EFFECTS OF HERBIMYCIN DERIVATIVES ON *src* ONCOGENE FUNCTION IN RELATION TO ANTITUMOR ACTIVITY

Sir:

Herbimycin A (**1**, Fig. 1) was originally isolated due to its herbicidal activity[1] and was later found to possess anti-tobacco mosaic virus and antitumor activities[2,3]. More recently, we reisolated this benzoquinonoid ansamycin antibiotic as an active substance which reversed the transformed morphology of temperature-sensitive Rous sarcoma virus-infected rat cells (ts/NRK) to the normal morphology at a permissive temperature (33°C)[4], concomitant with a drastic reduction in intracellular $p60^{src}$ kinase activity[5]. A variety of herbimycin A derivatives have been synthesized and examined for their possible antitumor activities elongating the life span of mice inoculated with Ehrlich ascites carcinoma (EAC) cells[6,7]. In the present paper, we describe the structure-activity relationships among these herbimycins (herbimycins

Fig. 1. The structure of herbimycin A (**1**) and numbering used in this study.

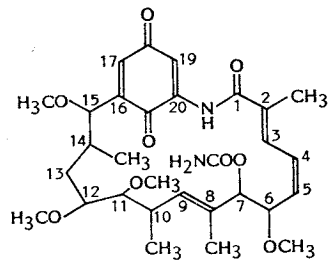


Table 1. Effect of herbimycins on growth and morphology of ts/NRK cells.

	Compound	Growth inhibition (IC_{50}, μg/ml)	Morphological reversion
1:	Herbimycin A	0.45[a]	+[b]
2:	Herbimycin B	5.0	+
3:	8,9-Epoxyherbimycin A	1.3	−
4:	8,9-Epoxyherbimycin A (epoxide stereoisomer of **3**)	20	−
5:	17-Cyclopropylaminoherbimycin A	3.0	+
6:	19-Dimethylaminoherbimycin A	>20	−
7:	19-Allylaminoherbimycin A	0.17	+
8:	19-Methylpiperazinoherbimycin A	7.2	−
9:	8,9-Epoxy-19-dimethylaminoherbimycin A	15	+
10:	8,9-Epoxy-19-cyclopropylaminoherbimycin A	10	+
11:	8,9-Epoxy-19-methylpiperazinoherbimycin A	2.8	+
12:	19-Dimethylamino-7-decarbamoyl-7,8-*O*-carbonyl-8,9-dihydro-8-hydroxyherbimycin A	>20	−
13:	19-Bromoherbimycin A (*)[c]	7.5	−
14:	9,19-Dibromo-7-decarbamoyl-7,8-*O*-carbonyl-8,9-dihydro-8-hydroxyherbimycin A	5.0	−
15:	8,9-Epoxy-19-bromoherbimycin A	8.0	−
16:	*N*-Acetylherbimycin A	5.2	+
17:	7-Decarbamoyl-19-chloroherbimycin A	6.8	−
18:	6-Chloro-6-demethoxyherbimycin A (*)	5.0	−
19:	4,5-Dichloro-4,5-dihydro-7-decarbamoyl-6-demethoxy-6-enoherbimycin A (*)	15	−
20:	2,3,4,5-Tetrahydroherbimycin A (*)	>20	−
21:	7-Decarbamoylherbimycin A (*)	7.0	−

[a] ts/NRK cells grown at 33°C, a permissive temperature for cell transformation, were plated at cell densities of 5×10^4 cells/2 ml/35 mm dish in duplicate and incubated overnight. Then each drug was added at serial 2-fold dilutions and the incubation was continued for 3 days; the cells were trypsinized and the cell numbers were counted with a Coulter counter. IC_{50} values (a concentration which inhibits cell growth by 50% of control) were obtained from graphic plots.

[b] The morphological reversion was examined as described previously[5] and in the text; + (positive), − (negative).

[c] Compounds which showed higher chemotherapeutic index than herbimycin A in mice with EAC[7].

A, B, and 19 derivatives of herbimycin A) with respect to the following activities; (1) reversing transformed cell morphology to the normal one in ts/NRK cells at the permissive temperature and (2) inhibiting cell growth and macromolecular syntheses under the same conditions. The results are discussed in comparison to their effects on EAC in mice.

The inhibitory activity of these compounds against growth of ts/NRK cells (activity **a**) were determined and the concentrations required for 50% growth inhibition (IC_{50}) are presented in Table 1. The activity of reversing the transformed cell morphology to the normal one (activity **b**) was determined by examining the cell morphology of all plates which were assayed for **a** activity under a phase contrast microscope between 1 and 2 days after the addition of drugs[5] at wide concentrations including IC_{50} values for each drug. The compound which reversed the cell morphology similar to that observed at a nonpermissive temperature (39°C) at any concentrations tested was referred to as positive in activity **b**. Herbimycin A and other positive analogs exhibited this activity at the concentrations near or greater than IC_{50} values. Both **a** and **b** activities are summarized in Table 1 and the following correlations between the structure and the activity were recognized.

1) *N*-Acetylation of herbimycin A (**16**) significantly decreased activity **a** but did not affect activity **b**. This modification is homologous to the structural difference from herbimycin A to macbecin II[8], a natural compound which is also as effective in reversing the transformed morphology as herbimycin A (data not shown). 2) Reduction of conjugated double bonds in position 2~5 (**20**) abolished both activities completely, indicating that either one or two double bonds in this position is necessary for both activities. 3) Replacement of 6-*O*-methyl to 6-chloride (**18**) diminished the activity **a** to one tenth that of herbimycin A and abolished activity of **b**. It should be noted that compound **18** has been reported to be one of the most effective derivatives prolonging the life span of mice inoculated with EAC[7]. 4) 7-Decarbamoylation (**21**) lowered the activity **a** and lost the activity **b** suggesting this moiety is important for both activities. The 19-chloro-7-decarbamoyl herbimycin A (**17**) likewise lost activity in both assays. 5) 8,9-Epoxide formation in combination with modification at 19-position gave interesting results as follows. 8,9-Epoxide formation alone (**3**) retained most of the **a** activity but lost the **b** activity. Its corresponding epoxide stereoisomer (**4**)†, however, lost both activities completely. Substitutions at 19-position affected both **a** and **b** activities depend on the substituents. Dimethylamino, or methylpiperazino, or bromo groups worked negatively in both activities (**6**, **8**, **13**), but the allylamino group (**7**), worked positively. On the basis of activity **b**/drug concentration, compound **7** was 2~3 times more active than herbimycin A and was the only derivative which was more active than the mother compound with respect to activity **b**. The combination of 8,9-epoxydation and 19-amino derivatization, such as dimethylamino (**9**) or methylpiperazino (**11**) recovered the activity **b**, although the latter was less effective. However, bromide incorporation into 19-position did not recover activity **b** (**15**). 6) Substitution by cyclopropylamino group at 17-position within the benzoquinone ring (**5**) lowered activity **a** but retained activity **b**.

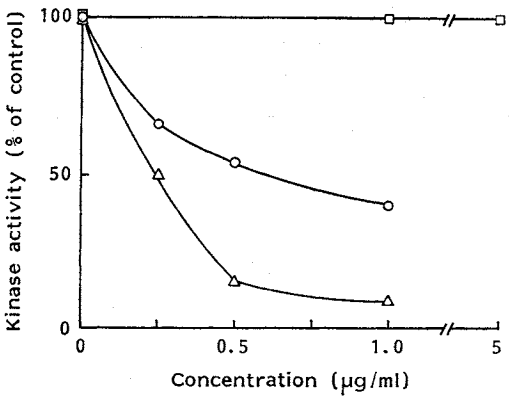
We hoped to find derivatives which have strong activity **b** and little or no activity **a**. However, the six derivatives (**5**, **7**, **9**, **10**, **11** and **16**), like herbimycins A and B (**2**), all reversed cell morphology (activity **b**) at the concentrations around or greater than IC_{50} values (activity **a**) of each compound.

To confirm that the activity reversing cell morphology is due to inhibition of intracellular $p60^{src}$ kinase[5], we chose compounds **7** and **18** to determine their possible inhibition of $p60^{src}$ kinase and compared the results with that of herbimycin A, because the derivative **7** was 2~3

† **4**: $[\alpha]_D^{20}$ +113° (*c* 0.5, $CHCl_3$); high resolution electron impact mass spectra (HREI-MS) 590.283, calcd for $C_{30}H_{42}N_2O_{10}$ (M^+) 590.284; ^1H NMR (400 MHz, $CDCl_3$) δ 0.80 (d, J=5.5 Hz, 14-CH_3), 1.09 (d, J=4.5 Hz, 10-CH_3), 1.33 (s, 8-CH_3), 1.40 (m, 10-H), 1.60 (m, 13-H), 1.65 (m, 14-H), 2.00 (d, J=~1.0 Hz, 2-CH_3), 2.89 (br d, J=8.5 Hz, 9-H), 3.48 (s, 15-OCH_3), 3.34 (s, 11 and 12-OCH_3), 3.40 (s, 6-OCH_3), 3.40 (m, 12-H), 3.78 (m, 11-H), 4.36 (m, 15-H), 4.68 (dd, J=7.5 and ~1.0 Hz, 6-H), 5.00 (br s, 7-$OCONH_2$), 5.57 (d, J=~1.0 Hz, 7-H), 5.83 (dd, J=11.5 and 7.5 Hz, 5-H), 6.48 (dd, J=11.5 and 11.5 Hz, 4-H), 6.63 (dd, J=2.0 and 2.0 Hz, 17-H), 6.95 (dd, J=11.5 and 1.0 Hz, 3-H), 7.36 (d, J=2.0 Hz, 19-H), 8.80 (br s, CONH).

times more potent than herbimycin A in both **a** and **b** activities, while the derivative **18** had 10 times less activity of **a**. Another purpose of this study was to find possible correlations between the antitumor activity toward EAC in mice and the activity **a** or **b**, because the derivative **18** was reported to be most potent in elongating the life span of mice inoculated with EAC[6,7]. As shown in Fig. 2, herbimycin A and derivative **7** inhibited intracellular $p60^{src}$ kinase in a concentration-dependent manner, while derivative **18** had no inhibitory activity. The results paralleled the **b** activity of these compounds (Table 1) and were consistent with the previous observation that morphological reversion in ts/NRK cells is closely correlated with inhibiting intracellular *src* kinase[5].

Fig. 2. Inhibition of intracellular $p60^{src}$ kinase activity in ts/NRK cells.



ts/NRK cells treated with either herbimycin A (**1**; ○), 19-allylaminoherbimycin A (**7**; △) or 6-chloro-6-demethoxyherbimycin A (**18**; □) at indicated concentrations for 15 hours at 33°C were lysed in KRUEGER's buffer and clarified as previously described[5]. TBR serum was added to the protein extracts (50 μg protein each) for the immunoprecipitation of $p60^{src}$. The immune complex was suspended in 30 μl of 20 mM Tris-HCl, pH 7.2, 10 mM $MgCl_2$, 1 μM γ-[^{32}P]ATP (5 μCi) and incubated at 20°C for 15 minutes. The resulting reaction mixture was electrophoresed in sodium dodecyl sulfate-polyacrylamide gel, autoradiographed, and the phosphorylated heavy chain bands of immunoglobulin were cut out, counted and plotted as percent of control value (1,900 cpm for net control).

The question which remained to be answered was what is the biochemical basis underlying the effectiveness of derivative **18** on EAC in mice. In order to get some information about this problem, we next examined the inhibitory effect of these compounds on macromolecular synthesis in ts/NRK cells. As shown in Table 2, both herbimycin A and derivative **7** inhibited DNA synthesis with little or no inhibition of RNA and protein syntheses at the concentrations corresponding to their IC_{50}, but the derivative **18** inhibited both DNA and RNA syntheses to almost an equal extent at its IC_{50}. Similar results were obtained with cultured Ehrlich carcinoma cells (data not shown). Therefore, the inhibition of RNA synthesis accompanying the inhibition of DNA synthesis may be important to the antitumor effect on EAC in mice. It is speculative to extrapolate the same mechanism to other derivatives which also showed high chemotherapeutic indices than herbimycin A with EAC in mice (**13**, **19**, **20** and **21**)[7]. But it is clear that the inhibitory activity of *src* oncogene function does not contribute to the higher chemotherapeutic effects on EAC in mice. Since herbimycin A was recently found to reverse various transformed phenotypes including anchorage independent cell growth property to normal ones[9], it will be promising to test herbimycin A

Table 2. Effect of herbimycins on macromolecular syntheses in ts/NRK cells.

Compound (concentration)	Inhibition (%)[a]		
	DNA	RNA	Protein
1: Herbimycin A (0.45 μg/ml)	85.5	7.0	11.0
7: 19-Allylaminoherbimycin A (0.17 μg/ml)	62.0	6.5	16.0
18: 6-Chloro-6-demethoxyherbimycin A (5.0 μg/ml)	82.0	67.0	24.0

[a] After the treatment of ts/NRK cells with each drug for 15 hours at 33°C, the cells were pulse-labeled with [^{3}H]thymidine for DNA, [^{3}H]uridine for RNA or [^{3}H]leucine for protein for 1 hour. Radioactivity in the acid-insoluble fraction of the cells was counted as described previously[10]. Radioactivity incorporated into acid-insoluble fraction of the control run was taken as 100% incorporation of each precursor and data which was equivalent to IC_{50} values of growth inhibition was indicated.

and its derivatives which inhibit the *src* function for chemotherapeutic activity with an animal tumor model system including *src* oncogene.

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