EFFECTS OF A NEW ADENOSINE DEAMINASE INHIBITOR, ISOCOFORMYCIN, ON TOXICITY, ANTITUMOR ACTIVITY AND TISSUE DISTRIBUTION OF FORMYCIN A AND 9-β-D-ARABINOFURANOSYLADENINE

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(Received for publication December 5, 1979)

Single intraperitoneal and intravenous injections of isocoformycin at 1,200 mg/kg did not cause the death of mice. Isocoformycin which inhibited adenosine deaminase enhanced significantly the toxicity of formycin A and ara-A at various combination ratios. Isocoformycin potentiated antitumor activity of formycin A and ara-A against L1210 leukemia. Formycin A and ara-A disappeared rapidly from the blood and tissues and could not be found in any tissues even 0.5 hour after a single intraperitoneal injection. However, when used in combination with isocoformycin both were detected in the blood and tissues, especially at high concentration in liver and kidney. These indicate that the deamination of formycin A and ara-A is blocked by isocoformycin *in vivo*.

Formycin A[1,2] and 9-β-D-arabinofuranosyladenine (ara-A)[3~5] are known as antitumor and antiviral agents. However, the antitumor activity of formycin A and ara-A against experimental tumor systems is relatively weak since they[6,7] are rapidly deaminated to formycin B (further oxidized to oxoformycin B) and ara-hypoxanthine respectively, which have a low toxicity and a low antitumor activity. Therefore, the antitumor activity of formycin A and ara-A is enhanced by an inhibitor of adenosine deaminase. In fact, coformycin[8,9], a potent adenosine deaminase inhibitor has been shown to potentiate the action of formycin A in inhibiting the growth of YOSHIDA sarcoma cells or EHRLICH carcinoma cells. In addition, it has also been reported that 2'-deoxycoformycin, another potent adenosine deaminase inhibitor enhances the antitumor activity and cytotoxicity of ara-A[10,11]. Recently, a new adenosine deaminase inhibitor, isocoformycin, was chemically synthesized by SHIMAZAKI *et al.*[12] It was a structural isomer of coformycin. It would be of interest, therefore, to examine whether isocoformycin enhances the antitumor activity of formycin A or ara-A. As reported in a previous paper[13], isocoformycin inhibited adenosine deaminase activity *in vitro* and *in vivo*, and showed a competitive type of inhibition with the substrate.

In this paper, we will report the enhancement of the acute toxicity and antitumor activity of formycin A and ara-A by isocoformycin, and the tissue distribution of formycin A and ara-A in mice after injection of each agent in combination with isocoformycin.

Materials and Methods

Chemicals

Isocoformycin was prepared in our laboratory by the method as described previously[12]. Formycin A was supplied by Meiji Seika Kaisha Ltd. 9-β-D-Arabinofuranosyladenine (ara-A) was kindly donated from National Cancer Institute (U.S.A.) and Heinrich Mack Nachf. (West Germany).

Isocoformycin was dissolved in physiological saline, and formycin A and ara-A were suspended in physiological saline containing 0.25% carboxymethylcellulose (CMC) prior to use (Formycin A and ara-A could be dissolved in saline at the concentration of about 5 mg/ml and about 0.5 mg/ml, respectively).

Animals

Female *ddY* mice weighing 20~23 g were used for toxicity study. Female BDF_1 (C57BL × DBA/2) mice (18~22 g) were used for antitumor activity and tissue distribution studies.

Toxicity study

Isocoformycin was injected intraperitoneally into *ddY* mice. For the study of the combined toxicity, isocoformycin and formycin A or ara-A were suspended in physiological saline containing 0.25% CMC and injected intraperitoneally into *ddY* mice at indicated doses. Experiments were carried out with 5 mice in each group. They were observed for three weeks after the injection.

Antitumor activity

L1210 leukemia has been maintained in ascitic form in BDF_1 mice. To three BDF_1 mice in each group were inoculated 1×10^5 cells of 7-day-old L1210 leukemia intraperitoneally. The combination treatment was made by intraperitoneal administration of combined agents at indicated schedules and dose levels after the tumor inoculation.

Antitumor activity was evaluated by the increase in life span (ILS%: T/C% − 100).

Tissue distribution study

Formycin A, ara-A alone, or in combination with isocoformycin at indicated doses was injected intraperitoneally into two BDF_1 mice in each group. Mice were sacrificed 0.5 hour, 2 hours and 4 hours after the administration. Blood was collected by cardiac puncture to heparinized syringes. Tissues (lung, liver, spleen and kidney) were taken from sacrificed mice, rinsed with cold 0.9% NaCl, rapidly blotted with filter paper, and weighed. All samples except blood were stored in a freezer (−20°C) until their assay. Each tissue except spleen was homogenized in one volume of cold 0.02 M phosphate buffer (pH 7.0) at 0°C. Spleen was homogenized in 4 volumes of the same buffer at 0°C. Moreover, to each blood and tissue homogenate an equal volume of methanol was added and centrifuged at 3,000 rpm for 10 minutes. The supernatant of each sample was used for assay.

Determination of formycin A, ara-A and isocoformycin

Concentrations of formycin A, ara-A and isocoformycin in all samples were determined with high pressure liquid chromatography (JASCO, FLC-A10). Analytical conditions were as follows: solid phase, JASCO CV-01 (2.3 mm × 500 mm); mobile phase, 0.01 M KH_2PO_4 (pH 6.0) for the analysis of formycin A, and the mixture of formycin A and isocoformycin, and 0.01 M KH_2PO_4 (pH 4.7) for the analysis of ara-A, and the mixture of ara-A and isocoformycin; flow rate, 0.5 ml/min; column pressure, 100 kg/cm^2; detector, UVIDEC-100 (wave length 280 nm); chart speed, 0.5 cm/min. Under these conditions, the lowest detectable concentrations of formycin A, ara-A and isocoformycin, in ml blood or g wet tissue are shown in the following table:

Agents	μg/ml or g tissue				
	Blood	Lung	Liver	Spleen	Kidney
Formycin A	20	40	40	100	40
Ara-A	50	100	100	250	100
Isocoformycin	10	20	20	50	20

Results

Toxicity of Isocoformycin in Combination with Formycin A or Ara-A

Acute toxicity of isocoformycin, formycin A, ara-A alone and isocoformycin in combination with formycin A or ara-A was examined. The results are shown in Table 1. All mice to which 1,200 mg/kg of isocoformycin was injected intraperitoneally tolerated the dose without any toxic symptoms. Single intravenous injection of 1,200 mg/kg also did not cause the death of mice (data not shown). Two and five out of 5 mice to which 400 mg/kg and 800 mg/kg of formycin A, respectively, were administered intraperitoneally died whereas 1,600 mg/kg of ara-A did not cause death of mice. From these results, the LD_{50} values of formycin A and ara-A by single intraperitoneal injection were roughly estimated to be about 400 mg/kg and over 1,600 mg/kg, respectively. Toxicity of formycin A and ara-A was enhanced by simultaneous injection of isocoformycin (Table 1). However, 50 mg/kg of formycin A and 200 mg/kg of ara-A in the presence of a large amount of isocoformycin such as 400 mg/kg did not cause death of mice.

All the dead mice were examined macroscopically. Hemorrhage in gastro-intestinal tracts (sometimes, in the lung) and atrophy of the thymus and spleen were observed.

Table 1. Acute toxicity of isocoformycin (ICFM) in combination with formycin A (FMA), or ara-A in mice.

Agents	Dose (mg/kg)	Mortality*			Agents	Dose (mg/kg)	Mortality*		
		7 days	14 days	21 days			7 days	14 days	21 days
ICFM	1,200	0/5	0/5	0/5	Ara-A	1,600	0/5	0/5	0/5
	400	0/5	0/5	0/5		800	0/5	0/5	0/5
FMA	800	5/5	—	—		400	0/5	0/5	0/5
	400	1/5	2/5	2/5		200	0/5	0/5	0/5
	200	0/5	0/5	0/5	Ara-A+ ICFM	800+100	5/5	—	—
	100	0/5	0/5	0/5		800+ 20	3/5	3/5	3/5
FMA+ ICFM	200+20	5/5	—	—		800+ 5	0/5	0/5	0/5
	200+ 5	5/5	—	—		400+400	2/5	2/5	2/5
	200+ 1	1/5	2/5	2/5		400+100	1/5	1/5	1/5
	100+100	3/5	3/5	3/5		400+ 20	0/5	0/5	0/5
	100+ 20	2/5	2/5	2/5		200+400	0/5	0/5	0/5
	100+ 5	0/5	0/5	0/5		200+100	0/5	0/5	0/5
	50+400	0/5	0/5	0/5					
	50+100	0/5	0/5	0/5					

* No. of mice died/No. of mice used within 7, 14 and 21 days after injection.

Treatment of Mouse L1210 with Formycin A or Ara-A in Combination with Isocoformycin

The results of combination treatment of formycin A or ara-A with isocoformycin against BDF_1 mice bearing L1210 leukemia are shown in Tables 2 and 3. The treatment was made once a day for 10 days, starting 2 hours after tumor inoculation (day 0~9), or once a day for 5 days, starting 24 hours after tumor inoculation (day 1~5). In the treatment on day 0~9 schedule, formycin A and ara-A showed weak activity against L1210 leukemia, which produced only 25~38% of ILS (2.5~5 mg/kg/day) and only 17% of ILS (50 mg/kg/day), respectively. On the other hand, the combination

Table 2. Combination effects of formycin A (FMA) and ara-A with isocoformycin (ICFM) on L1210 leukemia when given on day 0~9 schedule.

Agents	Dose (mg/kg/day)	MST (days)	ILS (%)
ICFM	200	8.3	4
	20	8.3	4
	2.5	8.0	0
	0.1	8.3	4
FMA	10	10.7	34
	5	11.0	38
	2.5	10.0	25
	1.25	10.0	25
FMA+ ICFM	5+20	14.0	75
	5+10	14.7	84
	5+5	14.0	75
	5+2.5	12.7	59
	5+1.25	14.7	84
	5+0.313	14.3	79
	5+0.078	14.0	75
	2.5+200	14.0	75
	2.5+20	13.3	66
	2.5+10	14.0	75
	2.5+5	13.0	63
	2.5+2.5	13.7	71
	2.5+1.25	12.7	59
	2.5+0.313	12.7	59
	2.5+0.078	12.7	59
Control	—	8.0	0
Ara-A	100	9.7	17
	50	9.7	17
	25	9.0	8
Ara-A+ ICFM	50+200	12.7	53
	50+20	11.7	41
	50+10	12.7	53
	50+5	11.7	41
	50+2.5	10.7	29
	50+0.5	10.3	24
	50+0.1	10.3	24
Control	—	8.3	0

Inoculum size: 10^5 cells/BDF_1 mouse, ip, 3 mice/group.
Therapy: Day 0~9 daily ip, starting 2 hours after tumor inoculation.
MST: Mean survival time.
ILS: Increase in life span.

Table 3. Combination effects of formycin A (FMA) and ara-A with isocoformycin (ICFM) on L1210 leukemia when given on day 1~5 schedule.

Agents	Dose (mg/kg/day)	MST (days)	ILS (%)
ICFM	200	8.3	−5
	20	8.7	0
	2.5	8.7	0
	0.1	8.3	−5
FMA	10	10.7	23
	5	11.0	26
	2.5	10.0	15
	1.25	9.3	7
FMA+ ICFM	2.5+200	10.7	23
	2.5+20	11.7	34
	2.5+10	11.7	34
	2.5+2.5	11.3	30
	2.5+0.5	13.7	57
	2.5+0.1	11.7	34
Ara-A	100	10.7	23
	50	10.0	15
	25	9.3	7
Ara-A+ ICFM	50+200	11.3	30
	50+20	11.7	34
	50+10	10.7	23
	50+2.5	12.0	38
	50+0.5	11.3	30
	50+0.1	10.0	15
Control	—	8.7	0

Inoculum size: 10^5 cells/BDF_1 mouse, ip, 3 mice/group.
Therapy: Day 1~5 daily ip, starting 24 hours after tumor inoculation.
MST: Mean survival time.
ILS: Increase in life span.

treatment of formycin A and ara-A with isocoformycin produced 59~84% and 41~53% of ILS, respectively. The degree of antitumor activity of combination of formycin A and ara-A with isocoformycin was independent of the increasing dose of inhibitor. Similar results were observed in the treatment on day 1~5 schedule (Table 3), although the degree of the effectiveness was weaker than that treated on day 0~9.

Isocoformycin showed no antitumor activity when given on day 0~9 or on day 1~5 schedule (Tables 2 and 3).

Tissue Distribution

Effect of isocoformycin on tissue distributions of formycin A and ara-A in mice was examined. The results are shown in Tables 4 and 5. Neither formycin A nor ara-A was detected in any of tissues examined (blood, lung, liver, spleen and kidney) 0.5 hour, 2 hours and 4 hours after an intraperitoneal administration of formycin A or ara-A alone (200 mg/kg). However they were detected in blood and tissues when given in combination with isocoformycin. Formycin A was found at higher level in liver, kidney and spleen than in blood and lung. In addition, it was observed that isocoformycin increased tissue levels of formycin A and ara-A with the increase in the amount of the inhibitor. Isocoformycin distributed in blood and tissues, but it remained in tissues at higher levels for longer periods when

Table 4. Blood and tissue levels of formycin A (FMA) and isocoformycin (ICFM) in mice after their combined administration.

Agents (mg/kg)	Tissues	Concentration in μg/g or ml					
		0.5 hr.		2 hrs.		4 hrs.	
		FMA	ICFM	FMA	ICFM	FMA	ICFM
FMA (200)	Blood	—*		—		—	
	Lung	—		—		—	
	Liver	—		—		—	
	Spleen	—		—		—	
	Kidney	—		—		—	
FMA+ICFM (200+20)	Blood	—	10	—	15	—	—
	Lung	—	—	—	—	—	—
	Liver	95	35	115	20	105	—
	Spleen	100	50	100	50	—	50
	Kidney	75	30	70	20	60	—
FMA+ICFM (200+100)	Blood	20	100	—	100	—	15
	Lung	60	50	—	40	—	20
	Liver	380	120	575	115	375	—
	Spleen	100	160	115	180	—	160
	Kidney	270	105	430	385	205	35
FMA+ICFM (200+500)	Blood	45	350	20	355	—	165
	Lung	135	450	90	440	75	160
	Liver	525	485	1,060	495	840	125
	Spleen	115	615	115	890	100	980
	Kidney	495	495	660	1,030	515	395

* Not detected.
Each value is arithmetic mean of 2 mice.

Table 5. Blood and tissue levels of ara-A and isocoformycin (ICFM) in mice after their combined administration.

Agents (mg/kg)	Tissues	Concentration in μg/g or ml					
		0.5 hr.		2 hrs.		4 hrs.	
		Ara-A	ICFM	Ara-A	ICFM	Ara-A	ICFM
Ara-A (200)	Blood	—*		—		—	
	Lung	—		—		—	
	Liver	—		—		—	
	Spleen	—		—		—	
	Kidney	—		—		—	
Ara-A+ ICFM (200+20)	Blood	50	10	—	—	—	—
	Lung	—	—	—	—	—	—
	Liver	260	20	—	—	—	—
	Spleen	250	50	—	—	—	—
	Kidney	330	55	100	—	—	—
Ara-A+ ICFM (200+100)	Blood	50	55	—	—	—	—
	Lung	—	20	—	—	—	—
	Liver	250	45	—	—	—	—
	Spleen	250	185	—	75	—	50
	Kidney	560	95	100	20	—	—
Ara-A+ ICFM (200+500)	Blood	60	355	—	30	—	—
	Lung	100	220	125	70	—	20
	Liver	1,100	415	305	40	100	—
	Spleen	605	730	600	575	250	250
	Kidney	880	735	585	80	235	20

* Not detected.
Each value is arithmetic mean of 2 mice.

combined with formycin A than when combined with ara-A.

Discussion

The rapid deamination of formycin A and ara-A can be prevented by an adenosine deaminase inhibitor and the combination with an inhibitor can produce a better therapeutic effect and also enhance toxicity. It is apparent that isocoformycin increases the toxicity of formycin A and ara-A, which are due to the inhibition of deamination of the agents by an inhibitor (Table 1). Both formycin A and ara-A can show only weak antitumor activity against L1210 leukemia, because of a strong adenosine deaminase activity in this tumor[14]. LEPAGE *et al.*[10] have noted that the combination treatment of ara-A with 2'-deoxycoformycin produces more than 100% ILS of mice with L1210 leukemia when the treatment was made every 3 hours, 8 times per day on days 1 and 4, or on days 1, 4 and 7 after tumor inoculation. LEE *et al.*[15] also obtained similar results in the same combination treatment in an intracerebral leukemia. In the present study, it was observed that isocoformycin enhanced antitumor activity of formycin A and ara-A against L1210 leukemia when given in combination (Tables 2 and 3). However, the effectiveness did not increase with increasing dose of the inhibitor, that is, isocoformycin showed the same degree of the enhancement in a wide range of its dose. The combination effect of ara-A and isocoformycin was not strong, as compared to the results of LEPAGE *et al.*[10] This might be considered to be mainly due to the difference of the treatment schedule. Therefore, in order to obtain a stronger combination effect, the frequency of the administration may

require further study. Both formycin A and ara-A were detected in blood and tissues when administered in combination with isocoformycin. It is apparent that the deamination of formycin A and ara-A is blocked by isocoformycin. This is supported by the data reported in a previous paper[13] indicating that isocoformycin significantly inhibited adenosine deaminase activities derived from various organs. Isocoformycin has a very low toxicity. With the respect to these, isocoformycin may be an interesting adenosine deaminase inhibitor to increase the antitumor and antiviral effects of formycin A and ara-A.

Acknowledgements

We wish to thank Meiji Seika Kaisha Ltd. for the generous gift of formycin A, and Dr. J. D. DOUROS of National Cancer Institute (U.S.A.) and Dr. W. FORSTER of Heinrich Mack Nachf. Chem. Pharmazentische Fabrik (West Germany) for the generous gift of ara-A.

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