Bioorganic & Medicinal Chemistry Letters 18 (2008) 6273–6278

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Dihydroxylphenyl amides as inhibitors of the Hsp90 molecular chaperone

Pei-Pei Kung *, Lee Funk, Jerry Meng, Michael Collins, Joe Zhongxiang Zhou, M. Catherine Johnson, Anne Ekker, Jeff Wang, Pramod Mehta, Min-Jean Yin, Caroline Rodgers, Jay F. Davies II, Eileen Bayman, Tod Smeal, Karen A. Maegley, Michael R. Gehring

Pfizer Global Research and Development, La Jolla Laboratories, 10770, Science Center Dr., San Diego, CA 92121, USA

ARTICLE INFO

Article history:
Received 25 August 2008
Revised 22 September 2008
Accepted 22 September 2008
Available online 26 September 2008

Keywords:
Dihydroxylphenyl amides
Hsp90
Inhibitor

ABSTRACT

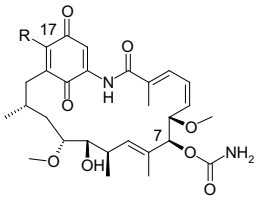
Information from X-ray crystal structures were used to optimize the potency of a HTS hit in a Hsp90 competitive binding assay. A class of novel and potent small molecule Hsp90 inhibitors were thereby identified. Enantio-pure compounds **31** and **33** were potent in PGA-based competitive binding assay and inhibited proliferation of various human cancer cell lines in vitro, with IC_{50} values averaging 20 nM.

Molecular chaperones are protein machines that are responsible for the correct folding, stabilization, and function of other proteins in the cell.[1] Many molecular chaperones and cocharperones have been identified to date. Among these, heat shock protein 90 (Hsp90) is the most extensively studied. This 90 kDa protein is an ATP-dependent molecular chaperone that has multiple identified client proteins involved in the development and progression of cancer.[2] Many of these client proteins are over-expressed in oncogenic settings and rely on Hsp90 for their proper folding. Therefore, Hsp90 has become a focus of interest as a potential anticancer drug target.

The natural product Hsp90 inhibitor geldanamycin (GA) and several semi-synthetic analogs (17-AAG and 17-DMAG, Fig. 1)[3a–c] have entered clinical trials, and initial encouraging results provide proof of concept for using Hsp90 inhibitors as cancer chemotherapy.[3a] However, these compounds have potential therapeutic limitations because of low solubility, liver toxicity, and extensive metabolism.[4a] Many of these issues could be associated with the quinone moieties that exist in these agents and have led us and others to identify non-quinone-containing, small molecule inhibitors of Hsp90 chaperone activity.

Our Hsp90 research program began with a high throughput screening campaign using a competitive binding assay in which the ability of test compounds to displace tritium-labeled 17-propylaminobenzoquinone ansamycin (17-PGA, Fig. 1) from Hsp90 was assessed. This effort led to the discovery of the tri-hydroxy-containing compound **1** (Table 1) which displayed a K_i of 200 nM in the competitive binding assay.[5] Unfortunately, the compound exhibited an IC_{50} greater than 20 μM in a cell-based assay which measured the degradation of the Hsp90 client protein Akt.[6a,6b] To facilitate potency improvements, we obtained a 1.4 Å co-crystal structure of compound **1** bound to Hsp90 and compared it to the crystal structures of other known Hsp90 inhibitors complexed with the protein. Such inhibitors include: GA,[7] purine analogs such as PU3,[8] the resorcinol-containing natural product radicicol,[9] and other resorcinol-containing small molecules[10a–d] (Fig. 2).

As shown in Figure 3 (PDB ID code: 3EKO), the amide carbonyl of **1** formed hydrogen bonds with the side chain of the Hsp90 Thr184 residue as well as with a water molecule that was conserved in many of the known co-crystal structures. The 2′ hydroxyl of **1** interacted with the same conserved water molecule along with the side chain of Asp93. In addition, the 4′ hydroxyl group of **1** formed hydrogen bonds with a second conserved water molecule and the Asn51 side chain of the Hsp90 protein. In contrast, the 6′ hydroxyl group interacted only with two non-conserved water molecules that were not observed in other Hsp90 inhibitor co-crystal structures. Collectively,



GA: R=OCH_3
17-AAG: R=-$NHCH_2CH{=}CH_2$
17-DMAG: R=-$NHCH_2CH2NMe_2$
17-PGA: R=-$NHCH_2CH2CH_3$

Figure 1. Structures of benzoquinone ansamycins.

* Corresponding author. Tel.: +1 858 526 4867; fax: +1 877 481 1781.
E-mail address: peipei.kung@pfizer.com (P.-P. Kung).

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doi:10.1016/j.bmcl.2008.09.081

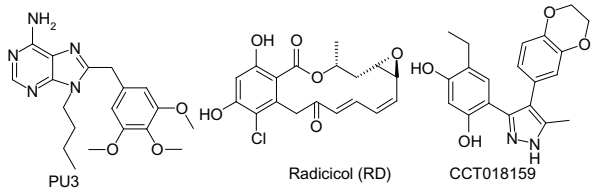


Figure 2. Structures of known Hsp90 inhibitors.

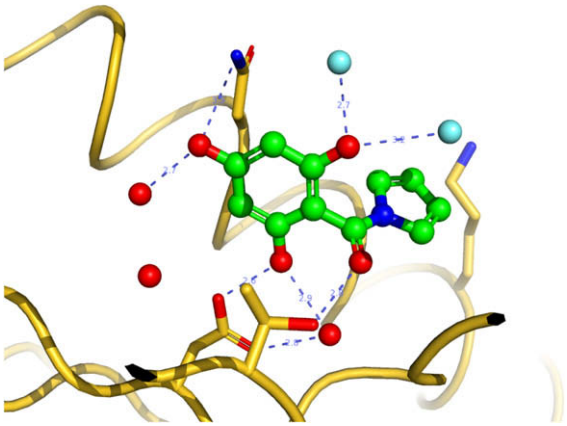


Figure 3. X-ray structure of **1** bound to the ATP binding site of human Hsp90. Red spheres represent structurally conserved water molecules and aqua spheres represent structurally non-conserved water molecules.

these observations suggested that the 6′ hydroxyl group of **1** was not critical for Hsp90 recognition, and this hypothesis was confirmed with the preparation of the resorcinol-containing compound **2** which displayed Hsp90 inhibition potency similar to that exhibited by **1** (Table 1).

Further analysis of the above co-crystal structure indicated that the pyrrole moiety of **1** occupied an area formed by mostly hydrophobic residues Met98, Ile96, Ala55, and Lys58 (Fig. 4). Of these, Lys58 appeared to be conformationally mobile, suggesting that moieties larger than the pyrrole group could be accommodated in this location. We therefore designed and synthesized a small library of compounds in which commercially available amines were used to replace the pyrrole moiety present in inhibitor **2**. In general, library compounds derived from primary amines were less potent Hsp90 inhibitors than those prepared from secondary amines (e.g., compare **3** and **4** with **5** and **7**, respectively). This phenomenon is likely due to the ability of the amide NH present in the former compounds to form an intra-molecular H-bond with the 2′-OH moiety which unfavorably positions the amide carbonyl relative to the orientation observed in the Hsp90 and compound **1** co-crystal structure.

Encouragingly, as shown in Table 1, many of the compounds that were prepared displayed Hsp90 inhibition activity that was equal to or better than that exhibited by compound **2** (compounds **5**–**13**). Inhibitors **7**, **10**, and **11** were considered to be particularly attractive leads due to their relatively small size, potent inhibition of Hsp90 activity, and <10 μM inhibition of Hsp90 activity in the cell-based Akt Lum assay. In order to further improve inhibitor potency, we subjected racemic compound **10** to preparative chiral SFC (supercritical fluid chromatography)[11] and obtained the corresponding single enantiomers **14** and **15**.[12] As shown in Table 1, the former compound was approximately 100-fold more potent than the latter in the competitive binding assay.

A 1.8 Å co-crystal structure of inhibitor **14** complexed with Hsp90 was obtained (Figs. 5 and 6) (PDB ID code: 3EKR) and the chirality of this molecule was thereby confirmed as the 'R' enantiomer.

Analysis of this co-crystal structure also identified a small unfilled hydrophobic pocket formed by Hsp90 residues Phe138,

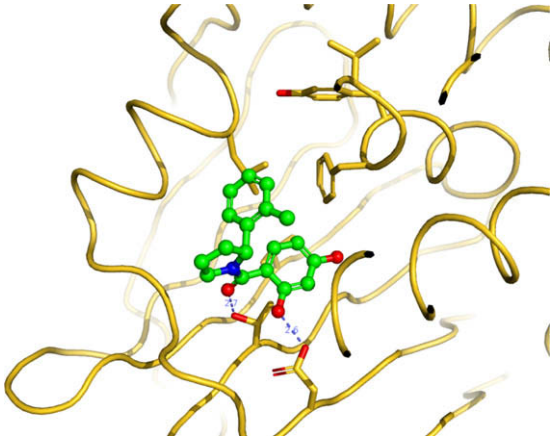


Figure 5. Binding interactions of **14** to Hsp90. The phenyl moiety of pyrrolidine amide moiety exposed to a lipophilic pocket formed by Tyr139, Val 136, and Gly135.

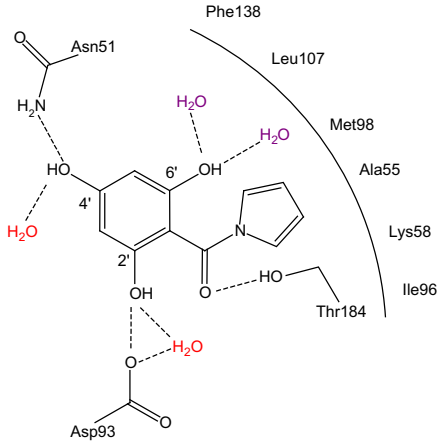


Figure 4. Schematic diagram showing the key interactions of **1** with Hsp90 enzyme.

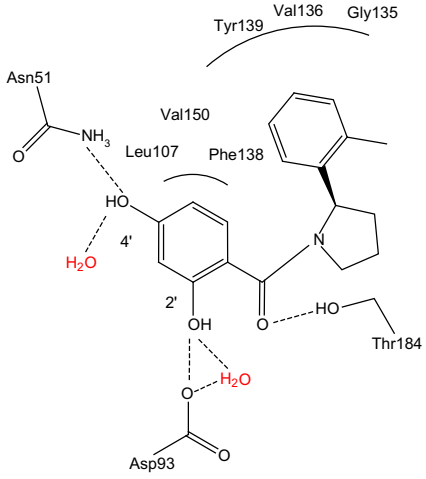
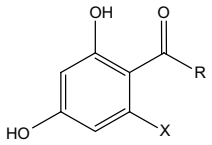
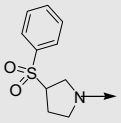
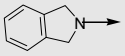
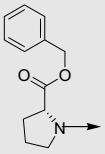
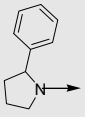
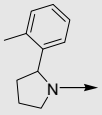
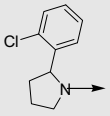
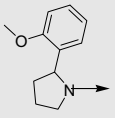
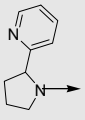
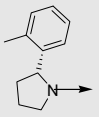
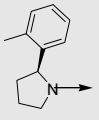


Figure 6. Schematic diagram shows the two unfilled lipophilic pockets.

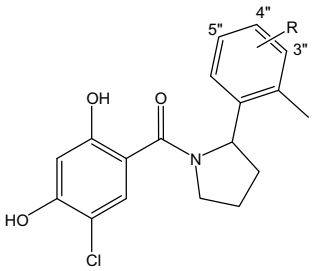
Table 1
SAR of 2,4-dihydroxyphenyl carboxyamides



Compound	R'	X	Enzyme IC_{50} (μM)	Enzyme K_i[a] (μM)	Cell IC_{50} (μM)
1		OH	0.4	0.2	>20
2		H	1.7	0.85	>20
3		H	200	101	ND
4		H	74	36.8	ND
5		H	3.2	1.6	>20
6		H	1.4	0.7	>50
7		H	0.06	0.03	7
8		H	0.5	0.25	ND
9		H	0.3	0.14	14
10		H	0.1	0.05	9.3
11		H	0.08	0.04	8.6
12		H	0.4	0.2	10.3
13		H	2.4	1.2	>20
14		H	0.06	0.03	4
15		H	5.6	2.8	>50

[a] Enzymatic K_i value was calculated from enzymatic IC_{50} value using Cheng–Prusoff equation.

Table 2
SAR of different carboxyl esters/amides of compound 16



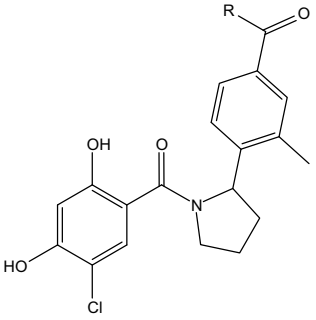
Compound	R	Location	Enzyme IC_{50} (μM)	Enzyme K_i[a] (μM)	Cell IC_{50}[a] (μM)
16	H	—	0.02	0.01	1
17	$COOCH_3$	5″	0.3	0.15	20
18	$CONHCH_2CH_3$	5″	1.4	0.7	ND
19	$COOCH_3$	4″	<0.02	<0.01	1
20	$CON(CH_3)_2$	4″	<0.02	<0.01	0.3
21	$CONHCH_2CH_3$	4″	0.04	0.02	4.8
22	$COOCH_3$	3″	<0.02	<0.01	1.6
23	$CON(CH_3)_2$	3″	0.2	0.1	2

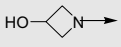
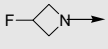
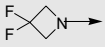
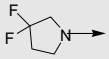
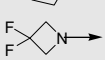
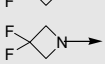
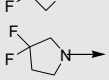
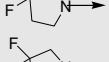
[a] Assay error for both assays are 10–15%.

Val150, and Leu107 near the 5′ position of the resorcinol ring of **14**. We therefore introduced a 5′-chloro moiety into the inhibitor design and observed a 10-fold improvement in cell IC_{50} values (compare **16** with **10**, Tables 1 and 2).[13] Similar SAR was also reported by others.[10a]

Additional analysis of the **14**-Hsp90 co-crystal structure identified a second larger unfilled pocket near the phenyl moiety formed by residues Tyr137, Val136, and Gly135. We therefore introduced simple esters and/or amides at three positions on the phenyl ring of **16** to determine whether these groups could improve inhibitor recognition of the Hsp90 protein. We envisioned that if these mod-

Table 3
SAR of 4-[1-(5-Chloro-2,4-dihydroxy-benzoyl)-pyrrolidin-2-yl]-3-methyl-phenyl carboxyamides



Compound	Chirality	R	Enzyme IC_{50} (μM)	Enzyme K_i[a] (μM)	Cell IC_{50}[a] (μM)
24	R/S		<0.02	<0.01	0.15
25	R/S		<0.02	<0.01	0.15
26	R/S		<0.02	<0.01	1.3
27	R/S		<0.02	<0.01	0.073
28	R/S		<0.02	<0.01	0.03
29	R/S		<0.02	<0.01	0.07
30	S		1.4	0.7	>20
31	R		<0.02	<0.01	0.02
32	S		0.3	0.15	11
33	R		<0.02	<0.01	0.027

[a] Assay error for both assays are 10–15%.

Table 4
IC_{50} of 17-DMAG, **31**, and **33** on expression of Hsp90 client proteins (Akt and Her2) and proliferation (MTT assay) in MDA-MB231 and A2058 cells

	IC_{50} (nM) for Hsp90 client protein degradation					EC_{50} (nM)
	MDA-MB231			A2058		Hsp70 (A2058)
	Akt	Her2	MTT	Akt	MTT	
DMAG	17.6	4.5	5.8	24.3	2.1	7.9
31	19.1	7.6	8.9	33.5	5.8	18.3
33	41.9	17	24.5	49.6	22.6	40.9

EC_{50} for induction of Hsp70 for 17-DMAG, **31**, and **33** in MDA-MB231.

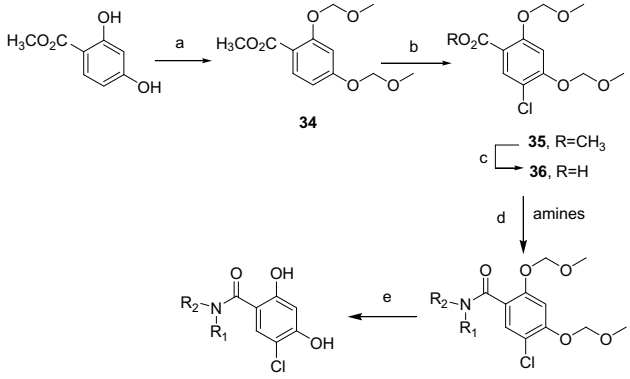
ifications were tolerated, the latter group could be rapidly optimized through parallel chemistry approaches. As shown in Table 2, appending such an ester or amide to the 5″ position of the phenyl ring of **16** was detrimental to Hsp90 recognition properties (compare **17** and **18** with **16**). In contrast, introducing these modifications at the 3″ or 4″ positions (compounds **19**–**23**) typically afforded potency improvements in both the competitive-binding and cell-based assays with activity in the former assessment often below the level of accurate quantitation (K_i < 0.01 μM). Compound **20**, bearing a 4″-dimethyl amide, was the most active compound of these molecules in the Akt Luminex assay.

Encouraged by this result, we synthesized a small library of compounds related to **20** containing di-substituted amides. As shown in Table 3, all of the tested molecules were extremely potent in the competitive binding assay with activities below the quantitation limit (**24**–**29**). They also typically displayed submicromolar Hsp90 inhibition activity in the cell-based assay. Two of the most active racemic compounds (**28** and **29**) were subjected to chiral SFC purification in order to characterize the corresponding enantiomers. As expected, potent Hsp90 inhibition activity was observed for only one of the purified enantiomers (compare **30** with **31** and **32** with **33**). The potent single enantiomers thus identified (**31** and **33**) were approximately 1000-fold more active in the Akt Luminex assay as compared with the original screening lead compound (compound **1**, Table 1).

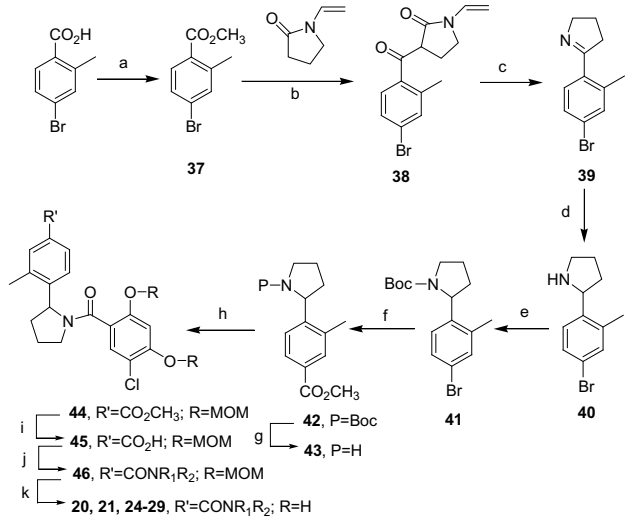
The ability of these two compounds to affect turnover of several Hsp90 client proteins in MDA-MB231 and A2058 cells was also assessed. As shown in Table 4, both molecules exhibited a well-established molecular signature[14,15] for Hsp90 inhibitors in these assessments (similar to that displayed by 17-DMAG).

The synthesis routes used to prepare compounds **16**–**23** and **24**–**29** described in this work are depicted in Schemes 1 and 2. The syntheses of similar substituted 2-methyl-phenyl-pyrrolidines using different starting materials are described in the supplementary material. Scheme 1 describes the synthesis of 5-chloro-2,4-bis methoxymethoxy-benzoic acid (compound **36**) as well as a general synthesis of the resorcinol amide Hsp90 inhibitors. Amide coupling reactions in the absence of the phenolic protecting groups resulted in low yields of the desired products. Compound **35** was synthesized from commercially available 2,4-dihydroxy-benzoic acid methyl ester by MOMCl protection of the phenols followed by chlorination of the aromatic ring to provide compound **35**.[16] Hydrolysis of **35** with lithium hydroxide provided compound **36** in 54% yield over three steps.

The 2-phenyl-pyrroline precursors were prepared from *N*-vinyl-pyrrolidin-2-one using the method reported by Maryanoff.[17] The imine intermediate **39** was then reduced with $NaBH_4$ to provide the desired intermediate **40**, 2-(4-bromo-2-methyl-phenyl)-pyrrolidine. Compound **41** was then carbonylated with CO_2 and subsequently trapped with methanol to give the desired product **42**, the boc protected 3-methyl-4-pyrrolidin-2-yl-benzoic acid methyl ester. Further acid de-protection of **42** gave the desired amine **43**. Compound **43** was coupled with compound **36** using HOBt/EDC mediated coupling condition to give compound **44**. Compound **44** was hydrolyzed to give compound **45**. A second amide coupling reaction of **45** and further de-protection of the MOM groups of



Scheme 1. Reagents and conditions: (a) MOMCl, DIEA, DMF, 23 °C, 12 h, 92%; (b) $Ca(OCl)_2$, acetic acid, acetone, 62%; (c) LiOH(aq) (2 M), MeOH, 23 °C, 12 h, 94%; (d) amines (both commercially available and synthetic amine from Scheme 2), 4-methylmorpholine, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride, 1-hydroxy benzotriazole, DMF, 23 °C, 12 h, 64.2%; (d) HCl (in dioxane, 4 M), DCM, 23 °C, 12 h, 82%.



Scheme 2. Reagents and conditions: (a) $SOCl_2$, MeOH, 40 °C, 12 h, 81%; (b) *N*-vinylpyrrolidin-2-one, THF, NaH, 80 °C, 2 h; (c) HCl (aq) (6 N), *i*-PrOH/THF, 90 °C, 12 h, 56%; (d) $NaBH_4$, MeOH/acetic acid, −40 °C, 2 h; (e) $(Boc)_2O$, K_2CO_3, DCM, 12 h, 23 °C, 81%; (f) $Pd(PPh_3)_4$, TEA, CH_3CN/MeOH, CO (100 psi), 65 °C, 24 h, 65%; (g) HCl (4 M in dioxane), 12 h, 23 °C, quant.; (h) compound **36**, 4-methylmorpholine, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride, 1-hydroxy benzotriazole, DMF, 23 °C, 12 h, 64.2%; (i) LiOH (2 M), dioxane, 40 °C, 90%; (j) amines, 4-methylmorpholine, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride, 1-hydroxy benzotriazole, DMF, 23 °C, 12 h, 64.2%; (k) HCl (4 M in dioxane), DCM, 23 °C, 12 h, 82%.

46 gave the desired products (compounds **20**, **21**, and **24**–**29**) shown in both Tables 2 and 3 in 50–80% yield.

In summary, a series of potent Hsp90 inhibitors was discovered by both focused library synthesis and structure-based design. The two lead compounds (**31** and **33**) from this series displayed equal or better potency compared with 17-DMAG in effecting the client proteins' degradation and cell proliferation in both A2058 and MDA-MB231 cell lines.

Acknowledgments

We thank the Pfizer La Jolla analytical group for chiral separation of racemic compounds. We also thank Drs. Peter Wells and Rob Kania for helpful discussions, Dr. Yinsheng Zhang for preparing the tritium-labeled 17-PGA, and Dr. Ketan Gajiwala for discussion of co-crystal structures and graphical support.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.09.081.

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5. Compounds were evaluated for potency against HSP-90 using a SPA (scintillation proximity assay) competition binding assay. Briefly, either full length or N-terminal HSP-90 that contains a 6-His tag on its C-terminus were bound to copper on Yttrium-silicate scintillant beads. Test compounds which bind to HSP-90 competed with tritiated propyl-Geldanamycin (^{3}H-pGA) at the ATP-binding site on HSP-90. For K_i determinations, % inhibition values are plotted vs. log inhibitor concentration and fit to four parameter logistic equation. The IC_{50} is converted to competitive K_i using Cheng–Prusoff equation, where K_i is the inhibition constant, L is the total concentration of pGA = 200 nM, and K_d is the dissociation constant for pGA = 240 nM.
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