BIOLOGICAL EFFECTS OF ACETOMYCIN

I. ACTIVITY AGAINST TUMOR CELLS *IN VITRO* AND *IN VIVO*

S. W. MAMBER†, J. D. MITULSKI, K. L. HAMELEHLE, J. C. FRENCH,
G. C. HOKANSON, J. L. SHILLIS, W. R. LEOPOLD,
D. D. VON HOFF†† and J. B. TUNAC

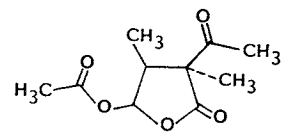
Warner-Lambert/Parke-Davis Pharmaceutical Research,
2800 Plymouth Road, Ann Arbor, MI 48105, U.S.A.
††University of Texas Health Science Center at San Antonio
and Cancer Therapy and Research Foundation of South Texas,
San Antonio, TX 78284, U.S.A.

(Received for publication August 18, 1986)

The antibiotic acetomycin was active *in vitro* against HCT-8 human colon adenocarcinoma cells (IC_{50}, 1.5 μg/ml) and L1210 murine leukemia cells (IC_{50}, 2.2 μg/ml). Acetomycin also had marked activity in the human tumor stem cell assay, with a 33% overall response rate ($\leq$30% survival) against 49 primary tumors. However, acetomycin was inactive in four *in vivo* tumor assay systems (L1210 and P388 leukemias, B16 melanoma and the MX-1 mammary xenograft system). This lack of *in vivo* activity may result from metabolic inactivation of acetomycin.

In the course of screening actinomycete cultures for novel antitumor agents, the fermentation broth of actinomycete WP-2661 showed inhibitory effects against HCT-8 human colon adenocarcinoma cells in an *in vitro* resting cell assay; the same broth lacked *in vitro* activity against proliferating L1210 murine leukemia cells. A crystalline compound isolated from the WP-2661 fermentation broth was identified as acetomycin (Fig. 1). Acetomycin first was isolated in 1958, and was shown to have antimicrobial activity[1]. A literature search indicated that acetomycin had not been evaluated for antitumor effects. The purpose of this research was to characterize the antitumor activities of acetomycin *in vitro* and *in vivo*.

Fig. 1. Structure of acetomycin.



Materials and Methods

Acetomycin

The antibiotic was obtained from fermentation broths of actinomycete culture WP-2661. The component responsible for the HCT-8 activity of the fermentation broth was extracted into ethyl acetate. A concentrate was purified by silica gel chromatography and crystallized from methanol. The crystalline product was identified as acetomycin based on spectral data. The relative stereochemistry of acetomycin was established by X-ray crystallography.

HCT-8 Resting Cell Bioassay

HCT-8 human colon adenocarcinoma cells (W. TOMPKINS, University of Illinois) were employed

† Present address: Bristol-Myers Company, Pharmaceutical Research and Development Division, 5 Research Parkway, P.O. Box 5100, Wallingford, CT 06492-7660, U.S.A.

in a disk-agar diffusion system for the detection of substances inhibitory to non-proliferating (resting) cells maintained in agar. Cells were maintained as monolayers in 150 cm^2 tissue culture flasks (Corning) using RPMI 1640 (Gibco) plus 10% fetal bovine serum (FBS; Armour), and containing gentamicin (Sigma), 50 μg/ml. Transfers into fresh media were made once each week.

For screening and/or assay, 50 ml of double-strength RPMI 1640 plus 20% FBS were mixed with 50 ml of 1.6% agar (Difco), which was kept molten at 45°C. Cell monolayers were trypsinized, and viable counts were obtained using a hemocytometer (trypan blue exclusion method). A cell suspension was added to the agar medium to yield a final cell count of 5×10^5 cells/ml. Petri dishes (150 mm diameter; Labtek) were promptly prepared by adding 20 ml of the agar media to each dish. After the agar solidified, paper disks (6.35 mm diameter; Schleicher and Schuell No. 740) impregnated with various samples of acetomycin (dissolved in dimethyl sulfoxide or ethanol) were spotted on the surface of the agar. Petri dishes were incubated at 37°C in a 5% CO_2 incubator for 48 hours. After incubation, the dishes were developed by first removing the disks and flooding the agar with 10 ml of phosphate - citrate buffer (0.3 M, pH 6.3) for 15 minutes. The buffer was decanted and the dishes were flooded with 10 ml of a solution of 0.05% resazurin for 10 minutes. The resazurin solution was removed and the dishes were incubated in a nitrogen atmosphere for 2 hours. Activity was determined by measuring the diameters of blue inhibition zones observed against a pink background. Mithramycin (Pfizer), 25~250 μg/ml, was the standard positive control.

In Vitro HCT-8 and L1210 Proliferating Cell Inhibition Assays

Assays against proliferating cells were employed to determine the concentration of acetomycin needed to inhibit 50% of cell growth relative to untreated controls (IC_{50}). IC_{50} assays against both HCT-8 human colon adenocarcinoma and L1210 murine leukemia cell lines were performed using methods described by LEOPOLD *et al.*[2]. Acetomycin solutions for these assays were prepared as 2-fold serial dilutions from dimethyl sulfoxide or ethanol stock solutions using cell culture media as the diluent.

In Vivo Antitumor Activities

Acetomycin was tested for *in vivo* activity against the L1210 and P388 leukemias, and against Ridgway osteogenic sarcoma, B16 melanoma, and the MX-1 mammary xenograft. All *in vivo* tests were performed in accordance with standard protocols[3,4], or as described by LEOPOLD *et al.*[2].

Human Tumor Stem Cell Assays

Cytotoxicity against a variety of primary human tumors was determined using a clonogenic assay technique[5,6]. Acetomycin was assayed against 49 tumors at a concentration of 10 μg/ml with continuous exposure[7].

Results

In Vitro Antitumor Assays

Acetomycin was active at 25 μg/ml (approximately 0.5 μg/disk) and exhibited a linear dose-response relationship in the HCT-8 resting cell agar diffusion assay (Fig. 2). In the HCT-8 and L1210 proliferating cell inhibition assays, IC_{50} values were 1.5 μg/ml and 2.2 μg/ml, respectively.

In Vivo Antitumor Assays

Acetomycin was inactive vs. L1210 leukemia, P388 leukemia, and B16 melanoma at maximum tolerated doses that ranged from 20~40 mg/kg/injection with qd×5 or qd×9 ip injection schedules. Acetomycin was inactive against the MX-1 mammary xenograft grown as a subrenal capsule implant; maximum tolerated levels were not realized in these tests. Marginal activity was obtained in the Ridgway osteosarcoma system, with a 44% T/C value at 12.5 mg/kg/injection.

Fig. 2. Inhibition of non-proliferating HCT-8 human colon adenocarcinoma cells by acetomycin in an agar bioassay system (6.35 mm paper disks).

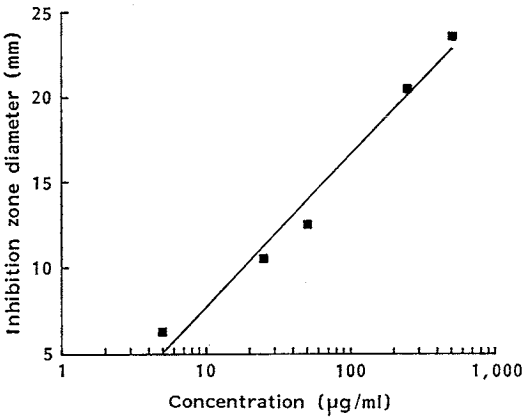


Table 1. Activity of acetomycin in human tumor stem cell assays.

Tumor	Sensitive/Total (%)[a]	
	≤50% Survival	≤30% Survival
Breast	8/14 (57)	6/14 (43)
Colon	0/2 (0)	0/2 (0)
Corpus uteri	1/2 (50)	0/2 (0)
Lung, bronchial	7/14 (50)	4/14 (29)
Ovary	7/15 (47)	5/15 (33)
Unknown[b]	1/2 (50)	1/2 (50)
Overall response	24/49 (49)	16/49 (33)

[a] Acetomycin was tested at a concentration of 10 μg/ml with continuous exposure.
[b] Unknown primary site, not adenocarcinoma.

Human Tumor Stem Cell Assays

The results of these assays are presented in Table 1. Of a total of 49 tumors, 24 (49%) demonstrated sensitivity based on reduction of colony formation to 50% or less relative to control values, while 16 (33%) showed reduction of colony formation to 30% or less of the controls. A response rate of 20~25% or better in such assays is considered to be significant with respect to the development of clinically useful antitumor agents[7] (also, D.D. VON HOFF, personal communication).

General Discussion

Acetomycin was detected by screening fermentation broths for substances active against non-proliferating HCT-8 cells but not active against proliferating L1210 cells *in vitro*. The purified material, however, proved to be active against both cell types, but the IC_{50} vs. proliferating HCT-8 cells was slightly less than that vs. L1210 cells. This was unusual, because the majority of known antitumor agents evaluated in these two cell lines have markedly greater potency against L1210 (unpublished observations). Furthermore, because acetomycin was capable of inhibiting both proliferating and non-proliferating cells, acetomycin may be acting as a cell cycle-nonspecific agent[8]. Acetomycin also had marked activity in the human tumor stem cell assay, with a 33% overall response rate at the 70% colony inhibition level. This would have ranked acetomycin as one of the 10 best compounds evaluated in a pilot screening study of the human tumor stem cell assay with 50 compounds, including a variety of established anticancer agents[7]. The results for acetomycin against both HCT-8 cells and in the stem cell assay suggest that acetomycin may have activity against solid tumors. Solid tumor cell lines such as HCT-8 thus may have utility as simple *in vitro* screens for the detection of novel substances with selective antitumor effects.

In contrast to the *in vitro* data, acetomycin lacked *in vivo* antitumor activity in four of five murine tumor systems evaluated. SHOEMAKER *et al.*[7] observed that a variety of compounds shown to be active in stem cell assays or in other *in vitro* assays are not active in *in vivo* systems such as P388 leukemia. It was hypothesized that for acetomycin, the lack of *in vivo* effects resulted from metabolic inactivation of this compound in animals. Accordingly, a series of experiments were performed to determine if acetomycin is readily inactivated enzymatically. These studies are reported in the following paper[9].

References

1) ETTLINGER, L.; E. GÄUMANN, R. HÜTTER, W. KELLER-SCHIERLEIN, F. KRADOLFER, L. NEIPP, V. PRELOG & H. ZÄHNER: Uber die Isolierung und Charakterisierung von Acetomycin. Helv. Chim. Acta 41:

216~219, 1958

2) LEOPOLD, W. R.; J. L. SHILLIS, A. E. MERTUS, J. M. NELSON, B. J. ROBERTS & R. C. JACKSON: Anticancer activity of the structurally novel antibiotic CI-920 and its analogs. Cancer Res. 44: 1928~1932, 1984
3) GERAN, R. I.; N. H. GREENBERG, M. M. MACDONALD, A. M. SCHUMAKER & B. J. ABBOTT: Protocols for screening chemical agents and natural products against animal tumors and other biological systems. Cancer Chemother. Rep. 3: 1~103, 1972
4) National Institutes of Health: *In vivo* cancer models, 1976–1982. NIH Publication No. 84-2635, 1984
5) HAMBURGER, A. W. & S. E. SALMON: Primary bioassay of human tumor stem cells. Science 197: 461~463, 1977
6) VON HOFF, D. D.; M. ROZENCWEIG & F. M. MUGGIA: The evaluation of cytotoxic drugs. Cancer Treat. Rev. 6 (Suppl.): 1~8, 1979
7) SHOEMAKER, R. H.; M. K. WOLPERT-DEFILIPPES, D. H. KERN, M. M. LIEBER, R. W. MAKUCH, N. R. MELNICK, W. T. MILLER, S. E. SALMON, R. M. SIMON, J. M. VENDITTI & D. D. VON HOFF: Application of a human tumor colony-forming assay to new drug screening. Cancer Res. 45: 2145~2153, 1985
8) HILL, B. T. & R. BASERGA: The cell cycle and its significance for cancer treatment. Cancer Treat. Rev. 2: 159~175, 1975
9) MAMBER, S. W.; J. D. MITULSKI, P. E. BORONDY & J. B. TUNAC: Biological effects of acetomycin. II. Inactivation by esterases *in vitro*. J. Antibiotics 40: 77~80, 1987