Vitamin A family compounds, estradiol, and docetaxel in proliferation, apoptosis and immunocytochemical profile of human ovary endometrioid cancer cell line CRL-11731

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Abstract: Endometrioid carcinoma represents approximately 10% of cases of the malignant ovarian epithelial tumors. According to literature, the vitamin A (carotenoids and retinoids) plays an essential role in cell proliferation, differentiation and apoptosis in both normal and neoplastic ovarian tissues. Apart from that, the retinoids alter a cytotoxic effect of chemotherapeutics, i.e. docetaxel, on ovarian cancer cell lines. Retinoids act on cancer cells throughout different mechanism than taxanes, so they may be the potential candidates for the new treatment strategies of ovarian cancer. The aim of the study was to determine the effects of vitamin A family compounds (retinol, β-carotene, lycopene, all-*trans* -, 9-*cis* – and 13-*cis* retinoic acid) on the growth and proliferation of CRL-11731 endometrioid ovary cancer cell line and on docetaxel and estradiol activity in this culture. The assay was based on [3H] thymidine incorporation and the proliferative activity of PCNA- and Ki 67-positive cells. The apoptotic index and expression of the Bcl-2 and p53 antigens in CRL-11731 cells were also studied. Among vitamin A family compounds retinol and carotenoids, but not retinoids, inhibited the growth of cancer cells in dose dependent manner. Only the concentration of 100 μM of docetaxel inhibited incorporation [3H] thymidine into CRL-11731 cancer cells. Retinol (33.4%±8.5), carotenoids (β-carotene 20 μM 4.7%±2.9, 50 μM 2.2%±0.9; lycopene 10 μM 7.6%±0.8, 20 μM 5.2%±2.5, 50 μM 2.9%±1.2), and 13-*cis* retinoic acid (19.7%±2.2) combined with docetaxel (100 μM) significantly decreased the percentage of proliferating cells (p<0.0001). The antiproliferative action of lycopene alone and in combination with docetaxel was also confirmed in immunohistochemical examination (decreased the percentage of PCNA and Ki67 positive cells). Also retinol (10 μM) and lycopene (20 and 50 μM) combined with estradiol (0.01 μM) statistically decreased the percentage of proliferating cells compared to the control (p<0.0001) and estradiol (p<0.01, p<0.0001) group (63.5%±14.8, 61.0%±20.6, 15.0%±5.5 respectively). In our experiments, the compounds tested induced an apoptotic effect. Docetaxel and estradiol increased the percentage of apoptotic cells (71% apoptotic cells after administration of 10 μM all-*trans* retinoic acid combined with 0.01 μM estradiol, p<0.0001). β-carotene, lycopene and all-*trans* retinoic acid alone and in combination with docetaxel were found to influence the expression of bcl-2 and p53 antigen in the cells examined. The results of our study justified an important role of vitamin A in the pathophysiology of the ovarian endometrioid cancer.

Key words: vitamin A, docetaxel, estradiol, CRL-11731, proliferation, apoptosis

Introduction

Ovarian carcinoma is the most fatal of the neoplasms that affect the female reproductive organs. Patients usually seek medical advice when cancer clinical advancement precludes complete recovery. Even though the subsequent stages of molecular mechanisms of carcinogenesis have been discovered, the etiology and pathology of ovarian carcinoma are still not fully elucidated. According to the type of carcinogenic cells, ovarian carcinomas can be divided mainly into: surface epithelial-stromal, sex cord-stromal, germ cell mucous tumors [1]. Endometrioid cancer is the third most common of the epithelial ovarian neoplasms [2], accounting for approximately 10% of cases [3]. This

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histological type originates from foci of endometriosis and endometrial cysts localized in the ovary [3]. The structural-histological similarity between endometrioid ovarian cancer and endometrioid uterine carcinoma suggests its development from malignant transformation of surface epithelium [4]. Wang *et al*. have proved that ovarian carcinoma is a heterogeneous pathology, in which histological phenotype correlates with certain phenomena on the molecular level [5], and that certain genetic changes may cause malignant transformation of endometriosis [6]. Although ovarian carcinoma responds to chemotherapy (paclitaxel, carboplatin) [7], its prognosis still remain poor due to recurrent nature. Therefore, a search is conducted for compounds/drugs showing other mechanisms, among which vitamin A derivatives play a major role [8].

Vitamin A (carotenoids and retinoids) is believed to have a fundamental role in proliferation, differentiation and apoptosis of ovarian cancer cells [9,10]. Since its active metabolites, mainly retinoic acid, by binding to specific nuclear receptors (RAR and RXR) activate transcription processes, retinoids seem to play chemopreventive and chemotherapeutic roles in ovarian carcinoma [11].

As revealed by epidemiological studies, also carotenoids, i.e. retinoid precursors, provided to the human body with plant and animal foods reduce the risk of ovarian cancer [12]. It has been shown that patients with advanced forms of carcinoma have lower serum carotenoid concentrations [13,14].

β-carotene, the most widely spread carotenoid belonging to the provitamin A group (split into two vitamin A molecules), acts as an antioxidant in the human body and can reach its target cells via blood in an unchanged form [15]. Among carotenoids that do not belong to the provitamin A group, lycopene is the most active antioxidant [16].

The study objective was to assess the effect of compounds belonging to the vitamin A family (retinol, β-carotene, lycopene, *trans* retinoic acid, 9-*cis* retinoic acid and 13-*cis* retinoic acid) on the growth and proliferation of the human ovarian endometrioid cancer cell line CRL-11731, and to evaluate the influence of the compounds tested on the activity of docetaxel and estradiol in the culture of cancer cells. We also made an attempt to elucidate the effect of the substances tested on the stimulation of the apoptotic pathway in ovarian endometrioid carcinoma.

Materials and methods

Chemicals. Retinol (all-*trans* retinol, Sigma) β–carotene (β-carotene, Sigma), lycopene (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), 17 β-estradiol (1,3,5 [10]-estratriene-3, 17 β-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), Ki 67: monoclonal mouse antibody (clone Ki 67), Bcl-2 oncoprotein: monoclonal mouse antibody (clone 12) and p53 protein: monoclonal mouse antibody (clone DO-7) was 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 17 β-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 cell line CRL-11731 of human ovary cancer (American Type Culture Collection, Rockville, MD). Cells were maintained in 75 cm^2 tissue culture flask (Sarsted, USA), under standard culture conditions; at 37°C, 5% CO$_2$ /95% air humidified incubator in 1:1mixture of MCDB 105 medium and 199 medium (Sigma-Aldrich, Poland), were renewal every 3-4 days. Culture media contained 15% of fetal bovine serum (Sigma-Aldrich, Poland), and enriched with 50 U/ml antibiotic, antimycotic solution (penicillin, streptomycin, amphotericin B (Sigma-Aldrich, Poland). When cells reached 80% confluence cells were rinse with 0.05% trypsin/0.02% EDTA solution (Sigma-Aldrich, Poland) to remove all traces of serum. After that culture were incubated witch 3 ml of trypsin -EDTA solution. Cells layer were observed under an inverted microscope and cells dispersed usually within 5 to 15 minutes of incubation. Cells were aspirate by pipeting with complete growth medium (6 to 8 ml). Appropriate medium were added for cell suspension in new culture vessels. Subcultivation ratio was 1:3. For experiment cells were collected and seeded in 6 well culture plates (Sarsted, USA). The cells were incubated for 24 h prior to treatment and allowed to proliferate to 70% – 80% confluence before commencement of each experiment, without serum in MCDB105/199 medium.

The methods for [3H] thymidine incorporation, immunocytochemical examinations and determination of apoptotic index were described previously in Folia Histochem Cytobiol. 2004;42:221-227 and Acta Biochim Polon. 2004;51:733-745.

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

The effects of retinol, carotenoids and retinoids as well as docetaxel and estradiol on [³H] thymidine incorporation into carcinoma cells.

Of the compounds of the vitamin A family, retinol and carotenoids were found to inhibit incorporation of labeled thymidine into cancer cells in a dose-dependent manner. Addition of 50 μM retinol to the cell culture nearly totally inhibited proliferation of the cells tested (Fig. 1).

Retinoids showed no such effects in a 24h culture (Fig. 2).

The concentrations of 0.0000001 to 1 μM docetaxel stimulated the proliferation of the cell line CRL-11731. We observed the inhibition of [³H] thymidine
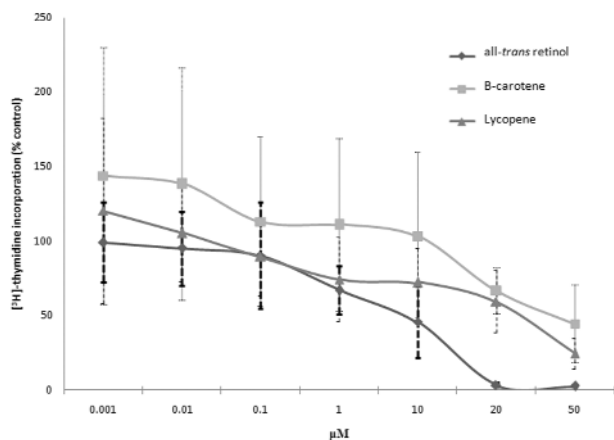
Fig. 1. Influence of retinol and carotenoids on [³H]-thymidine incorporation into CRL-11731 ovary cancer cells. Control = 100%. Exposure time 24 h. Data presented as mean values ± SD (n=4).

Fig. 2. Influence of retinoids on [³H]-thymidine incorporation into CRL-11731 ovary cancer cells. Control = 100%. Exposure time 24 h. Data presented as mean values ± SD (n=4).
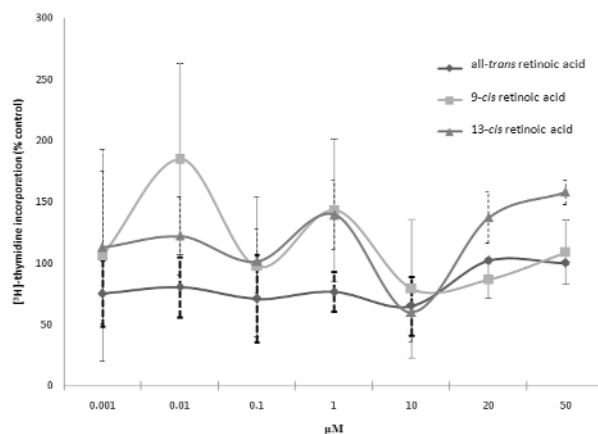

Fig. 3. Influence of docetaxel on [³H]-thymidine incorporation into CRL-11731 ovary cancer cells. Control = 100%. Exposure time 24 h. Data presented as mean values ± SD (n=4).

Fig. 4. Influence of estradiol on [³H]-thymidine incorporation into CRL-11731 ovary cancer cells. Control = 100%. Exposure time 24 h. Data presented as mean values ± SD (n=4).
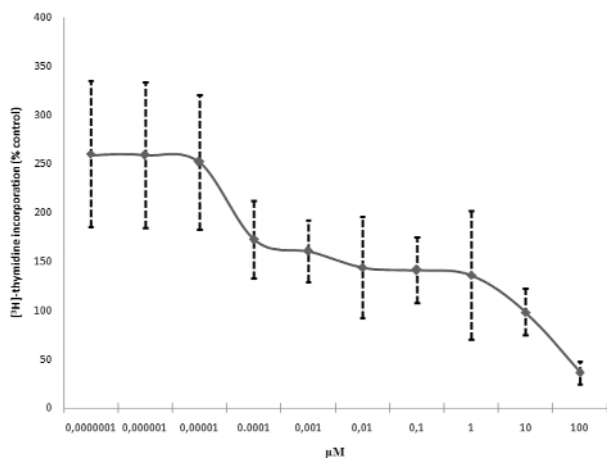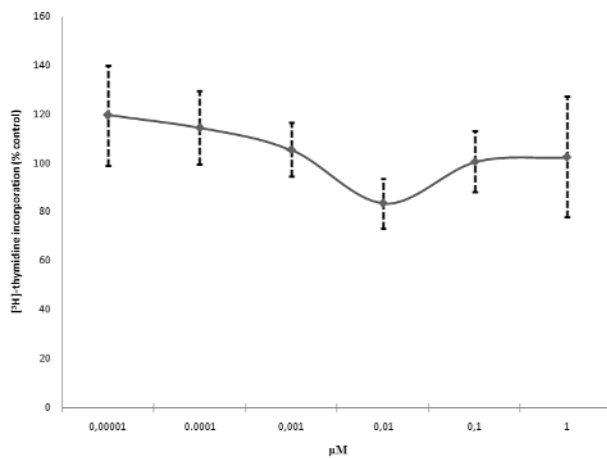
incorporation into cancer cells only after adding 100 μM docetaxel to the cell culture (36%) (Fig. 3). We used the same concentration of this chemotherapeutic in further study phases.

Estradiol used at a concentration of 0.01 μM slightly inhibited the proliferation of ovarian carcinoma cells (83%) (Fig. 4).

The effects of retinol, carotenoids and retinoids combined with docetaxel or estradiol on [³H] thymidine incorporation into carcinoma cells.

Retinol and carotenoids added to cell culture simultaneously with docetaxel (100 μM) statistically significantly reduced the percentage of proliferating cells as compared to the control and docetaxel group. Also retinol (10 μM) and lycopene (20 and 50 μM) added to the culture simultaneously with estradiol (0.01 μM) had

a similar effect. On the other hand, irrespective of the concentrations, β-carotene stimulated the growth of cancer cells in the presence of estradiol (Fig. 5).

Among retinoids, only 10 μM 13-*cis* retinoic acid statistically significantly inhibited the proliferation of cancer cells. Retinoids added to the culture in combination with estradiol stimulated the growth of the cell line tested (Fig. 6).

Immunohistochemical examinations

The antiproliferative actions of lycopene alone and in combination with docetaxel were also confirmed in immunohistochemical examinations (decreased percentage of PCNA and Ki67 positive ovarian cancer cells) (Table 1).

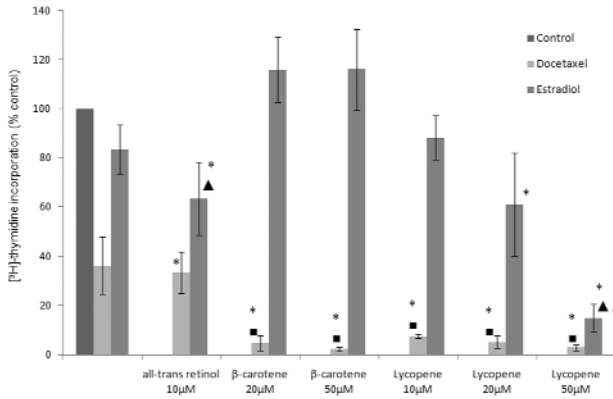In immunohistochemical examinations, β-carotene alone and combined with docetaxel decreased the per-

Fig. 5. Influence of retinol and carotenoids in combination with docetaxel and estradiol on [³H]-thymidine incorporation into CRL-11731 ovary cancer cells. Control = 100%. Exposure time 24 h. Data presented as mean values ± SD (n=4). Cocentrations: docetaxel 100 μM, estradiol 0.01 μM. * – p<0.0001 relative to the control group, ■ – p<0.0001 relative to the docetaxel group, ▲ – p<0.001 relative to the estradiol group, ▲▲ – p<0.0001 relative to the estradiol group.

Fig. 6. Influence of retinoids in combination with docetaxel and estradiol on [³H]-thymidine incorporation into CRL-11731 ovary cancer cells. Control = 100%. Exposure time 24 h. Data presented as mean values ± SD (n=4). Cocentrations: docetaxel 100 μM, estradiol 0.01 μM. * – p<0.0001 relative to the control group, ■ – p<0.0001 relative to the docetaxel group.

centage of bcl-2 positive and increased the percentage of p53 positive ovarian cancer cells. The same effect was also observed for all concentrations of lycopene alone and combined with docetaxel (Tab. 1). Among retinoids, only all-*trans* retinoic acid (0.01 and 10 μM) influenced the percentages of bcl-2 and p53 positive ovarian cancer cells (Table 2).

Assessment of the apoptotic index

All the compounds tested alone and in combination with docetaxel or estradiol stimulated the apoptotic pathway (Table 3 and 4). The percentage of apoptotic cells increased after addition of docetaxel, in some cases also when estradiol was added. We observed the greatest percentage of apoptotic cells (71%) after addition of 10 μM all-*trans* retinoic acid combined with 0.01 μM estradiol.

Discussion

The vitamin A family compounds take part in many physiological and pathological processes in the ovary [9,10].

The presence of carotenoid pigments in physiological and pathological ovarian tissues has been previously demonstrated by our team [17,18]. Furthermore, among the ovarian epithelial neoplasms, the endometrioid carcinoma tissue has the highest total carotenoid content (β-carotene, β-cryptoxanthin, lutein, zeaxanthin, canthaxanthin, epoxy-lutein, neoxanthin, violaxanthin and mutatoxanthin) and the highest percentage of the provitamin A group carotenoids (*e.g.* β-carotene) [17]. Thus, it is likely that carotenoids may have an influence on proliferation, differentiation and apoptosis
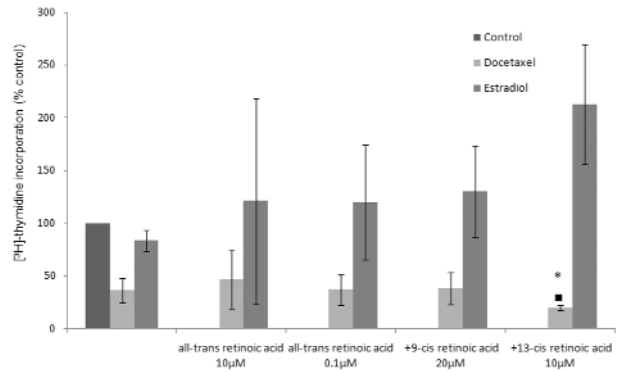
of the ovarian carcinoma cells. In addition, a coordinate inactivation of a group of 3p genes by genetic or epigenetic mechanism for example under-expressed putative tumor suppressor genes, i.e. retinoic acid receptor β (RAR β) may be an important factor for the pathogenesis of ovarian cancer [11]. It has also been demonstrated that β-carotene can reach target tissues in an unchanged form and undergo metabolic transformation into retinoids directly in the ovary [15].

The results of Wu *et al.* have shown various ways in which retinoids affect ovarian carcinoma cells. Retinoids were found to inhibit the growth of the cell line CA-OV3 but not SK-OV3. The mechanism was associated with blocking of the cell cycle in the G-0 and/or G-1 phase [19]. In a 48-hour experiment, 10 μM retinoic acid and 9-*cis* retinoic acid did not restrain the incorporation of a labeled thymidine into the cancer cells of six epithelial cell lines, whereas in the cell line OV 266 they had a stimulating effect. Both retinoic acid and 9-*cis* retinoic acid inhibited cell proliferation in approximately 80% only in the OV 167 and OV 177 cell lines [20]. The cancer cells used in our experiments originated from an aggressive ovarian endometrioid tumor (stage 3 grade 3) and were highly proliferative ones. We observed a similar effect of retinoids (10 μM) in a 24-hour culture of the cell line CRL-11731.

The cancer cell growth inhibition was well-defined after application of 10 μM retinol and 20 and 50 μM β-carotene and lycopene. Retinol and lycopene counteracted the stimulatory action of estradiol and β-carotene (irrespectively of concentrations), whereas retinoids enhanced this action. According to literature, β-carotene and lycopene are the most abundant carotenoids in the human tissues [21,22]. However, we did not confirm the presence of lycopene in the studied

Table 1. Percentage of PCNA, Ki67, Bcl-2 and p53 positive CRL-11731 ovary cancer cells.

	PCNA	Ki 67	bcl 2	p53
Control	65.7±2.1 ++/+++	59.0±1.7 ++/+++	23.3±10.3 ++	54.3±4.4 +/++/+++
Docetaxel	45.0±2.0 ++/+++	45.3±2.5 +++	25.0±2.0 ++/+++	55.3±1.5 +++
Estradiol	92.3±3.0 +++	86.0±2.6 +++	18.0±1.0 ++/+++	51.0±2.6 ++/+++
Retinol 10 μM	65.7±3.5 ++/+++	60.3±2.1 ++/+++	25.0±0.70 +/++	55.5±4.9 ++/+++
Retinol 10 μM + docetaxel	55.0±2.6 ++/+++	65.7±1.5 ++/+++	25.0±3.0 ++	65.0±2.6 +/++
Retinol 10 μM + estradiol	80.0±2.0 +++	80.5±0.7 ++/+++	20.7±1.5 +/++	65.0±4.0 +++
β-carotene 20 μM	65.7±3.5 ++/+++	91.7±1.5 +++	20.5±2.1 +++	65.0±3.0 ++/+++
β-carotene 50 μM	65.7±3.0 ++/+++	85.7±2.1 ++/+++	17.5±3.5 ++/+++	80.3±4.5 ++/+++ ******●●●●●■
β-carotene 20 μM + docetaxel	75.0±3.0 +++	96.0±1.0 +++	14.7±1.5 ++ *●	75.0±1.41 +++ ******●●●●●■
β-carotene 50 μM + docetaxel	85.7±4.0 +++	81.7±0.6 ++/+++	10.0±1.4 ++ ***●●	75.7±5.8 ++/+++ ******●●●●●■
Lycopene 10 μM	35.7±3.2 ++/+++ ***	34.3±2.1 +/++/+++ ***	7.5±0.7 ++ ****●●●	90.0±1.4 ++/+++ ******●●●●●■
Lycopene 10 μM + docetaxel	30.7±2.1 ++/+++ ***	31.3±0.6 ++/+++ ****	6.3±2.1 ++ ******●●●●●	90.3±2.1 +++ ******●●●●●■
Lycopene 20 μM	40.7±4.0 ++/+++ **	40.3±5.0 ++/+++ ***	10.9±2.6 ++ ***●●	90.3±2.1 +++ ******●●●●●■
Lycopene 20 μM + docetaxel	31.3±5.7 ++ ****	35.7±1.1 ++/+++ ***	8.0±1.0 +++ *****●●●●	85.7±2.1 +/++ ******●●●●●■
Lycopene 20 μM + estradiol	45.0±1.0 ++/+++ ****	43.3±2.1 ++/+++ **	22.5±1.5 ++	55.3±3.0 ++/+++
Lycopene 50 μM	35.7±2.5 ++/+++ ***	39.3±3.0 +/++/+++ ***	5.3±1.5 +/++ ******	96.3±2.1 ++/+++ ******●●●●●■
Lycopene 50 μM + docetaxel	35.0±1.0 ++/+++ ***	35.7±3.5 ++/+++ ***	3.3±1.1 ++/+++ ******●●●●●	86.7±2.1 +/++ ******●●●●●■
Lycopene 50 μM + estradiol	40.0±2.6 +++ ***	44.7±4.5 +++ **	25.6±1.3 ++	53.0±1.0 ++/+++

100 μM docetaxel, 0.01 μM estradiol; exposure time 24 h.
Data presented as mean values ± S.D. (n=4);
Statistically significant differences:
Relative to the control group:
*　　　　p<0.02
**　　　p<0.004
***　　　p<0.001
****　　p<0.0005
*****　　p<0.0004
******　p<0.0001

Relative to the docetaxel group:
●　　　　p<0.02
●●　　　p<0.001
●●●　　p<0.0005
●●●●　p<0.0004
●●●●●　p<0.00001
Relative to the estradiol group:
■　　　　p<0.0001
Immunostaining of PCNA, Ki67, Bcl-2 and p53 of ovary cancer cells:
+　　　　weak
++　　　mild
+++　　strong

Table 2. Percentage of PCNA, Ki67, Bcl-2 and p53 positive CRL-11731 ovary cancer cells.

	PCNA	Ki 67	bcl 2	p53
Control	65.7±2.1 ++/+++	59.0±1.7 ++/+++	23.3±10.3 ++	54.3±4.4 +/++/+++
Docetaxel	45.0±2.0 ++/+++	45.3±2.5 +++	25.0±2.0 ++/+++	55.3±1.5 +++
Estradiol	92.3±3.0 +++	86.0±2.6 +++	18.0±1.0 ++/+++	51.0±2.6 ++/+++
9-*cis* retinoic acid 10 μM	76.0±1.0 ++/+++	85.7±2.1 ++/+++	19.0±1.0 +/++	55.0±1.0 +++
9-*cis* retinoic acid 20 μM	85.7±1.5 +++	80.7±2.5 ++/+++	19.2±1.0 +/++	50.6±2.8 +++
9-*cis* retinoic acid 20 μM + docetaxel	86.0±2.0 +++	86.3±2.1 +++	19.3±3.05 +	45.0±2.0 +++
13-*cis* retinoic acid 10 μM	93.0±2.6 +++	75.3±1.5 +++	27.7±1.5 +/++	41.7±1.5 ++
13-*cis* retinoic acid 10 μM + docetaxel	64.7±0.6 ++/+++	65.0±3.0 +++	18.7±0.6 +	36.7±1.5 +
all-*trans* retinoic acid 0.01 μM	92.7±3.2 +++	80.5±3.0 +++	8.0±1.0 +/++*●	72.3±2.5 +++**■
all-*trans* retinoic acid 0.01 μM + docetaxel	70.7±1.5 +++	87.0±2.0 +++	6.1±2.1 ++/+++**●●	84.0±1.0 ++/+++***■■
all-*trans* retinoic acid 10 μM	63.7±1.1 ++/+++	83.0±4.6 +++	8.3±1.5 ++/+++*●	91.7±1.5 ++***■■
all-*trans* retinoic acid 10 μM + docetaxel	90.7±0.5 +++	85.3±1.5 +++	8.3±1.5 +/++*●	91.7±1.5 ++/+++***■■

100 μM docetaxel, 0.01 μM estradiol; exposure time 24 h.
Data presented as mean values ± S.D. (n=4);
Statistically significant differences:
Relative to the control group:
*   p<0.001
**   p<0.0005
***   p<0.0001
Relative to the docetaxel group:
●   p<0.001
●●   p<0.0005
●●●   p<0.0001

Relative to the estradiol group:
■   p<0.0005
■■   p<0.0001
Immunostaining of PCNA, Ki67, Bcl-2 and p53 of ovary cancer cells:
+   weak
++   mild
+++   strong

ovarian tissues [17]. This may result from the fact that lycopene acts as an intermediate link in the formation of carotenoids, such as β-carotene, or its role as a free radical scavenger [23].

In our study, only lycopene, apart from promoting apoptosis, affected the expression of PCNA and Ki67 antigens in the cancer cell line CRL-11731.

Retinoids have been shown to increase a cytotoxic effect of the chemioterapeutics, also docetaxel, in the cell lines of ovarian carcinoma [24]. Docetaxel is a semisynthetic anticancer agent which shows its action throughout binding to β-tubulins and stabilizing the microtubular network, thus blocking the cell cycle at G2-M phase and subsequent regulate the apoptosis of the cells [25,26], also in ovarian cancer cells [27].

The IC$_{50}$ concentrations for docetaxel were 0.8 nM

for CRL-11731 cells in 72 h experiments [28]. Our findings revealed that low doses of docetaxel (0.0000001 – 0.00001 μM) strongly stimulated the growth of ovarian cancer cells (24 h experiments).

Retinol, β-carotene, lycopene and 13-*cis* retinoic acid added to the cell culture simultaneously with docetaxel (100 μM) significantly reduced the percentage of proliferating cells and increased the percentage of apoptotic cells. As shown by *in vitro* studies, the inhibition of the growth of ovarian cancer cells could occur via a number of mechanisms, including apoptosis induction [20,29,30].

Programmed cell death, i.e. apoptosis, is a fundamental process of tissue development and homeostasis, stimulated by growth factors withdrawal, deregulation of the cell cycle, DNA damage or lack of the receptor-

Table 3. Influence of retinol β-carotene and lycopene alone and combined with docetaxel and estradiol on apoptosis in CRL-11731 ovary carcinoma cells.

	Control	Docetaxel	Estradiol	Retinol 10 μM	β-carotene 20 μM	β-carotene 50 μM	Lycopene 10 μM	Lycopene 20 μM	Lycopene 50 μM
Viable cells (%)	95.0±1.0	58.7±2.1	58.7±1.1	71.0±2.0	60.3±2.1	41.3±3.0	69.7±3.5	58.0±3.5	60.3±0.6
Apoptotic cells (%)	3.7±0.6	34.7±0.6 **	39.3±2.1 **	22.7±1.5 *	35.3±1.5 **	55.3±3.0 **●▲	29.7±2.5 **	40.7±2.1 **	36.7±1.5 **
Necrotic cells (%)	1.3±0.6	6.3±1.1	2.0±1.0	6.3±2.1	4.3±1.1	3.3±1.1	0.7±1.1	1.3±1.5	3.0±1.0
					+ Docetaxel				
Viable cells (%)				52.7±1.1	41.0±1.7	38.7±2.5	51.3±4.2	46.7±2.1	37.0±1.4
Apoptotic cells (%)				39.3±2.1 **■	50.0±2.0 **○	60.3±3.0 **●●	41.3±2.1 **	49.7±1.5 **	53.0±1.4 **●□
Necrotic cells (%)				8.0±1.0	9.0±1.0	1.0±1.0	8.3±1.5	3.7±1.1	10.0±2.8
					+ Estradiol				
Viable cells (%)				50.7±1.1	57.5±4.9	36.7±9.0	55.0±1.4	53.0±2.6	38.0±2.6
Apoptotic cells (%)				44.0±1.0 **■■	40.5±3.5 **	52.7±4.2 **	41.5±3.5 **	44.0±2.0 **	54.7±2.5 **▲□
Necrotic cells (%)				5.3±2.1	2.0±1.4	11.0±5.3	3.5±4.9	3.0±1.0	7.3±1.1

100% = viable (%) + apoptotic (%) + apoptopic/necrotic (%) + necrotic (%) cells. 100 μM docetaxel, 0.01 μM estradiol.
Exposure time 24 h. Data presented as mean values ±S.D. (n=4); Statistically significant differences:

*	p<0.001	relative to the control group
**	p<0.0001	
●	p<0.01	relative to the docetaxel group
●●	p<0.0001	
▲	p<0.002	relative to the estradiol group
■	p<0.01	relative to the retinol 10 μM group
■■	p<0.0003	
○	p<0.01	relative to the β-carotene 20 μM group
□	p<0.01	relative to the lycopene 50 μM group

ligand interaction [31,32,33]. Apoptosis may be induced in order to remove abnormal cells. The mechanism of apoptosis involves morphological and biochemical processes. Apoptosis is the highly regulated process involving the sequential activation of multiple proteins (caspases and proteases). Lack of proper apoptotic regulation can result in various diseases and may cause tumorgenesis of ovarian cells [34]. The initial development of ovarian carcinoma is associated with a high expression of Bcl-2 proteins and in some cases with a defect in p53 gene or its regulation. So these cells are resistant to DNA damage- induced apoptosis and cell cycle control. Programmed cell death in ovarian cancer can be related to cell cycle control [35] and a "cross-talk" with AP1 [36] and EGF [37].

Bcl-2 is one of the factors which strongly inhibits apoptosis. We observed an enhanced apoptotic effect of 20 μM 9-*cis* retinoic acid combined with docetaxel and estradiol. Also 10 μM of all-*trans* retinoic acid combined with estradiol increased the percentage of apoptotic cells. Nahme *et al.* concluded that ATRA synergistically enhanced docetaxel toxicity by down regulation of Bcl-2 expression [38]. Our results showed that apart from β-carotene and lycopene, only concentrations of all-*trans* retinoic acid used in the study decreased the percentage of bcl-2 positive cells and increased the percentage of p53 positive cells.

Also p53 has been reported to down regulate the antiapoptotic Bcl-2 protein [39]. It has been proposed that the low levels of p53 determine growth arrest and high levels determine apoptosis [40]. Thus, high levels of p53 are required for the transactivation of proapoptotic genes.

The process of apoptosis can also be initiated without Bcl-2 proteins [41].

Table 4. Influence of 9-*cis*-, 13-*cis*-, and all-*trans* retinoic acid alone and combined with docetaxel and estradiol on apoptosis in CRL-11731 ovary carcinoma cells.

	Control	Docetaxel	Estradiol	9-*cis* retinoic acid 10 μM	9-*cis* retinoic acid 20 μM	13-*cis* retinoic acid 10 μM	all-*trans* retinoic acid 0.1 μM	all-*trans* retinoic acid 10 μM
Viable cells (%)	95.0±1.0	58.7±2.1	58.7±1.1	39.7±1.5	34.0±4.0	61.7±0.6	53.0±4.2	47.5±0.7
Apoptotic cells (%)	3.7±0.6	34.7±0.6 *	39.3±2.1 *	52.7±3.8 *●	60.7±2.5 *●●▲	34.0±1.0 *	44.0±5.6 *	50.5±2.1 *
Necrotic cells (%)	1.3±0.6	6.3±1.1	2.0±1.0	7.7±2.3	5.3±1.5	4.3±1.1	3.0±1.4	2.0±1.4
						+ Docetaxel		
Viable cells (%)				38.3±2.1	17.7±3.5	37.7±2.1	29.0±0.0	31.5±3.5
Apoptotic cells (%)				56.0±3.6 *●	75.0±3.0 *●●■	52.3±1.5 *●○	57.5±2.1 *●	56.5±2.1 *●
Necrotic cells (%)				5.7±2.1	7.3±0.6	10.0±2.6	13.5±2.1	12.0±1.4
						+ Estradiol		
Viable cells (%)				50.0±1.7	31.0±3.6	56.0±1.7	42.0±2.0	24.5±0.7
Apoptotic cells (%)				46.0±1.0 *	57.7±2.5 *▲	36.7±1.5 *	57.0±1.0 *▲	71.0±1.4 *▲▲□
Necrotic cells (%)				4.0±1.0	11.3±1.1	7.3±3.0	1.0±1.7	4.5±0.7

100% = viable (%) + apoptotic (%) + apoptopic/necrotic (%) + necrotic (%) cells. 100 μM docetaxel, 0.01 μM estradiol.

Exposure time 24 h. Data presented as mean values ± S.D. (n=4); Statistically significant differences:

*	p<0.0001	relative to the control group
●	p<0.001	relative to the docetaxel group
●●	p<0.0001	
▲	p<0.001	relative to the estradiol group
▲▲	p<0.0001	
■	p<0.01	relative to the 9-*cis* retinoic acid 20 μM group
○	p<0.01	relative to the 13-*cis* retinoic acid 10 μM group
□	p<0.001	relative to the all-*trans* retinoic acid 10 μM group

The p53 pathway as well as estrogen signaling plays an important role for differentiation and growth of CRL-11731 cells [42].

The carcinogenesis in the ovary is affected by exo- and endogenous estrogens that act via estrogen receptors α and β (ERα and ERβ) [43]. Loss of ERβ expression is a key step in a malignant transformation process. ERβ upregulates apoptosis in ovarian cancer cells [44] and mediates estrogen-induced apoptosis [45]. In our study, among examined compounds, only retinol and lycopene overcame a proliferative action of estradiol in culture of ovary cancer cell line CRL-11731. A simultaneous application of lycopene and estradiol resulted in a presence of a highest percentage of apoptotic cells in the culture. The vitamin A family compounds role and the molecular events responsible for induction of apoptosis in CRL-11731 ovary cancer cells need some further investigations.

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