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Antitumor Effectiveness of Improsulfan on Nitrogen Mustard-resistant Yoshida Sarcoma Cells[1)]

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In the cross-resistance studies *in vitro* and *in vivo*, three sublines of Yoshida sarcoma with high resistance to nitrogen mustard (HN_2) were highly susceptible to improsulfan, and there was no significant difference in the extent of improsulfan uptake and its binding to desoxyribonucleic acid between the original and HN_2-resistant cells. The results obtained by the transport study of improsulfan at a dose range of 0.01 to 1 mM suggest that improsulfan uptake is non-active by Yoshida sarcoma cells. The factors of resistance to HN_2 demonstrated in these resistant cells may not serve as factors of resistance to improsulfan.

Keywords——antitumor activity; antitumor alkylating agent; drug resistance; drug uptake by tumor cells; tumor cells

Improsulfan, a methanesulfonic acid ester of aminoglycol, has been found to be effective on malignant lymphoma, leukemia and bronchogenic carcinoma, and have no severe toxicity in the tentative clinical evaluation.[3–5)] It was reported that some clinical cases resistant to other alkylating agents responded to this drug.[6,7)] Preclinically, Sakurai, *et al.*[8)] found that, among hundreds of the alkylating agents prepared by them, improsulfan was the first one to affect rat ascites hepatomas such as AH-66 and AH-7974 which are refractory to nitrogen mustard (HN_2) and its related compounds. The present communication deals with the effects of improsulfan on the sublines of Yoshida sarcoma which acquired resistance to HN_2, and improsulfan uptake by the original and HN_2-resistant Yoshida sarcoma cells.

Materials and Methods

Tumors, Animals, and Compounds——Yoshida sarcoma and its sublines which acquired resistance to HN_2 (RAc_{-13}, RAc_{-22}, and YSc_{-20}) were maintained by successive intraperitoneal inoculation in male Donryu rats at 6 weeks of age, supplied from Nihon Rat Co., Urawa. Improsulfan *p*-toluenesulfonate and HN_2 hydrochloride synthesized in this laboratories were used. ^{3}H-Improsulfan *p*-toluenesulfonate was obtained by exchanging the salt of ^{3}H-improsulfan hydrochloride which was tritiated by the Wilzbach method.[9)] When ^{3}H-improsulfan *p*-toluenesulfonate with specific activity of 12.2 mCi/mmole was hydrolyzed, dipropanolamine, a hydrolysis product, had more than 94% of the total radioactivity. Other compounds were obtained commercially.

1) This paper constitutes Part XI of a series entitled "Studies on Carcinostatic Substances." Part X: T. Okumoto and H. Imamura, *Chem. Pharm. Bull.* (Tokyo), **25**, 812 (1977).
2) Location: *Nishigahara 1–26–1, Kita-ku, Tokyo 114, Japan.*
3) M. Hirano, M. Miura, H. Kakizawa, A. Morita, T. Uetani, R. Ohno, K. Kawashima, H. Nishiwaki, and K. Yamada, *Cancer Chemother. Rep.*, **56**, 335 (1972).
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5) N. Gad-el-Mawla, M.E. Mahmoud, A. Osman, B. El-Morsi, M.M. El-Merzabani, M. Aboul-Enein, and Y. Sakurai, *Cancer Chemother. Rep.*, **59**, 367 (1975).
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7) Y. Wakabayashi and F. Takaku, *Rinsho Ketsueki*, **16**, 39 (1975).
8) M.M. El-Merzabani and Y. Sakurai, *Gann*, **56**, 589 (1965).
9) K.E. Wilzbach, *J. Am. Chem. Soc.*, **79**, 1013 (1957).

Cross-resistance Studies——As described in the previous paper,[10] 5×10^4 cells of each tumor in 1 ml of Eagle's minimum essential medium supplemented with 20% calf serum were incubated with the respective alkylating agent at 37° in sealed test tubes. Cell number was counted on the model B Coulter countr after a 48-hr culture, and the concentration of the agent corresponding to 50% inhibition (IC_{50}) was determined. Resistance index was estimated according to the following formula reported by Sakurai, *et al.*[11]

$$\text{resistance index} = \frac{IC_{50} \text{ of the agent to } HN_2\text{-resistant cells}}{IC_{50} \text{ of the agent to the original cells}}$$

In the *in vivo* studies, HN_2 or improsulfan was given intraperitoneally to rats 3 hr after intraperitoneal inoculation of 10^6 tumor cells. Survival days of tumor-bearing rats were noted and used as a measure of drug effect.

Transport Studies——Tumor cells harvested from the peritoneal cavity of tumor-bearing rats were suspended in ice-cold 0.9% NaCl solution, washed several times with 0.9% NaCl solution and resuspended in Hanks' balanced salt solution. ^{3}H-Improsulfan was added to this cell suspension at a required concentration and the mixture was incubated at 0° or 37° for a certain period. The tumor cells were washed twice with ice-cold Dulbecco's phosphate-buffered saline (PBS) containing 1 mM improsulfan, solubilized in 1 ml of the Beckman tissue solubilizer and then transferred to 10 ml of toluene scintillation fluid. Radioactivity was counted on a Beckman LS-100C scintillation spectrometer.

Binding of Improsulfan to DNA——Each tumor (4×10^6 cells) was suspended in 1 ml of Hanks' balanced salt solution containing 1 mM ^{3}H-improsulfan and incubated at 37° for 30 min. The tumor cells were washed twice with 5 ml of ice-cold PBS. The desoxyribonucleic acid (DNA) fraction was extracted by the method of Rowland.[12] To the washed tumor cells, 1.5 ml of ice-cold 10% (w/v) perchloric acid (PCA) was added. The precipitate was washed by 4 successive resuspensions and centrifugations in 2 ml of ice-cold 2% (w/v) PCA. Lipids were extracted first with 1 ml of ethanol, then twice with 2 ml of ethanol–chloroform mixture (1: 1) and finally with 1 ml of ethanol. The precipitate was resuspended in 2 ml of ice-cold N PCA, allowed to stand at 4° for 18 hr and washed once with 0.5 ml of 1 N PCA. For extraction of DNA, the precipitate was heated in 2 ml of 0.5 N PCA at 70° for 20 min. The DNA extract was neutralized with 10 N KOH and the precipitated potassium salt was removed. The content of DNA was determined by optical density at 260 mμ. For the determination of radioactivity, 0.5 ml of the DNA extract was added to 10 ml of toluene scintillation fluid containing 33% Triton X-100.

Results

Cross-resistance Studies

Fig. 1 shows the effects of improsulfan and HN_2 on the growth of the original and HN_2-resistant Yoshida sarcoma cells in suspension culture. These HN_2-resistant cells showed resistance index of 100 to 200 for HN_2 when it was estimated by comparison of the IC_{50} value of the drug to the original cells with those to HN_2-resistant cells. These HN_2-resistant cells had resistance index below 4 to improsulfan.

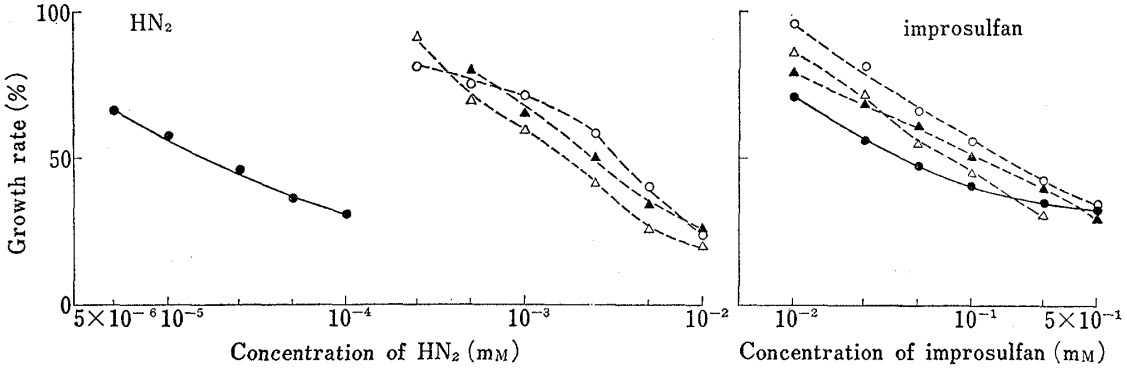

Fig. 1. Effect of Improsulfan and HN_2 on the Growth of the Original (●) and HN_2-resistant Yoshida Sarcoma Cells (○: RAc_{-13}, △: RAc_{-22}, ▲: YSc_{-20}) in Suspension Culture

Cell number was counted after a 48-hr culture.

10) H. Imamura, K. Ikegami, T. Okumoto, and N. Tanaka, *Yakugaku Zasshi*, **95**, 420 (1975).
11) Y. Sakurai and A. Moriwaki, *Gann*, **54**, 473 (1963).
12) G.F. Rowland, *Cancer Res.*, **29**, 391 (1969).

Table I shows the mean survival days of rats bearing the original or HN_2-resistant Yoshida sarcoma cells by administration of improsulfan or HN_2. The rats bearing the original cells had prolonged survival times by administration of these two drugs compared to the untreated control rats. Improsulfan increased significantly survival days of rats bearing HN_2-resistant cells which had no response to HN_2, but the rats bearing the original cells survived longer by administration of improsulfan than those bearing HN_2-resistant cells.

Table I. Survival Days of Rats Bearing the Original and HN_2-resistant Yoshida Sarcoma Cells by Adminstration of Improsulfan and HN_2

Tumor	Survival days (mean±S.E.)		
	Improsulfan (50 mg/kg)	HN_2 (0.25 mg/kg)	Untreated
RAc_{-13}	42.2±7.3[a] (2)	11.0±0.9	9.2±0.8
RAc_{-22}	38.2±6.4[a] (1)	12.0±0.9	10.7±0.3
YSc_{-20}	47.5±6.7[a] (3)	14.7±1.4	12.3±1.2
Original	54.5±5.5[a] (5)	45.3±9.3[a] (4)	8.5±0.6

Improsulfan and HN_2 were given intraperitoneally 3 hr after tumor inoculation. The numbers of rats surviving over 60 days are given in parentheses, and these rats were calculated as 60-day survivors. For each group, 6 rats were used.
a) $p < 0.05$ (statistical significance of difference from the untreated control rats by the 2-tailed t test).

Improsulfan Uptake and Its Binding to DNA in the Original and HN_2-resistant Yoshida Sarcoma Cells

As shown in Tables II and III, there was no significant difference in the extent of improsulfan uptake and its binding to DNA between the original and HN_2-resistant cells by exposure to 0.04 and 0.2 mM ^{3}H-improsulfan for 15 min, and 1 mM ^{3}H-improsulfan for 30 min, respectively.

Table II. Improsulfan Uptake by the Original and HN_2-resistant Yoshida Sarcoma Cells

Tumor	cpm/4×10^6 cells	
	0.04 mM ^{3}H-improsulfan	0.2 mM ^{3}H-improsulfan
RAc_{-13}	476	1928
RAc_{-22}		2103
Original	455	2085

After 4×10^6 cells were incubated in 1 ml of Hanks' balanced salt solution containing ^{3}H-improsulfan at 37° for 15 min, the tumor cells were washed three times with ice-cold PBS and collected on the glass-fiber filters (GF/C, Whatman). Radioactivity of the dried filter was measured. Each value represents the mean of 3 samples, and no statistically significant difference was obtained between the values of the original and HN_2-resistant cells.

Table III. Radioactivity Bound to DNA of the Original and HN_2-resistant Yoshida Sarcoma Cells after Their Exposure to ^{3}H-Improsulfan

Tumor	cpm/μg DNA
RAc_{-22}	28.1
YSc_{-20}	26.0
Original	27.6

The tumor cells were incubated with 1 mM ^{3}H-improsulfan at 37° for 30 min. Each value represents the mean of 3 samples, and no statistically significant difference was obtained between the values of the original and HN_2-resistant cells.

Improsulfan Uptake by the Original Yoshida Sarcoma Cells

Time courses of improsulfan uptake by Yoshida sarcoma cells at concentrations of 0.02 and 0.2 mM are shown in Fig. 2. Improsulfan uptake was temperature sensitive and reached a plateau within 3 min at 37°. Radioactivity in the tumor cells reached a plateau at 0° within 1 min.

The relationship between the ratio of radioactivity in the tumor cells (an intracellular volume of 2 $mm^3/10^6$ cells[13]) to that in an equivalent volume of medium and the concentration of ^{3}H-improsulfan used is presented in Fig. 3. At a dose range of 0.01 to 1 mM improsulfan, the ratio was approximately constant, indicating that improsulfan uptake increased

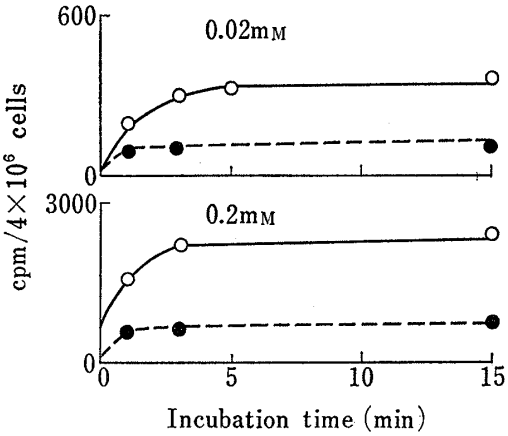

Fig. 2. Time Course of Improsulfan Uptake by Yoshida Sarcoma Cells at 0° (●) and 37° (○)

Yoshida sarcoma cells at a concentration of 4× 10^6 cells/ml were incubated with ^{3}H-improsulfan.

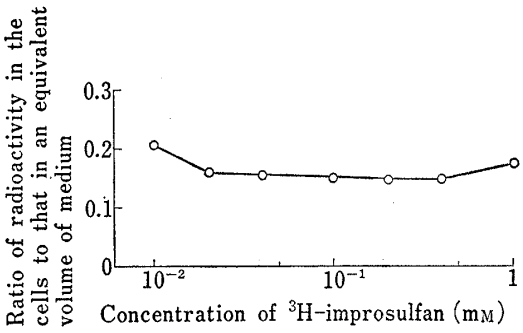

Fig. 3. Uptake of Improsulfan by Yoshida Sarcoma Cells

Yoshida sarcoma cells at a concentration of 4× 10^6 cells were incubated at 37° for 3 min with ^{3}H-improsulfan. The calculation of the ratio was based on an intracellular volume of 2 $mm^3/10^6$ cells. Each value represents the mean of 3 samples.

TABLE IV. Effects of Several Compounds on Improsulfan Uptake by Yoshida Sarcoma Cells

Compound	Concentration (mM)	0.01 mM ^{3}H-improsulfan		0.2 mM ^{3}H-improsulfan	
		% of control	P	% of control	P
2,4-Dinitrophenol	1	94	NS	82	NS
Potassium cyanide	5	92	NS	85	NS
Ouabain	0.5	95	NS	107	NS
Iodoacetate	1	211	<0.05	217	<0.05
Iodoacetate[a)]	1	104	NS	106	NS
Sodium fluoride	20			112	NS
N-Ethylmaleimide	1	20	<0.05	32	<0.05
N-Ethylmaleimide[b)]	1	100	NS	110	NS
L-Cysteine	0.2	108	NS	94	NS
HN_2	1	113	NS	108	NS

The tumor cells were preincubated at 37° for 60 min in Hanks' balanced salt solution containing the compounds. ^{3}H-improsulfan was added to this cell suspension and followed by incubation for 3 min at 37°. The data represent the mean of 3 samples, and were analyzed statistically by the 2-tailed t test.
NS: not significant.
a) The tumor cells preincubated with the compound were washed and then reincubated in fresh Hanks' balanced salt solution immediately before the addition of ^{3}H-improsulfan.
b) Simultaneous addition of the compound and ^{3}H-improsulfan.

13) J.H. Mulder and K.R. Harrap, *Europ. J. Cancer*, **11**, 373 (1975).

linearly with increasing drug concentration. The intracellular concentration never exceeded the concentration in the medium.

Effects of L-cysteine, HN_2 and several metabolic inhibitors on improsulfan uptake by Yoshida sarcoma cells are shown in Table IV. The tumor cells were preincubated with the test compounds at 37° for 60 min prior to the addition of ^{3}H-improsulfan, unless otherwise stated. When the tumor cells were treated with inhibitors of glycolysis, the uptake was enhanced by iodoacetate, inhibited by N-ethylmaleimide, and not affected by sodium fluoride. The reason for the enhancement by iodoacetate is not clear, but the enhancement was not observed when the tumor cells preincubated with iodoacetate were washed and reincubated in fresh medium before the addition of ^{3}H-improsulfan. Inhibition by N-ethylmaleimide could not be explained by its chemical reactivity with improsulfan, since the uptake was not inhibited by the simultaneous addition of N-ethylmaleimide and ^{3}H-improsulfan. Both iodoacetate and N-ethylmaleimide are sulfhydryl-reactive agents, but L-cysteine and HN_2 had no effect on improsulfan uptake. Dinitrophenol, potassium cyanide and ouabain had no significant inhibition of improsulfan uptake.

Discussion

It is well known that tumor cells acquire resistance to alkylating agents by repeated exposure to the chemicals. The various factors have been proposed to account for the development of drug resistance. The factors of resistance to HN_2 in HN_2-resistant Yoshida sarcoma cells were extensively examined by Inaba, *et al.*,[14–16] and the factors demonstrated in the HN_2-resistant sublines of Yoshida sarcoma used in the present study are as follows: (a) decrease of HN_2 transport which is an active process mediated by a transport carrier for choline, and (b) other mechanisms which prevent the binding of HN_2 to the cellular DNA. In the present cross-resistance studies *in vitro* and *in vivo*, HN_2-resistant Yoshida sarcoma cells with high resistance to HN_2 were highly susceptible to improsulfan, and there was no significant difference in the extent of the binding of improsulfan to DNA between the original and HN_2-resistant cells. Improsulfan uptake was also not suppressed in these resistant cells as shown previously by Inaba.[15] It is likely that the factors of resistance to HN_2 cited above do not serve as factors of resistance to improsulfan. But, the original cells were slightly more susceptible to improsulfan than HN_2-resistant cells. These results may indicate that these resistant cells have also other factors of resistance to alkylating agents. The effectiveness of improsulfan on these resistant cells suggests that tumor cells acquired resistance to an alkylating agent are not always fully cross-resistant with other alkylating agents.

On the other hand, improsulfan uptake by the original Yoshida sarcoma cells *in vitro* was temperature sensitive and was inhibited only by N-ethylmaleimide among the inhibitors of glycolysis examined. HN_2, L-cysteine and the other metabolic inhibitors used had no significant effect on improsulfan uptake. It does not exclude a possibility that inhibition of the uptake at a low temperature and by N-ethylmaleimide is due to a decrease of the chemical reactivity of improsulfan, and a competition for cellular binding sites with improsulfan and N-ethylmaleimide. If the effect of N-ethylmaleimide on improsulfan uptake is due to the competition, no inhibition of the uptake by two other sulfhydryl-reactive agents HN_2 and iodoacetate may be attributed to differences in the amount or type of cellular binding sites for these two agents and N-ethylmaleimide. The radioactivity of ^{3}H-improsulfan bound to the cell membrane at 37° was not assayed in the present study, the ratio of radioactivity

14) M. Inaba and Y. Sakurai, *Int. J. Cancer*, **7**, 430 (1971).
15) M. Inaba, *Int. J. Cancer*, **11**, 231 (1973).
16) M. Inaba and Y. Sakurai, The 30th Annual Meeting of the Japanese Cancer Association, Tokyo, 1971.

in the tumor cells to that in an equivalent volume of medium was approximately constant and improsulfan did not accumulate in the tumor cells at a dose range of 0.01 to 1 mM. These results suggest that improsulfan uptake is non-active by Yoshida sarcoma cells, although they can not discriminate between a carrier-mediated process and a simple diffusion process.

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