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Research Paper

The Tyr-Kinase Inhibitor AG879, That Blocks the ETK-PAK1 Interaction, Suppresses the RAS-Induced PAK1 Activation and Malignant Transformation

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KEY WORDS

AG879, ETK, RAS, PAK, transformation

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ABSTRACT

AG 879 has been widely used as a Tyr kinase inhibitor specific for ErbB2 and FLK-1, a VEGF receptor. The IC_{50} for both ErbB2 and FLK-1 is around 1 μM. AG 879, in combination of PP1 (an inhibitor specific for Src kinase family), suppresses almost completely the growth of RAS-induced sarcomas in nude mice. In this paper we demonstrate that AG 879 even at 10 nM blocks the specific interaction between the Tyr-kinase ETK and PAK1 (a CDC42/ Rac-dependent Ser/Thr kinase) in cell culture. This interaction is essential for both the RAS-induced PAK1 activation and transformation of NIH 3T3 fibroblasts. However, AG 879 at 10 nM does not inhibit either the purified ETK or PAK1 directly in vitro, suggesting that this drug blocks the ETK-PAK1 pathway by targeting a highly sensitive kinase upstream of ETK. Although the Tyr-kinases Src and FAK are known to activate ETK directly, Src is insensitive to AG 879, and FAK is inhibited by 100 nM AG 879, but not by 10 nM AG879. The structure-function relationship analysis of AG 879 derivatives has revealed that both thio and tert-butyl groups of AG 879, but not (thio) amide group, are essential for its biological function (blocking the ETK-PAK1 pathway), suggesting that through the (thio) amide group, AG 879 can be covalently linked to agarose beads to form a bioactive affinity ligand useful for identifying the primary target of this drug.

INTRODUCTION

PAK1, a member of CDC42/Rac-dependent Ser/Thr kinase family (PAKs), is activated by oncogenic RAS mutants such as v-Ha-RAS, and is essential for RAS-transformation of fibroblasts such as Rat-1 and NIH 3T3 cells.[1,2] Several distinct pathways appear to be essential for v-Ha-RAS-induced activation of PAK1 in these cells.[2] One of these pathways involves PI-3 kinase which produces phosphatidyl-inositol 3,4,5 trisphosphate (PIP3) that activates both CDC42 and Rac GTPases through a GDP-dissociation stimulator (GDS) called VAV. A second pathway involves PIX, an SH3 protein which binds a Pro-rich motif (residues 186-203, PAK18) located between the N-terminal GTPase-binding domain and C-terminal kinase domain of PAK1.[3] PIX binds another protein called CAT which is a substrate of Src family kinases.[4] A third pathway involves an SH2/SH3 adaptor protein called NCK.[5] The SH3 domain of NCK binds another Pro-rich motif of PAK1 located near the N-terminus, while the SH2 domain of NCK binds the Tyr-phosphorylated EGF receptor/ErbB1.[5] Thus, when ErbB1 is activated by EGF, PAK1 is translocated to the plasma membrane through NCK. The involvement of both Src family kinases and ErbB1 in the PAK1 activation is also supported by our finding that both PP1 (inhibitor of Src family kinases) and AG1478 (ErbB1-specific inhibitor) block the RAS-induced PAK1 activation and transformation in vitro and in vivo.[2,6] A fourth pathway involves ErbB2, a member of ErbB family Tyr kinases.[2] We have previously shown that AG 825 (ErbB2-specific inhibitor) blocks RAS-induced activation of PAK1 and malignant transformation with the IC_{50} around 0.35 μM.[2] A fifth pathway has been recently discovered in which RAS activates PAK1 through Tiam1, a Rac-specific GDS , in a PI-3 kinase-independent manner.[7] In this pathway, RAS directly binds Tiam1 which in turn activates Rac.[7]

Another possible pathway involves β1-integrin, FAK and ETK. β1-integrin activates the Tyr kinase FAK, which in turn phosphorylates and activates ETK,[8] a member of TEC/BTK family Tyr kinases.[9,10] ETK carries an N-terminal pleckstrin homology (PH) domain followed by a TEC homology domain.[9,10] Activated ETK binds PAK1 through the PH domain, phosphorylates and activates PAK1.[11] However, it still remains to be

clarified (1) whether RAS requires this integrin/FAK/ ETK pathway for its oncogenicity, and (2) how RAS activates this pathway.

To suppress the growth of RAS-induced sarcomas in vivo (in nude mice), we previously used AG 879, a Tyr-kinase inhibitor specific for ErbB2 and VEGF receptor FLK-1.[2,12,13] In this paper we demonstrate that AG879 inhibits selectively the activation of ETK (IC_{50} around 5 nM), blocking RAS-induced ETK-mediated activation (Tyr phosphorylation) of PAK1 to suppress RAS transformation.

MATERIALS AND METHODS

Cell Culture and Reagents. v-Ha-RAS-transformed NIH 3T3 fibroblasts (RAS cells) were grown in a standard medium, i.e., Dulbecco's modified Eagle's medium in the presence or absence of 10% fetal calf serum as described previously.[2,6] The Tyr kinase inhibitor AG879 and its derivatives (AG 306 and AG 1584) were synthesized as described previously.[12] The novel derivative GL-2002 was synthesized analogously, and full synthetic details will be published in due course. Two other AG 879 derivatives (AG 99 and AG 213) were purchased from Calbiochem (Croydon, Australia). The following antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz,CA): anti-PAK1 antibody, anti-phospho-Tyr antibody (PY99) and goat-anti-ETK antibody. The rabbit anti-FAK antibody was a generous gift of Dr. David Schlaepfer (The Scripps Research Institute, La Jolla, CA). The mouse anti-ETK antibody was purchased from Becton Dickinson Biosciences (North Ryde, NSW, Australia). The rabbit anti-ETK antibody was prepared as described previously.[14]

Assay for the Effect of AG 879 on Cell Growth. The effect of AG879 on anchorage-independent growth of RAS cells was determined by seeding 10^3 cells per plate into 0.35% top agar containing different concentrations of AG879 (from 1 nM to 1 μM) and incubating for 3 weeks as described previously.[2,6,15] At the end of 3 weeks, the colonies formed in the agar were stained and counted. The effect of AG 879 on anchorage-dependent growth of RAS cells and normal NIH/3T3 fibroblasts was examined by seeding 10^3 cells per plate in the medium containing 1–100 nM AG 879, incubating for 5 days and counting as described previously.[2,6,15]

PAK and ETK Kinase Assays. For the PAK kinase assay, RAS cells were serum-starved overnight, and then treated with different concentrations of AG879 for 1 hour as described in the text. The cells were lysed in the lysis buffer (40 mM HEPES, pH 7.4, 1% Nonidet P-40, 1 mM EDTA, 100 mM NaCl, 25 mM NaF, 100 μM $NaVO_3$, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 100 units/ml aprotinin). The lysates containing 1 mg of proteins (measured by Bradford assay) were immuno-precipitated with the anti-PAK1 antibody, and the immuno-precipitates were subjected to the PAK kinase assay as described previously.[1,2,6,16] The direct effect of AG 879 on PAK1 was determined in vitro, using GST-PAK1 fusion protein as described previously.[17] For ETK kinase assay, serum-starved RAS cells were lysed in a buffer containing 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10% glycerol, 1% Nonidet P-40, 10 mM NaF, 100 μM $NaVO_3$, 1 mM PMSF, and 100 units/ml aprotinin. The cell lysates were immuno-precipitated with the rabbit anti-ETK antibody, and the ETK kinase assay was performed as described previously[8,11] using the endogenous PAK1 associated with ETK as a substrate in the presence or absence of different concentrations of AG879 or its derivatives such as AG 1584. Immuno-blotting was performed to determine the protein levels for PAK1 and ETK (see below).

Immuno-Precipitation and Immuno-Blotting. Serum-starved RAS cells were treated with different concentrations of AG879 as indicated in the text. The cells were lysed in two different lysis buffers mentioned above. The cell lysates containing 1.5–2.0 mg of protein (measured by Bradford assay) were incubated with protein A-Sepharose beads (Amersham Pharmacia Biotech) and anti-PAK1, anti-ETK or anti-FAK antibodies separately.[2,8,11,18] The proteins in immuno-precipitates were separated on 4–12% NuPage gel (Invitrogen) electrophoresis and transferred to a nitrocellulose membrane (Micron Separations, Inc.). The membranes were blocked with 10% (w/v) skim milk in phosphate-buffered saline containing 0.04% Tween20 (PBST), followed by an incubation for 1 hr at room temperature with different first antibodies as described in the text. After washing with PBST, the blots were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit (Bio-Rad) secondary antibodies. The bound antibodies were visualised using ECL reagents (Amersham Pharmacia Biotech). Some membranes were stripped and reblotted[19] with different antibodies as described in the text.

The ETK Baculo Viral Construct and its Affinity-Purification. The plasmid encoding the full-length ETK (residues 1-674)[14] was constructed in pBacPAK8 transfer vector (Clontech, Palo Alto, CA) and recombinant virus was made by Dr. Chi-Ying F. Huang (NHRI, Taiwan). Sf9 insect cells infected with the recombinant virus were harvested and disrupted with ice-cold lysis buffer containing 10 mM Tris pH, 7.5; 130 mM NaCl; 1% Triton X-100; 10 mM NaF; 10 mM Na phosphate; 10 mM Na pyro-phosphate and protease inhibitor cocktail (Pharmingen, San Diego, CA). From the clear supernatant of the cell lysate obtained by centrifuging at 40,000xg for 45 min, ETK was affinity-purified by the 6xHis purification kits (Cat. No. 21474K, Pharmingen, San Diego, CA) according to the supplier's instruction.

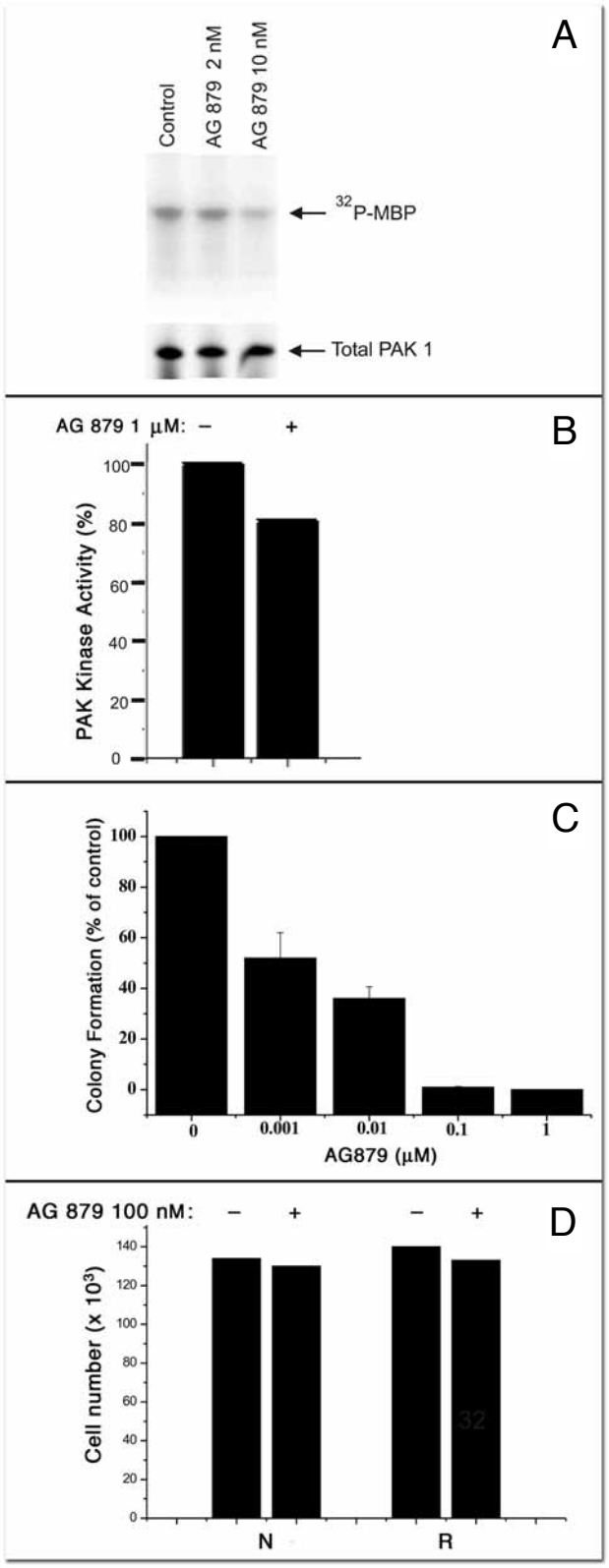
Autophosphorylation of Recombinant ETK Constructs In Vitro. GST fusion protein of human ETKC, a constitutively activated ETK mutant (residues 243–674) which lacks the N-terminal PH domain was affinity-purified from bacteria (E. coli). The GST-ETKC (0.6 μg) was incubated in the kinase buffer containing 10 μM ATP (with or without 5 μCi of [γ-^{32}P]-ATP) as described previously[8] in the presence or absence of AG879 (10 nM or 1–10 mM) at 37°C for 40 min. The auto-phosphorylation was then monitored by immuno-blotting the protein separated by the SDS-PAGE and transferred onto nitrocellulose with anti-phospho-Tyr antibody (or by radio-autography). Similarly 3 μg of full-length ETK purified from the insect cells was incubated in the buffer containing 30 mM PIPES, pH 7.0, 10 μM $MnCl^2$, 30 μM ATP, 5 μCi of [γ -^{32}P] ATP, 1 mM Na_3VO_4 for 20 min at 30°C, in the presence or absence of AG879 (10 nM–1 mM), and the auto-phosphorylation was measured by radio-autography of the proteins separated on SDS-PAGE.

Upregulation of ETK by RAS. The ETK protein levels were compared between normal NIH/3T3 cells and two distinct v-Ha-RAS transformed cell lines, excluding the possible clonal difference in the ETK levels: stable v-Ha-RAS cell line (RAS cells) and doxycycline-inducible v-Ha-RAS transformants prepared as described previously.[20,21] The lysates of both normal and RAS cells (20 μg protein of each) were subjected to SDS-PAGE and immuno-blotted by the anti-ETK antibody. In the case of doxycycline-inducible RAS transformants, cells were incubated for 2–3 days in the presence or absence of 2 μg/ml doxycycline, and each lysate (20 μg protein) was subjected to the SDS-PAGE/ immuno-blot with the anti-ETK.

RESULTS

AG879 (10 nM) Blocks the Activation of PAK1 and Suppresses RAS-Induced Malignant Transformation. AG879 was reported as an inhibitor specific for both ErbB2 and VEGF receptor FLK-1 (IC50 is around 1 mM)[12,13] and appears to be metabolically more stable than AG825 in vivo as it strongly suppresses the growth of RAS-sarcomas in nude mice.[2] However, the IC_{50} of AG879 for inhibiting PAK1 activation and RAS transformation in vitro still remained to be determined.

10 nM AG879 strongly blocks PAK1 kinase activity in RAS cells without affecting the PAK1 protein level (Fig. 1A). However, AG879 does not inhibit the kinase activity of the purified GST-PAK1 fusion protein directly even at 1 μM (Fig. 1B). These observations suggest that ErbB2 is not involved in the inactivation by AG879 of PAK1 in cells. Interestingly, AG879 also suppresses the anchorage-independent growth of RAS cells in soft agar (Fig. 1C). The IC_{50} of AG879 for the large colony formation is 1–10 nM. However, the inhibition of anchorage-independent growth by AG879 is not due to non-specific inhibition on cell growth, as at even 100



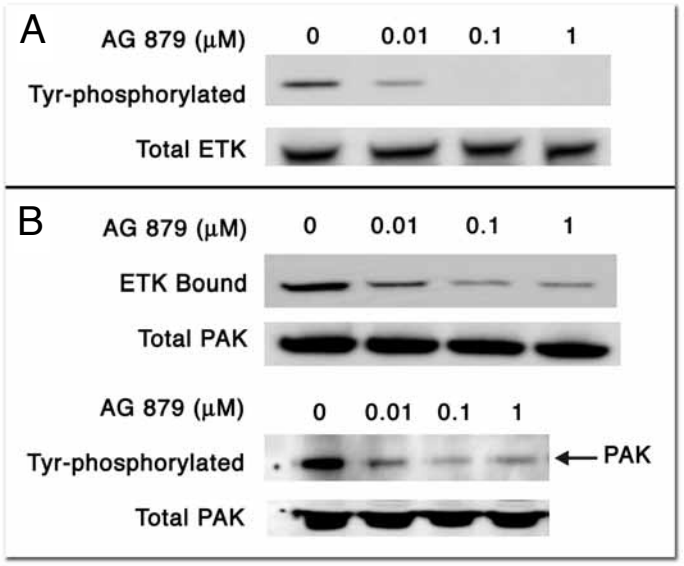


Figure 2. AG879 blocks the Tyr-phosphorylation of ETK and its association with PAK1. (A) Serum-starved RAS cells were first incubated with AG879 (0.01-1 μM) for 1hr. The cells lysates (CL) were then immuno-precipitated (IP) with the anti-ETK antibody as described under "Materials & Methods", followed by immuno-blot (IB) with anti-phospho-Tyr (PY) antibody. (B) Alternatively, the CL were IP with the anti-PAK1 (top panel) or anti-PY (bottom panel) antibodies, followed by IB with anti-ETK (top panel) or anti-PAK1 (bottom panel) antibodies. The total PAK1 protein level of the bottom panel was determined by IBing each CL directly. Similar results were obtained from two or three independent experiments.

nM AG879 does not affect the anchorage-dependent growth of either normal or RAS-transformed NIH 3T3 cells (Fig. 1D). These results suggest that the suppression by AG 879 of both RAS-induced malignant transformation and PAK1 activation is not due to blocking ErbB2, but another kinase(s) associated with PAK1.

AG879 Inhibits the Tyr-Phosphorylation of ETK and Its Association with PAK1. The Tyr-phosphorylation of PAK1 is required for its Ser/Thr kinase activity as the treatment of PAK1with a Tyr-phosphatase reduces its kinase activity.[22] Activated ETK associates with PAK1 through its PH domain and activates PAK1 by the Tyr phosphorylation.[11] To test whether the Tyr kinase activity of ETK is affected by AG879, serum-starved RAS cells were treated with AG879 (0.01–1 μM) in culture for 1hr. Cell lysates were then immuno-precipitated with the anti-ETK antibody, followed by immuno-blotting with the anti-phospho-Tyr or anti-ETK antibody. AG879 inhibits the Tyr-phosphorylation of ETK at 10 nM, but does not affect the ETK protein level (Fig. 2A). Furthermore, using the anti-PAK1 antibody, we found that AG879 significantly suppresses the ETK-PAK1 association (Fig.2B, top panel) and reduced the Tyr-phosphorylation of PAK1 in cells at 10 nM (Fig.2B, bottom panel). These results suggest that AG879 inhibits somehow the kinase activity of ETK, thus blocking its auto-phosphorylation, and association with PAK1, and the Tyr-phosphorylation of PAK1 by ETK.

AG879 Inactivates ETK In Vitro. To determine whether AG879 directly inhibits the kinase activity of ETK or not, RAS cells were lysed and

Figure 1. (A) AG879 inhibits the activation of PAK1 in cells. Serum-starved RAS cells were incubated with AG879 (0.01–10 μM) for 1hr. The cell lysates were subjected to PAK kinase assay as described under "Materials & Methods". 10 nM AG 879 clearly inhibited the PAK1 activity (phosphorylation of MBP) in cells (top panel). Similar levels of PAK1 protein were detected in all lanes as judged by immuno-blot (bottom panel). (B) PAK1 is not a direct target of AG 879. 1 μM AG 879 fails to inhibit significantly the kinase activity of GST-PAK1 in vitro. (C) AG879 inhibits anchorage-independent growth of RAS cells. RAS cells were planted in soft agar with or without AG879 (0.001—100 μM) as described under "Materials & Methods". The colony formation was measured in comparison with that of the control (non-treated) cells. Only large colonies consisting more than 100 cells per colony were counted. The presented values are the average of those obtained from two independent experiments. (D) AG 879 has no effect on the anchorage-dependent cell growth. The growth of either normal or RAS-transformed cells in liquid culture was monitored in the presence or absence of 100 nM AG 879 as described under "Materials & Methods".

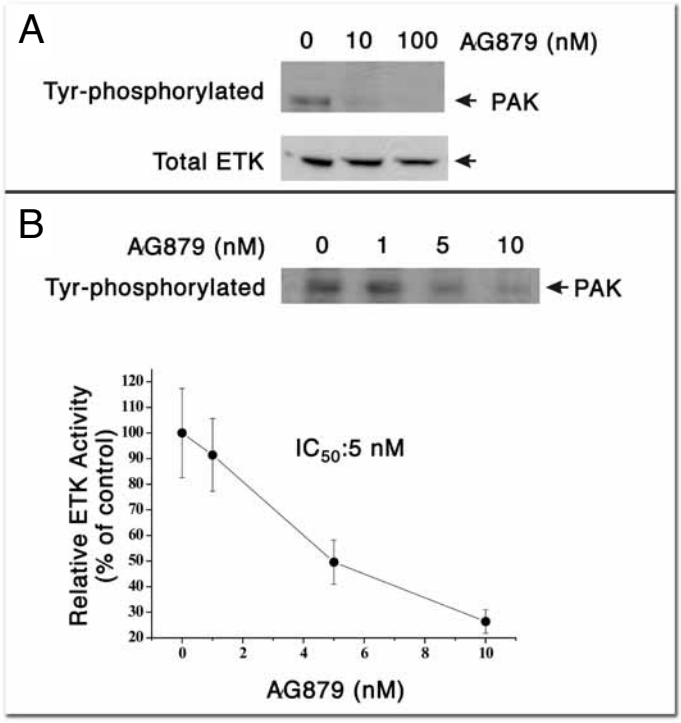


Figure 3. AG879 inhibits the kinase activity of ETK in vitro. The lysates of RAS cells were immuno-precipitated by anti-ETK antibody. The immuno-precipitates (IB) were subjected to an in vitro kinase assay in the presence or absence of AG879 (A, 10 or 100 nM; B, 1 to 10 nM) as described under "Materials & Methods". The ETK activity (Tyr-phosphorylation of PAK1) was monitored by immuno-blot (IB) with anti-PY antibody. Similar protein levels of ETK were detected in all lanes by IB with the anti-ETK antibody. Similar results were obtained from two independent experiments.

immuno-precipitated with the anti-ETK antibody. The immuno-precipitates were subjected to an in vitro kinase assay in the presence or absence of AG879 (0.001–10 μM) as described in the "Materials and Methods". AG879 (10 nM) strongly inhibits the Tyr-phosphorylation of PAK1 by

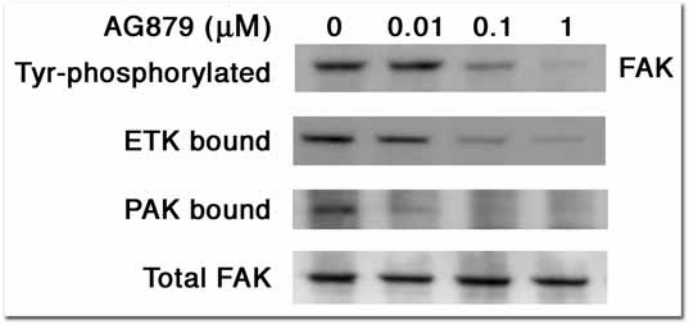


Figure 5. AG879 suppresses the Tyr-phosphorylation of FAK and its association with ETK and PAK1. Serum-starved RAS cells were incubated with AG879 (0.01–1 μM) for 1hr. The cell lysates were immuno-precipitated (IP) with anti-FAK antibody as described under "Materials & Methods", followed by immuno-blot (IB) with anti-phospho-Tyr (PY), anti-ETK or anti-PAK antibodies. Similar protein levels were detected in all lanes by IB with the anti-FAK antibody. Similar results were obtained from two independent experiments.

ETK (Fig.3A), and the IC_{50} for ETK is around 5 nM (Fig. 3B). Furthermore, AG 879 has no direct effect on any other members of TEC family kinases such as TEC, BTK or ITK, even at 10 μM in vitro (data not shown). These results suggest that ETK is so far most sensitive to the action of AG879. However, since the anti-ETK antibody could precipitates not only ETK itself, but also any proteins forming a tight complex with ETK such as PAK1 and FAK, we cannot exclude the possibility that the primary target of AG 879 might be a third Tyr-kinase which is associated with ETK, and responsible for the ETK activation.

AG879 (5 nM) Does Not Inhibit Recombinant ETK from Bacteria or Insect Cells. To clarify whether ETK itself is the primary target of ETK, two different recombinant ETK samples of human origin were purified from bacteria or insect cells: a constitutively activated mutant of ETK called ETKC (residues 243–674) which lacks the N-terminal PH domain purified from bacteria as a GST fusion protein, and full-length ETK purified from insect cells. Either the ETKC or the full-length ETK were not inhibited by AG 879 at 10 nM, although they were significantly inhibited at 1–10 μM (see Fig. 4). Furthermore, in vitro binding of PAK1 in RAS cell lysates to either the PH domain of ETK or a kinase-dead mutant of ETK (called

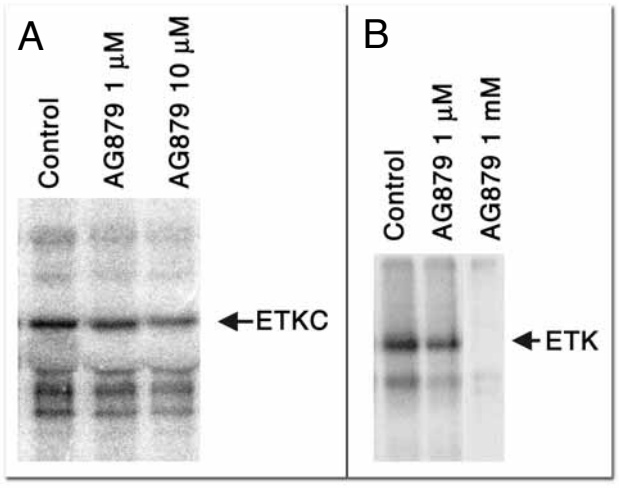


Figure 4. Recombinant ETK proteins alone are far less sensitive to AG 879 in vitro. (A) Effect of AG879 on the ETKC from bacteria. The GST-ETKC was auto-phosphorylated in the presence of AG 879 (0, 1 and 10 μM) in vitro as described under "Materials and Methods". (B) Effect of AG879 on full-length ETK from insect cells. The full-length ETK was auto-phosphorylated in the presence of AG 879 (0, 1 μM and 1mM) in vitro as described under "Materials and Methods".

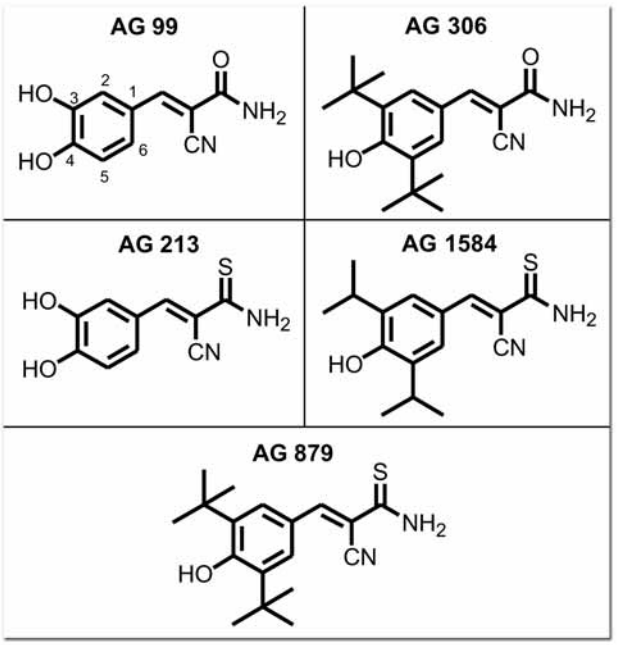


Figure 6. Chemical structure of AG 879 derivatives.

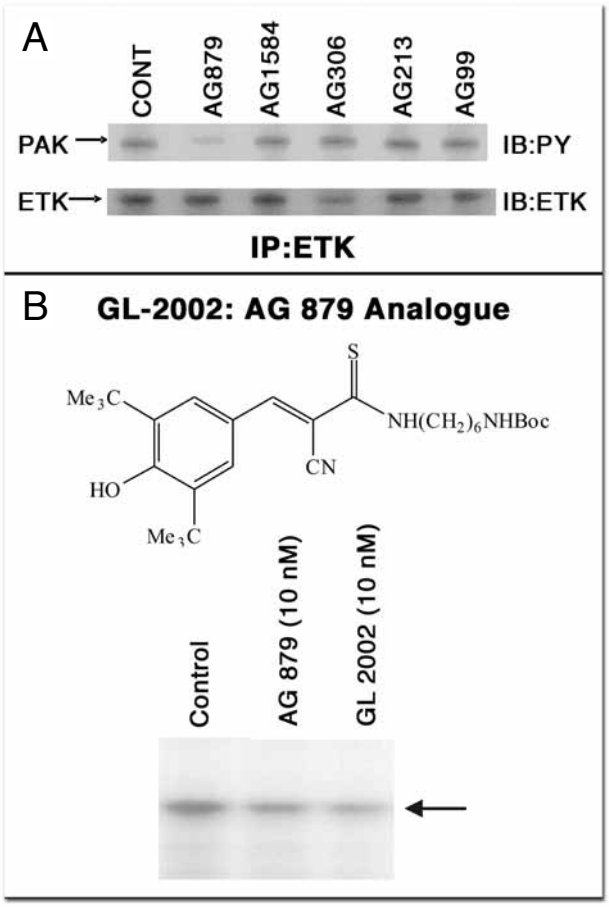


Figure 7. Anti-ETK activity of AG 879 derivatives. (A) The lysates of RAS cells were immuno-precipitated by anti-ETK antibody. The immuno-precipitates (IP) were subjected to an in vitro kinase assay in the absence of any drug (CONT) or presence of either AG879 (10 nM) or four other derivatives (10 μM) as described in Figure 3. Only AG 879 inhibits the ETK activity (Tyr-phosphorylation of PAK1) monitored by immuno-blot (IB) with anti-PY antibody. Similar protein levels of ETK were detected in all lanes by IB with the anti-ETK antibody. Similar results were obtained from two independent experiments. (B) After RAS cells were treated with either 10 nM GL-2002 or AG 879 for 1.5 hrs, each cell lysate was subjected to the in vitro PAK1 kinase assay described in Fig. 1B. GL-2002 strongly inhibited PAK1 activation as did AG 879. Similar results were obtained from three independent experiments.

ETK/KQ) as a GST-fusion protein was not inhibited by 10 nM AG879 (Hirokawa Y, He H, Maruta H, unpublished observation, 2002). These observations altogether suggest that the primary target of AG879 is not ETK itself, but rather its associated upstream activator to be identified.

AG879 Inhibits the Tyr-Phosphorylation of FAK and Its Association with ETK and PAK1. ETK is a cytoplasmic (non-receptor) Tyr kinase which is activated at the plasma membranes.[9,10] The N-terminus of FAK shares significant sequence homology with FERM domains, which are involved in linking cytoplasmic proteins to the membranes.[23,24] It was shown recently that the activation of ETK by extracellular matrix (ECM) is regulated by FAK through the interaction between the PH domain of ETK and the FERM domain of FAK, and that activated FAK binds ETK and elevates the Tyr-phosphorylation of ETK.[8]

To test whether the FAK-ETK interaction is affected by AG879, serum-starved RAS cells were treated with AG879 (0.01–1μM). Cell lysates were then immuno-precipitated with anti-FAK antibody, followed by blotting with anti-phospho-Tyr, anti-ETK or anti-PAK1 antibodies separately. As shown in Figure 5, AG879 suppresses both the Tyr-phosphorylation of FAK and its association with ETK at 100 nM, but not at 10 nM, whereas AG879 inhibits the FAK-PAK1 interaction at 10 nM. These results suggest that PAK1 associates with FAK probably through ETK, and PAK1 can no longer interact with FAK when the PAK1-ETK complex is disrupted by AG879.

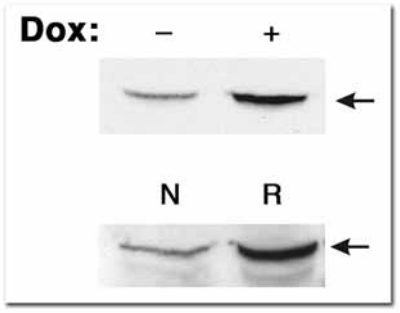


Figure 8. RAS-induced up-regulation of ETK. Upper panel: Up-regulation of ETK by the Doxycyline-induced v-Ha-RAS expression in normal NIH/3T3 cells. Dox-minus, the control (no doxycycline-added); Dox-plus, 2 μg/ml doxycycline added. Lower panel: Enhanced expression of ETK in v-Ha-RAS-transformants. N, normal NIH/3T3 cells; R, v-Ha-RAS-transformants. The arrow indicates the ETK band. Both stable and inducible RAS up-regulate the ETK protein level.

The Structure-Function Analysis of AG879 Derivatives in Inhibiting ETK. To determine which side chains of AG879 are essential for the ETK inhibition, and further screen for a more potent "ETK inhibitor", we have examined the anti-ETK activity of several AG879 derivatives shown in Figure 6. However, none of these derivatives other than AG879 itself inhibits ETK activity in vitro even at 10 μM (see Fig. 7A). These results indicate that at least both tert butyl groups at positions 3 and 5, and the thio group are absolutely essential for AG879 to inhibit ETK. Interestingly, the ErbB2-specific inhibitor AG825 is distantly related to AG879, but like AG306, lacks both the thio and tert butyl groups, and in fact shows no anti-ETK activity at even 1 μM in vitro (data not shown). However, when the free (thio) amino group of AG879 was alkylated with an amino-hexane chain, the resulting derivative called GL-2002 was still able to show a strong anti-ETK activity that blocks the PAK1 activation in RAS cells even at 10 nM (Fig. 7B), suggesting that, unlike other side chains, this free amino group of AG879 is not essential for its anti-ETK activity. Thus, we are currently generating a series of bioactive immobilized AG 879 (or its water-soluble N-hexylamine derivative, called GL-2003) by coupling them to agarose beads through the amino group so that we can use the AG879/GL-2003 bead as a ligand for fishing a high-affinity AG879-binding protein(s).

Upregulation of ETK Protein Level by RAS. How does RAS activate this integrin/FAK/ETK pathway? Although the whole picture of this mechanism still remains to be unveiled , we found that v-Ha-RAS upregulates the protein level of ETK several folds, using both doxycycline-inducible v-Ha-RAS transformants and stable v-Ha-RAS transformants derived from normal NIH/3T3 cells (see Fig. 8), clearly indicating that oncogenic RAS signalling involves ETK.

DISCUSSION

In this study, we have demonstrated that AG879 selectively inactivates the cytoplasmic Tyr kinase ETK with IC_{50} of about 5 nM. The inactivation of ETK by AG879 blocks the ETK-PAK1 interaction, thereby blocking the Tyr-phosphorylation of PAK1 and its kinase activity. Interestingly at this concentration AG879 does not inhibit directly any other kinases including FAK, PAK, ErbB2, ErbB1, TRK, TEC, BTK and ITK. However, since the IC_{50} of this drug for recombinant ETK proteins alone is 1–10 μM, instead of 5 nM, it is most likely that the primary target of AG879 is not ETK, FAK or ErbB2 themselves, but an as yet unidentified activator of ETK. Thus, we are currently identifying this highly AG879-sensitive

target among the kinases which are associated with ETK, Tyr-phosphorylate the kinase-dead (non-auto-phosphorylatable) mutant of ETK (ETK-KQ), and bind tightly to the AG879/GL-2003 beads. Our preliminary data suggest that the primary target is a Tyr-phosphorylated protein of 62 kDa (Hirokawa Y and Maruta H, unpublished observation).

ETK is required for the anchorage-independent and tumorigenic growth of human breast cancer cells.[11] Although ETK alone is not transforming, it enhances malignant transformation of NIH 3T3 cells caused by a partially activated c-Src mutant.[25] ETK can also be activated by Src family kinases and is responsible for Src activation of signal transducer and activator of transcription factor 3 (STAT3) and v-Src-induced transformation.[25] In this study we have established that ETK is essential for RAS transformation: the concentration of AG879 that inhibits both RAS-induced PAK1 activation and anchorage-independent growth is similar to the IC_{50} for ETK both in vitro and in vivo.

We have established here that RAS signalling involves ETK by demonstrating that RAS significantly up-regulates the ETK protein level. To understand further the detailed mechanism, we are currently investigating whether this regulation is at either transcriptional or translational levels or its stability (turn-over rate). In this context, it is worth noting that ETK is highly expressed in metastatic prostate and breast carcinoma cell lines such as PC3M which carry oncogenic Ki-RAS mutants.[8] Since RAS cells in general are both metastatic and angiogenic, it is conceivable that at least a part of the reason for the high expression of ETK in these cell lines might be due to the constitutive RAS activation. Thus, it would be of great interest to determine whether AG879 inhibits the metastasis of these RAS cancer cell lines in vivo.

It was suggested earlier that AG879 (also called SU0879) suppresses angiogenesis by blocking VEGF receptor FLK-1.[13] However, since the VEGF receptor also directly activates ETK[10] which is then inhibited by AG879 at 5 nM (200 times more sensitive to this drug than FLK-1), it is more likely that AG879 suppresses angiogenesis primarily by blocking ETK, rather than FLK-1. Since oncogenic RAS mutants up-regulate expression of VEGF through Raf-MEK-MAP kinase cascade,[26] and VEGF in turn activates ETK through FLK-1in endothelial cells, RAS transformation can induce angiogenesis through this paracrine pathway. Thus, it is conceivable that the suppression of RAS sarcomas growth by AG879 in mice[2] might be at least in part due to its anti-angiogenic action, (in addition to blocking the anchorage-independent growth of RAS cells per se). Interestingly it was recently shown that PAK1 is essential for angiogenesis. A cell-permeable peptide which blocks selectively the NCK-PAK1 interaction inhibits bFGF-induced angiogenesis.[27]

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