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Avermectins inhibit multidrug resistance of tumor cells

Yuri N. Korystov[a,*], Natalia V. Ermakova[a], Ludmila N. Kublik[a], Maria Kh. Levitman[a], Vera V. Shaposhnikova[a], Vladimir A. Mosin[b], Viktor A. Drinyaev[b], Elena B. Kruglyak[b], Tamara S. Novik[b], Tatiana S. Sterlina[b]

[a] *Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Pushchino, Moscow region 142290, Russian Federation*
[b] *NBC Farmbiomed, Selskokhozyaystvennaya st., 12a, 129343 Moscow, Russian Federation*

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Abstract

The modification of the sensitivity of Hep-2 and P388 tumor cells to taxol and vincristine, substrates of multidrug resistance proteins, by naturally occurring avermectins and the effect of avermectins on the accumulation of calcein in cells and the efflux of rhodamine 123 were studied. While avermectins did not affect the sensitivity of tumor cells to hydrogen peroxide and cisplatin, they significantly enhanced the sensitivity of cells of both wild-type and resistant strains to taxol and vincristine. The coefficients of modification for resistant strains were substantially higher. Avermectins suppressed the efflux of rhodamine 123 from cells and increased the accumulation of calcein in cells. The relative inhibitory activity of avermectins depended on the cell type and on the substrate of multidrug resistance proteins whose transport they suppressed (vincristine, taxol, rhodamine 123, calcein acetoxymethyl ester). The least active was avermectin B_1 or ivermectin; the most active avermectins varied depending on the substrate and the cell type. In the case of vincristine transport, the most active avermectin was almost by one order of magnitude more effective than the traditional inhibitor of multidrug resistance cyclosporin A. This property of avermectins can be used in tumor therapy by combining application of avermectins with antitumor preparations, the substrates of multidrug resistance proteins.

Keywords: Avermectins; Calcein; Multidrug resistance; Rhodamine 123; Tumor cells

1. Introduction

Avermectins are macrolide compounds produced by the fungus *Streptomyces avermitilis*. This fungus synthesizes a complex of eight individual avermectins (Burg et al., 1979). Both the avermectin complex as a whole and the individual avermectins possess antiparasitic activity (Burg et al., 1979; Ostlind and Long, 1979), which has attracted considerable attention among researchers. Because this activity is most pronounced in avermectin B_{1a} (Burg et al., 1979), commercial antiparasitic preparations based on avermectins of the B_{1a} group were developed: abamectin (80% of avermectin B_{1a} and 20% of avermectin B_{1b}) and ivermectin (a dehydrated derivative of avermectin B_{1a}) (Campbell, 1989; Roslavtsev, 1987). Recently, it was found that ivermectin is an inhibitor of P-glycoprotein, (Dider and Loor, 1996), an integral protein (170 kDa) of plasma membrane whose main function is the ATPase-dependent transport of foreign substances from the cell (Dradley et al., 1988; Gill et al., 1992). P-glycoprotein is one of the key factors of multidrug resistance of tumors, which severely restricts the effectiveness of chemotherapy (Lautier et al., 1996; Van der Heyden et al., 1995). The available inhibitors of multidrug resistance do not completely abolish it at clinically permissible concentrations (Fisher and Sikis, 1995). Therefore, a search for more effective and less toxic inhibitors of multidrug resistance proteins was performed. Ivermectin suppresses multidrug resistance four and nine times more effectively than the traditional inhibitors of P-glycoprotein verapamyl and cyclosporin A, respectively (Pouliot et al., 1997). It is believed that ivermectin, owing to its high efficacy and low toxicity, is an ideal agent for abolishing the multidrug

* Corresponding author. Tel.: +7-739465; fax: +7-827790553.
E-mail address: ykorystov@rambler.ru (Y.N. Korystov).

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resistance of tumor cells (Pouliot et al., 1997). Here, we tested whether naturally occurring avermectins can modify the sensitivity of tumor cells to the substrates of multidrug resistance proteins, taxol and vincristine (Nooter and Stoter, 1996). We also studied the effect of avermectins on the accumulation of calcein in tumor cells and the efflux of rhodamine 123.

2. Materials and methods

Human larynx tumor cell line Hep-2 cells in Dulbecco's Modified Eagle Medium (DMEM; Sigma, USA) supplemented with 10% fetal bovine serum (Sigma) and 40 μg/ml of gentamycin (Sigma) were seeded in 96-well plates at a concentration of 50,000 cell/ml, 0.1 ml to each well. After 24 h of incubation at 37 °C in 5% CO_2, the agents tested were added and the incubation was continued for an additional 2 days. After the termination of incubation, cells were stained for 10 min with 0.02% cresil violet (Sigma) in 20% ethanol. Then the dye solution was removed, the wells were washed with water, and a 0.1% SDS solution was added to extract the dye from the cells. Optical density was measured at 570 nm on a Multiscan Plus spectrophotometer. The effect of treatment was determined with the formula:

$$N_e/N_c = \{(D_e - D)/(D_c - D)\} \times 100\%$$

where D_e, D_c, D are the optical densities in experiment, control, and background density, respectively; and N_e/N_c reflects the cell survival: the ratio of viable cells in experiment and control.

The taxol-resistant strain Hep-2TR was prepared by seeding Hep-2 cells in plastic flasks (V 6 ml), 100,000/ml to each flask, in DMEM supplemented with 10% fetal bovine serum and 40 μg/ml of gentamycin. After a 24-h incubation at 37 °C, taxol (Sigma) at a final concentration of 10 nM was added. After 3 days, detached cells were removed from the exchange medium. Surviving cells grew further to form colonies. These cells were seeded and cultivated to obtain large amounts for use in experiments with Hep-2TR.

The activity of the transport proteins responsible for multidrug resistance in cells was determined from the rate of efflux of rhodamine 123 (ICN, USA). The theory and experimental details of the technique are reported elsewhere (Van der Heyden et al., 1995; Wielinga et al., 2000). The method is applicable only for cells adhering to the substrate. It was shown that the rate of rhodamine 123 efflux from cells obeys the exponential law $1/\tau=\{k+(V_{max}/K_M)\}/V_{in}$, where k is the constant of passive efflux of rhodamine 123 from cells, V_{max}/K_M is the characteristic of active transport, and V_{in} is the volume of cells. It was also shown that the ratio of the rate (τ) of rhodamine 123 efflux from cells under normal conditions to that after complete inhibition of active transport minus unity: $(R-1)_{max}$ is equal to the ratio of active to passive transport and, consequently, characterizes the activity of proteins providing multidrug resistance. We modified the method described in the above-cited papers in two points. First, cells were seeded not onto glass but onto plastic plates cut out of cell culture flasks. Second, cells loaded with rhodamine 123 were washed, and only after this stored at low temperature. Cells in which external rhodamine 123 is not washed away (as in the original method) become damaged upon storage, probably due to a photodynamic effect. Hep-2 cells ($1-1.5 \times 10^6$) in DMEM supplemented with 10% fetal bovine serum and gentamycin were seeded onto plates ($50 \times 9 \times 2$ mm) placed into dishes 6 cm in diameter and 8 ml in volume. After 24 h of incubation at 37 °C under on atmosphere of CO_2, the cells were washed in RPMI 1640 (Sigma) and loaded for 60 min at 37 °C with rhodamine 123 (0.5 μg/ml) in the presence of the inhibitor of transport proteins, cyclosporin A (Sigma; 3 μg/ml) in RPMI 1640 supplemented with 10% fetal bovine serum. After incubation, cells were washed (3×10 min) free from the dye with a cold (+2 °C) physiological saline containing 1% fetal bovine serum. After been washed, plates with cells in Hanks' solution (Sigma) supplemented with 0.5% serum were stored on ice until used. A washed plate with cells was placed into a cuvette of a MF44 Perkin Elmer fluorimeter, which contained Hanks' solution and 0.5% fetal bovine serum (3 ml). An increase in the amount of rhodamine 123 was determined under continuous stirring at 37 °C. The excitation and emission wavelengths were 488 and 520 nm, respectively. At the end of the experiment, cells were disrupted by adding 0.02% digitonin (Sigma) to determine the maximum amount of rhodamine 123 in cells.

In experiments in which the effect of inhibitors on the efflux of rhodamine 123 was tested, the inhibitor at a necessary concentration was added after a few minutes of normal efflux of rhodamine 123. Determination of the rate constant of rhodamine 123 efflux in the control and in the presence of the inhibitor on one plate diminished the error in determining their ratio, which is necessary to obtain $R-1$.

Lympholeukosis P388 cells were grown in the abdominal cavity of DBA2 male mice. Male mice, 8–10 weeks old (animal collection of Institute of Bioorganic Chemistry, Pushchino), were used. Mice were housed in groups of 20 per cage ($35 \times 25 \times 15$ cm). Housing conditions were thermostatically maintained at 22 ± 2 °C with a 12-h light–dark cycle. Animals were given free access to food and water. Mice were killed by cervical vertebra dislocation. The local ethics committee for animal use approved these experiments. Vincristine-resistant P388 cells were obtained by treating tumor-carrying mice with 1 mg/kg of vincristine (Gedeon, Richter; Hungary). Vincristine was injected intraperitoneally 1 day after inoculation of the tumor cells (10^6 cells per mouse). After cells increased in number, they were reinoculated and again 1 mg/kg of vincristine was injected. The procedure was repeated six times. The strain obtained was vincristine-resistant and was designated P388VR. Both

cell types were withdrawn from the abdominal cavity of mice 7 days after tumor inoculation (2×10^6 cells/mouse), washed with Hanks' solution, and placed in RPMI 1640 medium supplemented with 10% fetal bovine serum and gentamycin (40 μg/ml) at a concentration of 10^6/ml. The agents tested were added immediately prior to incubation at 37 °C. Cells were incubated for 22 h under an atmosphere of 5% CO_2. After the termination of incubation, the proportion of dead cells was determined by staining with 0.04% Trypan blue (Sigma). The survival of cells in the experiment relative to control was determined with the formula: S, $\% = N_e/N_c \times 100\%$, where N_e and N_c are the proportions of living cells in the experiment and control, respectively. The activity of the transport proteins in cells was determined by loading the cells with the readily permeable substrate of the transport proteins calcein acetoxymethyl ester (calcein AM; Molecular Probes, USA), as described in Wielinga et al. (1998). In cells, calcein AM is degraded by esterases to calcein (loaded fluorochrome), which leaves cells slowly.

On the basis of preliminary tests, the following protocol for the experiment with P388 cells was adopted. Cells withdrawn from the abdominal cavity of mice after 7 days of growth were washed in Hanks' solution, placed in RPMI 1640 medium supplemented with 10% fetal bovine serum, and added to the wells (2 cm in diameter) of a 12-well plate at a concentration of cells 1×10^6/ml (volume of medium in the cell 2 ml). After a 30-min incubation of cells at 37 °C, avermectins and 20 min later 1 μM calcein AM were added. After 10 min, cells were harvested and sedimented by centrifugation for 1.5–2 min at 12,000 × g. The medium was decanted, and the sediment was resuspended in a medium without phenol red, which contained a reduced content of calcium: 120 mM NaCl, 5 mM KCl, 0.4 M $MgCl_2$, 0.04 mM $CaCl_2$, 10 mM N-2 hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES, pH 7.4), 5 mM Na_2HPO_4, and 10 mM glucose. The factors that substantially affected the results were as follows. Some of the cells died during incubation at a lower density at the bottom, as well as during centrifugation if the growth medium was not supplemented with serum. If cells were sedimented at lower g values, the sediment was loose, and some cells were decanted together with the medium, which severely reduced the accuracy of the method. After the washing of cells, the amount of calcein in cells was determined by measuring fluorescence at the wavelengths of excitation and emission, 493 and 515 nm, respectively, using an MF-44 Perkin-Elmer fluorimeter.

Avermectin characterization. Ivermectin and chromatographic standards of avermectins were supplied by Merck (Darmstadt, Germany). High-performance liquid chromatography (HPLC) grade solvents were purchased from Merck. Individual avermectins A_1, A_2, B_1 and B_2 were isolated from aversectin C. Avermectins were obtained from a dry biomass of *S. avermitilis* (Drinyaev et al., 1999). The dried purified ethanolic extract of *S. avermitilis* mycelia named aversectin C contained about 86–90% of avermectins. The impurities consisted of avermectin-related compounds (5–9%, including oligomycin about 2%), neutral lipids (4%), proteins (< 1%) and salts (< 1%). HPLC chromatograms of aversectin C demonstrated the following avermectin profile: A_{1a}—9%; A_{1b}—4%; B_{1a}—36%; B_{1b}—6%; A_{2a}—19%; A_{2b}—4%; B_{2a}—21%; B_{2b}—1%. Individual avermectins were isolated by semi-preparative reverse-phase HPLC and/or preparative silica gel HPLC. After isolation, each avermectin was subjected to analytical HPLC to check its purity. Additionally, UV and MALDI-MS spectra of each sample were taken (data not shown). For each avermectin studied, UV spectra (not shown) revealed three maxima characteristic for avermectins: at 237, 243 and 252 nm. HPLC data showed the following purity and composition of isolated avermectins: A_1 component: A_{1a} (79%), A_{1b} (15%), and avermectin-related impurities (6%); B_1 component: B_{1a} (90%), B_{1b} (6%), and avermectin-related impurities (4%); A_2 component: A_{2a} (74%), A_{2b} (16%), and avermectin-related impurities (10%); B_2 component: B_{2a} (86%), B_{2b} (6%), and avermectin-related impurities (8%). The composition of ivermectin according to HPLC data was avermectin H_2B_{1a} (94%), avermectin H_2B_{1b} (3%), and impurities (3%). All individual avermectins were purified from oligomycin by column chromatography.

Reverse-phase HPLC analyses of aversectin C and isolated avermectins were performed with a Hewlett Packard (1050 series) liquid chromatograph (Darmstadt, Germany) equipped with a UV variable wavelength detector (set to 243 nm) operating online with a PC and using "Multichrom for Windows" software (Ampersand, Moscow, Russia) to acquire and process chromatographic data. The following chromatographic conditions were used: isocratic elution with a methanol/acetonitrile/water (65:20:15, v/v/v) mixture; an analytical Ultrasphere Octadecyl Silane (ODS, 4.6 × 250 nm) column with an ODS precolumn; and a flow rate of 1 ml/min. Individual avermectin components were identified by their retention times in comparison with authentic commercial standards and published data (Novak et al., 1993). Mass-spectra of aversectin C and isolated avermectins were obtained with a MALDI TOF VISION 2000 spectrometer (ThermoBioanalysis, UK) operating online with a PC. The UV laser was set to 337 nm and normally 10–60 shots were applied; a 2,5-dihydroxybenzoic acid (DHB) matrix, an accelerating voltage of 5 kV, and positive polarity were used. The mass (m/e) range measured was 0–5000. All avermectins studied were registered as sodium monovalent molecular cations. Typically, 5–20 pmol of avermectins were used for each MALDI-MS analysis. Under these conditions, practically no fragmentation of the avermectin molecule was observed.

Cisplatin (*cis*-Platinum(II)diamine dihydrochloride; Sigma) and 3% hydrogen peroxide were also used. Vincristine was dissolved in physiological saline supplemented with 0.9% benzyl alcohol, and avermectins were dissolved in ethanol. Ethanol at the maximum concentration used (0.1%) did not affect the survival of cells, which was 99 ± 3%.

All experiments were performed in no less than three replicates. In figures and tables, averaged values with mean square deviations are given.

3. Results

Avermectins purified from oligomycin at concentrations up to 1 μM did not affect the survival of Hep-2 cells. Cell survival at this concentration of avermectins A_1, A_2, B_1, and B_2 was $92 \pm 4\%$, $99 \pm 2\%$, $103 \pm 4\%$, and $97 \pm 2\%$, respectively. A slight decrease in survival was observed only in the case of avermectin A_1; however, at this concentration it was not used for modifying the tumor cell sensitivity, and at a concentration of 0.3 μM avermectin A_1 was nontoxic (the survival of tumor cells was $100 \pm 2\%$). At concentrations above 1 μM, avermectins reduced cell survival. Thus, at a concentration of 3 μM, avermectin B_1 decreased cell survival up to $84 \pm 2\%$. It was shown earlier that a complex of avermectins and individual avermectins A_1 and A_2 had a toxic effect on tumor cells at concentrations below 1 μM (Mosin et al., 1999). Probably this is due to contamination of the preparation by oligomycin.

As mentioned above, the derivative of avermectin B_1, ivermectin, is an effective inhibitor of P-glycoprotein (Pouliot et al., 1997). We tested whether naturally occurring avermectins could modify the sensitivity of tumor cells to the substrates of multidrug resistance proteins. Fig. 1 shows the effect of taxol on the survival of Hep-2, Hep-2TR, and Hep2TR cells in the presence of 0.3 μM avermectin B_1. It became evident that the strain Hep-2TR was more resistant to taxol than the original strain Hep-2: the concentration of taxol at which cell survival decreased by 50% (IC_{50}) was 1660 pM for Hep-2TR and as low as 30 pM for Hep-2 cells. Avermectin B_1 at a concentration of 0.3 μM reduced the IC_{50} of taxol to 1.4 pM Hep-2TR cells. Thus, at this concentration, avermectin B_1 increased the sensitivity of Hep-2TR cells to taxol more than thousandfold. Avermectin B_1 also increased the sensitivity of Hep-2 cells to taxol: the

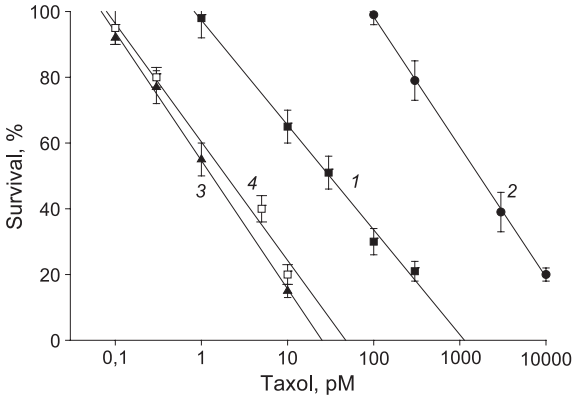

Fig. 1. Effect of taxol on the survival of Hep-2 (1) and Hep-2TR cells (2) and the effect of taxol in combination with avermectin B_1 at a concentration of 0.3 μM on the survival of Hep-2TR (3) and Hep-2 cells (4).

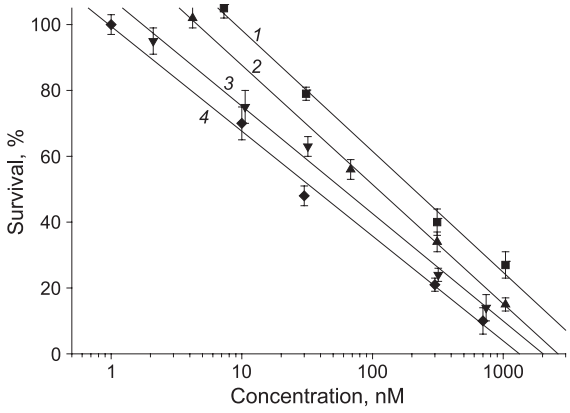

Fig. 2. Effect of avermectins B_1 (1), B_2 (2), A_1 (3), A_2 (4) on the survival of Hep-2 cells treated with taxol at a concentration of 5 pM. The survival of cells treated with taxol was taken to be 100% (75% of the control).

IC_{50} decreased to 2 pM. The strain Hep2TR was not stable long enough to determine the modifying activity of all avermectins. Therefore, further experiments were performed with Hep-2 cells, whose characteristics do not change with time. It is evident from Fig. 1 that avermectin B_1 substantially (15 times) increased the sensitivity of Hep-2 cells to taxol, and, consequently, the modifying activity of avermectins could also be reliably determined on the wild-type strain Hep-2.

At a concentration of 0.3 μM, the effect of avermectin B_1 was not maximal; as the concentration increased, the effect became more pronounced. Fig. 2 shows the effect of different avermectins on the survival of cancer cells treated with a low dose of taxol (5 pM). For all agents, the decrease in survival was nearly proportional to the logarithm of the concentration added. The concentration of the agents at which cell survival was modified by 50% (MC_{50}) are 200, 107, 59, and 35 nM for B_1, B_2, A_1, and A_2, respectively.

The taxol-resistant strain of Hep-2 cells appeared to be resistant to vincristine, too. The IC_{50} of vincristine for this strain was 151 nM, which was by one order of magnitude greater than that for the original Hep-2 cells (15.7 nM). Avermectin B_1 at a concentration of 0.3 μM decreased the IC_{50} of vincristine to 13 nM for Hep-2TR cells and to 4.3 nM for Hep-2 cells. The coefficient of modification of sensitivity to vincristine by avermectin B_1 (0.3 μM) for Hep-2TR cells was 12, which is much higher than the modification coefficient for Hep-2 cells, which was 3.6.

These results indicate that naturally occurring avermectins in the concentration range of 10–100 nM effectively modify the multidrug resistance of tumor cells. Avermectins of group A were more effective than avermectins of group B. Avermectins enhanced the effect of both taxol and vincristine. The taxol-resistant strain Hep-2TR appeared to be also resistant to vincristine, suggesting that drug resistance is due to the overexpression of multidrug resistance proteins in tumor cells.

The direct effect of avermectins on the transport systems providing multidrug resistance was studied by measuring the rate of efflux of rhodamine 123 from tumor cells. The data obtained for avermectin A_1 were compared with the effect of the known inhibitor of multidrug resistance, cyclosporin A. Fig. 3 shows the kinetics of changes in rhodamine 123 concentration in medium in the control (1) and after the addition of cyclosporin A (2) and digitonin (3). The total amount of rhodamine 123 in cells was determined from the difference in fluorescence intensity after the addition of digitonin and just after placing the cells into the cuvette. The relative amount of rhodamine 123 in cells (N) was determined with the formula $N = 1 - (I_t - I_0/I_{max} - I_0)$, where I_t, I_0, and I_{max} are the intensities of rhodamine 123 fluorescence at time t and 0 and the maximum fluorescence intensity after the addition of digitonin, respectively. As is evident from Fig. 4, the amount of rhodamine 123 in cells in the control and after the addition of cyclosporin A decreased exponentially and consequently the rate constant could be determined for characterization of this process. Cyclosporin A at a concentration of 0.8 μM lowered the rate constant of rhodamine 123 efflux from cells, on average, 3.2 times: $R - 1 = 2.2$ (Fig. 5).

Fig. 5 shows the dependence of the $R - 1$ value on the concentration of avermectin A_1 and cyclosporin A. In both cases, the dependence was nonlinear and went through the maximum. The maximum value was nearly equal in both cases; it is determined by the expression of transport proteins in cells, and the position of the maximum reflects the activity of the inhibitor. It is evident from Fig. 5 that avermectin A_1 was threefold more effective than cyclosporin A. The decrease in the $R - 1$ value with increasing concentration of the inhibitors is likely to be due to structural changes in the cell membrane induced by hydrophobic agents (formation of clusters), which increases the passive permeability of the membrane for rhodamine 123. This was confirmed by the observation that, at concentrations decreasing the $R - 1$ value, avermectin A_1 and cyclosporin A were toxic for cells.

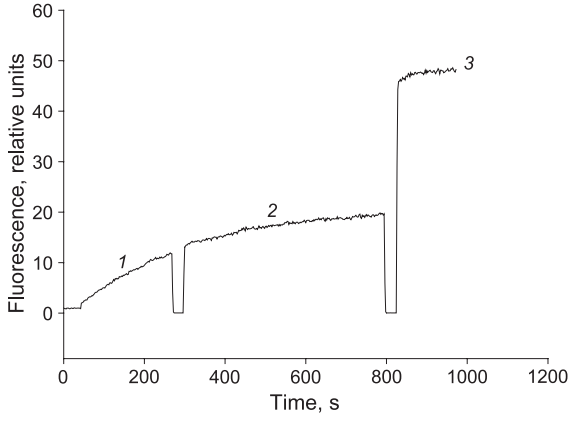

Fig. 3. Kinetics of efflux of rhodamine 123 from Hep-2 cells in the control (1) and after the addition of cyclosporin A at a concentration of 0.8 μM (2) and 0.02% digitonin (3).

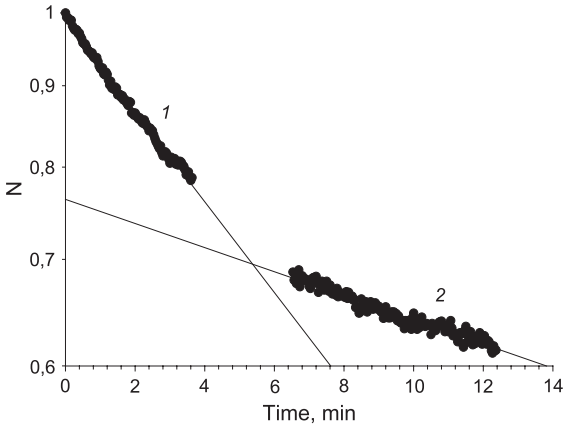

Fig. 4. Kinetics of efflux of rhodamine 123 from Hep-2 cells in the control (1) and after the addition of cyclosporin A at a concentration of 0.8 μM (2). Rate constant for efflux 2—0.065, 3—0.017 min^{-1}. $R - 1 = 2.8$. N is the amount of rhodamine 123 in cells relative to the initial level.

Avermectins A_2, B_1, and B_2 purified from oligomycin at concentrations of up to 1 μg/ml had no effect on the survival of P388 and P388VR cells. Avermectin A_1 did not affect survival at a concentration of 0.3 μM (97 ± 4%) and reduced it by 12% at a concentration of 1 μM.

Fig. 6 shows the effect of vincristine and vincristine in combination with avermectin B_1 (0.3 μM) on the survival of wild-type P388 cells. First, it should be noted that the dose-effect curves were nonlinear, because cell survival is determined by two different processes, cell division and cell death. In the control, the number of cells during incubation increased by 25–30%; therefore, cell survival should decrease due to the suppression of cell division by this value. Nearly the same effect was produced by vincristine at low concentrations. Thus, it can be said that low concentrations of vincristine suppressed cell division, and high concentrations of the compound induced cell death. The addition of avermectin B_1 at a concentration of 0.3 μM did not change

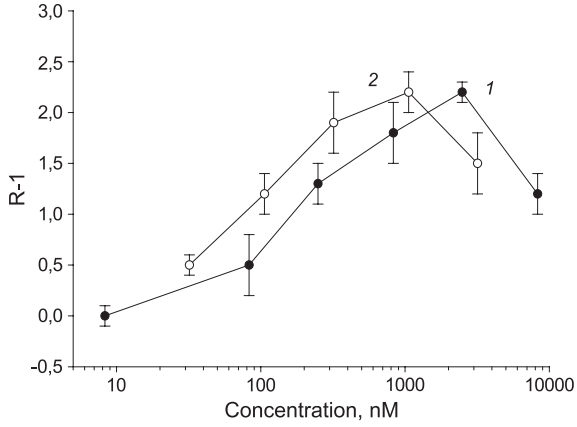

Fig. 5. Concentration dependence of the inhibition of the rate of rhodamine 123 efflux from Hep-2 cells by cyclosporin A (1) and avermectin A_1 (2).

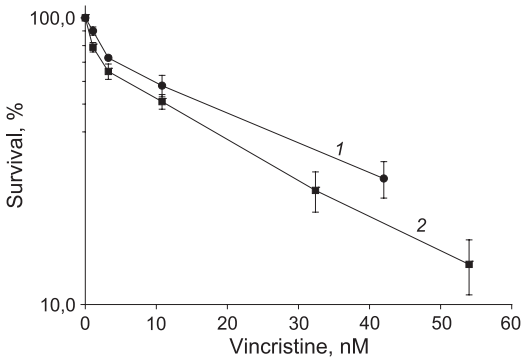

Fig. 6. Effect of vincristine (1) and vincristine in combination with avermectin B_1 at a concentration of 0.3 μM (2) on the survival of P388 cells.

the shape of the dose-response curve but increased the sensitivity of cells to vincristine: the IC_{50} value decreased from 17 to 11 nM. The low coefficient of modification of the sensitivity of P388 cells to vincristine by avermectin B_1 (1.6) may be due to a low level of expression of multidrug resistance proteins in these cells.

The IC_{50} value of P388 cells after a second, fourth, and sixth injection of vincristine to mice increased and was 170, 380, and 570 nM, respectively, which was 10, 22, and 33 times greater than the IC_{50} value for original wild-type P388 cells. After the sixth injection of vincristine, the sensitivity of the P388VR strain to vincristine remained unchanged upon reinoculation of mice over a period of 2 months. The P388VR strain was much more resistant to vincristine than the wild type also by the criterion of nuclear damage (data not shown).

Avermectin B_1 at a concentration of 0.3 μM reduced the IC_{50} of vincristine for P388VR cells to 35 nM, i.e., it increased the sensitivity of P388VR cells to vincristine 16 times. Thus, the coefficient of sensitivity modification of avermectin B_1 at a concentration of 0.3 μM for P388VR cells was one order of magnitude higher than for P388 cells. Consequently, the resistant strain P388VR could be successfully used for studying the modifying effect of various inhibitors of multidrug resistance proteins.

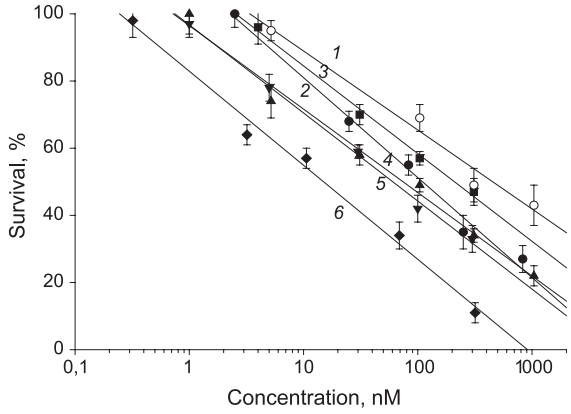

Fig. 7. Effect of ivermectin (1—○), cyclosporin A (2—●), and avermectins B_1 (3—■), B_2 (4—▲), A_2 (5—▼), and A_1 (6—◆) on the survival of P388VR cells in the presence of 100 nM of vincristine.

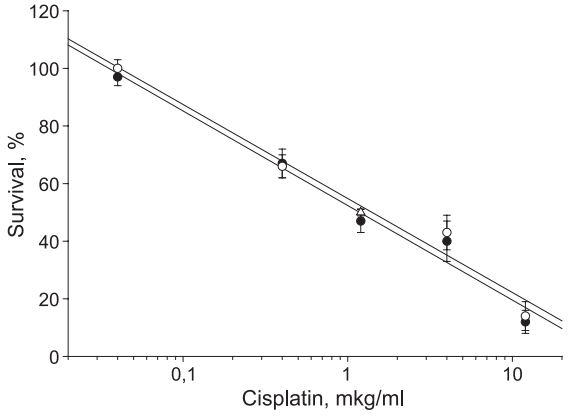

Fig. 8. Effect of cisplatin on the survival of P388 (1—●), P388VR (2—○), and P388VR cells in the presence of avermectin B_1 at a concentration of 0.3 μM (3—△).

Fig. 7 shows the concentration dependence of the effect of avermectins and cyclosporin A on the survival of P388VR cells treated with vincristine at a concentration of 100 nM. At this concentration, vincristine did not affect cell survival. After the addition of avermectins, cell survival decreased proportionally to the logarithm of avermectin concentration. The MC_{50} values were 15, 59, 74, and 209 nM for avermectins A_1, A_2, B_2, and B_1, respectively. The MC_{50} value for ivermectin was 460 nM and for cyclosporin A—107 nM.

Thus, all avermectins at nontoxic concentrations increase the sensitivity of tumor cells to vincristine. The modulating effect of avermectins varied greatly (from 15 to 460 nM). Among avermectins there were more and less active inhibitors of multidrug resistance than cyclosporin A. The most potent inhibitor for P388VR cells was A_1: it was seven times as effective as cyclosporin A. Ivermectin was the least

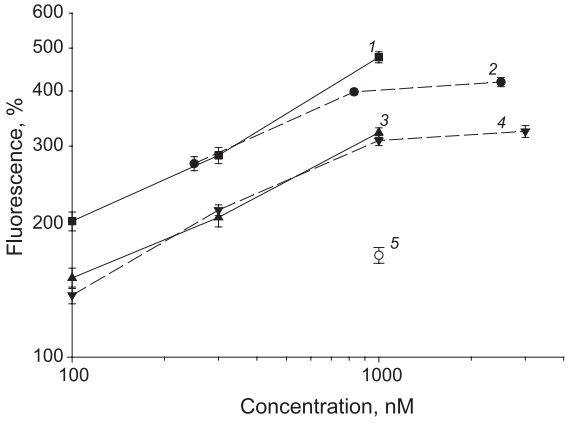

Fig. 9. Increase in the accumulation of calcein in P388VR cells in response to avermectin B_2 (1), cyclosporin A (2), avermectin A_2 (3), and ivermectin (4); 5—increase in the accumulation of calcein in P388 cells in response to avermectin B_2. The fluorescence of calcein in cells in the absence of avermectins was taken as 100%.

Table 1
Concentrations of the inhibitors at which calcein fluorescence in P388VR cells increased twofold (C_2)

Agent	Cycl A	Av A_1	Av A_2	Av B_1	Av B_2	Av C	Av 3	Iver
C_2, nM	108	210	240	385	104	144	144	270

active: it reduced cell survival by 50% at a concentration 4.3 times higher than that of cyclosporin A.

The sensitivity of P388VR cells to agents whose accumulation in tumor cells does not depend on multidrug resistance proteins, such as hydrogen peroxide (data not shown) and cisplatin, was similar to that of the original strain P388, and avermectin B_1 did not affect the sensitivity of cells to cisplatin (Fig. 8). These findings suggest that the resistance of P3888VR cells to vincristine is due to an increased content of multidrug resistance proteins, and the modifying effect of avermectin is related to the inhibition of this transport protein.

The inhibition of multidrug transport proteins, including P-glycoprotein and MRP1, by avermectins was determined using calcein AM. Fig. 9 shows an increase in the accumulation of calcein in cells treated with avermectins A_1, B_2, ivermectin, and cyclosporin A. Cyclosporin A appeared to be twice as effective as ivermectin. We examined whether the increase in calcein content in P388VR cells depended on the concentration of avermectins (data not shown). From these data, we determined the concentration at which calcein content increased twofold (Table 1). It is evident from Table 1 that some avermectins were less effective and others were more effective in inhibiting the calcein AM transport from cells than cyclosporin A. However, the relative activity of avermectins was different from that for inhibition of vincristine transport. Avermectin B_2 showed the highest relative activity in removing calcein AM.

4. Discussion

We tested for the first time the effect of natural avermectins on multidrug resistance of tumor cells. We showed that avermectins at nontoxic concentrations effectively inhibit multidrug resistance in vincristine-resistant lympholeukemic cells P388 and taxol-resistant larynx tumor cells Hep-2. The relative activity of avermectins was found depend on the cell type and the agent. Avermectin B_1 and ivermectin were the least active: the activity of the other avermectins varied depending on the substrate and the cell type. In some experimental systems, the most active avermectin was almost one order of magnitude more effective than the traditional inhibitor of multidrug resistance cyclosporin A. In principle, it is possible to select the most active avermectin for a particular type of resistant tumor cell and to use this drug in the therapy of these tumors.

The sensitivity to taxol and vincristine of all tumor cells was modified to a greater (resistant strains) or lesser extent (wild-type strains) by inhibitors of tumor multidrug resistance. In addition, these inhibitors also influenced the accumulation of calcein in cells and the efflux of rhodamine 123 from cells. These findings suggest that multidrug transport proteins were present in all cells examined. It is known that P-glycoprotein and MRP1 often occur in tumor cells, and that the multidrug resistance of P388 cells selected with respect to resistance to vincristine is mainly due to P-glycoprotein (Sarkadi and Muller, 1997). It is also known that calcein AM and rhodamine 123 are the substrates for P-glycoprotein and MRP1 (Essodaigui et al., 1998; Twentyman et al., 1994). This information and the data obtained in the present work indicate that avermectins may be inhibitors of both transport proteins. It was found earlier that one of the avermectins, ivermectin, is an inhibitor of P-glycoprotein and is even more efficient than cyclosporin A (Pouliot et al., 1997). Here, we showed that the relative efficacy of inhibition of tumor cell multidrug resistance by avermectins and cyclosporin A depends on the type of cells and the substrate of transport proteins. This can be explained by the fact that avermectins and cyclosporin A differ in specificity for transport proteins. Therefore, if the contribution of transport proteins to transport changes, due to a change in the substrate or the cell type, than their relative effectiveness would also change. Further studies are needed to estimate the effect of avermectins on each particular transport protein.

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