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SPECIFIC INHIBITION OF CYTOPLASMIC PROTEIN TYROSINE KINASES BY HERBIMYCIN A *IN VITRO*

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Abstract—Herbimycin A is an antibiotic which reverses transformation caused by various *src* related oncogenes. The reversion of transformation is restricted to cells transformed by tyrosine kinase coding oncogenes, and accompanies a decrease in kinase activity of the oncogene products. We have shown *in vitro* that herbimycin A directly inactivates $p60^{v\text{-}src}$ kinase by conjugating with SH group(s) of the kinase, raising the possibility that the molecular target of the antibiotic for reversion of v-*src* transformation is the $p60^{v\text{-}src}$ itself. To investigate the relevance of its *in vitro* tyrosine kinase inactivating activity to *in vivo* transformation reversing activity, we examined the specificity of herbimycin A for inhibition of cAMP-dependent kinase, protein kinase C and $p210^{bcr\text{-}abl}$ tyrosine kinase *in vitro*. Herbimycin A had no inhibitory effect on the activities of cAMP-dependent kinase or protein kinase C, whereas the SH-reagent *N*-(9-acridinyl)maleimide, which inactivates $p60^{v\text{-}src}$ *in vitro* by a mechanism similar to that of herbimycin A, blocked the two serine/threonine kinases. On the other hand, the activity of $p210^{bcr\text{-}abl}$ tyrosine kinase was inhibited by herbimycin A treatment. The results indicate that herbimycin A specifically binds to reactive SH group(s) of cytoplasmic protein tyrosine kinases, and confer the biochemical basis for its selectivity in reversing cell transformation.

It is now becoming increasingly evident that products of cellular proto-oncogenes are vital elements of signal transduction, cell growth or development which are highly regulated in normal states. Activation of proto-oncogenes by mutation or amplification can perturb regulated mechanisms and trigger uncontrolled proliferation of cells. Specific inhibitors of onogene products thus should be useful for understanding mechanisms of signal transduction and cell transformation, and may be developed into new types of antitumor drugs.

Herbimycin A is an antibiotic which reverses transformation caused by various tyrosine kinase coding oncogenes [1–4]. The reversion clearly associates with reduction of tyrosine kinase activities. Although the precise mechanism of kinase reduction in cells is yet to be revealed, we have shown that herbimycin A directly binds to reactive SH group(s) of $p60^{v\text{-}src}$ and blocks its function *in vitro* [5]. Some SH-reagents, by binding to the same site(s), can also inactivate $p60^{v\text{-}src}$ kinase *in vitro* [6], but lack activity to reverse transformation of cells. This suggests that herbimycin A is a specific inactivator of tyrosine kinases, and the reversion of the transformed phenotype is a result of selective impairment of function of the oncogene products.

Furthermore, herbimycin A has been shown to affect various events involving tyrosine phosphorylation. For example, in at least 90% of the human chronic myelogenous leukemias, and also in some acute lymphocytic leukemias, a portion of chromosome 9 including c-*abl* is translocated to chromosome 22, generating the Philadelphia chromosome. The translocation results in production of the *bcr-abl* fusion protein, with elevated protein tyrosine kinase activity [7–10]. Herbimycin A induces differentiation of K562 [11, 12] and other Philadelphia chromosome-positive human leukemias, but not those with a normal c-*abl* gene.† In addition, growth of leukemia cells with the structurally altered c-*abl* is extremely sensitive to herbimycin A. Treatment of K562 cells with herbimycin A results in rapid reduction of $p210^{bcr\text{-}abl}$ tyrosine kinase activity (unpublished observation), suggesting that herbimycin A acts through inactivation of the tyrosine kinase.

We thus attempted to assess the specificity of herbimycin A by investigating its effect on various protein kinases *in vitro*. Since herbimycin A is readily inactivated by reducing agents [5] required to maintain the activities of the purified kinases, we developed cell-free systems which permit detection of cAMP-dependent protein kinase or protein kinase C activity in crude preparations, without addition of reducing agents. Herbimycin A, unlike other SH-reagents and protein kinase inhibitors, was totally ineffective in reducing cyclic AMP (cAMP) dependent protein kinase or protein kinase C activity, but inactivated various cytoplasmic protein tyrosine kinases, including the $p210^{bcr\text{-}abl}$ of K562 cells. The results indicate that this antibiotic does react specifically with protein tyrosine kinases, and are in accord with the hypothesis that the *in vitro* mode of inactivation is the actual mechanism of reduction of tyrosine kinase activity in cells.

MATERIALS AND METHODS

Reagents. Herbimycin A was isolated as described

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† Y. Honma, personal communication.

previously [1]. *N*-(9-Acridinyl)maleimide (NAM*) was purchased from Dojin Laboratories (Kumamoto, Japan), staurosporine from the Sigma Chemical Co. (St Louis, MO, U.S.A.) and H-7 from Seikagaku Kogyo Co. Ltd (Tokyo, Japan). Monoclonal antibody to *abl* protein was a product of Oncogene Science, Inc. (Mineola, NY, U.S.A.).

cAMP-dependent protein kinase assay. NIH/3T3 cells infected with Schmidt Ruppin-D strain of Rous sarcoma virus were lysed with 20 mM Hepes, pH 7.4, 5 mM $MgCl_2$, 1 mM Na_3VO_4 and 1%(v/v) Triton X-100, containing 25 μg/mL each of protease inhibitors phenylmethylsulfonyl fluoride, antipain, leupeptin and pepstatin A. Tubes of the lysis mixture were kept on ice for 10 min then centrifuged at 15,000 *g* for 30 min. The supernatant was collected, and the protein concentration adjusted to 3.3 mg/mL with the lysis buffer. To 15 μL of this extract, 2.5 μL of various inhibitors dissolved in DMSO, and 2.5 μL of 200 μM cAMP in 20 mM Hepes, pH 7.4 were added. The reaction was started by adding 5 μL of [γ-^{32}P]ATP (50 μM, 10 μCi) in 20 mM Hepes, pH 7.4. After incubation for 20 min at 25°, the reaction was terminated by adding 12.5 μL of three-times concentrated sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer. The phosphorylated proteins were separated using 11% SDS–PAGE and visualized by autoradiography.

Protein kinase C assay. We modified the method of Chakravarthy *et al.* [13] for measuring protein kinase C activity directly in its native membrane-associated state. Our modified method permits specific detection of protein kinase C activity in post-nuclear fraction, without further addition of exogenous substrates or ultracentrifugation. v-*src* transformed or uninfected NIH/3T3 cells were incubated for 5 min on ice in a hypotonic buffer containing 1 mM Hepes, pH 7.4, 5 mM $MgCl_2$, and 25 μg/mL each of protease inhibitors phenylmethylsulfonyl fluoride, antipain, leupeptin and pepstatin A. The swollen cells were lysed by vortexing the lysis mixture for 2 min at room temperature. Following addition of Hepes buffer (200 mM, pH 7.4) to 20 mM, the homogenate was centrifuged at 500 *g* for 5 min to remove nuclei. The supernatant was collected, and additions were made to give a final concentration of 20 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 0.1 mM Na_3VO_4, 10 mM β-glycerophosphate, 1 mM NaF and 2.5 mg/mL protein. To 20 μL of this post-nuclear fraction, 3 μL of inhibitors dissolved in DMSO, 1 μL of 250 μM $CaCl_2$, and 1 μL of 25 μM TPA were added and incubated for 10 min at 25°. The background reaction mixture received 2 μL of 5 mM EGTA instead of $CaCl_2$ and TPA. The kinase reaction was started by addition of 5 μL [γ-^{32}P]ATP (60 μM, 10 μCi) and further incubated for 20 min at 25°. The reaction was terminated by adding 10 μL of four-times concentrated SDS–PAGE sample buffer, or by heating in a boiling water bath for 5 min. Heat-treated mixtures were cooled to room temperature, centrifuged at 15,000 *g* for 30 min, and the supernatants were mixed with 10 μL of four-times concentrated electrophoresis sample buffer. The phosphorylated proteins were separated using 10% SDS–PAGE and visualized by autoradiography.

p210$^{bcr\text{-}abl}$ kinase assay. Human chronic myelogenous leukemia K562 cells were lysed and centrifuged as described in cAMP-dependent protein kinase assay. The 15,000 *g* supernatant was precleared with formalin-fixed *Staphylococcus aureus*, and then incubated with a mouse monoclonal antibody to *abl* protein. The immune complex was colleted onto formalin-fixed *Staphylococcus aureus* with rabbit anti-mouse IgG as a second antibody, washed, suspended in 20 mM Hepes, pH 7.4, and then divided into 90 μL aliquots. Ten microliters of herbimycin A dissolved in DMSO at various concentrations were added to each aliquot, and the reaction mixtures were incubated at 25° for 2 hr. The immune complex was pelleted, washed with 20 mM Hepes, pH 7.4, and then assayed for p210$^{bcr\text{-}abl}$ autophosphorylating activity. The assay mixture in 20 μL contained 20 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 1 mM dithiothreitol and 10 μM [γ-^{32}P] ATP (10 μCi). After incubation for 20 min at 25°, the reaction was terminated by adding 10 μL of three-times concentrated SDS–PAGE buffer. The phosphorylated proteins were separated using 6% SDS–PAGE and visualized by autoradiography.

RESULTS

cAMP-dependent protein kinase

Phosphorylation of several proteins in v-*src* transformed cell lysate increased upon addition of cAMP (Fig. 1A). Herbimycin A had no effect on the enhanced phosphorylation of those proteins, but reduced the phosphorylation of a protein with a molecular mass of about 100 kDa. NAM, an SH-reagent which inhibits p60$^{v\text{-}src}$ kinase *in vitro* [6] apparently was not as selective since it did not discriminate between the cAMP-enhanced phosphorylation and the phosphorylation of the 100 kDa protein. Selectivity of two known protein kinase inhibitors, H-7 [14] and staurosporine [15] in this system was the converse of herbimycin A: they did not affect the phosphorylation of the 100 kDa protein, but reduced cAMP-enhanced phosphorylation. cAMP-dependent phosphorylation of proteins smaller than 35 kDa appeared to be particularly sensitive to H-7 and staurosporine action. The cAMP-enhanced bands were no longer detectable in the autoradiogram of the gels treated with 1 M KOH to enrich phosphotyrosine (Fig. 1B). The most prominent band after alkali treatment was a 53 kDa band, the phosphorylation of which was inhibited by herbimycin A. The 100 kDa band, although to a lesser extent, was also alkali-resistant and the inhibition by herbimycin A became more evident. The above results indicate that herbimycin A does not interfere with cAMP-dependent protein kinases, under a condition in which some other kinases are blocked.

* Abbreviations: BIPM, *N*-[*p*-(2-benzimidazolyl)phenyl] maleimide; EGTA, ethylene glycol-bis(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid; DMSO, dimethyl sulfoxide; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; IC_{50}, concentration which reduces the activity to 50% of control; NAM, *N*-(9-acridinyl)maleimide; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

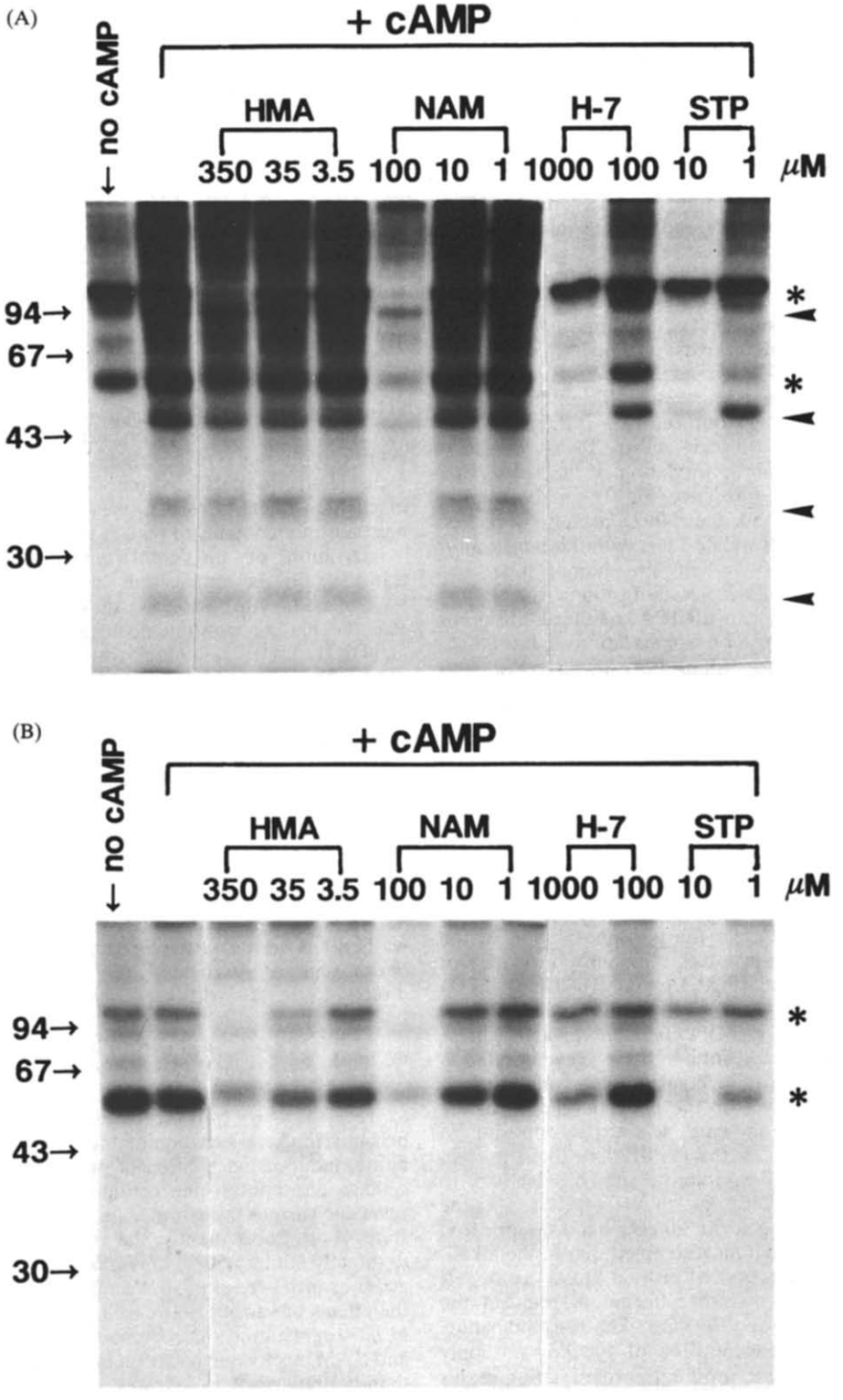


Fig. 1. Effects of herbimycin A, NAM, H-7 and staurosporine on cAMP-dependent protein kinase. Phosphorylation of v-*src* transformed cell extracts were performed as described in Materials and Methods, in the presence of indicated concentrations of inhibitors. HMA, herbimycin A; STP, staurosporine. The phosphorylation was analysed by SDS–PAGE (11% gel) and autoradiography. Shown are exposures of the gel before (A) and after (B) 1 M KOH treatment at 55° for 1 hr. Positions of cAMP-enhanced bands are indicated by arrowheads, and those of alkali-resistant bands by asterisks. Positions and sizes (kDa) of markers are as shown.

Protein kinase C

In a cell-free system using v-*src* transformed cell homogenate, phosphorylation of three proteins of about 80, 42 and 40 kDa increased in a TPA- and Ca^{2+}-dependent manner (Fig. 2A and B). Phosphorylation of a 100 kDa protein was also EGTA sensitive, but this phosphorylation reached its maximum level simply by omission of EGTA (Fig. 2A, lane "none") and did not increase upon further addition of TPA or Ca^{2+}, suggesting that protein kinase C is not involved in the increased phosphorylation of this 100 kDa protein.

To determine the effect of herbimycin A and other inhibitors on protein kinase C activity, compounds dissolved in DMSO were added to reaction mixtures. DMSO alone was apparently sufficient to activate protein kinase C in our cell-free system, and we were unable to observe clear TPA- or Ca^{2+}-dependency for phosphorylation of the 80, 42 and 40 kDa bands in its presence (Fig. 2A and B). Similar to the 100 kDa band, these three proteins appeared to be fully phosphorylated just with the omission of EGTA. However, when the homogenate was prepared from cells depleted of protein kinase C by prolonged treatment with TPA, neither DMSO nor TPA stimulated the phosphorylation of the 80, 42 and 40 kDa bands (data not shown). We thus concluded that the 80, 42 and 40 kDa proteins were phosphorylated by protein kinase C, and not by another cellular enzyme which is stimulated by DMSO. Because the phosphorylated protein band of 80 kDa was very prominent, we focused our attention on this band. This protein was heat-stable and is likely to be the 80 kDa protein which is a specific and physiological substrate of protein kinase C in many types of cells [16].

The addition of herbimycin A to the reaction mixture did not affect the phosphorylation of the 80 kDa protein, but reduced the phosphorylation of the 100 kDa band (Fig. 3A and B). The 100 kDa band is probably identical to the 100 kDa band observed in the cAMP-dependent protein kinase assay. Staurosporine inhibited the phosphorylation of the 80 kDa protein kinase C substrate with no demonstrable effect on the phosphorylation of the 100 kDa band, showing selectivity opposite to herbimycin A, as in the cAMP-dependent protein kinase assay. H-7 exhibited a similar selectivity to staurosporine (data not shown). After alkaline treatment of the gel, the 80 kDa band disappeared and the autoradiogram resembled that of the alkali-treated cAMP-dependent protein kinase assay gels (Fig. 3C). Here too, herbimycin A reduced the intensity of the 53 and 100 kDa alkali-resistant bands.

NAM, at a concentration of 100 μM, was only slightly inhibitory to protein phosphorylation in this experiment using v-*src* transformed cell homogenate. In an experiment using uninfected NIH/3T3 cells, however, the phosphorylation of both 80 and 100 kDa proteins was inhibited by NAM (Fig. 4A and B). The difference between the effects of NAM in v-*src* transformed and uninfected cell homogenate does not seem to be a result of different protein kinase C. We have observed that, at higher concentrations, NAM does inhibit protein kinase C in v-*src* transformed cell homogenate (data not shown). Since herbimycin A was also effective at a lower concentration in untransformed cells (compare the effect of 35 μM herbimycin A on the phosphorylation of the 100 kDa protein in Figs 3 and 4), but the effect of staurosporine was about the same, we speculate that v-*src* transformed cells are more abundant in NAM and herbimycin A binding proteins ($p60^{v\text{-}src}$, for instance). The results indicate that herbimycin A does not affect protein kinase C activity, under a condition in which some other kinases are inhibited.

$p210^{bcr\text{-}abl}$ kinase

We have shown that herbimycin A irreversibly inhibits $p60^{v\text{-}src}$ tyrosine kinase *in vitro* by binding to SH-group(s) of the kinase [4]. Since herbimycin A reversed the morphology of cells transformed by every cytoplasmic tyrosine kinase-coding oncogenes tested [1–4], we speculated that the antibiotic would inactivate other tyrosine kinases *in vitro* by a mechanism analogous to $p60^{v\text{-}src}$ inactivation.

Activation of the c-*abl* tyrosine kinase by translocation is implicated in several human leukemias [7–10]. K562 is an example of such Philadelphia chromosome-positive leukemias, with an activated tyrosine kinase, $p210^{bcr\text{-}abl}$. Herbimycin A rapidly reduces the elevated tyrosine kinase activity (unpublished observation), and induces erythroid differentiation of this cell line [11, 12]. We prepared $p210^{bcr\text{-}abl}$ immune complex from K562 cells, and examined the *in vitro* effect of herbimycin A on the kinase activity. The antibiotic reduced the autophosphorylating activity of the $p210^{bcr\text{-}abl}$ tyrosine kinase in a concentration dependent manner, as shown in Fig. 5. The IC_{50} value was about 3 μg/mL (5 μM) under the condition employed. Since the herbimycin A-treated immune complexes were washed before the kinase reaction, it is likely that herbimycin A inactivated $p210^{bcr\text{-}abl}$ by binding directly to the kinase.

DISCUSSION

In the course of screening for agents with activity to reverse v-*src* transformation, we found that herbimycin A, a benzoquinonoid ansamycin antibiotic, induced inactivation of $p60^{v\text{-}src}$ and reduced cellular phosphotyrosine content in cells, thereby reversing various transformed phenotypes to normal states [1–4]. Subsequently, the antibiotic was found to directly inhibit $p60^{v\text{-}src}$ kinase *in vitro*, possibly by acting as an SH-reagent [5]. We therefore investigated the effects of various SH-reagents on $p60^{v\text{-}src}$ kinase *in vitro* and found that compounds such as BIPM and NAM inactivated $p60^{v\text{-}src}$ at lower concentrations than herbimycin A [6]. However, BIPM and NAM did not reverse v-*src* transformation, indicating that the reversion requires specific impairment of the $p60^{v\text{-}src}$ function.

If the *in vitro* herbimycin A mode of $p60^{v\text{-}src}$ inactivation is the actual mechanism of reversion of transformation, the antibiotic should display high specificity *in vitro*, and have little or no effect on serine/threonine kinases. Also, since herbimycin A reverses the morphology of cells transformed by

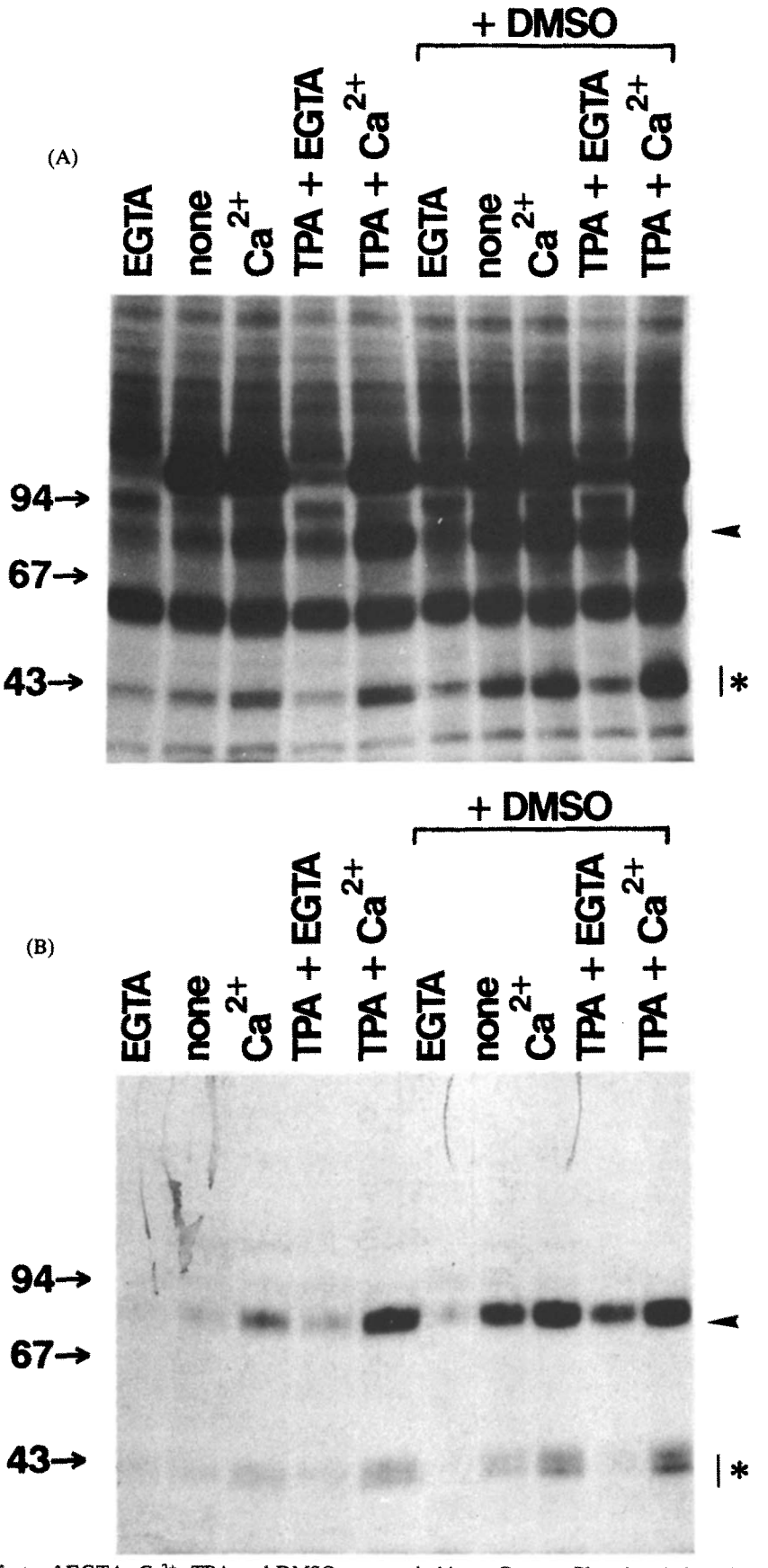
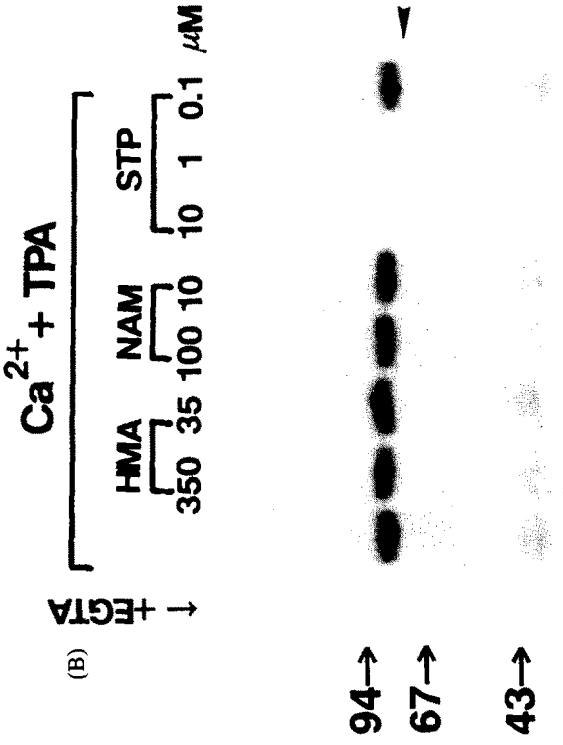
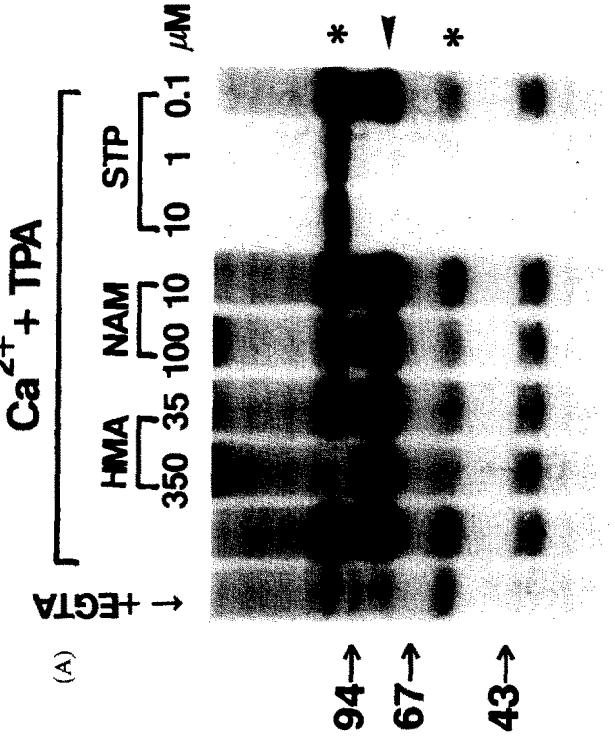


Fig. 2. Effects of EGTA, Ca^{2+}, TPA and DMSO on protein kinase C assay. Phosphorylation of v-*src* transformed cell homogenates was performed in the presence of indicated additions. The final concentrations were: EGTA, 333 μM; Ca^{2+}, 8.3 μM; TPA, 0.83 μM; DMSO, 10% (v/v). Reactions were stopped either by adding four-times concentrated sample buffer (A) or by heating in a boiling water bath for 5 min (B). The phosphorylation was analysed by SDS–PAGE (10% gel) and autoradiography. Position of the 80 kDa band is indicated by an arrowhead, and those of the 40 and 42 kDa bands by an asterisk. Positions and sizes (kDa) of markers are as shown.


(B)
+EGTA →
Ca2+ + TPA
HMA 350 35
NAM 100 10
STP 10 1 0.1 μM
94→
67→
43→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+EGTA →
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NAM 100 10
STP 10 1 0.1 μM
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67→
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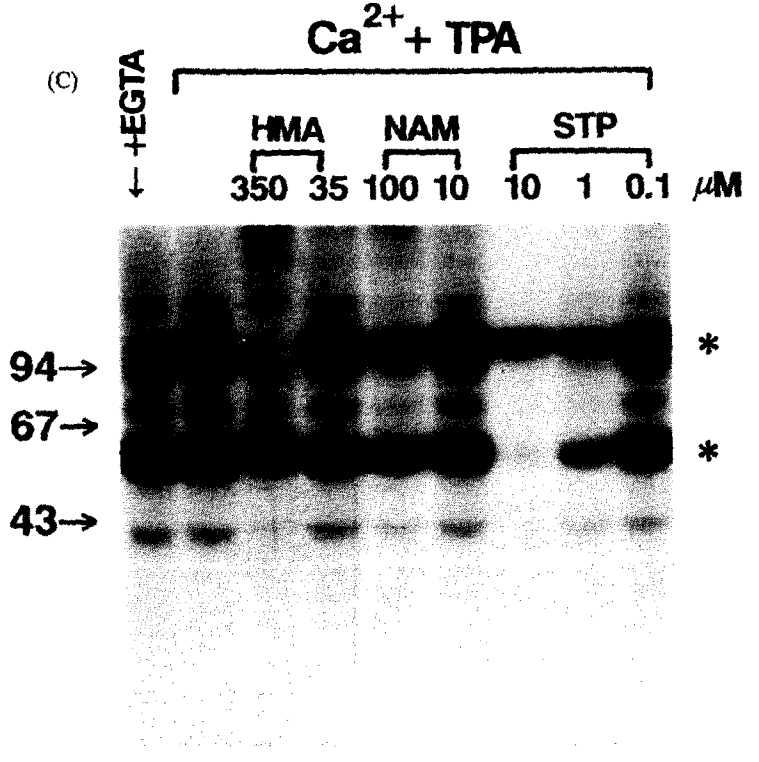

Fig. 3. Effect of herbimycin A, NAM and staurosporine on protein kinase C in v-*src* transformed cell homogenate. Assays were performed as described in Materials and Methods in the presence of indicated concentrations of inhibitors. HMA, herbimycin A; STP, staurosporine. Reactions were stopped either by adding four-times concentrated sample buffer (A) or by heating in a boiling water bath for 5 min (B). The phosphorylation was analysed by SDS–PAGE (10% gel) and autoradiography. Shown in (C) is the autoradiogram of gel (A) after alkali treatment. Position of the 80 kDa protein kinase C substrate is shown by the arrowhead, and those of alkali-resistant bands by asterisks. Positions and sizes (kDa) of markers are as shown.

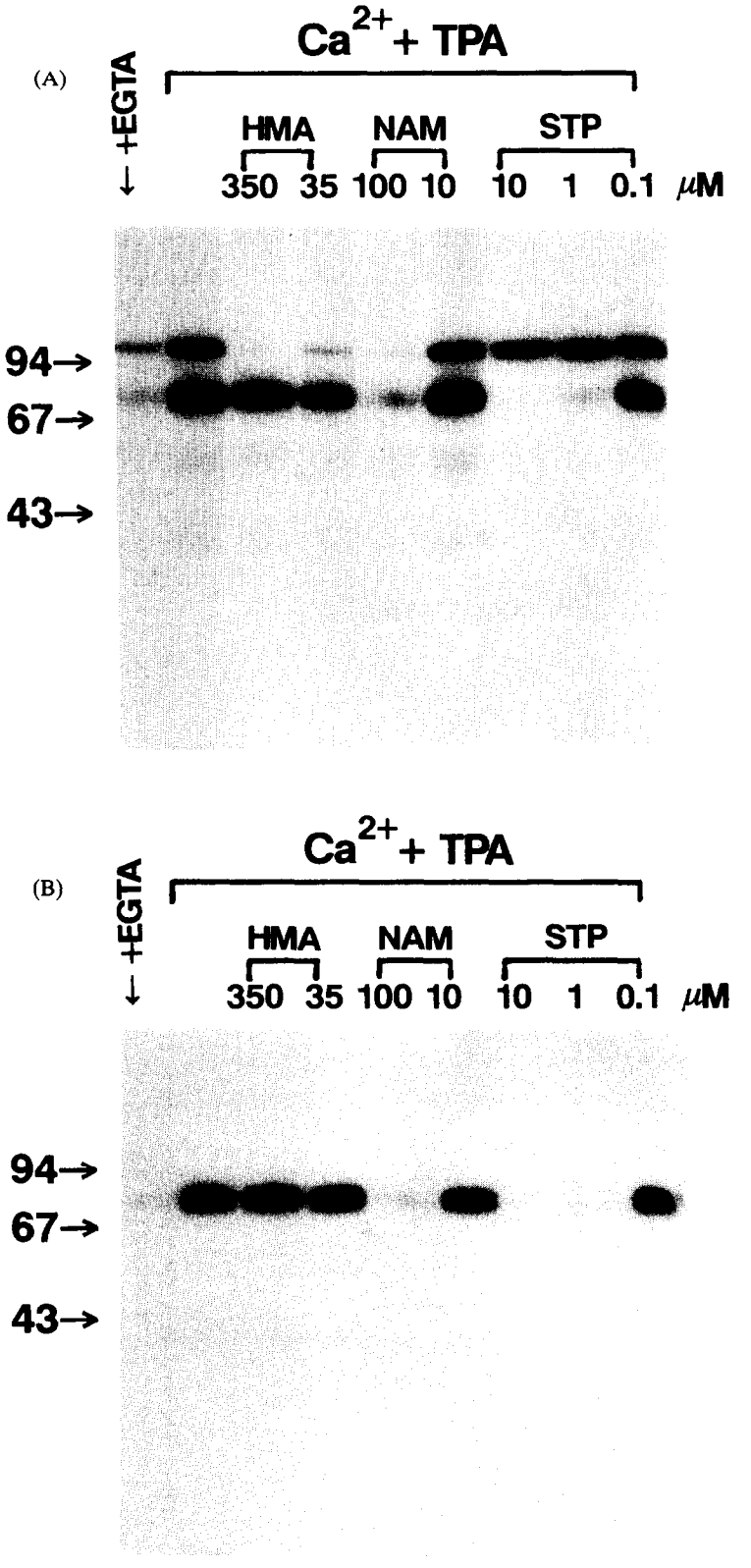

Fig. 4. Effect of herbimycin A, NAM and staurosporine on protein kinase C in untransformed cell homogenate. Assays were performed as described in Materials and Methods in the presence of indicated concentrations of inhibitors. HMA, herbimycin A; STP, staurosporine. Reactions were stopped either by adding four-times concentrated sample buffer (A) or by heating in a boiling water bath for 5 min (B). The phosphorylation was analysed by SDS–PAGE (10% gel) and autoradiography. Positions and sizes (kDa) of markers are as shown.

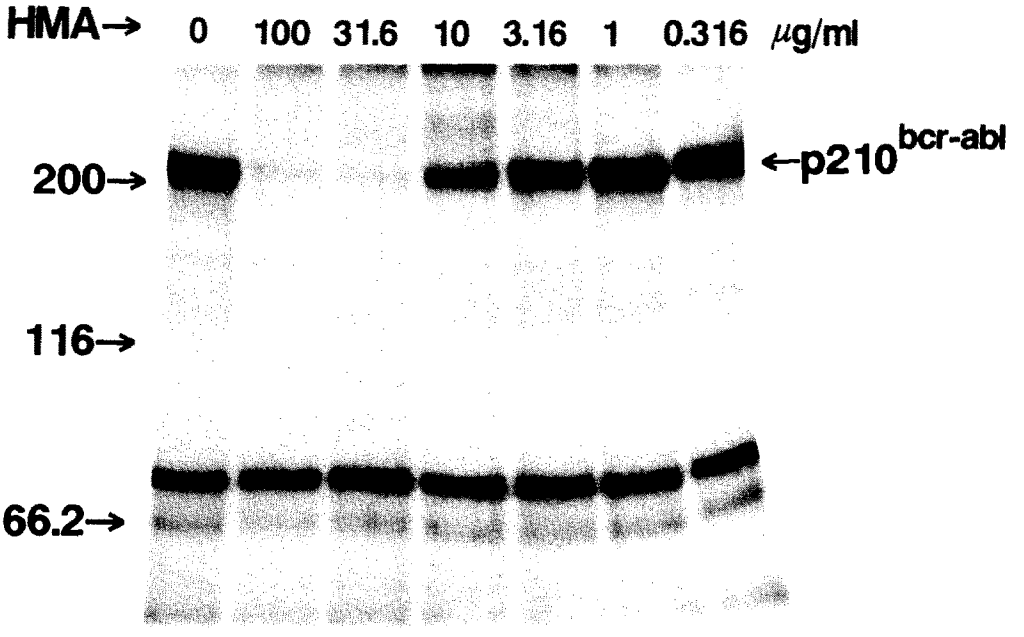

Fig. 5. Effect of herbimycin A on $p210^{bcr\text{-}abl}$ kinase. Immune complexes prepared from K562 cells were treated with indicated concentrations of herbimycin A and then assayed for $p210^{bcr\text{-}abl}$ autophosphorylating activity as described in Materials and Methods. The phosphorylation was analysed by SDS–PAGE (6% gel) and autoradiography. Positions and sizes (kDa) of markers are as shown.

various tyrosine kinase coding oncogenes, and the reduction of kinase activity and cellular phosphotyrosine content has been observed in every case examined [1–4 and unpublished data], the antibiotic should inactivate other tyrosine kinases *in vitro* by the same mechanism as it inactivated $p60^{v\text{-}src}$.

In this publication, we demonstrated by *in vitro* assays that herbimycin A is actually a specific inactivator of tyrosine kinases. In our cell-free assay systems, herbimycin A did not affect phosphorylation by cAMP-dependent protein kinase or protein kinase C, but reduced th phosphorylation of 53 and 100 kDa proteins which were alkali-resistant, indicating that inhibition of tyrosine phosphorylation took place in these assays without cAMP-dependent protein kinase or protein kinase C being affected. Furthermore, herbimycin A inactivated the $p210^{bcr\text{-}abl}$ tyrosine kinase in an immune complex assay, and although data are not shown, we have observed that herbimycin A inactivates tyrosine kinases $p120^{v\text{-}abl}$, $p130^{v\text{-}fps}$, and an activated form of $p60^{c\text{-}src}$ from human colon tumor cells *in vitro*. All these results indicate that herbimycin A favors inactivation of tyrosine kinases over other enzymes. NAM, which does not have any activity to reverse cell transformation, inactivated $p60^{v\text{-}src}$ [5] and $p210^{bcr\text{-}abl}$ (data not shown) by a mechanism similar to that of herbimycin A, but was not as selective and also inhibited cAMP-dependent protein kinase and protein kinase C. All these results, together with our previous data on intact cells, strongly suggest that herbimycin A selectively inactivates tyrosine kinases in cells. Additionally, it has recently been shown that herbimycin A inactivates two protein tyrosine kinases (Fyn and Lyn) in T cells, while being sparing of effect on protein kinase C and c-Raf, another protein serine/threonine kinase [17].

The specificity of enzyme inhibitors may best be demonstrated in assay systems using purified enzymes and substrates, and we are aware that data obtained from experiments that rely on crude preparations must be cautiously interpreted. We were obliged to adopt such assay systems for cAMP-dependent protein kinase and protein kinase C, since these kinases require reducing agents, which inactivate herbimycin A [5], to preserve their catalytic activities when purified. We examined effects of various specific inhibitors and activators on these assay systems (data not shown), and judged that the phosphorylations observed permit direct links to the enzymes intended to be measured. The kinase detection systems are now further modified, so that activities of cyclic nucleotide-dependent protein kinase, protein kinase C and protein tyrosine kinase can be detected simultaneously. Details and the validity of the system will be demonstrated elsewhere.

We speculate that herbimycin A binds irreversibly to thiol group(s) of tyrosine kinases as shown in Fig. 6 [5, 6 and this publication], although it appears that its reactivity as an SH-reagent is not as high as N-substituted maleimides, at least *in vitro*. The inactivation of immunoprecipitated tyrosine kinases by herbimycin A *in vitro* was dependent on the temperature and the period of pretreatment, and in most cases, did not occur when the mixture was put in an ice bath (data not shown). The reaction proceeded faster at higher temperatures, but since

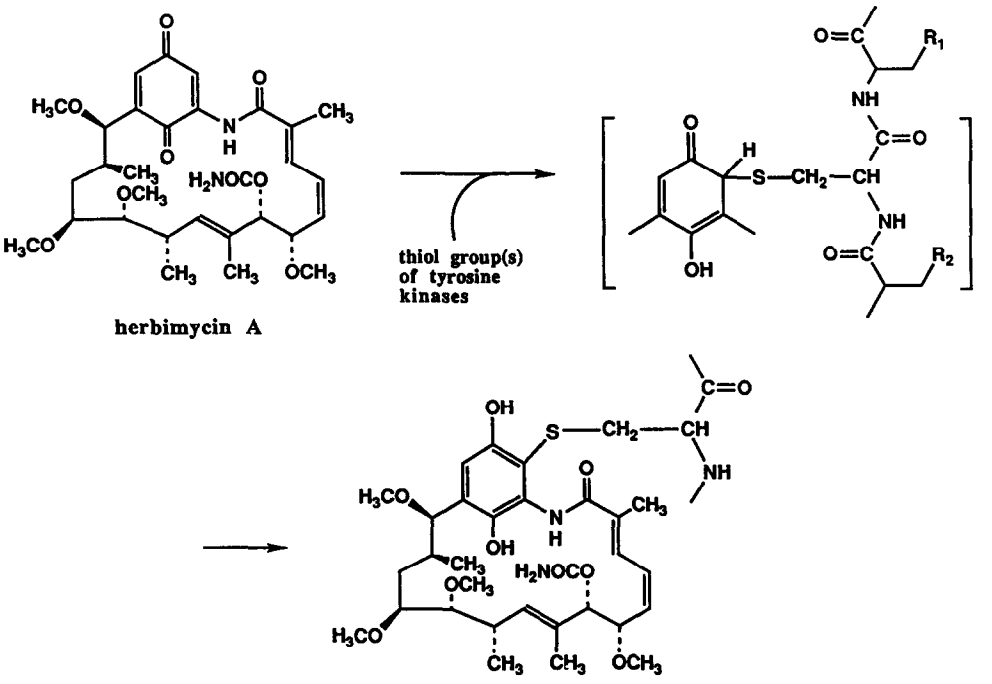

Fig. 6. Proposed mechanism of herbimycin A binding to thiol group(s) of protein tyrosine kinases.

incubation at higher temperatures alone inactivates kinases, we selected conditions where the effect of herbimycin A was most evident. The optimal condition varied among the kinases and also the antibodies used, and in the case of $p210^{bcr\text{-}abl}$ it was 25° for 2 hr, although the inactivation, to a lesser extent, can be detected within 15 min (data not shown). The length of time we chose for inactivation of $p210^{bcr\text{-}abl}$ might be somewhat disturbing, but it should be noted that as for $p60^{v\text{-}src}$ [5], $p120^{v\text{-}abl}$, $p130^{v\text{-}fps}$ and activated $p60^{c\text{-}src}$ (unpublished observations), immune complexes, which contain little contaminating enzymes, were *pre*incubated, and herbimycin A was washed away before the actual kinase reactions. These results suggest that the inactivation of $p210^{bcr\text{-}abl}$ kinase *in vitro* is a result of direct binding, rather than a secondary effect of herbimycin A. The low reactivity *in vitro* may partly explain the requirement for higher concentration of herbimycin A for inactivation of tyrosine kinases in cell-free systems than in intact cells, since cells are treated with herbimycin A at 37° and for a longer period of time. In addition, kinases in their native environment may be more accessible to herbimycin A than kinases in immune complexes.

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