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HIGH ANTI TUMOR ACTIVITY AGAINST RHABDOMYOSARCOMA CELLS AND LOW NORMAL CELLS CYTOTOXICITY OF HEAT SHOCK PROTEIN 90 INHIBITORS, WITH SPECIAL EMPHASIS ON 17-[2-(PYRROLIDIN-1-YL)ETHYL]-AMINNO-17-DEMETHOXYGELDANAMYCIN

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Rhabdomyosarcoma is a highly metastatic tumor, mostly observed in children and adolescence. When diagnosed at early stages it is mostly curable. However, in advanced or metastatic stages the 5-years survival rate is below 20%. Thus, new treatment strategies for this tumor are needed. In this paper we showed that HSP90 inhibitors, geldanamycin and its analogs, can profoundly affect proliferation of rhabdomyosarcoma cells. We also showed that blocking of HSP90 function induces apoptosis of tumor cells and downregulates expression of anti apoptotic protein AKT. Cells exposed to geldanamycin and its analogs exhibit strong reduction of MET receptor expression and subsequent inhibition of HGF-dependent tumor cells migration and invasion. Interestingly, at concentrations sufficient to block tumor cells growth and motility, the 17AEP-GA, 17AAG and 17DMAP-GA were not toxic or only slightly toxic toward normal hematopoietic, mesenchymal and endothelial cells. This could be due to low HSP90 expression both at mRNA and protein level in these cells. Collectively, our findings suggest that blocking HSP90 action through geldanamycins could be in the future a part of new therapeutic strategies in rhabdomyosarcoma treatment.

Key words: *HSP90, rhabdomyosarcoma, MET receptor, geldanamycin, 17AAG, 17AEP-GA*

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INTRODUCTION

Despite of the great progress made in the last decades in cancer treatment, most of the advanced and highly metastatic tumors are still incurable (1). This situation legitimizes every effort directed towards development of new, more potent and more specific treatment options.

Drugs that interfere with an activity of heat shock proteins, particularly heat shock protein 90 (HSP90), seem to fulfill this expectation. HSP90 is a molecular chaperone crucial for correct protein folding, assembly and transportation across cellular compartments. Recently, HSP90 has been shown to participate in malignant transformation through stabilization of mutated genes and enhancement of cancer cell survival under stress conditions (2). Proteins dependent on HSP90 for correct folding and expression include e.g. AKT, RAF and cyclin D1 (3, 4).

It seems possible to inhibit tumor cells growth and to induce apoptosis through blocking of these proteins. We suggest, therefore, that HSP90 inhibitors can serve as efficient agents for anti cancer therapy. Geldanamycin (GA) and its analogs are particularly interesting. GA is a naturally occurring ansamycin antibiotic isolated from *Streptomyces hydroscopicus* (5). GA possesses strong anti tumor activity (5), however, it also has intolerable toxicity (6). Thus, GA analogs have been developed with a comparable anti tumor activity but with fewer side effects. One of the GA derivatives, 17-allylaminogeldanamycin (17AAG),

has already entered clinical trials with promising preliminary results (7). However, 17AAG is known to have also a major drawback, namely it is water insoluble and it has to be dissolved in DMSO, which is known to cause several adverse effects such as cardiotoxicity, hepatotoxicity, nausea and vomiting (8).

Rhabdomyosarcoma (RMS) is a pediatric soft tissue sarcoma belonging to a family of small blue cell line tumors and it represents the largest subset of soft tissue sarcomas in this age group. RMS can be divided into two major subtypes based on chromosomal analysis - embryonal (ERMS) and alveolar (ARMS). They differ in clinical outcome with ERMS having more favorable prognosis (9). Nevertheless, if RMS is diagnosed at late stage (IV) or with metastatic disease the outcome is usually poor with only 20% of 5-years overall survival (9). Obviously, at this stage an effective treatment is not available now.

We have shown recently that GA inhibits growth of RMS cell lines (10). However, as mentioned earlier, GA also exhibits strong cytotoxic action towards normal cells, which excludes it from the use in a clinic. In order to look for new treatment options for late stage RMS, we compared the influence of five GAs (GA, 17AAG, 17DMAG, 17AEP-GA, 17DMAP-GA) on RMS cells. We paid particular attention to the influence of these compounds on tumor cells migration and invasion, two characteristics often associated with late stage and metastatic tumors. Moreover, we compared the cytotoxicity of these inhibitors towards normal cells.

METHODS

HSP90 inhibitors

Geldanamycin, 17-AAG (17-allylamino-17-demethoxygeldanamycin) – a GA analog, both insoluble in H₂O and soluble in DMSO. 17DMAG (17-(dimethylaminoethylamino)-17-demethoxygeldanamycin) - a water soluble 17-AAG analog; 17AEP-GA (17-[2-(pyrrolidin-1-yl)ethyl]aminino-17-demethoxygeldanamycin) – a water-soluble GA analog containing an alkylamino group instead of the methoxy moiety at C17; 17DMAP-GA (17-(dimethylaminopropylamino)-17-demethoxygeldanamycin) - a water soluble GA analog synthesized based on binding affinity to Hsp90.

GA, 17AAG, 17DMAG, 17AEP-GA and 17DMAP-GA were purchased from InvivoGen (USA).

Cell lines and primary cells

SMS-CTR (ERMS) and RH30 (ARMS) cell lines were used (cells obtained from dr Ratajczak's lab). Cells were maintained in DMEM (Gibco BRL) and supplemented with 10% heat-inactivated FBS (Gibco), 100 IU/ml penicillin, 10 mg/ml streptomycin (Gibco). The cells were cultured at 37°C, 5% CO₂, 95% humidity. They were split usually twice a week with medium change.

CD34⁺ cell were isolated from bone marrow using MiniMacs (Milteney Biotech, Germany). Mesenchymal stem cells were isolated by plastic adherence and cultured in Mesenchymal Medium (PAA, Germany). HUVEC cells were obtained by collagenase digestion of cord and cultured in EBM basal medium containing supplements (Clonetics, USA).

The permission of Bioethics Committee of the Jagiellonian University and informed consent of the donor were obtained for all procedures.

Cell proliferation/survival assay

The cell proliferation/survival assay was performed using CellTiter 96 Aqueous One Solution Kit accordingly to the manufacturer's recommendations (Promega) and cell counting. For MTT assay, cells (SMS-CTR, RH30, HUVEC and MSC), were seeded in 96-well plates at 10⁴/well in 200 µl of DMEM medium containing 10% FBS plus various concentrations of GAs (1, 10, 100 and 1000 nM). After 24, 48, 72 and 96 hours, 20 µl of CellTiter 96 Aqueous One Solution reagent were added to each well and plates were incubated for 3-4 hours. Subsequently, plates were read at 490 nm using the ELx800 Universal Microplate Reader (Bio-tech) and analyzed with KC4 v3.0 with PowerReports software. For cell counting, cells were seeded in 24-well plates at 5x10⁴/well in 500 µl of DMEM medium containing 10% FBS plus various concentrations of GAs (1, 10, 100 and 1000 nM). After 24, 48, 72 and 96 hours, cell were dissociated with 0.5% trypsin/EDTA and counted in a hemocytometer. Each experiment was repeated three times and each concentration was in triplicates.

Apoptosis assays

Apoptosis was evaluated by the Annexin-V binding assay, caspase-3 activation and PARP cleavage. The cells were harvested, washed in PBS and resuspended in binding buffer. The cell suspension was incubated with propidium iodide (PI) and fluorescein isothiocyanate (FITC)-Annexin V (BD Pharmingen) for 15 min at RT. Permeabilised cells were stained with anti PARP mAb or anti caspase-3 mAb for 30 min on ice (BD Pharmingen). Flow cytometric analysis was performed

(FACS Canto, Becton Dickinson Bioscience) within an hour. Experiments were performed twice.

RNA extraction and reverse transcription

Total RNA was extracted using RNeasy Mini Kit (Qiagen) followed by DNase treatment (Promega). The reverse polymerase transcription was performed using MMLV reverse transcriptase (Invitrogen) according to manufacturer's protocol.

Quantitative real time PCR analysis

HSP90 and MMP2 expression was determined by quantitative real time PCR analysis on ABI PRISM 7300 Sequence Detection System (Applied Biosystems) using a commercially available TaqMan PCR Master Mix and specific primers-probe set (HSP90-Hs00743767_sh, MMP2-Hs00234579_m1). The HSP90 expression level was normalized to the housekeeping gene GAPDH (Hs99999905_m1). Experiments were done two times.

Chemotaxis assay

The directional movement of cells towards HGF gradient in a presence or an absence of GAs was evaluated using modified Boyden's chamber with 8-8 mm pore polycarbonate membrane inserts (Costar Transwell; Costar-Corning) covered with 0.5% gelatin (Sigma). Cells were detached from the culture flasks with trypsin, incubated for 30 min with GAs (10 nM and 100 nM) and subsequently seeded into the upper chamber of an insert at a density of 2.5x10⁴ in 100 µl. The lower chamber was filled with pre-warmed medium containing HGF (20 ng/ml) or medium alone (DMEM with 0.5% BSA) as a control. After 24 hours, inserts were removed from the transwell and cells were fixed with methanol. The cells that did not migrate were scraped off with a cotton wool from the upper membrane whereas the cells that transmigrated to the lower side of the membrane were stained with Wright solution and counted under high power field (HPF) with inverted microscope. 5 fields were counted each time and the mean number of cells per HPF was calculated. Chemotaxis assay was done two times in duplicates.

Invasion assay

GFR Matrigel invasion inserts (BD Biosciences) were rehydrated with DMEM for 2 hours and transferred to the wells containing medium with chemoattractant (HGF 20 ng/ml) or medium alone (DMEM with 0.5% BSA) as a control. Cells were harvested from the culture flasks by trypsinization, washed and resuspended in DMEM medium containing 0.5% BSA. After 30 min of incubation with GAs (100 nM), they were seeded at a density of 2.5x10⁴ in 0.5 ml to the inside of the inserts. In experiments with inhibitors, cells were preincubated for 30 min at 37°C in the presence of 10 or 100 nM GA, 17AAG or 17-AEP-GA. After 24 hours the cells that invaded the Matrigel barrier towards either media alone or an HGF gradient were counted on the undersides of filters after fixation and staining with Wright solution. As a control of invasion the same number of control inserts (no GFR Matrigel coating) was used. Invasion assay was done two times in duplicates.

Western blot

Western blots were done on extracts prepared from the cells that were treated with different concentrations of GAs for 24 and 48 hours as described earlier (10). In short, RMS cells were lysed (for 10 min) on ice in M-Per lysing buffer (Pierce) 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 PVDF membrane (BioRad). Total level of MET, Hsp90, AKT proteins was assessed using primary rabbit polyclonal antibodies for MET and Hsp90 (Santa Cruz Biotech), AKT (Cell Signaling) and subsequently detected with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (Santa Cruz Biotech). The membranes were developed with an enhanced chemiluminescence (ECL) reagent (Amersham Life Sciences), dried, and subsequently exposed to the HyperFilm (Amersham Life Sciences). An equal loading in the lanes was evaluated by probing with an anti GAPDH antibody (Santa Cruz Biotech). Western blot analysis was performed twice.

Hematopoietic colony assay

2.5×10^3 CD34⁺ cells were cultured in MethoCult medium containing growth factors (Stem Cells Technologies) in a constant presence of GAs (1, 10 and 100 nM). Cells were seeded in duplicates and two experiments were performed. Cultures were incubated at 37°C in fully humidified atmosphere supplemented with 5% CO₂. Colony-Forming Units-Granulocyte-Macrophage (CFU-GM) and Burst-Forming Units-Erythrocyte (BFU-E) were identified and counted using an inverted microscope after 14 days of culture.

Statistical analysis

All results are presented as mean \pm SD. Statistical analysis of the data were performed using the t-Student test, with $p < 0.05$ considered significant.

RESULTS

Similar effect but different potency exhibited by geldanamycins on rhabdomyosarcoma cells growth

RMS cells were grown in a presence of various concentrations of GA and its analogs (from 1 nM to 1000 nM). All GAs strongly blocked RMS cells proliferation at 100 nM and 1000 nM. However, only GA, 17DMAG and 17AEP-GA induced significant growth arrest of tumor cells at doses of 10 nM or lower (Fig. 1A). IC₅₀ value was calculated for all drugs (Fig. 1B). IC₅₀ observed for 17DMAG and 17AEP-GA was similar to that for GA, indicating their high anti tumor activity. IC₅₀ of 17DMAP-GA and 17AAG was significantly higher in comparison to GA ($p < 0.05$).

Induction of rhabdomyosarcoma cells apoptosis by HSP90 inhibitors

We also became interested whether the inhibition of a cell growth is due to induction of apoptosis. Therefore, in order to answer this question we used Annexin V, activated caspase-3 and cleaved PARP protein staining to detect apoptotic cells after GAs treatment. After 24 hours of incubation with 100 nM GAs, RMS cells became Annexin V positive (Fig. 2A). As in previous experiments 17DMAG and 17AEP-GA were the strongest inducers of apoptosis. The results obtained for GA, 17AAG and 17AEP-GA were confirmed with caspase-3 and PARP staining, where a high number of cells positive for activated caspase-3 and cleaved form of PARP in 17AEP-GA treated group was observed (Fig. 2A). Number of cells positive for Annexin V,

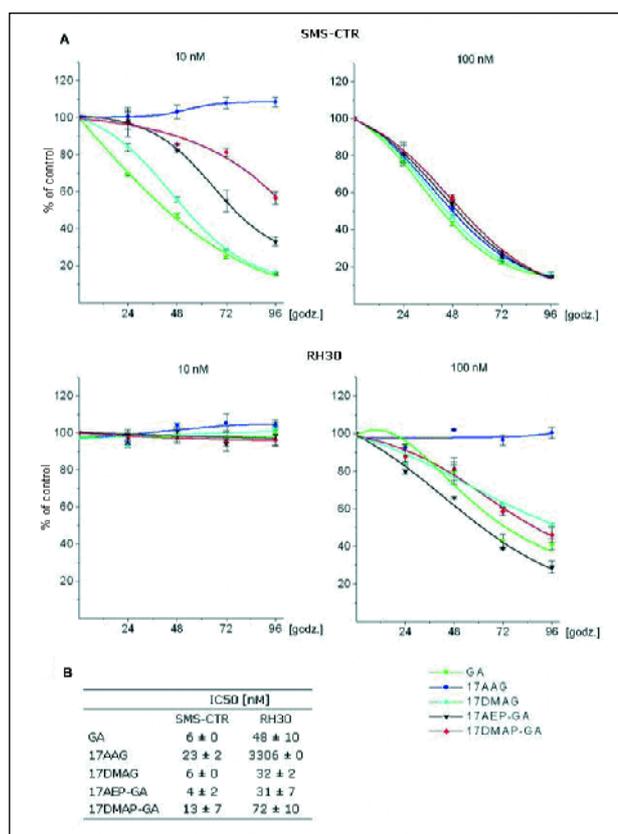


Fig. 1. Influence of GAs on RMS proliferation. Panel A - RMS cell lines were exposed to different concentrations of GAs and incubated for 24, 48, 72 and 96 hours and subsequently evaluated by MTT assay. Panel B - IC₅₀ calculated for all drugs revealed similar activity of GA and its analogs: 17DMAG and 17AEP-GA. Experiments were performed three times.

activated caspase-3 and cleaved PARP was further increased after 48 hours (Fig. 2A).

Since GA had been already shown to inhibit expression of HSP90 client protein AKT in RMS cells (10), we studied, in parallel to results obtained for Annexin V, caspase-3 and PARP staining, the expression pattern of AKT kinase in ERMS and ARMS cells after incubation with GAs. When the cells had been incubated for 24 hours with 100 nM of inhibitors, we noticed strong ablation of AKT expression (ERMS Fig. 2B and ARMS Fig. 2C). Partial reduction of this kinase expression was induced at 10 nM concentration (data not shown).

MET- induced responses are inhibited by geldanamycin and its analogs

MET receptor tyrosine kinase has been shown to be an important factor in RMS cell growth and motility (11). It has been proved to be a good target for anti RMS therapy (12). In our previous studies we had observed that GA inhibits MET expression and RMS cell responses to HGF (10). Here we looked if other HSP90 inhibitors will exert similar effects on HGF-MET signaling axis.

On the grounds of previous results, we chose three inhibitors for these experiments: GA, 17AAG (now in clinical trials) and 17AEP-GA, which had comparable to GA effects on RMS cells growth.

Because HGF-MET axis plays an important role in migration in normal and pathological conditions, we tested the

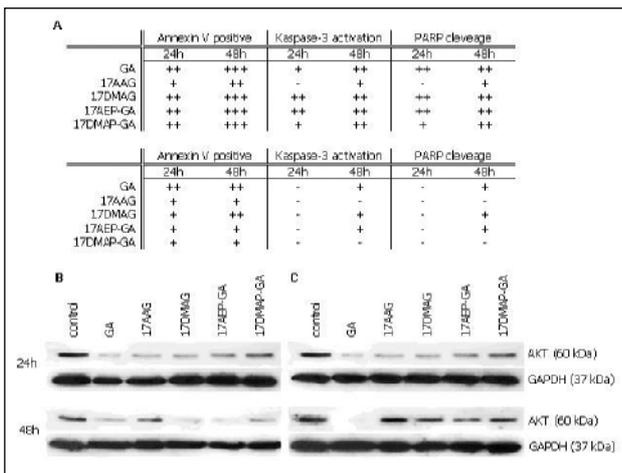


Fig. 2. Influence of GAs on RMS cells survival and Western blot analysis of total AKT expression after GAs treatment. Cells were incubated for 24 and 48 hours with GAs (100 nM) and stained against Annexin V, against activated form of caspase-3 and with antibodies directed toward cleaved form of PARP. „+”, „++”, „+++” denotes no cells with positive staining, „+” under 30% of positive cells, „++” between 30-50% of positive cells and „+++” above 50% of positive cells. 17DMAG, and 17AEP-GA were the strongest inducers of apoptosis (Upper panel A-SMS-CTR, lower panel A-RH30). Experiments were repeated twice. Cells were incubated for 24 and 48 hours with 100 nM of GAs. Strong downregulation of AKT expression was observed after 24 and 48 hours in both SMS-CTR (Panel B) and RH30 cells (Panel C). One representative experiment of three performed is shown.

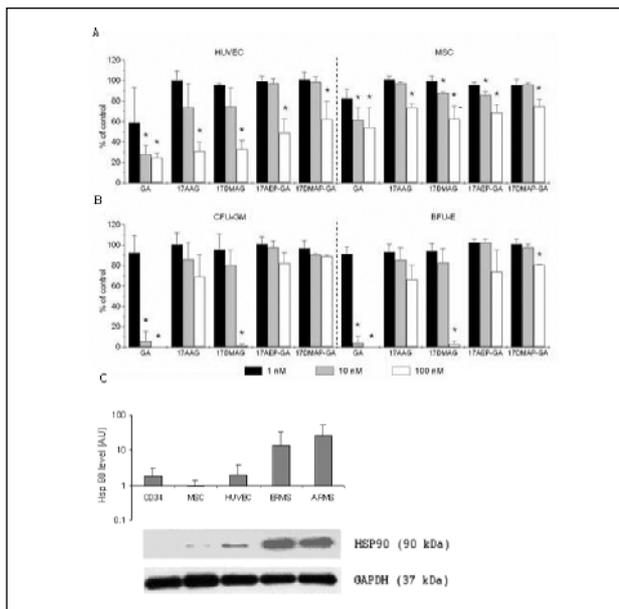


Fig. 4. Influence of GAs on normal cells. The effect of GAs on cells representing bone marrow environment: MSC and HUVEC (Panel A – MTT data) and bone marrow CD34⁺ (Panel B – colony formation data). HSP90 expression is significantly higher in RMS cells in comparison to normal cells, at both mRNA and protein level (Panel C). Experiments were performed two times. *;p<0.05

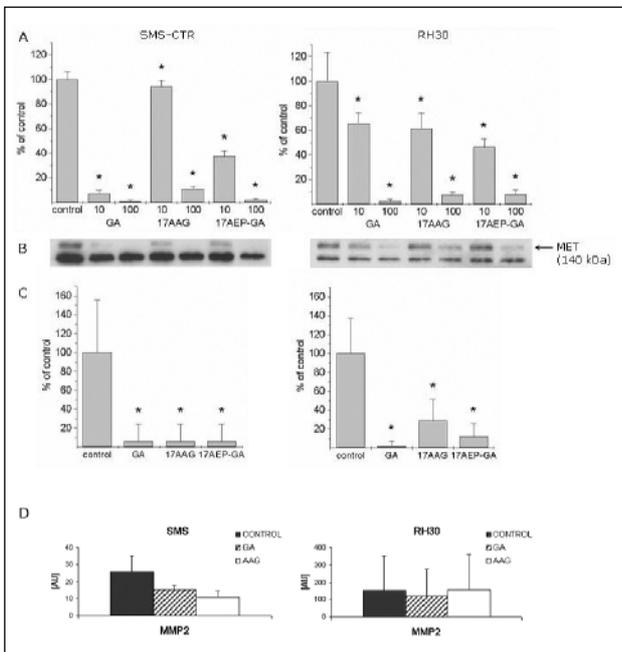


Fig. 3. Influence of GAs on HGF-MET axis. Panel A - Analysis of RMS chemotaxis toward HGF in a presence of selected GAs (10 and 100 nM). *;p<0.05. Panel B - Western blot analysis of MET expression. Exposure of RMS cells to 10 or 100 nM GA downregulated MET level in both SMS-CTR and RH30 cells. Panel C - Analysis of HGF-mediated invasion of RMS cells through GFR Matrigel invasion inserts in a presence of selected GAs (100 nM). *;p<0.05 Panel D - Influence of selected GAs on the mRNA level of MMP2. All assays were done two or three times.

influence of GAs on HGF - induced chemotaxis of RMS cells. We observed a strong inhibition of RMS cells migration towards HGF following GAs treatment (Fig. 3A), which correlated with a decreased level of MET expression (Fig. 3B).

HGF has been shown to induce metastatic potential of RMS cells due to upregulation of membrane and extracellular matrix (ECM) degrading enzymes (11). Thus, we tested the ability of GAs to inhibit transmigration of RMS cells through matrigel-coated inserts. We noticed that HGF - induced transmigration of RMS cells was almost completely impaired by HSP90 inhibitors (Fig. 3C). To explain this effect we studied the influence of GAs on the expression of extracellular matrix degrading enzymes, metalloproteinases. Using qRT-PCR analysis we noticed approximately 2 folds downregulation of MMP2 in GAs treated ERMS cells (Fig. 3D). The downregulation of MMP2 in ARMS cells, although noticed, was weak.

Influence of geldanamycins on normal cells

In order to test the effect of GAs on normal cells, we used purified bone marrow CD34⁺, mesenchymal stem cells (MSC) and endothelial cells (HUVEC). CD34⁺ cells, MSC and HUVEC were exposed to different concentrations of GAs (1 nM, 10 nM and 100 nM) and CFU-GM, BFU-E colony assay and MTT assay were performed. All GAs exhibited cytotoxic effect towards normal cells, however, to different degrees (Fig. 4A and B). GA and 17DMAG were most toxic among tested inhibitors regardless of a normal cell type, whereas 17AAG and 17AEP-GA possessed lowest toxicity toward BFU-E and CFU-GM.

Our data suggested that RMS cells were more sensitive to HSP90 inhibition in comparison to normal cells. In order to find the mechanism responsible for this observation, the level of HSP90 expression in these cells was examined. We found that the level of HSP90 expression was significantly higher in tumor cells than in normal cells of various lineages (Fig. 4C).

DISCUSSION

Recently, we have shown that GA, a potent HSP90 inhibitor blocks proliferation, induces apoptosis and inhibits human RMS cells migration and homing into bone marrow (10). However, because of its strong cellular toxicity, use of GA in clinical studies is not permitted. In the last few years, several new GA analogs that exhibit much less adverse effects but retain similar anti tumor potency to GA were developed (13, 14).

The goal of the present study was to compare efficacy of GA and four new HSP90 inhibitors in relation to growth and metastatic potential of RMS cells.

Firstly, the influence of GAs on RMS cells proliferation/survival was assessed. All GAs were able to inhibit tumor cells growth and maximum level of growth inhibition was more than 80% after 96 hours. In particular, 17DMAG and newly developed water-soluble 17AEP-GA blocked successfully cell growth in concentration range similar to GA. IC50 for these analogs did not statistically differ from IC50 for GA. At the same time IC50 for 17AAG and 17DMAP-GA were significantly higher in comparison to GA, indicating a lower inhibitory potential of these compounds. Our results are in agreement with the previous ones showing that GA and its analogs, especially 17AAG, are able to block proliferation of various tumors (13, 14). However, in contrast to already published data on other tumors (15, 16), the inhibition of RMS cells proliferation by 17AAG did not reach the level of GA as measured by MTT assay.

GA has been shown to block various oncoproteins including tyrosine kinase receptors (19), and among them, MET receptor (10). In this paper we report that all GAs partially inhibit MET expression at 10 nM and completely at 100 nM. Downregulation of MET receptor is paralleled by inhibition of HGF-dependent chemotaxis. Maulik and colleagues showed that nanomolar concentration of GA inhibits MET expression in SCLC cells within 24 hours (20). Similar concentrations of GA were necessary to downregulate EGF receptor expression in thyroid cancer cells (21). However, Xie and co-workers observed a decreased migration at femtomolar concentration of GA without changes in MET expression. They postulated that this phenomenon is due to targeting downstream MET signaling pathways (22). Since 100 nM GA, used in our study, was sufficient to block RMS cells migration, similarly to Maulik's report, we think that migration blockade of RMS cells is due directly to MET downregulation.

Metastasizing RMS has a very poor prognosis, therefore, any ways of blocking its invasion could be beneficial for patients. Here we show that GA, 17AAG and 17AEP-GA are able to inhibit not only chemotaxis but also HGF-dependent transmatrix migration of RMS cells. Our data shows also that GAs are also responsible for reduction of MMP2 expression in RMS cells. MMP2 is recognized as an important factor responsible for ECM degradation and tumor cells invasion (23) and its expression has been shown to be upregulated by HGF (11).

We postulate, therefore, that HGF-dependent invasion can be reduced due to decreased expression of MET receptor and MMP2.

Since bone marrow is composed of various cell types, the influence of HSP90 inhibitors on their growth is an important aspect when new anticancer drug is considered. Our results suggest that GAs, particularly 17AAG and 17AEP-GA, exhibit low cytotoxicity towards normal cells residing in the bone marrow. It is important when combined use of HSP90 inhibitors and standard chemotherapy is considered.

Taking into consideration the data discussed in the present study, we postulate that HSP90 inhibitors should be considered as new potential agents for the treatment of RMS. 17AEP-GA seems to be particularly interesting because it shows strong

inhibitory action towards both growth and migration of RMS cells, which is similar to the one observed for GA, but possesses much better bioavailability (it is water soluble) and reduced cytotoxicity towards normal cells.

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