In Vitro Effect of Knotolan, a New Lignan from *Abies sibirica*, on the Growth of Hormone-Dependent Breast Cancer Cells

O. S. Zhukova[1], L. V. Fetisova[1], A. V. Trishin[1], N. Yu. Anisimova[1], A. E. Shcherbakov[1], D. V. Yashunskii[2], D. E. Tsvetkov[2], V. M. Men'shov[2], M. V. Kiselevskii[1], and N. E. Nifant'ev[2]

Translated from *Kletochnye Tehnologii v Biologii i Medicine*, No. 2, pp. 78-81, April, 2010
Original article submitted September 3, 2009.

Here we present antiestrogenic effects of Knotolan®, a new dietary lignan from *Abies sibirica* raw material. Knotolan abolished growth-stimulating effects of 17β-estragiol on hormone-dependent MCF-7 cells.

Key Words: *lignans; phytoestrogens; breast cancer; Knotolan*

Lignans and isoflavonoids are the important groups of phytoestrogens (PE), natural compounds with estrogenic activity. Recently developed isoflavonoid bioactive additives (Glycine max, T. prateuse) exhibit positive effects in postmenopausal disorders [8,10].

The results of experimental studies suggest that lignans can exhibit tumor-protective properties and inhibit tumor growth. These effects are based on various mechanisms including antiestrogenic, antiangiogenic, antioxidant, and apoptosis-inducing activities these compounds [3,4,11]. Linseed, sunflower seed, wheat, rye, rice, and nuts are rich with lignan precursors. Lignans isolated from plant sources contain 2,3-dibenzylbutane, a product of dimerization of two cinnamic acid residues. In the organism of mammals, dietary lignans matairesinol and secoisolariciresinol are converted into enterodiol and enterolactone, respectively. Enterodiol can then be converted into enterolactone, a primary lignan that is present in the blood of mammals. Secoisolariciresinol predominates among vegetable lignans [11].

[1]N. N. Blokhin Cancer Research Center, Russian Academy of Medical Sciences; [2]N. D. Zelinskii Institute of Organic Chemistry, Russian Academy of Sciences, Moscow, Russia. ***Address for correspondence:*** kiselev@inbox.ru. M. V. Kiselevskii

Here we studied the effect of secoisolariciresinol (Knotolan®) isolated from a new nonfood vegetable raw material (*Abies sibirica* leaves) on proliferation of hormone-dependent breast cancer cells *in vitro*.

MATERIALS AND METHODS

Knotolan was obtained from *Abies sibirica* wood as described earlier [2]. Commercial enterolactone (Sigma) was used as the positive control. Knotolan and enterolactone were dissolved in ethanol and then in RPMI-1640. The final concentration of Knotolan and enterolactone in samples 1000-fold surpassed the concentration of 17β-estradiol (E2) and was 1 μM, because previous studies showed that higher concentrations of enterolactone produced a growth-stimulating effect *in vitro* [10].

Human breast cancer cells (MCF-7) obtained in Laboratory of Molecular Endocrinology, Institute of Carcinogenesis, N. N. Blokhin Cancer Research Center, were used as the test object. The number of estrogen receptors was determined by a modified radioligand method [7]. Specific binding was measured at saturating concentration of ^{3}H-labeled E2 (^{3}H-E2; GE Healthcare; specific radioactivity 85-95 Ci/mol) in the presence and absence of a 200-

0007-4888/10/1494-0511

fold excess of unlabeled hormone (diethylstilbestrol, Sigma).

The cells (MCF-7) were grown in RPMI-1640 medium without phenol red with 10 mM L-glutamine and 10% FCS containing estrogens. Before the experiment, the cells were washed with Hanks saline and harvested with Versene; the cell suspension was transferred to 96-well plates (1000 cells per well) in the above medium containing 5% FCS with estrogen and cultured for 48 h. Then, the medium was removed from the wells and the cells were washed with Hanks saline and then grown in a medium containing 5% FCS without estrogens for 72 h. The cells were than twice washed with physiological saline, 200 μl medium containing 5% FCS without estrogens was added to each well, and then the test compounds (effectors) dissolved in ethanol were added (final concentration of ethanol in samples did not exceed 0.001%). The effectors were added according to the following scheme: 1) E2; 2) Knotolan; 3) enterolactone; 4) Knotolan+E2; 5) enterolactone+E2.

The cells were incubated with E2, Knotolan, or enterolactone for 5 days. In case of combined treatment with lignan and E2, the cells were first incubated with lignan for 2 days and then E2 was added and incubation was continued for 3 days under the same conditions. The total duration of incubation was 5 days. Cells grown under the same conditions, but without effectors served as the control.

For evaluation of cell proliferation in samples, standard MTT test was used. Light absorption of stained dimethylsulfoxide solutions was measured on a Titertek Multiskan MCC/340 at λ=570 nm. Light absorption of solutions is proportional to the number of living cells in the sample. The results are presented as the ratio of light absorption in experimental samples ($A_{experiment}$) to that in the control ($A_{control}$) calculated by the formula: $A_{experiment}/A_{control}\times100\%$. Measurement error did not exceed 5%.

Microscopic examination was performed using an AxioImager inverted microscope (Zeiss).

RESULTS

Radioligand analysis of estrogen receptor binding capacity of MCF-7 cells revealed $(6.1\pm0.5)\times10^5$ ^{3}H-E2 binding sites per cells. These findings confirmed the presence of estrogen receptors in MCF-7 cells.

On day 5, incubation of cells with E2 in a concentration of 1 nM increased proliferation of hormone-dependent MCF-7 cells by 41.7% (Table 1, Fig. 1, *a*). Incubation with Knotolan or enterolactone in a concentration of 1 μM for 5 days had no effect on cell proliferation. Cell survival in this case was 98.0 and 99.6% of the control, respectively (Table 1, Fig. 1, *b*, *c*). Preincubation with Knotolan or enterolactone for 2 days followed by 3-day incubation with E2 did not stimulate cell proliferation, which corresponded to 99.8% and 101.5% cell survival (Table 1, Fig. 1, *e*, *f*). Thus, Knotolan was not inferior to enterolactone in inhibiting the growth-stimulating effect of E2.

These findings agree with the notion that molecular weight and the presence of the diphenol ring in the molecule of PE make them structurally similar to E2 [5]. These features enable the interaction of Knotolan and enterolactone with ligand-binding pocket of estrogen receptor. The capacity of Knotolan to abolish the growth-stimulating effect of E2, *i.e.* to exhibit its antiestrogen properties, was similar to the effect of enterolactone (Sigma).

Due to structural similarity with endogenous estrogens, lignans can act as estrogen agonists or antagonists. *In vitro* and *in vivo* studies showed biphasic character of the effect of enterolactone. *In vitro* studies showed that purified lignans compete with E2, stimulate binding of sex steroids with globulin, and inhibit binding of steroids. Moreover, lignans inhibit enzymes involved in the synthesis of steroids, *e.g.* aromatase, 5α-reductase, and 17β-hydroxysteroid dehydrogenase. It was hypothesized that enterolactone and enterodiol compete with androstenedione for substrate binding in the active center of the enzyme [4]. Despite the absence of consensus concept about the mechanisms underlying the effects of lignans, the data obtained on *in vivo* models suggest that lignans can modulate activity of estrogens via direct or indirect interactions with estrogen receptors. It was found that the presence of 10% crude or purified lignan extracts in rat ration reduced the expression of estrogen receptors α and β in the breast tissue [9].

On the basis of our findings we hypothesized that Knotolan (similarly as enterolactone) can bind to estrogen receptors of MCF-7 cells, thereby competing with E2. Thus, Knotolan abolishes the stimulating effect of E2 on proliferation of MCF-7 cells. It can be hypothesized that this protective effect can be realized *in vivo*.

TABLE 1. Influence of Effectors of Proliferation of MCF-7 Cells

Effector	Survival, %
E2	141.7±3.2
Knotolan	98.0±3.7
Enterolactone	99.6±4.1
Knotolan+E2	99.8±3.8
Enterolactone+E2	101.5±3.9

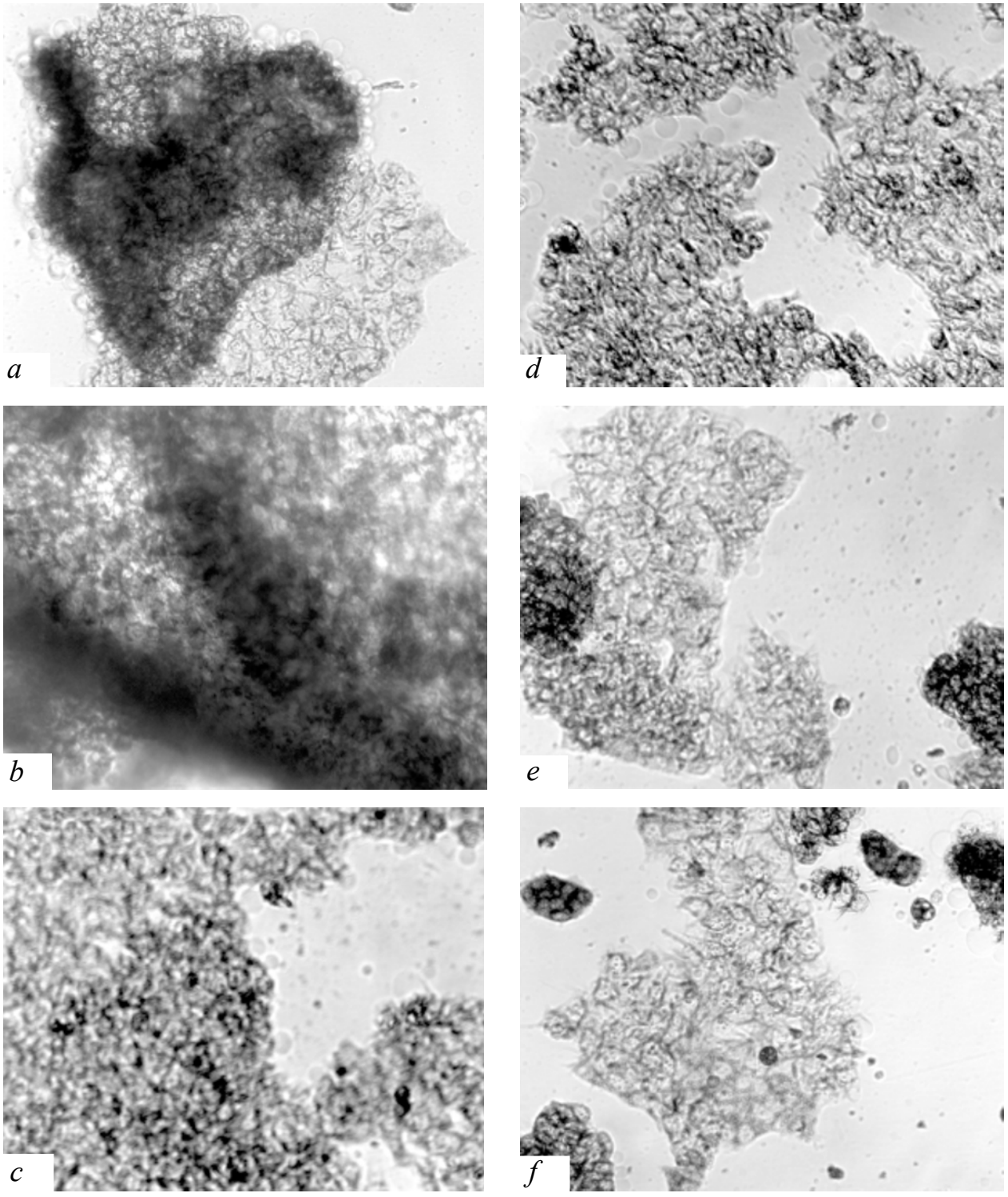

Fig. 1. MCF-7 cells on day 5 of incubation with the test substances after addition of MMT reagent (×200). *a*) control series; *b*) E2; *c*) enterolactone; *d*) Knotolan; *e*) E2+enterolactone; *f*) E2+Knotolan.

The available epidemiological data demonstrate the positive role of lignans during tumor growth [11]. Our findings suggest that Knotolan isolated from *Abies sibirica* can serve as the basis for the creation of a new plant preparation with protective properties.

REFERENCES

1. O. S. Zhukova, G. K. Gerasimova, I. Zh. Shubina, and M. V. Kiselevskii, *Byull. Eksp. Biol. Med*., **142**, No. 8, 198-201 (2007).
2. N. E. Nifant'ev, D. V. Yashunskii, V. M. Men'shov, *et al*., *A Method of Isolation of Secoisolariciresinol and Dihydroquer-*

cetin from Wood (*Variants*). RU 235966, 27.06.2009.
3. W. G. Helferich, J. E. Andrade, and M. S. Hoagland, *Inflammopharmacology,* 16, No. 52, 219-226 (2008).
4. P. J. Magee and I. R. Rowland, *Br. J. Nutr*., **91**, No. 4, 513-531 (2004).
5. K. Morito, T. Hirose, J. Kinjo, *et al., Biol. Pharm. Bull*., **24**, No. 4, 351-356 (2001).
6. N. Olea-Serrano, N. Devleeschouwer, G. Leclercq, and J. C. Heuson, *Eur. J. Cancer Clin. Oncol*., **21**, No. 8, 965-973 (1985).
7. N. M. Saarinen, A. Warri, S. I. Makela, *et al., Nutr. Cancer.* **36**, No. 2, 207-216 (2000).
8. G. Scambia, D. Mango, P. G. Signorile, *et al., Menopause,* **7**, No. 2, 105-111 (2000).
9. J. Teas, T. G. Hurley, J. R. Hebert, *et al., J. Nutr*., **139**, No. 5, 939-944 (2009).
10. K. P. Tan, J. E. Chen, W. E. Ward, and L. U. Thompson, *Exp. Biol. Med.* (*Maywood*), **229**, No. 2, 147-154 (2004).
11. A. L. Webb and M. L. McCullough, *Nutr. Cancer.* **51**, No. 2, 117-131 (2005).