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Can vitamin A modify the activity of docetaxel in MCF-7 breast cancer cells?

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Abstract: Docetaxel is one of the most effective chemotherapeutic agents in the treatment of breast cancer. On the other hand, the vitamin A family compounds play the essential roles in many biological processes in mammary gland. The aim of our study was to investigate the effect of all-*trans* retinol, carotenoids (β-carotene, lycopene) and retinoids (9-*cis*, 13-*cis* and all-*trans* retinoic acid) on the activity of docetaxel and to compare these effects with the estradiol and tamoxifen actions on human ER(+) MCF-7 breast cancer cell line. The evaluation was based on [^{3}H] thymidine incorporation and the proliferative activity of PCNA and Ki 67 positive cells. In our study, the incorporation of [^{3}H] thymidine into cancer cells was inhibited to 50% by 0.2, 0.5 and 1 μM of docetaxel in the 24-hour culture and addition of estradiol (0.001 μM) didn't influence the results. However, addition of tamoxifen caused a statistically significant decrease of the percentage of the proliferating cells in the culture medium with 0.2 and 0.5 μM of docetaxel (38.99 ± 2.84%, $p<0.01$ and 40.67 ± 5.62%, $p<0.01$) in comparison to the docetaxel only group. The above-mentioned observations were also confirmed with the use of the immunohistochemical investigations. Among the examined vitamin A family compounds, the simultaneous application of β-carotene (0.1 μM) and docetaxel (0.2 μM) resulted in a statistically significant reduction in the percentage of proliferating cells (40.25 ± 14.62%, $p<0.01$). Lycopene (0.1 μM), which stimulates the growth of breast cancer cells in a 24-hour culture, had an inhibitory effect (42.97 ± 9.58%, $p<0.01$) when combined with docetaxel (0.2 μM). Although, β-carotene and lycopene belong to the different chemical groups, they surprisingly had a similar inhibitory influence on both growth and proliferation of MCF-7 breast cancer cells when combined with docetaxel. The application of docetaxel either with β-carotene or lycopene had comparable inhibitory effect on breast cells growth and proliferation as tamoxifen. Therefore, it may suggest a possible important role of these carotenoids in the breast cancer therapy in women especially when docetaxel is applied.

Key words: Carotenoids - Retinoids - Docetaxel - Estradiol - Tamoxifen - MCF-7 - Proliferation - Ki 67 - PCNA

Introduction

Taxans, paclitaxel and docetaxel, are highly effective chemioterapeuthics commonly used against breast cancer. Docetaxel is a hemisyntetic taxan, which acts on microtubules similarly to paclitaxel [1,2]. However, it is more potent than paclitaxel both in *in vitro* and *in vivo* conditions. Therefore, it can be used in lower therapeutic dosages than paclitaxel [1,3]. It was proved that docetaxel through Bcl-2 protein phosphorilization had induced apoptosis of cancer cells in concentration 100 times lower than paclitaxel [4] and demonstrated higher effectiveness [5,6]. An uptake and a retention of docetaxel by cancer cells is better than paclitaxel [7]. The experiments conducted on many different cancers proved higher cytotoxicity of docetaxel in comparison to paclitaxel [8]. On the other hand, the resistance of breast cancer cells on decetaxel is observed and severe side effects are observed when docetaxel is used for long period and in high dosages [9]. Therefore, it seems to be important to find out the substances which could potentialize docetaxel anitcancer activity when it is used in low concentration. Taxans have high activity against breast cancer cells either as the single agent or in combination with other anticancer compounds [10]. Among many substances, the vitamin A family has ability to suppress growth and proliferation of breast cancer cells [11,12]. Vitamin A metabolites acts throughout RAR and RXR receptors belonging to group of steroid/thyroid receptors regulating genes expressions [13]. Therefore, retinoids may express their anti-cancer activity by means of the organ specific mechanisms [14]. It is

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belived that retinoids can suppress cancer growth by inhibition of cells proliferation, promotion of cells differentiation as well as induction of apoptosis. Taxans and retinoids have similar point of action, which is Raf-1. Retinoids potentialize the activation of Raf-1 induced by taxans [15]. Susceptibility of MCF-7 breast cancer cells on anticancer agents like taxans can be potentialized by preincubation with retinoic acid [16].

The aim of our study was to investigate the effect of all-*trans* retinol, carotenoids (β-carotene, lycopene) and retinoids (9-*cis*, 13-*cis*, and all-*trans* retinoic acid) on the activity of docetaxel and to compare these effects with the estradiol and tamoxifen actions on human ER(+) MCF-7 breast cancer cell line.

Materials and methods

Chemicals. Retinol (all-*trans* retinol, Sigma) β-carotene (β-carotene, Sigma), lycopen (Lycopene, Sigma), 9-*cis* retinoic acid (9-*cis* Retinoic acid, Sigma), 13-*cis* retinoic acid (Isotretinoin), all-trans retinoic acid (Tretinoin), tamoxifen (Citrate Salt Tamoxifen), 17β-estradiol (1,3,5 [10]-estratriene-3, 17β-diol) and docetaxel (Taxotere, ≥ 97% [HPLC]) were obtained from Sigma (St. Louis, MO, USA). The following antibodies: PCNA - Proliferating Cell Nuclear Antigen: monoclonal mouse antibody (clone PC 10) and Ki 67: monoclonal mouse antibody (clone Ki 67) were obtained from Dako (Glostrup, Denmark).

Retinol, carotenoids and retinoids were diluted in ethyl alcohol (lycopene in THF) and then in the culture medium, to final concentrations of 0.001 - 10 μM. Tamoxifen and 17β-estradiol were added to the culture at a concentration of 10 and 0.001 μM, respectively. Docetaxel was diluted in the culture medium to final concentrations of 0.00001, 0.0001, 0.001, 0.01, 0.1, 0.2, 0.5, 1.0 and 10.0 μM.

Culture of cell line MCF-7. The study was carried out on the hormone sensitive cell line (ER+) MCF-7 of human breast cancer (American Type Culture Collection, Rockville, MD) in DMEM medium (Sigma, St. Louis, MO, USA) supplemented with 10% FBS (Sigma, St. Louis, MO, USA), 50 μg/ml streptomycin, and 100 U/ml penicillin in 75 cm^2 plastic flasks (Nunc, Roskilde, Denmark), at 37°C, in a humid incubator with 5% CO_2/95% air. The cell line was passaged once a week. The cells for the experiment were obtained from passages 3-7 and inoculated in 24-well plates (Nunc, Roskilde, Denmark) at 5×10^4 cells/well and grown to 85% confluence in Dulbecco's modified Eagle's medium (DME/F12, Sigma, St. Louis, MO, USA) supplemented as above. During the experiments, cells were detached with 0.05% trypsin/0.02% EDTA (Sigma, St. Louis, MO, USA).

Experiments were conducted in plates in DME/F12 Ham (Sigma, St. Louis, MO, USA), supplemented with a synthetic substitute of CPSR-1 serum (Sigma, St. Louis, MO, USA). Incubation of the MCF-7 cells with the examined substances was performed for 24 hours.

[^{3}H]thymidine incorporation. Cell proliferation in the culture was assessed based on incorporation of [^{3}H]thymidine (Amersham, United Kingdom, specific activity 925 GBq/mmol), after incubation of the cell culture in the medium with or without the examined substances. Two hours prior to the termination of the experiment, [^{3}H] thymidine was added to the culture at 18.8 KBq/well. After 2 - 3 washings of the culture with cold phosphate buffer, tripsinisation and precipitation (3 washings with 10% trichloroacetic acid), the precipitate was flooded with Instagel scintillation fluid (Packard, Groningen, The Netherlands). Radioactivity was expressed in dpm per well.

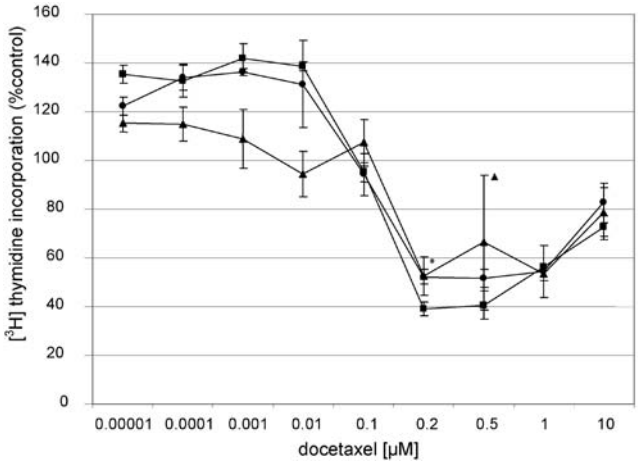

Fig. 1. The influence of docetaxel and docetaxel combined with estradiol or tamoxifen on [^{3}H] thymidine incorporation (in %) into MCF-7 breast cancer cells. Legend: ● - docetaxel, ■ - docetaxel + estradiol 0.001 μM, ▲ - docetaxel + tamoxifen 10 μM. Exposure time 24 hrs. Control = 100 %. Data presented as mean values ± SD (n = 4). (Estradiol = 152.37 ± 10.22%, tamoxifen = 56.17 ± 2.23%). ● - $p < 0.01$ relative to the docetaxel group 0.2 μM, ▲ - $p < 0.01$ relative to the docetaxel group 0.5 μM

Immunocytochemical examinations. Immunocytochemical examinations were carried out in chambers for histochemical examinations (Lab-tek 4 well chamber slide, Nunc, Naperville, IL, USA). Cell material was fixed with cytofix (Cytofix, Merck, Darmstadt, Germany). A 2-step streptavidin-biotin LSAB kit + HRP kit (with horse-radish peroxidase) was used for detection. The antigen-antibody reaction was visualized with the chromogen DAB (diaminobenzidine). The cells were counted with the use of Olympus MicroImage ImCD UDF morphometric program. The individual microscopic fields were photographed and then the cells were counted on the monitor and the percentage of immunopositive cells in comparison to all cells was established.

Statistical analysis. In all the experiments, mean values ± standard deviation (SD) for 4 measurements of each parameter were calculated. The Mann-Whitney test was used to perform statistical analysis.

Results

In our study the incorporation of [^{3}H]thymidine into cancer cells was inhibited by 0.2, 0.5 and 1 μM of docetaxel to 50% in the 24-hour culture. No stimulating effect of estradiol (0.001 μM) in the culture medium with such concentrations of chemotherapeutic agent was observed. The percentage of proliferating cells was 52.78, 66.45 and 53.61% respectively. However, in combination with tamoxifen we observed a statistically significant decrease of the percentage of the proliferating cells in the culture medium with 0.2 and 0.5 μM of docetaxel (38.99±2.84%, $p<0.01$ and 40.67±5.62%, $p<0.01$ compared to the docetaxel group) (Fig. 1). Such a result was also confirmed by immunocytochemical investigations.

Table 1. The influence of all-trans retinol. carotenoids and retinoids on [^{3}H] thymidine incorporation into MCF-7 breast cancer cells. Exposure time 24 hrs. Control = 100%. Data (% control) presented as mean values ± SD (n=4).

Group	Concentration (μM)				
	0.001	0.01	0.1	1	10
all-*trans* retinol	75.35 ± 15.51	81.10 ± 7.91	73.99 ± 7.61	79.46 ± 16.97	90.95 ± 10.49
β- carotene	94.09 ± 26.20	99.23 ± 21.56	83.79 ± 26.12	102.49 ± 16.68	94.46 ± 13.82
Lycopene	127.88 ± 20.29	112.40 ± 6.62	106.55 ± 12.67	124.00 ± 12.51	127.88 ± 20.29
9-*cis* retinoic acid	102.34 ± 5.73	100.93 ± 7.49	83.45 ± 10.06	97.48 ± 10.08	62.13 ± 14.69
13-*cis* retinoic acid	92.01 ± 18.49	86.34 ± 8.18	80.4 ± 13.83	78.05 ± 3.89	54.04 ± 7.48
all-*trans* retinoic acid	64.27 ± 5.10	68.83 ± 4.50	63.3 ± 5.23	71.012 ± 23.74	60.11 ± 8.39

Table 2. The influence all-*trans* retinol, carotenoids and retinoids combined with docetaxel and docetaxel with estradiol or docetaxel with tamoxifen on [^{3}H] thymidine incorporation into MCF-7 breast cancer cells. Exposure time 24 hrs. Control = 100 %. Data (% control) presented as mean values ± SD (n=4). * - p<0.01 relative to the docetaxel group 0.2 μM

	all-*trans* retinol	β- carotene	Lycopene	9-*cis* retinoic acid	13-*cis* retinoic acid	all-*trans* retinoic acid
	Concentration (μM)					
	0.1			10		
+ Dcetaxel 0.2μM	72.01 ± 12.23	40.25 ± 14.63*	42.97 ± 9.58*	63.89 ± 10.88	64.39 ± 4.42	49.17 ± 19.74
+Docetaxel 0.2μM +Estradiol 0.001μM	89.97 ± 2.31	87.86 ± 8.88	92.54 ± 0.72	65.92 ±5.62	71.88 ± 3.82	66.92 ± 6.98
+ Docetaxel 0.2μM + Tamoxifen 10μM	51.24 ± 5.71	85.50 ± 5.44	76.33 ± 4.14	74.19 ± 3.03	73.58 ± 3.07	81.48 ± 11.03

All-*trans* retinol, β-carotene and lycopene were used in concentration of 0.1 μM and retinoids in concentration of 10 μM in experiments with the exposition of MCF-7 cells lines on retinol, carotenoids and retinoids.

Only, 13-*cis* retinoic acid inhibited cancer cells growth in 54% during 24 hrs incubation (Table 1). Among the examined vitamin A family compounds β-carotene (0.1 μM) combined with docetaxel (0.2 μM) resulted in a statistically significant reduction in the percentage of proliferating cells (40.25 ± 14.62%, p<0.01). Lycopene (0.1 μM), which stimulated the growth of breast cancer cells in a 24-hour culture (Table 1), inhibited the growth of cancer cells (42.97±9.58%, p<0.01) when combined with docetaxel (0.2 μM) (Table 2).

The percentage of the cells demonstrating expression of PCNA and Ki 67 is presented in Table 3.

We observed the statistically significant values in the culture medium with docetaxel and 13-*cis* retinoic acid (Ki 67), and with docetaxel and all-*trans* retinoic acid (PCNA) compared to the docetaxel group 0.2 μM. They were also statistically significant values compared to the values in 13-*cis* retinoic acid and all-*trans* retinoic acid group (unpublished data).

Discussion

Docetaxel is one of the most active anticancer agents in the treatment of breast cancer. The mechanism of docetaxel activity include the influence on cell cycle regulation. Recent studies indicates that this activity of taxanes originate in part from induction of genes encoding enzymes which take part in proliferation and other anti-proliferative factors [17]. In our study we have shown, that its activity in MCF-7 breast cancer cell line was most effective in the 24-houres exposure of concentration of 0.2, 0.5 and 1 μM. Hill *et al.* [18] confirmed that 24-houres exposure of various tumor cells on increasing concentrations of docetaxel resulted in a plateau-shaped curve (IC_{50} ranged from 0.13 to 3.3 ng/ml). In our study a docetaxel dose-response curve shows that the growth of MCF-7 was inhibited by docetaxel with or without estradiol supplementation. The information about docetaxel activity in presence of estrogens is limited. Estrogens

Table 3. The influence of the studied compounds and their combinations on the percentage of PCNA- and Ki67-positive MCF-7 breast cancer cells. Exposure time 24 hrs. Data presented as mean values ± SD (n=4). Statistically significant differences relative to the control group, ns - statistically not significant.

Group	PCNA	Ki67
Control	85.75 ± 5.25	95.5 ± 2.75
Estradiol 0.001 μM	83.45 ± 2.75 ns	91.25 ± 3.33 ns
Tamoxifen 10 μM	49.3 ± 2.65 p < 0.0001	55.9 ± 2.85 p < 0.0001
Docetaxel 0.2 μM	70.35 ± 3.35 p < 0.001	60.7 ± 4.01 p < 0.0001
Docetaxel 0.2 μM + Estradiol 0.001 μM	45.02 ± 1.7 p < 0.0001	55.65 ± 3.53 p < 0.0001
Docetaxel 0.2 μM + Tamoxifen 10 μM	50.25 ± 2.48 p < 0.0001	45.73 ± 2.65 p < 0.0001
Docetaxel 0.5 μM	45.06 ± 1.65 p < 0.0001	55.4 ± 3.53 p < 0.0001
Docetaxel 0.5 μM + Estradiol 0.001 μM	31.75 ± 2.26 p < 0.0001	35.85 ± 3.2 p < 0.0001
Docetaxel 0.5 μM + Tamoxifen 10 μM	35.75 ± 2.65 p < 0.0001	45.13 ± 2.45 p < 0.0001
Docetaxel 1.0 μM	35.63 ± 4.26 p < 0.0001	62.25 ± 1.5 p < 0.0001
Docetaxel 1.0 μM + Estradiol 0.001 μM	20.5 ± 1.25 p < 0.0001	25.55 ± 2.01 p < 0.0001
Docetaxel 1.0 μM + Tamoxifen 10 μM	30.0 ± 1.25 p < 0.0001	35.2 ± 3.06 p < 0.0001

Table 4. The influence of the all-*trans* retinol, caretonoids and retinoids combined with docetaxel on the percentage of PCNA- and Ki67-positive MCF-7 breast cancer cells. Exposure time 24 hrs. Data presented as mean values ± SD (n = 4).

Group	PCNA	Ki67
Control	85.75 ± 5.25	95.5 ± 2.75
Docetaxel 0.2 μM	70.75 ± 3.26 p < 0.001	51.27 ± 2.02 p < 0.0001
Docetaxel 0.2 μM + all-*trans* retinol 0.1 μM	80.25 ± 2.05 ns	50.35 ± 1.05 p < 0.0001
Docetaxel 0.2 μM + β- carotene 0.1 μM	70.38 ± 2.17 p < 0.001	51.75 ± 2.35 p < 0.0001
Docetaxel 0.2 μM + Lycopene 0.1 μM	81.25 ± 2.35 ns	60.05 ± 4.26 p < 0.0001
Docetaxel 0.2 μM + 9-*cis* retinoic acid 10 μM	71.55 ± 3.05 p < 0.001	53.33 ± 15.27 p < 0.0001
Docetaxel 0.2 μM + 13-*cis* retinoic acid 10 μM	70.5 ± 2.5 p < 0.001	40.25 ± 10.25* p < 0.0001
Docetaxel 0.2 μM + all-*trans* retinoic acid 10 μM	52.45 ± 1.75** p < 0.0001	55.75 ± 3.53 p < 0.0001

Statistically significant differences relative to the control group, ns - statistically not significant, * - p<0.01, ** - p<0.001 - statistically significant differences relative to the docetaxel 0.2 μM group.

are important regulators of growth and differentiation in normal mammary gland and are important factors in development and progression of breast cancer [19]. Estradiol regulates the expression of estrogen responsive genes involved in regulation of cell growth, proliferation and differentiation and this regulation is mediated by estrogen receptors (ERα and ERβ) [20]. Brown *et al.* [21] demonstrated that 50% inhibitory concentration for docetaxel in ER+ (MCF-7) was 15 nmol/l versus 40 nmol/l in ER- (MDA-MB-231) cells. The results presented by Mueck *et al.* [22], concerning the influence of endogenous estradiol metabolite - 2-Methoxyestradiolu (2ME) in combination with docetaxel on MCF-7 breast cancer cells, demonstrated an additive effect. On the other hand, Kim *et al.* [23] revealed that estrogen administration decreased sensitivity both in ER+ and ER- breast cancer cells to anticancer drugs, for example, taxanes. The latest data revealed that 17-β estradiol significantly reduces cytotoxicity of taxanes (paclitaxel) in ERα+ breast cancer cells but has no influence on ERα- cells [24].

Ferlini *et al.* [25] observed that docetaxel in combination with tamoxifen more effectively inhibited the growth of ER- breast cancer cells than single agent. They observed the synergism when docetaxel was used at 0.2 and tamoxifen at 1 μM. To investigate the possible interaction between docetaxel and tamoxifen we used ER+ breast cancer cells. In our study tamoxifen significantly enhanced inhibitory effect of 0.2 and 0.5 μM docetaxel.

In vitro experiments demonstrated that compounds belonging into vitamin A family were able to inhibit growth of many cells lines and the effect was most pronounced in estrogen receptors positive cells (ER+) [26]. Lu *et al.* [27] indicated that there is a gene activation pathway in which ERα drives retinoic acid receptor α (RARα) transcription and RARα drives cellular retinoic acid binding protein II (CRABP II) transcription in MCF-7 cells. β-carotene and lycopene inhibited the growth of MCF-7 cells but in prolonged time of incubation (up to 9 days) [12]. Hirsch *et al.* [28] confirmed that these compounds inhibit estrogen signaling of 17β-estradiol in MCF-7 breast cancer cells. Although β-carotene and lycopene belong to the different chemical groups, they surprisingly inhibit the growth and proliferation of MCF-7 breast cancer cells in combination with docetaxel in 24-hours culture.

Retinoic acid, a major metabolite of vitamin A, inhibits growth and differentiation of many cancer cell

lines *in vitro* as well as inhibits cancer growth *in vivo*. In *in vitro* conditions, retinoids prevent or diminish the development of induced carcinogenesis in breast cells [29]. Budman and Calabro [10] observed that 9-*cis* retinoic acid synergized with docetaxel in MCF-7 cell line. Also all-*trans* retinoic acid act in this way [16]. In our study we didn't observe above-mentioned effect and didn't demonstrated significant influence of retinoids on response of MCF-7 cells to docetaxel. All-*trans* retinoic acid when combined with docetaxel decreased the percentage of proliferating cells but not in statistically significant way.

The examination of cells proliferation rate is very important prognostic and predictive factor in breast cancer. In our study, we didn't observe significant decrease of Ki 67 positive cells in comparison to PCNA positive cells when cells line were exposed to different concentrations of docetaxel. PCNA is essential component of the DNA replication events, so this gradually decrease means, that DNA replication lessen and result in slower cell proliferation. The percentage of Ki 67 and PCNA positive cells was comparable in cells line exposed to docetaxel in combination with either estradiol or tamoxifen. Both PCNA and Ki 67 are present in S, G1 and G2/M phase of the cell cycle [30,31]. The breast cancer cells are most sensitive to docetaxel in G2/M phase [32]. The highest percentage of Ki 67 and PCNA positive cells was observed in culture with 1 μM of docetaxel and 0.001 μM of estradiol. This results need some further investigations.

In vivo studies didn't demonstrated any correlation between percentage of Ki 67 positive cells and the response to docetaxel [32,33]. But Ki 67 is frequently evaluated after the neoadjuvant chemotherapy. Lee and coworkers [34] confirmed that most of the post-treatment ER+ breast tumors were negative for biological marker Ki 67. Therefore, Ki 67 proliferation index and ER status may be used as prognostic factors for a prediction of survival following neoadjuvant chemotherapy in breast cancer.

Our results confirmed that there is a hormonal control of growth and proliferation in the MCF-7 cells during exposition on docetaxel. We also found that there is dissociation between influence of carotenoids and retinoids on response of breast cancer cells to docetaxel. The application of docetaxel either with β-carotene or lycopene had comparable inhibitory effect on breast cells growth and proliferation as tamoxifen. Therefore, it may suggest a possible important role of these carotenoids in the breast cancer therapy in women especially when docetaxel is applied. There is evidence that β-carotene can bind the retinoid receptors without being cleaved to form vitamin A [35] so maybe this process is enhanced by docetaxel in MCF-7 cells.

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