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Effects of Hibarimicins and Hibarimicin-Related Compounds Produced by *Microbispora* on v-Src Kinase Activity and Growth and Differentiation of Human Myeloid Leukemia HL-60 Cells

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We studied the effects of hibarimicins and hibarimicin-related compounds produced by *Microbispora rosea* subsp. *hibaria* [glycosides (hibarimicins A, B, C, D, E, G, H and I) and aglycon (hibarimicinone)] or compounds produced by its mutants [glycosides (HMP-P4 and -Y6), aglycons (HMP-P1 and -Y1) and shunt products (HMP-M1, M2, M3 and -M4)] on v-Src tyrosine kinase and growth and differentiation of human myeloid leukemia HL-60 cells. Among them, hibarimicin B was a strong and the most selective v-Src kinase inhibitor with differentiation inducing activity of HL-60 cells. Hibarimicin E similarly induced HL-60 cell differentiation but had no v-Src kinase inhibitory activity. Hibarimicinone was the most potent v-Src kinase inhibitor, although less selective, and did not induce differentiation of HL-60 cells. Hibarimicin B competitively inhibited ATP binding to the v-Src kinase, but hibarimicinone showed noncompetitive inhibition. These two compounds, however, showed similar mixed types of inhibition against a Src substrate binding to the v-Src kinase. Altogether, these results suggest that signaling molecules other than Src might be more important in the differentiation induction of HL-60 cells.

Many oncogenes with tyrosine kinase activity are involved in tumorigenesis. Protein tyrosine kinase inhibitors may be useful in cancer chemotherapy and differentiation therapy[1]. Human myeloid leukemia cell line HL-60 cells can be differentiated from an immature form into a different cell type of the myelomonocytic lineage by a variety of agents[2,3]. The HL-60 cell has been used not only as a good model for studying the differentiation process but also as a good screening system for the development of the differentiation-inducing agents.

We previously reported that hibarimicins A, B, C, D and G produced by *Microbispora rosea* subsp. *hibaria* are potent v-Src-tyrosine kinase inhibitors. They are the first natural glycosides with selective Src-tyrosine kinase inhibitory action[4~6]. Searching for more potent and selective inhibitors, we studied the effects of many hibarimicins and hibarimicin-related compounds on the Src tyrosine kinase as well as growth and differentiation of human myeloid leukemia HL-60 cells. The compounds were obtained from the fermentation broth of *Microbispora rosea* subsp. *hibaria* (hibarimicins A, B, C, D, E, G, H and I) or its mutants (HMP-P4, Y6, M1, M2, M3 and -M4). Some of their aglycons (hibarimicinone and HMP-P1 and -Y1) were also studied. Chemical structures of test compounds are shown in Fig. 1.

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Fig. 1. Structures of hibarimicin and related compounds used in this study.

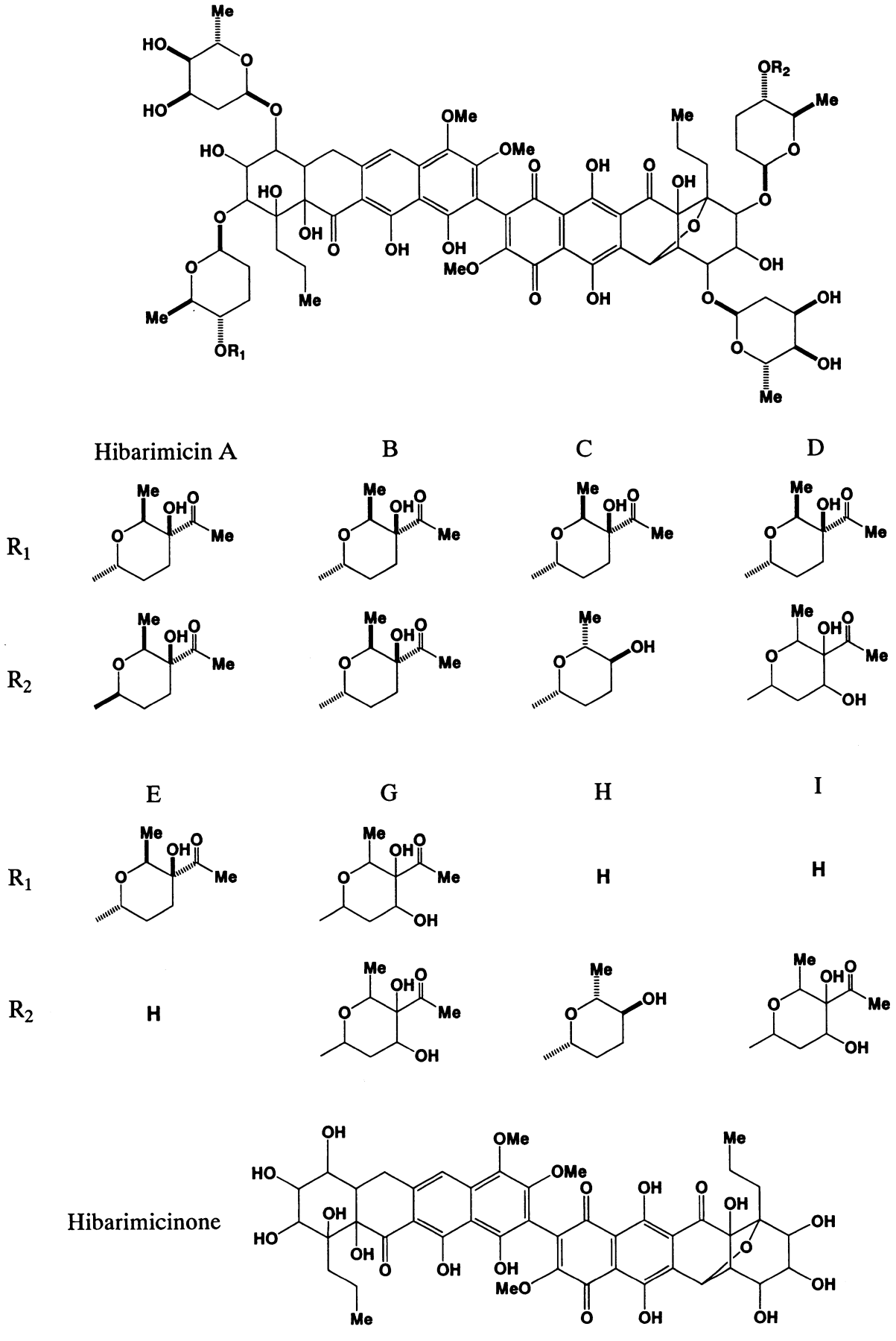
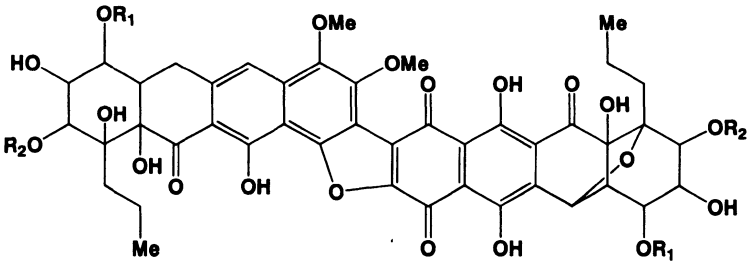
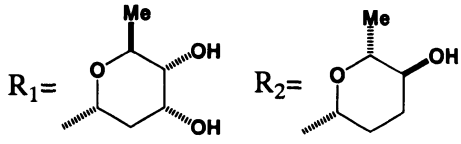


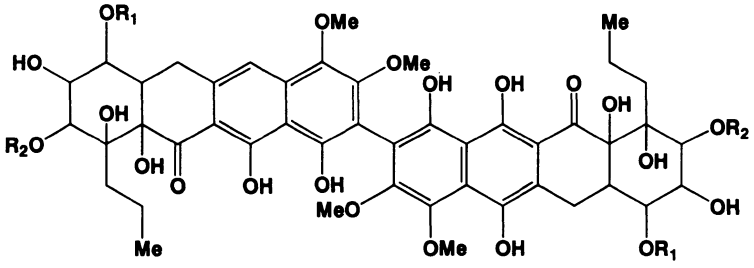
Fig. 1. (Continued).



HMP-P1 $R_1=R_2=H$

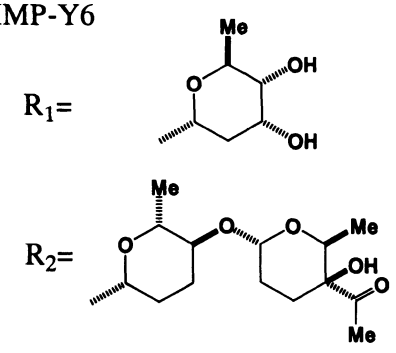
HMP-P4



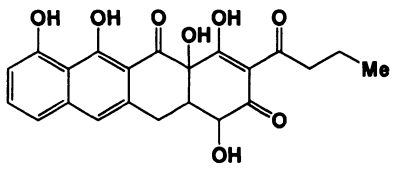


HMP-Y1 $R_1=R_2=H$

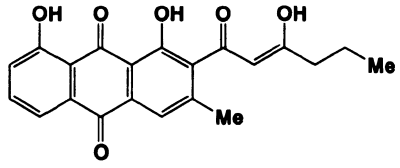
HMP-Y6



HMP-M1



HMP-M2



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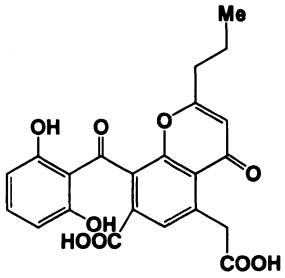
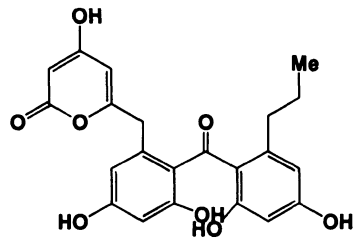


HMP-M4



Materials and Methods

Multiple Protein Kinase Assay

Multiple protein kinase assay was done according to the procedure reported[7,8].

v-Src Autokinase Assay

Protein lysate was prepared from v-*src*-transformed NIH3T3 cells cultured to near confluence on 90 mm dishes. The lysis buffer was composed of 20 mM HEPES (pH 7.4), 150 mM NaCl, 1% (v/v) Triton X-100, 1 mM EDTA, 0.1 mM Na_3VO_4, and 25 μg/ml each of antipain, leupeptin, pepstatin, and phenylmethylsulfonyl fluoride. The v-Src protein of the protein lysate (1 mg/ml) was

immunoprecipitated by incubation with 2 μg of anti-v-Src mouse monoclonal antibody, clone 327 (Calbiochem) at 4°C for 2 hours and subsequently with 40 μg of rabbit antimouse IgG and 250 μl of 10% (v/v) protein A Sepharose-4FF beads in STE buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA) at 4°C overnight. The immunoprecipitated beads were washed 3 times with lysis buffer without protease inhibitors, twice with STE buffer, and once with 20 mM HEPES buffer (pH 7.4).

The beads were resuspended in 250 μl of kinase buffer without the ATP mixture. To 17.5 μl of aliquots, 2.5 μl of DMSO with each compound or without (control) was added. The tubes were preincubated at room temperature for 30 minutes. Kinase reaction was started by adding 5 μl of ATP mixture and allowed to proceed for 20 minutes in a 25°C water bath. The kinase reaction mixture included the following: immunoprecipitated v-Src kinase protein corresponding to about 70 μg of the lysate protein, 5 μCi of ^{32}P-γ-ATP (1, 3, 10, 30, or 100 μM ATP), 20 mM HEPES (pH 7.4), 5 mM $MgCl_2$, 10% DMSO alone or with a concentration of hibarimicin B or hibarimicinone. The reaction was stopped by adding 4×SDS sample buffer. After boiling for 3 minutes in a water bath, the tubes were centrifuged. The supernatant was loaded and run on 9% SDS-polyacrylamide gel. The gel was analyzed by autoradiography and image analysis using BAS (Fuji Co.).

Src Substrate Peptide Phosphorylation Assay

The v-Src protein was immunoprecipitated as described above. The beads were resuspended in 300 μl of kinase buffer without the ATP mixture. To 12.5 μl of the aliquot, 2.5 μl of DMSO with each compound or without (control) and 5 μl of Src substrate peptide were added. The tubes were preincubated at room temperature for 30 minutes. Kinase reaction was started by adding 5 μl of the ATP mixture and was allowed to proceed for 20 minutes in a 25°C water bath. The kinase reaction mixture included the following: immunoprecipitated v-Src kinase protein corresponding to about 42 μg of lysate protein, 2.5 μCi ^{32}P-γ-ATP (10~300 μM ATP), a Src substrate peptide, p34cdc2[6-20] (U.B.I.; 15~150 μM), 20 mM HEPES (pH 7.4), 5 mM $MgCl_2$, 10% DMSO alone or with a concentration of hibarimicin B or hibarimicinone.

In ATP competition experiments, ATP concentration was 10, 30, 100, or 300 μM and the Src substrate peptide concentration was 150 μM. In Src substrate competition experiments, the Src substrate peptide concentration was 15, 25, 50, 85, or 150 μM and the final ATP concentration was 15 μM. The kinase reaction was stopped by adding 15 μl of 20% trichloroacetic acid and 10 μl of 0.5% bovine serum albumin. After centrifugation (15,000 r.p.m.×10 minutes), 40 μl of the aliquot was loaded onto P81 Whatman phosphocellulose paper (2×2 cm). The papers were washed four times with 75 mM phosphoric acid (250 ml each for 10 minutes) and once with 99.5% EtOH (200 ml for 5 minutes). They were then dried and the radioactivity was counted by Cerenkov counting.

Assay of Cell Growth and Differentiation of Leukemia Cells

Human myeloid leukemia HL-60 cells were cultured in suspension in RPMI 1640 medium supplemented with 10% fetal bovine serum. Cell numbers were counted with a Model Z1 Counter (Beckman-Coulter Electronics, Miami, FL, USA) after culture for 6 days. Granulocyte differentiation was colorimetrically measured by nitroblue tetrazolium (NBT) reduction as described in the previous report[9].

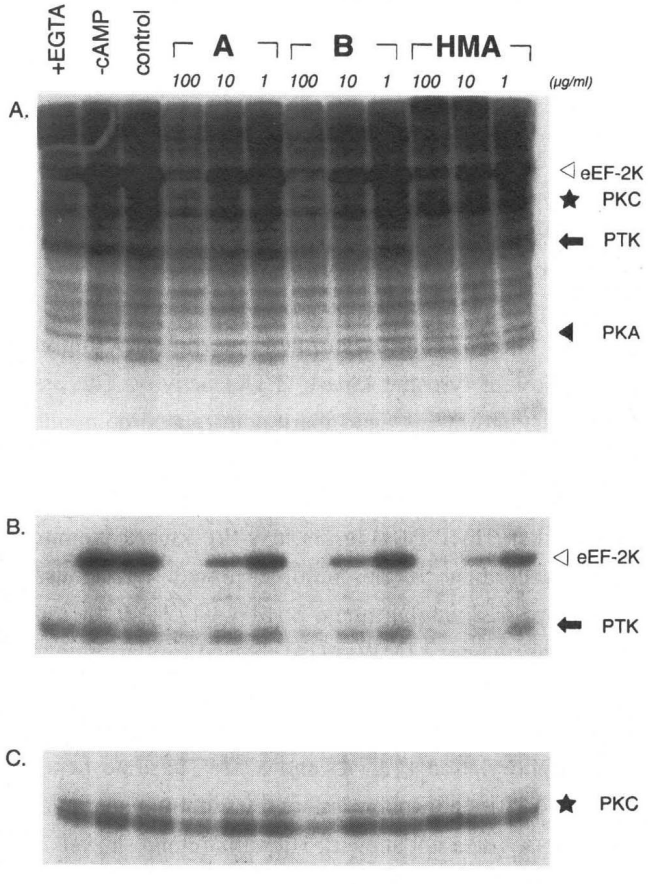
Results and Discussion

In a multiple protein kinase assay using the postnuclear fraction of v-src transformed NIH3T3 cells, not only hibarimicin A and B but also each of the corresponding aglycons, HMP-P1 and hibarimicinone, potently inhibited the Src protein tyrosine kinase (PTK) activity. The assay results of hibarimicins and hibarimicin-related compounds are summarized on Table 1. Hibarimicin B was stronger than hibarimicin A (Fig. 2) and hibarimicinone was stronger than HMP-P1 (Fig. 3) in v-Src kinase inhibitory activity according to the multiple protein kinase assay. When compared to hibarimicin B (MW=1724) at the molar level, hibarimicinone (MW=924) showed similar inhibition to v-Src kinase activity assayed using multiple protein kinase assay (Table 2) or a stronger inhibition to autophosphorylation (Fig. 4) and a Src substrate peptide, p34cdc2[6-20], phosphorylation by immunoprecipitated v-Src kinase (data not shown). Hibarimicinone showed 1.3- or 5.5-fold stronger inhibition against eEF-2K or PKC than hibarimicin B, respectively (Table 2). In autophosphorylation of immunoprecipitated v-Src kinase (Fig. 4), hibarimicinone (IC_{50}=10.5 μM) showed more than two-fold stronger inhibition than hibarimicin B (IC_{50}=23 μM). At higher ATP concentrations (30 and 100 μM ATP), hibarimicinone showed much stronger inhibition to v-Src kinase compared with hibarimicin B (data not shown). Collectively, hibarimicinone is a stronger v-Src kinase inhibitor than hibarimicin B, but the latter seems to be a more selective v-Src kinase inhibitor than its

aglycon, hibarimicinone.

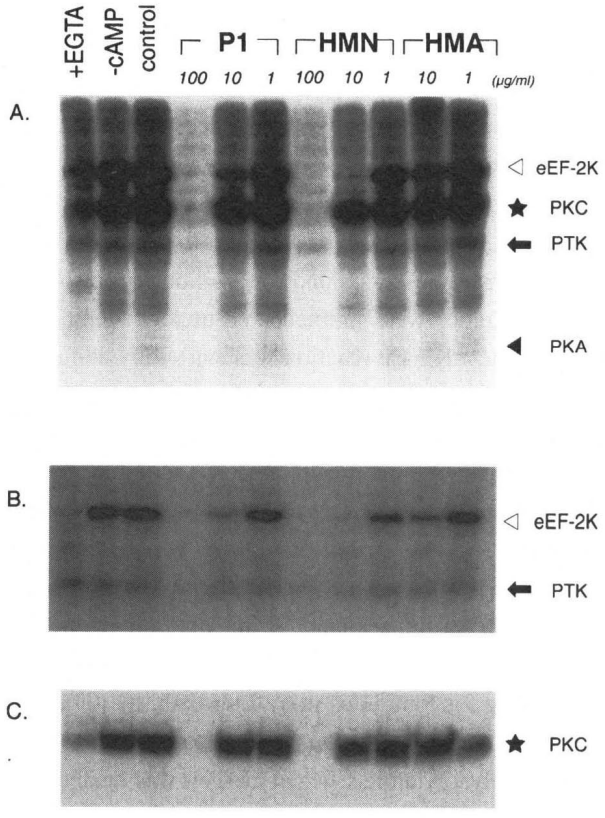
Hibarimicin B showed a competitive inhibition to ATP binding to the v-Src kinase (Fig. 5, panel A) as represented by increase of *Km* without change of Vmax. However, hibarimicinone showed a noncompetitive inhibition (Fig. 5, panel B) as represented by decrease of Vmax without change of *Km*. To a Src substrate peptide, p34cdc2[6-20], binding to v-Src kinase, however, both compounds showed similar, mixed types of inhibition as represented by increase of *Km* and decrease of Vmax (Fig. 6, panel A and B).

Fig. 2. Effects of hibarimicin A and B on multiple protein kinases.



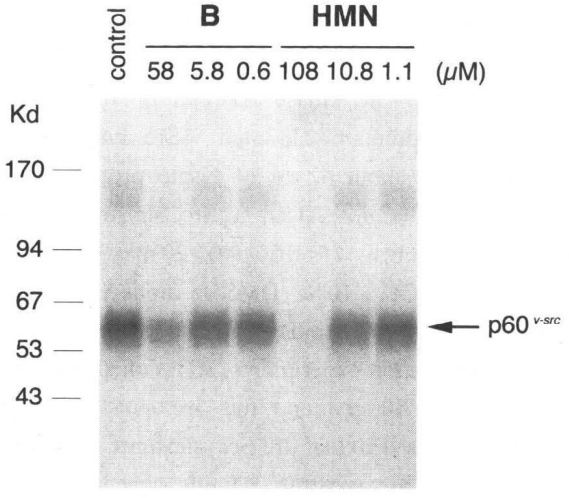
Protein kinase reaction was performed as described in Materials and Methods in the presence of indicated additions. The final concentrations were: EGTA, 0.5 mM; cAMP, 20 μM; and DMSO, 10%. The phosphorylated proteins were analyzed using SDS-PAGE (9% gel) and visualized by autoradiography. Shown are results before (A) and after (B) 1 N KOH treatment at 55°C for 2 hours to enrich phosphotyrosine or after (C) heat treatment. Arrowheads, stars, arrows, and open triangles represent PKA, PKC, PTK (v-Src protein tyrosine kinase) and eEF-2K (=CaMK-III) activities, respectively. Herbimycin A (HMA) was used as a positive control.

Fig. 3. Effects of aglycons HMP-P1 (P1) and hibarimicinone (HMN) on multiple protein kinases.



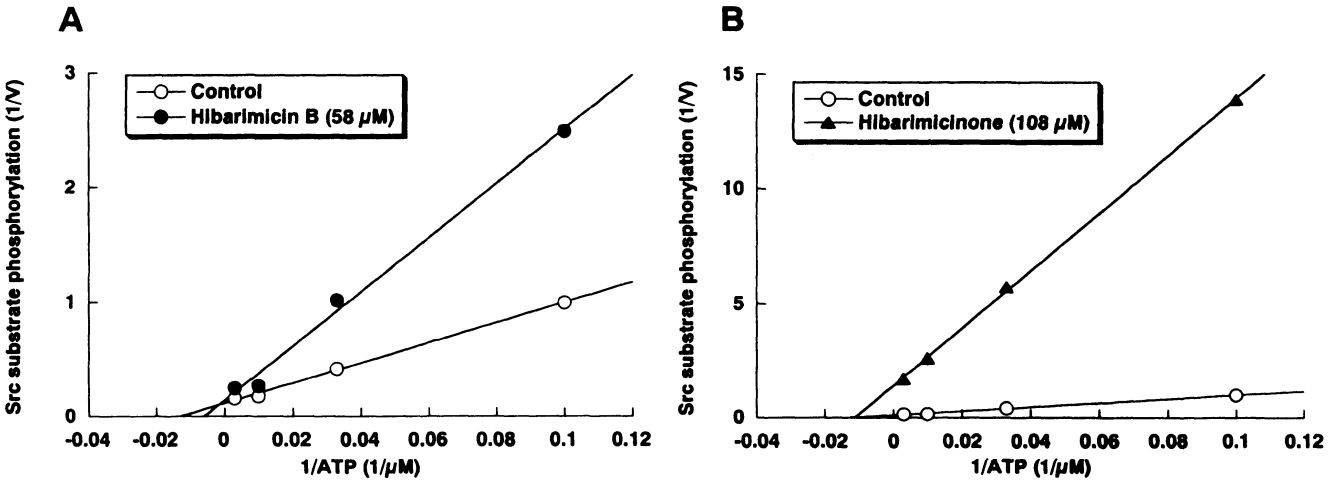
Symbols are the same as in Fig. 2.

Fig. 4. Effects of hibarimicin B (B) and hibarimicinone (HMN) on autophosphorylation of immunoprecipitated v-Src kinase.



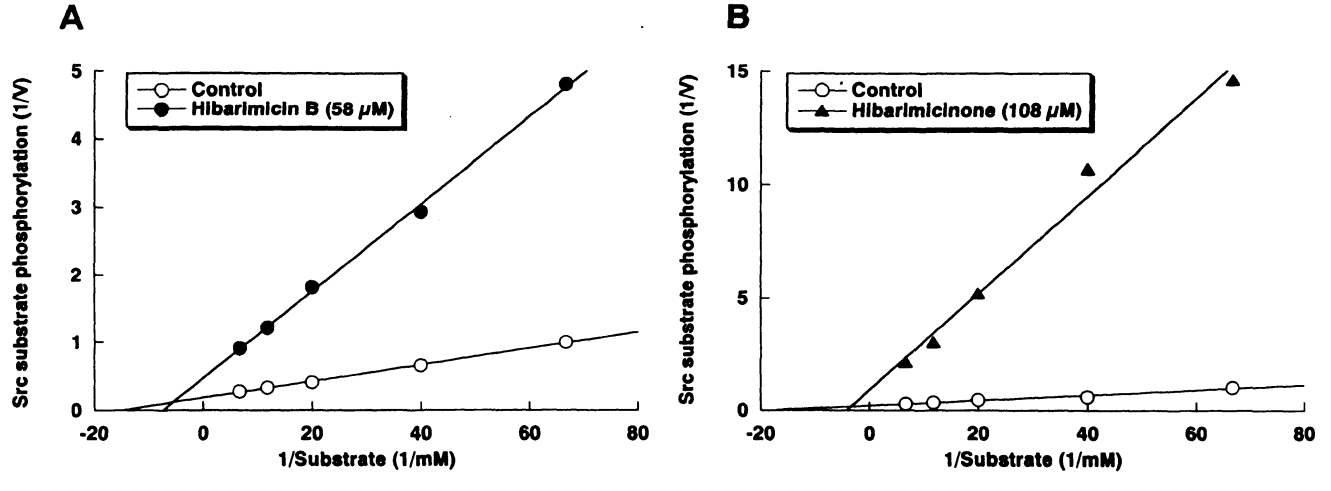
Arrow on the right indicates phosphoryated p60$^{v\text{-}src}$ protein. Positions of molecular size markers are shown on the left.

Fig. 5. Competitive inhibition of hibarimicin B (panel A) and noncompetitive inhibition of hibarimicinone (panel B) on ATP binding to immunoprecipitated v-Src kinase.



Plotted data were obtained from means of two independent experiments each in duplicate.

Fig. 6. Mixed type of inhibition of hibarimicin B (panel A) and hibarimicinone (panel B) on a Src substrate binding to immunoprecipitated v-Src kinase.



Plotted data were obtained from means of two independent experiments each in duplicate.

Similar findings were observed in the autophosphorylation of immunoprecipitated v-Src kinase (data not shown). These different inhibitory modes may explain why hibarimicinone showed much stronger inhibition to v-Src kinase than hibarimicin B at high ATP concentrations (30 and 100 μM ATP). How the differential inhibitory mode of the two compounds can be involved in various cell functions, however, remains to be studied.

Effects of hibarimicins and hibarimicin-related compounds on the growth of HL-60 cells are shown in panel A of Fig. 7.; their effects on the differentiation are shown in panel B of the figure and are summarized in Table 1. The differentiation-inducing and the growth-inhibitory actions of hibarimicins B and E seem to be qualitatively similar even though hibarimicin E is a little weaker in terms of concentration. Interestingly, hibarimicin E does not have

Fig. 7. Effects of hibarimicin-related compounds on the growth (panel A) and the differentiation (panel B) of HL-60 cells.

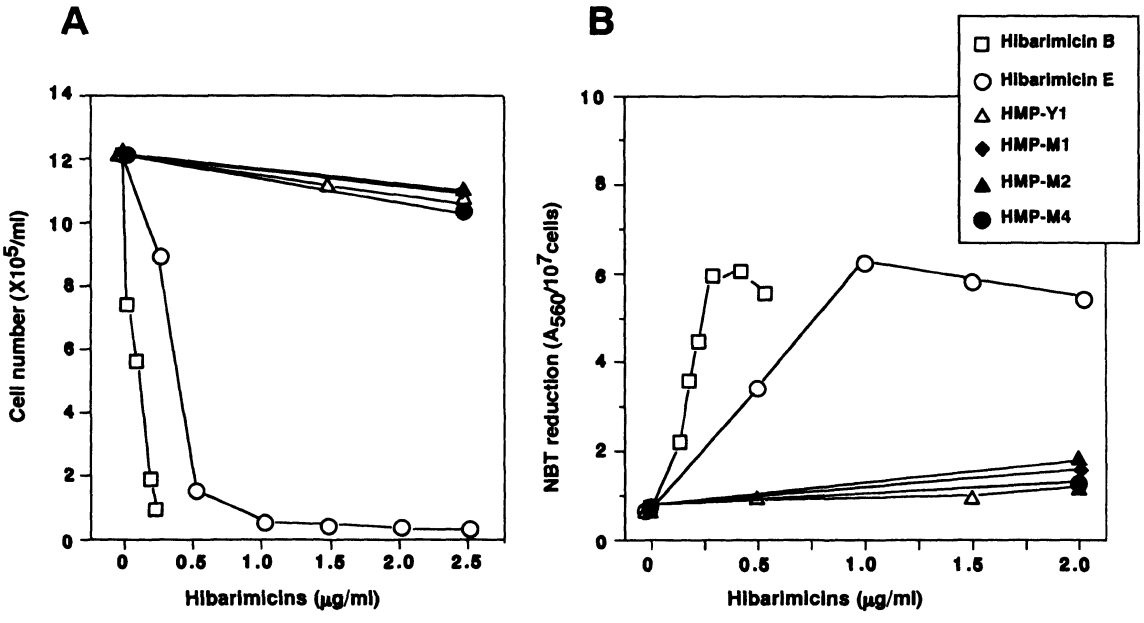


Table 1. Summary of effects of hibarimicins and hibarimicin-related compounds on v-Src protein tyrosine kinase and HL-60 cell differentiation.

Compound	Classification	v-Src tyrosine kinase inhibition	Induction of cell differentiation
Hibarimicin A	Glycoside (6 sugars)	1+*	2+
Hibarimicin B	Glycoside (6 sugars)	2+	2+
Hibarimicin C	Glycoside (6 sugars)	1+	2+
Hibarimicin D	Glycoside (6 sugars)	2+	2+
Hibarimicin E	Glycoside (5 sugars)	-	2+
Hibarimicin G	Glycoside (6 sugars)	1+	1+
Hibarimicin H	Glycoside (5 sugars)	1+	1+
Hibarimicin I	Glycoside (5 sugars)	1+	-
HMP-P4	Glycoside (4 sugars)	1+	1+
HMP-Y6	Glycoside (6 sugars)	-	1+
Hibarimicinone	Aglycon	3+	-
HMP-P1	Aglycon	2+	-
HMP-Y1	Aglycon	-	-
HMP-M1	Mutant product	1+	-
HMP-M2	Mutant product	1+	-
HMP-M3	Mutant product	-	-
HMP-M4	Mutant product	-	-

* + after number represents relatively stronger activity. -; no activity

Table 2. Inhibitory effects of hibarimicin B and hibarimicinone on several protein kinases using multiple protein kinase assay.

Compound	IC_{50} (μM)		
	PKC	PTK[a]	eEF-2K
Hibarimicin B	36	1.8	2.4
Hibarimicinone	6.5	1.2	1.9

[a] Tyrosine phosphorylation of 60 kDa bands (v-Src kinase) of alkali-treated gels was quantitated by densitometer and the IC_{50} was calculated.

v-Src kinase inhibitory action but hibarimicin B does (Table 1). Genistein, a broad-spectrum kinase inhibitor, induced granulocyte differentiation of HL-60 cells[10]. According to the previous report[9], v-Src tyrosine kinase inhibitory action of hibarimicins A, B, C and D did not show a close relationship in their IC_{50}s required for growth inhibition or differentiation induction of HL-60 cells. These results suggest that signaling molecule(s) other than Src might be more important in differentiation of these cells. HMP-Y1, M1, M2 and -M4 did not show significant influence on the differentiation and growth (Fig. 7). Even though aglycons of hibarimicins (hibarimicinone, HMP-P1 and -Y1) did not show HL-60 cell differentiating action, the presence of sugars of hibarimicins is apparently not always linked to HL-60 cell differentiation because a glycoside hibarimicin I, containing 5 sugars, did not have differentiation inducing action.

In conclusion, hibarimicinone was the most potent v-Src kinase inhibitor but was less selective than hibarimicin B and hibarimicin B was a strong and most selective v-Src kinase inhibitor with differentiation inducing activity of HL-60 cells. Hibarimicin E induced HL-60 cell differentiation but had no v-Src kinase inhibitory activity. These results suggest that the differentiation-inducing activity of hibarimicins is not directly associated with Src kinase-inhibiting activity, and may be associated with the modulation of other signaling pathway(s).

Acknowledgements

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growth and induces myelomonocytic differentiation of human myeloid leukemia HL-60 cells. Leukemia Res. 20: 491~497, 1996

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