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19-Allylaminoherbimycin A, an analog of herbimycin A that is stable against treatment with thiol compounds or granulocyte-macrophage colony-stimulating factor in human leukemia cells

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Abstract

Herbimycin A, a benzoquinonoid ansamycin antibiotic, reduces intracellular phosphorylation by some protein tyrosine kinases and inhibits the proliferation of malignant cells which express high tyrosine kinase activity. Herbimycin A inhibited the proliferation of human monoblastic leukemia U937 cells, but this inhibition was abrogated by the addition of granulocyte-macrophage colony-stimulating factor (GM-CSF). On the other hand, a derivative of herbimycin A, 19-allylaminoherbimycin A, inhibited the proliferation of such cells without interference by the addition of GM-CSF. Phosphorylation of MAP kinase and c-*myc* expression induced by GM-CSF in U937 cells were inhibited by both herbimycin A and 19-allylaminoherbimycin A. The time courses of growth inhibition showed that the growth-inhibitory activity of herbimycin A in U937 cells was initially potent, but gradually decreased in the presence of GM-CSF. Thiol compounds, glutathione (GSH) and 2-mercaptoethanol, abrogated the inhibition of the growth of U937 cells by herbimycin A, but not by 19-allylaminoherbimycin A, like GM-CSF. Intracellular GSH content in U937 cells was increased by treatment with GM-CSF, and decreased with herbimycin A, but returned to the control level with the addition of GM-CSF to herbimycin A. In thin-layer chromatography, after in vitro incubation with herbimycin A and GSH, nothing could be detected at the position of intact herbimycin A, while 19-allylaminoherbimycin A was stably detected. These findings suggest that changes in the intracellular concentration of GSH play a role in the abrogation of the inhibition of U937 cell growth by herbimycin A. In the presence of GSH, 19-allylaminoherbimycin A inhibited the proliferation of U937 cells and Philadelphia chromosome-positive K562 cells more effectively than herbimycin A. Since GSH plays a role in detoxicating several anticancer drugs, 19-allylaminoherbimycin A may have therapeutic advantages over herbimycin A against some types of leukemia.

Keywords: Herbimycin A; 19-Allylaminoherbimycin A; Glutathione; Granulocyte-macrophage colony-stimulating factor; Growth inhibition; U937 cell

1. Introduction

Herbimycin A is a benzoquinonoid ansamycin antibiotic isolated from *Streptomyces hygroscopicus* that is known to inhibit protein tyrosine kinase activities [1,2]. This compound reverses the v-*src* transformation of rat kidney cells by inhibiting its kinase activity [2] and effectively inhibits the growth of Philadelphia chromosome-positive leukemia cell lines, which exhibit *bcr-abl* rearrangement and express its fusion product, p210 or p190, with high tyrosine kinase activity [3,4]. Administration of herbimycin A has prolonged the survival of mice inoculated with mouse myeloid leukemia C1 cells that highly expressed v-*abl* oncogene [5] and SCID mice inoculated with human Philadelphia chromosome-positive cells [6]. Thus, herbimycin A might be useful in the chemotherapy of some types of leukemia.

Herbimycin A is believed to inhibit tyrosine kinases by binding to sulfhydryl group(s) of the kinases [7,8]. Sulfhydryl compounds such as GSH counteract the inhibitory effect of herbimycin A on v-*src* tyrosine kinase

Abbreviations: GSH, glutathione (reduced form); GM-CSF, granulocyte-macrophage colony-stimulating factor; NEM, N-ethylmaleimide; IC_{50}, concentration of drug required for 50% inhibition of cell growth

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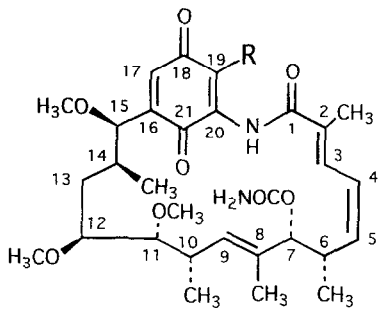

Herbimycin A: R = H
19-Allylaminoherbimycin A: R = CH_2CHCH_2NH

Fig. 1. Structures of herbimycin A and 19-allylaminoherbimycin A. The numbering system used in the present study is indicated.

activity [7]. Since GSH is a major non-protein intracellular sulfhydryl compound that plays a role in the detoxication of several anticancer agents [9], it may interfere with the therapeutic efficacy of herbimycin A. In this study, we found that GM-CSF, as well as thiol compounds, abrogated the inhibitory effect of herbimycin A on the growth of human monoblastic leukemia U937 cells, and that 19-allylaminoherbimycin A (Fig. 1) was resistant to this abrogation.

2. Materials and methods

2.1. Materials

Herbimycin A and its derivatives [10,11] were gifts from Dr. Satoshi Omura, Kitasato Institute, Tokyo, Japan. Human recombinant GM-CSF was purchased from Genzyme (Boston, MA), GSH was from Sigma (St. Louis, MO), and 2-mercaptoethanol was from Wako (Osaka, Japan).

2.2. Cells and cell cultures

Human myeloid leukemia U937 [12], THP-1 [13] and K562 cells [14] were cultured in suspension in RPMI 1640 medium supplemented with 10% fetal bovine serum and 80 μg/ml gentamicin at 37°C in a humidified atmosphere of 5% CO_2 in air.

2.3. Cell growth

Suspensions of cells were cultured with or without compounds in multiple dishes. Cell numbers were counted in a Model ZM Coulter Counter (Coulter Electronics, Luton, UK). Cellular viabilities after all of the treatments in this study were more than 90%, as determined by exclusion of Trypan blue.

2.4. Immunoblot analysis of MAP kinase

Cells were lysed in lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% SDS, 1% Triton-X, 0.5% sodium deoxycholate and 1 mM phenylmethylsulfonyl fluoride]. The cell lysates were mixed with sample buffer and separated on 12.5% SDS-polyacrylamide gel. The proteins were electrophoretically transferred from the gel onto Immobilon-P Transfer Membrane (Millipore, Bedford, MA), immunoblotted with monoclonal anti-MAP kinase (erk 2) antibody (UBI, Lake Placid, NY) and visualized with a biotin-avidin-alkaline phosphatase system (Vectastain ABC system; Vector, Burlingame, CA).

2.5. RNA extraction, Northern blotting, and hybridization

RNA was extracted by modification of the method of Chomczynski and Sacchi [15], using Isogen (Nippon gene, Toyama, Japan). Total RNA (20 μg/lane) was separated on 1.2% agarose-formaldehyde gel and transferred to a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). The membranes were hybridized with end-labeled probes as previously described [16]. Autoradiography was performed using a bioimage analyzer (Fujix BAS2000; Fuji Photo Film, Kanagawa, Japan). Amounts of individual mRNA were quantified using the bioimage analyzer, and were normalized to the amount of GAPDH mRNA.

2.6. Probes

A probe for GAPDH was purchased from Oncogene Science (Manhasset, NY). An oligonucleotide probe for c-*myc* was synthesized with a DNA synthesizer (Model 392; Applied Biosystems, Foster City, CA) based on the antisense sequence (AGGTGATCCAGACTCTGACCTT-TTGCCAGGAGCCTGCCT) derived from exon 3 of the human c-*myc* gene.

2.7. Cellular GSH content

Intracellular GSH contents were assayed as previously described [17,18]. Briefly, cell extracts were obtained with 0.5 ml of 1 M perchloric acid, and were neutralized with 2 M KOH/0.3 M MOPS buffer. The supernatant from the cell extract was used in the reaction mixture for GSH assay. The reaction mixture consisted of 700 μl of 125 mM sodium phosphate/6.3 mM Na-EDTA buffer, 100 μl of 6 mM dithionitrobenzoic acid (Wako), 200 μl of test sample, and 10 μl of 50 units/ml glutathione reductase (Wako). The change in absorbance at 412 nm was mea-

sured with a spectrophotometer (U-2000; Hitachi, Tokyo) over 2 min or until it exceeded 2.0.

2.8. Cellular thiol content

The total cellular thiol content was measured by a previously described method [19]. Briefly, 10^6 cells were suspended in 150 μl phosphate-buffered saline with 4.44 × 10^3 Bq [^{14}C]NEM (NEN Products, Boston, MA), which was adjusted to 3.7×10^4 Bq/μmol. After a 15-min incubation at room temperature, the reaction was stopped by the addition of 12 μl of unlabeled 100 mM NEM. Cells were then washed three times with phosphate-buffered saline, and the resulting cell pellet was solubilized with dimethyl sulfoxide and mixed with scintillation liquid. Incorporated radioactivity was counted in a scintillation counter (LS5800; Beckman, Fullerton, CA).

2.9. In vitro reaction of herbimycin A with GSH

Herbimycin A and 19-allylaminoherbimycin A were incubated with GSH in phosphate-buffered saline at 37°C for 2 h. Herbimycin A and its derivative were then extracted with chloroform-methanol (1:1) solution and separated by fluorescent thin-layer chromatography (Whatman, Clifton, NJ) as described previously [11]. Materials on the chromatography plate were visualized under an ultraviolet lamp.

2.10. Statistical evaluation

Statistical analyses were performed using Student's *t*-test.

3. Results

Herbimycin A inhibited the proliferation of human monoblastic leukemia U937 cells in a concentration-dependent manner, with an IC_{50} of 108.9 ng/ml (190 nM). However, GM-CSF significantly reversed this growth inhibition concentration-dependently (Fig. 2). Whereas herbimycin A inhibited the proliferation of another monoblastic leukemia THP-1 cells with an IC_{50} of 276.5 ng/ml (482 nM), GM-CSF also counteracted the inhibitory effect of herbimycin A on the proliferation of human monoblastic leukemia THP-1 cells (data not shown). Next, we examined the effect of several derivatives of herbimycin A on the growth of U937 cells in the absence or presence of GM-CSF. 19-Allylaminoherbimycin A inhibited the proliferation of U937 cells with an IC_{50} of 212.8 ng/ml (338 nM) (Fig. 2). GM-CSF only slightly counteracted the growth inhibition induced by 19-allylaminoherbimycin A. Other herbimycin A derivatives (8,9-epoxyherbimycin A, 17-cyclopropylaminoherbimycin A, 19-bromoherbimycin A, 2,3,4,5-tetrahydroherbimycin A and 4,5-dichloroherbimycin A) also inhibited the proliferation of U937 cells, with IC_{50} values of 960 ng/ml (1.62 μM), 679 ng/ml (1.08 μM), 1700 ng/ml (2.60 μM), 1035 ng/ml (1.79 μM) and 1427 ng/ml (2.22 μM), respectively. GM-CSF did not reverse the inhibition of cell growth caused by 8,9-epoxyherbimycin A, 17-cyclopropylaminoherbimycin A or 2,3,4,5-tetrahydroherbimycin A (data not shown).

GM-CSF has been reported to induce the proliferation of GM-CSF receptor-expressed mouse BaF3 cells by stimulating post-receptor signal transduction systems, such as those for MAP kinase and c-*myc* expression [20]. In U937 cells, GM-CSF also stimulated the phosphorylation of

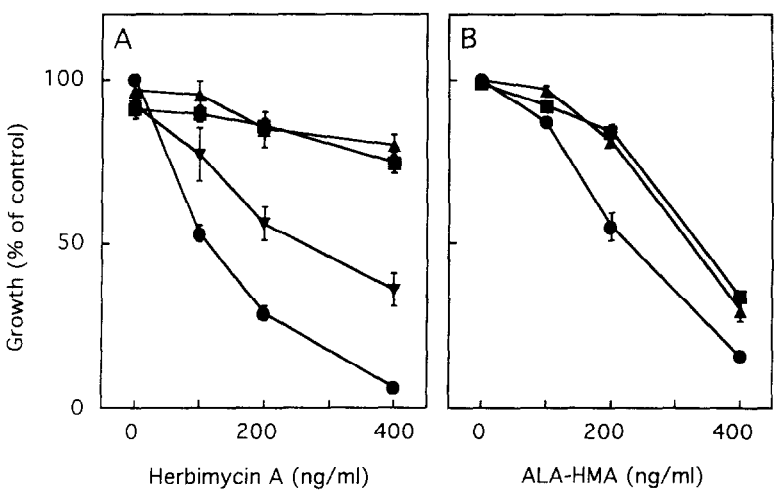

Fig. 2. Effects of herbimycin A and 19-allylaminoherbimycin A (ALA-HMA) in combination with GM-CSF on the growth of U937 cells. Cells (2×10^5cells/ml) were cultured with herbimycin A (A) or ALA-HMA (B) in combination with 0 (●), 0.05 (▼), 0.1 (▲) or 1 (■) ng/ml GM-CSF for 3 days. The test compounds were added the medium once at the beginning of culture. Values are the means ± S.D. of three separate experiments.

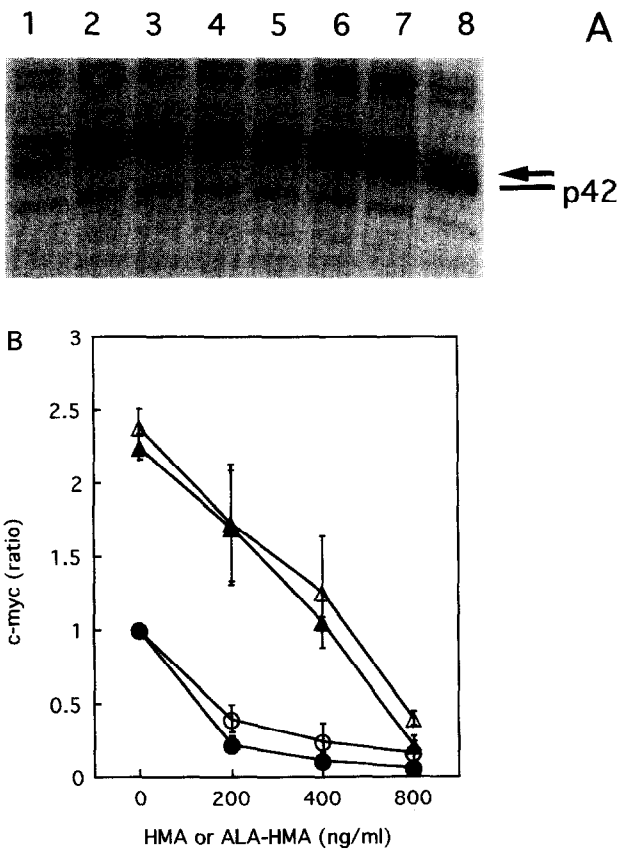

Fig. 3. Inhibition of GM-CSF-induced phosphorylation of MAP kinase (A) and expression of c-*myc* mRNA (B) by herbimycin A (HMA) and 19-allylaminoherbimycin A (ALA-HMA). (A) U937 cells were pretreated with herbimycin A (lanes 3–5) and 19-allylaminoherbimycin A (lanes 6–8) at concentrations of 200 (lanes 3, 6), 400 (lanes 4, 7) and 800 (lanes 5, 8) ng/ml for 4 h. Cells in lanes 1 and 2 were not pretreated with HMA or ALA-HMA. Cells were then stimulated with 1 ng/ml GM-CSF for 10 min (lanes 2–8). MAP kinase was identified by immunoblot with anti-$p42^{MAP\ kinase}$ antibody, and its phosphorylated form is indicated with the arrow. The viabilities of the cells after all the treatments were more than 90%. (B) U937 cells were pretreated with herbimycin A (●,▲) and 19-allylaminoherbimycin A (○,△) for 4 h. Cells were then stimulated with 1 ng/ml GM-CSF (▲,△) for 1 h, or left unstimulated (●,○). Relative amounts of mRNA of c-*myc* were determined as described in Section 2. Values are the means ± S.D. of three separate experiments.

MAP kinase and expression of the c-*myc* gene (Fig. 3). However, pretreatment with herbimycin A, as well as with 19-allylaminoherbimycin A, inhibited the phosphorylation of MAP kinase and c-*myc* mRNA expression induced by GM-CSF (Fig. 3). Thus, pretreatment with herbimycin A was effective in inhibiting the short-term signal transduction induced by GM-CSF. Fig. 4 shows the time courses of the growth inhibition in U937 cells induced by herbimycin A. At day 1, herbimycin A effectively inhibited the proliferation of cells despite the addition of GM-CSF. However, this inhibition induced by herbimycin A gradually decreased in the presence of GM-CSF. Thus, in the presence of GM-CSF, herbimycin A was initially effective, but then gradually became ineffective.

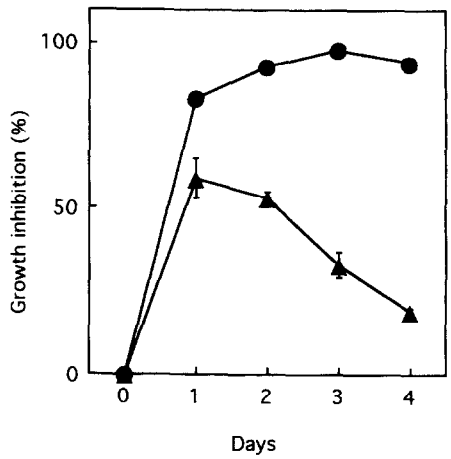

Fig. 4. Time courses of growth inhibition of U937 cells by herbimycin A in combination with GM-CSF. Cells (1×10^5 cells/ml) were cultured with herbimycin A (200 ng/ml) in the absence (●) or presence (▲) of 1 ng/ml of GM-CSF. The test compounds were added to the medium at the beginning of culture. Growth inhibition (%) was calculated by the formula, $(Cx-Tx)/(Cx-I)\times100$, where Cx is the cell number of the control culture on day x, Tx is that of the treated culture on day x, and I is the inoculum number on day 0. The number of cells in the control culture was 2.4 ± 0.1, 6.6 ± 0.2, 13.1 ± 0.4 and $21.8\pm0.6\times10^5$ cells/ml at days 1, 2, 3 and 4, respectively. Values are the means ± S.D. of three separate experiments.

The inhibitory effect of herbimycin A on the proliferation of v-*src*-transfected cells and Philadelphia-positive leukemia cells is abrogated by the addition of thiol compounds, such as GSH [7,8]. Therefore, we examined the effects of GSH and 2-mercaptoethanol in combination with herbimycin A. GSH and 2-mercaptoethanol abrogated the effect of herbimycin A, but not that of 19-allylaminoherbimycin A (Fig. 5). Thus, 19-allylaminoherbimycin A stably inhibited the proliferation of monoblastic U937 cells in the presence of GM-CSF or thiol compounds. Her-

Table 1
GSH and total thiol contents in U937 cells treated with GM-CSF and/or herbimycin A

Treatment	GSH content (nmol/10^7 cells)	Ratio (%)	Thiol content (nmol/10^7 cells)	Ratio (%)
None	44.0 ± 2.8	100	61.7 ± 2.5	100
GM-CSF	49.5 ± 1.1 [a]	113	71.7 ± 2.5 [d]	116
HMA	37.1 ± 1.4 [b]	84	59.1 ± 1.6	96
HMA + GM-CSF	43.1 ± 3.1 [c]	98	61.8 ± 1.1	100
ALA	37.7 ± 5.2	86	NT	
ALA + GM-CSF	39.2 ± 2.3	89	NT	

U937 cells were treated with 1 ng/ml GM-CSF and/or 400 ng/ml herbimycin A (HMA) or 400 ng/ml 19-allylaminoherbimycin A (ALA) for 6 h. Values are the means ± S.D. of three separate experiments. NT, not tested.

[a] significant increase comparing to the control ($P < 0.04$).

[b] significant decrease comparing to the control ($P < 0.02$).

[c] significant increase comparing to the cells treated with HMA alone ($P < 0.04$).

[d] significant increase comparing to the control ($P < 0.01$).

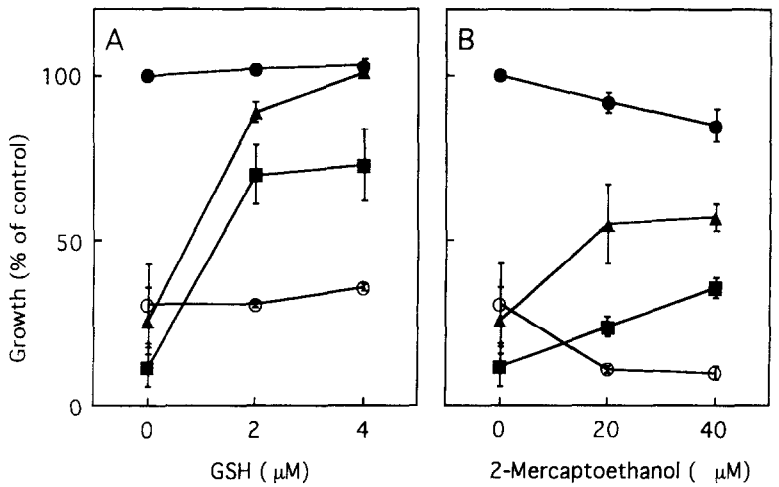

Fig. 5. Effects of GSH and 2-mercaptoethanol on the growth of U937 cells inhibited by herbimycin A and 19-allylaminoherbimycin A. Cells (2×10^5 cells/ml) were treated with GSH (A) or 2-mercaptoethanol (B) in combination with 0 (●), 200 (▲), 400 (■) ng/ml of herbimycin A, or 200 ng/ml of 19-allylaminoherbimycin A (○) for 3 days. Values are the means ± S.D. of three separate experiments.

bimycin A has been reported to prolong the survival of SCID mice inoculated with Philadelphia chromosome-positive leukemia cells, such as K562 cells [6]. 19-Allylaminoherbimycin A inhibited the proliferation of K562 cells, but was less effective than herbimycin A [4]. However, in the presence of GSH, 19-allylaminoherbimycin A was a more effective inhibitor than herbimycin A (Fig. 6).

We next investigated the effects of intracellular sulfhydryl compounds in U937 cells treated with GM-CSF. The GSH and total thiol contents in U937 cells were slightly increased by treatment with GM-CSF, and the GSH content decreased with herbimycin A. When GM-CSF was combined with herbimycin A, these values were elevated to the control levels (Table 1). In U937 cells treated with 19-allylaminoherbimycin A, the decrease of GSH content was not significant ($P = 0.1$). Since herbimycin A has been reported to be inactivated by the formation of an adduct with a thiol compound [7], we next examined whether herbimycin A and the derivative conjugated with GSH. After in vitro incubation with GSH and extraction with chloroform/methanol solution, the amount of herbimycin A eluted with chloroform was diminished (Fig. 7).

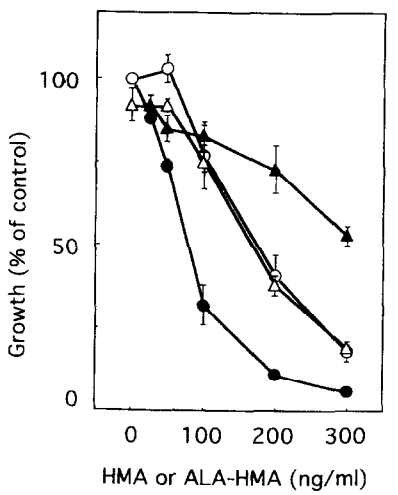

Fig. 6. Effects of GSH on the growth of K562 cells inhibited by herbimycin A or 19-allylaminoherbimycin A. Cells (5×10^4 cells/ml) were treated with herbimycin A (HMA) (●,▲) or 19-allylaminoherbimycin A (ALA-HMA) (○,△) in combination with 0 (●,○) or 20 (▲,△) μM GSH for 4 days. Values are the means ± S.D. of three separate experiments.

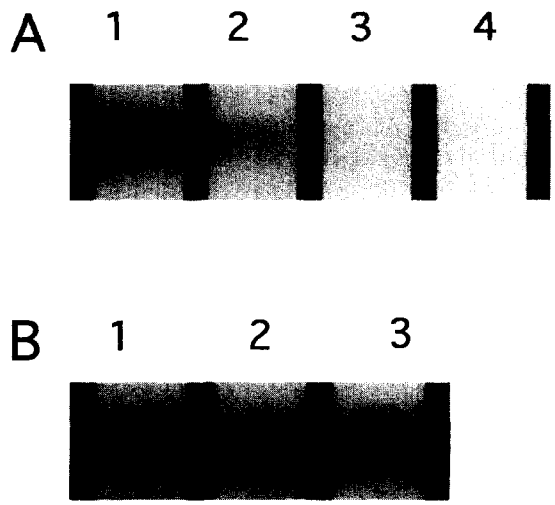

Fig. 7. In vitro treatment with herbimycin A and 19-allylaminoherbimycin A. (A) Herbimycin A at 100 μg/ml (174 μM) was incubated with 0 (lane 1), 100 (lane 2), 300 (lane 3) and 3000 (lane 4) μM GSH in phosphate-buffered saline. (B) 19-Allylaminoherbimycin A at 100 μg/ml (159 μM) was incubated with 0 (lane 1), 300 (lane 2) and 3000 (lane 3) μM GSH. Herbimycin A and 19-allylaminoherbimycin A were separated by thin-layer chromatography. The Rf values of herbimycin A and 19-allylaminoherbimycin A solubilized in phosphate-buffered saline and eluted with chloroform were 0.48 and 0.51, respectively, in a development solvent of benzene-acetone (7:3).

On the other hand, 19-allylaminoherbimycin A was stably detected in the presence of a high concentration of GSH. This result suggests that herbimycin A changes its solubility in chloroform by conjugating with GSH but 19-allylaminoherbimycin A does not.

4. Discussion

Herbimycin A and 19-allylaminoherbimycin A inhibited the proliferation of human monocytic leukemia U937 cells. We previously reported that kinase inhibitors (staurosporine, genistein and methyl 2,5-dihydroxycinnamic acid) inhibited the proliferation of U937 cells, and were more effective in the presence of GM-CSF [21]. In this study, GM-CSF effectively reversed the inhibition of proliferation induced by herbimycin A, but not that induced by 19-allylaminoherbimycin A. Modification of the chemical structure of tyrosine kinase inhibitors has been reported to change their specificity in inhibiting several kinases [22]. Therefore, we examined whether there were differences between herbimycin A and 19-allylaminoherbimycin A with regard to inhibiting the intracellular signal transduction of GM-CSF. The membrane-proximal region of the cytoplasmic domain of GM-CSF receptor β has been reported to be associated with JAK2 kinase and to lead to c-*myc* induction, while the distal region of the receptor induces activation of MAP kinase pathway [20,23]. Pretreatment with herbimycin A, as well as with 19-allylaminoherbimycin A, inhibited the induction of c-*myc* expression and phosphorylation of MAP kinase induced by GM-CSF (Fig. 3). These findings indicate that herbimycin A inhibited the main pathway of the post-receptor signal transduction of GM-CSF, as did 19-allylaminoherbimycin A. The time courses of growth inhibition showed that the activity of herbimycin A in inhibiting the proliferation of U937 cells was gradually decreased in the presence of GM-CSF (Fig. 4). These findings suggest that the presence of GM-CSF inactivates herbimycin A in U937 cells by degrees.

The inhibitory effect of herbimycin A is abrogated by the addition of thiol compounds to the culture medium [7,8], and hematopoietic cytokines such as interleukin-3 and interleukin-6 increase the intracellular GSH level in bone marrow cells [18]. Therefore, we examined the intracellular thiol contents in U937 cells. GM-CSF increased, and herbimycin A decreased, intracellular GSH contents (Table 1). The content of GSH in the culture with both GM-CSF and herbimycin A returned to control levels. Growth inhibition by herbimycin A was reversed by the addition of 2 μM GSH in U937 cells (Fig. 5), while v-*src*-transformed NIH/3T3 required 100 μM GSH for this reversal [7]. Thus, changes in the intracellular concentration of GSH may play a role in the abrogation of the inhibition of U937 cell growth by herbimycin A. Herbimycin A associated with GSH in vitro and changed its solubility in chloroform, but 19-allylaminoherbimycin A did not (Fig. 7). This suggests that herbimycin A easily forms conjugate with the sulfhydryl compound and that the conjugate formation contributes to reducing the activity. Inhibition of the proliferation of U937 cells by 19-allylaminoherbimycin A was not abrogated by the addition of GSH or 2-mercaptoethanol (Fig. 5). These findings indicate that 19-allylaminoherbimycin A is a stable analog of herbimycin A in the presence of thiol compounds. 19-Allylaminoherbimycin A inhibited the growth of Rous sarcoma virus-infected rat cells more effectively than herbimycin A, and inhibited the tyrosine kinase activity of p210, a *bcr-abl* product, in Philadelphia chromosome-positive K562 cells [24,25]. The highly polarized double bonds at positions 17 and 19 (Fig. 1) of the benzoquinone moiety of herbimycin A are considered to be highly reactive to the sulfhydryl group of thiols and protein tyrosine kinases [7,8]. It is interesting that changing position 19 to an allylamine group contributes to its stability without interfering with its inhibitory activity on tyrosine kinases. In the presence of GSH, 19-allylaminoherbimycin A inhibited the proliferation of monoblastic U937 cells and *bcr-abl* rearrangement-positive K562 cells more effectively than herbimycin A (Figs. 5 and 6). GSH, a major intracellular sulfhydryl, plays a role in the detoxication of several anticancer drugs and interferes with their therapeutic efficacy [9]. Therefore, the 19-allylamine derivative of herbimycin A may be useful in the treatment of some types of leukemia.

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