# PHARMACOLOGY AND TOXICOLOGY

# Influence of Estrogen Cytostatics on Activity of Plasma Membrane Enzymes 5'-Nucleotidase and $N^+$-$K^+$-ATPase

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We studied the effect of four conjugated synthetic derivatives of estrone and ethynylestradiol and bis-β-chloroethylamine-containing substance on activity of plasma membrane enzymes 5'-nucleotidase and $N^+$-$K^+$-ATPase. As differentiated from precursors, estrogen cytostatics decreased activity of plasma membrane enzymes. Reference preparations chlorophenacyl and estradiol had little effect on activity of 5'-nucleotidase and $N^+$-$K^+$-ATPase. These data suggest that damage to plasma membrane enzymes is related to the effect of estrogen cytostatic molecules. Test compounds produced an antiproliferative effect on estrogen-independent tumor cells, which strongly correlated with a decrease in activity of plasma membrane enzymes 5'-nucleotidase and $N^+$-$K^+$-ATPase. The derivative of ethynylestradiol with the cytostatic residue in the 3-position of the steroid nucleus (Po-714-11α) most significantly modulated enzyme activity.

**Key Words:** *estrogen cytostatics; plasma membrane enzymes; cytotoxicity*

To decrease general toxicity and increase selectivity of chemotherapeutics the bis-β-chloroethylamine group is attached to metabolites and biologically active substances during the synthesis of antitumor drugs with directed action. These preparations (sarcolysin, lophenal, and cyclophosphane) possess specific physicochemical properties, are characterized by particular distribution in tissues and specific metabolism, and differ from classic bis-β-chloroethylamines (embichin, dopan, and chlorbutin) in their effect and tolerability [2].

Cytostatics obtained from synthetic steroids with different hormonal or antihormonal activity were used for the therapy of hormone-dependent tumors. It was proposed that attachment of the hormone to the alkyl group can modify the distribution of a cytostatic in the organism, direct it to target tissues due to binding of the hormone molecule to receptors in hormone-sensitive tumor tissue, and provide high specificity of the preparation [5,6]. However, further studies showed that attachment of the steroid nucleus with various radicals affects physicochemical properties of the molecule and changes the interaction between cytostatic compounds and cells in hormone-independent tissues [4].

In the present work we studied estrogens transformed by the C-ring and containing the bis-β-chloroethylamine fragment. They possess antitumor activity in relation to transplanted estrogen-sensitive (rat breast carcinoma) and estrogen-independent tumors (mouse skin sarcoma).

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To evaluate the mechanisms of action of estrogen cytostatics not bound to cytosolic estradiol receptors (ER) we studied the effect of the most potent agents on proliferation of transformed L-929 fibroblasts from mouse skin sarcoma [2]. Test compounds produced a potent antiproliferative effect on ER-negative tumor cells. Therefore, the effect of bis-β-chloroethylamine-containing estrogen derivatives is not related to their interaction with the receptors. Transformed compounds were more potent than chlorophenacyl in inhibiting proliferation of fibroblasts. These results indicate that cytostatic activity of estrogen cytostatics is associated not only with the presence of bis-β-chloroethylamine groups, bit also with the complex action of steroid and cytostatic regions of the molecule. Published data show that estramustine phosphate (analogue of the test compounds) has cytotoxic properties and produces damage to the cell membrane [7]. We studied the effect of compounds on membrane-bound enzymes $N^+$-$K^+$-ATPase and 5'-nucleotidase involved in the regulation of energy metabolism, cell respiration, ion transport, and DNA synthesis. The inhibition of these enzymes can be followed by changes in physiological characteristics of the cell (shape, volume, intracellular pH) and its death. Chlorophenacyl (embichin) and 17β-estradiol served as reference preparations.

## MATERIALS AND METHODS

Activity of 5'-nucleotidase and $N^+$-$K^+$-ATPase was measured by the method of Emmelot and Boss [3]. The rate of hydrolysis of 5'-AMP and ATP catalyzed by 5'-nucleotidase and $N^+$-$K^+$-ATPase, respectively, was determined by the amount of inorganic phosphate (method of Lowry with modifications of V. M. Skulachev) [1].

To measure 5'-nucleotidase activity samples (1.8 ml) containing the homogenate in 50 mM Tris-HCl buffer (pH 7.5), KCl (final concentration 100 mM), and $MgCl_2$ (final concentration 5 mM) were preincubated at 37°C for 5 min. The substrate of 5'-nucleotidase (5'-AMP, 0.2 ml) was added to the mixture and incubated at 37°C for 15 min. The reaction was stopped by adding 10% trichloroacetic acid (TCA). Control experiments were performed with the substrate and enzyme.

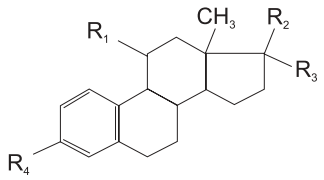


**Fig. 1.** Common formula and position of radicals in bis-β-chloroethylamine derivatives of transformed estrogens. The composition of radicals is shown in Table 1.

$N^+$-$K^+$-ATPase activity was determined by subtraction of $MgCl_2$-ATPase activity from total ATPase activity.

Samples (1.8 ml) were maintained at 37°C for 5 min (150 mM $MgCl_2$, 5 mM KCl, and 20 mM Tris-HCl buffer; pH 7.5). The substrate (ATP- $MgCl_2$) was added in a final concentration of 2.5 mM. The mixture was incubated at 37°C for 15 min. The reaction was stopped by 10% TCA. Control experiments were performed with the substrate and enzyme.

The test compounds (20 μl, final concentration $10^{-5}$ M) were added to the incubation mixture to estimate their influence on enzyme activity. Samples containing the incubation mixture and test compounds were maintained at 37°C for 5 min. The reaction was initiated by adding 0.2 ml homogenate. Enzyme activity was expressed in μg inorganic phosphate formed per 1 mg protein over 15 min.

## RESULTS

Bis-β-chloroethylamine estrogens derivatives (Fig. 1, Table 1) markedly decreased activity of plasma membrane enzymes (Fig. 2, *a*, *b*). 5'-Nucleotidase was suppressed by 34-94% after addition of estrogen cytostatics. Estradiol and chlorophenacyl decreased enzyme activity by 19 and 11%, respectively (Fig. 2, *a*).

After the addition of bis-β-chloroethylamine estrogens derivatives $N^+$-$K^+$-ATPase was suppressed by 43-82%. Estradiol and chlorophenacyl decreased enzyme activity by 14 and 7%, respectively (Fig. 2, *b*).

These data show that steroid and chlorophenacyl potentiated the effects of each other and inhibited enzymes. Activity of various enzymes decreased similarly after addition of the test compounds. We propo-

**TABLE 1.** Radical Composition of bis-β-Chloroethylamine Derivatives of Transformed Estrogens

| Derivative | R1 | R2 | R3 | R4 |
|---|---|---|---|---|
| Po-714 (11α) | OH | $CH_3COO$ | C=CH | CytO |
| Po-715 (11α) | HCOO | $CH_3COO$ | C=CH | CytO |
| Po-716 (11α) | $CH_3COO$ | $CH_3COO$ | C=CH | CytO |
| Po-728 (11α) | HCOO | =O | — | CytO |

**Note.** CytO group denotes structure $COXN(CH_2CH_2Cl)_2$, where X is $CH_2C_6H_4$; —, absence of radical.

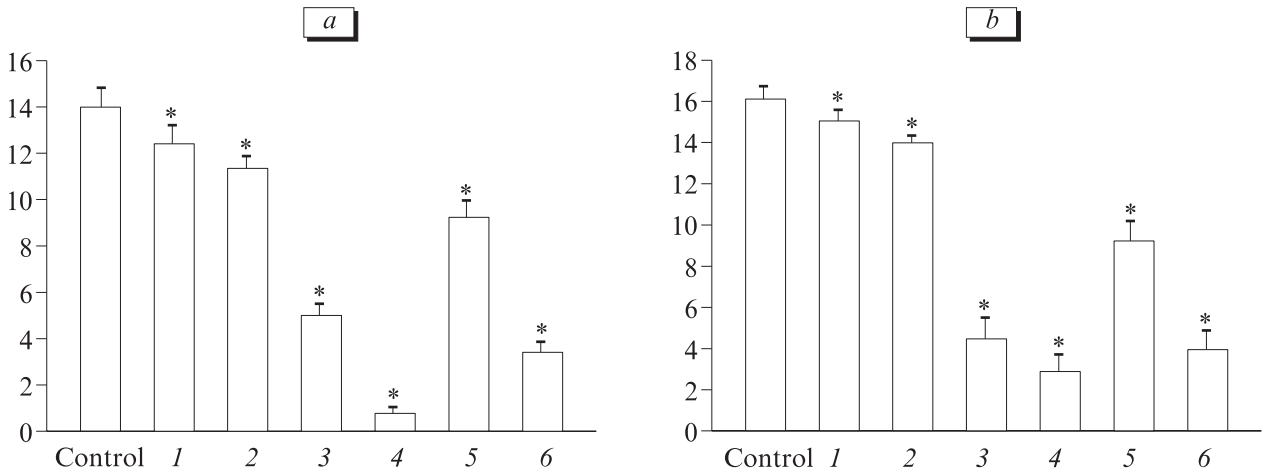


**Fig. 2.** Effect of estrogen cytostatics on activities of 5'-nucleotidase (*a*) and $N^+$-$K^+$-ATPase (*b*) in the homogenate of rat liver. Ordinate: enzyme activity, amount of inorganic phosphate (μg) formed per 1 mg protein over 15 min. Chlorophenacyl (*1*), estradiol (*2*), Po-715 (*3*), Po-714 (*4*), Po-716 (*5*), and Po-728 (*6*). *$p \leq 0.05$ compared to the control.

sed that this process does not depend on the nature of enzymes. However, the inhibition was specific relative to the nature of estrogen cytostatics and associated with an increase in the time of their interaction with the biological membrane.

The antiproliferative effect of compounds (5'-nucleotidase and $N^+$-$K^+$-ATPase) on estrogen-independent tumor cells closely correlated with inhibition of enzymes in plasma membranes of cells non-tropic for estrogens (k=0.96, k=0.85) [2]. No correlation was found between inhibition of enzymes and affinity for estrogen receptors [1]. Our results indicate that cytostatic activity of bis-β-chloroethylamine estrogen derivatives is not related to the effect of alkyl groups on the genome and interaction with the receptors. It is associated with direct damage to membrane-bound enzymes. This effect is determined by the presence of the steroid nucleus in estrogen cytostatics, which increases lipophilic activity of compounds and contributes to their accumulation in membranes.

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