The Effects of All-*Trans*-Retinoic Acid on Cell Cycle and Alkaline Phosphatase Activity in Pancreatic Cancer Cells

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Abstract: Pancreatic cancer is one of the tumors with the highest mortality, poorly responding to available chemotherapeutic agents. The objective of this study was to study the anticancer effects of all-*trans* retinoid acid, a functional form of vitamin A, on pancreatic cancer cells. Human pancreatic cancer MiaPaCa-2 cells were treated with 1, 5, 10, 20, 30, 40 and 50 μM ATRA for 1, 2, 3, 4, 5 or 6 d, respectively. Cell growth was determined by MTT viability assay. The cell cycle distribution and the alkaline phosphatase (ALP) activity were analyzed by flow cytometry and chemical analyzer, respectively. The results show that ATRA significantly inhibited the growth of MiaPaCa-2 cells at 40 and 50 μM. ATRA arrested pancreatic cancer cells at G0/G1 phase. The sub-G1 peak and DNA fragmentation were observed. There were time and dose dependent increases in alkaline phosphatase activity (ALP), an indicator of cell differentiation, upon treatment with ATRA when compared to controls. In conclusion, ATRA has an inhibitory effect on the cell growth of MiaPaCa-2, and its tumor suppressive effect is by means of cell cycle arrest and apoptosis induction.

Key Words: All-*trans*-retinoic acid, human pancreatic cancer cell, alkaline phosphatase, cell growth, cell cycle.

INTRODUCTION

Pancreatic cancer is one of the tumors with the highest mortality, poorly responding to available chemotherapeutic agents. According to the latest report (July, 2004), the incidence rates of pancreatic cancer in the Americans rank 11 and 9 for male and female cancer patients, and the death rates rank 4 for both sexes [1]. More than 30,000 patients died of pancreatic cancer in 2003 [2]. Pancreatic cancer is the most lethal abdominal malignancy; 99% of patients diagnosed with adenocarcinoma of the pancreas die within 5 years of diagnosis [3]. Conventional chemotherapy has shown only a minimal survival benefit for pancreatic cancer [4]. Specific therapies targeted to pancreatic cancer may improve the overall prognosis of this kind of the most deadly gastrointestinal malignancy.

Vitamin A (retinal), one of the necessary vitamins for humans, has an effect on a wide range of biological functions such as growth, differentiation, reproduction and vision [5]. At the cellular level, vitamin A is converted to all-*trans*-retinoic acid (ATRA) that in turn is isomerized to 9-cis-retinoic acid (9cRA), another active metabolite of vitamin A. The biological effects of ATRA and 9cRA are mediated by nuclear retinoic acid receptors (RARs) and retinoid X receptors (RXRs) [6].

ATRA is a well-established regulator of proliferation and differentiation of various types of cells including hepatocyte, breast carcinoma cells and so on [7, 8]. However, the biological functions and molecular effects of ATRA on pancreatic cancer cells are largely unknown. To investigate whether ATRA can inhibit pancreatic cancer progression, we studied the ATRA effects on human pancreatic carcinoma cell lines MiaPaCa-2.

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RESULTS

MTT Assay Results

Growth curves of MiaPaCa-2 cells treated with the indicated concentrations of ATRA for 1-6 d, were found to be time-dependent and dose-dependent inhibitory fashions (Fig. **1**). At concentrations 40 and 50 μM, ATRA significantly inhibited the growth of MiaPaCa-2 cells (P<0.05, Fig. **1**).

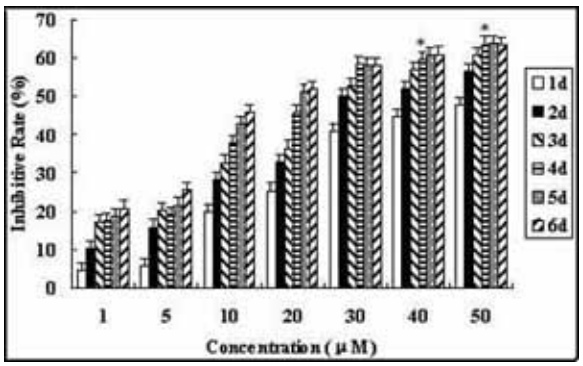

Fig. (1). Inhibitory effects of MiaPaCa-2 cells after exposure to ATRA over time. Cell growth was measured using the MTT assay. Means ± SEM represent four independent experiments. *P<0.05 compared to controls.

Flow Cytometric Analysis

In order to decipher the suppressive mechanisms of ATRA on pancreatic cancer MiaPaCa-2, we monitored change in the cell cycle distribution by flow cytometry. Treatment of ATRA resulted in an increase in the distribution of cells at G0/G1 phase (Fig. **2** and **3**). The sub-G1 peak (apoptosis peak) was observed (Fig. **2B** and **2C**).

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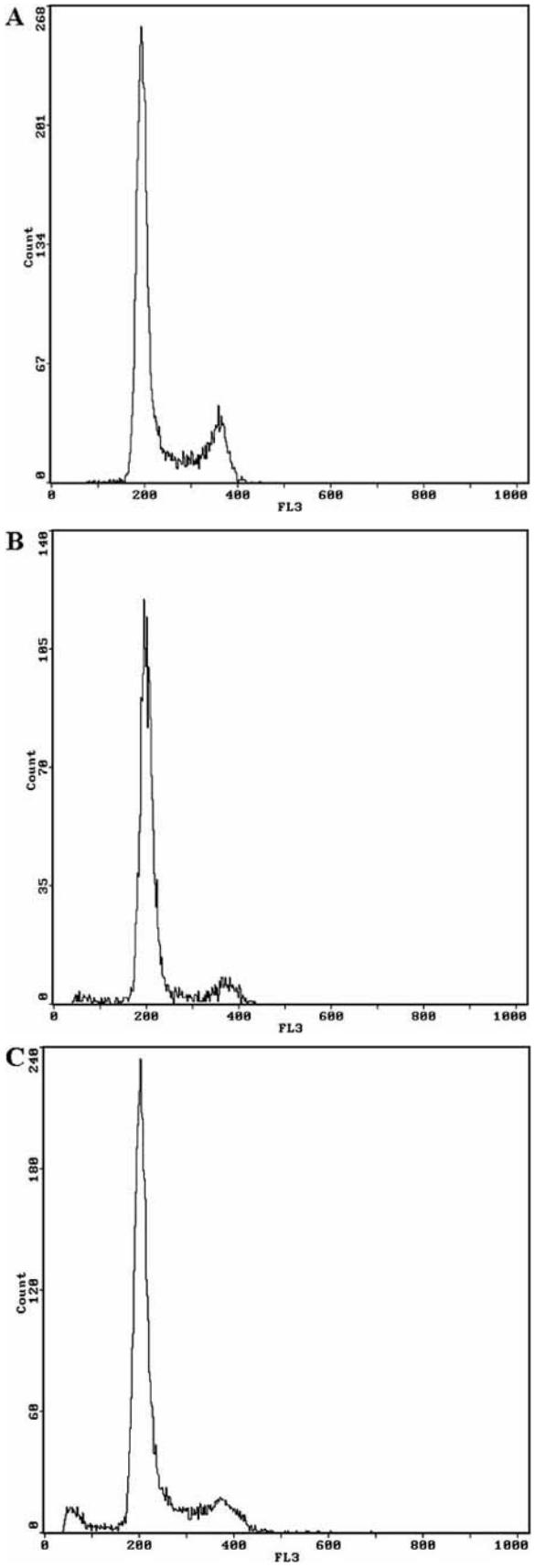

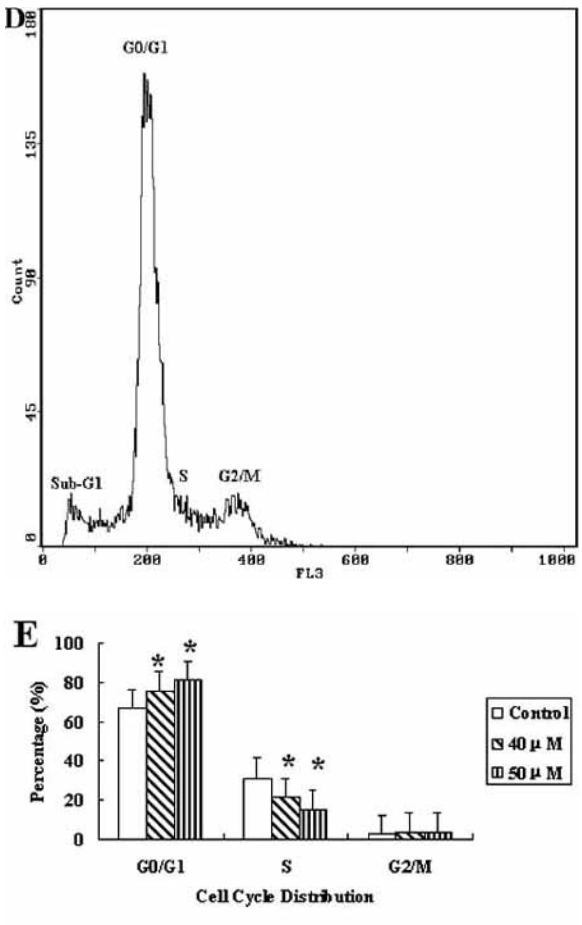

Fig. (2). Effects of ATRA on cell cycle distribution in MiaPaCa-2 cells. Cancer cells were arrested at G0/G1 phase when incubated with ATAR. (A) Untreated MiaPaCa-2 cells. G0/G1, 66.3%; S, 31.4%; G2/M, 2.2%. (B) MiaPaCa-2 cells were treated by 40 μM ATRA for 4 d. G0/G1, 75.6%; S, 21.2%; G2/M 3.2%. (C) MiaPaCa-2 cells were treated by 50 μM ATRA for 4 d. G0/G1, 81.1%; S, 15.2%; G2/M, 3.7%. (D) Apoptosis positive control. MiaPaca-2 cells were treated with 100 ng/ml tumor necrosis factor α for 4 d. The sub-G1 (apoptosis peak) is shown. (E) The changes of cell cycle distribution in pancreatic cancer cells treated by ATRA. $^{*}P<0.001$ compared to controls.

Determination of Alkaline Phosphatase Activity

In order to determine whether ATRA was capable of affecting the alkaline phosphatase (ALP) activity of Mia PaCa-2 cells, a dynamics assay was used. The results showed that ALP activity in MiaPaCa-2 cells was increased by ATRA (Fig. **4**). We were able to observe statistical significance between the ALP activity of control and treated cells.

DISCUSSION

Retinoic acid, a derivative of vitamin A, modulates cellular proliferation and differentiation in a variety of tissues in the adult organism as well as during embryogenesis [9]. Retinoids have recently received considerable attention in medical oncology [7,8,10,11]. Among all retinoids, ATRA is the most widely used. It was reported

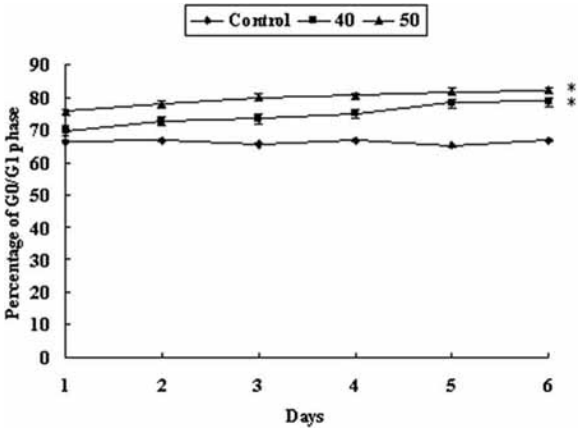

Fig. (3). The percentage of G0/G1 phase in human pancreatic cancer cells MiaPaca-2 treated by ATRA. The pancreatic cancer cells (10^6 cells/ml) were treated with 40 and 50 μM ATRA for 1, 2, 3, 4, 5 or 6 d. The medium containing 0.1% ethanol was defined as control. The cell cycle distribution was determined by flow cytometry as described in Section 2.3. Each point represents the mean ± SEM of three independent assays. * $P<0.001$, compared with control.

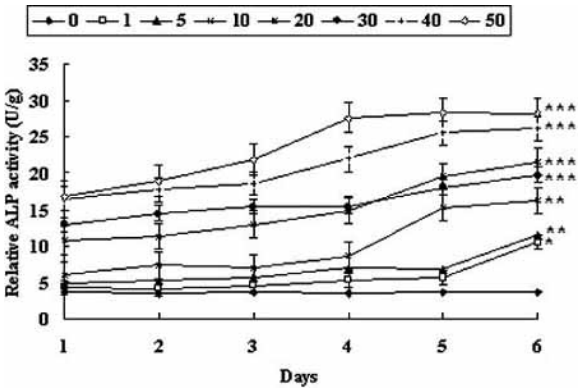

Fig. (4). Effects of ATRA on the ALP activity of MiaPaCa-2 cells. Cancer cells treated with 1, 5, 10, 20, 30, 40 and 50 μM ATRA for 1, 2, 3, 4, 5 or 6 d. Cells growing in ethanol (<0.1%) were used as the control group. The ALP activity was calculated as described in "Materials and methods". Means ± SEM represents four independent experiments. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, compared to controls.

that the multifunction of ATRA was mediated through retinoic acid receptors (RARs) and retinoid X receptors (RXRs) [6].

The prognosis for patients with pancreatic cancer is dismal; five-year survival is less than 5%, despite optimal surgical and chemotherapeutic treatments [3]. In the current study, we have shown that ATRA affects the growth of human pancreatic cancer cell lines MiaPaCa-2. Our data indicate that cell growth of MiaPaCa-2 was significantly inhibited by ATRA (Fig. **1**). ATRA is the natural ligand for RARs [12,13]. Most pancreatic cancer cell lines including MiaPaca-2 used express RARγ, but do not express RARβ [6, 14-16]. These imply that the suppressive growth effect of ATRA on MiaPaCa-2 cells may be due to RARγ.

One of the most possible mechanisms of chemotherapeutic drugs on cancer cells is to act on their cell cycle [17]. In this study, we found that ATRA arrested MiaPaCa-2 cells at G0/G1 phase (Fig. **2** and **3**). These suggest that one of the antitumorigenic mechanisms of ATRA may be derived from G0/G1 phase arrest. The same results of ATRA on the cell cycle distribution on other cancer cells have been reported. Retinoids cause an accumulation of cell in G0/G1 phase of the cell cycle in breast carcinoma MCF-7 cells and lymphoma HL-60 cells due to the downregulation of cyclin E (HL-60) or cyclin D1, cdk 2 and pRB protein levels (MCF-7) [18, 19]. This means that the retinoids might control different subsets of retinoid-responsive genes to induce growth inhibition in different cell types. The molecular mechanisms of the cell cycle blockage have been reported in recent years. Fujimoto *et al*. reported that the cell-cycle arrest in pancreatic cancer cells (MiaPaca-2 and others) induced by a retinobenzoic acid was accompanied by the reduction of retinoblastoma-gene product phosphorylation and an increase of two cyclin-dependent kinase inhibitors, p21 and p27 [20]. Another study showed that the cell cycle arrest in MiaPaca-2 cells by other reagents was associated with the stabilization of p21 and p27 [21]. The fact that ATRA induce G0/G1 blockage could imply that the combination treatment with cell cycle acting drugs might facilitate synergistic activities.

However, the antiproliferative mechanism of ATRA on pancreatic cancer appears to be dependent on the type of cell lines. A study involving another pancreatic cancer line DNA-G found that ATRA restrained cells at G2/M phase [22].

Another mechanism of chemicals on cancer cells is the induction of apoptosis. Regarding the effect of ATRA on cancer cells, Pettersson *et al*. found that it induced growth inhibition followed by apoptosis in pancreatic adenocarcinoma cell lines T3M-4 and BxPc-3 [23]. In this study, the sub-G1 peak (apoptosis peak) was observed when MiaPaca-2 cells were treated with ATRA (Fig. **2** and **3**). This implied that the apoptosis induction of ATRA on cancer cells depends on different kinds of cancers and/or different types of cell lines.

Cellular ALP is an important marker of differentiation in human malignancies [24]. Several studies found that ATRA upregulated the expression of ALP activity in cancer cells [25-28]. Its mechanism was thought to be associated with ATRA receptors RAR/RXR [26, 27]. About the association between the increase of ALP activity in human pancreatic cancer MiaPaCa-2 cells and the effect of induce differentiation, Yamada *et al*. reported that when a specific inhibitor of DNA methylation, 5-aza-2'-deoxycytidine, was used to induce differentiation of MiaPaCa-2 cells, the ALP activity was markedly increased [16]. In this study, we found that the ALP activity in MiaPaCa-2 cells was increased by ATRA (Fig. **4**). The significant enhancement of ALP activity was initiated at 1 μM ATRA. We noticed that the concentration of ATRA significantly suppressing the pancreatic cancer cells growth was much more higher than 1 μM (Fig. **1**). As a

result, our findings provided a useful and sensitive marker for monitoring the induced differentiation effect of inducers (e.g. ATRA) on pancreatic cancers.

In conclusion, we have shown that ATRA affects the growth, cell cycle distribution and ALP activity of human pancreatic cancer cell line MiaPaCa-2 cells. These results highlight the idea that retinoids are useful for preventing and/or treating patients with pancreatic carcinomas.

EXPERIMENTAL

Materials

ATRA and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO). Tumor necrosis factor was obtained from Boehringer Mannheim (Mannheim, Germany), while RPMI-1640 medium was from Life Technologies (Grand Island, NY). Human pancreatic carcinoma cell lines MiaPaCa-2 were obtained from the Department of General and Gastroenterological Surgery, Osaka Medical College (Takatsuki City, Japan). Alkaline phosphatase (ALP, EC 3.1.3.1) activity and total protein detection kits were purchased from Ningbo Asia-Pacific Biotechnology Ltd. (Ningbo, China).

MTT Assay

MiaPaCa-2 cells were cultured, respectively, at 37 ℃ in a humidified atmosphere of 5% CO_2 with RPMI-1640 Medium containing 10% fetal calf serum (FCS), 50 U/ml penicillin and 50 μg/ml streptomycin. Exponentially growing cancer cells were plated in 96-well microtiter plates at 10^4 cells/ml for the 1, 2, 3, 4, 5, and 6-d time-points. Serial dilutions of ATRA (1, 5, 10, 20, 30, 40 and 50 μM) dissolved in absolute ethanol were added to the appropriate well. After the cells were exposed to ATRA for the specific time, ATRA was removed, and the cell proliferation assay (MTT assay) was performed as previously described [29]. All manipulations of solutions and cell cultures were carried out in subdued light. ATRA-containing media were changed every other day. Control vehicles received ethanol, and the final concentration of ethanol in the medium did not exceed 0.1% [30]. Sets of 12 wells were used for each dose along with controls in this assay. The absorbance (A) was measured at 550 nm using a Wellscan (Labsystems, Santa Fe, NM). The mean A from the 12 wells was calculated. Growth inhibition was calculated in percent as follows: $[(A_{control} - A_{experiment}) / A_{control}] \times 100\%$ [29]. The mean of the four independent assays was determined to analyze the effect of ATRA on cell growth.

Cell Cycle Analysis

Cancer cells (10^6 cells/ml) were treated with various concentrations of ATRA for 1, 2, 3, 4, 5 or 6 d. Cells were harvested with 0.25% trypsin/0.05% EDTA and resuspended in phosphate buffered saline (PBS). Cells were fixed in ice-cold 70% ethanol and incubated in PBS containing 40 μg/ml RNase A for 30 min at room temperature. The DNA was stained with DNA-Prep Coulter Reagents Kit (Beckman Coulter, Inc., Miami, FL). DNA content of cells was analyzed on Coulter Flow Cytometer (Beckman Coulter, Inc., Miami, FL). The cell cycle distribution was estimated according to standard procedures [31]. The percentage of cells in different cell cycle phases (G0/G1, S, or G2/M phase) was calculated using Coulter Epicx XL-MCL DNA Analysis Software (Beckman Coulter, Inc., Miami, FL). The sub-G1 peak was considered as a measure of apoptosis [32, 33]. Three wells of a 6-well plate were used for each dose and timed treatment. Each experiments was conducted in triplicate.

Determination of Relative Alkaline Phosphatase Activities

MiaPaCa-2 cells were seeded at a density of 10^4 cells/ml and treated with 1, 5, 10, 20, 30, 40, and 50 μM ATRA for 1, 2, 3, 4, 5 or 6 d before being assayed for ALP activity. Cells were then dissolved with 0.25% sodium deoxycholate. Finally, the ALP activities were measured by a dynamics assay with Screen Master 3000 Semi-automatic Biochemistry Analyzer (Hospitex Diagnostics, Firenze, Italy). The relative enzyme activity was expressed as ALP/total protein (U/g) [34, 35]. Six wells of a 12-well plate were used for each dose and treated time. Four independent experiments were performed in this analysis.

Statistics

Statistical analysis was performed using SPSS version 10.0 (SPSS Inc. Chicago, IL). The Student's *t*-test was used for comparisons between groups. Significant differences were considered if $P<0.05$. All values were given as mean ± SEM.

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ABBREVIATIONS

A = Absorbance

ALP = Alkaline phosphatase

ATRA = All-*trans* retinoid acid

9cRA = 9-cis-retinoic acid

DMSO = Dimethylsulfoxide

EDTA = Ethylenediaminotetraacetic acid

FCS = Fetal calf serum

MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

PBS = Phosphate buffered saline

RARs = Retinoic acid receptors

RXRs = Retinoid X receptors

SPSS = Statistical package for social science

REFERENCES

[1] Jemal, A.; Clegg, L.X.; Ward, E.; Ries, L.A.G.; Wu, X.; Jamison, P.M.; Wingo, P.A.; Howe, H.L.; Anderson, R.N.; Edwards, B.K. *Cancer*, **2004**, *101*, 3.

[2] Jemal, A.; Murray, T.; Samuels, A.; Ghafoor, A.; Ward, E.; Thun, M.J. *C.A. Cancer J. Clin*., **2003**, *53*, 5.

[3] Lillemoe, K.D.; Yeo, C.J.; Cameron, J.L. *C.A. Cancer J. Clin.*, **2000**, *50*, 241.

[4] Yeo, C.J.; Abrams, R.A.; Grochow, L.B.; Sohn, T.A.; Ord, S.E.; Hruban, R.H. Zahurak, M.L.; Dooley, W.C.; Coleman, J.; Sauter, P.K.; Pitt, H.A.; Lillemoe, K.D.; Cameron, J.L. *Ann. Surg*., **1997**, *225*, 621.
[5] Blumentrath, J.; Neye, H.; Verspohl, E.J. *Cell Biochem. Funct*., **2001**, *19*, 159.
[6] Rosewicz, S.; Stier, U.; Brembeck, F.; Kaiser, A.; Papadimitriou, C.; Berdel, W.E.; Wiedenmann, B.; Riecken, E.O. *Gastroenterology*, **1995**, *109*, 1646.
[7] Chung, J.; Liu, C.; Smith, D.E.; Seitz, H.K.; Russell, R.M.; Wang, X.D. *Carcinogenesis*., **2001**, *22*, 1213.
[8] Sapi, E.; Flick, M.B.; Tartaro, K.; Kim, S.; Rakhlin, Y.; Rodov, S.; Kacinski, B.M. *Cancer Res*., **1999**, *59*, 5578.
[9] Gudas, L.J. *Cell Growth Differ*., **1992**, *3*, 655.
[10] Jetten, A.M.; Kim, J.S.; Sacks, P.G.; Rearick, J.I.; Lotan, D.; Hong, W.K.; Lotan, R. *Int. J. Cancer*, **1990**, *45*, 195.
[11] Miller, W.H. Jr. *Cancer*, **1998**, *83*, 1471.
[12] Giguere, V.; Ong, E.S.; Swgui, P.; Evans, R.M. *Nature*, **1987**, *330*, 624.
[13] Petkovich, M.; Brand, N.J.; Krust, A.; Chambon, P. *Nuture*, **1987**, *330*, 444.
[14] Vickers, S.M.; Sampson, L.K.; Ying, W.; Phillips, F.O. *J. Gastrointest. Surg*., **1997**, *1*, 174.
[15] Egawa, N.; Maillet, B.; VanDamme, B.; De Greve, J.; Kloppel, G. *Virchows Arch*., **1996**, *429*, 59.
[16] Yamada, T.; Ohwada, S.; Saitoh, F.; Adachi, M.; Morishita, Y.; Hozumi, M. *Anticancer Res*., **1996**, *16*, 735.
[17] Diaz, G.D.; Paraskeva, C.; Thomas, M.G.; Binderup, L.; Hague, A. *Cancer Res*., **2000**, *60*, 2304.
[18] Burger, C.; Wick, M.; Muller, R. J. *Cell Sci*., **1994**, *107*, 2047.
[19] Teixeira, C.; Pratt, M.A. *Mol. Endocrinol*., **1997**, *11*, 1191.
[20] Fujimoto, K.; Hosotani, R.; Doi, R.; Wada, M.; Lee, J.U.; Koshiba, T.; Miyamoto, Y.; Tsuji, S.; Nakajima, S.; Imamura, M. *Int. J. Cancer*, **1999**, *81*, 637.
[21] Nawrocki, S.T.; Sweeney-Gotsch, B.; Takamori, R.; McConkey, D.J. *Mol. Cancer Ther*., **2004**, *3*, 59.
[22] Kaiser, A.; Brembeck, F.H.; Nicke, B.; Wiedenmann, B.; Riecken, E.O.; Rosewicz, S. *J. Biol. Chem*., **1999**, *274*, 18925.
[23] Pettersson, F.; Colston, K.W.; Dalgleish, A.G. *Pancreas*, **2001**, *23*, 273.
[24] Dabare, A.A.; Nouri, A.M.; Cannell, H.; Moss, T.; Nigam, A.K.; Oliver, R.T. *Uro. Int*., **1999**, *63*, 168.
[25] Gianni, M.; Zanotta, S.; Terao, M.; Garattini, S.; Garattini, E. *Biochem. Biophys. Res. Commun*., **1993**, *296*, 252.
[26] Lenhard, J.M.; Weiel, J.E.; Paulik, M.A.; Furfine, E.S. *Biochem. Pharmacol*., **2000**, *59*, 1063.
[27] Paulik, M.A.; Lenhard, J.M. *Cell Tissue Res*., **1997**, *290*, 79.
[28] Yan, T.; Wergedal, J.; Zhou, Y.; Mohan, S.; Baylink, D.J.; Strong, D.D. *Growth Horm. IGF Res*., **2001**, *11*, 368.
[29] Guo, J.M.; Xiao, B.X.; Liu, D.H.; Grant, M.; Zhang, S.; Lai, Y.F.; Guo, Y.B.; Liu, Q. *Food Chem. Toxicol.*, **2004**, *42*, 1641.
[30] Rosewicz, S.; Riecken, E.O.; Stier, U. *FEBS Lett.,* **1995**, *368*, 45.
[31] Liu, H.S.; Chen, C.Y.; Lee, C.H.; Chou, Y.I. *Br. J. Cancer*, **1998**, *77*, 1777.
[32] Patel, V.; Senderowicz, A.M.; Pinto, D. Jr.; Igishi, T.; Raffeld, M.; Quintanilla-Martinez, L.; Ensley, J.F.; Sausville, E.A.; Gutkind, J.S. *J. Clin. Invest*., **1998**, *102*, 1674.
[33] Su, S.J.; Yeh, T.M.; Lei, H.Y.; Chow, N.H. *Clin. Cancer Res*., **2000**, *6*, 230.
[34] Herz, F.; Halwer, M. Exp. *Cell Res*., **1990**, *188*, 50.
[35] Honma, Y.; Takenaga, K.; Kasukabe, T.; Hozumi, M. *Biochem. Biophys. Res. Commun*., **1980**, *95*, 507.

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