ATP Analog Enhances the Actions of a Heat Shock Protein 90 Inhibitor in Multiple Myeloma Cells

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ABSTRACT
Heat shock protein (HSP) 90 regulates client oncoprotein maturation. The chaperone function of HSP90 is blocked by 17-N-allylamino-17-demethoxygeldanamycin (17-AAG), although it results in transcription and translation of antiapoptotic HSP proteins. Using three myeloma cell lines, we tested whether inhibition of transcription/translation of HSP or client proteins will enhance 17-AAG-mediated cytotoxicity. 8-Chloro-adenosine (8-Cl-Ado), currently in clinical trials, inhibits bioenergy production, mRNA transcription, and protein translation and was combined with 17-AAG. 17-AAG treatment resulted in HSP transcript and protein level elevation. In the combination, 8-Cl-Ado did not abrogate HSP mRNA and protein induction. HSP90 requires ATP to stabilize client proteins; hence, expression of signal transducer and activator of transcription 3 (STAT3), Raf-1, and Akt was analyzed. 17-AAG alone resulted in <10% change in STAT3, Raf-1, and Akt protein levels, whereas no change was observed for 4E-BP1. In contrast, the combination treatment resulted in a >50% decrease in client protein levels and marked hypophosphorylation of 4E-BP1. 8-Cl-Ado alone resulted in a <30% decrease of client proteins and 4E-BP1 hypophosphorylation. 8-Cl-Ado combined with 17-AAG resulted in more than additive cytotoxicity. In conclusion, 8-Cl-Ado, which targets transcription, translation, and cellular bioenergy, enhanced 17-AAG-mediated cytotoxicity in myeloma cells.

Introduction
The heat shock protein (HSP) family is a group of highly conserved constitutive and stress-inducible related proteins that act as molecular chaperones assisting in protein folding and stabilization (Hartl and Hayer-Hartl, 2002). Cytosolic HSP90α/β is the most abundant chaperone in the cell. An increase in the levels of this chaperone is further observed when the cell is subjected to physiological stress [including hypoxia, heat, heavy metals, and small molecules such as 17-N-allylamino-17-demethoxygeldanamycin (17-AAG)] (Whitesell and Lindquist, 2005). HSP90 is essential in the stabilization and functional conformation of stress-denatured client oncoproteins. Hence, inhibition of the refolding activity of HSP90 would result in the simultaneous down-regulation of signaling cascades (Sharp and Workman, 2006; Biamonte et al., 2010). HSP90 chaperone activity is an ATP-dependent process (Prodromou et al., 1997). A denatured client protein is recognized by cochaperones HSP70 and HSP40, priming it in preparation for loading onto HSP90 (Pratt and Toft, 2003), which is facilitated by the cochaperone HSP90/HSP70 organizing protein (Smith et al., 1993; Onuoha et al., 2008). Once the unfolded client interacts with HSP90, ATP binds to the ATP binding pocket in HSP90 and subsequent cochaperones bind to the client · HSP90 complex, facilitating its stabilization. A change in the HSP90 conformation triggers ATP hydrolysis, catalyzing the maturation and functional stability of the client protein (Whitesell and Lindquist, 2005; Biamonte et al., 2010).

Given the importance of the HSP90 chaperone cycle in the stability of oncoproteins, several inhibitors of HSP90 have been identified, including geldanamycin and its analog derivative 17-AAG. Similar to geldanamycin, 17-AAG is an ATP competitive inhibitor of HSP90 (Schnur et al., 1995). 17-AAG inhibits the chaperone function of HSP90 by mimicking ATP and occupying its place in the N-terminal ATP-ADP-binding pocket (Prodromou et al., 1997). Binding of
17-AAG to the ATP pocket in HSP90 results in an immature HSP90 · client complex. Hence, the HSP90 inhibitor interrupts the maturation and proper folding of the client protein by HSP90, leading to the proteasomal degradation of the client substrates (Neckers and Neckers, 2002; Whitesell and Lindquist, 2005). However, binding of 17-AAG or any other HSP90 inhibitor to the ATP pocket of the chaperone elicits a stress response resulting in the transcription activation of HSPs through heat shock factor (HSF-1) (Bagatell et al., 2000). HSF-1 is a transcription activator of all HSP genes (Wu, 1995). Under basal physiological conditions, HSP90 and other chaperones that are part of the repressing complex are bound to the transcription factor HSF-1, regulating its activation (Guo et al., 2001). The presence of stress inducers in the cell triggers the release of HSF-1 from its constitutive repressing complex, triggering transcription of the HSPs. Once released, HSF-1 trimerizes and is phosphorylated, resulting in its active conformation (Wu, 1995). Once activated, the HSF-1 trimer translocates to the nucleus where it binds the heat shock elements present in the promoter of the HSP genes, resulting in an overexpression of HSP proteins in the cell (Baler et al., 1993). The antiapoptotic nature of the HSPs (Beere et al., 2000; Pandey et al., 2000) allows the cell to evade cell death and become resistant to therapeutic agents. Multiple studies have reported that HSP overexpression facilitates resistance to chemotherapeutic agent-induced cytotoxicity in various malignancies (Demidenko et al., 2006; Martins et al., 2008).

The nucleoside analog 8-Cl-Ado is phosphorylated into its cytotoxic triphosphate 8-Cl-ATP. The accumulation of the cytotoxic metabolite results in a parallel decrease of the ATP cellular pools (Gandhi et al., 2001; Chen et al., 2009). 8-Cl-Ado gets incorporated into RNA during transcription, hindering this process (Stellrecht et al., 2003). In addition, this triphosphate inhibits ATP-dependent poly(A) tail synthesis, and, as a consequence, mRNA processing is inhibited (Chen and Sheppard, 2004; Chen et al., 2010), resulting in intracellular toxicity in several solid and hematological malignancies (Gandhi et al., 2001; Balakrishnan et al., 2005). This agent is currently in phase I clinical trials for the treatment of chronic lymphocytic leukemia.

HSP90 is overexpressed in several malignancies including multiple myeloma (MM) (Whitesell and Lindquist, 2005; Chatterjee et al., 2007; Richardson et al., 2011). Inhibition of HSP90 is a therapeutic strategy for the treatment of myeloma and other cancers. On the basis of the mechanism of action of 8-Cl-Ado, we hypothesized that 8-Cl-ATP will inhibit 17-AAG-mediated transcription activation of HSPs, will inhibit client protein translation, and may have more binding of 17-AAG to HSP90 due to ATP decline. Using three multiple myeloma cell lines as a model we tested our hypothesis by combining 8-Cl-Ado with 17-AAG.

Materials and Methods

Cell Lines. The MM.1S cell line was obtained from Drs. Nancy Krett and Steve Rosen (Robert H. Lurie Comprehensive Cancer Center, Northwestern University, Chicago, IL). The U266 and RPMI-8226 cell lines were obtained from Dr. William S. Dalton (H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL). The cell lines were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Waltham, MA) in the presence of 5% CO₂ at 37°C. Approximate doubling times for MM.1S, U266, and RPMI-8226 are 48, 36, and 24 h, respectively. Cells were routinely tested for Mycoplasma infection using a commercially available kit (Gen-Probe Inc., San Diego, CA).

Materials. 17-AAG was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in dimethyl sulfoxide at a concentration of 1 mM. 8-Cl-Ado was obtained from Dr. Vishnuvajjala Rao (Drug Development Branch, National Cancer Institute, Bethesda, MD) and dissolved in Milli-Q water (Millipore Corporation, Billerica, MA) at a concentration of 10 mM.

Radioactive Uridine and Leucine Incorporation. Global RNA synthesis and global protein synthesis were measured using [³H]uridine or [³H]leucine incorporation (37.2 or 149 Ci/mmol; Moravek Biochemicals, Brea, CA), respectively, as described before (Cervantes-Gomez et al., 2009). In brief, myeloma cells were left untreated or treated according to the drug schedule. Forty-five minutes before the end of incubation, the cells were labeled with the radioactive material at 37°C. The labeled samples were harvested, washed with ice-cold (4°C) phosphate-buffered saline, and transferred to glass fiber filters (Whatman, Clifton, NJ) using a Milipore vacuum manifold (Thermo Fisher Scientific). The filters were then washed with ice-cold (4°C) 0.4 N perchloric acid and rinsed once with 70% ethanol and dried overnight. The dried filters were transferred to scintillation vials containing 7 ml of high flash point cocktail scintillation fluid (Research Products International, Mount Prospect, IL). Data are expressed as a percentage of the untreated control.

Isolation of RNA and Quantitative Real-Time RT-PCR. Total RNA was isolated using a RNeasy Mini Kit (Qiagen, Valencia, CA). The relative transcript levels of gene expression were assessed using TaqMan One-Step RT-PCR Master Mix reagents (Applied Biosystems, Foster City, CA). Pre-designed primers and TaqMan probes for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), HSP90α (GenBank accession number NM_002093), HSP90β (GenBank accession number NM_002093), HSP70 (GenBank accession number NM_000520), HSC70 (GenBank accession number NM_000520), HSP27 (GenBank accession number NM_000835), HSF-1 (GenBank accession number NM_002093), HSF-2 (GenBank accession number NM_002093), and HSF-3 (GenBank accession number NM_002093) were purchased from Applied Biosystems. Contaminating DNA was removed from RNA preparations by using a commercially available RNAse-free DNase treatment and removal kit (Ambion, Austin, TX). Relative levels of gene expression were determined by using standard curves and normalized with the endogenous gene GAPDH. Experiments were done in triplicate, and the results are plotted as fold change in comparison with untreated cells.

Protein Extraction and Immunoblot Assays. After treatment, cells were lysed, protein concentration was determined, and immunoblot analyses were performed as described previously (Cervantes-Gomez et al., 2009). In brief, myeloma cells were treated as already mentioned; samples were harvested, centrifuged, and washed twice with phosphate-buffered saline. Cells were lysed using 1 tablet of Complete Mini Protease Inhibitor Cocktail (Roche, Indianapolis, IN) in 10 ml of M-PER Mammalian Protein Extraction Reagent manufactured by Thermo Fisher Scientific. For immunoblot analysis, protein samples were electrophoresed on Criterion Bis-Tris gels using the XT MOPS buffer kit (Bio-Rad Laboratories, Hercules, CA) and transferred to nitrocellulose membranes. Primary antibodies were purchased from the following sources: HSP90α/β, HSP70, HSC70, and HSP27 from Assay Designs (Ann Arbor, MI); STAT3, Akt, and GAPDH from Cell Signaling Technology (Danvers, MA); and Raf-1 (HSP00991918, m1) were purchased from Applied Biosystems. Contaminating DNA was removed from RNA preparations by using a commercially available RNAse-free DNase treatment and removal kit (Ambion, Austin, TX). Relative levels of gene expression were determined by using standard curves and normalized with the endogenous gene GAPDH. Experiments were done in triplicate, and the results are plotted as fold change in comparison with untreated cells.

Immunoprecipitation of HSP90 Protein Bound to [³H]17-AAG. Radioactive [allylamine-2,3-³H]17-AAG was synthesized by Moravek Biochemicals. MM.1S cells were incubated with [allylamine-2,3-³H]17-AAG in the presence or absence of 8-Cl-Ado for 20 h. Cells were lysed by sonication, samples were precleared, and protein samples were electrophoresed on Criterion bis-Tris gels using the XT MOPS buffer kit (Bio-Rad Laboratories, Hercules, CA) and transferred to nitrocellulose membranes. Primary antibodies were purchased from the following sources: HSP90α/β, HSP70, HSC70, and HSP27 from Assay Designs (Ann Arbor, MI); STAT3, Akt, and GAPDH from Cell Signaling Technology (Danvers, MA); c-Raf from BD Biosciences Pharmingen (San Diego, CA); β-actin from Sigma-Aldrich; and RNA Polymerase II from Covance (Princeton, NJ).
8-Cl-Ado Enhances the Actions of 17-AAG in Myeloma Cells

Results

Experimental Design. Myeloma cells (MM.1S, RPMI-8226, and U266) were either untreated or treated with 0.5 μM 17-AAG alone for 8 h, a combination treatment with 10 μM 8-Cl-Ado for 12 h followed by 0.5 μM 17-AAG for 8 h, or 10 μM 8-Cl-Ado alone for 20 h. These concentrations were selected on the basis of prior reports and are clinically achievable (Gandhi et al., 2001; Cervantes-Gomez et al., 2009). Two additional combination sequences were also evaluated. In one strategy, cells were treated with 0.5 μM 17-AAG alone for 20 h, a combination treatment with 0.5 μM 17-AAG added first for 8 h followed by 10 μM 8-Cl-Ado for 12 h, or 10 μM 8-Cl-Ado alone for 12 h. The last combination approach evaluated 0.5 μM 8-Cl-Ado and 0.5 μM 17-AAG added simultaneously for 20 h, including the two parallel conditions with the single agents alone.

Cytotoxicity of 8-Cl-Ado in Combination with 17-AAG in Myeloma Cells. Endogenous cell death was subtracted from each condition. Treatment with 17-AAG resulted in <5% cell death, whereas treatment with 8-Cl-Ado resulted in 26, 6, and 2% death in MM.1S, RPMI-8226, and U266 cells, respectively. In the same cell lines, the combination resulted in 55, 15, and 19% cell death (Table 1). The measured (observed) cytotoxicity for the combination condition was more than the expected (calculated) cell death and was significantly different in all cell lines (MM.1S, p = 0.019; RPMI-8226, p = 0.011; U266, p = 0.019). Because these results suggested more than an additive effect, different combination sequences for 8-Cl-Ado and 17-AAG were tested for cytotoxicity (data not shown). The cytotoxicity between the expected and observed cell death values was either not different or marginally different in the simultaneous combination of both drugs (MM.1S, p = 0.045; RPMI-8226, p = 0.29; U266, p = 0.047).

Effect of 8-Cl-Ado on Global RNA Synthesis. The MM.1S cell line was more sensitive to the RNA inhibitory actions of 8-Cl-Ado than RPMI-8226. A 25% decrease in global RNA synthesis was observed after 4 h, further decreasing to 50% after 8 h. Treatment with 8-Cl-Ado in RPMI-8226 cells caused only a 25% inhibition at both time points compared with the control (data not shown).

Effect of 8-Cl-Ado and 17-AAG on Constitutive and Stress-Inducible HSP mRNAs. Treatment with 17-AAG triggered the elevation of all constitutive and stress-inducible HSP mRNA ranging from more than 2- to 9-fold (Fig. 1). However, this induction persisted with the combination treatment in both cell lines, which implies that 8-Cl-Ado was not able to abrogate the HSP induction elicited by 17-AAG (Fig. 1, A, B, and D). In fact, the combination treatment further elevated the levels of HSP70 by 13- and 4-fold in MM.1S and RPMI-8226 cells, respectively (Fig. 1C). Likewise, in MM.1S cells, the mRNA levels of HSP27 were further induced in the combination treatment (Fig. 1E, p = 0.02). In RPMI-8226 cells, treatment with 17-AAG alone resulted in an 8-fold increase in HSP27 transcript levels that remained similar after 8-Cl-Ado addition (Fig. 1E). Treatment with 8-Cl-Ado as a single agent did not result in a decrease in the basal levels of any of the inducible or constitutive HSP transcript levels (Fig. 1, A–E).

Effect of 8-Cl-Ado on Constitutive and Stress-Inducible HSP Expression Levels Induced by 17-AAG. Immunoblots were performed to determine whether the observed increases in inducible HSP transcripts after combination treatment were obtained at the protein level (Fig. 2). HSP70 and HSP27 protein levels were increased with 17-AAG treatment in MM cells (Fig. 2A). Similar to HSP transcript levels, 17-AAG-induced expression of HSPs was not abrogated by 8-Cl-Ado addition. Treatment with 8-Cl-Ado alone did not affect the endogenous levels of HSPs in any cell line. The normalization, quantitation, and statistical analyses of three independent immunoblots indicated that 8-Cl-Ado was not able to abrogate 17-AAG-mediated induction of HSPs (Fig. 2B).

Effect of 8-Cl-Ado and 17-AAG Alone and in Combination on Client Protein Expression Levels. Oncoproteins, such as STAT3, Raf-1, and Akt, are part of redundant signaling cascades. 17-AAG and other HSP90 inhibitors are unique in their ability to simultaneously down-regulate numerous client oncoprotein substrates by increasing their

### Table 1

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<th>8-Cl-Ado, 10 μM</th>
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<tr>
<td></td>
<td>8 h</td>
<td>20 h</td>
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<td>26 ± 11</td>
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*p = 0.019

Materials and Methods. This compares the expected and observed cell death values was either not different or marginally different in the simultaneous combination of both drugs (MM.1S, p = 0.045; RPMI-8226, p = 0.29; U266, p = 0.047).
turnover rate (Neckers and Neckers, 2002). 8-Cl-Ado can inhibit transcription and translation processes because of its actions not only on RNA incorporation and termination but also on the decrease of endogenous ATP pool levels (Gandhi et al., 2001; Stellrecht et al., 2003; Chen et al., 2009). The combination of both agents resulted in a drastic decrease (75%) in STAT3, Raf-1, and Akt compared with that in untreated controls as well as the single agents in MM.1S and U226 cells; this decrease was minimal in the RPMI-8226 cell line (Fig. 3A). Three independent immunoblots showed that the combination of 8-Cl-Ado and 17-AAG resulted in a statistically significant decrease in STAT3, Raf-1, and Akt client protein levels in all of the MM cell lines evaluated compared with the untreated controls, with the exception of Raf-1 protein levels in RPMI-8226 cells (Fig. 3B).

Effect of 8-Cl-Ado and 17-AAG Alone and in Combination on STAT3, Raf-1, and Akt mRNA Levels. To investigate whether the reduction in STAT3, Raf-1, and Akt expression observed in the combination treatment was due to a decrease in their mRNA levels, real-time RT-PCR was performed (Fig. 4). There was no significant change in STAT3 (Fig. 4A), Raf-1 (Fig. 4B), or Akt (Fig. 4C) transcript levels with any of the treatments in either cell line. Although there was a small induction of Akt transcript in RPMI-8226, this proved to be significant (p = 0.02) (Fig. 4C). Therefore, the decrease in STAT3, Raf-1, and Akt protein levels in the combination treatment is not due to a change in their transcript levels.

Cellular Metabolism of 8-Cl-Ado and Its Effect on the Binding of [3H] 17-AAG to HSP90. In MM.1S cells, a parallel decrease in ATP pools is observed with accumulation of 8-Cl-ATP. After the first 2 h of treatment with 8-Cl-Ado, there is 100 μM 8-Cl-ATP accumulation with a 25% decrease in ATP levels. By 20 h, treatment with 8-Cl-Ado decreases ATP levels by 65%, with approximately 1 mM intracellular ATP remaining.

To determine whether addition of 17-AAG affected the accumulation of 8-Cl-ATP or the decrease in the ATP pool, the combination condition was also evaluated (Fig. 5, B and C). Treatment with 8-Cl-Ado caused a 57% decrease in ATP levels. The combination treatment resulted in an 83% drop of the ATP pool. The expected parallel accumulation of 8-Cl-ATP levels was surprisingly not observed. Addition of 17-AAG after 8-Cl-Ado treatment resulted in only 20 μM 8-Cl-ATP accumulation in the combination treatment compared with 60 μM 8-Cl-ATP after 8-Cl-Ado treatment (Fig. 5B).

In RPMI-8226 cells, after 8-Cl-Ado treatment, 8-Cl-ATP accumulation reached 110 μM with a slight decrease in the ATP pool. Addition of 17-AAG after 8-Cl-Ado treatment did not affect the levels of ATP to a great extent compared with 8-Cl-Ado alone (76 versus 64%). However, a decrease in 8-Cl-ATP levels was also observed compared with 8-Cl-Ado treatment alone (Fig. 5B). Overall, these data suggest that 17-AAG affects the metabolism of 8-Cl-Ado to its cytotoxic triphosphate form.
To bind to HSP90, 17-AAG competes with ATP (Schnur et al., 1995), inhibiting the stabilization of client proteins by HSP90 and resulting in client protein proteasomal degradation (Neckers and Neckers, 2002). The combination treatment resulted in a dramatic decrease in ATP levels compared with treatment with 8-Cl-Ado alone (Fig. 5C) as well as a marked decrease in the client protein levels (Fig. 3). A possible explanation for this result is that there is more binding of 17-AAG to HSP90 in the combination treatment because 8-Cl-Ado decreases the cellular ATP pool. For this reason, immunoprecipitation and immunoblot analyses of radioactive [3H]17-AAG bound to HSP90 were performed in MM.1S cells for the combination treatment of 8-Cl-Ado and [3H]17-AAG compared with [3H]17-AAG treatment alone (Fig. 5D). MM.1S cells were treated with 0.5 μM [3H]17-AAG alone or the combination of 10 μM 8-Cl-Ado plus [3H]17-AAG for 20 h. Each condition was performed twice in duplicate. The total amount of [3H]17-AAG in both condition treatments was similar (175,000 dpm). Immunoprecipitation of HSP90α/β in MM.1S cells treated with [3H]17-AAG alone determined that [3H]17-AAG is bound to HSP90α/β, and the complex can be pulled down and radioactivity analyzed (approximately 25,000 dpm). However, the combination condition did not show an increasing amount of [3H]17-AAG binding to HSP90α/β. In addition, immunoprecipitation of RNA Pol II protein indicated the absence of [3H]17-AAG bound to RNA Pol II because the radioactive count was determined to be approximately 50 dpm. These data indicate that a 57% decrease in ATP levels due to 8-Cl-Ado treatment (Fig. 5, A and C) does not increase the amount of [3H]17-AAG binding to HSP90α/β protein (Fig. 5D).

**Global Protein Synthesis and Role of Protein 4E-BP1.**
The effect of 8-Cl-Ado alone and in combination with 17-AAG on global protein synthesis (Fig. 6A) and the phosphorylation sta-
tus of 4E-BP1 (Fig. 6B), a repressor protein of mRNA translation, were analyzed. Radioactive leucine incorporation in untreated MM.1S and U266 cells had an average of 19,776 and 21,635 dpm, respectively. The global protein synthesis was decreased by 30% with 8-Cl-Ado treatment in both cell lines. However, the combination treatment resulted in a 50% decline of leucine incorporation compared with that in the untreated control in MM.1S cells, although no further decrease was observed in U266 cells. MM.1S cells were treated with 10 μM 8-Cl-Ado for different time points or starved for several days to be used as a positive control for hypophosphorylation of 4E-BP1, and U266 cells were treated with the two different sequential or simultaneous combinations of 8-Cl-Ado and 17-AAG to determine whether 17-AAG could further affect the phosphorylation status of 4E-BP1 (Fig. 6B). The multiple bands of 4E-BP1 represent its varied phospho forms and stacking of the bands represents its hypophosphorylated form (Gingras et al., 1998, 2001). Treatment of MM.1S cells with 8-Cl-Ado resulted in a time-dependent decrease in 4E-BP1 phosphorylation levels, with a more pronounced hypophosphorylated form of 4E-BP1 appearing at 24, 48, and 72 h. In U266 cells, treatment with 17-AAG for 8 or 20 h did not affect the hyperphosphorylation status of 4E-BP1 as shown by its multiple bands. Treatment with 8-Cl-Ado for 12 or 20 h resulted in a decrease in phosphorylation levels in 4E-BP1. However, combination treatment with 8-Cl-Ado and 17-AAG resulted in further hypophosphorylation of 4E-BP1 as shown by the absence of multiple bands. As expected, starvation of MM.1S cells (positive control) for 24 and 48 h resulted in hypophosphorylation of 4E-BP1.

**Discussion**

Inhibition of HSP90 is an attractive therapeutic strategy for the treatment of myeloma. Preclinical and clinical studies...
Fig. 4. Effect of 8-Cl-Ado and 17-AAG alone and in combination on STAT3, Raf-1, and Akt client protein mRNA levels in MM.1S and RPMI-8226 cells. STAT3 (A), Raf-1 (B), and Akt (C) mRNA levels were measured using real-time RT-PCR and are represented as fold change in comparison with the untreated control. GAPDH was used as the endogenous gene for normalization, and each column represents the mean ± S.D. of triplicate experiments. Statistical values comparing the combination condition with 17-AAG alone, 8-Cl-Ado alone, or the untreated control are shown.

Fig. 5. Cellular metabolism of 8-Cl-Ado and its effect on the binding of [3H]17-AAG to HSP90. A, accumulation of 8-Cl-ATP and concomitant ATP concentration decrease. MM.1S cells were treated with 10 μM 8-Cl-Ado as a function of time. Cells were harvested at the indicated times, and 8-Cl-ATP (∆) and ATP (○) levels in the cells were measured. Each time point was performed in triplicate, and data represent means ± S.D. B and C, 8-Cl-Ado metabolism to 8-Cl-ATP and ATP accumulation in the absence or presence of 17-AAG. D, immunoprecipitation of HSP90 bound to [3H]17-AAG. MM.1S cells were treated with 0.5 μM [3H]17-AAG in the absence or presence of 8-Cl-Ado for 20 h and then were lysed and immunoprecipitated for HSP90. Similar experiments were performed using RNA Pol II as a negative control for nonspecific [3H]17-AAG binding. Each column represents the mean ± S.D. of two independent experiments done in duplicate.
suggest that HSP90 inhibitors increase cytotoxic susceptibility in myeloma cells when combined with other forms of therapy, such as conventional chemotherapy, radiation, or targeted therapy (Richardson et al., 2011). The ability of 8-Cl-Ado to inhibit 17-AAG-mediated transcription induction of HSPs, leading to increased cytotoxicity, was evaluated on the basis of the following mechanisms of action of this ATP analog (Stellrecht et al., 2003; Chen and Sheppard, 2004; Chen et al., 2010). First, the cytotoxic metabolite of 8-Cl-Ado, 8-Cl-ATP, can be incorporated into the body of RNA, leading to mRNA synthesis inhibition (Stellrecht et al., 2003). Second, 8-Cl-Ado treatment depletes cellular ATP pools in several cell lines such as breast cancer (Stellrecht et al., 2010), mantle cell lymphoma, and multiple myeloma (Gandhi et al., 2001), as well as primary chronic lymphocytic leukemia cells (Balakrishnan et al., 2005). ATP is a substrate for RNA synthesis, and hence its decline will affect transcription. Third, 8-Cl-ATP can inhibit mammalian polyadenylation, a required step for transcript function and stability (Chen and Sheppard, 2004; Chen et al., 2010).

Therefore, because of its inhibitory actions on RNA synthesis and stability, 8-Cl-Ado was selected as the agent to combine with 17-AAG. However, real-time RT-PCR data demonstrated that 8-Cl-Ado was not able to inhibit the transcription of any of the stress-inducible or constitutive HSP mRNA levels (Fig. 1); rather there was an induction of the HSP70 mRNA level when 8-Cl-Ado and 17-AAG drugs were combined (Fig. 1C). As mentioned previously, the HSP70 gene has some singularities that distinguish it from the rest of the HSP genes. It is primed for rapid induction because HSP70 transcription is initiated and paused until HSF-1 regulates the binding of HSF-1 to the HSP70 DNA promoter, resulting in elevated induction of HSP70 mRNA. Studies investigating the induction of the HSP response in Drosophila and humans have reported that a decrease in the intracellular ATP pools stimulates the binding of HSF-1 to the HSP70 DNA promoter, although this is not sufficient to trigger HSP70 transcription (Winegarden et al., 1996). However, a second stimulus (such as 17-AAG treatment) augments the heat shock stress response, resulting in elevated induction of HSP70 mRNA (Bagatell et al., 2000). One of the mechanisms by which 8-Cl-Ado exerts cytotoxicity is through depletion of the ATP pools; hence, these observations may explain why the combination of 8-Cl-Ado followed by 17-AAG results in an increased level of HSP70 mRNA.

Similar to HSP transcripts, myeloma cells treated with 8-Cl-Ado did not indicate a reduction in STAT3, Raf-1, or Akt mRNA levels (Fig. 4). A possible explanation for the inability
of 8-Cl-Ado to decrease HSP or client protein mRNA levels is that the length of the transcript could be an important feature. For example, HSP70, HSP27, and Raf-1 transcript length is reported to be 2.2, 2.2, and 2.8 kilobases, respectively (Hunt and Morimoto, 1985). In contrast, c-Met, an oncogene whose expression promotes tumorigenesis in multiple human cancers, is 8 kilobases long (Miller et al., 1996). The c-Met transcript is depleted by 8-Cl-Ado treatment followed by reduced protein expression (Stellrecht et al., 2007).

Hence, it may be possible that incorporation of 8-Cl-ATP is increased with long transcripts, leading to its inhibitory actions in mRNA synthesis.

Another possibility is that inducible gene transcription is not affected by transcription inhibition. For example, as described in an earlier report for actinomycin D (Cervantes-Gomez et al., 2009), DRB (Gomes et al., 2006), α-amanitin (Ljungman et al., 1999), and the cyclin-dependent kinase inhibitor roscovitine (Dey et al., 2008), RNA Pol II inactivation does not always result in transcription inhibition of some of the stress-inducible genes such as HSP70, p53, p21, and PUMA.

For HSPs, a lack of decrease in mRNA level was consistent with a lack of decline of all HSP expression; however, despite no change in mRNA levels, a decrease in client protein levels is measured in MM cells after 8-Cl-Ado treatment, which is further reduced when combined with 17-AAG (Fig. 3). The 8-Cl-Ado-mediated decrease in the endogenous ATP pool (Fig. 5, A and C) could account for the decrease in client protein levels. The possibility that ATP depletion could increase the binding of 17-AAG to HSP90, increasing the turnover rate of the client proteins, was contemplated as a potential mechanism of action by which the 8-Cl-Ado and 17-AAG combination resulted in a more pronounced decrease in client proteins and in increased cell death (Fig. 5D). Results indicated that when cells are treated with [3H]17-AAG or 8-Cl-Ado in combination with [3H]17-AAG, there is no further increase in the binding of [3H]17-AAG to HSP90. This result suggests that the levels of ATP in the cell do not affect binding affinity of [3H]17-AAG for HSP90.

Of interest, a study investigating the effect of ATP depletion in the stabilization of the client protein ErbB2 by HSP90 concluded that a decrease in the levels of ATP results in the disruption of the HSP90 - ErbB2 complex and ErbB2 degradation (Peng et al., 2005). The authors of this study lowered the ATP concentration in the cell by inhibiting glycolysis and mitochondrial respiration using the pharmacological inhibitors 2-deoxy-d-glucose or antimycin A, respectively. They concluded that depletion of cellular ATP levels alters stabilization and maturation of ErbB2 by HSP90, and this depletion of the client protein ErbB2 was similar to that obtained when cells were treated with geldanamycin. A similar decrease in protein levels was observed for the other two client proteins evaluated in this study, Raf-1 and Akt, after ATP depletion due to treatment with 2-deoxy-d-glucose or antimycin A. This result indicates that a decrease in cellular ATP concentration mimics the mechanism by which HSP90 inhibitors cause cytotoxicity in the cell. Hence, the decrease in client protein levels observed with 8-Cl-Ado treatment alone could be a result of its effect on ATP depletion.

An additional plausible mechanism by which 8-Cl-Ado could be leading to decreased client protein levels, without affecting transcript levels, is by negatively affecting protein synthesis, a process that requires high cellular energy. Protein translation is regulated when the cell undergoes some kind of stress that perturbs homeostasis, and the mammalian target of rapamycin (mTOR) pathway is sensitive to changes in cellular ATP levels. Protein 4E-BP1 regulates translation, and its activity is in turn regulated by the upstream serine/threonine kinase mTOR (Gingras et al., 1998). The translation-repressing activity of 4E-BP1 is regulated by phosphorylation at four specific amino acid residues (Gingras et al., 1998, 2001). In its hypophosphorylated state, 4E-BP1 represses initiation of translation by binding to the eukaryotic initiation factor 4E (Gingras et al., 1998). In contrast, hyperphosphorylation of 4E-BP1 by upstream kinases such as mTOR results in the release of eukaryotic initiation factor 4E, allowing translation initiation (Gingras et al., 2001). Similar to the known inhibitor starvation, treatment with 8-Cl-Ado results in hypophosphorylation of 4E-BP1 (Fig. 6B). The hypophosphorylated status of 4E-BP1 could initially be attributed to the decrease in ATP concentration. This, in principle, could decrease kinase activity as a result of substrate availability. However, with another ATP congener, a decline in ATP did not result in a global decrease in protein phosphorylation (Ghias et al., 2005). In addition, in U266 cells treated with 8-Cl-Ado and 17-AAG in sequential or simultaneous combination, 4E-BP1 was further hypophosphorylated compared with treatment with the drugs as single agents (Fig. 6B). Therefore, further studies addressing the effect of 8-Cl-Ado alone and in combination with 17-AAG on 4E-BP1 and global protein translation are needed. Consistent with the consequence of 4E-BP1 hypophosphorylation, there was a global decline in protein synthesis. Although the mechanism by which 8-Cl-Ado treatment leads to hypophosphorylation of the translation repressor 4E-BP1 was not elucidated in this current study, this negative effect on the translation process could be another potential mechanism by which treatment with 8-Cl-Ado affects protein translation.

In summary, although the mechanism by which 8-Cl-Ado and 17-AAG interact remains to be fully elucidated and tested in samples of patients with MM, the combination of transcription/translation inhibitors that cause ATP depletion such as 8-Cl-Ado could be used in combination with 17-AAG as a strategy to decrease client protein levels, resulting in an increase in cytotoxicity regardless of the high expression levels of the antiapoptotic HSP expression levels. In addition, different time schedules and combination sequences were also evaluated for the two drugs (data not shown), which resulted in a molecular and cytotoxic response similar to that of the combination strategy presented in this study. Because the agents evaluated in this investigation are either approved drugs or in clinical trials, these approaches may be applied in the clinic to the design of new drug combination strategies.

Authorship Contributions

**Participated in research design:** Cervantes-Gomez, Nimmanapalli, and Gandhi.

**Conducted experiments:** Cervantes-Gomez.

**Performed data analysis:** Cervantes-Gomez and Gandhi.

**Wrote or contributed to the writing of the manuscript:** Cervantes-Gomez and Gandhi.
References
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