Hydrophilic Hexapeptide Imunofan as a Hyperactive Regulator of Transport Proteins for Multiple Drug Resistance

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Hydrophilic hexapeptide Imunofan produces a modulatory effect on multiple drug resistance transport proteins depending on their functional activity. The hexapeptide inhibited multiple drug resistance proteins during substrate transport, but increased their activity in the latent period. By the inhibition of multiple drug resistance, specific activity of Imunofan more than 1000-fold surpassed that of other substrate analogues. Inhibitory analysis showed that regulatory effect of this hexapeptide depends on protein kinase C.

Key Words: *multiple drug resistance; Imunofan; ATP-dependent transport proteins; protein kinase C; regulatory peptide*

Multiple drug resistance (MDR) of tumor cells and microorganisms to several cytostatic and antimicrobial drugs is one of the urgent problems of modern biology and medicine. As distinct from drug resistance of cells induced by resistance gene mutation, MDR develops due to activation of various ATP-dependent transport proteins of the ABC family [3]. These proteins provide elimination of various toxic compounds (*e.g.*, chemotherapeutic drugs) from the cell. This process sharply decreases the effectiveness of chemotherapy.

The development of drugs preventing MDR was based on the synthesis of substrate analogues acting as competitive inhibitors of drugs. Various hydrophobic cyclic peptides, *e.g.* cyclosporine A and its chemical derivatives, are used as substrate analogues in experimental medicine. Substrate analogues in effective doses have a potent toxic effect and, therefore, cannot be used for the therapy [6].

Another approach to develop new drugs preventing MDR is based on the use of hydrophilic regulatory peptides. Previous studies showed that hydrophilic regulatory hexapeptide Imunofan (arginyl-α-aspartyl-lysyl-valyl-tyrosyl-arginine) inhibits MRP (multiple resistance-associated protein, ABC family) [1]. Regulatory activity of this peptide in cells of the lymphoid, reticuloendothelial, and antioxidant systems is well studied [2]. Regulatory properties of Imunofan are taken into account for the correction of immune and antioxidant dysfunction in patients [2]. However, the regulatory effect of Imunofan on transport proteins for MDR remains unknown.

Here we studied regulatory activity and mechanism of the effect of hydrophilic hexapeptide on function of MDR transport proteins. The knowledge of hexapeptide properties is required for preclinical evaluation of its effectiveness during combination chemotherapy to prevent MDR.

MATERIALS AND METHODS

Experiments were performed on cultured Hep-2 human laryngeal cancer cells.

Hep-2 cells were inoculated in Petri dishes with DMEM supplemented with 10% fetal bovine serum

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(1.5 million cells per dish; 3 glass plates were put on the bottom of each Petri dish) and maintained in a CO_2 incubator.

Activity of MDR transport proteins in cells was estimated by the rate of rhodamine 123 (R123) efflux [8].

Fluorescence was measured on a Perkin-Elmer MF44 spectrophotometer at 37°C and constant agitation. The rhodamine excitation and emission wavelengths were 488 and 520 nm, respectively. The cells were loaded with fluorochromes. Cyclosporine A in a concentration of 1 μg/ml was added to prevent fluorochrome elimination from cells. These cells were washed and stored on ice to estimate the rate of R123 efflux. Hep-2 cells (1-1.5 million) fixed to a transparent plastic plate were placed in a cuvette. During study of R123 efflux from Hep-2 cells the exciting light did not pass through cells and therefore only fluorescence of extracellular R123 (in the medium) was recorded. The rate of fluorochrome release was initially measured in control samples and then the test agent was added and the increase in R123 concentration in the medium was measured. The effect of treatment was evaluated by the ratio between efflux rates in the control and after addition of the test agent. The effects of the peptide were compared with those of cyclosporine A.

For evaluation of the regulatory properties and effect of protein kinase C on Imunofan activity, H8 and/or hexapeptide were added to dishes 9 h after cell inoculation. After 13-h incubation, the cells were loaded with R123 (0.5 μg/ml) for 1 h. Experiments were performed in the medium used for cell growth. After loading, the cells were washed and stored in cold until measurements of R123 efflux rate. Cyclosporine (0.3 μg/ml) served as an inhibitor of R123 transport.

RESULTS

The inhibitory effect of Imunofan on transport proteins 1000-fold exceeded that of cyclosporine (Fig. 1). Imunofan in a concentration of 0.12 nM inhibited R123 efflux from Hep-2 cells. Cyclosporine A in a concentration of less than 10 nM was ineffective. Imunofan and cyclosporine A were most effective in concentrations of 1-3 nM and 0.8-2.5 μM, respectively.

Imunofan decreased activity of transport proteins starting from concentrations of 10^{-10} M. The coefficient of maximum inhibition of R123 efflux for Imunofan was slightly lower compared to that for cyclosporine A (2.8±0.2 and 3.2±0.1, respectively). Published data show that cyclosporine A inhibits 2 transport proteins (P-glycoprotein [Pgp] and MRP) in Hep-2 cells, while the regulatory hexapeptide modulates function of only MRP [1,7]. The concentration dependence for the influence of the test compounds on R123 efflux was studied in some independent experiments.

The concentration dependences illustrated the maximum coefficient of R123 efflux inhibition in cells. The appearance of this maximum was associated with the fact that the test compounds not only inhibit ATP-dependent active transport of R123, but also increase passive transport through the membrane into the extracellular space. The latter effect became more significant with increasing the concentration to 3 nM. These changes were accompanied by a decrease in R. It is evident that the coefficient of R123 efflux inhibition in cells does not depend on passive transport under the influence of compounds in concentrations ~10^{-10} M.

These data show that activity of Imunofan surpasses that of cyclosporine A by at least 1000 times. MDR inhibitors effective in low concentrations were unknown until the present time. The hexapeptide is more specific than other competitive analogues. For example, active specific MRP inhibitor probenecid inhibits transport proteins only in a concentration of 1 mM. The dose of probenecid exceeds that of Imunofan by 6 orders of magnitude [4].

The observed properties of Imunofan are directly related to the mechanism of action of regulatory peptides. The effects of these peptides are realized via the cell signaling system (*e.g.*, protein kinase C) [5]. We studied the regulatory effect of Imunofan on MDR proteins and evaluated the role of protein kinase C in this process.

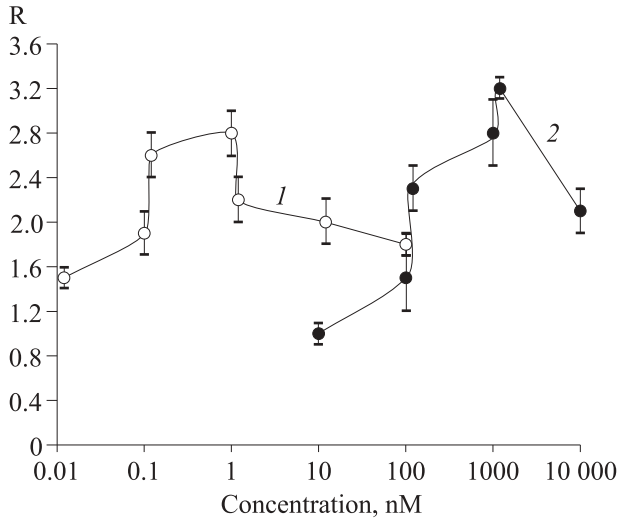

Fig. 1. Concentration dependence for the inhibition of R123 efflux from Hep-2 cells by Imunofan (*1*) and cyclosporine A (*2*). R, inhibition coefficient of R123 efflux from cells.

TABLE 1. Effects of Imunofan and H8 on the Rate of R123 Efflux from Hep-2 Cells (*M*±*m*)

Conditions	k'	k_i'	R
Control, cyclosporine A (*n*=15)	$47\pm3\times10^{-3}$	$22\pm2\times10^{-3}$	2.3±0.1
Imunofan, 0.3 ng/ml (*n*=15)	$40\pm4\times10^{-3}$	$11{,}7\pm0.9\times10^{-3}$	3.5±0.3
Imunofan, 100 ng/ml (*n*=15)	$44\pm2\times10^{-3}$	$13\pm0.7\times10^{-3}$	3.5±0.17
H8, 15 μM (*n*=15)	$46\pm6\times10^{-3}$	$20\pm6\times10^{-3}$	2.3±0.3
H8, 30 μM (*n*=15)	$42\pm3\times10^{-3}$	$20\pm3\times10^{-3}$	2.3±0.3
H8 (30 μM)+Imunofan (0.3 ng/ml, *n*=15)	$50\pm10\times10^{-3}$	$30\pm9\times10^{-3}$	1.9±0.3

Note. H8, protein kinase C inhibitor; k', estimated constant of the rhodamine efflux rate; k_i', constant of the rhodamine efflux rate in the presence of inhibitor; R, ratio between the transport rates.

Active transport of substrates by MDR proteins is characterized by the difference between the ratio of the transport rates in the absence and presence of inhibitors (R) and 1:R-1 [9]. $R=V_1/V_2$, where V_1 and V_2 are the rates of R123 efflux from cells under control conditions and in the presence of inhibitors, respectively.

The hexapeptide in concentrations of 0.3 and 100 ng/ml increased R-1 by 1.9 times (Table 1), which was mainly related to a decrease in the constant of the rhodamine efflux rate in the presence of inhibitor (k_i'). Imunofan produced the inhibitory effect only during active transport of the substrate (R123, Fig. 1), while in the latent phase after preincubation of cells with cyclosporine A Imunofan activates the system of transport proteins.

Hence, the regulatory effect of Imunofan depends on functional activity of MDR transport proteins. The hexapeptide inhibits proteins of the ATP-dependent transport system during substrate transport, but increases protein activity in the latent period. Substrate analogues acting as competitive inhibitors do not have this property.

Published data show that transport properties of MDR proteins depend on protein kinase C activity [5]. Activation of protein kinase C is followed by the increase in substrate efflux from the cell into the extracellular space. We evaluated the role of protein kinase C in the regulation of transport proteins by Imunofan (Table 1). Protein kinase C inhibitor abolished the regulatory effect of Imunofan. Moreover, the influence of this hexapeptide was suppressed by protein kinase C inhibitor.

Our results indicate that hydrophilic hexapeptide exhibits high regulatory activity relative to MDR transport proteins. Hence, Imunofan holds promise for the prevention of MDR. Inhibitory analysis shows that the effect of Imunofan is realized via protein kinase C.

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