3,3',5-Triiodo-L-thyronine inhibits ductal pancreatic adenocarcinoma proliferation improving the cytotoxic effect of chemotherapy

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Abstract

The pancreatic adenocarcinoma is an aggressive and devastating disease, which is characterized by invasiveness, rapid progression, and profound resistance to actual treatments, including chemotherapy and radiotherapy. At the moment, surgical resection provides the best possibility for long-term survival, but is feasible only in the minority of patients, when advanced disease chemotherapy is considered, although the effects are modest. Several studies have shown that thyroid hormone, 3,3',5-triiodo-L-thyronine (T3) is able to promote or inhibit cell proliferation in a cell type-dependent manner. The aim of the present study is to investigate the ability of T3 to reduce the cell growth of the human pancreatic duct cell lines chosen, and to increase the effect of chemotherapeutic drugs at conventional concentrations. Three human cell lines hPANC-1, Capan1, and HPAC have been used as experimental models to investigate the T3 effects on pancreatic adenocarcinoma cell proliferation. The hPANC-1 and Capan1 cell proliferation was significantly reduced, while the hormone treatment was ineffective for HPAC cells. The T3-dependent cell growth inhibition was also confirmed by fluorescent activated cell sorting analysis and by cell cycle-related molecule analysis. A synergic effect of T3 and chemotherapy was demonstrated by cell kinetic experiments performed at different times and by the traditional isobologram method. We have showed that thyroid hormone T3 and its combination with low doses of gemcitabine (dFdCyd) and cisplatin (DDP) is able to potentiate the cytotoxic action of these chemotherapeutic drugs. Treatment with 5-fluorouracil was, instead, largely ineffective. In conclusion, our data support the hypothesis that T3 and its combination with dFdCyd and DDP may act in a synergic way on adenopancreatic ductal cells.


Introduction

Thyroid hormone, 3,3',5-triiodo-L-thyronine (T3) is a lipophilic molecule produced by the thyroid gland that plays an important role in development, differentiation, and metabolism in many tissues and cell types in mammals (Lazar 1993). T3 exerts its effects at genomic and non-genomic levels. The genomic effects of thyroid hormones are mediated by nuclear interaction with thyroid receptors (TRs), which are transcriptional factors (Lazar 1993) belonging to the nuclear receptor superfamily (Evans 1988, Glass 1994, Mangelsdorf et al. 1995, Ribeiro et al. 1995). The ligand–receptor complex binds to specific consensus sequences, called thyroid hormone-responsive elements, located on the promoter region of target genes. This interaction leads to chromatin modification and gene transcription regulation (Umesono et al. 1991). The non-genomic effects of thyroid hormone have been described at the plasma membrane level, in the cytoplasm, and in the cellular organelles. These effects are usually associated with secondary messengers signaling pathways and might in some cases persist in genetically modified mice lacking the classical nuclear receptors (Davis & Davis 1996, D’Arezzo et al. 2004). Numerous studies have shown that T3 is able to promote or inhibit cell proliferation in a cell type-dependent manner (Ledda-Columbano et al. 2005, Misiti et al. 2005, Verga Falzacappa et al. 2006). A thyroid hormone-mediated growth arrest has been observed in different cell lines including rat chondrocyte primary culture (Ballock et al. 2000), murine neuroblastoma N2a-β cells that overexpress the thyroid hormone receptor β1 isof orm (Perez-Juste & Aranda 1999, Garcia-Silva et al. 2002), murine embryonic carcinoma P19 cells (Nygard et al. 2003), and rat oligodendrocyte precursor cells (Tokumoto et al. 1999). An important report has demonstrated that cell proliferation induced by T3 in rat liver is associated with nodule regression and reduction of hepatocellular carcinomas, therefore, this hormone exerts anti-carcinogenic effects in the liver also (Ledda-Columbano et al. 2000). Several data showed that T3 does not inhibit DNA synthesis. Analysis of the cell cycle phases demonstrated, indeed, an increment in the...
number of cells in G0/G1 with increasing cell density and proliferation block (Lebel et al. 1994).

The pancreatic adenocarcinoma is an aggressive and devastating disease, which is characterized by invasiveness, rapid progression, and profound resistance to actual treatments, including chemotherapy and radiotherapy (Kopper et al. 2005). This neoplasia represents the fifth leading cause of cancer death in the western population, having an average survival of 3–6 months after diagnosis and a 5 years survival rate under 5% (Rosenberg 2000). At the moment, surgical resection provides the best possibility for long-term survival (Edge et al. 1993, Allema et al. 1995, Nitecki et al. 1995, Conlon et al. 1996, Mosca et al. 1997, Yeo et al. 1997, 1999, Allison et al. 1998, Finlayson & Birkmeyer 2003), but it is feasible only in the minority of patients (Halloran et al. 2004) and with several collateral risks (Halloran et al. 2002). In advanced disease, chemotherapy is also considered although the anti-proliferative effects are modest. Intrinsic limitations of chemotherapy might be endogenous or due to resistance of pancreatic tumor cells to anti-cancer drugs. At the present time, gemcitabine (dFdCyd), cisplatin (DDP), and fluoropyrimidine 5-fluorouracil (5-FU) are considered the conventional drugs of choice in the treatment of pancreatic cancer (Symon et al. 2002, Eisold et al. 2004, Halloran et al. 2004). dFdCyd inhibits the ribonucleotide reductase and DNA synthesis causing a redistribution of cells into the early S-phase of cell cycle with a radiosensitizing and cytotoxic activity (Huang et al. 1991, Ruiz van Haperen et al. 1993, van Mooresel et al. 2000, Pauwels et al. 2005). DDP is one of the most potent anti-tumor agents known, displaying clinical activity against a wide variety of solid tumors. Its cytotoxic action is mediated by its interaction with DNA to form intrastrand crosslink DNA adducts, which activate several signal transduction pathways, including those involving ataxia telangiectasia-and RAD3-related protein (ATR), p53, p73, and mitogen activated protein kinase (MAPK), and culminate in the activation of apoptosis (Siddik 2003). The fact that DDP-induced DNA damage activates a number of pathways has been borne out from several investigations. One of these pathways culminates in the activation of cell cycle checkpoints, which temporally induce a transient S-phase arrest, followed by inhibition of the Cdc2-cyclin A or B kinase activity to affect a durable G2/M arrest (Shi et al. 1994, Shapiro & Harper 1999). The fluoropyrimidine 5-FU is an anti-metabolite drug that works by incorporating into macromolecules, such as DNA and RNA, and by inhibiting their normal function (Longley et al. 2003). 5-FU exposure resulted in a statistically significant G1/S accumulation (Mirjolet et al. 2002). It has been demonstrated that synergistic interactions between dFdCyd, DDP (Symon et al. 2002), and other drugs are able to potentiate apoptosis in pancreatic cancer (Giovannetti et al. 2004, Modrak et al. 2004). Since the treatment of pancreatic cancer is actually limited and inefficacious, novel therapeutic strategies for the treatment of this cancer patients have to be developed and the utilization of hormones with this purpose is currently under study (Ledda-Columbano et al. 2000, Giovannetti et al. 2004, Halloran et al. 2004, Modrak et al. 2004). A growing body of evidence suggests that different systemic hormones and peptide growth factors may inhibit cancer cell proliferation, representing potential therapeutic agents for the treatment of cancer. Recent reports indicate that the tumor necrosis factor α, and the sex steroid hormone progesterone inhibit breast cancer cell proliferation, and that the peroxisome proliferator-activated receptor γ ligand induces growth arrest in pancreatic cancer cells (Lee et al. 2003). In the present study, we have shown that the thyroid hormone T3 inhibits the proliferation of the pancreatic cancer cell line hPANC–1 and Capan1, and demonstrated that T3 and its combination with either dFdCyd or DDP may potentiate the cytotoxic action of chemotherapy.

Materials and Methods

Cell culture

Human pancreatic ductal cell lines hPANC–1 (aneuploid), HPAC (diploid), and Capan1 (aneuploid) (CRL–1496, HTB–79, and CRL–2119, were purchased from (ATCC; American Type Culture Collection, Manassas, VA, USA). The hPANC–1 cells were maintained as monolayer culture in Dulbecco’s modified Eagle’s medium high glucose (4.5 g/l; Cambrex Corp., East Rutherford, NJ, USA) containing 10% FBS (Cambrex Corp.) and 1-glutamine 2 mM, and supplemented with antibiotics 50 µg/ml streptomycin and 100 µg/ml penicillin. The HPAC cells were maintained in a 1:1 mixture of Dulbecco’s modified Eagle’s medium high glucose (4.5 g/l; Cambrex Corp.) and 1-glutamine 2 mM, and supplemented with antibiotics 50 µg/ml streptomycin and 100 µg/ml penicillin. The HPAC cells were grown under the same culture conditions as treated cells but in the absence of drugs. Final concentrations of NaCl 0.95% and dimethyl sulfoxide (DMSO) were identical in every culture, irrespective of the particular treatment group.

Chemicals

T3 was obtained from Sigma–Aldrich and solubilized in NaOH 0.1 M as stock solution 10−3 M, stored at −20 °C. dFdCyd (Gemzar; Eli Lilly, S.p.A., Italy) was dissolved in 0.9% sodium chloride water at concentration of 0.1334 M as stock solution and stored at 4 °C for 48 h. DDP was...
purchased from Sigma and solubilized in water at 25 °C. Apparent molar solubilities were 0.77 × 10^{-2} M after 24 h. 5-FU (Sigma) was solubilized in DMSO (77 mM) and stored at +4 °C for 4 months protected from light.

**Cell growth**

Cell growth rate was assessed by determining cell number in a Thomas’s hemocytometer. Human PANC-1 and Capan1 cells were plated at a density of 3 × 10^4 in 24-multiwells as a monolayer and after 24 h they were exposed to different doses of T3 (from 10^{-3} to 10^{-9} M). At 1, 2, and 3 days of continuous exposure, viable cells were harvested and counted. Cell number was determined and data are expressed as means ± S.D.

**Cell proliferation ELISA, BrdU**

Cell proliferation was quantified by Cell Proliferation ELISA, 5-bromo-2'-deoxyuridine (BrdU; colorimetric) kit (Roche Applied Science). The hPANC-1 cells were plated at a density of 2 × 10^5 in a 96-well plate. Chemotherapeutic drugs and hormone T3 were added separately or in combination. Pharmaceutical treatments were added once at the beginning of the treatment, while T3 was added at 24, 48, and 72 h. During this labeling period, the pyrimidine analogue BrdU was incorporated in place of thymidine into the DNA of the proliferating cells. After removing the culture medium, cells were fixed and the DNA was denatured in one step by adding FixDenat solution.

The anti-BrdU-conjugated with peroxidase bound to the BrdU was incorporated in newly synthesized cellular DNA. The immune complexes were detected by subsequent substrate reaction. The reaction product was quantified by measuring the absorbance at the respective wavelength using an ELISA reader. The developed color and thereby the absorbance values directly correlate to the rate of DNA synthesis and thereby to the number of proliferating cells in the respective microcultures.

**Cell cycle analysis**

Cell cycle was studied using both iodide propidium (PI) staining (Sigma–Aldrich) and flow cytometry analysis. Treated and untreated hPANC-1, Capan1, and HPAC cell lines were harvested, washed in cold PBS, fixed in 70% ethanol for at least 1 h, and, after removing alcoholic fixative, stained with a solution containing 50 mg/ml PI and 75 KU/ml RNase (Sigma–Aldrich) in PBS for 30 min at room temperature in the dark. Twenty thousand events per sample were acquired using a FACScan cytofluorimeter (Becton Dickinson, Sunnyvale, CA, USA). The percentages of the cell cycle distribution were estimated on linear PI histograms using the MODFIT software (Verity Software House, Inc, Topsham, ME, USA).

**RNA isolation and RT-PCR analysis**

Total cellular RNA was isolated from hPANC-1 cells using RNAeasy kit (Qiagen) according to the manufacturer’s protocol. RNA (2 μg) was then subjected to reverse transcription (RT) using a cDNA synthesis kit OmniScript (Qiagen). Amplification was performed for 30 and 33 cycles for the hTRβ1 and α1 isoforms respectively, at the denaturing temperature of 94 °C for 5 min; annealing temperature (52 °C for TRβ1 and 58 °C for TRα1) for 1 min and an extension temperature of 72 °C for 15–60 s. PCR products were electrophoresed onto a 1-5% agarose gel containing ethidium bromide (0.5 μg/ml) and visualized under u.v. light. GAPDH expression levels were analyzed as control for mRNAs quality. PcDNA1/TRβ1 and α1 expression vectors (kindly obtained from Prof. Paul M Yen) were used as positive control to verify if primers were specific for the different TR isoforms. The molecular weight of transcripts was controlled by the 1 kb Plus DNA ladder (Invitrogen, Life Technologies).

**Protein assay and western blot analysis**

Approximately, 5 × 10^6 cells were incubated for 30 min at 4 °C in lysis buffer (1% Tween 20, 10% glycerol, 150 mM NaCl, 50 mM HEPES pH 7.0, 1 mM MgCl2, 1 mM CaCl2, 100 mM NaF, 10 mM Na3P2O7, 2 mM NaVO3, 1 mM phenylmethylsulphonyl fluoride, protease inhibitors). The lysates were centrifugated at 16 000 × g for 30 min and the total cellular protein content was measured using the Bradford method (Bio-Rad). Seventy microgram of proteins per sample were loaded onto a 12% SDS-polyacrylamide gel, electrophoresed, and then blotted onto nitrocellulose membrane (Bio-Rad). As a loading control, proteins were stained with Ponceau. Filters were blocked for non-specific reactivity by incubation at 4 °C in 5% non-fat dry milk dissolved in Phosphate Buffered Saline PBS 1× (Tris–HCl pH 8.0 10 mM, NaCl 150 mM), Tween 20 0.05% and then probed with mouse anti-TRβ1 (Santa Cruz Biotechnology, Inc.; Santa Cruz, CA, USA, 1:500); rabbit anti-TRα1 (Santa Cruz Biotechnology, Inc.; 1:500); mouse anti-cyclin D1 (BD Pharmingen; 1:250); mouse anti-cyclin E (BD Pharmingen, 1:250); mouse anti-p27kip (BD Pharmingen, 1:250); rabbit anti-p21 (Santa Cruz Biotechnology, Inc.; 1:1000); mouse anti-β-Actin (Sigma–Aldrich; 1:1000). After three washes in PBS 1× and Tween 20 0.1%, the membranes were incubated with the secondary antibodies (anti-rabbit, anti-mouse, 1:4000; Sigma) for 45 min at room temperature. Immunoreactivity was visualized by the ECL immunodetection system (Amersham Corp; Arlington Heights, IL, USA) following manufacturer’s instructions. The molecular weights of the proteins were controlled by the prestained SDS-PAGE marker (Bio-Rad) or by ECL DualVue western blotting marker (Amersham Corp).
Immunoprecipitation

hPANC-1 cells were plated at a density of $3 \times 10^5$ in 100 mm dishes and exposed to T3 for the indicated times. The cellular pellets were incubated for 15 min in lysis buffer containing NP40 1%, phenylmethylsulphonyl fluoride (PMSF) 0.2 mM, NaF 10 mM, pepstatin 0–7 μg/ml, aprotinin 25 μg/ml in PBS 1X. After 10 min on ice, samples were sonicated and centrifuged at 12 000 g for 15 min. The total cellular protein content was measured using the Bradford method (Bio-Rad). For CDK4-cyclin D1 or CDK2-cyclin E immunoprecipitations, 400 and 250 μg cell lysates were incubated for 1 h with 30 μl G-protein or A-protein respectively (Sigma–Aldrich) to reduce non-specific cell lysate-G/A protein binding (preclearing). Then, the cell lysates were incubated with mouse anti-CDK4 or rabbit anti-CDK2 (BD Pharmingen, 1 μg) and freshly prepared G/A-protein (30 μl) overnight at 4°C. The immunoprecipitates were electrophoresed onto a 12% SDS-polyacrylamide gel and blotted onto nitrocellulose membrane.

Analysis of the phosphorylated pRb and H1 proteins

The cells were plated at a density of $3 \times 10^5$ in a 100 mm dishes and exposed to T3 for the indicated times. After washing in ice-cold PBS 1X, the cells were incubated for 20 min in ice-cold lysis buffer (50 mM Tris–HCl pH 7.2, 1% NP40, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 1 mM Na3VO4, 1 mM PMSF, 1 mM NaF, 25 μg/ml aprotinin, 40 nM leupeptin and 2 μg/ml pepstatin). The cell lysates were centrifugated at 16 000 g for 15 min at 4°C and the total cellular protein content was measured using the Bradford method (Bio-Rad). Fifty micrograms of protein were electrophoresed onto an 8% SDS-polyacrylamide gel and blotted onto nitrocellulose membrane (Bio-Rad). Filters were blocked with 5% non-fat dry milk dissolved in TBS 1X (Tris–HCl pH 8.0 10 mM, NaCl 150 mM), Tween 20 0.05% for 1 h at room temperature and then probed with mouse anti-human-pRb antibody (BD Pharmingen, 1:250: the anti-pRb antibody recognized the hypophosphorylated and the phosphorylated (ppRB) forms of the protein) and rabbit anti-human pH1 antibody (1:500) diluted in 5% (for pRb) and 1-25% (for pH1) non-fat dry milk in PBS 1X-Tween 20 0.1% overnight at 4°C under gentle rocking.

Quantitative analysis of synergism by isobologram

The traditional isobologram method was used to analyze the effect of the chemotherapeutic–T3 combinations. Drug concentrations producing the same 40% of growth inhibition at 24 h of exposure (IC40) were selected to outline the line of additivity: dFdCyd 20 nM, DDP 5000 nM, and T3 $10^{-7}$ M. Since below these concentrations the effect of the two drugs was linear, fractions of these concentrations were combined to observe which one was able to get the same effects as 20 nM dFdCyd, 5000 nM DDP or $10^{-7}$ M T3. If the results of combination treatments were coincident with the line of additivity, the action of the two drugs has to be considered additive, if located above the line of additivity the combination is antagonistic and finally if below the line the interaction can be considered synergic.

Statistical analysis

The data were presented as means ± s.d. A comparison of the individual treatment was conducted using Student’s t-test or, if there were more than two groups, by one-way ANOVA, followed by Dunnett or Tukey post hoc analyses. A P-value < 0.05 was considered significant.

Results

Expression of thyroid hormone receptors in human adenocarcinoma cell lines.

We first confirmed the presence of the main thyroid hormone receptor α1 and β1 isoform mRNA and protein levels in the hPANC-1, Capan1 and, HPAC cell lines selected for this study (Fig. 1). Total RNA was reverse transcribed and amplified by PCR using distinct sets of primers designed in the specific regions of each isoform. Two PCR products (160 and 324 bp) revealed the presence of TRα1 and TRβ1 mRNA and, their specificity was controlled by amplification of pcDNA1/TRα1 and β1. In addition, the TRα1 (48 kDa) and TRβ1 (52 kDa) proteins were detected by western blot (Fig. 1). For this experiment, we used different controls to validate the antibody specificity: as positive control we utilized C32 nuclear extract (Santa Cruz Biotechnology, Inc.) and, as negative control we used human insulinoma cell line CM transfected with siRNA SmartPool THRB1 (Dharmacon, Lafayette, CO, USA; Verga Falzacappa et al. 2007) versus its wild-type control. We used the same extracts (hCM wt and hCM TRβ1siRNA) also to validate the antibody against TRα1. The expression level of TRα1 was lower than TRβ1 and not influenced by the RNA interference supporting the specificity of the antibodies utilized. Our results indicate that the cell lines examined express the main α1 and β1 isoforms of TR, resulting in suitable experimental models for our study.

T3 treatment inhibits cell growth in human pancreatic adenocarcinoma cell lines

The thyroid hormone effects on cell growth of hPANC-1, Capan1, and HPAC cell lines were determined by culturing the cells in the presence or in the absence of different T3 concentrations (ranging $10^{-3}$–$10^{-9}$ M) and by counting the number of adherent cells in treated versus control samples (Fig. 2). The T3 $10^{-8}$ and $10^{-9}$ M doses showed a low inhibitory effect. The T3 $10^{-5}$, $10^{-6}$, and $10^{-7}$ M doses

showed a much stronger anti-proliferative effect and, the dose 10^{-5} M was selected for our study because it was the strongest and the best one to stress the effect in our in vitro cell system. The 10^{-3} and 10^{-4} M doses were toxic for the cells (data not shown). To establish the period of time with the maximum T_3 effect, we performed a kinetic analysis at different times: 24, 48, and 72 h in the different lines. An inhibitory effect on cell growth was detected after hormone treatment in hPANC-1 and Capan1 cells, at all the considered times, while the same effect was not observed in HPAC cells (data not shown). In hPANC-1 cells (Fig. 2a) the anti-proliferative effect was 30% after 24 h and reached a maximum value of 45% after 72 h of T_3 treatment. A comparable anti-proliferative effect was observed using the same hormone dose in Capan1 cells too (Fig. 2b).

In order to determine which phase of the cell cycle was affected by T_3 treatment, we have analyzed the cell cycle distribution in hPANC-1, Capan1, and HPAC cells by PI staining and flow cytometry applying the MODFIT LT™ software to each PI histogram. The analysis, performed at different times (24, 48, and 72 h) after T_3 exposure, revealed an accumulation of the...
hormone-treated hPANC-1 cells in the diploid and aneuploid G1 phase (Table 1), similar results were obtained with the Capan1 cells. As shown in Table 1, the cells were accumulated in the G1 phase of the cell cycle (68% compared with 44% of control cells) already at 24 h. After 72 h of hormone treatment more than 80% of the cells were blocked in G1 compared with the 47% of control cells. The arrest in the G1 phase was associated with a concomitant depletion of cells from both the S and G2/M cell cycle compartments. No effect was observed on the HPAC cell line. The subsequent experiments reported in this paper, have been performed mainly on hPANC-1 cells because the Capan-1 cell line always showed similar results.

T3 inhibits the expression of the D1 and E cyclins and induces the expression of p21 and p27 cdk inhibitors (CDKI) involved in G0/G1 arrest

In order to investigate at the molecular level the anti-proliferative effect observed, we studied the influence of T3 treatment on the expression of the main proteins that control the cell cycle progression. In particular, western blotting analysis of cyclins and CDKI involved in the G0/G1 phase transition was performed on the cells on examination. Immunoblots (Fig. 3) showed a significant decrease of cyclin D1 (36 kDa) and E (55 kDa) expression levels in hPANC-1 cells after 24 and 48 h of T3 treatment. The reduction observed reached a maximum after 48 h of T3 exposure for cyclin D1 (95%) and for cyclin E (40%), with the accumulation of cells in the G1 phase consistently observed. Moreover, the hormone treatment caused a significant increase of the CDKIs p21cip1 (35%) and p27kip1 (200%) expression levels versus control at the same times. Western blot for β-actin (44 kDa) was used as control for protein loading.

T3 inhibits the kinase activity of cyclin–CDK complexes

To elucidate the significance of the reduced expression levels of cyclins D1 and E, we verified the T3 effects on the cyclin D1-CDK4 and cyclin E-CDK2 complexes. The hPANC-1 cells were treated with T3 $10^{-6}$ M for 24 and 48 h and the
The percentages in the cell cycle compartment were estimated by applying the MODFIT software to each DNA histogram. The flow cytometric analysis of DNA content was performed after PI staining, before (C, controls) and after treatment with T3 (10⁻⁵ M) for the indicated times. The percentages in the cell cycle compartment were estimated by applying the MODFIT software to each DNA histogram. The flow cytometric analysis of DNA content was performed after PI staining, before (C, controls) and after treatment with T3 (10⁻⁵ M) for the indicated times. The percentages in the cell cycle compartment were estimated by applying the MODFIT software to each DNA histogram.

Table 1 Cell cycle analysis in hPANC-1, Capan1, and HPAC cells. Data are means ± S.E.M. of three separate experiments with similar results.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Diploid</th>
<th>Aneuploid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G1</td>
<td>S</td>
</tr>
<tr>
<td>hPANC-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>Control</td>
<td>43·99±0·3</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>51·00±1·2</td>
</tr>
<tr>
<td>48</td>
<td>Control</td>
<td>40·00±0·9</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>52·24±0·9</td>
</tr>
<tr>
<td>72</td>
<td>Control</td>
<td>50·00±1·8</td>
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<tr>
<td></td>
<td>T3</td>
<td>60·00±1·0</td>
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<tr>
<td>Capan1</td>
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<td></td>
</tr>
<tr>
<td>24</td>
<td>Control</td>
<td>44·00±1·1</td>
</tr>
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<td></td>
<td>T3</td>
<td>68·00±0·3</td>
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<tr>
<td>48</td>
<td>Control</td>
<td>42·00±0·8</td>
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The flow cytometric analysis of DNA content was performed after PI staining, before (C, controls) and after treatment with T3 (10⁻⁵ M) for the indicated times. The percentages in the cell cycle compartment were estimated by applying the MODFIT software to each DNA histogram.

cyclin–CDKs association was analyzed by co-immunoprecipitation experiments. The cyclin D1–CDK4 complex formation was completely inhibited by 24 h of hormone treatment, suggesting that cyclin D1 levels were not sufficient to promote its association with the kinase CDK4 in the T3-treated cells. The IgG levels were used as a control for protein pull down (data not shown). Even the cyclin E–CDK2 complex formation was completely inhibited by 24 h of T3 treatment, although a weak signal was detectable after 48 h in T3-treated cells compared with control (Fig. 4a). Consistent with the inhibitory effect observed on the cyclin D1–CDK4 and cyclin E–CDK2 complex formation, a reduction in

![Figure 3](https://www.endocrinology-journals.org)
CDKs activity has been observed. The kinase activity was tested by western blotting analysis of the phosphorylation of pRB and H1 histone proteins, the main substrates of cyclin D1-CDK4 and cyclin E-CDK2 complexes respectively. The hPANC-1 cells were treated with the same dose of T3 for 24 and 48 h for pRB and H1 histone protein detection. As shown (Fig. 4b) T3 treatment reduced the phosphorylation of pRB and the effect was accompanied by a concurrent increase in the hypophosphorylated form. Thus, T3 maintains the activation of hypophosphorylated pRB by inhibiting its association with E2F factor and the G1 to S transition. In addition, the histone H1 phosphorylation, which is performed by the cyclin E–CDK2 complex, was little affected after 24 h, while the reduction was more evident after 48 h of T3 treatment, thus indicating a further signal that attends to G0/G1 growth arrest.

Analysis of synergism

We tested the hPANC-1 cell line for its sensitivity to the three drugs with a clinical relevance to pancreatic cancer, dFdCyd, DDP and 5-FU, and their IC50 is shown (Fig. 5a–c). The variations in the proliferative activity of these cells were measured using the BrdU cell proliferation assay and expressed as percentage of viability. The sensitivity of the hPANC-1 cells to the selected drugs was different; the IC50 value for dFdCyd was 75 nM, while the IC50 of the DDP was approximately 5000 nM and, for 5-FU was 500 μM. We analyzed also the IC50 of the T3 (>10^{-4} M) to make uniform the following experiments (Fig. 5d). The T3 inhibition effect was evaluated by BrdU cell proliferation assay, obtaining a maximum value of 35% after 72 h of T3 treatment; with this method we always got a lower percentage of cell proliferation inhibition in comparison with the value obtained by counting the number of adherent cells. Subsequently, we analyzed the effects of different concentration of drugs (dFdCyd 1 and 10 nM, DDP 10 and 100 nM, 5-FU 0.5 and 10 μM) alone and in combination with T3, using always much lower doses.

Figure 4 T3 inhibits the formation of cyclin E-CDK2 and cyclin D1–CDK4 complexes and their kinase activities. hPANC-1 cells were treated with T3 10^{-5} M for 24 and 48 h. The cyclinD1–CDK4 and cyclin E–CDK2 complexes were detected by immunoprecipitation (IP) analysis, using anti-CDK4 and anti-CDK2 antibodies, followed by immunoblot analysis of cyclin D1 and cyclin E in treated sample and in control (C) untreated sample (a). The kinase activity was evaluated by western blotting (WB) analysis of the pRB and H1 histone phosphorylation levels (b).

Figure 5 Effects of chemotherapic treatments and T3 on growth of hPANC-1 at 24 h of exposure (IC50). hPANC-1 cells were plated in 96-well plates and incubated for 24 h with increasing concentrations of gemcitabine (a), cisplatin (b), 5-fluorouracil (c), and T3 (d). All the data are represented as means ± S.D. and were the results of five individual experiments at least. A comparison of the individual treatments was conducted using Student’s t-test.
of chemotherapeutics than the IC₅₀ values. The T₃ hormone showed the maximum inhibitory effect combined with dFdCyd 10 nM reaching a value of 73% cell inhibition after 72 h, while with dFdCyd 1 nM the effect was maximum (33%) after 48 h of combined treatment (Fig. 6a). The thyroid hormone showed an inhibitory effect even when combined with DDP 100 and 10 nM, although in this case the combination treatments were less effective than with dFdCyd because their potency decreased with time (Fig. 6b). The analysis of two 5-FU concentrations did not get a good inhibitory effect on our cell models and in addition did not improve the effect of T₃ (Fig. 6c).

The isobologram method was used to confirm the synergism observed between the chemotherapeutic drugs (dFdCyd and DDP) and T₃ at the IC₄₀ level to minimize the toxic effects of drugs (Loewe 1953, Cusinato et al. 2006). Experimental combination therapy data points were located well below the expected additive line at each IC₄₀ value for both combinations indicating the presence of a strong synergism across a broad range of doses and agents (Fig. 6d and e).

To evaluate whether the different sensitivity of the cells to treatment with dFdCyd and DDP alone or in combination with T₃ was associated with different cell cycle perturbations, we analyzed cell cycle by PI staining and flow cytometry (Table 2).

dFdCyd 10 nM and T₃ treatments when used separately, produced a similar (more than 60%) block in the aneuploid G₁ phase of cell cycle versus control cells (41%) at 24 h. Consequently, T₃ treatment, used alone,
led to a stronger G1 arrest reaching 84% after 72 h, compared with 68% in the dFdCyd-treated cells (control value 42%). Moreover, T3 caused a persistent accumulation also in the diploid G1 peak, while dFdCyd exposure revealed different effects on the diploid population; 24 h after treatment the amount of S-phase cells was 67% compared with 46% in the control cells. In the following 72 h, the S-phase percentage decreased from 67 to 35% (control value 39%) concurrently with a 19% increase in G1 phase, indicating that cells were able to recover from the dFdCyd-induced damage. dFdCyd 10 nM was more effective when administered with T3, determining the maximum inhibitory effect on cell proliferation. The histograms of DNA content revealed that the cells were arrested in G1 phase in both diploid and aneuploid cell populations, reaching 79 and 100% respectively, at 72 h, compared with control cells (50 and 42% respectively), suggesting that dFdCyd 10 nM administered in combination with T3 was able to completely impair DNA synthesis, thus demonstrating the synergistic effect of this drug with T3. dFdCyd alone at the dose of 1 nM did not affect significantly the cell cycle. In contrast, when used in combination with T3 for 24 h the cell cycle was perturbed. In fact, a significant G1 increase in the aneuploid compartment compared with control cells was observed (75 versus 41% respectively), thus strongly supporting the hypothesis that T3 could promote cell cycle G1 arrest.

DDP alone did not alter the cell cycle distribution of the diploid cell population, while it induced S-phase accumulation of cells in the aneuploid population (66%) after 24 h treatment that was recovered within the subsequent 72 h. The combined treatments of T3 with DDP was less effective than the ones with dFdCyd; indeed when cells were treated with DDP plus T3, the cells were blocked in G1 phase (74%) at 24 h, but within the following 48 h a fraction of cells returned to the G1 phase (68% compared with 46% in the dFdCyd-treated cells (control value 39%) concurrently with a 19% increase in the aneuploid peak compared with control cells was observed (75 versus 41% respectively), thus strongly supporting the hypothesis that T3 could promote cell cycle G1 arrest.

An additional set of experiments was carried out to confirm the ability of T3 treatment to modulate the expression of proteins that control cell cycle progression in combination with dFdCyd and DDP at 24 and 48 h. Western blotting analysis of the main cell cycle-related molecules (Fig. 7) revealed that T3 in combination with chemotherapy treatments induced a further decrease in both cyclin D1 and cyclin E expression reaching a maximum at 24 h of exposure.

Untreated (control) and treated cells (T3, dFdCyd 10 nM, dFdCyd 10 nM+T3, dFdCyd 1 nM, dFdCyd 1 nM+T3, DDP 100 nM and DDP 100 nM+T3) were harvested, fixed in ethanol 70% and stained with PI at the indicated times. The percentages in the cell cycle compartments were estimated by applying the MODFIT software to each DNA histogram.
while these combination treatments did not improve the increase of CDKI p21cip1 and p27kip1 expression observed with T3 treatment alone at the indicated times (Table 3).

Discussion

Recent reports have shown that several natural peptides, including thyroid hormones, are able to inhibit cancer cell proliferation (Lee et al. 2003, Saji et al. 2005, Vesely 2005, Yano et al. 2006). In the present study, we have demonstrated that T3 hormone exposure exerts an anti-proliferative effect on human ductal pancreatic adenocarcinoma cell growth, and that it is able to increase the effect of dFdCyd and DDP acting synergistically with them. Availability of several new active drugs has improved the efficacy of combination regimens, and increased the response rate of refractory tumors, including pancreatic cancer (Symon et al. 2002, Kulke 2003, Macdonald 2004).

Figure 7 Effects of low doses of gemcitabine, cisplatin, and drug T3 combined treatments on cell cycle related proteins. hPanc-1 cells were plated in the presence of T3 10^{-5} M, gemcitabine 1 nM, and 10 nM, cisplatin 100 nM and their combinations for 24 h (a) and 48 h (b). Western blotting analyses were performed as described in Materials and Methods. Densitometric absorbance values from three separate experiments were averaged (±S.D.) after they had been normalized to β-actin for equal loading. Data are presented in the histograms as percentages of control (100%) on x-axis (a and b). All the data are presented as means ± S.D. and are the results of three individual experiments at least. A comparison of the individual treatment was conducted using one-way ANOVA followed by Dunnett post hoc test. *P<0.05; †P<0.01. A P-value <0.05 was considered significant. C, control.

Table 3 Summary of the effects of gemcitabine (dFdCyd) and cisplatin (DDP) and their combination with T3 on protein expression

<table>
<thead>
<tr>
<th>Protein</th>
<th>T3</th>
<th>dFdCyd 10 nM</th>
<th>dFdCyd 1 nM</th>
<th>DDP 100 nM</th>
<th>dFdCyd 10 nM+T3</th>
<th>dFdCyd 1 nM+T3</th>
<th>DDP 100 nM + T3</th>
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<td>Cyclin D1</td>
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<td>P21</td>
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<td>P27</td>
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↓, ↑, or – indicates a decrease, increase, or no change respectively, in protein expression relative to control cells.
As a first step, we have investigated the effect of different T3 concentrations on cell growth, and the dose of $10^{-3}$ M was chosen for our study to stress the observed effect in our cell systems. We have used pharmacological concentrations of T3, even though it could cause long-term side effects such as hyperthyroidism, tachycardia, astenia, loss of weight, atrial fibrillation etc. (McKeown et al. 2005). All these complications can be managed by pharmacological therapy in contrast with the inefficacy of treatments for this cancer. The translation in vivo of these results obtained with in vitro cell systems of course will need a dose response analysis to choose the right concentration. At the moment surgical resection provides the best possibility for long-term survival, but is feasible only in the minority of patients and it is not without risk. In advanced disease chemotherapy is considered, although the effects are still modest. Pancreatic cancer is a tumor that represents the fifth leading cause of cancer death in the western population with an average survival after diagnosis of 3–6 months and a 5 years survival rate under 5%.

Three human cell lines hPANC-1 (aneuploid), Capan1 (aneuploid), and HPAC (diploid) have been used as experimental models to investigate the T3 effects on ductal pancreatic adenocarcinoma cell proliferation. The hPANC-1 and Capan1 cells were responsive to T3 treatment, HPAC cells were, instead, not influenced by the hormone treatment. The T3-dependent cell growth inhibition was also confirmed by FACS analysis indicating a substantial reduction of cell population entering in S phase and evidencing an increase in the percentage of resting cells in G0/G1 phase of the cell cycle, as already reported for Neuro-2a cells, (Lebel et al. 1994, Garcia-Silva et al. 2002) rat chondrocyte primary culture (Ballock et al. 2000), murine embryonic carcinoma P19 cells (Nygard et al. 2003), and rat oligodendrocyte precursor cells (Tokumoto et al. 1999). It is widely recognized that anti-proliferative agents can induce cell cycle perturbations associated with growth arrest (Otto et al. 1996, Marchal et al. 2004). In this context our results indicated that T3 not only exerts an anti-proliferative action on our cells, but is also able to alter the cell cycle distribution. The transition between G1 and S phase is one of the most important regulated steps in the cell cycle process and the entering S phase is strictly regulated both in the early and the late G1 by the balance between cell cycle activators (CDKs) and cell cycle inhibitors (CDKIs; Park & Lee 2003, Deshpande et al. 2005). According to these observations, the inhibition of the formation of cyclin D1–CDK4 and cyclin E–CDK2 complexes required for CDKs activity has been observed in T3-treated cells and this effect has been shown to be a consequence of the reduced levels of D1 and E cyclins, already observed at 24 h of hormone treatment. The Cip/Kip family of CDKI, which are targeted by different growth inhibitory and differentiation signals, includes p21$^{\text{Cip1}}$, p27$^{\text{Kip1}}$, and p57$^{\text{Kip2}}$ that bind to and inhibit all the G1 cyclin–CDK complexes. In agreement with the important role of CDKIs on cell cycle progression inhibition, we found that T3 induces a strong and significative increase in p21$^{\text{Cip1}}$ and p27$^{\text{Kip1}}$ expression levels in hPANC-1 cells. This effect might be associated with the reduction in pRB and H1 histone phosphorylation caused by T3 treatment, because of the ability of CDKIs to bind CDK2 and CDK4 and to inhibit their kinase activity. One of the molecular events required for cell cycle progression is the inactivation by hyperphosphorylation of the pRB family proteins once activated (Herwig & Strauss 1997); pRB associates with E2F factor and represses the transcription of target genes involved in the progression through the G1/S restriction point of the cell cycle. Phosphorylation of both the pRB family member and the histone H1 is catalyzed by the CDK kinase; therefore, the significant T3-dependent decrease in pRB and H1 histone phosphorylation we observed was found to be directly due to the cyclin D1-CDK4 and cyclin E-CDK2 complex activity reduction. Once we had characterized the T3 effect on proliferation at the molecular level, we analyzed the capacity of T3 to improve the sensibility to chemotherapy when used in combination treatments. To this aim, we have selected drugs that are known to have clinical relevance to pancreatic cancer, namely dFdCyd, DDP, and 5-FU. The intrinsic toxicity of these drugs was examined after 24 h treatments to evaluate the IC50, so that for the combination experiments we could choose drug concentrations much below the IC50, thus minimizing the toxic effects of treatments. dFdCyd 10 nM was already effective in inhibiting the cell growth, but the addition of T3 treatment enhanced significantly its chemosensitivity; on the other hand dFdCyd 1 nM showed a little increase in toxicity only if combined with T3.

We obtained good results also with the combination of DDP 100 nM and T3. The cytotoxic effect of DDP was constant during the first 48 h treatment, but after 72 h the cells began to grow again slowly; this is different from treatment with dFdCyd that showed an increase of cytotoxicity until the end of treatment. Recent studies suggested that the overexpression of cyclin D1 could contribute chemoresistance to DDP therapy in pancreatic cancer cells since dual roles of cyclin D1 in promoting cell proliferation and inhibiting drug-induced apoptosis have been shown (Biliran et al. 2005). It was reported that the inhibition of cyclin D1 expression, using an anti-sense strategy, not only suppressed pancreatic cancer cell growth, but also potentiated the anti-proliferative effect of DDP (Kornmann et al. 1999). Overall, these studies suggested that cyclin D1 exerts a protective effect against drug-induced cytotoxicity. The molecular mechanisms of cyclin D1-mediated chemoresistance, however, remains to be identified (Biliran et al. 2005). In agreement with this data, we have demonstrated that T3 decreased cyclin D1 levels and we can speculate that the increase of the anti-proliferative effect of DDP induced by T3 is associated to this T3-dependent cyclin D1 inhibition.

Chemotherapy with 5-FU was largely ineffective in hPANC-1 cells probably because of inactivation in this cell line of the $p53$ gene, which is implicated in cell cycle arrest.
The p53 tumor suppressor gene is functionally inactivated in about 50% of all human malignancies, including up to 60% of pancreatic cancer (Ghaneh et al. 2002). Indeed several reports have suggested that the p53 status of the tumor cells may be an important response determinant to 5-FU-based chemotherapy (Ahnen et al. 1998, Lenz et al. 1998, Eisold et al. 2004). Even though we already knew that expression of wt-p53 was required for 5-FU-induced apoptosis and to potentiate the 5-FU-cytotoxicity (Lowe et al. 1993, Bunz et al. 1999, Eisold et al. 2004), we decided to verify if the combination 5-FU and T3 could inhibit the tumor proliferation in a p53-independent manner (Dinda et al. 2002). We have shown that T3 was able to enhance the inhibitory effect of chemotherapy on cell growth at 24, 48 and 72 h, and the existence of synergism between T3 and the selected drugs has been demonstrated by the traditional analysis of isobologram. Drug concentrations producing the same 40% of growth inhibition at 24 h of exposure (IC40) were selected to outline the line of additivity: dFdCyd 20 nM, DDP 5000 nM, and T3 $10^{-4}$ M. dFdCyd and DDP were already effective as anti-proliferative agents, but the addition of T3 treatment enhanced significantly their chemosensitivity. The drug combination doses, indeed, were plotted well below the additive line suggesting that lower doses of drug combinations were able to produce the same IC40 as the individual drug. The effect of T3 hormone in combination with dFdCyd 1 nM, 10 nM, and DDP 100 nM on cell cycle distribution and protein expression has also been evaluated using FACS and western blotting analysis. FACS analysis of cells treated with dFdCyd 10 nM revealed an arrest of cells in G1 phase in both diploid and aneuploid cell populations at 72 h, while only the diploid cell population was arrested in S phase at 24 h of exposure. dFdCyd 1 nM did not affect the cell cycle significantly.

Our data confirm that the cytotoxic effects of dFdCyd are cell line specific and dependent on drug concentration and exposure time (Mose et al. 2003). It has been reported, indeed, that in some human solid tumors, cells were blocked in the S phase depending on the exposure time and chemotherapeutic concentration (Cronauer et al. 1996, Ng et al. 2000). In contrast, other authors reported an arrest in G1 phase only depending on the dFdCyd concentration. In our hands, dFdCyd 10 and 1 nM showed an inhibitory interaction with T3 rising during the time of exposure, inducing a greater cell arrest in G1. The data obtained with the FACS analysis were in accordance with a further decrease of cyclins D1 and E and with an additional increase of p27 expression levels observed in the combination treatments. On the other hand, the p21 regulatory protein was not modulated significantly. Considering our data, we hypothesize that the G1 phase accumulation induced by T3 treatment could lead to the enhancement of dFdCyd efficacy. FACS analysis of hPANC-1 cells treated with DDP 100 nM showed an S phase accumulation of aneuploid population cells after 24 h treatment that was recovered within the subsequent 72 h. The cell cycle distribution of the diploid cell population was not altered. Other groups have shown that high DDP concentrations caused an accumulation of cells in S/G2 phase (Chang et al. 2002, Mack et al. 2003), whereas low DDP doses induced an increase in p21 expression suggesting an arrest in G1 (Lanzi et al. 1998, Crescenzi et al. 2006). In our system the T3-DDP combination treatment blocked the aneuploid cell population in G1 phase at 24 h, but the cells then progressed in S phase within 48 h and entered in G1 phase at 72 h. These observations suggest that cells were able to recycle through the cell cycle after the G1 block and are consistent with the reduced anti-proliferative effect caused by DDP plus T3. This DDP dependent G1 block was proportional with the reduction of D1 and E cyclins we observed and with the increase of p21 and p27 expression levels; in combined treatments, the cyclins reduction was only enhanced at 24 h, while p21 and p27 returned to basal levels within 24 h, consistent with the cells’ ability to recycle.

In conclusion, this study characterizes the potential mechanisms involved in the synergistic effect of thyroid hormone T3 with dFdCyd and DDP against in vitro models of pancreatic cancer and provides the experimental basis for the rational development of these combinations for the treatment of this malignancy.

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