BIOLOGICAL STUDIES ON FORMYCIN AND FORMYCIN B

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The antitumor effect, distribution among various organs and toxicity of formycin and formycin B are studied. Formycin inhibits of EHRLICH carcinoma and L-1210, with stronger activity on the solid form than on the ascites form. Formycin B shows no inhibition of EHRLICH carcinoma or L-1210. The distribution among various organs of mice was examined after subcutaneous injection with ^{3}H-formycin or ^{3}H-formycin B. On the basis of radioactivity, formycin shows high concentrations in spleen and kidney, and formycin B is found in peritoneum, urinary bladder, kidney and spleen. By testing the effect on *X. oryzae*, low antibacterial activity was shown in various organs. When the lethal dose of formycin was injected in dogs, liver damage was found. Formycin B gives a marked reduction in white blood cells, though this reduction is recovered.

Formycin inhibits EHRLICH carcinoma in mice and mouse leukemia L-1210, but formycin B does not show any inhibition of these animal tumors. The toxicity of formycin to mice is relatively slight and that of formycin B is extremely small. Both formycin and formycin B inhibit multiplication of influenza A_1 virus in cells of chick chorioallantoic membrane[1,2]. Thus, formycin has shown interesting antitumor activity, and formycin B antiviral effects.

As shown in a paper reporting the antitumor effect of bleomycin A[3,4], information of distribution of an antitumor substance in various organs is helpful in finding sensitive tumors. Therefore, the distribution of formycin and formycin B in various organs was examined.

In this paper, the antitumor effect, distribution in various organs and toxicities of formycin and formycin B are reported.

Materials

Formycin and ^{3}H-formycin: Formycin was prepared by Meiji Seika Co. and recrystallized three times from water. It was tritiated by Dr. KOMAI, Isotope Division in National Institute of Health, Tokyo. ^{3}H-Formycin was diluted two times with cold formycin before use. The radioactivity of ^{3}H-formycin employed was 8.31×10^6 cpm/mg. Formycin B was obtained by chemical deamination of formycin and was tritiated by Dr. KOMAI. ^{3}H-Formycin B thus obtained was diluted two times with cold formycin B and used at 9.30×10^6 cmp/mg.

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Results

The Effect on EHRLICH Carcinoma

The effect on the ascites type and the subcutaneous solid form was examined. Two million tumor cells were intraperitoneally inoculated in mice (dd/Y-F strain) and 2 hours thereafter the first intraperitoneal injection of formycin was given. Thereafter the same dose was injected intraperitoneally daily. A total of 10 injections were given. The control mice died in 16~18 days after inoculation of the tumor cells. A daily dose of 40 mg/kg was lethal. Two mice died on the 3rd day after inoculation and two died on the 12th and 14th day after inoculation. A daily dose of 20 mg/kg seemed to also be toxic. All mice died 7~42 days after inoculation, but there was no increase in the ascites. A daily dose of 10 mg/kg was effective. Two mice survived 50 days after inoculation and others died 35~49 days after inoculation. A daily dose of 5 mg/kg was also effective. One survived 50 days after inoculation and others died 26~51 days after inoculation. A daily dose of 2.5 mg/kg was also effective. One survived 50 days and others died after 15~49 days. The mean survival period on various dosages was as follows: 21.2 days at 20 mg/kg, 42.5 days at 10 mg/kg, 37.5 days at 5 mg/kg, 33.5 days at 2.5 mg/kg, 15.2 days at 1.25 mg/kg, 16.7 days at 0 mg/kg. A daily dose of 1.25 mg/kg was not effective.

These results indicate that formycin inhibits of EHRLICH carcinoma, but the effect is not a strong one. Two million cells were subcutaneously inoculated to mice (dd/Y-F strain) and 24 hours thereafter the first intraperitoneal injection of formycin was performed. Thereafter, the same dose was injected intraperitoneally daily. A total of 10 injections were given. Each tumor was taken on the 15th day of treatment and weighed. The result is shown in Table 1. All mice except two daily injected with 20 mg/kg survived on the 15th day of the treatment. A daily dose of 20 mg/kg showed 88.7 % inhibition and 10 mg/kg 78.0 % inhibition of the tumor. Even the dose of 1.25 mg/kg daily showed 60.7 % inhibition. Thus the minimal effective dose for the subcutaneous solid tumor was smaller than that for the ascites form.

Table 1. Effect of formycin on subcutaneous solid type of EHRLICH carcinoma

Daily dose of formycin mg/kg	Weight of each tumor (mg)						
	20	10	5	2.5	1.25	0.625	0
1	death	300	300	500	500	790	1,100
2	death	300	400	500	600	880	1,300
3	200	400	400	600	700	1,100	1,300
4	200	400	600	650	700	1,100	1,300
5	200	490	600	700	720	1,100	1,900
6	200	580	700	700	1,000	1,200	2,800
7	270	650	700	970	1,200	1,400	2,900
8	400	700	710	980	1,200	1,400	3,000
9							3,000
10							3,100
Average	245	477	551	673	852	1,121	2,170
Inhib. %	88.7	78.0	74.6	68.9	60.7	48.3	0

2×10^6 cells of EHRLICH tumor cells were subcutaneously inoculated to mice.

The Effect on L-1210

The BDF_1 mice supplied by Dr. YAMAMOTO, the Institute of Medical Sciences, University of Tokyo were used, and 1×10^5 tumor cells were intraperitoneally inoculated. The first injection was made 2 hours after the inoculation of L-1210 cell and repeated daily. A total of 10 injections were per-

formed. Each group contained 5 mice. The mean survival time of the control was 8.6 days. A daily dose of 40 mg/kg was toxic. This dose was injected 5 times and the mean survial period was 6.0 days. The daily injection of 20 mg/kg was also toxic. This dose was injected 7 times and the mean survival period was 8.7 days. As shown in Table 2, 10 mg/kg, 5 mg/kg or 2.5 mg/kg daily was effective and the survival period was longer than that of the non-treated mice. In another experiment, a daily dose of 1.25 mg/kg showed no significant effect. A daily dose of 2.5~80 mg/kg of formycin B were inactive against the ascites form of EHRLICH carcinoma and L-1210.

Table 2. Effect of formycin against L-1210

Dose mg/kg	No. of injection	T/C survival days	T/C value %
40	5	6.0/8.6	69.7 Toxic
20	7	8.7/8.6	101.1
10	10	13.0/8.6	151.1
5	10	12.0/8.6	139.5
2.5	10	10.5/8.6	122.0
1.25	10	9.5/9.0	105.5

Treatment : given once daily for 10 days starting 2 hours after tumor cell (1×10^5 cells/mouse) inoculation.

Distribution of Formycin and Formycin B among Various Organs of Mice

Cold formycin, 1 mg, and 1 mg of ^{3}H-formycin were dissolved in 0.5 ml of distilled water; 0.25 ml of this solution were injected subcutaneously in each of two mice. In this experiment, the residual amount in the syringe was not determined. One hour after the injection, the mice were sacrificed and brain, heart, lung, spleen, liver, kidney, alimentary tract, testis, bone, muscle, skin, feces, urine and blood were taken, ground, and assayed for radioactivity and antibacterial activity to *X. oryzae*. The same experiment was done with ^{3}H-formycin B. In the case of formycin B, the residul amount in the syringe was washed out with distilled water and the radioactivity was determined. Thus, the actual amount injected was determined. The results are shown in Tables 3 and 4.

In the case of formycin B, 448 mcg was left in the syringe and the total amount injected in two mice was 1,552 mcg. The total radioactivity recovered in mice was 91 %. In the case of ^{3}H-formycin, the residual amount in the syringe was not determined, and the total radioactivity recovered was 55.64 %. The main reason for this low recovery must be the ^{3}H-formycin which remained in the syringe.

In experiments shown in Table 3, the activity on *X. oryzae* was measured by the disc method using formycin as the standard, and in Table 4 formycin B was employed as the standard. In the disc method, potencies of formycin, formycin B and oxoformycin B are as follows: If formycin is taken as the standard, formycin is 1,000 mcg (potency)/mg (weight), formycin B 1,560 mcg (potency)/mg, oxoformycin B 10 mcg (potency)/mg; if formycin B is taken as the standard, then formycin is 640 mcg (potency)/mg, formycin B 1,000 mcg (potency)/mg, oxoformycin B 6 mcg (potency)/mg.

As shown in Table 4, formycin B shown by the activity on *X. oryzae* is much less than that shown by radioactivity in various organs. It indicates that most of formycin B was converted to oxoformycin B in 1 hour after the injection. Compared

Table 3. Distribution of formycin among organs and tissues of mice

Mice were sacrificed at 1 hour after the injection.
Total c.p.m. of ^{3}H-formycin injected was 8.31×10^{6}.
Total activity of formycin injected was 2,000 mcg.

Organs	Weight	cpm ($\times10^{5}$)	Total mcg*	mcg/g*	Determined by the activity on *X. oryzae*	
					Total mcg	mcg/g
Liver	2.7 g	2.39	28.7	10.6	12.0	4.4
Spleen	0.2	2.48	29.9	149.5	10.0	50.0
Kidney	0.6	5.03	60.5	100.6	8.5	14.2
Testis	0.2	0.06	0.8	4.0	1.3	6.5
Lung	0.3	0.14	1.8	6.0	4.7	15.7
Heart	0.2	0.18	2.2	11.0	4.8	24.0
Brain	0.8	0.16	1.6	2.0	0	—
Alimentary tract	6.0	3.23	38.9	6.5	52.9	8.8
Peritoneum	2.2	3.75	45.1	20.5	62.8	28.5
Skin	6.0	3.75	45.1	7.5	88.8	14.8
Muscle	11.0	9.59	115.4	10.5	208.0	18.9
Bone *etc.*	8.0	3.10	37.4	4.7	40.0	5.0
Feces	9.2 (ml)	1.93	23.2	—	26.7	—
Urine	0.4 (ml)	10.09	121.4	303.5	213.8	534.5
Blood	0.4 (ml)	0.33	13.8	34.5	19.5	48.8
Total :		46.21	565.8		753.8	
Recovery :		55.6 %			37.7 %	

* Calculated as formycin.

Table 4. Distribution of formycin B among organs and tissues of mice

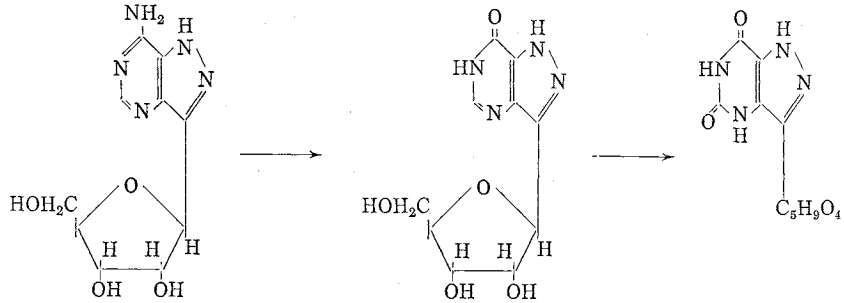
Mice were sacrificed at 1 hour after the injection.
Total c.p.m. injected was 7.22×10^{6}.
Total formycin B injected was 1,552 mcg.

Organs	Weight	c.p.m. ($\times10^{5}$)	Total mcg	mcg/g*	Activity on *X. oryzae* mcg/g*
Liver	2.10 g	1.53	33.00	15.7	<0.6
Spleen	0.28	0.50	10.60	37.8	2.2
Kidney	0.60	1.08	23.34	38.8	1.6
Stomach	0.33	0.17	3.73	11.3	—
Alimentary tract	3.70	5.42	116.70	31.5	—
Testis	0.60	0.25	5.36	8.8	0.7
Lung	0.27	0.19	4.04	14.9	—
Heart	0.21	0.16	3.33	15.8	—
Brain	0.80	0.18	3.77	4.7	0.01
Urinary bladder	0.03	0.06	1.31	43.6	—
Tongue	0.19	0.13	2.69	13.8	—
Peritoneum	0.90	2.67	57.36	63.7	13.5
Skin	6.40	5.31	114.19	17.8	—
Muscle	4.20	4.05	87.07	20.7	—
Feces	18.60 (ml)	0.80	17.21	—	—
Urine	0.72 (ml)	42.72	918.50	—	750.0
Blood	3.00 (ml)	0.40	2.56	—	1.8
Total :		65.62	1,438.36		
Recovery :		90.87 %			

* Calculated as formycin B.

to formycin B, formycin shows much stronger activity against *X. oryzae* in the various organs.

As reported in a previous paper, formycin and formycin B are metabolized in mice and rabbits to oxoformycin B[5] as follows:



Using the radioactivity of various organs as a measure of organ uptake, formycin shows the highest concentration such as 100~150 mcg/g in spleen and kidney and the next high concentration (10~21 mcg/g) in peritoneum, heart, muscle and liver. In spleen, kidney and liver, the amount found by radioactivity is larger than the amount shown by the activity on *X. oryzae*. In heart, muscle, skin and lung, the amount shown by radioactivity is about half or less than half the amount shown by the activity on *X. oryzae*. In peritoneum, alimentary tract, bone and testis, the amount shown by radioactivity is slightly less than the amount shown by the activity on *X. oryzae*. These differences are considered to be due to conversion of formycin to formycin B, oxoformycin B, and formycin phosphates. In EHRLICH carcinoma cells, formycin taken into cells is phosphorylated to mono-, di- and tri-phosphates. The monophosphate shows the same activity against *X. oryzae* as formycin, but the other phosphates show much less activity.

If the radioactivity in various organs shown in Table 4 is considered to indicate amounts of formycin B taken into the organs, then, formycin B shows the highest concentration (63.7~43.6 mcg/g) in peritoneum and urinary bladder, and next highest concentrations (38.8~31.5 mcg/g) in kidney, spleen and alimentary tract. It shows 20.7~8.8 mcg/g in muscle, skin, heart, liver, lung, stomach, tongue and testis. The lowest concentration is in brain. The activity on *X. oryzae* is highest in the peritoneum and suggests that conversion of formycin B to oxoformycin B is the slowest in this organ. Urine taken 1 hour after the injection showed activity on *X. oryzae*, indicating that about 80 % of formycin B exists in urine in this form. In organs, most of the formycin B is converted to oxoformycin B and the activities against *X. oryzae* are none or very slight. This difference between the antibacterial activity of urine and that of organs suggests that an enzyme oxidizing formycin B might be an inducible one. When 100 mg/kg of cold formycin B was subcutaneously injected to mice, then the amounts shown in urine by the activity on *X. oryzae* after various times was as follows: 1,500 mcg/ml at 1 hour after injection, 475 mcg/ml at 2 hours, 48 mcg/ml at 4 hours, 17.7 mcg/ml at 6 hours and 12.5 mcg/ml at 8 hours.

The rapid fall of the antibacterial activity after 1 hour can perhaps be considered as due to conversion of formycin B to oxoformycin B.

Toxicity of Formycin and Formycin B to Mice and to Dogs

All mice tolerated intravenous or intraperitoneal injection of 125 mg/kg of formycin and LD_{50} by the intravenous or intraperitoneal injection was 250~500 mg/kg. It showed a higher toxicity by the subcutaneous injection with an LD_{50} of 125~250 mg/kg. Orally, mice tolerated 250 mg/kg of formycin and LD_{50} was about 1,000 mg/kg. The intraperitoneal or subcutaneous injection and the oral administration of formycin caused delayed death in mice; mice died 5 days after the intraperitoneal injection and 10~20 days after subcutaneous injection or oral administration. Formycin thus shows delayed death and similarly to most antitumor substances, when daily injected, death occurs when the total amount reaches the lethal dose. When 25 mg/kg was injected daily, all mice died after 8~9 days of injection and daily injection of 12.5 mg for 10 days caused no deaths.

Formycin B killed no mice after intravenous, intraperitoneal or subcutaneous injection of 1,000 mg/kg or after oral administration of 1,000 mg/kg. However, daily injection of 400 mg/kg of formycin for 10 days was lethal. During the injection, weight was lost and among 4 mice injected one died on the day of the last injection and three others died 1~5 days thereafter. Daily injection of 200 mg/kg of formycin B for 10 days caused no deaths, but during the injection period, the body weight decreased. After cessation of the injection, the weight rapidly increased. Daily injection of 100 mg/kg caused no weight decrease and no deaths.

Table 5. Data of examination of two dogs intravenously injected with 20 mg/kg of formycin

	Dog No. 1		Dog No. 2	
	0*	1 day*	0	1 day
Red cell ($\times 10^3$)	6,690	11,020	7,790	12,360
White cell	7,000	5,300	11,000	7,300
Neutrophile(%)	65	71	64	77
Segment	32	43	38	45
Stab	33	28	26	32
Lymphocyte	27	17	34	15
Eosinophile	0	2	0	0
Basophile	0	2	0	6
Monocyte	8	8	2	2
G.O.T. (u/ml)	24	52	34	26
G.P.T. (u/ml)	7.0	>140	13	>106
Urea N (mg/dl)	8.5	19.5	8.0	28.5
Na (meq/L)	151	145	148	145
K (meq/L)	4.7	4.0	4.5	3.5
Cl (meq/L)	110	—	117	109
Body weight (kg)	8.0	7.4	8.0	7.2

* 0 means the examination done just before the injection. One day means the examination data about 24 hours after the injection. Two days after the injection, the dogs died (the weight of No. 1 was 7.0 kg and No 2, 6.9 kg).

The intravenous injection of 20 mg/kg of formycin to two dogs was lethal on the third day of the injection. Before death, jaundice was observed and the G.O.T. and G.P.T. in blood were highly elevated, indicating liver function damage. At autopsy, hemorrhage was observed in the intestinal mucosa and yellow spots were observed in the liver. The blood picture in two dogs is shown in Table 5.

Fifty mg/kg of formycin B was intravenously injected to two dogs. The white cel. count was reduced markedly on 3rd to 9th day after injection and the dogs lost appetite. However, thereafter, the white cell count recovered rapidly and became

normal. As shown in Table 6, other toxic signs were not observed. In another dog, 10 mg/kg of formycin B was injected twice a week. No toxic signs were observed when the 11th injection was finished, but 3 days after the 12th injection a slight hair loss was observed and no further injection was made. The dogs were alive and the hair loss recovered. No other toxic signs were observed.

Table 6. The toxicity of formycin B to dogs by the intravenous injection of 50 mg/kg

	Dog No. 1							Dog No. 2				
	0	1	2	3	6	9	15	0	3	6	14	30
Red cell ($\times 10^3$)	8,320	7,920	7,830	8,670	8,160	5,640	7,210	8,360	7,270	7,110	7,370	7,340
White cell	16,000	11,500	6,500	5,000	1,650	4,400	7,900	11,350	3,350	5,700	7,800	9,500
Neutrophile(%)	79.0	87.0		79.0	57.0	64.0	69.0	66.0	79.0	80.0	75.0	77.0
Segment	51.0	61.0		54.0	38.0	41.0	56.0	44.5	54.0	56.0	47.0	54.0
Stab	28.0	26.0		25.0	19.0	23.0	13.0	21.5	25.0	24.0	28.0	23.0
Lymphocyte	18.0	11.0		20.0	41.0	36.0	21.0	25.5	18.0	13.0	21.0	13.0
Eosinophile	0	0		0	0	0	10.0	6.5	1.0	7.0	3.0	10.0
Basophile	0	0		0	0	0	0	0	0	0	0	0
Monocyte	3.0	2.0		1.0	0	0	0	2.0	2.0	0	1.0	0
G.O.T. (u/ml)	26				25	29	29	46	25		29	40
G.P.T. (u/ml)	23				19	30	31	31	31		23	26
Urea N (mg/dl)	16.0				9.0	20.0	17.5	23.0	21.5		16.0	20.5
Na (meq/L)	145				148	148	148				145	145
K (meq/L)	4.3				3.6	3.9	4.4				4.3	4.4
Cl (meq/L)	109				116	113	117				109	110
Body weight (kg)	9.8	9.0	8.8	8.6	8.9	9.4	9.8	9.2	8.0	8.1	9.0	9.6

Discussion

Formycin shows inhibition of EHRLICH carcinoma and mouse leukemia L-1210. The data presented in this paper indicate a stronger effect on the subcutaneous tumor than on the ascites form. As described in a previous paper[6], formycin taken into EHRLICH carcinoma cells is rapidly phosphorylated and in the medium surrounding the cells it is rapidly deaminated to formycin B. If the same thing happens in the ascites form, then formycin outside of the cells is rapidly converted to formycin B and this rapid conversion to formycin B may be the reason for the weaker effect on ascites form than on the subcutaneous solid form.

In a disc or cylinder plate method using *X. oryzae* as the test organism, the activity ratio of formycin, formycin B and oxoformycin B is as follow: 1 : 1.56 : 0.01. One hour after the subcutaneous injection of formycin B, almost none of the antibacterial activity was found in the extract of various organs except peritoneum. It indicates that in organs formycin B was rapidly oxidized to oxoformycin B and this oxidation is slow in peritoneum. Urine excreted 1 hour after the injection showed antibacterial activity and indidates that formycin B is excreted in urine immediately after the injection. The enzyme oxidizing formycin B to oxoformycin B may be induced by formycin B. After the conversion to oxoformycin B, radioactivity of ^{3}H-formycin B is divided into two portions: ^{3}H-water and ^{3}H-oxoformycin B. Therefore, the radioactivity in various organs after the injection of ^{3}H-formycin B does not show the exact distribution of formycin B among various organs. But, if the radioactivity of the organs is assumed to show the distribution of formycin B, then, it is suggested that the injected formycin B gives the highest concentration in peritoneum and urinary bladder, and the next highest concentration in kidney, spleen and alimentary tract. By testing the effect on *X. oryzae*, low but recongnizable antibacterial activity was shown in kidney and spleen. It is not certain that the anti-

bacterial activity of kidney is due to formycin B in kidney tissue or in urine. The antibacterial activity in spleen suggests that the oxidation of formycin B in this organ may be slower than in others except peritoneum.

As reported in a previous paper[6], formycin taken into EHRLICH carcinoma cells is phosphorylated to mono-, di- and tri-phosphates, and the phosphorylation stimulates the transport of formycin from the medium into the cells. Formycin monophosphate has almost the same antibacterial activity as formycin in the disc plate assay, but the diphosphate and the triphosphate have much weaker activity. Formycin in the medium surrounding the EHRLICH carcinoma cells, as described above, is rapidly deaminated to formycin B which has stronger activity in the disc plate method. These conversions of formycin to the phosphates and formycin B make difficulty to understand the results shown in Table 3. However, it may be said that on the basis of high radioactivity, formycin injected shows the highest concentration in spleen and kidney and the but next highest concentration in peritoneum.

Formycin triphosphate which has been reported to inhibit PRPP synthesis and to be incorporated into nucleic acids. It shows the accumulative toxicity which are generally shown by antitumor substances. In dogs, when the lethal dose is injected, liver damage is the main cause of death. Formycin B has extremely low toxicity to mice, but it kills mice when 400 mg/kg daily is injected. Formycin B causes marked reduction of white blood cells, neutrophile leucocytes, though this reduction is soon recovered. When the white blood cell count decreased, the dogs lost appetite and weight. But along with the recovery of blood cells, the weight increased.

Formycin B does not show any inhibitory effect of EHRLICH carcinoma and L-1210, but the toxicity test in dogs indicates its specific toxicity on white blood cells, especially neutrophiles.

The general course of finding and evaluating antitumor substances which should be applied to cancer patients has been as follows: An antitumor substance is selected by the effect on tumors in mice and rats; the toxicity is examined in dogs, rats and sometimes in monkies. Generally the value of LD_{50} in dogs is about 10 times smaller than that in mice and antitumor substances cause delayed death of animals. After toxicity study, the substance is tried in cancer patients selected randomly. Substances which showed no effect on an animal tumor have been discarded without trial to a cancer patient. From this view point, formycin B must be discarded, and formycin can be selected as a substance which is worth the clinical study.

However, all known antitumor substances which are used clinically do not discriminate between cancer cells and normal cells. In this point, it is interesting that formycin B shows toxicity only in the reduction of white blood cells, especially of neutrophiles, and this reduction is reversible. Though formycin B shows no effect on EHRLICH carcinoma and L-1210, its selective toxicity on neutrophiles suggests that formycin B would be worth clinical trial in myeloic leukemia.

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