

The Geldanamycins Are Potent Inhibitors of the Hepatocyte Growth Factor/Scatter Factor-Met-Urokinase Plasminogen Activator-Plasmin Proteolytic Network¹

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ABSTRACT

The Met receptor tyrosine kinase and its ligand, hepatocyte growth factor/scatter factor (HGF/SF), have been implicated in human tumor development and metastasis. HGF/SF induces the expression of urokinase plasminogen activator (uPA) and the uPA receptor (uPAR), important mediators of cell invasion and metastasis. We have developed a cell-based assay to screen for inhibitors of this signaling system using the induction of endogenous uPA and uPAR and the subsequent conversion of plasminogen to plasmin as the biological end point. Assay validation was established using a neutralizing antiserum to HGF/SF and a uPA inhibitor (B428), as well as inhibitors of the MKK-MAPK1/2 pathway, shown previously to be important in the induction of uPA and uPAR. Using this assay, we found several classes of molecules that exhibited inhibition of HGF/SF-dependent plasmin activation. However, we discovered that certain members of the geldanamycin family of anisamycin antibiotics are potent inhibitors of HGF/SF-mediated plasmin activation, displaying inhibitory properties at femtomolar concentrations and nine orders of magnitude below their growth inhibitory concentrations. At nanomolar concentrations, the geldanamycins down-regulate Met protein expression, inhibit HGF/SF-mediated cell motility and invasion, and also revert the phenotype of both autocrine HGF/SF-Met transformed cells as well as those transformed by Met proteins with activating mutations. Thus, the geldanamycins may have important therapeutic potential for the treatment of cancers in which Met activity contributes to the invasive/metastatic phenotype.

INTRODUCTION

The product of the *met* proto-oncogene is the transmembrane tyrosine kinase p190^{Met} (1), which has been identified as the receptor for HGF/SF³ (2). HGF/SF is a polypeptide growth factor produced predominantly by cells of mesenchymal origin, which elicits a variety of effects on target cells expressing Met *in vitro*, including the induction of cell proliferation, migration/invasion, and morphogenesis (reviewed in Refs. 3 and 4). *In vivo*, the HGF/SF-Met signaling pathway plays an important role during embryological development, tissue regeneration/repair, wound healing, and angiogenesis (reviewed in Ref. 5). In addition to its roles in normal physiological processes, it has now been established that aberrant HGF/SF-Met signaling plays a critical role in the development and progression of primary tumors and secondary metastases (reviewed in Ref. 6). For example, we have previously shown that the coexpression of Met and HGF/SF in the same cell results in the acquisition of both tumorigenic and metastatic

properties when these cells are injected into athymic nude mice (7-10). More recently, activating Met mutations have been identified in human patients with papillary renal carcinoma (11), and the introduction of these mutations into Met cDNA results in transforming, tumorigenic, and metastatic properties in mouse cell lines (12, 13). When the same mutations are introduced into mice as transgenes, the founders develop tumors that metastasize to secondary sites (13). In addition, there are numerous reports demonstrating an increased expression of Met and/or HGF/SF in a variety of human tumors, often associated with increased tumor grade and poor prognosis (reviewed in Ref. 13). Thus, inhibitors of the HGF/SF-Met signaling system would be useful for the treatment of a wide variety of human tumors and/or metastasis.

Cell invasion is a major component of the complex multistep process of tumor metastasis. Invasion requires both cell motility and degradation of the surrounding ECM, the latter of which is mediated by a number of proteolytic enzymes (reviewed in Ref. 14). We and others have shown that HGF/SF stimulation of a variety of cells expressing Met induces the expression of the serine protease urokinase (uPA) and its receptor (uPAR), resulting in an increase of uPA at the cell surface (4, 7, 15) through a pathway involving MAPK1/2 signaling (16). Although uPA is directly involved in the degradation of some components of the ECM, such as fibronectin, most of its ECM/BM-degrading properties are believed to arise through its ability to cleave plasminogen into the broader specificity protease plasmin (17, 18). Like uPA, the active plasmin protease is predominantly associated with the cell surface, but its broader substrate specificity allows for plasmin to play a more direct role in ECM/BM degradation. In addition, plasmin can activate metalloproteinases, proteases with potent ECM/BM-degrading capabilities (reviewed in Ref. 19). Because uPA plays a central role in catalyzing ECM/BM degradation, it is not surprising that a strong association between uPA expression and induction of the invasive/metastatic phenotype has been demonstrated (reviewed in Ref. 20). Thus, activation of the uPA-plasmin proteolytic network is likely of great importance for HGF/SF-mediated cell invasion and metastasis.

Using the induction of uPA/uPAR and subsequent conversion of plasminogen to plasmin as a biological end point, we have developed a cell-based assay to screen for inhibitors of the HGF/SF-Met-mediated signaling pathways that lead to activation of plasmin protease activity. Among a number of interesting inhibitors of this pathway, we show that the geldanamycin family of anisamycin antibiotics inhibit HGF/SF-Met-mediated plasmin activation. The geldanamycins inhibit HGF/SF-mediated cell motility and invasion associated with a reduction in Met expression, and may have potential as a therapeutic in preventing invasion and metastasis associated with aberrant signaling through HGF/SF-Met.

MATERIALS AND METHODS

Cell Lines. The following cell lines used in this study were obtained from the American Type Culture Collection, Rockville, MD and were cultured as recommended by the supplier: HT-29, human colon adenocarcinoma; A431,

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³ The abbreviations used are: HGS/SF, hepatocyte growth factor/scatter factor; ECM, extracellular matrix; uPA, urokinase plasminogen activator; uPAR, uPA receptor; BM, basement membrane; FBS, fetal bovine serum; MDCK, Madine Darby canine kidney; MAP, mitogen-activated protein; MKK, MAP kinase kinase; LF, lethal factor; PA, protective antigen; SRB, sulforhodamine B; GI, growth inhibitory.

human epidermoid carcinoma; A549, human lung carcinoma; SKLMS-1, human leiomyosarcoma; EMT6, mouse mammary carcinoma; U-118, human glioma; C127-Met^{mtu}, nontransformed immortalized cells established from a mouse mammary tumor engineered to express high levels of Met^{mtu} (9); and C127-Met^{mtu}/HGF^{hu}, C127 cells expressing both Met^{mtu} and human HGF/SF (9). Human renal carcinoma ARZ-2 cells were a kind gift from J. Gnarr (Louisiana Medical School, New Orleans, LA) and were cultured in DMEM supplemented with 10% FBS. A clone of the MDCK cell line, MDCK-2, cells were a kind gift from I. Tsarfaty (Tel Aviv University, Tel Aviv, Israel). MDCK-1 cells have been previously described as a variant MDCK cell line insensitive to HGF/SF (21). Both MDCK variants were cultured in DMEM + 10% FBS. NIH3T3 (490) cells were obtained from D. Blair (Frederick, MD). NIH3T3 cells transformed by Tpr-Met (22), mutationally activated Met (Met L1213V/M1628T; Ref. 11), and a mutationally activated Trk-Met chimera (Trk-Met L1213V/M1628T; Ref. 12) have been previously described.

Reagents. Human plasminogen and a specific plasmin chromophore substrate (Chromozyme PL) were purchased from Boehringer Mannheim. Human uncleaved HGF/SF was purified from the supernatant of transformed NIH3T3 cells engineered to overexpress the factor as previously described (23). Polyclonal antiserum (NCI-53) was raised against HGF/SF by immunization of rabbits with full-length purified human HGF/SF. The uPA inhibitor B428 (24) was a kind gift from Dr. B. Littlefield (Eisai Research Institute, Andover, Ma). The MKK inhibitor PD 98059 was purchased from New England Biolabs. Anthrax LF and PA were a kind gift from Dr. S. Leppla (Institute of Dental Research, NIH, Bethesda, MD). Drugs were obtained from the Drug Synthesis and Chemistry Branch of the National Cancer Institute (Rockville, MD).

The HGF/SF-Met-uPA-Plasmin Cellular Assay. The following procedure was used to determine the effect of test reagents on HGF/SF-mediated plasmin activation and cell growth. MDCK-2 cells were seeded at 1500 cells/well of a 96-well microtiter plate and grown overnight in DMEM/10% FBS growth medium. Duplicate plates were made for the determination of plasmin activation and cell growth. Drugs were serially diluted from stock concentrations in DMEM/10% FBS media and added to the relative wells. A 1:100 dilution of the NCI-53 neutralizing antiserum to human HGF/SF was added to the relevant wells as a standard control on each microtiter plate. Immediately after drug or reagent addition, HGF/SF (10 units/ml) was added to all wells (with the exception of wells used to calculate basal growth and plasmin activation). Twenty-four h after drug/HGF/SF addition, one of two duplicate plates was processed for the determination of plasmin activity as follows. Wells were washed twice with DMEM (without phenol red; Life Technologies, Inc.), and 200 μ l of reaction buffer [50% (v/v) 0.05 units/ml plasminogen in DMEM (without phenol red), 40% (v/v) 50 mM Tris buffer (pH 8.2), and 10% (v/v) 3 mM Chromozyme PL in 100 mM glycine solution] were added to each well. The plates were then incubated at 37°C, 5% CO₂ for 4 h, at which time the absorbances generated were read on an automated spectrophotometric plate reader at a single wavelength of 405 nm. The determination of cell growth on a duplicate plate was performed by measuring SRB staining of cellular proteins as described previously (25). In brief, cells were fixed *in situ* with 50% trichloroacetic acid, and the plates were washed five times with deionized water and dried. SRB [100 μ l/well, 0.4 (w/v) in 1% acetic acid] was added to each well and incubated for 30 min at room temperature. Unbound SRB was removed by washing three times with 1% acetic acid. Plates were then air-dried, and bound stain was solubilized with 10 mM Tris. Absorbances were read on an automated spectrophotometric plate reader at a single wavelength of 570 nm.

Plasmin activation ($A_{405\text{ nm}}$) was first normalized for the amount of protein in each well. After subtracting the background plasmin activity of unstimulated control cells, percent inhibition of chromozyme production [% chromozyme inhibition (CI)] for all test agents was calculated relative to HGF/SF-stimulated cells in the absence of the drug. IC₅₀ values (concentration of drug inhibiting HGF/SF response by 50%) and GI₅₀ values (concentration of drug inhibiting growth by 50%) were then calculated for each drug or reagent from plotted graphs. For the calculation of GI₅₀, one replica plate per experiment was fixed before 24-h HGF/SF stimulation to determine the growth at $t = 0$ (= 0% growth).

Cell Scattering, Branching Morphogenesis/Invasion, and Motility Assays. MDCK-2 cells were used in cell scattering assays as previously described (26). Drugs were diluted in growth media containing serially diluted

human HGF/SF to determine the ability of the drugs to inhibit cell scattering over 24 h.

Branching morphogenesis/invasion in a three-dimensional Matrigel matrix was analyzed as described previously (6). In brief, cells at a density of 50,000 cells/ml in DMEM + 10% FBS media were mixed with an equal volume of Matrigel (Becton Dickinson), plated at 0.1 ml/well of a 96-well culture plate, and incubated for 20 min at 37°C/5% CO₂ to allow gel formation. Growth media containing 200 units/ml of human HGF/SF with or without drugs at various concentrations were then added to each well. After 48 h, representative fields of view were photographed.

Cell motility assays were performed using 24-well transwell units with 8- μ m polycarbonate filters (Costar) as previously described (27). In brief, 1×10^4 cells (in 100 μ l) were plated onto the upper surface of the filter in DMEM media + 1% BSA in the absence or presence of drug. The filter was then lowered into the lower compartment containing DMEM + 1% FBS media \pm 200 units/ml human HGF/SF in the absence or presence of drug. After 16 h of incubation at 37°C/5% CO₂, cells were fixed in methanol and stained with Diff-Quick (Dade, Aguada, Puerto Rico). Nonmigratory cells on the upper filter surface were removed using a cotton swab, and the total number of cells on each filter were counted at $\times 200$ magnification using a phase-contrast microscope accommodated with an ocular grid.

Western Blotting. Western analysis was performed essentially as described previously (6, 28) with the following modifications. In brief, cells were grown to ~50% confluence in DMEM/10% FBS growth medium before treatment with HGF/SF (100 units/ml) \pm inhibitors at the indicated concentrations for 24 h. At the end of the incubation period, cells were lysed by washing twice with ice cold PBS and resuspending in lysis buffer [20 mM PIPES (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1.5 mM MgCl₂, 1% Triton-X-100, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 100 μ M pervanadate, and 0.1% SDS]. Cell lysates were clarified by centrifugation at 15,000 g for 15 min at 4°C, and protein determination was performed on the soluble protein supernatants using a standard assay (Pierce). Ten μ g of cell lysates were resuspended 3:1 into 4 \times reducing or nonreducing Laemmli buffer [\pm DTT, respectively; 0.4 M Tris-HCl (pH 6.8), 8% SDS, 39% glycerol, 0.04% bromophenol blue \pm 0.4 M DTT], boiled for 5 min, and resolved by SDS-PAGE. Proteins were then transferred to nitrocellulose, and blots were stained with Ponceau S stain to visualize protein bands and ensure equal protein loading. Blots were then washed and blocked for 1 h with a 5% solution of BSA in TBS buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% Tween 20]. Blots were then probed for 1 h using either 2 μ g/ml of a rabbit antihuman uPA antiserum (American Diagnostica), 1 μ g/ml of a rabbit anti-human uPAR antiserum (American Diagnostica), 1 μ g/ml of a rabbit anti-mouse Met antiserum (clone SP-260, Santa Cruz, CA), or 1 μ g/ml of a rabbit antihuman Met antiserum (clone C-28, Santa Cruz, CA). Blots were then probed with a 1:15,000 dilution of a goat antirabbit antiserum coupled to peroxidase (Sigma), followed by detection using the enhanced chemiluminescence system (Amersham) and X-ray film development. Protein bands were quantified using a Linocolor scanner and Quantiscan analysis software.

RESULTS

MDCK Cells Display Optimal HGF/SF-mediated uPA Activation. The ability of HGF/SF to induce the conversion of exogenous plasminogen to plasmin was tested in a variety of cell lines to obtain the optimal response before inhibitor screening. Fig. 1A shows the relative responses of different cell lines to 10 units/ml HGF/SF above control untreated cells. Of the various cell lines tested, a clone of the MDCK canine kidney cell line (MDCK-2) was found to display the greatest increase in plasmin activity following 24-h stimulation with HGF/SF. Doses ranging from 1 to 10000 units/ml HGF/SF were tested in MDCK-2 cells. Ten units/ml HGF/SF were found to be submaximal and were used in subsequent experiments. The maximum response (4.5-fold increase) was observed using 32 units/ml HGF/SF (Fig. 1B). Higher doses (>1000 units/ml) induced plasmin activation to a lesser extent, and the dose-response curve displayed typical bell-shaped characteristics (data not shown). An additional variant MDCK cell clone (MDCK-1) displayed no uPA-plasmin response to HGF/SF

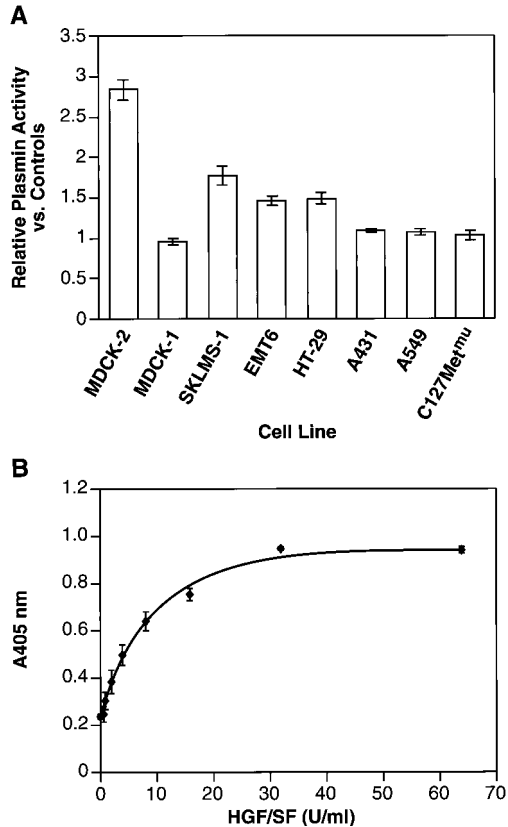


Fig. 1. A, HGF/SF-mediated plasmin activation in various cell lines after 24-h stimulation with 10 units/ml HGF/SF. The results are expressed relative to unstimulated control cells. B, dose response for HGF/SF-mediated plasmin activation in MDCK-2 cells after 24 h. Plasmin activation was determined by using a specific plasmin chromophore substrate and measuring absorbance at 405 nm as described in "Materials and Methods." Error bars, the SE from the mean of multiple experiments ($n > 3$).

concentrations up to 10,000 units/ml consistent with its previously reported insensitivity to HGF/SF (21). SKLMS-1, EMT6, HT-29, A431, A549, and C127 cells displayed between ~1.1–1.7-fold increases in plasmin activity following 10 units/ml HGF/SF treatment (Fig. 1A). MDCK-2 cells were, therefore, used in further studies because they gave the best response to HGF/SF. In addition, these cells scatter in response to HGF/SF (26) and, therefore, provide an additional means to study inhibition of HGF/SF-Met signaling.

Inhibition of the HGF/SF-Met-uPA-Plasmin Network by Neutralizing HGF/SF Antiserum and a Known uPA Inhibitor (B428). To validate the efficiency and specificity of this cellular assay, we examined the inhibition of HGF/SF-induced plasmin activation by reagents expected to display inhibitory properties. An HGF/SF neutralizing polyclonal antiserum (NCI-53) was first tested for inhibition of plasmin activation. A 1:100 dilution of this serum added at the time of HGF/SF treatment inhibited the response by ~90% (Fig. 2A). HGF/SF had no effect on MDCK-2 cell proliferation as previously observed (3, 21). The NCI-53 antiserum displayed no cytotoxic/cytostatic properties and had no effect on the basal level of plasmin activity displayed by MDCK-2 cells (data not shown), demonstrating that both cell growth and basal uPA/plasmin activity are HGF/SF-independent. The NCI-53 antiserum was included as an inhibitory control in all assays in the search for inhibitors.

We also tested the inhibitory properties of the known uPA inhibitor, B428 (24), in this assay. B428 inhibited both basal (represented by >100% inhibition) and HGF/SF-induced plasmin activity at concentrations where little effect on cell proliferation were observed (Fig. 2B; 24). Furthermore, B428 displayed no toxicity and was a more potent inhibitor if added only during the second stage, where cells are incubated for 4 h with exogenous plasminogen after the 24-h period of HGF/SF stimulation (see "Materials and Methods"). Because B428 is a competitive inhibitor of uPA activity (24), this is likely due to B428 directly inhibiting uPA activity. Thus, the reduced efficacy of B428

Fig. 2. Inhibition of HGF/SF-mediated plasmin activation in MDCK-2 cells by: A, neutralizing HGF/SF antiserum; B, the uPA inhibitor B428; C, the MKK inhibitor PD 98059; and D, the MKK protease anthrax LF. Cells were incubated with 10 units/ml HGF/SF in the absence or presence of various concentrations of the different reagents as indicated. Plasmin activation was determined by using a specific plasmin chromophore substrate and measuring absorbance at 405 nm as described in "Materials and Methods." The left axes show the percent inhibition of plasmin activity relative to HGF/SF-stimulated control cells (except for in A, in which plasmin activity is expressed relative to nontreated control cells). The right axes show percent growth relative to HGF/SF-stimulated control cells (except again in A, in which growth is expressed relative to nontreated control cells). In B, B428 was added either simultaneously with HGF/SF for 24 h (first stage) or during the 4-h incubation with exogenous plasminogen (second stage), see "Materials and Methods". Error bars, the SE from the mean of multiple experiments ($n > 3$).

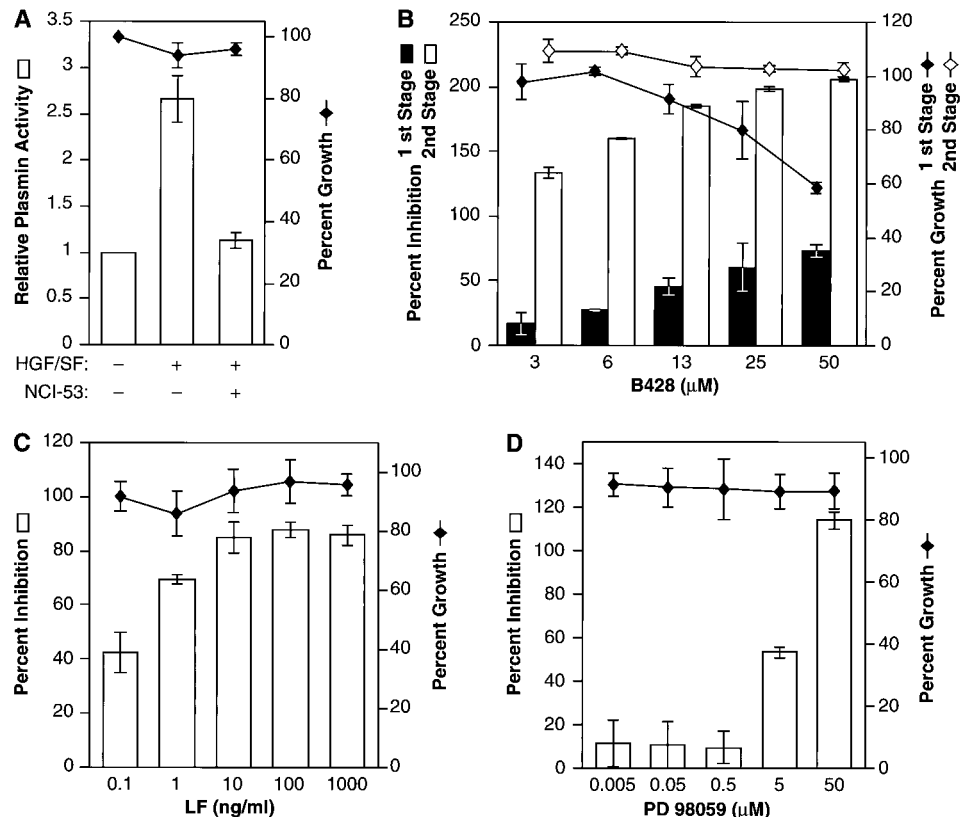
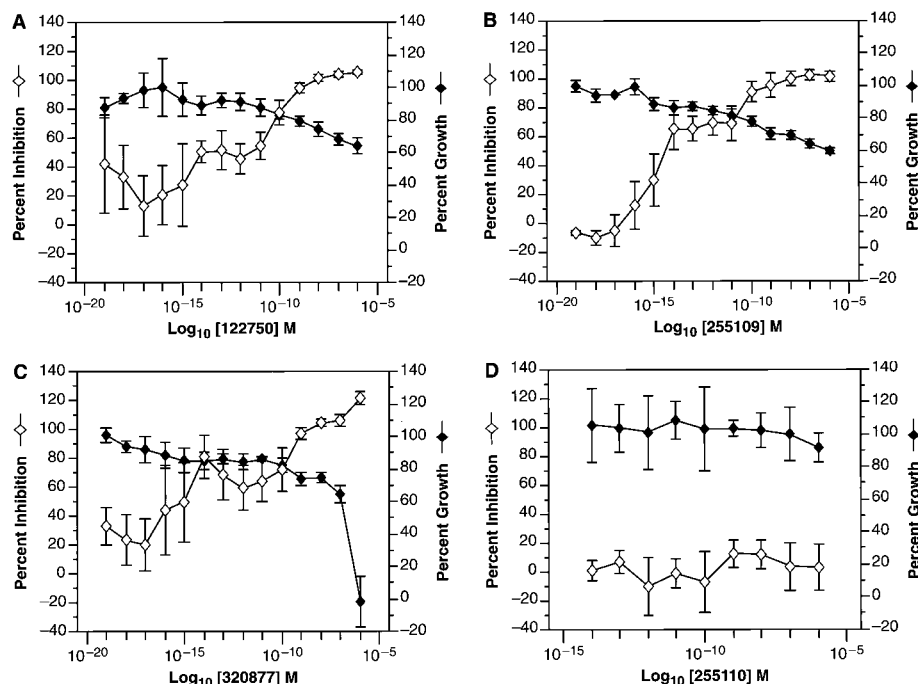


Fig. 3. Inhibition of HGF/SF-mediated plasmin activation by the geldanamycins in MDCK-2 cells. Cells were incubated with 10 units/ml HGF/SF in the presence or absence of different concentrations of drugs as indicated (A, 122750; B, 255109; C, 320877; D, 255110). Plasmin activity was assayed as described in "Materials and Methods." The *left axes* show the percent inhibition of plasmin activity relative to HGF/SF-stimulated control cells. The *right axes* show the percent growth relative to HGF/SF-stimulated control cells. Error bars, the SE from the mean of multiple experiments ($n > 3$).



when added during the first stage is likely due to the washing steps before plasminogen addition. Interestingly, B428 does not prevent the scattering of MDCK cells in response to HGF/SF (data not shown), indicating that scattering occurs independently of uPA/plasmin activation.

Inhibitors of the MAP Kinase Pathway Inhibit HGF/SF-mediated Plasmin Generation. It has previously been suggested that activation of the MAP kinase 1/2 pathway plays an important role in the induction of uPA expression (16, 29–31). We tested the activity of two known inhibitors of the MAP kinase pathway in this assay, anthrax LF and PD 98059. LF proteolytically inactivates MKK by cleaving within the amino terminus (32). Thus, by the addition of LF together with PA, which allows LF to enter cells through cell surface PA-receptors (33, 34), the MKK-MAP kinase signaling pathway is inactivated. Treatment of MDCK-2 cells with LF and PA resulted in a dose-dependent inhibition of HGF/SF-mediated plasmin activation with minimal effects on cell proliferation (Fig. 2C). Similarly, the MKK inhibitor PD 98059 (35) inhibited plasmin activation in response to HGF/SF with little effect on cell proliferation during the 24-h period (Fig. 2D). These results show that HGF/SF-mediated uPA/plasmin activation requires activation of the MKK-MAP kinase pathway and furthermore demonstrates the ability of this assay to detect inhibitors of multiple targets within the HGF/SF-Met-uPA-plasmin signaling pathway.

The Geldanamycins Are Potent Inhibitors of the HGF/SF-Met-uPA-Plasmin Proteolytic Network. Approximately 1000 compounds were tested for inhibitory properties using the cell-based screen in MDCK-2 cells. Our major interest was to identify compounds that prevented plasmin activation but had insignificant cytotoxic or cytostatic activities. Whereas three fluorinated steroids and two staurosporine analogues as well as others were identified as displaying these properties⁴, geldanamycin (National Service Center No. 122750) and a geldanamycin analogue (National Service Center No. 255109) were discovered as highly potent inhibitors in this assay (Fig. 3, A and B, respectively).

Both 122750 and 255109 inhibited HGF/SF-dependent plasmin activation in a dose-dependent manner with maximal inhibition observed at nm concentrations, but some inhibitory activity was retained at even femtomolar concentrations. 122750 or 255109 displayed only minimal dose-dependent cytotoxicity at concentrations where significant inhibition of HGF/SF-mediated plasmin activation was apparent. The IC_{50} values for 122750 and 255109 were calculated as 2.83×10^{-14} M and 1.13×10^{-14} M, whereas the GI_{50} values were 3.91×10^{-6} M and 4.9×10^{-6} M, respectively. Thus, an extraordinary 8-log differential exists between the IC_{50} for plasmin activation and GI_{50} for growth inhibition for these geldanamycins. We tested 10 additional geldanamycin analogues and found varying degrees of inhibition (Table 1). For example, 320877 was the most potent inhibitor of those tested, with an IC_{50} value of 3.04×10^{-15} M, whereas 255110 displayed inhibitory properties only at cytotoxic concentrations ($IC_{50} = 7.84 \times 10^{-6}$ M; Fig. 3, C and D, respectively and Table 1). This suggests that certain structural-functional relationships exist within these geldanamycin analogues that govern their relative abilities to inhibit HGF/SF-mediated uPA/plasmin activation.

Geldanamycins Inhibit HGF/SF-mediated uPA/uPAR Induction and Down-Regulate Met Expression. Because the geldanamycins were identified as inhibitors of HGF/SF-mediated uPA-depen-

Table 1. Inhibition of cell growth (GI_{50}) and HGF/SF-mediated plasmin activation (IC_{50}) by geldanamycin analogues

Geldanamycin National Service Center no.	GI_{50} (M)	IC_{50} (M)
320877	4.99×10^{-7}	3.04×10^{-15}
255105	3.11×10^{-6}	5.52×10^{-15}
255109 ^a	4.90×10^{-6}	1.13×10^{-14}
122750 ^a	3.91×10^{-6}	2.83×10^{-14}
330507	6.88×10^{-6}	1.35×10^{-10}
255104	1.02×10^{-6}	2.38×10^{-9}
330499	3.04×10^{-6}	6.67×10^{-9}
330512	7.92×10^{-6}	6.01×10^{-8}
330500	4.23×10^{-6}	1.05×10^{-7}
265482	7.82×10^{-6}	2.61×10^{-6}
255112	3.00×10^{-6}	6.84×10^{-6}
255110	3.00×10^{-6}	7.84×10^{-6}

⁴ C. P. Webb, A. Monks, C. D. Hose, and G. F. Vande Woude, unpublished observations.

^a Compounds initially identified from the screen.

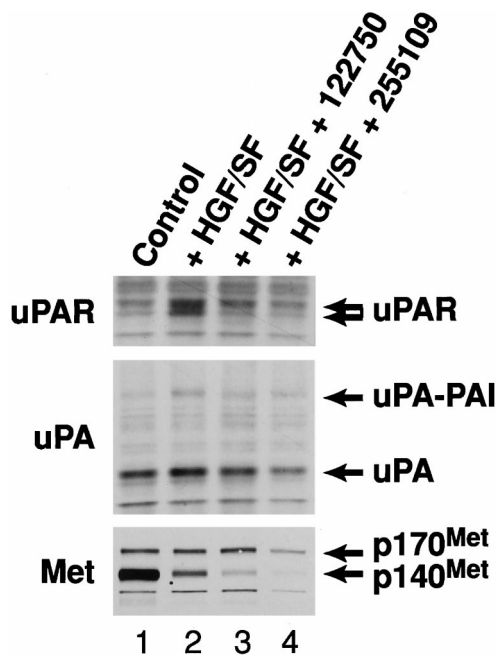


Fig. 4. Western blot analysis of uPAR, uPA, and Met expression in SKLMS-1 cells after 24-h HGF/SF stimulation in the presence or absence of the geldanamycins. Lane 1, control cells; Lane 2, cells treated with 100 units/ml HGF/SF; Lane 3, cells treated with 100 units/ml HGF/SF + 100 nM 122750; Lane 4, cells treated with 100 units/ml HGF/SF + 100 nM 255109. The positions of the relative proteins discussed in the text are indicated.

dent plasmin activation, we investigated their effect on uPA and uPAR protein expression. Western blot analysis demonstrated that whereas uPA and uPAR protein expression were induced in human SKLMS-1 cells after 24-h HGF/SF stimulation as previously observed (6; Fig. 4, top and middle panel, Lanes 1 and 2), both 122750 and 255109 inhibited the induction of uPA and uPAR (Lanes 3 and 4, respectively). HGF/SF induced uPA expression by 1.4-fold above control cells, but this was inhibited by 122750 and 255109 (1.0-fold and 0.6-fold relative to control unstimulated cells, respectively). Both compounds also inhibited the formation of the uPA-plasminogen-activator inhibitor complex induced by HGF/SF (6). uPAR expression was induced 2.5-fold in the presence of HGF/SF, and this was inhibited by both 122750 and 255109 (1.6-fold and 0.8-fold relative to control unstimulated cells, respectively). It should be noted, however, that these effects were only observed at nanomolar concentrations (data not shown), suggesting that the inhibition of HGF/SF-mediated uPA/uPAR induction may not be the sole mechanism by which the geldanamycins inhibit HGF/SF-mediated plasmin activation at lower concentrations (Table 1). Identical results were obtained when using the MDCK-2 cells originally screened (data not shown).

Geldanamycin has been shown to down-regulate the expression of a number of proteins, including tyrosine kinase molecules, such as the ErbB2 oncoprotein (36). To determine whether the geldanamycins down-regulate endogenous Met expression in a similar fashion, we performed Western blot analysis in the presence and absence of the geldanamycins in SKLMS-1 cells (Fig. 4, bottom panel). SKLMS-1 cells express high levels of both the p170^{Met} precursor and the mature p140^{Met} β -chain (Lane 1). HGF/SF stimulation results in a reduction in p140^{Met} expression due to increased receptor turnover (Lane 2; Ref. 37). However, HGF/SF treatment in the presence of either 122750 or 255109 resulted in an even greater reduction in p140^{Met} expression (Lanes 3 and 4, respectively). At 100 nM, treatment with 255109 induced a near complete loss of p140^{Met} expression, as well as a reduction in the expression of the p170^{Met} precursor. Treatment with 100 nM 122750 down-modulated the expression of p140^{Met} alone. In

addition, both compounds reduced Met expression in SKLMS-1 and MDCK-2 cells when added in the absence of HGF/SF (data not shown), demonstrating that ligand stimulation is not necessary for this effect and that geldanamycin-mediated Met down-regulation occurs in multiple cell types. However, loss of Met expression was not observed at subnanomolar concentrations (data not shown), suggesting that at lower concentrations, the geldanamycins function to inhibit plasmin activation independently of Met down-regulation.

The Geldanamycins Inhibit HGF/SF-mediated Cell Motility and Branching Morphogenesis/Invasion. We tested the ability of the geldanamycins to inhibit HGF/SF-mediated motility and invasion in responsive cell lines *in vitro*. Both 122750 and 255109 were potent inhibitors of HGF/SF-mediated MDCK-2 cell scattering (Fig. 5A). Concentrations of 122750 and 255109 as low as 1 nM inhibited scattering (data not shown). At 100 nM, both 122750 and 255109 displayed noticeable cytotoxicity consistent with that observed during the cell-based plasmin activation assay (compare Fig. 5A and Fig. 3, A and B). To ensure that the inhibitory properties were not selective to MDCK-2 cells, we performed branching morphogenesis/invasion assays using human SKLMS-1 cells and a three-dimensional Matrigel ECM. At 100 nM, both 122750 and 255109 inhibited HGF/SF-mediated branching/invasion of SKLMS-1 cells (Fig. 5B). In addition, in human glioblastoma cells (U118) and human renal cell carcinoma cells (ARZ-2), which efficiently branch/invade in response to HGF/SF (27, 38), this response was inhibited by the geldanamycins (data not shown). We also tested the ability of the geldanamycins to inhibit HGF/SF-mediated motility (chemotaxis) across 8- μ m filters in SKLMS-1 cells (Fig. 5C). Both compounds inhibited HGF/SF-mediated SKLMS-1 cell motility at 100 nM, with 255109 displaying the greatest degree of inhibition. However, the effects on cell motility and branching morphogenesis/invasion were only observed at concentrations >1 nM (data not shown). These data demonstrate that the geldanamycins are potent inhibitors of HGF/SF-mediated cell motility and branching morphogenesis/invasion.

Reversion of the HGF/SF-Met-mediated Transformed Morphology by the Geldanamycins. We tested the ability of the geldanamycins to revert the phenotype of NIH3T3 cells transformed with the *tpr-met* oncogene, a mutationally activated Met molecule (Met L1213V/M1628T) and a mutationally activated Trk-Met chimera, which is activated independently of ligand stimulation (Trk-Met L1213V/M1628T). Treatment of NIH3T3 cells transformed by these various Met oncogenes with 100 nM of either 122750 or 255109 resulted in a reversion of the transformed phenotype, which was observed as a flattening of the cell morphology and a reduction in the number of highly refractile pseudopods (Fig. 6).

Western blot analysis on whole cell lysates from each of these cell lines before and after geldanamycin treatment demonstrated that this was associated with a significant reduction in the ectopic expression of the respective Met proteins (Fig. 7). Collectively, these data demonstrate that the geldanamycins revert the Met-transformed phenotype and down-regulate Met expression independently of endogenous promoter activity and autocrine HGF/SF-Met signaling.

DISCUSSION

Activation of the uPA/uPAR/plasmin proteolytic network has been shown to play a key role in tumor invasion and dissemination of various malignancies (reviewed in Refs. 39 and 40). For example, the role of the uPA fibrinolytic network in tumor malignancy was shown in uPA $-/-$ mice, in which there was a dramatic reduction in the progression of chemically induced malignant melanomas (41). In addition, levels of expression of uPA and uPAR serve as prognostic markers in various malignancies in which high levels of expression

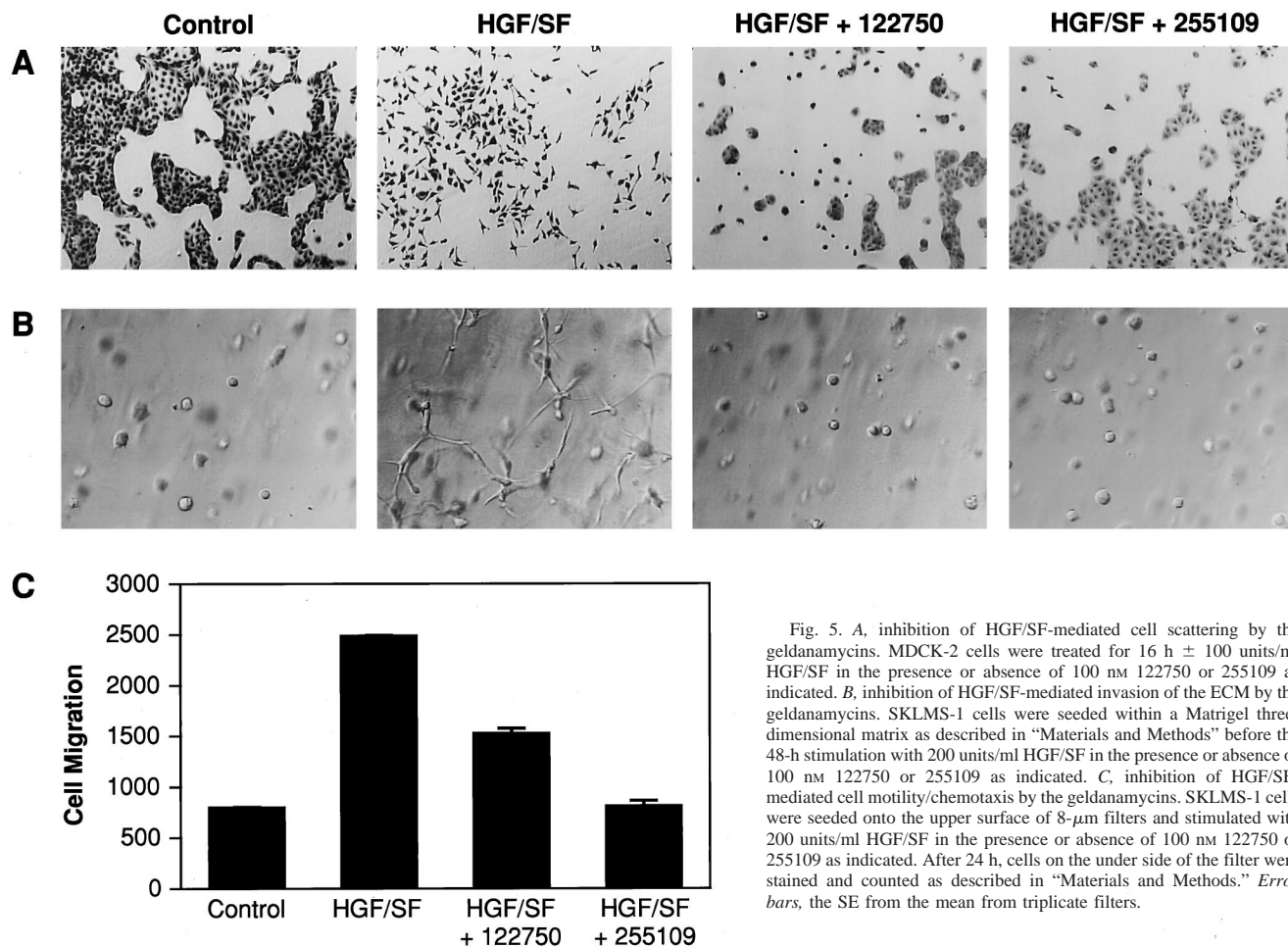


Fig. 5. *A*, inhibition of HGF/SF-mediated cell scattering by the geldanamycins. MDCK-2 cells were treated for 16 h \pm 100 units/ml HGF/SF in the presence or absence of 100 nM 122750 or 255109 as indicated. *B*, inhibition of HGF/SF-mediated invasion of the ECM by the geldanamycins. SKLMS-1 cells were seeded within a Matrigel three-dimensional matrix as described in "Materials and Methods" before the 48-h stimulation with 200 units/ml HGF/SF in the presence or absence of 100 nM 122750 or 255109 as indicated. *C*, inhibition of HGF/SF-mediated cell motility/chemotaxis by the geldanamycins. SKLMS-1 cells were seeded onto the upper surface of 8- μ m filters and stimulated with 200 units/ml HGF/SF in the presence or absence of 100 nM 122750 or 255109 as indicated. After 24 h, cells on the under side of the filter were stained and counted as described in "Materials and Methods." Error bars, the SE from the mean from triplicate filters.

are often associated with a poor prognosis (42, 43). Likewise, there is considerable evidence demonstrating a key role for the HGF/SF-Met signaling system in the etiology of human tumors and in particular, their progression to highly malignant and metastatic cancers (reviewed in Ref. 13). We and others have previously shown that levels of uPA and uPAR protein expression are increased after HGF/SF stimulation (6, 15), resulting in increased cell surface-bound uPA and plasmin activation (6). Thus, we have developed an effective cell-based assay to screen for inhibitors of the HGF/SF-Met-uPA-plasmin network, which encompasses all of the steps after HGF/SF stimulation leading to activation of the plasmin proteolytic system. This assay allows for the identification of inhibitors of multiple molecular targets in the context of a single assay in viable cells, as opposed to screening for inhibitors of a defined target *in vitro*. This not only allows for the identification of a wide range of inhibitors that may be of therapeutic value in the treatment of invasive cancers, but may also allow for the identification of novel molecular targets that function within the HGF/SF-Met-uPA-plasmin network.

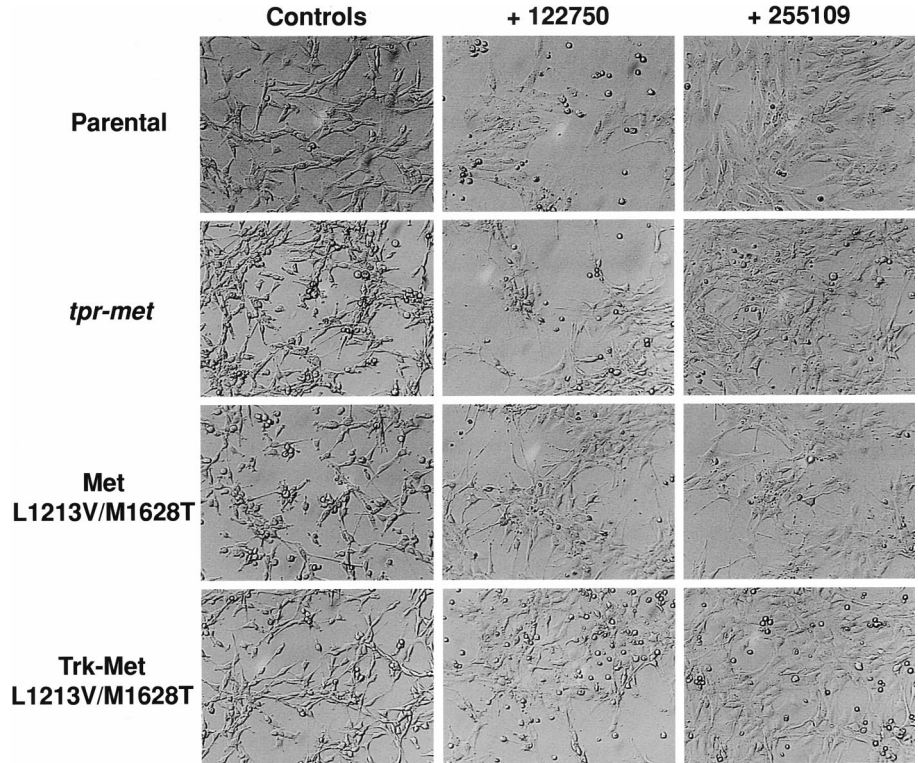
To validate the assay, we demonstrated that a polyclonal neutralizing antiserum against HGF/SF (NCI-53) and a proven uPA inhibitor (B428; Ref. 24) inhibited HGF/SF-dependent plasmin activation (Fig. 2, *A* and *B*). B428 competitively binds and selectively inhibits uPA catalytic activity and has virtually no effect on the tissue-type plasminogen activator (24). Coupled with our results, this suggests that the activation of plasmin after HGF/SF stimulation in MDCK cells occurs predominantly through uPA. We also show that the MAP kinase 1/2 pathway is important for HGF/SF-mediated plasmin activation because both the MKK inhibitor (PD 98059) and anthrax LF inhibited HGF/SF-mediated plasmin activation. This supports previ-

ous findings reporting the role of the MKK/MAP kinase 1/2 pathway in the regulation of uPA expression (16, 29–31). Recently, we have shown the importance of the Raf-MKK-MAPK kinase 1/2 pathway in the acquisition of the metastatic phenotype in NIH3T3 cells (28). Thus, agents that inhibit this important signaling pathway could be useful as anti-invasive drugs and ultimately useful for the treatment of tumor progression and metastasis. In this regard, B428 has already been shown to inhibit tumor growth and metastasis in a variety of experimental systems *in vivo* (44, 45).

Some of the compounds identified also served to validate the assay. For example, a series of fluorinated steroids and some staurosporine analogues were identified as weak to moderate inhibitors of HGF/SF-mediated plasmin activation.⁴ The fluorinated steroids appear to block uPA/uPAR expression, but do not effect the expression or activation of the Met receptor.⁴ Staurosporine and its analogues are known inhibitors of protein kinase C isoforms and have shown potential as anticancer drugs (46), although their precise mechanism of action in relation to their inhibitory properties in the HGF/SF-Met-uPA-plasmin assay remains unclear.

The geldanamycins were identified as potent inhibitors in this assay with some inhibitory activity at femtomolar concentrations. Complete inhibition of HGF/SF-mediated plasmin activity was observed at >1 nM, a concentration that was sufficient to inhibit HGF/SF-induced motility and invasion in various cell types (Fig. 3, *A* and *B* and Fig. 5). The geldanamycin family of anisamycin antibiotics were first identified as inhibitors of the Src family of tyrosine kinases (47). More recently, however, they have been shown to strongly bind the heat shock protein, Hsp90 (48, 49). Hsp90 is a molecular chaperone that,

Fig. 6. Reversion of the transformed phenotype associated with aberrant Met signaling in NIH3T3 cells by the geldanamycins. Cells stably expressing the various transforming Met genes were treated for 24 h with 100 nM 122750 or 255109 as indicated before phase-contrast photography.



in association with other proteins, serves to ensure the correct folding of several regulatory and signal transduction proteins (50). Geldanamycin has been shown to interfere with the chaperone function of Hsp90, leading to the destabilization and degradation of several key cellular proteins, including pp60^{V-src} (48), ErbB2 (51), Raf-1 (52), and mutated p53 (53). Although further work is required to detail the mechanisms by which geldanamycin inhibits the HGF/SF-Met-uPA-plasmin response, we have shown that Met is down-regulated after exposure to nanomolar concentrations of geldanamycin. This suggests that Met degradation is controlled, in part, by Hsp90 or related proteins and provides a partial explanation for its inhibitory properties

in this cellular assay. However, because some inhibition of HGF/SF-mediated plasmin activity is also observed at concentrations where there is no apparent effect on Met expression (<1 nM), it is likely that additional targets lying within the HGF/SF-Met-uPA-plasmin pathway are inhibited by geldanamycin. For example, Raf-1 function is inhibited by geldanamycin (52), and based upon the role of the Raf-MKK-MAPK1/2 pathway in HGF/SF-mediated uPA/uPAR induction (Fig. 2, C and D; Ref. 16), this pathway is likely to be influenced by geldanamycin.

The loss of Met expression that occurs after treatment with nanomolar concentrations of geldanamycin results in the concomitant inhibition in the uPA/uPAR response after HGF/SF stimulation. Analogues of geldanamycin are presently under consideration for trials in human cancer patients based upon their limited cytotoxic properties (54). We have shown that there is a vast difference between the concentrations of certain (but not all) geldanamycins required for cytotoxic effects (GI₅₀ typically ~10⁻⁶ M) and those necessary for inhibition of HGF/SF-mediated plasmin activation (IC₅₀ typically ~10⁻¹⁴ M). Moreover, our results suggest that tumors in which aberrant HGF/SF-Met signaling has been implicated should be considered for geldanamycin-based therapy. For example, germ-line and sporadic activating mutations in Met have been identified in patients with papillary renal carcinoma (10). The same mutations in murine Met transform rodent cell lines and mediate tumorigenesis and metastasis in mice (11, 12). We have shown that the geldanamycins revert the transformed phenotype associated with these activating Met mutations, with a concomitant reduction in the expression of the mutant Met proteins. These findings suggest that geldanamycin and its analogues may be effective as anti-invasive/metastatic agents.

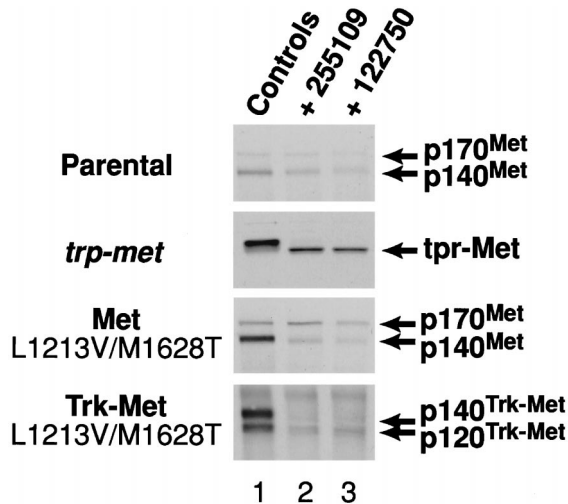


Fig. 7. Western blot analysis of ectopically expressed transforming Met proteins in NIH3T3 cells after 24-h geldanamycin treatment. Upper panel, control NIH3T3 cells; second panel, *tpr-met* transformed NIH3T3 cells; third panel, NIH3T3 cells transformed by a double-point mutation in murine Met; bottom panel, NIH3T3 cells transformed by a double-point mutation in a murine Trk-Met chimera. Lane 1, no treatment; Lane 2, 100 nM 255109; Lane 3, 100 nM 122750. The positions of the relative proteins are indicated.

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The Geldanamycins Are Potent Inhibitors of the Hepatocyte Growth Factor/Scatter Factor-Met-Urokinase Plasminogen Activator-Plasmin Proteolytic Network

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