EVALUATION OF SUCCINYL NEOCARZINOSTATIN *IN VIVO*

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The toxicity of the bis-succinyl derivative of the protein antibiotic, neocarzinostatin, was compared with the parent compound, neocarzinostatin (NCS), in rats. The derivative was found to be about two to five fold more active than NCS *in vivo*. The antitumor activity in rats bearing eleven distinct YOSHIDA hepatoma ascitic cell lines was tested under four possible combinations with regard to sites of drug and tumor cell administration. The results indicate that the antitumor spectrum of the derivative had changed slightly. Antitumor activity in mice was also tested with L1210 and P388 lymphatic leukemia, and with B16 melanocarcinoma. When the effect of the derivative was compared with parental NCS at the molecular level with respect to the inhibition of DNA synthesis *in vitro*, the specific activities of the two were found to be almost identical. These results were interpreted to indicate that the succinyl derivative of NCS was more stable to inactivation and proteolytic break-down *in vivo* than NCS as observed previously in *in vitro* studies.

We reported previously the preparation and chemical and biological characterization of the bis-succinyl (N^{α}Ala 1, N^{ε}Lys 20) neocarzinostatin derivative of the antitumor antibiotic, neocarzinostatin (NCS). It was found in comparison with NCS that modification of the two amino groups in NCS did not alter the effect of the drug against cultured cell lines at 0.25 μg/ml level, but a considerable decrease was observed in antibacterial activity[1,2]. Furthermore, the stability of the derivative against proteolytic digestion by blood or serum enzymes was enhanced due to succinylation of the free amino groups in NCS[3,4]. These results prompted an investigation of the potential usefulness of the derivative *in vivo*.

In the present communication we wish to report the toxicity and antitumor activity of the succinyl derivative of neocarzinostatin in comparison with NCS. Furthermore, the action of the two compounds upon DNA synthesis was also studied with a cultured lymphoblastoid cell line.

Materials and Methods

Succinyl NCS: Bis-succinyl neocarzinostatin (SUC) was prepared as described previously[2]. NCS (Lot. T-58) was obtained from Kayaku Antibiotic Research Laboratories, Tokyo, and the same lot was used throughout the investigation.

Toxicity: Acute toxicity of SUC and NCS was examined with a single dose of drug given to female Donryu rats (*ca.* 150 g, five or six rats per group) which had been inoculated intraperitoneally (i.p.) with 10^6 YOSHIDA sarcoma cells 72 hours prior to the tests. The administration of the drugs was carried out *via* i.p., or i.v. (tail), at doses of 0.63, 1.25, 2.5, 5, 10 and 20 mg/kg or orally at doses of 5, 10, 50, 100, 250 and 500 mg/kg, respectively. Cytological observations of the tumor cells were also conducted microscopically at about $\times 100$ after staining with WRIGHT-GIEMSA solution.

Antitumor Activity in Rats: Assays were employed to establish the antitumor spectrum of SUC

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in comparison with NCS. The tumors maintained in Donryu rats as ascitic hepatomas were: AH13, AH41C, AH44, AH60C, AH66, AH66F, AH109A, AH130, AH272, AH414, and AH7974. Each tumor is unique in its oncological properties *in vivo*[5]. They were obtained from ascitic fluid and inoculated either i.p. (10^6 cells), or i.v. (10^7 cells) (tail). SUC or NCS was dissolved in saline and administered 72 hours later. The concentration of drug used was based on the maximum tolerable dose (MTD) as described above, *i.e.*, one tenth of MTD was given once daily for 10 days. The route of drug administration was either i.p. or i.v. and the inoculation sites of the tumors were also either i.p. or i.v., namely the combinations of the sites of administrations (tumor/drug) were i.p./i.p., i.p./i.v., i.v./i.p. and i.v./i.v. Six female rats were used per group. Animals were observed over a period of 60 days.

Antitumor Activity in Mice: The procedures employed were described in a previous publication[6] and the assays were carried out by the Drug Research and Development Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md., U.S.A. The strains of mice used for the expriments were BDF_1 (male), DCF_1 (male) and BDF_1 (female), while the tumor lines used were L1210, P388 (both leukemia) and B16 (melanocarcinoma). Inoculations were made intraperitoneally in all three cases. Cells were obtained (1) by adding 10 ml of a cold HANKS' balanced salt solution to 1 g of non-necrotic tumor (solid mass of B16) and homogenizing followed by filtration with wire gauze and implantation of 0.5 ml, or (2) from the ascitic fluid (L1210 and P388) derived from the tumor-bearing mice with an inoculum size of 10^5 (L1210) and 10^6 (P388) cells. The drug was dissolved in saline and administered i.p. once daily at doses of 2.0, 1.0, 0.5, 0.25 and 0.13 mg/kg, respectively for 9 days beginning one day after tumor inoculation. The effect of the drug was measured by the prolongation [on day 20 (L1210), on day 30 (P388), or on day 60 (B16)] of the median survival time (B16 and P388) and mean survival time (L1210) over control. The data are expressed as percent of test/control.

Inhibition of DNA Synthesis: The effect of SUC and NCS on DNA synthesis was compared *in vitro*. Cells used were a lymphoblastoid cell line, P3HR-1, which originated from human Burkitt's lymphoma, and were maintained in floating culture in RPMI-1640 (Gibco Inc., Grand Island, N.Y.) with 10% fetal calf serum[1]. An initial cell density of 2×10^5 cells/ml were used. 6-[^{3}H] Thymidine, (specific activity, 1.40 μCi/μ mole; Daiichi Pure Chemical Inc. Co., Tokyo) was added at a final concentration of 0.3 μCi/ml to the media. Incorporation of tritium-labeled thymidine into DNA was studied in the presence and absence of NCS or SUC. The incorporation into DNA was terminated by addition of 30% trichloroacetic acid to a final concentration of 5% and the trichloroacetic acid precipitable samples were filtered on glass-fiber filters (GB-100, Toyo Scientific Inc. Co., Tokyo) and washed with 5% trichloroacetic acid followed by washing with cold ethanol and ethyl ether. Radiolabel on the filter pads was measured by liquid scintillation counting.

Results

Toxicity

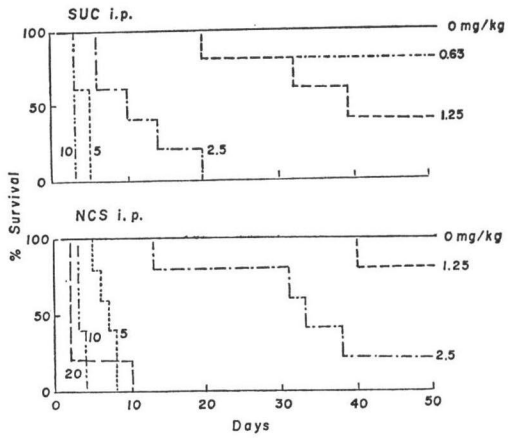
The doses of NCS and SUC given i.p. and the percent survival-days for rats are shown in Fig. 1; the median LD_{50} and average survival days are summarized in Table 1. When a dose of 5 mg/kg of the derivative was given i.p., edema in the intestines and shrinkage of the spleen were observed. Cytologically the predominant effects observed on the tumor cells, which persisted for 24~72 hours, were cell swelling and chromosomal abnormalities. Intravenous administration of SUC even at 1 mg/kg, resulted in death of rats; cytological effects against ascitic tumor cells were only noted at 10 mg/kg or more, however. Orally the maximum tolerable dose seen with SUC was at 100 mg/kg with no cytological effects ob-

Table 1. Toxicity of neocarzinostatin and its succinyl derivative in rats.

Evaluation methods	NCS	SUC
Average survival (Days)		
Dose, i.p. one injection only:		
2.5 (mg/kg)	>32	9.2
5.0 (mg/kg)	6.8	4.2
LD_{50} (mg/kg, i.p.)	1.72	0.96

served. It is noteworthy that there was a considerable deviation in survival time with respect to the toxicity of both drugs (Fig. 1). The death of rat due to the toxicity of NCS or SUC occurred even after 30 days following administration of the drug.

Fig. 1. Comparison of toxicity of neocarzinostatin and its succinyl derivative as judged by survival days.
NCS and SUC mean neocarzinostatin and its succinyl derivative.



Antitumor Activity

The results with SUC and NCS are summarized in Tables 2 and 3. Both drugs were effective only in a single system; i.p.-tumor/i.p.-drug. In the studies with 6 or 7 of the tumor strains, more than 50% of the rats tested showed a greater than 300% increase in their expected life-span. On the other hand, with 5 tumor strains (SUC) or three tumor lines (NCS) there was more than a 200% increase. Details are seen in Table 2. In the case of AH41C, the effect of SUC was found to be positive cytologically but prolongation of the life-span was moderate (50% of rats showed more than 200% increase in the expected life-span); a finding which is similar to that observed with NCS. In rats with AH-109A tumor, SUC possessed an antitumor activity greater than NCS; thus more than 50% of the rats exhibited 200% prolongation of the expected life span.

As seen in Table 2, a 0.1 mg/dose of SUC was almost equivalent to NCS employed at 0.5 mg/dose. In all other combinations (i.p./i.v., i.v./i.p. and i.v./i.v.), the antitumor effects of NCS and SUC were about the same at 0.5 and 0.1 mg/kg dose, respectively.

The effect of SUC on three different tumor systems in mice is shown in Table 3. The derivative possessed activity on L1210, P388 and the B16 melanocarcinoma which was comparable to NCS[7]. In fact, three complete cures out of six mice injected with the P388 strain were seen. Death due to

Table 2. Increase of life span of Donryu rats bearing various type of ascitic form of YOSHIDA hepatoma (AH-) by treatment of neocarzinostatin (NCS) and its succinyl derivative (SUC)

Sites of injections		Drug	Dose per injection[a]	13	130	272	44	66F	66	7974	41C	60C	109A	414
Tumor	Drug													
ip	ip	SUC	0.1	+	+	±	+	+	±	+	±	±	±	+
		NCS	0.5	+	+	±	+	+	+	+	±	±	−	+
ip	iv	SUC	0.1	−	−	−	−	−	−	−	−	−	−	−
		NCS	0.5	±	−	−	−	−	±	−	−	−	−	−
iv	ip	SUC	0.1	−	−	−	+	−	−	±	−	−	−	NT[b]
		NCS	0.5	−	−	−	+	−	−	−	−	−	−	NT
iv	iv	SUC	0.1	−	−	−	+	−	−	−	−	−	−	NT
		NCS	0.5	−	±	−	±	−	−	−	−	−	−	NT

a) Dose/injection expressed in mg/kg. All drug injections were once daily, for ten days.
b) Not tested. + sign indicate at least 50% of rat survived over sixty days (about 300% increase of life span of the control). ± signs indicate that more than 50% of the treated rats survived 200% of the life span of the control (100%).

Table 3. Antitumor effects of succinyl neocarzinostatin in mice bearing leukemia L1210 and P388 and melanocarcinoma B16

Tumors	Dose per injection[a)]	Toxicity day survivor at day 5	Animal weight difference (g)	Tumor evaluation[b)]		Survival
			(test-cont)	Test	Control	Test/Control(%)
B16 melanocarcinoma	2.0	10/10	−3.6	36.0	20.7	173
	1.0	10/10	−2.4	35.4	20.7	171
	0.5	10/10	−0.8	40.3	20.7	194
	0.25	10/10	−0.9	37.8	20.7	182
	0.13	10/10	+0.2	34.0	20.7	164
L1210 leukemia	2.0	6/6	−2.4	9.8	9.4	104
	1.0	6/6	−1.9	13.5	9.4	143
	0.5	6/6	−2.2	13.7	9.4	145
	0.25	6/6	−2.1	12.0	9.4	127
	0.13	6/6	−2.3	12.5	9.4	132
P388	2.0	6/6	−2.1	29.7	11.6	256*
	1.0	6/6	−1.2	15.0	11.6	129
	0.5	6/6	−0.4	22.0	11.6	189
	0.25	6/6	−0.1	19.0	11.6	163
	0.13	6/6	+0.7	17.0	11.6	146

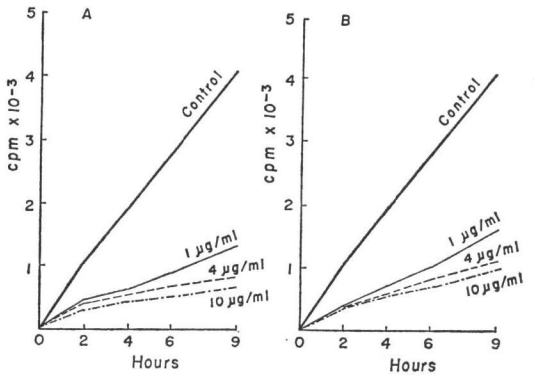
a) mg SUC-NCS/kg. b) Survival days.
* Three mice were completely cured. Other details are in the text.

the toxic effect of the drug was not observed at the dose levels used.

Effect on DNA Synthesis

As shown in Fig. 2A and 2B the inhibitory effect of NCS and SUC on DNA synthesis as judged by the rate of thymidine incorporation was almost the same.

Fig. 2. (A) Incorporation of [^{3}H]-TdR into DNA (5% trichloro acetic acid insoluble fraction) and effect of neocarzinostatin in lymphoblastoid P3HR-1 cells. (B) Incorporation of [^{3}H]-TdR into DNA and effect of succinyl neocarzinostatin in P3HR-1 cells. Control; no drug. Two experiments were averaged. The radioactivity count (cpm) was expressed per tube.



Discussion

The results of the experiments on DNA synthesis in mammalian cells in culture show that both SUC and NCS exhibit the same inhibitory activity to the same extent (at 1,4,10 μg/ml) (Fig. 2. A, B). On the other hand, a considerable difference was observed in their activity *in vivo* (Table 1, Fig. 1. A, B). Namely, in comparison with NCS there was about a two fold increase in the toxicity and five fold increase in the antitumor activity of SUC (Table 2). These results are in accord with the fact that SUC exhibited greater stability in serum or blood *in vitro*[4]. This can be explained by the fact that succinylation of the lysine at position 20 in NCS may prevent proteolysis. Perhaps such a modification of the enzyme binding site may render SUC unfavorable as substrate for the serine type proteolytic enzymes[3,4]. Analogously, NCS undergoes inactivation at a slower rate in serum or blood in the

presence of DFP (diisopropyl fluorophosphate), which is an inhibitor of serine-type proteolytic enzymes[3].

The results shown in Table 2 indicate that either NCS or SUC is most effective when the drug is given very close to or *in situ* with the tumor cells (i.p./i.p.). We interpret this finding to mean that NCS or SUC has a very short half-life *in vivo*, because of the instability due to both proteolytic degradation[3,4] and extremely rapid renal clearance[8]. When NCS or SUC is given at site remote from target tumor site (*eg.* i.p./i.v. or i.v./i.p.), the drug is inactivated and also excreted into urine before reaching the tumor site, thus it exhibited little effect. When the drug is given i.v. and tumors inoculated i.v., the extremely short half-life of the drug in plasma[3,8,9] also resulted little effect because most of the AH-tumor cells would manage to escape from the susceptible phase of the cell cycle (S-phase and G-2) thus unaffected.

The present results indicate, therefore, that NCS and also SUC should be more effective, in view of its short half-life, when administered directly into the tumor or very near to the tumor cells in order to facilitate direct contact. The results also reveal that SUC possessed a similar antitumor spectrum to NCS which may be due to similar distribution *in vivo* because of similar physicochemical properties and molecular weight. The strongly hydrophilic nature of NCS[10] is not changed by the addition of two succinyl residues which are also hydrophilic.

The observation that both NCS and SUC inhibit DNA synthesis in a lymphoblastoid cell line suggests to us that other biological activities (*eg.* DNA degradation, inhibition of mitosis) exhibited by SUC may also be comparable to those found in studies with NCS using HeLa cells[11]. By contrast, however, the altered pharmacokinetic properties, namely increased *in vitro* stability[4] and also enhanced *in vivo* activity of the modified derivative have been clarified. Based on these results, more advantageous dose regimen, different from that of NCS, for SUC should be designed in its clinical applications.

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