BIOLOGICAL EFFECTS OF ACETOMYCIN

II. INACTIVATION BY ESTERASES *IN VITRO*

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Acetomycin has antitumor activity *in vitro* but not *in vivo*. HCT-8 human colon adenocarcinoma assays in the presence of a drug metabolizing system (rat liver S9 fraction) demonstrated that liver enzymes inactivated acetomycin. The structure of acetomycin suggested that an esterase could be the key inactivating enzyme. Assays with porcine liver esterase (EC 3.1.1.1) showed that this enzyme rapidly abolishes the activity of acetomycin against HCT-8 cells. The potential utility of acetomycin as an antitumor agent thus depends on finding a means of preventing esterase inactivation.

The antibiotic acetomycin[1] was shown to have activity against HCT-8 human colon adenocarcinoma cells, L1210 murine leukemia cells and human tumor stem cells *in vitro*, but was inactive against four *in vivo* tumor models[2]. In attempting to explain the good *in vitro* HCT-8 activity but poor *in vivo* effects, HCT-8 bioassays were conducted in the presence of an *in vitro* drug metabolizing system. Initial experiments indicated that a component of rat liver S9 fraction other than mixed-function oxidases was capable of readily inactivating the compound. Because acetomycin contains an acetate ester functionality, it was hypothesized that an esterase in the rat liver homogenate was the inactivating factor. This report demonstrates that acetomycin is rapidly inactivated by a purified porcine esterase *in vitro*.

Materials and Methods

Bioassay System

The test system was a disk-agar diffusion assay to detect the inhibition of non-proliferating (resting) HCT-8 human colon adenocarcinoma cells in a 0.8% agar medium[2]. Briefly, paper disks (6.35 mm diameter) saturated with a test solution were spotted onto the surface of an agar petri dish seeded with 5×10^5 HCT-8 cells/ml. For these studies, samples were spotted in duplicate onto each of two separate petri dishes. After incubation at 37°C for 48 hours, inhibition zones were detected by a staining technique employing resazurin.

Acetomycin

The compound was obtained from the fermentation of actinomycete culture WP-2661[2]. Dimethyl sulfoxide was the solvent for the preparation of stock and test solutions.

Biochemical Reagents

Rat liver S9 fraction (Aroclor-induced) was prepared in accordance with the methods of AMES *et al.*[3]. The initial protein concentration of the S9 fraction was about 40 mg/ml. S9 fraction was used either directly by suspending aliquots in 0.2 M sodium phosphate buffer, pH 7.5, or as a component of a mixed-function oxidase drug metabolizing system (S9 mix). The S9 mix formula was that of MOREAU *et al.*[4]. Bovine serum albumin (Sigma) was solubilized at 10 or 40 mg/ml concentrations

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Table 1. The effects of S9 mix, S9 fraction and various proteins on the inhibition of resting HCT-8 cells by acetomycin.

Acetomycin (1 mg/ml) dissolved in:	Protein concentration (mg/ml)	Inhibition zone diameter (mm) (6.35 mm paper disks)
100% Dimethyl sulfoxide	0	26
Sodium phosphate buffer, 0.2 M, pH 7.5	0	25
S9 Mix	5.7	0
S9 Fraction - buffer (1 : 4)	10	0
Bovine serum albumin (in buffer)	10	30
Bovine serum albumin (in buffer)	40	24
Fetal bovine serum (in buffer)	9~12.5	30
Casein hydrolysate, 5% (in buffer)	2.4	25

Solvent controls all were negative. Mithramycin, 250 μg/ml, yielded zones in exceeding of 20 mm.

Fig. 1. Inactivation of acetomycin (1 mg/ml) by S9 fraction, as determined by inhibition of HCT-8 cells *in vitro*.

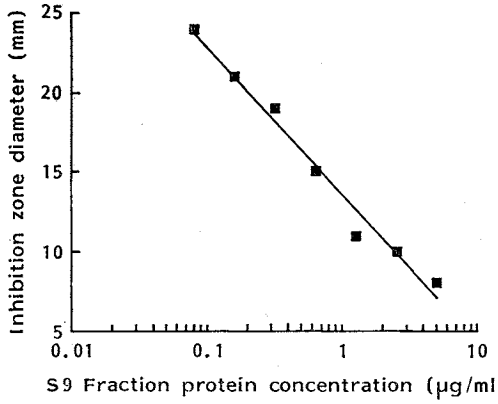
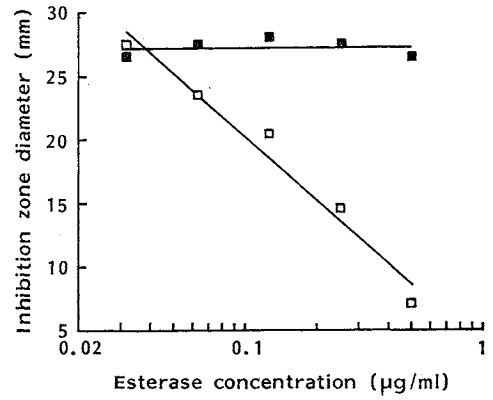


Fig. 2. Inactivation of acetomycin (1 mg/ml), as determined by inhibition of HCT-8 cells *in vitro*, by varying concentrations of porcine liver esterase (□) and by trichloroacetic acid-inactivated esterase (■).



in 0.2 M sodium phosphate buffer, pH 7.5. Casein hydrolysate (Oxoid) was prepared as a 5% solution in distilled water. Porcine liver esterase (EC 3.1.1.1; Sigma) had a rated activity of 160 μmol of substrate (ethyl butyrate) converted per mg protein per minute at pH 8 and 25°C. Esterase solutions were prepared in 0.2 M sodium phosphate buffer, pH 8.0.

Inactivation of Acetomycin in the Presence of Rat Liver Homogenate (S9 Fraction)

To determine the effects of hepatic drug metabolism on the activity of acetomycin against HCT-8 cells *in vitro*, acetomycin was added to varying concentrations of either S9 mix or S9 fraction in buffer to achieve a final acetomycin concentration of 1 mg/ml. Test solutions were incubated at room temperature for 15 minutes prior to saturating paper disks and spotting them onto the surface of agar dishes seeded with HCT-8 cells.

Acetomycin solutions of 1 mg/ml dissolved in 10 or 40 mg/ml of bovine serum albumin, in fetal bovine serum, or in 5% casein hydrolysate also were tested to determine if proteins other than rat liver homogenates could affect acetomycin activity vs. HCT-8.

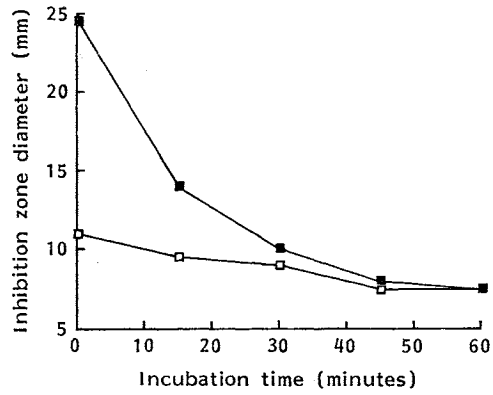
Inactivation of Acetomycin by Porcine Liver Esterase

Acetomycin, 1 mg/ml, was dissolved in solutions of 0.2 M sodium phosphate buffer, pH 8, containing porcine liver esterase at concentrations ranging from 0.008~1.0 mg/ml. Test solutions either were spotted immediately against HCT-8 cells, or were incubated at room temperature for varying periods of time prior to dipping and spotting disks. For some experiments, esterase was inactivated by the addition of trichloroacetic acid (final acid concentration was 2%).

Results

Both S9 mix and S9 fraction minus cofactors were capable of inactivating acetomycin (Table 1 and Fig. 1). This indicated that mixed-function oxidase activities were not a factor in acetomycin inactivation. Moreover, neither bovine serum albumin, fetal bovine serum, nor casein hydrolysate had any effects on the activity of acetomycin against HCT-8 cells (Table 1). These results established that the inactivating factor was present in the 9,000×*g* supernatant of rat liver homogenate, and that an enzyme (esterase) was likely to be involved, rather than a phenomenon such as nonspecific protein binding.

Fig. 3. Time course for the inactivation of acetomycin (1 mg/ml) by 1 mg/ml of porcine liver esterase (□) and by esterase with trichloroacetic acid added at defined time intervals in order to inactivate the enzyme (■).



The results of assaying acetomycin in the presence of porcine liver esterase are shown in Fig. 2. These results confirmed the role of esterase as the drug-inactivating enzyme. By adding trichloroacetic acid to inactivate the esterase, acetomycin retained its inhibitory effects against HCT-8 cells (Fig. 2). To determine the amount of time required for inactivation, a time-course evaluation was performed. The results (Fig. 3) showed that acetomycin-inactivation was a relatively rapid process; within 30~45 minutes, the inhibition zone diameters of trichloroacetic acid-treated samples of acetomycin plus esterase were essentially equal to those observed in control (acid-free) samples.

Discussion

These experiments clearly show that porcine liver esterase is capable of rapidly inactivating acetomycin *in vitro*. It should be noted that the inactivation of acetomycin by esterase is not a unique occurrence. Other antibiotics, such as chloramphenicol and erythromycin, can be inactivated by esterases of microbial and mammalian origin[5~7]. The detoxication of other drugs, such as aspirin and heroin, and the pyrethroid pesticides, also is accomplished *via* the action of esterases[6].

More importantly, the data suggest that esterase inactivation *in vivo* is a major factor in the lack of antitumor effects in the animal models evaluated. Therefore, chemical modification of acetomycin to prevent its inactivation by esterases should be investigated. Alternatively, it may be possible to administer acetomycin in the presence of an esterase inhibitor in order to promote *in vivo* activity. Such studies are warranted in view of the *in vitro* inhibitory effects of acetomycin against both proliferating and non-proliferating human colon adenocarcinoma cells, as well as its marked activity in human tumor stem cell assays[2].

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