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Effect of docetaxel and human beta-defensin-2 on proliferation of anaplastic thyroid carcinoma KTC-2 cells

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Summary. In present research we have studied the effect of docetaxel in ultralow concentrations (0.1-10 nM) and recombinant human beta-defensin-2 (hBD-2) in nanomolar concentration range on proliferation and viability of anaplastic thyroid carcinoma cells of KTC-2 line, and expression of some cell cycle regulatory factors. It has been shown that docetaxel, hBD-2 and combination of these agents significantly inhibited proliferation of KTC-2 cells via down-regulation of the cyclin E expression.

Keywords: thyroid, anaplastic carcinoma, cell cycle, human beta-defensin-2, docetaxel.

Taxanes are compounds that can interact with microtubules and are considered as promising carcinostatics. They are already being used for treatment of some malignant tumors [1-3], including anaplastic thyroid carcinoma (ATC) [4-8]. However, their use is limited due to high toxicity and insufficient efficacy. Therefore, studies aimed on determination of taxanes minimal effective concentrations capable of initiating cancer cell proliferation blockage as well as senescence and death with insignificant damage to normal tissues and the search for effective combinations of taxanes with other anticancer compounds are of high concern.

Human beta-defensins (hBDs) belong to a family of cationic antimicrobial peptides and represent an important component of innate immunity. Apart from direct antimicrobial activity, defensins provide a link between innate and acquired immunity and modulate immune response toward invading bacteria, are involved in inflammatory responses and wound healing, and could play a role in oncogenesis [9, 10]. In particular, hBD-1 is supposed to possess anticancer activity: its expression is downregulated in renal and prostate tumors [11], while induction of hBD-1 expression results in cancer cell death [12]. Another beta-defensin, hBD-2, may cause opposite effects on cancer cell growth dependent on its concentration. Recently it has been shown that hBD-2 may regulate proliferation and viability of human lung adenocarcinoma cells of A549 line

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and human epidermoid carcinoma of A431 line in a concentration-dependent manner. It also stimulates cancer cell growth in low nanomolar range (1-10 nM) and causes its significant suppression at higher concentrations (100-1000 nM) via cell cycle arrest at G1/S checkpoint [13]. The effects of hBD-2 on growth patterns of human thyroid cancer cells remain unstudied.

Therefore, the aims of this work were to study the effect of ultralow concentrations of docetaxel on anaplastic thyroid cancer KTC-2 cells proliferation, examine the ability of hBD-2 to regulate KTC-2 cell growth, and analyze the role of human beta-defensin-2 as a cell cycle regulator in combination with ultralow docetaxel concentrations.

Materials and Methods

Cell line

Anaplastic thyroid cancer KTC-2 cell line introduced into culture by Dr. J. Kurebayashi (Kawasaki Medical School, Okayama, Japan) was kindly provided by professor V.A. Saenko and professor S. Yamashita (Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan).

KTC-2 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 [14]. In brief, *E.coli* BL21(DE3) cells transformed with GST-hBD-2-recombinant plasmid were induced with 1 mM IPTG 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 the 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 UV absorbance at 280 nm using spectrophotometer Nanodrop-1000 (Labtech, USA).

Direct cell counting

To study the effect of Dtx and/or hBD-2 on cell proliferation, KTC-2 cells were routinely cultured in 24-well plates ($5x10^4$ cells per well) to nearly 50% confluence. Culture medium was then replaced with fresh DMEM supplemented with 2.5% FBS and rec-hBD-2 was added into cell cultivation medium in concentrations of 100, 500, 1000 nM. The cells were cultured for 48 hours after that. Following the treatment, cells were washed with PBS, detached with trypsin, and counted in hemocytometer. The percentage of dead cells was analyzed using trypan blue staining.

MTT assay

To evaluate the effect of Dtx and/or hBD-2 on cell viability, MTT-test has been applied [15]. KTC-2 cells were seeded into 96-well plates ($7x10^3$ cells per well) and incubated with these agents in DMEM supplemented with 2.5% FBS 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.

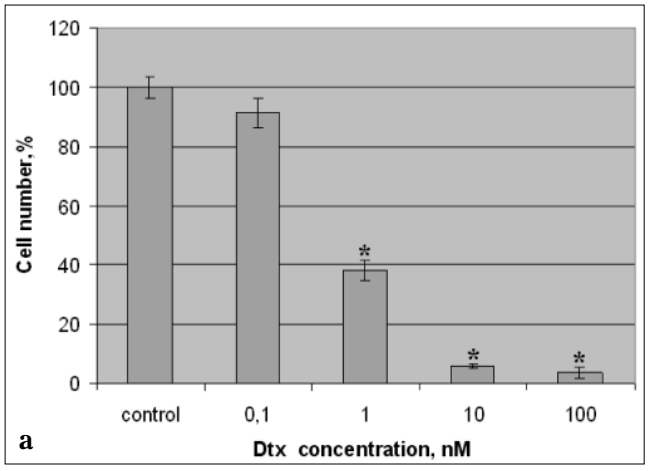
Western blotting

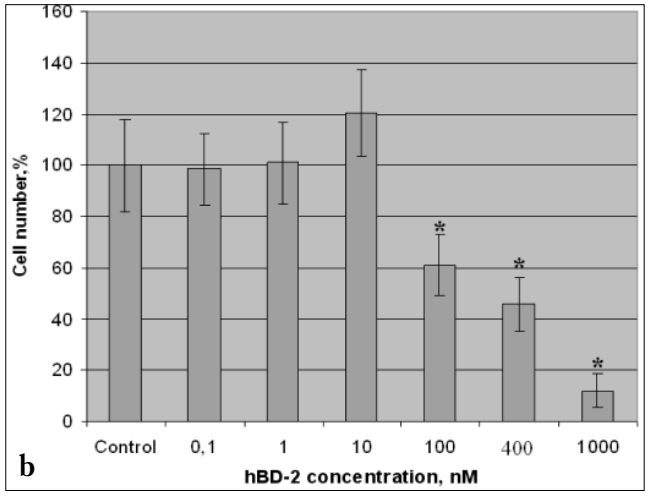
To analyze the expression level of some signal pathway components involved in cell cycle regulation, KTC-2 cells were cultured in 6-well plates and treated with Dtx and/or hBD-2 for 48 hours as described above, washed with PBS and lyzed in RIPA buffer with protease and phosphatase inhibitors. The proteins 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. The blots were then incubated with primary Abs, and later with secondary polyclonal HRP-conjugated anti-rabbit IgG or anti-mouse IgG Abs (DakoCytomation, Denmark). The ECL western blotting detection system (Amersham Pharmacia Biotech) was used to reveal immunoreactivity. The antibodies against p53 (IEPOR, Ukraine), cyclin E (Santa-Cruz, USA), and MoAbs against beta-actin (Sigma, USA) were used. All antibodies were used at working dilutions according to manufacturer instructions.

Statistical analysis

The data are reported as the mean ± m of values obtained from four independent experiments.

Data on direct cell counting and MTT were analyzed by Student's *t*-test to assess the statistical significance of the difference between the groups. A statistically significant difference was considered to be present at $p<0.05$.





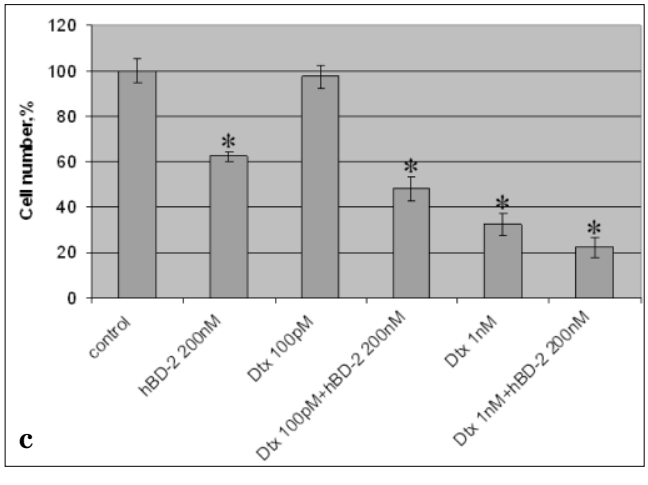


Figure 1. Effect of docetaxel (a), rec-hBD-2 (b) and their combination (c) on KTC-2 cells proliferation (direct cell counting)
M±m, n=4. *differences are significant compared to the control; p<0.05.

Results and Discussion

The aim of our study was to analyze the influence of ultralow docetaxel concentrations (tens and hundreds of thousand times lower than the doses used in clinical practice [16]) and rec-hBD-2 in physiologic (nanomolar) concentrations on anaplastic thyroid cancer cell growth *in vitro*.

The study of Dtx effect on KTC-2 cells proliferation has shown that the cells are sensitive even to ultralow Dtx concentrations: the counts of viable KTC-2 cells after 48 hours of incubation with 1 nM and 10 nM decreased by 2.5-fold and 20-fold respectively ($p<0.05$) (**Fig. 1, a**).

Our data have shown that rec-hBD-2 exerted no effect on proliferation of KTC-2 cells at low concentrations (1-10 nM), while at higher concentrations (100-1000 nM) it significantly suppressed cell proliferation in a concentration dependent manner ($p<0.05$) (**Fig. 1, b**).

To study the combined effects of the agents, we chose a concentration of rec-hBD-2 equal to 200 nM, as 100 nM of hBD-2 is the initial (boundary) concentration at which its effect was observed, and at higher rec-hBD-2 concentrations the additive effects of defensin and Dtx would be difficult to demonstrate.

Combined treatment of KTC-2 cells with Dtx and rec-hBD-2 showed significant additive proliferation-suppressing effect of these compounds (**Fig. 1, c**).

The study of effects of Dtx, rec-hBD-2 and their combination upon KTC-2 cells viability

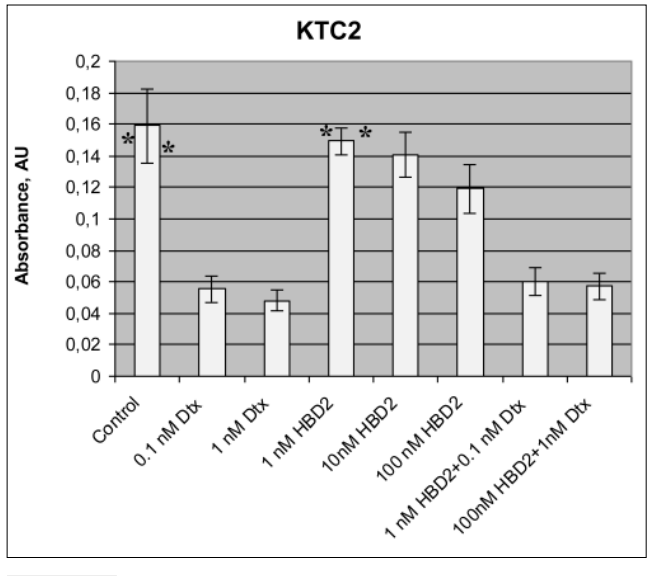


Figure 2. Effect of docetaxel, rec-hBD-2 and their combination on KTC-2 cells viability (MTT assay)
M±m, n=4. *differences are significant compared to the control; p<0.05

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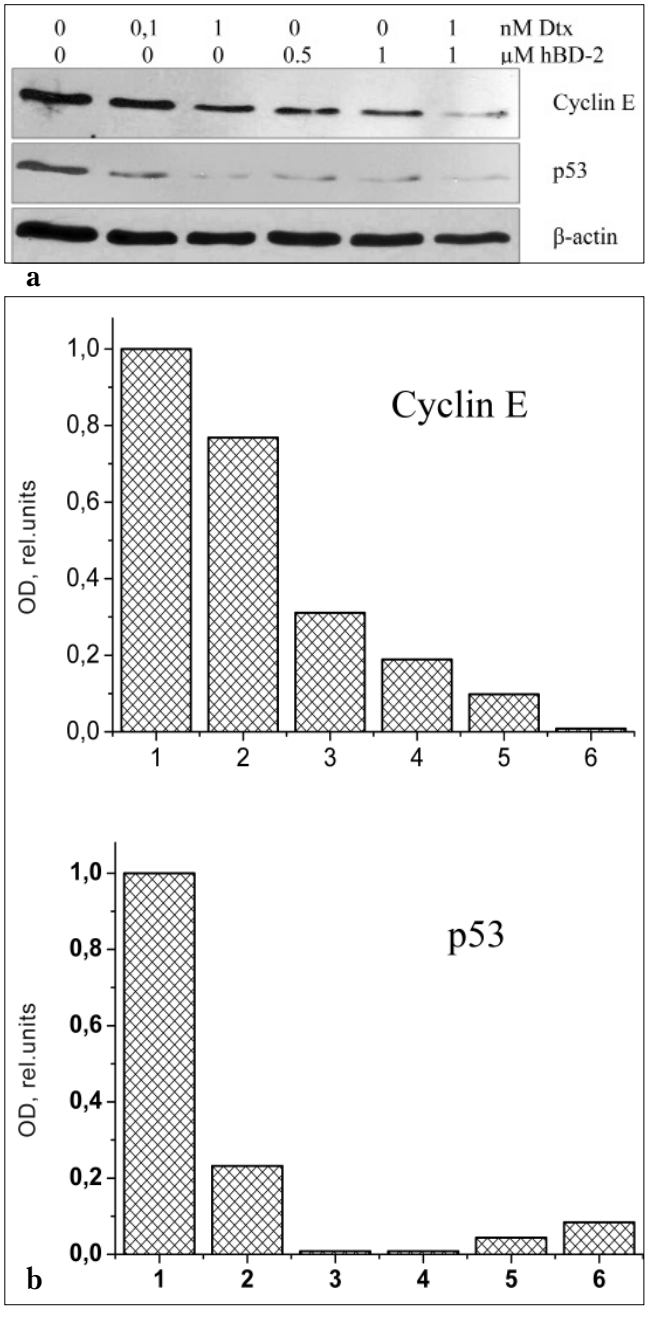

Figure 3. Effect of docetaxel, rec-hBD-2 and their combination on cyclin E and p53 expression in KTC-2 cells

a – Western blotting data; b – graphic representations of western blotting data normalized by β-actin and calculated with the use of GelPro v.3.2 program

demonstrated significant decrease of viable cell counts at 0.1-1 nM of Dtx ($p<0.05$), a concentration dependent effect of defensin at 1-100 nM and the absence of additive effect of these two agents toward anaplastic thyroid cancer cells viability **(Fig. 2)**.

So, we have shown for the first time that rec-hBD-2 is capable of affecting anaplastic thyroid cancer cell growth in a concentration-dependent manner typical for this defensin toward cancer cells of other histologic origin [13]. An additive effect of physiologic (nanomolar) concentrations of hBD-2 and taxanes toward suppression of KTC-2 cell proliferation is of potential clinical importance: it seems reasonable to analyze an efficacy of taxane-based cancer therapy in combination with natural inducers of hBD-2 expression (i.e. vitamin D_3).

To study the effect of rec-hBD-2 on the protein expression, we used this peptide at higher concentrations (500-1000 nM) because herein deeper mechanisms are involved.

The analysis of Dtx effects on cell cycle regulatory mechanisms has revealed the significant decrease of p53 expression – tumor suppressor protein that controls cell cycle under the stress conditions [17], in KTC-2 cells treated with 0.1 nM Dtx, while nearly complete down-regulation of p53 has been registered in the cells treated with 1 nM Dtx or 0.5 μM rec-hBD-2 **(Fig. 3)**.

The study of cyclin E expression, which in combination with cyclin-dependent kinase CDK2 phosphorylates (and inactivates) another tumor suppressor protein – retinoblastoma protein (pRb) [18], has shown that Dtx significantly suppressed cyclin E expression, especially at concentration of 1 nM (Fig. 3). More pronounced cyclin E down-regulation has been observed in KTC-2 cells treated with 0.5 μM and 1 μM rec-hBD-2, while combined use of 1 μM hBD-2 and 1 nM Dtx caused nearly complete suppression of cyclin E expression (Fig. 3).

It is known that tumor suppressor protein p53 after its phosphorylation under stress conditions transactivates genes of CDK inhibitors resulting in cell cycle arrest [18]. Significant down-regulation of p53 caused by Dtx evidences that this protein is not involved in proliferation cease of KTC-2 cells treated with ultralow taxanes concentrations. It seems to be more likely that suppression of KTC-2 cell proliferation upon Dtx and/or hBD-2 action might be related to down-regulation of cyclin E expression that could lead to subsequent pRB activation [18].

Conclusions

1. Docetaxel effect in ultralow concentrations (0.1-10 nM) results in significant suppression of KTC-2 cells proliferation and viability.
2. Recombinant hBD-2 is capable to affect growth patterns of anaplastic thyroid cancer cells in a concentration-dependent manner.

3. Combined effect of Dtx and rec-hBD-2 results in significant additive suppression of KTC-2 cells proliferation and down-regulation of cyclin E expression.

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Вплив таксанів та дефенсину hBD-2 на проліферацію клітин анапластичної карциноми щитоподібної залози лінії КТС-2

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Резюме. У роботі було досліджено вплив доцетакселю в наднизьких концентраціях та рекомбінантного бета-дефенсину-2 людини (hBD-2) на проліферативну активність і життєздатність клітин анапластичної карциноми щитоподібної залози лінії КТС-2 та на експресію деяких регуляторних факторів клітинного циклу. Показано, що дія доцетакселю, hBD-2 та комбінації цих двох агентів призводила до значного зниження проліферації клітин КТС-2 шляхом пригнічення експресії цикліну Е.

Ключові слова: щитоподібна залоза, анапластична карцинома, клітинний цикл, бета-дефенсин-2 людини, доцетаксель.

Влияние доцетакселя и дефенсина hBD-2 на пролиферацию клеток анапластической карциномы щитовидной железы линии КТС-2

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Резюме. В работе исследовано влияние доцетакселя в сверхнизких концентрациях (0,1-10 нМ) и рекомбинантного бета-дефенсина-2 человека (hBD-2) в наномолярном диапазоне концентраций на пролиферативную активность и жизнеспособность клеток анапластической карциномы щитовидной железы линии КТС-2 и экспрессию некоторых регуляторных факторов клеточного цикла. Показано, что действие доцетакселя, hBD-2 и их комбинации приводит к значительному угнетению пролиферации клеток КТС-2 путем снижения экспрессии циклина Е.

Ключевые слова: щитовидная железа, анапластическая карцинома, клеточный цикл, бета-дефенсин-2 человека, доцетаксель.