RESORTHIOMYCIN, A NOVEL ANTITUMOR ANTIBIOTIC

III. POTENTIATION OF ANTITUMOR DRUGS AND ITS MECHANISM OF ACTION

MAKOTO TAHARA, AKIHIRO TOMIDA, TOSHIO NISHIMURA, HIDEYO YAMAGUCHI and HIDEO SUZUKI*

Institute of Applied Microbiology, University of Tokyo, Yayoi, Bunkyo-ku, Tokyo 113, Japan

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Resorthiomycin suppressed the clonogenic activity of a multidrug-resistant mutant cell line of Chinese hamster V79 cells more potently than its parental cells. Moreover, resorthiomycin at 40 μg/ml potentiated the cytotoxic activity of vincristine and actinomycin D on V79 cells over 3-fold. Uptake of [^{3}H]actinomycin D into V79 cells was stimulated 2-fold by 40 μg/ml of resorthiomycin during 2 hours incubation. On the other hand, incorporation of [^{3}H]thymidine and [^{3}H]uridine into mouse leukemia L5178Y cells was inhibited in a dose-dependent manner at resorthiomycin concentrations ranging from 5 to 40 μg/ml. In ATP-depleted L5178Y cells, membrane transport of [^{3}H]thymidine and 2-[^{3}H]deoxyglucose was strongly suppressed by resorthiomycin. These results suggest that resorthiomycin acts on the plasma membrane and perturbes some membrane function.

Resorthiomycin (6-acetyl-4-(3-hydroxybutyl)-2-methyl-5-methylthioresorcinol, Fig. 1) was isolated from a strain of *Streptomyces* 45H-6 by our screening method using cultured tumor cells including drug-resistant mutants. The production, purification and structure elucidation was described in preceding papers[1,2]. In this publication we report the biological activity and the mechanism of action of resorthiomycin.

Materials and Methods

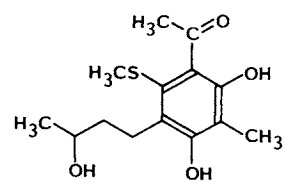
Chemicals

[*methyl*-^{3}H]Thymidine (25 Ci/mmol), [5,6-^{3}H]uridine (42 Ci/mmol), L-[2,3-^{3}H]alanine (54 Ci/mmol) and [^{3}H]actinomycin D (3.6 Ci/mmol) were purchased from Amersham Japan, Tokyo and 2-[^{3}H(G)]deoxy-D-glucose (7.1 Ci/mmol) was from New England Nuclear, Boston, Mass. Vincristine sulfate, doxorubicin hydrochloride, methotrexate and 5-fluorouracil were obtained from Wako Pure Chemical Inc., Tokyo and actinomycin D from Banyu Pharmaceutical Co., Tokyo. Resorthiomycin was purified as described in a previous paper[1] and dissolved in methanol. When resorthiomycin was added to a culture medium, it was diluted with phosphate-buffered saline (PBS) and the same amount of methanol was added to control cultures (final concentration less than 0.5%).

Cells

Mouse leukemia L5178Y cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum and Chinese hamster V79 cells were grown in EAGLE's minimum essential medium with 10% calf serum in a humidified atmosphere of 5% CO_2 at 37°C. A doxorubicin-resistant mutant strain

Fig. 1. Chemical structure of resorthiomycin.



* To whom requests for reprints should be addressed.

of V79 cells (V79/ADM) was established by stepwise selection during subculturing in increasing concentrations of doxorubicin. The mutant cell line used in the present experiments is approximately 150-fold more resistant to doxorubicin as compared with the parental cell line and shows a typical phenotype of multidrug resistance, while it is peploymycin-supersensitive[3].

Macromolecular Synthesis of L5178Y Cells

L5178Y cells (1.4×10^5 cells/ml) in a 96-well microplate (180 μl of cell suspension/well) were preincubated with 20 μl of various concentrations of resorthiomycin for 5 hours at 37°C and then 10 μl of tritium-labeled precursors diluted with PBS were added (1 μCi/ml). After 1 hour incubation, cells were harvested on glass fiber filter, washed 3 times with PBS and twice with cold 5% TCA. Radioactivity was measured in a scintillation counter. In another series of experiments, resorthiomycin was washed out from the culture medium before an addition of [^{3}H]thymidine to examine the reversibility of the cytotoxic effect of resorthiomycin.

Transmembrane Transport of [^{3}H]Thymidine and 2-[^{3}H]Deoxyglucose in L5178Y Cells

Transmembrane transport of exogenous radioactive substrates was measured according to the method of PLAGEMANN *et al.*[4]. Briefly, L5178Y cells ($5 \sim 10 \times 10^5$/ml) were deprived of intracellular ATP by incubating with 5 mM NaCN and 5 mM monoiodoacetic acid for 10 minutes at 37°C. Then a 0.5-ml volume of the cell suspension containing an indicated concentration of resorthiomycin, was mixed with 50 μl of [^{3}H]thymidine or 2-[^{3}H]deoxyglucose to give a final concentration of 10 μCi/ml. The reaction was carried out on a 0.5-ml layer of an oil mixture (silicon oil - paraffin liquid, 4 : 1) in an Eppendorf tube and stopped by precipitating the cells into the bottom of the tube through the oil layer by rapid centrifugation using an Eppendorf centrifuge 5412[5]. The radioactivity of 5% TCA-soluble materials was measured in Hionic Fluor (Packard).

Colony Formation of V79 Cells

To a culture medium of Chinese hamster V79 cells which had been plated on 60 mm plastic dishes ($200 \sim 300$ cells/plate) and incubated for 20 hours, resorthiomycin and other drugs were added. After $7 \sim 8$ days of incubation, cells were fixed with 10% formaldehyde solution and stained with crystal violet to count and score numbers of colonies.

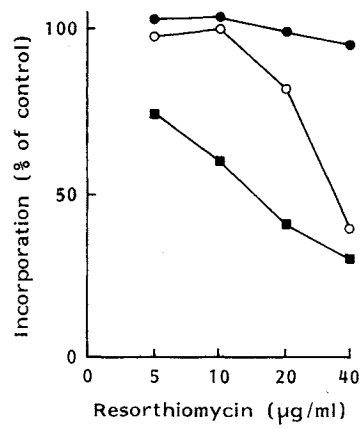
Uptake of [^{3}H]Actinomycin D by V79 Cells

V79 cells (2×10^5/ml/well) were incubated with [^{3}H]actinomycin D (0.5 μCi/ml) for 1 or 2 hours at 37°C in the presence or absence of 40 μg/ml of resorthiomycin. Then cells were washed twice with cold PBS, harvest after trypsinization, and solubilized with Protozol (NEN, Mass.). The radioactivity was measured in Scintizol EX-H (Dojin, Kumamoto-city).

In all experiments in this paper, the mean values of three determinants were calculated.

Fig. 2. Incorporation of [^{3}H]alanine, [^{3}H]thymidine and [^{3}H]uridine into L5178Y cells in the presence of various concentrations of resorthiomycin.

● Alanine, ■ thymidine, ○ uridine.



Results

Effects of Resorthiomycin on Macromolecular Synthesis by L5178Y Cells

Resorthiomycin at levels of $5 \sim 40$ μg/ml inhibited, dose-dependently, the incorporation of [^{3}H]thymidine and [^{3}H]uridine into the cold TCA-insoluble fraction of L5178Y cells when they were pretreated for 5 hours with the antibiotic, while

Fig. 3. Effect of resorthiomycin on transmembrane transport of [^{3}H]thymidine and 2-[^{3}H]deoxyglucose in ATP-depleted L5178Y cells.

(A) [^{3}H]Thymidine, (B) 2-[^{3}H]deoxyglucose.
○ Control, △ 1 μg/ml, ▲ 10 μg/ml, ■ 30 μg/ml.

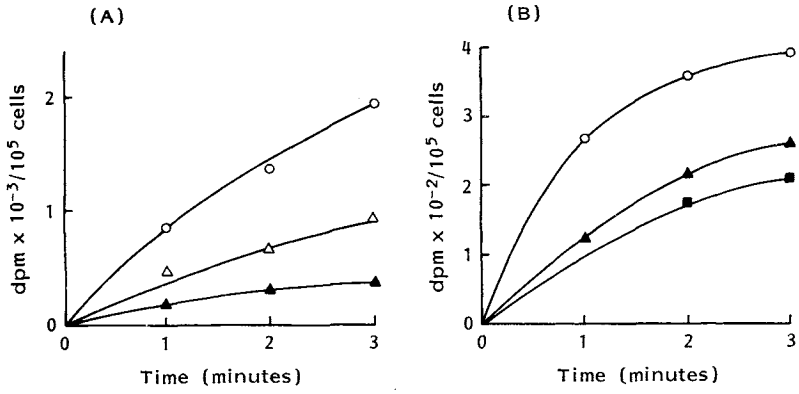


Fig. 4. Effect of resorthiomycin on colony formation by V79/S and V79/ADM cells.

○ V79/S, △ V79/ADM.

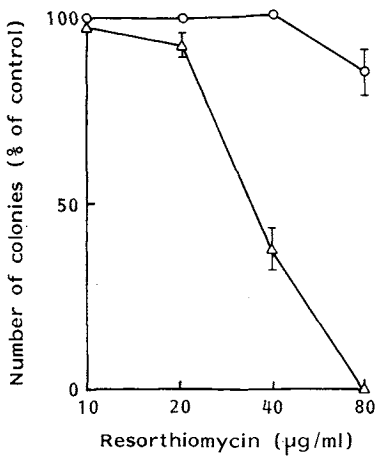
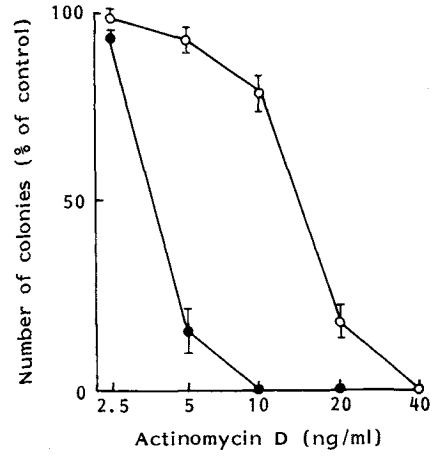


Fig. 5. Potentiation of the cytocidal activity of actinomycin D on V79/S cells by resorthiomycin.

○ 0 μg/ml, ● 40 μg/ml of resorthiomycin.



it was without effect on the incorporation of [^{3}H]alanine at these concentrations (Fig. 2). The inhibitory activity of resorthiomycin on [^{3}H]thymidine incorporation was readily reversed after removal of the antibiotic (data not shown).

Transmembrane Transport of [^{3}H]Thymidine and 2-[^{3}H]Deoxyglucose in ATP-depleted Cells

The effect of resorthiomycin on the transmembrane transport of exogenous substrates mediated by energy-independent passive diffusion were studied using ATP-depleted L5178Y cells. In these cells, resorthiomycin reduced the initial rate of transmembrane transport of [^{3}H]thymidine and 2-[^{3}H]deoxyglucose at 1 and 10 μg/ml, respectively, to about 50% of the level for the control (Fig. 3).

Comparison of Cytocidal Activity of Resorthiomycin against V79/S and V79/ADM Cells

Since resorthiomycin has been shown to inhibit colony formation of multidrug-resistant human

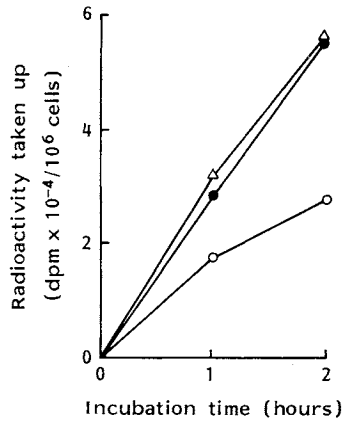
Table 1. Effect of resorthiomycin on the cytocidal activity of several antitumor drugs against V79/S cells.

	Resorthiomycin (μg/ml)		
	0	20	40
Actinomycin D	14.0[a] (1)[b]	—	3.8 (3.7)
Vincristine	17.3 (1)	10.9 (1.6)	5.1 (3.4)
Doxorubicin	40.6 (1)	42.9 (0.9)	41.7 (1.0)
Methotrexate	15.8 (1)	15.5 (1.0)	12.0 (1.3)
5-Fluorouracil	10.5 (1)	10.9 (1.0)	10.3 (1.0)

[a] IC_{50} expressed as ng/ml except in case of 5-fluorouracil (μM).
[b] Values in parenthesis represent the potentiation index.

Fig. 6. Uptake of [^{3}H]actinomycin D in V79 cells.

Resorthiomycin (40 μg/ml) was added to cells simultaneously with (●) or 5 hours before (△) [^{3}H]actinomycin D. Control (○).



hepatoma PLC/PRF/5 cells to a greater extent than its parental cells[1], another cell line with multidrug resistance was employed in this study to test whether this was also inhibited. As shown in Fig. 4, colony formation of parental Chinese hamster V79 (V79/S) cells was not inhibited by resorthiomycin at 40 μg/ml and slightly inhibited at 80 μg/ml, whereas that of the multidrug-resistant mutant (V79/ADM) was strongly or completely inhibited in the presence of 40 or 80 μg/ml of the antibiotic, respectively.

Potentiation by Resorthiomycin of the Cytocidal Activity of Some Other Antitumor Drugs against V79/S Cells

The effect of several clinically useful antitumor drugs on colony formation by V79/S cells was examined in the presence or absence of resorthiomycin. As illustrated in Fig. 5, the inhibitory activity of actinomycin D was significantly enhanced by the addition of 40 μg/ml of resorthiomycin. IC_{50} of actinomycin D was 14.0 ng/ml in the absence of resorthiomycin, while the value decreased to 3.8 ng/ml in the presence of 40 μg/ml of resorthiomycin. A similar potentiation by resorthiomycin was also exhibited when combined with vincristine, but the antibiotic had no effect on the cytocidal activity of doxorubicin, methotrexate or 5-fluorouracil. The IC_{50} values of these antitumor drugs for V79/S cells in the presence or absence of resorthiomycin are summarized in Table 1, along with the potentiation ratio.

Effect of Resorthiomycin on Uptake of Actinomycin D by V79/S Cells

The rate of uptake of actinomycin D by V79 cells was examined in the presence or absence of resorthiomycin. As shown in Fig. 6, resorthiomycin at 40 μg/ml significantly increased the intracellular level of [^{3}H]actinomycin D in V79 cells during the 2-hour incubation period. Preincubation of cells with resorthiomycin for 5 hours did not affect the enhancement of intracellular accumulation of [^{3}H]actinomycin D.

Discussion

The occasional occurrence of cancer cells acquiring resistance to anticancer drugs in cancer patients has become an increasingly serious problem in cancer chemotherapy. With the aim of overcoming this problem, we have been exploring the molecular mechanisms of drug resistance developing in malignant cells[6~9] and searching for new microbial products with selective toxicity against such resistant cancer cells. In the course of our screening program, COTC[10] and lactoquinomycins[11,12] were discovered in this

laboratory.

More recently, a new antitumor antibiotic was isolated from the culture broth of a strain of *Streptomyces* sp. The antibiotic exhibited preferential cytotoxicity on multidrug-resistant human hepatoma PLC/PRF/5 cells together with an inhibitory activity on [^{3}H]thymidine incorporation into mouse leukemia L5178Y cells[1]. The structure of the antibiotic is quite unique containing a 6-substituted benzene ring and has designated resorthiomycin on the basis of its chemical structure[2].

In addition to the cytotoxic activity preferentially exhibited against multidrug-resistant Chinese hamster V79 cells (V79/ADM) (Fig. 4), resorthiomycin enhanced the cytocidal activity of other antitumor drugs (actinomycin D and vincristine) on V79/S cells, although it had no effect on the activity of others (doxorubcin, methotrexate and 5-fluorouracil, see Table 1).

Resorthiomycin caused a 2-fold increase in the intracellular level of [^{3}H]actinomycin D in V79 cells at 40 μg/ml (Fig. 6). In ATP-depleted L5178Y cells, the initial rate of transmembrane transport of [^{3}H]thymidine and 2-[^{3}H]deoxyglucose was reduced by resorthiomycin, to approximately 50% at drug levels of 1 and 10 μg/ml, respectively (Fig. 3). However, the inhibitory effect of resorthiomycin was reversed when the antibiotic was removed from the medium before introduction of [^{3}H]thymidine. These results suggest the possibility that resorthiomycin binds reversibly to the cytoplasmic membrane and modulates the permeability to various chemical substances. The precise mechanism of the membrane action of this antibiotic warrants further investigation.

Acknowledgments

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