Abstract. Objective: Most chemotherapeutic regimens for pancreatic cancer (PC) use combination therapy. 2-Methoxyestradiol (2-ME2) is a natural estrogen metabolite with proven tumor-inhibiting effect as a single agent. The aim of this study was to determine whether a combination of 2-ME2 with other established chemotherapeutic compounds increases its tumor-inhibiting effect on human PC cells. Materials and Methods: The human PC cell lines AsPC-1 and MiaPaCa-2 were treated with 2-ME2 alone or in combination with different doses of gemcitabine, cisplatin, cetuximab, 5-fluorouracil and paclitaxel in vitro (range: 0.5-5 μM). FACS analysis and nuclear staining were used to reveal apoptotic cells and cell-cycle changes after treatment. Subsequent in vivo experiments were performed on a subcutaneous tumor model in nude mice using AsPC-1 cells. Results: A tumor-reductive effect of 2-ME2 was found in both human PC cell lines. The combination of 2-ME2 with other agents resulted in additive growth inhibition of both cell lines through the induction of apoptosis and cell-cycle arrest. The growth inhibition was confirmed in vivo. After 32 days’ treatment, gemcitabine alone showed no effect on tumor growth at a dose of 75 mg/kg body-weight. However, 2-ME2 at a daily dose of 2 mg per animal led to a growth inhibition of 63% with no evident toxicity. The combination of 2-ME2 and gemcitabine caused a growth-inhibition of 83%. Major toxicity was observed in the combination group, with six deaths out of eight animals in this group. Conclusion: 2-ME2 can be successfully combined with other chemotherapeutic agents. However, toxicity in the in vivo experiment is strong and requires further investigation.

The prognosis of patients with pancreatic cancer remains poor with a five-year survival of all patients of only 0.2%, and new therapeutic regimens may improve survival in combination with surgical resection (1). However, there is still a need to improve the current conservative treatment for pancreatic cancer. While the postoperative application of gemcitabine appeared to significantly delay the development of recurrent disease after complete resection of pancreatic cancer compared with observation alone (1), most chemotherapeutic regimens use combinations of different compounds to reduce side-effects and to enhance efficacy. A positive effect on toxicity and efficacy was observed after treatment of advanced pancreatic cancer with a triple combination of irinotecan, gemcitabine and 5-fluorouracil (2).

A compound which is currently under clinical investigation for a variety of different cancer types is the physiological estrogen metabolite 2-methoxyestradiol (2-ME2). Physiologically, serum levels of 2-ME2 range from 10 pg/ml in young men to 3768 pg/ml in pregnant women (3, 4). 2-ME2 is a competitive inhibitor and a substrate of cytoplasmic catechol-O-methyl-transferase, which is found in blood vessel endothelia, fibroblasts and particularly in tumour cells (5), and is also an inhibitor of tyrosine hydroxylase, with very low affinity to the oestrogen receptor (6, 7). Extended in vitro and in vivo experiments have shown that 2-ME2 inhibits the growth of a large variety of cancer entities (8, 9). In human pancreatic cancer cells, strong induction of apoptosis was observed after treatment with 2-ME2, which was confirmed in vivo in a lung metastasis model in nude mice (10). Furthermore, 2-ME2 was able to inhibit tumour growth of multi-chemoresistant human pancreatic and gastric cancer cells (11).

This study performed in vitro and in vivo experiments of combined treatment modalities with 2-ME2 and different chemotherapeutic compounds on human pancreatic cancer cells.
Materials and Methods

Cell lines and proliferation assays. 2-ME2, obtained from EntreMed Inc. (Rockville, MD, USA), was dissolved in absolute ethanol to give a 20 nM solution which was subsequently diluted with saline to obtain a stock solution of 660 μM. The solution was aliquoted and stored at −20°C. 16-Epiestriol (16-E) was obtained from Sigma (Deisenhofen, Germany) and dissolved, diluted, and stored as 2-ME2. For each experiment, a new aliquot was thawed and used.

The human pancreatic cancer cell lines AsPC-1 and MiaPaCa-2 were used for all experiments. They were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). AsPC-1 cells were grown in modified RPMI-1640 medium. MiaPaCa-2 cells were grown in a modified DMEM. Both media were supplemented with 10% foetal bovine serum, 1% glutamine, 1% non-essential amino acids, 1% sodium pyruvate, antibiotics, and antimycotics. They were grown in an incubator at 37°C with 5% CO2. Culture medium was refreshed twice per week and a 1:5 subculture split was performed once per week. Cells were seeded from a subconfluent monolayer in 24-well plates. After 48 h, cells were incubated with 2-ME2 and combinations with gemcitabine, cisplatin, cetuximab, 5-fluorouracil, and paclitaxel at doses ranging from 0.1 to 5 μM.

Immunofluorescence. Nuclear staining and fluorescence microscopy was used to visualise apoptotic nuclei. The procedure was performed according to the accompanying protocol. Briefly, cells were seeded on coverslips in 50 mm plates at quantity of 104 cells per plate. The experiments were performed in triplicate. Cells were incubated for 48 h before treatment. The doses of 2-ME2 or 16-E were chosen according to the IC50 in the proliferation analysis. Therefore, 0.7 μM of 2-ME2 or 16-E were used for the AsPC-1 cell line, and 0.6 μM 2-ME2 or 16-E for the MiaPaCa-2 cell line. Three days after incubation with 2-ME2 or 16-E, the cells were fixed in acetic acid and absolute methanol then air dried. Hoechst staining solution (Hoechst-No. 33258) was added and the samples were stored at −20°C until use. Cell counts were performed using a fluorescence microscope. At least 300 cells were counted in randomly determined high power fields. The average number of apoptotic cells was calculated.

FACS analysis. The cells were grown in 6-well plates and treated as described in the section “Cell lines and proliferation assays”. The experiments were performed in duplicate. After three days of incubation, cells were trypsinized, washed in PBS, fixed in 70% ice-cold ethanol for 60 min and stored at 4°C until used. The procedure for FACS analyses was performed according to a protocol previously described (1, 2). Briefly, fixed cells were incubated with 1 mg/ml of RNase (Sigma Chemical Co., Deideshofen Germany) for 15 min at room temperature. Thereafter, 0.5 ml propidium iodide (PI, Sigma; 100 μg/ml PBS) was added and cells left for 15 min at room temperature in the dark. Cells were washed once in PBS and kept at 4°C in the dark until measurement. A total of 20,000 cells were analysed using a FACS scan flow cytometer (Becton-Dickinson).

In vivo experiments. For the in vivo experiments, 32 female athymic nude mice (Charles River, Hannover, Germany) were used. After intraperitoneal anesthesia, the animals underwent a laparotomy and 2x106 AsPC-1 cells/animal were injected into the pancreatic tail. Three days after surgery, the animals were randomized into four groups. The groups were: control group, 2-ME2 monotherapy, gemcitabine monotherapy, and combination of 2-ME2 plus gemcitabine. 2-ME2 was administered orally at a dose of 2 mg daily/mouse, whereas gemcitabine was applied twice a week intraperitoneally at a dose of 75 mg/kg body weight.

The animals were killed 32 days after cell injection using an overdose of anaesthesia (Halothane 99%, Cat-Nr. B-4388 of Sigma) and cervical dislocation. The tumours were removed carefully from the pancreas and were measured. The spherical volume was calculated with the formula $\frac{4}{3} \pi r^3$, where $r$ is the radius of the tumour.

Statistics. The statistical evaluation of the proliferation analyses was carried out using the H-test of Kruskal and Wallis. The Wilcoxon test for independent random samples, based on the ranking test of Mann and Whitney, was applied for further testing to restrict significance. The conditions for the test were always fulfilled, e.g. the continuous distribution function and two independent coincidental random samples of measured values from the fundamental totality with a similar or identical distribution form.

Student’s t-test was used to statistically evaluate the animal experiments.

Results

Figure 1 shows the results of the proliferation analyses after incubation of cells with 2-ME2 alone and in combination with gemcitabine, cisplatin, paclitaxel, cetuximab, and 5-fluorouracil. A strong dose-dependent growth inhibition in both cell lines was observed. A dose of 5 μM reduced the growth rate by 65.1% in AsPC-1 cells. MiaPaCa-2 cells were even more sensitive with growth inhibition of 94.3% at the same dose. After incubation with a low dose of 1.0 μM 2-ME2, the growth rate was reduced by 66.7% after five days. The control, oestrogen 16-E, showed no effect on tumour growth at the same doses. For the combination treatments with other chemotherapeutic agents a dose of 0.5 μM 2-ME2 was chosen, which resulted in growth inhibition of 11.8% in AsPC-1 cells and 29.8% in MiaPaCa-2 cells after monotherapy. In the combination treatments of 2-ME2 and the other compounds, additive growth inhibition was found to different extents in all cases. The most effective growth inhibition was observed when 2-ME2 was combined with paclitaxel, 5-fluorouracil and cisplatin. The combination of 2-ME2 and gemcitabine as well as cetuximab appeared to be less effective.

FACS analyses were performed to reveal a potential additional induction of apoptosis when 2-ME2 was combined with other agents. The number of apoptotic cells is listed in Table I. Induction of apoptosis was observed after treatment with 2-ME2 alone. Further induction of apoptosis was observed after combination with 5-fluorouracil, paclitaxel, and gemcitabine. Cetuximab was not tested because of the weak growth inhibition in the proliferation analysis. Addition of cisplatin to 2-ME2 resulted in further induction of apoptosis in MIAPaCa-2 cells. No such effect was observed in AsPC-1 cells.
Figure 1. Proliferation analyses of the cell lines AsPC-1 (A-F) and MiaPaCa-2 (G-L) after treatment with the control estrogen 16-epiestriol or 2-ME2 alone at 0.5 μM or in combination with chemotherapeutic compounds. 2-ME2 was given at a dose of 0.5 μM for all combinations. A, G: 2-ME2 and 16-epiestriol alone. Numbers without letters indicate the concentrations of 2-ME2 alone; B, H: 2-ME2 alone and combined with gemcitabine; C, I: 2-ME2 alone and combined with cisplatin; D, J: 2-ME2 alone and combined with paclitaxel; E, K: 2-ME2 alone and combined with cetuximab; F, L: 2-ME2 alone and combined with 5-FU. C: Control non-treated cells; E: 16-epiestriol with different concentrations (E1=1.0 μM; E2=1.5 μM; E3=5.0 μM); M: 2-methoxyestradiol 0.5 μM; G: gemcitabine 3.0 ng/ml; Cis: cisplatin 10 ng/ml; Pac: paclitaxel 0.1 pg/ml; Cet: cetuximab 20 nM/ml; FU: 5-fluorouracil 20 ng/ml.
To confirm the data obtained from the FACS analysis, immunohistochemical nuclear staining as described in Materials and Methods was performed, which confirmed the induction of apoptosis after therapy with 2-ME2 alone and with the various combinations (data not shown). Both cell lines showed typical morphological patterns of apoptosis in the nuclei after treatment with 2-ME2, e.g. cyto-pleomorphy, karyolysis and karyorrhexis, as well as detached nuclear fragments. Additionally, and according to the compound of treatment, more or less distinctive changes of the cell form occurred as well as a loss of cell integrity.

The in vivo experiments were carried out using the AsPC-1 cells, which were injected into the pancreatic tail as described in the Materials and Methods. The tumour mass of the animals in each treatment group, which consisted of 8 animals per group, was determined. Figure 2 shows the average tumour volume after 32 days of treatment. The animals of the non-treated control group showed a continuous increase in tumour size, with an average volume of 165.1 mm³ 32 days after tumour cell injection. Tumours of the animals treated with gemcitabine alone had an average volume of 172.3 mm³ (p=0.8). In comparisons, 2-ME2 alone resulted in a significant reduction of the average tumour volume to 61.2 mm³, which was statistically significant (p=0.05). The combination therapy of 2-ME2 and gemcitabine led to the maximum tumour reduction, to an average of 29 mm³. However, six out of eight animals of this treatment group died during the period of treatment. Thus, meaningful statistical evaluation was not impossible.

Discussion

2-ME2 has been shown to be highly effective for the treatment of a large variety of cancer types (8), including pancreatic cancer (10, 11, 13-16). The sensitivity of the cancer cells appears to be very similar between different cell lines in vitro and in vivo. Even pancreatic cancer cells that are highly resistant to different commonly used chemotherapeutic compounds underwent apoptotic cell death to the same degree as the parental chemosensitive cells (11). The results of the current study confirmed previously published data. Low doses of 2-ME2 significantly reduced cell growth in vitro in both cell lines. Several studies have already evaluated combined treatments of 2-ME2 and other compounds. Increased induction of apoptosis has been observed in human breast cancer cells when 2-ME2 was combined with bis(ethyl)norspermine (17). The microtubule stabilizer laulimalide was able to cause synergistic growth inhibition when combined with 2-ME2 (18).

In pancreatic cancer cells, 2-ME2 was able to up-regulate members of the death receptor family. Furthermore, it was shown that 2-ME2 in combination with Fas ligand (FasL)/tumour necrosis factor-related apoptosis-inducing ligand increased apoptotic cell death. In vivo 2-ME2 and tumour necrosis factor-related apoptosis-inducing ligand reduced the tumour volume in a murine pancreatic cancer model (14). Androgen deprivation combined with 2-ME2 caused increased growth inhibition of hormone-dependent prostate tumours in vivo (19). The microtubule-disrupting agents, paclitaxel and vinorelbine, in combination with 2-ME2 showed synergistic growth inhibition of breast cancer cells in vitro (20). Also in head and neck squamous cancer cells, the effect of 2-ME2 on growth inhibition were enhanced significantly in vivo when paclitaxel was added (21). The chemotherapeutic agents epirubicin and carboplatin have been successfully combined with 2-ME2 for the treatment of ovarian cancer (22). The combination of docetaxel with 2-ME2 overcomes the protective effect of VEGF in both in vitro and in vivo models in breast cancer, which led to synergistic growth inhibition (23). For the first time, an effect of systemic adenoviral gene transfer was shown when an intravenously injected p53-expressing adenovirus was combined with oral application of 2-ME2 in a murine lung metastasis model (24). The combination of 2-ME2 and a systemic adenoviral gene transfer showed additive growth inhibition of lung metastases. A clinical trial was

![Figure 2. Tumour size in mm³ after 32 days of treatment with 2-ME2 alone, gemcitabine alone, or the combination of both compounds. C: Control non-treated mice; G: gemcitabine; M: 2-ME2.](image-url)
conducted using a combination of 2-ME2 and docetaxel on metastatic breast cancer (25). The treatment was well tolerated, with some elevation of transaminases, but the effect on tumour growth was below the expected therapeutic range. A possible reason for the relatively low effect on tumour growth could be the low serum concentration of the compound. The maximum tolerated dose was not identified in this study.

In the current study, the proof of induction of apoptosis was carried out with two different methods, FACS analysis and immunohistochemical staining (Hoechst). Both methods showed strong induction of apoptosis after treatment with 2-ME2, which could be increased with the addition of gemcitabine, 5-fluorouracil, paclitaxel and, in part, cisplatin. A difference in the number of apoptotic cells was observed between both cell lines after combination with 2-ME2 and cisplatin. As yet, there is no explanation for this phenomenon. A possible reason could be the more aggressive behaviour of MiaPaCa-2 cells in comparison to the AsPC-1 cells. The effect of cisplatin on cell death is at least partially due to the induction of apoptosis together with other mechanisms (26).

Gemcitabine induced apoptotic cell death which was enhanced when combined with apigenin (27). In nude mice, the combination treatment significantly reduced the tumour volume in a subcutaneous pancreatic cancer xenograft model using the MiaPaCa-2 cells. 5-Fluorouracil induced induction of apoptosis in relation to the p53 mutational status. Depletion of the p53 gene resulted in decreased induction of apoptosis after treatment with 5-fluorouracil (28). No induction of apoptosis was observed after treatment with cetuximab alone. However, when combined with irinotecan, cetuximab potentiated the in vitro antiproliferative and proapoptotic effect of irinotecan, which was confirmed in vivo in an orthotopic tumour model of thyroid cancer in nude mice (29).

For the in vivo experiments, AsPC-1 cells were used after our experience of their tumourigenicity in nude mice. As expected, the group treated with 2-ME2 alone showed growth inhibition of 63.4% when compared to non-treated control mice. Previous experiments gave similar results in different tumour types (10, 24, 30, 31).

In summary, this study showed that combination treatments of 2-ME2 with other compounds appear to enhance the efficiency of growth inhibition of human pancreatic cancer cells. The combination of 2-ME2 with gemcitabine seems to be of particular interest because gemcitabine is widely used as monotherapy for adjuvant and palliative therapy for pancreatic cancer. The combination of 2-ME2 and gemcitabine or other chemotherapeutic compounds may have clinical application and should be evaluated in clinical trials.

Acknowledgements

This work was partly supported by EntreMed Inc., Rockville, MD, U.S.A.

References


