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Geldanamycin derivative inhibition of HGF/SF-mediated Met tyrosine kinase receptor-dependent urokinase-plasminogen activation

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Abstract—Ansamycins, including geldanamycin and the derivative 17-allylamino-17-demethoxygeldanamycin, and radicicol are known for their ability to tightly bind to the ATP-binding site of the amino-terminal domain region of heat shock protein 90. We have found that geldanamycin and some of its derivatives can inhibit hepatocyte growth factor/scatter factor-mediated Met tyrosine kinase receptor-dependent urokinase-plasminogen activation at femtomolar levels. Assessment is made of structural requirements for such an activity and evidence is given that distinguishes the target of such an activity from that of heat shock protein 90.

1. Introduction

Signaling via the Met protooncogene, the tyrosine kinase receptor for hepatocyte growth factor/scatter factor (HGF/SF), is critical for normal physiological, as well as pathological, processes. The Met signal transduction pathway is involved in aspects of embryogenesis, proliferation, motility, differentiation, and cellular survival. Dysregulation of Met signaling has been found to contribute to the development and growth of primary tumors and their metastases. Evidence for this feature is multifold.[1] Inappropriate expression of Met is found in many types of human cancers (www.vai.org/vari/metandcancer). Cell lines coexpressing Met and HGF/SF become tumorigenic and metastatic in nude mice.[2] Transgenic mice having HGF/SF inappropriately directed to various tissues developed diverse tumors.[3] Importantly, activating mutations of the Met receptor have been found in human carcinomas.[1,4] Recent work has shown that Met signaling pathway is a potential therapeutic target for cancer therapy. Studies have shown the efficacy of Met-directed ribozyme and antisense strategies for reduction of Met and HGF/SF expression, tumor growth, and metastatic tumor potential.[5–8] NK4, a HGF/SF fragment possessing its amino-terminal four-kringle domain, is a competitive HGF/SF antagonist for the Met receptor[9] and has been demonstrated to inhibit tumor invasion and metastasis, as well as tumor angiogenesis.[10] Monoclonal antibodies directed to HGF/SF neutralize its activity with inhibition of human xenograft tumor growth in athymic nu/nu mice.[11] The indole-based receptor tyrosine kinase inhibitors, K252a and PHA-665752, inhibit Met kinase activity and Met-driven tumor growth and metastatic potential.[12,13]

A recent study screened for inhibitors of the Met receptor signal transduction pathway.[14] HGF/SF induces the expression of the urokinase plasminogen activator (uPA) and its receptor (uPAR), mediators of cell invasion and metastasis. A cell-based assay utilizing the induction of uPA and uPAR, and subsequent conversion of plasminogen to plasmin allowed the screening of compounds for inhibitory properties in MDCK-2

Keywords: Geldanamycin; Hepatocyte growth factor/scatter factor; Urokinase-plasminogen activation; Met tyrosine kinase receptor.

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cells. Geldanamycin (**1**) and some derivatives thereof were found to exhibit high inhibitory activity: at femtomolar concentrations. More recently, this exquisite inhibitory activity has been extended to include additional activities of the invasion complex, notably the in vitro invasion of human tumor cells through three-dimensional Matrigel.[15] No loss of Met expression was observed at lower than nanomolar concentrations, indicating that the observed inhibitory activity was independent of down-regulation of the Met receptor.

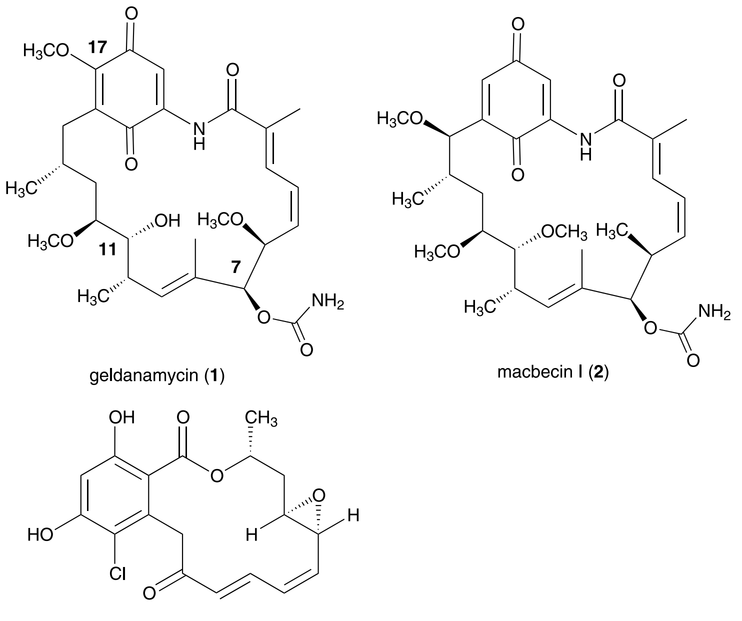
Geldanamycin and 17-alkylamino-17-demethoxygeldanamycin derivatives are best known for their ability to bind to the ATP-binding site of the amino-terminal domain region of heat shock protein 90 (hsp90).[16–20] Hsp90 belongs to the structural protein family of GHKL ATPases.[21] This abundant protein helps in the regulation of activity, turnover, and trafficking of various critical proteins. It facilitates in the folding and regulation of proteins in cellular signaling, such as transcription factors, steroid receptors, and protein kinases.[22–25] The function of hsp90 is blocked by ansamycin natural products, such as geldanamycin[26] and macbecin I (**2**),[27,28] as well as radicicol (**3**)[29,30] (Fig. 1). It is the blockage of hsp90 function that has been ascribed to the antitumor effect of 17-allylamino-17-demethoxygeldanamycin (**4**), a drug now used in clinical trials.[31,32]

The target(s) for disruption of the Met signal transduction pathway at femtomolar levels in tumor cells by geldanamycin and its derivatives has remained unknown. The above-described disruption of hsp90 function is an effect known for this ansamycin class of drugs at higher concentrations, that is, micromolar and greater. We assess here the structure–activity relationship of geldanamycin derivatives for an unknown target(s) and provide further evidence that distinguishes the femtomolar target(s) from hsp90.

2. Results

2.1. Chemistry

Different classes of geldanamycin derivatives were synthesized for this study. 17-Alkylamino-17-demethoxygeldanamycin derivatives can bind hsp90 with high affinity inhibiting its function, and numerous derivatives of this class were prepared (Fig. 2). The 17-methoxy group of geldanamycin is easily displaced by primary amines. 17-Allylamino-17-demethoxygeldanamycin (**4**) and 17-(2-dimethylaminoethyl)amino-17-demethoxygeldanamycin (**5**), each of which is currently in clinical trials[33,34] (NCI protocol ID's are available at: http://cancer.gov/clinicaltrials), were prepared in near quantitative yield from geldanamycin by reaction with an appropriate amine. Similarly prepared in high yield were the known 17-amino-17-demethoxygeldanamycin (**6**),[35] 17-(2-chloroethyl)amino-17-demethoxygeldanamycin (**7**), 17-(2-fluoroethyl)amino-17-demethoxygeldanamycin (**8**),[36] as well as the new derivatives 17-(2-acetylaminoethyl)amino-17-demethoxygeldanamycin (**9**), 17-(6-acetylaminohexyl)amino-17-demethoxygeldanamycin (**10**), 17-(6-biotinylaminohexyl)amino-17-demethoxygeldanamycin (**11**), 17-(8-acetamido-3,6-dioxaoctylamino)-17-demethoxygeldanamycin (**12**), and 17-carboxymethylamino-17-demethoxygeldanamycin (**13**). The 17-methoxy group of geldanamycin has been found to be displaceable by the secondary amine azetidine and 17-*N*-azetidinyl-17-demethoxygeldanamycin (**14**) was so prepared.[35] In a similar fashion, treatment of geldanamycin

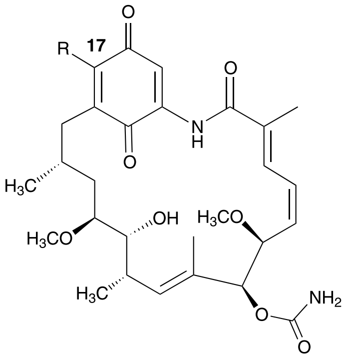


geldanamycin (**1**)

macbecin I (**2**)

radicicol (**3**)

Figure 1. Geldanamycin, macbecin I, and radicicol.



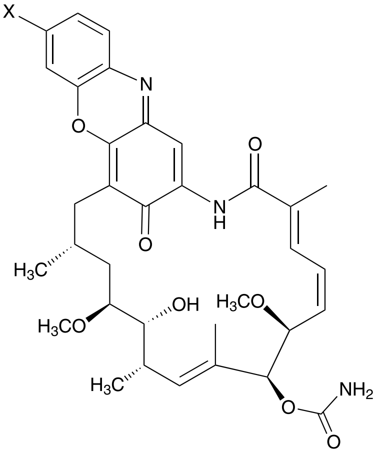
4 R = -NHCH$_2$CH=CH$_2$
5 R = -NHCH$_2$CH$_2$N(CH$_3$)$_2$
6 R = -NH$_2$
7 R = -NHCH$_2$CH$_2$Cl
8 R = -NHCH$_2$CH$_2$F
9 R = -NHCH$_2$CH$_2$NHC(O)CH$_3$
10 R = -NH(CH$_2$)$_6$NHC(O)CH$_3$
11 R = -NH(CH$_2$)$_6$NH-biotinyl
12 R = -NH(CH$_2$CH$_2$O)$_2$CH$_2$CH$_2$NHC(O)CH$_3$
13 R = -NHCH$_2$CO$_2$H
14 R = -NCH$_2$CH$_2$CH$_2$-
15 R = -NCH$_2$CH$_2$-

Figure 2. 17-Amino-17-demethoxygeldanamycin derivatives.

with aziridine gave the new compound 17-*N*-aziridinyl-17-demethoxygeldanamycin (**15**).

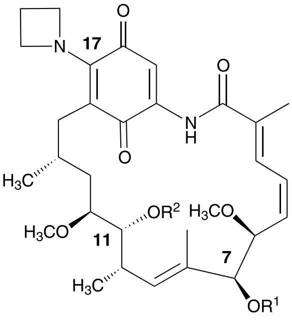
Two derivatives of the geldanamycin derivative class, geldanoxazinone, were synthesized (Fig. 3). Such derivatives can be prepared by acid-catalyzed condensation of geldanamycin with a 2-aminophenol.[37] 5-Bromo-2-aminophenol and 5-iodo-2-aminophenol were thus used to prepare adducts **16** and **17** in 60% and 44% yield, respectively.

In an effort to investigate the effect of modification of the ansa ring of geldanamycin on activity, an active 17-aminosubstituted-17-demethoxygeldanamycin derivative, 17-*N*-azetidinyl-17-demethoxygeldanamycin (**14**), was used for making such changes (Fig. 4). The 11-hydroxyl group of the latter compound could be esterified with acetic anhydride and 4-dimethylaminopyridine to provide 11-*O*-acetyl-17-*N*-azetidinyl-17-demethoxygeldanamycin (**18**).[35] The 7-urethane group of compound **14** could be removed per slight modification of the literature procedure[35] by treatment with potassium *tert*-butoxide in *tert*-butanol (in lieu of the solvent dimethylsulfoxide, which gave a lower product yield) to provide 17-*N*-azetidinyl-7-decarbamoyl-17-demethoxygeldanamycin (**19**).



16 X = -Br
17 X = -I

Figure 3. Geldanoxazinones.



14 R^1 = -C(O)NH$_2$; R^2 = -H
18 R^1 = -C(O)NH$_2$; R^2 = -C(O)CH$_3$
19 R^1 = -H; R^2 = -H

Figure 4. 17-*N*-Azetidinyl-17-demethoxygeldanamycin derivatives.

Finally, dihydrogeldanamycin (**20**), the hydroquinone version of geldanamycin, and the geldanamycin-related benzoquinone ansamycin macbecin I (**2**), were prepared as per reported methods [sodium dithionite reduction of geldanamycin and ferric chloride oxidation of macbecin II (**21**), respectively] (Fig. 5).

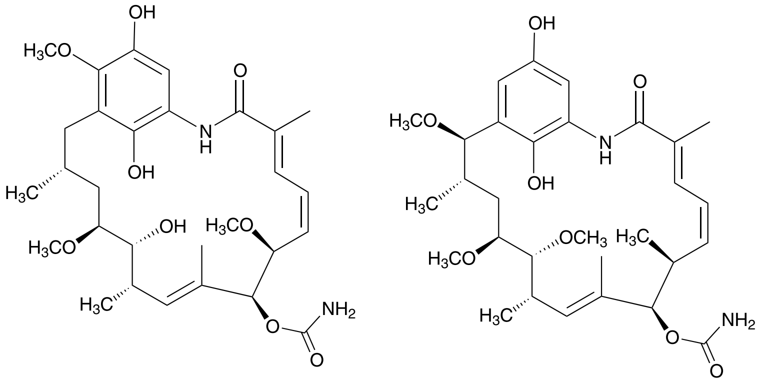
2.2. Biological evaluation

The aforementioned compounds were investigated for their inhibitory effect on the HGF/SF-Met-induced urokinase plasminogen activation (uPA) pathway, as per a previously described method.[14] The 17-alkyl- and 17-cycloalkyl-amino-17-demethoxygeldanamycin derivatives **4–8**, **14**, and **15** were found to be active at low concentrations for the inhibitory effect on the Met-uPA pathway in the cell-based assay, whereas compounds **9–13** were inactive. Table 1 shows the uPA inhibition index for the compounds investigated. Dramatically, the compounds fall into two groups: those with an index greater than 12 and those less than 8. The former group displays activity well into the femtomolar range.

The bromo-substituted and iodo-substituted geldanoxazinones **16** and **17**, respectively, were found to be inhibitory to the Met signaling pathway only at nanomolar concentrations, that is, <8 IC$_{50}$ (Table 1).

The modifications introduced to the ansa ring of a highly active compound **14** (>15 IC$_{50}$) led to the formation of compounds **18** and **19** that exhibited only <8 IC$_{50}$ Met-uPA signaling inhibitory activity (Table 1).

Testing of the inhibitory activity of the dihydroquinones dihydrogeldanamycin (**20**) and macbecin II (**21**) was



dihydrogeldanamycin (**20**) macbecin II (**21**)

Figure 5. Dihydroquinone ansamycins.

Table 1. uPA-plasmin inhibition index of compounds

Compound	Chemical name	uPA-plasmin inhibition index[a]
8	17-(2-Fluoroethyl)amino-17-demethoxygeldanamycin	19
4	17-Allylamino-17-demethoxygeldanamycin	18.0
15	17-*N*-Aziridinyl-17-demethoxygeldanamycin	15.7
6	17-Amino-17-demethoxygeldanamycin	15.3
14	17-*N*-Azetidinyl-17-demethoxygeldanamycin	15
5	17-(2-Dimethylaminoethyl)amino-17-demethoxygeldanamycin	14.9
1	Geldanamycin	14.3
7	17-(2-Chloroethyl)amino-17-demethoxygeldanamycin	14.0
20	Dihydrogeldanamycin	12.7
18	11-*O*-Acetyl-17-*N*-azetidinyl-17-demethoxygeldanamycin	7.9
3	Radicicol	7.9
21	Macbecin II	6.5
2	Macbecin I	6.4
13	17-Carboxymethylamino-17-demethoxygeldanamycin	6.3
9	17-(2-Acetylaminoethyl)amino-17-demethoxygeldanamycin	5.8
17	5′-Iodogeldanoxazone	5.8
12	17-(8-Acetamido-3,6-dioxaoctylamino)-17-demethoxygeldanamycin	5.8
19	17-*N*-Azetidinyl-7-decarbamyl-17-demethoxygeldanamycin	5.7
11	17-(6-Biotinylaminohexyl)amino-17-demethoxygeldanamycin	5.5
10	17-(6-Acetylaminohexyl)amino-17-demethoxygeldanamycin	5.3
16	5′-Bromogeldanoxazone	5.3

[a] uPA-plasmin inhibition index or IC$_{50}$ is the negative log of the drug concentration at which 50% inhibition of uPA occurs when MDCK cells are treated with HGF/SF.[14] Compounds with IC$_{50}$ higher than 12 are referred to fM-GAi, while compounds with index lower than 8 belong to nM-GAi.

done and compared with those of their benzoquinone analogs, geldanamycin and macbecin I (**3**). Additionally, testing was done of radicicol (**3**). Though radicicol[29] and macbecins I[27] and II[15] are known for their affinity for hsp90, each of these compounds exhibited poor activity in our HGF/SF-induced uPA-plasmin assays. However, dihydrogeldanamycin (**20**) was found to be $>$12 IC$_{50}$ and active, as was seen also with geldanamycin (Table 1).

3. Discussion

Investigations into the therapeutic potential of geldanamycin and its derivatives have been focusing primarily on biological processes where hsp90 plays a critical role.[33,38,39] Multiple proteins critical in cancer cell survivability and proliferation are dependent on this chaperone protein.[32,40] The ability of geldanamycin derivatives to block the function of hsp90 has led to clinical investigations of 17-*N*-allylamino-17-demethoxygeldanamycin (**4**) for cancer treatment.[32,33,39] Preliminary reports of clinical trials have indicated that the drug has efficacy as an anticancer therapeutic. It has been reported that hepatic toxicity is dose-limiting. Non-dose-limiting toxicities have been reported to include anemia, anorexia, nausea, emesis, and diarrhea.[32,33]

Our results indicate that various geldanamycin derivatives are inhibitors of HGF/SF-induced Met-dependent urokinase activation in MDCK cells and in cancer cell lines in a fashion that is distinct from the traditional hsp90 binding and inhibition paradigm. Our lines of evidence for this characteristic are as follows.

Geldanamycin derivatives can act as inhibitors of the Met-urokinase-plasminogen activation pathway at concentrations far below that needed for inhibition of hsp90 function. Although currently we cannot exclude the possibility that some isoforms of hsp90 or a specific complex of drug and hsp90 are contributing to this exquisite sensitivity, our highly active compounds exhibit this activity at femtomolar levels, well below the nanomolar concentrations that have been ascribed to hsp90 inhibition. Indeed, our most active compound, 17-(2-fluoroethyl)amino-17-demethoxygeldanamycin (**8**), exhibited activity to 10^{-19} M concentrations. Of the 21 compounds tested for their inhibitory effect on the HGF/SF-Met-induced urokinase plasminogen activation (uPA) pathway in our cell-based assays, nine of these had inhibitory activity in the 10^{-12} to 10^{-19} M range. The remaining 12 compounds had inhibitory activity in a distinct 10^{-5} to 10^{-8} M range.

Additionally, we have found that the activity seen does not always correlate with affinity to human α-hsp90. The known hsp90 binding compounds, geldanamycin (**1**),[26,16,41,19,42] 17-allylamino-17-demethoxygeldanamycin (**4**),[18,42,43] and 17-(2-dimethylaminoethyl)amino-17-demethoxygeldanamycin (**5**),[20] were found to be highly active compounds in our cell-based assays. However, the known hsp90-binding compounds, macbecin I (**2**),[27] macbecin II (**21**),[15] and, in particular, the hsp90 high affinity compound radicicol (**3**),[19,42] were found to have low inhibitory activity in our assays of the HGF/SF-Met-induced uPA pathway.

Further evidence distinguishing the mode of action of high activity compounds from acting via hsp90 comes from the observed effect of our compounds on hsp90 and Met expression. The reported cellular effect of geldanamycin is such that hsp90 is usually up-regulated and that of Met expression is down-regulated in vitro.[44,45,15] We have observed this effect of geldanamycin on hsp90 and Met expression levels only at higher concentrations (<8 IC_{50}). At subnanomolar concentrations (>12 IC_{50}), where uPA activity remains inhibited, we did not detect any change of either hsp90 or Met expression.[15]

Finally, evidence for a non-hsp90 direct effect of our active compounds comes from the effect of tested compounds on tumor cell invasion and proliferation. High activity geldanamycin derivatives not only inhibit uPA activity at femtomolar level, they also inhibit tumor cell invasion in vitro.[15] However, proliferation is inhibited only at nanomolar levels, the same concentration levels of low activity geldanamycin derivatives.[14] This suggests that geldanamycin inhibits proliferation and invasion by several mechanisms. Thus, while proliferation may be affected via inhibition of hsp90 function, invasion may be affected by geldanamycin interaction with unknown target(s). In support of this hypothesis, the MDCK cells were intentionally cultured in the presence of macbecin II (**21**). This ansamycin inhibits both invasion and proliferation activity at nanomolar level. MDCK cells were maintained at the highest non-toxic concentrations of macbecin II (**21**) (3 μM) for several months. Under these conditions, both Met and hsp90 returned to parental levels and Met responsiveness to HGF/SF was restored, but hsp90 appeared to remain in complex with macbecin. Strikingly, the uPA-plasmin sensitivity to geldanamycin in the macbecin II-treated cells was the same as that in the parental MDCK cells. HGF/SF could still significantly upregulate uPA activity and this could also be inhibited by geldanamycin at femtomolar levels. These studies have further confirmed the fact that geldanamycin inhibits HGF/SF-induced uPA activity through non-hsp90 target(s).[15]

Although the unknown target(s) of our active geldanamycin derivatives remain to be identified, our data suggest some structure–activity relationships. Some 17-*N*-aminoderivatized-17-demethoxygeldanamycin compounds were found to be active in our cell-based assays, but others were not. The latter includes those with longer 17-*N*-aminosubstitutions, for example, **9**–**12**, in addition to the carboxylate derivative **13**.

In regard to ansa ring modifications, when the 7-urethane group was removed from the active geldanamycin derivative **14**, the resulting decarbamoylated compound **19** was found to be inactive. The urethane functionality is known to undergo hydrogen bonding interactions with several aminoacid residues in the crystal structures of geldanamycin and geldanamycin derivative **5** complexed with the amino-terminal domain of hsp90.[16,20] Additionally, it has been reported by Schnur et al. that the 7-urethane was needed for anti-erbB-2 activity.[35] The 7-urethane of geldanamycin derivatives is buried deep in the ATP-binding site of hsp90. Thus, it is possible that the binding site for geldanamycin of our unknown target(s) for Met function may share similarities with this binding area of hsp90. We additionally modified the active geldanamycin derivative **14** with acetylation of the 11-hydroxyl group to give **18**, which was inactive in our cell-based assays.

As mentioned, the activity we observed showed differences with the known relative affinity of compounds with hsp90. Although the binding site on the target(s) of our cell-based uPA assays remains unknown, the site may possibly also be an ATP-binding site, albeit with some differences. It has recently been reported by Kamal et al. that a high-affinity conformation of hsp90 in tumor cells accounts for the tumor selectivity of 17-*N*-allylamino-17-demethoxygeldanamycin (**4**) and radicicol (**3**).[43] The hsp90 of the tumor cells forms multichaperone complexes, whereas normal tissue hsp90 is not so complexed. It is not possible to speculate if such target(s) is so complexed changing the conformation of the geldanamycin-binding site.

That the Met uPA-plasmin and invasion pathway is so exquisitely sensitive to our active compounds is also suggestive that such compounds may play a catalytic role in the disruption of the pathway. Dihydrogeldanamycin (**20**) was found to be active in our assays, albeit slightly less so than geldanamycin itself. The former has been reported to be air-oxidizable to the latter,[36] and thus this cannot be discounted as a possible contributing factor to dihydrogeldanamycin's observed activity. However, the related ansamycins macbecin I (**2**) and its reduction product macbecin II (**21**) were both observed to be inactive. Both of these latter compounds bind hsp90.[27,15]

It may be that the active ansamycin derivatives participate in a catalytic electron-transfer redox process and that the oxidation–reduction potential between dihydrogeldanamycin (**20**) and geldanamycin is critical for it to be able to do so. The potential between the two macbecins may be sufficiently different not to allow this. It has been reported that geldanamycin can participate in the generation of superoxide via a redox process.[46]

The low concentrations we have found that are needed to arrest the Met signaling responsible for the invasive and metastatic behavior of solid tumors makes the active compounds of our cell-based assays attractive as potential drugs. The low concentrations needed may eliminate the observed dose-dependent toxicities of geldanamycin derivatives. Successful identification and isolation of the unknown targets of such derivatives may allow a better design of compounds that may be efficacious in blocking this Met signaling pathway.

4. Conclusions

Geldanamycin and some 17-amino-17-demethoxygeldanamycin derivatives were found to block hepatocyte growth factor/scatter factor-mediated Met tyrosine kinase receptor-dependent urokinase-plasmin activation and scattering at femtomolar levels. In contrast, other ansamycins (macbecins I and II), geldanamycin derivatives, and radicicol required concentrations several logs higher (greater than nanomolar) to effect such inhibition. Additionally, the ability of tested compounds to effect this inhibition was discordant with the known ability to bind hsp90. These results are indicative of a novel target(s) for hepatocyte growth factor/scatter factor-mediated urokinase-plasmin activation.

5. Experimental

5.1. General methods

Melting points are uncorrected. Infrared spectra were recorded on a Matton Galaxy Series FTIR 3000 spectrophotometer. Ultraviolet–visible spectra were recorded on a Hitachi U-4001 spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded on Varian Inova-600, UnityPlus-500, VRX-500, or VRX-300 spectrometers. The numbering used in assignments is based on the geldanamycin ring system unless otherwise indicated. Mass spectra were performed by the MSU Mass Spectrometry Facility. Geldanamycin and macbecin II were provided by the National Cancer Institutes. Macbecin I was synthesized from macbecin II as per published procedure.[47] Radicicol was obtained commercially (Sigma-Aldrich). Anhydrous solvents were purified as per standard methods.

5.2. 17-Allylamino-17-demethoxygeldanamycin (4)[35,36]

(+)-Geldanamycin (5.1 mg, 9.0 μmol) was stirred with allylamine (10.0 μl, 0.13 mmol) in chloroform (1.5 ml) at room temperature. Upon complete conversion of geldanamycin shown by thin-layer chromatography (18 h), the mixture was washed with brine, dried over anhydrous sodium sulfate, and concentrated. Separation by flash chromatography on silica gel (1:2 hexane/ethyl acetate) gave the product as a purple solid (5.3 mg, 99%). IR (KBr) (cm^{-1}) 3464, 3333, 2958, 2929, 2825, 1728, 1691, 1652, 1571, 1485, 1372, 1323, 1189, 1101, 1057; UV (95% EtOH) (nm) 332 ($\varepsilon = 2.0 \times 10^4$); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 9.14 (s, 1H), 7.28 (s, 1H), 6.93 (br d, $J = 11.5$ Hz, 1H), 6.56 (br dd, $J = 11.5$, 11.0 Hz, 1H), 6.38 (br t, $J = 6.0$ Hz, 1H), 5.94–5.81 (m, 3H), 5.30–5.24 (m, 2H), 5.17 (s, 1H), 4.82 (br s, 2H), 4.29 (br d, $J = 10.0$ Hz, 1H), 4.21 (br s, 1H), 4.18–4.08 (m, 2H), 3.55 (ddd, $J = 9.0$, 6.5, 2.0 Hz, 1H), 3.43 (ddd, $J = 9.0$, 3.0, 3.0 Hz, 1H), 3.34 (s, 3H), 3.25 (s, 3H), 2.72 (dqd, $J = 9.5$, 7.0, 2.0 Hz, 1H), 2.63 (d, $J = 14.0$ Hz, 1H), 2.34 (dd, $J = 14.0$, 11.0 Hz, 1H), 2.00 (br s, 3H), 1.78 (d, $J = 1.0$ Hz, 3H), 1.78–1.74 (m, 2H), 1.74–1.67 (m, 1H), 0.99–0.95 (m, 6H); ^{13}C NMR ($CDCl_3$, 125 MHz, assignment of protonated carbons aided by DEPT) δ 183.8 (18-C), 180.9 (21-C), 168.4 (1-C), 156.0 (7-O_2CNH_2), 144.6 (17-C), 141.2 (20-C), 135.8 (5-C), 134.9 (2-C), 133.7 (9-C), 132.7 (8-C), 132.5 (2′-C), 126.9 (4-C), 126.5 (3-C), 118.5 (3′-C), 108.8 (19-C), 108.7 (16-C), 81.6 (7-C), 81.4 (12-C), 81.2 (6-C), 72.6 (11-C), 57.1 (6- or 12-OCH_3), 56.7 (6- or 12-OCH_3), 47.8 (1′-C), 35.0 (13-C), 34.3 (15-C), 32.3 (10-C), 28.4 (14-C), 22.9 (14-CH_3), 12.8 (8-CH_3), 12.6 (2-CH_3), 12.3 (10-CH_3); HRMS (FAB) $[M+H]^+$ calcd for $C_{31}H_{44}N_3O_8$, 586.3129; found 586.3120.

5.3. 17-(2-Dimethylaminoethyl)amino-17-demethoxygeldanamycin (5)[48]

N,*N*-Dimethylethylenediamine (6.0 μl, 0.055 mmol) was added to a solution of (+)-geldanamycin (4.3 mg, 7.7 μmol) in chloroform (1.0 ml). The mixture was stirred at room temperature. Upon complete conversion of geldanamycin shown by thin-layer chromatography (4 h), the mixture was washed with 0.5% aqueous sodium hydroxide solution and brine, dried over anhydrous sodium sulfate, and concentrated. Separation by flash chromatography on silica gel (9:1 ethyl acetate/methanol) gave the product as a purple solid (4.5 mg, 95%). IR (KBr) (cm^{-1}) 3462, 3329, 2932, 2871, 2824, 2774, 1733, 1690, 1653, 1565, 1485, 1373, 1321, 1253, 1188, 1100, 1055; UV (95% EtOH) (nm) 332 ($\varepsilon = 1.7 \times 10^4$); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 9.18 (s, 1H), 7.24 (s, 1H), 7.04 (br t, $J = 5.0$ Hz, 1H), 6.94 (br d, $J = 11.5$ Hz, 1H), 6.57 (br dd, $J = 11.5$, 11.0 Hz, 1H), 5.90 (br d, $J = 9.5$ Hz, 1H), 5.84 (dd, $J = 11.0$, 10.0 Hz, 1H), 5.17 (s, 1H), 4.75 (br s, 2H), 4.42 (br s, 1H), 4.29 (br d, $J = 10.0$ Hz, 1H), 3.70–3.42 (m, 3H), 3.57 (br dd, $J = 9.0$, 6.5 Hz, 1H), 3.34 (s, 3H), 3.25 (s, 3H), 2.72 (dqd, $J = 9.5$, 7.0, 2.0 Hz, 1H), 2.67 (d, $J = 14.0$ Hz, 1H), 2.55 (t, $J = 5.5$ Hz, 2H), 2.38 (dd, $J = 14.0$, 11.0 Hz, 1H), 2.25 (s, 6H), 2.01 (br s, 3H), 1.83–1.68 (m, 3H), 1.78 (br s, 3H), 0.98 (d, $J = 7.0$ Hz, 3H), 0.95 (d, $J = 6.5$ Hz, 3H); MS (FAB) $[M+H]^+$ found 617.

5.4. 17-Amino-17-demethoxygeldanamycin (6)[36,49,50]

Concentrated aqueous solution of ammonia (28%, 0.70 ml, 0.010 mol) was added to a solution of (+)-geldanamycin (5.0 mg, 9.0 μmol) in acetonitrile (5.0 ml) at room temperature. The yellow solution turned slowly dark red. Upon complete conversion of geldanamycin shown by thin-layer chromatography (5 h), the mixture was partitioned between ethyl acetate and brine. The organic phase was washed with brine, dried over anhydrous sodium sulfate, and concentrated. Separation of the solid residue by flash chromatography on silica gel (1:2 hexane/ethyl acetate) gave the product as a dark red solid (4.6 mg, 95%). IR (KBr) (cm^{-1}) 3452, 3339, 2957, 2931, 2825, 1721, 1692, 1617, 1591, 1495, 1374, 1323, 1250, 1190, 1133, 1101, 1055; UV (95% EtOH) (nm) 328 ($\varepsilon = 2.0 \times 10^4$); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 9.08 (s, 1H), 7.26 (s, 1H), 6.95 (br d, *J* = 11.5 Hz, 1H), 6.56 (br dd, *J* = 11.5, 11.0 Hz, 1H), 5.89–5.82 (m, 2H), 5.37 (br s, 2H), 5.17 (s, 1H), 4.73 (br s, 2H), 4.29 (br d, *J* = 10.0 Hz, 1H), 3.98 (br s, 1H), 3.59 (ddd, *J* = 9.0, 6.5, 2.0 Hz, 1H), 3.42 (ddd, *J* = 9.0, 3.0, 3.0 Hz, 1H), 3.34 (s, 3H), 3.25 (s, 3H), 2.75 (dqd, *J* = 9.5, 7.0, 2.0 Hz, 1H), 2.65 (d, *J* = 14.0 Hz, 1H), 2.01 (br s, 3H), 1.97–1.75 (m, 4H), 1.79 (d, *J* = 1.0 Hz, 3H), 0.99–0.97 (m, 6H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 183.1, 180.4, 167.9, 156.1, 146.0, 140.4, 135.8, 135.0, 134.0, 133.0, 126.9, 126.6, 110.3, 108.6, 81.9, 81.2, 81.1, 72.2, 57.1, 56.8, 35.0, 34.7, 32.2, 28.7, 23.8, 12.8, 12.5, 12.2; HRMS (FAB) $[M+H]^+$ calcd for $C_{28}H_{40}N_3O_8$, 546.2816; found 546.2818.

5.5. 17-(2-Chloroethyl)amino-17-demethoxygeldanamycin (7)[50]

Sodium hydroxide aqueous solution (2.80 M, 0.75 ml, 2.1 mmol) was added to a mixture of (+)-geldanamycin (11.7 mg, 0.021 mmol) and 2-chloroethylamine hydrochloride (242 mg, 2.1 mmol) in acetonitrile (3.0 ml). The mixture was stirred at room temperature. Upon complete conversion of geldanamycin shown by thin-layer chromatography (20 h), the mixture was partitioned between ethyl acetate and brine. The organic phase was washed with brine, dried over anhydrous sodium sulfate, and concentrated. Separation by flash chromatography on silica gel (1:2 hexane/ethyl acetate) gave the product as a purple solid (12.0 mg, 95%). IR (KBr) (cm^{-1}) 3334, 2938, 2874, 2822, 1733, 1696, 1653, 1577, 1489, 1375, 1325, 1274, 1190, 1136, 1101, 1060; UV (95% EtOH) (nm) 332 ($\varepsilon = 1.9 \times 10^4$); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 9.09 (s, 1H), 7.29 (s, 1H), 6.94 (br d, *J* = 11.5 Hz, 1H), 6.56 (ddd, *J* = 11.5, 11.0, 1.0 Hz, 1H), 6.35 (br t, *J* = 5.0 Hz, 1H), 5.87 (br d, *J* = 9.5 Hz, 1H), 5.85 (br dd, *J* = 11.0, 10.0 Hz, 1H), 5.18 (s, 1H), 4.72 (br s, 2H), 4.30 (br d, *J* = 10.0 Hz, 1H), 4.03 (br s, 1H), 3.94–3.83 (m, 2H), 3.75–3.67 (m, 2H), 3.56 (ddd, *J* = 9.0, 6.5, 2.0 Hz, 1H), 3.43 (ddd, *J* = 9.0, 3.0, 3.0 Hz, 1H), 3.35 (s, 3H), 3.26 (s, 3H), 2.73 (dqd, *J* = 9.5, 7.0, 2.0 Hz, 1H), 2.70 (d, *J* = 14.0 Hz, 1H), 2.24 (dd, *J* = 14.0, 11.0 Hz, 1H), 2.01 (br s, 3H), 1.78 (d, *J* = 1.0 Hz, 3H), 1.80–1.75 (m, 2H), 1.75–1.68 (m, 1H), 1.00–0.96 (m, 6H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 183.8, 181.2, 168.3, 155.9, 144.7, 140.8, 135.9, 135.0, 133.6, 132.9, 127.0, 126.5, 110.0, 109.1, 81.6, 81.4, 81.2, 72.7, 57.1, 56.7, 46.9, 42.7, 35.1, 34.4, 32.4, 28.8, 23.0, 12.8, 12.6, 12.5; MS (FAB) $[M+H]^+$ found 608.

5.6. 17-(2-Fluoroethyl)amino-17-demethoxygeldanamycin (8)[36]

Sodium hydroxide aqueous solution (1.10 M, 0.53 ml, 0.58 mmol) was added to a mixture of (+)-geldanamycin (5.5 mg, 9.8 μmol) and 2-fluoroethylamine hydrochloride (65 mg, 0.59 mmol) in acetonitrile (1.0 ml). The mixture was stirred at room temperature. Upon complete conversion of geldanamycin shown by thin-layer chromatography (12 h), the mixture was partitioned between ethyl acetate and brine. The organic phase was washed with brine, dried over anhydrous sodium sulfate, and concentrated. Separation by flash chromatography on silica gel (1:2 hexane/ethyl acetate) gave the product as a purple solid (5.7 mg, 98%). IR (KBr) (cm^{-1}) 3465, 3330, 2954, 2927, 2873, 1728, 1691, 1653, 1576, 1487, 1375, 1323, 1255, 1190, 1103, 1051; UV (95% EtOH) (nm) 332 ($\varepsilon = 1.7 \times 10^4$); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 9.10 (s, 1H), 7.29 (s, 1H), 6.94 (br d, *J* = 11.5 Hz, 1H), 6.57 (br dd, *J* = 11.5, 11.0 Hz, 1H), 6.36 (br t, *J* = 5.0 Hz, 1H), 5.88–5.83 (m, 2H), 5.18 (s, 1H), 4.75 (br s, 2H), 4.69–4.57 (m, 2H), 4.30 (br d, *J* = 10.0 Hz, 1H), 3.94–3.76 (m, 2H), 3.56 (br d, *J* = 9.0 Hz, 1H), 3.43 (ddd, *J* = 9.0, 3.0, 3.0 Hz, 1H), 3.35 (s, 3H), 3.26 (s, 3H), 2.73 (dqd, *J* = 9.5, 7.0, 2.0 Hz, 1H), 2.70 (d, *J* = 14.0 Hz, 1H), 2.30 (dd, *J* = 14.0, 11.0 Hz, 1H), 2.01 (br s, 3H), 1.80–1.76 (m, 2H), 1.78 (d, *J* = 1.0 Hz, 3H), 1.75–1.68 (m, 1H), 0.99 (d, *J* = 7.0 Hz, 3H), 0.97 (d, *J* = 6.5 Hz, 3H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 183.8, 181.2, 168.4, 156.0, 144.9, 140.9, 135.9, 135.0, 133.6, 132.8, 127.0, 126.5, 109.7, 109.1, 81.6, 81.5 (d, *J* = 170 Hz), 81.4, 81.2, 72.6, 57.2, 56.7, 46.0 (d, *J* = 20 Hz), 35.1, 34.3, 32.4, 28.8, 23.0, 12.8, 12.6, 12.5; HRMS (FAB) $[M]^+$ calcd for $C_{30}H_{42}FN_3O_8$, 591.2956; found 591.2952.

5.7. 17-(2-Acetylaminoethyl)amino-17-demethoxygeldanamycin (9)[36]

N-Acetylethylenediamine (90%, 10.0 μl, 0.094 mmol) was added to a solution of (+)-geldanamycin (5.0 mg, 8.9 μmol) in chloroform (1.0 ml) at room temperature. Upon complete conversion of geldanamycin shown by thin-layer chromatography (10 h), the mixture was washed with distilled water, dried over anhydrous sodium sulfate, and concentrated. Separation by flash chromatography on silica gel (ethyl acetate) gave the product as a purple solid (4.5 mg, 80%). IR (KBr) (cm^{-1}) 3449, 3338, 2932, 2881, 2824, 1718, 1685, 1654, 1569, 1487, 1374, 1323, 1269, 1189, 1102, 1057; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 9.12 (s, 1H), 7.23 (s, 1H), 6.94 (br d, *J* = 11.5 Hz, 1H), 6.63 (br t, *J* = 5.0 Hz, 1H), 6.56 (br dd, *J* = 11.5, 11.0 Hz, 1H), 5.88 (br d, *J* = 9.5 Hz, 1H), 5.84 (dd, *J* = 11.0, 10.0 Hz, 1H), 5.80 (br t, *J* = 6.0 Hz, 1H), 5.17 (s, 1H), 4.72 (br s, 2H), 4.29 (br d, *J* = 10.0 Hz, 1H), 4.17 (br s, 1H), 3.77–3.62 (m, 2H), 3.58–3.46 (m, 3H), 3.42 (ddd, *J* = 9.0, 3.0, 3.0 Hz, 1H), 3.34 (s, 3H), 3.25 (s, 3H), 2.73 (dqd, *J* = 9.5, 7.0, 2.0 Hz, 1H), 2.64 (d, *J* = 14.0 Hz, 1H), 2.33 (dd, *J* = 14.0, 11.0 Hz, 1H), 2.01 (s, 3H), 2.00 (d, *J* = 1.0 Hz, 3H), 1.80–1.76 (m, 2H), 1.78 (d, *J* = 1.0 Hz, 3H), 1.74–1.67 (m, 1H), 0.98 (d, *J* = 7.0 Hz, 3H), 0.95 (d, *J* = 6.5 Hz, 3H); HRMS (FAB)

$[M+H]^+$ calcd for $C_{32}H_{47}N_4O_9$, 631.3343; found 631.3344.

5.8. 17-(6-Acetylamino-1-hexyl)amino-17-demethoxygeldanamycin (10)

A solution of (+)-geldanamycin (5.7 mg, 0.010 mmol) and *N*-(6-aminohexyl)acetamide[51] (5.5 mg, 0.035 mmol) in chloroform was stirred at room temperature. Upon complete conversion of geldanamycin shown by thin-layer chromatography (20 h), the mixture was washed with distilled water, dried over anhydrous sodium sulfate, and concentrated. Separation by flash chromatography on silica gel (ethyl acetate) gave the product as a purple solid (5.7 mg, 82%). IR (KBr) (cm^{-1}) 3445, 3323, 3202, 2931, 2865, 2824, 1723, 1687, 1653, 1562, 1486, 1371, 1322, 1256, 1188, 1135, 1106; UV (95% EtOH) (nm) 333 ($\varepsilon = 1.2 \times 10^4$); ^1H NMR ($CDCl_3$, 500 MHz) δ 9.17 (br s, 1H), 7.26 (s, 1H), 6.94 (br d, J = 11.5 Hz, 1H), 6.57 (br dd, J = 11.5, 11.0 Hz, 1H), 6.26 (br t, J = 5.0 Hz, 1H), 5.89 (br d, J = 9.5 Hz, 1H), 5.85 (dd, J = 11.0, 10.0 Hz, 1H), 5.42 (br s, 1H), 5.18 (s, 1H), 4.73 (br s, 2H), 4.31 (br s, 1H), 4.29 (br d, J = 10.0 Hz, 1H), 3.59–3.39 (m, 4H), 3.35 (s, 3H), 3.27–3.19 (m, 2H), 3.25 (s, 3H), 2.74 (dqd, J = 9.5, 7.0, 2.0 Hz, 1H), 2.66 (d, J = 14.0 Hz, 1H), 2.39 (dd, J =14.0, 11.0 Hz, 1H), 2.01 (br s, 3H), 1.96 (s, 3H), 1.80–1.75 (m, 2H), 1.78 (d, J = 1.0 Hz, 3H), 1.73–1.62 (m, 3H), 1.55–1.47 (m, 2H), 1.46–1.33 (m, 4H), 0.99 (d, J = 7.0 Hz, 3H), 0.95 (d, J = 6.5 Hz, 3H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 183.9, 180.7, 170.0, 168.4, 156.0, 144.9, 141.5, 135.9, 135.0, 133.8, 132.8, 127.0, 126.6, 108.7, 108.4, 81.7, 81.5, 81.2, 72.7, 57.2, 56.7, 45.8, 39.4, 35.1, 34.4, 32.4, 29.7, 29.6, 28.6, 26.5, 26.4, 23.4, 22.9, 12.8, 12.6, 12.4; HRMS (FAB) $[M+H]^+$ calcd for $C_{36}H_{55}N_4O_9$, 687.3969; found 687.3967.

5.9. (+)-Biotin 17-(6-aminohexyl)amino-17-demethoxygeldanamycin amide (11)

1,6-Diaminohexane (10.0 mg, 0.086 mmol) was added to a solution of (+)-geldanamycin (5.0 mg, 8.9 μmol) in chloroform (1.0 ml) at room temperature. Upon complete conversion of geldanamycin shown by thin-layer chromatography (20 h), the mixture was washed with 0.5% aqueous sodium hydroxide solution and brine, dried over potassium carbonate, and concentrated. The resulted dark purple solid was then stirred overnight with (+)-biotin *N*-hydroxysuccinimide ester (3.0 mg, 8.8 μmol) in DMF (1.0 ml). Removal of the solvent and separation by flash chromatography on silica gel (9:1 ethyl acetate/methanol) gave the product as a purple solid (6.5 mg, 85%). IR (KBr) (cm^{-1}) 3327, 2931, 2864, 1709, 1651, 1562, 1485, 1371, 1325, 1255, 1099, 731; ^1H NMR ($CDCl_3$, 500 MHz) δ 9.19 (s, 1H), 7.24 (s, 1H), 6.94 (br d, J = 11.5 Hz, 1H), 6.56 (br dd, J = 11.5, 11.0 Hz, 1H), 6.28 (br t, J = 5.0 Hz, 1H), 5.87 (br d, J = 9.5 Hz, 1H), 5.84 (dd, J = 11.0, 10.0 Hz, 1H), 5.88–5.77 (m, 2H), 5.17 (s, 1H), 5.15 (br s, 1H), 4.87 (br s, 2H), 4.50 (dd, J = 7.5, 5.0 Hz, 1H), 4.32–4.29 (m, 2H), 4.23 (br s, 1H), 3.58–3.41 (m, 4H), 3.34 (s, 3H), 3.26 (s, 3H), 3.24–3.20 (m, 2H), 3.17–3.12 (m, 1H), 2.91 (dd, J = 13.0, 5.0 Hz, 1H), 2.75–2.69 (m, 2H), 2.66 (d, J = 14.0 Hz, 1H), 2.38 (dd, J = 14.0, 11.0 Hz, 1H), 2.21–2.15 (m, 2H), 2.01 (br s, 3H), 1.78 (d, J = 1.0 Hz, 3H), 1.78–1.32 (m, 17H), 0.98 (d, J = 7.0 Hz, 3H), 0.95 (d, J = 6.5 Hz, 3H); HRMS (FAB) $[M+H]^+$, calcd for $C_{44}H_{67}N_6O_{10}S$, 871.4592; found 871.4619.

5.10. 17-[2-[2-(2-Acetylaminoethoxy)ethoxy]ethyl]amino-17-demethoxygeldanamycin (12)

A mixture of 2,2′-(ethylenedioxy)bis(ethylamine) (56.0 μl, 0.38 mmol), acetic anhydride (46.0 μl, 0.48 mmol), and triethylamine (73.2 μl, 0.52 mmol) in chloroform (1.0 ml) was stirred for 1 h at room temperature and then concentrated to dryness under high vacuum. The colorless solid residue was then stirred with (+)-geldanamycin (4.0 mg, 7.1 μmol) in chloroform (1.0 ml). Upon complete conversion of geldanamycin shown by thin-layer chromatography (20 h), the mixture was washed with distilled water, dried over anhydrous sodium sulfate, and concentrated. Separation by flash chromatography on silica gel (5:1 ethyl acetate/methanol) gave the desired product as a purple solid (1.1 mg, 21%). IR (KBr) (cm^{-1}) 3446, 3336, 2960, 2929, 2877, 1727, 1689, 1655, 1566, 1487, 1375, 1325, 1261, 1190, 1103, 1057; ^1H NMR ($CDCl_3$, 300 MHz) δ 9.17 (s, 1H), 7.25 (s, 1H), 6.94 (br d, J = 11.5 Hz, 1H), 6.78 (br t, J = 5.0 Hz, 1H), 6.57 (br dd, J = 11.5, 11.0 Hz, 1H), 6.36 (br s, 1H), 5.89 (br d, J = 9.5 Hz, 1H), 5.85 (dd, J = 11.0, 10.0 Hz, 1H), 5.18 (s, 1H), 4.74 (br s, 2H), 4.29 (br d, J = 10.0 Hz, 1H), 4.26 (br s, 1H), 3.78–3.40 (m, 14H), 3.35 (s, 3H), 3.25 (s, 3H), 2.78–2.64 (m, 2H), 2.39 (dd, J = 14.0, 11.0 Hz, 1H), 2.01 (br s, 3H), 1.98 (s, 3H), 1.78–1.67 (m, 3H), 1.78 (d, J = 1.0 Hz, 3H), 0.99–0.94 (m, 6H); HRMS (FAB) $[M+H]^+$ calcd for $C_{36}H_{55}N_4O_{11}$, 719.3867; found 719.3864.

5.11. 17-Carboxymethylamino-17-demethoxygeldanamycin (13)

(+)-Geldanamycin (3.1 mg, 5.5 μmol) was stirred at room temperature with glycine sodium salt (10.7 mg, 0.11 mmol) in a mixture of ethanol (1.2 ml) and water (0.3 ml). Upon complete conversion of geldanamycin shown by thin-layer chromatography (3 h), the purple mixture was acidified with diluted hydrochloric acid and partitioned between chloroform and distilled water. The organic phase was dried over anhydrous sodium sulfate and concentrated. Separation by flash chromatography on silica gel (1:1 ethyl acetate/methanol) gave the product as a purple solid (3.2 mg, 96%). IR (KBr) (cm^{-1}) 3446, 3305, 2929, 2875, 1734, 1693, 1655, 1618, 1574, 1485, 1394, 1319, 1267, 1139, 1072; ^1H NMR ($CDCl_3$, 500 MHz) δ 8.91 (s, 1H), 7.25 (s, 1H), 6.83 (br s, 1H), 6.80 (br d, J = 11.5 Hz, 1H), 6.60 (br dd, J = 11.5, 11.0 Hz, 1H), 5.86–5.80 (m, 2H), 5.16 (s, 1H), 4.95 (br s, 2H), 4.33–4.21 (m, 2H), 4.27 (br d, J = 10.0 Hz, 1H), 3.54 (dd, J = 9.0, 2.0 Hz, 1H), 3.42 (ddd, J = 9.0, 3.0, 3.0 Hz, 1H), 3.33 (s, 3H), 3.25 (s, 3H), 2.70 (dqd, J = 9.5, 7.0, 2.0 Hz, 1H), 2.59 (d, J = 14.0 Hz, 1H), 2.27 (dd, J = 14.0, 11.0 Hz, 1H), 2.07 (br s, 3H), 1.80–1.75 (m, 2H), 1.62–1.54 (m, 1H), 1.77 (br s, 3H), 0.98 (d, J = 7.0 Hz, 3H), 0.91 (d,

$J = 6.5$ Hz, 3H); HRMS (FAB) $[M+H]^+$ calcd for $C_{30}H_{42}N_3O_{10}$, 604.2870; found 604.2867.

5.12. 17-(1-Azetidinyl)-17-demethoxygeldanamycin (14)[52]

Azetidine (4.0 µl, 0.059 mmol) was added to a solution of (+)-geldanamycin (7.5 mg, 0.013 mmol) in dichloromethane (1.5 ml) with stirring. Upon complete conversion of geldanamycin shown by thin-layer chromatography (40 min), the mixture was washed with brine, dried over anhydrous sodium sulfate, and concentrated. Separation by flash chromatography on silica gel (1:2 hexane/ethyl acetate) gave the product as a deep purple solid (7.7 mg, 98%). IR (in CH_2Cl_2) (cm^{-1}) 3422, 3075, 3049, 2986, 1733, 1686, 1651, 1605, 1540, 1486, 1420, 1375, 1283, 1260, 1103, 1047; ^1H NMR ($CDCl_3$, 500 MHz) δ 9.16 (s, 1H), 7.10 (s, 1H), 6.92 (br d, $J = 11.5$ Hz, 1H), 6.56 (br dd, $J = 11.5$, 11.0 Hz, 1H), 5.92 (br d, $J = 9.5$ Hz, 1H), 5.82 (dd, $J = 11.0$, 10.0 Hz, 1H), 5.15 (s, 1H), 4.79 (br s, 2H), 4.72–4.58 (m, 4H), 4.28 (br d, $J = 10.0$ Hz, 1H), 3.54 (br d, $J = 9.0$ Hz, 1H), 3.43 (ddd, $J = 9.0$, 3.0, 3.0 Hz, 1H), 3.34 (s, 3H), 3.24 (s, 3H), 2.71 (dqd, $J = 9.5$, 7.0, 2.0 Hz, 1H), 2.59 (d, $J = 14.0$ Hz, 1H), 2.42 (quintet, $J = 8.0$ Hz, 2H), 2.23 (dd, $J = 14.0$, 11.0 Hz, 1H), 2.00 (br s, 3H), 1.78 (br s, 3H), 1.77–1.73 (m, 2H), 1.69–1.62 (m, 1H), 0.97 (d, $J = 7.0$ Hz, 3H), 0.94 (d, $J = 6.5$ Hz, 3H); ^{13}C NMR ($CDCl_3$, 125 MHz, assignment of protonated carbons aided by DEPT and HMQC) δ 185.8 (18-C), 178.4 (21-C), 168.4 (1-C), 156.0 (7-O_2CNH_2), 145.9 (17-C), 140.5 (20-C), 135.5 (5-C), 135.1 (2-C), 134.0 (9-C), 132.6 (8-C), 126.7 (4-C), 126.6 (3-C), 109.6 (19-C), 109.2 (16-C), 81.8 (7-C), 81.6 (12-C), 81.3 (6-C), 72.5 (11-C), 58.9 (1′- and 3′-C), 57.1 (6- or 12-OCH_3), 56.7 (6- or 12-OCH_3), 35.1 (13-C), 34.1 (15-C), 32.3 (10-C), 28.1 (14-C), 22.9 (14-CH_3), 18.4 (2′-C), 12.7 (8-CH_3), 12.6 (2-CH_3), 12.2 (10-CH_3); MS (FAB) $[M+H]^+$ found 586.

5.13. 17-(1-Aziridinyl)-17-demethoxygeldanamycin (15)

Aziridine[53] (0.30 ml, 5.80 mmol) was added to a solution of (+)-geldanamycin (5.8 mg, 0.010 mmol) in dichloromethane (2.0 ml). The mixture was stirred at room temperature. Upon complete conversion of geldanamycin shown by thin-layer chromatography (25 min), the mixture was washed with brine, dried over anhydrous sodium sulfate, and concentrated. Separation by flash chromatography on silica gel (1:2 hexane/ethyl acetate) gave the product as an orange solid (5.6 mg, 95%). IR (KBr) (cm^{-1}) 3438, 3338, 3192, 2925, 2827, 1736, 1701, 1687, 1644, 1585, 1517, 1457, 1367, 1272, 1192, 1112; ^1H NMR ($CDCl_3$, 500 MHz) δ 8.77 (s, 1H) 7.27 (s, 1H), 6.91 (br d, $J = 11.5$ Hz, 1H), 6.55 (br dd, $J = 11.5$, 11.0 Hz, 1H), 5.86–5.80 (m, 2H), 5.17 (s, 1H), 4.80 (br s, 2H), 4.30 (br d, $J = 10.0$ Hz, 1H), 3.52 (ddd, $J = 9.0$, 6.5, 2.0 Hz, 1H), 3.42–3.37 (m, 2H), 3.34 (s, 3H), 3.27 (s, 3H), 2.73 (dqd, $J = 9.5$, 7.0, 2.0 Hz, 1H), 2.57 (d, $J = 14.0$ Hz, 1H), 2.50 (dd, $J = 14.0$, 11.0 Hz, 1H), 2.44–2.33 (m, 4H), 2.00 (br s, 3H), 1.80–1.76 (m, 2H), 1.77 (br s, 3H), 1.75–1.69 (m, 1H), 0.99–0.96 (m, 6H); ^{13}C NMR ($CDCl_3$, 125 MHz, assignment of protonated carbons aided by DEPT) δ 183.8 (18-C), 183.2 (21-C), 168.3 (1-C), 156.0 (7-O_2CNH_2), 152.7 (17-C), 138.8 (20-C), 136.1 (5-C), 134.9 (2-C), 133.3 (9-C), 133.1 (8-C), 127.0 (4-C), 126.4 (3-C), 125.4 (16-C), 111.6 (19-C), 81.6 (7-C), 81.1 (12-C), 81.1 (6-C), 72.7 (11-C), 57.2 (6- or 12-OCH_3), 56.7 (6- or 12-OCH_3), 35.1 (13-C), 33.6 (15-C), 32.3 (10-C), 29.2 (17-NCH_2), 28.9 (14-C), 23.3 (14-CH_3), 12.9 (8-CH_3), 12.5 (2-CH_3), 12.4 (10-CH_3); HRMS (FAB) $[M+H]^+$ calcd for $C_{30}H_{42}N_3O_8$, 572.2926; found 572.2968.

5.14. 3-Bromo-4-nitrophenol and 3-bromo-6-nitrophenol

Fuming nitric acid (3.8 ml, 89 mmol) in glacial acetic acid (12 ml) was added for over 35 min to a solution of 3-bromophenol (15.2 g, 87.9 mmol) in glacial acetic acid (60 ml) in a flask with a surrounding ice bath. The reaction mixture was stirred at room temperature for an additional 30 min, poured on ice, and then concentrated in vacuo. Medium pressure chromatography on silica gel (1:2 ethyl acetate/hexanes) allowed separation of products 3-bromo-4-nitrophenol (3.47 g, 15.9 mmol, 18% yield); mp 130–131 °C, following recrystallization from ether/hexanes (reported mp 130–131 °C[54] and 131 °C[55]); ^1H NMR (DMSO-d_6, 500 MHz) δ 7.99 (d, 1H, $J = 9$ Hz), 7.18 (d, 1H, $J = 3$ Hz), 6.91 (dd, 1H, $J = 9$, 3 Hz); and 3-bromo-6-nitrophenol (1.94 g, 8.90 mmol, 10% yield, following recrystallization from ether/hexanes); mp 41.5–42.5 °C (reported mp 42–45 °C[56] and 42 °C[55]); ^1H NMR ($CDCl_3$, 500 MHz) δ 10.60 (s, 1H), 7.95 (d, 1H, $J = 9$ Hz), 7.35 (d, 1H, $J = 2$ Hz), 7.11 (dd, 1H, $J = 9$, 2 Hz); ^{13}C NMR ($CDCl_3$; assignments aided by HMQC) δ 122.9 (C-2), 123.8 (C-4), 126.0 (C-5), 132.2 (C-3), 132.7 (C-6), 155.2 (C-1); IR (KBr) (cm^{-1}) 3450 (broad), 1612, 1578, 1527, 1475, 1311, 1235, 1186, 900.

5.15. 2-Amino-5-bromophenol

3-Bromo-6-nitrophenol (0.292 g, 1.34 mmol) was stirred in a 0.5% aqueous sodium hydroxide solution (30 ml). Sodium hydrosulfite (2.00 g of 85%, 9.76 mmol) was added to the reaction flask and this solution was stirred at room temperature for 15 min. The reaction flask was then acidified with diluted hydrochloric acid until a pH of 5 was obtained. The reaction mixture was then extracted with diethyl ether (3× 40 ml), the combined organic layers were dried over anhydrous sodium sulfate and concentrated to provide crude 2-amino-5-bromophenol, which was recrystallized from ethyl ether/hexanes to provide the pure product (0.151 g, 0.80 mmol, 60% yield); mp 125–127 °C (dec.) (reported mp 149.5–150.5 °C[57]); ^1H NMR (CD_3CN, 500 MHz) δ 7.08 (br s, 1H), 6.82 (d, 1H, $J = 2$ Hz), 6.78 (dd, 1H, $J = 8$, 2 Hz), 6.56 (d, 1H, $J = 8$ Hz), 4.03 (br s, 2H); IR (KBr) (cm^{-1}) 3496 (broad), 3377, 3298, 1598, 1502, 1431, 1269, 1210, 916, 877.

5.16. 5′-Bromogeldanoxazinone (16)[37,14]

A mixture of (+)-geldanamycin (21.8 mg, 0.039 mmol) and 2-amino-5-bromophenol (14.6 mg, 0.078 mmol) in glacial acetic acid (2.0 ml) was stirred at 78 °C under nitrogen for 19 h, then cooled, and concentrated. Separation of the deep orange residue by flash chromatography on silica gel (hexane:ethyl acetate) gave a crude product contaminated with unreacted (+)-geldanamycin. This solution was then dissolved in chloroform and subjected to preparative HPLC separation (Waters Nova-Pak Silica 6 μm 7.8 × 300 mm column, 2.0 ml/min, 2:3 $CHCl_3$/EtOAc) to afford the product as a bright orange powder (16.4 mg, 60% yield); mp 274–278 °C (dec.) (lit.[37] mp 275–278 °C). IR (KBr) (cm^{-1}) 3442, 3342, 3209, 2954, 2926, 2878, 1734, 1700, 1615, 1583, 1507, 1384, 1314, 1192, 1111, 1061 (lit.[37] 1605, 1585, 1505); ^1H NMR ($CDCl_3$, 500 MHz) δ 9.13 (br s, 1H), 8.33 (s, 1H), 7.73 (d, $J = 8.5$ Hz, 1H), 7.60 (d, $J = 2.0$ Hz, 1H), 7.53 (dd, $J = 8.5$, 2.0 Hz, 1H), 7.03 (br d, $J = 11.5$ Hz, 1H), 6.60 (br dd, $J = 11.5$, 11.0 Hz, 1H), 5.96 (br d, $J = 9.5$ Hz, 1H), 5.86 (dd, $J = 11.0$, 10.0 Hz, 1H), 5.21 (s, 1H), 4.72 (br s, 2H), 4.35 (br d, $J = 10.0$ Hz, 1H), 4.25 (br s, 1H), 3.64 (br dd, $J = 9.0$, 6.5 Hz, 1H), 3.46 (ddd, $J = 9.0$, 3.0, 3.0 Hz, 1H), 3.37 (s, 3H), 3.27 (s, 3H), 2.82–2.71 (m, 3H), 2.08 (br s, 3H), 1.98–1.86 (m, 2H), 1.85–1.77 (m, 1H), 1.81 (d, $J = 1.0$ Hz, 3H), 1.01 (d, $J = 7.0$ Hz, 3H), 0.99 (d, $J = 6.5$ Hz, 3H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 180.7, 168.4, 156.0, 148.5, 145.0, 143.5, 136.8, 135.5, 135.3, 133.9, 133.0, 132.9, 130.9, 129.1, 126.7, 125.2, 119.3, 117.5, 112.9, 81.9, 81.3, 81.2, 72.2, 57.1, 56.8, 35.3, 33.1, 32.2, 29.4, 27.6, 23.3, 12.8, 12.7, 12.1; HRMS (FAB) $[M+H]^+$ calcd for $C_{34}H_{41}BrN_3O_8$, 698.2077; found 698.2080.

5.17. 3-Iodo-4-nitrophenol and 3-iodo-6-nitrophenol

Fuming nitric acid (3.0 ml, 75 mmol) in glacial acetic acid (12 ml) was added for over 25 min to a solution of 3-iodophenol (15.03 g, 68.3 mmol) in glacial acetic acid (60 ml) in a flask with a surrounding ice bath. The reaction mixture was stirred at room temperature for an additional 30 min, poured on ice and then concentrated in vacuo, taken up with water (150 ml), and extracted with methylene chloride (2× 300 ml), and the combined methylene chloride layers were dried over anhydrous magnesium sulfate and evaporated to give 17 g organic residue. Medium pressure chromatography on silica gel (1:2 ethyl acetate/hexanes) allowed the separation of products 3-iodo-4-nitrophenol (6.93 g, 26.1 mmol, 38% yield); mp 121–123 °C; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.98 (d, 1H, $J = 9$ Hz), 7.54 (d, 1H, $J = 3$ Hz), 6.92 (dd, 1H, $J = 9$, 3 Hz), 5.54 (br s, 1H); IR (KBr) (cm^{-1}) 3150 (broad), 1600, 1580, 1512, 1404, 1336, 1298, 1212, 1121, 1023, 870; and 3-iodo-6-nitrophenol (3.07 g, 11.6 mmol, 17% yield); mp 92–94 °C, following recrystallization from methylene chloride/hexanes (reported mp 96 °C[58]); ^1H NMR ($CDCl_3$, 300 MHz) δ 10.53 (s, 1H), 7.76 (d, 1H, $J = 9.0$ Hz), 7.59 (d, 1H, $J = 2.0$ Hz), 7.33 (dd, 1H, $J = 9.0$, 2.0 Hz); ^{13}C NMR ($CDCl_3$; assignments aided by HMQC) δ 105.2 (C-3), 125.6 (C-5), 129.2 (C-2), 129.7 (C-4), 133.4 (C-6), 154.6 (C-1); IR (KBr) (cm^{-1}) 3430 (broad), 1604, 1571, 1518, 1463, 1317, 1225, 1172, 1055, 888; Anal. Calcd for $C_6H_4INO_3$: C, 27.19; H, 1.52; N, 5.29. Found: C, 27.36; H, 1.57; N, 5.15.

5.18. 2-Amino-5-iodophenol

3-Iodo-6-nitrophenol (0.993 g, 3.75 mmol) was stirred in an aqueous sodium hydroxide solution (0.06 M, 100 ml water). Sodium hydrosulfite (4.62 g of 85%, 22.6 mmol) was added to the reaction flask and this solution was stirred at room temperature for 40 min. The reaction flask was then cooled with a surrounding ice bath and acetic acid was added until a pH of 5–6 was obtained. The reaction mixture was then extracted with methylene chloride (3× 200 ml), the combined organic layers were dried over anhydrous magnesium sulfate and concentrated to provide crude 6-amino-3-iodophenol (0.533 g, mp 99.5–100.5 °C), which was recrystallized from ethyl ether/hexanes to provide the pure product (0.463 g, 1.97 mmol, 53% yield); mp 126–128 °C (dec.) (reported mp 141 °C[59]); ^1H NMR (CD_3CN, 500 MHz) δ 7.04 (br s, 1H), 6.97 (d, 1H, $J = 2$ Hz), 6.95 (dd, 1H, $J = 8$, 2 Hz), 6.45 (d, 1H, $J = 8$ Hz), 4.05 (br s, 2H); IR (KBr) (cm^{-1}) 3455 (broad), 3380, 3305, 1714, 1504, 1430, 1365, 1279, 1257, 1223, 890; Anal. Calcd for C_6H_6INO: C, 30.66; H, 2.57; N, 5.96. Found: C, 30.65; H, 2.42; N, 5.92.

5.19. 5′-Iodogeldanoxazinone (17)

A mixture of (+)-geldanamycin (4.8 mg, 8.6 μmol) and 2-amino-5-iodophenol (4.0 mg, 0.017 mmol) in glacial acetic acid (1.0 ml) was stirred at 78 °C under nitrogen for 20 h, then cooled, and concentrated. Separation of the deep orange residue by flash chromatography on silica gel (hexane/ethyl acetate) gave a crude product contaminated with unreacted (+)-geldanamycin. This mixture was then dissolved in chloroform and subjected to preparative HPLC separation (Waters Nova-Pak Silica 6 μm 7.8 × 300 mm column, 2.0 ml/min, 2:3 $CHCl_3$/EtOAc) to afford the product as a bright orange powder (2.8 mg, 44%). IR (in CH_2Cl_2) (cm^{-1}) 3139, 3076, 3048, 2995, 2967, 1733, 1684, 1599, 1580, 1496, 1447, 1423, 1260, 1098; ^1H NMR ($CDCl_3$, 500 MHz) δ 9.12 (br s, 1H), 8.30 (s, 1H), 7.79 (d, $J = 2.0$ Hz, 1H), 7.71 (dd, $J = 8.5$, 2.0 Hz, 1H), 7.55 (d, $J = 8.5$ Hz, 1H), 7.01 (br d, $J = 11.5$ Hz, 1H), 6.59 (br dd, $J = 11.5$, 11.0 Hz, 1H), 5.94 (br d, $J = 9.5$ Hz, 1H), 5.84 (dd, $J = 11.0$, 10.0 Hz, 1H), 5.20 (s, 1H), 4.71 (br s, 2H), 4.33 (br d, $J = 10.0$ Hz, 1H), 4.24 (br s, 1H), 3.63 (ddd, $J = 9.0$, 6.5, 2.0 Hz, 1H), 3.45 (ddd, $J = 9.0$, 3.0, 3.0 Hz, 1H), 3.36 (s, 3H), 3.26 (s, 3H), 2.79–2.70 (m, 3H), 2.06 (br s, 3H), 1.97–1.84 (m, 2H), 1.82–1.74 (m, 1H), 1.80 (d, $J = 1.0$ Hz, 3H), 0.99 (d, $J = 7.0$ Hz, 3H), 0.97 (d, $J = 6.5$ Hz, 3H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 180.8, 168.4, 156.0, 148.7, 144.9, 143.3, 136.9, 135.6, 135.3, 135.0, 133.9, 133.6, 133.0, 131.0, 126.8, 126.6, 125.2, 117.5, 112.9, 96.6, 81.9, 81.4, 81.3, 72.2, 57.1, 56.8, 35.4, 33.0, 32.3, 27.7, 23.3, 12.8, 12.6, 12.2; HRMS (FAB) $[M+H]^+$ calcd for $C_{34}H_{41}IN_3O_8$, 746.1938; found 746.1937.

5.20. 11-*O*-Acetyl-17-(1-azetidinyl)-17-demethoxygeldanamycin (18)[35]

17-(1-Azetidinyl)-17-demethoxygeldanamycin (3.2 mg, 5.5 μmol) was stirred with acetic anhydride (5.2 μl, 0.055 mmol) and DMAP (7.3 mg, 0.060 mmol). Upon complete conversion of starting material shown by thin-layer chromatography (40 h), the mixture was washed with brine, dried over anhydrous sodium sulfate, and concentrated. Separation by flash chromatography on silica gel (1:2 hexane/ethyl acetate) gave the product as a purple solid (3.2 mg, 93%). IR (in CH_2Cl_2) (cm^{-1}) 3686, 3536, 3420, 3069, 3052, 2930, 1734, 1689, 1649, 1601, 1585, 1549, 1486, 1435, 1374, 1273, 1102; ^1H NMR ($CDCl_3$, 500 MHz) δ 9.37 (s, 1H), 7.13 (br s, 1H), 6.94 (s, 1H), 6.50 (ddd, $J = 11.5$, 11.0, 1.0 Hz, 1H), 5.81 (dd, $J = 11.0$, 7.5 Hz, 1H), 5.45 (br s, 1H), 5.28 (br d, $J = 10.0$ Hz, 1H), 5.04 (dd, $J = 8.0$, 3.5 Hz, 1H), 4.64–4.54 (m, 4H), 4.48 (br d, $J = 7.5$ Hz, 1H), 3.63 (br s, 1H), 3.33 (s, 3H), 3.31 (s, 3H), 2.85–2.77 (m, 1H), 2.71 (br d, $J = 10.0$ Hz, 1H), 2.38 (quintet, $J = 8.0$ Hz, 2H), 2.06–2.00 (m, 1H), 1.98 (br s, 3H), 1.97 (s, 3H), 1.71–1.56 (m, 2H), 1.68 (br s, 3H), 1.28–1.18 (m, 1H), 0.96–0.93 (m, 6H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 186.2, 178.0, 170.6, 169.2, 155.7, 145.6, 140.4, 135.6, 134.8, 132.9, 128.3, 126.2, 109.2, 108.6, 80.0, 79.2, 78.4, 75.1, 58.5, 57.6, 56.1, 35.8, 33.0, 30.1, 29.7, 21.6, 20.9, 18.5, 15.6, 14.1, 12.3; HRMS (FAB) $[M+H]^+$ calcd for $C_{33}H_{46}IN_3O_9$, 628.3234; found 628.3237.

5.21. 17-(1-Azetidinyl)-7-decarbamyl-17-demethoxygeldanamycin (19)[52,35]

Potassium *tert*-butoxide (5.3 mg, 0.045 mmol) was added to a solution of 17-(1-azetidinyl)-17-demethoxygeldanamycin (5.0 mg, 8.5 μmol) in *tert*-butanol (4.0 ml) under nitrogen atmosphere. The reaction mixture was stirred at room temperature for 1 h and then quenched by partitioning between ethyl acetate and brine. The organic layer was washed with brine, dried over anhydrous sodium sulfate, and concentrated. Separation by flash chromatography on silica gel (hexane/ethyl acetate) gave the product as a purple solid (4.4 mg, 95 %). IR (KBr) (cm^{-1}) 3461, 3330, 2955, 2927, 2871, 2826, 1685, 1652, 1539, 1489, 1404, 1381, 1287, 1255, 1191, 1136, 1106; ^1H NMR ($CDCl_3$, 500 MHz) δ 9.16 (s, 1H), 7.09 (s, 1H), 6.90 (br d, $J = 11.5$ Hz, 1H), 6.54 (br dd, $J = 11.5$, 11.0 Hz, 1H), 5.98 (dd, $J = 11.0$, 10.0 Hz, 1H), 5.70 (br d, $J = 9.5$ Hz, 1H), 4.72–4.59 (m, 4H), 4.16 (br d, $J = 10.0$ Hz, 1H), 3.98 (s, 1H), 3.52 (dd, $J = 9.0$, 2.0 Hz, 1H), 3.41 (ddd, $J = 9.0$, 3.0, 3.0 Hz, 1H), 3.34 (s, 3H), 3.23 (s, 3H), 2.73 (dqd, $J = 9.5$, 7.0, 2.0 Hz, 1H), 2.57 (d, $J = 14.0$ Hz, 1H), 2.42 (quintet, $J = 8.0$ Hz, 2H), 2.23 (dd, $J = 14.0$, 11.0 Hz, 1H), 2.01 (d, $J = 1.0$ Hz, 3H), 1.77–1.71 (m, 2H), 1.74 (d, $J = 1.0$ Hz, 3H), 1.70–1.62 (m, 1H), 0.97 (d, $J = 7.0$ Hz, 3H), 0.94 (d, $J = 6.5$ Hz, 3H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 185.8, 178.4, 168.6, 145.8, 140.5, 137.2, 136.1, 134.8, 132.0, 126.9, 125.9, 109.5, 109.2, 81.8, 80.5, 80.3, 72.9, 58.9, 56.7, 56.3, 34.9, 34.2, 32.2, 28.2, 22.9, 18.4, 12.6, 12.4, 11.8; MS (FAB) $[M+H]^+$ found 543.

5.22. 17,21-Dihydrogeldanamycin (20)[36]

(+)-Geldanamycin (3.5 mg, 6.2 μmol) was dissolved in ethyl acetate (2.5 ml) and then aqueous solution (2.5 ml) of sodium dithionite (~85%, 0.50 g, 2.4 mmol) was added. The mixture was stirred at room temperature. Upon complete conversion of geldanamycin shown by thin-layer chromatography (1 h), the organic layer was separated, washed with brine, dried over anhydrous sodium sulfate, and concentrated. Separation of the solid residue by flash chromatography on silica gel (3:1 hexane/ethyl acetate) afforded a pale yellow solid (3.3 mg, 94%). ^1H NMR ($CDCl_3$, 500 MHz) δ 8.34 (s, 1H), 8.08 (s, 1H), 8.02 (br s, 1H), 6.76 (br d, $J = 11.5$ Hz, 1H), 6.37 (br dd, $J = 11.5$, 11.0 Hz, 1H), 5.94 (br d, $J = 9.5$ Hz, 1H), 5.64 (dd, $J = 11.0$, 10.0 Hz, 1H), 5.04 (br s, 1H), 4.95 (s, 1H), 4.65 (br s, 2H), 4.29 (br d, $J = 10.0$ Hz, 1H), 3.81 (s, 3H), 3.61 (br d, $J = 9.0$ Hz, 1H), 3.43 (br d, $J = 9.0$ Hz, 1H), 3.33 (s, 3H), 3.21 (s, 3H), 2.79–2.74 (m, 2H), 2.35 (br d, $J = 14.0$ Hz, 1H), 1.82–1.65 (m, 3H), 1.76 (br s, 6H), 0.92 (d, $J = 6.5$ Hz, 3H), 0.86 (d, $J = 7.0$ Hz, 3H); HRMS (FAB) $[M]^+$ calcd for $C_{29}H_{42}N_2O_9$, 562.2890; found 562.2886.

5.23. HGF/SF-Met-uPA-plasmin cell-based assay

Cells were seeded in 96-well plates at 1500 cells/well (with the exception of SK-LMS-1 cells, which were seeded at 5000 cells/well) to detect color intensity, either with MTS (Promega) for cell growth determination or via Chromozyme PL (Boehringer–Mannheim) for uPA-plasmin activity measurement. Cells were grown overnight in DMEM/10% FBS, as previously described.[14] Drugs were dissolved in DMSO, serially diluted from stock concentrations into DMEM/10% FBS medium, and added to appropriate wells. Immediately after the addition of drugs or reagents, HGF/SF (60 ng/ml) was added to all wells (with the exception of wells used as controls to calculate basal growth and uPA-plasmin activity levels). Twenty-four hours after the addition of drugs and HGF/SF, plates were processed for the determination of uPA-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 U/ml plasminogen in DMEM (without phenol red), 40% (v/v) 50 mM Tris buffer (pH 8.2), and 10% (v/v) of 3 mM Chromozyme PL (Boehringer–Mannheim) in 100 mM glycine solution] was 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. uPA-plasmin inhibition index or IC_{50} is the negative $\log_{10}$ of the concentration at which uPA-plasmin activity is inhibited by 50%.[14]

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