

# Labeling of v-Src and BCR-ABL tyrosine kinases with [<sup>14</sup>C]herbimycin A and its use in the elucidation of the kinase inactivation mechanism

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## Abstract

The ansamycin antibiotic, herbimycin A, selectively inactivates cytoplasmic tyrosine kinases, most likely by binding irreversibly to the reactive SH group(s) of kinases. To further investigate the mechanism of herbimycin A action, we attempted to label tyrosine kinases with [<sup>14</sup>C]herbimycin A. p60<sup>v-src</sup> and p210<sup>BCR-ABL</sup> in immune complexes were labeled with [<sup>14</sup>C]herbimycin A, demonstrating that the antibiotic binds directly to tyrosine kinases. Digestion of [<sup>14</sup>C]herbimycin A-labeled p60<sup>v-src</sup> with *Staphylococcus aureus* V8 protease revealed that the herbimycin A binding site is within the C-terminal 26-kDa fragment of p60<sup>v-src</sup>, which contains the tyrosine kinase domain. Herbimycin A treatment inhibited labeling of p60<sup>v-src</sup> by [<sup>14</sup>C]fluorosulfonylbenzoyl adenosine, an affinity labeling reagent of nucleotide binding sites, indicating that herbimycin A-modified p60<sup>v-src</sup> cannot interact with ATP. The results suggest that herbimycin A inactivates tyrosine kinases by binding directly to the kinase domain, thereby inhibiting access to ATP.

**Key words:** Tyrosine kinase; Herbimycin A

## 1. Introduction

The ansamycin antibiotic, herbimycin A, blocks many biological events mediated by tyrosine kinases [1]. It selectively reduces tyrosine kinase activities both in cells and in cell-free systems [1–6]. Although the precise mechanism of herbimycin A action is not fully understood, the inhibitor irreversibly inactivates tyrosine kinases in vitro, and its action is abrogated by sulfhydryl compounds [6]. The most logical mechanism of such effect is conjugation between herbimycin A and reactive SH group(s) of kinases.

In this study, we present confirming evidence for direct and irreversible binding of herbimycin A to p60<sup>v-src</sup> and p210<sup>BCR-ABL</sup> using [<sup>14</sup>C]herbimycin A. V8 protease cleavage of [<sup>14</sup>C]herbimycin A-labeled p60<sup>v-src</sup> revealed that the binding site is within the tyrosine kinase domain. Binding of herbimycin A to p60<sup>v-src</sup> appeared to obstruct access of ATP, which is likely to be the mechanism of inhibition. As herbimycin A is expected to bind and inac-

tivate other cytoplasmic tyrosine kinases in the same manner, radioisotope-labeled herbimycin A may be useful as an affinity labeling reagent for some tyrosine kinases.

## 2. Materials and methods

### 2.1. Materials

[methyl-<sup>14</sup>C]Methionine was a product of American Radiolabeled Chemicals Inc. (St. Louis, MO). [<sup>14</sup>C]FSBA was from NEN Research Products. Monoclonal antibodies against Src (clone 327) and Abl (clone 24–21) were purchased from Oncogene Science Inc. (Mineola, NY). Herbimycin A was prepared as described previously [7]. [<sup>14</sup>C]Herbimycin A was prepared biosynthetically by adding [methyl-<sup>14</sup>C]methionine to herbimycin A-producing cultures, and was purified using high-performance liquid chromatography prior to use in these experiments. The specific activity of [<sup>14</sup>C]Herbimycin A was 10–20 mCi/mmol, depending on culture conditions.

### 2.2. Labeling of tyrosine kinases by [<sup>14</sup>C]herbimycin A

NIH3T3 cells infected with the Schmidt-Ruppin D strain of Rous sarcoma virus (NIH3T3/v-src cells) or K562 cells were lysed in 20 mM HEPES, pH 7.4, 1 mM EDTA, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, 150 mM NaCl, and 1% Triton X-100 containing 25 µg/ml each of the protease inhibitors antipain, leupeptin, and pepstatin A. Lysates were centrifuged at 15,000 × g for 30 min, split into two halves, and then each half was incubated with monoclonal antibody against Src (NIH3T3/v-src cells) or ABL (K562 cells) protein with rabbit anti-mouse IgG as a second antibody. The immune complexes were collected onto formalin-fixed *Staphylococcus aureus*, washed and suspended in 20 mM HEPES, pH 7.4. [<sup>14</sup>C]Herbimycin A dissolved in DMSO was added to the immune complexes and incubated at 25°C for 1 h. The final concentrations of [<sup>14</sup>C]herbimycin A and DMSO were 10 µg/ml and 10% (v/v), respectively. The immune complexes were washed with RIPA buffer (20 mM

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**Abbreviations:** FSBA, fluorosulfonylbenzoyl adenosine; HEPES, *N*-(hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; EDTA, ethylene glycol-bis(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid; DMSO, dimethyl sulfoxide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

HEPES, pH 7.4, 1 mM EDTA, 0.1 mM  $\text{Na}_2\text{VO}_4$ , 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS), and the labeled proteins were separated by 9% ( $\text{p60}^{\text{v-src}}$ ) or 7.5% ( $\text{p210}^{\text{BCR-ABL}}$ ) SDS-PAGE and visualized using a Fujix BAS2000 Bio-imaging Analyzer (Fujix Photo Film Co., Tokyo, Japan). [ $^{14}\text{C}$ ]Herbimycin A-labeled  $\text{p60}^{\text{v-src}}$  was excised from the gel, subjected to peptide mapping with *Staphylococcus aureus* V8 protease [8], and the labeled fragment was visualized using BAS2000.

### 2.3. Labeling of $\text{p60}^{\text{v-src}}$ with [ $^{14}\text{C}$ ]FSBA

$\text{p60}^{\text{v-src}}$  immune complexes were treated with various concentrations of herbimycin A for 30 min at 25°C, washed, and suspended in 90  $\mu\text{l}$  of 20 mM HEPES, pH 7.4. 10  $\mu\text{l}$  of [ $^{14}\text{C}$ ]FSBA (1  $\mu\text{Ci}$ , 18.8 nmol) in DMSO was added to the suspensions, and the mixture was incubated for 1 h at 30°C. The treated immune complexes were washed with RIPA buffer and separated by 7.5% SDS-PAGE. The gel was dried without staining and analyzed using BAS2000.

## 3. Results

### 3.1. Labeling of $\text{p60}^{\text{v-src}}$ and $\text{p210}^{\text{BCR-ABL}}$ with [ $^{14}\text{C}$ ]herbimycin A

As shown in Figs. 1A and 2, proteins of about 60 kDa and 210 kDa were labeled when immune complexes prepared, respectively, with anti-Src and anti-Abl monoclo-

nal antibodies were treated with [ $^{14}\text{C}$ ]herbimycin A. Since the bands were not observed in control immune complexes without monoclonal antibodies (Figs. 1A and 2, left lanes) they are probably  $\text{p60}^{\text{v-src}}$  and  $\text{p210}^{\text{BCR-ABL}}$ , respectively. The [ $^{14}\text{C}$ ]herbimycin A-labeled  $\text{p60}^{\text{v-src}}$  was excised from the gel and subjected to V8 protease analysis. The result shown in Fig. 1B indicates that herbimycin A binds to the carboxyl-terminal 26-kDa V2 fragment [9], which contains the tyrosine kinase domain.

### 3.2. Labeling of $\text{p60}^{\text{v-src}}$ with [ $^{14}\text{C}$ ]FSBA

5'-FSBA is an irreversible analog of ADP, ATP, NAD or NADP and is known to bind to nucleotide sites of various proteins [10,11]. As shown in Fig. 3,  $\text{p60}^{\text{v-src}}$  in the immune complex was labeled when incubated with [ $^{14}\text{C}$ ]FSBA. Another protein of about 53 kDa was also labeled, but this band was observed in the control lane without anti-Src antibody, and is probably IgG heavy chain. Pretreatment of the immune complex with herbimycin A inhibited the labeling of  $\text{p60}^{\text{v-src}}$  by [ $^{14}\text{C}$ ]FSBA. We confirmed that there was no loss of v-Src protein during incubation with herbimycin A using immune

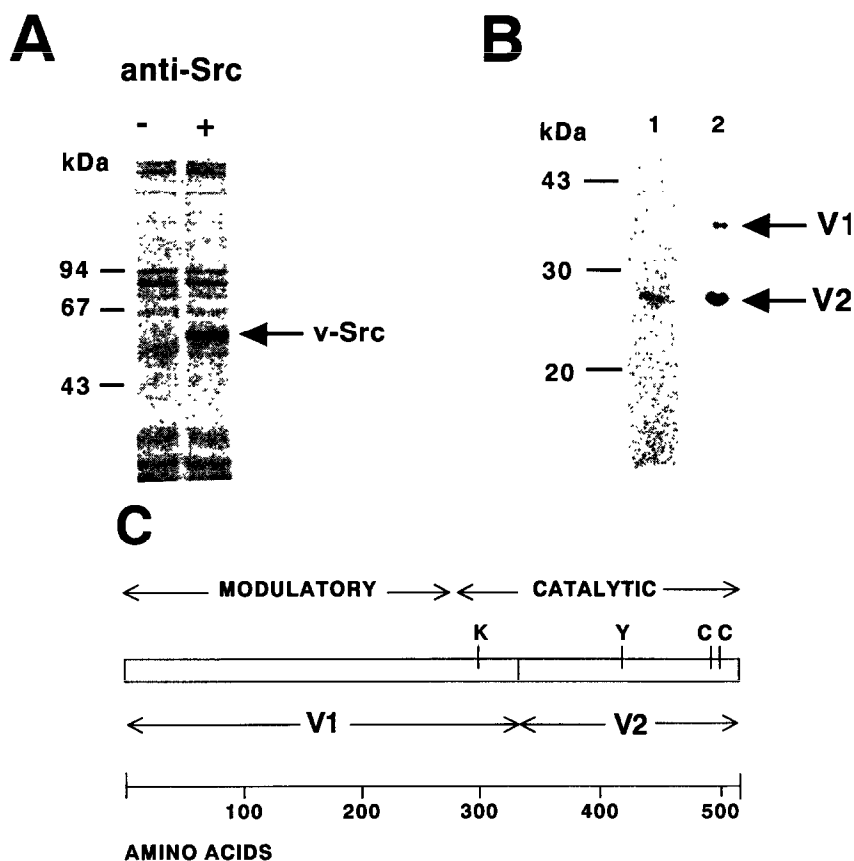


Fig. 1. Labeling of  $\text{p60}^{\text{v-src}}$  by [ $^{14}\text{C}$ ]herbimycin A. (A) v-Src was immunoprecipitated from NIH3T3/v-src cell extract and incubated with [ $^{14}\text{C}$ ]herbimycin A as described in the text. The treated immune complex was subjected to 9% SDS-PAGE and analyzed using a Fujix BAS2000 Bio-imaging Analyzer. The left lane is a control without anti-Src antibody. Positions and sizes of molecular markers are shown to the left. (B) v-Src was cut out from the gel and subjected to peptide mapping with *Staphylococcus aureus* V8 protease and visualized using the Fujix analyzer. Lanes: 1, [ $^{14}\text{C}$ ]herbimycin A-labeled v-Src; 2, [ $^{35}\text{S}$ ]methionine-labeled v-Src. Positions of V1 and V2 fragments are indicated on the right. Molecular markers are shown on the left. (C) V8 cleavage map of v-Src. Shown are positions of the ATP binding site (K), autophosphorylation site (Y) and conserved cysteines (C).

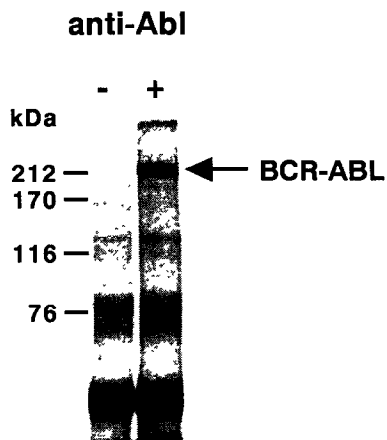


Fig. 2. Labeling of p210<sup>BCR-ABL</sup> by [<sup>14</sup>C]herbimycin A. p210<sup>BCR-ABL</sup> was immunoprecipitated from K562 cell extracts and incubated with [<sup>14</sup>C]herbimycin A as described in the text. The treated immune complex was subjected to 7.5% SDS-PAGE and analyzed using the Fujix analyzer. The left lane is a control without anti-Abl antibody. Positions and sizes of molecular markers are shown to the left.

complex prepared from [<sup>35</sup>S]methionine-labeled cells (data not shown). The results suggest that the nucleotide pocket is masked when p60<sup>v-src</sup> is modified with herbimycin A.

#### 4. Discussion

The tyrosine kinase inhibitor, herbimycin A, has been proved to be a useful reagent for analyzing various biological events that involve tyrosine phosphorylation [12–18]. It inactivates tyrosine kinases both in intact cells and cell-free in vitro systems. Previous experiments suggested that the most likely mechanism of inactivation is conjugation between herbimycin A and the reactive SH group(s) of kinases [5,6,19]. In this study, we used [<sup>14</sup>C]herbimycin A to present strong evidence for direct binding of herbimycin A to p60<sup>v-src</sup> and p210<sup>BCR-ABL</sup>. Although the precise position was not conclusively determined, binding of herbimycin A to the tyrosine kinase domain was confirmed by V8 protease cleavage of [<sup>14</sup>C]herbimycin A-labeled p60<sup>v-src</sup>.

Considering that herbimycin A inactivates many tyrosine kinases, the two conserved cysteine residues in the kinase domain (Cys<sup>487</sup> and Cys<sup>498</sup> in p60<sup>v-src</sup>) seem the most probable binding sites. The significance of the conserved cysteine residues in tyrosine kinases was recently demonstrated by Veillette et al. [20], who altered the cysteines in p56<sup>lck</sup> to alanine by site-directed mutagenesis. Mutation of either of the conserved cysteines in p56<sup>lck</sup> (Cys<sup>464</sup> and Cys<sup>475</sup>) abolished catalytic activity of Lck. Furthermore, mutation of Cys<sup>475</sup> resulted in reduced half-life of Lck. Herbimycin A-treatment also induces inactivation and degradation of cytoplasmic tyrosine kinases, and their results support the hypothesis that her-

bimycin A acts by modifying the conserved cysteine residues of tyrosine kinases.

As mentioned above, we and other investigators observed that herbimycin A promotes degradation of tyrosine kinases in several cells [4,12,17], and such degradation, in some cases, was presumed to be the mechanism of its action. For example, June et al. reported that herbimycin A did not inhibit Fyn tyrosine kinase in vitro, and that reduction in protein amount rather than specific activity accounts for the inhibition of Fyn-dependent signal transduction by herbimycin A [12]. It should be noted, however, that their kinase inhibition experiment was performed at 4°C, and conjugation of herbimycin A to tyrosine kinases in cell-free systems almost always requires incubation at higher temperatures [1]. We speculate from our experimental results that the primary mechanism of herbimycin A is the direct inactivation of kinase activity, at least for cytoplasmic tyrosine kinases. Degradation may also contribute, however, and appears to be the main mode of action against EGF receptor tyrosine kinase of A431 cells (unpublished observations). In this case, herbimycin A does not inhibit the tyrosine kinase activity of the EGF receptor, but decreases EGF receptor number by promoting degradation of immature receptors, resulting in an altered response of the cells to EGF. The mechanism of accelerated degradation has not been conclusively determined but we speculate that it is a consequence of direct binding of herbimycin A to EGF receptors during the maturation process. It is possible that in some cells, herbimycin A-modified tyrosine kinases are recognized as abnormal proteins and thus selectively degraded.

Labeling of p60<sup>v-src</sup> by [<sup>14</sup>C]FSBA, an affinity labeling reagent of nucleotide sites, was inhibited by preincubation with herbimycin A, implying that the nucleotide binding site is masked in the herbimycin A-treated p60<sup>v-src</sup>. The ATP binding site of p60<sup>v-src</sup> is Lys<sup>295</sup>, which is about 200 residues N-terminal from the predicted her-

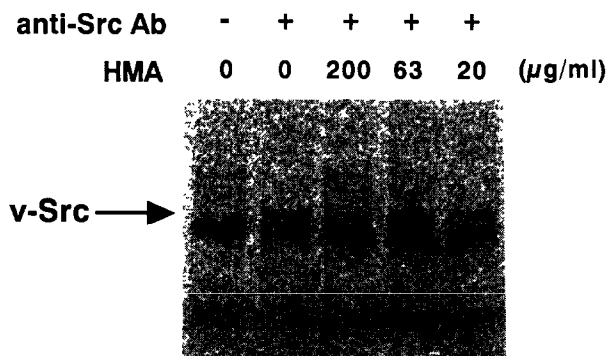


Fig. 3. Inhibition of [<sup>14</sup>C]FSBA binding to p60<sup>v-src</sup> by herbimycin A. v-Src immune complexes were pretreated with various concentrations of herbimycin A, and then incubated with [<sup>14</sup>C]FSBA (1 μCi, 18.8 nmol in a volume of 100 μl). Treated immune complexes were subjected to 7.5% SDS-PAGE and analyzed using the Fujix analyzer. The leftmost lane is a control without anti-Src antibody. HMA, herbimycin A.

bimycin A binding sites. Although not close to the ATP binding site in the primary structure, we speculate that the conserved cysteines are positioned within or in the vicinity of the nucleotide pocket, and that herbimycin A binding impairs ATP access, consequently inactivating tyrosine kinase activity. It is possible that the tertiary conformation of the kinases is critical for herbimycin A action. In this report, we have presented data of herbimycin A binding to activated forms of tyrosine kinases. According to the current model, normal forms of tyrosine kinases are negatively regulated, due to changes in tertiary structure resulting from the interaction of phosphorylated C-terminal tyrosine with the SH2 domain in the amino terminal region. Whether the conserved cysteines in such forms of tyrosine kinases are accessible to herbimycin A is not clear at present.

Of the known tyrosine kinase inhibitors, herbimycin A is the only compound reported to bind covalently to the target enzymes. [ $^{14}\text{C}$ ]Herbimycin A may thus be useful as an affinity labeling reagent for some tyrosine kinases, and we are currently investigating such use.

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