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EFFECTS OF HERBIMYCIN A AND VARIOUS SH-REAGENTS ON $p60^{v\text{-}src}$ KINASE ACTIVITY IN VITRO

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Summary Herbimycin A is an antibiotic which reverses transformation caused by src family oncogenes. It inactivates $p60^{v\text{-}src}$ in vitro, possibly by binding to reactive SH-group(s) of the kinase. We examined effects of various SH-reagents on $p60^{v\text{-}src}$ and observed that N-[p-(2-benzimidazolyl)phenyl]maleimide (BIPM) or N-(9-acridinyl)maleimide (NAM) were potent inactivators of the kinase, whereas N-ethylmaleimide (NEM) required high concentrations, and iodoacetamide was totally ineffective in reducing the kinase activity. Pretreatment of $p60^{v\text{-}src}$ immune-complex with NEM and iodoacetamide, however, protected the kinase from inactivation by herbimycin A, BIPM, and NAM. The results suggest that SH-group(s) to which herbimycin A binds is not essential for the kinase activity, but is positioned in the vicinity of the active center.

Transformation of cells by Rous sarcoma virus is mediated by the product of v-src oncogene, $p60^{v\text{-}src}$, which is a tyrosine kinase attached to the cytoplasmic face of the plasma membrane (reviewed in 1). Expression of $p60^{v\text{-}src}$ kinase activity is essential and sufficient for both initiation and maintenance of the transformed state (1). In the course of screening for agents which reverse v-src transformation, we found that herbimycin A (Fig 1), a benzoquinonoid ansamycin antibiotic, induced inactivation of $p60^{v\text{-}src}$, thereby reversing various transformed phenotypes to normal states (2, 3). The antibiotic inactivated

Abbreviations: BIPM, N-[p-(2-benzimidazolyl)phenyl]maleimide; DMSO, dimethyl sulfoxide; Hepes, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid; IC_{50}, concentration which reduces the activity to 50% of the control; Mes, 2-(N-morpholino)-ethansulfonic acid; NAM, N-(9-acridinyl)maleimide; NEM, N-ethylmaleimide; TBR, tumor-bearing rabbit.

0006-291X/90 $1.50

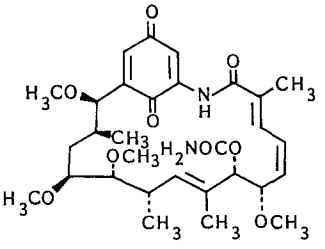


Figure 1. Structure of herbimycin A.

$p60^{v\text{-}src}$ kinase activity *in vitro*, possibly by binding to SH-group(s) of the kinase (4). In order to gain further insight into the herbimycin A mode of $p60^{v\text{-}src}$ inactivation, we compared its effects on the kinase with those of known SH-reagents.

MATERIALS AND METHODS

Materials

Herbimycin A was isolated as described previously (2). BIPM (5) was purchased from Kanto Chemicals Co., Tokyo, Japan, NAM (6) from Dojin Laboratories, Kumamoto, Japan, NEM from Nacalai Tesque, Kyoto, Japan, and iodoacetamide from Wako Pure Chemical Industries, Ltd., Osaka, Japan. TBR serum (#1011) and monoclonal antibody 327 were products of Transformation Research, Inc., Framingham, MA, and Oncogene Science, Inc., Mineola, NY, respectively. Formalin-fixed *Staphylococcus aureus* (Pansorbin) was a product of Calbiochem-Behring, La Jolla, CA. [gamma-^{32}P] ATP was obtained from ICN Radiochemicals, Irvine, CA.

Methods

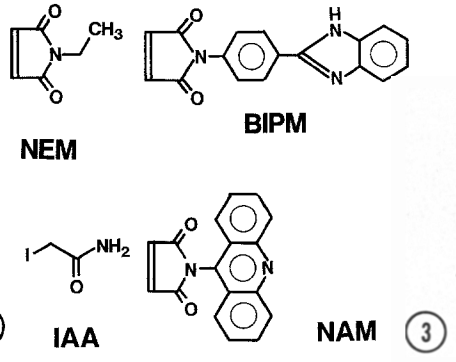
NIH/3T3 cells infected with Schmidt Ruppin-D strain of Rous sarcoma virus were lysed in 20mM Hepes, pH 7.4, 1mM EDTA, 0.1mM Na_3VO_4, 150mM NaCl, and 1% Triton X-100 containing 25µg/ml each of protease inhibitors phenylmethylsulfonyl fluoride, antipain, leupeptin, and pepstatin A. The lysate was centrifuged at 15,000 x g for 10 min, and the supernatant was incubated with TBR serum 1011 or with monoclonal antibody 327 plus rabbit anti-mouse IgG. The immune-complexes were collected onto formalin-fixed *Staphylococcus aureus*, washed, and suspended in 20mM Hepes, pH 7.4 (for treatment with herbimycin A or iodoacetamide) or 20mM Mes, pH 6.0 (for treatment with BIPM, NAM, or NEM). To a 90µl suspension, 10µl of SH-reagent dissolved in DMSO was added, and the mixture was incubated at 25°C for 30 min. The immune-complex was washed and assayed for kinase activity as previously described (4), except that 1mM dithiothreitol was included in the reaction mixture.

RESULTS

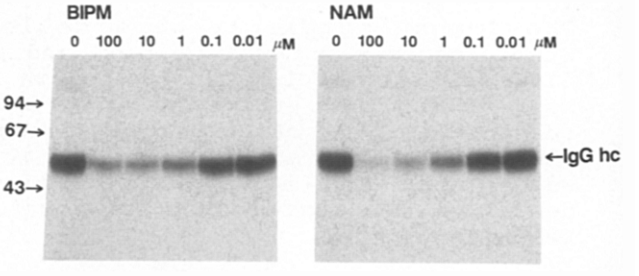
Effects of SH-inhibitors on $p60^{v\text{-}src}$ kinase

We examined effects of four SH-reagents, iodoacetamide, NEM, BIPM, and NAM on $p60^{v\text{-}src}$ kinase activity *in vitro*. The

structures of the four compounds are shown in Fig 2. BIPM and NAM reduced the TBR IgG heavy chain phosphorylating activity with IC_{50} of about 1μM under the condition employed (Fig 3). The two compounds also inactivated, at similar concentrations, $p60^{v\text{-}src}$ autophosphorylating activity in immune-complexes prepared with monoclonal antibody 327 (data not shown). On the other hand, more than 60% of the IgG heavy chain kinase activity in the immunoprecipitate of TBR serum still remained after 10mM NEM treatment (Fig 4, second lane from the left). We raised the concentration to 100mM, but the inhibition never exceeded 45% (data not shown). NEM was able to completely destroy the autophosphorylating activity in monoclonal antibody 327 immunoprecipitates, but the complete inhibition required high concentrations (IC_{50}=5mM, Fig 5). Treatments of the immune-complexes with N-substituted maleimides were performed at pH 6.0 to minimize possible reaction with amino-groups, but treatments at pH 7.4 gave similar results (data not shown). Another SH-reagent, iodoacetamide, was totally ineffective in reducing



(2)



(3)

Figure 2. Structures of iodoacetamide, NEM, BIPM and NAM. IAA; iodoacetamide.

Figure 3. Effects of BIPM and NAM on $p60^{v\text{-}src}$ kinase. Immune-complex prepared with TBR serum was treated with indicated concentrations of BIPM or NAM for 30 min, washed, and then assayed for $p60^{v\text{-}src}$ kinase activity. Positions and sizes (kDa) of markers are shown to the left. IgG hc; IgG heavy chain.

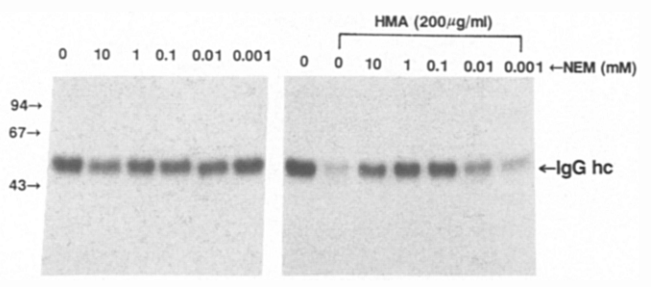


Figure 4. Effect of NEM on inactivation of $p60^{v\text{-}src}$ kinase (TBR IgG heavy chain phosphorylation) by herbimycin A. Immune-complex prepared with TBR serum was treated with indicated concentrations of NEM for 30 min, washed, and incubated without or with 200μg/ml herbimycin A for 30 min. The immune-complex was then washed and assayed for $p60^{v\text{-}src}$ kinase activity. Positions and sizes (kDa) of markers are shown to the left. IgG hc; IgG heavy chain.

either IgG heavy chain kinase (Fig 6, second lane from the left) or autophosphorylating activity (data not shown).

Effect of NEM and iodoacetamide pretreatment on inactivation of $p60^{v\text{-}src}$ kinase by herbimycin A, BIPM, and NAM

A trivial explanation for the incapability of NEM and iodoacetamide to inactivate $p60^{v\text{-}src}$ kinase activity is that these compounds do not bind to $p60^{v\text{-}src}$. To test this hypothesis, the immune-complexes were pretreated with NEM or iodoacetamide before treatment with $p60^{v\text{-}src}$ inactivating SH-reagents. As shown in Fig 4 and 5, $p60^{v\text{-}src}$ kinase in the immune-

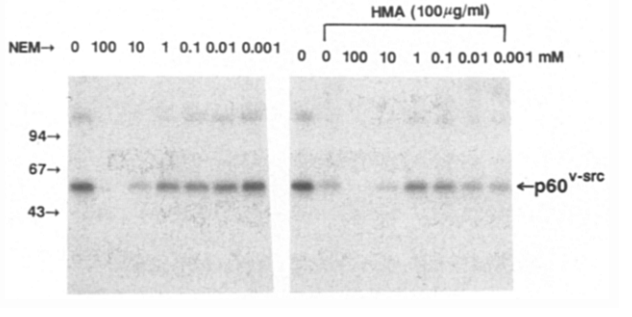


Figure 5. Effect of NEM on inactivation of $p60^{v\text{-}src}$ kinase (autophosphorylation) by herbimycin A. Immune-complex prepared with monoclonal antibody 327 was treated with indicated concentrations of NEM for 30 min, washed, and incubated without or with 100μg/ml herbimycin A for 1h. The immune-complex was then washed and assayed for $p60^{v\text{-}src}$ kinase activity. Gels were treated with 1M KOH at 55°C for 2h to enrich phosphotyrosine before autoradiography. Positions and sizes (kDa) of markers are shown to the left.

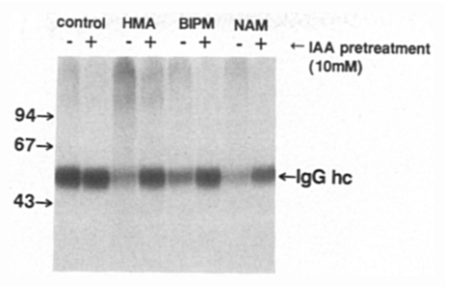


Figure 6. Effect of iodoacetamide on inactivation of $p60^{v\text{-}src}$ kinase by herbimycin A, BIPM and NAM. Immune-complex prepared with TBR serum was either untreated or treated with 10mM iodoacetamide for 30 min, washed, and then incubated with 200μg/ml herbimycin A, 100μM BIPM, or 100μM NAM for 30 min. The immune-complex was then washed and assayed for $p60^{v\text{-}src}$ kinase activity. Positions and sizes (kDa) of markers are shown to the left. IgG hc; IgG heavy chain.

complexes became less susceptible to inactivation by herbimycin A after NEM treatment. The protective effect was evident at 0.1mM NEM, which was considerably lower than the concentration required to inactivate autophosphorylating activity. Figure 6 shows the protective effect of iodoacetamide from inactivation by herbimycin A, BIPM, and NAM. These results indicate that NEM, iodoacetamide, herbimycin A, BIPM, and NAM all bind to same cysteine residue(s) in $p60^{v\text{-}src}$, but with different effects on kinase activity.

DISCUSSION

We previously reported that herbimycin A can directly inactivate $p60^{v\text{-}src}$ tyrosine kinase *in vitro*, and demonstrated the likelihood that the inactivation occurs through conjugation between the antibiotic and SH-group(s) of the kinase (4). Although the existence of an SH-group in $p60^{v\text{-}src}$ crucial for its function has not been demonstrated, $p60^{v\text{-}src}$ requires reducing agents for preservation of its activity, and we expected that known SH-reagents would also inactivate $p60^{v\text{-}src}$ kinase activity like herbimycin A. BIPM and NAM were found to be potent inactivators of $p60^{v\text{-}src}$ kinase, but contrary to our expectation,

NEM and iodoacetamide were not as effective. However, pretreatment of $p60^{v\text{-}src}$ immune-complex with NEM and iodoacetamide protected the kinase from inactivation by herbimycin A, BIPM, and NAM. The results suggest that the five compounds all bind to the same SH-group(s) in $p60^{v\text{-}src}$, but affect the kinase activity differently. Inactivation of autophosphorylating activity by high concentrations of NEM appears to result from binding to different site(s), which may not be SH-groups, since the protective effect from herbimycin A was observed at lower concentrations (Fig 5). The reason for such variation in degrees of kinase inactivation among SH-reagents is not clear at present, but differences in molecular sizes seem to contribute to the variation. We speculate that the SH-group(s) in $p60^{v\text{-}src}$ to which herbimycin A binds is not essential for kinase activity, but is positioned in the vicinity of the active center. Binding of a compound above a certain size to such a site may impair $p60^{v\text{-}src}$ function, possibly by inhibiting the access of ATP.

Although BIPM and NAM inactivated $p60^{v\text{-}src}$ at concentrations lower than herbimycin A, they did not reverse transformation by v-src. We have observed in vitro that herbimycin A is much more specific for tyrosine kinases than other $p60^{v\text{-}src}$ inactivating SH-reagents (manuscript submitted). Determining the precise mechanism of herbimycin A action may offer further insights into the structure and regulation of tyrosine kinases, and may provide new hints for developing tyrosine kinase inhibitors.

Acknowledgments

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