# ANTITUMOR PROTECTIVE PROPERTY OF AN ISOPRENOID ANTIBIOTIC, ASCOFURANONE

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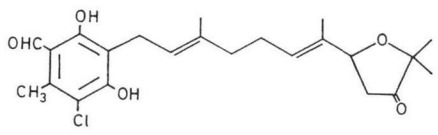
Ascofuranone (AF) showed an antitumor protective effect on L-1210 leukemia when AF was administered once 7 days before tumor challenge. However, effect was not elicited when host mice were treated with AF simultaneously with tumor challenge. AF pretreatment on day 7, 5 and 3 before tumor challenge protected the host from the ascites form of S-180. AF also retarded tumor growth when administered once daily for 5 consecutive days 24 hours after transplantation, but antitumor effect was not seen with combined treatments before and after the transplantation. Similar results were noted with Ehrlich ascites carcinoma.

AF treatment of normal mice enlarged the solid lymphoid organs without affecting body weight gain. The splenocytes derived from AF-treated mice lowered mitogenic response to phytohemagglutinin, while the mitogenic response to concanavalin A and lipopolysaccharide was unaffected.

Ascofuranone (AF, Fig. 1) is an isoprenoid antibiotic produced by a phytopathogenic fungus, *Ascochyta visiae*. The present authors have reported elsewhere that AF affected lipid metabolism in rats by reducing serum lipids.[2,3] Our previous study led us to investigate how tumor growth was affected by alteration in lipid metabolism caused by AF.

We are reporting the antitumor protective effect of AF using a mouse syngeneic tumor, L-1210 and the allogenic tumors sarcoma-180 (S-180) and Ehrlich carcinoma. The effects of AF on organ weights and mitogenic responses are also presented in this paper.

Fig. 1. Structure of ascofuranone.




## Materials and Methods

### Animals

6 Week-old female $BDF_1$ and ICR mice were purchased from Japan Charles River Co. and 6 week-old male *dd*Y mice, from Shizuoka Agricultural Cooperative Association for Laboratory Animals. They were given a commercial pellet diet (Nihon Clea, grade CE-2) and tap water *ad libitum*.

### Chemicals

AF used in this study was in the form of micronized crystalline powder (diameter 2 $\mu$m) with a purity above 99%. Mitomycin C (MMC) was obtained from Kyowa Hakko Co. and mitogens, phytohemagglutinin (PHA) from Wellcome (HA 16/17; purified phytohemagglutinin), concanavalin A (con A) from Sigma (C2010; type 4, highly purified) and lipopolysaccharide (LPS) from Sigma (L4505; *Salmonella enteritidis*). Other chemicals were of the highest grade in purity. For intraperitoneal injection (i.p.) AF was suspended in the vehicle indicated in the legend with the aid of a Tefron homogenizer because of its insolubility in water. The control mice received only the vehicles. The injection volume was 0.2 ml/mouse. For an *in vitro* study, AF was dissolved in methanol and added to the incubation medium.

Tumors

Ascites forms of L-1210, S-180 and Ehrlich carcinoma were used for the evaluation of antitumor property.

Mitogen Response[4]

6 Week-old female $BDF_1$ mice (n=2), were administered i.p. with AF three times on day 7, 5 and 3 before assay. The mice were sacrificed by cervical dislocation and the spleens were removed. The spleens were placed in a Petri dish containing 5 ml of Eagle essential medium (MEM, Nissui Seiyaku Co.) and teased with the flat bottom of a plastic syringe. The cells were treated with tris-ammonium chloride to remove erythrocytes[5] and the splenocytes obtained were suspended in RPMI-1640 medium (Nissui Seiyaku Co.) supplemented with 10% fetal calf serum (Flow Laboratories, FCS). The splenocyte suspension ($2\times10^6$ cells/ml), 0.1 ml, was put into the wells of a plastic plate and diluted with RPMI-1640 (0.1 ml) containing mitogens. The splenocytes were cultured at 37°C for 3 days on a $CO_2$ incubator and then [$^3$H]thymidine (2 $\mu$Ci/ml) in 50 $\mu$l RPMI-1640 medium was added. They were incubated at 37°C for additional 6 hours and filtered. After washing with water, the filters were dried and the radioactivity counted with a scintillation counter.

## Results

### Protective Effect of AF on L-1210 Leukemia

L-1210 leukemia was so malignant that inoculation of $10^2$ cells was sufficient to kill all mice within 16 days. When AF, 400 mg/kg, was injected once i.p. into the hosts 7 days before the tumor challenge, one half of the treated mice in experiment 1 and 5 out of 12 in experiment 2 survived longer than 60 days (Table 1). The protective effect was dependent on the inoculum size, since the effect was no longer noted with inoculum of over $10^3$ cells/mouse.

A number of investigators have pointed out the importance of timing in studying the protective effect of cytotoxic antitumor agents on rodent tumors.[6~9] Therefore, the protective effect was compared between treatments before and simultaneous with the tumor challenge. As shown in Table 2, the effect was dependent on the timing of

Table 1. Antitumor protective activity of AF against leukemia L-1210.

| Tumor cells inoculated (cells/mouse) | Groups | 60-Day survivors[a] | |
|---|---|---|---|
| | | Experiment 1 | Experiment 2 |
| $10^2$ | Control | 0 / 6 | 0 / 12 |
| | AF | 3 / 6* | 5 / 12* |
| $10^3$ | Control | 0 / 6 | 0 / 12 |
| | AF | 1 / 6 | 1 / 12 |
| $10^4$ | Control | 0 / 6 | 0 / 12 |
| | AF | 0 / 6 | 0 / 12 |

[a]) Survival/total. * $p<0.05$ in $\chi^2$ test.

6 Week-old $BDF_1$ female mice were used. AF was suspended in 1% tragacanth gum and injected i.p. 7 days prior to i.p. challenge with L-1210 cells. The injection volume was 0.2 ml/mouse. The control mice received 0.2 ml of 1% tragacanth gum i.p.

Table 2. Effect of dose-schedule on the antitumor protective property of AF.

| Groups | Schedule (on day) | Life span[a] (days) | 60-Day survivors |
|---|---|---|---|
| Experiment 1 | | | |
| Control | — | 13.4±0.37 | 0 |
| AF | −8, −4 | 13.3±0.44 | 4* |
| | 0 | 13.4±0.68 | 0 |
| Experiment 2 | | | |
| Control | — | 13.4±0.74 | 0 |
| AF | −8, −4 | 14.1±2.47 | 5* |
| | 0 | 14.5±1.22 | 0 |

[a]) mean±SD. * $p<0.05$ in $\chi^2$ test.

5 Week-old female $BDF_1$ mice were used (n=20). AF, 400 mg/kg, was suspended in 1% tragacanth gum and injected i.p. either 8 and 4 days before, or simultaneously with the tumor cell challenge. On day 0, L-1210 cells ($10^2$ cells/mouse) were inoculated i.p. The control mice were injected i.p. with 1% tragacanth gum three times on days 8, 4 before and simultaneously with tumor cell challenge.

doses; simultaneous treatment showed little effect while some pretreated mice survived.

Antitumor Protective Effect against S-180 and Ehrlich Carcinoma

These tumors were less malignant than L-1210. AF again suppressed the growth of S-180 when injected i.p. on day 7, 5 and 3 before tumor challenge (Table 3, Experiments 2 and 4). This protective effect depended on dose; AF was ineffective at doses below 100 mg/kg. AF retarded tumor growth when treatment was initiated 24 hours after tumor transplantation once daily, i.p., for 5 consecutive days. However, MMC was more effective than AF (Table 3, Experiment 1). When efficacy was compared

Table 3. Antitumor activity of AF against S-180.

| Experiment No. | Groups | Schedule (on day) | Life span[a)] (days) | T/C (%) | 60-Day survivors |
|---|---|---|---|---|---|
| 1 | Control | +1 ~ +5 | 20.6± 3.48 | 100 | 0 / 30 |
| | AF 300 mg/kg | | 36.3±13.78** | 176 | 6 / 30 |
| | MMC 1 mg/kg | | 40.7±11.36** | 198 | 4 / 10 |
| 2 | Control | −7, −5, −3 | 19.7± 2.65 | 100 | 0 / 10 |
| | AF 300 mg/kg | | 43.7±16.11** | 218 | 4 / 10 |
| 3 | Control | −7, −5, −3, +1, +3 | 24.7± 3.95 | 100 | 0 / 10 |
| | AF 300 mg/kg | | 33.7±16.11* | 148 | 2 / 10 |
| 4 | Control | −7, −5, −3 | 21.1± 2.95 | 100 | 0 / 8 |
| | AF 25 mg/kg | | 18.9± 4.14 | 90 | 0 / 8 |
| | 50 mg/kg | | 20.8± 3.39 | 99 | 0 / 8 |
| | 100 mg/kg | | 21.5± 3.96 | 102 | 0 / 8 |
| | 200 mg/kg | | 36.0±19.07* | 171 | 2 / 8 |
| | 400 mg/kg | | 48.0±13.39** | 227 | 3 / 8 |

a) mean±SD. *$p<0.05$ and **$p<0.01$ in Student's t-test.

AF was suspended in PBS(−) containing 0.5% Tween-80 and the suspension were i.p. injected according to the indicated schedule. 6 Week-old female ICR mice were used throughout this study. MMC was dissolved in 0.85% saline and i.p. injected.

On day 0, S-180 cells ($2\times10^6$ cells/mouse) were intraperitoneally implanted into the mice.

Table 4. Antitumor activity of AF against Ehrlich ascites carcinoma.

| Experiment No. | Groups | Schedule (on day) | Life span[a)] (days) | T/C (%) | 60-Day survivors |
|---|---|---|---|---|---|
| 1 | Control | −7, −5, −3 | 18.4± 1.85 | 100 | 0 / 8 |
| | AF 25 mg/kg | | 18.5± 3.55 | 101 | 0 / 8 |
| | 50 mg/kg | | 19.8± 1.28 | 108 | 0 / 8 |
| | 100 mg/kg | | 27.0±18.48 | 147 | 1 / 8 |
| | 200 mg/kg | | 34.2±20.04 | 185 | 2 / 8 |
| | 400 mg/kg | | 23.0± 5.29 | 125 | 0 / 8 |
| 2 | Control | +1 ~ +5 | 18.8± 2.49 | 100 | 0 / 29 |
| | AF 300 mg/kg | | 26.2±10.31** | 139 | 1 / 30 |
| | MMC 1 mg/kg | | 45.6±15.49** | 243 | 4 / 10 |

a) mean±SD. *$p<0.05$ and **$p<0.01$ in Student's t-test.

6 Week-old female *ddY* mice were used. Ehrlich ascites carcinoma cells ($1\times10^6$ cells/mouse) were implanted i.p. on day 0. The agents were injected i.p. according to the indicated schedules. AF was suspended in PBS (−) containing 0.5% Tween-80 and MMC was dissolved in 0.85% saline. The control mice were injected i.p. with PBS (−) containing 0.5% Tween-80.

between treatments before and after challenge, the former appeared to be more effective than the latter, and no additional effect was noted with combined treatment before and after challenge (Table 3, Experiment 3).

A similar antitumor protective effect of AF was noted against Ehrlich carcinoma (Table 4). AF was less effective against Ehrlich carcinoma than against S-180. The optimal dose of MMC again produced the best result in this experiment.

## Tumor Rechallenge of 60-Day Survivors

The protective effect of AF on experimental tumors suggests that AF enhances the resistance of hosts to tumor transplantation. Therefore, experiments were performed to determine whether the survivors acquired specific transplantation resistance to the tumor with which they were first challenged (Table 5).

Table 5. Tumor rechallenge of 60-day survivors.

| Tumors | Groups | n | Survivors |
|---|---|---|---|
| L-1210 | Control | 30 | 0 |
| | Survivors | 22 | 0 |
| S-180 | Control | 26 | 0 |
| | Survivors | 24 | 11* |

* $p<0.01$ in $\chi^2$ test.

AF treated 60-day survivors were rechallenged i.p. with their cured tumors. Inoculum sizes were $10^2$ cells/mouse for L-1210 and $10^6$ cells/mouse for S-180. Age-matched mice were used as controls.

Table 6. Effect of AF on weights of solid lymphoid organs.

| | Control | AF |
|---|---|---|
| Body weight gain (g/mouse/week) | 4.2±0.71 | 4.4±0.38 |
| Thymus (mg/mouse) | 65±5.4 | 77±10.0* (118%) |
| Spleen (mg/mouse) | 118±28.6 | 164±2.63* (139%) |

* $p<0.05$ in Student's t-test. (n=5).

6 Week-old male *dd*Y mice were used. AF was suspended in PBS (−) containing 0.5% Tween-80 and AF, 300 mg/kg, was injected i.p. on days 5, 4 and 3 before sacrifice. The control mice were injected i.p. only the vehicle. The figures represent the mean±SD.

Table 7. Lowered mitogenic response to PHA in the splenocytes derived from AF-treated mice.

| AF dosed (mg/kg) | Mitogens (μg/ml) | [$^3$H]Thymidine incorporated (cpm) | Activity (%) |
|---|---|---|---|
| 0 | None | 1,174± 481 | 100 |
| 100 | | 1,254± 725 | 107 |
| 400 | | 782± 43 | 67 |
| 0 | con A 2.0 | 49,574±4,039 | 100 |
| 100 | | 48,314±3,234 | 97 |
| 400 | | 41,763±1,573 | 84 |
| 0 | PHA 2.0 | 6,131±1,200 | 100 |
| 100 | | 4,739±1,453 | 77 |
| 400 | | 3,054± 279* | 50 |
| 0 | LPS 10.0 | 24,248±2,614 | 100 |
| 100 | | 22,849± 126 | 94 |
| 400 | | 26,000±1,629 | 107 |

* $p<0.02$ (Students t-test; n=3). Figures represent the mean±SD.

Female $BDF_1$ mice (n=2) were used. AF was injected i.p. on days 7, 5 and 3 before assay. The splenocytes were incubated in the presence or absence of mitogens at 37°C for 3 days in a $CO_2$ incubator. Then, [$^3$H]thymidine was added and the cultures were incubated for an additional 6 hours. The cells were harvested and the [$^3$H]thymidine incorporated was counted.

The survivors of L-1210 demonstrated the same life span as age-matched controls after rechallenge with $10^2$ L-1210 cells. Eleven of 24 mice which had survived the first challenge with S-180 rejected S-180 when rechallenged.

### Effect of AF on Immune Organ Weights

The above result indicated that AF did not always endow 60-day survivors with specific transplantation resistance to the first-challenge tumors. Thus the effect on solid lymphoid organs was examined using normal mice. As shown in Table 6, treatment caused enlargement of spleen and thymus without affecting body weight gain.

### Effect of AF on Mitogenic Response

The splenomegaloic effect of AF suggested the possibility that AF modulated the mitogenic response of splenocytes *in vivo*. AF was administered i.p. three times on day 7, 5 and 3 before sacrifice and the mitogenic response of splenocytes examined (Table 7). AF treatment resulted in a slight decrease of [$^3$H]-thymidine incorporation in the absence of mitogens but had virtually no effect on the mitogenic responses to con A and LPS. However, the PHA response was dose-dependently decreased in the splenocytes.

## Discussion

It is generally accepted that the host immune system rejects with difficulty malignant leukemia L-1210 because of its close similarity in antigenicity. KOSHIMURA *et al.*, have shown that some immunopotentiators endowed $BDF_1$ mice with the ability to reject $10^2 \sim 10^3$ L-1210 cells, when they were previously immunized with large numbers of MMC-treated L-1210 cells.[10] KATAOKA *et al.*, have demonstrated also that the combination of immunopotentiators with glutaraldehyde-treated con A-bound L-1210 cells (L-1210 vaccine) efficiently protected $BDF_1$ mice from tumor death.[11] These protective effects were tumor-specific, since the combined treatments failed to protect the hosts from P-388 leukemia. Consequently, it was shown that a suitable combinations of immunopotentiators with vaccination endow the host specific tumor immunity leading to rejection of transplanted L-1210 cells.

However, the present study clearly indicated that a single AF treatment gives the host the capability to resist tumor growth and led to a complete regression at significant rates. It is likely that the mechanism of action is host-mediated, but in contrast with combined treatments of immunopotentiators with vaccination, tumor protection by AF showed no need of vaccination. Moreover, 60-day survivors treated with these immunopotentiators acquired transplantation resistance to their cured tumors whereas those treated with AF did not.

The protective effect of AF was manifested again against S-180 and Ehrlich carcinoma. Although these tumors were less malignant than L-1210, a protective effect was noted using larger inoculum sizes than those for L-1210. Therefore, it seems that AF treatment before tumor challenge potentiates host resistance to tumor transplantation. However, the mechanism of host resistance by AF is still obscure. The fact that AF enlarged spleen and thymus in mice suggests the involvement of the immune system in its protective effect. Natural killer cells are the most plausible candidates for activation by AF. The effect of AF on natural killer activity will be presented in a subsequent paper. If this is the case, AF could induce interferon. In addition, AF might have some effect on a metastasis model for NK cells have been reported to concern the inhibition of metastasis.[12] These possibilities are now under investigation.

Both host-mediated antitumor effect and splenomegaloic effect shown by AF suggest that the treatment modulates mitogenic responses of splenocytes, because it is believed that they occur through blastgenesis of lymphocytes similar to the mitogenic responses. Unexpectedly, AF treatment suppressed the PHA response to mouse splenocytes, although it showed no effect on blastgenesis of tumoricidal effectors such as macrophages and NK cells. It is likely that host-mediated antitumor effect is due to inhibition of immunosuppressive mechanisms necessary for tumor growth. However, the relationship between

PHA response and blastgenesis of suppressor cells is unknown.

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