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17-N-Allylamino-17-demethoxygeldanamycin induces a diverse response in human acute myelogenous cells

Jennifer M Napper and Vincent E Sollars

Department of Biochemistry and Microbiology, Joan C. Edwards School of Medicine, Marshall University, Huntington, WV, USA

Abstract

The goal of this study was to ascertain the specific effects of 17-N-Allylamino-17-demethoxygeldanamycin (17-AAG) treatment in human acute myelogenous leukemia (AML). Four human leukemia cell lines were treated with varying doses of 17-AAG followed by analysis of toxicity, apoptosis, proliferation, and cell cycle. Cell cycle analysis revealed that the cells accumulate in G₂/M phase within 96 hours of treatment, although the effect was not equivalent among the cell lines. p21, p53 expression and MDR1 activity were among the possible mechanisms uncovered for the differing responses. Exploiting these differences may allow for more effective combinatory treatments in patients with AML.

Keywords

AML; 17-AAG; G₂/M arrest; Hsp90; CDC2; MDR1; p53

1. Introduction

Hsp90 is a constitutively expressed protein that makes up 1–2% of cellular protein [1]. It is a member of the molecular chaperone protein family which are involved in protein folding and quality control. In times of stress, Hsp90 expression is up-regulated to assist the cell's recovery by stabilizing misfolded proteins. Under normal conditions, Hsp90 is required for the activation of many signaling proteins including protein kinases and transcription factors [2]. In cancer, it acts to stabilize a variety of mutated and over-expressed signaling proteins that are required for cancer cell survival [3]. As a result, cancer cells become more dependent on Hsp90, which makes them more sensitive to inhibitors than normal cells. Thus, Hsp90 has become an exciting new target in chemotherapies.

Geldanamycin is an ansamycin antibiotic that inhibits Hsp90 by competitively binding in the ATP binding pocket located in the N-terminal domain of the protein. Although it showed promise as an anticancer agent, it was eventually determined that the drug was highly hepatotoxic. 17-AAG, an analogue of geldanamycin, was developed in order to improve the

Corresponding Author: Vincent E Sollars, Department of Biochemistry and Microbiology, Joan C. Edwards School of Medicine, Marshall University School of Medicine, One John Marshall Drive, Huntington, WV 25755 USA, Tel: 304.696.7357, Fax: 304.696.7207, sollars@marshall.edu.

Conflicts of interest

none.

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therapeutic index of geldanamycin. These drugs ultimately cause the proteasomal degradation of Hsp90 client proteins by inhibiting the ATPase activity necessary for Hsp90 to function as a chaperone [4,5]. Because Hsp90 clients include many signaling proteins, inhibitors such as 17-AAG, can have an impact on multiple signaling pathways making them desirable therapeutic agents. [extensively reviewed in reference 6]

Recently, a phase I clinical trial investigating alvespimycin treatment, another geldanamycin derivative, in AML showed complete remission in 3 out of 17 patients and 1 patient achieved a 50% reduction in bone marrow blasts [7]. These results demonstrate that Hsp90 inhibition can produce clinically relevant effects, however, determining the mechanisms behind the positive responses will improve treatment strategies for AML patients in the future. Consequently, the goal of this study was to ascertain the specific effects of Hsp90 inhibition treatment in human AML. To that end, the human leukemia cell lines: HL-60, KG-1a, THP-1 and Kasumi-3 cells, which represent a variety of AML subtypes, were studied. Apoptosis, proliferation, cell cycle, and differentiation studies were performed after exposure to 17-AAG for various periods of time. Our data indicate that there is a diverse response among these AML cell types to 17-AAG treatment. These findings suggest that tailoring treatment on an individual basis may prove to be more effective in treating AML with 17-AAG.

2. Materials and Methods

2.1. Materials

p21 (clone CP36, CP74) and GAPDH (clone 6C5) antibodies were purchased from Millipore (Temecula, CA). Secondary antibody used with p21 was purchased from Abcam (Cambridge, MA). CDC2 and CDC25c (clones pstaire & H-6) antibodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). p53 antibody (clone DO-1) was a kind gift from Dr. Pier Palo Claudio. Clone DO-1 recognizes amino acids 21–25, in the transactivating domain of the protein and in our hands resulted in a single band. This epitope makes it specific for the full length isoforms α , β , γ and the truncated isoform Δ p53 [8,9]. Rabbit secondary antibody was purchased from Cell Signaling (Boston, MA) and mouse secondary was purchased from Amersham Biosciences. 17-AAG was purchased from A. G. Scientific, (San Diego, CA). Rh123 and Verapamil were purchased from Sigma (St. Louis, MO)

2.2. Cell Culture

All cell lines were purchased from American Type Culture Collection (Manassas, VA) and grown in the recommended culture medium and incubated at 37°C with 5% CO₂.

2.3. Apoptosis Studies

Cells were seeded at 2×10^5 cells/mL and treated with vehicle, 2 or 3 μ M of 17-AAG. After 48 hours, cells were labeled with pacific blue conjugated annexin V (Molecular Probes, Eugene OR) and 7-aminoactinomycin D (BD Pharmingen, San Jose, CA) according to the Annexin V product sheet. Fluorescence was then measured by flow cytometry on a BD FACSaria flow cytometer. Data was analyzed using Flowjo 8.8.6 (Mac version).

2.4. Proliferation Studies

Cell Trace CSFE Proliferation Kit was purchased from Molecular Probes (Eugene, OR). The basic protocol was followed as given on the product information sheet to load the cells with dye. The cells were then seeded at 2×10^5 cells/mL and were treated with vehicle, 2 or 3 μ M 17-AAG. HL-60 cells and THP-1 cells were treated for 48 hours and Kasumi-3 cells were treated for 72 hours before collection. KG-1a cells were treated for 48 hours, counted and reseeded at 2×10^5 cells/mL in fresh treated medium. 48 hours later (96 hours total) the cells

were collected. All cells were fixed in 2% paraformaldehyde at time of collection for at least 18 hours before measuring fluorescence by flow cytometry. Median fluorescent values were determined using Flowjo 8.8.6 (Mac version).

2.5. Cell cycle

Cells were seeded at 2×10^5 cells/mL and treated with vehicle, 0.25, 0.50, 1.0, 2.0 or 3.0 μ M of 17-AAG. After 48 hours, HL-60, THP-1 and Kasumi-3 cells were collected and fixed in 70% ethanol. KG-1a cells were counted and resuspended at 2×10^5 cells/mL in fresh treated medium. After an additional 48 hours, the cells were also fixed in 70% ethanol. DNA staining was performed by addition of 50 μ g/mL of propidium iodide and 250 μ g/mL of RNase A (both purchased from Sigma, St. Louis, MO) in PBS and incubated at 37°C for 30 minutes. Cells were then placed on ice and fluorescence was measured by flow cytometry. Subsequent data was analyzed using ModFit LT for MacIntel.

2.6. Western Blotting

Protein was extracted by resuspension of the cells in RIPA lysis buffer [25mM Tris pH 7.4, 1% triton X100, 1% SDS, 1% Na Deoxycholate, 150mM NaCl + Halt protease inhibitor (Thermoscientific, Rockford IL)]. Protein was quantified using BCA Protein Assay Kit (Pierce, Rockford, IL). 50 μ g of protein was loaded onto a 10% polyacrylamide gel and subjected to electrophoresis. Protein was then transferred to a nitrocellulose membrane and blocked with 5% milk in 0.1% tween in tris-buffered saline (TBST). Antibodies were incubated over night at 4°C in blocking solution (except for CDC25c which was incubated in block) at 1:1000 dilution. Washes and secondary incubations (1:3000 dilution) were performed at room temperature. Membranes were stripped by gently rocking in medium stripping buffer (200 mM glycine, 0.1% SDS, & 1% tween, pH2.2) for five minutes two times at room temperature. The membrane was then washed by rocking in TBST four times for five minutes each. The immunoreactive bands were visualized using chemiluminescence (ECL) detection reagents or Super Signal West Pico (thermoscientific, Rockford, IL). The bands on the autoradiograms were quantified using Quantity One 4.5.2 software program from Bio-Rad.

2.7. Rh123 efflux assay

Rh123 efflux assay was performed as previously described [10]. Briefly, cells were seeded at 2×10^5 cells/mL. Rh123 was added at a final concentration of 200 ng/mL for Rh123 positive samples. Verapamil, at a final concentration of 50 μ M, was added to the appropriate cultures at this time. Cells were incubated at 37°C for one hour. The cells were then washed once with PBS and resuspended in fresh medium with or without Verapamil. After 90 minutes incubation the cells were spun down and resuspended in FACS buffer for analysis by flow cytometry as described above.

2.8. Verapamil combined with 17-AAG treatments

Cell cycle profile and apoptosis assay was performed as described above with the following treatments: 2×10^5 cells/mL were seeded into a six well plate and treated with vehicle, 50 μ M Verapamil, 3 μ M 17-AAG or both. After 24 hours a portion of the culture was harvested and fixed as described above for cell cycle. 24 hours later, 1×10^5 cells were taken and analyzed for apoptosis as described above.

2.9. Statistical analysis

All statistical analysis was performed using SigmaStat, Systat Software, Inc. Analysis of variance was performed on all data sets where normality and equal variance assumptions were met. Student's T-test was performed with Tukey's correction for multiple comparisons. Significance was assigned for all data with $p < 0.05$. Cell cycle data sets were analyzed using

Dunnett's method, comparing each group to the control. For all other non-normal data, ANOVA on Ranks was performed. Pairwise testing was performed using Dunn's method for multiple comparisons.

3. Results

3.1. Human leukemia cells have varying susceptibilities to 17-AAG treatment

A 17-AAG toxicity assay was performed on the four AML cell lines. Cells were seeded at 2×10^5 cells/mL and incubated with varying concentrations of 17-AAG ranging from 2 μ M to 15 μ M for 48 hours. At this time, erythrosin B dye exclusion counts were performed. The results illustrate that there are distinct differences in the susceptibility of these cell lines to 17-AAG treatment (Fig. 1A). KG-1a cells are still able to proliferate as demonstrated by an increase in cell number at lower treatment levels. In fact, these cells did not attain an apparent static state until they were treated with 8 μ M of 17-AAG. On the other hand, Kasumi-3 cells exhibit a much higher susceptibility to apoptosis than the other cell lines, as can be seen by the low cell counts even at the lowest concentrations of treatment. These cells also continued to decline in cell number with greater doses of 17-AAG, unlike the other three cell lines which were able to sustain a constant cell concentration at higher doses.

To determine if 17-AAG was inducing apoptosis, annexin V studies were performed. It should be noted that in phase I clinical trials, the circulating concentration of 17-AAG in peripheral blood was less than 3.5 μ M [11]. Because of this, the rest of the experiments performed in this study centered around the physiologically relevant concentrations of 2 and 3 μ M. The cells were seeded as described above and treated with 0, 2 and 3 μ M of 17-AAG. After 48 hours the cells were collected and labeled with annexin V and 7-aminoactinomycin D as a dead cell counter stain. HL-60, KG-1a and THP-1 cells showed significant apoptosis and/or cell death, however there was only about a 5% increase (Fig. 1B). On the other hand, treatment induced a greater percentage of cellular apoptosis and death with short-term treatment in Kasumi-3 cells (Fig. 1B). These data indicate that Kasumi-3 cells are much more vulnerable to short term 17-AAG treatment than the other cell lines.

Due to the resistance of KG-1a cells to treatment, they were exposed to 17-AAG for 96 hours with a re-treatment after 48 hours in order to obtain the similar results as the other cell lines. All the other cell lines were treated for 48 hours unless otherwise noted.

Dye dilution assays were performed next to confirm that treatment slowed proliferation of the cells. Kasumi-3 cells doubling time is quite long, ranging from 50–60 hours. Therefore, in this particular study, proliferation rate for this cell line was assessed after 72 hours to give the cells ample time to double. The proliferation rate of HL-60, THP-1 and Kasumi-3 cells was decreased significantly by up to 60% with 2 and 3 μ M treatment (Fig. 1C). KG-1a cells exhibit a significant dose dependent reduction in proliferation rate, 20% for 2 μ M and 45% for 3 μ M. These data indicate that even with more stringent treatment, KG-1a cells were still less responsive to 17-AAG treatment when compared to the other cell lines.

3.2. 17-AAG treatment causes G₂/M arrest

The cells were treated with a range of 17-AAG concentrations and cell cycle was assessed using flow cytometry. HL-60 cells arrest in G₁ at the lower doses of treatment, but with increasing concentrations a 2.0 to 2.5-fold increase in percentage of cells in the G₂/M phase compared to the vehicle control was seen (Fig. 2A). Not surprisingly, KG-1a cells show a 2-fold increase in G₂/M only with the highest dose of 3 μ M (Fig. 2B). KG-1a cells' lack of response in the lower concentrations of treatment provides further evidence of their resistant phenotype. THP-1 cells however, arrested at the lowest concentrations tested in this study, with

a 3-fold increase in G₂/M phase. This occurred with no concurrent change in G₁ phase (Fig. 2C). 14% of Kasumi-3 cells accumulated in G₂/M phase with the highest dose, which was very low compared to the other cell lines (Fig. 2D). This result was surprising given that this cell line thus far had been most susceptible to treatment. This suggests an alternate mechanism of cell death for Kasumi-3 cells and illustrates the complexity of the cell lines' responses to 17-AAG treatment.

3.3. 17-AAG depletes CDC25c and CDC2 cell cycle protein levels

CDC25c and CDC2 were recently described as Hsp90 client proteins and subsequently down regulated with 17-AAG treatment in glioblastoma and lung cancer [12,13]. These two proteins are very important regulators of G₂/M progression [14]. In all of the AML cell lines, both proteins were diminished with Hsp90 inhibition (Fig. 3A, B, C and D). According to the densitometry results, CDC2 and CDC25c proteins were degraded significantly in THP-1, Kasumi-3, and HL-60 cells treatment. However, only CDC2 was degraded to a significant extent in KG-1a cells at the highest concentration tested. It is interesting that the two proteins are degraded to a greater degree in HL-60 cells considering THP-1 cells undergo cell cycle arrest much more readily. This is suggestive of an additional mechanism facilitating G₂/M arrest in THP-1 cells.

3.4. p21 is up-regulated with 17-AAG treatment in THP-1 cells

p21 is an important protein that is up-regulated in times of stress by p53 [15,16]. Recently, p21 has been shown to be up-regulated independently of p53 in chronic lymphocytic leukemias with geldanamycin treatment [17]. p21 expression promotes cell cycle arrest and differentiation and has been shown to disrupt CDC25c interactions with CDC2 [18]. To determine whether p21 plays a role in the observed G₂/M arrest, protein levels were measured by western blot in the four cell lines. p21 protein levels were persistently elevated in treated THP-1 cells, which was not apparent in the other cell lines (Fig. 4). Up-regulation of p21 provides a possible mechanism for the high propensity of THP-1 cells to arrest in the G₂/M phase of the cell cycle with 17-AAG treatment.

3.5. p53 is significantly down-regulated with 17-AAG treatment in Kasumi-3 cells

Wild-type p53 is known to help facilitate apoptosis in response to chemotherapeutic drugs [19,20]. Therefore, it was hypothesized that Kasumi-3 cells' high sensitivity to 17-AAG treatment was due to wild type p53 expression. Western blotting was performed to assess p53 expression in all of the cell lines. The antibody used in this study recognizes an epitope in the N-terminal region of p53 (21–25 aa), which makes it is specific for the full length isoforms α , β , γ and the truncated isoform Δ p53 [8,9]. A recent study revealed that AML patients have higher levels of Δ p53 compared to α p53 in their leukemic cells. However, after receiving chemotherapy, there was a fast up-regulation of α p53 protein levels and p53 target genes [9]. This indicates the importance of the α p53 isoform in the chemotherapeutic response of AML patients. Indeed, p53 protein was highly expressed, in Kasumi-3 cells as compared to the p53 over-expressing cell line Wi38 (Fig. 5A). The other cell lines did not express detectable levels of p53 as previously reported [21–23] and only a single band was detected in Kasumi-3 cell lysate.

Western blotting also revealed that p53 levels were significantly reduced with Hsp90 inhibition in Kasumi-3 cells (Fig. 5B). This is consistent with mutant p53 being a known Hsp90 client which is degraded upon Hsp90 inhibition [24–27]. This evidence, suggests that Kasumi-3 cells harbor mutant p53 and not wild type. Sequencing of the p53 cDNA revealed that Kasumi-3 cells do indeed express mutant forms of p53 (data not shown). Studies investigating these mutations are currently underway. p53 mutations and deletions occur in over 50% of all cancers [28] and has been shown to protect cancer cells from apoptosis [29–31]. It is conceivable that

degradation of mutant p53 could be a possible mechanism for Kasumi-3 cells' high sensitivity to 17-AAG treatment.

3.6. KG-1a cells have P-glycoprotein(P-gp) activity facilitating their resistance to 17-AAG treatment

P-gp is an ATP-binding cassette (ABC) transporter that allows for efficient efflux of substrates from cells. It has been well characterized as a mechanism in multi-drug resistance (MDR), due to its inherent ability to clear chemotherapeutic agents from cells before they can inflict harm. KG-1a cells are known to have active P-gp [32]. In order to determine if this was the mechanism causing KG-1a cells' resistant phenotype, the efflux capacity of the different cell lines were measured. KG-1a cells have P-gp activity, as illustrated by decreased fluorescence in non-inhibited cells (Fig. 6A, black histogram). This effect was ablated when 50 μ M of verapamil (Vp), a specific P-gp inhibitor, was added to the cell culture (Fig. 6A, shaded histogram). THP-1 and HL-60 cells however, were not able to efflux Rh123 to a significant degree (Fig. 6B and C, black histograms). Results shown are representative of three independent experiments.

KG-1a cells were then treated with a combination of 17-AAG and verapamil to determine whether inhibition of P-gp would result in a more susceptible phenotype. Cells were seeded as described above and treated with vehicle, 50 μ M verapamil, 3 μ M 17-AAG or a combination of both reagents. The cell cycle profile of each treatment group was measured after 24 hours. Treatment with 17-AAG and verapamil alone did result in a slight increase in G₁ phase. This effect is not surprising since verapamil has been previously shown to inhibit growth of primary AML cells in vitro [33,34]. However, this effect is ablated when the cells are treated with both reagents (Fig. 6D). In fact, a 2-fold increase in G₂/M phase of the cell cycle was observed, which is more indicative of the phenotype displayed by the other cell lines in this study. There was also a significant increase in dead and apoptotic cells after 48 hours of treatment (Fig. 6E). Not only was the increase significant when compared to vehicle control, it was also significant when comparing to cells treated with Vp or 17-AAG alone. These results illustrate that P-gp activity is clearly playing a role in KG-1a cells resistance to 17-AAG treatment.

4. Discussion

Our data indicates that the response of AML cell lines to 17-AAG treatment is diverse and that Hsp90 operates to stabilize cancer cells by varied mechanisms in AML. Previous studies have shown that 17-AAG sensitivity is variable among primary AML cells treated in vitro. This effect was correlated to Hsp90 expression, which was also found to be heterogeneous among the samples tested [35]. Another study confirmed this observation showing that heat shock protein expression is highly variable among AML patients [36]. Both studies reported that high expression of heat shock proteins was associated with lower complete remission rates, illustrating the importance of heat shock proteins in AML. Our results add to the current known data by offering further mechanisms to explain the diversity of sensitivities of AML cells.

We have observed that 17-AAG treatment inhibits AML cell growth, which was previously reported [35]. Further analysis revealed the cells undergo G₂/M arrest, although, this response was certainly not equivalent. We observed a 5–25% increase in G₂/M phase upon 17-AAG treatment among the cell lines tested. KG-1a cells required a more aggressive treatment regimen to achieve cell cycle arrest. THP-1 cells exhibited the highest susceptibility, undergoing a high percentage of G₂/M accumulation at even the lowest doses. HL-60 cells started off arresting in G₁ phase at lower doses, however, began accumulating in G₂/M at higher concentrations of treatment. G₁ arrest has been observed in malignant pleural mesothelioma and in Jurkat cells [37,38] with 17-AAG treatment. The exact mechanism of this result has yet to be discovered, but our results suggest that with higher doses of treatment, these examples may have been forced into G₂/M arrest as well. Kasumi-3 cells also show a small, but significant

accumulation in G₂/M phase. These cells however, display a much higher level of apoptosis with 48 hours treatment as compared to the other cell lines (Fig. 1B). This indicates that there is an alternate mechanism that is directing them to undergo apoptosis instead of cell cycle arrest.

p21 is an important effector in cell cycle arrest. Therefore, p21 expression was measured in the cell lines to determine its possible role. Our results indicate that THP-1 cells up-regulate p21 with 17-AAG treatment, which is not seen in the other cell lines. This result correlates with THP-1 cells undergoing G₂/M arrest more readily. It is tempting to speculate that p21 expression is the driving force behind this effect, however more studies are needed to confirm this hypothesis.

Our data suggest that targeting mutant p53 with 17-AAG may prove to be an effective therapy in that subpopulation of AML patients. Evidence presented in this study indicate Kasumi-3 cells harbor mutant p53. Some forms of mutant p53 have been shown to protect cancer cells against apoptosis and enhance their tumorigenic potential [29]. Loss of mutant p53, as seen with 17-AAG treatment (Fig. 5B), could possibly alleviate this protection from apoptosis causing the cells to die instead of undergo cell cycle arrest. Additional studies, such as siRNA knock down of p53, are needed to support this hypothesis. Mutant p53 could be an important biomarker for 17-AAG treatment in AML as well as other cancers.

A previous study published by Ryningen et. al. [39] illustrated that low Bcl-2 versus Bax ratios were associated with high in vitro apoptosis of primary AML cells. They also found that low Hsp70 levels was related to low vitality of the cells. It is possible that upon added stress of Hsp90 inhibition, Kasumi-3 cells may succumb to treatment due to an unfavorable ratio of these proteins. Further studies need to be performed to determine the mechanism for Kasumi-3 cells' high sensitivity to Hsp90 inhibition.

This study shows that a possible reason for the lackluster response of 17-AAG in clinical trials is due to the heterogeneous nature of AML. Among the cell lines in this study, a diverse response was elicited by 17-AAG treatment. Three different possible mechanisms for this diversity were observed. Clinical trials are ongoing investigating 17-AAG as a single agent, as well as in combination with other chemotherapeutic agents. Specific biomarkers, such as mutant p53 or P-gp, will aid in targeting patients that will benefit from 17-AAG treatment either alone or in combination with another drug. More studies need to be conducted to determine more biomarkers to aid in the efficacy of treatment.

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Figure 1.

17-AAG treatment inhibits proliferation in human leukemia cells and causes apoptosis. (A) Cells were seeded at a density of 2×10^5 cells/mL at indicated 17-AAG concentrations. Cell counts were performed after 48 hours. (B) Cells were treated with 0, 2 or 3 μM of 17-AAG for 48 hours. Apoptosis was assessed using annexin V and 7-aminoactinomycin D. Graphs show percentage of apoptotic and dead cells combined at the various concentrations. (C) Cells were loaded with CFSE dye and treated with 17-AAG as outlined in the materials and methods. Median fluorescence was measured by flow cytometry. Proliferation rate was assessed by calculating the median fluorescence percentage of untreated values to treated values. All results depicted here are representative of three separate experiments performed in triplicate (mean + S.E.M.). For statistical significance, analysis of variance was performed between the treatments followed by pair-wise comparison with Tukey's test (except panel (B) THP-1 cells, Kruskal-Wallis one way analysis of variance on ranks was performed followed by Dunn's method for pair-wise testing). (*) designates significance of $p < 0.05$ when data is compared to the vehicle control, (**) designates significance of $p < 0.05$ when 3 μM treatment is compared to the 2 μM treated cells.

Figure 2.

17-AAG treatment causes arrest in G₂/M phase of the cell cycle. Cells were treated as outlined in the materials and methods with the indicated concentrations of 17-AAG. Cell cycle was measured by flow cytometry. Graphs illustrate percentage of cells in each phase. Results depicted here are the mean values representative of three separate experiments performed in triplicate. For statistical significance, analysis of variance was performed between the treatments (except Kruskal-Wallis one way analysis of variance on ranks was performed for KG-1a cells) followed by Dunnett's method. (*) indicates $p < 0.05$ as compared to the vehicle control.

Figure 3.

CDC2 and CDC25c levels are diminished with 17-AAG treatment. Cells were treated with 0, 2 and 3 μM 17-AAG as outlined in the materials and methods. Total cell lysates were subjected to SDS PAGE analysis on a 10% polyacrylamide gel and blotted onto a nitrocellulose membrane. Each panel shows a representative western blot after probing with CDC2 and CDC25c antibodies. Graphs represent densitometry from 3–4 experiments (mean + S.E.M.). For statistical significance of KG-1a, THP-1, and Kasumi-3 CDC2 densitometry, analysis of variance was performed between the treatments followed by pair-wise comparison with Tukey's test. With HL-60 and CDC25c expression in Kasumi-3 cells' densitometry, Kruskal-Wallis one way analysis of variance on ranks was performed followed by Tukey's test for pair-wise testing. (*) indicates $p < 0.05$ as compared to the vehicle control.

Figure 4.

THP-1 cells up-regulate p21 with 17-AAG treatment. Cells were treated with 17-AAG as outlined in the material and methods. Total cell lysates were western blotted and probed with p21 antibody. The blot shown is representative of three blots all exhibiting the same effect.

Figure 5.

p53 is significantly down-regulated with 17-AAG treatment in Kasumi-3 cells. (A) untreated cellular protein was blotted and probed with p53 antibody. WI-38 cells transfected with a p53 over-expression vector was used as a positive control. (B) Kasumi-3 cells were treated for 48 hours with 0, 2 and 3 μM 17-AAG. p53 levels were measured by western blot. A representative blot is shown. Densitometry from four independent trials is graphed in panel under blot (mean + S.E.M.). For statistical significance, analysis of variance was performed between the treatments followed by pair-wise comparison with Tukey's test. (*) indicates $p < 0.05$ as compared to the vehicle control.

Figure 6.

KG-1a cells possess P-gp activity. Cells were loaded with Rh123 dye in the presence or absence of 50 μ M verapamil(Vp) for an hour. Dye efflux was measured by flow cytometry. Each panel shows a representative graph of fluorescence from three independent trials. (A) KG-1a, (B) HL-60, and (C) THP-1. (D) KG-1a cells were treated with 50 μ M of Vp alone, 3 μ M of 17-AAG or a combination of both. After 24 hours, cell cycle was measured by flow cytometry. Results depicted are the means from three independent trials each performed in triplicate. For statistical significance, analysis of variance was performed between the treatments followed by pair-wise comparison with Tukey's test. (E) Cells from the same cultures were incubated an additional 24 hours. At this time, they were labeled with annexin V and 7-aminoactinomycin D. Graph shows percentage of cells undergoing apoptosis and cellular death. Data is from three independent trials (mean + S.E.M.). Kruskal-Wallis one way analysis of variance on ranks was performed followed by Tukey's test for pair-wise testing. (*) indicates $p < 0.05$ as compared to the vehicle control (**) indicates $p < 0.05$ as compared to single treatment.