HSP90 antagonist, geldanamycin, inhibits proliferation, induces apoptosis and blocks migration of rhabdomyosarcoma cells *in vitro* and seeding into bone marrow *in vivo*

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In this study, geldanamycin (GA) was found to have an antiproliferative effect on both embryonal and alveolar rhabdomyosarcoma (RMS) cell lines. The maximum level of inhibition reached 80% for both embryonal and alveolar RMS. After GA treatment, cells also became apoptotic as judged by Annexin V-positive staining, activation of caspase-3 pathway and poly(ADP ribose) polymerase cleavage. GA was responsible for the arrest of RMS cells in both G_1 and G_2/M phases of the cell cycle. G_1 blockade, however, was transient and was seen only in the first 24 h of GA treatment. RMS often gives distant metastases to various organs including bone marrow. RMS cells express high levels of MET receptor and respond to hepatocyte growth factor with increased motility. In our study, we found that GA decreased the level of MET expression and inhibited the chemotaxis of RMS cells toward the hepatocyte growth factor gradient. GA also blocked the homing of RMS cells into bone marrow of severe combined immune deficient mice. In all our experiments embryonal RMS cell lines were significantly more sensitive, and lower concentrations of GA were sufficient to block embryonal RMS cell proliferation, induce apoptosis and inhibit motility. Our data show that the HSP90 inhibitor GA has the potential to become a new drug in RMS treatment. It blocks RMS proliferation, decreases cell survival and inhibits motility of RMS cells. *Anti-Cancer Drugs* 18:1173–1181
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Anti-Cancer Drugs 2007, **18:**1173–1181

Keywords: chemotaxis, geldanamycin, MET, rhabdomyosarcoma, tumor growth

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Received 19 January 2007 **Revised form accepted** 25 June 2007

Introduction

Rhabdomyosarcoma (RMS) is a relatively uncommon tumor but it still presents an important clinical problem in infants and children, as it represents the largest subset of soft tissue sarcomas among these patients [1]. It occurs in the first two decades of life, with most cases being diagnosed in children before their 10th birthday [2]. Two major subtypes of RMS result from chromosomal aberrations: embryonal RMS (ERMS) without genetic translocations, and alveolar RMS (ARMS) with Pax3–FKHR [$t(2:13)(q35:q14)$] and Pax7–FKHR [$t(1:13)(p36:q14)$] fusion proteins [1]. The two RMS subtypes, ERMS and ARMS, have different biological structures and localization. ERMS is the more frequent type of RMS and usually occurs in young children less than 10 years old and frequently involves the head and neck region and the genitourinary system (urinary bladder, prostate) [3]. ERMS recapitulates phenotypically and biologically some features of embryonic skeletal muscles. Prognosis for ERMS patients is usually better than for ARMS patients, particularly in younger children. ARMS growth is more rapid; it usually presents as high stage tumors, seen also in adults. It is often located in the extremities but some studies have found no favored site of origin for this subtype [4,5]. ARMS has a higher risk of relapse and metastasis. ARMS cells resemble lymphoma cells and show signs of partial skeletal muscle differentiation. The new fusion genes present in ARMS are very potent transcription factors that contain a DNA-binding domain of a *PAX* gene and a transcriptional activation domain of a *FKHR* gene [6–10]. These proteins are responsible for the upregulation of the genes involved in cell proliferation, survival and motility [11–13].

Geldanamycin (GA) is a relatively new drug that has been shown in various cell systems (lung cancer, neuroblastoma and B cell chronic lymphocytic leukemia) to inhibit tumor cell proliferation, survival and motility [14,15]. GA belongs to the family of ansamycin antibiotics and it has been shown to bind to HSP90, a member of a heat shock protein family that plays a critical role in tumor cell growth and survival [14,15]. HSP90 is responsible for the correct folding of target proteins including AKT, cyclin-dependent kinases and RAF-1 [16–18]; all these proteins

are involved in transmitting signals that stimulate cell proliferation and survival. By disrupting the adenosine triphosphate-binding packet of HSP90, GA causes misfolding and production of inactive proteins, which are subsequently targeted for degradation [14,15]. This results in a block of cell growth.

RMS treatment involves chemotherapy that is often combined with radiation and surgery. This results in high morbidity and deformation, and in particular affects very young children. The prognosis is poor for newly diagnosed high-risk patients and for those who develop recurrent tumors. Therefore, new treatment strategies are needed [19]. The presumption that all patients with RMS have micrometastatic disease at diagnosis further strengthens the rationale for multidisciplinary therapy. In this study, we looked at the effects of GA on RMS cells *in vitro* and *in vivo*. Our data show that GA could become a potential new drug in RMS treatment, which blocks RMS proliferation, decreases RMS cells survival and inhibits motility of tumor cells.

Methods

Cell lines

All RMS cell lines used in this study (SMS-CTR, RD-ERMS, RH30 and RH18-ARMS) were maintained in Dulbecco's-modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, New York, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 100 IU/ml penicillin and 10 mg/ml streptomycin (Gibco). Cells were cultured at 37°C, 5% CO_2 and 95% humidity. They were split usually twice a week with medium change.

Cell proliferation assessment by MTT assay

The MTT assay was performed according to the manufacturer's recommendations (Promega, Madison, Wisconsin, USA). Briefly, cells were seeded in 96-well plates at 10^4 cells/well in 100 µl of DMEM medium containing 10% fetal bovine serum plus various concentrations of GA (1, 10, 100 and 1000 nmol/l). After 24, 48, 72 and 96 h, 20 µl of CellTiter 96 Aqueous One Solution (Promega) reagent were added to each well and plates were incubated for 3–4 h. Subsequently, plates were read at 490 nm using the EL × 800 Universal Microplate Reader (Bio-tek, Vinooski, Vermont, USA) and analyzed with KC4 v3.0 with PowerReports software (Bio-tek).

Apoptosis assays

Apoptosis was evaluated by the Annexin V-binding assay, caspase-3 activation and poly(ADP ribose) polymerase (PARP) cleavage. Cells were harvested, washed in phosphate-buffered saline (PBS) and resuspended in appropriate binding buffer. For Annexin V staining, the cell suspension was incubated with propidium iodide (PI) and fluorescein isothiocyanate (FITC)–Annexin V (BD Pharmingen, San Diego, California, USA) for 15 min at room temperature. For caspase-3 and PARP staining, cells were permeabilized for 20 min, washed and subsequently stained with anti-caspase-3 or with anti-PARP monoclonal antibodies for 30 min on ice (both antibodies from BD Pharmingen). Flow cytometric analysis was performed (FACS Canto; Becton Dickinson Bioscience, San Jose, California, USA) within 1 h.

Cell cycle analysis

Cell cycle was analyzed as described previously [20]. Briefly, cells were harvested and washed with PBS. Cells were fixed with cold 75% ethanol in PBS and incubated for at least 12 h at 4°C before staining. Samples were treated with RNAse (50 µl/ml in PBS; Sigma, St Louis, Missouri, USA) for 30 min at 37°C and then incubated with PI (50 µg/ml in sodium citrate; Sigma) for 30 min at room temperature. Subsequently, cells were analyzed using FACS Canto flow cytometer.

RNA extraction and reverse transcription

Total RNA was extracted using RNeasy Mini kit (Qiagen, Valencia, California, USA) followed by DNAse treatment (Promega). The reverse polymerase transcription was performed using Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, California, USA) according to the manufacturer's protocol.

Quantitative real-time PCR analysis

Cyclin expression was determined by quantitative real-time PCR analysis on an ABI PRISM 7300 Sequence Detection System (Applied Biosystems, Foster City, California, USA) using a commercially available SYBR Green PCR Master Mix (Applied Biosystems). The primers sequences were as follows: 5′-CCTGCATTT GGCTGTGAACTAC-3′ forward and 5′-ACGGGTGTT CGACTTCAAAAG-3′ reverse for cyclin A_2; 5′-GCAT GTTCGTGGCCTCTAAGAT-3′ forward and 5′-GCCAC ATCTACGTGTCGAAGAG-3′ reverse for cyclin D_1; and 5′-GGATGCAGAAGGAGATCACTG-3′ forward and 5′-C GATCCACACGGAGTACTTG-3′ reverse for β-actin. The primers were designed with Primer Express software (Applied Biosystems). The mRNA expression level for all samples was normalized to the housekeeping gene *β-actin*.

Chemotaxis assay

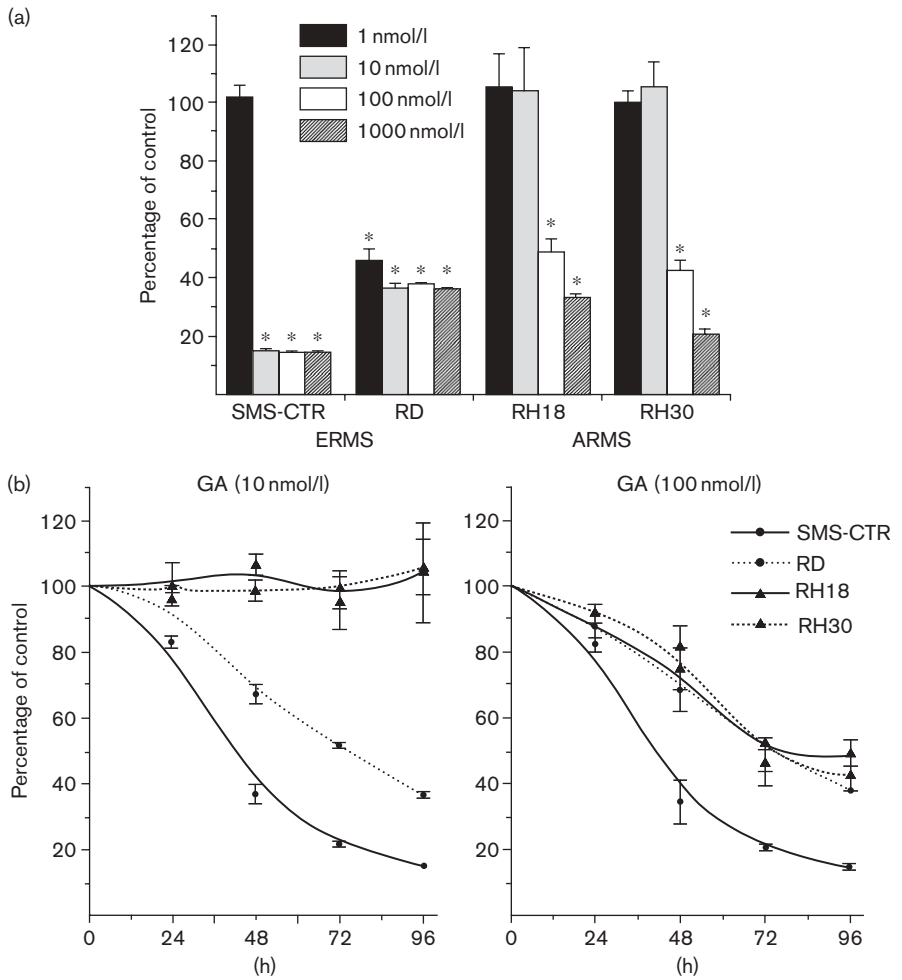
The directional movement of cells toward a hepatocyte growth factor (HGF) gradient in the presence or absence of GA was evaluated using a modified Boyden's chamber with 8-µm pore polycarbonate membrane inserts (Costar Transwell; Costar-Corning, Cambrige, Massachusetts, USA). Cells detached with 0.25% trypsin were incubated for 30 min with 10 or 100 nmol/l of GA and subsequently seeded into the upper chamber of an insert at a density of 2.5×10^4 in 100 µl. The lower chamber was filled with prewarmed medium containing HGF at 10 ng/ml concentration. Bovine serum albumin (0.5%) DMEM medium was used as a negative control. After 24 h, inserts

were removed from the transwell and cells were fixed with methanol. Cells that did not migrate were scraped off with cotton wool from the upper membrane, and cells that had transmigrated to the lower side of the membrane were stained with Wright solution and counted under a high power field with an inverted microscope. Five fields were counted each time and the mean number of cells per high power field was calculated.

Western blot

Western blots were performed on extracts prepared from cells that were treated with different concentrations of GA for 24 and 48 h as described previously [21]. Briefly, RMS cells were lysed (for 10 min) on ice in M-Per lysing buffer (Pierce, Rockford, Illinois, USA) containing protease and phosphatase inhibitors (Sigma). Subsequently, the extracted proteins were separated on a 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel and fractionated proteins were transferred into a polyvinylidene diflouride membrane (BioRad, Hercules, California, USA). Total levels of MET, AKT and STAT3 proteins were assessed using primary rabbit polyclonal antibodies for MET (Santa Cruz Biotech, Santa Cruz, California, USA), AKT and STAT3 (Cell Signaling, Denvers, Massachusetts, USA), and they were detected subsequently with horseradish peroxidase-conjugated goat antirabbit IgG secondary antibody (Santa Cruz Biotech). The membranes were developed with an enhanced chemiluminescence reagent (Amersham Life Sciences, Little Chalfont, UK), dried, and subsequently

Fig. 1



RMS cells respond to GA treatment in a concentration-dependent and time-dependent manner. (a) RMS cell lines were exposed to different concentrations of GA and incubated for 96 h. Subsequently, the MTT assay was performed. ERMS cells were more sensitive and responded more robustly to GA treatment than ARMS cells. *$P<0.05$. (b) To evaluate the time–response profile, RMS cell lines were exposed to 10 nmol/l GA (left panel) and 100 nmol/l GA (right panel) for 0, 24, 48, 72 and 96 h, and were subsequently evaluated by the MTT assay. At both GA concentrations, cells responded in a time-dependent manner. Only the ERMS cells, however, exhibited decreased proliferation when 10 nmol/l of GA was used. ARMS, alveolar rhabdomyosarcoma; ERMS, embryonal rhabdomyosarcoma; GA, geldanamycin.

exposed to the HyperFilm (Amersham Life Sciences). An equal loading in the lanes was evaluated by probing with an anti-β-actin antibody (Santa Cruz Biotech).

Short-term tumor cells engraftment assay

SMS-CTR (5×10^6) and RH30 cells (5×10^6) were either incubated or not incubated for 6 h with 100 nmol/l of GA. Subsequently, cells were washed and they were injected supraorbitally into 6–8 weeks severe combined immune deficient (SCID) mice, treated a day earlier with 300 cGy. Each experimental group consisted of 5–10 animals and experiments were repeated twice. After 24 h, mice were killed and bone marrow cells from two legs were harvested. Cells from each leg were kept separately and total RNA was isolated using the RNAeasy kit (Qiagen). The reverse polymerase transcription was performed using Moloney murine leukemia virus reverse transcriptase (Invitrogen). The level of RMS cells homing to bone marrow was evaluated by real-time PCR using a human glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific primersprobe set (Hs99999905_m1; Applied Biosystems). Control experiments showed no cross-reactivity with murine GAPDH.

For FACS analysis RMS cells were stained for 10 min with 1 μmol/l calcein (Molecular Probes, Eugene, Oregon, USA), washed and 5×10^6 cells were injected as described above.

Results were assessed in a modified two-way factorial analysis of variance model. The modification was based on incorporating a mouse as a random factor. With this model we studied mean differences (and associated 95% confidence intervals) between cells treated with GA and untreated in each of the two cell lines. At this stage, when calculating the confidence intervals, we used the Tukey–Kramer correction. Next, the effect of an injection of GA was assessed by calculating the expression 2^-difference between the two treatments.

Statistical analysis

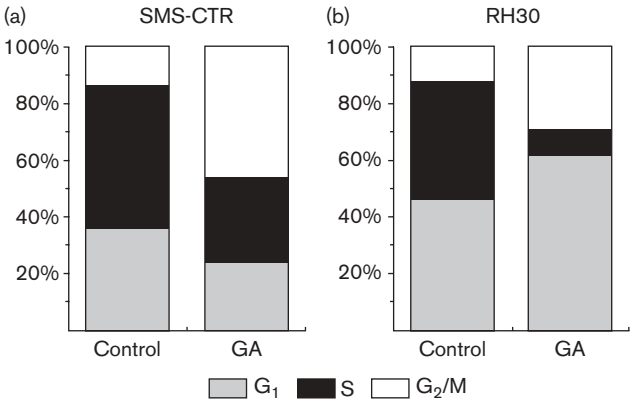
Statistical analysis, unless otherwise indicated, was performed using one-way, nonpaired Student's *t*-test, with SAS 9.1 (TS1M3) (licensed to Jagiellonian University School of Medicine, Site 0092550001).

Results

Geldanamycin inhibits rhabdomyosarcoma cell proliferation in a concentration-dependent and time-dependent manner

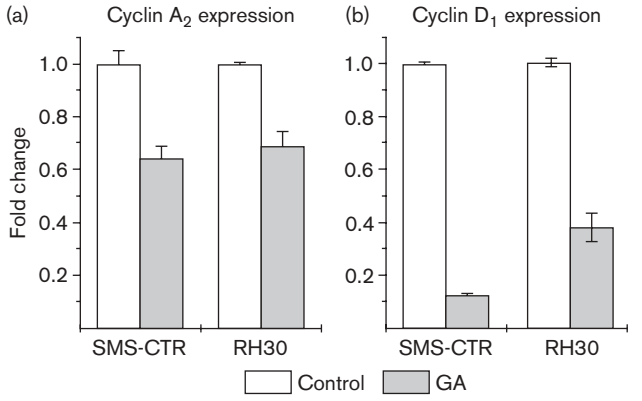
In this study, our research centered on the influence of the HSP90 inhibitor GA on the proliferation of RMS cells. Cells were incubated for 24, 48, 72 and 96 h with different concentrations of GA ranging from 1 to 1000 nmol/l. We noticed that both ERMS and ARMS cell lines responded to the GA treatment with decreased proliferation (Fig. 1). The RMS subtypes, however, responded with a different sensitivity. Accordingly, the ERMS cells were significantly more susceptible to GA than ARMS cells (Fig. 1a). The ERMS proliferation was already inhibited with 1 nmol/l GA

Fig. 2



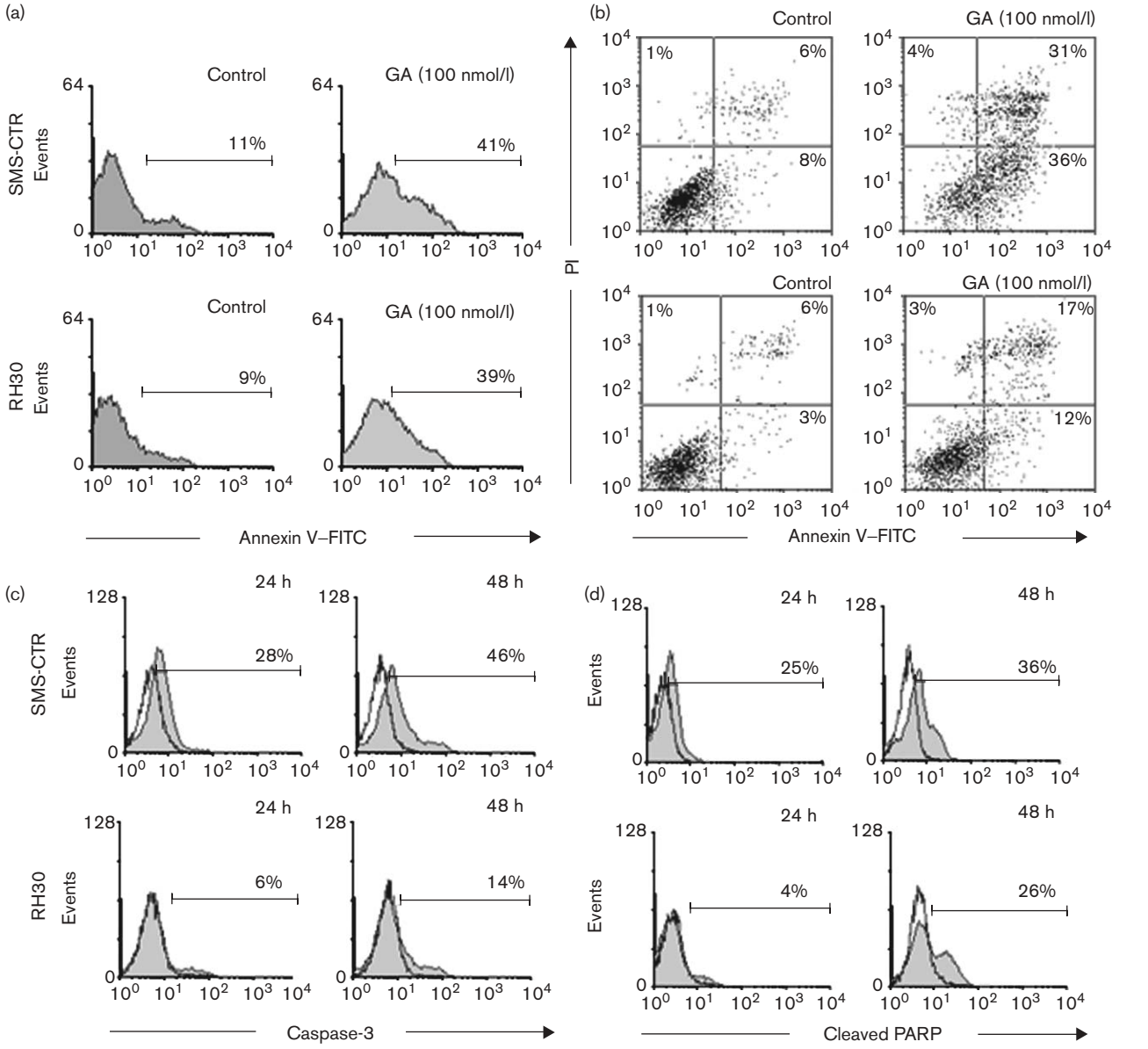
Cell cycle analysis of GA-treated RMS cells. Analysis was performed using the propidium iodine method. To synchronize cells they were incubated for 24 h in serum-free medium containing 0.5% BSA. Next, cells were cultured for 24 h in DMEM + 10% FBS-containing GA. We noticed that GA arrested both SMS-CTR and RH30 cells in the G_2/M phase (white box), it also arrested RH30 cells in the G_1 phase of the cell cycle (gray box). Moreover, different sensitivities between SMS-CTR (a) and RH30 (b) were observed. One representative experiment of the three performed is shown. BSA, bovine serum albumin; DMEM, Dulbecco's-modified Eagle's medium; FBS, fetal bovine serum; GA, geldanamycin; RMS, rhabdomyosarcoma.

Fig. 3



Real-time RT-PCR analysis of cyclin A_2 and cyclin D_1 expression after GA treatment. Cells were synchronized for 24 h in a medium supplemented with 0.5% BSA and subsequently transferred into DMEM + 10% FBS and treated with 100 nmol/l of GA for 24 h. Both SMS-CTR and RH30 cells exhibited a decrease in the expression of cyclin A_2 (a), and cyclin D_1 (b), with a stronger inhibition seen in the SMS-CTR cells. Each bar represents the mean value ± SD. *$P < 0.05$. BSA, bovine serum albumin; DMEM, Dulbecco's-modified Eagle's medium; FBS, fetal bovine serum; GA, geldanamycin.

Fig. 4



GA influence on the survival of RMS cells. (a) Cells were incubated for 24 h with GA (10 or 100 nmol/l) and stained with Annexin V. (b) Cells were incubated for 24 h with 100 nmol/l GA and double stained with Anexin V–FITC and PI. (c) Cells were incubated for 24 and 48 h with 100 nmol/l GA, and stained with antibodies against the activated form of caspase-3. (d) Cells were incubated for 24 and 48 h with 100 nmol/l GA, and stained with antibodies directed toward the cleaved form of PARP. Stained cells were analyzed with FACS Canto flow cytometer. All experiments were repeated at least two times and representative results are shown. FITC, fluorescein isothiocyanate; GA, geldanamycin; PARP, poly(ADP ribose) polymerase; RMS, rhabdomyosarcoma.

(RD cells) and 10 nmol/l GA (SMS-CTR). Correspondingly, to achieve a similar level of inhibition for ARMS cells, at least a 10 times higher concentration of GA (100 nmol/l) was needed.

Response to GA treatment was also time dependent with strongest inhibition seen after 96 h of incubation (Fig. 1b). Again, differences in sensitivity to GA between subtypes were observed.

As the response patterns to GA of the ARMS cell lines RH30 and RH18 and the ERMS cell lines SMS-CTR and RD did not differ significantly within each subtype, in all further experiments RH30 and SMS-CTR cell lines were used.

Cell cycle arrest of geldanamycin-treated rhabdomyosarcoma cells

Decreased cell growth can be a result of inhibition of proliferation and/or increased apoptosis. First, we deter-

mined the effect of GA on the cell cycle. To obtain cells at approximately the same point in the cell cycle RMS cells were synchronized by culture in serum-free medium for 24 h before experiments. Subsequently, cells were transferred to growth medium containing 100 nmol/l GA for 24 h and stained with PI. We noticed the higher susceptibility of ERMS cells to the cell cycle inhibition by GA. For both tumor cell types, cells were arrested in the G_2/M phase, but in the case of ERMS, 50% of cells were in G_2/M; only 25% of ARMS cells behaved accordingly (Fig. 2).

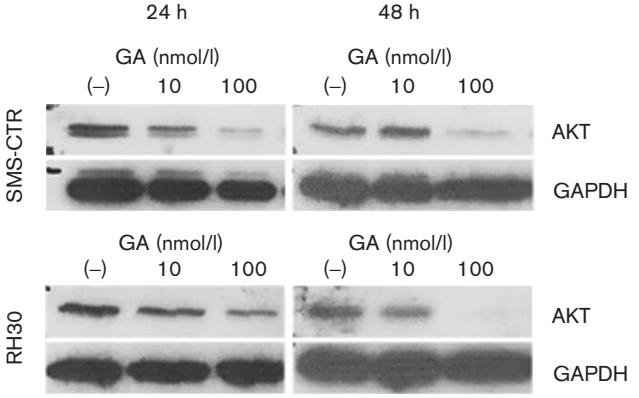
To find out the reason for the accumulation of cells in G_2/M phase we studied the expression of cyclins at the mRNA level after incubation with GA. We examined at the expressions of different cyclins. We found a significantly decreased level of mRNA for cyclin A_2 ($t_1 = 11.48$; $P = 0.0277$ for SMS-CTR and $t_1 = 7.51$; $P = 0.0422$ for RH30) and D_1 ($t_1 = 254.43$; $P = 0.0013$ for SMS-CTR and $t_1 = 52.23$; $P = 0.0061$ for RH30). The levels of other cyclins remained unchanged. ERMS again responded to the lower concentration of GA with a more profound decrease in the level of cyclin-specific mRNA (Fig. 3).

Geldanamycin induces rhabdomyosarcoma cell apoptosis

Decreased cell proliferation after applied treatment can be a result of increased apoptosis. To test this possibility we used Annexin V staining. We found that after a 24-h incubation with 10 and 100 nmol/l of GA, RMS cells underwent apoptosis (Fig. 4a). ERMS cells responded rapidly to a 10 nmol/l concentration of GA and the number of apoptotic cells increased by 100% compared with the untreated control. In contrast, ARMS cells did not respond to 10 nmol/l GA with increased apoptosis. The apoptotic cells were seen only after treatment with 100 nmol/l of GA. To distinguish between the early and late apoptotic and necrotic cells we performed a more detailed analysis using double staining with Anexin V and PI (Fig. 4b). The percentages of early and late apoptotic ERMS cells were more than two times those of ARMS cells. The percentages of necrotic cells were similar in both treated cell lines.

To study the mechanism of GA-induced apoptosis we examined the activation of caspase-3 and PARP cleavage, as well as the total level of AKT kinase expression in treated cells. Both ERMS and ARMS responded to GA. We, however, noticed a higher percentage of cells with activated caspase-3 (Fig. 4c) and cleaved PARP (Fig. 4d) in ERMS cells than in ARMS cells after 24 and 48 h. We also found that the amount of AKT protein decreased in both ERMS and ARMS cells (Fig. 5). We also observed a slight decrease in the expression of STAT3 protein in ERMS cells (data not shown).

Fig. 5



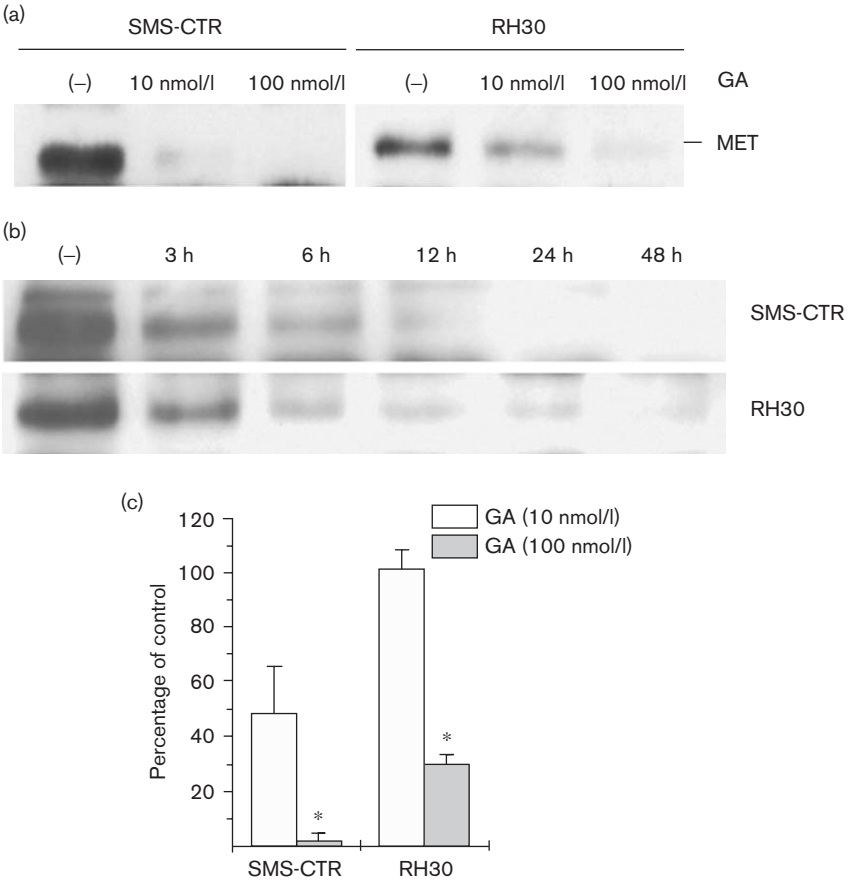
Western blot analysis of total AKT expression after GA treatment. Cells were incubated for 24 and 48 h with 10 or 100 nmol/l of GA. Strong downregulation of AKT expression was observed in SMS-CTR cells after 24 and 48 h (upper panel). In RH30 cells AKT was donwregulated only slightly after 24 h and profoundly after 48 h (lower panel). GA, geldanamycin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Geldanamycin downregulates MET expression in rhabdomyosarcoma cells and their chemotaxis toward hepatocyte growth factor

In this study, we tested if GA could downregulate the expression of MET protein and subsequently the responsiveness of RMS to the HGF gradient. We treated RMS cells with 10 and 100 nmol/l of GA for 24 and 48 h, and analyzed MET expression by Western blotting. We found that GA reduces expression of MET in both ERMS and ARMS cell lines. The reduction was more prominent in ERMS cells (Fig. 6a). Correspondingly, the mRNA level for MET was unchanged (data not shown) confirming that the HSP90 inhibitor blocks its expression at the protein level. Downregulation of MET expression was time dependent, with a profound decrease already observed after 6 h of GA treatment, followed by a further reduction (Fig. 6b).

We attempted to ascertain if the reduced level of MET expression positively correlates with the responsiveness of RMS cells to the HGF gradient. The RMS cells were preincubated for 30 min with 10 or 100 nmol/l of GA and chemotaxis was carried out over a period of 24 h. Cells that did not migrate were removed from the membrane, and the remaining cells were stained and counted in five high-resolution fields under inverted microscope. The chemotaxis followed the MET expression pattern. Direct movement of ERMS cells was reduced by approximately 50% with 10 nmol/l of GA ($t_1 = 4.48$; $P = 0.0699$) and almost completely with 100 nmol/l of the drug ($t_1 = 65.67$; $P = 0.0048$). ARMS cells were less sensitive but at 100 nmol/l the chemotaxis was inhibited by more than 50% ($t_1 = 15.67$; $P = 0.0203$) (Fig. 6c). These data

Fig. 6



Influence of GA on the HGF–MET axis. (a) Western blot analysis of MET expression. Exposure of RMS cells to 10 or 100 nmol/l GA downregulated MET levels in both SMS-CTR and RH30 cells. The experiment was repeated twice with similar results. (b) Time course of MET downregulation after 3, 6, 12, 24 and 48 h treatment with 100 nmol/l of GA. (c) Analysis of RMS chemotaxis toward HGF in the presence of GA. Cells were incubated for 30 min with 10 or 100 nmol/l of GA and subsequently loaded into an upper transwell chamber. Chemotaxis was carried out over a period of 24 h. Cells exhibiting chemotaxis were stained and counted in five high-power fields under a light-inverted microscope. Chemotaxis of SMS-CTR cells was inhibited by 50 ± 10% with 10 nmol/l of GA and blocked completely with 100 nmol/l GA. The RH30 cells were less sensitive to GA, and chemotaxis was inhibited by 35 ± 5% after exposure to 100 nmol/l GA. $P < 0.05$. GA, geldanamycin; RMS, rhabdomyosarcoma.

showed that GA could be used to block HGF-dependent metastasis of RMS cells.

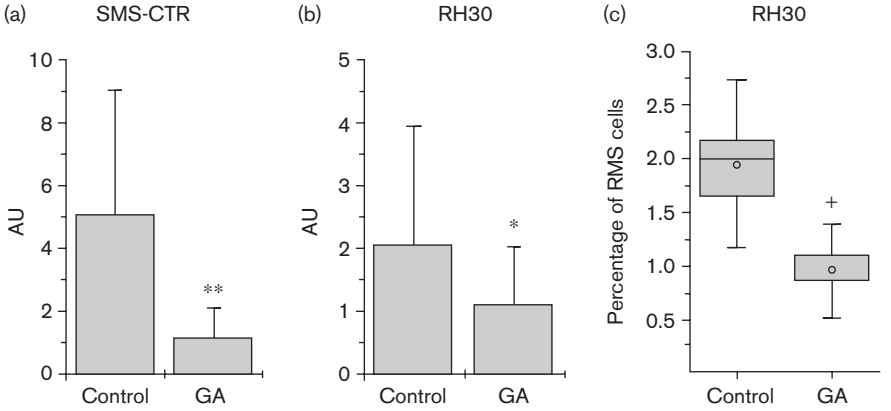
Decreased seeding of human rhabdomyosarcoma cells into bone marrow of SCID mice

In the chemotaxis assay we have shown that GA inhibits the migration of RMS cells toward the HGF gradient *in vitro*; therefore, we attempted to learn if GA could also inhibit the homing of RMS cells to bone marrow *in vivo*. RMS cells of both subtypes, pretreated with GA for 6 h, were injected into sublethally irradiated SCID mice and 24 h later a level of tumor cells homing was assessed. We looked for human cells in murine bone marrow using human-specific primers against GAPDH.

The overall effect of GA on RMS cell homing was significant ($F_{1,17} = 9.35$; $P = 0.0071$), indicating that tumor cells treated with GA have a decreased potential to seed into bone marrow (Fig. 7). We observed that the preincubation of SMS-CTR cells with GA results in the reduction of their homing by more than seven times (as compared with cells without GA) (Fig. 7a). In the case of the RH30 line the results were qualitatively similar but to a lesser extent (Fig. 7b). Reduction of homing in treated cells was nearly half of that observed in the control group, but the result was not statistically significant.

To confirm that GA-treated RMS cells had a lower seeding efficiency into bone marrow we labeled RH30 cells with calcein and injected then into SCID mice. After 24 h, we harvested the bone marrow and examined for the presence of labeled cells. The percentage of calcein-positive cells in mice bone marrow in the GA-treated group was two times lower than in untreated control (Fig. 7c).

Fig. 7



GA inhibits the seeding of RMS cells into bone marrow *in vivo*. SCID mice were injected with RMS cells only (control) or with RMS cells incubated with 100 nmol/l of GA. After total RNA extraction, human RNA was detected using human-specific GAPDH primers. (a) Level of GAPDH expression after injection with SMS-CTR cells. (b) Level of GAPDH expression after injection with RH30 cells. (c) Percentage of calcein-labeled RH30 cells present in the bone barrow of SCID mice assessed by flow cytometry. Experiments were repeated twice, the results were similar. $^{**}P<0.0001$, $^{*}P=0.0919$, $^{+}P<0.05$. AU, arbitrary unit; GA, geldanamycin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RMS, rhabdomyosarcoma; SCID, severe combined immune deficient.

Discussion

In this study, we tested GA as a new potential therapeutic for the treatment of RMS. We determined for the influence of GA on RMS cell proliferation, apoptosis and motility, and compared the sensitivity of both the ERMS and ARMS subtypes to GA.

We found that GA strongly inhibits proliferation of RMS cell lines in a concentration-dependent and time-dependent manner. ERMS cells were sensitive to concentrations as low as 1–10 nmol/l, in contrast to ARMS cells requiring 10 or even 100 times higher concentrations of this drug. We also noticed differences in the time courses of growth inhibition between the two RMS subtypes. Accordingly, whereas low concentrations of GA (10 nmol/l) decreased the proliferation of ERMS cells by more than 60% after 48 h, ARMS proliferation was not affected by this concentration of GA. A 48-h treatment of ARMS cells with high doses of GA (100–1000 nmol/l), nevertheless, profoundly inhibited their growth rate. The difference in response to GA between ERMS and ARMS can be potentially explained by the differences in the level of HSP90 in these cells. Experiments performed to check the level of HSP90 in different types of RMS do not, however, show any differences (unpublished, manuscript in preparation). Correspondingly, we also observed a significantly increased number of apoptotic cells as judged by Annexin V, activated caspase-3 and cleaved PARP-positive RMS cells after GA treatment. Again, treatment of ERMS cells with GA resulted in a higher number of apoptotic cells. Similar observations were made for other tumors, including neuroblastoma [18,22].

Inhibition of cell growth can be a result of the blocking of cell cycle progression, increased apoptosis or both. Our data showing that GA downregulates the expression of cyclin D_1 and cyclin A_2 mRNA, and causes profound cell cycle arrest in G_1 and G_2/M phases of the cell cycle and the almost complete depletion of RMS cells in the S phase correspond to earlier reports about a role of D_1 and A_2 cyclins in RMS growth. In the previously published work the expression levels of cyclins D and A were increased in RMS cells [23]. Cyclin D_1 was also shown to be important in preventing skeletal muscle cell differentiation [24] and cyclin D_1-associated kinase inhibitors were demonstrated to decrease the proliferation of RMS cells [25]. As the expression of cyclin D_1 is regulated by the phosphatidylinositol 3-kinase/AKT pathway, blocking of AKT protein expression by the GA analog, 17-AAG, downregulated the cyclin D_1 level [26]. In our study, we found that GA suppresses AKT expression in RMS cells. We hypothesize that GA-mediated downregulation of AKT expression, by blocking HSP90 and AKT interaction, inhibits cyclin D_1 expression and induces the cell cycle arrest of RMS cells. Inhibition of AKT expression can also cause the apoptosis and death of RMS cells. We have also observed a slight decrease in the expression of the STAT3 protein, which is responsible for cell proliferation, their differentiation and survival. Reduced levels of STAT 3 along with decreased AKT expression after GA treatment can explain increased levels of apoptotic RMS cells. Bringing all the data together, we postulate that the growth of RMS cells is suppressed owing to both apoptosis and cell cycle arrest.

Metastatic RMS has a very poor prognosis and is predominantly fatal. Several factors, including the

HGF–MET axis, have been shown to be important for regulating the growth and metastatic behavior of RMS cells [13,27]. HSP90 is responsible for the correct folding of several receptor tyrosine kinases, including MET [15]. Blocking of HSP90 and misfolding of MET leads to its degradation and cell surface depletion [28]. In this study, we found that GA downregulated MET protein expression in a concentration-dependent and time-dependent manner, and inhibited in-vitro migration of RMS cells toward the HGF gradient. The inhibitory effect of GA on the migration of RMS cells was also seen in an in-vivo assay using SCID mice, in which GA significantly decreased the seeding of RMS cells into bone marrow. As bone marrow involvement is a poor prognostic factor, inhibiting the bone marrow homing of RMS cells by GA is of clinical importance.

In this study, we have shown that GA exerts several potential therapeutic effects on RMS cells. It blocks RMS cells proliferation, survival, motility and seeding into bone marrow. Taken together, our data indicate that GA has the potential to become a novel drug for RMS treatment.

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