Effects of 17-Allylamino-17-Demethoxygeldanamycin (17-AAG) on Pediatric Acute Lymphoblastic Leukemia (ALL) with Respect to Bcr-Abl Status and Imatinib Mesylate Sensitivity

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ABSTRACT

As more and more effective targeted therapeutics have been developed to treat adults with cancer, it is of critical importance to devise appropriate *in vitro* experimental models to study their use in pediatric patients. Acute lymphoblastic leukemia (ALL) with Bcr-Abl translocation is one of the most difficult to treat and deadly diseases in children. The targeted kinase inhibitor imatinib mesylate has been shown to induce an initial response but resistance often develops. Recently, the geldanamycin family of antibiotics has been found to induce apoptosis in many malignant cells, including adult CML and AML. We describe experiments in which 17-allylamino-17-demethoxygeldanamycin (17-AAG) was evaluated in the context of Bcr-Abl and resistance to imatinib mesylate. Pediatric ALL cell lines with varying Bcr-Abl status and imatinib mesylate sensitivity were generated and their growth inhibition by 17-AAG was studied *in vitro*. Western blots were used to follow the changes in proteins that correlate with cell survival. Results show that apoptosis was induced in all lines with an increased 50% inhibitory concentration (IC₅₀) for Bcr-Abl positive but imatinib mesylate-resistant cells. Addition of

17-AAG greatly increased imatinib sensitivity *in vitro*. A decrease in p53, survivin, Her2/neu, and WT1 was seen in cells that expressed these proteins. With some notable exceptions, when combined with 17-AAG, the IC₅₀ of most of the common chemotherapeutic agents decreased. We describe an experimental approach to investigate the complex interaction between Bcr-Abl status, imatinib mesylate sensitivity, and 17-AAG in pediatric ALL. Information from such an approach will provide means to devise combined treatment approaches and to follow their effectiveness *in vitro*. (*Pediatr Res* 57: 430–437, 2005)

Abbreviations

ALL, acute lymphoblastic leukemia
AML, acute myeloid leukemia
CML, chronic myeloid leukemia
FISH, fluorescent *in situ* hybridization
IC₅₀, 50% inhibitory concentration
Ph, Philadelphia

Despite the impressive therapeutic advances of the last 30 years, a quarter of the children with ALL still die from their disease (1). Attempts to cure the patients at greatest risk of treatment failure have included intensification of chemotherapeutic regimens and the use of bone marrow transplantation. In recent years, an increasing knowledge of the molecular mechanisms involved in leukemic cell growth has shifted the focus of new antileukemic drug development from empirical antineoplastics to agents that act on specific molecular targets. Such

targets include individual proteins that regulate signal transduction, apoptosis, and cell cycle progression and those that confer oncogenic potential such as Bcr-Abl (2).

The Philadelphia chromosome is a product of a t(9;22)(q34;q11) translocation that fuses the c-Abl gene on chromosome 9 with the Bcr gene on chromosome 22 (3). This abnormality is observed in 3–5% of children with ALL, and it is one of the most difficult to treat among all childhood leukemias (1,4). The use of the novel kinase inhibitor imatinib mesylate (Gleevec, STI571; Novartis, Basel, Switzerland) has shown effectiveness in relapsed Ph+ ALL (5), but adult studies have shown significant potential for recurrence (6).

It is widely known that the survival and proliferation of leukemic cells are facilitated by an interactive network of signaling pathways (7). Studies on solid tumors provide evidence that the disruption of one or two members of this network may not be enough to completely abrogate the malig-

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nant phenotype (8). Therefore, it stands to reason that drugs that are capable of interacting with multiple components in signal transduction pathways of leukemic cells can effectively overcome the limitations associated with single targeted therapeutics. The benzoquinone ansamycin antibiotics represent a class of drugs that is capable of affecting multiple targets that are critical for the survival and proliferation of malignant cells (9).

Geldanamycin (GA) and its less toxic derivative, 17-allylamino-17-demethoxygeldanamycin (17-AAG), are representatives of a group of such agents. They have been shown to achieve significant antitumor effects by their tenacity in binding to heat shock protein 90 (Hsp90), a molecular chaperone (9–11). Under normal circumstances, Hsp90 functions as a chaperone for a number of kinases, cell surface receptors, and transcription factors that are involved in cell signaling, proliferation, and cell survival (12,13). Interaction with Hsp90 not only facilitates intracellular trafficking but also helps such molecules to avoid abnormal folding and aggregation so they remain in active configuration (13,14). Association with Hsp90 also protects the client proteins from endogenous proteases and allows them to maintain their respective growth promoting functions (15,16).

The effects of 17-AAG on cell functions are of particular interest with respect to Ph⁺ leukemias as Bcr-Abl has been shown to be a client protein for Hsp90 (16–21). Treatment with 17-AAG results in significant down-regulation of intracellular levels of Bcr-Abl and the induction of apoptosis (13,20). So far, investigations into the effects of 17-AAG have been performed mostly with adult leukemias such as CML blasts, AML cell lines, and the Bcr-Abl–positive variant of the erythroleukemic cell line K562 (13,20).

This report describes our initial findings regarding the apoptotic function of 17-AAG, its combined effect with imatinib, and other chemotherapeutic agents. In addition, we describe 17-AAG–induced changes in some of the intracellular proteins that have been implicated in the survival and growth of leukemic cells. Results presented indicate the potential of 17-AAG to be an effective antileukemic agent in pediatric patients alone or in combination with other chemotherapeutic agents. We also provide evidence that 17-AAG has the potential to sensitize imatinib treatment in Bcr-Abl–positive leukemias.

MATERIALS AND METHODS

Cells and cell lines. After approval from the institutional review board and written informed consent was gained from subjects, leukemic and normal bone marrow cells were obtained. All cell lines were established from peripheral blasts from pediatric patients who were initially diagnosed with pre-B ALL and had subsequently relapsed after conventional chemotherapy. Cell lines 1 and 3 were established from patients with pre-B ALL that had no reported Bcr-Abl fusion product. Cell line 2 was established from a patient with Ph⁺ pre-B ALL who relapsed after chemotherapy. Cell line 4 was established from a Ph⁺ pre-B ALL patient who relapsed after conventional chemotherapy and bone marrow transplant. The patient was then treated with imatinib, went into remission but relapsed again while solely on imatinib for 6 mo. The cell line was established at this point in time. All these cells were maintained in culture for at least 6 mo and single-cell subcloned at least twice. Cell lines were maintained in OPTI-MEM culture media (Invitrogen, Burlington, ON, Canada) with 5% FCS (Invitrogen). Stromal cells from normal marrow cells were established as described previously (22). Bcr-Abl status in the ALL cell lines were analyzed by FISH using DNA probes (LSI Bcr/Abl ES dual-color translocation probe; Vysis, Downers Grove, IL). The spanning Abl probe (orange) is approximately 650 kb extending from an area centromeric of the

Ass gene to well telomeric of the last Abl exon. The Bcr probe (green) is approximately 300 kb beginning between Bcr exons 13 and 14 and extending well beyond the m-bcr region. Hybridization procedure was carried out as described by the probe manufacturer.

Cell growth inhibition assay. ALL cells were cultured in 96-well plates at 10⁴ cells/well with appropriate drug treatment (see individual figure legends) to a final volume of 200 μ L/well. After 4 d in culture, viable cells were quantified by Alamar blue assay (Medicorp, Montreal, QC, Canada), according to the manufacturer's protocol. Percentage survival was calculated as follows:

$$\% \text{ survival} = \frac{\text{Absorbance 570nm} - 620\text{nm test well}}{\text{Absorbance 570nm} - 620\text{nm DMSO control well}} \times 100$$

For chemosensitivity assays, individual agents were diluted and added alone or in combination with 17-AAG (0.1 μ M final concentration) to 96-well plates with 10⁴ cells/well. Final volume in all wells was again 200 μ L. Cell growth was measured after 4 d in culture by Alamar blue assay and an IC₅₀ for each agent with and without 17-AAG was calculated.

Antileukemic agents. 17-AAG was purchased from A.G. Scientific, Inc. (San Diego, CA), dissolved in DMSO at a concentration of 17 mM, aliquoted, and stored at –20°C. Imatinib mesylate and all chemotherapeutic agents were obtained from the oncology pharmacy of the Alberta Children's Hospital.

Detection of apoptosis. ALL cells were grown in 24-well plates at 10⁵ cells/well with and without 1 μ M 17-AAG. After 24 h in culture, the cells were stained with a dye (Apo-percentage, Accurate Chemical & Scientific Corporation, Westbury, NY) that specifically detects apoptotic cells. The staining process was done according to the manufacturer's instructions. Representative areas were photographed using a camera attached to an inverted microscope (Zeiss AxioCam, Carl Zeiss Canada, Toronto, ON, Canada).

Immunoblotting. Cells were cultured in 24-well plates as described above in apoptosis assay. After 12–24 h in culture, the cells were harvested and cell lysates were analyzed by Western blot technique. Following are the antibodies to the proteins investigated and their corresponding manufacturers: c-Abl (Oncogene, San Diego, CA), Hsp90 (Stressgen, Victoria, BC, Canada), Her2/neu (DakoCytomation, Mississauga, ON, Canada), WT1 (DakoCytomation), p53 (Oncogene), survivin (R & D Systems, Minneapolis, MN), and actin (Sigma Chemical Co., Saint Louis, MO).

RESULTS

Bcr-Abl status of cell lines. FISH analysis of exponentially growing cells was carried out as described in "Materials and Methods." Data presented in Figure 1A show that cell line 1 has two copies each of Bcr and Abl, cell line 3 has multiple copies of Bcr and Abl, and both cell lines 2 and 4 have Bcr-Abl fusions. Proteins in the lysates from these cell lines were separated by SDS-PAGE and analyzed for proteins that reacted with anti c-Abl antibodies by Western blot analysis. Results shown in Figure 1B show cell lines 1 and 3 with a protein band of a lower molecular weight corresponding to c-Abl (145 kD) and lines 2 and 4 with a larger protein band corresponding to the Bcr-Abl fusion protein (approximately 190 kD), confirming the FISH analysis data.

Sensitivity to 17-AAG treatment. In the next set of experiments, we analyzed how these four cell lines responded to 17-AAG. Cells were incubated with increasing concentrations of 17-AAG and cell viability was measured by Alamar blue assay. Results presented in Figure 2 show that 17-AAG was able to inhibit the growth of these cells irrespective of their Bcr-Abl status, with an IC₅₀ of approximately 1 μ M. The IC₅₀ for cell line 4 was found to be higher, at 2 μ M. This figure also shows that, in this concentration range, 17-AAG has no effect on normal stromal cells. We then looked at the effect of 17-AAG in cells treated with imatinib. Our previous experiments have shown that under our culture conditions, imatinib treatment remains specific at 0.1 μ M concentrations and, at higher doses (>1 μ M), Bcr-Abl–negative cells and normal

Figure 1. Bcr-Abl status of cell lines. (A) FISH analysis on exponentially growing cells. Bcr stains green. Abl stains orange. This figure shows that cell line 1 has 2 copies of Bcr and Abl, cell line 3 has multiple copies of Bcr and Abl and cell lines 2 and 4 have Bcr-Abl fusion genes. (B) Western blot analysis of cell lysates probed with anti-c-Abl confirms results. Cell lines 1 and 3 have c-Abl protein and cell lines 2 and 4 have the larger Bcr-Abl protein.

Figure 2. Sensitivity to 17-AAG treatment. Cell lines were treated with varying concentrations of 17-AAG (10–0.01 μ M) for 96 h. Cell survival was measured by Alamar blue assay. Percentage cell survival was determined by the absorbance of Alamar blue by treated cells compared with DMSO-treated control cells. IC_{50} for cell lines 1, 2, and 3 is shown to be 1 μ M and for cell line 4 is about 2 μ M. Normal stroma was unaffected by 17-AAG treatment at these concentrations. Representative results were obtained in three separate experiments.

cells show nonspecific cell death (data not shown). Figure 3 shows the effects of these two drugs together on all four cell lines. As expected, the Bcr-Abl-negative cell lines 1 and 3 were not affected by imatinib mesylate. Inhibition of growth

Figure 3. Sensitivity to imatinib and 17-AAG combined treatment. Cell lines were treated with 0.1 μ M imatinib alone, 1.0 μ M 17-AAG alone, and 0.1 μ M imatinib plus 1.0 μ M 17-AAG. After 96 h, cell survival was measured by Alamar blue assay. Percentage cell survival was determined by the absorbance of Alamar blue by treated cells compared with DMSO-treated control cells. Cell lines 1 and 3 show resistance to imatinib, and combined treatment of 17-AAG and imatinib does not increase the effectiveness of 17-AAG alone. Cell line 2 shows sensitivity to imatinib and combined treatment of imatinib and 17-AAG increases the effectiveness in comparison to either drug alone. Cell line 4 shows decreased sensitivity to imatinib; however, combined treatment of 17-AAG and imatinib increases the effectiveness of both drugs alone. Representative results were obtained in three separate experiments. * $p < 0.05$.

was induced by 17-AAG in these two cell lines, which was not altered by the addition of imatinib. However, the combined effect was significantly higher ($p < 0.05$) in the Bcr-Abl cell lines (lines 2 and 4) compared with either drug alone. In the imatinib mesylate-sensitive cell line 2, a significant combined effect was seen that decreased the percentage survival from approximately 45% to <10% at the concentrations tested. In cell line 4, which shows a greater resistance to imatinib mesylate, cell survival was reduced to about 40% with the combination of drugs, compared with 70% with 17-AAG alone.

17-AAG induces apoptosis in ALL cell lines. The results displayed in Figure 4 confirm that the ALL cells are undergoing apoptosis as a result of the treatment with 17-AAG. After incubation with 1 μ M 17-AAG for 24 h, cells were stained with an apoptosis-specific stain. Compared with control cells, cells treated with 17-AAG are highly stained in all four cell lines.

17-AAG induces changes in protein levels that have been implicated in leukemia cell growth, survival, and apoptosis. A number of intracellular proteins are involved in the sustained and unregulated growth of leukemia cells and some of these proteins have been shown to be clients of Hsp90. We wanted to investigate how Bcr-Abl status and sensitivity to imatinib may differ in regulation of these proteins in response to 17-AAG. Cell lines 1–4 were treated with 17-AAG for 12–24 h and the cell lysates were compared with that of untreated cells by Western blot analysis. Results are shown in Figure 5. It was found that Bcr-Abl cell lines (2 and 4) down-regulated p53 and Her2/neu. Survivin and WT1 were found to be reduced in all four cell lines in the presence of 17-AAG. We observed no

Figure 4. Induction of apoptosis by 17-AAG. Cell lines were treated overnight with 1.0 μ M 17-AAG or no treatment and stained with a dye that selectively stains cells undergoing apoptosis. Representative areas were photographed under an inverted microscope. All four cell lines show stained cells, indicating apoptosis in the treatment group compared with no treatment control. Data representative of three separate experiments.

Figure 5. Differential protein expression after treatment with 17-AAG. Cell lines were treated with 1.0 μ M 17-AAG or no treatment for 12–24 h and cell lysates were prepared. Protein expression was determined by Western blot analysis by probing with antibodies to Hsp90, Her2/neu, WT1, p53, and survivin. Actin was included as a protein loading control. Results from experiments with no treatment were identical to those treated with appropriate dilutions of DMSO (results not shown).

significant changes in Hsp90 under this particular experimental condition. Actin levels also remained constant indicating that the loss of these proteins is not secondary to cell death.

17-AAG alters the effect of chemotherapeutic agents. In the next series of experiments, we examined how 17-AAG modifies the effect of selective antileukemic chemotherapeutic agents based on the Bcr-Abl status and imatinib sensitivity of the particular cell type. Cells were incubated with increasing

concentrations of chemotherapeutic agents, with or without 0.1 μ M of 17-AAG, for 4 d. The cell proliferation was measured by Alamar blue assay and the IC_{50} for each drug was calculated. Table 1 shows how the IC_{50} for each agent changed in the presence of 17-AAG for a given cell line. As a general trend, the combination with 17-AAG appears to decrease the IC_{50} for most of the agents. However, both Bcr-Abl cell lines show no change with cytarabine chemosensitivity. Vincristine sensitivity was not altered in one of the Bcr-Abl negative lines (line 1) and in line 4, which is Bcr-Abl positive and is resistant to imatinib. All four cell lines were fairly resistant to topotecan with an IC_{50} >50–100 μ g/mL. However, in cell line 2, IC_{50} for topotecan was substantially reduced to 0.01 μ g/mL in the presence of 17-AAG. A similar trend was also seen with cyclophosphamide where in lines 1 and 2, the IC_{50} changed significantly in the presence of 17-AAG.

DISCUSSION

Clinical studies have shown that the Philadelphia chromosome translocation, t(9;22)(q34;q11), is an independent risk factor in childhood ALL (23). At the molecular level this results in the fusion of Bcr and Abl sequences leading to a chimeric mRNA and the Bcr-Abl protein. The leukemia-promoting function of this protein resides in its deregulated tyrosine kinase activity (24). Imatinib mesylate is an inhibitor of Bcr-Abl tyrosine kinase that has been shown to be highly effective in the treatment of Bcr-Abl-positive CML (25). Imatinib has also been shown to induce undetectable minimal residual disease in a small cohort of pediatric patients with Ph+ leukemia (26). However, in patients with acute leukemias, emergence of treatment resistance has been a major problem (27–32). A number of recent publications have described the possible mechanisms by which imatinib resistance may be generated (27,33–35). These include overexpression, mutations in Bcr-Abl gene, and the loss of Bcr-Abl expression. This emphasizes the importance of exploring novel therapeutic agents in the treatment Ph+ leukemia.

The ansamycin antibiotic GA and its derivative 17-AAG bind to Hsp90, causing destabilization of various Hsp-dependent oncogenic molecules, including Bcr-Abl, that facilitate survival and proliferation of malignant cells. This well-known property of 17-AAG suggests it has potential to interfere with the cellular machinery involving Ph+ cells and the development of imatinib resistance. It has been thought previously that benzoquinone ansamycins inhibited Bcr-Abl enzyme activity by direct binding through SH groups in the kinase. Recently, a report by An and colleagues (36) has provided an alternative explanation showing that Bcr-Abl protein in leukemic cells exists as a stable complex with Hsp90, and this association is interrupted by GA. It was also noted that this dissociation is followed by a rapid decline in the steady state level of Bcr-Abl protein. Current data indicate that gene amplification that results in stable and increased levels of Bcr-Abl protein as well as mutations in the drug binding region are largely responsible for the induction of imatinib mesylate resistance (37,38). Using retroviral-mediated transfection experiments in the murine hematopoietic cell line Ba/F3, Gorre

Table 1. 17-AAG and other chemotherapeutic agents

Cell line	Chemotherapy	IC ₅₀ (μg/mL)	IC ₅₀ + 0.1 μM 17-AAG (μg/mL)	Effect of 17-AAG on IC ₅₀
1	Cytarabine	10	0.5	Decrease
	Carboplatinum	0.5	0.1	Decrease
	Topotecan	50	0.01	Decrease
	Vincristine	2	2	No change
	Cyclophosphomide	>100	0.01	Decrease
	Cisplatin	<0.01	<0.01	—
	Methotrexate	<0.01	<0.01	—
	Etoposide	90	9	Decrease
2	Cytarabine	30	30	No change
	Carboplatinum	0.1	<0.01	Decrease
	Topotecan	100	0.01	Decrease
	Vincristine	1	0.1	Decrease
	Cyclophosphomide	50	0.01	Decrease
	Cisplatin	<0.01	<0.01	—
	Methotrexate	<0.01	<0.01	—
	Etoposide	1	0.1	Decrease
3	Cytarabine	0.1	<0.01	Decrease
	Carboplatinum	0.5	0.01	Decrease
	Topotecan	>100	>100	—
	Vincristine	5	0.5	Decrease
	Cyclophosphomide	>100	>100	—
	Cisplatin	<0.01	<0.01	—
	Methotrexate	<0.01	<0.01	—
	Etoposide	10	5	Decrease
4	Cytarabine	5	5	No change
	Carboplatinum	1	0.5	Decrease
	Topotecan	>100	>100	—
	Vincristine	10	10	No change
	Cyclophosphomide	>100	>100	—
	Cisplatin	0.01	<0.01	Decrease
	Methotrexate	0.05	0.01	Decrease
	Etoposide	30	1	Decrease

Cell lines were treated with increasing concentrations (concentration range of 0.01–100 μg/mL) of common pediatric chemotherapeutic agents, including cytarabine, carboplatinum, topotecan, vincristine, cyclophosphomide, cisplatin, methotrexate, and etoposide, with and without 0.1 μM 17-AAG. IC₅₀ was calculated for chemotherapy agent alone or with 0.1 μM 17-AAG.

and colleagues (17) have investigated the effect of 17-AAG on cells that have been rendered imatinib mesylate resistant by mutant Bcr-Abl. This study has found that 17-AAG affected both wild-type Bcr-Abl as well as mutant Bcr-Abl containing (and consequently imatinib mesylate resistant) cells and its activity is substantially increased in the latter. In addition, using the phosphorylation status of CRCL, which is an indicator of downstream Bcr-Abl signaling, the significant functional impact of 17-AAG on these imatinib-resistant cells has also been demonstrated. Based on these observations, a rationale for the use of 17-AAG in the clinical setting with respect to imatinib resistance has been proposed (17). A similar conclusion was also drawn from the extensive studies of Nimmanapalli and colleagues (20), who investigated the effect of 17-AAG on human leukemic cells. The facts that 17-AAG and imatinib mesylate inhibit Bcr-Abl activity by different mechanisms and 17-AAG is effective in cells with molecular mechanisms that instill resistance to imatinib provide a rationale for the use of 17-AAG in the treatment of children with Bcr-Abl-positive leukemias, with or before development of imatinib resistance.

The purpose of this study is to develop an experimental model to investigate the complex interaction between Bcr-Abl, imatinib resistance, and 17-AAG with special reference to Ph+ pediatric ALL. As an initial step, we have established cell lines from children who have been diagnosed with the most common form of ALL, pre-B ALL. We have then selected the lines to represent Ph– as well as Ph+ with differential sensitivity to imatinib mesylate. As a control, we have also included a line (cell line 3) that has multiple Bcr and Abl copies but no fusion protein (Fig. 1). We then investigated the effect of 17-AAG on these cell lines. Previous *in vitro* studies have shown that 17-AAG is effective against many different types of cancer cells. This includes human AML HL-60, K562, CML blasts (20), human prostate cancer (39), breast cancer (40), colon cancer (41), melanoma (42), and ovarian cancer cells (43). The parent compound of 17-AAG, GA, has also been shown to induce apoptosis in neuroblastoma cells (44). Our results, presented in Figure 2, show the effectiveness of 17-AAG against all four cell lines, with an IC₅₀ of approximately 1 μM. Cell line 4, which expresses Bcr-Abl and is resistant to imatinib, showed a marginally enhanced IC₅₀ of about 2 μM.

Topaly and co-workers (45) have reported that cross-resistance between the two compounds in Bcr-Abl overexpressing cells have resulted in an increased IC_{50} for 17-AAG. This is thought to be due to the requirement for increased doses of 17-AAG to inhibit of Hsp90 in the presence of increased Bcr-Abl protein. In our studies, line 4 did not express increased Bcr-Abl protein as judged by Western blot analysis (Fig. 1B). We are currently investigating the presence of other variables, such as a mutation in this line or another mechanism, responsible for this phenomenon. We chose normal stromal cells to look at the nonspecific effects of 17-AAG. These cells are nontransformed but, like the cell lines, show significant cell division and growth in culture. In addition, unlike normal lymphocytes, stromal cells do not undergo rapid cell death in culture. Results, shown in Figure 2, demonstrate that at 1 μ M concentration normal stromal cells are unaffected by 17-AAG.

Ansamycins cause the degradation of a group of proteins that require Hsp90 for conformational maturation (46,47). We have chosen to investigate the changes in five distinct proteins in the presence of 17-AAG based on relevant previous studies and their ability to provide specific *in vitro* data to follow drug action (see below for a detailed discussion). These include Hsp90, to show that the effects are not due to nonspecific degradation of Hsp90; HER2/neu; WT1 and p53, for their previously reported role in leukemia cell growth; and, finally, survivin for its ability to gauge changes in antiapoptotic potential in malignant cells.

Previous studies have shown a change in a number of critical regulators of cell growth and apoptosis after exposure to 17-AAG. For example, Basso and co-workers (48) have shown a sharp decrease in Akt protein levels within 12–24 h of exposure to 17-AAG. Hostein and colleagues (41) have investigated the effect of 17-AAG on a number of critical signal transduction components of malignant cells. This includes the demonstration of a decrease in c-Raf-1, N-ras, Ki-ras, and c-Akt and the inhibition of ERK-1/2 phosphorylation. We investigated how some other intracellular proteins that have been shown to confer malignant potential are altered in the presence of 17-AAG. This is to provide insight into the unique mechanism that may be operational in each cell line. We have found that the expression of Her2/neu is decreased in cell lines 2 and 4. However, examination of a large number of pediatric ALL lines and fresh leukemic cells showed that Her2/neu has no direct relationship to the presence of Bcr-Abl fusion proteins (data not shown). In this study, the two cell lines that expressed Her2/neu showed a decrease in the level of this protein. Similar findings have been reported for other malignant cells (49). It is not clear whether this effect is secondary to the disruption of Her2/neu–Hsp90 complex or due to some yet unidentified mechanism.

The tumor suppressor gene product p53 is an important regulator of cellular response to stress and DNA damage. In normal cells, p53 is found in very low levels due to its rapid degradation but becomes stable in response to different stress stimuli (50). The mutational status of p53 in the cell lines used in this study is currently being investigated. However, a direct influence of Bcr-Abl on cellular p53 levels in lines 2 and 4 cannot be ruled out. For example, a marked down-regulation of

p53 level in a Bcr-Abl-expressing cells has been reported previously (51). Results shown in Figure 5 indicate a) an increased p53 baseline presence in lines 1 and 3 and b) a decrease in cellular p53 level after 17-AAG treatment in cell lines 2 and 4. This observation is somewhat unexpected as previous studies have shown that Hsp90 mediated stabilization is found mostly in mutant p53 containing cells (52). On the other hand, p53 is a structurally unstable protein that undergoes conformational changes under a wide variety of physiologically stressful conditions. Therefore, it is possible that in these transformed cells Hsp90 dependency exists for p53 that can be interfered with by 17-AAG, but further experiments are needed to test this possibility. Gene sequencing and transfection experiments are currently in progress, with the specific aim of understanding the effect of Bcr-Abl on p53 stability and sensitivity to 17-AAG.

Expression of WT1 has been described in ALL and AML and the increased levels of WT1 mRNA has been shown to be associated with a worse long-term outcome in some studies (53). Whether the increased WT1 expression is a nonspecific phenomenon resulting from malignant transformation or whether it has implications for leukemic growth is controversial. In our studies, a decrease in WT1 protein level has been noted in all cell lines in the presence of 17-AAG (Fig. 5). However, interaction of WT1 with HSP90 has not been widely reported. Our results could also reflect the finding that the expression of WT-1 frequently disappears from bone marrow of leukemia patients in complete remission (54).

Survivin, an inhibitor of apoptosis (IAP) gene family protein, is overexpressed in almost all malignant cells with critical roles in mitotic control and apoptosis inhibition (55). In HeLa and B lymphoblastoid Raji cells, the disruption of the survivin–Hsp90 interaction has been shown to destabilize survivin, and to initiate mitochondrial apoptosis (56). Our data show a reduction of survivin after treatment with 17-AAG (Fig. 5), providing further evidence for a similar mechanism in these cells. It has been suggested that in cancer cells the Hsp90-survivin interaction may provide a broad permissive environment inhibiting apoptosis and eliminating mitotic checkpoints. In this regard, antagonism of this interaction by 17-AAG may provide a means to critically lower this antiapoptotic threshold and promote targeted destruction of leukemic cells.

Imatinib has been shown to induce a high rate of remissions in CML, but its effectiveness in Ph+ ALL is not long lasting (57). One viable strategy to overcome the generation of resistance to imatinib mesylate is to take advantage of other vulnerabilities of the Bcr-Abl protein, such as its dependence on the molecular chaperone Hsp90. A number of recent studies have shown effectiveness of 17-AAG against many adult leukemias with Bcr-Abl (17,58). However, cross-resistance of imatinib and 17-AAG in imatinib-resistant cells that over express Bcr-Abl has also been reported (45).

In children, drug-related toxicities often limit dose escalation that is needed to treat aggressive malignancies such as Bcr-Abl-positive leukemia. To explore this, we examined the *in vitro* cytotoxic effects of 17-AAG in combination with commonly used antileukemic agents. Results presented in Table 1 show the reduction of IC_{50} of many of these agents in all

cell lines. However, some interesting variations were also noted. For example, irrespective of their imatinib sensitivity status, both Bcr-Abl positive cell lines showed no change in cytarabine chemosensitivity. Interestingly, a synergistic effect of cytarabine with imatinib has been reported previously (59). All cell lines needed fairly high concentrations of topotecan with an $IC_{50} > 50\text{--}100\text{ }\mu\text{g/mL}$. However, in cell line 2, which is Bcr-Abl positive and sensitive to imatinib, significant cytotoxicity was seen with much less topotecan (IC_{50} approximately $0.01\text{ }\mu\text{g/mL}$). Overall, the addition of 17-AAG appears to reduce the IC_{50} of all chemotherapeutic agents tested but significant individual variability is also seen in their response patterns. Reasons for this finding may be complex. It is conceivable that these cell lines have unique molecular properties in addition to their Bcr-Abl status and such differences could differentially affect their ability to be killed by different chemotherapeutic agents (60,61). Secondly, the differential usage of distinct Hsp90-dependent or -independent apoptotic pathways used by different antileukemic agents could also affect the final sensitivity to that particular drug in the presence of 17-AAG. Finally, the patients from whom these cell lines were derived may have been exposed to different levels of these drugs giving rise to other variables between them such as the expression of multi-drug resistance (MDR) proteins. We believe that the experimental model suggested in this study will provide an effective way to further study these questions and to evaluate *in vitro* drug sensitivity profiles on individual patients.

In summary, we have explored an experimental model to study some of the complex issues that arise in the use of targeted therapeutics. We have shown that 17-AAG induces apoptosis in pediatric leukemic cells and that a combination of imatinib and 17-AAG can potentially be used before the emergence of resistance to imatinib in Bcr-Abl cells. We have also identified the expression of proteins such as survivin that can be used to follow the activity of 17-AAG *in vitro*. Finally, we demonstrated the feasibility of a combined treatment protocol that can be derived from analyzing the chemosensitivity profiles of different agents. It is possible that the individual lines we selected may not be completely representative of all the phenotypes possible for this chromosomal abnormality. Studies are currently in progress with a larger number of pediatric ALL lines as well as with fresh leukemic cells to further understand such possibilities.

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