The anticancer effects of 2-methoxyestradiol on human huh7 cells in vitro and in vivo

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ABSTRACT

Hepatocellular carcinoma (HCC) is associated with a poor prognosis. 2-methoxyestradiol (2-ME) is currently under preclinical evaluation as a treatment for many malignancies, but the utility of the drug in terms of HCC treatment remains unclear. Here, we explored the effect of 2-ME on human huh7 cell proliferation and apoptosis and discuss the possible molecular mechanisms involved. The MTT assay showed that proliferation was markedly inhibited by 2-ME (at 5, 10, 15, and 20 μmol/L) in a time- and dose-dependent manner. Moreover, flow cytometry indicated that 2-ME induced cell cycle arrest at the G2/M phase, and early apoptosis. We used Western blotting and PCR to detect the expression of vascular endothelial growth factor (VEGF) and Bcl-2; 2-ME decreased the mRNA/protein expression levels of both effectors. Furthermore, 2-ME remarkably suppressed xenograft tumor growth in nude mice, and no visible toxicity was observed in either the liver or kidneys. Immunohistochemically, the Bcl-2 and VEGF expression levels were significantly lower than those of controls. Thus, 2-ME inhibited huh7 cell proliferation, promoted apoptosis, and suppressed xenograft tumor growth in nude mice, perhaps reflecting the effects of the drug on VEGF and Bcl-2 expression.

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1. Introduction

2-ME is a natural metabolite of the endogenous, human estrogen 17β-estradiol in lacks estrogenic activity. 2-ME exhibits antiproliferative, antiangiogenic, and antitumor properties, thus acting against cancer in several ways [1,2]. The drug is potentially cytotoxic toward carcinomas of the prostate and breast, head-and-neck squamous cell carcinoma and nasopharyngeal carcinoma, both in vivo and in vitro [3–6], inducing G2/M arrest and apoptosis of many actively dividing cell types.

HCC is associated with a poor prognosis and a high mortality rate. 2-ME is an effective antitumor agent, but any effect on HCC remains unknown. Therefore, we explored whether 2-ME at different concentrations was cytotoxic toward human huh7 cells and the mechanisms underlying the antiproliferative and apoptotic effects. Our work will facilitate the use of 2-ME to treat HCC.

2. Materials and Methods

2.1. Chemicals and reagents

2-ME (Sigma, St. Louis, MO, USA) was stored as a stock solution of 300 μmol/L in dimethyl sulfoxide (DMSO) at 4 °C and diluted to various concentrations with Dulbecco’s Minimal Essential Medium (DMEM) containing 10% (v/v) calf serum (Gibco, Grand Island, NY, USA) prior to use. MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was obtained from Goodbio Technology (Wuhan, China). The Annexin V-FITC assay kit was the product of Bestbio Biotechnology (Shanghai, China).

2.2. Cell lines and cell culture

The hepatoma cell line huh7 was obtained from the Department of Oncology, Zhongnan Hospital, Wuhan University, Wuhan, China and cultured in RPMI1640 medium (Sigma) with 10% (v/v) fetal bovine serum, 50 mg/mL streptomycin, 50 IU/mL penicillin, and 2 mM glutamine under 5% (v/v) CO2 in a humidified atmosphere at 37°C.

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2.3. Cell proliferation assay

The in vitro inhibitory effect of 2-ME on cell proliferation was determined with the aid of the MTT assay. Briefly, huh7 cells (1 \( \times 10^6 \) mL/well) were seeded into 96-well microtiter plates. After 24 h, the culture medium was discarded, the cells exposed to 2-ME (5, 10, 15, or 20 \( \mu \)mol/L) and then incubated at 37 °C for 24, 36, and 48 h. MTT solution (20 \( \mu \)L of a 5 mg/mL solution in phosphate-buffered saline [PBS]) was added to each well and the plates incubated for an additional 4 h at 37 °C. The MTT solution was then aspirated. To solubilize formazan crystals formed within viable cells, 200 \( \mu \)L DMSO was added to each well and the optical density at 570 nm determined with the aid of a multiwell plate reader (Tecan, Grödig/Salzburg, Austria). Each essay was performed in triplicate. The results were expressed as inhibition rates (IRs): IR = (A − B)/A \( \times 100\% \) where A and B were the absorbances of control and test groups after 24, 36, and 48 h of incubation, respectively.

2.4. Flow cytometry

Huh7 cells were seeded into 6-well microtiter plates and, when the cells attained about 80% confluence, 2-ME was added to 0, 5, 10, 15, or 20 \( \mu \)mol/L. After 24 h, single-cell suspensions were prepared via trypsin digestion and washed twice with PBS. The cells were then centrifuged at 2,000 rpm for 5 min and fixed in 70% (v/v) ethanol at 4 °C overnight. The cells were resuspended in PBS to 1 \( \times 10^6\) cells/mL and RNase (final concentration 20 \( \mu \)g/mL) and propidium iodide (PI) staining solution (0.3 mL of a solution of 50 mg/mL) were added; staining proceeded at room temperature for 30 min in the dark. The fluorescence intensities were measured by flow cytometry. Each assay was performed in triplicate. Cell cycle status was analyzed using Cell Quest/Modfit software.

2.5. Annexin V/PI staining

To quantify apoptosis, we used an Annexin V-FITC kit as described by the manufacturer. Briefly, huh7 cells were seeded into six-well microtiter plates (4 \( \times 10^5 \) cells/plate), incubated for 24 h with different concentrations of 2-ME, washed twice with cold PBS and resuspended in binding buffer to a concentration of 1 \( \times 10^6\) cells/mL. After incubation, 100 \( \mu \)L amounts of solutions were transferred to 5-mL culture tubes, and 5-\( \mu \)L amounts of an Annexin V-FITC solution and 10-\( \mu \)L amounts of a propidium iodide (PI) solution then added. The tubes were gently centrifuged and incubated for 15 min at room temperature in the dark. Then, 400 \( \mu \)L binding buffer was added to each tube and the cells immediately subjected to flow cytometry (Partec, Muenster, Germany). Data were analyzed with the aid of Cell Quest software.

2.6. Western blotting

Huh7 cells in the logarithmic growth phase were treated with different concentrations of 2-ME and, after 24 h, the cells were collected. Nuclear proteins were extracted with the aid of the Nonenzy Cell Detach Solution and a Nuclear-Cytosol Extraction kit (Applygen, Beijing, China) according to the manufacturer’s instructions. Protein concentrations were determined using a BCA protein assay kit (Jiancheng, Nanjing, China). Fifty microgram amounts of protein were electrophoresed on 12% (w/v) SDS polyacrylamide gels, transferred to nitrocellulose membranes, blocked in 5% (w/v) nonfat dry milk in TBS overnight at 4 °C, washed three times with TTBS (for 5 min each time), incubated with anti-VEGF and anti Bcl-2 antibodies (1:1,000 dilution) for 1 h at room temperature, washed three times with TBST, incubated with anti-rat IgG (1:5,000 dilution, KPL, Los Angeles, USA) for 1 h at room temperature, washed three times with TBST, and signals were detected using the ECL-plus Western blotting system (GE Healthcare, formerly Amersham, Little Chalfont, UK). The expression levels of target proteins was densitometrically determined and normalized to those of β-actin. All assays were performed in triplicate.

2.7. Real-time PCR

Total RNA was extracted from homogenized ischemic myocardia using the Trizol reagent (Invitrogen, Grand Island, NY, USA). The levels of mRNAs encoding specific genes were measured at various timepoints using an ABI Prism 7500 Sequence Detection System (Applied Biosystems [ABI], Carlsbad, USA) and normalized to the levels of β-actin mRNA. Primers were designed using the ABI-Perkin Elmer guidelines. The PCR primer sequences were: VEGF forward, 5'-GGGACAGAAGCAGCCATGAA-3' and reverse 5'-AGATG TCCACCGGGTTCTCA-3'; Bcl2 forward 5'-CAGGAAGGGGGCGAT-3' and reverse, 5'-CTGGGGCTTCTATCCCTC-3'; and β-actin forward 5'-CATAGAGGAAAGCTGTGCT-3' and reverse 5'-GGTGAAGGTAG TTCCGTGA-3'. All assays were performed in triplicate.

2.8. Tumor xenograft growth in nude mice

Male BALB/c nude mice weighing about 20 g (4–6 weeks of age) were obtained from the Center for Animal Experiments of Wuhan University and housed in laminar-flow cabinets under specific pathogen-free conditions with food and water available ad libitum. The protocol was approved by the Experimental Animal Care Committee (IACUC) (approval number: S013150467) of Renmin Hospital, Wuhan University. Huh7 cells (200 \( \mu \)L, 5 \( \times 10^6 \)) in the logarithmic phase of growth were subcutaneously injected into the backs of the animals. Seven days later, 32 mice with visible tumor masses 6–8 mm in diameter were randomized to four groups of eight mice each, ear-tagged (Day 1), and followed-up individually. The control (and three) experimental groups received intraperitoneal saline at 10 mg/kg/day, or 2-ME at 10, 20, or 30 mg/kg/day. Tumor volume (TV) and body weight were recorded every 5 days. TV was calculated in mm3 as \((a \times b)^2/2\), where a and b were the shortest and longest diameters, respectively. TV measurements were performed in duplicate and the means recorded. All mice were sacrificed via orbital sinus bleeding on Day 30, and the tumors dissected and weighed. The extent of liver and renal injury was determined by measuring the levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), and serum creatinine (Cr).

Tumors from each group were fixed in 10% (v/v) formalin, embedded in paraffin, and cut into 5-\(\mu\)m-thick serial sections for hematoxylin-and-eosin (H&E) staining. VEGF and Bcl-2 expression levels were evaluated immunohistochemically employing rat anti-human VEGF and Bcl-2 antibodies (Zhongshan Goldenbridge, China). Positive cell percentages were measured using a CM1AS image analysis system (Beihang University, China).

2.9. Statistical analysis

All statistical analyses were performed with the aid of the SPSS ver. 20.0 statistical package. All data are presented as the means ± SDs of those of at least three independent experiments. Student’s t-test was used to compare between-group means. When more than two means were compared, we employed one-way ANOVA followed by a multiple comparisons test. Statistical significance is indicated by *, P < 0.05; **, P < 0.01; and ***, P < 0.001.
3. Results

3.1. Inhibitory effects of 2-ME on huh7 cells

The inhibitory effect of 2-ME in terms of hepatoma cell proliferation in vitro was assessed after 24 h of drug exposure followed by 24 h of culture in drug-free medium. As shown in Table 1, 2-ME inhibited cell growth in a dose-dependent manner (P < 0.01).

3.2. Effect of 2-ME on the cell cycle and apoptosis

After exposure to 2-ME at different concentrations, the cell cycle was arrested in G2/M in most huh7 cells; the numbers of S-phase cells decreased significantly (Table 2). 2-ME induced apoptosis (P < 0.01 compared to the control), as confirmed using Annexin V/PI staining to detect phosphatidylserine externalization onto the cell membrane. Fig. 1 show the early apoptosis rate was 11.32% at 5 μmol/L 2-ME, and 32.75% at 20 μmol/L 2-ME; the late apoptosis rate increased from 7.17 to 18.07%.

3.3. 2-ME inhibits mRNA/protein expression of VEGF and Bcl-2

To investigate further the apoptotic mechanism of 2-ME, we measured the expression levels of VEGF and Bcl-2. Huh7 cells were exposed to different concentrations of 2-ME for 24 h, and VEGF and Bcl-2 expression evaluated via RT-qPCR and Western blotting. Fig. 2 shows that the expression levels fell significantly (P < 0.05 compared to control) as the 2-ME dose increased; Western blotting (Fig. 2A) showed that the protein expression data mirrored the mRNA expression data (P < 0.05; Fig. 2B).

3.4. Effects of 2-ME on tumor development in vivo

The effects of 2-ME on huh7 cell growth in vivo were examined in xenografts developing in BALB/c nude mice after subcutaneous tumor cell injection. Tumor volumes were clearly reduced in mice treated with 10, 20, or 30 mg/kg/day 2-ME at days 5, 10, 15, 20, 25, and 30 compared to the control group (Fig. 3A). After treatment, the mean tumor weight and volume in 2-ME-treated mice were significantly lower than in control mice (P < 0.05, Fig. 3B).

3.5. Pathological tumor features

H&E staining of tumor sections showed that the cell density decreased significantly as the 2-ME dose increased, compared with the control. The expression levels of Bcl-2 and VEGF were measured immunohistochemically (Fig. 4A). The expression levels in the 10, 20, and 30 mg/kg/day 2-ME groups were all significantly lower than the control group; maximally so in the latter group (Fig. 4B). Thus, the expression levels fell in a dose-dependent manner.

3.6. Side-effects

No death or toxic sign was observed in any mouse. The serum ALT, AST, BUN, and Cr levels did not differ between test and control mice (all P > 0.05, Table 3), indicating the absence of liver or kidney injury. In addition, there was no significant difference in body weight among the four groups (data not shown).

4. Discussion

In recent years, 2-ME has been shown to inhibit tumor growth in general [1,2] and, in particular, prostate [3], bladder [4], breast [5], esophageal [6], nasopharyngeal [7], and several other carcinomas [8–19]: no data on HCC are available. Our results suggest that 2-ME may be active against HCC. Ricker [8] exposed several human head-and-neck squamous cell carcinoma lines to 2-ME (0–50 μmol/L) or paclitaxel (0–1 μmol/L) for 72 h; the drugs (alone or in combination) inhibited cell growth. We found that 2-ME significantly inhibited huh7 cell proliferation in a time- and dose-dependent manner.

Angiogenesis is essential during normal development [20] and also if cancers are to progress; cancer cells require high levels of nutrients and oxygen during proliferation. Angiogenesis is associated with the proliferation of cancers greater than 1 mm in length. If angiogenesis is low, the tumor is relatively hypoxic; VEGF promotes angiogenesis facilitating tumor growth. VEGF is highly expressed in many tumors including gastric and breast cancers. We found that when Huh7 cells were exposed to different concentrations of 2-ME, VEGF mRNA and protein level decreased as the drug dose increased; similar results were obtained in vivo. Thus, 2-ME may downregulate VEGF expression.

Cell cycle dysfunction is the main reason why tumor cells proliferate. Naga [10] found that 2-ME was cytotoxic to HepG2 cells, blocking the cycle at G2/M. Reiner [3] found that 2-ME induced androgen-independent prostate cancer cell arrest in G2/M. Our results were similar. When Huh7 cells were exposed to various levels of 2-ME, the proportion of S-phase cells decreased and that of G2/M-phase cells gradually increased; 2-ME arrested the cycle in G2/M.

Apoptosis plays important roles in eliminating invasions by multicellular organisms and in compromising the growth, differentiation and proliferation of unwanted (damaged and infected) cells [21]. We used Annexin V/PI staining to detect apoptosis induced by 2-ME. After exposure to 2-ME at various concentrations, the early apoptosis rates were 11.32 and 32.75% at 5 and 20 μmol/L 2-ME, respectively. Also, in vivo, the mean xenograft tumor weight and volume in 2-ME-treated mice were significantly lower than those of controls.

Apoptosis is normally strictly regulated; defective regulation triggers certain diseases, such as cancers and autoimmune and neurodegenerative conditions. The Bcl-2 protein family (which includes both pro- and anti-apoptotic proteins) plays an important role in apoptosis; the dynamic balance between family proteins either promotes or inhibits death. Mitochondria also play an important role, engaging in cascade amplification of irreversible hydrolysis mediated by activated caspase.

Bcl-2 (a proto-oncogenic protein) is found in the outer...
mitochondrial membrane, the endoplasmic reticulum, and the nuclear membrane, inhibiting apoptosis by preventing cytochrome C release from mitochondria into the cytoplasm, wherein cytochrome C promotes apoptosis. In addition, Bcl-2 is protective; high-level expression triggers nuclear accumulation of glutathione, changing the redox balance in a manner that reduces caspase activity. Western blotting and PCR showed that VEGF mRNA/protein expression decreased as the 2-ME concentration increased; the in vivo results were similar. Thus, 2-ME induced huh7 cell apoptosis, perhaps by inhibiting Bcl-2 protein expression.

Fig. 1. Apoptosis of huh7 cells exposed to different concentrations of 2-ME for 24 h, with quantitative data.

Fig. 2. The VEGF and Bcl-2 mRNA and protein levels of huh7 cells exposed to different concentrations of 2-ME for 24 h. A: Cells were treated with 0, 5, 10, 15 or 20 µmol/L of 2-ME for 24 h, followed by Western blotting using appropriate antibodies. B: Total RNA was extracted, and the VEGF and Bcl-2 mRNA levels quantified via real-time PCR and normalized to the β-actin mRNA levels. The data are means ± SDs (n = 3); *P < 0.05.
We found that 2-ME exhibited anti-gastric cancer effects both in vitro and in vivo. Thus, 2-ME may usefully treat HCC. However, the underlying molecular mechanism(s) remain to be elucidated.

Author contributions

Conceptualization, hai tao; Formal analysis, hai tao; Funding acquisition, xiaoan yang; Investigation, hai tao and juanjuan mei; Methodology, juanjuan mei and xiaoan yang; Project administration, juanjuan mei and xiaoan yang; Resources, juanjuan mei and xiaoan yang; Software, xiaoan yang; Writing-review & editing, hai tao and juanjuan mei.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.02.068.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.02.068.

Conflicts of interest

The authors declare no conflict of interest.

References


Fig. 3. The effects of 2-ME on huh7 xenograft tumor volume and weight. *P < 0.05 compared to the untreated control.

Fig. 4. Pathological features of xenograft tumors from mice that were and were not treated with 2-ME. Mice with xenografts, established as described in the Materials and Methods, were treated with saline (control group); or 10, 20, or 30 mg/kg/day 2-ME (test groups) for 30 days. VEGF and Bcl-2 expression levels in tumor tissues assessed both immunohistochemically and quantitatively. *P < 0.05 compared to control.

Table 3

The effects of 2-ME on mouse hepatic and renal functions. Means ± SDs are shown; n = 8 mice/group. The groups were treated as follows: 10 mg/kg/day saline; or 10, 20, or 30 mg/kg/day 2-ME. No among-group differences in the ALT, AST, BUN, or Cr levels were apparent (P > 0.05).

<table>
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<th>Group</th>
<th>n</th>
<th>ALT (u/l)</th>
<th>AST (u/l)</th>
<th>BUN (μmol/l)</th>
<th>Cr (μmol/l)</th>
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<tr>
<td>0</td>
<td>8</td>
<td>25.68 ± 2.25</td>
<td>104.46 ± 12.74</td>
<td>4.78 ± 0.53</td>
<td>17.34 ± 3.49</td>
</tr>
<tr>
<td>10 mg/kg 2-ME</td>
<td>8</td>
<td>23.75 ± 2.05</td>
<td>104.96 ± 13.91</td>
<td>5.13 ± 0.51</td>
<td>18.65 ± 2.98</td>
</tr>
<tr>
<td>20 mg/kg 2-ME</td>
<td>8</td>
<td>24.56 ± 3.63</td>
<td>99.72 ± 15.63</td>
<td>4.89 ± 0.62</td>
<td>16.42 ± 3.18</td>
</tr>
<tr>
<td>30 mg/kg 2-ME</td>
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<td>98.34 ± 17.46</td>
<td>5.25 ± 0.77</td>
<td>19.35 ± 3.57</td>
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cell line HepG2, Pharmacology 84 (1) (2009) 9–16.


