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TAMOXIFEN IS A Na^+–ANTAGONISTIC INHIBITOR OF Na^+/K^+–TRANSPORTING ATPase FROM TUMOUR AND NORMAL CELLS

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INTRODUCTION

Tamoxifen is the most widely used antihormonal agent for the treatment of estrogen receptor-positive breast cancer.[1] Tamoxifen controls breast cancer cell replication by blocking the binding of estrogen to the estrogen receptor and preventing estrogen-stimulated growth.[2] It displaces the growth-promoting natural hormone estradiol-17β from its protein receptor and, consequently, its principal mode of action has been thought to be as an antiestrogen.[2]

However, the antiestrogenic activity of tamoxifen is insufficient to explain its cytotoxic effects.[3] Actually, several estrogen receptor-independent actions of antiestrogens have been observed. These include calmodulin-antagonism in cyclic AMP phosphodiesterase activity[4,5] and in protein kinase C activity,[6–10] inhibition of cholesterol biosynthesis,[11] changes in the physical properties of membrane lipid bilayer,[12,13] suppression of polypeptide chain elongation in eukaryotic protein biosynthesis,[14] and inhibition of the Ca^{2+}-transport system of brain microsomes.[15]

As emphasized by Wakeling,[16] alternative mechanisms independent of estrogen receptors may contribute significantly to the response of breast cancer to tamoxifen. The serum and tissue concentrations of tamoxifen in patients are sufficiently high (>5 μM) that the estrogen-irreversible antiproliferative action of tamoxifen, observed *in vitro*, may also be important *in vivo*. The antiestrogenic activity, may thus be only one component, albeit an important one, affecting the response of breast tumours

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Abbreviations: Tamoxifen, [*Z*-1[p-dimethylaminoethoxy-phenyl]-1,2-diphenyl-1-butene]; EMAC, Ehrlich mouse ascites carcinoma; Na/K-ATPase, Na^+/K^+-transporting ATPase (EC 3.6.1.37).

to tamoxifen therapy.[16] More specifically, in 1989 Tang *et al.*[17] felt it to be possible to define experimental systems which exclude estrogen receptor-mediated events and thus allow the elucidation of new aspects of tamoxifen action which may reveal that this agent is broader in its action than previously suspected.

Such a system appears to be the Na^+K^+-pump of plasma membrane because it plays an important role in normal and cancer cell proliferation as deduced by Repke elsewhere.[18] Especially, the available evidence seemed to him to support the belief that the enhancement of Na^+K^+-pump power in a mitotic cell is a primary event in the mainline sequence between growth stimulation, signal transmission, macromolecule synthesis, and cell multiplication.[18-20] So, the biochemical machinery in the Na^+K^+-pump, the Na^+K^+-ATPase, has emerged as a potential target for cytostatic and cancerostatic agents.[18] Since initial work has shown us that tamoxifen is a rather strong, digitalis-unlike inhibitor of Na/K-ATPase from human tissues,[21] we have performed a more detailed analysis of the molecular mechanimsm of tamoxifen's inhibiting interaction with the enzyme, the results of which are reported here.

MATERIALS AND METHODS

Cell Cultures

Ehrlich mouse ascites carcinoma (EMAC) cells were obtained from the intraperitoneal cavity of mice about 6 days after transplantation and cultured in 24-well microliter plates (5×10^4 cells in 0.5 ml Eagle MEM/well) in the presence of the compounds for two days and the cell number was then evaluated with a cell counter (PS-4, Medicar Budapest). Increase in the cell number of the controls was about threefold at this time.[22] The IC_{50}, values were read from dose-response curves.

Preparation of Na/K-ATPase and Determination of Activity

The enzyme from EMAC cells was prepared via isolation of the plasma membranes followed by treatment of the membranes with deoxycholate as previously described.[23] The human tissue sources were cardiac ventricle muscle and brain cortex taken within 12 h of post mortem. The enzymes were prepared as previously described.[24,25] Their activity was assessed in the optical test system as detailed elsewhere,[26] except with omittance of $(NH_4)_2SO_4$ from the incubation medium in the analysis of the impact of ionic composition on the inhibitory potency of tamoxifen.

Determination and Expression of Inhibitor Potency

Under the conditions chosen the concentration of the inhibitors was greatly in excess of the concentration of the enzymes such that the concentration of free inhibitor was approximately equal to the concentration applied. The concentration of a compound C producing half-maximum inhibition, $K_{0.5}$, was calculated according to

$$I_\infty = \frac{[C]^h}{[C]^h + (K_{0.5})^h}$$

TABLE 1
Comparison of the potency of the isomers of tamoxifen (T) and of ouabain in inhibiting proliferation of EMAC cells or activity of Na/K-ATPase from various origin.

Test object	Inhibitor	$K_{0.5}$ (μM)	n
Proliferation of	trans-T	8.5±4.8[a]	–
EMAC cells	cis-T	7.7±2.7[a]	–
	ouabain	160±9[a]	–
Na/K-ATPase from	trans-T	5.6±0.5	2.0±0.4
EMAC cells	cis-T	4.9±0.4	2.5±0.4
	ouabain	46.5±2.7	1.0
Na/K-ATPase from	trans-T	7.0±0.8	2.9±0.3
human cardiac	cis-T	6.0±1.2	1.9±0.5
muscle	ouabain[b]	0.027±0.003	0.8±0.05
Na/K-ATPase from	trans-T	4.5±0.7	2.4±0.5
human brain	cis-T	4.2±0.4	2.4±0.6
cortex	ouabain[b]	0.030±0.003	0.7±0.3

[a]IC_{50} values (μM) [b]Values taken from[26]

in which denote I_∞ is the equilibrium value for the inhibition and h is the Hill coefficient.

Inhibitors Studied

The tamoxifen isomers and derivatives shown in Tables 1 and 2 were provided by Dr. Raymond McCague, Cancer Research Campaign Laboratory, Institute of Cancer Research, Sutton, Surrey (England). Ouabain was from a commercial source. The compounds were dissolved in pure dimethyl sulfoxide and transferred in this solution into the assay medium.

RESULTS AND DISCUSSION

The comparison of the inhibitory potency of the tamoxifen isomers (Table 1) shows that the two isomers almost equally strongly inhibit the proliferation of Ehrlich mouse ascites carcinoma (EMAC) cells and the activity of Na/K-ATPase from EMAC cells as well as from human cardiac muscle and from human brain cortex. Since the trans-isomer acts antiestrogenic, whereas the cis-isomer is estrogenic,[27] the findings prove the absence of an antiestrogenic mechanism as underlying the inhibitory action on EMAC cell proliferation. While the cardiac glycoside ouabain very much differentiates as to effectiveness, the tamoxifens show an almost equal inhibitory potency within the four systems.

TABLE 2
Comparison of the potency of tamoxifen derivatives (for structure see[5]) in inhibiting the activity of Ca^{2+} calmodulin-dependent cAMP phosphodiesterase (A)[a] or Na/K-ATPase from human brain cortex (B).

Inhibitor	A IC_{50} μM	B $K_{0.5}$ μM	B n
cis-Tamoxifen	6.5±0.52	4.2±0.40	2.4±0.6
trans-Tamoxifen	6.75±1.06	3.7±0.66	1.8±0.6
4-Hydroxy trans-tamoxifen	19.0±2.85	6.9±0.29	2.1±0.2
4-Iodo trans-tamoxifen	2.3±0.42	0.99±0.03	1.7±0.1
Pyrrolidino analogue of 4-iodo trans-tamoxifen	1.45±0.08	0.75±0.06	1.3±0.1

[a]Data taken from Rowlands *et al.*.[5]

The comparison of the inhibitory action of the tamoxifen isomers and of three derivatives of trans-tamoxifen on calmodulin-dependent cyclic AMP phosphodiesterase[5] or on Na/K-ATPase (Table 2) reveals a graded, but continuous parallelism. Remarkably, the dimethylamino group in the side chain of tamoxifen is equi-effectively replaceable by the pyrrolidino group. This indicates that is not the configuration but the cationic property of the side chain that is all-important. Supporting this interpretation, the chloroethoxy derivative of 4-iodotrans-tamoxifen proved to be inactive in inhibiting Na/K-ATPase up to the limit of solubility, i.e. 10 μM (not shown). Taking all findings together, the striking unspecificity of tamoxifen's inhibitory effects on the various tissues indicates the involvement of a common simply constructed anionic point of interaction on the various targets. In the case of Na/K-ATPase from tumour and normal cells, this acceptor place appears to be the Na^+ binding site, whose occupancy by Na^+ initiates the activity of the enzyme. This hypothesis results from the following interrelationships.

The inhibitory action of tamoxifen on Na/K-ATPase is independent of K^+-concentration, but significantly decreased by an elevation of Na^+-concentration concomitant with an increase of the Hill coefficient (Table 3). These findings appear to mean that tamoxifen competes with Na^+ for the Na^+ binding site, and that, at higher Na^+ concentrations, tamoxifen tends to blockade both sets of Na^+ sites on the diprotomeric enzyme.

This interpretation is supported by comparable, comprehensively documented findings with other, likewise cationic inhibitors, of Na/K-ATPase such as ethylenediamine,[28] some guanidinium compounds[29] and amiloride derivatives.[30] These organic cations have been classed as competitive Na^+ antagonists which bind to negatively charged carboxyl residues of the enzyme protein. Due to their size they block Na^+ binding and thus interrupt the activity of Na/K-ATPase.[28–30] Interestingly enough,

TABLE 3
Impact of variation in the composition of incubation medium on inhibitory effect of trans-tamoxifen on activity of Na/K-ATPase from human brain cortex in absence of 22 mM NH_4^+ otherwise routinely present in the optical test medium.

Variable component (mM)			$K_{0.5}$	n
[KCl]	[NaCl]	[ATP]	(μM)	
5	5	2	1.9±0.3	1.5±0.4
5[a]	5	2	2.9±0.7	1.3±0.5
5	5	1	3.7±0.3	2.0±0.3
1	80	2	7.3±0.3	2.9±0.4
5	80	2	7.1±0.1	3.6±0.2
32	80	2	6.9±0.4	3.7±0.7
50	50	2	5.6±0.2	2.9±0.3

[a]Additionally 90 mM choline chloride

contrary to the action of choline (cf. Table 3) some cancerostatically active phosphocholine derivatives, e.g. hexadecyl phosphocholine, are also Na^+ antagonistic inhibitors of Na/K-ATPase.[31]

In conclusion, our observations add to the knowledge that micromolar concentrations of tamoxifen may have multivarious points of attack as reviewed in the introduction. Specifically. Na/K-ATPase has been shown here to belong to the class of estrogen-irreversible target sites of tamoxifen[5-11] and to possess between all targets the highest tamoxifen affinity at the low Na^+ concentration in the cytoplasm from where tamoxifen elicits all of its effects. Remarkably enough, the cationic side chain of tamoxifen is not involved in high affinity binding to the estrogen receptor, but it is essential for effecting cytotoxicity.[5] In conclusion, the inhibition of Na/K-ATPase by tamoxifen could possibly be involved in eliciting its cancerostatic and cytotoxic actions.

Na/K-ATPase of human cardiac muscle has been known for some time to be the molecular point of attack of digitalis compounds such as ouabain which, via inhibition of the Na^+/K^+ pump, elicit the therapeutically desired inotropic action (reviewed in[32]). The serum and tissue concentrations of tamoxifen in patients receiving tamoxifen treatment are sufficiently high, i.e. >5 μM,[16] such that an inotropic tamoxifen action appears to be not impossible. However, as yet only a decrease in adverse events and in mortality related to coronary heart disease has been reported.[33]

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