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Inhibitory Effects of Four Isoabrins on the Growth of Sarcoma 180 Cells

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ABSTRACT

The four isoabrins were shown to be capable of inhibiting the growth of tumor cells *in vivo* when one-fifth of their median lethal dose was used. From the *in vitro* experiments, the doses required for 50% inhibition of protein biosynthesis are 3.2 pg, 45 ng, 32 ng, and 10 ng/ml for abrin-a, -b, -c, and -d, respectively. Except for abrin-b, a good correlation between the inhibitory effects of abrins on the tumor growth and protein biosynthesis was observed. These isoabrins show a moderate inhibitory effect on DNA biosynthesis.

INTRODUCTION

Abrin, a highly toxic protein in the seeds of *Abrus precatorius*, was found to have a strong inhibitory effect on the growth of Ehrlich ascites tumor cells by inhibiting protein biosynthesis (5, 11, 16). In addition, several laboratories showed that abrin was capable of preventing the growth of Yoshida sarcoma cells (3), leukemia cells (17), and human cancer cells (2, 4). The toxic protein was found to consist of subunits A and B, which were linked by a disulfide bond (15). Subunit B was able to carry the toxin and bind on cell surface receptors (14), and Subunit A then penetrated into the cytoplasm and exerted protein biosynthesis inhibition by inactivating the 60S ribosomal subunit (18).

In the previous investigation, the abrin, isolated early from the jequiriti bean (9, 15), was further separated into 2 abrins (8, 19). Recently, 4 isoabrins (abrin-a, -b, -c, and -d) were purified from the seeds of *Abrus precatorius*, and their physicochemical properties were characterized in our laboratory (7). It is the purpose of the present investigation to compare their antitumor activities and biological properties in order to better understand the nature of the cancerostatic properties of abrins.

MATERIALS AND METHODS

Materials

[*methyl*-^{3}H]Thymidine (specific activity, 2 Ci/mmol) and L-[4,5-^{3}H] leucine (specific activity, 67 Ci/mol) were obtained from New England Nuclear, Boston, Mass. GF/A glass microfiber paper was purchased from Whatman Ltd., England. Roswell Park Memorial Institute Tissue Culture Medium 1640 and minimum essential medium (Eagle) without leucine were obtained from Grand Island Biological Co., Grand Island, N.Y.

Animals and Tumors

Male noninbred N:NIH(S) white male mice and Sarcoma 180 tumor cells were used throughout the present experiments. The Sarcoma 180 tumor cells were maintained by serial i.p. transplantation into mice weighing 20 ± 2 g. After 0.3 ml of ascites fluid containing 3×10^7 tumor cells was injected i.p. into mice, the ascites were formed within 10 days. The ascites fluid containing 10^8 cells/ml on the seventh to ninth days was used for these experiments.

Antitumor Tests

***In Vivo* Treatment.** Ascites fluid (0.3 ml) was inoculated i.p. into mice weighing 20 ± 2 g, and 1 to 2 hr after tumor transplantation, one-fifth of median lethal dose of isoabrins was injected i.p. The growth of tumor cells was evaluated by determining the life span. The survival values do not include those long-term survivors during 60-day observation.

***In Vitro* Treatment.** Tumor cells in ascites fluid were washed 3 times and diluted to a final concentration of 3×10^7 cells/ml with phosphate-buffered saline (0.01 M phosphate buffer, pH 7.4, containing 0.14 M NaCl). An equal volume of various amounts of isoabrins in phosphate-buffered saline was added, and the reaction mixtures were incubated at 37° for 30 min. The treated tumor cells were then washed twice and resuspended in phosphate-buffered saline to the concentration of 4×10^7 cells/ml. 0.5 ml of the cell suspension was inoculated i.p. into mice.

Test of Protein Biosynthesis Inhibition

Experiment 1. The washed tumor cells were suspended in leucine-free minimum essential medium containing 10% calf serum to a final concentration of 5×10^6 cells/ml. After the tumor cell suspension was mixed with an equal volume of abrins at the dose of 2 μg/ml, [^{3}H]-leucine was added immediately at the concentration of 1 μCi/ml. The reaction mixtures were incubated at 37° in a shaking water bath. After various periods of time, an aliquot (0.4 ml) of reaction mixture was removed, added into 1 ml of 0.1 N KOH, and incubated at room temperature for 30 min. Cold trichloroacetic acid was then added to a final concentration of 10% (w/w). The precipitated materials were collected on glass microfiber paper, washed twice with 5% cold trichloroacetic acid and once with absolute ethanol, and air dried. The leucine incorporation was obtained by measuring the radioactivity with a Beckman LS250 scintillation counter.

Experiment 2. The washed tumor cells were suspended in leucine-free medium and preincubated with isoabrins at various concentrations at 37° for 30 min. Then 50 μl of [^{3}H]leucine (μCi/ml) were added to a reaction mixture (0.4 ml) containing 1.5×10^6 cells and further incubated at 37° for 1 hr. The reaction was stopped by addition of KOH, and the reaction mixture was treated by the procedures as described in Experiment 1.

Experiment 3. The washed tumor cells were mixed with an equal volume of isoabrins (abrin-a, 2 ng/ml; abrin-b, -c, and -d, 2 μg/ml) and incubated at 37°. At various incubation times, an aliquot of reaction mixture (0.4 ml) was removed and the tumor cells were washed twice with 3 ml of phosphate-buffered saline containing 0.01 M galactose. The treated cells were resuspended in 0.4 ml of leucine-free incubation medium, and after the addition of 50 μl of [^{3}H]leucine (10 μCi/ml), the reaction mixture was incubated continuously at 37° for 1 hr. The preparation of samples for measuring the degree of [^{3}H]leucine incorporation was the same as described in Experiment 1.

Test of DNA Biosynthesis Inhibition

The experimental procedures were similar to those of Experiment 1 for testing the inhibition of protein biosynthesis, but [^{3}H]leucine and leucine-free medium were replaced by [^{3}H]thymidine and Roswell Park Memorial Institute Tissue Culture Medium 1640, respectively.

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RESULTS

Effect of Isoabrins on the Growth of Sarcoma 180 Cells

The growth of Sarcoma 180 cells was inhibited significantly in the isoabrin-treated mice, as shown in Table 1. The life span of the treated groups was also increased from 71% to more than 400%. Except for abrin-b, the long-term survival mice were observed in the treated groups.

The life spans of the mice that received isoabrin-pretreated tumor cells are given in Table 2. The results showed that the dosage of abrin-b required for the inhibition of tumor growth at the experimental conditions was much higher than that of others. Although the *in vivo* host toxicity of abrin-a was not much higher than that of other abrins (7), the inhibitory activity of abrin-a on the growth of tumor cells *in vitro* was much stronger than that of others. The concentration of abrin-a for complete inhibition of tumor growth was about one-thousandth of the concentration of other isoabrins required for the inhibition of tumor growth.

Table 1

Effect of isoabrins on the survival time of mice inoculated with Sarcoma 180 cells[a]

Agent	Dose injected (μg)	No. of mice	Life span (days)	No. of long-term survivors[b]
None		8	16.1 ± 1.6[c]	0
Abrin-a	0.04	7	34.2 ± 14.2[d](111)[e]	3
Abrin-b	0.10	5	32.6 ± 12.8[d](102)	0
Abrin-c	0.06	7	43.0 ± 8.2[f](167)	4
Abrin-d	0.12	4	27.6 ± 13.2[g] (71)	1

[a] The survival values do not include long-term survivors during 60-day observation.
[b] Increase in life span of more than 400% in comparison to untreated group.
[c] Mean ± S.E.
[d] Significantly different by analysis of variance (ANOVA) and least significant difference analysis ($p < 0.01$).
[e] Numbers in parentheses, percentage of increase.
[f] Significantly different ($p < 0.001$).
[g] Significantly different ($p < 0.05$).

Table 2

The survival time of mice inoculated with Sarcoma 180 cells pretreated with various concentrations of isoabrins in vitro[a]

Agents	No. of mice	Survival time (days)	Increase in life span (%)
None	10	15.1 ± 2.6[b]	
Abrin-a			
0.2 ng/ml	3	All survived	
0.02 ng/ml	3	All survived	
0.002 ng/ml	3	29.5 ± 9.5[c], 1 survived	95
0.0002 ng/ml	3	23.7 ± 1.5[d]	57
Abrin-b			
2.0 μg/ml	3	All survived	
0.2 μg/ml	4	21.8 ± 4.3[d]	44
0.02 μg/ml	6	16.5 ± 4.0	
0.002 μg/ml	3	16.0 ± 2.6	
Abrin-c			
0.2 μg/ml	3	All survived	
0.02 μg/ml	6	25.8 ± 2.2[c], 2 survived	71
0.002 μg/ml	3	20.0 ± 2.0[e]	32
0.0002 μg/ml	3	14.0 ± 0.0	
Abrin-d			
0.2 μg/ml	3	All survived	
0.02 μg/ml	6	31.5 ± 0.7[c], 4 survived	108
0.002 μg/ml	3	18.1 ± 1.7	
0.0002 μg/ml	3	18.3 ± 4.5	

[a] The pretreated tumor cells (2×10^7) were suspended in 0.5 ml of phosphate-buffered saline and injected i.p.
[b] Mean ± S.E.
[c] Significantly different by analysis of variance (ANOVA) and least significant difference analysis ($p < 0.001$).
[d] Significantly different ($p < 0.01$).
[e] Significantly different ($p < 0.05$).

Test of Protein Biosynthesis Inhibition

Experiment 1. As shown in Chart 1, when 1 μg of abrins per ml was added to a suspension of tumor cells, the incorporation of [^{3}H]leucine was strongly inhibited after a lag period of about 30 min.

Experiment 2. From the results of Chart 2, the doses required for 50% inhibition of protein biosynthesis were calculated to be 3.2×10^{-3}, 45, 32 and 10 ng/ml for abrin-a, -b, -c, and -d, respectively. It was also found that, even at a concentration as high as 100 μg/ml. *Abrus* agglutinin, another lectin extracted from the jequiriti bean (7), did not inhibit the protein biosynthesis of tumor cells. It was coincident with the fact that *Abrus* agglutinin was nontoxic to mice (7).

Experiment 3. The results, shown in Chart 3, indicated that isoabrin-mediated inhibition of cellular protein biosynthesis was dependent on the preincubation time. At least 15 min of preincubation was needed for the inhibition of leucine incorporation caused by abrin-a and -d in tumor cells. The inhibitory effect of abrin-b and -c was gradually increased, and the maximum inhibition was reached after 60 min of incubation.

Test of DNA Biosynthesis Inhibition

As shown in Chart 4, the incorporation of labeled thymidine was slightly inhibited by the isoabrins at the concentration of 1

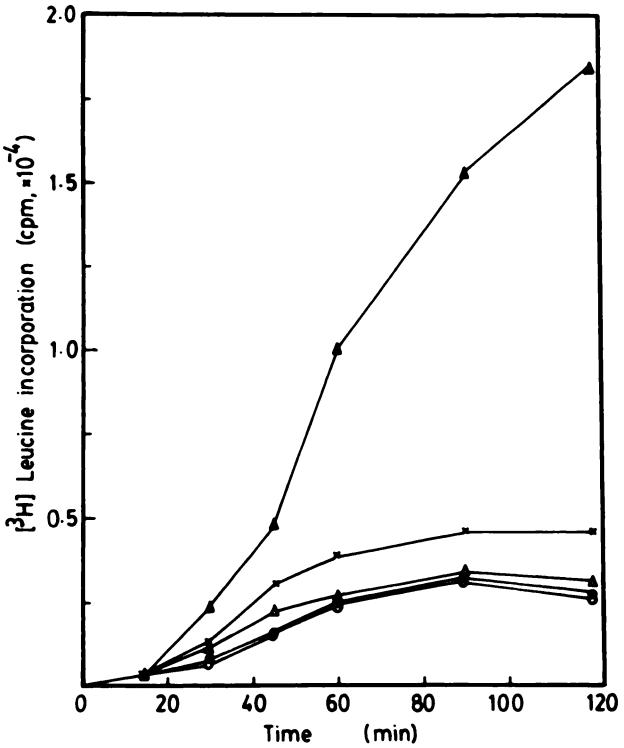

Chart 1. The effect of abrin-a, -b, -c, and -d on the protein biosynthesis of tumor cells. The reaction mixture (0.4 ml) containing tumor cells (1×10^6), toxin (1 μg/ml), and [^{3}H]leucine (1 μCi/ml) was incubated at 37°. At the time indicated, the acid-precipitable radioactivity was measured. ▲, control; ●, abrin-a; ×, abrin-b; △, abrin-c; ○, abrin-d.

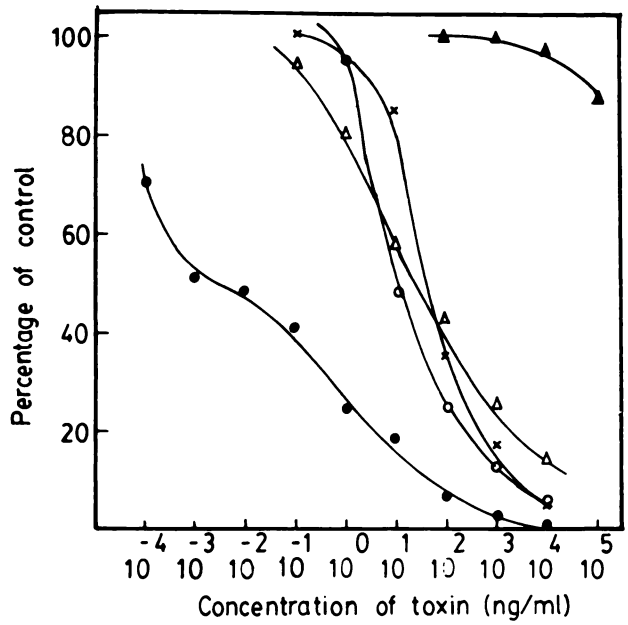

Chart 2. The effects of various amounts of abrin-a, -b, -c, and -d and *Abrus* agglutinin on the protein biosynthesis of tumor cells. After the tumor cells were preincubated with various amounts of lectins for 30 min at 37°, 0.5 μCi of [^{3}H]leucine was added. The incubation was carried out for another hr, and the acid-precipitable radioactivity was measured. The radioactivity of the control was measured to be 20,000 cpm. ●, abrin-a; ×, abrin-b; △, abrin-c; ○, abrin-d; ▲, *Abrus* agglutinin.

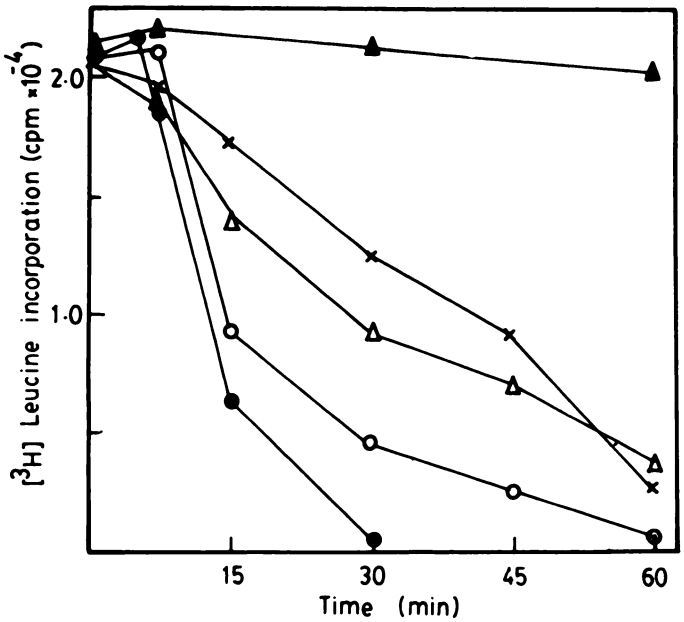

Chart 3. The effect of preincubation time of isoabrins with tumor cells on the inhibition of protein biosynthesis. The tumor cells were treated with isoabrins for various times, and at the end of each incubation, they were washed with phosphate-buffered saline containing 10 mM galactose. The incorporation of leucine was determined by addition of 0.5 μCi of [^{3}H]leucine and incubation at 37° for 1 hr. ▲, control; ●, 2 ng of abrin-a; ×, 2 μg of abrin-b; △, 2 μg of abrin-c; ○, 2 μg of abrin-d.

μg/ml, and the lag period was about 1 hr which was longer than that for the inhibition of leucine incorporation. The extent of inhibition of abrin-a was 40% at 2 hr of incubation, while that of abrin-b, -c, and -d was about 20%.

DISCUSSION

Although certain lectins have tumor-supressive and differential toxic effects on transformed cells *in vivo* (12, 13), the killing mechanisms are in most cases not known completely. In this report, we found that all 4 isoabrins, at the dosage of one-fifth of their medium lethal doses, were able to inhibit the tumor growth *in vivo* (Table 1). For comparative study of the inhibitory effects of isoabrins on the tumor cells, the *in vitro* experiments were then carried out. The results shown in Charts 1 and 4 indicate that the effects of 4 isoabrins on the protein and DNA biosynthesis are similar to those reported by Lin *et al.* (6) and Olsnes *et al.* (16). Since the inhibition of DNA biosynthesis is less profound and much slower than that of protein biosynthesis, it indicates that the primary effect of isoabrins is probably on the translational level in the cell. However, it is possible that isoabrins may act on the DNA polymerase directly, since it has been shown that several lectins are able to specifically inhibit the DNA polymerase in the cell-free system of human neuroblastoma cells (1).

The results shown in Table 2 and Chart 2 indicate that the inhibitory effects of isoabrins on the tumor growth *in vitro* were closely correlated to those on the protein biosynthesis. It is interesting to note that abrin-a is the most potent inhibitor of tumor growth and protein biosynthesis among the isoabrins, and further investigation is required to understand these phenomena. The low efficiency of abrin-b on the inhibition of tumor growth *in vitro* may be due to its instability; it has been noted that, after storage at physiological pH and 4°, abrin-b tends to denature and precipitate within 2 to 3 days, while other isoabrins can be stored much longer under the same conditions. Since a 2-step model has been proposed to elucidate the entry of abrin and ricin into cells (16), the differences in the degree of inhibition of protein biosynthesis and tumor growth among the isoabrins also may be due to their different binding capacities, penetrating rates, and inhibiting potencies. As shown in

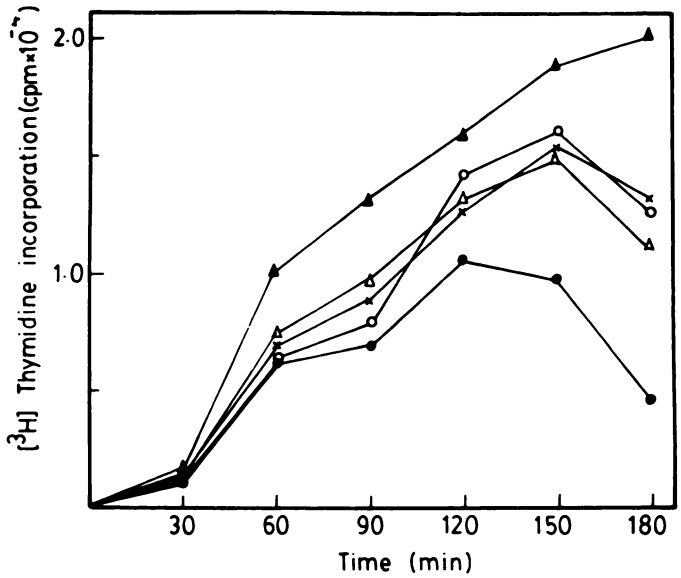

Chart 4. The effect of abrin-a, -b, -c, and -d on the DNA biosynthesis of tumor cells. The reaction mixture (0.4 ml) containing tumor cells (1 × 10^6), toxin (1 μg/ml), and [^{3}H]thymidine (2.5 μCi/ml) was incubated at 37°. At the time indicated, the acid-precipitable radioactivity was measured. ▲, control; ●, abrin-a; ×, abrin-b; △, abrin-c; ○, abrin-d.

Chart 3, the [^{3}H]leucine incorporation of the tumor cells, which were preincubated with abrin-a or -d for 30 min, is completely inhibited; however, under the same conditions, the tumor cells pretreated with abrin-b or -c still possess 70% of their ability to incorporate [^{3}H]leucine. These results indicate that the reaction rate of abrin-b and -c is much slower than that of abrin-a and -d. This may be due to the fact that both abrin-a and -d bind specifically to galactose residues of membrane receptors and possess a stronger binding affinity and a faster penetrating rate.

Since abrin did not interfere primarily with the synthesis of nucleic acids and depressed the level of peripheral leukocytes, it was suggested by Fostad *et al.* (2) that abrin might be a useful agent, particularly in combination with other antitumor agents, in treatment of slowly growing human cancer. In addition, it has been demonstrated recently that the antitumor activity of methotrexate and chlorambucil increased when they were covalently linked to abrin or *Abrus* agglutinin (10). Thus, utilizing the selectivity of lectin to tumor cells is a permissive approach to improve the efficacy of antitumor drugs.

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