CHEMICAL AND BIOLOGICAL CHARACTERIZATION OF SUCCINYL NEOCARZINOSTATIN

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The proteinous antibiotic neocarzinostatin was succinylated using succinic anhydride, and two *bis*-succinyl compounds, neocarzinostatin derivatives (SUC-I and SUC-II), were obtained. The biological and chemical properties of SUC-I and SUC-II were investigated after separation by column chromatography. The only free amino groups in neocarzinostatin were succinylated completely in both derivatives. Amino terminal analysis, electrophoretic mobility and amino acid analysis were the same for both derivatives. However, only SUC-II was biologically active when assayed on three human cell lines and *Sarcina lutea*. Analysis by UV spectroscopy and circular dichroism indicated a difference in the molecular conformation, perhaps a slightly losened structure in SUC-I as compared with SUC-II. These isologous derivatives with different biological and optical activity have also been observed in neocarzinostatin. SUC-I seems to be a counterpart of "pre-NCS", an inactive form of NCS. SUC-II differs from NCS in being completely devoid of cytolytic and cytocidal activity. The retention of biological activity in *bis*-N-succinyl neocarzinostatin, in which all amino groups are bolocked, indicates that in NCS free amino groups are not essential for activity.

Among proteinous antibiotics[1~3] with antitumor activity*, neocarzinostatin (NCS) has been extensively studied. The amino acid sequence[4] and conformation[5] have recently been reported by the present author and others. Clinical reports[6,7] have indicated greatest effectiveness of the drug in acute myelogenous leukemia. A recent report[7] indicated complete remission in 45% of the cases treated with NCS alone at a dosage of 2~3 mg/day.

In the present investigation, NCS was subjected to chemical modification for two reasons: (a) to find a more beneficial derivative, and (b) to investigate structural requirement for activity.

Chemical characterization[8] and amino acid sequence[4] studies showed that there are only two free amino groups in the molecule, one as an N-terminal amino group on an alanyl residue, the other as an ε-amino group on lysine at the 20th position. Previously[9], we reported that the N-terminal amino group on alanine was unnecessary but the ε-NH_2 of lysine is required for the activity, a conclusion based on modification of the compound by fluorescein thiocarbamylation, dinitrophenylation[8] and deamination[9]. However, the present study has shown that when both of the amino functions were modified by succinyl groups, the antibiotic still retained biological activity against bacteria and mammalian cells. Thus, neither of the free amino groups are needed for activity provided that desuccinylation is not occurring. Two derivatives, SUC-I and II, were separated and studied. They are identical in gross chemical properties but differ spectroscopically and biologically.

Abbreviations used are: NCS; neocarzinostatin. Dansyl; 1-dimethylamino-naphthalene-5-sulfonyl. CM; Carboxymethyl. SUC-I and SUC-II; succinylated neocarzinostatin I and II respectively. CD; circular dichroism.

* Representatives of these with acidic nature are macromomycin[1], actinoxanthin[2], and lymphomycin[3].

Materials and Methods

Neocarzinostatin. Crude neocarzinostatin (Lot. 720756), obtained from Kayaku Antibiotic Research Laboratory (Tokyo, Japan), was purified in this laboratory by CM-cellulose chromatography[8]. The purity was confirmed by electrophoresis on cellulose acetate membranes (pH 4.6 and 8.6) and end-group analysis (alanine) by the dansyl-procedure[10,11] as described below.

Succinylation and purification of succinyl derivatives[12,13]. The succinyl derivatives were obtained according to the method published earlier[13]. The mole ratio of succinic anhydride to mole of amino group was 1.87 and the reaction was carried out at about 12°C and pH 7.2~8.0 for 30 minutes followed by dialysis and separation using CM-cellulose column (1.5×31.5 cm). The two major derivatives, SUC-I and SUC-II, were obtained in 16 and 56% yield by elution with 0.05 M acetic acid. Unreacted NCS was less than 11%.

Dansylation[10]. The procedures of dansylation and identification of the dansyl amino acids were those described by WOODS and WANG[11]. The samples of protein were dissolved in 20 μl of 0.2 M $NaHCO_3$ solution. A 20-μl portion of dansyl chloride (Sigma Chemical, St. Louis, Mo., or Seikagaku Kogyo Ltd., Tokyo) solution (0.5% in acetone) was added and the reaction mixture allowed to stand at room temperature for 3 hours. The mixture was dried *in vacuo* over P_2O_5 and NaOH pellets. The dried material was hydrolysed in 6 N HCl at 110°C *in vacuo* for 17 hours followed by drying. Identification was carried out on a polyamide sheet (Polyamide UF plate, Wako Pure Chem. Ind. Ltd., Osaka, Japan).

Electrophoresis. Cellulose acetate membranes (Separax, Fuji Film Inc., Tokyo, Japan) were used. Electrophoresis was carried out under several conditions in the pH range of 4.3~8.8. Typical runs at pH 4.8 and 8.6 were as follows. [pH 4.8] : 0.05 M Na acetete buffer, 0.34 mA/cm, 200 V for 1 hour; [pH 8.6] : 0.04 M tris-veronal buffer, 0.14 mA/cm, 200 V for 50 minutes. The cellulose acetate membranes have a length of 11 cm and were fixed with 25% sulfosalicylic acid solution for about 30 minutes after the run, then stained either with 0.4% Coomassie brilliant blue (Sigma Chemical Co.) or ponceau 3R (Tokushu Chemical, Tokyo, Japan) in 7% acetic acid. The trichloroacetic acid solution (5%) was used to rinse the strips.

Amino acid analysis. Amino acid analysis was performed according to the method of MOORE and STEIN[14]. About 1 mg of succinylated NCS or control NCS were hydrolysed in double distilled 6N HCl, at 110°C *in vacuo*, for 18 hours. The hydrolysate after drying and redissolving in 0.01 M HCl was applied to an amino acid analyzer.

Optical measurements. In order to characterize the conformation of the derivatives, ultraviolet absorption spectra and circular dichroism of the derivatives were measured in a physiological buffer solution (0.15 M NaCl in 0.01 M Na phosphate buffer, pH 7.0) using Hitachi Model 124 (double beam) spectrophotometer and JASCO ORD/UV-5 with CD attachment, calibrated with D-10-camphor sulfonic acid (0.06%) and D-glucuronolacton (0.03%). The concentration of the derivatives was 0.4 mg/ml for CD and 0.057 mg/ml for ultraviolet adsorption measurements.

Biological activity. The biological activity of NCS and its succinylated derivatives was examined with three different mammalian cell lines of human origin and a strain of bacteria. He cell line, supplied by Prof. J. SATO of Okayama Univ. Medical School, Okayama, Japan, was derived from a human hepatoma specimen with an epithelial morphology and has been passaged for two years. Flow 2000 is a cell strain derived from human embryonic lung (Flow Laboratory, Bethesda, Md.) having fibroblastic morphology and diploid nature. P3HR-1 is a clonal cell line derived from P3J line which originated from BURKITT's lymphoma[15]. He and and P3HR-1 cells were maintained and grown in EAGLE's minimum essential medium (Gibco, Grand Island, N.Y.) enriched with bovine serum (obtained locally). Flow 2000 cells were carried similarly but enriched with 10% fetal calf serum (Microbial Associates or Flow Laboratories, Bethesda, Md.). Assays using He and Flow 2000 cells, both of which grow as a monolayer, were carried out using a Lab-Tek Microchamber (4 chamber type, 1.94 cm^2 each, Miles

Laboratories Inc., Ill.) under 5 % CO_2 atmosphere. P3HR-1 cells were grown and assayed in the stationary floating state in a rubber stoppered test tube (1.5×10 cm). To each chamber or the test tube was added 0.9 ml of cell suspension containing 5.4×10^4 He cells, 1.8×10^4 Flow 2000 or 2.2×10^5 P3HR-1 cells. Two to three hours were allowed for cells to attach to the glass surface. Then the compound dissolved in 0.1 ml of PBS (1/100 M phosphate buffer, 0.15 M NaCl, pH 7.0) was added and incubated at 37°C. The inoculum has a cell population equivalent to one fifth to one fourth of the saturation density for the given surface area or volume. The doubling times of He, Flow 2000 and P3HR-1 cell are approximately 36, 20 and 20 hours respectively. The saturation densities of the cells under the present conditions were: He cells; $1.5\times10^5/cm^2$, Flow 2000 cells; $1.5\times10^5/cm^2$, P3HR-1; $8.5\times10^5/ml$, and they reached the saturation density in about two to three days in all cases. Viability was more than 95 % in all cases. The He and Flow 2000 cells were examined for morphological changes (appearance of atypical epithelial or atypical fibroblastic character, swelling of cell volume and budding in He or Flow 2000), cell proliferation (inhibition of cell multiplication) and cytotoxicity (cell lysis, cell death or cell shrinkage in volume) under a low-power microscope. The effect on P3HR-1 cells was quantitated by cell count and percent viability. The cell counts were carried out using a hemocytometer under a light microscope. The viable cells were judged by trypan blue staining (exclusion method). Colony formation of these cell lines has not been well worked out and thus could not be used in this study. *Sarcina lutea* PCI-1001 is one of the most susceptible bacteria to NCS[16]. The assay was carried out on a nutrient agar plate (Nissan, Nissui Seiyaku K.K., Tokyo, Japan) which was preseeded with an aliquot of fully grown *S. lutea* (seed culture) suspended in trypticase soy broth (Difco, Detroit, Mich.). Each paper disc was moistened with an aliquot of the solution containing an appropriate amount of the compound and then placed on a agar plate followed by incubation at 4°C for 2 hours to allow the compound to diffuse. The agar plates were then incubated at 37°C overnight. The inhibition zone diameters on the agar plate were compared with those of standard NCS. The log of concentration of standard NCS and the diameter (mm) of inhibition zones exhibited a linear relationship up to a concentration of 100 μg/ml.

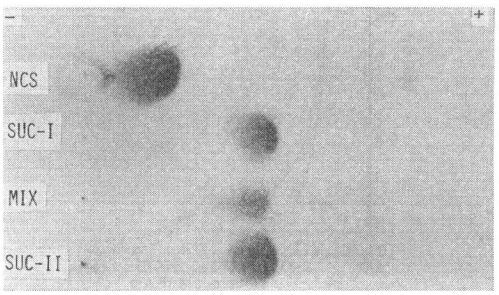
Results

Electrophoresis of Succinyl NCS

Electrophoresis on cellulose acetate strips between pH 4.3 and 8.8 revealed that SUC-I and SUC-II had the same electrophoretic mobility, but both were definitely different from NCS and more acidic (Fig. 1). These results indicate that both SUC-I and SUC-II have the same net charge.

Fig. 1. A representative electrophoresis of neocarzinostatin and *bis*-succinyl derivatives.

The run was carried out on a cellulose acetate membrane at pH 4.6, for 1 hour, at room temperature and 200V. The staining was with coomassie blue in 7 % acetic acid. MIX is a mixture of SUC-I and II.



Amino Terminal Analysis by Dansyl Procedure

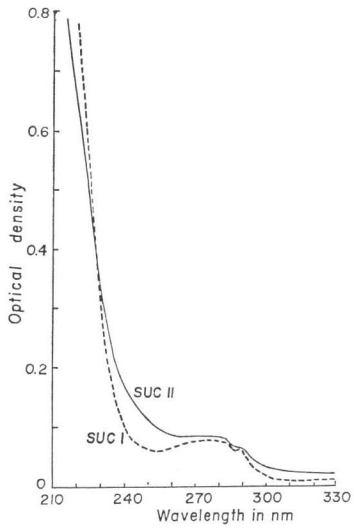
The analysis of the dansylated materials showed that only NCS yielded dansyl-alanine and ε-dansyl-lysine, while neither SUC-I nor SUC-II gave any detectable dansyl derivatives. These results were in agreement with the increased electrophoretic character of SUC-I and SUC-II.

Table 1. Amino acid composition

Amino acids	Number of residues per mole of protein			
	NCS[a]	NCS[b]	SUC-I[b]	SUC-II[b]
Lys	1	1.02	1.00	1.03
His	0	0.00	0.00	0.00
Arg	3	2.82	2.88	2.62
Asp	11	11.7	12.1	11.9
Thr*	12	11.5	12.0	11.5
Ser*	10	9.99	10.2	9.75
Glu	5	5.00	5.32	5.00
Pro	4	4.80	4.82	4.54
Gly	15	14.0	14.5	14.2
Ala	17	16.4	16.9	16.0
1/2 Cys	4	3.10	3.65	3.76
Val*	12	11.5	11.8	11.5
Met	0	0.00	0.00	0.00
Ile	1	0.95	1.01	1.00
Leu	6	5.70	5.76	5.74
Tyr	1	1.04	1.10	1.04
Phe	5	4.37	4.75	4.62
Trp*	2	—	(2.0)	(2.0)
Total	109			

a) Calculated from the amino acid sequence (15).
b) Found in the present analyses.
* Values of Thr, Ser and Val were corrected by 2%, 10%, 8% respectively to that obtained in the experiments. Trp values by spectroscopy.

Fig. 2. Ultraviolet absorption spectra of SUC-I and SUC-II at 0.057 mg/ml in 0.01M Na-phosphate buffer (pH 7.0) containing 0.15 M NaCl



Amino Acid Analysis

The results of amino acid analyses showed that SUC-I, SUC-II and NCS were essentially identical within experimental error (Table 1). The succinyl groups were hydrolysed easily in 6 N HCl at 110°C as expcected. The results proved that SUC-I and SUC-II although blocked at amino functions retained the rest of the peptide linkages intact.

Ultraviolet Absorption and CD Spectra

Differences in UV absorption of the two derivatives between 235 nm and 260 nm, and below 225 nm are clearly seen (Fig. 2). CD Patterns of the two (Fig. 3) are similar to those of NCS[5,17] with a through at about 263 nm, and a red-shift in λ_{min} in SUC-I (SUC-II, 266 nm to SUC-I, 274 nm). A decrease of 21% in the magnitude of ellipticity at about 265 nm of SUC-I from that of SUC-II (calculation based on the original CD spectra) was observed which resulted in the apparent shift in λ_{min} of $[\theta]$ to 274 nm (see arrows in Fig. 3). These differences in the optical properties of the derivatives probably relate to a difference in their conformation.

Biological Activity

A summary of the assays is shown in Table 2.

Fig. 3. Ultraviolet CD spectra of SUC-I and SUC-II at 0.4 mg/ml in 0.01 M Na phosphate buffer (pH 7.0) containing 0.15 M NaCl. Arrows show the minimum points of the troughs.

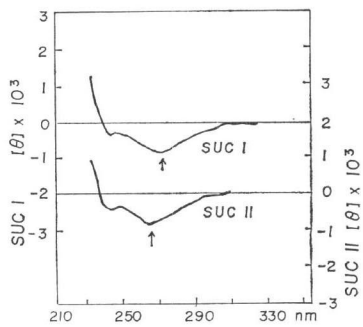


Table 2. Minimum inhibitory concentration

Cell	Drugs (μg/ml)		
	NCS	SUC-I	SUC-II
S. lutea[a)]	<0.25	>100	<0.40
He[b)]	<0.25	>4.0	<0.25
Flow[b)]	<0.25	>4.0	≦0.25

a) Drugs were assayed on an agar plate for complete growth inhibition of *Sarcina lutea* as described in the text.

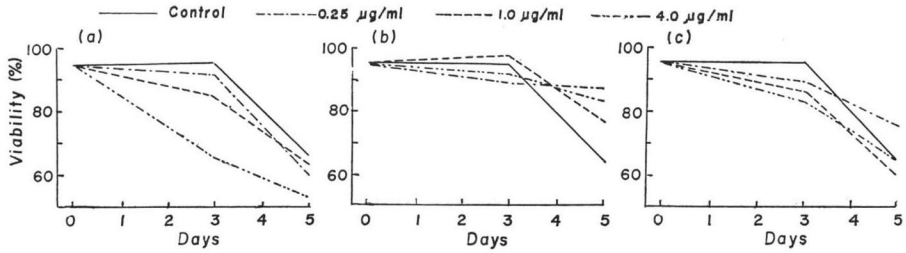
b) He cells and Flow 2000 were subjected to drug treatment and assayed by morphological changes such as atypical cell feature for a certain cell line, increase or shrinkage in cell volume, formation of granules or budding. Effects of drugs were judged positive when about more than 90% of the population exhibited morphological change at day 3 and later. These morphological changes were accompanied by slower multiplication of cells. See details in the text.

He cells: Since assays were carried out with 4-fold diluted samples (4.0, 1.0, 0.25 and 0 μg/ml), the determination of the exact minimal effective concentration to produce any specific morphological change was not attempted. The cells in the Lab-Tek chambers were incubated at 37°C, and as described above examined *in situ* and compared with control twice daily. Without the drugs, the cells were homogeneous with typical epithelial features. They reached saturation density in two to three days. Parental NCS exerted its action at 0.25 μg/ml even at day 1 (24 hours later) when elongated or enlarged cell morphology became apparent. NCS at 4.0 μg/ml was enough to cause cell shrinkage at day 3. The inhibitory effect on cell multiplication of SUC-II at 0.25 μg/ml was clear from the sparse cell population at day 3, whereas at higher doses (1.0 and 4.0 μg) enlarged or elongated cell structure and budding of cells were typical characteristics. By day 5, these morphological changes of He cells appeared also at 0.25 μg/ml. No cell shrinkage at any dose level with SUC-II was observed. Based on these observations the activity of SUC-II seems to be about one fourth that of NCS. SUC-I was not active at the dose levels tested.

Flow 2000: These fibroblastoid cells were grown also in the Lab-Tek microchamber and the effect of the drug was observed as described above *in situ*. The effect of NCS on the inhibition of cell multiplication was clear at 1 μg/ml after 24 hours and at 0.25 μg/ml after 48 hours (day 2), whereas the effect of SUC-II became apparent at day 3 at all doses (0.25~4.0 μg/ml). The cytotoxicity of NCS became clear from shrunken cells at 4.0 μg/ml by day 2 and at 1.0 μg/ml by day 4~5, but SUC-II failed to show cell shrinkage at 4.0 μg/ml even after 6 days. No effect was observed for SUC-I.

P3HR-1: The results from the average of triplicate experiments showed that at day 3 NCS exhibited a dose response in viable counts (cytocidal toxicity) at 1.0 and 4.0 μg/ml (Fig. 4) as judged by trypan blue staining. SUC-I and SUC-II derivatives resulted no cell-killing. At day 5, cell death became progressively dominant and thus no clear "dose responese" is seen

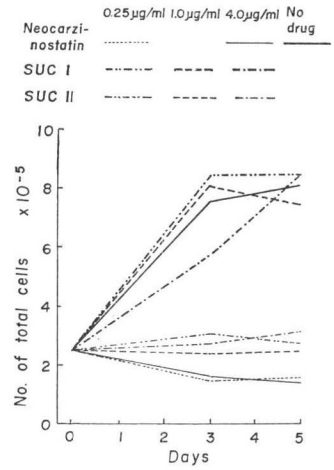
Fig. 4. Viability of P3HR-1 cells.
All values are average of three runs. The values at 0 are for the inoculum. Cells were treated with (a) neocarzinostatin, (b) SUC-I, and (c) SUC-II.



for NCS (Fig. 4a). SUC-I, which is considered an inactive derivative, seems to show enhanced cell viability (75~90 %) at all concentrations tested at day 5 even when the cells without drug started to die (viability of 64 %). SUC-II at lower concentrations (0.25 and 1.0 μg/ml) also seemed to show higher viability than that of NCS.

When the total numbers of cells were counted it was found that SUC-II inhibited mitosis completely at 0.25 μg/ml as well as higher doses because the number of cells did not increase from that of inoculum (Fig. 5). The SUC-II derivative seems as active as NCS at 0.25 μg/ml in its cytostatic action (Fig. 5) but devoid of cytolytic action. A decrease in the total number of cells when treated with NCS (Fig. 5) seems to be caused by cell lysis (see discussion). The phenomenon of decreased cell population was not observed with SUC-II at sixteen times higher dose level than NCS. Enlarged cell volume was observed at all doses of SUC-II and at low doses (0.25 and 1.0 μg/ml) of NCS in P3HR-1 line.

Fig. 5. Drug treatments and numbers of total counts of P3HR-1 cells. The percent viability in Fig. 3 is based on this total cell number.



Sarcina lutea: SUC-II retained about 20% of the growth inhibitory activity of the original NCS on the basis of the procedure described above. SUC-I lost its activity completely even at 100 μg/ml while SUC-II was still active at 0.4 μg/ml.

Discussion

Two Succinylated Derivatives, SUC-I and SUC-II

The blocking of amino groups in SUC-I and SUC-II was revealed by the dansyl procedure and electrophoresis. The amino acid analysis proved that SUC-I and SUC-II were identical. This indicates that the entire polypeptide chain of SUC-I or SUC-II was identical to that of parental NCS (see Table 1). The difference between SUC-I and SUC-II, in addition to the elution profile of CM-cellulose column chromatography as described before[13], was found in the optical properties. In the CD study, SUC-I appears to have a looser gross structure than SUC-II. The trough in ellipticity of SUC-II near 266 nm decreased considerably; which could be interpreted as a loosened state (exposed to the exterior of the molecule) of a chromophore residue, most likely a phenylalanyl residue[5]. In addition UV absorption of the two derivatives differed considerably which could indicate considerable alteration in the conformation[18,19]. $\Delta\varepsilon_{\text{mole(SUC-I-SUC-II)}}$ at about 250 nm is too large to account for a perturbed single chromophore residue (*e.g.* exposure to aqueous environment of tryptophan) although this may contribute a part of it*. Another residue which might be responsible for this could be tyrosine residue at 32. But it seems still fixed (buried state) in SUC-I from the CD data due to the trough at 274 nm, and thus it should not influence the chromicity in that region. Then the most likely element for these alterations in UV absorption is an altered conformation of the back bone

* Tryptophan 46, which is involved in the biological activity, is more or less buried, while the other tryptophan residue 79 is exposed to the solvent. The latter residue is not involved in the biological activity[20,21].

peptide chromophore[18,19]. The above results suggest that the biologically inactive SUC-I is a loosened form of SUC-II. This seems to be similar to the relationship between NCS and pre-NCS. In careful chromatography on CM-cellulose columns, NCS separates into pre-NCS and NCS. Pre-NCS is not active and SUC-I may represent the succinylation of this component. Conformational, chemical and biological analysis of NCS and pre-NCS is very similar to the present case (MAEDA *et al.* unpublished).

Biological Activities

SUC-II remained effective against bacteria and mammalian cells, but its potency is about 20~25 % of NCS. Contrary to NCS, no lytic effect was observed in mammalian cell systems with SUC-II. The enlarged cells seen after treatment with SUC-II may be an indication of protein-synthesis with inhibition of DNA synthesis and mitosis[22~25].

The human embryonic lung cell, Flow 2000, was also susceptible to SUC-II but only after three days at all three dose levels tested. Although a good growth of the cells was seen until day 2, there was an indication of slightly enlarged cell volume (structure) whereas NCS retarded growth more and gave a sparse cell population at day 1 at 1.0 and 4.0 μg/ml. At 4.0 μg/ml of NCS, the cells began to show shrunken cell volume at day 2. An arbitrary minimum effective concentration of SUC-II for any morphological change of 90 % of the cells appeared to be about 0.25 μg/ml or less although most of the cells seem viable even at day 7 in spite of retarded growth. These diploid fibroblastoid cells seem relatively resistant to the toxicity of SUC-II. NCS is, however, toxic to this non-malignant cell line as well as the hepatoma derived He cells non-selectively, contrary to an earlier report[16].

In contrast to the cell line above, P3HR-1 yielded a more quantitative results. SUC-II at 4 μg/ml yielded a similar degree of viability as with NCS at 1 μg/ml in the P3HR-1 cell line (Fig. 4a, c). As shown in Fig. 5, a definite cytostatic effect was seen with NCS and SUC-II. The total number of cells, when treated with 0.25 μg/ml of SUC-II, remained unchanged indicating complete inhibition of mitosis (and perhaps DNA synthesis) similar to the report for NCS by SAIRENJI *et al.*[24] and KAWAI *et al.*[23] A concentration of 2.0 μg/ml of NCS was required for the complete inhibition of mitosis of HeLa S_3 cells, and lower concentrations (0.2~0.1 μg/ml) resulted in only temporary inhibition (HOMMA *et al.*[22]). This difference in the concentration may be due to timing difference. In my experiment, drug treatment was performed at an early period when the cell population was still sparse and perhaps at early log phase while in their experiment cells were two days old and at their late exponential growth. In other experiments using NCS and almost fully grown (two-day old) He cells, it was found that five to ten times higher drug concentration than the present dose level was required to observe the activity of the drug.

A slight decrease (10 %) in the total number of cells, presumably due to cell lysis, when P3HR-1 cells were treated with NCS at 0.25 to 4.0 μg/ml, appears significant (Fig. 5) and was observed also in other experiments[23,24]. But this decrease was not observed when the same cells were treated with 4.0 μg/ml of SUC-II, suggesting a lessened cytocidal toxicity (cell killing and lysis) for SUC-II (Fig. 4c). SUC-I has lost activity here again, but an interesting feature of SUC-I is the enhanced cell viability (Fig. 4b) over that of the controls.

Structure-Activity Relationship

As described above, SUC-II (*bis*-N-succinyl neocarzinostatin), retained biological activity despite its complete loss of free amino groups. These results confirm the previous finding that the N-terminal amino group of alanine is not required for the activity of NCS, and also indicate that the ε-amino group of lysine 20 is not essential for NCS activity, assuming that no reconversion of SUC-II to NCS occurs during the test.

Furthermore, the removal of the positive charges of amino groups and addition of negative charges have altered electronic properties of NCS, and consequently should have influenced the pharmacology such as stabilization against proteolysis or different permeability and circulation

properties. The fact that toxicity and activity are not modified in exactly parallel fashion indicates that the derivative should be evaluated *in vivo*.

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