism 3.0 software (Graphpad Software Inc., San Diego, CA, USA).

Results

Cytotoxic effect of 2HBZ and ASA

Cytotoxic effects of the phenolic compound, 2HBZ, were compared to those of ASA on HT-1080 cells, using the MTT assay and a flow cytometry-based annexin-V assay. Both compounds caused a degree of mitogenic activity at low concentrations that was negated at concentrations greater than 100 μM for 2HBZ and 400 μM for ASA (Fig. 2a). Cytotoxic effects of 2HBZ on our cells were clearly both time- and dose-dependent (Fig. 2a), and this has subsequently been confirmed on a number of other cell lines, including HeLa and S180 (data not shown). Furthermore, 2HBZ demonstrated 5-fold reduction in LD_{50} value when compared to ASA, as measured by annexin-V assay (Fig. 2b). Figure 3 shows an example of the annexin-V/propidium iodide plots for 2HBZ and ASA in the cells after treatment for 48 h.

Morphological assessment of apoptosis induced by 2HBZ and ASA

MTT and flow cytometric results clearly showed the cytotoxic effects of 2HBZ as illustrated in Figs 1 and 2. In

Figure 3. Examples of annexin V/propidium iodide (PI) dot plots. Illustrating differential effects of 2HBZ and ASA in HT-1080 cells at concentrations of 100, 200 and 400 μM after exposure for 48 h.

Figure 4. Immunohistochemical detection of apoptotic HT-1080 cells using annexin-V labelling. 2HBZ was significantly more toxic than ASA at each concentration and exposure period tested.

addition, we conducted further investigations to elucidate the apoptotic effect of 2HBZ and ASA on morphology of HT-1080 cells. Figure 4 shows immunofluorescent labelling with annexin-V of treated cells in the early stages of apoptotic cell death as elucidated by the labelling of the flagged phosphatidylserine (bright area) and in the later stages of apoptosis as indicated by the cell shrinkage and nuclear condensation after exposure to 400 μM 2HBZ for 24 and 48 h.

Cells stained with haematoxylin/eosin and methyl green/pyronin Y showed the classical composite morphological signs of apoptosis, now induced by 400 μM 2HBZ (Fig. 5a). These include membrane blebbing, cytoplasmic shrinkage and loose pseudopodial attachment. It is clear that the cells were more sensitive to 2HBZ compared to ASA at 400 μM and 24 h treatment. Scanning electron micrographs showed further evidence of apoptotic effects of 2HBZ. Untreated HT-1080 cells had a flat monolayer structure (Fig. 5b) and retained attachment to the surface as well as their pseudopodia attached to each other. These features disappeared in cells treated with ASA and 2HBZ and were replaced by the appearance of apoptotic bodies (Fig. 3b). The morphological results showed strong agreement with immunocytochemical and morphological evidence of apoptosis induced by 2HBZ.

Detection of apoptosis by caspase-3 activity

Caspase-3 activity assays were also performed, using a specific fluorescent substrate, PhiPhiLux-G₁D₂. This detects specific activity of terminal caspase and here was quantified using flow cytometry, based on the reaction of active caspase-3 with the cell-permeable PhiPhiLux®

Figure 5. Morphological evidence of apoptosis induced by 2HBZ and ASA. (a) HT-1080 cells stained with haematoxylin-eosin (left) and methyl green/pyronin Y (right) after exposure to 2HBZ and ASA for 24 h (magnification = ×200). (b) Scanning electron micrographs of treated HT-1080 cells after exposure to 2HBZ for 24 h.

G₁D₂ substrate. Each substrate molecule contains two fluorophores quenched by a linker sequence that is specifically cleaved by caspase-3. Figure 6 shows caspase-3 activity in the cells treated with 2HBZ and ASA for 48 h. The results indicate that both compounds induce caspase-3-mediated apoptosis and that 2HBZ was able to induce caspase activation at much lower concentrations than ASA.

Western blot analysis of p53, p21 and Bax proteins

Given the perturbations in the cell cycle induced by 2HBZ, Western blot analysis was performed on cell lysates derived from HT-1080 cells to establish whether p53, p21 and Bax protein expressions were altered after exposure to 2HBZ. Figure 7 shows that 2HBZ upregulated expression of all of these proteins after exposure for 24 h. Expression was induced by at least 50% when compared to untreated control samples. These data suggest

Figure 6. Comparison of quantification of apoptosis in HT-1080 cells after exposure to 2HBZ and ASA for 24 h. Mean values ($\pm$ SE $n = 4$) of labelled caspase-3 with fluorescent substrate, PhiPhiLux- G_1D_2 .

that 2HBZ promotes cell cycle arrest, probably mediated by induction of p21, and induces a p53-dependent mechanism of cell killing, potentiated by induction of Bax.

Cell cycle analysis of HT-1080 cells treated with 2HBZ

Cell cycle analysis was performed on HT-1080 cells with and without addition of 2HBZ. Results clearly demonstrate that exposure to 2HBZ caused loss of cells in S-phase with concomitant increase in both G_1 and G_2 phases. These effects became apparent after 12 h exposure to 2HBZ and preceded the characteristic development of a sub G_0/G_1 peak associated with apoptosis.

Discussion

Experimental *in vitro* assessment of utility of ASA as an anti-cancer agent has been mostly performed using relatively high concentrations (5–50 mM) of this compound (7,9,21,22). However, high concentrations of ASA *in vivo* are contraindicated because of reported complication of impaired cardiac function, GI bleeding and perforation (23–25). In this context, *in vivo* relevance and significance

Figure 7. Expression of pro-apoptotic proteins in treated and untreated HT-1080 cells with 400 μ M 2HBZ and 400 μ M ASA for 24 h. (a) p21, p53 and Bax were identified using Western blot analysis and quantified by optical density (OD) measurements. Relative changes in protein expression induced by 2HBZ and ASA were expressed as normalized percentages based on the control (no drug) Western blot %OD values for ASA and 2HBZ were: p21 = 77.8 ± 4.1 and 154.1 ± 6.2 ; p53 = 114.5 ± 16.9 and 200.5 ± 12.5 and Bax = 139.3 ± 14.9 and 253.5 ± 30.5 respectively. (b) Cell cycle analysis revealed that 2HBZ resulted in decrease of S phase cells and concomitant increase in both G_1 and G_2 prior to induction of apoptosis. This finding concurs with the protein analyses, which clearly show that 2HBZ induces cell cycle arrest proteins, p21, p53 and Bax.

of reports on effects of high *in vitro* concentrations of ASA have been questioned (26,27). Pharmacologically, therapeutic concentration of ASA required to achieve selective inhibition of cyclo-oxygenase-2 transcription in fibroblasts and endothelial cells is 40–50 μM (28). In this study, we report the cytotoxic profile of an ASA analogue, 2HBZ. 2HBZ is a novel phenolic-based compound chemically close in structure to ASA. Notably, we have shown that 2HBZ was approximately 5 times more cytotoxic to HT-1080 cells than ASA. It inhibited them in a time- and dose-dependent manner with mean LD_{50} value of 210 μM . However, further studies are required to define the precise molecular targets of 2HBZ that result in induction of apoptosis. In addition, we have shown recently that 2HBZ is more cytotoxic than ASA in further cell types including primary chronic lymphocytic leukaemia cells (data not shown).

Induction of apoptosis in target cells is a key requirement for development of novel anti-cancer therapeutic agents (29,30). Hence, 2HBZ has been shown to possess the desired apoptotic features for possible development as a chemotherapeutic agent. Evidence from the immunochemical assays was validated by morphological evidence of apoptosis in HT-1080 cells. Furthermore, detection of apoptosis by annexin-V/propidium iodide labelling and caspase-3 activity assay also provided evidence concerning the apoptotic potential of 2HBZ. Indeed, these two assays were crucial in demonstrating proof of apoptotic induction by 2HBZ. Results of this study have shown that 2HBZ induced caspase-3 activation and induced apoptosis in HT-1080 cells. 2HBZ at 400 μM caused significantly higher levels of apoptosis when compared to ASA. Cell cycle arrest accompanied by activation of p21, p53 and Bax proteins provides evidence of a p53-dependent mechanism of cell killing by 2HBZ. However, a more thorough analysis of the precise molecular targets of 2HBZ in HT-1080 cells is warranted.

In conclusion, this study has demonstrated the anti-cancer potential of the phenolic compound, 2HBZ, in HT-1080 a human fibrosarcoma cell line. Further studies are currently in progress in primary human leukaemia cells and normal haematopoietic cells to establish whether this class of compounds has a favourable therapeutic index for treatment of leukaemias and possibly other human cancers.

Acknowledgements

We thank Dr Andrew Waggett and Dr Steve Luckman, School of Biosciences, Cardiff University for sharing Western blot equipment and for the gift sample of HT-1080 cell lines respectively.

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