

## Effects of Heavy Water (D<sub>2</sub>O) on Human Pancreatic Tumor Cells

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**Abstract.** *Background:* Pancreatic cancer constitutes an entity which is difficult to treat and, therefore, mostly fatal. Since heavy water (deuterium oxide, D<sub>2</sub>O) was shown to be active in various cancer cell lines *in vitro* and *in vivo*, we now investigated its effects in human pancreatic tumor cells. *Materials and Methods:* The cytotoxic effects of D<sub>2</sub>O were examined in three pancreatic cancer cell lines (AsPC-1, BxPC-3 and PANC-1). Induction of apoptosis was determined by Hoechst/propidium iodide double staining and cell cycle distribution was investigated by FACS analysis. *Results:* Employing a clonogenic assay, D<sub>2</sub>O yielded IC<sub>50</sub> values of 15%, 18% and 27% in AsPC-1, PANC-1 and BxPC-3 cells, respectively, and led to the induction of apoptosis when compared to untreated controls. Moreover, D<sub>2</sub>O caused a cell cycle arrest in the G2-M-phase (BxPC-3, PANC-1) or in the S-phase (AsPC-1). *Conclusion:* It is hoped that D<sub>2</sub>O might offer an additional option for the treatment of pancreatic carcinomas.

Pancreatic cancer occurs with an incidence of 2 to 10 per 100,000 persons per year. Because of its unspecific symptoms, it is often diagnosed in an extremely late phase of the disease, mostly after formation of metastases. Therefore, therapy modalities are quite poor at the time of diagnosis. Only in less than 20% of patients, can pancreatic

carcinomas be removed by surgery. Individuals suffering from inoperable tumors receive palliative therapy, including chemotherapy and radiation therapy. However, the treatment options are very limited and most patients die within months after diagnosis.

Incubation of tumor cells with various concentrations of D<sub>2</sub>O leads to inhibition of cell proliferation and might, therefore, help in the chemotherapeutic treatment of human tumors (1). D<sub>2</sub>O, known as heavy water, contains a neutron and a proton in its hydrogen atoms and shows a variety of different biological activities from normal (light) water. The concentration-dependent induction of apoptosis and arrest of cell cycle transition were described for murine astrocytoma cells by Uemura and co-workers (2). Although several authors have described antitumor effects *in vitro* and in animals after administration of D<sub>2</sub>O (3, 4), only a few studies investigated the cellular events. Somlyai and co-workers even state that naturally occurring deuterium might be essential for normal cell growth; in addition, they show that proliferation of tumor cells can be suppressed by deuterium-depleted water (5).

The aim of this study was the elucidation of the cytotoxic and biochemical effects of D<sub>2</sub>O in three different human pancreatic carcinoma cell lines (AsPC-1, BxPC-3 and PANC-1). The growth inhibitory effects and the inhibition of colony formation of these tumor cells were, therefore, examined. Moreover, induction of programmed cell death and effects on the cell cycle phase distribution were investigated in all cell lines.

We focused on the effects of D<sub>2</sub>O on human pancreatic carcinoma cells as a possible additional option for the treatment of these tumor entities. Due to the limited number of chemotherapeutic regimens, which are active against this malignancy, a new approach, like the use of D<sub>2</sub>O, might offer an alternative for the treatment of pancreatic cancer.

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**Key Words:** Heavy water (deuterium oxide, D<sub>2</sub>O), apoptosis, cell cycle distribution, pancreatic cancer, AsPC-1, BxPC-3 and PANC-1 cells.

## Materials and Methods

**Chemicals.** Deuterium oxide ( $D_2O$ ) was obtained from CU Chemie Uetikon AG (Uetikon am See, Switzerland). For the treatment of cells with  $D_2O$ , dry powder culture medium was dissolved in  $D_2O$  and supplements were added. The  $D_2O$ -containing medium was then mixed with aqueous cell culture media in the designated concentrations (5-50%).

**Cell culture.** The AsPC-1, BxPC-3 and PANC-1 human pancreatic adenocarcinoma cell lines were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA). The cells were maintained in a humidified atmosphere containing 5%  $CO_2$  using a Cytoperm 8080 incubator (Heraeus, Vienna, Austria). AsPC-1 and BxPC-3 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 1% sodium pyruvate and 1% penicillin-streptomycin. PANC-1 cells were grown in DMEM high glucose medium containing 10% heat-inactivated fetal calf serum and 1% penicillin-streptomycin. All pancreatic adenocarcinoma cells were grown in a monolayer culture using 25-cm<sup>2</sup> tissue culture flasks and were periodically detached from the flask surface using 2.5% trypsin-ethylenediamine tetraacetic acid (trypsin-EDTA) solution. All media and supplements were obtained from Gibco Life Technologies Ltd. (Paisley, Scotland, Great Britain). Cell counts were determined using a CC-108 microcellcounter (Sysmex, Kobe, Japan). Cells in the logarithmic phase of growth were used for all studies described.

**Growth inhibition assay.** Logarithmically-growing cells ( $0.5 \times 10^6$ ) were seeded in 25-cm<sup>2</sup> tissue culture flasks and allowed to attach overnight. Then the cells were incubated with various concentrations of  $D_2O$  for 5 days. After detaching the cells by trypsin treatment, the  $IC_{50}$  value of  $D_2O$  was determined. The cells were counted using the Sysmex CC-108 microcellcounter (Sysmex, Kobe, Japan) and the results were calculated as percent of control values. All experiments were performed in duplicate and were repeated twice.

**Clonogenic assay.** Logarithmically-growing cells were plated in 24-well plates ( $0.5 \times 10^3$  –  $1.0 \times 10^3$  cells per well) and, after 24 hours, incubated with various concentrations of  $D_2O$  (5-50%) for 7 days under cell culture conditions. After staining with crystal violet, colonies (>50 cells) were counted using an inverted microscope (Olympus) at 40x magnification. The results were calculated as percent of the control values. All experiments were performed in triplicate and were repeated twice.

**Hoechst dye 33258 and propidium iodide double staining.** Cells were seeded in 12-well plates ( $4 \times 10^4$  cells per well). After 24 hours,  $D_2O$  was added in concentrations ranging from 5% to 30% and the cells were incubated for 5 days. The cells were then washed with phosphate-buffered saline, detached with 2.5% trypsin and centrifuged twice at 800 rpm for 3 min in an Eppendorf microfuge. After resuspending, the cells were stained with Hoechst 33258 (Sigma, St. Louis, MO, USA) and propidium iodide (Sigma) (final concentrations: 5  $\mu$ g/ml and 2  $\mu$ g/ml, respectively) and incubated at 37°C for 1 hour. Subsequently, the cells were examined by fluorescence microscopy (Zeiss Axiovert 35) equipped with appropriate DAPI filters for Hoechst 33258 and PI. The cells were photographed with an Olympus camera using Kodak Ektachrome

P1600 films (Kodak, Rochester, NY, USA). This method allows a distinction to be made between apoptosis and necrosis. The cells were judged according to their morphology and the integrity of their cell membranes, which can easily be seen after propidium iodide staining. All experiments were performed in duplicate and were repeated twice.

**Analysis of cell cycle distribution.** Logarithmically-growing pancreatic carcinoma cells ( $0.5 \times 10^6$ ) were seeded in 25-cm<sup>2</sup> tissue culture flasks and were allowed to attach overnight. Then fresh supplemented medium containing 50%  $D_2O$  was added and the cells were incubated for 72 hours. After trypsin-EDTA treatment, the cells were detached and the cell count was determined. The cells were washed with 5 ml cold PBS, centrifuged at 600 rpm, resuspended, and fixed in 3 ml cold ethanol (70%) for 30 minutes at 4°C. After two washing steps in cold PBS, RNase A and propidium iodide were added to a final concentration of 50  $\mu$ g/ml each and incubated at 4°C for 1 hour before measurement.  $2 \times 10^6$  cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and cell cycle distribution was calculated with ModFit LT software (Verity Software House, Topsham, ME, USA). All experiments were performed in duplicate and were repeated twice.

**Statistical calculations.** Dose response curves were calculated using the Prism 3.03 software (GraphPad Software Inc., San Diego, CA, USA). Statistical differences were calculated by unpaired *t*-test.

## Results

**Growth inhibition assay.** The growth inhibitory effects of  $D_2O$  in pancreatic cancer cells were evaluated after 5 days of incubation with concentrations of  $D_2O$  ranging from 5-50%.  $IC_{50}$  values ( $D_2O$  concentration which causes a 50% inhibition of tumor cell colony growth) were determined for all three pancreatic carcinoma cell lines. AsPC-1 and PANC-1 cells were inhibited by  $D_2O$  with  $IC_{50}$  values of 23% and 26%, respectively. Furthermore,  $D_2O$  inhibited the growth of BxPC-3 cells with an  $IC_{50}$  value of 48%. The results are shown in Figure 1.

**Clonogenic assay.** Cells were incubated with 5-50% of  $D_2O$  for 7 days, as described in the Methods section.  $IC_{50}$  values were determined for all three pancreatic carcinoma cell lines. In AsPC-1 and PANC-1 cells,  $D_2O$  yielded  $IC_{50}$  values of 15% and 18%  $D_2O$ , respectively. BxPC-3 cells showed an  $IC_{50}$  value of 27%. Again, similarly to the growth inhibition assay, BxPC-3 cells yielded a higher  $IC_{50}$  value than observed for PANC-1 or AsPC-1 cells. The results are shown in Figure 2.

**Induction of apoptosis.** The induction of programmed cell death was determined morphologically after incubation of cells with 5-30%  $D_2O$  for 24 hours. 9.1 to 11.9 % of control cells showed morphological signs of apoptosis. These numbers could be significantly increased in AsPC-1 and PANC-1 cells. In AsPC-1 cells, incubation with 30%  $D_2O$

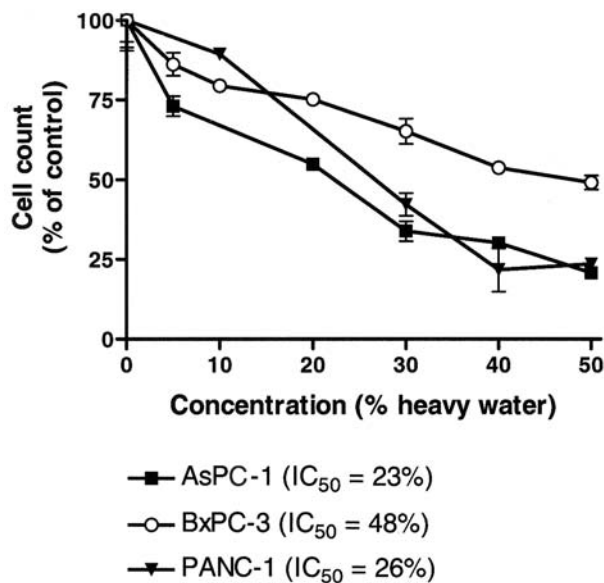


Figure 1. Growth inhibition assay in AsPC-1, BxPC-3 and PANC-1 human pancreatic cancer cells. Logarithmically-growing cells were seeded in 25-cm<sup>2</sup> tissue culture flasks and incubated with various concentrations of D<sub>2</sub>O for 5 days. Cells were counted using the Sysmex CC-108 microcellcounter. Results were calculated as percent of control values. All experiments were performed in duplicate and were repeated twice. Data are mean  $\pm$  S.D. of two or more determinations.

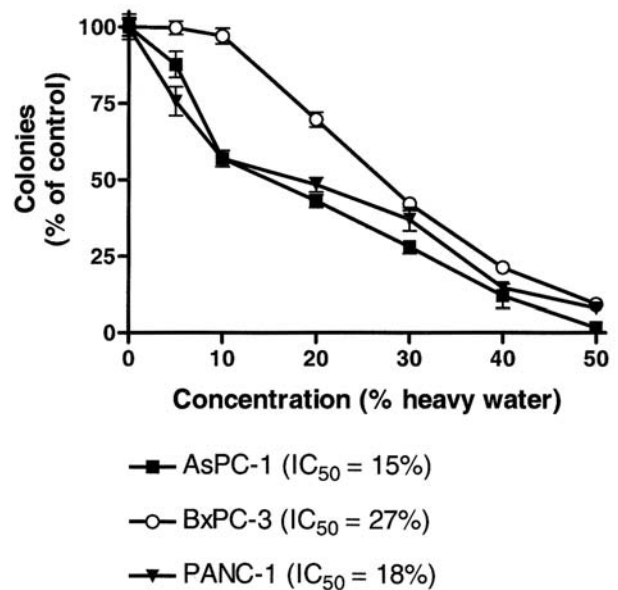


Figure 2. Clonogenic assay in AsPC-1, BxPC-3 and PANC-1 human pancreatic cancer cells. Logarithmically-growing cells were plated in 24-well plates and incubated with various concentrations of D<sub>2</sub>O for 7 days under cell culture conditions. After staining with crystal violet, colonies (> 50 cells) were counted using an inverted microscope. Results were calculated as percent of control values. All experiments were performed in triplicate and were repeated twice. Data are mean  $\pm$  S.D. of two or more determinations.

increased the number of apoptotic cells up to 22%, whereas in PANC-1 cells, incubation with 20% D<sub>2</sub>O led to 16% of apoptotic cells. In BxPC-3 cells, treatment with D<sub>2</sub>O did not induce apoptosis. The results are depicted in Figure 3.

**Cell cycle distribution.** Treatment with 50% D<sub>2</sub>O for 72 hours caused a remarkable change in the cell cycle distribution of all three pancreatic carcinoma cell lines. Untreated AsPC-1 control cells showed a cell distribution of 62.6%, 23.5% and 13.9% in the G<sub>0</sub>-G<sub>1</sub>-, S- and G<sub>2</sub>-M-phases of the cell cycle, respectively. After treatment with 50% D<sub>2</sub>O, a significant decrease in the G<sub>0</sub>-G<sub>1</sub>-phase to 23.8% and a significant increase of cells in the S-phase to 55.9% could be observed.

In the BxPC-3 cell line, untreated control cells were to 37.2%, 55.5% and 7.3% in the G<sub>0</sub>-G<sub>1</sub>-, S- and G<sub>2</sub>-M-phases of the cell cycle, respectively. D<sub>2</sub>O treatment significantly decreased the percentage of cells in the G<sub>0</sub>-G<sub>1</sub>-phase to 22.3%, whereas cells accumulated to 29.4% in the G<sub>2</sub>-M-phase. In PANC-1 cells, similarly to the BxPC-3 cell line, D<sub>2</sub>O treatment significantly depleted cells in the G<sub>0</sub>-G<sub>1</sub>-phase and caused an accumulation of cells in the G<sub>2</sub>-M-phase, suggesting a growth arrest after the G<sub>2</sub>-M-phase of the cell cycle. The results are summarized in Table I.

## Discussion

In the present study, it was shown that D<sub>2</sub>O effectively inhibited the growth and colony formation of three different human pancreatic carcinoma cell lines. D<sub>2</sub>O also induced apoptosis in two of the three cell lines tested and arrested AsPC-1 cells in the S-phase and BxPC-3 and PANC-1 cells in the G<sub>2</sub>-M-phase of the cell cycle, respectively.

The effects of D<sub>2</sub>O on mammalian cells have been described as early as 1936 by Fischer (6), but it was Rothstein and colleagues who observed characteristic changes in cell morphology and metabolism in response to treatment with heavy water. They suggested that DNA metabolism might be affected by D<sub>2</sub>O (7). Gross and Harding finally proved that D<sub>2</sub>O indeed inhibited DNA synthesis in sea urchin eggs. Deuterated cells failed to divide and showed a decreased incorporation rate of <sup>3</sup>H-labelled thymidine (8). In recent years, the biological and antineoplastic effects of D<sub>2</sub>O have been further characterized. In several cell lines, D<sub>2</sub>O caused mitotic arrest and inhibited DNA and protein synthesis (9-11). Uemura and co-workers revealed that D<sub>2</sub>O induced apoptosis *via* the activation of caspases in murine glioblastoma cells and treatment with heavy water also caused mitotic arrest in this cell line (2). Here, we investigated the

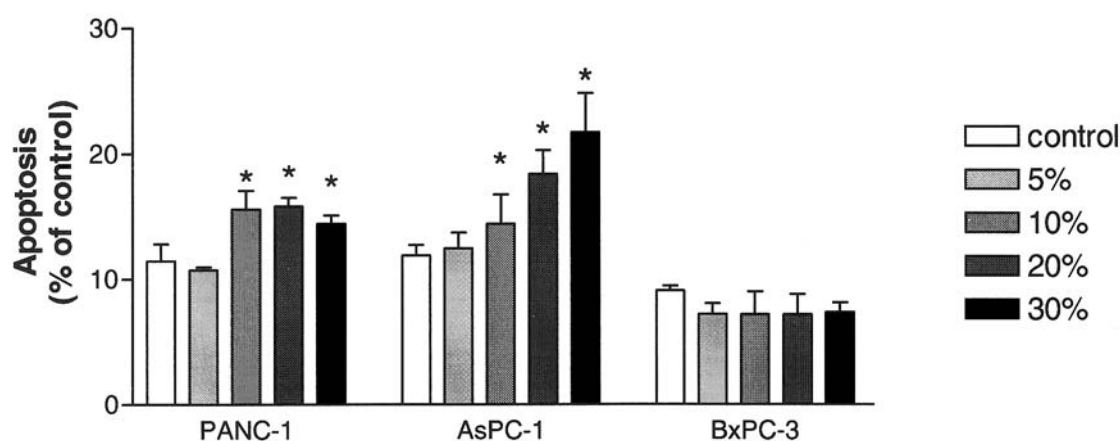


Figure 3. Induction of apoptosis in AsPC-1, BxPC-3 and PANC-1 human pancreatic cancer cells. Cells were seeded in 12-well plates and incubated with various concentrations of D<sub>2</sub>O for 5 days. Cells were judged according to their morphology and the integrity of their cell membranes, which can easily be seen after propidium iodide staining. All experiments were performed in duplicate and were repeated twice. Data are mean  $\pm$  S.D. of two or more determinations. \*Significantly different from untreated control cells ( $p < 0.05$ )

Table I. Cell cycle distribution in AsPC-1, BxPC-3 and PANC-1 human pancreatic cancer cells.

	AsPC-1		BxPC-3		PANC-1	
	Co	+50% D <sub>2</sub> O	Co	+50% D <sub>2</sub> O	Co	+50% D <sub>2</sub> O
% G0-G1	62.6	23.8*	37.2	22.3*	43.1	14.8*
% S	23.5	55.9*	55.5	48.3	37.8	51.5
% G2-M	13.9	20.3	7.3	29.4*	19.1	33.7*

Data are means of two or more determinations.

\*Significantly different from untreated control cells ( $p < 0.05$ )

effects of D<sub>2</sub>O in human pancreatic cancer cells. Our results are concordant with these previous findings and we can show that D<sub>2</sub>O is also active against pancreatic cancer cells *in vitro*.

Although D<sub>2</sub>O was able to induce apoptosis in the PANC-1 and AsPC-1 cell lines, this is probably not the solely mechanism of cytotoxicity since no induction of apoptosis could be observed in the BxPC-3 cell line.

Although some studies suggest that a certain deuteration grade of water is essential for normal cell growth and division (5), D<sub>2</sub>O has been reported to exert antineoplastic effects *in vitro* and *in vivo*. It delayed the growth of human oropharyngeal squamous cell cancer and colon cancer in nude mice (3) and synergistically potentiated the effects of clinically well-established cytostatic compounds such as 5-fluorouracil, bleomycin and methotrexate *in vivo* (4, 14). Several studies revealed that a moderate body deuteration had little or no adverse

effects and was well-tolerated by mice and rats (15, 16). On the other hand, D<sub>2</sub>O has been reported to enhance IgE-mediated immune response in rats due to its interaction with microtubules, an effect which has not yet been investigated in humans (17, 18).

Our results show that D<sub>2</sub>O is a useful agent against human pancreatic carcinoma cells *in vitro*, a fact that makes it a potential candidate for the treatment of pancreatic tumors. However, *in vivo* studies are needed to determine the value of D<sub>2</sub>O for the treatment of these, mostly fatal, tumors.

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