**T3 preserves ovarian granulosa cells from chemotherapy-induced apoptosis**

Cecilia Verga Falzacappa1,4, Eleonora Timperi1, Barbara Bucci2, Donatella Amendola2, Piero Piergrossi2, Davide D’Amico3, Maria Giulia Santaguida4, Marco Centanni4 and Silvia Misiti1

1Dipartimento di Medicina Sperimentale, Cattedra di Endocrinologia, Sapienza Università di Roma, c/o Servizio Speciale Malattie della Tiroide, Viale Regina Elena 324, 00161 Rome, Italy
2Centro Ricerc, Ospedale San Pietro FBF, Rome, Italy
3Dipartimento di Medicina Molecolare, Sapienza Università di Roma, Rome, Italy
4Dipartimento di Scienze e Biotecnologie Medico Chirurgiche, Sapienza Università di Roma, Latina, Italy

(Correspondence should be addressed to S Misiti; Email: silvia.misiti@uniroma1.it)

**Abstract**

Infertility is a dramatic and frequent side effect in women who are undergoing chemotherapy. Actual strategies are mainly focused on oocyte cryopreservation, but this is not always a suitable option. Considering the key role that granulosa cells play in follicle life, we studied whether thyroid hormone 3,5,3'-triiodothyronine (T3) protects rat ovarian granulosa cells from chemotherapy-induced apoptosis. To this aim, a cell line was established from fresh isolated rat granulosa cells and named rGROV. Cells were exposed to paclitaxel (PTX) and T3, and apoptosis, cell viability, and cell cycle distribution were analyzed under different conditions. First, the integrity of the steroidogenic pathway was demonstrated, and the presence of thyroid receptors, transporters, and deiodinases was confirmed by quantitative PCR. Cells were then exposed to PTX alone or contemporary to T3. MTT and TUNEL assays revealed that while there was a relevant percentage of dying cells when exposed to PTX (40–60%), the percentage was sensibly reduced (20–30%) in favor of living cells if T3 was present. Cell cycle analysis showed that cells exposed to PTX alone were first collected in G2 and then died by apoptosis; on the other hand, the T3 granted the cells to cycle regularly and survive PTX insult. In addition, western blot and FCM analyses confirmed that caspases activation, casp 3 and Bax, were downregulated by T3 and that Bcl2 and cyclins A and B together with cdk1 were upregulated by T3. In conclusion, we demonstrated that thyroid hormone T3 can counteract the lethal effect of taxol on granulosa cells.

**Introduction**

Exposure to chemotherapy causes various adverse effects on the ovaries, including premature ovarian failure and infertility. Over the last decades, great attempts have been made to minimize the toxic effects of chemotherapy on the ovaries (Meirow & Nugent 2001, Ben-Aharon et al. 2010, Tomao et al. 2010). Moreover, cancer-related infertility is still considered a marginal aspect of the quality of life in cancer patients. Adjuvant chemotherapy causes a progressive dose-related depletion of ovarian follicles and granulosa cells leading to oligoamenorrhea and subsequent chemotherapy-induced amenorrhea (CIA). This condition can be strongly invalidating when occurring in premenopausal women, being related to a rapid depletion of the oocyte reserve, mediated by the apoptotic cell death and ovarian atrophy with disappearance of primordial follicles. The incidence of CIA varies with the type, dosage, and schedule of chemotherapy and is directly related to age (Padmanabhan et al. 1986, Swain et al. 2010). Reversible amenorrhea will result when maturing follicles are destroyed, differently when all primordial follicles are destroyed, permanent amenorrhea, or premature ovarian failure will happen. Recent data suggest that the major concern of patients receiving chemotherapy is that of premature menopause and infertility (Maltaris et al. 2008, Kim et al. 2011). This is becoming a more pressing issue as, over the last 30 years, there has been a trend toward delaying child bearing. Although loss of fertility is an important issue for young cancer survivors, there is often little discussion about fertility preservation before initiation of adjuvant therapy. The impact of infertility on the quality of life of the patient who survives breast cancer explains the increase in the demand for fertility preservation techniques. Neither embryo nor oocyte cryopreservation is a practical option for women who cannot delay cancer treatment. These aspects are more relevant in breast cancer patients, mainly in the early phase of the disease, due to the high probability to retain reproductive possibilities also after aggressive and integrated therapies. In addition, breast cancer is the most common cancer in fertile premenopausal women and constitutes...
an emerging reproductive problem for three different reasons: the increasing rate of young patients in fertile age, the lack of specific guidelines for gonadic failure prevention after chemotherapy, and the conflicting and unresolved debates about the prognosis and risks of pregnancy occurring in women after breast cancer (De la Haba-Rodriguez & Calderay 2010).

Chemotherapeutic agents can be grouped depending on the severity of their impact on ovarian reserve. Taxanes are classified as high risk, provoking amenorrhea in 62–83% of cases (Najafi et al. 2011, Pérez-Fidalgo et al. 2010). Paclitaxel (PTX) and mitoxantrone, frequently used drugs for the treatment of advanced breast cancer, are microtubule-stabilizing agents, which kill malignant cells by inducing both cellular necrosis and apoptosis. They exert their cytotoxic effects via different mechanisms, including the inhibition of microtubule reorganization during interphase followed by cell cycle arrest, cell cycle nonspecific interaction with DNA and DNA cleavage, and the activation of several caspases (Blagosklonny & Fojo 1999). It has been demonstrated that the cytotoxic effects of PTX are due to apoptosis (Georgadis et al. 1997, Fan 1999, Stumm et al. 2004), being crucial its action on Bcl2 and consequently on Bax.

Apoptosis has been identified as a key event in the depletion of oocytes and follicles in both aged and chemotherapy-treated women (Fujino et al. 1996, Perez et al. 2007a,b). The main compromised compartment is the granulosa cell layer. It is well known, in fact, that ovarian granulosa cells play a key physiological role in supporting the development and selection of the ovarian follicle by controlling oocyte maturation and by producing the steroid hormones that are critical for maintenance of the ovarian cycle (Richards & Hedin 1988). Indeed, follicular atresia is initiated by apoptosis of the granulosa cells (Amsterdam & Selvaraj 1997).

Thyroid hormones play important roles in proliferation and apoptosis, as reviewed by Oetting & Yen (2007). However, little information is available concerning their role in proliferation and apoptosis of granulosa cells, although a link between thyroid and ovaries has widely been observed (Stradtman 1993, Wakim et al. 1995, Doufas & Mastorakos 2000). Our previous work has demonstrated how the granulosa cell population can be considered a thyroid hormone target, being as how their survival is induced by 3,5,3′-triiodothyronine (T₃) under specific circumstances via cell cycle and metabolism regulation (Verga Falzacappa et al. 2009), strengthening our consideration of this hormone as a survival factor. In addition, a recent work has evidenced an antiapoptotic action on rat granulosa cells exerted by T₃ together with FSH (Zhang et al. 2011). The purpose of this study was to elucidate the effect of T₃ on granulosa cell proliferation and apoptosis after treatment with chemotherapeutic agents, demonstrating that T₃ is able to protect rat ovarian granulosa cells from chemotherapy-induced apoptosis.

Materials and Methods

Chemicals

T₃ was obtained from Sigma–Aldrich. PTX was purchased from Teva Pharmaceutical Industries – Teva Italia (Milan, Italy).

Animals

Female Wistar rats (about 18 days old) were used as cell donors. The animals had free access to tap water and pelleted food throughout the course of the study. The Local Animal Ethics Committee approved all experiments.

Isolation and culture of rat granulosa cells

Ovaries were isolated from female Wistar rats by standard surgical procurement followed by needle puncture and follicular fluid collection. In brief, animals anesthetized with ketamine 70 mg/kg + domitor 0.5 mg/kg injected i.p. were killed by CO₂ inhalation. For the exposure of the whole ovaries, the abdominal wall was opened via a midline incision. The whole ovaries were excised and transferred to a Petri capsule containing DMEM:F12 (Lonza, Basel, Switzerland); the visible follicles were punctured with a 25 gauge needle and the follicular fluid was collected. The fluid was centrifuged at 250 g for 5 min, and the pellet was resuspended in DMEM:F12. rGROV cells were then cultured in DMEM:F12 supplemented with 10% fetal bovine serum (FBS), 1–glutamine 2 mmol/l, and penicillin 100 μg/ml–streptomycin 50 μg/ml in 24 multiwells (BD Biosciences, San Jose, CA, USA), expanded, and freeze-dried in FBS containing DMSO 10%. Then cells were thawed and cultured for various cycles at 37 °C in a humidified atmosphere of 5% CO₂.

PTX or vehicle alone was added to culture medium 24 h after seeding. The hormonal treatment was performed by adding T₃ or vehicle alone to culture medium contemporary to PTX. Every 24 h, fresh aliquots of T₃ (10⁻³ M) were added to culture medium in all the experiments.

RNA isolation and quantitative RT-PCR analysis

Total cellular RNA was isolated from rGROV cells using SV Total RNA Isolation Kit (Promega), according to the manufacturer’s instructions. RNA (1 μg) was subjected to RT using a cDNA synthesis kit OmniScript (Qiagen). cDNA was amplified to determine expression by real-time PCR as described previously (Canettieri et al. 2008) using the primer pairs listed in Table 1. All primers were synthesized by MWG Oligo Synthesis Report (Eurofins MWG Operon, Edersberg, Germany).

Cell growth analysis

Cell growth was analyzed by determining Trypan Blue-negative cell number in a Thomas’s hemocytometer. PTX 7 μM was added together with the hormone. At 24, 48,
MTT assay

Cell viability in the apoptosis studies was assessed by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Promega). Cells were plated in 96 multwells and treated as described previously. A solution of a tetratetrazolium salt was added to the culture medium and, after 3 h, the metabolic formazan product was solubilized in an organic solution. After 1 h of solubilization, the absorbance at 570 and 630 nm were recorded using a 96-well plate reader.

Cell cycle and apoptosis analyses

The cell cycle and apoptosis were evaluated using PI staining and flow cytometry (FCM) analysis. Adherent cells alone for cell cycle or floating and adherent cells for apoptosis were harvested, fixed in 70% ethanol, and, after removing alcoholic fixative, stained with a solution containing 50 μg/ml PI (MP Biomedicals, Santa Ana, CA, USA) and 75 KU/ml RNase (Sigma–Aldrich) in PBS 1× for 30 min at room temperature in the dark.

Samples were then measured using a FACScan cytofluorimeter (Becton Dickinson, Sunnyvale, CA, USA). Twenty thousand events per sample were acquired. The percentages of the cell cycle distribution were estimated on linear PI histograms using the MODFIT Software (Verity Software House, Topsham, ME, USA). Scattering light was used to denote the cell size.

Table 1 qPCR primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>rFSHR</td>
<td>F: TGGATTTGGAAGACCTGGAGAA</td>
</tr>
<tr>
<td></td>
<td>R: AATTTCTGACATTTGCTGGATTT</td>
</tr>
<tr>
<td>rTHRA1</td>
<td>F: AGACGACAGCTGTGCTGGATTT</td>
</tr>
<tr>
<td>rStaR</td>
<td>F: GTGGATTTGGAAGACCTGGAGAA</td>
</tr>
<tr>
<td>rCyp17a1</td>
<td>F: CTGGATTTGGAAGACCTGGAGAA</td>
</tr>
<tr>
<td>rHsd3b</td>
<td>F: ATCCACCCCGCTGGCTGGATTT</td>
</tr>
<tr>
<td>rp450cc</td>
<td>F: GCTTACCGGTCTTGATTTG</td>
</tr>
<tr>
<td>rMCt8</td>
<td>F: GCAAGTGGAGTCTCAAGCA</td>
</tr>
<tr>
<td>rDIO1</td>
<td>F: GCTTCCACAGCTGACTCCCTTT</td>
</tr>
<tr>
<td>rDIO2</td>
<td>F: CTCTTACGCGCTAACAAAGGTT</td>
</tr>
<tr>
<td>rDIO3</td>
<td>F: CCCATGGTGTCCATTTG</td>
</tr>
<tr>
<td>rCyp19a</td>
<td>F: GTCAAGTGGGCTGGCTGGATTT</td>
</tr>
<tr>
<td>rTHRB</td>
<td>F: AGGACCCAGCATGACCTACACTCTTTTTT</td>
</tr>
</tbody>
</table>

TUNEL assay

The induction of apoptosis was evaluated by TUNEL assay (Roche Diagnostics) using FCM. Briefly, trypsinized adherent cells and floating cells were pooled, washed once with PBS (Lonza), and fixed in 4% paraformaldehyde (Sigma–Aldrich) for 30 min. Samples were then permeabilized in 0.1% Triton X-100 (Sigma–Aldrich) in 0.1% sodium citrate (Sigma–Aldrich) and washed with PBS (Lonza). Each sample was incubated in 50 μl reaction mixture (terminal deoxynucleotidyl transferase, TdT, and fluorescein-UTP) for 1 h at 37 °C, washed in PBS (Lonza), and then measured by FCM at the indicated hours.

Active caspase assay

The activation of caspasas has been evaluated by flow cytometry, using the CaspGlow Red Active Caspase Staining Kit (MBL, 4-5-3 Sakae, Naka-ku, Nagoya, Japan). Briefly, cells were cultured and exposed to PTX and T3 alone or combined as described previously. After 48 h of culture, supernatant and cells were collected, and the centrifuged pellets were stained with Red-VD-FMK as indicated by the manufacturer, 1 h, 37 °C, 5% CO2. Then cells were resuspended in the provided buffer and samples were analyzed by FCM using the FL-2 channel.

Western blot

Cells were lysed and the total cellular protein content was measured using the Bradford method (Bio–Rad). Total extracts (40 μg) per sample were loaded onto an 8–10–12% SDS–polyacrylamide gel, electrophoresed, and then blotted onto nitrocellulose membranes (Bio–Rad). Membranes were incubated 16 h at 4 °C with TRA/B (1:500; Santa Cruz Biotechnology, Inc.), FSHR (1:400; Santa Cruz Biotechnology, Inc.), FSHR (1:500; Santa Cruz Biotechnology, Inc.), cyclin A (1:500; Santa Cruz Biotechnology, Inc.), cyclin B (1:500; Santa Cruz Biotechnology, Inc.), cdk1 (1:500; Santa Cruz Biotechnology, Inc.), Bax (1:500; BD Pharmingen), caspase 3 (1:500; Santa Cruz Biotechnology, Inc.), Bcl2 (1:500; BD Pharmingen), and 1 h at RT with β-actin (1:1000; Sigma). After three washes, the membranes were incubated with the secondary HRP antibodies (antimouse and anti-rabbit; Sigma) 1:4000 for 45 at RT. Immunoreactivity was visualized by the ECL immunodetection system (Amersham Corp.) following the manufacturer’s instruction. The relative band intensity was evaluated by densitometric analysis (TotalLab, Nonlinear Dynamics, Newcastle, UK) and normalized to β-actin.

Hormonal assays

Culture media were collected. The secreted 17β-estradiol (E2) content was measured using a competitive chemiluminescent immunoassay (Roche) and normalized for the milligram of total protein obtained from cells as described.
previously. To test the ability of the cells to secrete E2 in response to testosterone, the cells were shifted in a serum-free medium and exposed to testosterone 10 μM for the last 6 h of a 48 h cell culture. Then E2 was measured.

Statistical analysis
The data were presented as mean ± s.d. A comparison of the individual treatment was conducted by Student's t-test or, if there were more than two groups, by one-way ANOVA, followed by Dunnett or Tukey's post hoc analyses.

Results
Cell line establishment and characterization
Rat granulosa cells were obtained from follicle injection as described in the Materials and Methods section. Cells were then cultured and passed through various freezing and thawing passages, maintaining their typical morphology and aspect, and the cell line rGROV has been established (Fig. 1A). To ascertain that the main features of granulosa cells were not altered, steroidogenesis key genes have been analyzed by qRT-PCR and compared with rat follicles. As shown in Fig. 1B, the amplicons for cyp19, cyp17a, p450 scc, StAR, 3BHSD, and FSHR were present. When compared with rat follicles, the resultant genes were lower (about 1/5th ± 1/10th); this in accordance with the E2 production.

To consider the gene expression and hormone production levels as significant, they were also compared with non-steroidogenic tissues (thyroid) and resulted much higher (about 20 times). The receptor for FSH, which is usually lost during cell culture, was found to be believable even at the protein level. To confirm that the steroidogenic pathway was also functioning, the ability of the cells to synthesize and secrete E2 was analyzed at different time points. The most representative 48-h point is shown in Fig. 1C; as evident, the hormone was present in the cell-collected media, in a quantity lower when compared with follicles, but significantly higher when compared with nonsteroidogenic tissues and media alone. In addition, the ability of the cells to secrete E2 in response to testosterone alone has been successfully tested (Fig. 1D), and the resultant rGROV cells were suitable for our following experiments.

Thyroid hormone receptors, transporters, and deiodinases are expressed in rGROV cells
To assess that our cell model was appropriate for thyroid hormone action studies, the key molecules in the ‘thyroid hormone machinery’ were searched for both mRNAs and
protein levels. As shown in Fig. 1D, qRT-PCR showed that the cells express levels of the thyroid receptors α and β, the T3 transporter MCT8, and the selenoenzymes deiodinases DIO2 and DIO3, at lower levels than a control thyroid cell line, but easily detectable and sometimes even comparable. In addition, TRα/β presence was confirmed by western blot analysis.

**T3 grants cell cycle progression against PTX**

It is well known that PTX can alter cell cycle in almost every cell (Blagosklonny & Fojo 1999). In particular, its ability to induce at first a G2 arrest that can be resolved in apoptotic death has been diffusively proved. Given these considerations, our first goal was to assess whether T3 could contrast PTX action on cell cycle and see where this eventual action could lead. To this aim, first cell cycle analysis was performed through flow cytometry on adherent cells at 6, 12, 24, and 48 h. As shown in Fig. 2A, cell cycle analysis showed that PTX produced the characteristic G2 block already at 12 h (20%), which increased at 24 h up to 35%. On the other hand, when T3 was administrated contemporary to PTX, the cells were well distributed through the various cell cycle phases. In particular, the G2 block was not relevant even at 24 h (15%) and a transient S-phase accumulation was evident (30%), thus indicating that cells were able to overcome the PTX-induced DNA damage and cycle regularly. Otherwise, at 48 h, the difference in G2 population was totally overcome.

**T3 can protect rGROV cells from PTX apoptosis thus preserving cell viability**

PI staining and TUNEL assay were then performed to investigate apoptosis. To this aim, adherent and floating cells were pooled and either PI staining or TUNEL assay was then performed as described in the Materials and Methods section. As shown in panel B, even though a significant amount of apoptosis was revealed in the PTX cells (20%, 24 h and 60%, 48 h), the PTX+T3 sample did not have such a massive amount of apoptotic cells (13%, 24 h and 20%, 48 h). The hormone-treated cells in fact, despite the PTX presence, were mainly vital and regularly cycling. The presence of T3 alone was also tested and, as shown, the hormone-treated cells cycle regularly without significant alteration in phase distribution, except of the 48-h point in which it is evident that cells have been speeded up by T3. Taking together the cell cycle and the apoptosis panels, data showed how T3 can counteract the ability of PTX to induce G2 accumulation at first, consequently counteracting the strong apoptosis induced by PTX at 48 h. TUNEL assay was performed also by immunofluorescence on rGROV cells exposed to PTX alone or contemporary to T3, for 24 and 48 h (data not shown), and the results were consistent with the FCM analysis. Coherently, cell metabolism analyzed by MTT (Fig. 3B) confirmed that the presence of T3 could decrease the percentage of nonviable cells by about 10% at 24 h and 30% at 48 h, ensuring cell viability to be similar to control cells.
As our preface is to determine whether T₃ can preserve ovarian cells from a chemotherapy-induced apoptosis in a mammalian cancer treatment regimen, it is an essential condition that T₃ could not protect the mammalian cancer cells as well. To exclude this chance, two different human mammalian cancer cells, namely T47D and MCF7, were exposed to the same experimental condition as rGROV, and cell viability was tested, too. As shown, in both cases, the ability of PTX to affect cell viability was not overcome by T₃, thus granting the chemotherapy efficacy on the cancer cells.

T₃ prevents caspases activation by PTX

It is well known that caspase activation is one of the key events in apoptosis. To better analyze the T₃ antiapoptotic effect on rGROV cells, caspase activation was analyzed by FCM as described in the Materials and Methods section. As shown, when active caspases were measured by FCM, while in the PTX-treated sample there was a positivity of the FL-2 signal, the presence of T₃ maintained the caspase activation at a lower level, comparable to the control one, in accordance with the TUNEL data.

T₃ regulates cell cycle molecule expression

To investigate deeper into the mechanisms underlying the T₃ ability to counteract PTX action on the cell cycle, the G2 cyclins A and B1, and their partner CDK1 levels were analyzed by western blot (Fig. 4A). In accordance with the cell cycle data, the cell cycle molecules were strongly downregulated by PTX, mainly CYCA1 and CDK1, but the presence of the hormone was able to ensure that the said molecule levels were as high as control, thus allowing cell cycling. To investigate the mechanism of this regulation, cell cycle molecule expression was also analyzed by RT-PCR at earlier time points (data not shown), and a trend coherent with the proteic one was observed, thus suggesting that T₃ may act at different regulation levels.

T₃ regulates apoptotic factors

To moreover confirm the ability of T₃ to regulate the apoptotic process, the proapoptotic factors Bax and Casp 3 and the antiapoptotic factor Bcl2 levels were analyzed by western blot (Fig. 4B). As shown, PTX could sensibly upregulate the expression of both the proapoptotic factors and downregulate the expression of the antiapoptotic Bcl2. However, the presence of T₃ maintained the levels of both Bax and active Casp 3 as low as the control ones, and the Bcl2 levels as high as the control, which is consistent with T₃ antiapoptotic action.

Figure 3 Caspases and vitality analyses. Cells were exposed to PTX alone or contemporary to PTX and T₃ for 24 and 48 h. (A) Active caspases assay: floating and adherent pooled cells were stained for active caspases and the fluorescence positivity was measured by FCM. Geo mean obtained in the FL2 channel was compared with control, the increment is shown in the histogram, as mean ± S.D., and is the result of at least five independent experiments. A comparison of the individual treatment was conducted using one-way ANOVA and Tukey’s post hoc test. The pairing was significantly effective (P<0.0001) and the means were significantly different (P<0.0001 PTX vs control and P<0.005 PTX+T₃ vs PTX). (B) MTT assays were performed as described in the Materials and Methods section. Data are presented as percentage of the OD (570 nm) control value (taken as 100%) on the y axis, as mean ± S.D., and are the results of at least five independent experiments. A comparison of the individual treatment was conducted by using one-way ANOVA and Tukey’s post hoc test. The pairing was significantly effective (P<0.0001) and the means were significantly different (P<0.0001 PTX vs control 24 h; P<0.0001 PTX vs control 48 h).
Discussion

Although CIA represents a severe side effect of cancer treatment, the concept of intervention on the ovary to protect ovarian follicular and steroidogenic function from chemotoxicity remains unpopular. Very few models of protection of the ovary from chemotherapy toxicity have been reported in the literature (Morita et al. 2000, Perez et al. 2007a,b, Yeh et al. 2008, Gonfloni et al. 2009, Salih 2011). In this context, one of the major compartments deserving such protection is represented by granulosa cells, which are mainly responsible for hormone production and thus follicular progression. Those cells did indeed initiate the death process that is responsible for atresia. Protection of granulosa cells by direct intervention on the survival pathways might constitute another means for chemoprotection of the ovary.

Our previous study on granulosa cancer cells COV 434 (Verga falzacappa et al. 2009) together with the data from Zhang et al. (2011) gave strong indications of the ability of T3 to counteract apoptosis in those cells. Nonetheless, growing evidence indicates that the thyroid hormones might induce cell survival against various insults, both physiological and pharmacological, in different cellular systems (Laoag-Fernandez et al. 2004, Sukocheva & Carpenter 2006, Verga Falzacappa et al. 2006).

We investigated the effects of T3 treatment on granulosa cell survival after chemotherapy treatment, focusing on the molecular aspects of these effects. To this aim, we established a new nontumoral cell line named rGROV cells. Granulosa cells were isolated from follicles obtained by a 18-day-old rat females and settled for cell culture. Their ability to secrete E2 and the good expression of the main steroidogenic genes were tested. This study demonstrates for the first time that thyroid hormone T3 is able to block the cytotoxic effect of taxanes on rGROV granulosa cells, granting the cells to cycle regularly, escaping apoptosis.

As taxanes represent a highly affective agent concerning ovarian function and are often used in breast cancer treatment, we utilized PTX for our study. The ppc of PTX for breast cancer treatment is 175 mg/m², corresponding to 4-7 μM (as reported in the Pharmacopeia); so we decided to use this dose for our experiments. PTX is well known to induce cell cycle arrest at the metaphase–anaphase transition of mitosis (Blagosklonny & Fojo 1999). Inhibition of mitotic progression and arrest correlates with PTX cytotoxicity and the mitotic cells are the most sensitive to PTX arrest (Jordan et al. 1996). In accordance, we observed a strong G2 block in the cycling cells exposed to PTX. T3 is considered a primary liver mitogen (Francavilla et al. 1994, Malik et al. 2003) and its action on various cell cycle molecules has been demonstrated (Barrera-Hernandez et al. 1999, Pibiri et al. 2001, Verga Falzacappa et al. 2006). In our cell system, T3 was able to contrast the G2 arrest induced by PTX. As this action was accompanied by a strong regulation of the G2 regulator molecules, we can speculate that even a T3 direct action on the cell cycle could be implied in its survival effect against PTX.

When PTX is administered to cycling cells, apoptosis does not usually occur immediately but starts after PTX exposure of at least 16 h or longer, when slippage from mitosis may...
occur. Our results obtained by cytofluorimetry indicate that apoptosis occurs already after the first 24 h of PTX treatment but is evident only at 48 h. Coherently with the FCM indication, TUNEL assay revealed a strong antiapoptotic action of T3 on PTX-treated cells. Also, the metabolic assay revealed how, acting on both cell cycle and apoptosis, T3 was able to grant a vital cell metabolism.

The ability of T3 to act as an antiapoptotic factor has been demonstrated recently in diverse cell systems (Laoag-Fernandez et al. 2004, Sukocheva & Carpenter 2006, Verga Falzacappa et al. 2006) and different pro- and antiapoptotic factors have been evidenced as targets of T3 action. Caspase 3 is the most characterized effector caspase, required for granulosa cell apoptosis, as follicles from caspase 3 null ovariies do not show granulosa cell apoptosis in response to serum starvation (Matikainen et al. 2001). In this work, we demonstrated how the caspase 3 active form is strongly downregulated by T3, even when there is a tight activation signal triggered by PTX.

PTX cytotoxicity involves signaling distinct from DNA-damaging drugs, but common to all microtubule-active drugs with the specific hallmark of Bcl2 hyperphosphorylation, resulting in increased levels of free Bax, favoring apoptosis. Moreover, Bax is one of the proapoptotic Bcl2 family members that has been demonstrated to be deeply implied in the ovarian apoptotic process. Its prominent role in oocyte death sustains DNA damage induced by both chemotherapeutic agents and aging (Greenfield et al. 2007). Inactivation of the Bax gene noticeably delayed ovarian senescence in female mice and reduced the number of oocytes undergoing spontaneous and chemotherapy-induced apoptosis. In addition, accumulation of both mRNA and proteins has been documented in oocytes of aged mice (Kujjo et al. 2010). Our previous data have already demonstrated how Bax could be targeted by T3 while exerting its antiapoptotic action (Verga Falzacappa et al. 2006); in this work, we demonstrate how the strong increment of free Bax provoked by the presence of the chemotherapeutic agent is sensibly diminished by the presence of T3, in accordance with the TUNEL and MTT result. In addition, the Bcl2 protein was also regulated. The caspases activation was also tested and resulted to be regulated by T3; thus, we hypothesize that T3 act also on the apoptotic pathway.

Compelling evidence indicates that PTX kills cells through the induction of apoptosis. The action of PTX on the cell cycle and apoptosis has been deeply discussed in the literature and it is somehow still not completely clear, as reviewed in Wang et al. (2000). However, it is noteworthy that these two processes are connected and involve various pathways. In particular, it has been shown that high concentrations of PTX cause massive microtubule damage, regulate gene expression, and activate kinases. Among genes that have been shown to be upregulated by PTX, Bax promotes apoptosis, whereas cyclin B1, together with its counterpart cdc2/cdk1, activates p34cdc2, which in turn facilitates the apoptotic process. All these events cooperate to finally activate caspases and lead to apoptosis. Considering this picture, we can speculate that all the changes we observed at the molecular levels are deeply interconnected and act together to lead to the final event of apoptotic cell death.

Taken together, our results suggest that the thyroid hormone T3 can exert a strong pro-survival action on granulosa cells and overcome the PTX insult, enabling the cells to remain vital and cycling.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

Author contribution statement

C V F and S M conceived and designed the experiments. C V F, E T, D A, D D A and P P performed the experiments. C V F, S M, and B B analyzed the data. S M, M C, and PP contributed reagents/materials/analysis tools. C V F and S M wrote the paper. M C and M G S did critical reading.

Acknowledgements

The authors would like to thank Drs Silvia Salatino and Luca Mauri for their technical assistance in hormonal assay and Drs Chiara Napoletano and Ilaria Zizzari for their technical assistance.

References

Barrera-Hernandez G, Soo Park K, Dace A, Zhan Q & Cheng SY 1999 Thyroid hormone-induced cell proliferation in GC cells is mediated by changes in G1 cyclin/cyclin-dependent kinase levels and activity. Endocrinology 11 5267–5274. (doi:10.1210/endo.11.10.5267)


Greenfield CR, Babus JK, Furth PA, Marion S, Hoyer PB & Flaws JA 2007 Paclitaxel treatment of breast cancer cell lines modulates Fa/ps ligand expression and induces apoptosis which can be inhibited through the CD40 receptor. Oncology 66 101–111. (doi:10.1159/000077435)


Received in final form 17 August 2012
Accepted 21 August 2012
Made available online as an Accepted Preprint 21 August 2012