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Combined effect of docetaxel and human-beta-defensis-2 on viability of follicular thyroid cancer cells

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Summary. The effect of low docetaxel (Dtx) concentrations in combination with recombinant human beta-defensin-2 (rec-hBD-2) on viability of cultured follicular thyroid carcinoma WRO cells has been studied. Treatment with 0.1 nM Dtx caused insignificant decrease of WRO cell viability while 1 nM Dtx caused statistically significant cytotoxic effect. Incubation of WRO cells with 0.1 or 1 nM Dtx resulted in significant up-regulation of key proteins involved in cell cycle control, in particular, cyclin D1, p53 protein, and $p21^{Waf1/Cip1}$. Treatment of WRO cells with 10 nM rec-hBD-2 resulted in stimulation of cell viability, while their incubation with 100 nM rec-hBD-2 resulted in significant decrease of cell viability. The most significant suppression of WRO cell viability was observed in the case of combined use of 1 nM rec-hBD-2 and 0.1 nM Dtx. So, we have shown that in cultured follicular thyroid cancer cells mitogenic concentrations of rec-hBD-2 could potentiate growth-suppressive effects of the lowest Dtx concentrations.
Keywords: follicular thyroid cancer, docetaxel, recombinant human beta-defensin-2, cell viability.

Follicular thyroid cancer (FTC) cases yields up to 10-15% from all malignant thyroid tumors. This well differentiated tumor type is characterized by the presence of microfollicular regions, vascular and capsular invasions, and is capable to metastasize [1]. FTC is characterized by mutations in such genes as *Ras* (30-45% of cases), *PTEN* (10-15%), *PIK3CA* (5-15%), *IDH1* (5-25%) and deletions in *PTEN* gene (30%). Due to the mentioned genetic defects, in FTC cells MAPK and PI3K/Akt signal cascades acquire constitutive character and independence from growth factors [1].

Treatment of FTC patients includes thyroidectomy and irradiation with radioiodine as well as chemotherapy with the use of modern cytostatics for the treatment of radioiodine-resistant tumor types. Although FTC could be successfully treated by surgical intervention, the development of alternative ap-

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proaches for treatment of radioiodine-resistant metastatic thyroid cancer remains highly actual.

Among anticancer agents used in clinical practice one should mention taxanes – alkaloids of pacific yew (*Taxus brevifolia*) – paclitaxel and docetaxel. Their mechanism of action is realized via binding to the tubulin and consequent acceleration of its polymerization rate. Therefore microtubules become stabilized with the following disorder of maturation spindle and cell cycle arrest in G2/M checkpoint. Taxanes are effective against many cancer types [2-4], however, their usage is known to cause severe side effects. That's why for decreasing taxane-related toxicity it was reasonable to study the treatment regimens based on low taxane doses in combination with other antimitotic preparations.

In the study we have aimed to analyze an antiproliferative effect of taxane in combination with human beta-defensin-2 (hBD-2), small cationic peptide of innate immunity system with wide spectrum of activities. As other members of defensin family, hBD-2 exerts direct antimicrobial action, but it is also capable to control the growth of human tumor cells, including thyroid carcinoma cells, in a concentration-dependent manner via cell cycle regulation [5,6]. In previous studies we have shown that some hBDs could potentiate antimitogenic effects of taxanes *in vitro* [7, 8]. Moreover, in recent study it has been reported [9] that overexpression of alpha-defensins (DEFA) and MAP2 (microtubule-associated protein) in tumors of breast cancer patients may serve as an effective marker of complete pathologic response on neoadjuvant therapy with paclitaxel.

The aim of our work was to study *in vitro* the influence of taxane in low concentrations on viability of follicular thyroid carcinoma cells of WRO line and expression of some regulatory cell cycle factors in taxane-treated WRO cells and to evaluate cytotoxic effects of docetaxel in combination with recombinant hBD-2 (rec-hBD-2).

Materials and Methods

Cell line. Follicular thyroid cancer cell WRO line was kindly provided by Professor V.A. Saenko and Professor S. Yamashita (Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan).

WRO cells were cultured in DMEM culture medium supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin G sodium, 100 μg/mL streptomycin sulfate in humidified 5% CO_2 atmosphere at 37°C.

Docetaxel (Dtx) («Wako Chemicals», Japan) was dissolved in DMSO and then added into culture medium. Control samples were treated with equal amount of DMSO without Dtx.

Preparation of recombinant hBD-2. To study the effect of exogenous defensin upon cell growth, we have used the rec-hBD-2 expressed in bacterial cells as GST-hBD-2 fusion protein and purified by standard procedure as described earlier [10]. In brief, *E.coli BL21(DE3)* cells transformed with GST-hBD-2-recombinant plasmid were induced with 1 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) for 6 hours, pelleted by centrifugation, resuspended in lysis buffer (50 mM Tris-HCl, pH 7.6; 250 mM NaCl; 1% Triton X-100 and a mix of protease and phosphatase inhibitors), and disrupted using ultrasound disintegrator (UD-11 Automatic, Poland). Cell lysate was then applied to affine chromatography on glutathione-agarose column (GE Healthcare, Sweden) with following cleavage of defensin from fusion protein by thrombin digestion. hBD-2 peptide was further purified by reverse phase chromatography on Sep-Pack C18 cartridge (Waters, USA), vacuum-dried, and re-dissolved in acidified water. Protein concentration was determined by hBD-2 extinction coefficient at 280 nm using spectrophotometer Nanodrop-1000 (Labtech, USA).

MTT assay. To evaluate the effect of Dtx and/or rec-hBD-2 on cell viability, MTT-test has been applied [11]. WRO cells were seeded into 96-well plates (7x103 cells per well) and incubated with the agents in serum-free DMEM for 48 hours. The cells were then routinely treated with MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) by standard protocol, and colorimetric reaction was evaluated with the use of ELISA reader (Awareness Technology Inc, USA) at λ=545.

Western blotting. To analyze the expression level of proteins involved in cell cycle regulation, WRO cells were cultured in 6-well plates and treated with Dtx for 48 hours as described above, washed with PBS and lysed in RIPA (Radio-Immunoprecipitation Assay) buffer with protease and phosphatase inhibitors. The proteins were separated by 9-22% gradient SDS-PAAG electrophoresis and transferred to nitrocellulose membrane Hybond-ECL, RPN3032D (Amersham Biosciences, USA). Nonspecific binding sites were blocked with 1X PBS-T, 5% BSA solution for 1 h. Then blots were incubated with primary antibodies (Abs), and later with secondary polyclonal HRP-conjugated anti-rabbit IgG or anti-mouse IgG Abs (DakoCytomation, Denmark). The antibodies against p53 (IEPOR,

Ukraine), cyclin D1 («Cell Signaling Technology», USA), $p21^{WAF1}$ (Oncogene, USA), PARP («Santa Cruz Biotechnology», USA), and MoAbs against beta-actin (Sigma, USA) were used. All antibodies were used at working dilutions according to manufacturer instructions. The ECL western blotting detection system (Amersham Pharmacia Biotech) was used to reveal immunoreactivity.

Statistical analysis. The data are reported as the mean±m of values obtained from four independent experiments. Data on MTT were analyzed by Student's t-test to assess the statistical significance of the difference between the groups. The differences were considered statistically significant at $p<0.05$.

Results and Discussion

The study of cytotoxic effects of taxanes *in vitro* has been performed with the use of docetaxel (Dtx) at final concentrations of 0.1 and 1 nM in the cell incubation medium. This protocol was based on our earlier observation that thyroid cancer cells are sensitive to low doses of this cytostatic which caused antiproliferative effects starting from 0.1 nM concentrations in anaplastic thyroid cancer cells of KTC-2 line [7]. Follicular thyroid carcinoma cells of WRO line were found to be more resistant to Dtx action than KTC-2 cells, and statistically significant cytotoxicity has been observed at 1 nM Dtx concentration while 0.1 nM Dtx caused insignificant decrease of WRO cell viability (**Fig. 1**). Relative resistance of WRO cells to Dtx action was supported by the data of Western blotting – no degradation of caspase substrate PARP has been observed in WRO cells incubated with 0.1 or 1 nM Dtx for 48 hours (**Fig. 2a,b**).

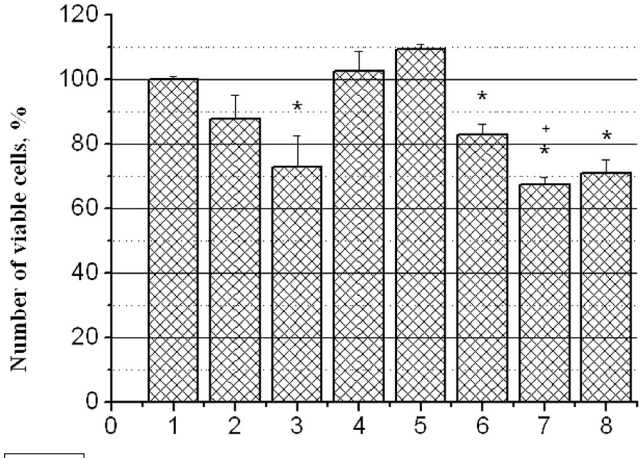

Fig. 1. Influence of docetaxel, rec-hBD-2 and their combination on viability of WRO cells. The cells were cultured in 96-well plates in serum-free DMEM in the presence of Dtx and/or hBD-2 for 48 hours. Cell viability was evaluated by MTT analysis. The data of 4 independent experiments are presented as M±m. * – The difference is significant compared to the control, $P<0.05$; + – The difference is significant compared to corresponding Dtx concentration without rec-hBD-2, $P<0.05$.
1 – control; 2 – 0.1 nM Dtx; 3 – 1 nM Dtx; 4-6 – 1 nM, 10 nM, and 100 nM rec-hBD-2 respectively; 7 – 0.1 nM Dtx + 1 nM rec-hBD-2; 8 – 1 nM Dtx + 100 nM hBD-2.

Next, we have studied expression levels of some cell cycle regulator proteins, in particular, cyclin D1, p53, and $p21^{Waf1/Cip1}$ in WRO cells incubated with low Dtx concentrations. According to the data of Western blotting (Fig. 2a,b), action of 0.1 and 1 nM Dtx resulted in significant up-regulation of cyclin D1, a protein playing an important role in initiation of cell division, and p53 protein, the main cell cycle controller. Along with this, in WRO cells treated with Dtx, we have detected increased expression of $p21^{Waf1/Cip1}$ protein (Fig. 2a,b), which is directly regulated via p53-dependent transcription [12].

Thus, inhibition of WRO cell proliferation in the presence of Dtx probably due to stabilization (and accumulation) of p53 and subsequent expression CDK-inhibitor – $p21^{Waf1/Cip1}$ which stops the cell cycle at G1/S stage [12]. Additional suppression of cell proliferation in the presence of the Dtx and hBD-2 can be explained by the activation of the pRb, as previously shown [13].

Then we have analyzed a combined influence of Dtx and hBD-2 on viability of follicular thyroid carcinoma cells. As it has been shown earlier [6], hBD-2 is capable to control the growth of cultured human thyroid cancer cells in a concentration-dependent manner and caused proliferative effects in

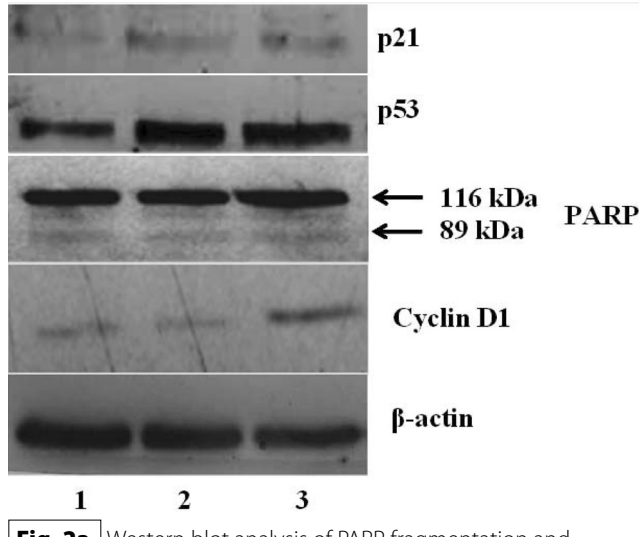

Fig. 2a. Western blot analysis of PARP fragmentation and expression of cyclin D1, p53, p21 in WRO cells treated or not treated with Dtx;
1 – control; 2 – 0.1 nM Dtx; 3 – 1 nM Dtx.

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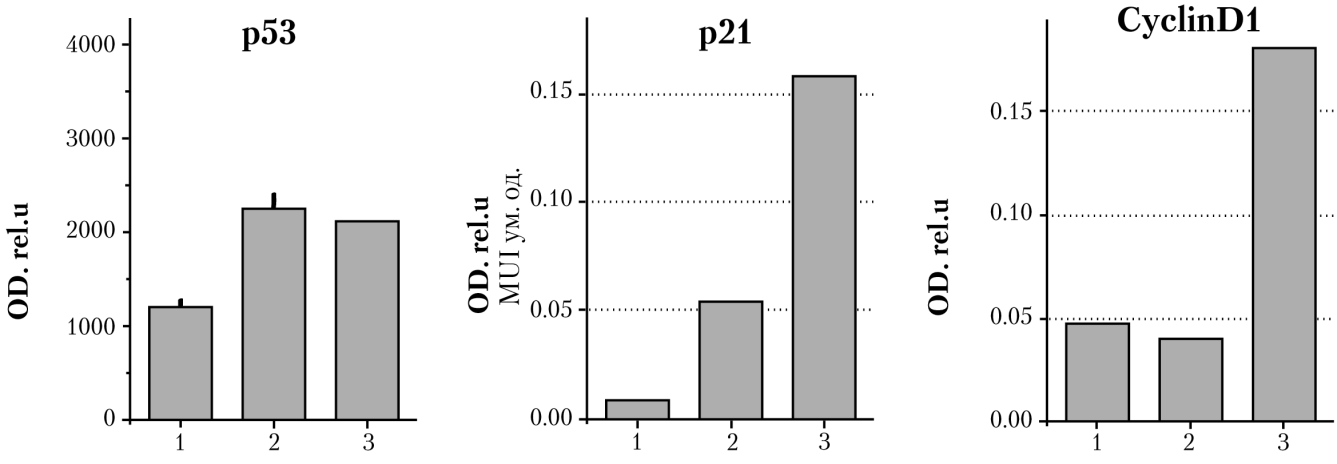

Fig. 2b. Graphic presentation of Western blot data calculated with the use of GelPro 3.1.
1 – control; 2 – 0.1 nM Dtx; 3 – 1 nM Dtx.

concentration range of 0.1-10 nM and significant suppression of cell proliferation in concentrations higher than 100 nM. From three studied cell lines (TPC-1, KTC-2 and WRO) the follicular thyroid carcinoma cells were found to possess the lowest sensitivity to growth suppressing concentrations of the defensin, but were the most responsive to its mitogenic concentrations [6]. Indeed, at concentration of 10 nM rec-hBD-2 stimulated WRO cell viability, while an action of 100 nM rec-hBD-2 caused significant decrease in cell viability ($p<0.05$) (Fig. 1, columns 4-6). In the case of combined use of 1 nM rec-hBD-2 and 0.1 nM Dtx there has been registered a significant suppression of WRO cell viability ($p<0.05$) (Fig. 1, column 7). At the same time combined action of 100 nM rec-hBD-2 and 1 nM Dtx did not differ from cytotoxic action of 1 nM Dtx alone (Fig. 1, column 8). So, 1 nM rec-hBD-2 significantly enhanced growth-suppressive effect of the lowest Dtx concentration. Such effects could be possibly related to the fact that in the presence of low mitogenic concentrations of the defensin (1-10 nM) the larger percent of cells are undergoing mitosis and are targeted by docetaxel which causes cell cycle arrest at G2/M checkpoint.

Thus, the presented data evidence on higher effectiveness of low docetaxel concentrations in combination with low nanomolar concentrations of hBD-2. It's necessary to note that similar synergistic effects of defensins and taxanes have been shown in the research of biological activity of recombinant human beta-defensin-3 [8]. For better understanding of the perspectives for combined use of defensins and taxanes in thyroid cancer patients it is necessary to perform further research of biological activities of these peptide antibiotics and prognostic significance of their expression in human tumors.

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Вплив комбінованого застосування доцетакселу та бета-дефенсину-2 людини на життєздатність клітин фолікулярного раку щитопобідної залози

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Резюме. У роботі було досліджено вплив наднизьких концентрацій доцетакселу (Dtx) в комбінації з рекомбінантним бета-дефенсином-2 людини (rec-hBD-2) на життєздатність культивованих клітин фолікулярного раку щитоподібної залози людини лінії WRO. Тоді як дія 0,1 нМ Dtx не чинила значного впливу на життєздатність клітин, Dtx у дозі 1 нМ виявляв значний цитотоксичний ефект. Інкубація клітин WRO з 0,1 або 1 нМ Dtx призводила до значного підвищення експресії ряду білків, задіяних у контролі клітинного циклу, а саме, цикліну D1, p53 та $p21^{Waf1/Cip1}$. Дія rec-hBD-2 в дозі 10 нМ на клітини WRO мала наслідком стимуляцію життєздатності клітин, тоді як у присутності 100 нМ rec-hBD-2 було відмічено значне пригнічення життєздатності клітин. Найзначніше зниження життєздатності клітин WRO було відмічено за умов комбінованої дії 1 нМ rec-hBD-2 та 0,1 нМ Dtx. Таким чином, показано, що в культивованих клітинах фолікулярного раку щитоподібної залози мітогенні концентрації rec-hBD-2 підсилюють ріст-супресувальні ефекти найнижчих концентрацій доцетакселу.

Ключові слова: фолікулярний рак щитоподібної залози, доцетаксел, рекомбінантний бета-дефенсин-2 людини, життєздатність клітин.

Влияние комбинированного применения доцетаксела и бета-дефенсина-2 человека на жизнеспособность клеток фолликулярного рака щитовидной железы

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Резюме. Исследовали комбинированный эффект препаратов доцетаксела (Dtx) и рекомбинантного бета-дефенсина-2 человека (рек-hBD-2) на жизнеспособность клеток фолликулярного рака щитовидной железы линии WRO. Действие Dtx в дозе 0,1 нМ не оказывало значительного влияния на жизнеспособность клеток, а в присутствии Dtx в дозе 1 нМ наблюдали значительный цитотоксический эффект. Инкубация клеток WRO с 0,1 или 1 нМ Dtx приводила к значительному повышению экспрессии ряда белков-регуляторов клеточного цикла, а именно, циклина D1, p53 и $p21^{Waf1/Cip1}$. Действие rec-hBD-2 в дозе 10 нМ на клетки WRO приводило к стимуляции их жизнеспособности, в то время как в присутствии 100 нМ rec-hBD-2 было отмечено значительное угнетение жизнеспособности клеток. Наибольшее снижение жизнеспособности клеток WRO было отмечено в условиях комбинированного действия 1 нМ rec-hBD-2 и 0,1 нМ Dtx. Таким образом, показано, что в культивированных клетках фолликулярного рака щитовидной железы митогенные концентрации rec-hBD-2 усиливают рост-супрессирующий эффект низких концентраций доцетаксела.

Ключевые слова: фолликулярный рак щитовидной железы, доцетаксел, рекомбинантный бета-дефенсин-2 человека, жизнеспособность клеток.