Preparation of Succinyl Neocarzinostatin

HIROSHI MAEDA
Department of Microbiology, Kumamoto University Medical School, Kumamoto, Japan

Received for publication 8 November 1973

All amino groups in a proteinous antitumor antibiotic, neocarzinostatin, were reacted with succinic anhydride yielding bis-N-succinyl neocarzinostatin which retained biological activity in vitro against human cell lines and a bacterium. Amino groups in neocarzinostatin do not appear to play an important role in the activity.

Of the many high-molecular-weight antibiotics with antitumor activity, neocarzinostatin (NCS) has been studied most. The amino acid sequence and its conformation have recently been reported by this author et al. (8, 9). The effectiveness of the drug is most impressive when used in patients with acute leukemia, and complete remission in 50% of the patients treated with NCS alone has recently been reported (4).

In the present investigation, NCS was subjected to chemical modification with two purposes: (i) to look for a more beneficial derivative, and (ii) to investigate the structural requirements for activity. Chemical investigation has shown previously that there are only two free amino groups in the molecule, alanine 1 and lysine 20 (7, 9).

The chemical modification was carried out as follows (1, 6). A 1.35-mg (15 μmol) amount of succinic anhydride (Wako Pure Chemical Ind., Osaka, Japan) was added to a stirred solution of 0.15 M NaCl (5 ml) at 12°C containing 45 mg (4 μmol) of NCS (obtained from Kayaku Antibiotic Research Laboratories, Tokyo, and purified by O-(carboxymethyl) (CM)-cellulose chromatography in this laboratory. The pH of the solution was maintained between 7.2 and 8.0 with additions of 0.2 M NaHCO₃. The succinylation was terminated by dialysis against 0.05 M acetic acid at 2°C. The NCS derivative was purified by column chromatography on a CM-cellulose column (1.5 by 31.5 cm). Succinylated NCS was eluted first by using 0.05 M acetic acid, whereas untreated NCS in the same condition was eluted at higher pH (3.4 to 3.5) with 0.1 M acetic acid buffer (Fig. 1). As shown in Fig. 1, two major fractions, SUC-I and SUC-II, were obtained. Amino acid analysis (10) and amino-terminal determination by the dansyl chloride procedure (2, 11) showed no difference between the two; that is, the amino acid composition was the same as the original NCS, and the amino-terminal alanine and lysine 20 were found to be blocked by the succinyl group. Electrophoresis by using cellulose acetate membranes indicated identical mobility of the two in pH 4.3 to 8.8 buffer, but a definite change from NCS. The difference of SUC-I and SUC-II seems to be due to the conformation of the two derivatives. (The evidence is based on ultraviolet spectroscopy and circular dichroism. SUC-I has loosened conformation in comparison with

![Figure 1](http://aac.asm.org/)

**Fig. 1.** A column chromatography of succinylated neocarzinostatin made by using CM-cellulose. A vertical arrow indicates the position at which the original neocarzinostatin was eluted. Peaks I and II were designated as SUC-I and SUC-II. Overall recovery from the column was about 60%, and SUC-I and SUC-II constituted 16 and 56%, respectively. Other minor peaks were not investigated. In the fractionation, 2 ml/tube and 6 min each were allowed.
SUC-II [H. Maeda, 1974, J. Antibiot., in press].] In careful chromatography on CM-cellulose, NCS separates into pre-NCS and NCS. Pre-NCS is not active and SUC-I may be succinylation of this component. NCS preparation, free of pre-NCS, seems to generate pre-NCS during the recycled purification process (H. Maeda and J. Meienhofer, manuscript in preparation). Detailed physical and chemical characteristics will be published elsewhere.

Biological activities of SUC-II were detectable at 0.4 µg/ml (an arbitrary minimal inhibitory concentration) against Sarcina lutea (5), a gram-positive bacterium, and at 20 to 25% of the NCS dose against human lymphocytic cell clone P3HR-1 derived from a Burkitt lymphoma (3), as judged by cell multiplication. The bacteriological assay was carried out by using a nutrient agar and paper disks, whereas the tests with cells of P3HR-1 were carried out with Eagle minimal essential medium and 10% bovine serum in rubber-stoppered test tubes to which appropriate amounts of the drug were added. After incubation at 37°C, the cells were counted with a hemocytometer. SUC-II at 0.25 µg/ml or higher concentrations showed only cytostatic effect and, unlike NCS, no lytic effect was observed even at 16 µg/ml. SUC-I did not show any activity. In addition to the effect on the floating cell culture of P3HR-1, similar results were obtained with other human cell lines when tested at 0.25 µg/ml or higher concentration.

The results confirm that not only the N-terminal amino group, as described before, but also the ε-amino group of lysine do not play an important role in the activity. Furthermore, the increased negative charges of the derivatives should have altered the effects on the pharmacology of the drug such as its stability, permeability, and circulation (transport) properties. Therefore, these results warrant a further evaluation of SUC-II in vivo. Detailed accounts of chemical and biological characteristics of SUC-I and SUC-II will be published elsewhere.

I would like to thank Yasuo Koyama for his generous supply of NCS, Kenji Kuromizu, and Carol Moriarty of The Children’s Cancer Research Foundation in Boston for the amino acid analyses and for confirming the results of our dansyl chloride experiments. I would also like to thank Junichi Haga of our department for his excellent technical assistance in the experiments using P3HR-1 cells, and Yorio Hinuma for his advice in various aspects of this work.

LITERATURE CITED