Heat Shock Protein 90 Inhibitor 17-Dimethylaminoethylamino-17-Demethoxygeldanamycin Enhances EphA2+ Tumor Cell Recognition by Specific CD8+ T Cells

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Abstract

EphA2, a member of the receptor tyrosine kinase family, is commonly expressed by a broad range of cancer types, where its level of (over)expression correlates with poor clinical outcome. Because tumor cell expressed EphA2 is a non-mutated “self” protein, specific CD8+ T cells are subject to self-tolerance mechanisms and typically exhibit only moderate-to-low functional avidity, rendering them marginally competent to recognize EphA2+ tumor cells in vitro or in vivo. We have recently reported that the ability of specific CD8+ T cells to recognize EphA2+ tumor cells can be augmented after the cancer cells are pretreated with EphA2 agonists that promote proteasomal degradation and up-regulated expression of EphA2/class I complexes on the tumor cell membrane. In the current study, we show that treatment of EphA2+ tumor cells with the irreversible heat shock protein 90 inhibitor, 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG), similarly enhances their recognition by EphA2-specific CD8+ T-cell lines and clones in vitro via a mechanism that is dependent on proteasome and transporter-associated protein function as well as the retrotranslocation of EphA2 into the tumor cytoplasm. When 17-DMAG and agonist anti-EphA2 monoclonal antibodies are coapplied, T-cell recognition of tumor cells is further increased over that observed for either agent alone. These studies suggest that EphA2 represents a novel heat shock protein 90 client protein and that the treatment of cancer patients with 17-DMAG–based “pulse” therapy may improve the antitumor efficacy of CD8+ T effector cells reactive against EphA2-derived epitopes. [Cancer Res 2009;69(17)6995–7003]

Introduction

EphA2, a member of the receptor tyrosine kinase family of molecules, is a 130-kDa (type I) glycoprotein that mediates intercellular interactions via binding to its ligands ephrin A1, A3, A4, and A5 expressed on an opposing cell surface (1). This receptor tyrosine kinase is expressed at low levels on a broad range of epithelial tissues in normal adults, including lung, spleen, kidney, and liver (2), where it is primarily localized to sites of cell-to-cell contact and plays a role in contact inhibition of cell growth/migration that is critical for the organization and formation of epithelial layers in EphA2+ tissues (3). In addition to epithelial cells, activated endothelial cells also express EphA2 in association with tissue neovascularization in adults (4).

In contrast to nontransformed cells, EphA2 is commonly overexpressed in a range of cancer types, including melanoma and many carcinomas (5–12), where it serves as an oncoprotein and a facilitator of metastasis (3, 13). Clinical observations suggest that the level of EphA2 overexpression by tumor cells is an indicator of poor prognosis, because it has been linked to reduced time to disease recurrence and enhanced disease progression and metastatic spread (7, 9, 14, 15).

As a consequence, EphA2 represents an attractive target for therapeutic intervention in the majority of patients with solid tumors, with several treatment strategies considered for translation into the clinic. One strategy involves the implementation of agents [agonist monoclonal antibody (mAb) or recombinant ligands] that promote the proteasome-mediated degradation of EphA2 protein, thereby limiting its oncogenic function (16, 17). We have recently determined that such reagents also promote a corollary enhancement in tumor cell presentation of EphA2 peptides in tumor cell MHC class I complexes, thereby facilitating tumor cell recognition and eradication by low-to-modest avidity CD8+ T cells (18). Because EphA2-specific CD8+ T cells have been detected in the peripheral blood of patients with renal cell carcinoma (RCC; ref. 8), prostate carcinoma (19), or glioma (20), and the frequencies of these protective T cells would be anticipated to be augmented as a consequence of active vaccination (2, 21), combinational therapies that sensitize EphA2+ tumors for specific CD8+ T-cell eradication may yield enhanced clinical benefits in the cancer setting (22).

Interestingly, many receptor tyrosine kinase serve as client proteins for the molecular chaperone heat shock protein 90 (HSP90), a protein designed to stabilize and refold denatured proteins into their native conformations to preserve their function and utility in normal and stressed cells (23). HSP90 is commonly overexpressed in tumor cells, where it is believed to protect client oncogenic/survival proteins that support tumor progression and metastasis, in part, by preventing their proteasome-dependent destruction (24). Our current report suggests that EphA2 represents a previously unknown HSP90 client protein. Furthermore, treatment of tumor cells with 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG), a well-tolerated clinical inhibitor of HSP90 (24, 25), results in the proteasome-dependent degradation of tumor EphA2 and in augmented tumor cell recognition by anti-EphA2 CD8+ T cells in vitro.  

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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**Materials and Methods**

**Cell lines and media.** SLR20 (EphA2+, HLA-A2+; ref. 8), SLR22 (EphA2+, HLA-A2+; ref. 8), and SKOV3 (EphA2+, HLA-A2+; kindly provided by Dr. Nora Disis, University of Washington) as well as the SLR20.A2 (EphA2+, HLA-A2+) and SKOV3.A2 (EphA2+, HLA-A2+) cell lines (established via transduction of the corresponding parental cell lines with a recombinant retrovirus encoding HLA-A2.1 provided by Dr. Peter Cresswell, Yale University; ref. 19) and EphA258-66 (IMNDMPIYM; ref. 8), EphA259-72 (GFEKAVFII; ref. 8), and EphA2883-891 (TLADFDPRV; ref. 8) were synthesized using 9-fluorenylmethoxycarbonyl chemistry by University of Pittsburgh Cancer Institute Peptide Synthesis Facility as described previously (18). The purity of peptides was >96% based on high-performance liquid chromatography, with peptide identity validated by tandem mass spectrometric analyses of peptides was >96% based on high-performance liquid chromatography, with peptide identity validated by tandem mass spectrometric analyses done by the University of Pittsburgh Cancer Institute Protein Sequencing Facility (a shared resource). The ICP471-35 and ICP4735-1 synthetic peptides (26) were kindly provided by Dr. Peter Cresswell.

**Western blot.** RCC cell lines at 80% to 90% confluency were incubated with 17-DMAG (10-1,000 nmol/L) in 2% human serum-supplemented RPMI 1640 for 24 to 48 h. To assess the effect of proteasome function, endosomal acidification, and retrotranslocation in EphA2 protein degradation promoted by the HSP90 inhibitor, MG-132 (5-10 μmol/L; Sigma-Aldrich), chloroquine (30-100 μmol/L; Sigma-Aldrich), and Pseudomonas aeruginosa exotoxin A (ExoA; 10-50 μg/mL; Sigma-Aldrich), respectively, were added at the initiation of 24 h tumor cell cultures as indicated in individual experiments. Harvested cells were then incubated with lysis buffer (1% Triton X-100, 150 mmol/L NaCl, 10 mmol/L Tris-HCl (pH 7.4), 1 mmol/L EDTA, 0.2 mmol/L sodium orthovanadate, 0.5% NP-40 in PBS; all reagents from Sigma-Aldrich) containing a protease inhibitor cocktail (Complete mini; Roche Diagnostic) for 30 min at 4°C. Lysates were cleared by centrifugation at 13,500 × g for 10 min, and proteins in the lysate were resolved by SDSPAGE before electrophoretic analysis. Anti-EphA2 antibody and horseradish peroxidase–conjugated goat anti-rabbit antibody (both from Santa Cruz Biotechnology) were used to detect EphA2. mAbs against transporter-activated protein (TAP)–1 and TAP-2 (NOB-1 and NOB-2, respectively, were kindly provided by Dr. Soldano Ferrone, University of Pittsburgh), with horseradish peroxidase–conjugated goat anti-mouse IgG (Santa Cruz Biotechnology) used to probe blots. Probed proteins were visualized by Western Lighting chemiluminescence detection kit (PerkinElmer) and exposed to X-Omat film (Eastman Kodak) for 1 to 5 min.

**Flow cytometry.** Control or treated RCC cells were harvested with trypsin-EDTA (Invitrogen), washed, and then incubated with anti-EphA2 mAb (BD6; Upstate Biologicals) or anti-pan class I mAb (W6/32; Serotec) for 30 min at 4°C. After washing with PBS/0.02% bovine serum albumin/0.02% NaN₃, cells were stained with FITC-conjugated anti-mouse IgG (MP

**Figure 1.** HSP90 inhibitor 17-DMAG promotes the loss of tumor EphA2 protein (via degradation) in a dose-, time-, and proteasome-dependent manner. A, EphA2+ SLR20 RCC line was incubated in the absence or presence of 17-DMAG (10-1,000 nmol/L) for 24 or 48 h at 37°C before generation of cell lysates and Western blot analysis to determine levels of EphA2 protein expression. β-Actin was monitored as an internal control protein. B, SLR20 cells were treated as above, with cell surface expression of EphA2 protein monitored by flow cytometry. Differences in tumor cell mean fluorescence intensity (MFI) expression of EphA2 were significant for 17-DMAG-treated versus control untreated tumor cells evaluated in flow cytometry–based assays. P = 0.008 at 24 h for 500 nmol/L 17-DMAG treated (MFI = 28 ± 13) versus untreated (MFI = 60 ± 12). C, SLR20 cells were treated with 500 nmol/L 17-DMAG in the absence or presence of MG-132 (5-10 μmol/L) or chloroquine (30-100 μmol/L) before Western blot analysis to analyze the dependence of EphA2 (versus control β-actin) protein loss on proteasome function or endosomal acidification, respectively. Representative of four independent experiments.
Biologically (for 30 min at 4°C). Cells were washed twice and then analyzed using a LSRII flow cytometer (BD Biosciences). Isotype control mAb was mlgG1 (Sigma-Aldrich).

**Proteasome function analysis.** SLR20 cells were transfected with the proteasome sensor vector (PSV; BD Biosciences) using Lipofectamine 2000 (Invitrogen) and selected in cultures containing G418 (Invitrogen), thus generating SLR20.PSV cells. PSV expresses a fluorescent substrate for the proteasome (26), which accumulates in the cytoplasm of cells if proteasome function is inhibited. SLR20.PSV cells were grown to 80% to 90% confluency before being cultured in the absence or presence of 17-DMAG or the proteasome inhibitors MG-132 (Sigma-Aldrich) or PS-341 (bortezomib; kindly provided by Dr. Ram Ganapathi, Cleveland Clinic Foundation) at the indicated concentrations for 24 h at 37°C and 5% CO₂ tension. Fluorescence was detected in the FITC bandwidth (488 nm) by flow cytometry.

**T-cell lines and clones.** Bulk CD8⁺ T-cell lines and clones specific for EphA258-66 or EphA2883-891 were generated as described previously (18). Briefly, mature dendritic cells were developed from peripheral blood mononuclear cells (obtained with written consent under an institutional review board–approved protocol) isolated from normal HLA-A2+ donors in mononuclear cells (obtained with written consent under an institutional review board–approved protocol) isolated from normal HLA-A2+ donors in

**Results**

**HSP90 inhibitor 17-DMAG induces EphA2 degradation that may be blocked by inhibitors of proteasome function but not endosomal acidification.** The EphA2 (over)expressing RCC cell line SLR20 was incubated in the absence or presence of 17-DMAG (0-1000 nmol/L) for 24 to 48 h. The resultant cells were then analyzed for EphA2 protein levels by Western blotting (total protein; Fig. 1A) and flow cytometry (cell surface protein; Fig. 1B). In both cases, tumor EphA2 levels were reduced at both 24 and 48 h post-treatment with 17-DMAG treatment (IC₅₀ ~ 250 nmol/L), although the pool of EphA2 protein most sensitive to 17-DMAG effects may be intracellular given the somewhat greater degree of reduction noted in the Western blotting–based versus flow cytometry–based assays. Inclusion of the proteasome inhibitor MG-132 blocked the ability of 17-DMAG to promote EphA2 protein
loss (Fig. 1C), suggesting that HSP90 effects on EphA2 are at least partially proteasome-dependent. In contrast, inclusion of chloroquine, which disrupts endosomal acidification (28), failed to affect 17-DMAG–induced degradation of EphA2 protein (Fig. 1C). Single and combinational (17-DMAG + inhibitors) drug treatments did not lead to tumor cell death based on retention of control h-actin signal in the Western blot experiments and appropriate forward/side scatter gating profiles in the flow cytometry assays (Fig. 1; data not shown).

Treatment of tumor cells with HSP90 inhibitor does not significantly alter major components of the tumor MHC class I antigen processing machinery. Because our ultimate goal was to discern potential augmentation in immune recognition of EphA2+ tumor cells after treatment with 17-DMAG, we next assessed the effect of drug treatment on the expression and/or function of components of tumor cell MHC class I antigen processing machinery (APM). Proteasome activity was analyzed using a model employing SLR20 cells transfected with a PSV (SLR20.PSV cells), in which intracellular fluorescent protein accumulates when proteasome function is inhibited. As depicted in Supplementary Fig. S1A, the proteasome inhibitors MG-132 and PS-341 both increased the fluorescence of SLR20.PSV cells, whereas 17-DMAG had minimal effect even after 48 h at concentrations in excess of that required to promote EphA2 degradation (as shown in Fig. 1). TAP levels (both TAP-1 and TAP2) were also shown to be unaffected by 17-DMAG treatment (Supplementary Fig. S1B). Finally, tumor cell surface MHC class I levels were shown to be unaltered by 17-DMAG as assessed by flow cytometry using a pan-class I–reactive mAb (Supplementary Fig. S1C). These results suggest that 17-DMAG does not have a deleterious effect on tumor cell MHC class I/peptide complex generation and corollary expression of such complexes on the tumor cell surface.

17-DMAG treatment of tumor cells transiently enhances tumor recognition by bulk CD8+ T cells specific for EphA2. Bulk CD8+ T-cell lines and clones reactive against EphA2 were generated from normal HLA-A2+ donors using an in vitro stimulation protocol employing autologous dendritic cells pulsed either EphA258-66 or EphA2883-891 (HLA-A2–presented) peptide epitopes.

Figure 3. 17-DMAG promotes enhanced recognition of the EphA2+, HLA-A2+ ovarian carcinoma cell line SKOV3.A2 in vitro. A, SKOV3.A2 tumor cells were analyzed for expression of cell surface HLA-A2 and EphA2 proteins by flow cytometry using specific mAbs (open histograms) versus isotype control mAbs (filled histograms). To assess the effect of HSP90 inhibition, SKOV3.A2 tumor cells were untreated or treated with 17-DMAG (10–1,000 nmol/L) for 24 h and analyzed for EphA2 expression by flow cytometry (B) and Western blotting (C) as well as for their ability to be recognized by bulk anti-EphA258-66 T cells (D). In the flow cytometry analyses, P = 0.001 for 500 nmol/L 17-DMAG treated (MFI = 74 ± 16) versus untreated (MFI = 190 ± 18). In T-cell assays, cell-free supernatants were harvested after a 24 h coculture period (T cells (10⁵)+tumor cells (10⁴) ± 500 nmol/L 17-DMAG pretreatment) and analyzed for IFN-γ content by specific ELISA. EphA258-66 peptide-pulsed control SKOV3.A2 cells were used to establish the “maximal” level of T cell reactivity. E, SKOV3 or SKOV3.A2 tumor cells were untreated or treated with 500 nmol/L 17-DMAG ± MG-132 (10 μmol/L) or chloroquine (100 μmol/L) or ExoA (50 μg/mL) for 24 h before being used as targets for bulk anti-EphA258-66 CD8+ T cells (10⁶) responses monitored using IFN-γ ELISA. D and E, light gray columns, SKOV3; black columns, SKOV3.A2. All ELISA data are mean ± SD of triplicate assay determinations. Representative of one of three experiments. *, P < 0.05 for all indicated comparisons.
as stimulator cells. After two or three rounds of identical restimulation, responder CD8+ T cells were assessed for recognition of RCC cell lines (control or 17-DMAG pretreated) in IFN-γ ELISPOT assays. As shown in Fig. 2, bulk peptide-primed CD8+ T cells recognized EphA2+, HLA-A2+ RCC lines [SLR22 (Fig. 2A) and SLR20.A2 (Fig. 2B; Supplementary Fig. S2)] in a manner that was class I–restricted (inhibited by W6/32 mAb; Fig. 2A). These same T-cell lines reacted poorly against the EphA2+ but HLA-A2− SLR20 cell line (Fig. 2B). When SLR20.A2 was pretreated with 17-DMAG for 24 h before coculture with bulk (and cloned) T cells reactive against either of the EphA2 peptides, tumor cell recognition by T cells was significantly enhanced (P < 0.05; Fig. 2B; Supplementary Fig. S2B). However, this enhanced immune recognition was transient in nature, because tumor cells pretreated for 48 h with 17-DMAG were recognized to a degree that was comparable with untreated tumor cells (Fig. 2B).

To determine whether 17-DMAG could sensitize tumor cells of an alternate lineage to anti-EphA2 CD8+ T-cell recognition, we untreated tumor cells (Fig. 2B). 17-DMAG promotes enhanced recognition by CD8+ T cells capable of recognizing peptides derived from both extracellular (EphA258-66) and intracellular (EphA2883-891) domains of this receptor tyrosine kinase, this suggests that both domains of this transmembrane protein must become accessible for proteasomal processing into peptides that are then conveyed via TAP into the endoplasmic reticulum for loading into nascent HLA-A2 complexes in tumor cells. Current paradigms (29–31) suggest that cytosolic access for the extracellular domains of transmembrane proteins may be accomplished through a retrotranslocation process involving sec61-dependent “ratcheting” of the target protein into the cytoplasm where it may become a substrate for the proteasome. To test this hypothesis, we added the sec61 inhibitor ExoA (27) to tumor cells with inclusion of mAb W6/32 during the coculture period (Fig. 3D). Because the ability of 17-DMAG to promote EphA2 degradation in tumor cells was inhibited by MG-132 but not by chloroquine, we next evaluated the effect of these agents on anti-EphA2 CD8+ T-cell recognition of HSP90 inhibitor–treated tumor cells. As shown in Fig. 3E, enhanced T-cell recognition of 17-DMAG–treated SKOV3.A2 cells was effectively ablated by the proteasome inhibitor but not by the lysosomotropic drug (chloroquine). Notably, MG-132 also reduced basal recognition of SKOV3.A2 cells by anti-EphA2 T cells (Fig. 3E), supporting that the normal loading of tumor cell class I complexes with EphA2 peptides occurs via a proteasome-dependent pathway. 17-DMAG treatment of tumor cells enhances tumor recognition by a low-avidity CD8+ T-cell clone specific for EphA2 via a retrotranslocation- and TAP-dependent mechanism. Because 17-DMAG promotes enhanced recognition by CD8+ T cells capable of recognizing peptides derived from both extracellular (EphA258-66) and intracellular (EphA2883-891) domains of this receptor tyrosine kinase, this suggests that both domains of this transmembrane protein must become accessible for proteasomal processing into peptides that are then conveyed via TAP into the endoplasmic reticulum for loading in nascent HLA-A2 complexes in tumor cells. Current paradigms (29–31) suggest that cytosolic access for the extracellular domains of transmembrane proteins may be accomplished through a retrotranslocation process involving sec61-dependent “ratcheting” of the target protein into the cytoplasm where it may become a substrate for the proteasome. To test this hypothesis, we added the sec61 inhibitor ExoA (27) to tumor cells
during culture with 17-DMAG before their analysis in Western blotting and T-cell assays. As shown in Fig. 4A, ExoA inhibits 17-DMAG–induced degradation of tumor cell EphA2 protein. Notably, ExoA also prevents enhanced recognition of SLR20.A2 tumor cells by anti-EphA2 (clone 15/9) CD8+ T cells after 24 h treatment with 17-DMAG (Fig. 4B and C) but does not negatively affect the recognition of EphA2 peptide-pulsed tumor cells (Fig. 4B). Similar results were obtained using a bulk anti-EphA258-66 CD8+ T-cell line and the SKOV3.A2 tumor cell line (Fig. 3E).

The dependence of the anti-EphA2 T-cell sensitizing effects of 17-DMAG on tumor cell TAP function was next addressed. We observed that cotreatment of tumor cells with 17-DMAG and a NH2-terminal fragment of the ICP47 protein (ICP471-35), a (Herpes simplex) viral inhibitor of TAP (27), ablated enhanced T-cell recognition when compared with tumor cells treated with 17-DMAG alone (P < 0.05). This effect was specific, as inclusion of a scrambled peptide ICP35-1 (bearing the reverse AA sequence of ICP471-35) exhibited no such inhibitory effect (Fig. 4C).

Combined treatment of EphA2+, HLA-A2+ tumor cells with both 17-DMAG and agonist anti-EphA2 mAb208 results in superior recognition by a low-avidity CD8+ T cells specific for EphA2. As we have reported previously that EphA2 agonist mAb208 promotes the proteasomal destruction of tumor EphA2 protein and enhances specific CD8+ T-cell recognition of treated tumor cells (18), we next investigated whether the combined use of both 17-DMAG and mAb208 would result in an even greater degree of tumor cell recognition by specific T cells when compared with either treatment modality alone. SLR20.A2 cells were cultured for 24 h in the absence or presence of 17-DMAG, mAb208 or control IgG, with IFN-γ ELISPOT assays subsequently done using the 15/9 CD8+ T-cell clone and a bulk anti-EphA2883-891 T cells, respectively, in IFN-γ ELISPOT assays.

* P < 0.05 for all indicated comparisons. Representative of one of three independent experiments.
either of the EphA2 epitopes) after SLR20.A2 cells are pretreated with both 17-DMAG and mAb208 versus either reagent alone (P < 0.05; Fig. 5C and D).

**Discussion**

Immunotherapies (including cancer vaccines) designed to stimulate specific T-cell–mediated immunity have thus far yielded rather modest objective clinical response rates despite their ability to enhance circulating frequencies of tumor-specific T cells in many treated patients (32). Because most tumor-associated antigens are nonmutated ("self") proteins, treatment-enhanced T cells are believed to be derived from a low-to-moderate avidity repertoire that has survived negative selection (33, 34). These T cells may become effectively activated by (peptide-based) vaccines, but they frequently fail to recognize tumor cells that naturally present low stochastic frequencies of relevant MHC/tumor peptide complexes (35). Poor immune reactivity of tumor cells may be further exacerbated by defects in the tumor APN (36, 37).

If tolerance selection restricts the antitumor CD8+ T-cell repertoire to a degree that limits their clinical utility, we hypothesized that tumor cells might instead be manipulated to exceed the cognate antigen threshold requirements for effective immune surveillance. In particular, we believe that by conditionally enhancing the proteasomal processing of tumor antigens, such as EphA2, the level of class I/EphA2 peptide complexes might be (at least transiently) increased on the tumor cell surface, allowing for improved recognition by modest avidity anti-EphA2 CD8+ T cells (22). Indeed, in the current study, we have determined that (a) EphA2, which is commonly overexpressed in a broad range of cancer types, is a novel client protein of HSP90 and that HSP90 inhibitors, such as 17-DMAG, promote tumor cell EphA2 degradation; (b) 17-DMAG treatment of EphA2+ tumor (RCC and ovarian carcinoma) cells improves recognition by low-avidity anti-EphA2 CD8+ T cells; (c) enhanced T-cell recognition of 17-DMAG–treated tumor cells is MIC-dependent and appears unrelated to tumor cell expression of costimulatory/coinhibitory molecules, which remains unchanged on HSP90 antagonism (Supplementary Fig. S3); and (d) EphA2+ tumor cell recognition by specific CD8+ T cells may be further enhanced by combined treatment with 17-DMAG and EphA2 agonists (versus treatment with either single modality).

Notably, tumor recognition by T cells reactive against peptides found in the extracellular (EphA258-66) as well as intracellular (EphA2369-377) domains of the target protein was improved by treatment with 17-DMAG. This suggests the superior processing/presentation of peptides derived from the full-length, transmembrane protein may be anticipated as a consequence of drug application. Mechanistically, we observed that enhanced T-cell recognition of 17-DMAG–treated tumor cells was ablated on inclusion of proteasome (MG-132), TAP (ICP471-35), or sec61 (P. aeruginosa ExoA) inhibitors. In contrast, there was minimal effect associated with the inclusion of the lysosomotropic agent chloroquine, which interferes with endosomal acidification and lysosomal processing of protein antigens. These data suggest that the major pool of EphA2 protein undergoing (constitutive as well as) enhanced proteasomal processing as a consequence of 17-DMAG inhibition likely enters the tumor cytosol via a retrotranslocation event (27, 29–31). At present, we cannot distinguish whether this pool of EphA2 protein derives from an early endosomal compartment (internalized after interaction with the EphA2 ligands coexpressed by adjacent tumor cells that is unaffected by chloroquine) and/or from newly synthesized, misfolded EphA2 proteins within the exocytic pathway. However, given the observed quantitative variance in 17-DMAG–induced EphA2 degradation as imaged using flow cytometry versus Western blotting analyses, and the apparent synergy of agonist mAb208 and 17-DMAG in promoting EphA2 protein loss and enhanced T-cell recognition, it could be suggested that mAb208 primarily affects the membrane pool of EphA2 protein, whereas 17-DMAG primarily affects the intracellular pool of this protein. In either case, enhanced EphA2 peptides would then appear to be integrated into "empty" class I complexes after TAP-dependent transfer into the MHC class I loading compartment.

Although several HSP90 inhibitors (including geldanamycin, 17-allylamino,17-demethoxygeldanamycin, and 17-DMAG) have now entered advanced clinical trials, to our knowledge, very few articles have addressed the potential effects of HSP90 inhibitors on tumor cell recognition by T cells. In this regard, Castilleja and colleagues reported that the HSP90 inhibitor geldanamycin induced degradation of nonmutated HER-2/neu in a treated ovarian carcinoma cell line, resulting in enhanced recognition by a HLA-A2–restricted CTL line reactive against the HER-2/neu369-377 epitope (38). They also noted that the level of HLA-A2 class I molecules expressed by their tumor cell line was increased after treatment (38). Although this latter result is somewhat in contrast to our current results, the mechanistic paradigm(s) for HSP90 inhibitor effects on immune augmentation appear consistent in the two studies. In marked contrast, Callahan and colleagues reported that H-2Ld expression was reduced on cells treated with HSP90 inhibitors radicicol, geldanamycin, or 17-allylamino,17-demethoxygeldanamycin due to the inhibited loading of peptides into MHC class I complexes and that radicicol treatment of SV40 large T antigen–expressing SVB6 cells impaired their recognition by an anti-T antigen T-cell clone (K11; ref. 39). However, the doses of HSP90 inhibitors used by these investigators were high compared with those used (50 and 16 times) in our study and by Castilleja and colleagues (38), respectively. Such drug excess could result in adverse effects at the level of the tumor cell APM or in the expression of alternate integrins/adhesion molecules required for cognate T-cell recognition. Moreover, although T antigen may interact with HSP90, its degradation is only modestly induced by HSP90 inhibitors (40), which could limit the pool of derivative T antigen peptides for the consequent loading of MHC class I complexes required for specific T-cell reactivity to occur.

Our data suggest that 17-DMAG enhances tumor cell recognition by EphA2-specific CD8+ T cells in a transient manner, with improved recognition observed 24 h, but not 48 h, after drug treatment. This contrasts slightly with the inhibitory effects of 17-DMAG on tumor EphA2 expression levels, which were comparable or somewhat greater at 48 h versus 24 h. One possible explanation for this dichotomy may involve differential rates at which the diverse array of HSP90 client proteins undergo proteasomal destruction on application of 17-DMAG.6 As a result, the competitor substrates for proteasomal processing of EphA2 protein may vary over time after drug treatment. If this hypothesis is correct, EphA2 may be more efficiently processed and/or be less effectively competed for loading MHC class I complexes during the

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6 M. Kawabe et al., unpublished results.
first 24 h of HSP90 inhibitor administration. In extended studies, we noted that the ability of 17-DMAG–treated tumor cells to be loaded by exogenous peptide was somewhat diminished when compared with untreated tumor cells (Supplementary Fig. S4) but that this was not differential at the 24 h versus 48 h time points post-DMAG application (Supplementary Fig. S4A) and that this did not appear to reflect any change in the prevalence of "empty" HLA-A2 complexes on the tumor cell surface as a consequence of drug treatment (Supplementary Fig. S4B). We interpret these results to suggest that the average affinity of presented peptides (including EphA2 epitopes) in tumor cell HLA-A2 (and corollary stability of MHC complexes that limit loading of exogenous peptides) may be increased as a consequence of 17-DMAG treatment (for 24 or 48 h). These findings appear to mitigate concerns that the class I APM increased as a consequence of 17-DMAG treatment (for 24 or 48 h). Therefore, Rafl is a client protein of HSP90.7 Hence, HSP90 inhibitors may antagonize this signaling cascade (42) and consequently limit EphA2 transcription by promoting Rafl degradation. We are currently in the process of analyzing such possibilities to establish an optimal treatment regimen capable of improving tumor cell presentation of EphA2 epitopes to the immune system.

Based on our current and recently published studies (18), both EphA2 agonists (mAb, recombinant EphA1-Fc) and 17-DMAG (alone or in combination) are capable of enhancing anti-EphA2 T-cell recognition of (EphA2+, HLA-A2+) tumor cell lines. In the clinic, EphA2 agonists would be expected to be superior with regard to specificity in targeting the EphA2 antigen versus multiple client proteins in the case of HSP90 inhibitors. However, the promiscuous and coordinate effects of HSP90 inhibitors on multiple oncoprotein clients would argue for the use of these drugs in combinational immunotherapies targeting multiple antigens (such as EphA2, epidermal growth factor receptor, HER-2/neu, etc.). HSP90 inhibitors may be preferred to agonists based on their selective accumulation in tumor versus normal EphA2– tissue sites. For example, 17-allylamino,17-demethoxygeldanamycin binds to tumor cell-derived HSP90 up to 2 logs more tightly than it does to normal cell-derived HSP90 (43). Furthermore, in mouse models, 17-DMAG persists within tumor lesions far longer than in normal tissues, with detectable levels selectively observed in tumors 48 h after a single i.v. administration of drug (44). This suggests that drug dosing far below the maximum tolerated dose may be capable of modulating T-cell recognition of cancer cells within the tumor microenvironment in vivo. However, it is also possible that the application of 17-DMAG and/or EphA2 agonists may promote increased immune recognition of normal EphA2– tissues that express a fully functional class I APM. This raises the specter of autoimmune pathology that would need to be closely monitored in prospective clinical trials applying these agents, particularly should they be combined. In this regard, no untoward immune-mediated effects on patient EphA2– organs (lungs and kidney) have been reported in clinical trials employing HSP90 inhibitors to date (45), and EphA2 agonist therapies have yet to be investigated in phase I trials.

In conclusion, our results suggest that an effective combinational immunotherapy for clinical translation (22) may be defined by “pulse” 17-DMAG administration to improve EphA2– tumor cell recognition by CD8+ T cells that have been elicited previously in response to EphA2-based vaccination or that are provided via adoptive transfer. Such combinational approaches might be further improved by inclusion of type I or II IFN coadministration to further improve tumor cell APM function and the MHC class I (and II) presentation of targeted epitopes by tumor cells in vivo (46, 47).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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