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Suppressive Effect of Cycloheximide Cinnamates on Immune Response and Tumor Growth in Mice——A Structure-activity Relationship with Respect to the Substituent on the Benzene Ring of the Cinnamic Acid Moiety[1)]

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The immunosuppressive and tumor-inhibitory activities in mice of cycloheximide cinnamates depended on the structure and position of a substituent on the benzene ring of the cinnamate moiety. When the levels of antibody production and tumor growth rate in the treated mice were analyzed statistically in relation to Hansch's hydrophobic constant π for each substituent, the π-response curves for the two biological responses were found to be similar to each other, suggesting that the two pharmacological activities depend on a common process which is influenced by the hydrophobicity of the cycloheximide cinnamates.

Keywords——cycloheximide cinnamates; substituents on benzene ring; synthesis of cycloheximide cinnamates; immunosuppression; inhibition of tumor growth; subcellular protein synthesis; mouse; structure-activity relationship; Hansch's hydrophobic substituent constant (π); regression analysis

Cycloheximide (CHI), known as an inhibitor of protein synthesis, has also been shown to inhibit the immune response.[3)] The author found that some cinnamic esters of CHI were more potent than CHI as inhibitors of antibody production in the mouse, and that the inhibitory activity of the esters was modified by changing the structure or position of the substituent on the benzene ring of the cinnamate moiety.[1)]

In the present study, the author attempted to analyze structure-activity relationships of the immunosuppressive and tumor-inhibitory activities of CHI cinnamates with a variously substituted benzene ring. For this purpose the author synthesized 10 CHI cinnamates including five new esters (ester No. 2, 3, 6, 15 and 16) and tested the two biological activities of 16 CHI cinnamates.[4)] Structure-activity relationships were examined in relation to Hansch's substituent constant (π) that determines the hydrophobic character of a molecule with that substituent.[5)] The results showed that both activities had a common relationship with π values of substituents, indicating that the hydrophobic character of the esters might be one of the important factors determining the two suppressive activities. In contrast to CHI, any of the CHI cinnamates did not inhibit *in vitro* protein synthesis by the subcellular system of mouse liver when added as fine suspensions.

1) Part of this report was presented at the 94th Annual Meeting of the Pharmaceutical Society of Japan, Sendai, April 1974.
2) Location: *2-2-50, Kawagishi, Toda-shi, Saitama.*
3) W.J. Cooney and S.G. Bradley, *Antimicrob. Agents Chemother.*, **1962**, 1; M. Ellenrieder, *Klin. Wochenschr.*, **45**, 1159 (1967); E.A. Mel'nikova, *Antibiotiki*, **15**, 823 (1970) [*C.A.*, **73**, 128958*t* (1970)]; J.E. Foker, *Diss. Abstr. Int. B*, **31**, 1360 (1970).
4) Six (ester No. 5, 7, 8, and 12—14; see Table I) of the 16 CHI cinnamates were generously donated by Mr. Y. Sugawara, Drug Metabolism Research Laboratory of Tanabe Seiyaku Co., Ltd.
5) C. Hansch, R.M. Muir, T. Fujita, P.P. Maloney, F. Geiger, and M. Streich, *J. Am. Chem. Soc.*, **85**, 2817 (1963); M.S. Tute, "Advances in Drug Research," Vol. 6, ed. by N.J. Harper and A.B. Simmonds, Academic Press, London, 1971, p. 1.

Materials and Methods

Synthesis of CHI Cinnamates——A substituted benzoyl chloride was reduced by the Rosenmund reaction[6] to a benzoyl aldehyde, which in turn condensed with malonic acid to yield a substituted cinnamic acid.[7] The reaction of a cinnamoyl chloride with CHI by Egawa's procedure[8] gave a CHI cinnamate (I). *o*-CH_3S-benzoic acid was obtained by methylation[9] of thiosalicylic acid with CH_3I. The products were purified by recrystallization or by silica gel column chromatography, and identified by infrared spectrum (IR) and mass spectrometric analysis. Melting points of the synthesized CHI cinnamates are listed in Table I.

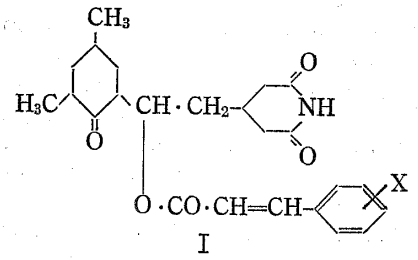

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Chart 1

Mice——Male albino dd mice (for the immune response) and male ICR mice (for the tumor growth response), each aged four weeks, were purchased from Shizuoka Agricult. Coop. Assoc. Lab. Animals and CLEA Japan Inc., respectively.

TABLE I. List of CHI Cinnamates Tested in the Present Study

Ester No.	Substituent (X)	π[a]	mp (°C)
1	H	0.00	157—159
2	CH_3, *o*	0.68	134—137
3	*m*	0.51	140—141
4	*p*	0.52	127—128
5	Cl, *o*	0.59	56— 58
6	*m*	0.76	158.5
7	*p*	0.70	162—163
8	*o*, *p*	1.25[b]	124—126
9	NH_2, *p*	−1.23[c]	101—104
10	NO_2, *o*	−0.23	129—132
11	*m*	0.11	159
12	*p*	0.24	185—187
13	OCH_3, *o*	−0.33	54— 56
14	*p*	−0.04	120—121
15	SCH_3, *o*	0.61[b]	66— 67
16	*t*-C_4H_9, *p*	1.68[d]	82— 85

a) substituent constant determined by Fujita, *et al.* (T. Fujita, J. Iwasa and C. Hansch, *J. Am. Chem. Soc.*, **86**, 5175 (1964)) with 1-octanol and water as the solvent system (parent compound: phenoxyacetic acid)
b) calculated from the data of Leo, *et al.* (A. Leo, C. Hansch and D. Elkins, *Chem. Rev.*, **71**, 525 (1971)) (parent compound:phenoxyacetic acid for ester No. 8 and benzene for No. 15)
c) parent compound: benzene
d) determined by Metcalf and Fukuto (R.L. Metcalf and T.R. Fukuto, *J. Econ. Entomol.*, **55**, 340 (1962))

Immune Response——On day 0, groups of five mice were immunized by *i.p.* injection of 1.2×10^9 erythrocytes of Sprague-Dawley rats (CLEA Japan Inc.). The mice were treated *i.p.* from day 0 to 3 with 25 mg/kg per day of a CHI cinnamate suspended in a saline solution of 0.5% carboxymethylcellulose (The test compound was first homogenized in a small amount of Tween 80 on an agate mortar and then suspended in 0.5% carboxymethylcellulose). On day 6, blood samples were obtained from the animals *via* the femoral vessels by making a deep incision at the inguinal region, and hemagglutinin titers of the serum samples were assayed with the Microtiter (Cooke Engineering Co.). The titer was expressed as $\log_2$ of the highest serum dilution producing agglutination.

6) R.P. Barnes, "Organic Synthesis," 2nd ed., Col. Vol. III, ed. by E.C. Horning, John Wiley and Sons, Inc., New York, 1964, p. 551.
7) E.K. Thayer, "Organic Synthesis," 2nd ed., Coll. Vol. I, ed. by H. Gilman and A.H. Blatt, John Wiley and Sons, Inc., New York, 1964, p. 398.
8) Y. Egawa, S. Oshima, and S. Umezawa, *J. Antibiotics* (Tokyo), Ser. A, **18**, 171 (1965).
9) T. Hashimoto, H. Kitano, and K. Fukui, *Nippon Kagaku Zasshi*, **89**, 810 (1968).

Tumor Growth Response——Groups of five mice were injected *s.c.* on day 0 with 2×10^6 of Ehrlich ascites cells at the inguinal region, and injected *i.p.* from day 1 to 7 with 25 mg/kg per day of a CHI cinnamate. On day 10, the mice were sacrificed and wet weights of the solid tumors were determined.

Subcellular Protein Synthesis——To examine possible effects of the esters on protein synthesis, the mouse liver subcellular system described by Bennett, *et al.*[10] was used. Ribosomes were prepared by the method of Kaji, *et al.*[11] Protein contents of the pH 5 fraction were determined by Lowry's method[12] using ovalbumin as a standard protein. The reaction mixture contained the following: pyruvate kinase, 5 μg; phosphoenolpyruvate, 2.5 μmoles; ATP, 0.25 μmole; GTP, 7.5 nmoles; 21 unlabeled amino acids, 25 nmoles each; leucine-U-^{14}C (New England Nuclear; specific activity: 315 mCi/mmole), 0.1 μCi; ribosomes, 10—20 absorbancy (at 260 nm) units; pH 5 fraction 4—8 mg; a test compound, 50—200 μg in a final volume of 1.0 ml of the standard buffer of Bennett, *et al.*[10] A CHI cinnamate was added to the incubation mixture after homogenization with an ultrasonic disrupter (Model UR-200 P, Tomy Seiko Co.) in a small volume of the standard buffer. All the incubation mixtures were prepared in duplicate. After incubation at 37° for 40 min, 1 ml of 1 N NaOH was added to stop the reaction. One hr later, protein was precipitated by addition of 1 ml of 2.5 M perchloric acid and washed successively with 3 ml each of 0.5M perchloric acid containing 0.1M unlabeled leucine (three times), propanol: ether (1: 2, v/v) and propanol: ether: chloroform (2: 2: 1, v/v). The washed protein was dissolved in 1 ml of Hyamine (Horiba; 10-X, OH type) and, after addition of 15 ml of the toluene-scintillation mixture (DPO 4 g and POPOP 100 mg in 1 liter of toluene), counted with a Horiba liquid scintillation spectrometer.

Results

Effect of CHI Cinnamates on the Immune Response and Tumor Growth

Effects of CHI cinnamates in both the production of hemagglutinins to rat erythrocytes and the growth of transplanted Ehrlich cells in mice are shown in Table II. Both the hemagglutinin titer and the tumor weight of treated mice tended to vary in parallel depending on the structure and position of a substituent on the benzene ring of the cinnamate moiety.

Table II. Effect of CHI Cinnamates on the Immune Response and Tumor Growth

Ester No.	Substituent (X)	Hemagglutinin titer	Tumor weight (g)
1	H	3.4±1.2[a]	3.40±0.38[a]
2	CH_3, *o*	6.6±0.6	3.34±0.34
3	*m*	4.6±1.1	2.60±0.35
4	*p*	4.4±0.9	2.05±0.28
5	Cl, *o*	4.5±1.0	2.88±0.21
6	*m*	0 ± 0	3.29±0.23
7	*p*	3.2±1.2	1.00±0.10
8	*o*, *p*	5.6±0.7	3.56±0.23
9	NH_2, *p*	6.0±0.8	3.61±0.16
10	NO_2, *o*	6.4±0.5	4.12±0.38
11	*m*	2.8±0.6	2.73±0.21
12	*p*	2.8±1.2	2.53±0.40
13	OCH_3, *o*	7.4±0.4	4.52±0.14
14	*p*	6.0±0.6	3.26±0.52
15	SCH_3, *o*	5.2±0.5	3.35±0.45
16	*t*-C_4H_9, *p*	6.8±0.5	2.79±0.46
CHI		5.4±1.0	4.03±0.23
Control		6.2±0.2	4.58±0.11

Groups of five mice were treated *i.p.* with 25 mg/kg per day of CHI cinnamates on day 0 to 3 (mice immunized with rat erythrocytes) or on day 1 to 7 (Ehrlich cell-transplanted mice).
a) mean ± standard error (n=5)

10) L.L. Bennett, Jr., V.L. Ward, and R.W. Brockman, *Biochim. Biophys. Acta*, **103**, 478 (1965).
11) A. Kaji, T. Otaka, and H. Kaji, "Seikagaku Jikken Koza," Vol. 7, ed. by The Japanese Biochemical Society, Tokyo Kagaku Dojin, Tokyo, 1975, pp. 19—21.
12) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

Analysis

The correlation coefficient between the titer and the tumor weight was 0.67 ($p<0.01$, Fig. 1) when the data of ester No. 6, which inhibited the immune response only, were omitted.

When the observed hemagglutinin titers (Fig. 2) and tumor weights (Fig. 3) were plotted against Hansch's substituent constant π (Table I), a U-shaped curve was obtained in either case. Within a certain range of π values (between approximately -0.3 and 0.8) the curves were parabolic, although some esters were dislocated considerably from the curves (for example, ester No. 6 in immune response and ester No. 7 in tumor growth response). Outside the "parabolic" region, the esters exerted little effect, except for ester No. 16 which inhibited tumor growth response.

The best-fit equations for the two U-shaped curves were sought by the minimum square method. For the inhibition of immune response,

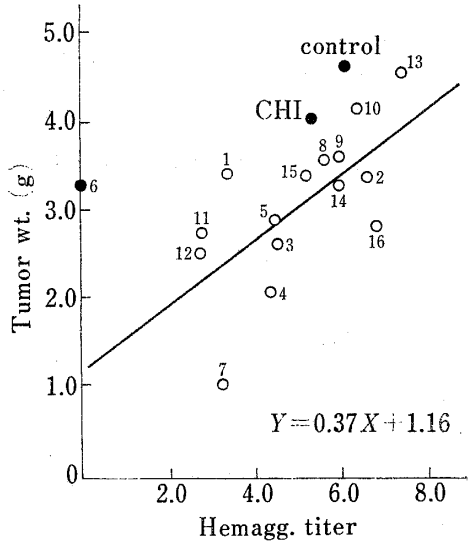

Fig. 1. Correlation between the Immunosuppressive and Tumor-inhibitory Activities of CHI Cinnamates

Numerals in the Fig. are the Nos. of the CHI cinnamates listed in Table I.
●, the data omitted for calculation of the correlation coefficient

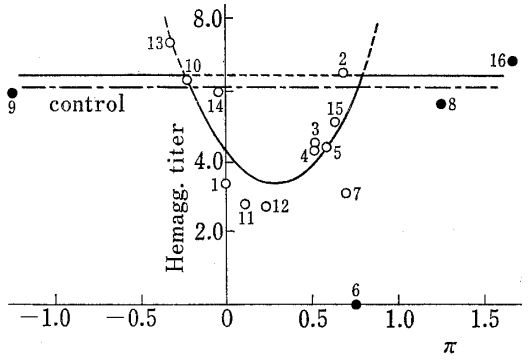

Fig. 2. Relation of the Immunosuppressive Activity of CHI Cinnamates to Hansch's π Value of the Substituent

Numerals in the Fig. are the Nos. of the CHI cinnamates listed in Table I.
●, the data omitted for calculation of the parabolic curve

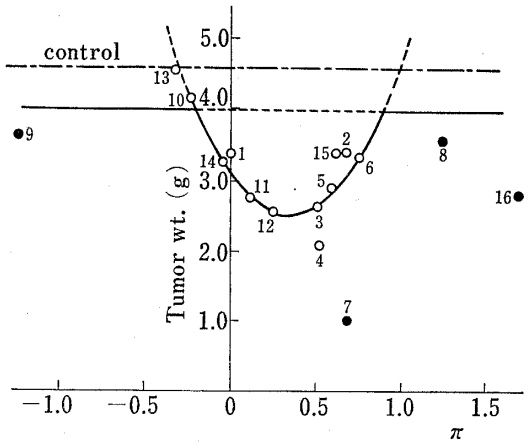

Fig. 3. Relation of the Tumor-inhibitory Activity of CHI Cinnamates to Hansch's π Value of the Substituent

Numerals in the Fig. are the Nos. of the CHI cinnamates listed in Table I.
●, the data omitted for calculation of the parabolic curve

i) within the "parabolic" region, a quadratic equation

$$y = 11.37\pi^2 - 6.30\pi + 4.22$$
$$= 11.37(\pi - 0.28)^2 + 3.55$$

(correlation coefficient $r=0.76$, $p<0.05$)

where y is hemagglutinin titer and π is Hansch's substituent constant, was obtained from the data of esters No. 1—5, 7 and 10—15, and

ii) outside the "parabolic" region,

$$y = 6.4$$

was obtained from the data of esters No. 2, 8—10, 13, 14 and 16. For the inhibition of tumor growth, the respective equations were

i) $y = 4.85\pi^2 - 3.25\pi + 3.08$

$= 4.85(\pi - 0.34)^2 + 2.54$

($r = 0.92$, $p < 0.005$; obtained from the data of esters No. 1—6 and 10—15)

where y is tumor weight, and

ii) $y = 3.95$

(obtained from the data of esters No. 8—10 and 13).

Effect of CHI Cinnamates on Subcellular Protein Synthesis

Because CHI cinnamates were practically insoluble in water in contrast to CHI, fine suspensions were prepared to test possible effects on *in vitro* protein synthesis. As shown in Table III, none of the esters inhibited protein synthesis by the mouse liver subcellular system. Among the esters tested, only ester No. 10 was soluble in ethanol. Therefore, effect of ester No. 10 on protein synthesis was examined in a medium containing ethanol (final concentration: 0.7% (v/v)), in which an inhibition of protein synthesis by CHI could be observed. As shown in Table IV, ester No. 10 was again without effect on protein synthesis.

Table III. Effect of CHI Cinnamates on Subcellular Protein Synthesis

Ester No.	Substituent (X)	^{14}C-Leucine incorporated (pmole/tube)	
		100 μg/ml	200 μg/ml
1	H	3.39	5.46
2	CH_3, *o*	4.38	6.66
3	*m*	3.81	6.12
4	*p*	4.12	6.33
5	Cl, *o*	4.06	6.07
6	*m*	4.25	6.26
7	*p*	4.16	6.63
8	*o*, *p*	4.42	5.74
9	NH_2, *p*	3.55	4.59
10	NO_2, *o*	3.95	5.83
11	*m*	4.06	6.31
12	*p*	3.96	6.37
13	OCH_3, *o*	4.30	6.15
14	*p*	4.22	6.56
15	SCH_3, *o*	4.11	6.15
16	*t*-C_4H_9, *p*	3.74	6.71
CHI		2.86[a]	1.44[b]
Control		4.03	6.40

a) 50 μg/ml (29.1% inhibited)
b) 100 μg/ml (77.5% inhibited)

Table IV. Effect of Ester No. 10 on Subcellular Protein Synthesis in Medium Containing 0.7% Ethanol

Addition	Concn. (μg/ml)	^{14}C-Leucine incorporated (pmole/tube)	Inhibition (%)
Ester No. 10[a]	100	1.01	0
CHI	100	0.21	76.3
Control	—	0.90	—

a) CHI-*o*-nitrocinnamate

Discussion

A common structure-activity relationship between two pharmacological actions shared by a series of related compounds is suggestive of a common mechanism or process involved in the two biological responses to these compounds. Immunosuppression and tumor inhibition are two separate biological responses, but they are often affected by common cytostatic agents, because the immune response and tumor growth both require vigorous proliferation of cells. Cellular proliferation naturally involves protein synthesis. Since CHI has been known to inhibit protein synthesis, it seemed likely that the CHI cinnamates elicited the immunosuppressive and tumor-inhibitory properties through their actions as inhibitors of protein synthesis. Therefore, it was of some interest to examine whether a similar structure-activity relationship could be observed when these CHI cinnamates were tested in a cell-free system of protein synthesis. However, none of these compounds were inhibitory when added to the *in vitro* system as fine suspensions. It could not be decided whether they were actually inert or they simply did not get into solution to give an inhibitory concentration. Ester No. 10, which is soluble in a dilute ethanol solution, was dissolved in the incubation medium containing 0.7% ethanol, but did not inhibit protein synthesis whereas CHI did inhibit under the same conditions. Ester No. 10 happened to be inactive in the two *in vivo* systems described above, which may or may not be related to its inertness in the *in vitro* system of protein synthesis. Ennis[13] showed that esterification (acetylation and benzoylacetylation) of the hydroxyl group of CHI greatly diminishes the inhibitory effect on protein synthesis in the cell-free system from rat liver. It could be assumed that cinnamic esters of CHI have to be hydrolyzed to free CHI in order to inhibit protein synthesis. Such hydrolysis would easily take place *in vivo*.

Hansch's hydrophobic substituent constant π is defined as follows,[5]

$$\pi_X = \log P_X - \log P_H$$

where P_H is a partition coefficient between two immiscible solvents of a parent compound and P_X is that of a X-substituted derivative. Thus, π represents a change in hydrophobicity (or lipophilicity) induced by substitution on the parent compound. Fujita, *et al.*[14] measured partition coefficients of 203 substituted aromatic compounds in the 1-octanol and water system, and calculated π values, which were approximately constant with each substituent for eight different series of parent compounds. The fact that the two π-response curves obtained in the present study was alike and U-shaped for both the immune response and tumor growth could be taken to indicate that, ouside the "parabolic" region of π, the esters possess neither of the suppressive activities under the conditions used, and that, within the "parabolic" region, both suppressive activities exhibit a maximum at a certain π value (around 0.3) and gradually decrease on either side.

All these results point to the importance of an optimal lipid-solubility of the CHI cinnamates in determining their pharmacological activities. It is likely that affinity to drug receptors or permeability through membranes becomes a limiting factor for their activities, because drug receptors and biological membranes are supposed to be of lipoid nature. In addition, the enzymatic hydrolysis *in vivo* of the esters to free cinnamates and CHI might also depend on the hydrophobic character of the substrates. The fact that the π values giving the maximum suppressive activity in the two systems were approximately equal (0.28 for the immune response and 0.34 for the tumor growth response) may suggest that a common process may be involved in eliciting the two suppressive activities.

The present study adds another example of structure-activity relationship which show the importance of proper hydrophobicity of a compound in eliciting its pharmacological

13) H.L. Ennis, *Biochem. Pharmcol.*, **17**, 1197 (1968).
14) T. Fujita, J. Iwasa, and C. Hansch, *J. Am. Chem. Soc.*, **86**, 5175 (1964).

activity. When the essential structure for a pharmacological activity is known, it seems to be one useful approach to prepare its derivatives with different hydrophobicities and analyze the structure-activity relationship in order to arrive at most effective compounds.

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