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Antitumor Effect of Bisbenzylisoquinoline Alkaloids[1]

Hiroyuki Kuroda, Shozo Nakazawa,[2a] Ken Katagiri, Osamu Shiratori,[2b] Mutsuo Kozuka, Kazuyoshi Fujitani, and Masao Tomita[2a]

Kyoto College of Pharmacy[2a] and Shionogi Research Laboratory[2b]

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Twenty-three kinds of bisbenzylisoquinoline alkaloids including tetrandrine, isotetrandrine, and berbamine were studied on various biological activities. We made comparisons in biological activities, for examples, on bacterial cells, blood cells, tumor cells, and in the whole mouse, in order to obtain better suggestion concerning the correlation between activity and chemical structure. From the results of the present investigation, it is suggested that at the present stage a definite structure-activity relationship, as reported by Kupchan, *et al.*, is hardly delineated, though certain structure requirements for the manifestation of the antitumor activities were noticed.

Since Kupchan, *et al.* reported in 1966 that bisbenzylisoquinoline (biscoclaurine) alkaloids such as thalidasine, thalicarpine, and tetrandrine have antitumor activity, studies on the antitumor effect of these alkaloids have been made actively.[3] Recently, Gralla, *et al.*[4] carried out preclinical toxicity experiments with tetrandrine on beagle dogs and rhesus monkeys. Also there is a report that cepharanthine is effective in recovery of immune function at the time of administration of antitumor agents as well as in prevention of leukopenia.[5] The report suggests that the alkaloids have not only direct action on tumors but also may be a participant of the immunological mechanism of hosts against tumors and others.

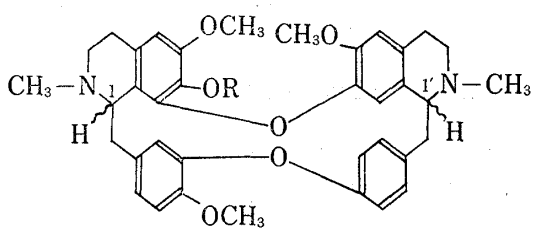
The recent report by Kupchan, *et al.*[6] refers to the correlation between antitumor activity of bisbenzylisoquinoline alkaloids and their chemical structures, but their report seems to contradict in some points with the results which we obtained from our similar but independent experiment made at the same time. This may be due to the difference between experimental procedures of Kupchan and us. In this paper are reported the results of our various bioassays *in vitro* and *in vivo* of 23 alkaloids including tetrandrine, isotetrandrine, and berbamine.

Materials and Methods

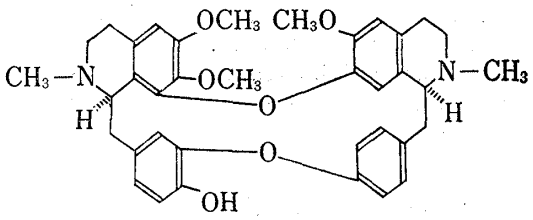
1. Samples Tested——Bisbenzylisoquinoline alkaloids used in this experiment are shown in Fig. 1. For *in vitro* assays the samples were dissolved in absolute methanol and subjected to serial twofold dilutions, and for *in vivo* assays those were neutralized with sodium bicarbonate after dissolving in dilute hydrochloric acid.

2. Antitumor Effect——a) Cytotoxic Effect Against Cultured Cells: HeLa and HeLa-S_3 cells were obtained from Dr. N. Ishida, Tohoku University (Japan). The cells were suspended in Eagle's minimum essential medium (MEM) containing 10% bovine serum, diluted to the concentration of 1×10^5 cells/ml, and transferred to small test tubes. Three cultures thus prepared were employed as a set for each experiment.

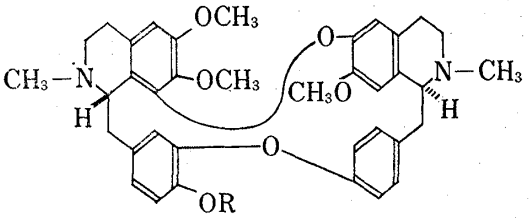
1) A part of this work was presented at the 94th Annual Meeting of Pharmaceutical Society of Japan, Sendai, April 1974, and at the 33rd Annual Meeting of the Japanese Cancer Association, Sendai, October 1974.
2) Location: *a) Higashiyama-ku, Kyoto, 607, Japan; b) Fukushima-ku, Osaka, 553, Japan.*
3) *a)* S.M. Kupchan, T.H. Yang, G.S. Vasilikiotis, M.H. Barnes, and M.L. King, *J. Am. Chem. Soc.*, **89**, 3075, (1967); *b) Chem. & Eng. News*, **44**, [51], 64, (1966); *ibid.*, **50**, [9], 58, (1972); R.E. Perdue and J.L. Hartwell, *Morris Arbor. Bull.*, **20**, 35, (1969); M.P. Cava and A. Afzali, *J. Org. Chem.*, **40**, 1553, (1975).
4) E.J. Gralla, G.L. Coleman, and A.M. Jonas, *Cancer Chemother. Rep.* [Part III], **5**, 79, (1974).
5) K. Nomoto, R. Makidono, and K. Takeya, *Nippon Rinsho* (Tokyo), **31**, 3548, (1973).
6) S.M. Kupchan and H.W. Altland, *J. Med. Chem.*, **16**, 913, (1973).



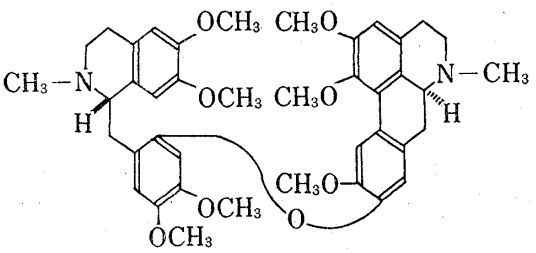
1–S, 1′–S R=CH_3 tetrandrine (**1a**)
tetrandrine dimethiodide (**1a***)
1–R, 1′–S R=CH_3 isotetrandrine (**1b**)
1–S, 1′–S R=H fangchinoline (**1c**)



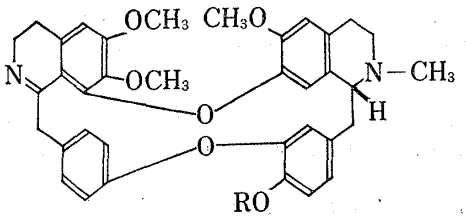
berbamine (**3**)



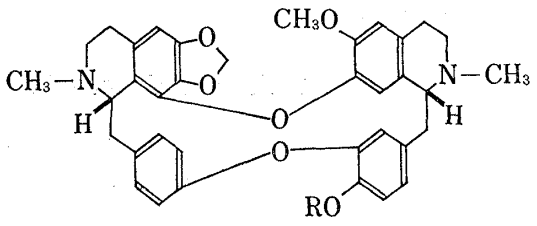
R=H thalicberine (**5a**)
R=CH_3 O-methylthalicberine (**5b**)



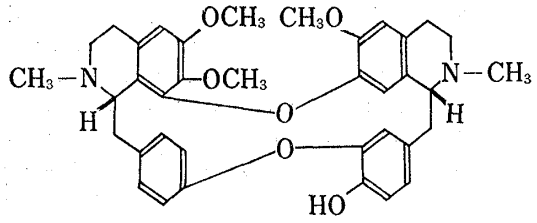
thalicarpine (**7**)



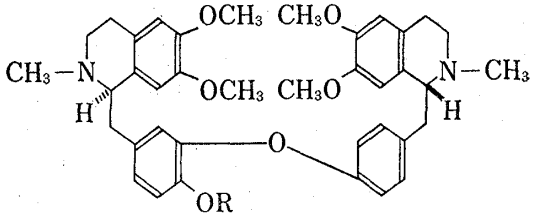
R=CH_3 epistephanine (**9a**)
R=H hypoepistephanine (**9b**)



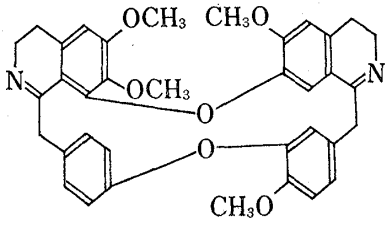
R=CH_3 cepharanthine (**2a**)
R=H cepharanoline (**2b**)



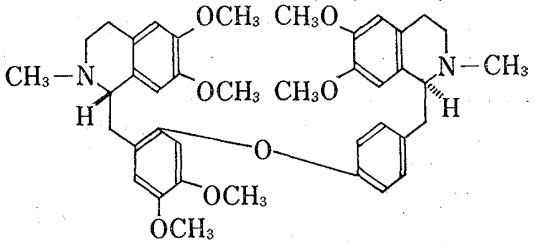
oxyacanthine (**4**)
oxyacanthine dimethiodide (**4***)



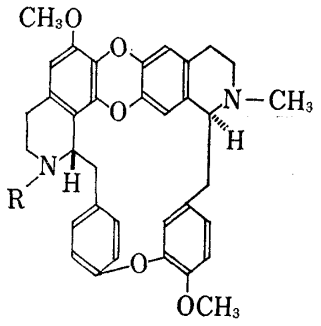
R=H dauricine (**6a**)
R=CH_3 O-methyldauricine (**6b**)



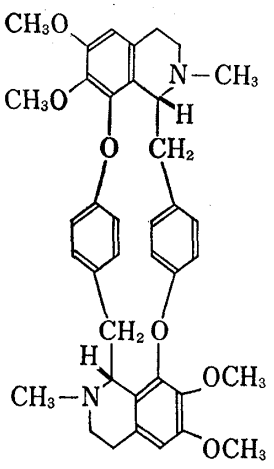
stebisimine (**8**)



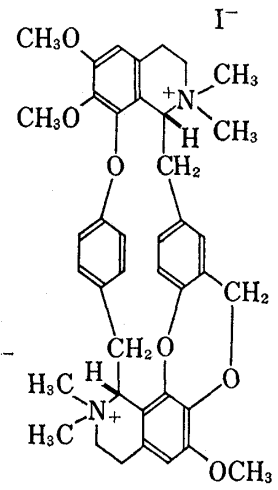
tetramethylmagnolamine (**10**)



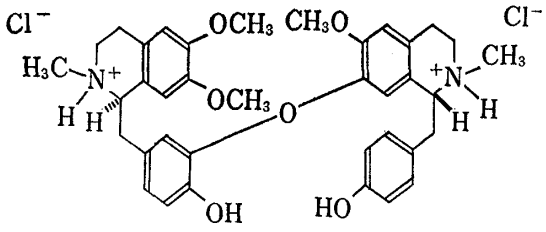
R=H trilobine (**11a**)
R=CH_3 isotrilobine (**11b**)



cycleanine (**12**)



insularine dimethiodide (**14**)



isoliensinine dihydochloride (**13**)

Fig. 1. The Bisbenzylisoquinoline Alkaloids Tested

Each sample was added to the cell cultures on the second day of cultivation. After 2 days of incubation with the sample, the cell population was measured by means of an electronic device, the TOA micro-cell counter (TOA Electronics, Kobe, Japan). The effective dose for 50% growth inhibition (ED_{50}) was determined by plotting the logarithmic curve of the drug concentration against the growth rate.

b) Effect against Ehrlich Ascites Carcinoma: Ehrlich ascites carcinoma cells were inoculated to ddYS mice. After 7 days, tumor cells were taken out and adjusted to the cell number of 2×10^6 cells/mouse. The cell suspension was inoculated intraperitoneally to experimental animals (ddYS male mice weighing 20 ± 0.5 g) divided into groups of 10 each. From 24 hours after the inoculation, each sample was injected to mice intraperitoneally once a day for consecutive 7 days. Observation was continued for 60 days, and the body weight gain, life-prolongation effect, and survival rate during the observation were compared with those of the control.

c) Effect against Sarcoma-180 Solid Type: Sarcoma-180 was inoculated to ICR/JCL mice. The cells 2×10^6 cells/mouse, were inoculated subcutaneously to the right inguinal region of experimental mice (ICR/JCL male mice weighing 20 ± 0.5 g) divided into groups of 10 each. From 24 hours after the inoculation of tumor cells, each sample was injected intraperitoneally once a day for consecutive 7 days. The inhibitory rate of tumors by each sample was obtained in comparison of the average weight of tumors removed 15 days after the inoculation with that in the control.

3. Antibacterial Activity *in Vitro*——The antibacterial activity *in vitro* of each sample was determined against gram-positive and gram-negative bacteria which had been preserved in Department of Microbiology, Kyoto College of Pharmacy. The determination was made according to the method authorized by the Japan Society of Chemotherapy.

4. Toxicological Tests——a) LD_{50} for the Mouse: LD_{50} of each sample by intraperitoneal injection was determined using ddYS mice (weighing 20 g) divided into groups of 5 each according to Lichfield-Wilcoxon's method.

b) Hemolysis: After incubation with each sample at 37° for 2 hours, the hemolysis of red blood cells of rabbits, which had been previously adjusted to 3%, was compared macroscopically with that of the control.

Results

1. Relationship between Structure and Cytotoxic Activity of Bisbenzylisoquinoline Alkaloids

The results of cytotoxicity assay on cultured cells are shown in Table I.

TABLE I. Comparative *in Vitro* Cytotoxicity of Bisbenzylisoquinoline Alkaloids against Cultured Cells

Samples	Cells (ED_{50}, μg/ml)	
	HeLa	HeLa-S_3
Tetrandrine **1a**	4.4	1
Tetrandrine dimethiodide **1a***	>30	
Isotetrandrine **1b**	5.8	10
Fangchinoline **1c**	4.1	
Cepharanthine **2a**	5.5	7
Cepharanoline **2b**	>30	
Berbamine **3**		>10
Oxyacanthine **4**		3
Oxycanthine dimethiodide **4***	>30	
Thalicberine **5a**	13	
O-Methylthalicberine **5b**		
Dauricine **6a**		10
O-Methyldauricine **6b**	11	
Thalicarpine **7**		2.5
Stebisimine **8**	16	
Epistephanine **9a**	14	
Hypoepistephanine **9b**	12	
Tetramethylmagnolamine **10**	13	
Trilobine **11a**		1.1
Isotrilobine **11b**		2
Cycleanine **12**	12	
Isoliensinine dihydrochloride **13**	16	
Insularine dimethiodide **14**	>30	

There was difference in the effect on HeLa-S_3 cells between tetrandrine (**1a**) and isotetrandrine (**1b**), both of which have the same two-dimensional structure except the absolute configuration of C-1. This suggests that the configuration is related to activity. On the other hand, berbamine (**3**) which is 12′-O-norisotetrandrine had no activity, but oxyacanthine (**4**), which is an isomer different in the position of two phenyl ether linkages, was obviously more active.

In the effect against HeLa cells, no remarkable difference was observed between **1a** and **1b**, contrary to the effect against HeLa-S_3 strains. Further, it seems from the comparison of **1a** with fangchinoline (**1c**) that the substituent at the position 7 is whether a hydroxyl or methoxyl group has no relation to the presence of activity. As for the substituent at the position 12′, there is difference in activity between cepharanthine (**2a**) and cepharanoline (**2b**), whereas no difference between epistephanine (**9a**) and hypoepistephanine (**9b**). Compounds (**9a**, **9b**) and stebisimine (**8**) showed weak activity on HeLa cells, while quaternary ammonium salts such as tetrandrine dimethiodide (**1a***) and oxyacanthine dimethiodide (**4***) had no longer any activity.

A few examples will suffice. Comparison of **1a** and **1a*** indicated that the quaternization reduced the cytotoxicity. Such characteristics are also recognized when **4** and **4*** are compared.

2. Effect against Animal Tumors and Acute Toxicity

Effects against ascites tumor from Ehrlich ascites carcinoma (EAC) and against solid tumor from Sarcoma-180 (S-180) are shown in Table II, along with LD_{50} by intraperitoneal injection. The effect against Ehrlich ascites carcinoma is represented in the dose (mg/kg/day) enough to prolong the life of 50% of mice for 60 days after inoculation of tumor. And the effect against Sarcoma-180 solid type is represented in the dose (mg/kg/day) to inhibit the tumor weight gain of tumors removed 15 days after inoculation under 50% of the tumor weight of the control. LD_{50} was obtained by one shot of intraperitoneal injection of each sample represented in mg/kg.

TABLE II. Results of *in Vivo* Assay of Bisbenzylisoquinoline Alkaloids

Samples	EAC[a] (mg/kg)	S-180[b] (mg/kg)	LD_{50} (mg/kg)	Hemolysis
1a	50	62.5	280	+
1a*	—	NT	7	NT[c]
1b	100	125	160	—
1c	—	NT	> 50	NT
2a	30	100	125	—
3	—	—	75	—
4	—	—	50	—
4*	—	NT	9	NT
5a	62.5	—	>125	—
5b	—	NT	125	NT
6a	>100	—	>125	—
6b	>100	—	>125	—
9a	—	NT	NT	NT
9b	—	NT	NT	NT
11b	30	25	115	+
13	—	NT	NT	NT
14	—	NT	10	NT

a) EAC: dose [mg/kg/day] for 50% surviving at 60 days after Ehrlich ascites carcinoma inoculation intraperitoneally in mice
b) S-180: dose [mg/kg/day] for 50% inhibition against Sarcoma-180 subcutaneously inoculation in mice
c) NT: not tested

Three samples of **1a**, **1b**, and isotrilobine (**11b**) were similarly effective against both tumors of ascites type (EAC) and solid type (S-180). Compound (**2a**) and thalicberine (**5a**) were effective against EAC, but slightly effective against S-180 solid tumor. In order to grasp the correlation of antitumor activity with toxicity, the therapeutical effect against EAC was studied by administration of each sample at 1/4 of LD_{50}. ED_{50}/LD_{50} values of **1a**, **2a**, **5a**, and **11b** were under 1/4, but the effective dose of **1b** approximated closely to the lethal dose. Fig. 2 shows the therapeutical effect of each sample against EAC, which was given at 1/4 of LD_{50}. As the result, it was found that **1a** and **1b** are quite different in antitumor activity.

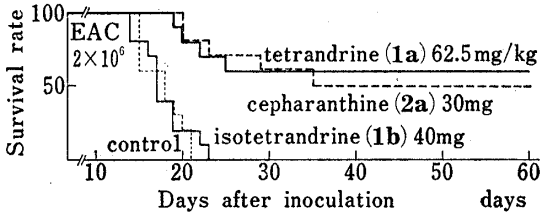


Fig. 2. Antitumor Effect of **1a**, **1b** and **2a** on Ehrlich Ascites Carcinoma in Mice [1/4 LD_{50} × 7 days, *i.p.*]

From Table I and II, a parallelism may be drawn between the effect against HeLa cells and the antitumor activity *in vivo*, with a few exceptions, *i.e.*, **1c**, **4**, and **9a** showed considerable cytotoxic effect *in vitro*, but no antitumor activity *in vivo*.

Fig. 3 shows the therapeutic effect of **5a** and O-methylthalicberine (**5b**) against EAC.

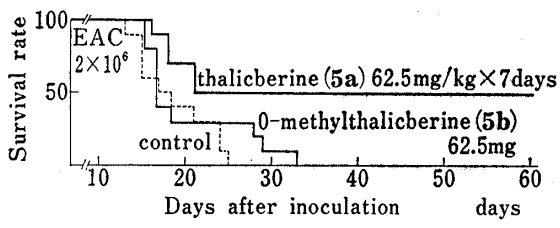


Fig. 3. Antitumor Effect of **5a** and **5b** on Ehrlich Ascites Carcinoma in Mice

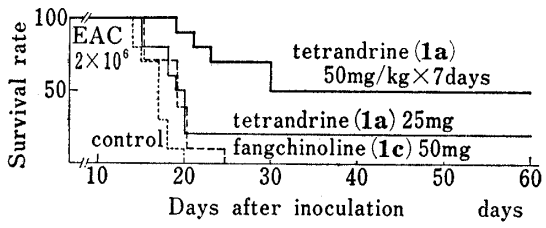


Fig. 4. Antitumor Effect of **1a** and **1c** on Ehrlich Ascites Carcinoma in Mice

Compound (**5a**), in which the substituent at the position 12 is hydroxyl group, had antitumor activity, but **5b** having a methoxyl group instead was non-effective at the same dose with **5a**. Compound (**1c**), which corresponds to 7-O-nor-**1a**, was effective against HeLa cells *in vitro* as much as **1a**. The antitumor activity on EAC *in vivo* was observed only in **1a**, but **1c** had no effect (Fig. 4).

Compounds (**1a***, **4***), and insularine dimethiodide (**14**), which showed no cytotoxic effect against HeLa cells *in vitro*, were non-effective against tumors either *in vivo*, but their acute toxicity on mice was very strong.

As is seen from Table I and II, three biological activities of cytotoxicity on HeLa cells, acute toxicity on mice, and antitumor activity *in vivo* ran parallel in **1a**, **1b**, **2a**, **4**, **5a**, **11b**, *etc.*, but not in **1c**, **9a** **9b**, quaternary salts, *etc.*

3. Antibacterial Activity *in Vitro*

Table III shows the antibacterial activity of each sample on various bacteria.

TABLE III. Antibacterial Effect of Bisbenzylisoquinoline Alkaloids

Organisms	**1a**	**1b**	**2a**	**3**	**4**	**5a**	**6b**	**11b**
Staphylococcus aureus 209-P JC	62.5	125	31.25	250	500	250	250	31.25
TERAJIMA	250	250	125	500	500	1000	1000	250
epidermidis	62.5	125	31.25	125	125	250	250	62.5
Sarcina lutea ATCC-9341	15.6	62.5	7.8	500	62.5	500	500	7.8
Bacillus subtilis ATCC-6633	62.5	62.5	31.25	500	125	500	250	15.6
anthracis	500	500	250	500	500	1000	500	125
Escherichia coli NIH JC-2	1000	1000	1000	1000	1000	1000	1000	500
Klebsiella pneumoniae	500	500	500	500	500	500	500	500

[MIC μg/ml]

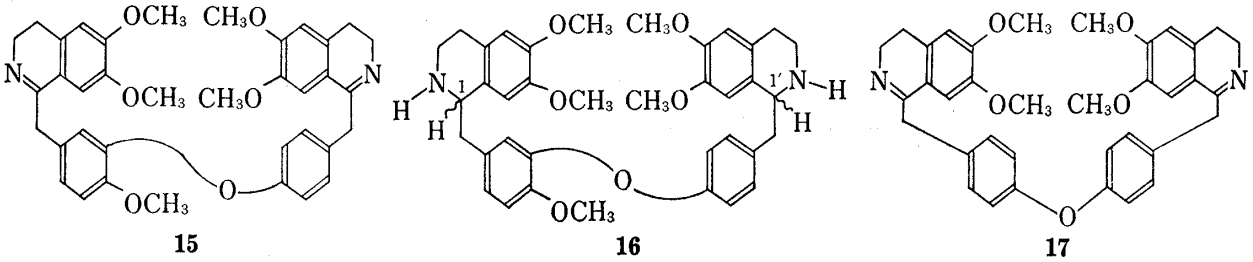
Bisbenzylisoquinoline alkaloids used in this experiment were not active on gram-negative bacteria such as *Escherichia coli*, but some of the above alkaloids had weak activity on gram-positive bacteria such as *Staphylococcus aureus*. Compounds (**1a**, **2a**, and **11b**) had also antibacterial activity almost in parallel with antitumor activity *in vivo*, but **5a** had only antitumor activity. Compound (**4**), which was high in acute toxicity as well as cytotoxicity on HeLa cells and had no antitumor activity *in vivo*, showed neither antibacterial activity.

4. Hemolysis of Red Blood Cells of the Rabbit

After incubation of each sample with red blood cells at 37° for 2 hours, the hemolysis was observed macroscopically. Hemolysis of red blood cells was remarkable with **1a** and **11b** at the level of 30 μg/ml, but under the same conditions, no hemolysis was observed with **1b**, **2a**, **3**, **4**, **5a**, and dauricine (**6a**) at the level of 100 μg/ml.

Discussion

Kupchan, *et al.*[6] studied the correlation between antitumor activity of bisbenzylisoquinoline alkaloids and their chemical structures, using intramuscular carcinosarcoma-256 (Walker-256) of rats, and reached the following conclusions: (i) monomeric benzylisoquinolines and aporphines showed no antitumor activity on Walker-256, (ii) O-methyldauricine (**6b**) and thalidasine[3a] were active on Walker-256, and this result suggests that the macrocyclic ring may have no relation to the activity, (iii) bis(benzyl-3,4-dihydroisoquinoline) synthesized (Fig. 5 **15**, **16**, and **17**) showed the activity, and from this, it seems that methylation of N atoms have nothing to do with the activity, and it can be said that the activity is not stereospecific, because **15** and **17** were active and the enantiomer of tetrandrine (**1a**), pheanthine



15 16 17

Fig. 5. Related Structures of Tumor Inhibitory Bisbenzylisoquinolines[6]

was active.[7] However, the study by Kupchan, *et al.* was made by bioassay of only Walker-256 and at doses very close to the lethal dose. For example, the difference in activity between tetrandrine (**1a**) and isotetrandrine (**1b**) shown in Fig. 2 was diminished by using the dose of 2 mg/mouse (100 mg/kg). This result, both **1a** and **1b** appeared effective, might be misleading. We made comparisons in biological activities, for example, on bacterial cells, blood cells, tumor cells, and in the whole mouse, in order to obtain better suggestion concerning the correlation between activity and chemical structure.

TABLE IV. Summarized Results of the Experiments

Samples	Cells		EAC	S-180	Bacteria[a]	Toxicity
	HeLa	HeLa-S_3				
1a	##	##	##	##	+	−
1a*	−		−			##
1b	##	+	+	−	+	+
1c	##		−			−
2a	##	##	##	−	##	+
2b	−					
3		−	−	−	−	##
4		##	−	−	−	##
4*	−		−			##
5a	+		+	−	−	−
5b			−			
6a		+	−	−		−
6b	+		−	−	−	−
7		##				
8	+					
9a	+		−			−
9b	+		−			−
10	+					
11a		##				
11b		##	##	##	##	+
12	+					
13	+		−			−
14	−		−			##

##: strongly, +: weakly, −: no effect
a) antibacterial effect against *Staphylococcus aureus* 209-P JC

Table IV summarized the results of this experiment.

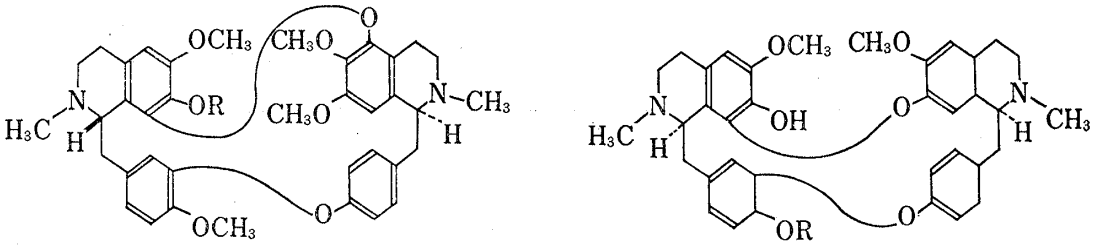
Of these substances, tetrandrine (**1a**), cepharanthine (**2a**), thalicberine (**5a**), and isotrilobine (**11b**) were effective against tumors *in vivo*. The results obtained above led us to the following conclusion: (i) substances exhibiting antitumor activity *in vivo* has S,S configuration at C-1,1′, except cepharanthine (**2a**), (ii) whether the substituents at position 12′ and 7 are hydroxyl or

7) J.L. Hartwell and B.L. Abott, *Advan. Pharm. Chemother.*, **7**, 117, (1969).

methoxyl group, has influence on manifestation of antitumor activity *in vitro* and *in vivo*, and (iii) remarkable differences were found between tetrandrine (**1a**) and isotetrandrine (**1b**) in such points as the effect against S-180 solid type, hemolysis, sensitivity to HeLa-S_3 cells, and ED_{50}/LD_{50} value against EAC. Therefore, in combination with (i), it is suggested that the stereochemistry at position C-1 and C-1′ has influence on the activity of bisbenzylisoquinolines having two or more phenyl ether bonds.

However, our results contain some contradictions: (i) of substances having antitumor activity on EAC *in vivo*, only tetrandrine (**1a**) and isotrilobine (**11b**) were effective against S-180 solid tumor, (ii) thalicberine (**5a**) showed no antibacterial activity, and on the contrary, berbamine (**3**) showed only antibacterial activity, and (iii) fangchinoline (**1c**) and oxyacanthine (**4**) were strong only in cytotoxic effect against HeLa cells, which was not in parallel with antitumor activity *in vivo*.

Kupchan, *et al.* formed their conclusion in a simplified treatment in sample choice and bioassay methods. They may possibly have overlooked little differences in correlation between the delicate and complicated whole structure, and substituents. Mitscher, *et al.*[8] pointed out that there is difference in antibacterial activity against *Mycobacterium smegmatis* between thalidasine (**18**), and thalrugosidine (**19**) as well as between obamegine (**20**), and thalrugosine (**21**). From this, it is suggested that substituents at position 7 and 12 are closely



18: R=CH_3
19: R=H
20: R=H
21: R=CH_3

Fig. 6. Related Structures of Bisbenzylisoquinoline Alkaloids[8]

related to biological activity. It should be noted here that the influence of the substituents at positions 7 and 12 (or 12′) on biological activity may not be generalized over all of the bisbenzylisoquinoline alkaloids studied so far.

From the results of the present investigation, it is suggested that at the present stage a definite structure-activity relationship, as reported by Kupchan, *et al.*, is hardly delineated, though certain structural requirements for the manifestation of the antitumor activities were noticed. To validate these structural requirements, further studies including distribution and metabolism of the alkaloids *in vivo* must be conducted.

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8) L.A. Mitscher, Wu-nan Wu, R.W. Doskotch, and J.L. Beal, *Chem. Comm.*, **1971**, 589.