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ORIGINAL ARTICLE

2-Methoxyestradiol inhibits hepatocellular carcinoma cell growth by inhibiting Cdc25 and inducing cell cycle arrest and apoptosis

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Abstract
Purpose 2-Methoxyestradiol (2-ME) is a physiological metabolite of estrogen, which can inhibit growth of many types of tumor cells, including hepatocellular carcinoma, both in vitro and in vivo. The exact mechanisms of its action are still unclear. We have studied the mechanisms of growth inhibition of several of human and rat hepatoma and normal liver cells by 2-ME.
Methods Human (Hep3B, HepG2, PLC/PRF5) and rat (McA-RH7777, JM-1) hepatoma and normal rat (CRL-1439) and human (CRL-11233) liver cell lines were cultured in vitro, in presence of 2-ME, and its IC50s were determined. Cell cycle arrest, Cdc25 phosphatase inhibition and apoptosis induction were studied. Finally, the effect of 2-ME on the growth of JM-1 rat hepatoma cells in rat liver was determined in vivo.
Results The IC50 range for growth inhibition of hepatoma cells was found to be between 0.5 and 3 μM. In contrast, normal rat hepatocytes and liver cell lines were resistant to 2-ME up to 20 μM. JM-1 cells were arrested in the G2/M phase of the cell cycle. Cdc25A and Cdc25B, cell cycle controlling phosphatases, activities were inhibited in vitro and 2-ME was found to likely bind to their catalytic site cysteines. As a consequence, their cellular substrates Cdk1 and Cdk2 were tyrosine phosphorylated. Caspase-3 was cleaved suggesting apoptotic cell death. Moreover, growth of JM-1 tumors, which were transplanted into rat liver, was also inhibited by treatment with 2-ME in vivo.
Conclusions 2-Methoxyestradiol is a selective, potent and relatively non-toxic hepatoma growth inhibitor both in vitro and in vivo. Cell cycle arrest of hepatoma cells was likely mediated by binding and inactivation of the Cdc25 phosphatases and induction of apoptosis.

Keywords 2-Methoxyestradiol · HCC cells · Cell cycle arrest · Apoptosis · Cdc25 inhibition

Abbreviations

HCC	Hepatocellular carcinoma
WB	Western blot
EGF	Epidermal growth factor
2-ME	2-Methoxyestradiol

Introduction

Estrogens promote growth, especially of breast and endometrial cancers [1, 2]. Therefore, antagonism of estrogen has been used as a potential therapeutic strategy for these cancers [3]. 2-Methoxyestradiol (2-ME) is a physiological metabolite of the endogenous estrogens estradiol-17β and 17-ethinylestradiol [4]. Serum concentrations of 2-ME range between 10 and 3,768 pg/ml in men and pregnant women, respectively [5]. 2-ME was found to inhibit growth of tumors of breast, lung, pancreas, brain, liver, and prostate both in vitro and in vivo [6–11]. It does not bind to estrogen receptor and hence the growth inhibition is receptor

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independent [12]. It has several different proposed mechanisms of action, which appear to be tumor specific. 2-ME has anti-angiogenic effects [6, 13]. In some tumor cells, 2-ME was found to inhibit tubulin polymerization [14] causing cell cycle arrest in the M-phase. However, cell cycle arrest in the M-phase has also been seen in leukemia cells treated with 2-ME, without tubulin depolymerization [15]. Various types of tumor cells also undergo apoptosis in response to 2-ME [8, 10, 11]. In breast, liver or colorectal cancer cells, JNK is phosphorylated and activated, which was correlated with phosphorylation and activation of Bcl2 [8]. Reactive oxygen species were generated by 2-ME in leukemia cells [16]. In vivo studies of 2-ME in mice, rat and dogs show no or very little toxicity, even at higher doses [6, 17]. Normal cells are unaffected by 2-ME, indicating a selectivity for tumor cells [18].

Previous studies [10] have also shown that 2-ME inhibits growth of several HCC cell lines, likely by induction of apoptosis. In the present report, we studied the inhibitory effects of 2-ME on hepatocellular carcinoma (HCC) cells both in vitro and in vivo, specifically concentrating on its effects on the cell cycle controlling Cdc25 phosphatases.

The cyclin-dependent kinases (Cdk) have important functions in the progression of the eukaryotic cell cycle [19]. One of the major mechanisms of cell cycle progression is the regulation of the activities of Cdk1, Cdk2, and Cdk4 by phosphorylation and dephosphorylation. The Cdc25 phosphatases dephosphorytate tyrosine residues on these Cdks and thereby activate them. Mammalian cells express three Cdc25 proteins, Cdc25A, Cdc25B and Cdc25C. Cdc25A mainly controls G1/S progression, whereas Cdc25B and Cd25C predominantly activate G2/M transition [20, 21]. Mutation analysis has recently revealed that Cdc25A by itself can control both the G1/S and G2/M phases and is sufficient for executing a normal cell cycle [22–25]. Cdc25A and Cdc25B can also behave as oncogenes [26]. Elevated Cdc25A and Cdc25B mRNA and protein levels have been found in many human tumor types [27–29], which make them attractive targets for anticancer therapies.

Materials and methods

Rat hepatocyte isolation, primary culture and DNA synthesis

About 8–12 weeks old rats were used in our experiments. The procedure for isolation of rat hepatocytes, measurement of DNA synthesis, and primary culture conditions were as described below. Male Fisher F344 rats (average body weight 200 g) were purchased from Charles River laboratories (Wilmington, MA). They were caged in an air-conditioned facility with a 12 h illumination cycle and free access to food and water. Hepatocytes were isolated by a two-step collagenase perfusion technique [30]. Isolated primary hepatocytes were suspended in serum-free WE medium (Invitrogen Inc., Carlsbad, CA), at a concentration of 1.75×10^5 viable cells/ml. As much as 3.5×10^5 viable cells were plated per tissue culture dish (35 mm diameter) coated with 1 μg/dish of either bovine plasma fibronectin, which corresponded to a 90% confluence after cell attachment and spreading. Mouse EGF (Roche Diagnostics, Indianapolis, IN) was added to the cultures at a concentration of 10 ng/ml. 2-ME (0, 0.06, 0.13, 1, 2.5, 5 and 20 μM) was added to the hepatocytes 3 h after plating and was labeled with ^{3}H-thymidine (6.7 Ci/m mole, 5 μCi/dish, NEN, Waltham, MA) between 48 and 72 h after plating, at the peak of DNA synthesis. The medium was aspirated and hepatocytes were washed with 5 ml of Hanks' buffered saline solution, containing 1 mM non-radioactive thymidine. About 1 ml of cold 10% trichloroacetic acid (TCA) was added to each dish and placed at 4°C for 1 h. TCA was aspirated and 1 ml of 0.5 N NaOH was added to lyse the cells. An aliquot (0.1 ml) was used for measuring protein concentration, using the Bradford protein assay. Then 0.25 ml of 50% TCA was added to 0.9 ml of the cell lysate and kept at 4°C for 30 min. Samples were then filtered through Whatman GF/C glass filter. Radioactivity (cpm) incorporated into the cold TCA-insoluble fraction retained on the glass filter was measured in a liquid scintillation counter. All results were normalized with protein concentrations.

Culture of cell lines

Human (Hep3B, HepG2, PlC/PRF5) or rat (McA-RH7777, JM-1) hepatoma cell lines (ATCC, Manassas, VA) were grown as monolayer cultures in minimal essential medium supplemented with 10% fetal bovine serum (Invitrogen Inc., Carlsbad, CA) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Normal rat (CRL-1439) and human (CRL-11233) liver cell lines were grown in ATCC-suggested culture medium. Medium was changed after 3 days and cells were trypsinized and subcultured after 6 days. For DNA synthesis inhibition assay, the JM-1 hepatoma cells were plated and cultured under identical conditions to primary hepatocytes in serum-free WE culture medium. Increasing concentrations of 2-ME (0, 0.06, 0.13, 1, 2.5 and 5 μM) were added to the cultures at 3 h after cell plating and DNA synthesis was measured by ^{3}H-thymidine incorporation between 24 and 48 h after cell plating. For cell growth inhibition assays, cells were plated at 2×10^4 cells/well in 24-well dishes (Corning, Inc., Scientific Products Division, Corning, NY). After cell attachment for a day, the medium was replaced with growth medium with or without the compounds. After 3 days of culture, the medium was

removed and the cells were immediately washed with ice-cold phosphate-buffered saline (PBS) to terminate growth. Cells were then harvested and stored at −80°C until used. Cell number was measured by a DNA fluorimetric assay with Hoechst 33258, as previously described [31].

Cell lysate preparation, Western blots and immunoprecipitation

Lysate proteins (40 μg) were separated on a 10% gel by SDS-polyacrylamide gel electrophoresis. The proteins were electro-blotted onto a polyvinylidene difluoride membrane (Amersham, Arlington Heights, IL). The membrane was incubated for 1 h at room temperature in TBST buffer (10 mM Tris–HCl, PH 8.0, 150 mM NaCl, 0.05% Tween-20), containing 1% bovine serum albumin (Fraction V, Sigma, St Louis, MO). The membrane was subsequently incubated for 1 h with antibody diluted in the same buffer. It was then washed four times with TBST (without BSA) and then incubated for another 1 h with horseradish peroxidase conjugated secondary antibody (Amersham, Arlington Heights, IL), which was diluted in TBST–BSA. The membrane was again washed four times with TBST. Enhanced chemiluminescence reagent (NEN Life Science, Boston, MA) was used for detection.

Western blot and immunoprecipitation protocols were followed as described previously [32, 33]. Briefly, cells were plated at 5×10^4 cells/well density in 6-well tissue culture plates. After 24 h, the culture medium was replaced by control medium or medium containing 2-ME at various concentrations and incubated for different times. They were then rinsed with phosphate-buffered saline and lysed in 100 μl of RIPA buffer (150 mM NaCl, 50 mM Tris–HCl pH 8.0, 0.1% SDS, 1% Triton X-100, 1 mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin). The lysates were cleared by centrifugation at 12,000 rpm for 5 min in a micro-centrifuge. Protein concentration of the lysates was determined by Bio-Rad assay (Bio-Rad, Hercules, CA).

For immunoprecipitation, 200 micrograms of cell lysate was incubated with antibody and protein A-agarose (Sigma, St Louis, MO) overnight at 4°C. Then the immunoprecipitate was washed three times with RIPA buffer. The washed precipitate was suspended in SDS-sample buffer and analyzed by Western blotting.

For the apoptosis assay, JM-1 cells were treated with increasing concentrations (0, 0.06, 0.12, 0.25 and 0.5 μM) of 2-ME for 24 h. Cell lysates were prepared and was analyzed on Western blots, which were probed with anti-caspase-3F (Cell Signaling, Waltham, MA) and β-actin control (Sigma, St Louis, MO) antibodies.

The antibodies used in these experiments (Cdk2, Cdk4, MKP1, Cdc25A antibodies from Santa Cruz Biotechnology, Santa Cruz, CA; anti-phospho-Cdk1 from Cell Signaling, Waltham, MA; anti-biotin and β-actin from Sigma, St Louis, MO) were commercially obtained.

Cell cycle analysis

Exponentially growing JM-1 tumor cells were treated with 0.5 or 1 μM of 2-ME for 24 h. Cells were harvested and stained with 50 μg/ml propidium iodide and 250 μg/ml RNase (Sigma Chemicals co. St Louis, MO) for 30 min. Fluorescence activated cell sorting (FACS) analysis was done with a Becton Dickinson FACS Star (Franklin Lakes, NJ). Cell numbers in each phase of cell cycle was calculated by the WinMDI software. Average fractions of cells in the G1 phase were calculated from three measurements.

PTP activity assay

PTP activity of liver protein lysates was measured by using the substrate 3-O-methyl fluorescein phosphate (OMFP) (Molecular Probes, Eugene, Oregon), as previously described [34]. The Cdc25A, Cdc25B2, Cdc25C, were a gift from Dr. J. Rudoph (Duke University, N. Carolina) and MKP1 phosphatase was obtained commercially (Upstate, Lake Placid, NY). 2-ME was solubilized in DMSO, and all reactions including controls were performed in 1% DMSO. The final incubation mixture (150 μl) was optimized for enzyme activity and consisted of 30 mM Tris (pH 8.5), 75 mM NaCl, I mM EDTA, 0.33% BSA and 1 mM DTT. Reaction was initiated by adding enzyme. Fluorescence emission from the product was measured over a 10–60 min time period at room temperature in a multi well plate reader. The reaction was linear over the time period of the experiment and was directly proportional to both enzyme and substrate concentration. Half-maximal inhibition constant was calculated by curve fit by Cricket Graph III program.

Competition of Cpd 5-Bt and 2-ME binding to Cdc25A or Cdc25B

Cdc25A or Cdc25B (180 ng) was incubated in a 10 μl reaction volume for 18 h at 4°C, with 1 μM Cpd 5-Bt (a biotin-tagged Cpd 5 derivative) and 2-ME at a concentration of 0, 10 and 100 μM or MKP1 protein (10 μM). Cpd 5-Bt bound to Cdc25A/B was immuoprecipitated with anti-Biotin antibody (Sigma, St Louis, MO) and the immunoprecipitated proteins were probed on Western blots with anti-Cdc25A or anti-Cdc25B antibodies.

In vivo treatment of transplantable liver tumors

Fischer (F344 male) rat HCC-derived JM-1 cell line [35] was grown in MEM + 10%FBS culture medium up to

semi-confluency. Cells were harvested by trypsinization (0.2%) for 5 min at 37°C, washed with ice-cold PBS, centrifuged (500*g*, 10 min), and resuspended in ice-cold PBS at a concentration of 10^6 cells/ml. A quantity of 1 ml of the cell suspension containing 10^6 cells was injected into a Fischer rat liver through its mesenteric vein. Tumor cell transplanted rat were divided into two groups (three rats per group). One group was treated intraperitoneally with 10 mg/kg body weight of 2-ME dissolved in DMSO, every other day, for a total of five injections. Treatment was started the day after cell transplantation. The other group was treated similarly with the solvent DMSO as control. Animals were killed 2 weeks after cell transplantation and the numbers of tumor foci visible were counter per liver. The diameter of the tumors was also measured by a slide calipers.

Statistical analysis

Data were analyzed statistically by *t* test and significance was determined by calculating the *p* value by Microsoft Excel program.

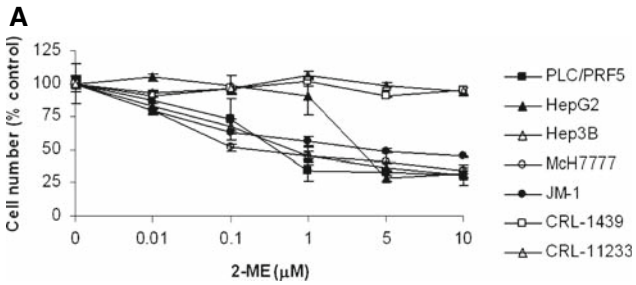
Results

Growth inhibition of HCC cell lines in vitro

2- Methoxyestradiol has been reported previously to inhibit growth of several HCC cell lines in vitro [10]. We first tested its effect on the HCC cells in our collection and culture conditions. The human Hep3B, HepG2, PLC/PRF5 HCC cell lines and the rat JM-1 and McA-RH7777 HCC lines were cultured in MEM medium containing 10% FBS. Increasing concentrations of 2-ME (0, 0.01, 0.1, 1, 5 and 10 μM) was added after cell plating. The number of cells remaining on the culture dish after 3 days was determined. IC50 values were calculated from the growth inhibition graph. 2-ME was found to be a potent growth inhibitor of all the HCC cell lines tested in this experiment and its IC50 values ranged from 0.5 to 3 μM for the various cell lines (Fig. 1). On the contrary, the normal rat (CRL-1439) and human (CRL-11233) liver cell lines were relatively resistant (IC50 > 10 μM) to 2-ME.

Specificity of growth inhibition

The growth-inhibitory effect of 2-ME on HCC cells was previously reported to be selective for the tumor cells and not normal human hepatocytes [18]. Therefore, in order to determine specificity of inhibition, we compared its growth inhibitory effects on our JM-1 rat HCC cells and on freshly isolated normal rat hepatocytes. Normal adult rat hepatocytes



B

	IC50 (μM)
PLC/PRF5	0.5
Hep3B	1
McH7777	1
JM-1	1
HepG2	3
CRL-1439	>10
CRL-11233	>10

Fig. 1 Growth inhibition of human hepatoma, rat hepatoma, normal rat liver and human liver cells. Human hepatoma (PLC/PRF5, HepG2, Hep3B), rat hepatoma (McA-RH7777, JM-1), human liver (CRL-11233) and rat (CRL-1439) liver cell lines were treated with increasing concentrations of 2-ME (0, 0.01, 0.1, 1, 5 and 10 μM). **a** Cell numbers remaining on the dish was determined and expressed as a percentage of untreated control. **b** IC50 of 2-ME for the cell lines were calculated from the growth inhibition results

do not proliferate in culture. However, they undergo DNA synthesis in response to a mitogen, like EGF. We compared EGF-induced DNA synthesis in isolated rat hepatocytes and the rat HCC cell line JM-1 cells under identical culture and assay conditions. Increasing concentrations of 2-ME (0, 0.06, 0.13, 1, 2.5, 5 and 20 μM) was added at the start of culture to the isolated rat hepatocytes or JM-1 rat HCC cells, each plated on fibronectin matrix and cultured on WE medium without serum but containing EGF. DNA synthesis was measured by incorporation of ^{3}H-thymidine between 48 and 72 h after cell plating. A gradual decrease in DNA synthesis was found in the JM-1 cells with increasing concentrations of 2-ME. The IC50 was about 125 nM. However, the adult rat hepatocytes were resistant to 2-ME-mediated DNA synthesis inhibition up to the highest tested concentration of 20 μM (Fig. 2).

G2/M phase cell cycle arrest

No cell cycle specific arrest was evident in previous studies of 2-ME on the HCC cells SK-Hep1, Hep3B or PLC/PRF5. An increase in the sub-G1 cell population, which is characteristic of apoptosis, was evident [18]. In other studies, 2-ME was found to arrest cells in mitosis with or without tubulin de-polymerization [15, 36]. We studied the effect of 2-ME on the cell cycle of the JM-1 rat hepatoma cells in culture. JM-1 cells growing in exponential phase in MEM

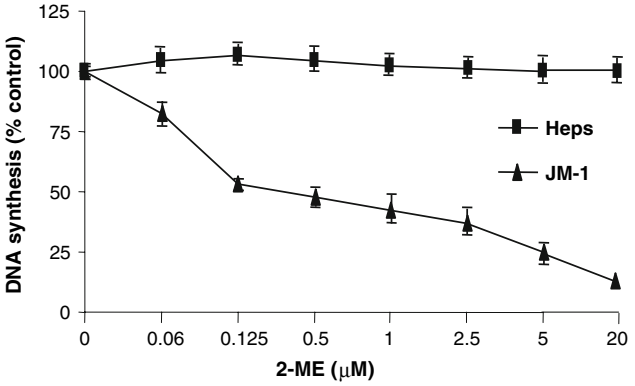


Fig. 2 Differential inhibition of rat hepatocytes and hepatoma cells. Rat hepatocytes and hepatoma cells (JM-1) were cultured in serum-free medium in presence of EGF (10 ng/ml). Cells were treated with increasing concentrations (0, 0.0625, 0.125, 0.5, 1, 2.5, 5, and 20 μM) of 2-ME, and DNA synthesis was measured by ^{3}H-thymidine incorporation into the cell DNA

medium containing FBS were treated with 0.5 or 1 μM of 2-ME for 24 h. The cell DNA was stained with propidium iodide and the relative cell populations in various phases of the cell cycle was determined by FACS analysis. 2-ME was found to preferentially arrest the JM-1 cells in the G2/M phase of the cell cycle (Fig. 3). The population of cells in the G1 phase decreased and that in the G2/M phase increased with increasing 2-ME concentration.

Inhibition of cell-free Cdc25 phosphatases

Since 2-ME blocks cell cycle of JM-1 cells, we studied its effects on the cell cycle controlling Cdc25 phosphatases. We initially determined the enzymatic activities of cell-free Cdc25A, Cdc25B and Cdc25C phosphatases, either in presence or in absence of 2-ME. Increasing concentrations of 2-ME were added to the Cdc25 enzymes and incubated at room temperature for 1 h before adding the phosphatase substrate OMFP. The fluorescent OMF generated by the enzymatic activities was assayed every 5 min. The phosphatase activities of Cdc25A and Cdc25B were found to be inhibited by 2-ME with an IC50 of about 1 μM. However, Cdc25C was poorly inhibited with an IC50 of 10 μM (Fig. 4).

Binding of 2-ME to Cdc25A and Cdc25B

We previously found that PD 49, a biotin-tagged derivative of Cpd 5, likely bound to the catalytic cysteine of Cdc25B [37]. PD 49 had the thioethanol side-chain of Cpd 5 substituted by Br. For the current study, we have used a biotin-tagged Cpd 5 derivative (Cpd 5-Bt), which had the thioethanol side-chain [38]. We assume that Cpd 5-Bt will identically bind to the catalytic cysteine as PD 49. We explored whether both Cpd

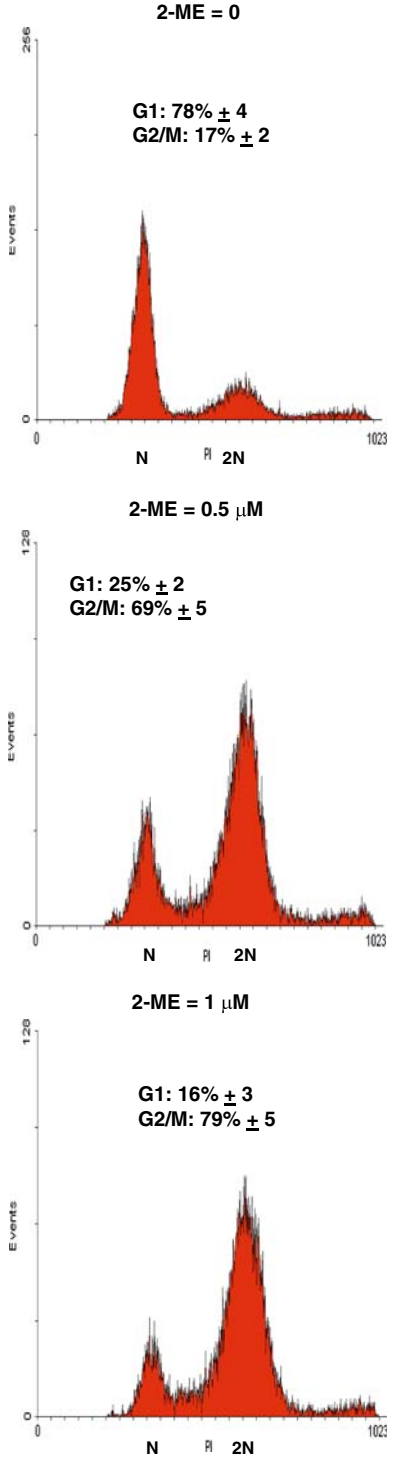


Fig. 3 Inhibition of cell cycle by 2-ME. JM-1 cells were treated with 0, 0.5 or 1 μM of 2-ME for 24 h. The cell DNA was labeled with propidium iodide and was analyzed by FACS. The fraction of cells in different phases of the cell cycle was determined from the graphs by the WinMDI software

5-Bt and 2-ME might bind to the catalytic cysteine in Cdc25A or Cdc25B. Cpd 5-Bt was incubated with Cdc25A or Cdc25B in the presence of increasing concentrations of

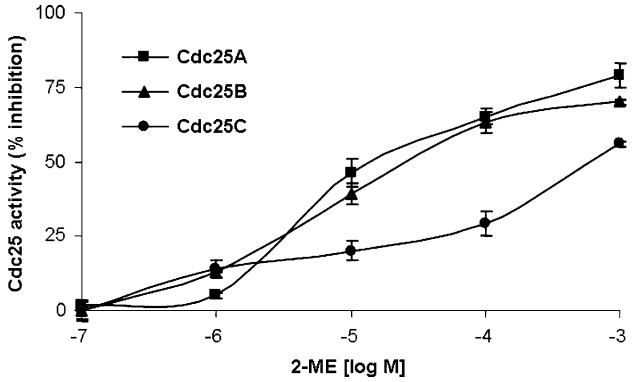


Fig. 4 Inhibition of Cdc25A, Cdc25B and Cdc25C phosphatase activities. Phosphatase activity of the Cdc25 phosphatases was determined by a dephosphorylation assay using OMFP as a substrate. The enzymes were incubated with increasing concentrations (0, 0.01, 0.1, 1, 10, 100 μM) of 2-ME for 1 h at 37°C. Substrate was added and OMF produced was assayed every 5 min. Activity was determined and expressed as % inhibition of control without 2-ME

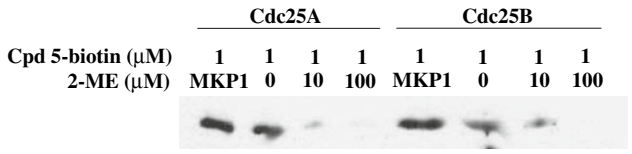


Fig. 5 2-ME and Cpd 5-Bt compete for binding to Cdc2A or Cdc25B. Cpd 5-Bt (1 μM) was incubated at 4°C for 18 h, with 180 ng of either Cdc25A or Cdc25B, in the presence of 10 μM MKP1 (*lane 1*) or increasing concentrations (0, 10 and 100 μM) of 2-ME (*lanes 2–4*). Enzyme bound Cpd 5-Bt was immunoprecipitated with anti-biotin antibody. The immunoprecipitated proteins were run on Western blots, and the blots were probed with either Cdc25A or Cdc25B antibody

2-ME. The Cpd 5-Bt-Cdc25 complex was immunoprecipitated with anti-biotin antibody and Cdc25 protein in the immunoprecipitate was visualized on Western blots, using anti-Cdc25 antibody. Increasing concentrations of 2-ME were found to decrease the binding of Cpd 5-Bt to both Cdc25A and Cdc25B (Fig. 5). A tenfold excess of 2-ME almost completely inhibited Cpd 5-biotin binding to Cdc25A. However, appreciable binding to Cdc25B was still found at the same ratio of 2-ME and Cpd 5-biotin. A 100-fold excess of 2-ME was necessary in that case. This suggested a competitive binding by 2-ME likely to the catalytic cysteine of either Cdc25A or Cdc25B. MKP1, another dual specificity phosphatase, even at 10 μM, did not compete with Cpd 5-Bt for binding to Cdc25A or Cdc25B (Fig. 5, lanes 1 and 5).

Cellular markers for Cdc25 inhibition

Growth-inhibitory tyrosine phosphorylation of the cell cycle kinases Cdk1, Cdk2 and Cdk4 are known to be

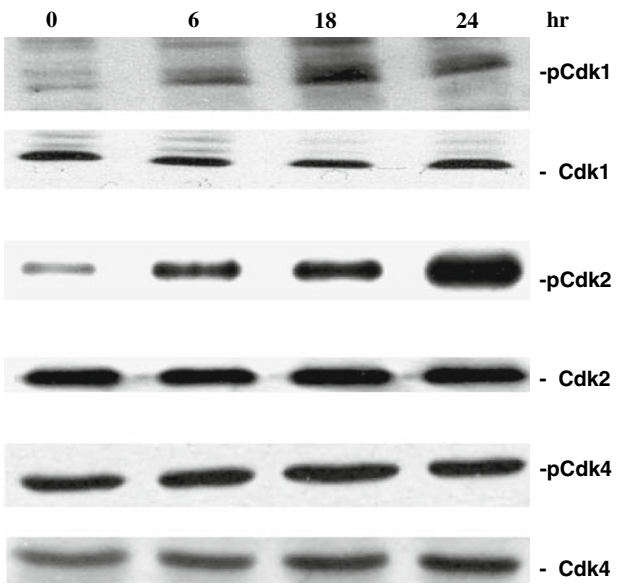


Fig. 6 2-ME induced tyrosine phosphorylation of Cdk 1, Cdk2 but not Cdk4 in JM-1 cells. JM-1 cells were treated with 1 μM of 2-ME for 6, 18 or 24 h. Treated or untreated lysates were immunoprecipitated using, either Cdk2 or Cdk4 antibodies. The immunoprecipitates were run on Western blots, which were probed with anti-phospho-tyrosine antibody. Tyrosine phosphorylated Cdk1 was assayed on Western blots with anti-pCdk1 antibody. Unphosphorylated Cdk1, Cdk2 and Cdk4 were probed as controls

induced in cells as a consequence of inhibition of the Cdc25 phosphatase activities [19]. Therefore, we treated JM-1 hepatoma cells with 2-ME (1 μM) for 6, 18 and 24 h and assayed for tyrosine phosphorylation of Cdk1, 2 and 4 after either first immunoprecipitating the Cdk2 or Cdk4 with their corresponding antibody, and then analyzing them on Western blots, which were probed with phosphotyrosine antibody, or probing Western blots directly with tyrosine-specific pCdk1 (Y15). We found that the tyrosine phosphorylation of Cdk1 and Cdk2 but not Cdk4 were induced by 2-ME treatment (Fig. 6), suggesting that 2-ME inhibited the Cdc25 phosphatases in the JM-1 cells.

Induction of apoptosis

2-Methoxyestradiol has been previously found to activate caspase and induce apoptosis in several hepatocellular carcinoma cells [10, 18]. Therefore, we studied apoptosis induction in JM-1 hepatoma cells by assaying caspase-3 cleavage. Cells were treated with increasing concentrations (0, 0.0625, 0.125, 0.25 and 0.5 μM) of 2-ME and cell lysates were run on Western blots, which were probed with caspase-3 fragment (caspase 3-F) antibody. Caspase-3F was found to increase dose dependently (Fig. 7), suggesting induction of apoptosis in the 2-ME-treated JM-1 cells.

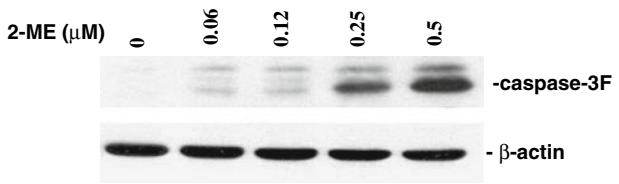


Fig. 7 Induction of apoptosis. JM-1 cells were treated with increasing concentrations (0, 0.06, 0.12, 0.25, 0.5 μM) of 2-ME for 24 h. The cell lysates were analyzed on Western blots, which were probed with anti-caspase-3 fragment (caspase-3F) and β-actin control antibodies

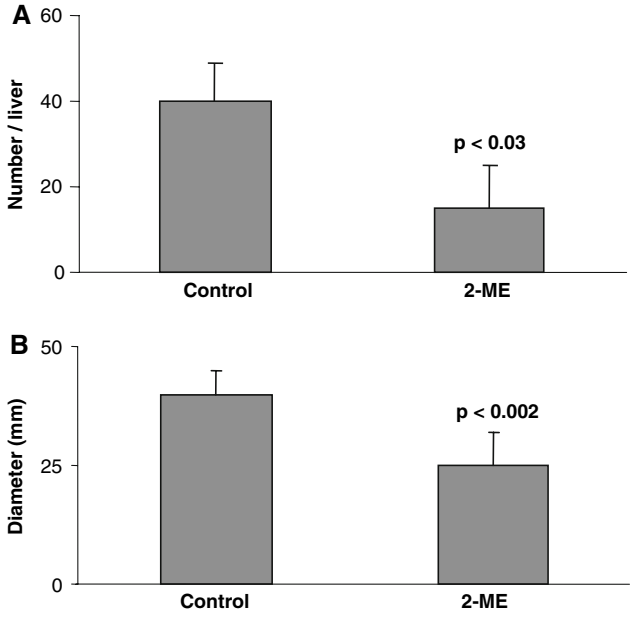


Fig. 8 Inhibition of transplantable rat liver tumors. One million JM-1 cells grown in culture, washed and suspended in PBS were transplanted into Fischer rat (six rats) livers by injecting (10 mg/Kg body weight) through the mesenteric vein. Two days after cell transplantation, rats were injected every other day (total of five injections) intraperitoneally either with 2-ME (three rats), which was dissolved in DMSO, or DMSO (solvent control) (three rats). Rats were killed 2 weeks after cell transplantation and the number of tumors per liver and average tumor diameter was counted

Treatment of transplanted JM-1 rat liver tumors

Since the growth of the JM-1 rat HCC cell line was inhibited efficiently in culture, we assayed JM-1 cell growth in rat liver in vivo. JM-1 cells were transplanted into rat liver by injecting them through their mesenteric vein. Intraperitoneal treatment (10 mg/kg body weight, every other day) of the rats with 2-ME was started 2 days after cell transplantation. The dose and treatment regiment was chosen to be similar to the one used by us for other Cdc25 inhibitors [47]. A total of five treatments were given and the animals were killed after 2 weeks. A control group was similarly treated with a solvent control. The number of tumor foci per rat liver and their average diameters was measured in the 2-ME-treated rats and compared to the solvent-treated controls. The solvent- and 2-ME-treated rats had an average of 40 and 15 tumor foci per liver, with an average tumor diameter of 40 and 25 mm, respectively Both the average number of tumors per liver and the tumor diameters were found to be decreased in the treated rats and the differences were statistically significant with a p value less than 0.03 and 0.002, respectively (Fig. 8).

Discussion

2-Methoxyestradiol has been shown to inhibit growth of various tumor cells, including HCC, both in vitro and in vivo [6–11]. Different mechanisms of action have been found for various tumor types. These include p53 dependent or independent apoptosis [16, 39], anti-angiogenesis [13], and inhibition of tubulin polymerization [14, 36]. We have found that 2-ME efficiently inhibited growth (IC_{50} = 0.5–3 μM) of three human (Hep3B, HepG2 and PLC/PRF5) and two rat (McA-RH7777 and JM-1) hepatoma cell lines (Fig. 1) in vitro. The p53 gene in these hepatoma cell lines were either normal (HepG2), deleted (Hep3B), or mutated (PLC/PRF5). A sixfold difference in sensitivity was found between Hep3B (deleted p53) and HepG2 (normal p53), perhaps suggesting a role of p53 in sensitivity. However, these were not isogenic cell lines and therefore, the differences in sensitivity may be due to other genetic factors.

An anti-cancer drug should ideally selectively kill the tumor and spare normal tissue. However, they are generally also toxic to normal cells and this limits their useful dose range. The appropriate control for JM-1 rat hepatocellular carcinoma cells would be rat hepatocytes. However, rat hepatocytes do not grow in culture and hence cannot be compared with the tumor cells by an in vitro growth assay. Mitogens, like EGF, are able to induce DNA synthesis in both rat hepatocytes and JM-1 tumor cells in culture. Therefore, we used the EGF-mediated DNA synthesis assay to compare normal adult rat hepatocytes with identically cultured JM-1 hepatoma cells. By this assay, we found the IC_{50} of 2-ME in JM-1 cells to be 125 nM, compared to >20 μM for the normal hepatocytes. No appreciable inhibition of DNA synthesis was found in normal hepatocytes, even at the highest 2-ME concentration tested (20 μM) (Fig. 2). Therefore, normal hepatocytes were at least 160-fold more resistant to 2-ME than the JM-1 hepatoma cells. Moreover, the normal rat (CRL1426) and human (CRL11233) liver cell lines were also resistant to 2-ME mediated growth inhibition (Fig. 1). These results suggest that 2-ME mediated growth inhibition is probably specific for tumor cells. 2-ME has also been found by others to be non-toxic to normal cells [18]. Hence, it is currently being evaluated in clinical trials as an anti-cancer drug

[40, 41]. Up to 6 mg/day has been used in a Phase I human study without reaching maximum tolerated dose [41]. However, its plasma concentrations are low, suggesting poor bioavailability [42]. Efforts are on going to improve that by re-formulation and structural modifications [43].

2-Methoxyestradiol was previously found to induce cell cycle arrest, which was dependent on cell type. For example, no cell cycle arrest was evident in several hepatoma cell lines [18]. However, it caused G2 phase arrest of other cells with or without tubulin de-polymerization [15, 36]. We used the rat hepatoma JM-1 cells to study cell cycle effects mediated by 2-ME, since they can be studied both in vitro and in vivo. A bi-phasic cell cycle arrest in both G1 and G2 phase was found in this cell line (Fig. 3). This would suggest that kinases and/or phosphatases regulating the cell cycle might be possible candidate targets for being affected by 2-ME. When we examined Cdc25 phosphatases (Fig. 4) and their substrate Cdks (Fig. 6), we found that they were indeed inhibited by 2-ME action. 2-ME is known to affect several cellular proteins. However, this was the first time that it was found to inhibit Cdc25 phosphatases. In cell free enzyme assays, Cdc25A and Cdc25B (IC_{50} = 1 μM) isoforms were found to be 10-fold more sensitive than Cdc25C (IC_{50} = 10 μM). It was interesting to note that the IC50 observed with the cell free Cdc25 enzymes was similar to that of the HCC cell lines. Although the enzyme-active site amino acid sequence and structure of different Cdc25 isoforms are very similar, the amino terminal regions probably modulate binding to substrates [44]. Hence, the observed difference in binding of 2-ME to the three Cdc25 isoforms may likely be due to this reason.

We have previously found that a biotin labeled Cpd 5 with a Br side chain likely binds to the catalytic cysteine of Cdc25B [37]. The catalytic site structure of the three Cdc25 isozymes are very similar and only minor difference in IC50 of Cdc25A and Cdc25B were found in our previous studies with Cpd 5 and its derivatives. Therefore, we considered Cdc25A inhibition by PD49 to be similar to that of Cdc25B. In a later study, we found that a biotin labeled Cpd 5 with a thioethanol side chain (Cpd 5-Bt) also likely binds to catalytic cysteine of Cdc25B [38]. The IC50 of 2-ME for the cell-free Cdc25 enzymes were found to be about 1 μM for Cdc25A/B and 10 μM for Cdc25C. These approximately agree with the IC50s observed for the hepatoma cell lines (Fig. 1). However, the binding affinity of 2-ME to Cdc25A and Cdc25B was found to be quite different (Fig. 5). The binding of biotin-Cpd 5 to Cdc25A could be competed efficiently with 10 μM of 2-ME (a 10-fold excess concentration). Comparatively, biotin-Cpd 5 binding to Cdc25B could only be competed with 100 μM of 2-ME (a 100-fold excess). Thus, binding affinity of 2-ME to Cdc25B is probably 10-fold stronger than to Cdc25A.This may reflect influence of structural differences between the two iso-enzymes on the binding affinity.

The Cdc25 phosphatases usually dephosphorylate phospho-tyrosines of the cell cycle controlling kinases Cdk1, 2 and 4 within the cell and thereby activate them. Inhibition of Cdc25 phosphatases by 2-ME was therefore, expected to induce tyrosine phosphorylation of Cdks. We found that Cdk1 and Cdk2 but not Cdk4 were tyrosine phosphorylated, when JM-1 cells were treated with 2-ME in vitro (Fig. 6). The reason why phospho-Cdk4 induction was not induced by 2-ME was not clear. It might possibly be due to more efficient Cdc25A-mediated dephosphorylation of Cdk4 than Cdk2.

Use of TUNNEL and annexin V immunocytochemical assays, led to the conclusion in an earlier study that 2-ME induced apoptosis in hepatoma cells [18]. In our study we found support for this with a biochemical assay for apoptosis. Caspase-3 is a critical mediator of apoptosis, which is responsible for the proteolytic cleavage of many key proteins such as the nuclear enzyme poly (ADP-ribose) polymerase (PARP) [45]. Activation of caspase 3 requires proteolytic processing of its inactive zymogen into activated p17 and p12 fragments [46]. In our experiments, caspase 3 was found to be efficiently cleaved in 2-ME-treated JM-1 cells (Fig. 7), which would possibly lead to apoptosis.

We have previously established a model for growing hepatocellular carcinoma cells (JM-1) in Fischer rat liver [47]. The rats, which contained growing JM-1 cells, were treated either with a 10 mg/kg body weight dose of 2-ME or solvent control, every other day for a total of five treatments. They were subsequently killed 2 weeks after cell transplantation and the number of liver tumor foci and their diameters were measured and compared to the solvent control. The solvent- and 2-ME-treated rats had an average of 40 and 15 tumor foci per liver respectively, and a tumor diameter of 40 and 25 mm, respectively. Both of these differences were statistically significant (Fig. 8).

2-Methoxyestradiol has previously been reported to have low toxicity. The experiments reported here show that it has selective growth inhibitory actions against hepatoma cells in vitro compared to normal hepatocytes and liver cell lines, and also inhibits growth of a transplantable hepatoma in vivo. It induces apoptosis in vitro and antagonizes the action of the cell cycle controlling Cdc25 family of dual specificity protein phosphatases. This represents a new approach to HCC treatments and holds promise for in vivo testing if it is found to be safe in people.

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