AMINOPEPTIDASE ACTIVITY OF AN ANTITUMOR ANTIBIOTIC, C-1027

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An antitumor antibiotic C-1027, a complex protein consisting of an apoprotein and a non-covalently bound chromophore, showed some aminopeptidase activity, 1/15 (on the basis of activity per mg protein) that of porcine kidney enzyme [E.C. 3.4.11.2] by use of L-phenylalanyl 4-methyl-coumaryl-7-amide as the substrate. Neither the apoprotein alone nor the chromophore alone were active. Amastatin and bestatin but not leupeptin inhibited the activity. The enzyme activity of the holo-antibiotic, as opposed to that of the porcine kidney enzyme, was readily lost by UV irradiation, indicating that the intact structure of the chromophore was needed to maintain the native conformation of the holo-antibiotic. The cytotoxicity of the holo-antibiotic, but not that of the chromophore, to Ehrlich carcinoma cells *in vitro* was reduced to 1/5 by 1 μg/ml of amastatin which alone had no effect on cell growth. The porcine aminopeptidase was not cytotoxic at all even at higher concentrations (higher enzyme activities/ml). Amastatin possibly occupied the catalytic domain of the holo-antibiotic, interfering with the binding of the holo-antibiotic with some cell-surface protein(s). Amastatin did not inhibit the holo-antibiotic to cleave isolated DNA.

Antibiotic C-1027 is a new member of the group of antitumor antibiotics which includes neocarzinostatin (NCS), macromomycin (MCM), actinoxantin (AXN) and some others[1]. Each member of this group consists of a labile, cytotoxic chromophore capable of cleaving DNA *in vitro* and *in vivo* and an apoprotein (*ca.* 110 amino acid residues) which specifically binds to and stabilizes the cognate chromophore[2~5]. The chromophores have been thought to be solely responsible for the antitumor activity, while the apoproteins for simply stabilizing the chromophores[1,5,6]. However, when we reviewed the data on the chemotherapeutic effectiveness of MCM and its sister antibiotic auromomycin (AUR, see below)[7], we became to suspect that the apoprotein should play a more important role than being a simple stabilizer of the chromophore. MCM and AUR are isolated from the same fermentation broths and differ only in the chromophore/apoprotein ratio[a] and in the binding strength between the chromophore and the apoprotein[b]; a) is *ca.* 6 times larger in AUR than in MCM, while b) is loose in AUR and tight in MCM[8]. It was MCM, rather than AUR, that gave higher increased life span (ILS) values when tested on some tumor-inoculated mice[7], suggesting some roles of the apoprotein in the therapeutic effects. ZAHEER *et al.* reported some peptidase activity with MCM apoprotein[9]. However, it is evident that their preparation

was not pure apoprotein but closely resembled authentic macromomycin in view of its antibacterial activity (Fig. 5 in ref 9) and their purification procedures. The tightly bound chromophore of MCM can be removed only by extraction with an appropriate organic solvent, leaving the true apoprotein which has no antibacterial activity[6]. By mixing the true apoprotein with the chromophore at appropriate molar ratios in aqueous solution, NAOI *et al.* succeeded in reconstituting the complexes which were indistinguishable from MCM as well as from AUR[6].

We report tht antibiotic C-1027 (holo-antibiotic) had some aminopeptidase activity but neither its chromophore alone nor its apoprotein alone had it and that the cytotoxity of the holo-antibiotic to Ehrlich carcinoma cells *in vitro* was lowered by amastatin, an inhibitor of aminopeptidase[10]. A possible role(s) of the apoprotein in the chemotherapeutic effects of the holo-antibiotic is discussed.

Materials and Methods

Antibiotic C-1027 (holo-antibiotic) and C-1027-AG (apoprotein) were purified as reported[1,11]. A fresh methanol-extract of the holo-antibiotic was used as the chromophore. Aminopeptidase activity was determined as reported by use of L-phenylalanyl 4-methyl-coumaryl-7-amide (Phe-MCA) as the substrate[12]; in brief, a reaction mixture, 400 μl, contained 200 μl of buffer A (100 mM Tris-HCl, pH 8.0, 200 mM NaCl and 20 mM $CaCl_2$), 40 μl of 2.5 mM Phe-MCA in 30.5% DMSO, 120 μl of H_2O (solvent for a test compound, if desired) and added 40 μl of antibiotic C-1027 solution (or its substitute). A control run included porcine microsomal aminopeptidase ([E.C. 3.4.11.2], Sigma L5006) as a substitute for the antibiotic. The reaction was initiated by immediate mixing after the addition of the last solution, allowed to proceed at 37°C for 30 minutes and terminated by mixing with 400 μl of 10% SDS. The mixture was diluted with 3.0 ml of 100 mM Tris-HCl, pH 9.0, and submitted to fluorometry of the released 7-amino-4-methyl-coumarin (AMC), monitored at excitation and emission maxima of 380 nm and 460 nm, respectively, on a Hitachi F-4000 fluorescence spectrophotometer. One nmol AMC/ml corresponds to 110.2 emissions.

Ehrlich carcinoma cells (ATCC CCL 77) were grown in DULBECCO's modified Eagle medium (DMEM, Nissui Pharm. Co., Tokyo) supplemented with 100 μg/ml kanamycin (Meiji Seika Kaisha, Ltd., Tokyo) and 5% heat-inactivated calf serum (Gibco Lab., Grand Island, NY) at 37°C in 5% CO_2-containing humidified air. To initiate a test, cells were seeded at a densily of 1×10^4 cells/1.5 ml/well in Costar 12-well tissue culture clusters (day 0). The cells received a test compound(s) on day 1 and were incubated further until day 3 without changing the medium. Cell growth was determined by cell counting in a Coulter counter or by staining cells with crystal violet followed by photometry of the dye taken up by the cells[13].

Results and Discussion

Aminopeptidase Activity of Antibiotic C-1027

Antibiotic C-1027 (holo-antibiotic) had aminopeptidase activity, 1/15 that of porcine microsomal aminopeptidase on the basis of activity/mg protein, while neither the apoprotein alone nor the chromophore alone was active (Table 1). The holo-antibiotic showed no other enzyme activity (trypsin, elastase, chymotrypsin and aminopeptidase B) by use of Boc-Gln-Ala-Arg-MCA, succinyl-Ala-Pro-Ala-MCA, succinyl-Leu-Leu-Val-Tyr-MCA and Arg-β-naphthylamine as the substrate, respectively. The aminopeptidase activity of the holo-antibiotic was rapidly lost upon UV irradiation, while that of the procine microsomal enzyme was only slightly affected (Fig. 1). The chromophore has been reported to be very labile to UV irradiation and heating[1]. Degradation of the chromophore by UV irradiation should have resulted in the loss of native conformation of the holo-antibiotic and in turn, the loss of the catalytic activity. Thus it seemed reasonable that the apoprotein shared little homology in the amino acid sequence

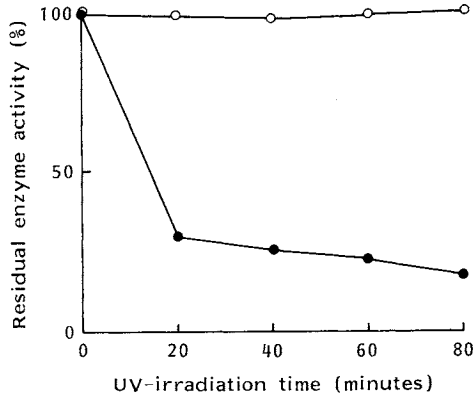
Table 1. Aminopeptidase activity of antibiotic C-1027.

	Amount (μg/reaction mixture)	AMC produced	
		pmol/minute	pmol/minute/μg[a]
Holo-antibiotic	4	40	10.1
	20	190	9.5
	40	340	8.5
Apoprotein	4	ns	
	20	0.1	ns
	40	0.1	
Chromophore	0.4	0.2	
	0.8	0.2	ns
	1.6	0.3	
APase[b]	0.8	148	177
	1.6	244	146
	3.2	344	102

[a] Weight (μg) of the antibiotic or a substitute added to the reaction mixture.
[b] Porcine microsomal leucine aminopeptidase (Sigma).
ns: Not significant.

Fig. 1. UV-sensitivity of the aminopeptidase activity of antibiotic C-1027.

● Antibiotic C-1027 (holo-antibiotic). ○ Porcine microsomal leucine aminopeptidase.



The antibiotic and the porcine enzyme were dissolved at 1 mg/ml and 5 U/ml, respectively, in buffer A. The solutions (2 mm depth in vials) were placed 15 cm under a UV lamp (NEC-GL-15).

Table 2. Sensitivity of the aminopeptidase activity of antibiotic C-1027 to various inhibitors.

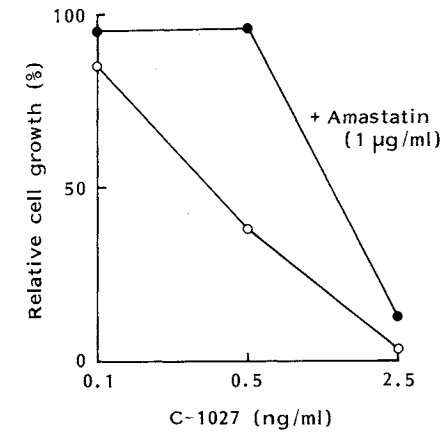
Amount (μg/reaction mixture)	Amastatin			Bestatin			Leupeptin	
	4	0.8	0.16	4	0.8	0.16	40	4
AMC produced (pmol/minute)	10	12	29	36	50	93	141	146
Inhibition (%)	93	92	81	76	67	38	5	2

with general amino-peptidases. Amastatin and bestatin but not leupeptin[14] inhibited the amino-peptidase activity of the holo-antibiotic (Table 2).

Amastatin Protected Ehrlich Carcinoma Cells from the Cytotoxic Effect of the Holo-antibiotic *In Vitro*

Inhibition by amastatin of the cytotoxic effect of the holo-antibiotic on Ehrlich carcinoma cells *in vitro*, as shown in Fig. 2, strongly suggested some contribution of the enzyme activity to the cytotoxicity of the holo-antibiotic. A simple interpretation would be that the holo-antibiotic digests some cell-surface protein(s) through its aminopeptidase activity, endangering the cell viability, while amastatin inhibits the digestion and thereby rescues the cells. This mechanism seemed unlikely, however,

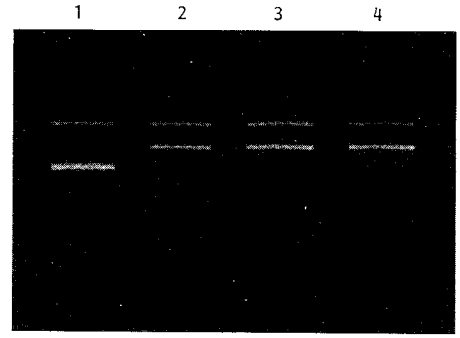
Fig. 2. Inhibition by amastatin of the cytotoxic effect of antibiotic C-1027 on Ehrich carcinoma cells *in vitro*.



Experiments were conducted as given in Materials and Methods. The closed and open circles indicate C-1027 holo-antibiotic with and without amastatin, respectively.

because the porcine aminopeptidase was not cytotoxic even at much higher concentrations (higher enzyme activities/ml). Amastatin could fill the catalytic region of the holo-antibiotic as a fake substrate and interfere with the contact of the antibiotic with some cell-surface proteins. The cytotoxicity of the isolated chromophore (methanol extract of the holo-antibiotic), on the other hand, was not lowered by amastatin. The result was as expected, because the free chromophore should penetrate into cells by diffusion.

Fig. 3. DNA strand scission by antibiotic C-1027 in the presence or absence of amastatin.



Strand scission of DNA by antibiotic C-1027 *in vitro* under various conditions were analyzed as reported[2] except that pBR322 DNA was used instead of ϕX174 RF DNA. Lane 1: pBR322 DNA alone, lane 2: pBR322 DNA + C-1027 (1 μg/ml), lane 3: pBR322 DNA + C-1027 (1 μg/ml) + amastatin (4 μg/ml), lane 4: pBR322 DNA + C-1027 (1 μg/ml) + amastatin (10 μg/ml).

A Possible Role for the Apoprotein as a Targeting Vehicle for the Chromophore

A question which remains to be answered is whether the holo-antibiotic as a whole enters into the cell (endocytosis) or is stuck on the cell surface, releasing the chromophore which alone travels as far as the cell chromosome (chromophore-release). Endocytosis has been suggested for NCS, another member of this group of antibiotics[15], and our data (Fig. 3) seem to support this mechanism. If "chromophore-release" holds true, amastatin, as a fake receptor, should facilitate chromophore-release and enhance, rather than lower, the cytotoxicity. Amastatin neither enhanced nor inhibited degradation of isolated DNA by the holo-antibiotic (Fig. 3). Strand scission of DNA by the chromophore is thought to be the major factor for the cytotoxicity of the holo-antibiotic[2], as with other members of this group of antibiotics. For an antitumor drug, an important characteristic is selective toxicity to tumor cells, in other words, chemotherapeutic effect. ILS of tumor-inoculated animals by a drug could be an index. The higher ILS values obtaind with MCM over AUR, as discussed above, must prove a role for the apoprotein as a targeting vehicle. Chemotherapeutic effectiveness of this group of antibiotics must therefore depend on the protein-protein recognition between the holo-antibiotics and the cellular proteins which are involved in binding on the cell surface, endocytosis, intracellular transport and release of the chromophore. Cloning of C-1027 apoprotein gene is in progress[16], which study may enable to produce more effective holo-antibiotics with modified apoproteins.

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