Introduction

A variety of molecular targeted drug screening assays have been developed by the Developmental Therapeutics Program in collaboration with various investigators. A number of additional assay are currently under development. These vary in format, ranging from simple cell-free assays to those involving cell lines engineered to express particular reporter systems. The screening assays are intended for use in conjunction with chemical libraries to support discovery of novel molecular targeted agents, or drug leads, relevant to treatment of cancer, AIDS-associated malignancies, HIV-1 disease or opportunistic infections. For a recent review of the process, see Shoemaker, et al. Application of High-Throughput, Molecular-Targeted Screening to Anticancer Drug Discovery. Current Topics in Medicinal Chemistry 2:229-246, 2002. A downloadable copy of this review is available at: <http://spheroid.ncifcrf.gov/stb/staff/shoemaker/shoemaker.cfm>.

Data generated during assay development (screening data on the NCI Training Set) and results from high-throughput screening of the NCI Diversity Set are available on this page.

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The Geldanamycins Are Potent Inhibitors of the Hepatocyte Growth Factor/Scatter Factor-Met-Urokinase Plasminogen Activator-Plasmin Proteolytic Network[1]

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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/SF[3] (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^{mu}, nontransformed immortalized cells established from a mouse mammary tumor engineered to express high levels of Met^{mu} (9); and C127-Met^{mu}/HGF^{hu}, C127 cells expressing both Met^{mu} and human HGF/SF (9). Human renal carcinoma ARZ-2 cells were a kind gift from J. Gnarra (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 chimer (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 μl 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% CO_2 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 μl/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. IC_{50} values (concentration of drug inhibiting HGF/SF response by 50%) and GI_{50} values (concentration of drug inhibiting growth by 50%) were then calculated for each drug or reagent from plotted graphs. For the calculation of GI_{50}, 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% CO_2 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-μm polycarbonate filters (Costar) as previously described (27). In brief, 1×10^4 cells (in 100 μl) 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 ± 200 units/ml human HGF/SF in the absence or presence of drug. After 16 h of incubation at 37°C/5% CO_2, 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 ×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) ± 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 $MgCl_2$, 1% Triton-X-100, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 100 μM 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 μg of cell lysates were resuspended 3:1 into 4× reducing or nonreducing Laemmli buffer [± DTT, respectively; 0.4 M Tris-HCl (pH 6.8), 8% SDS, 39% glycerol, 0.04% bromphenol blue ± 0.4 M DTT], boiled for 5 min, and resolved by SDS-PAGE. Proteins were then transferred to nitrocellulose, and blots were stained with Ponseau 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 μg/ml of a rabbit antihuman uPA antiserum (American Diagnostica), 1 μg/ml of a rabbit antihuman uPAR antiserum (American Diagnostica), 1 μg/ml of a rabbit antimouse Met antiserum (clone SP-260, Santa Cruz, CA), or 1 μg/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. 1*A* 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. 1*B*). 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

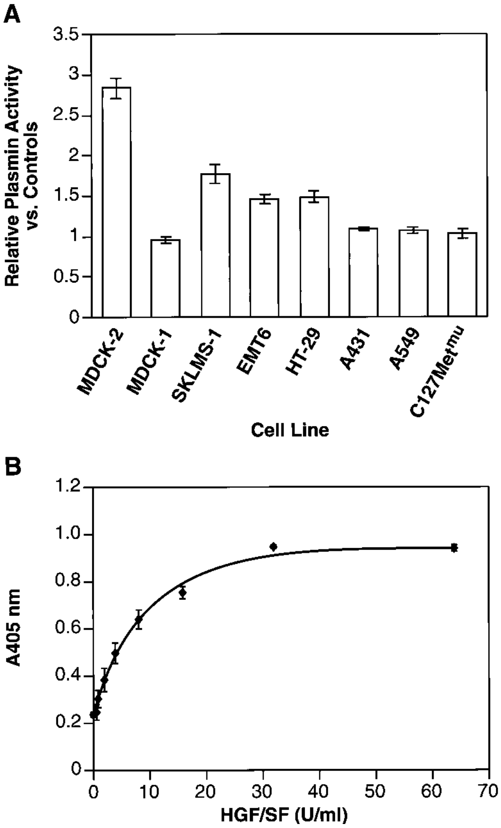

Fig. 1. *A,* HGF/SF-mediated plasmin activation in various cell lines after 24-h stimulation with 10 units/ml HGF/SF. The results are expressed relative to unstimulated control cells. *B,* dose response for HGF/SF-mediated plasmin activation in MDCK-2 cells after 24 h. Plasmin activation was determined 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. 1*A*). 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. 2*A*). 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. 2*B*; 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

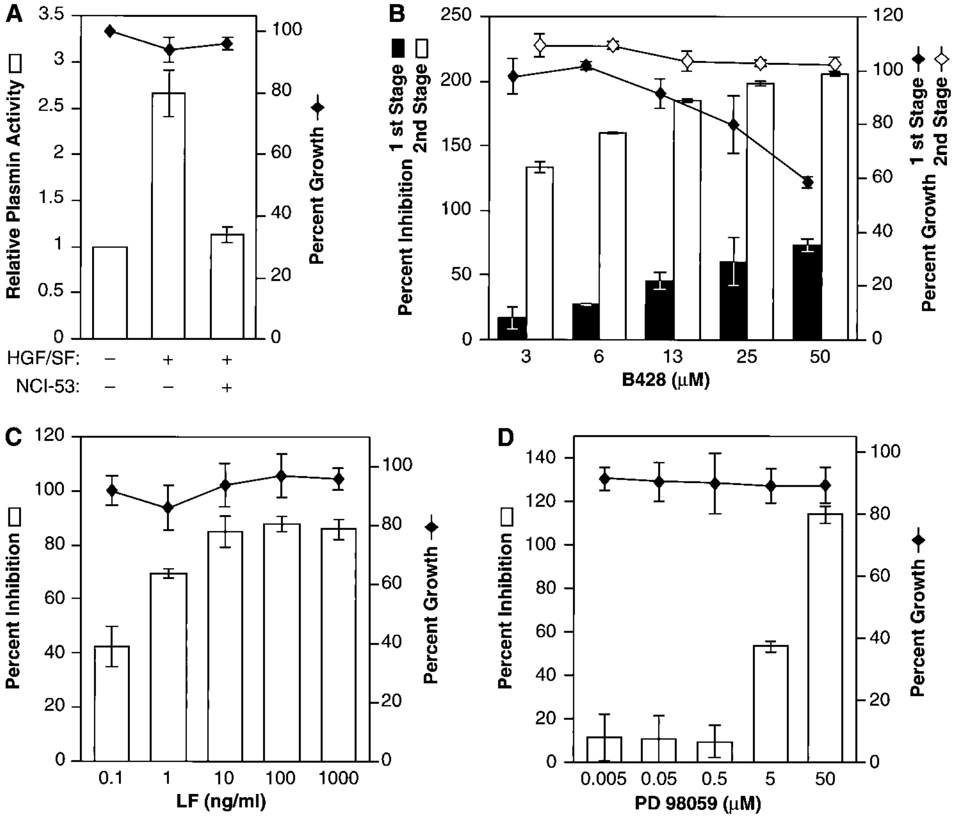

Fig. 2. Inhibition of HGF/SF-mediated plasmin activation in MDCK-2 cells by: *A,* neutralizing HGF/SF antiserum; *B,* the uPA inhibitor B428; *C,* the MKK inhibitor PD 98059; and *D,* the MKK protease anthrax *LF.* Cells were incubated with 10 units/ml HGF/SF in the absence or presence of various concentrations 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 ($n > 3$).

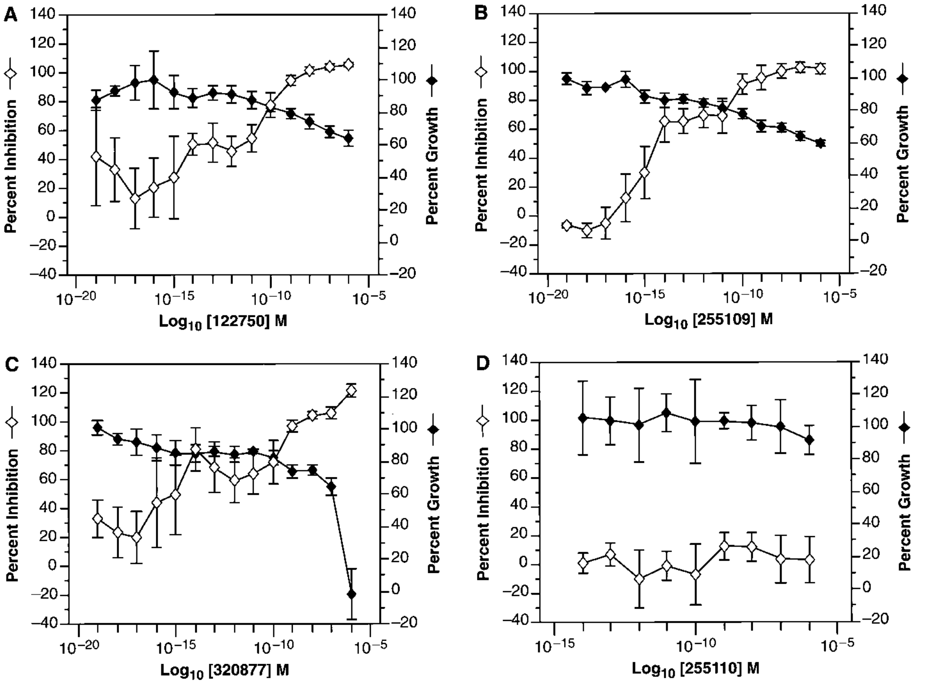

Fig. 3. Inhibition of HGF/SF-mediated plasmin activation by the geldanamycins in MDCK-2 cells. Cells were incubated with 10 units/ml HGF/SF in the presence or absence of different concentrations of drugs as indicated (*A,* 122750; *B,* 255109; *C,* 320877; *D,* 255110). Plasmin activity was assayed as 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. 2*C*). 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. 2*D*). 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 properties[4], 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}

[a] Compounds initially identified from the screen.

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

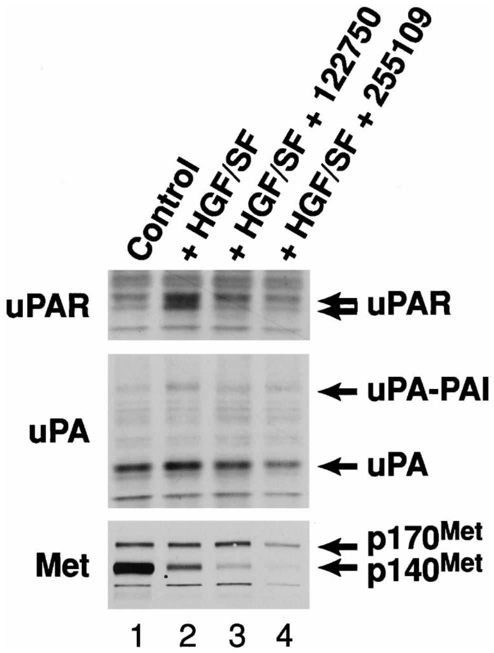

Fig. 4. Western blot analysis of uPAR, uPA, and Met expression in SKLMS-1 cells after 24-h HGF/SF stimulation in the presence or absence of the geldanamycins. *Lane 1,* control cells; *Lane 2,* cells treated with 100 units/ml HGF/SF; *Lane 3,* cells treated with 100 units/ml HGF/SF + 100 nM 122750; *Lane 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. 5*A*). 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. 5*A* 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. 5*B*). 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-μm filters in SKLMS-1 cells (Fig. 5*C*). 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

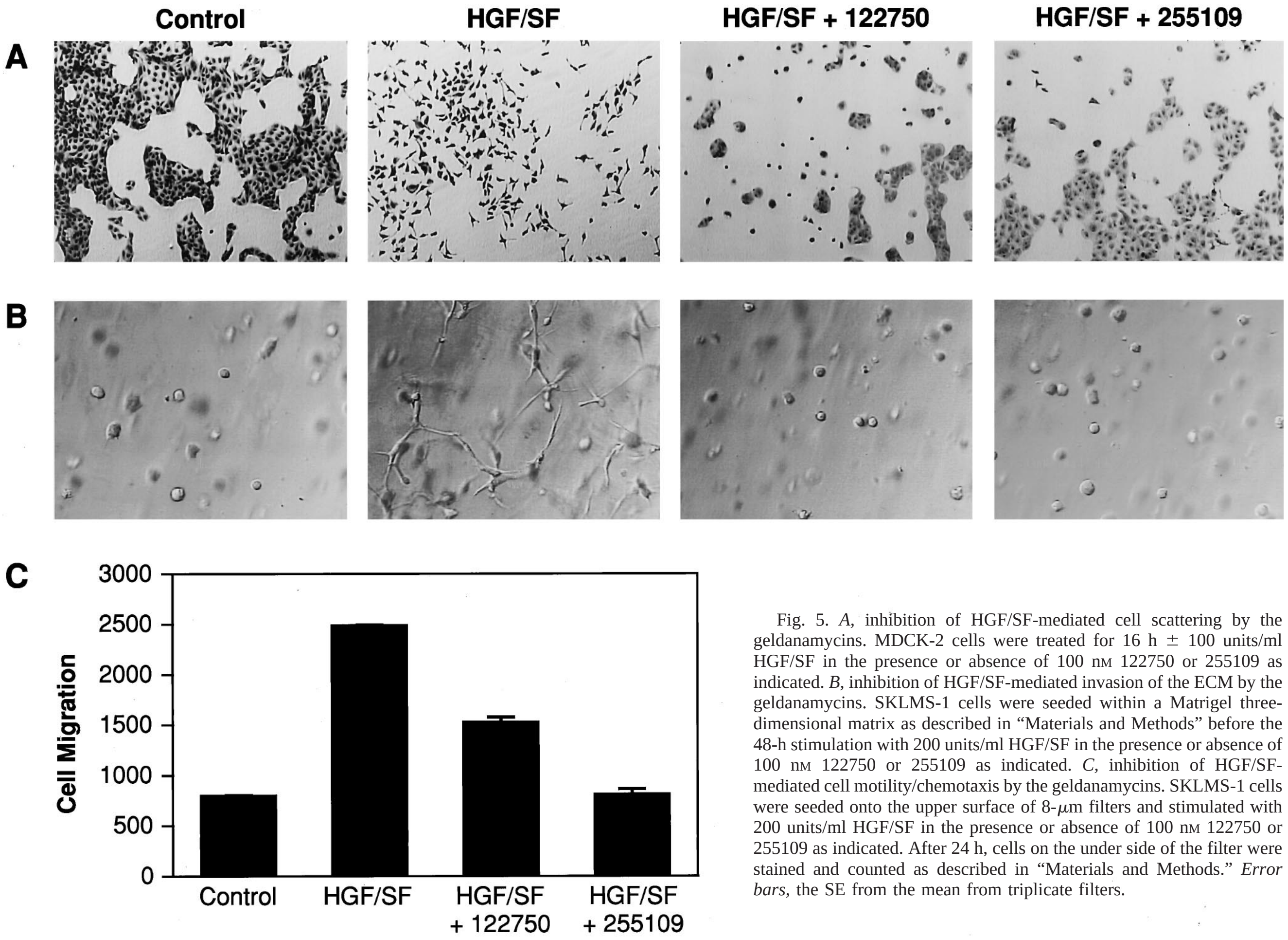

Fig. 5. *A*, inhibition of HGF/SF-mediated cell scattering by the geldanamycins. MDCK-2 cells were treated for 16 h ± 100 units/ml HGF/SF in the presence or absence of 100 nM 122750 or 255109 as indicated. *B*, inhibition of HGF/SF-mediated invasion of the ECM by the geldanamycins. SKLMS-1 cells were 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-μm 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 previous 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,

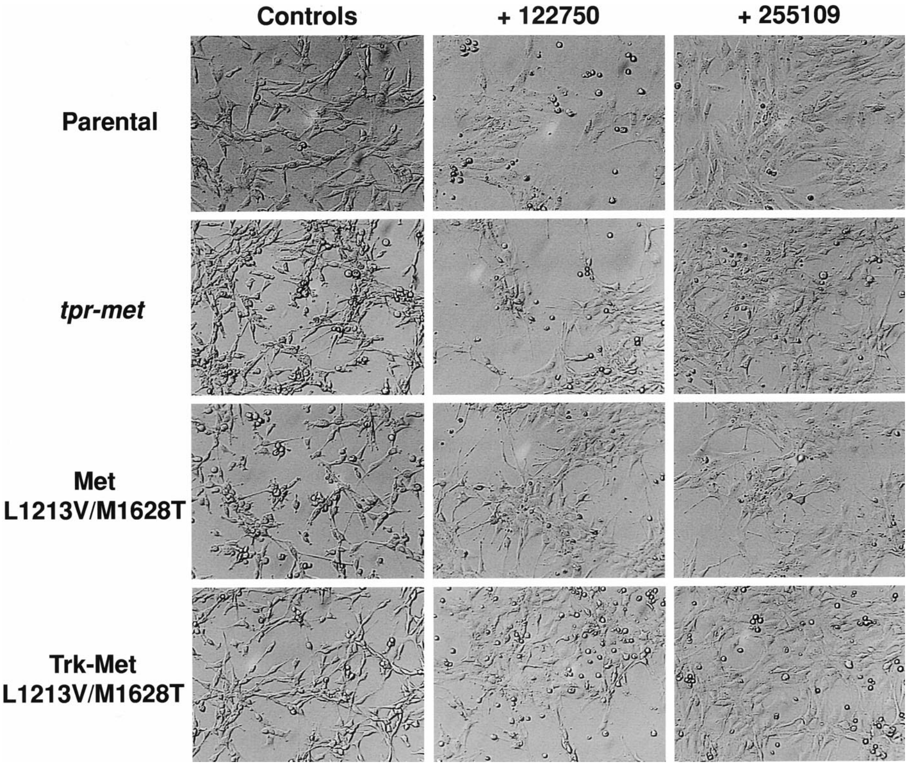

Fig. 6. Reversion of the transformed phenotype associated with aberrant Met signaling in NIH3T3 cells by the geldanamycins. Cells stably expressing the various transforming Met genes were treated for 24 h with 100 nM 122750 or 255109 as indicated before phase-contrast photography.

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$^{v\text{-}src}$ (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 (GI$_{50}$ typically $\sim10^{-6}$ M) and those necessary for inhibition of HGF/SF-mediated plasmin activation (IC$_{50}$ typically $\sim10^{-14}$ 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.

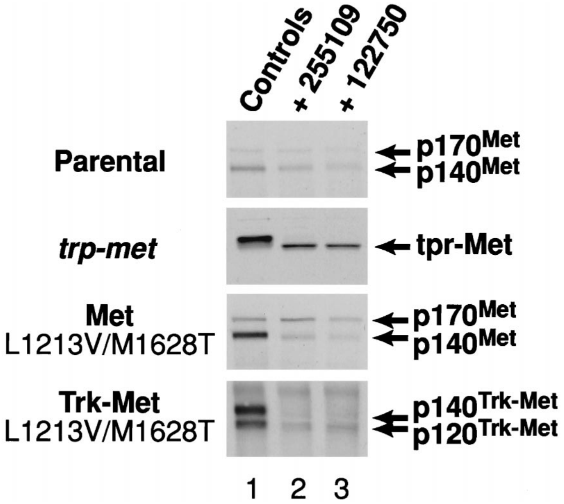

Fig. 7. Western blot analysis of ectopically expressed transforming Met proteins in NIH3T3 cells after 24-h geldanamycin treatment. *Upper panel,* control NIH3T3 cells; *second panel, tpr-met* transformed NIH3T3 cells; *third panel,* NIH3T3 cells transformed by a double-point mutation in murine Met; *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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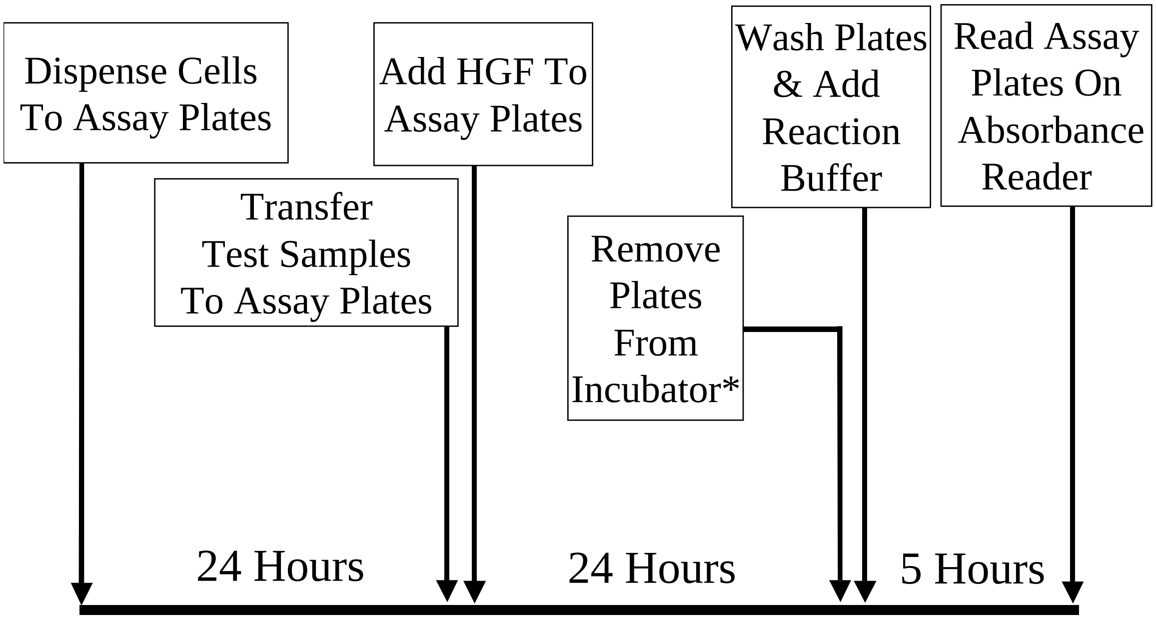
REFERENCES

1. Park, M., Dean, M., Cooper, C. S., Schmidt, M., O'Brien, S. J., Blair, D. G., and Vande Woude, G. F. Mechanism of met oncogene activation. Cell, *45:* 895–904, 1986.
2. Bottaro, D. P., Rubin, J. S., Faletto, D. L., Chan, A. M., Kmiecik, T. E., Vande Woude, G. F., and Aaronson, S. A. Identification of the hepatocyte growth factor receptor as the c-met proto-oncogene product. Science (Washington DC), *251:* 802–804, 1991.
3. Gherardi, E., Sharpe, M., Lane, K., Sirulnik, A., and Stoker, M. Hepatocyte growth factor/scatter factor (HGF/SF), the c-met receptor and the behaviour of epithelial cells. Symp. Soc. Exp. Biol., *47:* 163–181, 1993.
4. Vande Woude, G. F., Jeffers, M., Cortner, J., Alvord, G., Tsarfaty, I., and Resau, J. Met-HGF/SF. Tumorigenesis, Invasion, and Metastasis. *In:* G. R. Bock, and J. A. Goode (eds.), CIBA Foundation Symposium: Plasminogen-Related Growth Factors, Vol. 212, pp. 119–132. West Sussex: John Wiley & Sons Ltd., 1997.
5. Rubin, J. S., Bottaro, D. P., and Aaronson, S. A. Hepatocyte growth factor/scatter factor and its receptor, the c-*met* proto-oncogene product. Biochim. Biophys. Acta, *1155:* 357–371, 1993.
6. Jeffers, M., Rong, S., and Vande Woude, G. F. Hepatocyte growth factor/scatter factor-Met signaling in tumorigenicity and invasion/metastasis. J. Mol. Med., *74:* 505–513, 1996.
7. Jeffers, M., Rong, S., and Vande Woude, G. F. Enhanced tumorigenicity and invasion-metastasis by hepatocyte growth factor/scatter factor-Met signaling in human cells concomitant with induction of the urokinase proteolysis network. Mol. Cell. Biol., *16:* 1115–1125, 1996.
8. Rong, S., Bodescot, M., Blair, D., Dunn, J., Nakamura, T., Mizuno, K., Park, M., Chan, A., Aaronson, S., and Vande Woude, G. F. Tumorigenicity of the *met* proto-oncogene and the gene for hepatocyte growth factor. Mol. Cell. Biol., *12:* 5152–5158, 1992.
9. Rong, S., Segal, S., Anver, M., Resau, J. H., and Vande Woude, G. F. Invasiveness and metastasis of NIH 3T3 cells induced by Met-hepatocyte growth factor/scatter factor autocrine stimulation. Proc. Natl. Acad. Sci. USA, *91:* 4731–4735, 1994.
10. Jeffers, M., Rong, S., Oskarsson, M., Anver, M., and Vande Woude, G. F. Autocrine hepatocyte growth factor/scatter factor-Met signaling induces transformation and the invasive/metastatic phenotype in C127 cells. Oncogene, *13:* 853–861, 1996.
11. Schmidt, L., Duh, F-M., Chen, F., Kishida, T., Glenn, G., Choyke, P., Scherer, S. W., Zhuang, Z., Lubensky, I., Dean, M., Allilmets, R., Chidambaram, A., Bergerheim, U. R., Feltis, J. T., Casadevall, C., Zamarron, A., Bernues, M., Richard, S., Lips, C. J. M., Walter, M. M., Tsui, L-C., Geil, L., Orcutt, M. L., Stackhouse, T., Lipan, J., Slife, L., Brauch, H., Decker, J., Niehans, G., Hughson, M. D., Moch, H., Storkel, S., Lerman, M. I., Linehan, W. M., and Zbar, B. Germ-line and somatic mutations in the tyrosine kinase domain of the *Met* proto-oncogene in papillary renal carcinomas. Nat. Genet., *16:* 68–73, 1997.
12. Jeffers, M., Schmidt, L., Nakaigawa, N., Webb, C. P., Weirich, G., Kishida, T., Zbar, B., and Vande Woude, G. F. Activating mutations for the met tyrosine kinase receptor in human cancer. Proc. Natl. Acad. Sci. USA, *94:* 11445–11450, 1997.
13. Jeffers, M., Fiscella, M., Webb, C. P., Anver, M., Koochekpour, S., and Vande Woude, G. F. The mutationally activated Met receptor mediates motility and metastasis. Proc. Natl. Acad. Sci. USA, *95:* 14417–14422, 1998.
14. Johnsen, M., Lund, L. R., Romer, J., Almholt, K., and Dano, K. Cancer invasion and tissue remodeling: common themes in proteolytic matrix degradation. Curr. Opin. Cell Biol., *10:* 667–671, 1998.
15. Pepper, M. S., Matsumoto, K., Nakamura, T., Orci, L., and Montesano, R. Hepatocyte growth factor increases urokinase-type plasminogen activator (u-PA) and u-PA receptor expression in Madin-Darby canine kidney epithelial cells. J. Biol. Chem., *267:* 20493–20496, 1992.
16. Ried, S., Jager, C., Jeffers, M., Vande Woude, G. F., Graeff, H., Schmitt, M., and Lengyel, E. Activation mechanisms of the urokinase-type plasminogen activator promoter by hepatocyte growth factor/scatter factor. J. Biol. Chem., *274:* 16377–16386, 1999.
17. Blasi, F. Urokinase and urokinase receptor: a paracrine/autocrine system regulating cell migration and invasiveness. Bioessays, *15:* 105–111, 1993.
18. Ellis, V., Pyke, C., Eriksen, J., Solberg, H., and Dano, K. The urokinase receptor: involvement in cell surface proteolysis and cancer invasion. Ann. N. Y. Acad. Sci., *667:* 13–31, 1992.
19. Kugler, A. Matrix metalloproteinases and their inhibitors. Anticancer Res., *19:* 1589–1592, 1999.
20. Reuning, U., Magdolen, V., Wilhelm, O., Fischer, K., Lutz, V., Graeff, H., and Schmitt, M. Multifunctional potential of the plasminogen activation system in tumor invasion and metastasis. Int. J. Oncol., *13:* 893–906, 1998.
21. Webb, C. P., Lane, K., Dawson, A. P., Vande Woude, G. F., and Warn, R. M. C-Met signaling in an HGF/SF-insensitive variant MDCK cell line with constitutive motile/invasive behaviour. J. Cell Sci., *109:* 2371–2381, 1996.
22. Park, M., Dean, M., Kaul, K., Braun, M. J., Gonda, M. A., and Vande Woude, G. F. Sequence of *met* proto-oncogene cDNA has features characteristic of the tyrosine kinase family of growth-factor receptors. Proc. Natl. Acad. Sci. USA, *84:* 6379–6383, 1987.
23. Rong, S., Oskarsson, M., Faletto, D., Tsarfaty, I., Resau, J. H., Nakamura, T., Rosen, E., Hopkins, R. F., and Vande Woude, G. F. Tumorigenesis induced by co-expression of human hepatocyte growth factor and the human *met* proto-oncogene leads to high levels of expression of the ligand and receptor. Cell Growth Differ., *4:* 563–569, 1993.
24. Towle, M. J., Lee, A., Maduakor, E. C., Schwartz, C. E., Bridges, A. J., and Littlefield, B. A. Inhibition of urokinase by 4-substituted benzo[b] thiophene-2-carboxamidines: an important new class of selective synthetic urokinase inhibitor. Cancer Res., *53:* 2553–2559, 1993.
25. Monks, A., Scudiero, D., Skehan, P., Shoemaker, R., Paull, K., Vistica, D., Hose, C., Langley, J., Cronise, P., Vaigro-Wolff, A., Gray-Goodrich, M., Campbell, H., Mayo, J., and Boyd, M. Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. J. Natl. Cancer Inst., *83:* 757–766, 1991.
26. Stoker, M., Gherardi, E., Perryman, M., and Gray, J. Scatter factor is a fibroblast-derived modulator of epithelial cell mobility. Nature (Lond.), *327:* 239–242, 1987.
27. Koochekpour, S., Jeffers, M., Rulong, S., Klineberg, E., Taylor, G., Hudson, E. A., Resau, J. H., and Vande Woude, G. F. Met and hepatocyte growth factor/scatter factor expression in human gliomas. Cancer Res., *57:* 5391–5398, 1997.
28. Webb, C. P., Van Aelst, L., Wigler, M. H., and Vande Woude, G. F. Signaling pathways in *Ras*-mediated tumorigenicity and metastasis. Proc. Natl. Acad. Sci. USA, *95:* 8773–8778, 1998.
29. Lengyel, E., Stepp, E., Gum, R., and Boyd, D. Involvement of a mitogen-activated protein kinase signaling pathway in the regulation of urokinase promoter activity by c-Ha-ras. J. Biol. Chem., *270:* 23007–23012, 1995.
30. Lengyel, E., Gum, R., Stepp, E., Juarez, J., Wang, H., and Boyd, D. Regulation of urokinase-type plasminogen activator expression by an ERK1-dependent signaling pathway in a squamous cell carcinoma cell line. J. Cell. Biochem., *61:* 430–443, 1996.
31. Simon, C., Juarez, J., Nicolson, G. L., and Boyd, D. Effect of PD 098059, a specific inhibitor of mitogen-activated protein kinase kinase, on urokinase expression and *in vitro* invasion. Cancer Res., *56:* 5369–5374, 1996.
32. Duesbery, N. S., Webb, C. P., Leppla, S. H., Gordon, V. M., Klimpel, K. R., Copeland, T. D., Ahn, N. G., Oskarsson, M. K., Fukasawa, K., Paull, K. D., and Vande Woude, G. F. Proteolytic inactivation of MAP-kinase-kinase by anthrax lethal factor. Science (Washington DC), *280:* 734–737, 1998.
33. Gordon, V. M., Leppla, S. H., and Hewlett, E. L. Inhibitors of receptor-mediated endocytosis block the entry of *Bacillus anthracis* adenylate cyclase toxin but not that of *Bordetella pertussis* adenylate cyclase toxin. Infect. Immun., *56:* 1066–1069, 1988.
34. Milne, J. C., Furlong, D., Hanna, P. C., Wall, J. S., and Collier, R. J. Anthrax protective antigen forms oligomers during intoxication of mammalian cells. J. Biol. Chem., *269:* 20607–20612, 1994.
35. Alessi, D. R., Cuenda, A., Cohen, P., Dudley, D. T., and Saltiel, A. R. PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and *in vivo*. J. Biol. Chem., *270:* 27489–27494, 1995.
36. Miller, P., DiOrio, C., Moyer, M., Schnur, R. C., Bruskin, A., Cullen, W., and Moyer, J. D. Depletion of the erbB-2 gene produce p185 by benzoquinoid ansamycins. Cancer Res., *54:* 2724–2730, 1994.
37. Jeffers, M., Taylor, G. A., Weidner, K. M., Omura, S., and Vande Woude, G. F. Degradation of the met tyrosine kinase receptor by the ubiquitin-proteasome pathway. Mol. Cell. Biol., *17:* 799–808, 1997.
38. Koochekpour, S., Jeffers, M., Wang, P. H., Gong C., Taylor, G. A., Roessler, L. M., Stearman, R., Vasselli, J. R., Stetler-Stevenson, W. G., Kaelin, W. G., Jr., Linehan, W. M., Kausner, R. D., Gnarra, J. R., and Vande Woude, G. F. The von Hippel-Lindau tumor suppressor gene inhibits hepatocyte growth factor/scatter factor-induced invasion and branching morphogenesis in renal carcinoma cells. Mol. Cell. Biol., *19:* 5902–5912, 1999.
39. Andreasen, P. A., Kjoller, L., Christensen, L., and Duffy, M. J. The urokinase-type plasminogen activator system in cancer metastasis: a review. Int. J. Cancer, *72:* 1–22, 1997.
40. Kwaan, H. C. The plasminogen-plasmin system in malignancy. Cancer Metastasis Rev., *11:* 291–311, 1992.
41. Shapiro, R. L., Duquette, J. G., Roses, D. F., Nunes, I., Harris, M. N., Kamino, H., Wilson, E. L., and Rifkin, D. B. Induction of primary cutaneous melanocytic neoplasms in urokinase-type plasminogen activator (uPA)-deficient and wild-type mice: cellular blue nevi invade but do not progress to malignant melanoma in uPA-deficient animals. Cancer Res., *56:* 3597–3604, 1996.
42. Duffy, M. J., Maguire, T. M., McDermott, E. W., and O'Higgins, N. Urokinase plasminogen activator: a prognostic marker in multiple types of cancer. J. Surg. Oncol., *71:* 130–135, 1999.
43. Blasi, F. The urokinase receptor. A cell surface, regulated chemokine. APMIS, *107:* 96–101, 1999.
44. Alonso, D. F., Farias, E. F., Ladeda, V., Davel, L., Puricelli, L., and Bal de Kier Joffe, E. Effects of synthetic urokinase inhibitors on local invasion and metastasis in a murine mammary tumor model. Breast Cancer Res. Treat., *40:* 209–223, 1996.
45. Rabbani, S. A., Harakidas, P., Davidson, D. J., Henkin, J., and Mazar, A. P. Prevention of prostate-cancer metastasis *in vivo* by a novel synthetic inhibitor of urokinase-type plasminogen activator (uPA). Int. J. Cancer, *63:* 840–845, 1995.
46. Gescher, A. Analogs of staurosporine: potential anticancer drugs? Gen. Pharmacol., *31:* 721–728, 1998.
47. Uehara, Y., Murakami, Y., Mizuno, S., and Kawai, S. Inhibition of transforming activity of tyrosine kinase oncogenes by herbimycin A. Virology, *164:* 294–298, 1988.
48. Whitesell, L., Mimnaugh, E. G., De Costa, B., Myers, C. E., and Neckers, L. M. Inhibition of heat shock protein HSP90-pp60v-src heteroprotein complex formation by benzoquinone ansamycins: essential role for stress proteins in oncogenic transformation. Proc. Natl. Acad. Sci. USA, *91:* 8324–8328, 1994.
49. Roe, S. M., Prodromou, C., O'Brien, R., Ladbury, J. E., Piper, P. W., and Pearl, L. H. Structural basis for inhibition of the Hsp90 molecular chaperone by the antitumor antibiotics radicicol and geldanamycin. J. Med. Chem., *42:* 260–266, 1999.
50. Scheibel, T., and Buchner, J. The Hsp90 complex—a super-chaperone machine as a novel drug target. Biochem. Pharmacol., *56:* 675–682, 1998.
51. Miller, P., Schnur, R. C., Barbacci, E., Moyer, M. P., and Moyer, J. D. Binding of benzoquinoid ansamycins to p100 correlates with their ability to deplete the erbB2 gene product p185. Biochem. Biophys. Res. Commun., *201:* 1313–1319, 1994.
52. Schulte, T. W., Blagosklonny, M. V., Ingui, C., and Neckers, L. Disruption of the Raf-1-Hsp90 molecular complex results in destabilization of Raf-1 and loss of Raf-1-Ras association. J. Biol. Chem., *270:* 24585–24588, 1995.
53. Blagosklonny, M. V., Toretsky, J., and Neckers, L. Geldanamycin selectively destabilizes and conformationally alters mutated p53. Oncogene, *11:* 933–939, 1995.
54. Supko, J. G., Hickman, R. L., Grever, M. R., and Malspeis, L. Preclinical pharmacologic evaluation of geldanamycin as an antitumor agent. Cancer Chemother. Pharmacol., *36:* 305–315, 1995.

MET-HGF/SF Assay Timeline



Incubate Plates At 37^oC, 5%CO_2, Ambient O_2

*Sister plates processed for SRB toxicity assay

MOLECULAR SCREEN for MET-HGF/SF INHIBITORS

Reagents Needed:

a. **Dulbecco's Modified Eagle Medium (DMEM) (Gibco, BRL catalog # 31053-028) without phenol red.**
b. **L-Glutamine**
c. **Fetal Bovine Serum**
d. **Recombinant Human HGF(Hepatocyte Growth Factor recombinant human-rhHGF): (R&D Systems, cat#294-HG, Lot#GJ120051, 25ug vial)**
e. **PBS**
f. **Chromozym PL (Boehringer Mannheim catalog # 378 461, 20mg/vial**
    1. **Glycine (Sigma G-8790, 100g, Lot#10k0236)**
    2. **Polyoxyethylenesorbitan Monolaurate, Sigma P-7949, 100ml, Lot 69H0143**
g. **Human Plasminogen (Boehringer Mannheim, cat# 874 477, 20 U/vial)**
    1. **Sodium Phosphate (Sigma S-8282, 500g, Lot # 59H04591**
    2. **Sodium Chloride (Chempure, 500g, cat# 832-006, Lot# M272KMJC**
h. **Tris buffer (Sigma, 500g, T-1503, Lot# 100H5606)**
i. **2M HCL**
j. **Trichloroacetic Acid**
k. **Acetic Acid**
l. **Sulforhodamine B (SRB)**
m. **Cell culture grade water**

Reagent Preparation:

1. **Media(DMEM): supplemented with 1% L-glutamine and 10% Fetal Bovine Serum (5ml L-glutamine, 50ml Serum to every 500ml bottle).**

2. HGF: prepare a 160ng/ml HGF solution, for a final plate well concentration of 40ng/ml(1:4). Prepare aliquots by making a 1% (v/v) serum/PBS solution (100µlin 10ml PBS). Use two vials (25,000ng each) and dilute in 5.5ml of 1% serum/PBS. From this make 5 – 1ml aliquots, each 1ml aliquot is 9090.9ng/ml. Freeze aliquots at –80ºC (note: stable at –80ºC in 1% Serum PBS solution for ~ 2months). Aliquots to be diluted with complete DMEM media. Add 1 aliquot to 55.8ml complete media (total = 56.8ml), giving a final solution concentration of 160ng/ml HGF.
3. Chromozym PL: prepared fresh as a 3mM stock solution in 100mM glycine solution (7.5mg/ml glycine, 0.2% w/v Tween-20), stable at 4ºC for ~ 2 weeks.
    - i. Glycine solution: prepared 82.5mg glycine in 11ml (cell culture grade water) plus 220µl-*Tween* (Polyoxyethylenesorbitan Monolaurate).
    - ii. Chromozym PL: prepared fresh, 20mg vial Chromozym in 10.5ml Glycine solution(i).
4. Human Plasminogen: prepared as a 30 U/ml stock solution in 20mM sodium phosphate, pH 7.5, & 100mM NaCl buffer. Add 667µlof sodium phosphate-sodium chloride buffer to plasminogen vial, (now at a concentration of 20 U/vial = 30 U/ml). Make up 6 – 1µlaliquots to be frozen at -20ºC. Aliquots are stable at -20ºC for several months (avoid freeze thaw cycles). Dilute 100µl aliquot in 60ml incomplete media (no serum, no l-glutamine).
    - i. Sodium Phosphate: prepare buffer with 24mg sodium phosphate in 10ml (cell culture grade) water.
    - ii. Sodium Chloride: add 58mg NaCl to above Sodium Phosphate buffer.
5. Tris buffer: prepared as a 50mM stock solution at pH of 8.2. To prepare, dissolve 605.5mg Tris and 584mg NaCl in ~ 50mls water. Adjust pH to 8.2 with 2M HCL. Bring total volume up to 100ml with water. Store at 4ºC.
6. Trichloroacetic Acid: use cold 50% w/v solution (500g of TCA dissolved in 1000ml of water).
7. Acetic Acid: prepare a 1% solution (10ml of acetic acid added to 900ml of water).
8. Sulfurorhodamine B (SRB): 0.4% SRB in 1% acetic acid solution.
9. Reaction Buffer: (*prepared just prior to addition to plates*)

(10%) 3mM Chromozym PL stock solution. ---------------------10.5ml
(50%) 0.05 U/ml Plasminogen in phenol red free DMEM.-----52.5ml
(40%) 50mM Tris buffer. --42.0ml
100% ---Reaction Buffer--- 105ml
(***enough for 3 chromozym plates)

Cell Line Inoculation:

Experiment on 384 well plate: (using the entire plate), it is best to do experiments in increments of 3 chrom-3 srb plates, (due to reagents quantities).

A. Trypsinize Madin-Darby Canine Kidney Cells (MDCK) by aspirating media from flask, rinse flask with 5ml of PBS 2 times, aspirating after each rinse. Rinse flask with 3ml of trypsin, aspirate trypsin from flask and add 3ml of trypsin to flask. Place in incubator for 10-15 minutes.
B. Detach cells from flask by tapping and suspend cells in appoximately 10ml of complete DMEM w/o phenol red.
C. Centifuge cells at 1000rpm for 5minutes.
D. Aspirate supernatant from cell pellet and suspend cells in approximately 10ml of complete DMEM w/o phenol red.
E. Count cells with hemocytometer to determine number of cells/ml you have in the suspension.
F. Dilute cells in complete DMEM w/o phenol red to obtain a final cell density of 1,000 for 384well plates.

G. Inoculate cells onto 384 well, flat bottom tissue culture plates by adding 40µlof cell suspension to all 384wells of the plate using the Beckman Biomek 2000.
H. Place inoculated plates in a 37ºC, 5% CO2 incubator overnight (24hrs).

Drug & HGF Addition Preparations**: (See attached diagram for plate format.)**

Prepare <u>Geldanamycin</u>: (concentrations prepared are 25µM, 2.5µM, 0.25µM, 0.025µM, 0.0025µM)
(50µl/10ml(1/200) and then 20µl/80µl(1/4) -> (1/200)(1/4)=1/400 –> Geld)
(25µl/5ml(1/200) and then 20µl/80ml(1/4) -> (1/200)(1/4)=1/400 –> 17-A)

a. Prepare 5 conical tubes with 10ml in first tube and 9ml in last four.
b. Add 50µlof compound (122750) to first tube, and mix.
c. Transfer 1ml to tube 2, and mix.
d. Repeat step (c.) 3 times serially.

Prepare <u>17-Aminodemethoxygeldanamycin</u>: (concentrations prepared are 50 µM, 5.0 µM, 0.5 µM, 0.05 µM, 0.005 µM)

e. Prepare 5 conical tubes with 5ml in first tube and 4.5ml in last four.
f. Add 50µl of compound (255109) to first tube, and mix.
g. Transfer 0.5ml to tube 2, and mix.
h. Repeat step (g.) 3 times serially.

Prepare <u>HGF</u>: Add 1 aliquot of HGF to complete media, yielding a final HGF concentration of 160ng/ml (plate conc. = 40ng/ml).

Drug & HGF Addition: **(See attached diagram for plate format.)**
*****Completed on Beckman Biomek.**

A. Add media to drug dilution plates. Add 40 µl media to cells only wells and 20 µl of media to cells + HGF wells on test plates.
B. Transfer drugs from diversity plate to drug dilution plate.
C. Dilute drugs on drug dilution to give final plate concentration of 50µM and 1uM. (1:4 into plate)
D. Add 20 µl of drugs to each well of test plates: (2 test plates = 1 chromozym, 1 protein), for each diversity plate. Two wells for each concentration of each drug.
E. Add controls to test plates according to attached diagram. Add 20 µl of the three concentrations of Geldanamycin and 17-aminodemethoxy-geldanamycin to each of the designated wells. (8 wells of each concentration). See attached diagram.
F. After all drugs have been added, add 20µl of HGF to all wells except cells only wells.
G. Label plates with diversity plate #, date, SRB or Chromozym. Incubate plates at 37ºC, 5%CO2 for 24 hours. (Strip plates on side, so lids can be matched with appropriate plate.)

Chromozym PL Measurement:

1. **Mix reaction buffer as follows (volumes are for 3-384well plates):**
    - **a. 52.5ml of 0.05 U/ml plasminogen**
    - **b. 42.0ml of 50mM Tris buffer**
    - **c. 10.5ml of 3mM Chromozym PL**
2. **After a 24hr drug and HGF exposure time, remove the Chromozym PL plates from the incubator and remove drug/media from the plates by inverting over a dishpan.**
3. **Wash Chromozym PL plates by adding 80µl/well DMEM** w/o **phenol red & 1% L-glutamine (**_NO SERUM_**) to each well of the Chromozym PL plates. Remove the 1st wash by inverting the plate over the dishpan.**
4. **Repeat step 3, for a 2nd time (2 washes total).**

5. **Add 80µl/well of reaction buffer to each well of the Chromozym PL plates, and place in the incubator at 37ºC, 5% CO_2 for 5hrs.**
6. **Read Chromozym PL plates at 405nm and store optical densities on a disk.**

Protein Measurement: **% Growth (Toxicity)**

A. Fix protein plates after a 24hr drug and HGF exposure time, by adding 20 µl of cold 50% TCA to each well of the plates. Refrigerate for 1hour at 4ºC.
B. Remove plates from refrigerator and wash 3 times with water. Allow plates to dry.
C. Stain plates by adding 50µl of 0.4%SRB to each well of the plates. Leave stain on plates for 15 minutes.
D. Remove excess stain by rinsing plates 3 times with 1% acetic acid
E. Allow plates to dry.
F. Solubilize bound stain by adding 50µl of 50µM Tris to each well. Shake plate on an orbital shaker and read plates at 550nm and store optical densities on a disk.

Data Analysis:

1. CHROMOZYM PL ABSORBANCE

A. Experimental Data (Avg & Std)

- **Cells Only**
- **Cells & HGF**
- **Cells & Test Compound & HGF**
- **Cells & Geldanamycin & HGF**
- **Cells & 17A-Geldanamycin & HGF**

B. Calculated Fields

- **HGF Induction = Cells & HGF / Cells Only**
- **Geldanamycin Induction = 0.25 µM Geldanamycin / Cells Only**
- **Test Induction = Test / Cells Only**
- **Fold Induction = Test Induction / HGF Induction**
- **Z Prime = 1-((3*SD Cells Only+3*SD Cells & HGF)/ABS(Cells Only - Cells & HGF))**

2. GROWTH (for DIV, TRN, and Titration Retest)

C. Experimental Data (Avg & Std)

- **Cells Only**
- **Cells & HGF**
- **Cells & Test Compound & HGF**
- **Cells & Geldanamycin & HGF**
- **Cells & 17A-Geldanamycin & HGF**

D. Calculated Fields

- **Percent Growth = Test / Cells & HGF * 100**