THE HUMAN TUMOR-EGG HOST SYSTEM

IV. DISCOVERY OF A NEW ANTI-TUMOR AGENT, COMPOUND 593 A*

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A new crystalline anti-tumor agent, compound 593A, has been discovered in fermentation broths of *Streptomyces griseoluteus*. Its presence in the fermented broth was detected by the human tumor-egg host system which was used also to help guide fermentation and isolation studies. On a weight basis, against the human adenocarcinoma (H. Ad.) #1 in the embryonated egg, anti-tumor compound 593A is about 100 times as potent as hadacidin, about 4 times as potent as sodium L-tenuazonate, and about one-half as potent as mitomycin C. It also inhibits, in the egg, growth of the human sarcoma (H. S.) #1, and metastasis but not primary growth of the human epidermoid carcinoma (H. Ep.) #3. Anti-tumor compound 593A has the empirical formula $C_7H_{11}ON_2Cl$.

We have discovered a new anti-tumor agent, compound 593A. A product of actinomycete fermentation, anti-tumor compound 593A was detected in the fermented broth by the human tumor-egg host system which also helped to guide its isolation as s pure crystalline compound. KB cell and bacterial test systems provided supplementary guidance for these studies.

Cultures shown to produce anti-metabolite antibiotic activity in their fermentations were among those selected for testing for anti-tumor activity in the egg. The fermented broth of one such culture, isolated from soil collected in the area of Richmond, Union of South Africa, and identified as *Streptomyces griseoluteus*, was active in the egg, although its anti-tumor and anti-metabolite activities were subsequently found to be from different substances. The anti-tumor substance has been purified, and tentatively designated anti-tumor compound 593A.

Materials and Methods

Fermentation Broths

Procedures described by STAPLEY *et al.*[1] were used for selecting actinomycetes producing anti-metabolic antibiotic substances. The cultures were grown from vegetative inocula[1] in 250 ml Erlenmeyer flasks containing 50 ml of medium of the following composition: glucose, 1 %; peptone, 0.5 %; yeast extract, 0.3 %; NaCl, 1.27 %; KCl, 0.072 %; $FeSO_4$·

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$(NH_4)_2SO_4 \cdot 6H_2O$, 0.035 %; $MgCl_2 \cdot 2H_2O$, 0.532 %; $CaCl_2 \cdot 2H_2O$, 0.073 %; distilled water to volume. The pH was adjusted to 7.4 before sterilization. The flasks were incubated at 28°C on a 150 rev./min. rotary shaker. After 48~72 hours the broths were clarified and sterilized as described previously[2]. The sterile filtrates were subdivided for storage at −20°C until tested.

Biological Test Systems

The human adenocarcinoma (H. Ad.) #1[3] was used for testing fermentation broths and for guidance of fermentation and chemical studies. Purified preparations of anti-tumor compound 593A were tested also against the human epidermoid carcinoma (H. Ep.) #3[4] and the human sarcoma (H. S.) #1[4]. Tests against H. Ad. #1 were as previously described[2] with treatment 3 or 4 days after implantation of tumor, and harvesting on the 7th day after treatment.

Results were expressed as follows: ten eggs were sacrificed at time of treatment to determine the mean weight of the tumor. The value obtained was subtracted from the mean weight of treated and control tumors at time of harvest. Thus we obtained a measure of the actual increase in weight of the tumors during the treatment period. The percent growth inhibition ($100-T/C \times 100$) compares the increase in weight of treated tumors with the increase in weight for the control tumors. The percent growth inhibition for embryos was determined in a similar manner.

Because incubating eggs implanted with either H. Ep. #3 or H. S. #1 for more than 8 days resulted in a high mortality of embryos, we treated the eggs 2 days after implanting these tumors, and harvested them 5 days after treatment. For these tumors also, results were expressed in terms of percent growth inhibition.

We also studied the effect of anti-tumor compound 593A on metastasis of H. Ep. #3. This tumor, unlike H. Ad. #1 and H. S. #1, spontaneously metastasizes from its site of implantation on the chorioallantoic membrane to the chick embryo[5]. After determining the effect of treatment on primary growth of the H. Ep. #3 tumor, we implanted a portion of lung tissue from each harvested embryo into a fresh group of 10-day eggs. We incubated these eggs for 8 days and then weighed the resulting tumors to obtain an estimate of the amount of metastasis to the lung.

Cytotoxic activity in tube cultures was determined as previously described[6] using EAGLE's KB cell carcinoma[7].

Results

The fermentation broth of a culture subsequently identified as *Streptomyces griseoluteus*, active against *Escherichia coli* in a synthetic but not in a complex

Table 1. Activity of *Streptomyces griseoluteus* broth against H. Ad. #1 in the embryonated egg*

Broth filtrate	Dilution	Deaths	Per cent growth inhibition	
			Embryo	Tumor
1	1/2	2/18	7	80
	1/4	2/12	3	56
2	None	3/12	7	80
	1/2	1/12	6	23

* Each egg was treated with 1 ml broth filtrate at the dilution indicated. Six eggs were used per treatment level. The data from all tests at each treatment level were pooled.

Table 2. Time-activity study on broth of *S. griseoluteus* grown in a 150 gallon fermentor.

Sample time Hr.	Activity of broth		
	H. Ad. #1 ID[a] (ml/egg)	KB CE[b] (μl/ml)	*E. coli* Zone diameter[c] (mm)
24	>2.0	>100	0
36	N. T.[d]	3~10	0
48	1.5	<3	0
72	0.5	<3	0
96	0.5	<3	24
120	0.25	<3	30
144	0.25	<3	31

a) Dose found to inhibit tumor growth 60 % or greater. Highest level tested was 2 ml/egg.
b) Cytotoxic end-point. Lowest level tested was 3 μl/ml.
c) Inhibition zone produced with 13 mm filter paper discs in agar plate assay against *Escherichia coli* in a chemically defined medium 1).
d) Not tested.

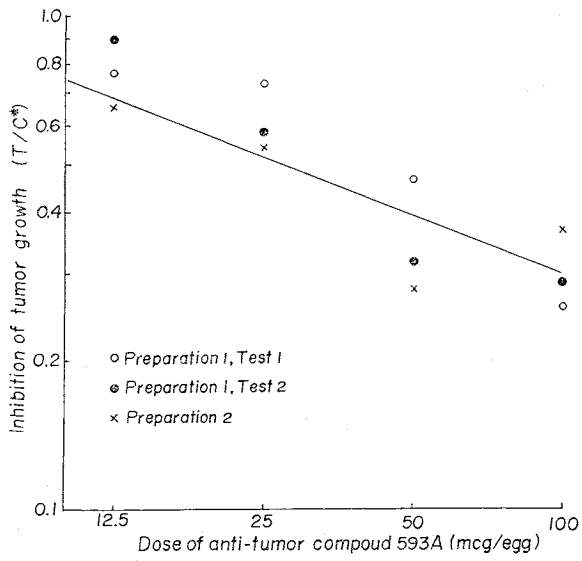
Table 3. Anti-tumor activity against H. Ad. #1 in the egg and cytotoxic activity against KB cells for representative fractions of broth of *S. griseoluteus*.

Perparation	Dose (mg/egg)	Mortality	% Growth inhibition		Cytotoxic ED_{50} (mcg/ml)
			Embryo	Tumor	
I Lyophillized filtered broth*	10	4/24	−5	61	10~30
II Absolute ethanol soluble of I	5	2/7	−1	72	10
	1.6	1/8	6	41	
	0.5	0/8	11	20	
III Butanol extract (at pH 7.0) of II	1.5	5/8	15	56	1
	0.5	0/8	−3	50	
IV Crude crystals from III	0.05	2/8	−1	51	0.1
	0.025	2/8	−8	56	
V HCl salt of recrystalized IV	0.025	1/8	−17	83	0.03

* Pooled results from 4 different test days.

medium, was found to inhibit the growth of H. Ad. #1 tumor in the embryonated egg. Anti-tumor activity was reproduced in two consecutive shake flask fermentations (Table 1). The fermented broths were active also against KB cells. To provide material for isolation of anti-tumor compound 593A, a large fermentation batch (150 gallons or 568 liters) was prepared. Activities against both H. Ad. #1 and KB cells were detected at 48 hours, but activity against *E. coli* was not detected until 96 hours (Table 2) indicating either the presence of more than one active constituent or different sensitivities of the test systems to the active material. A 50-fold concentration of activity against *E. coli* was not active against H. Ad. #1 nor against KB cells showing that the antibiotic and anti-tumor substances were different entities. Because some concentrates were found to be cytotoxic to KB cells but not active against H. Ad. #1, isolation of the anti-tumor factor, especially in the early stages, was guided primarily by the H. Ad. #1-egg host system with supplmentary assays in KB cells.

Fig. 1. Dose-response plot of anti-tumor compound 593A hydrochloride tested against H. Ad. #1 in the egg. Two preparations were used.
* T/C corrected for initial weight of tumor.



The procedure for isolating purified anti-tumor compound 593A and the activities of representative fractions prepared during the isolation are summarized in Table 3. Lyophilized filtered whole broth (I) active at 10 mg/egg, was extracted with absolute ethanol. The ethanol soluble fraction (II) was then con-

centrated to dryness *in vacuo*, dissolved in water and extracted at pH 7.0 with an equal volume of butanol. Concentration of the wet butanol soluble extract *in vacuo* to an aqueous solution, followed by lyophilization, resulted in fraction III. Subsequent batches yielded a crystalline fraction (IV) which separated from the aqueous solution resulting from concentration of the butanol extract. Recrystallization of IV from methanol and conversion to the hydrochloride salt gave the purified crystalline preparation of anti-tumor compound 593A (V).

Table 4. Effect of anti-tumor compound 593A against H. S. #1 and H. Ep. #3 tumors in the embryonated egg.

Tumor	Test	Dose (mcg/egg)	Mortality	% Growth inhibition		
				Embryo	Tumor	Metastasis*
H. S. #1	1	150	6/8	[6]	[74]	—
		100	4/8	24	91	—
		50	0/8	19	94	—
		25	0/8	4	55	—
	2	50	1/8	16	89	—
		25	1/8	−2	61	—
H. Ep. #3	1	100	3/8	10	1	68
		50	1/8	13	36	69
		25	0/8	18	17	48
	2	50	0/8	23	25	90
		25	1/8	8	5	41

* H. S. #1 does not metastasize in the chick embryo.

Table 5. Antibacterial activity of anti-tumor compound 593A

Test organism	Inhibition zone diameter (mm)* with 1 mg/ml
Vibrio percolans MB-1272	19
Brucella bronchiseptica MB-965	14
Alcaligenes faecalis MB-10	12
Staphylococcus aureus MB-698	17
Proteus vulgaris MB-1012	10
Streptococcus faecalis MB-753	8

* Assays were performed with 7 mm filter paper discs on Nutrient Agar containing 0.2% yeast extract; plates were incubated overnight at 37°C.

Fractions II, III and IV showed progressively increasing potencies against H. Ad. #1 in the egg. Preparation V, the hydrochloride salt of the recrystallized product, was active at 25 mcg per egg, representing a purification of over 400-fold compared to broth. The correlation of cytotoxic activity against KB cells *in vitro* with activity against H. Ad. #1 in the egg for these preparations is also shown in Table 3; the hydrochloride salt of the recrystallized product was cytotoxic at a level of 0.03 mcg/ml.

The ED_{60} (dose which inhibited tumor growth 60%) estimated from results obtained with two different crystalline hydrochloride salt preparations tested against H. Ad. #1 was 49.5 mcg per egg (Fig. 1).

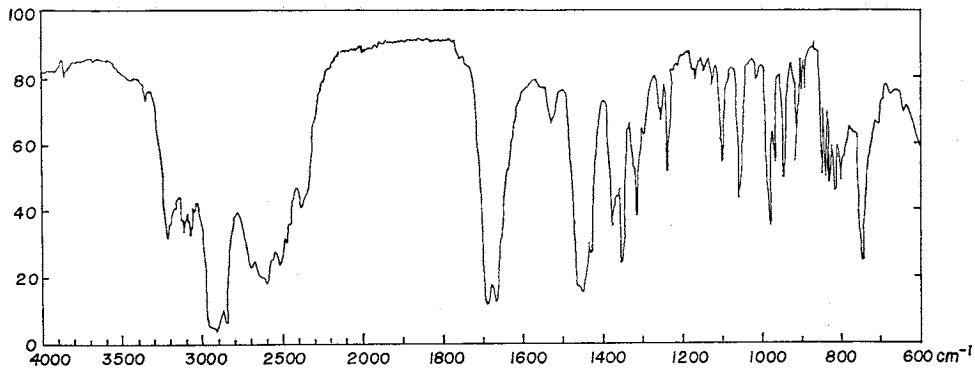
Anti-tumor compound 593A inhibited the growth of H. S. #1 (Table 4). It was ineffective against the growth of H. Ep. #3 primary tumor although metastasis was inhibited (Table 4).

In the mouse, anti-tumor compound 593A inhibited the Krebs 2 solid tumor (T/C =0.4 at 5×0.2 mg/kg intraperitoneally), but not Sarcoma 180 (solid and ascites). The tolerated dosages in mice were as follows: single intravenous, 4 mg/kg; single intraperitoneal, 5 mg/kg; daily intraperitoneal, 0.2 mg/kg.

A personal communication from Dr. GEORGE TARNOWSKI, Sloan-Kettering Institute, reports that anti-tumor compound 593A inhibited the growth of WALKER 256 carcinosarcoma in the rat and of RIDGWAY osteogenic sarcoma in the mouse.

Although the anti-metabolite antibiotic activity of the fermentation broth of *S. griseoluteus* was not associated with purified anti-tumor compound 593A, the latter

Fig. 2. Anti-tumor compound 593A. Infrared spectrum of hydrochloride salt as Nujol mull.



has some anti-bacterial activity as shown in Table 5.

A hydrochloride salt was obtained by evaporation of a dilute hydrochloric acid solution of the purified isolate. The anti-tumor compound 593A hydrochloride was crystallized from methanol. No melting point was observed below 330°C. $[\alpha]_D^{25}$ +11.0° (*c* 10.036 mg/ml water).

Anal. Calcd. for $C_7H_{12}ON_2Cl_2$: C 39.83, H 5.73, O 7.58, N 13.27, Cl 33.59.
Found : C 40.23, H 5.85, O 8.5, N 12.76, Cl 32.82.

Equivalent weight by titration: Calcd: 211. Found 215 (pH 1/2=5.4).

Anti-tumor compound 593A free base was obtained from the hydrochloride.

Anal. Calcd. for $C_7H_{11}ON_2Cl$: Cl 20.30. Found: Cl 19.07.

The infrared spectrum of the hydrochloride salt of anti-tumor compound 593A in a mineral oil (Nujol) mull is shown in Fig. 2.

Discussion

Isolation of anti-tumor compound 593A has demonstrated once more the utility of the human tumor-egg host system for detecting potential anti-tumor agents among products of microbial fermentation and for helping to guide the isolation of the active component. Previously use of this system led to the discovery of the anti-tumor agent, hadacidin[2] and the discovery of the anti-tumor properties of tenuazonic acid[6], both products of mold fermentation.

On a weight basis, against H. Ad. #1 in the egg, anti-tumor compound 593A is about 100 times as potent as hadacidin[2], about four times as potent as sodium L-tenuazonate[8], and about one-half as potent as mitomycin C[9]. It is much more cytotoxic than either hadacidin or tenuazonic acid[6]. Our tests show it to be about one-third as cytotoxic as mitomycin C against KB cells.

Anti-tumor compound 593A, though not inhibitory to primary growth of H. Ep. #3, inhibits metastasis of the tumor to chick embryo lung. This type of action could be important as an adjunct to present methods of therapy which focus on the primary tumor.

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References

1) STAPLEY, E. O.; T. C. DEMNY, A. K. MILLER & H. B. WOODRUFF : Histidomycin. I. Production by *Nocardia histidans* and biological characterization. Antimicr. Agents & Chemoth.-1966 : 595～602, 1967
2) GITTERMAN, C. O.; E. L. DULANEY, E. A. KACZKA, D. HENDLIN & H. B. WOODRUFF : The human tumor-egg host system. II. Discovery and properties of a new anti-tumor agent, hadacidin. Proc. Soc. Exp. Biol. & Med. 109 : 852～855, 1962
3) TOOLAN, H. W. : Permanently transplantable human tumors maintained in conditioned heterologous hosts : H. Chon. #1, H. Ep. #4, and H. Ad. #1. Cancer Res. 17 : 418～420, 1957
4) TOOLAN, H. W. : Transplantable human neoplasms maintained in cortisone-treated laboratory animals : H. S. #1; H. Ep. #1; H. Ep. #2; H. Ep. #3; and H. Emb. Rh. #1. Cancer Res. 14 : 660～666, 1954
5) GITTERMAN, C. O.; S. V. LUELL & R. N. ARISON : Invasive and metastatic properties of human transplantable tumors in the chick embryo. Proc. Amer. Assoc. Cancer Res. 9 : 24, 1968
6) GITTERMAN, C. O.; E. L. DULANEY, E. A. KACZKA, G. W. CAMPBELL, D. HENDLIN & H. B. WOODRUFF : The human tumor-egg host system. III. Tumor-inhibitory properties of tenuazonic acid. Cancer Res. 24 : 440～443, 1964
7) EAGLE, H. : Propagation in a fluid medium of a human epidermoid carcinoma, Strain KB. Proc. Soc. Exp. Biol. Med. 89 : 362～364, 1955
8) GITTERMAN, C. O. : Anti-tumor, cytotoxic, and antibacterial activities of tenuazonic acid and congeneric tetramic acids. J. Med. Chem. 8 : 483～486, 1965
9) WAGNER, A. F. & C. O. GITTERMAN : Methyl mitomycin. Antibiot. & Chemoth. 12 : 464～468, 1962