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Research Article

Novel actions of tyrphostin AG 879: inhibition of RAF-1 and HER-2 expression combined with strong antitumoral effects on breast cancer cells

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Abstract. Binding of growth factors to cell surface receptors activates protein tyrosine kinases (PTKs) that initiate cascades of downstream signaling events including the mitogen-activated protein (MAP) kinase cascade. This study reports that the PTK inhibitor AG 879 inhibits proliferation of human breast cancer cells through an effect involving inhibition of MAP kinase activation, but which cannot be explained by effects of AG 879 on its known PTK targets. Instead, AG 879 markedly inhibits expression of the RAF-1 gene, which encodes an upstream MAP kinase kinase kinase. Additionally, expression of HER-2, but not of other genes tested, is inhibited by this compound. These novel effects have to be considered when using AG 879 as a TRK-A and HER-2 inhibitor but may have useful therapeutic implications.

Key words. AG 879, HER-2, MAP kinase, RAF-1, breast cancer, apoptosis, proliferation, gene expression

Protein tyrosine kinases (PTKs) activate multiple signaling pathways that are important to cell division and survival [1]. Binding of ligands to growth factor receptors activates receptor autophosphorylation events that create binding sites for the SH2 domain of growth factor receptor-bound protein 2 (Grb2). The SH3 domain of Grb2 recruits guanine nucleotide exchange factors, which catalyze exchange of GDP for GTP in RAS. GTP-bound RAS activates a number of downstream signaling effectors, including the mitogen-activated protein kinase kinase kinase (MAPKKK) RAF-1. RAF-1 activates the MAPK kinases MEK1 and 2 and these, in turn, activate the MAPKs ERK-1 and 2 (extracellular signal-regulated kinase 1 and 2) [2]. Activated, dually phosphorylated ERKs translocate to the nucleus [3–5], where they stimulate cell cycle progression [6–8]. Additionally, ERKs activate the MAPK-activated protein kinase-1, Rsk, which catalyzes phosphorylation of Bad at serine 112 [9]. A number of agents targeting either RAS or downstream kinases of the MAPK cascade have been developed, and some are in clinical trials as cancer chemotherapeutic agents [2].

PTKs are frequently constitutively activated in cancers and many PTKs have been identified as oncogenes [1]. A number of PTK inhibitors have been developed for experimental or clinical use. A series of tyrphostin (AG) compounds designed to inhibit PTKs by binding to their substrate-binding sites have been developed, while other inhibitors including genistein target the ATP-binding site [10, 11]. PTK inhibitors as well as antibodies targeting PTKs have found use in cancer chemotherapy. Examples include the use of STI571 (Gleevec) in chronic myeloid leukemia and certain other malignancies, of Iressa in non-small-cell lung cancer and of monoclonal antibodies against the HER2/erbB2/neu EGF receptor family tyrosine kinase in HER-2-overexpressing breast cancer [2, 12–16].

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Many kinase inhibitors show a broader spectrum of inhibitory activities than originally conceived [17–19]. Thus, STI571 also inhibits c-kit and is therefore also useful for treatment of gastrointestinal stromal tumors [20]. Additionally, geldanamycin and related molecules, originally thought of as tyrosine kinase inhibitors, have been found to exert antitumoral effects by interacting with heat shock protein 90 (Hsp90), thereby targeting Hsp90 client proteins like RAF-1 and HER-2 to degradation by the proteasome [21–23].

This work now shows that the tyrphostin compound AG 879 effectively inhibits proliferation of human breast cancer cells. Moreover, it inhibits activation of ERK-1/2 and its effects on proliferation are, to a large extent, mimicked by the MEK inhibitor UO126. The inhibition of ERK-1/2 activation in serum-stimulated cells shows that AG 879 inhibits downstream MAPK signaling through the many receptors that are activated by growth factors present in serum. Thus, although AG 879 is a useful inhibitor of TRK-A [24–29] and HER-2 [30–35], these activities alone seemed unlikely to account for the inhibition of serum-stimulated ERK activation. This was further underlined by the finding that the effects of AG 879 were not reproduced by other selective inhibitors of these PTKs. Instead, studies using immunoblotting and real-time PCR documented that AG 879 inhibited expression of the upstream kinase RAF-1 at the protein and mRNA level. The effects on RAF-1 mRNA levels were negated in the presence of the transcriptional inhibitor actinomycin, showing that the effect occurred at the transcriptional level. Additionally, AG 879 inhibited expression of HER-2 but not of other genes, including c-jun, PEA-3 or NQO1. The effects of this compound on RAF-1 and HER-2 expression complicate its use as a selective PTK inhibitor but may be therapeutically useful.

Materials and methods

Cell culture and treatments

MCF-7 and MDA-MB-231 cells were cultured as described elsewhere [36]. SK-BR-3 cells were cultured in McCoy's medium containing Glutamax (Gibco, Paisley, UK) and 10% fetal calf serum (FCS). Cells were exposed to AG 879, AG 825, AG 9, genistein, K252a, MG132 or UO126 (Calbiochem, San Diego, Calif.), dissolved in DMSO (final concentration: 0.1%), or to 0.1% DMSO alone. Additionally, cells were treated with actinomycin [dissolved in phosphate-bufferd saline (PBS)], alone or in combination with AG 879. Cells for MTT assays and BrdU incorporation studies were grown in 96-well dishes, cells for RNA extraction or Western blotting were grown in plastic petri dishes and cells for annexin V-Fluos or hematoxylin staining were grown on sterile glass slides. All experiments were repeated at least three times.

Cell proliferation, DNA synthesis and apoptosis assays

MTT assays were carried out essentially as described elsewhere [37]. Briefly, cells were grown in 96-well plates containing 100 µl medium per well. Ten microliters of MTT solution (5 mg/ml in PBS) was added to each well and incubation continued for 4 h at 37 °C. Subsequently, 100 µl 10% SDS in 0.01 M HCl was added. After incubation at 37 °C overnight, absorption was measured at 550 nm in an ELISA reader using a reference filter of 690 nm.

Bromodeoxyuridine incorporation into DNA was measured using an ELISA kit (Cell Proliferation Kit; Roche, Basel, Switzerland) and results were read at an absorption of 370 nm using a 550-nm filter for reference.

For annexin V-Fluos-propidium iodide staining [38], cells grown on sterile glass slides were quickly rinsed in medium without serum, followed by a brief wash in incubation buffer (10 mM Hepes, pH 7.4, containing 140 mM NaCl and 5 mM $CaCl_2$). Subsequently, cells were incubated for 25 min at room temperature (RT) in annexin V-Fluos solution (Roche; diluted 1:50 in incubation buffer), fortified with 1 µg/ml propidium iodide. They were then rinsed in incubation buffer, postfixed in 3.7% paraformaldehyde for 10 min, rinsed in Tris-buffered saline (TBS) and mounted in antifade medium. The percentage of apoptotic (green-, but not red-fluorescent) cells was determined by counting cells in coded specimens in a fluorescence microscope. In addition, cells grown on sterile slides were fixed in 3.7% paraformaldehyde, stained with hematoxylin, coded and mitotic figures counted using a ×60 oil immersion objective. Between 1300–1700 cells were counted in each experiment.

BrdU incorporation, annexin and hematoxylin staining experiments were performed after 24 h of culture, while MTT assays were performed after 46 h. MTT and BrdU assay values are given as means ± SD of six individual determinations, while annexin values represent means ± SD of five determinations. Statistics were computed by the Mann-Whitney U test.

Immunoprecipitations

Cells were lysed in 10 mM TrisHCl pH 7.6, containing 1% Triton X-100, 50 mM sodium chloride, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 20 mM sodium β-glycerophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM sodium vanadate and a complete tablet of protease inhibitors (Roche), placed on ice for 15 min and centrifuged at 14,000 g. Aliquots of 0.5 mg protein were immunoprecipitated using 5 µg RAF-1 or HER-2 antibody (BD Biosciences, Heidelberg, Germany) per milliliter overnight at 4 °C. Subsequently, 100 µl of a 50% Protein A-Sepharose slurry (Pharmacia, Uppsala, Sweden) was added and incubation continued for 2 h. Following centrifugation the pellet was solubilized in SDS sample buffer and submitted to SDS-PAGE and immunoblotting.

SDS-PAGE and immunoblotting

Extraction, SDS-PAGE and electroblotting were performed as described previously [39]. Immunoblots were stained with antibodies recognizing active, dually phosphorylated ERK-1/2 (Cell Signaling, Beverly, Mass.), RAF-1, HER-2, Hsp70, Hsp90 or ERK-2 (BD Biosciences) using chemiluminescent detection [39].

Real-time RT-PCR

Cells were harvested with a rubber policeman and total RNA prepared using the trizol reagent. Reverse transcription employed the Superscript II kit following the manufacturer's (Invitrogen, Carlsbad, Calif.) recommendation for random hexamer RT and PCR except that 2.5 units of AmpliTaq Gold polymerase (Roche) were used. Initially, all primer sets were tested at different $MgCl_2$ concentrations and at different annealing temperatures to obtain specific amplification without primer-dimer formation. PCR reactions were done on a LightCycler (Roche) using the SYBR green I Fast start kit (Roche). Reactions were carried out in 20-µl volumes consisting of 1× Fast Start Master SYBR Green I Mix (which includes Taq DNA polymerase, reaction buffer, dNTPs and SYBR Green I), 3–5 mM $MgCl_2$ and 0.5 µM of primer. Each run consisted of serial dilutions (×10) of cDNA to create a standard curve with at least five samples of cDNA from each group. In each reaction, 2 µl cDNA was amplified. The amplification program was as follows: Preincubation for Fast Start Polymerase activation at 95 °C for 10 min, followed by 45 amplification cycles of denaturation at 5 s (20 °C/s), annealing at 59–63 °C for 10 s (20 °C/s), elongation at 72 ºC for 4–10 s (20 °C/s) depending on the primer set, and acquisition of fluorescence at 72 °C. After the end of the last cycle, the melting curve was generated by starting the fluorescence acquisition at 65 °C and taking measurements every 0.1 s until 97 °C was reached. The experiment was repeated three times for each primer set. RNA integrity was checked using a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, Calif.). Primers used included: RAF-1 (left: 5′-TTCTGTAGATGCGCAAGT GG-3′, right: 5′-GGCTGTTTGGTGCCTTATGT-3′), NQ O1 (left: 5′-CCAGAAAGGACATCACAGG-3′, right: 5′-CACAAGGTCTGCGGCTTCC-3′,), PEA-3 (left: 5′-AG GAGACGTGGCTCGCTGA-3′, right: 5′-GGGGCTGT GGAAAGCTAGGTT-3′), c-Jun (left: 5′-TTGTTTGT TTGGGTATCCTG-3′, right: 5′-ATGCAGAAAAGAG GTTAGGG-3′), HER-2 (left: 5′- TATGGCTGCCTCT TAGACCATGTCCGG-3′, right: 5′- TTGGGACTCTTG ACCAGCACGTTCCGA-3′).

Results

Effects of AG 879 on human breast cancer cells

An MTT assay was first employed for screening for effects of AG 879 on MCF-7 cells. Calibrations showed that the absorption values of the MTT assay correlated well with cell numbers over the relevant range studied (0–32,000 cells, $r^2 = 0.974$). AG 879 dose-dependently reduced MCF-7 cell numbers (fig. 1) and showed already a significant effect at 0.4 µM. This potent effect contrasted to that of the control tyrphostin compound AG 9, which showed no significant effect at concentrations up to 20 µM (fig. 1). Similarly, the HER-2-selective inhibitor (AG 825) as well as genistein showed only marginal effects on the cells at concentrations up to 20 µM (fig. 1). Additionally, the TRK-A inhibitor K252a only reduced the number of MCF-7 cells to 85.1 ± 6.4% of controls at its maximally effective dose (0.3 µM) and did not show increased effectiveness at 0.6 µM. Combined treatment of cells with AG 825 and K252a did not reveal any potentiating effects (data not shown). Together, these results show that AG 879 possesses a unique inhibitory effect on MCF-7 cells that is neither reproduced by other tyrosine kinase inhibitors nor readily explainable by its

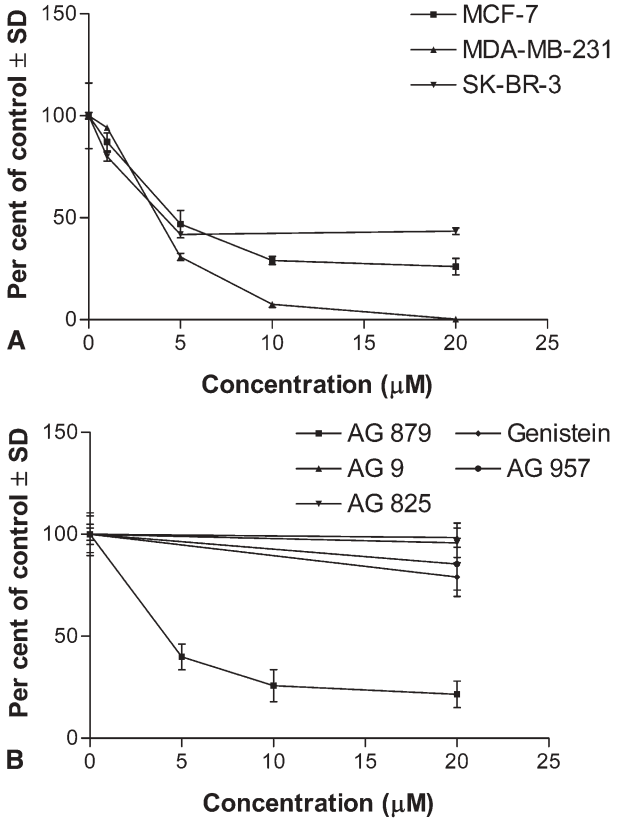

Figure 1. Effect of AG 879 on numbers of human breast cancer cells. All MTT readings were made 46 h after the addition of drugs and are expressed as percent of controls in which cells were exposed to only the solvent (0.1% DMSO). Separate studies documented that this concentration of DMSO did not affect the results. Data are given as means ± SD of five duplicates. (*A*) Dose-response curve from MTT assays demonstrating that AG 879 potently reduces numbers of MCF-7, MDA-MB-231 and SK-BR-3 cells. (*B*) MTT dose-response curves (MCF-7 cells) demonstrate that AG 879 is the most effective agent out of a number of PTK inhibitors tested.

known effects on HER-2 or TRK-A tyrosine kinases, but may reflect effects on an unidentified kinase. In addition, AG 879 showed a similarly potent effect on MDA-MB-231 cells, which, unlike MCF-7 cells, contain mutated p53 and lack estrogen receptors, and on SK-BR-3 cells, which overexpress HER-2 (fig. 1).

Studies of the mechanism by which AG 879 decreased breast cancer cell numbers showed that it reduced the frequency of mitotic figures by 70% (fig. 2) and decreased DNA synthesis to 14.5 ± 6.0% of controls. In addition, AG 879 induced apoptosis to a low, but significant, extent (fig. 2).

AG 879 inhibits activation of ERK-1/2

Studies on the effects of AG 879 on activation of the MAPK cascade employed immunoblotting using antibodies specifically recognizing active, dually phosphorylated forms of ERK-1 (p44) and ERK-2 (p42). Treatment with AG 879 for 24 h inhibited activation of ERK-1/2 (fig. 3). Reprobing the blots with a monoclonal antibody that recognized ERK-2, irrespective of its phosphorylation status, showed that the protein levels of this kinase were not changed (fig. 3). Interestingly, however, shorter times of treament with AG 879 caused a transient increase in ERK activation that peaked by 2 h and subsequently declined to undetectable levels by 24 h. After 6 h of treatment, levels of phosphorylated ERKs were still above control levels (fig 4).

Inhibition of ERK activation inhibits proliferation of serum-stimulated breast cancer cells

To determine if the effects of AG 879 on ERK activation were responsible for its biological effects, we examined MCF-7 cells for effects of the MEK inhibitor UO126 [40]. The inhibitor markedly reduced cell numbers and mitotic figures (fig. 2). Thus, these data show that the effects of AG 879 are to a large extent mimicked by UO126 and, hence, can be ascribed to inhibition of ERK activation. This is further underlined by the observation that, when the drugs were combined, UO126 only marginally increased the effect of AG 879 (fig. 2).

Effects of AG 879 on protein levels of the Hsp 90-client proteins RAF-1 and HER-2

The observation that treatment with AG 879 inhibited activation of ERK-1/2 in serum-stimulated cells suggested that its effect was not restricted to inhibition of a limited number of PTKs, but was of a more global nature. We therefore examined if AG 879 affected the levels of the upstream MAPKKK RAF-1. Immunoblottings revealed that AG 879 induced a marked decrease in RAF-1 protein levels (fig. 3).

RAF-1 is a client protein to the chaperone Hsp90. Geldanamycin and related tyrosine kinase inhibitors have been found to affect the stability of RAF-1 by interacting

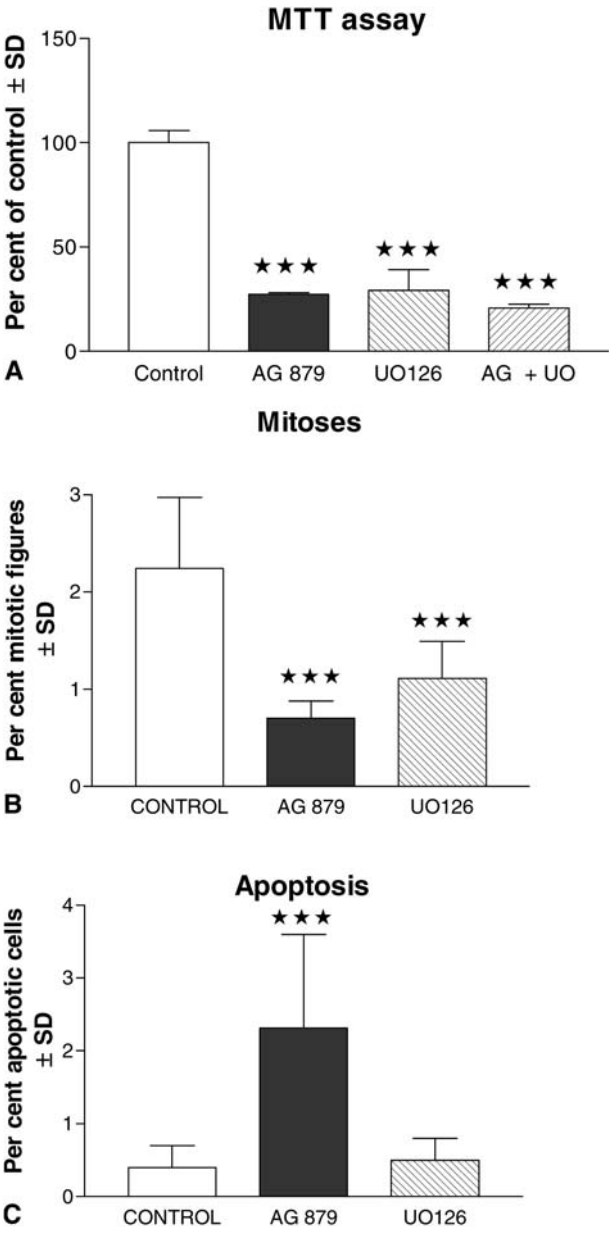

Figure 2. Mechanism of action of AG 879 and comparisons to the effects of the MEK inhibitor UO126. ***$p < 0.001$ compared to controls. (*A*) MTT assay demonstrating that AG 879 and UO126 (both at 20 µM) potently reduce numbers of MCF-7 cells and that combined treatment with the drugs (AG + UO) only produces a minor additional effect. MTT readings were made 46 h after drug addition and are expressed as in figure 1. (*B*) Counting of mitotic figures showed that both AG 879 and UO126 (both at 20 µM) significantly inhibit proliferation of serum-stimulated MCF-7 cells. Mitoses were counted 24 h after addition of drugs or solvent (0.1% DMSO) and the counts are expressed as a percentage of all cells. (*C*) Apoptosis, as analyzed by annexin V binding to living cells, shows that 20 µM AG 879 induces a small, but significant, increase in the percentage of apoptotic cells 24 h after addition of drugs, whereas 20 µM UO126 does not significantly affect the apoptotic index.

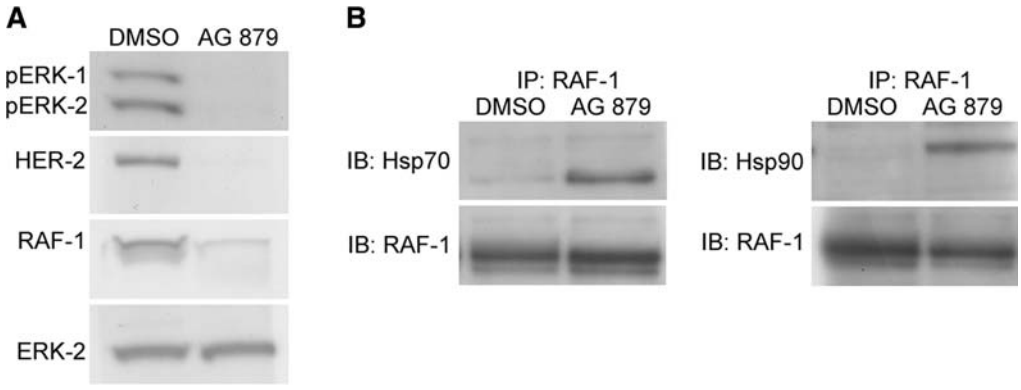

Figure 3. Immunoblotting and immunoprecipitation studies on the effects of AG 879 on MAPK cascade enzymes and their association with heat shock proteins. (*A*) Immunoblotting studies showing that AG 879 decreases phosphorylation/activation of ERK-1 and 2 and decreases protein levels of RAF-1 and HER-2, but not of ERK-2, in MCF-7 cells. Cells were exposed to the drug or solvent (DMSO) for 24 h and similar results were obtained with 5 or 20 µM AG 879. (*B*) Extracts of serum-stimulated MCF-7 cells, exposed to DMSO alone or AG 879 (20 µM) for 24 h, were immunoprecipitated (IP) with RAF-1 antibody and submitted to immunoblotting for Hsp70 or Hsp90. Subsequently, blots were reprobed with a RAF-1 antibody as a loading and recovery control. Note that the RAF-1 levels on the blot reflect the loading of equivalent amounts of RAF-1 onto the gels and that this does not represent a comparison of RAF-1 protein levels between treated and control cells. Treatment with AG 879 markedly increases association between RAF-1 and both heat shock proteins.

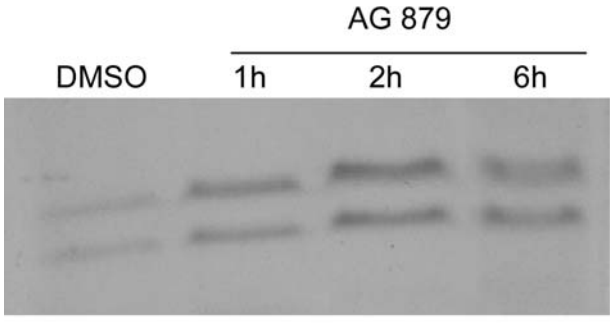

Figure 4. Immunoblots showing early time-course effects of AG 879 on ERK activation. MCF-7 cells treated with 20 µM AG 879 for 1, 2, 4 and 6 h or with vehicle (DMSO) for 6 h. Note that AG 879 induces a transient activation of ERK1/2 and that the levels are still above control levels after 6 h of exposure to the drug.

with Hsp90, thereby targeting RAF-1 to degradation by the proteasome [21, 22]. In addition, a similar mechanism accounts for downmodulation of another Hsp90 client protein, HER-2 [23]. Our immunoblotting studies revealed that AG 879 also downmodulated HER-2 protein levels (fig. 3).

Immunoprecipitations were then used to determine whether, like geldanamycin, AG 879 acted by decreasing the association between Hsp90 and RAF-1. Surprisingly, AG 879 markedly increased the association between RAF-1 and Hsp90 (fig. 3). The increased association was observed both after 6 and 24 h of exposure to the drug. Similarly, treatment with AG 879 for 24 h (6 h was not examined) increased the association between RAF-1 and Hsp70 (fig. 3). Moreover, the proteasome inhibitor MG132 failed to inhibit the AG 879-induced reduction in protein levels of RAF-1 (data not shown). These data show that AG 879 acts differently to drugs inhibiting the association between Hsp90 and its client proteins and that mechanisms other than proteasomal degradation must be responsible for the AG 879-induced decrease in RAF-1 levels.

AG 879 decreases expression of RAF-1 and HER-2, but not of other genes tested

Real-time PCR was employed next to determine whether the inhibitor affected levels of mRNAs encoding RAF-1 and HER-2. This approach demonstrated a marked decrease in levels of both mRNAs after 24 h of exposure to the drug (fig. 5). Even after 6 h of exposure to AG 879, a clearcut decrease in RAF-1 mRNA levels was observed (HER-2 was not studied in this context) (fig. 5). Since AG 879 has been found to decrease electrophile-induced expression of NAD(P)H:quinone oxidoreductase 1 (NQO1) [41], levels of this mRNA were also examined in AG 879-treated MCF-7 cells. However, if anything, NQO1 mRNA levels were marginally increased by AG 879 (fig. 5), showing that this compound does not generally decrease mRNA levels. This result concurs with the observation that AG 879 does not reduce the basal expression of NQO1 [41]. Similarly, AG 879 did not decrease mRNA levels of c-jun or PEA-3 (fig. 5), underlining that the effect was not due to a general depression of mRNA levels. The effect of AG 879 on HER-2 mRNA levels could also be reproduced in HER-2-overexpressing SK-BR-3 cells (fig. 5).

MCF-7 cells were then treated with AG 879 in the presence or absence of the transcriptional inhibitor actinomycin. As expected, actinomycin per se markedly decreased mRNA levels for RAF-1 and HER-2, documenting its efficiency as a transcriptional inhibitor. In the presence of actinomycin, AG 879 did not affect levels of

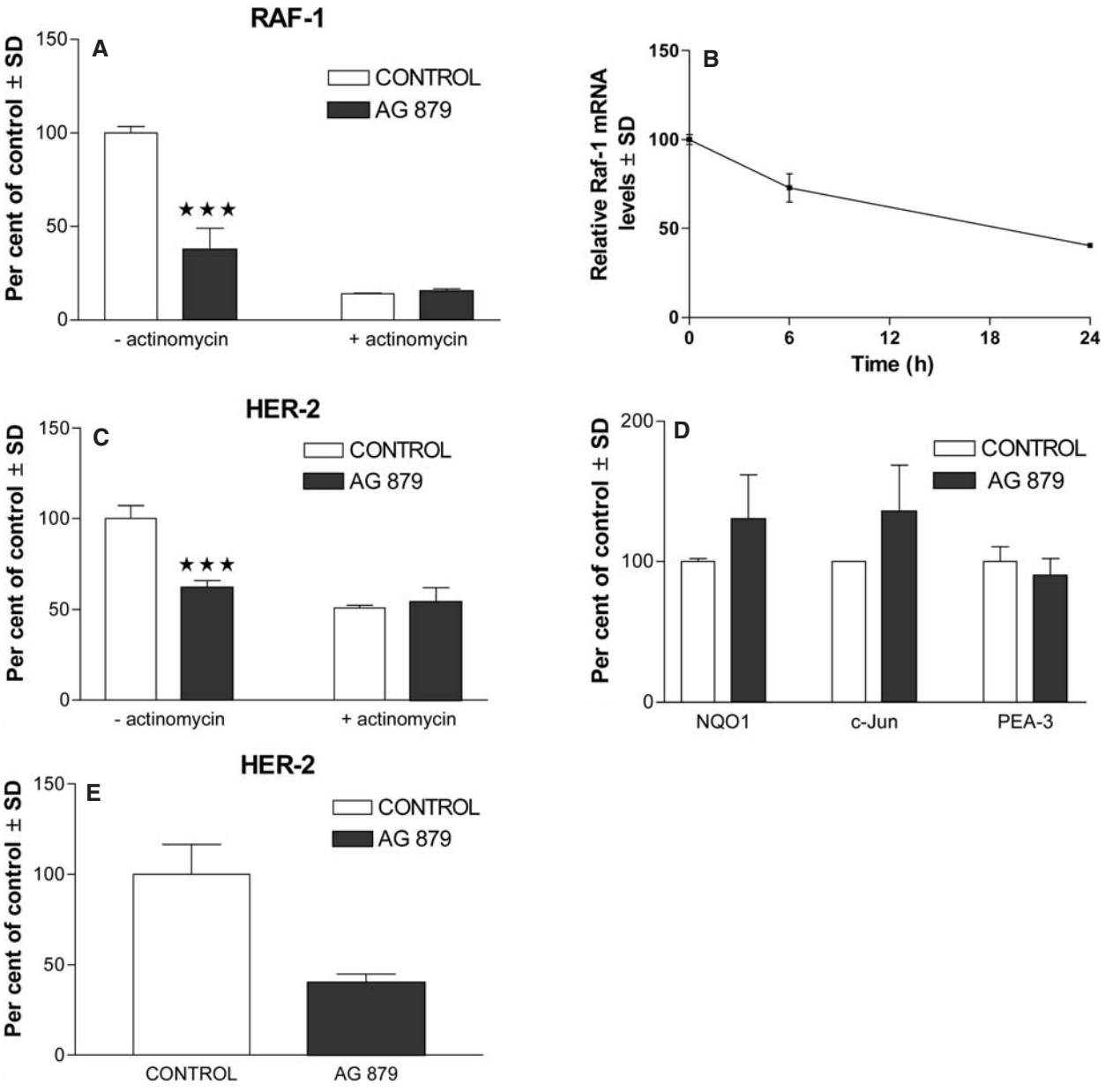

Figure 5. Real-time PCR demonstrating effects of AG 879 on mRNA levels of RAF-1, HER-2, NQO1, c-Jun and PEA-3. ***$p < 0.001$ compared to controls. (*A*) AG 879 (5 µM) significantly reduces levels of RAF-1 mRNA in the absence, but not in the presence of actinomycin, suggesting an effect at the level of transcription. (*B*) Effects of 20 µM AG 879 on levels of RAF-1 mRNA after 6 and 24 h treatment. (*C*) AG 879 (5 µM) significantly reduces levels of HER-2 mRNA in the absence, but not in the presence of actinomycin, suggesting an effect at the level of transcription. (*D*) AG 879 (5 µM) fails to decrease mRNA levels of NQO1, c-Jun or PEA-3. The same RNA preparations from serum-stimulated MCF-7 cells exposed to 5 µM AG 879 or DMSO were used in the experiments depicted in A, C and D. (*E*) AG 879 (20 µM) decreases HER-2 mRNA levels in overexpressing SK-BR-3 cells. Results are expressed relative to mRNA levels in DMSO-treated controls.

either RAF-1 or HER-2 mRNAs (fig. 5). Thus, AG 879 inhibits expression of the RAF-1 and HER-2 genes but does not affect the stabilities of RAF-1 or HER-2 mRNAs.

Discussion

These results show that AG 879 exerts potent antitumoral effects on human breast cancer cells. Since previous data have demonstrated that interference with HER-2 does not noticeably affect survival of cells that, like MCF-7 and MDA-MB-231, do not overexpress this kinase [42], an inhibitory effect of AG 879 on the tyrosine kinase activ-

ity of HER-2 was not expected to be responsible for the effects noted on these cells. This was confirmed by use of another selective HER-2 inhibitor, AG 825, which, in the relevant concentration range, did not affect MCF-7 cell numbers significantly. AG 879 also inhibits the NGF receptor TRK-A. However, the selective TRK inhibitor K252a did not reproduce the effects of AG 879. Instead, AG 879 was found to interfere with activation of the MAPKs ERK-1 and 2. Moreover, inhibition of ERK activation by UO126 to a large extent mimicked the effects of AG 879. Since AG 879 inhibited ERK activation in serum-stimulated cells, this effect was unlikely to be related to inhibition of any specific PTK but, rather, involved a more general action on the MAPK cascade. This was borne out by the observation that AG 879 strongly inhibited protein expression of the upstream MAPKKK RAF-1. Multiple experiments showed that this effect was not due to a geldanamycin-like effect but, instead, reflected decreased levels of mRNA encoding RAF-1. Most probably, the decreased RAF-1 mRNA and protein levels are responsible for the decreased activation of ERK-1 and 2 observed. Interestingly, AG 879 increased the association between RAF-1 and the chaperone Hsp90. The mechanism behind this is unknown but such an association may serve to stabilize RAF-1 and may contribute to the transient increase in ERK activation observed during short-term (1–6 h) treatment with the drug. Importantly, RAF-1 mRNA levels were found to be reduced after 6 h of treatment with the drug while ERK activation was still above control levels. These observations show that decreases in RAF-1 precede decreased ERK activation, suggesting that the effects on RAF-1 are upstream of the effects on ERK activation. Thus, although AG 879 induces an increased RAF-1:Hsp90 association, this effect is not sufficient to prevent a marked decrease in RAF-1 levels and in ERK activation 24 h after exposure to the drug.

We also observed that AG 879 markedly decreased mRNA levels of HER-2 in both MCF-7 cells and in HER-2-overexpressing SK-BR-3 cells. Studies using the transcriptional inhibitor actinomycin directly documented that AG 879 inhibited expression of the RAF-1 and HER-2 genes. AG 879 possibly interferes with the activation or availability of transcription factors that regulate RAF-1 and HER-2 gene expression. Previous studies have documented that AG 879 interferes with electrophile-induced, but not with basal, expression of NQO1 [41]. The studies here confirm that AG 879 does not inhibit basal expression of this gene. Similarly, no inhibition of the expression of two transcription factors, c-jun and PEA-3, could be detected. Thus, the effects of AG 879 are not attributable to a general depression of transcriptional activities. AG 879 is commonly used in doses between 20–100 μM [24, 25, 27, 29, 43–47] for up to 3 days of incubation and several direct observations document an absence of toxic effects under these conditions [25, 43, 45], although in one study [47], reductions in viable cell numbers were observed at doses at or above 50 μM. In the current studies, maximal effects were recorded in the 5–20 μM range.

In conclusion, the data presented here show that AG 879 can inhibit ERK activation via a mechanism involving reduced expression of the upstream kinase RAF-1 and that this effect occurs in cells stimulated with the multitude of growth factors that are present in FCS. This novel action must be taken into consideration when interpreting data using AG 879 as a selective PTK inhibitor. More importantly, however, these data point to a novel and therapeutically useful application of this type of drug, namely the specific transcriptional repression of molecules, which like RAF-1 and HER-2, are important to tumor growth and survival.

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