

β -Hydroxyisovalerylshikonin Is a Novel and Potent Inhibitor of Protein Tyrosine Kinases

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β -Hydroxyisovalerylshikonin (β -HIVS), a compound isolated from *Lithospermium radix*, most efficiently induced cell-death in two lines of lung cancer cells, namely, NCI-H522 and DMS114, whereas shikonin was effective against a wide variety of tumor cell lines. During our studies of the mechanism of action of β -HIVS on tumor cells, we found that this compound inhibited protein tyrosine kinase (PTK) activity. The tyrosine kinase activities of a receptor for EGF (EGFR) and v-Src were strongly inhibited and that of KDR/Flk-1 was weakly inhibited by β -HIVS. The inhibition by β -HIVS of the activities of EGFR and v-Src was much stronger than that by shikonin. The IC₅₀ values of β -HIVS for EGFR and v-Src were approximately 0.7 μ M and 1 μ M, respectively. Moreover, the inhibition of v-Src by β -HIVS was non-competitive with respect to ATP. These results strongly suggest that the action of β -HIVS, as well as that of shikonin, involves the inhibition of PTK, and they also suggest the possibility of producing a novel group of PTK inhibitors based on shikonin as the parent compound.

Key words: β -Hydroxyisovalerylshikonin — Shikonin — Tyrosine kinase inhibitor — EGFR — v-Src

Lithospermium radix is traditional oriental medicinal herb and its roots were used in ancient Japan to prepare an ointment for treatment of cuts and burns. Shikonin is a component of *L. radix* that exhibits antitumor activity in mice implanted with sarcoma 180 tumor cells.¹⁾ Shikonin has also been shown to inhibit the activities of topoisomerases I²⁾ and II,³⁾ and to have an antioxidative protective effect against reactive oxygen species.⁴⁾ However, the mechanisms responsible for these activities of shikonin are unknown.

We found previously that, when present at low concentrations between 0.01 μ M and 1 μ M, β -HIVS, another constituent of *L. radix*, inhibited the growth of various lines of cancer cells derived from human tumors, such as leukemia HL-60 and U937 cells and melanoma VMRC-MELG cells.⁵⁾ We also showed that treatment of HL-60 cells with β -HIVS induced apoptosis and that such apoptosis was preceded by activation of MAP kinases, such as ERK2, JNK1, and p38.⁵⁾

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Abbreviations: β -HIVS, β -hydroxyisovalerylshikonin; EGFR, a receptor for EGF; PTK, protein tyrosine kinase; ECL, electrochemiluminescence; DMEM, Dulbecco's modified Eagle's medium; HEPES, *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]; PBS, phosphate-buffered saline; DTT, dithiothreitol; WGA, wheat germ agglutinin; VEGF, vascular endothelial growth factor.

As part of our efforts to determine the mechanisms of inhibition of cell growth and induction of apoptosis by β -HIVS, we examined the effects of β -HIVS on the induction of cell death in 37 lines of cancer cells derived from human solid tumors and on the activities of various protein kinases. We report here that β -HIVS is a novel inhibitor of the PTK activities of proteins such as EGFR, v-Src, and KDR/Flk-1 and, moreover, that its inhibitory activity for EGFR and v-Src is much stronger than that of shikonin.

MATERIALS AND METHODS

Reagents and antibodies β -HIVS was isolated from the plant *Lithospermium radix* as described previously.⁵⁾ β -HIVS and shikonin were dissolved in ethanol and diluted with ethanol to make working-strength solutions. The monoclonal antibody against phosphotyrosine (PY20) was purchased from ICN Biomedicals, Inc. (Aurora, OH). The ECL Western Blot Kit was obtained from Dupont NEN (Boston, MA). Geneticin G418 was purchased from Calbiochem (La Jolla, CA). The catalytic subunit of protein kinase A isolated from bovine heart was from Sigma Chemical Co. (St. Louis, MO). [γ -³²P]ATP (3000 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK).

Cell lines and cell culture Human epidermoid carcinoma A431 cells were provided by the Japanese Cancer Research Bank (Tokyo). Other lines of human cancer cells

and their sources have been described elsewhere.⁶⁾ SR-3Y1 cells that stably expressed v-Src were kindly donated by Dr. H. Hanafusa of the Osaka Bioscience Institute (Osaka). All cell lines with the exception of KDR/Flk-1-NIH3T3 cells were maintained in RPMI 1640 medium or DMEM plus 10% fetal calf serum in a humidified incubator in an atmosphere of 5% CO₂ in air at 37°C. KDR/Flk-1-NIH3T3 cells were cultured in DMEM plus 10% fetal calf serum, supplemented with 200 μ g/ml geneticin (G418).⁷⁾

Quantitation of cell death The effects of β -HIVS and shikonin on various lines of cells derived from human solid tumors were evaluated with a panel of human cancer cell lines, that was established by the Anticancer Drug Screening Committee organized by the Cancer Chemotherapy Center of the Japanese Foundation for Cancer Research (Tokyo).⁶⁾ The various lines of cells were treated with β -HIVS or shikonin for 48 h and proliferation was assessed with sulforhodamine B, as described by Skehan *et al.*⁸⁾ The concentrations of β -HIVS and shikonin that caused a decrease of 50% in the number of seeded tumor cells (LC₅₀) were determined for each cell line and the mean values of log₁₀LC₅₀ (MG-MID) were calculated. The difference between log₁₀LC₅₀ for each cell line and MG-MID was calculated.

Detection of protein kinase SR-3Y1 cells that stably express v-Src were homogenized in hypotonic buffer that contained 1 mM HEPES, pH 7.4, 5 mM MgCl₂ and 25 μ g/ml each of the protease inhibitors antipain, leupeptin, and pepstatin A. The homogenate was centrifuged at 500g for 5 min and the supernatant was used as the cell lysate. Phosphorylation reactions were performed in a standard buffer for assays of protein kinases, which contained 20 mM HEPES, pH 7.7, 10 mM MgCl₂, 0.1 mM Na₃VO₄, 10 mM β -glycerophosphate, 1 mM NaF, 2.5 mg of cell proteins as lysate, and various activators or inhibitors of kinase activities, as described previously.⁹⁻¹¹⁾ Each reaction was initiated by the addition of [γ -³²P]ATP (10 μ Ci) and, after incubation at 25°C for 15 min, each reaction mixture was fractionated by SDS-PAGE (9% polyacrylamide). Phosphorylated proteins were visualized by autoradiography.

Quantitation of the PTK activity of EGFR EGFR was isolated from A431 cells by affinity chromatography on wheat germ agglutinin-agarose (Wako Pure Chemical Industries, Ltd., Osaka). The isolated EGFR was incubated with assay buffer, as described above, that contained [γ -³²P]ATP. The reaction was terminated by addition of one-tenth volume of SDS loading buffer that contained 0.6 M Tris-HCl, pH 6.8, 20% SDS, saturated sucrose, 10% mercaptoethanol, and 0.02% bromphenol blue and the reaction mixture was fractionated by SDS-PAGE (7.5% polyacrylamide gel). After SDS-PAGE, bands of phosphorylated EGFR were visualized by autoradiography and quanti-

fied with the Storm 830 system (Amersham Pharmacia Biotech).

Quantitation of the PTK activity of v-Src The monoclonal antibody against v-Src was mixed with protein A-Sepharose (Santa Cruz Biotechnology, Santa Cruz, CA) and incubated at 4°C for 1 h. The protein A-Sepharose with bound Mab was washed with assay buffer by centrifugation, mixed with a lysate of SR-3Y1 cells that stably expressed v-Src, and incubated at 4°C for 1 h. The suspension of protein A-Sepharose with bound v-Src was washed twice by centrifugation with the assay buffer and used as a source of tyrosine kinase activity. It was incubated with substrate "Raytide"; Oncogene Research Products, Cambridge, MA, with or without inhibitor, and [γ -³²P]ATP in assay buffer at 30°C for 10 min. The reaction was terminated by the addition of phosphoric acid and an aliquot of the reaction mixture was spotted on p81 filter paper (Whatman International, Ltd., Maidstone, England). The radioactivity incorporated into "Raytide" was determined by liquid scintillation counting.

Detection of PTK activity in KDR/Flk-1-NIH3T3 cells KDR/Flk-1-NIH3T3 and Flt-1-NIH3T3 cells, which over-expressed KDR/Flk-1 and Flt-1, respectively, were prepared as described by Seetharam *et al.*¹²⁾ After treatment of these cells with β -HIVS or shikonin, cells were washed twice with ice-cold PBS and lysed by addition of 100 μ l of sample buffer for SDS-PAGE [62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% glycerol, and 50 mM DTT]. Samples that contained 20 μ g of protein were subjected to SDS-PAGE (7.5% polyacrylamide gel) and bands of proteins were transferred electrophoretically to a polyvinylidene difluoride membrane (Millipore Co., Bedford, MA). The membrane was then probed with the monoclonal antibody against phosphotyrosine (PY20) by the standard method. Bands of immunoreactive proteins were detected with an ECL Kit.

RESULTS

Effects of β -HIVS on various lines of cells derived from solid tumors The structure of β -HIVS, as compared to

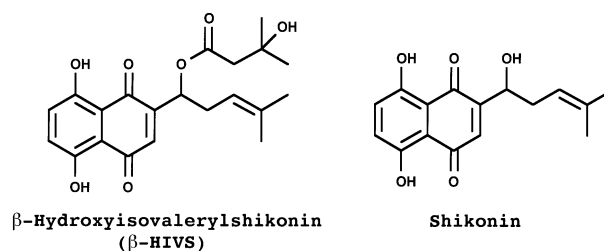


Fig. 1. Structures of β -HIVS and shikonin.

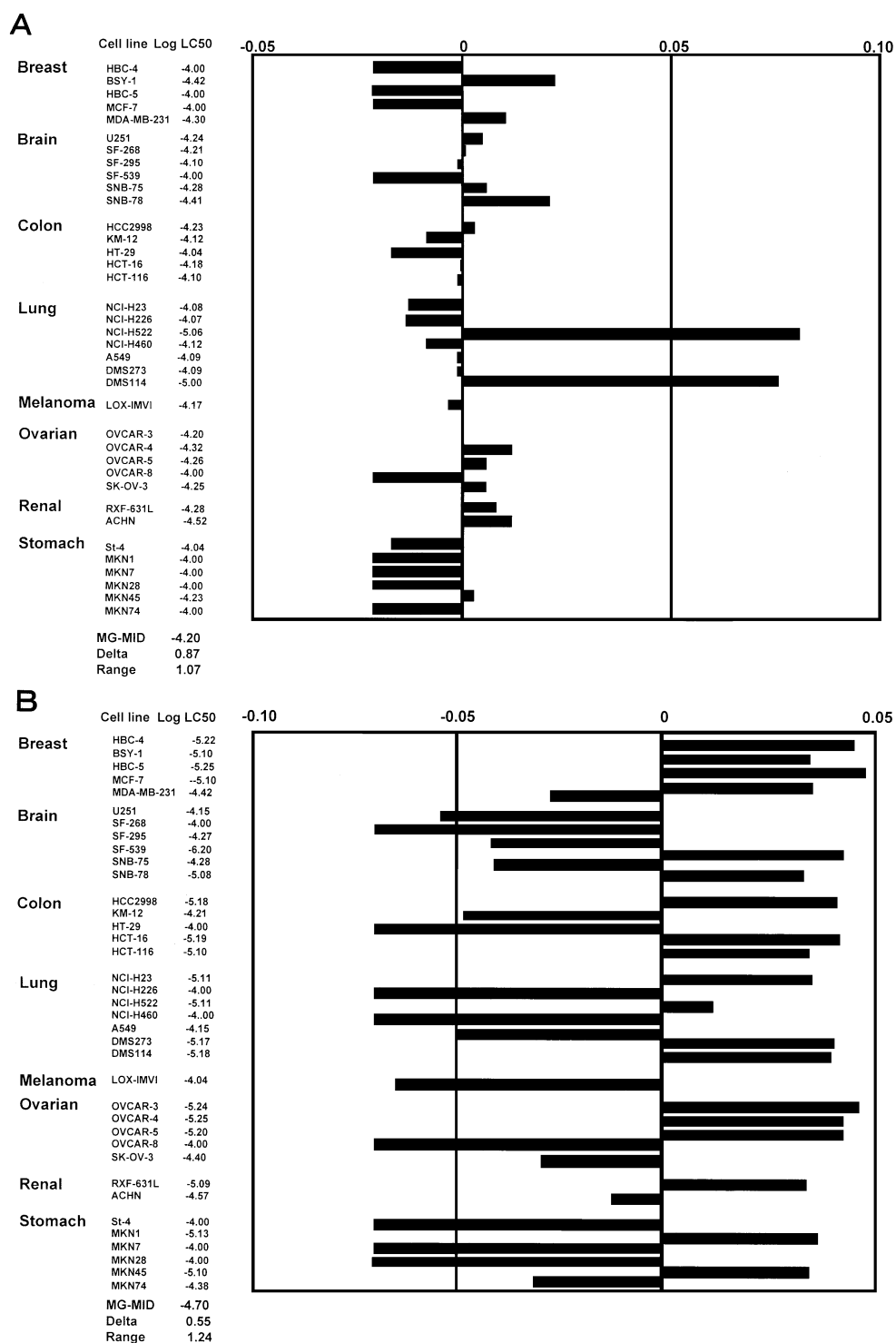


Fig. 2. Effects of β -HIVS and shikonin on 37 lines of cancer cells derived from human solid tumors. Cultured cells were treated with β -HIVS (A) or shikonin (B) for 48 h and proliferation was assessed as described in "Materials and Methods." The concentration of β -HIVS or shikonin that decreased the number of viable cells to 50% of the number of seeded cells (LC_{50}) was determined for each cell line and the values of $\log_{10}LC_{50}$ are indicated. Differences between $\log_{10}LC_{50}$ for each cell line and the mean value of $\log_{10}LC_{50}$ are represented by bars. The scale indicates the difference between these logarithms.

that of shikonin, is shown in Fig. 1. Fig. 2A shows the effect of β -HIVS on the proliferation of a variety of cell lines derived from human solid tumors. As is evident from the figure, β -HIVS efficiently induced cell-death in two lines of lung cancer cells, namely, NCI-H522 and DMS114 cells. Furthermore, two lines of breast cancer cells, three lines of brain cancer cells, three lines of ovarian cancer cells, and two lines of renal cancer cells were moderately sensitive to β -HIVS (Fig. 2A). The effects of β -HIVS on these cell lines were quite different from those of shikonin. As shown in Fig. 2B, shikonin induced cell death in a wide variety of tumor cell lines. This difference of antitumor activity between β -HIVS and shikonin was presumably due to the presence of the β -hydroxyisovaleryl substituent on shikonin.

Effects of β -HIVS on protein kinases In an attempt to clarify the mechanism responsible for the induction of cell-death in tumor cells by β -HIVS, we examined whether this compound might affect the activities of protein kinases using a method that allows detection of activities of several protein kinases simultaneously.⁹ When β -HIVS and shikonin were added separately at 2.6 μ M and 3.5 μ M, respectively, to a lysate of v-Src-expressing SR-

3Y1 cells and phosphorylation was allowed to proceed, the intensities of bands of proteins phosphorylated by PTK decreased markedly (Fig. 3B). By contrast, the intensities of bands of proteins phosphorylated specifically by protein kinase A, protein kinase C, and eukaryotic elongation factor-2 kinase, and calmodulin-dependent protein kinase III were not significantly altered by β -HIVS or shikonin at 2.6 μ M and 3.5 μ M, respectively (Fig. 3A). These results suggested that β -HIVS and shikonin each selectively inhibited PTK activities. To ascertain the selectivity of β -HIVS for PTK, the catalytic subunit of protein kinase A was treated with β -HIVS under the same conditions as described above, but practically no change in its activity was observed on treatment with as much as 100 μ M β -HIVS (results not shown).

Effects of β -HIVS on the PTK activity of EGFR To examine whether β -HIVS and shikonin might affect the PTK activity of EGFR, we isolated EGFR by affinity chro-

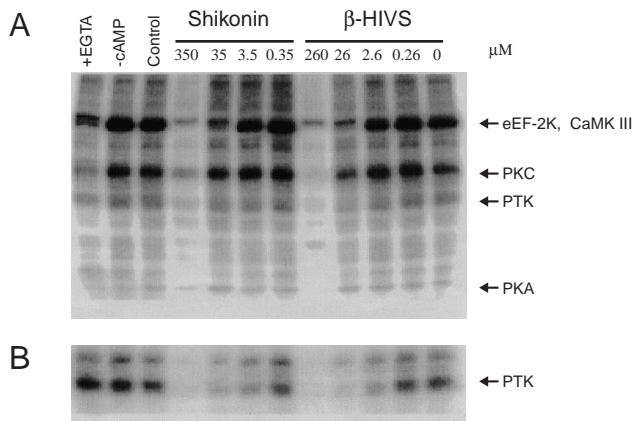


Fig. 3. Effects of β -HIVS and shikonin on the activities of various protein kinases in a lysate of v-Src-expressing SR-3Y1 cells. Phosphorylation in the presence of [γ -³²P]ATP was allowed to proceed as described in "Materials and Methods" with the indicated additions to reaction mixtures. The final concentrations of EGTA and cAMP were 0.5 mM and 20 μ M, respectively. Phosphorylated proteins were analyzed by SDS-PAGE and autoradiography. PKA indicates protein kinase A; PKC, protein kinase C; eEF-2K, CaMK III, eukaryotic elongation factor-2 kinase and calmodulin-dependent protein kinase III, respectively. A, Bands of proteins that were phosphorylated by various protein kinases are indicated by arrows and were identified by reference to Fukazawa *et al.*⁹ B, The gel shown in A was exposed to 1 M KOH at 55°C for 2 h to increase the intensity of signals due to phosphorylated tyrosine.

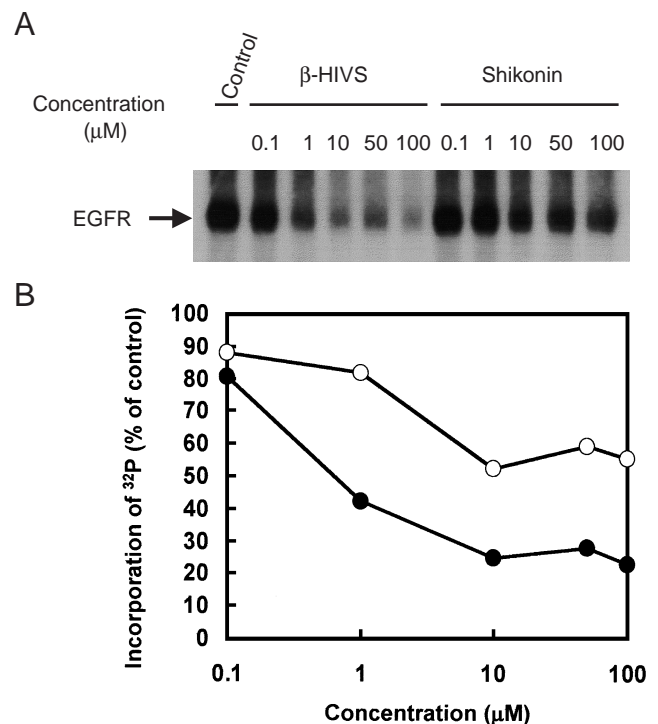


Fig. 4. Effects of β -HIVS and shikonin on the PTK activity of EGFR. EGFR was isolated from A431 cells by affinity chromatography on WGA-agarose and incubated with [γ -³²P]ATP in the presence of β -HIVS or shikonin as described in "Materials and Methods." A, Samples were analyzed by autoradiography after SDS-PAGE. B, PTK activity, as shown by the results in A, was quantified as described in "Materials and Methods." ●, in the presence of β -HIVS; ○, in the presence of shikonin. The results are representative of the results of three separate experiments that gave similar results.

matography on WGA-agarose from A431 cells, which express high levels of EGFR. We treated the isolated EGFR with these agents at various concentrations and analyzed the autophosphorylating activity of EGFR by SDS-PAGE. As shown in Fig. 4A, this activity was markedly and dose-dependently inhibited by β -HIVS. Inhibition of tyrosine phosphorylation was evident even at 0.1 μ M β -HIVS. Shikonin was a much less effective inhibitor of the tyrosine kinase activity of EGFR than was β -HIVS (Fig. 4A). A concentration of shikonin of more than 10 μ M was required for inhibition of the PTK activity of EGFR (Fig. 4A). The IC_{50} of β -HIVS for EGFR was estimated to be approximately 0.7 μ M (Fig. 4B). The inhibitory effect of shikonin on EGFR was so weak that it was impossible to estimate the IC_{50} for EGFR.

Effects of β -HIVS on the PTK activity of v-Src To examine the effects of β -HIVS and shikonin on tyrosine kinase activities in further detail, we lysed v-Src-expressing SR-3Y1 cells and purified v-Src by mixing a cell lysate with the anti-v-Src-specific antibody that had been bound to protein A-conjugated Sepharose. Then the v-Src-bound Sepharose was used as a source of v-Src. As shown in Fig. 5, the tyrosine kinase activity of v-Src was inhibited by β -HIVS in a dose-dependent manner. The IC_{50} of β -HIVS for v-Src was estimated to be approximately 1.0 μ M. The PTK activity of v-Src was also inhibited by shikonin, but the activity did not fall below 50%, indicating that the inhibitory effect of shikonin on v-Src was much weaker than that of β -HIVS.

Effects of β -HIVS on VEGF receptors We examined the

effects of β -HIVS on one type of VEGF receptor for VEGF, KDR/Flk-1, using KDR/Flk-1-NIH3T3 cells, which overexpress KDR/Flk-1. When KDR/Flk-1-NIH3T3 cells were treated with β -HIVS or with shikonin, the PTK kinase activity of KDR/Flk-1 was inhibited significantly (Fig. 6A). However, a concentration of β -HIVS of more than 1 μ M (IC_{50} was practically 2.5 μ M) was needed to inhibit the tyrosine kinase activity of KDR/Flk-1, suggesting that KDR/Flk-1 is more resistant to β -HIVS than EGFR and v-Src. Unlike the effects on EGFR and v-Src, the inhibitory effect of β -HIVS on the PTK activity of KDR/Flk-1 in KDR/Flk-1-NIH3T3 cells was very similar to that of shikonin (Fig. 6B). The PTK activity of the first receptor for VEGF, Flt-1, in Flt-1-overexpressing NIH3T3 cells was also inhibited by β -HIVS but the IC_{50} was greater than 5 μ M (results not shown).

Analysis of the mechanism of inhibition of v-Src by β -HIVS We examined the mechanism of inhibition of PTK activity by β -HIVS using v-Src-bound Sepharose as the source of v-Src, with various concentrations of ATP and a

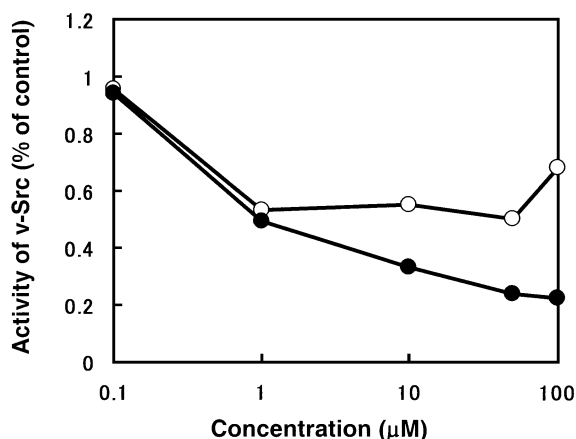


Fig. 5. Effects of β -HIVS and shikonin on the PTK activity of v-Src. Isolated v-Src was incubated with [γ - 32 P]ATP in the presence of β -HIVS or shikonin and then PTK activity was measured in terms of the incorporation of 32 P from [γ - 32 P]ATP into "Ray-tide," as described in "Materials and Methods." ●, in the presence of β -HIVS; ○, in the presence of shikonin. The results shown are the means of results from two samples in each case.

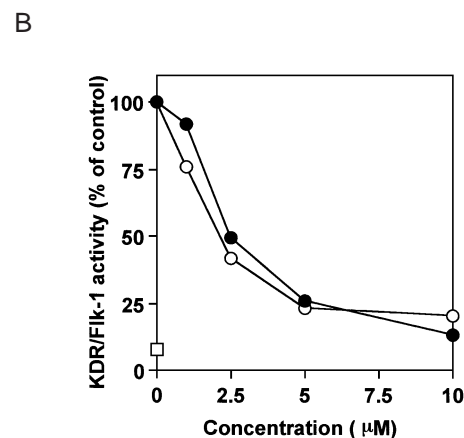
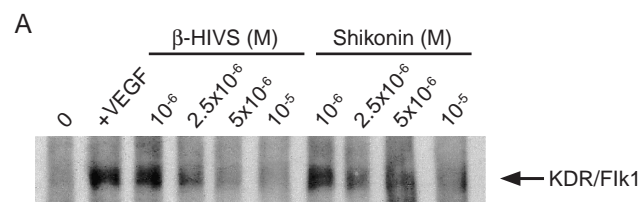


Fig. 6. Effects of β -HIVS and shikonin on the PTK activity of KDR/Flk-1-NIH3T3 cells that overexpressed KDR/Flk-1. Cells were treated with β -HIVS and shikonin as described in "Materials and Methods." A, Samples were analyzed by autoradiography after SDS-PAGE. B, PTK activities, as indicated by results in A, were quantified as described in "Materials and Methods." ●, in the presence of β -HIVS; ○, in the presence of shikonin. The results are representative of the results of three separate experiments that gave similar results.

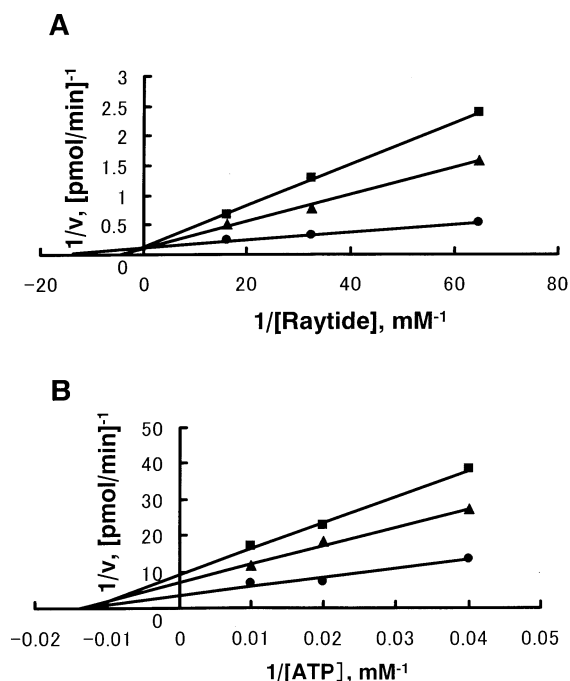


Fig. 7. Lineweaver-Burk plot for the phosphorylation of "Raytide" by v-Src. v-Src-bound Sepharose was used as the source of v-Src tyrosine kinase. Incorporation of ^{32}P from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ into "Raytide" was analyzed at various concentrations of "Raytide" (A) and ATP (B). ●, in the absence of β -HIVS; ▲, in the presence of $1\ \mu\text{M}$ β -HIVS; ■, in the presence of $10\ \mu\text{M}$ β -HIVS.

synthetic substrate, "Raytide," of v-Src. As is evident from Fig. 7A, the tyrosine phosphorylation of "Raytide" by v-Src was competitively inhibited by β -HIVS. In contrast to many inhibitors of PTK such as genistein, STI571, ZD1839, and SU5416,¹³⁻¹⁷ β -HIVS did not compete with ATP (Fig. 7B).

DISCUSSION

Our evaluation of the cell-death inducing effects of β -HIVS on human cancer cells suggested that the mechanism of action of β -HIVS might be different from those of antitumor drugs described to date. The lines of human cancer cells affected by β -HIVS were quite different from those affected by shikonin. The β -hydroxyisovaleryl group of β -HIVS might change shikonin's affinity for or diffusion through the plasma membrane of tumor cells. Our results suggest that it might be possible to modify the specificity of the cell-death-inducing effects of shikonin derivatives by changing the substituents on shikonin.

We demonstrated that β -HIVS inhibited PTKs such as EGFR and v-Src. PTKs play important roles in many cell-

signaling pathways. In many cases, PTKs are located upstream in signal-transduction pathways that control growth, cell death, and transformation. Once EGF has bound to its receptor EGFR, tyrosine kinase is activated, with subsequent activation of Ras and the MAP kinase signaling pathway.^{18, 19} In addition to EGFR, a cellular proto-oncogene c-Src is overexpressed and strongly activated in various human cancers.²⁰ Thus, PTKs, including EGFR and c-Src, are considered potential targets for anti-tumor drugs.^{13, 14, 21} We postulate that the first target of β -HIVS during the inhibition of cell-growth and induction of apoptosis in tumor cells might be PTKs located in the plasma membranes, although the subsequent signaling pathways are unknown at the present stage of investigation.

Numerous inhibitors for various PTKs have been developed and, in some cases, clinical trials are yielding promising results.^{13, 14, 21, 22} PTK inhibitors include flavones, such as quercetin²³; isoflavones, such as genistein²⁴; quinazolines, such as PD0165557²⁵ and ZD1839²⁶; phenylaminopyrimidines, such as STI571¹⁴; forms of benzylidene malonitrile, such as tryphostine and AG537²⁷; and oxindoles, such as PD146568, SU5416, and SU6668.^{15, 28, 29} Erbstatin³⁰ and lavendastin-A,³¹ which have a phenolic hydroxyl group, are non-selective inhibitors of EGFR.³² Herbimycin A, an irreversible specific inhibitor of intracellular v-Src, is a benzoquinoid ansamycin antibiotic.³³ We are the first to demonstrate that shikonin and its derivative β -HIVS inhibit PTKs.

It is likely that most of the above-mentioned inhibitors bind to the ATP-binding pocket of the PTKs. The inhibition of the PTK activity of EGFR by genistein,²⁴ erbstatin,³² lavendastin-A,³² PD158780, and PD0165557²⁵ was reported to involve competition with ATP, as does inhibition of v-Src by quercetin.²³ STI571 and SU6668 compete with ATP for the ATP-binding site of the catalytic domain of Bcr-Abl and a receptor for fibroblast growth factor, respectively.^{14, 15} In contrast to these inhibitors, β -HIVS did not compete with ATP in its inhibition of v-Src. Such inhibitors of PTKs have the advantage that they do not need to compete with ATP at millimolar levels in the intracellular environment. Hydroxynaphthalene derivatives, such as 2-carbonyl-3,6-dihydroxynaphthalene,³⁴ and 5-S-glutathionyl-N- β -alanyl-L-dopa¹⁷ have already been developed as inhibitors of PTKs that act non-competitively with respect to ATP.

EGFR was more sensitive to β -HIVS than were v-Src and KDR/Flk-1. The IC_{50} of β -HIVS for EGFR was estimated to be approximately $0.7\ \mu\text{M}$. This value is similar to values of $3.3\ \mu\text{M}$ for the action of AG537 on EGFR²⁷ and of $3\ \mu\text{M}$ for the action of quercetin on v-Src,²³ but is higher than the value of $0.65\ \text{nM}$ for the action of PD0165557 on EGFR.²⁵ Although EGFR was inhibited by β -HIVS and the inhibition of cell-growth of various tumor cell lines might be at least partly due to the inhibition of

the PTK activity of EGFR, the sensitivities to β -HIVS of tumor cell lines examined in the present study have no correlation with the levels of EGFR in the cells. The levels of EGFR in the lung cancer NCI-H522 and DMS114 cells, which were most efficiently inhibited by β -HIVS, are lower than those in the other tumor cell lines used in the present study (Yamori, T., unpublished data). In the case of carcinoma A431 cells, the IC_{50} value of β -HIVS for the cell-growth inhibition was calculated to be approx. 11 μM , which is markedly higher than the IC_{50} value of β -HIVS for the PTK activity of EGFR. By contrast, the IC_{50} value of β -HIVS for the cell-growth inhibition of SR-3Y1 cells was estimated to be approx. 1.2 μM , which is similar to the IC_{50} value of β -HIVS for the PTK activity of v-Src. These results may be due to differences in the accessibility to β -HIVS of EGFR and v-Src in the intracellular environment.

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