Significance of heat-shock protein (HSP) 90 expression in acute myeloid leukemia cells

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Abstract The 90-kDa heat shock protein (HSP90) is implicated in the conformational maturation and stabilization of a variety of client proteins with receptor and signal transduction functions. The objective of this study was to assess its expression in primary acute myeloid leukemia (AML) cells and to evaluate its biological and clinical significance. The *in vitro* effects of 17-AAG, a selective inhibitor of HSP90, was also evaluated. Cells from 65 patients with newly diagnosed AML were studied. The expression of HSP90 correlated with that of CD34, p170, and bcl-2 proteins but not with white cell counts, FAB or WHO subtype, or cytogenetics. HSP90 levels were also higher in samples exhibiting an autonomous growth in liquid culture or forming spontaneous colonies. A concomitant constitutive activation of the extracellular signal-regulated kinase and phosphatidylinositol 3-kinase/AKT pathways was observed in a majority of samples and was significantly correlated with HSP90 expression. All patients received induction chemotherapy. The percentages of HSP90-, CD34-, bcl-2-, and p170-positive cells were higher in patients who did not attain complete remission. Survival was also shorter in patients with high levels of HSP90. *In vitro* exposure of leukemic cells to 17-allylamino-demethoxy geldanamycin (17-AAG) resulted in inhibition

of growth in liquid and clonogenic cultures and in apoptosis, at concentrations which in most cases were not toxic for normal CD34-positive or progenitor cells. The concentration inhibiting 50% growth at 72 h in liquid culture correlated with HSP90 expression. Our study suggests that HSP90 is overexpressed in poor-prognosis AML cells and plays a role in cell survival and resistance to chemotherapy. Targeted therapy with 17-AAG represents a promising antileukemic strategy in adult AML.

Keywords HSP90 · Expression · Acute Myeloid Leukemia

Introduction

Heat shock proteins (HSP) are a group of structurally unrelated proteins, which act as molecular chaperones to ensure the proper folding of synthesized proteins or their refolding under denaturing conditions (Lindquist and Craig 1998). They also play a role in protein degradation via the proteasome machinery (Schneider et al. 1996). A member of the HSP family, HSP90, is abundantly expressed in the cytoplasm of most human cells, even in the absence of stress. HSP90 exists in two main isoforms (HSP90 α , inducible, and HSP90 β , constitutive; Young et al. 2001). It exerts its role by forming a multiprotein complex with ATPase activity, in cooperation with cochaperones, including HSP70 (Young et al. 2001; Hernandez et al. 2002). HSP90 clients are implicated in cell cycling, receptor function, signal transduction, and apoptosis (Stancato et al. 1993; Sato et al. 2000; Fujita et al. 2002; Basso et al. 2002; Pratt and Toft 2003). In cancer cells, HSP90 is necessary for the maintenance of transformed proteins such as mutated c-kit, flt3, and bcr-abl (An et al. 2000; Minami et al. 2002; Fumo et al. 2004). Indeed, high HSP90 α or HSP messenger ribonucleic acid

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(RNA) levels have been reported in many cancer cells, such as pancreatic carcinomas, breast cancer, ovarian cancer, lung cancer, renal cancer, and gastric cancer (reviewed in Ochel and Gademan 2002). Limited data are available regarding the expression of HSP90 in leukemic cells: High expression of HSP90 protein and HSP90 α RNA has been reported by Yufu et al. (1992) in leukemia cell lines and a small series of acute leukemia cells. We recently described the high expression of HSP including HSP90 in a larger series of 110 patients with acute myeloid leukemia (AML), which was associated with a trend to poor prognosis in patients exhibiting the highest percentage of HSP27-, HSP70-, and HSP90-positive cells (Thomas et al. 2005). In addition, some studies showed that HSP90 detected in cancer cells is mostly in an activated (complexed) form, whereas in nonmalignant cells, only a small part of HSP90 is activated (Kamal et al. 2003).

HSP90 activation and functional properties necessitate the binding of adenosine triphosphate (ATP) to a specific pocket. The benzoquinone ansamycins herbimycin A and geldanamycin are potent inhibitors of HSP90, by binding tightly to the ATP pocket and preventing the formation of an active HSP90 complex (Grenert et al. 1997). The less toxic geldanamycin derivative 17-allylamino-demethoxy geldanamycin (17-AAG) presents a much higher (up to 100-fold) affinity for HSP90 complexes than for uncomplexed HSP90, which confers to this drug a highly specific antitumoral activity (Schultze and Neckers 1998). 17-AAG exhibits a potent activity against leukemic cell lines, particularly those harboring a FLT3 mutation (Minami et al. 2002). In this paper, we report on the biological and clinical significance of HSP90 expression in AML cells and on the antiproliferative and proapoptotic activity of 17-AAG.

Materials and methods

Patients and treatments

Sixty-five adult patients aged 21 to 78 years (median 60 years) with AML at diagnosis were included into this study, after giving an informed consent. Diagnosis was performed by cytological and cytochemical methods and confirmed by flow cytometry, according to the World Health Organization recommendations (Harris et al. 1999). Cytogenetics were available in 63 cases, 36 presenting with normal karyotypes, three with t(8:21) translocation, one with chromosome 16 inversion, nine with complex or chromosomes 5 or 7 anomalies, and 14 with other anomalies. FLT3 internal tandem duplication (ITD) was tested as described (Libura et al. 2003) and was detected in 4 of 40 tested samples. All patients received an intensive induction. Patients up to 60 years were treated according to the Groupe Ouest Est des Leucémies et Autres Maladies du Sang (GOELAMS)

2001 protocol, with an induction by idarubicin 12 mg m⁻² day⁻¹ for 5 days or daunorubicin 60 mg m⁻² day⁻¹ for 3 days and cytarabine 200 mg m⁻² day⁻¹ for 7 days, with a second induction by idarubicin 8 mg m⁻² day⁻¹ for 2 days or daunorubicin 35 mg m⁻² day⁻¹ for 2 days and cytarabine 2 g m⁻² day⁻¹ for 4 days when the blast percentage at day 15 was higher than 5%. Patients in remission received then a first consolidation by idarubicin 12 mg m⁻² day⁻¹ for 2 days or daunorubicin 60 mg m⁻² day⁻¹ for 2 days and cytarabine 100 mg m⁻² day⁻¹ for 5 days, followed by a second consolidation by idarubicin 12 mg m⁻² day⁻¹ for 2 days or daunorubicin 60 mg m⁻² day⁻¹ for 2 days and cytarabine 6 g m⁻² day⁻¹ for 4 days. According to randomization, patients received finally one or two autologous peripheral stem cell transplantations after conditioning by busulphan (16 mg/kg) and melphalan 200 mg/m² (single or second transplant) or melphalan (200 mg/m²; first of two transplants). Patients with a human leukocyte antigen-identical sibling received after the first consolidation an allogeneic stem cell transplantation, with the exception of those with a favorable karyotype. A total of six patients received an allogeneic stem cell transplantation (four in first and two in second remission). Patients more than 60 were treated according to the GOELAMS SA2000 protocol, with an induction by idarubicin 8 mg m⁻² day⁻¹ for 5 days, cytarabine 100 mg m⁻² day⁻¹ for 7 days, and lomustine 45 mg/m², followed after remission by a maintenance treatment with methotrexate and 6-mercaptopurine, with six reinduction treatments by idarubicin and cytarabine every 3 months for a total of 18 months.

Acute myeloid leukemia cells

Cells were collected by bone marrow aspiration into heparin-containing vials, separated on a Ficoll gradient (Eurobio, Les Ulis, France), washed twice with phosphate-buffered saline (PBS), resuspended in Roswell Park Memorial Institute medium (RPMI) 1640 (Eurobio), and incubated for 2 h at 37°C on sterile plastic dishes. Nonadherent cells were then recovered, washed twice in PBS, and immediately processed for further studies.

Normal Bone Marrow cells

Cells were collected by bone marrow aspiration from 12 normal bone marrow donors into heparin-containing vials, separated on a Ficoll gradient (Eurobio, Les Ulis, France), washed twice with PBS, and resuspended in RPMI 1640 (Eurobio).

CD34+ cells

Normal CD34-positive cells were isolated from six normal bone marrow donors, by an immunomagnetic method using

the direct CD34 Progenitor Cell Isolation Kit (Miltenyi Biotec). More than 90% of the isolated cells were CD34+ after cytometry analysis. These cells were cultured in STEM α .A medium (STEM α , Saint-Clément-les-Places, France) in the presence or absence of 17-AAG.

Cultures

Short-term liquid cultures

Leukemic cells were incubated in RPMI 1640 at 37°C in a fully humidified atmosphere with 5% CO₂ in the absence or presence of different concentrations of 17-AAG (or dimethylsulfoxide [DMSO] for control) for 24, 48, and 72 h.

17-AAG was purchased from Sigma-Aldrich (St. Louis, MO, USA), diluted in DMSO, and stored at -20°C before use. After these treatments, cells were washed in PBS, and viable cells were enumerated using a trypan blue exclusion test.

All experiments were performed in triplicate.

Clonogenic assays

Normal mononuclear cells were incubated in triplicate in methylcellulose and growth factor-containing culture medium (STEM α .ID). In some experiments, cultures were performed in the presence of 17-AAG (or DMSO alone for control), which was added into the culture medium at the appropriate final concentration. Granulocyte-macrophage (GM) colony-forming units (CFU) and erythroid blood burst-forming units (BFU-E) were scored after 14 days of incubation. Leukemic colonies (CFU-L) were grown in STEM α .I medium without growth factors to assess spontaneous proliferation and were scored after 7 days.

Antigen expression and flow cytometry

Blast cells were identified by CD45/side scatter analysis.

For intracellular antigen detection, cells were first incubated for 15 min at room temperature with CD45-PE-Cy5 antibody (Beckman-Coulter France, Villepinte, France), washed, and fixed with 3.7% formaldehyde for 20 min. Then, two different protocols were used (Krutzig 2003). The staining of intracellular proteins HSP90, bcl-2, extracellular signal-regulated kinase (ERK) and p-ERK was performed after permeabilization in 0.2% Triton X100 (15 min at room temperature) using conjugated primary antibodies: phycoerythrin (PE)-conjugated antibody for Hsp90 (F-8, sc13-119, Santa Cruz Biotechnology, Santa Cruz, CA, USA), fluorescein isothiocyanate (FITC) conjugated antibody for Bcl-2 (DakoCytomation, Glostrup, Denmark), and PE-conjugated and FITC-conjugated antibodies to ERK and p-ERK, respectively (Santa Cruz Biotechnology). Cells were incubated for 1 h at room

temperature, washed, and resuspended in PBS before analysis. Unconjugated rabbit antibodies were used to Akt and p-Akt (Cell Signaling Technology, Beverly, MA, USA) and to phosphatidylinositol 3-kinase (PI3K; p110 δ , Upstate Biotechnologies, Lake Placid, NY, USA). After CD45 staining and fixation, cells were chilled for 1 min on ice, resuspended in ice-cold methanol, and incubated for 30 min on ice. After washing and incubation for 5 min in an incubation buffer, cells were then incubated with 10 μ l primary antibody at the appropriate titration for 45 min at room temperature, washed, and incubated for 30 min with the FITC-conjugated secondary antibody (goat anti-rabbit, Caltag Laboratories, San Francisco, CA, USA). Controls were performed with an isotypic PE- or FITC-labeled antibody (Beckman-Coulter, France). Finally, cells were resuspended in 1% formaldehyde prior to flow cytometry analysis. Controls were performed with an appropriate nonconjugated isotypic antibody.

Surface antigens were assessed using anti-CD34-FITC- and p170-PE-conjugated antibodies (Beckman Coulter).

Flow cytometry analysis was performed with an EPICS XL (Beckmann-Coulter) flow cytometer. At least 10,000 events were analyzed. Results were expressed as percentage of positive cells or mean fluorescence intensity.

In normal bone marrow, expression of HSP90 in CD34-positive cells was performed using a triple staining technique. Cells were first stained for surface antigens (CD34 and CD45) and secondarily permeabilized as described before for intracellular HSP90 staining.

Apoptosis

Morphology of apoptotic cells

Cytospins preparations of the cell suspensions were stained with Wright-Giemsa, and the morphology of apoptotic cells was determined by light microscopy. Five different fields were selected for counting at least 500 cells.

Annexin V staining

Untreated and drug-treated cells were incubated with Annexin V fluorescein and propidium iodide (PI) (DakoCytomation) in a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer. After incubation for 15 min in the dark, cells were analyzed by flow cytometry. Live cells were determined by PI exclusion. Early apoptotic fraction was determined by annexin V-positive and PI-negative stains.

Activated caspase-3 expression

Cytospins were also used to study the caspase-3 activation by the alkaline phosphatase-anti-alkaline phosphatase

technique. We used an amplification combination of alkaline phosphatase and the avidin-biotinylated enzyme complex (ABC) techniques Vectastain Universal mouse and rabbit kit (Vector Laboratories, Burlingame, CA, USA). Cytospins were fixed for 90 s with acetone at room temperature. They were rehydrated in PBS for 5 min. Nonspecific binding was blocked with horse serum during 20 min. Then, slides were incubated for 30 min with polyclonal rabbit anti-active caspase-3 (Cell Signaling Technology), washed twice in PBS for 5 min and incubated with biotinylated horse anti-mouse or rabbit secondary antibody for 30 minutes, rinsed again for 5 min in PBS, and reincubated for 30 min with biotinylated alkaline phosphatase complex (ABC reagent). After a new step, washing in PBS slides were incubated with an appropriate enzyme substrate solution (Fast red) until optimal red granular reaction (20–30 min) was achieved. Slides were rinsed, respectively, with PBS and distilled water before a counterstain nuclear step consisting of incubation for 7 min with Meyer's hematoxylin (Dako). After washing in distilled water, PBS, and distilled water again, slides were finally mounted in Fluorotech aqueous media (Valbiotech, Paris, France). Controls consisted of replacement of primary rabbit antibody by an irrelevant antibody of the same isotype. Slides were examined using $\times 10$ magnification by two observers. Cells were considered stained if any diffuse reddish cytoplasmic staining could be identified. A scale of three levels of red staining was used to assess intensity of staining: 0 for no staining, 1 for weak staining, 2 for strong staining, and 3 for very strong staining. The percentage of positive cells (strong or very strong staining) was determined after counting 100 cells.

Statistical analysis

A Mann–Whitney rank-sum test was used to compare the means of two groups. Proportions were compared by Chi-square test (or Fisher's test when a group comprised less than 10 units). Correlations were performed using a Spearman rank correlation test.

Survival curves were plotted according to the Kaplan–Meier method. Survival duration of different groups was compared by the log-rank test.

Statistical tests and data plots were performed using the Prim5 software (GraphPad Software, San Diego, CA, USA).

Results

Expression of HSP90 in AML cells

HSP90 expression was heterogeneous in primary AML cells. The percentage of HSP90-positive cells was between 11% and

97% with a median value of 41%. There was no correlation of HSP90 expression to clinical or biological characteristics such as age, presence of extramedullary disease, WHO subtype, CD34 expression, or cytogenetics and a borderline correlation with white blood cell count ($r=0.33$, $p<0.01$).

However, a significant correlation was observed between the expression of HSP90 and that of other markers associated with poor prognosis and resistance to chemotherapy. The percentage of HSP90-positive cells was correlated with that of bcl-2-positive cells ($p<0.01$) and p170-positive cells ($p<0.0005$). The expression of signal transduction proteins was also correlated with that of HSP90. Forty-four cases were positive for PI3K and pAKT expression (at least 20% positive cells). The percentage of HSP90-positive cells was significantly higher in these cases than in those without PI3K/AKT activation ($70\pm 23\%$ vs $39\pm 29\%$, $p<10^{-4}$). The difference was not significant for the AKT common form ($51\pm 30\%$ vs $39\pm 32\%$). Forty cases were also positive for pERK, and exhibited a significantly higher percentage of HSP90-positive cells than those negative ($69\pm 25\%$ vs $36\pm 27\%$, $p<10^{-4}$).

Autonomous growth in the liquid culture and in semisolid medium was studied in 41 samples. After 3 days of culture in the growth factor-deprived medium, the number of viable cells was correlated with the percentage of HSP90-positive cells ($r=0.8$, $p<10^{-5}$). The yield of colonies after the 7-day culture in the semisolid medium in the absence of growth factors was also proportional to the number of HSP90-positive cells ($r=0.92$, $p<10^{-5}$; Fig. 1). Eleven cases were considered not forming spontaneous colonies (less than 10 colonies per 10^5 cells), whereas 30 formed colonies (mean number 325 ± 206 per 10^5 cells). The percentages of HSP90+ and bcl2+ cells were significantly higher in colony-forming cases ($68\pm 23\%$ and $52\pm 27\%$, respectively, vs $23\pm 5\%$ and $19\pm 13\%$, respectively, $p<10^{-5}$ in both cases). Although more weakly, Bcl-2 expression and spontaneous CFU-L formation were also correlated ($r=0.52$, $p<0.05$).

With regard to prognosis, a complete remission (CR) was obtained by induction treatment in 45 of 65 patients. The percentage of HSP90-positive blast cells at diagnosis was significantly lower in the samples from patients in remission than in those from patients who did not obtain CR ($38\pm 29\%$ vs $73\pm 20\%$, $p<10^{-5}$). Other factors associated with CR were low expression of gp170 ($p<10^{-5}$), bcl-2 ($p<10^{-5}$), and CD34 ($p<10^{-2}$). It is interesting to note that the percentage of PI3K, pAKT, and pERK was also significantly lower in samples from patients who went into CR ($p<10^{-5}$). Detailed results are given in Fig. 2. Finally, autonomous growth of leukemic cells in the clonogenic assay was also associated with decreased CR rate: 16 of 30 patients whose leukemic cells formed spontaneous colonies obtained remission vs 10 of 11 whose cells did not exhibit

Fig. 1 Correlations between the expression of HSP90 and spontaneous growth in the liquid culture (*top panel*) and between the expression of HSP90 and spontaneous leukemic colony formation in the semisolid medium (*bottom panel*). Study was performed in 41 AML samples

spontaneous colony formation ($p < 0.05$). Disease-free survival (DFS) in the 45 patients in remission was not different according to HSP90 expression, although there was a trend for longer DFS in patients with low HSP90 levels (<40% vs $\geq 40\%$ positive cells, $p = 0.09$). On the contrary, overall

Fig. 2 Expression of HSP90 and CD34, P170, bcl-2, and signal transduction proteins according to the remission status after induction treatment. Protein expression was measured by flow cytometry and expressed as percentage of positive cells

Fig. 3 Overall survival according to HSP90 expression; patients with less than 40% HSP90-positive cells: *solid line*; patients with greater than or equal to 40% HSP90-positive cells: *dotted line*

survival was significantly longer in patients with less than 40% HSP90-positive cells than in patients with greater than or equal to 40% positive cells ($p < 0.05$, Fig. 3). Other factors associated with survival were age, cytogenetics, expression of bcl-2, p170 pAKT, ERK, and pERK (data not shown).

Expression of HSP90 in CD34+ cells from normal bone marrow

HSP90 expression was evaluated in 12 normal bone marrow samples. A homogeneous staining was observed in CD34-positive cells with a percentage of HSP90-CD34-positive cells between 28% and 40% (median value of 36%).

In vitro effects of HSP90 inhibition

Effects of 17-AAG on leukemic cells

The susceptibility of AML cells to 17-AAG, estimated by the IC₅₀ at 72 h, was highly variable, with a range between 0.05 and 18 μM and a median of 1.5 μM . The IC₅₀ was inversely correlated with the percentage of HSP90+ cells ($p < 10^{-4}$, Fig. 4a). In the 25 cases with the highest expression of HSP90 (more than 50% positive cells), the IC₅₀ was only $0.97 \pm 0.7 \mu\text{M}$, whereas it was $9.7 \pm 5.7 \mu\text{M}$ in the 16 cases with the lowest HSP90 expression. Although less significant, there was also a positive correlation between IC₅₀ and the percentage of bcl-2-positive cells ($r = 0.38$, $p < 10^{-5}$). There was no correlation of IC₅₀ with other biological characteristics, including cytogenetics. However, the IC₅₀ for the four cases with FLT3-ITD was less than 1 μM .

As assessed by annexin V binding, 17-AAG induced apoptosis in AML cells. The percentage of apoptotic cells after a 48-h culture in the presence of 5 μM 17-AAG varied between 30% and 96%, with a mean of 72%, and was inversely correlated with IC₅₀ (Fig. 4b). Accordingly, the

Fig. 4 **a** Correlations between IC₅₀ and HSP90 expression after exposing AML cells to increasing concentrations of 17-AAG for 72 h. HSP90 expression was assessed by flow cytometry and expressed as percentage of positive cells. **b** Correlation between IC₅₀ and percentage of apoptotic cells after exposing AML cells to 5 μ M 17-AAG for 72 h. Apoptosis was assessed by the annexin V technique

percentage of apoptotic cells was higher in the samples with high HSP90 expression ($86 \pm 24\%$) than in those with low HSP90 expression ($37 \pm 18\%$). Activated caspase 3 staining confirmed the apoptotic death of leukemic cells, with percentages of leukemic cells very similar to those obtained by the annexin V technique.

The effects of 17-AAG were investigated in the 31 samples forming spontaneous colonies in the semisolid medium. The mean number of colonies ($\pm$ SD) at day 7 was 376 ($\pm$ 206) in controls and, respectively, 71 ($\pm$ 59) and 3 ($\pm$ 6) in the presence of 5 and 7.5 μ M 17-AAG ($p < 10^{-5}$). Colony growth was completely inhibited at a concentration of 10 μ M. Details are presented in Fig. 5.

Normal CD34-positive cells and progenitor cells

The effects of increasing concentrations of 17-AAG on normal hematopoietic cells are presented in Table 1. Up to

Fig. 5 Effects of different concentrations of 17-AAG on spontaneous growth of leukemic cells ($n=31$, corresponding to samples forming spontaneous colonies in semisolid medium)

10 μ M, there was no significant toxicity of the drug on survival of CD34-positive cells in the liquid culture (in the absence of growth factors) or on granulo-monocytic and erythroblastic colonies. At 20 μ M, we observed a decrease of 40–50% in CD34-positive cell survival or colony formation.

Discussion

In this study, we show that HSP90 is expressed in most AML specimens, at variable levels. We have previously reported on the biological and prognostic value of HSP27, HSP70, HSP90, and HSP110 expression. High percentage of HSP90-positive cells was associated with low differentiation and low remission rate. Of note, it was also associated with high levels of p170 and Bcl-2 proteins (Thomas et al. 2005). We confirm here our findings and show that expression of HSP90 is also correlated with other significant biological features such as spontaneous growth of leukemic cells in liquid culture and spontaneous colony formation and constitutive activation of PI3K/AKT and ERK pathways. Autonomous growth of AML cells has

Table 1 Effects of 17-AAG on normal progenitors and CD34+ cells

	17-AAG concentration (μ M)				
	0	1	5	10	20
CFU-GM ^a	210	ND	205	209	79
BFU-E ^a	288	ND	281	279	134
CD34 ^b					
% cell surviving	93	93	92	89	67
% annexin V+	5	8	7	10	28

ND Not done

^a Number of colonies after 14-day culture in methylcellulose

^b 48-h culture

been associated with poor response to chemotherapy (Lowenberg et al. 1993; Bradbury et al. 1997) and with high expression of bcl-2 (Campos et al. 1993; Bradbury et al. 1997). We show here a very significant correlation between this phenomenon and the expression of HSP90. Furthermore, autonomous growth was readily inhibited by 17-AAG, even in samples with high expression of Bcl-2. Constitutive activation of transduction pathways, particularly the PI3K-Akt pathway (Xu et al. 2003; Kubota et al. 2004; Sujobert et al. 2005), is involved in survival and proliferation of primary AML cells, as well as the overexpression of bcl-2 protein (Bradbury et al. 1997). Data presented here suggest an implication of HSP90 in sustained activation of transduction pathways and its role in the phenomenon of spontaneous proliferation.

The drug 17-AAG is a promising anticancer agent and is presently used in clinical phase I–II trials in solid tumors and hematological malignancies (Goetz et al. 2003; Goetz et al. 2005). In leukemias, cytotoxic effects have been demonstrated in vitro against myeloid and lymphoid lines (Yao et al. 2003; Rahmani et al. 2003; George et al. 2004; George et al. 2005; Radjukovic et al. 2005). Of note, these effects are particularly evident in cell lines exhibiting specific mutations of HSP90 client oncoproteins such as mutated FLT3 and BCR-ABL. However, few data are available regarding its effect on primary leukemic cells, and investigators have focused on leukemias with FLT3 or BCR-ABL anomalies. In this study, we show on a large unselected series that AML cells from patients at diagnosis exhibit a significant, although variable, susceptibility to 17-AAG. The effect was significantly correlated with a higher expression of HSP90. We confirm here on clinical samples the ability of 17-AAG to induce apoptosis. Several mechanisms have been proposed to explain the biological consequences of 17-AAG inhibition such as a down-regulation of Raf-1, N-Ras, and Ki-ras (Hostein et al. 2001). Downregulation and phosphorylation inhibition of Akt by 17-AAG has also been reported in colon adenocarcinoma and HL60 cell lines (Hostein et al. 2001; Nimmanapalli et al. 2003), resulting in the activation of the mitochondrial pathway of apoptosis (Nimmanapalli et al. 2003). An alternative or complementary explanation to the effect of 17-AAG on AML cells resides in its implication in the regulation of proteins controlling apoptosis. The treatment of HL60 cells with 17-AAG results in a conformational change of Bax associated with apoptosis, while Bax-deficient cells are resistant to 17-AAG. Moreover, HL60 cells overexpressing Bcl-2 or Bcl-xL are not susceptible to 17-AAG, but this resistance is overcome by a Bcl-2 inhibitor (Nimmanapalli et al. 2003).

Finally, we show that high expression of HSP90 was predictive for poor prognosis in our series, mainly by decreasing the probability to obtain a remission. A high

expression of HSPs, including HSP90, has been associated with drug resistance in cancer cells (Ochel and Gademan 2002). Several drug resistance mechanisms are likely to act concomitantly in leukemic cells from patients refractory to chemotherapy. Indeed, we observed that other established (P170 and CD34) or more recently described (activation of multiple transduction pathways; Kornblau et al. 2006) markers of poor prognosis were frequently coexpressed in HSP-positive cells. Nevertheless, we did not observe a correlation with cytogenetics, and targeting HSP90 and signal transduction pathways could improve treatment outcome in patients with poor prognosis anomalies.

In conclusion, our data obtained on primary patient cells support the hypothesis that 17-AAG is a targeted agent potentially useful for AML therapy. Although active as a single agent, it should be more effective in combination with drugs targeting specific oncoproteins (Hostein et al. 2001; Radjukovic et al. 2005) or activated pathways (Kubota et al. 2004; Sujobert et al. 2005). Moreover 17-AAG exhibits additive activity with cytarabine and may contribute to overcome resistance to these drugs (Mesa et al. 2005). The assessment of HSP90 levels may be of help to elicit patients more likely to benefit of such a therapy.

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