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Advances in Brief

Identification of a Geldanamycin Dimer That Induces the Selective Degradation of HER-Family Tyrosine Kinases[1]

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Abstract

Geldanamycin (GM) is a natural antibiotic that binds Hsp90 and induces the degradation of receptor tyrosine kinases, steroid receptors, and Raf. It is a potent inhibitor of cancer cells that overexpress HER-kinases, but its effects on other important proteins may cause significant toxicity and limit its clinical use. We report the synthesis and identification of a GM dimer, GMD-4c, which had selective activity against HER-kinases. Selectivity was a function of linker length and required two intact GM moieties. GMD-4c is a potent inducer of G_1 block and apoptosis of breast cancer cell lines that overexpress HER2, but does not appreciably inhibit the growth of 32D cells that lack HER-kinases. GMD-4c could be useful in the treatment of carcinomas dependent on HER-kinases.

Introduction

HER-family transmembrane receptor tyrosine kinases play an important role in transducing extracellular growth signals and when activated can be oncogenic (1, 2). Overexpression of HER1 and HER2 occurs in a variety of human malignancies. In breast cancer, overexpression of HER2 is associated with a poor prognosis (2). HER1 and HER2 are attractive targets for therapeutic development. Antibodies against each of these receptors have been shown to have antitumor effects in animal models (3). Recently, an anti-HER2 antibody was shown to be effective in the treatment of breast cancers in which HER2 is overexpressed (2, 4). However, therapeutic effects were seen in only a minority of patients and were usually short-lived. Other, more effective methods for HER2 inhibition are needed.

GM[3] and herbimycin A are benzoquinoid ansamycin antibiotics (5). This class of drugs binds to a specific pocket in the chaperone protein Hsp90 (6, 7). Occupancy of this pocket by the drug leads to the degradation in the proteasome of a subset of proteins that require Hsp90 for conformational maturation (8–11). These include the HER- and insulin-receptor families of tyrosine kinases, Raf-1 serine kinase, and steroid receptors. The addition of GM to tumor cells leads to a Rb-dependent G_1 growth arrest and apoptosis.[4] HER-kinases are the most sensitive targets of GM, and tumor cell lines in which HER2 is overexpressed are inhibited by especially low concentrations of the drug (12–14). These findings imply that GM and related drugs may be useful in the treatment of a variety of tumors. An analogue of GM, 17-allylaminoGM, is currently under Phase I clinical trials. However, the number of important signaling molecules that are affected by ansamycins suggests that they may have untoward toxicity.

We have endeavored to synthesize derivatives of GM that have a narrower spectrum of action and greater selectivity. Signaling via the HER-kinases may require their association with Hsp90. This chaperone is required for *sevenless* (*Drosophila* epidermal growth factor receptor-family member) signaling (15). v-Src associates with Hsp90 and is very sensitive to GM (6). Both the sensitivity of v-Src to ansamycins and its association with Hsp90 depend on the presence of the catalytic domain but do not require catalytic activity (16, 17). The sensitivity of HER2 to GM also requires the catalytic domain (12). However, a direct interaction of Hsp90 and HER-kinases has not been convincingly demonstrated.

These data suggested to us that Hsp90 is likely to interact with the catalytic domain of HER-kinases. Because HER-kinases undergo dimerization on activation, we speculated that each element of the HER-kinase dimer interacts with Hsp90. Accordingly, it seemed possible that a GMD might be able to interact with both subunits of the HER-kinase dimers. Here, we report the synthesis and evaluation of several GMDs and the identification of a GMD, GMD-4c, which induces the selective degradation of HER-kinases.

Materials and Methods

Cell Lines. The human breast cancer cell lines MCF-7 and SKBR-3 were obtained from American Type Culture Collection (Manassas, VA) and maintained in DME/F12 (1:1) supplemented with 10% heat-inactivated FBS (Gemini Bioproducts), 2 mM glutamine, and 50 units/ml each of penicillin and streptomycin, in a humidified 5% CO_2/air atmosphere at 37°C. The murine hematopoietic cell line 32D was kindly provided by Dr. Yosef Yarden (The Weizmann Institute of Science, Rehovot, Israel) and maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS, 2 ng/ml interleukin 3 (R&D Systems, Inc.), 2 mM glutamine, and 50 units/ml each of penicillin and streptomycin.

Antibodies. Polyclonal antibodies against HER2 (c-18), HER3 (C-17), Raf-1 (c-12), and PI3 kinase (p85) (Z-8) were purchased from Santa Cruz Biotechnology, Inc. The HER2 monoclonal antibody (Ab-5) for immunoprecipitation was from Oncogene. A monoclonal antibody against ER (clone H-151) was from *Stress*Gen Biotechnology Corp. A polyclonal antibody against the α-subunit of IGF-IR was kindly provided by Dr. L-H. Wang (Mt. Sinai Medical Center, New York, NY).

GM and Its Analogues. GM was kindly provided by Drs. David Newman and Edward Sausville (Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda, MD), dissolved in 100% DMSO, and stored at −20°C. The GM analogues were prepared according to S. Kuduk *et al.*[5] using the method of Schnur *et al.* (13). Briefly, the GMDs were prepared by treatment of GM with 0.5 equivalent of the appropriate diamine in DMSO. The ansa-ring-opened GMDs (GMD-a and GMD-aa) were prepared by methanolysis (NaOMe/methanol) of the GMD-4c. GM-quinone was synthesized by first

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[3] The abbreviations used are: GM, geldanamycin; GMD, GM dimer; ER, estrogen receptor; IGF-I, insulin-like growth factor I; IGF-IR, IGF-I receptor; FBS, fetal bovine serum; RB, retinoblastoma protein.

[4] M. Srethapakdi and N. Rosen, manuscript in preparation.

[5] S. D. Kuduk *et al.*, unpublished data.

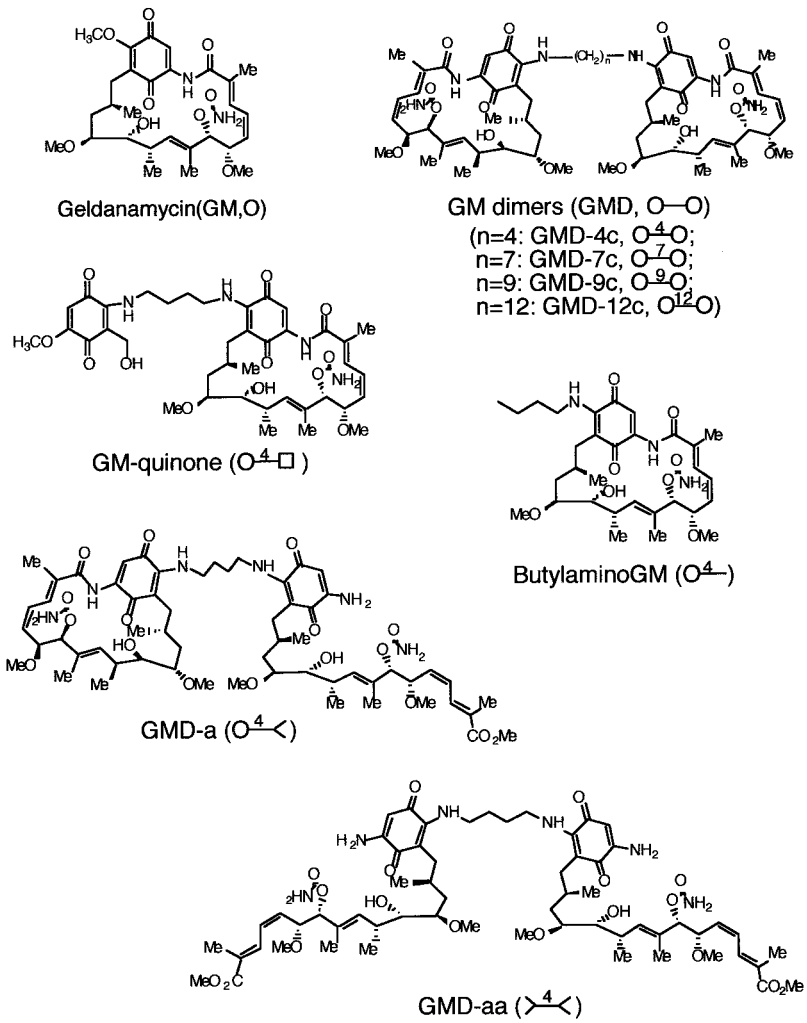

Fig. 1. Chemical structures of GM and its analogues, constructed according to "Materials and Methods." ○, GM moiety; –, carbon linker; □, quinone; < or >, GM moiety with an opened ansa-ring.

treating GM with excess 1,4-diamobutane, then the addition of 2-methoxy-1-hydroxymethylquinone.

Cell Growth Experiments. Cells were plated in 6-well tissue-culture plates (Corning Glass) at 20,000 cells/well. Two days after plating, cells were treated with different concentrations of drugs or the vehicle DMSO (0.1%). MCF-7 and SKBR-3 cells were treated for 4 days. Medium with the appropriate drug or vehicle was changed every 2 days. Cells were trypsinized, collected, and counted on a Coulter counter. IC_{50} for cell growth is designated as the amount of each drug needed to inhibit cell growth by 50% compared with the control vehicle. 32D cells were treated and counted every day for 3 consecutive days using a hematocytometer.

Immunoprecipitation and Immunoblotting. MCF-7 cells were washed twice with ice-cold PBS, collected by scraping, and transferred into microcentrifuge tubes. For immunoblotting (HER2, HER3, Raf-1, ER), cells were lysed with SDS lysis buffer [50 mM Tris-HCl (pH 7.5), 2% SDS, 10% glycerol, and 1 mM DTT], boiled for 10 min, and sonicated briefly. For immunoprecipitation (HER2 and IGF-IR), cells were lysed with NP40 lysis buffer [50 mM Tris-HCl (pH 7.5), 1% NP40, 150 mM NaCl, 1 mM Na_3VO_4, 40 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of aprotinin, leupeptin, and soybean trypsin inhibitor] for 20 min at 4°C. Cell lysates were cleared by centrifugation at 14,000 × *g* for 15 min at 4°C in a microcentrifuge. Supernatants were collected as the experimental samples. Protein concentration in each sample was determined using the BCA kit (Pierce Chemical Co.), according to the manufacturer's instructions. For detecting IGF-IR, samples were immunoprecipitated with anti-IGF-IR antibody. Immunocomplexes were collected on protein A-Sepharose beads (Pharmacia) and washed three times with the lysis buffer. Samples were subjected to SDS-PAGE, electrotransferred to nitrocellulose membranes, detected using the ECL kit (Amersham Corp.), according to the manufacturer's protocol, and quantitated using the Gel Doc 1000 (Bio-Rad Laboratories). IC_{50} for protein degradation is designated as the amount of each drug needed to decrease 50% of the protein (HER2 or Raf-1) compared with the control in MCF-7 cells after a 24-h treatment.

Pulse-labeling and Pulse-chase Experiments. To study the effects of GM and GMD-4c on protein synthesis, MCF-7 cells were pulse-labeled with

Table 1 *The effects of GM analogues on protein down-regulation and cell growth*

Drug[a]	MCF-7 IC_{50}[b] (HER-2) (nM)	MCF-7 IC_{50}[b] (Raf-1) (nM)	MCF-7 IC_{50}[c] (growth inh.) (nM)	SKBR-3 IC_{50}[c] (growth inh.) (nM)
GM (○)	45	200	25	3
GMD-4c (○4○)	60	2200	100	20
GMD-7c (○7○)	70	500	600	200
GMD-9c (○9○)	500	3800	700	500
GMD-12c (○12○)	750	3500	700	650
ButylaminoGM (○4—)	80	350	600	350
GM-quinone (○4□)	55	350	350	60
GMD-a (○4<)	500	3500	650	250
GMD-aa (>4<)	>5000	>5000	>2000	>2000

[a] See Fig. 1 for the structure of each drug.

[b] MCF-7 cells were treated with various concentrations of each drug; IC_{50} for each protein is designated as the amount of each drug needed to decrease the steady-state level of either HER2 or Raf-1 to 50% of the control after 24 h of treatment.

[c] MCF-7 and SKBR-3 cells were treated with different concentrations of each drug for 4 days; IC_{50} is designated as the amount of each drug needed to inhibit cell growth by 50% compared with the control after the 4-day treatment. The numbers were the average of three different experiments.

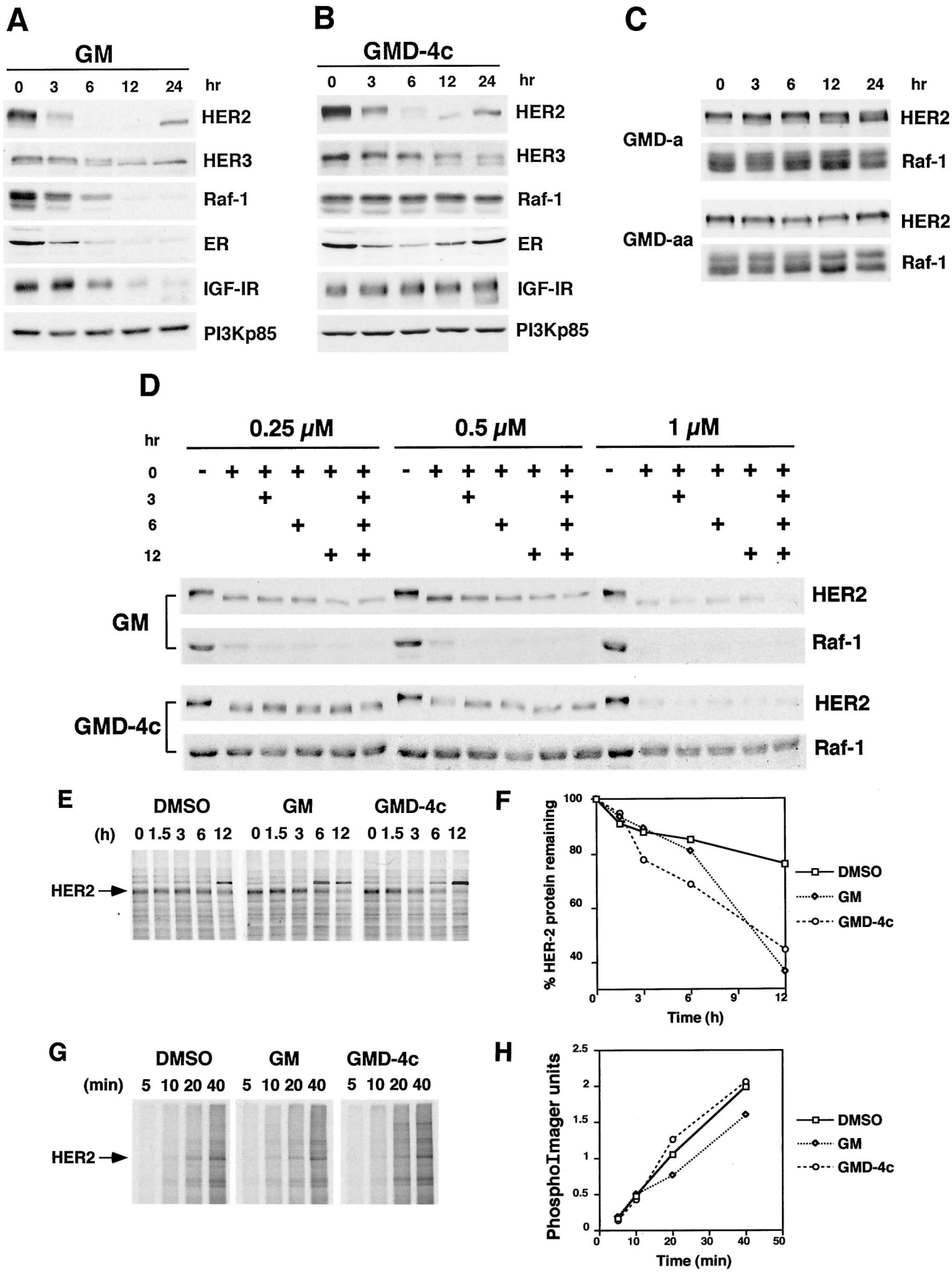

Fig. 2. The effects of GM and its analogues on the expression of the cellular proteins of MCF-7 cells. To investigate the effects of GM and its analogues on the steady-state levels of proteins, cells were treated with various drugs (each 1 μM) for different periods of time (*A*, *B*, and *C*), or cells were treated with either GM or GMD-4c, which was added multiple times according to the schedule shown on the *top* (*D*). + and hr, the time when the drug was added. Cells were treated for a total of 24 h. Total cell lysates were then extracted, subjected to SDS-PAGE, transferred to nitrocellulose membrane, and analyzed by immunoblotting using specific antibodies against each protein. To investigate the effect of GMD-4c on HER2

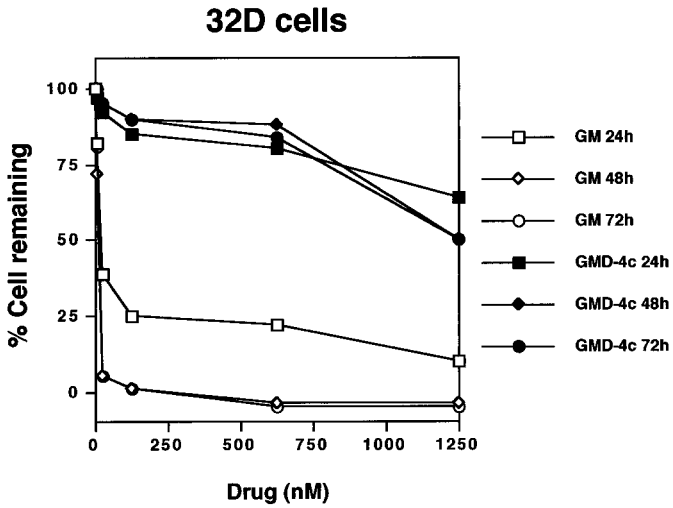

Fig. 3. The effects of GM and GMD-4c on the growth of the hematopoietic 32D cells, which lack HER-kinases. 32D cells were treated with various concentrations of either GM or GMD-4c for different periods of time. Cells were counted using a hemacytometer. The results are the average of three different experiments.

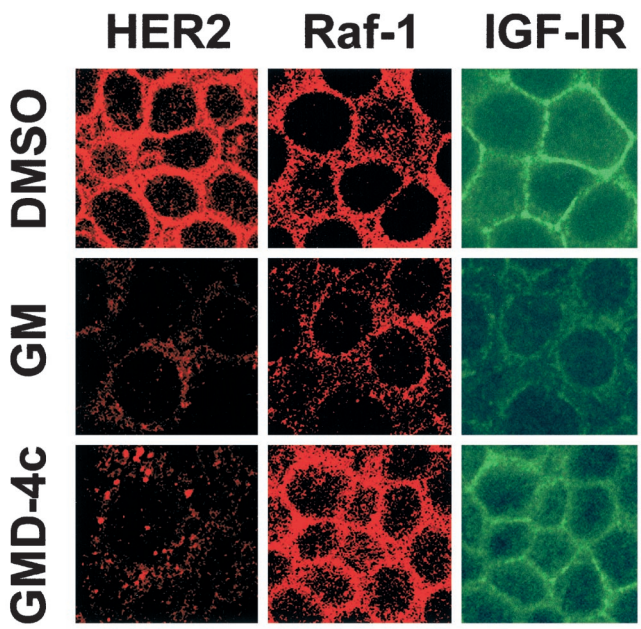

Fig. 4. Immunohistochemical analysis of the effects of GM and GMD-4c on the expression of HER2, Raf-1, and IGF-IR in MCF-7 cells. Cells were treated with either GM (1 μM), GMD-4c (1 μM), or the control vehicle DMSO (0.1%) for 24 h. Cells were then fixed and immunostained for HER2, Raf-1, and IGF-IR, according to "Materials and Methods."

[^{35}S]protein-labeling mix (NEN; 100 μCi/ml, 1175 Ci/mmol) in methionine/cysteine-free media for increasing amounts of time in the presence of either GM (1 μM), GMD-4c (1 μM), or the carrier (DMSO, 0.1%). To study the effects of GM and GMD-4c on protein degradation, MCF-7 cells were pulse-labeled to isotopic equilibrium with [^{35}S]protein-labeling mix (NEN; 100 μCi/ml, 1175 Ci/mmol) in methionine/cysteine-free medium mixed with regular medium/5% FBS (9:1) for 14 h and chased with unlabeled methionine/cysteine (150 μg/ml) in the presence of either GM (1 μM), GMD-4c (1 μM), or the carrier (DMSO, 0.1%) for a period of 12 h. Cells were collected at different time points from the pulse-labeling and pulse-chase experiments and lysed with NP40 lysis buffer. Samples containing equal amounts of protein (300 μg) were immunoprecipitated for HER2 and subjected to 7% SDS-PAGE. Gels were dried and exposed to X-ray films. HER2 bands were quantitated using Bio-Rad Gel Doc 1000.

Immunohistochemistry. Cells were grown and treated on fibronectin-coated coverslips placed in multiwell plates. Cells were fixed for 20 min at −20°C in 100% methanol, rehydrated in PBS for 10 min at room temperature, and blocked for 30 min at 37°C in a blocking solution consisting of 2% BSA, 10% normal goat serum, and 0.05% Tween 20 in PBS. HER2 and Raf-1 were immunodetected with antibodies (SC284 and SC133, respectively) from Santa Cruz Biotechnology, Inc., and IGF-IR was immunodetected with an antibody (Ab1) from Calbiochem Oncogene Science. Slides were incubated with a 1:1000 dilution of the primary antibody in blocking buffer for 1 h at room temperature, followed by an incubation with Alexa 546-coupled goat antirabbit IgG secondary antibody (A-1 I010; Molecular Probes) at a 1:50 dilution in blocking buffer, or fluoresceine isothiocyanate-coupled goat antimouse IgG (F276; Molecular Probes) at a 1:100 dilution. Slides were washed three times with 1 ml of 0.5% BSA in 0.05% Tween 20 in PBS in between and after incubations with primary and secondary antibodies. DNA was stained with bisbenzimide, which was included in the secondary antibody solution (3 μg/ml final concentration). Coverslips were mounted on glass slides using Vectashield (Vector Laboratories, Inc.) to prevent quenching of fluorescence. Immunofluorescence was detected with a Zeiss epifluorescence microscope at ×40 and ×100, using appropriate filters for detection of rhodamine and bisbenzimide, and via confocal microscopy.

Results and Discussion

We synthesized a series of GMDs covalently joined by alkylamino linkers of varying chain length (Fig. 1). The linker is bonded to the 17-carbon of both GM moieties. The crystal structure of GM bound to Hsp90 shows that the 17-carbon is the only one not buried in the binding pocket (7). The activities of GMDs were compared with those of GM and assessed in terms of efficiency of induction of down-regulation of the HER2 and Raf-1 protein kinases in MCF-7 cells (Table 1). The properties of these GMDs were found to vary as a function of chain length. GM itself causes the induction of HER2 degradation with an IC_{50} of 45 nM. Dimers with linkers of four to seven carbons retain activity against HER2 (IC_{50}, 60–70 nM). Dimers with longer linkers lose activity; the 12-carbon-linked compound has an IC_{50} of 750 nM.

The four-carbon-linked dimer, GMD-4c, has selective activity. GM causes Raf-1 degradation with an IC_{50} of 200 nM. GMD-4c is much less active, with an IC_{50} of 2200 nM. Selectivity is lost with increasing chain length; the seven-carbon-linked-dimer (GMD-7c) retains activity against HER2 (IC_{50}, 70 nM) and is only slightly less active than GM against Raf-1 (IC_{50}, 500 nM). As linker carbons increase to more than eight, activity against both targets declines in parallel. The properties of GMD-4c were examined in greater detail. GM causes the degradation over time of HER-kinases, Raf-1, the ER, and, more slowly, the IGF-IR (Fig. 2*A* and Fig. 4, and HER1 and HER4 data not shown). GMD-4c reduces HER2 expression with the same kinetics both on immunoblot and by immunohistochemical analysis (Fig. 2*B*). GMD-4c also decreases HER3 expression (Fig. 2*B*) and HER1 and HER4 expression (data not shown). GMD-4c is not selective for individual members of the HER-kinase family. However, under these conditions (1 μM, 24-h treatment), GMD-4c does not affect Raf-1 or IGF-IR expression. ER levels declined transiently but returned to

protein degradation, cells were pulse-labeled with [^{35}S]protein-labeling mix (NEN; 100 μCi/ml, 1175 Ci/mmol) in methionine/cysteine-free medium mixed with regular medium/5% FBS (9:1) for 14 h and chased with unlabeled methionine/cysteine (150 μg/ml) in the presence of either GM (1 μM), GMD-4c (1 μM), or the carrier (DMSO, 0.1%) for a period of 12 h. Cell lysates from different time points were immunoprecipitated for HER2 protein, subjected to SDS-PAGE, followed by autoradiography (*E*), and quantitated by the Bio-Rad gel doc (*F*). To investigate the effect of GMD-4c on HER2 protein synthesis, cells were pulse-labeled with [^{35}S]protein labeling mix (NEN; 100 μCi/ml, 1175 Ci/mmol) in methionine/cysteine-free media for an increasing amount of time in the presence of either GM (1 μM), GMD-4c (1 μM), or the carrier (DMSO, 0.1%). Cell lysates from different time points were immunoprecipitated for HER2 protein, subjected to SDS-PAGE, followed by autoradiography (*G*), and quantitated by the Bio-Rad gel doc (*H*).

baseline by 24 h (Fig. 2*B*). Both GM and GMD-4c do not affect PI3-kinase (PI3Kp85) expression. A faster migrating HER2-immunoreactive band appeared after 12 h of treatment with either drug, but more prominently with GMD-4c (Fig. 2, *A* and *B*). This form accumulates in intracellular vesicles and corresponds to immature HER2. Glycosylation studies revealed that this HER2 form is partially glycosylated and sensitive to endoglycosidase H (data not shown; Ref. 18).

Additional GM derivatives were synthesized to explore the mechanism of selectivity (Fig. 1). ButylaminoGM, a molecule in which the four-carbon linker is attached to only one geldanamycin residue, and a four carbon-linked heterodimer of GM and a quinone (GM-quinone) are modestly weaker than GM against both HER2 and Raf-1; they are not selective (Table 1). GMD-aa, a GMD-4c derivative in which the ansa-ring of each of the GM moieties is opened, is inactive (Fig. 2*C*). GMD-a, a dimer in which the ring of only one of the GM moieties is open has much reduced activity against both targets (Table 1; IC_{50}, HER2 500 nM, and IC_{50}, Raf-1 3500 nM; Fig. 2*C*). These data suggest that the selectivity of GMD-4c depends on both GM moieties. This apparent selectivity could be a property of a weaker or more rapidly metabolized drug that might seem to have selective activity against the most sensitive target (HER2). To address this question, GM and GMD-4c were added to cells at different concentrations and frequencies (Fig. 2*D*). Even when GMD-4c was added at high concentrations four times in 12 h, it retained selectivity.

To investigate the mechanism by which GMD-4c down-regulates HER-family protein expression, we tested the effects of GM or GMD-4c on HER2 protein degradation and synthesis by pulse-chase and pulse-labeling experiments. Both GMD-4c and GM accelerated the degradation of HER2 protein (Fig. 2, *E* and *F*) and did not affect its rate of synthesis (Fig. 2, *G* and *H*). These results indicate that GMD-4c, like GM (9), affects HER2 expression by inducing protein degradation.

GMD-4c was a potent inhibitor of growth of breast cancer cells containing HER-kinases (Table 1), with an IC_{50} of 100 nM against MCF-7 cells compared with an IC_{50} of 25 nM for GM and 650 nM for GMD-a. SKBR-3, a cell line in which the *HER2* gene is amplified and the protein is highly overexpressed, is especially sensitive to both GMD-4c and GM (Table 1; IC_{50}, 20 nM and 3 nM, respectively). Most epithelial cancer cell lines express one or more members of the HER-kinase family. To assess whether the effects of GMD-4c on cells were specific, we used the 32D hematopoietic cell line (19). None of the members of the HER-kinase family are expressed in this murine interleukin-3-dependent myeloid progenitor cell line. GM is a potent inhibitor of 32D (IC_{50}, 3 nM), but GMD-4c does not appreciably affect its growth at concentrations up to 1 μM (Fig. 3).

Thus, GMD-4c induces the selective degradation of HER-family kinases and specifically inhibits the growth of HER-kinase containing tumor cell lines. Because its effects on other key signaling proteins are attenuated, GMD-4c is likely to be much less toxic than GM. This work supports the idea that selective ansamycins with a different, more restricted spectrum of targets than the parent molecules can be synthesized. In this case, the mechanism of selectivity is not yet known, but depends on the presence of both GM moieties and is a function of the linker length. GMD-4c may selectively interact with HER-kinase dimers, but it is also possible that it preferentially interacts with different Hsp90-family members than GM. This work represents a new strategy for abrogating growth receptor function in human tumors.

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