

# Activated B-RAF Is an Hsp90 Client Protein That Is Targeted by the Anticancer Drug 17-Allylamino-17-Demethoxygeldanamycin

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## Abstract

**Hsp90 is a ubiquitously expressed molecular chaperone that folds, stabilizes, and functionally regulates many cellular proteins. The benzoquinone ansamycin 17-allylamino-17-demethoxygeldanamycin (17-AAG) is an anticancer drug that disrupts Hsp90 binding to its clients, causing their degradation through the ubiquitin-dependent proteasomal pathway. The protein kinase B-RAF is mutated in ~7% of human cancers. The most common mutation (~90%) is <sup>V600E</sup>B-RAF, which has constitutively elevated kinase activity, stimulates cancer cell proliferation, and promotes survival. Here, we show that <sup>V600E</sup>B-RAF is an Hsp90 client protein that requires Hsp90 for its folding and stability. <sup>V600E</sup>BRAF is more sensitive to degradation by 17-AAG treatment than <sup>WT</sup>B-RAF and we show that the majority of the other mutant forms of B-RAF are also sensitive to 17-AAG-mediated proteasomal degradation. Our data show that B-RAF is an important target for 17-AAG in human cancer. (Cancer Res 2005; 65(23): 10686-91)**

## Introduction

The molecular chaperone Hsp90 is part of a multiprotein complex with cochaperones, such as cdc37, p60Hop, and Hsp70 (1). This complex folds, stabilizes, and functionally regulates many client proteins, including many protein kinases that are implicated in cancer (2, 3). Because Hsp90 inhibition induces degradation of its client proteins, it has attracted considerable interest as a therapeutic target for anticancer drugs (2, 3). The most advanced of these is the benzoquinone ansamycin 17-allylamino-17-demethoxygeldanamycin (17-AAG), an agent that has shown promising activity in human tumor xenograft models (4, 5) and is now undergoing phase II clinical trials (6, 7). The combinatorial degradation of several oncogenic proteins seems to be a therapeutic advantage of Hsp90 inhibition (8) but it also masks the identification of specific target clients that could play a key role in particular diseases.

The protein kinase C-RAF is an important Hsp90 client protein (9). C-RAF is a component of the RAS/RAF/MEK [mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinase]/ERK signaling module, a pathway that regulates cell fate, and the activity of which is elevated in ~30% of human cancers (10). The C-RAF-related protein B-RAF is mutated in ~70% of human melanomas and a range of other cancers (11, 12). The most common

B-RAF mutant is a glutamic acid for valine substitution at position 600 (<sup>V600E</sup>B-RAF; previously incorrectly assigned as <sup>V599E</sup>B-RAF; ref. 11), resulting in a kinase with elevated activity that stimulates constitutive signaling, proliferation, and survival, establishing that B-RAF is a human oncogene and novel therapeutic target (10). Over 50 other B-RAF mutations have been described, the majority of which cluster to two regions of the kinase domain, the glycine-rich loop and activation segment (12). Mostly, these also have elevated kinase activity (high or intermediate levels) but a small number have reduced kinase activity or lack kinase activity altogether. The reduced activity mutants still signal to ERK because they activate C-RAF, which then directly activates MEK signaling (13).

Here, we show that 17-AAG stimulates B-RAF degradation in cancer cells and that mutant forms of B-RAF are more sensitive to 17-AAG than the wild-type protein. B-RAF binds to Hsp90 and targeting B-RAF with 17-AAG inhibits signaling in cancer cells. Thus, B-RAF is an Hsp90 client protein and an exciting downstream target of the Hsp90 inhibitor 17-AAG.

## Materials and Methods

**Expression vectors and cell culture techniques.** Cells are routinely cultured in DMEM/10% fetal bovine serum (FBS; A375, CHL, SW620, 501MEL, COLO829, WM266.4, and SK-MEL-2) or RPMI 1640/10% FBS (H508), or 50:50 DMEM/RPMI 1640 10% FBS with 10 mmol/L HEPES and ITS supplement (Sigma, Poole, United Kingdom) at 37°C in 10% CO<sub>2</sub>. COS cells were cultured, transfected, and protein extracts were prepared as described (14); cellular sensitivity to 17AAG was measured by sulforhodamine B dye. The percentage control *A*<sub>540</sub> is plotted against the logarithm of the drug concentration and analyzed by nonlinear regression to a four-variable logistic equation (Graphpad Prism, Graphpad Software, Inc., San Diego, CA). Expression vectors for B-RAF and the mutants have been described (13). RAF kinase activity was measured in a kinase cascade assay with glutathione *S*-transferase (GST)-MEK, GST-ERK, and myelin basic proteins as sequential substrates (13). For coimmunoprecipitation assays, cells were extracted as described (13) and standard approaches were used. The following antibodies were used: mouse monoclonal anti-B-RAF (Santa Cruz Biotechnologies, Calne, United Kingdom), rabbit polyclonal anti-B-RAF (R. Marais), mouse anti-RAF-1 (Signal Transduction Laboratories, Cowley, United Kingdom), mouse monoclonal anti-myc (clone 9E10; The Institute of Cancer Research Hybridoma Unit, London, United Kingdom), rabbit polyclonal anti-p42 ERK2 (C. Marshall, The Institute of Cancer Research), mouse monoclonal anti-ppERK1/2 (Sigma), rabbit polyclonal anti-ppMEK1/2 (New England Biolabs, Hitchin, United Kingdom), and mouse anti-cdc37 (Affinity Biolabs, Exeter, United Kingdom), mouse anti-Hsp70, and anti-Hsp90 (Stressgen, York, United Kingdom). 17-AAG was obtained from Dr. Ed Sauseville (National Cancer Institute, Bethesda, MD).

## Results and Discussion

**Oncogenic B-RAF is hypersensitive to 17-allylamino-17-demethoxygeldanamycin.** To determine whether B-RAF is an

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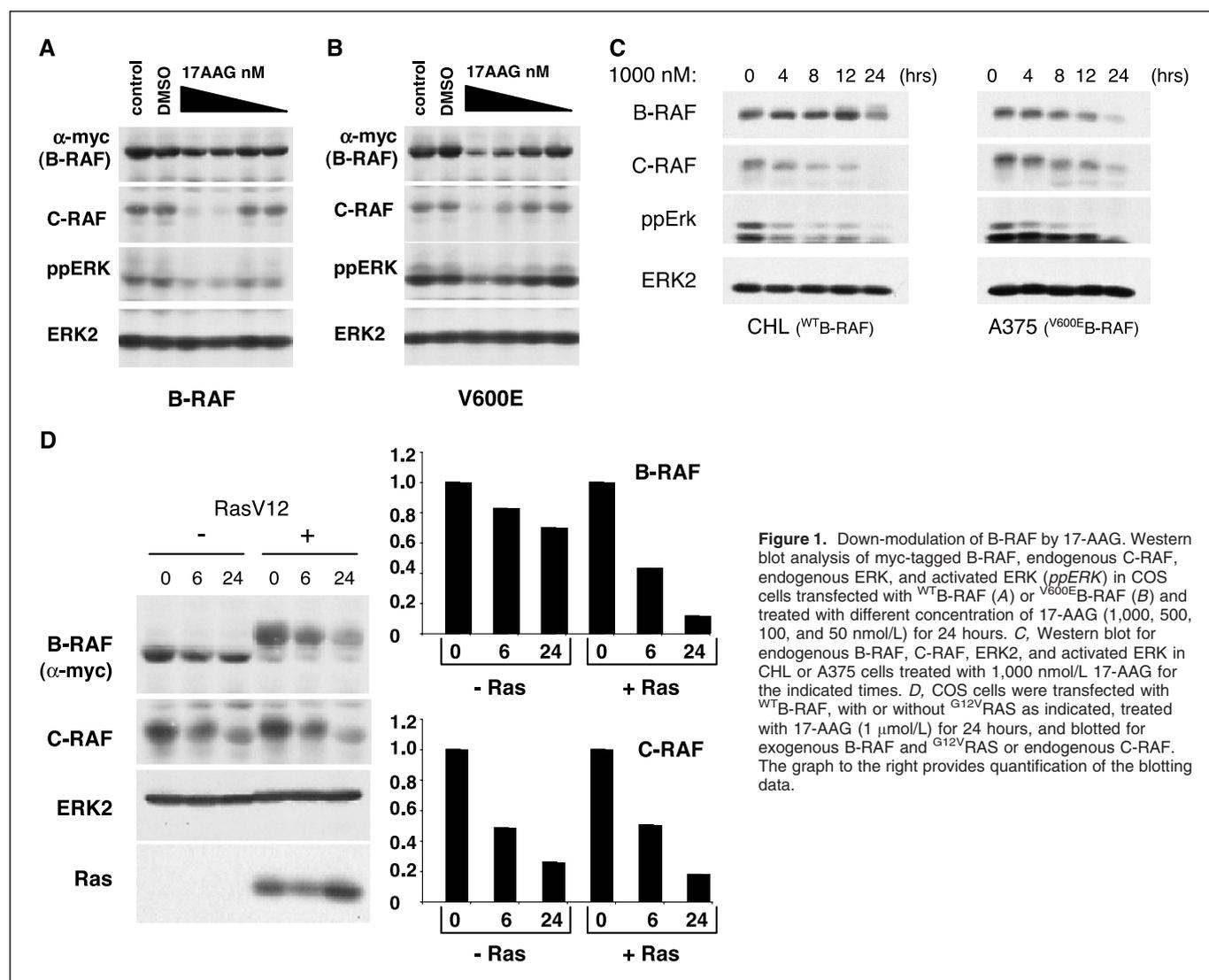
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Hsp90 client protein, we tested its sensitivity to 17-AAG using C-RAF as a control because C-RAF is closely related to B-RAF and is a known Hsp90 client protein. 17-AAG caused a reduction in endogenous C-RAF protein levels in COS cells in a concentration-dependent manner (Fig. 1A). This was a specific effect because there was no reduction in ERK2 protein levels in the same samples. However, as expected, the reduction in C-RAF was accompanied by decreased ERK phosphorylation (Fig. 1A). In contrast to C-RAF, transiently expressed myc-epitope-tagged <sup>WT</sup>B-RAF was relatively stable, with only a small reduction in protein levels even at 1,000 nmol/L 17-AAG (Fig. 1A). Importantly, myc-tagged <sup>V600E</sup>B-RAF was significantly more sensitive to 17-AAG than <sup>WT</sup>B-RAF in these cells, its sensitivity paralleling that of C-RAF (Fig. 1B).

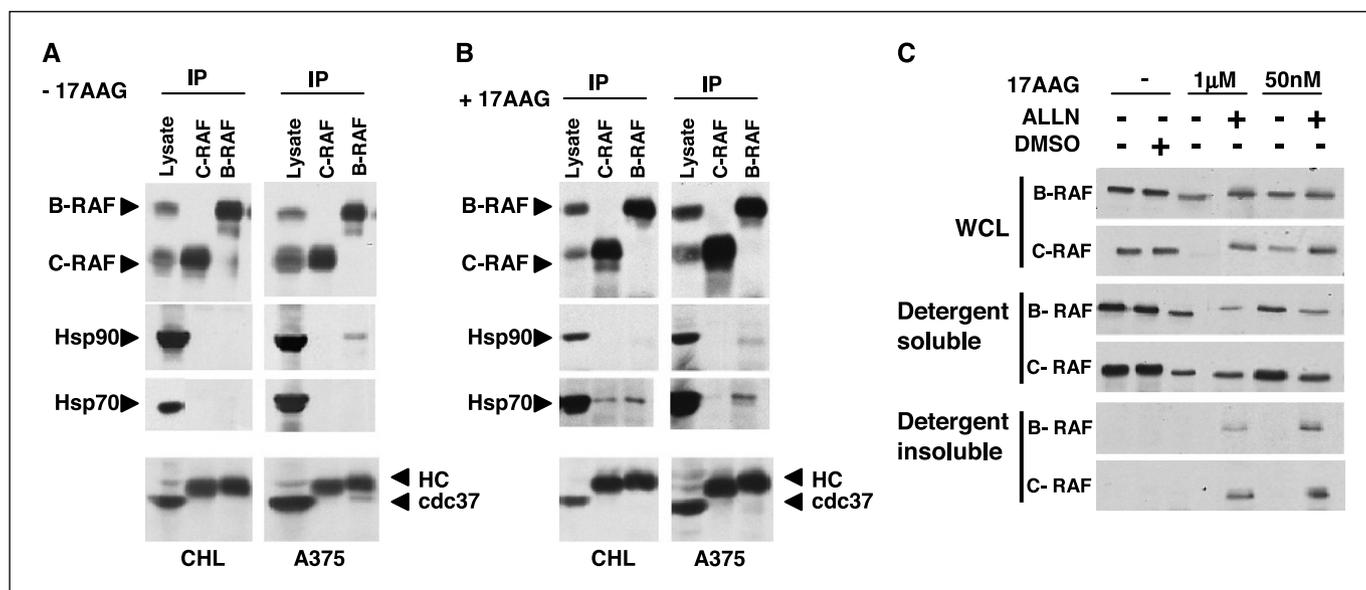
Next, we examined endogenous proteins in melanoma cell lines, using a line in which B-RAF is wild type (CHL cells) and another that carries a <sup>V600E</sup>B-RAF mutation (A375 cells) for comparison (11). 17-AAG caused a time-dependent reduction in C-RAF levels in both cell lines but did not affect ERK2 levels (Fig. 1C), demonstrating that the effect on C-RAF was specific. However, B-RAF is lost more rapidly in A375 cells than in CHL cells (Fig. 1C), suggesting that endogenous <sup>V600E</sup>B-RAF is more

sensitive to 17-AAG than <sup>WT</sup>B-RAF. Importantly, there was a 17-AAG concentration-dependent reduction in ERK activity in both lines (Fig. 1C) and we showed previously that ERK activity is absolutely dependent on <sup>V600E</sup>B-RAF in A375 cells, whereas it seems to primarily depend on C-RAF in CHL cells (15). Thus, 17-AAG can inhibit ERK signaling whether it is downstream of C-RAF or <sup>V600E</sup>B-RAF. These data also suggest that active B-RAF is more sensitive to 17-AAG than inactive B-RAF. In agreement with this, when <sup>WT</sup>B-RAF is activated by coexpression with <sup>G12V</sup>RAS, its protein levels are greatly reduced by 17-AAG treatment (Fig. 1D).

**B-RAF is an Hsp90 client protein.** Our data suggest that B-RAF is an Hsp90 client protein and that oncogenic B-RAF is more dependent on this chaperone for its folding, stability, and/or function than the wild-type protein. We tested whether endogenous B-RAF and Hsp90 associate with each other in intact CHL and A375 cells. We were unable to detect Hsp90 in B-RAF immunoprecipitates from CHL cells but did observe Hsp90 in B-RAF immunoprecipitates from A375 cells (Fig. 2A). Treatment of cells with 17-AAG reduced Hsp90 binding to B-RAF in A375 cells (Fig. 2B). The kinase-specific Hsp90 cochaperone p50<sup>cdc37</sup> was found to also coprecipitate with <sup>V600E</sup>B-RAF in A375 cells but



**Figure 1.** Down-modulation of B-RAF by 17-AAG. Western blot analysis of myc-tagged B-RAF, endogenous C-RAF, endogenous ERK, and activated ERK (*ppERK*) in COS cells transfected with <sup>WT</sup>B-RAF (A) or <sup>V600E</sup>B-RAF (B) and treated with different concentration of 17-AAG (1,000, 500, 100, and 50 nmol/L) for 24 hours. C, Western blot for endogenous B-RAF, C-RAF, ERK2, and activated ERK in CHL or A375 cells treated with 1,000 nmol/L 17-AAG for the indicated times. D, COS cells were transfected with <sup>WT</sup>B-RAF, with or without <sup>G12V</sup>RAS as indicated, treated with 17-AAG (1  $\mu$ mol/L) for 24 hours, and blotted for exogenous B-RAF and <sup>G12V</sup>RAS or endogenous C-RAF. The graph to the right provides quantification of the blotting data.



**Figure 2.** B-RAF is an Hsp90 client protein. *A* and *B*, endogenous B-RAF and C-RAF were immunoprecipitated (IP) from CHL and A375 cells that were untreated (*A*) or treated with 17-AAG (1  $\mu$ M/L, 4 hours; *B*) and the complexes were immunoblotted for B-RAF, C-RAF, Hsp90, Hsp70, and cdc37, which is right below the heavy chain (HC) of the antibody. *C*, WM266.4 cells were untreated or treated with 17-AAG as indicated (24 hours) in the absence or presence of ALLN (100  $\mu$ M/L). Whole cell lysates (WCL) or the separated detergent soluble and insoluble fractions were Western blotted for B-RAF or C-RAF.

this interaction was disrupted following treatment with 17-AAG (Fig. 2*A* and *B*). Surprisingly, we did not observe any binding of C-RAF to Hsp90 in either cell line either in the absence or presence of 17-AAG (Fig. 2*A* and *B*). Thus, it is difficult to conclude that  $^{WT}$ B-RAF is not an Hsp90 client protein. One possibility is that  $^{WT}$ B-RAF forms a very transient complex with Hsp90, making it difficult to detect under these conditions. Alternatively, B-RAF may only form a complex with Hsp90 when activated or mutated and it is only then that it becomes sensitized to 17-AAG. In contrast to Hsp90, Hsp70 did not bind to B-RAF in untreated CHL or A375 cells, but was recruited to  $^{WT}$ B-RAF and  $^{V600E}$ B-RAF following 17-AAG treatment (Fig. 2*A* and *B*).

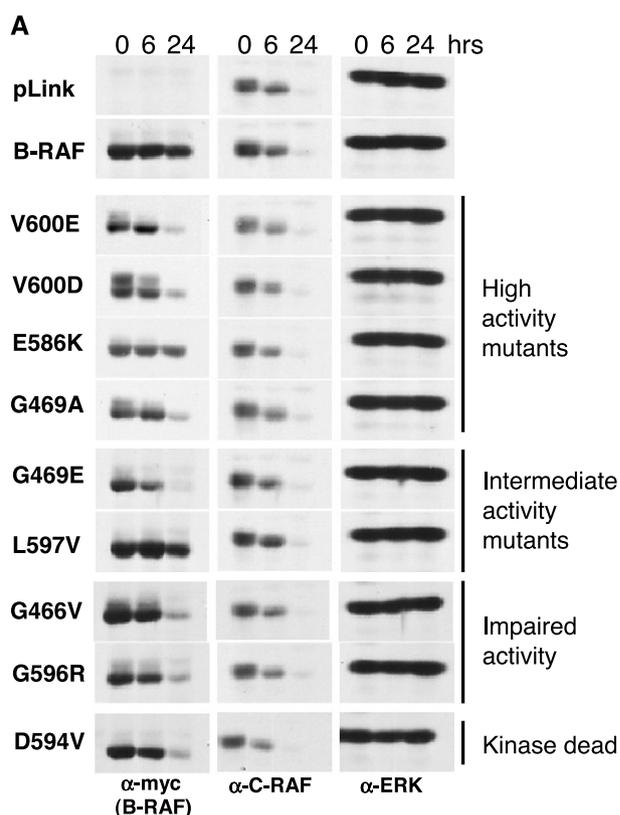
Finally, we tested the role of the proteasome in 17-AAG-mediated B-RAF degradation. The proteasome inhibitor ALLN suppressed 17-AAG-induced B-RAF and C-RAF degradation in WM266.4 cells ( $^{V600D}$ B-RAF; Fig. 2*C*). When lysates from these cells were separated into detergent-soluble and detergent-insoluble fractions, there was a clear increase in RAF isoforms in the insoluble fraction, a hallmark of 17-AAG-mediated proteasomal degradation. Together, these data show that B-RAF is an Hsp90 client protein that is targeted for degradation through the proteasomal pathway by 17-AAG.

**Most, but not all, B-RAF mutants are hypersensitive to 17-allylamino-17-demethoxygeldanamycin.** Next, we tested a selection of other B-RAF mutants present in human cancers. Transiently expressed myc-epitope-tagged versions of  $^{V600D}$ B-RAF and  $^{G469A}$ B-RAF, two high-activity mutants (~700- and 230-fold activated, respectively; ref. 13) are as sensitive to 17-AAG as  $^{V600E}$ B-RAF in COS cells (Fig. 3*A*). Similarly, the intermediate activity mutant  $^{G469E}$ B-RAF, the impaired-activity mutants  $^{G596R}$ B-RAF and  $^{G466V}$ B-RAF, and the kinase-inactive mutant  $^{G594V}$ B-RAF were hypersensitive to 17-AAG (Fig. 3*A*). However, the high-activity mutant  $^{E586K}$ B-RAF (130-fold activated) and the intermediate activity mutant  $^{L597V}$ B-RAF (~65-fold activated) were not hypersensitive to 17-AAG, displaying similar rates of protein depletion as were observed with  $^{WT}$ B-RAF (Fig. 3*A*).

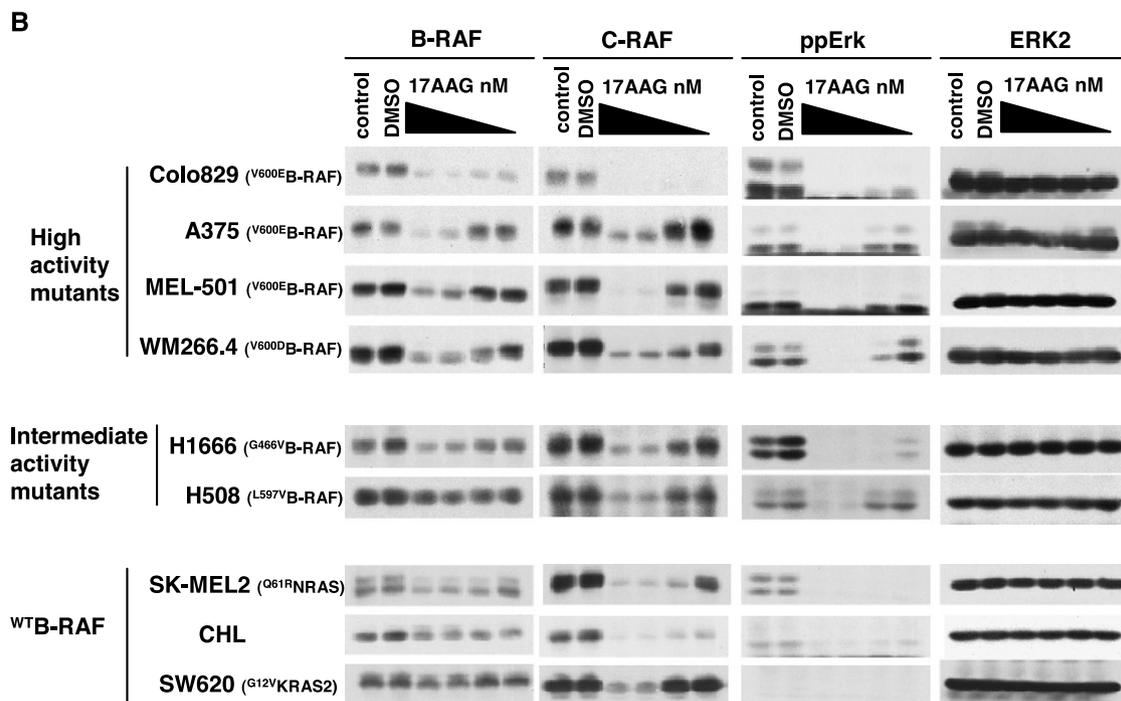
Similar results were observed with endogenous RAF proteins in human cancer cell lines. Endogenous  $^{V600E}$ B-RAF was hypersensitive to 17-AAG in three cancer cell lines (Colo829, A375, and 501MEL), endogenous  $^{V600D}$ B-RAF was hypersensitive in WM266.4 cells, and  $^{G466V}$ B-RAF was hypersensitive in H1666 cells (Fig. 3*B*). In contrast, but in agreement with our COS cell data,  $^{L597V}$ B-RAF was not hypersensitive in H508 cells and  $^{WT}$ B-RAF was not hypersensitive in CHL or SW620 cells (Fig. 3*B*). Surprisingly,  $^{WT}$ B-RAF seemed to be more sensitive to 17-AAG in SK-MEL2 cells than in CHL or SW620 cells. However, whereas  $^{WT}$ B-RAF is not active in SW620 or CHL cells, it is activated in SK-MEL2 cells, which carry a  $^{Q61R}$ N-RAS mutation (Fig. 3*C*). Thus, in agreement with our COS cell data, we conclude that activated endogenous  $^{WT}$ B-RAF is more sensitive to 17-AAG than nonactivated endogenous  $^{WT}$ B-RAF.

Note that the level of kinase activity correlates with the levels of B-RAF expression (Fig. 3*B* and *C*) and is directly affected by 17-AAG treatment. Furthermore, there is a close correlation between B-RAF kinase activity and the reduction in ERK phosphorylation in Colo829, A375, and WM266.4 cells, confirming that oncogenic B-RAF drives ERK signaling in cells.

Next, we tested the sensitivity of different cell lines to Hsp90 inhibition by measuring their proliferation in the presence of 17-AAG. All of the cancer lines that we tested were relatively sensitive to 17-AAG (see ref. 5 for data on a large panel of cell lines) and we observe that the cells that harbor mutant B-RAF are no more sensitive to 17-AAG than cells in which B-RAF is wild type (Table 1). Thus, there is no correlation between B-RAF mutation status and the sensitivity of the cells to 17-AAG. This result may seem counterintuitive at first sight but it is consistent with our observations. We have shown that C-RAF,  $^{V600E}$ B-RAF, and, importantly, activated  $^{WT}$ B-RAF are relatively sensitive to 17-AAG (Fig. 1*A* and *D*). Clearly, in melanoma cells harboring mutations in B-RAF, ERK signaling depends on the mutant protein so cells would be sensitive to 17-AAG. Similarly, in the lines where B-RAF is not mutated, ERK will be activated

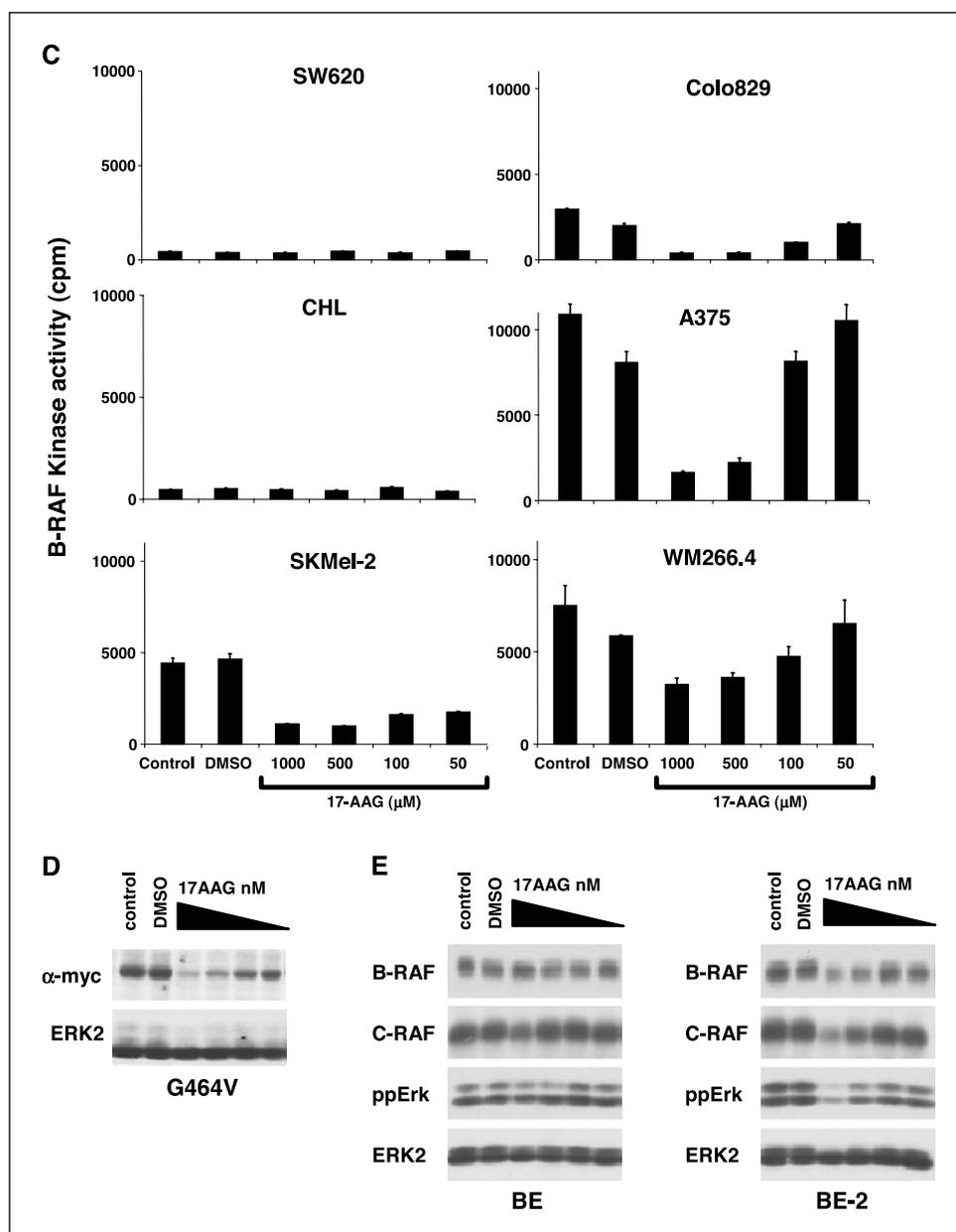


**Figure 3.** Susceptibility of mutant forms of B-RAF to 17-AAG. **A**, COS cells were transiently transfected with vector control or the indicated myc-tagged B-RAF mutant proteins and treated with 17-AAG (1 μmol/L) for the indicated times and blotted for myc-tagged B-RAF, endogenous C-RAF, and total ERK2 (as a loading control). **B**, WM266.4, Colo829, A375, Mel501, SK-Mel2, CHL, SW620, H1666, and H508 cells were treated with various concentrations of 17-AAG as in Fig. 1A and Western blotted for endogenous B-RAF, C-RAF, ERK2, and activated ERK.



downstream of either C-RAF or activated <sup>WT</sup>B-RAF and both of these are sensitive to 17-AAG. These studies show that 17-AAG is a versatile drug that will inhibit ERK signaling in the majority of cell lines because it targets C-RAF, active B-RAF, and most of the mutant forms of B-RAF.

It is intriguing that B-RAF is almost completely lost in many of the 17-AAG-treated lines because <sup>WT</sup>B-RAF expressed from the nonmutated allele would be expected to be relatively resistant to 17-AAG. We attribute this to the fact that in many samples from cancer patients, the mutant *B-RAF* allele is amplified relative to its



**Figure 3** Continued. *C*, B-RAF kinase activity from CHL, SW620, SKMEL-2, WM266.4, A375, and Colo829 cells following 17-AAG treatment at the indicated concentrations for 24 hours. *D*, COS cells expressing myc-tagged  $G^{464V}$ B-RAF were treated with 17-AAG as in Fig. 1A and blotted for myc-tagged B-RAF and endogenous ERK2. *E*, BE cells or BE cells expressing NQO1/DT-diaphorase (BE-2) were treated with various concentrations of 17-AAG as in Fig. 1A and Western blotted for endogenous B-RAF, C-RAF, ppERK, and ERK2 (loading control).

wild-type counterpart (16). Indeed, in many of the lines we tested, the mutant allele constitutes the major variant in sequencing electroferograms (data not shown), indicating that mutant allele amplification has occurred.

We have shown that  $V^{600E}$ B-RAF, the most clinically relevant mutant, is hypersensitive to 17-AAG and that six of the other eight mutants we tested are also hypersensitive to this drug. Thus, mutant B-RAF from the majority of human cancer patients will be sensitive to 17-AAG. Intriguingly, there does not seem to be a correlation to the position of the mutation and sensitivity to 17-AAG. Mutants involving glycine-rich loop residues (G466V, G469A, and G469E); the activation segment residues (D594V, G596R, V600E, and V600D); and mutants with high (G469A, V600E, and V600D), intermediate (G466V), and reduced (G469E and G596R) kinase activity are all hypersensitive. Structural studies have revealed that B-RAF is held in an inactive conformation by an unusual interaction between the glycine-rich loop and the activation segment

(13). The mutations that occur in these regions in cancer are thought to disrupt this interaction, destabilizing the inactive conformation and allowing the active conformation to predominate (13).

Our studies suggest that it is the adoption of the active conformation rather than the level of activity that determines 17-AAG sensitivity. We note that the kinase-inactive mutant  $D^{594V}$ B-RAF is hypersensitive to 17-AAG, demonstrating that kinase activity is not required for sensitivity. However, at least two of the mutants,  $L^{597V}$ B-RAF and  $E^{586K}$ B-RAF, were not hypersensitive despite the fact that one (L597V) is in the activation segment and has intermediate activity and the other (E586K) is an activation loop mutant with high activity. It is unclear why these mutants lack sensitivity. They may adopt a unique active conformation that is insensitive to 17-AAG or they may fold into the active form in a manner that is independent of Hsp90. Importantly, these mutations could provide a mechanism whereby B-RAF could become resistant to 17-AAG in the clinic and they are being further investigated.

**Table 1.** Cellular sensitivity to 17-AAG treatment

Cell line	IC <sub>50</sub> (nmol/L 17-AAG)	B-RAF status
Sk-Mel-2	8.7	WT
SW620	17.0	WT
CHL	18.2	WT
Colo829	15.4	V600E
501Mel	18.5	V600E
A375	20.0	V600E
WM266.4	19.0	V600D
NC-H1666	2.9	G466V

NOTE: Cell proliferation was measured by sulforhodamine B staining in the presence of a range of 17-AAG concentrations.

Another route to 17-AAG resistance for B-RAF could be intrinsic to the cancer cells. We find that <sup>G464V</sup>B-RAF is sensitive to 17-AAG when exogenously expressed in COS cells (Fig. 3D), whereas endogenous <sup>G464V</sup>B-RAF is not sensitive to 17-AAG in BE cells (Fig. 3E). However, BE cells have a destabilizing point mutations in DT-diaphorase/DT-diaphorase and so lack this enzyme activity (17). Consequently, their ability to convert 17-AAG to the active metabolite is reduced and consequently their client proteins are relatively resistant to 17-AAG-mediated degradation (5). However, when BE cells are engineered to express functioning NQO1/DT-diaphorase (BE-2 cells; ref. 17), <sup>G464V</sup>B-RAF becomes more sensitive

to 17-AAG. These data confirm that <sup>G464V</sup>B-RAF is an Hsp90 client protein and suggest that the loss of NQO1/DT-diaphorase could result in the development of clinical resistance.

In summary, this study shows that B-RAF is an Hsp90 client protein. Our data show that active B-RAF and most of the mutant forms of B-RAF from human cancer are more sensitive to the anticancer drug 17-AAG than inactive <sup>WT</sup>B-RAF. The sensitivity of the mutants seems to be determined by their conformation rather than activation status and these findings clearly have therapeutic implications in melanoma and other cancers in which this pathway plays a role. In particular, our results suggest that melanoma cells will be sensitive to 17-AAG whether they rely on B-RAF or C-RAF for signaling to ERK. Of note are the findings that melanoma cells display greater than average sensitivity to 17-AAG than most other cells in the National Cancer Institute 60 human tumor cell panel and human melanoma xenografts respond to 17-AAG (18). Finally, prolonged stable disease has been reported in two patients with advanced metastatic malignant melanoma in a phase I clinical trial of weekly administered 17-AAG (19), demonstrating the potential of this anticancer agent.

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