INHIBITORS OF PROTEOLYTIC ENZYMES PREVENT THE INACTIVATION BY BLOOD OF THE PROTEIN ANTIBIOTIC NEOCARZINOSTATIN AND ITS SUCCINYL DERIVATIVE

Sir:

The protein antibiotic neocarzinostatin (NCS) and its succinyl derivative (bis N-succinyl derivative, SUC-NCS) were inactivated *in vitro* by blood or blood serum or cell homogenate with concomitant breakdown in molecular size through proteolysis[1,2]. The antibiotic is composed of all L-type amino acids as revealed by the experiment with D-amino acid oxidase[3], structural analysis (for susceptibility to proteolytic enzymes)[4], and studies on the biogenesis (biosynthesis *via* m-RNA→polysome)[5]; however, its very rigid conformation[6] prevents the usual hydrolysis by trypsin and chymotrypsin unless the protein is unfolded by reduction of disulfide bonds followed by alkylation[7].

Recently we have reported that both the inactivation and degradation can be inhibited by diisopropylfluorophosphate (DFP) and N-ethylmaleimide (NEM)[1]. DFP is a well known inhibitor of serine type proteolytic enzymes with extremely high toxicity to the nervous system, and NEM is an alkylating agent for SH groups, thus DFP and NEM are unfavorable candidates for combination chemotherapy with NCS.

We now wish to report that various protease inhibitors of microbial origin[8] can protect NCS from inactivation. Furthermore, SUC-NCS, whose activity against bacteria is 25~50% of NCS *in vitro*[9] has about five-times the antitumor activity and toxicity of NCS in rats bearing hepatoma cells (MAEDA *et al.*, in preparation). It has been shown clearly that the rate of inactivation by blood is much slower than for NCS.

Neocarzinostatin and the succinyl derivative were obtained as described previously[9]. Microbial protease inhibitors were obtained from Dr. MATSUSHIMA. Trasylol was obtained from Bayer Leverkusen, Germany and used after membrane filtration on UM-10 (Amicon, Ltd., Bedford, Mass, USA.) to remove unidentified low molecular weight chromophore and after five-fold concentration to yield 25,000 kallikrein inactivating units/ml.

Rabbit blood samples were used fresh with 10 units of heparin/ml. One volume of NCS or SUC-NCS dissolved in 0.15 M NaCl - 0.01 M phosphate buffer (PBS, pH 7.0) at 300 μg/ml or 600 μg/ml respectively, was added to two volumes of inhibitor-treated blood. The inhibitor-treated blood samples were prepared by mixing an equal volume of blood and the inhibitor solution, which contained 10 mg/ml of either pepstatin, leupeptin or antipain, or concentrated Trasylol as described, in PBS. The inhibitor and blood were reacted for 30 minutes at 37°C before mixing with the antibiotics. After incubation of the reaction mixures at 37°C, each sample was applied to discs in quadruple and assayed on MÜLLER-HINTON agar plates seeded with *Sarcina lutea* PCI 1001 subsequently allowing diffusion for 2~4 hour at 4°C and incubation at 37°C overnight. The residual potency of the antibiotic activity was quantitated by the diameter of inhibition zone around the discs as described previously[9].

The results showed less inactivation by blood with SUC-NCS than with NCS as shown in Figs. 1 and 2. Although not dramatic, the effect of the inhibitors either single or in combination is seen for all inhibitors tested. When the inhibitors were combined with NEM (all inhibitors used at 0.05% final concentration) less inactivation of NCS or SUC-NCS was

Fig. 1. Effect of protease inhibitors on inactivation of neocarzinostatin and its succinyl derivative by blood.

The blood was treated with one of the inhibitors and then was incubated with the antibiotic at the indicated time. T, P, A, L and C show Trasylol, pepstatin, antipain, leupeptin and control (none) respectively.

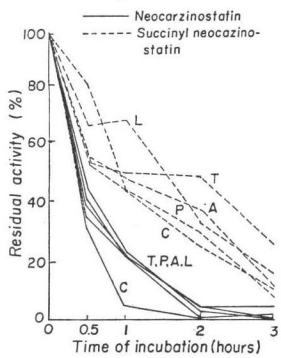
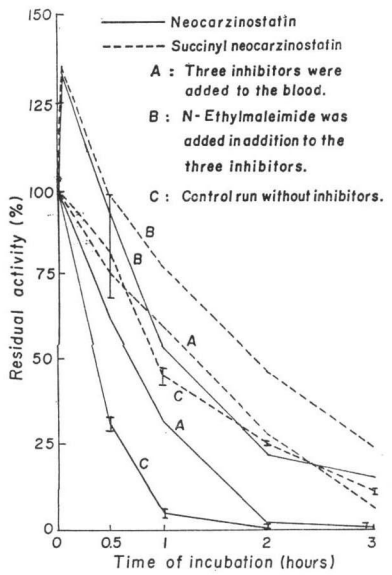


Fig. 2. Effect of protease inhibitors on inactivation of neocarzinostatin and its succinyl derivative.

The treatments were similar to Fig. 1, except that a mixture of three inhibitors (pepstatin, antipain and leupeptin) was added to the blood with or without N-ethylmaleimide.



observed. A considerable enhancement of the activity at 2 minutes compared to the value at 30 minutes in the presence of NEM may be explained in conjunction with the serum activation as reported.[1] No appreciable activation of NCS or SUC-NCS at about 2 minutes without NEM was observed in the presence of three microbial inhibitors (not shown in Fig. 2). The decreased rate of inactivation was noticed even with a single inhibitor, and a slow inactivation was noted for SUC-NCS. This may explain the 5-fold increase in *in vivo* activity of SUC-NCS (MAEDA *et al.*, in preparation).

The present results, therefore, suggest a possible application of these microbial inhibitors, which have very low toxicity in animals, in tumor chemotherapy in combination with protein antibiotics. Furthermore, it has been clearly demonstrated that blocking by succinylation of the ε-NH_2 of lysine 20 results in the masking of the enzyme binding site for protein hydrolysis with a prolonged half-life of the derivative *in vivo*.

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