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DIFFERENTIAL EFFECTS OF LOW AND HIGH DOSES OF TAXOL IN ANAPLASTIC THYROID CANCER CELLS: POSSIBLE IMPLICATION OF THE PIN1 PROLYL ISOMERASE

V.M. Pushkarev[1,], D.V. Starenki[2], V.A. Saenko[3], V.V. Pushkarev[1], O.I. Kovzun[1], M.D. Tronko[1], I.D. Popadiuk[1], S. Yamashita[3]*

[1]*V.P. Komisarenko Institute of Endocrinology & Metabolism, AMS of Ukraine, Kyiv 04114, Ukraine*
[2]*Health Sciences University of Hokkaido, Hokkaido 061-0293, Japan*
[3]*Nagasaki University, Nagasaki 852-8523, Japan*

Aim: **To study the molecular mechanisms of dose-dependent effects of an anticancer drug, Taxol, on the cell cycle machinery and apoptosis-related proteins in thyroid anaplastic cancer cell lines ARO and KTC-2.** ***Materials and Methods:*** **Western blot analysis was used for the detection of various proteins and of their phosphorylated forms.** ***Results:*** **Low dose of Taxol that cause apoptosis (25 nM) enhanced Rb protein phosphorylation, decreased the expression of cyclin-dependent kinase inhibitors $p27^{KIP1}$ and $p21^{WAF1}$, and potentiated the accumulation of phosphorylated p53 and of the prolyl isomerase Pin1. High Taxol doses (100 and 1000 nM) that cause necrosis-like cell death drastically decreased Pin1 level in both cell lines.** ***Conclusion:*** **Low doses of Taxol promoted G_1/S transition, thus exhibiting mitogen-like effect. Drug-induced Pin1 accumulation could probably facilitate this transition and in parallel contribute to apoptosis via the p53/p73-dependent mechanism. At higher doses of Taxol, there was a dramatic decrease of Pin1 levels which may be a reason for G_2/M cell cycle arrest.**

Key Words: **Taxol, anaplastic thyroid cancer, cell cycle, prolyl isomerase Pin1, apoptosis.**

The Chernobyl accident led to the increase in thyroid cancer incidence in Ukraine. There is also a rise in anaplastic thyroid carcinoma (ATC), which is a relatively rare (1–10% of all malignant thyroid tumors) yet one of the most aggressive types of human cancer with an extremely poor prognosis [1, 2].

Taxol (paclitaxel, docetaxel) is an anticancer drug, successfully used for treatment of lung, breast, ovarian, head and neck cancer [3]. Attempts are made to extend the range of malignancies that could be treated by Taxol, including anaplastic thyroid cancer [4, 5].

To increase Taxol efficacy, it is essential to understand fine biochemical changes induced by the drug in thyroid cancer cells. Taxol is well known to cause microtubules hyperpolymerization that in turn results in cell cycle alterations [3, 6] but the detailed mechanisms of its effects on cell cycle machinery are still unclear.

The peptidyl-prolyl cis/trans isomerase Pin1, a member of the parvulin family of peptidyl-prolyl isomerases, specifically binds phosphorylated Ser/Thr-Pro motifs and catalyzes the cis/trans isomerization of the peptide bond. Various transcription factors and regulators have been identified as substrates for Pin1. It enhances AP-1 activity through isomerization of both c-Jun and c-Fos and stabilizes the tumor suppressors p53 and p73 [7]. Pin1 overexpression in human cancers is important for the activation of multiple oncogenic pathways. Its deletion suppresses the ability of certain oncogenes to induce cancer in mice [8, 9]. Pin1 has been recently implicated in cell cycle control. It affects cyclin D1 and cyclin E, c-Myc, p53 and p73 expression and stability, interacts with a series of mitotic phosphoproteins, including Plk1, Cdc25 [10], Wee1 [11], and, possibly, the pRb/E2F complexes [12].

In human prostate cancer cells Pin1 mediates Taxol-induced apoptosis via modulation of phosphorylated Bcl-2 [13] but exact mechanisms of drug-induced apoptosis and cell cycle perturbations remain obscure.

This study was set out to clarify the mechanisms of Taxol effects on the early stages (G_1/S transition) of cell cycle in human ATC cell lines ARO and KTC-2.

MATERIALS AND METHODS

Cell lines and conditions of culturing. Human ATC cell line ARO were initially provided by J.A. Fagin (Memorial Sloan-Kettering Cancer Center, New York, NY, USA). KTC-2 cell line was established at Kawasaki Medical School (Okayama, Japan).

Cells were grown in RPMI 1640 medium supplemented with 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin (all reagents from Invitrogen Life Technologies, Paisley, UK) in a 5% CO_2 humidified atmosphere at 37 °C. After 2 days incubation, when the culture reached about 80% confluence, cells were washed twice with phosphate-buffered saline (PBS) (pH 7.4) at 37 °C, and a fresh medium was added to each dish. Cells were incubated for an additional 24 h, exposed to the drug as described below, and then collected at different time intervals.

Preparation of cell extracts. Cells were washed twice with an ice-cold PBS supplemented with sodium pyrophosphate and orthovanadate, scraped with a rubber policeman, collected in 1 ml PBS, and centrifuged for 3 min at 1000 rpm at 4 °C. The pellet was

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*Correspondence: Fax: +38044430-36-94
E-mail: endo@i.kiev.ua

Abbreviations used: ATC – anaplastic thyroid carcinoma; CDK – cyclin-dependent kinase; CHK1 – checkpoint kinase; pRb – retinoblastoma protein.

then resuspended in 200 µl of the lysis buffer (Cell Signaling Technology, USA) containing a cocktail of protease and phosphatase inhibitors. After 15 min on ice, lysates were centrifuged for 15 min at 15,000 g and stored at –80 °C until use. Protein concentration was determined with bicinchoninic acid assay reagent kit (Sigma, St. Louis, MO, USA) according to manufacturer's protocol.

Western blotting. Total cell lysates were boiled in the sample buffer (100 mM Tris-HCl, 4% sodium dodecyl sulfate, 0.2% bromophenol blue, 20% glycerol, 10% dithiothreitol) and resolved in 7.5–15% gradient SDS-PAGE gels (Biocraft, Tokyo, Japan). The single 8% and 15% acrylamide concentration gels were used when better separation of high- and low-molecular weight proteins, respectively, was required. 40 µg of total protein were applied per each lane. Proteins were transferred onto 0.2-µm nitrocellulose membranes (Millipore Corp., Bedford, MA, USA) by semi-dry blotting. Membranes were blocked with Tris-buffered saline / 0.1% Tween 20 containing 5% nonfat dry milk and incubated with primary antibodies (Pin1, phospho-pRb, phospho-c-Abl, phospho-CHK1, PARP, rapamicin, $p27^{KIP1}$, $p21^{WAF1}$ were from Cell Signaling Technology, USA; phospho-p53, β-actin — from Santa Cruz Biotechnology, USA) at 4 °C overnight. After washing three times with Tris-buffered saline / 0.1% Tween-20, the blots were incubated with horseradish peroxidase-conjugated species-specific secondary antibody (Cell Signaling Technology) for 1 h at room temperature and then again washed three times. Complexes were visualized using ECL reagents (Amersham, Arlington Heights, USA) in a LAS-3000 imaging system (Fujifilm, Tokyo, Japan).

Statistical analysis. All data were expressed as a mean ± SE. Differences between groups were examined for statistical significance using Student's *t* test. $P < 0.05$ denoted the presence of a statistically significant difference.

RESULTS AND DISCUSSION

Taxol effects on early cell cycle events. Cell entry into the S-phase of cell cycle (DNA replication) starts with mitogen/c-Myc-induced phosphorylation of the retinoblastoma protein (pRb) and that of two members of pocket protein family, p107 and p130, by the cyclin/CDK complexes, namely by the cyclin D/CDK4/6 ([14], for review). As a result, pRb affinity to the E2F transcription factor family, which play a key role in G_1/S transition, decreases and released E2F upregulates the expression of the cyclin E and cyclin A genes. Next, the cyclin E/CDK2 complex phosphorylates other amino acid residues pRb. Thus, pRb phosphorylation results in transcription initiation and accumulation of cyclins, CDKs and of other factors promoting cell cycle and determining the checkpoint-1 transition and entrance to S-phase [14].

In ARO and KTC-2 cells exposed to low doses of Taxol (15–25 nM), which cause *bona fide* apoptosis [5], the enhancement of pRb phosphorylation on Ser795 and Ser807/811 residues was observed (Fig. 1 (1) and 1 (2)). Phosphorylation of Ser795 was registered after 6 h of cell exposure to Taxol whereas that of Ser807/811 after 12 h incubation. Perhaps these modifications take place successively and comprise the initial events of Rb phosphorylation. The maximal phosphorylation of both sites was observed at 24 h followed by a slight decrease at 36 h (see Fig. 1 (1) and 1 (2)). Such intensive modifications may represent the dissociation of pRb/E2F and activation of the E2F/DNA-polymerase complexes under these conditions. The c-Myc level in untreated cell was rather high, as it is in most human cancers [15]. In ATC cells, further c-Myc accumulation was seen only after 36 h of cell incubation with Taxol (Fig. 1 (6)).

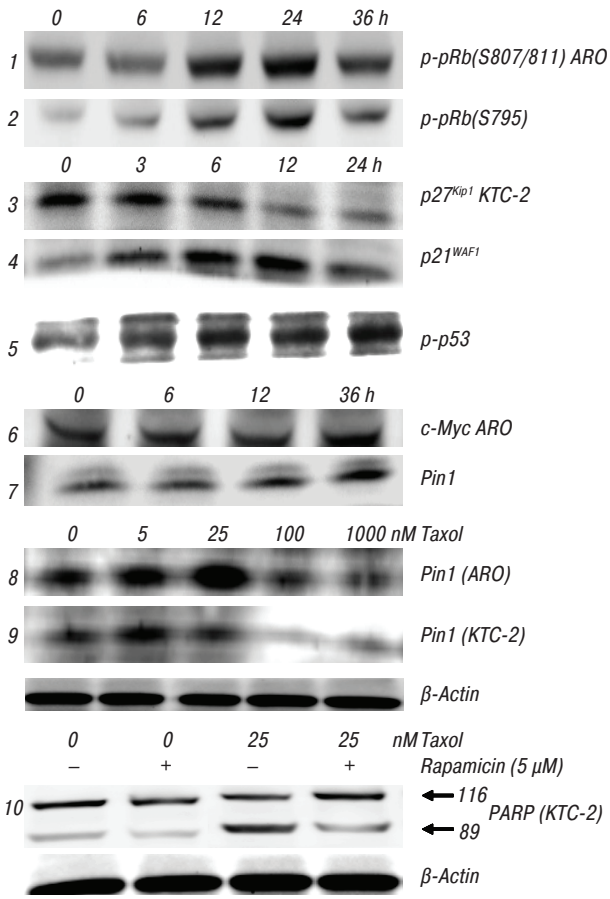

Fig. 1. Effects of Taxol on cell cycle regulatory and apoptosis-related proteins. 1–7 — time course of Taxol (25 nM) effects on protein expression and phosphorylation; 8–9 — effect of different Taxol concentration on Pin1 expression after 36 h of cell exposure to the drug. 10 — effect of rapamycin on Taxol-induced PARP cleavage. Arrows show the position of intact PARP (116 kDa) and its larger fragment (89 kDa). Results are representative of least two experiments

In both cell lines, after 6 h of treatment with Taxol we observed, simultaneously with pRb phosphorylation, the decrease in the level of a CDK inhibitor and thus the negative regulator of G_1/S transition, $p27^{KIP1}$ (Fig. 1 (3)). It possibly occurs as a result of proteosomal degradation after $p27^{KIP1}$ phosphorylation by the cyclin E/CDK2 complex [14]. Degradation of $p27^{KIP1}$ that binds and suppresses the cyclin E/CDK2 and cyclin A/CDK2 complexes, which play an important role

in the late G_1- and S-phases, creates the conditions for transition through these stages of cell cycle.

In contrast to $p27^{KIP1}$, the level of another key CDK inhibitor, $p21^{WAF1}$, increased during the first hours of cell incubation with Taxol. It decreased significantly only after 12 h of incubation with the drug (Fig. 1 (4)). This probably can be explained by the differential temporal roles of $p21^{WAF1}$. At the early stages of cell cycle it stabilizes and promotes cyclin D/CDK4/6 translocation to nucleus ([16], for review) while after the breakdown of these complexes $p21^{WAF1}$ manifests its inhibitory properties on the cyclin E/CDK2 and cyclin A/CDK2 complexes.

We also did not observe checkpoint kinase CHK1 phosphorylation, i. e. activation (data not shown), which is required for cell cycle arrest [17].

Thus, Taxol at low concentrations, on the one hand, enhanced pRb phosphorylation that causes the release of transcription factor E2F. On the other hand, it did not activate CHK1 and decreased the levels of G_1/S transition inhibitors, $p27^{KIP1}$ and $p21^{WAF1}$. Taken together, these findings suggest that Taxol displays a mitogen-like effect by stimulating the initial stages of cell cycle in ATC cells. The significance of such stimulation is unclear, but one can expect that cell cycle progression is a necessary condition of Taxol-induced apoptosis [5, 18]. Our data showed that low concentrations of Taxol, which initiated true apoptosis in ATC cells, did not arrest cell cycle [5]. High concentrations of the drug (100–1000 nM) do arrest cell cycle in G_2/M phase and induce mitochondrial collapse and necrosis-like death in ATC cells [5].

The role of peptidyl-prolyl cis/trans isomerase Pin1 in Taxol-dependent apoptosis. Pin1 is one of the key cell cycle regulators [19], but its role in apoptosis, cell proliferation and survival is still controversial. Some reports have claimed proapoptotic effects of Pin1 [20–22], while others described its involvement in cell survival and oncogenesis [23–25].

A growing body of evidence indicates that Pin1 plays a major role in the G_0/G_1/S transition, and its levels in normal cells are significantly elevated at the G_1/S phase [19, 26]. Pin1 is also overexpressed and positively regulates cyclin D1 by transcriptional activation and posttranslational stabilization in breast [26, 27] and thyroid tumors [28].

Fig. 1 (7) and 2 (1) shows that Taxol caused the increase of Pin1 level in ATC cells. Notably, the mode of Pin1 expression resembled that of c-Myc (Fig. 1 (6)). In ARO cells, the level of Pin1 was rather high in control and increased at low Taxol doses (Fig. 1 (8) and 2 (2)). At apoptotic concentration of Taxol (25 nM), the expression of Pin1 was maximal with subsequent sharp decrease at higher drug concentrations (100 or 1000 nM). In KTC-2 cells Taxol induced a slight accumulation of Pin1, which also dropped dramatically at higher Taxol doses (Fig. 1 (9)).

Pin1 is known to activate a tumor suppressor p53 and other proteins of p53 family, in particular p73 [29]. In response to DNA damage p53/p73 are phosphorylated by c-Abl, p38MAPK and other protein kinases on Ser and Thr residues that allow the interaction of p73 with Pin1. The latter catalyzes prolyl isomerization and results in conformational changes, acetylation, stabilization, and functional activation of p73 [29]. In KTC-2 cells Taxol stimulated phosphorylation of both c-Abl (data not shown) and p53 (Fig. 1 (5)). p53 activation in response to genotoxic or other stress, depending on strength of such challenge, may generally cause two different effects: cell cycle arrest or apoptosis. $p21^{WAF1}$ is an antiapoptotic factor and its decrease (see Fig. 1 (4)) may sensitize cells to apoptosis [30]. Thus, it is possible that Pin1 could act as a p53 stabilizer thereby activating p53-dependent apoptotic pathway. In ARO cells, which carry mutant *TP53* gene, Pin1 could stabilize a structural and functional homolog of p53, p73. p73 is highly expressed in a significant proportion of anaplastic thyroid cancers whereas it is undetectable in normal thyroid epithelium or in papillary and follicular thyroid cancer cells [31]. It is noteworthy that maximal expression of Pin1 coincides with most efficient proapoptotic Taxol concentration [5], and that rapamycin, a clinically important antibiotic and immunosuppressant, which also inhibits Pin1 and other peptidyl-prolyl cis/trans isomerases [32], significantly suppressed Taxol-induced poly(ADP-ribose)-polymerase (PARP) cleavage, a hallmark of apoptosis (Fig. 1 (10)). Pin1 can directly participate in Taxol-dependent apoptosis by preserving drug-induced Bcl-2 phosphorylation [5, 13].

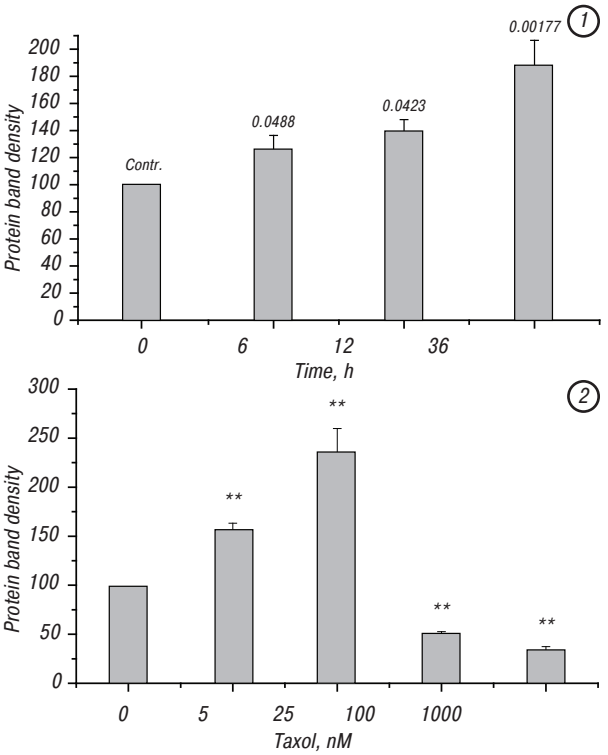

Fig. 2. Dependence of Pin1 accumulation on Taxol concentration and time of cells exposition to the drug. 1 — time course of Taxol (25 nM) effects on Pin1 expression in ARO cells. Figures on the plot columns show *P* value; 2 — effect of Taxol concentration on Pin1 content in ARO cells. $^{**}P < 0.01$; n = 3.

It is interesting that at high Taxol concentrations, at which the drug is likely to cause forms of cell

death other then apoptotic, Pin1 level was drastically decreased (Fig. 1 (8) and 1 (9)). Exact mechanisms and significance of such decrease are not clear at a moment. It is known that Pin1 stimulates cell cycle transition by increasing cyclin D expression, through activation of a key phosphatase cdc25C and inhibition of Wee1 kinase. Together these changes result in the activation of the cyclin B/cdc2 — the main G_2/M driving complex [11, 19]. Our data imply that Taxol-dependent decrease of Pin1 could be a reason for G_2/M cell cycle arrest caused by the drug.

Overall, the results presented here indicate that Taxol at low, apoptotic concentrations exhibited mitogen-like properties, facilitating G_1/S cell cycle transition in anaplastic thyroid cancer cells. Moreover, low doses of Taxol increased Pin1 expression, which could be involved in drug-induced apoptosis via the p53/p73-dependent mechanism. In addition, the increased Pin1 level can probably promote cell cycle transition. Finally, we demonstrated that high Taxol doses resulted in a dramatic reduction of Pin1 level that may represent the mechanism by which Taxol induces G_2/M cell cycle arrest.

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РАЗЛИЧИЕ ЭФФЕКТОВ НИЗКИХ И ВЫСОКИХ ДОЗ ТАКСОЛА В КЛЕТКАХ АНАПЛАСТИЧЕСКОГО РАКА ЩИТОВИДНОЙ ЖЕЛЕЗЫ: ВОЗМОЖНОЕ УЧАСТИЕ ПРОЛИЛ-ИЗОМЕРАЗЫ PIN1

Цель: **изучить молекулярный механизм дозозависимых эффектов противоопухолевого препарата таксола на клеточный цикл и ассоциированные с апоптозом белки в клетках линий ARO и KTC-2 анапластического рака щитовидной железы.** ***Материалы и методы:*** **для количественного анализа содержания различных белков и их фосфорилированных форм использовали Вестерн блоттинг.** ***Результаты:*** **при действии таксола в дозе 25 нмоль/л, которая вызывает апоптоз клеток ARO и KTC-2, отмечается усиление фосфорилирования белка Rb, снижение экспрессии ингибиторов циклинзависимых киназ $p27^{KIP1}$ и $p21^{WAF1}$, а также накопление фосфорилированного p53 и пролил-изомеразы Pin1. Высокие дозы таксола (100 и 1000 нмоль/л), вызывающие некрозоподобную гибель клеток, заметно снижают уровень Pin1 в обеих клеточных линиях.** ***Выводы:*** **низкие дозы таксола способствуют переходу из G_1 в S-фазу клеточного цикла, что свидетельствует о митогенподобном действии препарата. Индуцированное таксолом накопление изомеразы Pin1, возможно, облегчает этот переход и параллельно участвует в апоптотических процессах через p53/p73-зависимый механизм. При более высоких дозах препарата отмечают существенное снижение уровня Pin1, что может быть одной из причин задержки клеточного цикла на стадии G_2/M.**

Ключевые слова: **таксол, анапластический рак щитовидной железы, клеточный цикл, пролил-изомераза Pin1, апоптоз.**