Rationally Designed High-Affinity 2-Amino-6-halopurine Heat Shock Protein 90 Inhibitors That Exhibit Potent Antitumor Activity

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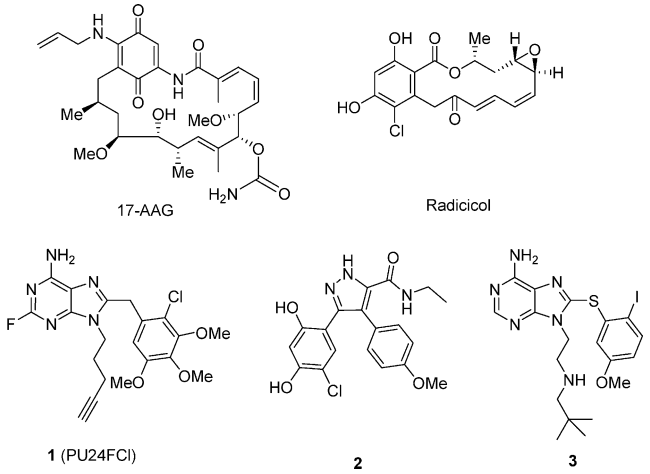
Heat shock protein 90 (Hsp90) is a molecular chaperone protein implicated in stabilizing the conformation and maintaining the function of many cell-signaling proteins. Many oncogenic proteins are more dependent on Hsp90 in maintaining their conformation, stability, and maturation than their normal counterparts. Furthermore, recent data show that Hsp90 exists in an activated form in malignant cells but in a latent inactive form in normal tissues, suggesting that inhibitors selective for the activated form could provide a high therapeutic index. Hence, Hsp90 is emerging as an exciting new target for the treatment of cancer. We now report on a novel series of 2-amino-6-halopurine Hsp90 inhibitors exemplified by 2-amino-6-chloro-9-(4-iodo-3,5-dimethylpyridin-2-ylmethyl)purine (**30**). These highly potent inhibitors (IC_{50} of **30** = 0.009 μM in a HER-2 degradation assay) also display excellent antiproliferative activity against various tumor cell lines (IC_{50} of **30** = 0.03 μM in MCF7 cells). Moreover, this class of inhibitors shows higher affinity for the activated form of Hsp90 compared to our earlier 8-sulfanylpurine Hsp90 inhibitor series. When administered orally to mice, these compounds exhibited potent tumor growth inhibition (>80%) in an N87 xenograft model, similar to that observed with 17-allylamino-17-desmethoxygeldanamycin (17-AAG), which is a compound currently in phase I/II clinical trials.

Introduction

As evidence accumulates that many mutated or overexpressed signaling proteins in malignant cells are dependent on the molecular chaperone protein Hsp90 for their stabilization and function, this chaperone has rapidly become the focus of drug discovery research efforts.[1] Indeed, Hsp90 has emerged as an important cancer target because of its key participation in regulating clinically validated oncogenic proteins such as HER-2, ER, AR, and Bcr-Abl, as well as other signaling proteins that play critical roles in malignancy, such as Raf-1, p-Akt, Cdk-4, mutant p53, and Flt-3.[2] These Hsp90 "client" proteins regulate many cellular processes important to the survival of cancer cells, including growth, cell cycle progression, and apoptosis. Another important rationale for targeting Hsp90 is to achieve simultaneous destruction of several signaling proteins in tumor cells so as to overcome the inevitable drug resistance associated with the activation of redundant signaling pathways.[3] Finally, our recent discovery[4] of the preponderance of a high affinity form of Hsp90 in tumor cells as compared to normal cells makes the Hsp90 pathway an even more attractive target for cancer therapy because of the resulting increase in therapeutic index that might be obtained from tumor cell selectivity. The Hsp90 cellular protein family consists of four members, Hsp90α, Hsp90β, Grp94, and Trap1. Structural studies reveal that Hsp90 is composed of three highly conserved domains:[5] (1) The N-terminal lobe that contains an adenine nucleotide binding pocket which is responsible for the ATPase activity of Hsp90,[6] (2) the highly charged "middle region" that plays an important role in modulating the ATPase activity,[7] (3) the C-terminal lobe that contains an additional ATP binding pocket identified by its ability to bind the antibiotic novobiocin.[8] Activation of Hsp90 results in the formation of complexes with a series of cochaperones including Aha1, p23, Hop, Cdc37/p50, Hsp70, and Hsp40 to form a superchaperone complex that interacts with the client proteins.[4,9] Inhibition of the ATPase activity with high affinity natural product inhibitors results in proteasome-mediated degradation of the client proteins and subsequent interruption of their signaling function.[3] Several of these natural product inhibitors of Hsp90 ATPase activity have been reported, including the ansamycin antibiotic geldanamycin, geldanamycin analogues, and radicicol.[10] A semisynthetic product of geldanamycin, 17-allylamino-17-desmethoxygeldanamycin (17-AAG),

Chart 1



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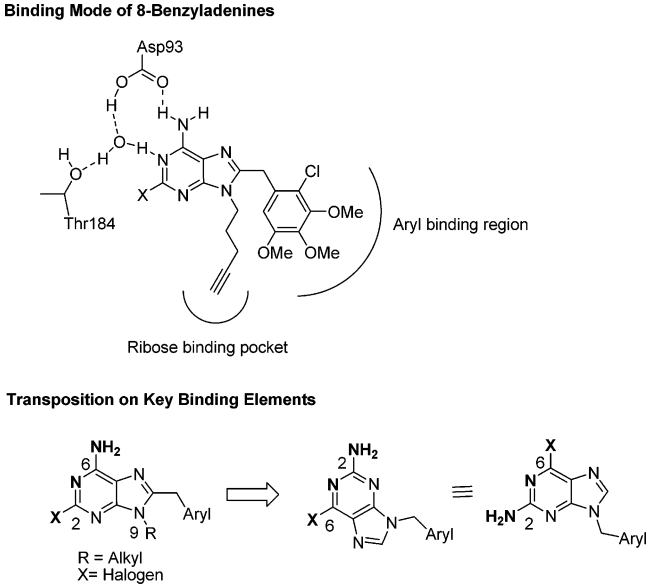


Figure 1. Rational design of 2-amino-9-benzylpurines from 6-amino-8-benzylpurines.

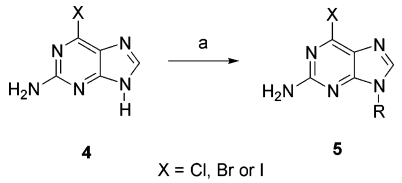
is currently in phase I/II clinical trials for cancer.[11] Although this compound represents a major advancement in Hsp90-directed therapy, natural product derivatives based on geldanamycin engender a number of difficulties.[12] In particular, geldanamycin derivatives present significant formulation and delivery challenges associated with their relative insolubility in aqueous media. Moreover, the dose-limiting toxicities appear to be hepatotoxicity due to the presence of the reactive quinone moiety. These reasons have encouraged researchers to develop second-generation small molecule inhibitors that are structurally unrelated to the first-generation natural products. We and others have recently identified a number of synthetic analogues of Hsp90 inhibitors.[13] The first synthetic purine-based inhibitor analogues, designed by Chiosis et al.[13q,m] and represented by **1** (PU24FCl), showed low micromolar in vitro potency but still exhibited some in vivo efficacy.[13n] Drysdale et al.[13h] described pyrazole-based inhibitors such as compound **2**, which displayed excellent binding potency and cell-based activity.[13o] However, no in vivo efficacy data on these compounds was reported. Finally, Biamonte et al.[13p] and He et al.[13r] recently disclosed the 8-(phenylsulfanyl)purine series, exemplified by compound **3**, with ionizable groups appended at the end of the N(9) substituents, which showed improved oral bioavailability and measurable antitumor activity. Since compound **3** showed only moderate efficacy in human xenograft models, we continued with our efforts to develop inhibitors with improved in vivo potency and selectivity toward the activated form of Hsp90. Herein, we discuss these efforts and disclose a new series of exceptionally potent and orally bioavailable Hsp90 inhibitors.

Design Strategy

Our goal was to design a more potent series of Hsp90 inhibitors based on the reported structural information[14] and on the information gained by several laboratories[13] from working with 8-benzyl[13a,g] and 8-sulfanylpurines[13a] (Figure 1). The X-ray structure of **1** cocrystallized with Hsp90[14a] indicates that it binds to the N-terminal ATP-binding site. As expected, the adenine ring of **1** binds in the same manner as the adenine ring of ATP. The 9-pentynyl group emulates the ribose group and forms a hydrophobic interaction with the side chain of Leu107. Although the 8-benzyl group of **1** was initially designed to occupy the phosphate-binding region, it actually projects toward a pocket that is not occupied by any of the natural products ATP, geldanamycin,[14b] or radicicol.[14c] In this paper, we will refer to this pocket as the "aryl-binding pocket". Structure−activity relationship (SAR) studies revealed that the N(1) and 6-NH_2 groups are essential for activity, since they form two strong hydrogen bonds with the 'hinge-region' of Hsp90. Addition of a 2-fluoro or 2-chloro substituent on the adenine ring improves the potency by about 2-fold.[13m] The aryl group tolerates various substitution patterns, but a 2-halo-5-methoxy[13p] or 2-halo-3,4,5-trimethoxy[13m] was preferred. Furthermore, the aryl group must be connected to the purine ring by a linker of exactly one atom (CH_2 or S). This places the aryl group six bonds away from the NH_2 group, and we surmised that this distance is an essential element of the pharmacophore. Finally, the N(9) side chain tolerates a wide range of modifications. In our design of new Hsp90 inhibitors, we sought to capture the critical interactions of 8-benzylpurines by rearranging the substituents around the purine ring, as indicated in Figure 1. The first modification entailed shifting the aryl binding moiety from the C(8) position to the N(9) position. Taken alone, this transformation resulted in molecules that did not inhibit Hsp90. However, when the shift of the aryl binding moiety to the N(9) position was combined with a commensurate shift of the NH_2 group from the 6- to the 2-position so as to re-established the overall six-bond distance between the 6-NH_2 group and the aryl group, the newly formed 2-amino-6-halo-9-benzylpurines exhibited outstanding Hsp90 inhibitory activity, resulting in a marked increase in in vitro and in vivo potency.

We also wanted to examine a broad range of 9-aryl groups including heteroaryls in order to introduce diverse pharmaceutical properties that could yield compounds with drug-like properties. For example, our recent experiences with 8-benzyladenines showed that they lacked significant solubility in aqueous media, which led to poor oral absorption.[13p] We reasoned that 9-benzyladenines may also be insufficiently soluble and further refined the design of this new series of inhibitors by replacing the substituted phenyl ring (e.g. 2-halo-3,4,5-phenyl) with a substituted pyridine ring.

Scheme 1[a]



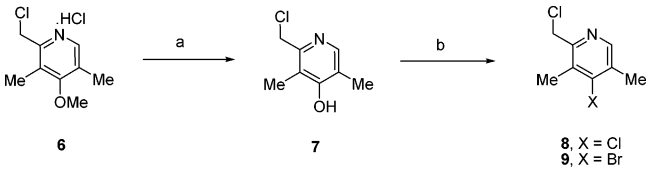
[a] Reagents and conditions: (a) K_2CO_3, DMF, NaI, R−L (L is a leaving group).

Chemistry

We prepared 2-amino-6-halo-9-substituted purine Hsp90 inhibitor analogues simply by alkylating the 2-amino-6-halo-purine **4** with an appropriate electrophile in the presence of K_2CO_3 and NaI in warm DMF, which gave the desired N(9) regioisomer **5** as the predominant product (Scheme 1).

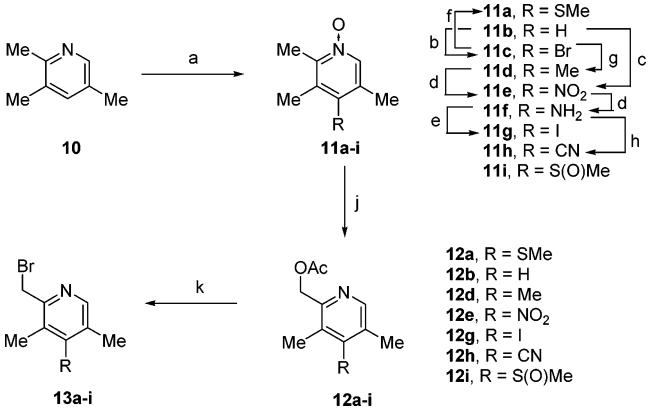
The electrophiles, 2,5-dimethoxybenzyl chloride, 3,4,5-trimethoxybenzyl chloride, and 2-chloromethyl-4-methoxy-3,5-dimethylpyridine **6** hydrochloride are commercially available. Compounds **7**−**9** were prepared from 2-chloromethyl-4-methoxy-3,5-dimethylpyridine **6** following reported procedures.[15] Demethylation of the hydrochloride salt of **6** in refluxing toluene gave 2-chloromethyl-4-hydroxy-3,5-dimethylpyridine **7**, which was deoxyhalogenated with $POCl_3$ or $POBr_3$ to provide the

Scheme 2[a]



[a] Reagents and conditions: (a) toluene, 120 °C; (b) $POCl_3$ or $POBr_3$, 110 °C, 1.5 h.

Scheme 3[a]



[a] Reagents and conditions: (a) *m*CPBA, CH_2Cl_2, 0 °C; (b) Br_2, K_2CO_3, CCl_4, rt; (c) HNO_3, H_2SO_4, 0−100 °C; (d) 10% Pd/C, EtOH, H_2, 60 psi; (e) HBF_4, $NaNO_2$, KI; (f) MeSNa, THF, 70 °C; (g) $Pd[P(Ph_3)]_4$, $AlMe_3$, THF, 70 °C; (h) HBF_4, $NaNO_2$, CuCN; (i) *m*CPBA, CH_2Cl_2 (**11a** → **11i**), rt; (j) Ac_2O, 90 °C; (k) K_2CO_3, MeOH, 50 °C; PPh_3, CBr_4, CH_2Cl_2, rt.

corresponding 4-halo compounds chloromethyl-4-chloro-3,5-dimethylpyridine **8** and chloromethyl-4-bromo-3,5-dimethylpyridine **9** (Scheme 2). 2-Bromomethylpyridines were prepared from 2,3,5-trimethylpyridine-*N*-oxide **11b**, in three to five steps using the sequence depicted in Scheme 3. The key step involved rearrangement of the 2-methylpyridine *N*-oxide derivative to the 2-acetoxymethylpyridine derivative using acetic anhydride.[16] Subsequent deacetylation and bromination with PPh_3/CBr_4 provided the 4-bromomethylpyridines electrophiles **13a**−**i**. The intermediate 2,3,5-trimethylpyridine *N*-oxide **11b** could be functionalized in a variety of ways. Bromination provided the intermediate 2,3,5-trimethyl-4-bromopyridine *N*-oxide **11c**. Treatment of the 4-bromopyridine **11c** with $AlMe_3$ in the presence of Pd(0) followed by three-step sequence depicted in Scheme 3, gave the 3,4,5-trimethylpyridine derivative **13d**.[17] Similarly, reaction of **11c** with MeSNa provided the 4-thiomethylpyridine **11a**. Nitration of **11b** gave the 4-nitropyridine **11e**, which was reduced to the 4-aminopyridine **11f** and subjected to a Sandmeyer reaction to yield the 4-iodopyridine **11g**. The three-step sequence of rearrangement, deacetylation, and deoxybromination was applied to **11a**−**g** to provide the bromomethylpyridines **13a**−**g**.

The 5-chloromethylpyridine **15**, a regioisomer of **6** in which the CH and N atom are formally permuted, was prepared in three steps starting from pyridoxine hydrochloride (Scheme 4). The intermediate chloromethylpyridine **14** was readily prepared by the reduction of pyridoxine hydrochloride with hydrazine followed by chlorination with thionyl chloride using reported procedures.[18] Methylation of the 3-hydroxypyridine **14** under Mitsunobu conditions gave the 3-methoxy-5-chloromethylpyridine **15**. Alkylation of 2-amino-6-chloropurine with electrophile **15** using the standard conditions gave compound **16**.

Modifications at the C(6)-position and substitutions at N(9) of the purine ring were made at a later stage of the synthesis (Schemes 5 and 6). Aromatic ring halogenation of the compound **17** with NCS or NBS or NIS gave the corresponding 2-halo-3,4,5-trimethoxybenzyl derivatives **18a**−**c**. Nucleophilic displacement of C(6) chlorine atom in compounds **18a** and **18b** provided compounds **19a**−**d**. Methylation of **18a** with $AlMe_3$ in the presence of Pd(0) gave 6-methyl derivative **19f**.[17] Alkylation of 2-aminopurine with 3,4,5-trimethoxybenzyl chloride followed by bromination gave **19e**. Demethylation of **20** with BBr_3 provided the 4-hydroxy analogue **21**, which was alkylated with MeI or EtI in the presence of K_2CO_3 to give the alkylated compounds **22** and **23**. Oxidation of **20** gave the *N*-oxide **24**, which was treated with $SOCl_2$ to give the chlorinated compound **25**.[19]

Results and Discussion

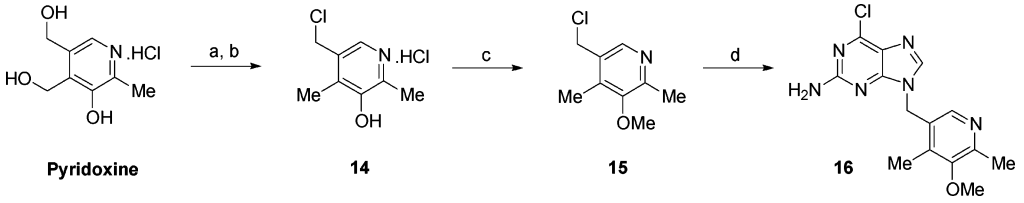
The primary screening assay for structure−activity optimization studies measured HER-2 degradation in MCF7 cells. HER-2 was chosen because it is a well-established oncology target[20] that is exquisitely dependent on Hsp90. The activity of each compound was measured by its ability to induce degradation of HER-2 using a flow cytometric assay.[4,10a] The IC_{50} values reported are the average of two or three experiments and are within 30% agreement. Additional in vitro assays used to evaluate the Hsp90 inhibitors are a cell lysate binding assay to determine the binding affinity to the activated form of Hsp90 and a MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium] assay[4,10a] to determine their cytostatic/cytotoxic activity to cancer cells.

To evaluate our design concept, we selected 2-amino-6-chloropurine as our first adenine-binding moiety. The selection was based on an earlier finding[13a,m] that the halogen at the 2-position of the purine ring is important for increased potency. Our initial selections of N(9) substituents, which are believed to occupy the ATP-ribose binding pocket, were 2,5-dimethoxybenzyl and 3,4,5-trimethoxybenzyl moieties, the same moieties that were optimized in the 6-amino-8-phenylsulfenylpurine series.[13a,m] We were gratified to see that the initial compounds **26** and **17** showed HER-2 degradation activity of 3.0 and 2.0 μM, respectively.

These compounds exhibited approximately 2−3-fold enhanced potency as compared to similar compounds prepared in our 6-amino-8-phenylsulfanylpurine series.[13p] With this initial success, we then optimized the series on the basis of HER-2 degradation activity.

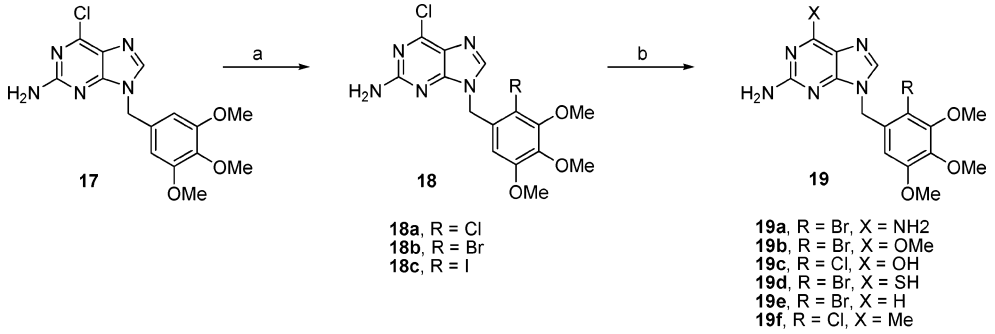
We first examined the effect of halogenation on the benzyl ring of compound **17** to determine if changes in activity were similar to those observed in the 6-amino-8-phenylsulfenylpurine series.[13m] As expected, introduction of a halogen at the 2-position of the benzyl ring increased the potency by 5−40-fold (compounds **18a**−**c**, Table 1). Optimization of over 30 benzyl derivatives indicated that the 2-halo-3,4,5-trimethoxybenzyl is the optimal benzyl derivative at the N(9) position. To understand the binding role of the chlorine atom at the C(6) position of the purine ring, we next investigated the size and electronics requirements of moieties at the C(6) position (Table 2). Replacement of chlorine with bromine (compound **27**) gave a slight improvement in the potency. However, replacing the chlorine with other substituents such as NH_2 (**19a**), OCH_3 (**19b**), OH (**19c**), SH (**19d**), H (**19e**), or CH_3 (**19f**) resulted in decreased potency. Interestingly, the loss of potency observed with hydrogen (**19e**) and methyl (**19f**) moieties at the C(6) position suggested that halogen not only contributes to the hydrophobic interactions but also provides electron-withdrawing characteristics that effect the electrostatic interaction of the 2-NH_2 group with the adenine binding site. As these benzyl analogues (**18a**

Scheme 4[a]



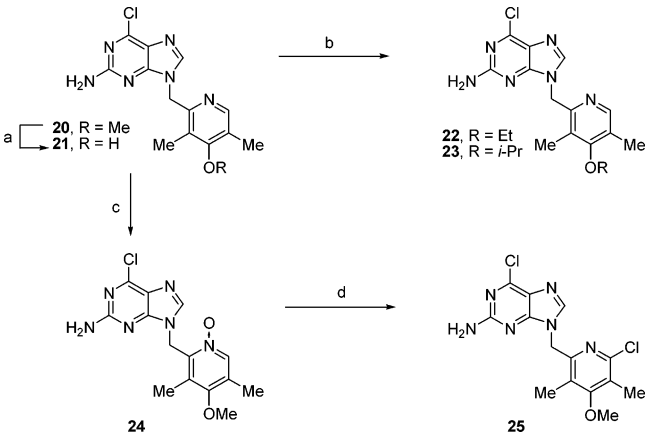
[a] Reagents and conditions: (a) 95% NH_2NH_2, reflux; (b) $SOCl_2$, DMF, 80 °C; (c) PPh_3, DIAD, MeOH, rt; (d) K_2CO_3, DMF, NaI, 2-amino-6-chloropurine, 40 °C.

Scheme 5[a]



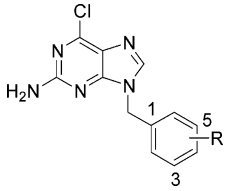
[a] Reagents and conditions: (a) NBS or NCS or NIS, CH_2Cl_2, rt; (b) NH_3 in MeOH, sealed-tube (**19a**); NaOMe, MeOH, 70 °C (**19b**); 1 N HCl, H_2O, 100 °C (**19c**); thiourea, EtOH, 80 °C (**19d**); $Pd[P(Ph)_3]_4$, Me_3Al, THF, 70 °C (**19f**).

Scheme 6[a]



[a] Reagents and conditions: (a) BBr_3, CH_2Cl_2, 0 °C to rt. (b) when R = H, CH_3I, or C_2H_5I, K_2CO_3, DMF, 40 °C; (c) when R = CH_3, *m*CPBA, CH_2Cl_2, 0 °C; (d) $SOCl_2$, 100 °C.

Table 1. Optimization of the N(9) Benzyl Substituents



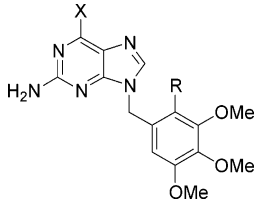
compd	R	HER-2 degradation IC_{50} (μM)[a]
26	2,5-dimethoxy	3.0
17	3,4,5-trimethoxy	2.0
18a	2-chloro-3,4,5-trimethoxy	0.05
18b	2-bromo-3,4,5-trimethoxy	0.07
18c	2-iodo-3,4,5-trimethoxy	0.34

[a] The IC_{50} values are the average of two or three replications and are within 30% agreement.

and **18b**) fulfilled our requirement of low nanomolar cellular activity, we turned our attention to optimizing the pharmacokinetic parameters of these analogues.

One important goal of this project was to develop an orally administrable compound. Accordingly, compounds were initially screened for solubility in serum and simulated gastric and intestinal fluids. Subsequently, the pharmacokinetic properties of the compounds were evaluated in mice at a dose of 100 mg/kg as an oral suspension formulated in PWD.[21] Serum samples were obtained during the first 2 h and concentrations were measured by HPLC fitted with a UV detector and quantified. Under these conditions, compound **18a** was observed only in very small amounts (<1000 ng/mL at 100 mg/kg dose) in the plasma. We attributed the poor oral absorption of **18a** to its low aqueous solubility (<1 μg/mL). Similar results were also observed with other benzyl derivatives, **18b** and **18c**. Having had limited success with N(9) benzyl substituted analogues, we then replaced the N(9)-benzyl group with pyridylmethyl, hoping that the pyridine ring would maintain the proper combination of aromaticity and lipophilicity while at the same time introducing aqueous solubility to the molecule. Replacement of the benzyl group of compound **18a** with a (3,5-dimethyl-4-methoxypyridine)methyl group gave compound **20**, which retained the potency (HER-2 IC_{50} of 0.03 μM). Having identified an active 9-heteroaryl moiety, we now focused our SAR studies on optimizing the potency by varying substituents on the heteroaryl ring. Starting with the 4′-position, demethylation of the 4′-OMe of compound **20** to 4′-OH compound **21** led to a substantial drop in potency (>3000-fold). Similarly, replacement of the methoxy group with an ethoxy (**22**) or isopropyloxy group resulted in a 50-fold potency loss (Table 3). In contrast, introduction of a bromo (**29**) or iodo (**30**) substituent showed a 2−3-fold improvement in potency. A chlorine atom had no effect on the potency. Although this hydrophobic binding region tolerated bulkier groups such as bromine and iodine, the 50-fold loss in potency observed with alkyl substituents such as ethyl (**22**) and isopropyl (**23**) indicated a linear space limitation for this domain. Further support for the presence of a hydrophobic region was indicated by the significant loss of

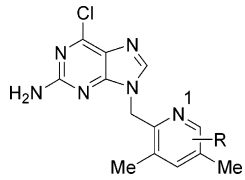
Table 2. Optimization of the C(6) Substituents



compd	R	X	HER-2 degradation IC_{50} (μM)[a]	compd	R	X	HER-2 degradation IC_{50} (μM)[a]
18b	Br	Cl	0.07	**19f**	Cl	Me	1.8
27	Br	Br	0.055	**19c**	Cl	OH	0.7
19a	Br	NH_2	17.0	**19d**	Br	SH	100.0
19b	Br	OMe	45.0	**19e**	Br	H	50.0

[a] The IC_{50} values are the average of two or three replications and are within 30% agreement.

Table 3. Optimization of the Pyridine Ring Substituents



compd	R	HER-2 degradation IC_{50} (μM)[a]	compd	R	HER-2 degradation IC_{50} (μM)[a]
17-AAG	−	0.007	**28**	4-Cl	0.038
PU24FCl	−	1.7	**29**	4-Br	0.015
3	−	0.09	**30**	4-I	0.009
16	−	0.02	**31**	4-NO_2	1.0
20	4-OMe	0.03	**32**	4-NH_2	5.80
21	4-OH	100.0	**33**	4-CN	0.99
22	4-OEt	1.5	**34**	4-Me	0.042
23	4-O-iPr	1.5	**35**	4-SMe	0.027
24	1-Oxa-4-OMe	0.08	**36**	4-S(O)Me	>10.0
25	4-OMe-6-Cl	0.09	**37**	4-H	7.5

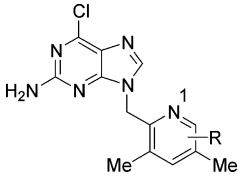
[a] The IC_{50} values are the average of two or three replications and are within 30% agreement.

activity observed for the 4′-unsubstituted analogue **37**. Strong electron-withdrawing or -donating 4′-substituents such as CN (**33**), $S(O)CH_3$ (**36**), NO_2 (**31**), or NH_2 (**32**) also resulted in significant loss in potency. As expected, small hydrophobic groups such as CH_3 (**34**) and SCH_3 (**35**) maintained the potency. To further probe the available space around the pyridyl ring, we prepared 6-chloro (**25**) and 1-oxide (**24**) analogues of compound **20**. The slight drop in the potency as compared to compound **20** indicates that the space around the pyridyl ring is limited.

To confirm that the degradation of HER-2 in cells occurs via Hsp90 inhibition, we analyzed the binding interaction of these analogues with Hsp90. The competitive binding assay using recombinant Hsp90 and a biotinylated geldanamycin probe showed that these compounds bound to Hsp90 within a narrow IC_{50} range from 0.7 to 0.9 μM. The insensitive nature of this assay—due to the absence of the full complement of Hsp90 cochaperones[4]—made it unsuitable to generate meaningful structure−activity relationships. As a result we switched to a cell lysate binding assay,[4] which comprised the full complement of cochaperones complexed with Hsp90 and provided high affinity binding activity. In this assay, our compounds showed excellent correlation between the cell lysate binding activity and the HER-2 degradation activity in cells (Table 4).

Selected compounds were also evaluated for their ability to inhibit the proliferation of two HER-2 expressing human breast

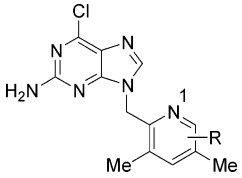
Table 4. In Vitro Binding Affinity



compd	R	IC_{50} (μM)[a] HER-2 degradation	rHsp90 binding	lysate binding
17-AAG	-	0.007	0.7	0.02
PU24FCl	-	1.7	6.0	2.5
3	-	0.09	0.9	0.15
16	-	0.02	0.9	0.018
20	4-OMe	0.03	0.9	0.02
24	1-oxa-4-OMe	0.08	0.8	0.03
25	4-OMe-6-Cl	0.09	NA[b]	0.025
28	4-Cl	0.035	NA	0.01
29	4-Br	0.015	NA	0.002
30	4-I	0.009	NA	0.003
34	4-Me	0.042	NA	0.035
35	4-SMe	0.027	NA	0.04
36	4-S(O)Me	>10.0	NA	10.0
37	4-H	7.5	NA	4.0

[a] The IC_{50} values are the average of two or three replications and are within 30% agreement. [b] NA: data not available.

Table 5. Cell Viability Data



compd	R	MTS IC_{50} (μM)[a] MCF7	BT474
17-AAG	−	0.01	0.01
PU24FCl	−	1.2	NA[b]
3	−	0.5	0.1
16	−	0.15	0.05
20	4-OMe	0.1	0.1
24	1-oxa-4-OMe	0.1	0.15
25	4-OMe-6-Cl	0.5	0.5
28	4-Cl	0.1	0.3
29	4-Br	0.03	0.05
30	4-I	0.03	0.03
34	4-Me	0.3	0.08
35	4-SMe	0.5	0.09

[a] The IC_{50} values are the average of two or three replications and are within 30% agreement. [b] NA: data not available.

cancer cell lines, MCF7 and BT474 (Table 5), using the MTS assay. The IC_{50}s ranged from 0.03 to 0.5 μM, and the results also correlated well with those obtained from the HER-2 degradation assay as well as with the Hsp90 cell lysate competitive binding assay.

A measure of in vitro therapeutic index for this class of compounds was determined by comparing their activity in normal and cancer cells using lysate binding[10a] and cell proliferation/cytotoxicity assays. Relative binding affinities for a subset of compounds were compared in cell lysates extracted from MCF7 breast cancer cells to cell lysates extracted from normal human dermal fibroblasts (NDF). The compounds exhibited 7−400-fold greater affinity for the Hsp90 complex in the cancer cell line over the normal cells (Table 6). It was gratifying to note that the higher affinity of binding to the activated Hsp90 complex in cancer cells translated to selective

Table 6. Cell Lysate Binding Selectivity in Normal Cells vs Tumor Cells

compd	IC_{50} (μM)[a] NDF	MCF7	fold selectivity
17-AAG	1.0	0.02	50
PU24FCl	18.0	2.5	7
20	0.4	0.020	20
29	0.8	0.002	400
30	1.0	0.003	333

[a] The IC_{50} values are the average of two or three replications and are within 30% agreement.

Table 7. Selective Antiproliferative Activity in Tumor Cells vs Normal Cells

compd	IC_{50} (μM)[a] RPTEC	HT29	MCF7	selectivity HT29	MCF7
17-AAG	0.1	0.002	0.01	50	10
20	0.6	0.13	0.1	5	6
28	5.0	0.5	0.1	10	50
29	1.3	0.13	0.03	10	43
30	0.15	0.023	0.03	7	5
35	2.3	0.2	0.5	12	5

[a] The IC_{50} values are the average of two or three replications and are within 30% agreement.

Table 8. Mouse Oral Pharmacokinetic Properties and Stability in HLM

compd[a]	C_{max} (μg/mL)	T_{max} (h)	AUC (μg h/mL)	$T_{1/2}$ (h)	HLM[b] % parent remaining
16	28.2	0.1	9.0	0.9	83
20	10.0	0.1	4.8	0.8	86

[a] Dose of 100 mg/kg as a 0.1 N aqueous HCl solution. [b] Drug incubated for 1 h in HLM (human liver microsomes) at 37 °C.

antiproliferative activity in human breast (MCF7) and colon (HT29) carcinoma cells over normal human renal proximal epithelial cells (RPTEC) (Table 7). In cell growth assays, the compounds were 5−50-fold selective in inhibiting cancer cell growth over that of even highly proliferative untransformed cells, suggesting that a reasonable therapeutic index could be expected in vivo.

As these compounds bound to the N-terminal ATP binding site of Hsp90, we were interested in evaluating the specificity for Hsp90 over other ATP binding proteins. The selectivity profile of compound **20** was tested at a single concentration of 10 μM in a small panel of kinases: aurora-A, CHK2, IKKα, MAPK1, MAPK2, MEK1, PDK1, Plk3, PI3k, c-Raf, and cSrc. No significant inhibition was observed on any of the targets after 1 h of incubation with 10 μM ATP concentration (data not shown).

Pharmacokinetic Studies. The compounds in Tables 3−5 that met our potency and binding criteria (<100 nM in both) were screened for their solubility in simulated gastric (pH 2.0) and intestinal (pH 6.5) fluids and in pooled normal mouse serum (pH 7.4). Compounds **16** and **20** showed good overall solubility (gastric, 451 and 240 μg/mL; intestinal, 234 and 87 μg/mL; and serum, 186 and 176 μg/mL, respectively) and hence were selected for the mouse in vivo pharmacokinetic evaluation. In the pharmacokinetic study Balb/C mice were administered a single 100 mg/kg oral dose of each compound as a 0.1 N aqueous HCl solution (pH 1.2). Blood samples were drawn at 5, 15, 30, 60, and 120 min, and total parent drug concentration was determined in serum by HPLC−UV. Terminal half-life and AUC values (Table 8) were estimated using a noncompartmental method (WinNonlin, Version 4.1). Both compounds **16** and **20** showed good oral exposure in mice.

Table 9. In Vivo Antitumor Activities of Selected Compounds in N87 Xenografts

compd	dose (mg/kg)	schedule	%TGI[a]	*t*-test (*p* value)
17-AAG[b]	90	ip qdx5	70	0.02
16[c]	60	po qdx5	83	0.001
20[c]	125	po qdx5	87	0.0001

[a] TGI, tumor growth inhibition. [b] 17-AAG was given intraperitoneally in PMSE. [c] Compound administered as a 0.1 N aqueous HCl solution.

These compounds were also screened for metabolic stability in human liver microsomes (HLM). Upon incubation with HLM, low intrinsic metabolism was observed with both compounds, which suggests potentially high oral bioavailability in humans.

In Vivo Antitumor Activity. On the basis of their encouraging pharmaceutics profiles, compounds **16** and **20** were selected for further evaluation in a mouse xenograft cancer model. The human stomach carcinoma N87 was selected for the evaluation due to its relatively high expression of HER-2, which is a sensitive Hsp90 client protein. Tumor fragments were implanted subcutaneously and treatment started once the tumor size reached 80−100 mm^3. Following once daily oral dosing of compound **16** at 60 mg/kg and compound **20** as a mesylate salt at 125 mg/kg on a weekly 5-day-on and 2-day-off schedule for 5 weeks, both compounds showed statistically significant tumor growth inhibition (Table 9). Both compounds were well-tolerated, exhibiting no treatment-related toxicity at these doses other than minimal weight loss (3−5%) over the course of the study.

Conclusion

In summary, we rationally designed and identified a novel 2-amino-6-halo-9-substituted purine scaffold that exhibits potent Hsp90 inhibitory activity. Compounds of this structural series also significantly bias the binding selectivity toward the activated form of the Hsp90 complex, which is reflected in increased tumor cell retention and, subsequently, more effective tumor cell killing. Among these highly selective inhibitors, compounds **16** and **20** displayed a superior overall profile as measured by in vitro potency, pharmaceutical properties, and in vivo oral efficacy. This new series presented an additional advantage in that it significantly simplified the synthetic chemistry by reducing the number of synthetic steps and avoided the difficult coupling reaction between 8-bromoadenine and the thiophenols.[22] More detailed biochemical, biological, pharmacokinetics, and pharmacodynamic characterizations of these compounds, as well as additional data from a range xenograft models will be published separately.[23,24]

Experimental Section

Chemistry. General Methods. All reactions were carried out with continuous stirring under an atmosphere of nitrogen. Commercial reagents and solvents were used as received without further purification or drying. ^1H and ^{13}C NMR spectra were obtained using a Bruker-400 spectrometer. Chemical shifts are reported in parts per million relative to tetramethylsilane as internal standard. Thin-layer chromatography (TLC) was performed on 250 μM silica gel plates (Whatman 4861-820). Flash chromatography was run using EM Science silica gel (230−400 mesh). Compound purity was determined by HPLC analysis using a C18 reverse phase column (Agilent Zorbax 300SB-C18; 5 μm; 4.6 mm × 150 mm) with an Agilent 1100 series system attached to a Hewlett-Packard chromatograph manager. A gradient was applied between solvent A (0.1% TFA in H_2O) and solvent B (0.5% TFA in CH_3CN) increasing the proportion of A linearly from 5% ($t = 0$) to 100%

($t = 7.00$ min), with a constant flow rate of 1 mL/min. The samples were diluted to 0.1−1 mg/mL in MeOH or CH_3CN and the injection volumes were typically 10 μL. Elemental analyses were obtained from Robertson Microlit Laboratories (Madison, NJ) and were within 0.4% of theoretical values.

2-Chloromethyl-4-chloro-3,5-dimethylpyridine (8). A suspension of **7**[15] (10 g, 58.3 mmol) in $POCl_3$ (30 mL, 551 mmol) was stirred at 110 °C for 1.5 h. The resulting mixture was cooled to room temperature and poured onto ice water and the pH was slowly adjusted to 10 with KOH. The compound was extracted into chloroform and dried over $MgSO_4$. The solvent was evaporated under reduced pressure and the crude product **8** (8.1 g, 74% recovered yield) was used in the next step without purification: HPLC t_R 5.54 min; ^{1}H NMR ($CDCl_3$) δ 8.24 (s, 1H), 4.71 (s, 2H), 2.48 (s, 3H), 2.36 (s, 3H).

4-Bromo-2-chloromethyl-3,5-dimethylpyridine (9). A neat mixture of **7**[15] (8.2 g, 47.8 mmol) and $POBr_3$ (60 g, 209 mmol) was stirred at 130 °C for 3 h. The resulting mixture was cooled to room temperature and poured onto ice water and the pH was slowly adjusted to 10 with KOH. The compound was extracted into chloroform and dried over $MgSO_4$. The solvent was evaporated under reduced pressure and the crude product was purified by flash chromatography to give **9** (8.7 g, 78%): HPLC t_R 6.03 min; ^{1}H NMR ($CDCl_3$) δ 8.20 (s, 1H), 4.62 (s, 2H), 2.50 (s, 3H), 2.38 (s, 3H).

2,3,5-Trimethyl-4-methylsulfanylpyridine *N*-Oxide (11a). A solution of **11c** (1.00 g, 4.63 mmol) in THF (25 mL) was treated in a pressure vessel with MeSNa (0.57 g, 8.13 mmol) at 110 °C for 16 h. The vessel was cooled to room temperature and the reaction mixture was transferred to a round-bottomed flask. The solvent was evaporated under reduced pressure and the crude product was purified by flash chromatography to give **11a** (0.74 g, 87%): HPLC t_R 5.30 min; ^{1}H NMR ($CDCl_3$) δ 8.07 (s, 1H), 2.57 (s, 3H), 2.52 (s, 3H), 2.42 (s, 3H), 2.23 (s, 3H).

2,3,5-Collidine *N*-Oxide (11b). A solution of **10** (6.10 g, 50 mmol) in dichloromethane (50 mL) was cooled in an ice bath, treated with *m*CPBA (19 g, 55 mmol) in three portions, and allowed to warm to room temperature. The mixture was extracted with dichloromethane, washed with saturated $NaHCO_3$ and water, and dried over $MgSO_4$ to give **11b** (4.8 g, 70%): HPLC t_R 3.96 min; ^{1}H NMR ($CDCl_3$) δ 8.03 (s, 1H), 6.90 (s, 1H), 2.47 (s, 3H), 2.31 (s, 3H), 2.24 (s, 3H); MS m/z 138.2 $(M + H)^+$; R_f (20% MeOH/EtOAc) 0.35.

4-Bromo-2,3,5-collidine *N*-Oxide (11c). To a suspension of **11b** (1.3 g, 10 mmol) and K_2CO_3 (2.9 g, 20 mmol) in 10 mL of CCl_4, was added dropwise 20 mL (20 mmol) of a 1 M bromine solution in CCl_4. The reaction mixture was heated at reflux for 2 h, cooled to room temperature, extracted with ethyl acetate, washed with saturated $NaHCO_3$ (10 mL) and then water, and dried over $MgSO_4$. The solvent was evaporated under reduced pressure and the crude product was purified by flash chromatography (10% MeOH/EtOAc) to afford **11c** (1.05 g, 51%): HPLC t_R 5.23 min; ^{1}H NMR ($CDCl_3$) δ 8.06 (s, 1H), 2.56 (s, 3H), 2.43 (s, 3H), 2.31 (s, 3H); R_f (20% MeOH/EtOAc) 0.45.

2,3,4,5-Tetramethylpyridine *N*-Oxide (11d). To a mixture of **11c** (2 g, 9.2 mmol) and tetrakis(triphenylphosphino)palladium (80 mg, 4 mol %) in 20 mL of dry THF was added a toluene solution of trimethylaluminum (2 M, 7.6 mL, 15.2 mmol) under nitrogen. The solution was heated at reflux for 4 h, diluted with toluene (20 mL), and quenched with 4 mL of methanol followed by 6 mL of aqueous ammonium chloride (24 mmol). The resulting mixture was further heated to reflux for 2 h, cooled, and filtered through Celite. The filtrate was concentrated, and the residue was taken into dichloromethane, washed with water, dried over $MgSO_4$, filtered, and concentrated to afford **11d** (0.8 g, 57%): HPLC t_R 4.18 min; ^{1}H NMR ($CDCl_3$) δ 8.05 (s, 1H), 2.55 (s, 3H), 2.27 (s, 3H), 2.22 (s, 3H), 2.20 (s, 3H).

2,3,5-Trimethyl-4-nitropyridine *N*-Oxide (11e). To a suspension of **11a** (3.77 g, 28 mmol) in concentrated H_2SO_4 (8 mL) cooled to 0 °C was slowly added dropwise fuming HNO_3 (5 mL, 100 mmol). The resulting solution was heated at 100 °C for 24 h and then cooled to room temperature, poured onto ice, and extracted with chloroform. The organic layer was washed with water followed by saturated $NaHCO_3$, dried over $MgSO_4$, filtered, and concentrated to give **11e** (4.89 g, 97%): R_f (MeOH/EtOAc 1:9) 0.7; HPLC t_R 4.75 min; ^{1}H NMR ($CDCl_3$) δ 8.08 (s, 1H), 2.50 (s, 3H), 2.27 (s, 3H), 2.23 (s, 3H); MS m/z 183.1 $(M + H)^+$.

2,3,5-Trimethylpyridin-4-ylamine *N*-Oxide Hydrochloride (11f). A suspension of **11e** (4.2 g, 23 mmol) in 60 mL of conc. HCl/EtOH (1:11) was hydrogenated over 10% Pd/C (0.42 g) at a 60 psi H_2 pressure for 3 h. The reaction mixture was filtered through Celite and evaporated to give **11f** as a light yellow solid (4.30 g, quantitative yield): HPLC t_R 4.75 min; ^{1}H NMR (DMSO-d_6) δ 8.28 (s, 1H), 7.24 (s, 2H), 2.50 (s, 3H), 2.12 (s, 3H), 2.11 (s, 3H); MS m/z 153.2 $(M + H)^+$.

4-Iodo-2,3,5-trimethylpyridine *N*-Oxide (11g). To a cold (5 °C) solution of **11f** (1.9 g, 10 mmol) and HBF_4 (3.6 mL, 20 mmol) in 50 mL of water was added a solution of $NaNO_2$ (1.04 g, 20 mmol) in 5 mL of water dropwise to give a dark yellow solution. Potassium iodide (2.3 g, 13.9 mmol) was slowly added in several portions to the suspension and the mixture turned dark brown. The reaction mixture was stirred at room temperature for an additional 5 min and then heated to 60 °C for 10 min. The mixture was cooled to room temperature and the pH was adjusted to 10 with KOH. The organics were extracted with chloroform, washed with saturated $NaHCO_3$ and then water, dried over $MgSO_4$, and concentrated to give **11g** (0.37 g, 14%): HPLC t_R 5.57 min; ^{1}H NMR ($CDCl_3$) δ 8.07 (s, 1H), 2.62 (s, 3H), 2.56 (s, 3H), 2.38 (s, 3H); MS m/z 264.1 $(M + H)^+$.

4-Cyano-2,3,5-trimethylpyridine *N*-Oxide (11h). Compound **11h** was prepared in 17% yield from **11f** and CuCN in HBF_4 following the procedure described for **11g** but replacing KI with CuCN: HPLC t_R 4.41 min; ^{1}H NMR ($CDCl_3$) δ 8.10 (s, 1H), 2.55 (s, 3H), 2.49 (s, 3H), 2.45 (s, 3H); MS m/z 162.2 $(M + H)^+$.

4-Methanesulfinyl-2,3,5-trimethylpyridine *N*-Oxide (11i). To solution of 2,3,5-trimethyl-4-methylsulfanylpyridine *N*-oxide **11a** (0.26 g, 1.4 mmol) in 10 mL of CH_2Cl_2 was added *m*CPBA (1.0 g, 4.1 mmol). After 10 min at room temperature the mixture was extracted with dichloromethane, washed with $NaHCO_3$ and water, and dried over $MgSO_4$. The solvent was removed and the residue was purified by flash chromatography (10% MeOH/EtOAc) to give **11i** (0.24 g, 86%): HPLC t_R 3.07 min; ^{1}H NMR ($CDCl_3$) δ 8.07 (s, 1H), 2.93 (s, 3H), 2.58 (s, 3H), 2.52 (s, 6H).

Acetic Acid 3,5-Dimethyl-4-methylsulfanylpyridin-2-ylmethyl Ester (12a). A solution of **11a** (240 mg, 1.3 mmol) in acetic anhydride (3 mL) was heated at reflux for 0.5 h. Excess acetic anhydride was evaporated under reduced pressure and the residue was purified by flash chromatography to afford **12a** (292 mg, quantitative yield): HPLC t_R 4.34 min; ^{1}H NMR ($CDCl_3$) δ 8.27 (s, 1H), 5.20 (s, 2H), 2.57 (s, 3H), 2.46 (s, 3H), 2.25 (s, 3H), 2.10 (s, 3H).

Acetic Acid 3,5-Dimethylpyridin-2-ylmethyl Ester (12b). The title compound **12b** was prepared in quantitative yield from compound **11b** (150 mg, 1.1 mmol) using the method described for the synthesis of **12a**: HPLC t_R 2.92 min; ^{1}H NMR ($CDCl_3$) δ 8.29 (d, $J = 1.2$ Hz, 1H), 7.33 (d, $J = 1.2$ Hz, 1H), 5.21 (s, 2H), 2.34 (s, 3H), 2.32 (s, 3H), 2.13 (s, 3H).

Acetic Acid 3,4,5-Trimethylpyridin-2-ylmethyl Ester (12d). The title compound **12d** was prepared in 46% yield from compound **11d** using the method described for the synthesis of **12a**: HPLC t_R 3.84 min; ^{1}H NMR ($CDCl_3$) δ 8.20 (s, 1H), 5.22 (s, 2H), 2.26 (s, 3H), 2.25 (s, 3H), 2.21 (s, 3H), 2.11 (s, 3H).

Acetic Acid 4-Iodo-3,5-dimethylpyridin-2-ylmethyl Ester (12g). The title compound **12g** was prepared in 50% yield from **11g** using the method described for the synthesis of **12a**: HPLC t_R 2.91 min; ^{1}H NMR ($CDCl_3$) δ 8.26 (s, 1H), 5.32 (s, 2H), 2.47 (s, 3H), 2.41 (s, 3H), 2.24 (s, 3H); MS m/z 306.0 $(M + H)^+$.

Acetic Acid 4-Cyano-3,5-dimethylpyridin-2-ylmethyl Ester (12h). The title compound **12h** was prepared in 63% yield from **11h** using the method described for the synthesis of **12a**: HPLC t_R 5.37 min; ^{1}H NMR ($CDCl_3$) δ 8.47 (s, 1H), 5.25 (s, 2H), 2.57 (s, 3H), 2.53 (s, 3H), 2.14 (s, 3H); MS m/z 205.0 $(M + H)^+$.

Acetic Acid 4-Methanesulfinyl-3,5-dimethylpyridin-2-ylmethyl Ester (12i). The title compound **12i** was prepared in 76% yield from **11i** using the method described for the synthesis of **12a**: HPLC t_R 3.74 min; ^{1}H NMR ($CDCl_3$) δ 8.30 (s, 1H), 5.24 (s, 2H), 2.90 (s, 3H), 2.57 (s, 3H), 2.56 (s, 3H), 2.13 (s, 3H).

2-Bromomethyl-3,5-dimethyl-4-methylsulfanylpyridine (13a). A mixture of **12a** (0.5 g, 2.2 mmol) and K_2CO_3 (0.57 g, 4.4 mmol) in methanol (5 mL) was stirred at 50 °C for 0.5 h. The solvent was evaporated and the residue was taken into chloroform and washed with water to give (3,5-dimethyl-4-methylsulfanylpyridin-2-yl)-methanol (0.4 g, quantitative yield): HPLC t_R 3.92 min. The crude mixture was used without any further purification.

A solution of (3,5-dimethyl-4-methylsulfanylpyridin-2-yl)methanol (0.4 g, 2.2 mmol) and triphenylphosphine (1.0 g, 4.0 mmol) in dichloromethane (6 mL) was cooled to 0 °C. A solution of CBr_4 (1.0 g, 3.0 mmol) in dichloromethane (2 mL) was added dropwise and the mixture was stirred for 1 h at room temperature. The solvent was evaporated under reduced pressure and the crude product was purified by flash chromatography to give **13a** (0.48 g, 90%): HPLC t_R 4.90 min; ^{1}H NMR ($CDCl_3$) δ 8.26 (s, 1H), 4.59 (s, 2H), 2.62 (s, 3H), 2.47 (s, 3H), 2.27 (s, 3H); MS *m*/*z* 246.13 $(M + H)^+$.

2-Bromomethyl-3,5-dimethylpyridine (13b). The title compound **13b** was prepared in quantitative yield from **12b** using the method described for the synthesis of **13a**: HPLC t_R 3.90 min; ^{1}H NMR ($CDCl_3$) δ 8.24 (d, $J = 1.2$ Hz, 1H), 7.30 (d, $J = 1.2$ Hz, 1H), 4.60 (s, 2H), 2.41 (s, 3H), 2.32 (s, 3H).

2-Bromomethyl-3,4,5-trimethylpyridine (13d). The title compound **13d** was prepared in 57% yield from **12d** using the method described for the synthesis of **13a**: HPLC t_R 3.97 min; ^{1}H NMR ($CDCl_3$) δ 8.18 (s, 1H), 4.63 (s, 2H), 2.35 (s, 3H), 2.48 (s, 3H), 2.24 (s, 3H).

2-Bromomethyl-3,5-dimethyl-4-nitropyridine (13e). The title compound **13e** was prepared in 53% yield from **12e** using the method described for the synthesis of **13a**: HPLC t_R 6.21 min; ^{1}H NMR ($CDCl_3$) δ 8.46 (s, 1H), 4.64 (s, 2H), 2.38 (s, 3H), 2.33 (s, 3H).

2-Bromomethyl-4-iodo-3,5-dimethylpyridine (13g). The title compound **13g** was prepared in 60% yield from **12g** using the method described for the synthesis of **13a**: HPLC t_R 5.95 min; ^{1}H NMR ($CDCl_3$) δ 8.14 (s, 1H), 4.67 (s, 2H), 2.59 (s, 3H), 2.45 (s, 3H); MS *m*/*z* 326.07 $(M + H)^+$.

2-Bromomethyl-4-cyano-3,5-dimethylpyridine (13h). The title compound **13h** was prepared in 30% yield from **12h** using the method described for the synthesis of **13a**: HPLC t_R 6.12 min; ^{1}H NMR ($CDCl_3$) δ 8.45 (s, 1H), 4.61 (s, 2H), 2.64(s, 3H), 2.55 (s, 3H).

2-Bromomethyl-4-methanesulfinyl-3,5-dimethylpyridine 1-Oxide (13i). The title compound **13i** was prepared in 46% yield (over two steps) from **12i** using the method described for the synthesis of **13a**: HPLC t_R 4.33 min. ^{1}H NMR ($CDCl_3$) δ 8.27 (s, 1H), 4.60 (s, 2H), 2.90 (s, 3H), 2.62 (s, 3H), 2.57 (s, 3H).

5-Chloromethyl-3-methoxy-2,4-dimethylpyridine (15). To a cold suspension of **14**[18] (11.6 g, 77.9 mmol), triphenylphosphine (26.8 g, 102 mmol), and MeOH (3.3 g, 100 mmol) in dry THF (150 mL) was slowly added a solution of diisopropyl azodicarboxylate (20.2 g, 100 mmol) in 80 mL of THF over a period of 0.5 h. After 2 h at room temperature, the solvent was removed under reduced pressure and ether (300 mL) was added to the residue to precipitate the triphenylphosphine oxide. The triphenylphosphine oxide was removed by filtration and the ether filtrate was subjected to an acid−base extraction to give 6.2 g of a crude brown solid. Filtration through a small plug of silica gel gave **15** (4.85 g, 45%): HPLC t_R 2.87; ^{1}H NMR ($CDCl_3$) δ 8.18 (s, 1H), 4.58 (s, 2H), 3.74 (s, 3H), 2.51 (s, 3H), 2.36 (s, 3H).

6-Chloro-9-(5-methoxy-4,6-dimethylpyridin-3-ylmethyl)-9*H*-purin-2-ylamine (16). A suspension of 2-amino-6-chloropurine (4.0 g, 236.0 mmol), compound **15** (4.65 g, 250.0 mmol), and K_2CO_3 (1.0 g, 72.0 mmol) in DMF (30 mL) was heated at 70 °C for 4 h under N_2 atmosphere. The solvent was evaporated under reduced pressure and the residue was extracted with EtOAc, washed with water, and dried over $MgSO_4$. The solvent was evaporated under reduced pressure and the crude product was purified by flash chromatography to give **16** (6.2 g, 82%): mp 210−215 °C; HPLC t_R 3.74 min; ^{1}H NMR ($CDCl_3$) δ 8.25 (s, 1H), 7.63 (s, 1H), 5.39 (s, 2H), 5.11 (s, 2H), 3.73 (s, 3H), 2.55 (s, 3H), 2.22 (s, 3H). Anal. ($C_{14}H_{15}ClN_6O$) C, H, N.

6-Chloro-9-(3,4,5-trimethoxybenzyl)-9*H*-purin-2-ylamine (17). A suspension of 2-amino-6-chloropurine (4.8 g, 28.4 mmol), 3,4,5-trimethoxybenzyl chloride (6.2 g, 28.7 mmol), and K_2CO_3 (1.0 g, 72.0 mmol) in DMF (30 mL) was heated at 70 °C for 4 h under N_2 atmosphere. The solvent was removed under reduced pressure and the residue was extracted with EtOAc, washed with water, and dried over $MgSO_4$. Evaporation and flash chromatography gave **17** (6.94 g, 70%): HPLC t_R 4.91 min; ^{1}H NMR ($CDCl_3$) δ 7.76 (s, 1H), 6.51 (s, 2H), 5.18 (s, 2H), 5.12 (s, 2H), 3.85 (s, 3H), 3.84 (s, 6H); HRMS calcd for $C_{15}H_{16}ClN_5O_3$ 348.0869 $(M - H)^-$, found 348.0867.

6-Chloro-9-(2-chloro-3,4,5-trimethoxybenzyl)-9*H*-purin-2-ylamine (18a). A solution of **17** (2.47 g, 7.0 mmol) and *N*-chlorosuccinimide (1.0 g, 7.5 mmol) in acetic acid (40 mL) was stirred at 90 °C for 0.5 h. The acetic acid was removed under reduced pressure and the residue was extracted with EtOAc and washed with water. Flash chromatography (3:1 EtOAc/hexanes) gave the title compound **18a** as a white solid (2.09 g, 77%); mp 154−155 °C; HPLC t_R 5.63 min; ^{1}H NMR ($CDCl_3$) δ 7.83 (s, 1H), 6.68 (s, 1H), 5.51 (s, 2H), 5.23 (s, 2H), 3.94 (s, 3H), 3.90 (s, 3H), 3.80 (s, 3H). Anal. ($C_{15}H_{15}Cl_2N_5O_3$) C, H, N.

6-Chloro-9-(2-bromo-3,4,5-trimethoxybenzyl)-9*H*-purin-2-ylamine (18b). To a solution of **17** (2.3 g, 6.6 mmol) in 1 M acetate buffer (30 mL), THF (60 mL), and MeOH (60 mL) was added bromine (1.4 g, 8.7 mmol) dropwise over a period of 5 min. The volatile solvents were removed under reduced pressure and the aqueous residue was extracted with EtOAc. Flash chromatography (3:1 EtOAc/hexanes) gave the title compound **18b** (2.08 g, 72%) as a white solid: mp 184−186 °C; HPLC t_R 5.74 min; ^{1}H NMR ($CDCl_3$) δ 7.85 (s, 1H), 6.66 (s, 1H), 5.32 (s, 2H), 5.11 (s, 2H), 3.90 (s, 3H), 3.87 (s, 3H), 3.76 (s, 3H). Anal. ($C_{15}H_{15}BrClN_5O_3$) C, H, N.

6-Chloro-9-(2-iodo-3,4,5-trimethoxybenzyl)-9*H*-purin-2-ylamine (18c). The title compound **18c** was prepared in 22% yield by halogenation of **17** with *N*-iodosuccinimide using the same procedure described for **18a**: mp 187−190 °C; HPLC t_R 5.89 min; ^{1}H NMR ($CDCl_3$) δ 7.87 (s, 1H), 6.67 (s, 1H), 5.33 (s, 2H), 5.22 (s, 2H), 3.91 (s, 3H), 3.88 (s, 3H), 3.77 (s, 3H); HRMS calcd for $C_{15}H_{15}ClIN_5O_3$ 475.9981 $(M + H)^+$, found 475.9960.

9-(2-Bromo-3,4,5-trimethoxybenzyl)-9*H*-purine-2,6-diamine (19a). A solution of **18b** (35 mg, 0.08 mmol) in MeOH was treated with 3 mL of 7 N NH_3 in MeOH in a pressure vessel at 90 °C for 16 h. The vessel was cooled to room temperature and the reaction mixture was transferred to a round-bottom flask. The solvent was evaporated under reduced pressure and the crude product was purified by preparative TLC (EtOAc:hexane 3:1) to give **19a** (16 mg, 48%): mp >250 °C; HPLC t_R 4.88 min; ^{1}H NMR (DMSO-d_6) δ 7.67 (s, 1H), 6.71 (s, 2H), 6.60 (s, 1H), 5.84 (s, 2H), 5.17 (s, 2H), 3.80 (s, 3H), 3.76 (s, 3H), 3.66 (s, 3H); HRMS calcd for $C_{15}H_{17}BrN_6O_3$ 409.0618 $(M + H)^+$, found 409.0621.

6-Methoxy-9-(2-bromo-3,4,5-trimethoxybenzyl)-9*H*-purin-2-ylamine (19b). A solution of **18b** (7.0 mg, 0.02 mmol) and MeO^-Na^+ (10.0 mg, 0.18 mmol) in MeOH (1 mL) was heated to reflux for 1 h. The mixture was quenched with 1 N NH_4Cl and the solvent was removed. The residue was taken into dichloromethane and washed with water, dried over $MgSO_4$, and concentrated. The crude product was purified by preparative TLC to give **19b** (3 mg): HPLC t_R 5.23 min; ^{1}H NMR ($CDCl_3$) δ 7.69 (s, 1H), 6.58 (s, 1H), 5.33 (s, 2H), 4.88 (s, 2H), 4.11 (s, 3H), 3.93 (s, 3H), 3.89 (s, 3H), 3.74 (s, 3H); HRMS calcd for $C_{16}H_{18}BrN_5O_4$ 424.0615 $(M + H)^+$, found 424.0600.

2-Amino-9-(2-chloro-3,4,5-trimethoxybenzyl)-9*H*-purin-6-ol (19c). A suspension of **18a** (37 mg, 0.1 mmol) in 1 N HCl (2 mL) was heated at reflux for 2 h, at which point the mixture became homogeneous. The solvent was evaporated under reduced pressure and the solid was washed with EtOAc to give **19c** (30 mg, 85%):

mp 215−230 °C; HPLC t_R 4.60 min; ^{1}H NMR (DMSO-d_6) δ 10.61 (s, 1H), 7.82 (s, 1H), 6.65 (s, 1H), 6.60 (s, 2H), 5.15 (s, 2H), 3.81 (s, 3H), 3.77 (s, 3H), 3.69 (s, 3H); HRMS calcd for $C_{15}H_{16}ClN_5O_4$ 366.0964 $(M + H)^+$, found 366.0955.

6-Mercapto-9-(2-bromo-3,4,5-trimethoxybenzyl)-9*H*-purin-2-ylamine (19d). A suspension of **27** (0.3 g, 0.6 mmol) and thiourea (0.2 g, 2.6 mmol) in EtOH (5 mL) was heated at reflux for 1 h. The reaction mixture was cooled to room temperature and the solvent was removed under reduced pressure and the solid residue was washed with water and EtOAc to give **19d** (0.17 g, 63%): mp 200−215 °C; HPLC t_R 4.93 min; ^{1}H NMR (DMSO-d_6) δ 11.95 (s, 1H), 7.82 (s, 1H), 6.85 (s, 2H), 6.60 (s, 1H), 5.15 (s, 2H), 3.80 (s, 3H), 3.78 (s, 3H), 3.71 (s, 3H). Anal. ($C_{15}H_{16}BrN_5O_3S$) C, H, N.

9-(2-Bromo-3,4,5-trimethoxybenzyl)-9*H*-purin-2-ylamine (19e). A suspension of 2-aminopurine (100 mg, 1.1 mmol), 3,4,5-trimethoxybenzyl chloride (200 mg, 1.1 mmol), and K_2CO_3 (100 mg, 7.2 mmol) in DMF (3 mL) was heated at 70 °C for 4 h under a nitrogen atmosphere. The solvent was evaporated under reduced pressure and the residue was extracted with EtOAc, washed with water, and dried over $MgSO_4$. Evaporation and flash chromatography gave 9-(3,4,5-trimethoxybenzyl)-9*H*-purin-2-ylamine (120 mg, 36%): HPLC t_R 5.06 min; ^{1}H NMR ($CDCl_3$) δ 8.74 (s, 1H), 7.74 (s, 1H), 5.52 (s, 2H), 5.20 (s, 2H), 5.04 (s, 2H), 3.85 (s, 3H), 3.83 (s, 6H).

The title compound **19e** (17 mg, 27%) was prepared by halogenation of 9-(3,4,5-trimethoxybenzyl)-9*H*-purin-2-ylamine (50 mg, 0.2 mmol) with bromine (75 mg, 0.5 mmol) using the procedure described for **18b**: mp 204−205 °C; HPLC t_R 6.69 min; ^{1}H NMR ($CDCl_3$) δ 8.70 (s, 1H), 7.85 (s, 1H), 6.66 (s, 1H), 5.34 (s, 2H), 5.11 (s, 2H), 3.91 (s, 3H), 3.88 (s, 3H), 3.75 (s, 3H). Anal. ($C_{15}H_{17}N_5O_3$) C, H, N.

6-Methyl-9-(2-chloro-3,4,5-trimethoxybenzyl)-9*H*-purin-2-ylamine (19f). To a mixture of **18a** (72 mg, 0.2 mmol) and tetrakis-(triphenylphosphino)palladium (22 mg, 0.02 mmol) in 20 mL of dry THF was added 0.22 mL (0.44 mmol) of a 2 M solution of trimethylaluminum in toluene under nitrogen. The solution was heated at reflux for 4 h, diluted with toluene (5 mL), and quenched with 0.5 mL of methanol followed by ammonium chloride (1 mmol of 1.0 N solution). The resultant mixture was heated at reflux for 2 h and the hot mixture was filtered through Celite. The filtrate was concentrated, and the residue was taken into dichloromethane, washed with water, dried over $MgSO_4$, filtered, and concentrated to afford **19f** (34 mg, 50%): mp 163−164 °C; HPLC t_R 4.80 min; ^{1}H NMR ($CDCl_3$) 7.82 (s, 1H), 6.65 (s, 1H), 5.35 (s, 2H), 5.10 (br s, 2H), 3.95 (s, 3H), 3.90 (s, 3H), 3.75 (s, 3H), 2.65 (s, 3H). Anal. ($C_{16}H_{18}ClN_5O_3 \cdot 0.3HCl$) C, H, N.

6-Chloro-9-(4-methoxy-3,5-dimethylpyridin-2-ylmethyl)-9*H*-purin-2-ylamine (20). A suspension of 2-amino-6-chloropurine (21.77 g, 128 mmol), K_2CO_3 (53.5 g, 387 mmol), NaI (1.92 g, 12.8 mmol), and compound **6** (29.9 g, 128 mmol) in DMF (650 mL) was heated at 40 °C with stirring under nitrogen. After 6 h, the reaction mixture was cooled and the inorganic solids were filtered and washed with DMF. Dilution with 700 mL of water induced the crystallization of the desired isomer **20** (21.8 g, 63%): R_f 0.20 (EtOAc); mp 192−193 °C; ^{1}H NMR (DMSO-d_6) 8.08 (s, 1H), 8.02 (s, 1H), 6.84 (s, 2H), 5.36 (s, 2H), 3.74 (s, 3H), 2.30 (s, 3H), 2.16 (s, 3H); ^{13}C NMR (DMSO-d_6) 163.81, 160.23, 155.01, 153.26, 149.65, 149.19, 144.65, 125.61, 123.71, 123.64, 60.30, 45.69, 13.29, and 10.65. Anal. ($C_{14}H_{15}ClN_6O$) C, H, N.

2-(2-Amino-6-chloropurin-9-ylmethyl)-3,5-dimethylpyridin-4-ol (21). The title compound **21** (0.14 g, 47%) was prepared by alkylation of 2-amino-6-chloropurine (0.17 g, 1.0 mmol) with 2-chloromethyl-4-hydroxy-3,5-dimethylpyridine **7**[15] (0.2 g, 1.2 mmol) as the alkylating agent using the procedure described for the synthesis of **17**: mp 255−270 °C; HPLC t_R 3.64 min; ^{1}H NMR (DMSO-d_6) δ 10.95 (s, 1H), 8.09 (s, 1H), 7.51 (s, 1H), 6.93 (s, 2H), 5.25 (s, 2H), 2.03 (s, 3H), 1.89 (s, 3H). Anal. ($C_{13}H_{13}ClN_6O \cdot 0.3H_2O$) C, H, N.

6-Chloro-9-(4-ethoxy-3,5-dimethylpyridin-2-ylmethyl)-9*H*-purin-2-ylamine (22). To a suspension of **21** (50 mg, 0.16 mmol) and K_2CO_3 (67 mg, 0.5 mmol) in DMF (5 mL) was added ethyl iodide (31 mg, 0.2 mmol) dropwise. After stirring at room temperature for 0.5 h, the mixture was diluted with ethyl acetate, washed with water, and dried over $MgSO_4$. The solvent was evaporated under reduced pressure and the crude product was purified by flash chromatography to give **22** (26 mg, 50%): mp 200−210 °C; HPLC t_R 4.32 min; ^{1}H NMR ($CDCl_3$) δ 8.21 (s, 1H), 7.90 (s, 1H), 5.34 (s, 2H), 5.12 (s, 2H), 3.90 (q, 2H), 2.31 (s, 3H) 2.26 (s, 3H), 1.44 (t, 3H). Anal. ($C_{15}H_{17}ClN_6O \cdot 0.1\ H_2O$) C, H, N.

6-Chloro-9-(4-isopropoxy-3,5-dimethylpyridin-2-ylmethyl)-9*H*-purin-2-ylamine (23). The title compound **23** was obtained by the reaction of **21** (50 mg, 0.16 mmol), K_2CO_3 (67 mg, 0.5 mmol), and isopropyl iodide (34 mg, 0.2 mmol) in DMF (3 mL) followed by purification by flash chromatography in the similar manner described for **22** (22 mg, 40%): mp 177−178 °C; HPLC t_R 4.57 min; ^{1}H NMR ($CDCl_3$) δ 8.17 (s, 1H), 7.89 (s, 1H), 5.32 (s, 2H), 5.06 (s, 2H), 4.20 (sept., J = 6.2 Hz, 1H), 2.26 (s, 3H) 2.22 (s, 3H), 1.28−1.30 (d, J = 6.2 Hz, 6H). Anal. ($C_{16}H_{19}ClN_6O$) C, H, N.

6-Chloro-9-(4-methoxy-3,5-dimethyl-*N*-oxypyridin-2-ylmethyl)-9*H*-purin-2-ylamine (24). A solution of **20** (4.2 g, 13.0 mmol) in dichloromethane (100 mL) was cooled with an ice-bath, treated with *m*CPBA (4.2 g, 18.3 mmol) in three portions, and allowed to warm to room temperature. The mixture was extracted with dichloromethane, washed with saturated $NaHCO_3$ (10 mL), and dried over $MgSO_4$. The solvent was evaporated under reduced pressure and the crude product was purified by flash chromatography to give **24** (3.1 g, 69%): mp 190−200 °C; HPLC t_R 4.43 min; ^{1}H NMR ($CDCl_3$) δ 8.55 (s, 1H), 8.06 (s, 1H), 5.52 (s, 2H), 5.07 (s, 2H), 3.76 (s, 3H), 2.61 (s, 3H), 2.25 (s, 3H). Anal. ($C_{14}H_{17}ClN_6O_2 \cdot 0.1H_2O$) C, H, N.

6-Chloro-9-(6-chloro-4-methoxy-3,5-dimethylpyridin-2-ylmethyl)-9*H*-purin-2-ylamine (25). A suspension of **24** (0.5 g, 1.5 mmol) in $POCl_3$ (5 mL) was heated at 90 °C for 2 h. The resulting mixture was cooled to room temperature and poured onto ice water and the pH was slowly adjusted to 10 with 1 N KOH. The compound was extracted into chloroform and dried over $MgSO_4$. The solvent was evaporated under reduced pressure to give 2**5** (90 mg, 24%): mp 212−213 °C; HPLC t_R 5.74 min; ^{1}H NMR ($CDCl_3$) δ 7.92 (s, 1H), 5.28 (s, 2H), 5.06 (s, 2H), 3.73 (s, 3H), 2.29 (s, 3H), 2.28 (s, 3H). Anal. ($C_{14}H_{14}Cl_2N_6O$) C, H, N.

6-Chloro-9-(2,5-dimethoxybenzyl)-9*H*-purin-2-ylamine (26). The title compound **26** (0.7 g, 70%) was prepared by alkylation of 2-amino-6-chloropurine (0.5 g, 3.0 mmol) with chloromethyl-2,5-dimethoxyphenyl (0.6 g, 3.2 mmol) as the alkylating agent using the procedure described for **17**: HPLC t_R 5.29 min; ^{1}H NMR ($CDCl_3$) δ 7.82 (s, 1H), 6.85−6.84 (d, 1H), 6.82−6.82 (d, 2H), 5.18 (s, 2H), 5.16 (s, 2H), 3.80 (s, 3H), 3.75 (s, 3H). Anal. ($C_{14}H_{14}ClN_5O_2$) C, H, N.

6-Bromo-9-(2-bromo-3,4,5-trimethoxybenzyl)-9*H*-purin-2-ylamine (27). The title compound **27** (0.62 g, 73%) was prepared by halogenation of 6-bromo-9-(3,4,5-trimethoxybenzyl)-9*H*-purin-2-ylamine (0.7 g, 1.8 mmol) with bromine (0.6 g, 3.9 mmol) using the procedure described for **18b**: HPLC t_R 5.79 min; ^{1}H NMR ($CDCl_3$) δ 7.88 (s, 1H), 6.68 (s, 1H), 5.33 (s, 2H), 5.19 (s, 2H), 3.93 (s, 3H), 3.90 (s, 3H), 3.79 (s, 3H); HRMS calcd for $C_{15}H_{15}Br_2N_5O_3$ 471.9614 $(M + H)^+$, found 471.9619.

6-Chloro-9-(4-chloro-3,5-dimethylpyridin-2-yl methyl)-9*H*-purin-2-ylamine (28). The title compound **28** (11.7 g, 88%) was prepared by alkylation of 2-amino-6-chloropurine (7 g, 41 mmol) with **8** (8.2 g, 43 mmol) as the alkylating agent using the procedure described for **17**: HPLC t_R 5.17 min; mp 229−235 °C; ^{1}H NMR ($CDCl_3$) δ 8.24 (s, 1H), 7.90 (s, 1H), 5.40 (s, 2H), 5.07 (s, 2H), 2.49 (s, 3H), 2.37 (s, 3H); HRMS calcd for $C_{13}H_{12}Cl_2N_6$ 321.0428 $(M - H)^-$, found 321.0420.

6-Chloro-9-(4-bromo-3,5-dimethylpyridin-2-ylmethyl)-9*H*-purin-2-ylamine (29). The title compound **29** (0.6 g, 76%) was prepared by alkylation of 2-amino-6-chloropurine (0.36 g, 2.1 mmol) with **9** (0.55 g, 2.4 mmol) as the alkylating agent using the procedure described for **17**: mp 215−225 °C; HPLC t_R 5.30 min; ^{1}H NMR ($CDCl_3$) δ 8.19 (s, 1H), 7.88 (s, 1H), 5.41 (s, 2H), 5.06 (s, 2H), 2.53 (s, 3H), 2.39 (s, 3H). Anal. ($C_{13}H_{12}BrClN_6$) C, H, N.

6-Chloro-9-(4-iodo-3,5-dimethylpyridin-2-ylmethyl)-9*H*-purin-2-ylamine (30). The title compound **30** (320 mg, 70%) was prepared by alkylation of 2-amino-6-chloropurine (190 mg, 1.1 mmol) with **13g** (600 mg, 1.6 mmol) as the alkylating agent using the same procedure described for **14**: mp 195−220 °C; HPLC t_R 5.36 min; ^{1}H NMR ($CDCl_3$) δ 8.08 (s, 1H), 7.86 (s, 1H), 5.41 (s, 2H), 5.04 (s, 2H), 2.57 (s, 3H), 2.41 (s, 3H). Anal. ($C_{13}H_{12}ClIN_6$) C, H, N.

6-Chloro-9-(3,5-dimethyl-4-nitropyridin-2-ylmethyl)-9*H*-purin-2-ylamine (31). The title compound **31** (460 mg, 69%) was prepared by alkylation of 2-amino-6-chloropurine (350 mg, 2.0 mmol) with **13e** (570 mg, 2.3 mmol) as the alkylating agent using the procedure described for **17**: mp 240−250 °C; HPLC t_R 6.21 min; ^{1}H NMR ($CDCl_3$) δ 8.40 (s, 1H), 7.94 (s, 1H), 5.40 (s, 2H), 5.05 (s, 2H), 2.40 (s, 3H), 2.27 (s, 3H). Anal. ($C_{13}H_{12}ClN_7O_2$) C, H, N.

6-Chloro-9-(3,5-dimethyl-4-aminopyridin-2-ylmethyl)-9*H*-purin-2-ylamine (32). A suspension of **31** (200 mg, 0.6 mmol) and an excess of sodium hydrosulfite (310 mg, 3.0 mmol) in methanol was stirred for 2 days at room temperature. The inorganic salts were filtered through Celite and concentrated, and the residue was purified by preparative TLC (100% EtOAc) to give **32** (73 mg, 40%): HPLC t_R 3.54 min; ^{1}H NMR ($CDCl_3$) δ 8.05 (s, 1H), 7.83 (s, 1H), 5.31 (s, 2H), 5.05 (s, 2H), 4.08 (s, 2H), 2.12 (s, 6H); HRMS calcd for $C_{13}H_{14}ClN_7$ 304.1072 $(M + H)^+$, found 304.1071.

6-Chloro-9-(3,5-dimethyl-4-cyanopyridin-2-ylmethyl)-9*H*-purin-2-ylamine (33). The title compound **33** (16 mg, 57%) was prepared by alkylation of 2-amino-6-chloropurine (16 mg, 0.1 mmol) with **13h** (20 mg, 0.09 mmol) as the alkylating agent using the procedure described for **17**: mp 200−220 °C; HPLC t_R 5.19 min; ^{1}H NMR ($CDCl_3$) δ 8.41 (s, 1H), 7.94 (s, 1H), 5.40 (s, 2H), 5.05 (s, 2H), 2.70 (s, 3H), 2.53 (s, 3H); HRMS calcd for $C_{14}H_{12}ClN_7$ 312.0076 $(M - H)^-$, found 312.0766.

6-Chloro-9-(3,4,5-trimethylpyridin-2-ylmethyl)-9*H*-purin-2-ylamine (34). The title compound **34** (43 mg, 21%) was prepared by alkylation of 2-amino-6-chloropurine (110 mg, 0.65 mmol) with **13d** (150 mg, 0.7 mmol) as the alkylating agent using the same procedure described for **17**: mp 200−220 °C; HPLC t_R 3.90 min; ^{1}H NMR ($CDCl_3$) δ 8.18 (s, 1H), 7.84 (s, 1H), 5.38 (s, 2H), 5.08 (s, 2H), 2.29 (s, 3H), 2.27 (s, 3H), 2.22 (s, 3H). Anal. ($C_{14}H_{15}ClN_6$) C, H, N.

6-Chloro-9-(3,5-dimethyl-4-methylsulfanylpyridin-2-ylmethyl)-9*H*-purin-2-ylamine (35). The title compound **35** (36 mg, 67%) was prepared by alkylation of 2-amino-6-chloropurine (30 mg, 0.18 mmol) with **13a** (40 mg, 0.16 mmol) as the alkylating agent using the procedure described for **17**: mp 170−180 °C; HPLC t_R 4.61 min; ^{1}H NMR ($CDCl_3$) δ 8.24 (s, 1H), 7.87 (s, 1H), 5.36 (s, 2H), 5.00 (s, 2H), 2.61 (s, 3H), 2.47 (s, 3H), 2.26 (s, 3H). Anal. ($C_{14}H_{15}ClN_6S$) C, H, N.

6-Chloro-9-(4-methanesulfinyl-3,5-dimethylpyridin-2-ylmethyl)-9*H*-purin-2-ylamine (36). The title compound **36** (12 mg, 38%) was prepared by alkylation of 2-amino-6-chloropurine (16 mg, 0.1 mmol) with **13i** (21 mg, 0.09 mmol) as the alkylating agent using the procedure described for **17**: mp 190−200 °C; HPLC t_R 4.02 min; ^{1}H NMR ($CDCl_3$) δ 8.23 (s, 1H), 7.91 (s, 1H), 5.39 (s, 2H), 5.09 (s, 2H), 2.90 (s, 3H), 2.68 (s, 3H), 2.54 (s, 3H); HRMS calcd for $C_{14}H_{15}ClN_6OS$ 349.0644 $(M - H)^-$, found 349.0643.

6-Chloro-9-(3,5-dimethylpyridin-2-ylmethyl)-9*H*-purin-2-ylamine (37). The title compound **37** (42 mg, 69%) was prepared by alkylation of 2-amino-6-chloropurine (30 mg, 0.18 mmol) with **13b** (40 mg, 0.16 mmol) as the alkylating agent using the procedure described for **17**; mp 180−190 °C; HPLC t_R 3.76 min; ^{1}H NMR ($CDCl_3$) δ 8.21 (d, $J = 1.4$ Hz, 1H), 7.89 (s, 1H), 7.30 (d, $J = 1.4$ Hz, 1H), 5.32 (s, 2H), 5.05 (s, 2H), 2.36 (s, 3H), 2.29 (s, 3H). Anal. ($C_{13}H_{13}ClN_6$) C, H, N.

Biochemical Assays. rHsp90 Competitive Binding Assay. Five micrograms of purified rHsp90 protein (Stressgen, BC, Canada, #SPP-770) in phosphate-buffered saline (PBS) was coated on 96-well plates by incubating overnight at 4 °C. Unbound protein was removed, and the coated wells were washed twice with 200 μL of PBS. DMSO controls (considered as untreated samples) or test compounds were then added at 100, 30, 10, 3, 1, or 0.3 μM dilutions (in PBS), and the plates were mixed for 30 s on the plate shaker and then incubated for 1 h at 37 °C. The wells were washed twice with 200 μL of PBS, biotinylated-geldanamycin (biotin−GM) was added, and the plates were incubated for 1 h at 37 °C. The wells were washed again twice with 200 μL of PBS before the addition of 20 μg/mL streptavidin−phycoerythrin (PE) (Molecular Probes, Eugene, OR) and incubation for 1 h at 37 °C. The wells were washed again twice with 200 μL of PBS. The relative fluorescence units (RFU) were measured using a SpectraMax Gemini XS spectrofluorometer (Molecular Devices, Sunnyvale, CA) with an excitation of 485 nm and emission of 580 nm; data were acquired using SOFTmaxPRO software (Molecular Devices Corp., Sunnyvale, CA). The background was defined as the RFU generated from wells that were not coated with Hsp90 but were treated with the biotin−GM and streptavidin−PE. Percent inhibition of binding for each sample was calculated from the background subtracted values as follows: % binding inhibition = [untreated RFU − sample RFU]/untreated RFU] × 100. IC_{50} was defined as the concentration of the compound at which there was 50% inhibition of the biotin−GM binding to rHsp90.

Her-2 Degradation Assay. MCF7 breast carcinoma cells (ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 10 mM HEPES and plated in 24-well plates (50% confluent). Twenty-four hours later (cells are 65−70% confluent), test compounds were added at 1000 nM and six half-log dilutions down to 3 nM and incubated overnight for 16 h. The wells were washed with 1 mL of PBS, and 200 μL trypsin was added to each well. After trypsinization was complete, 50 μL of FBS was added to each well. Then 200 μL of cells was transferred to 96-well plates The cells were pipetted up and down to obtain a single cell suspension. The plates were centrifuged at 2500 rpm for 1 min using a Sorvall Legend RT tabletop centrifuge (Kendro Laboratory Products, Asheville, NC). The cells were then washed once in PBS containing 0.2% BSA and 0.2% sodium azide (BA buffer). PE conjugated anti-Her-2/neu antibody (Becton Dickinson, #340552) or PE conjugated anti-keyhole limpet hemacyanin (KLH) (Becton Dickinson, #340761) control antibody was added at a dilution of 1:20 and 1:40, respectively (final concentration was 1 μg/mL), and the cells were pipeted up and down to form a single cell suspension and incubated for 15 min. The cells were washed twice with 200 μL of BA buffer, resuspended in 200 μL of BA buffer, and transferred to FACSCAN tubes with an additional 250 μL of BA buffer. Samples were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) equipped with argon ion laser that emits 15 mW of 488-nm light for excitation of the phycoerythrin fluorochrome; 10 000 events were collected per sample. A fluorescence histogram was generated and the mean fluorescence intensity (MFI) of each sample was determined using Cellquest software. The background was defined as the MFI generated from cells incubated with control IgG-PE and was subtracted from each sample stained with the Her-2/neu antibody. Cells were always incubated with DMSO as untreated controls, since the compounds were resuspended in DMSO. Percent degradation of Her-2 was calculated as follows: % Her-2 degradation = (MFl Her-2 untreated cells − MFI Her-2 sample)/(MFI Her-2 untreated cells) × 100. EC_{50} was defined as the concentration of the compound at which there was 50% degradation of the Her-2/neu protein.

Cell Lysate Binding Assay. MCF7 cell lysates were prepared by douncing in lysis buffer (20 mM Hepes, pH 7.3, 1 mM EDTA, 5 mM $MgCl_2$, 100 mM KCl) and then incubating with or without test compound for 0.5 h at 4 °C, followed by incubation with biotin−GM linked to BioMag streptavidin magnetic beads (Qiagen) for 1 h at 4 °C. Tubes were placed on a magnetic rack, and the unbound supernatant was removed. The magnetic beads were washed three times in lysis buffer and boiled for 5 min at 95° C in SDS−PAGE sample buffer. Samples were analyzed on SDS protein gels, and Western blots were done for rHsp90. Bands in the Western blots were quantitated using a Bio-Rad Fluor-S MultiImager, and the percent inhibition of binding of rHsp90 to the biotin−GM was

calculated. The IC_{50} reported is the concentration of test compound needed to cause half-maximal inhibition of binding.

MTS Assay. Measurement of Cytotoxicity of HSP90 Inhibitors. Cells were seeded in 96-well plates at 2000 cells/well and allowed to adhere overnight in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The final culture volume was 100 μL. Viable cell number was determined by using the Celltiter 96 AQ_{ueous} Non-radioactive Cell Proliferation Assay (Promega, Madison WI). The MTS/PMS (phenazine methosulfate) solution was mixed at a ratio of 20:1, and 20 μL was added per well to 100 μL of culture medium. MTS is a tetrazolium dye that is converted to a formazan product by dehydrogenase enzymes of metabolically active cells, which is measured at 490 nm absorbance after 2−4 h using a multiwell plate spectrophotometer. Background was determined by measuring the 490 nm absorbance of cell culture medium and MTS−PMS in the absence of cells. The background value was subtracted from all values. Percent viable cells was calculated as follows: % viable cells = $(A_{490\ sample}/A_{490\ untreated}) \times 100$. IC_{50} was defined as the concentration that gave rise to 50% viable cell number.

In Vivo Studies. Mouse PK Study. Six to eight week old Balb/C and nu/nu athymic female mice were obtained from Harlan Sprague Dawley (Indianapolis, IN). The mice were maintained in sterilized filter-topped cages or ventilated caging in a room with a 12 h light/dark cycle. Irradiated pelleted food (Harlan Teklad #7912) and autoclaved deionized water were provided ad libitum. Animals were identified by the use of individually numbered ear tags. Experiments were carried out under institutional guidelines for the proper and human use of animals in research established by the Institute for Laboratory Animal Research (ILAR). The test compounds were formulated (10 mg/mL) and a single dose was administered orally (po) at a dose volume of 10 mL/kg. Blood was collected from the retroorbital sinus in a 75 mm capillary tube. The blood was transferred to 1.5 mL conical tube and allowed to clot. Serum was stored at −20 °C until analysis.

Mouse Tumor Study. Tumor fragments (approximately 2 mm^3) or 5×10^6 tumor cells were inoculated subcutaneously in the right or left flank of the animal. Mice with established tumors (50−200 mm^3) were selected for study ($n = 7-10$/treatment group). Tumor dimensions were measured using calipers, and tumor volumes were calculated using the equation for an ellipsoid sphere $(l \times w^2)/2 =$ mm^3, where l and w refer to the larger and smaller dimensions collected at each measurement. The test compounds were formulated and administered orally (po) at a dose volume of 10 mL/kg. The vehicle alone was administered to control groups. Animals were dosed 5 days per week (Monday through Friday) for 4−6 consecutive weeks. Animals were weighed and the tumors were measured twice per week. Mice were followed until tumor volumes in the control group reached approximately 1000 mm^3 and were sacrificed by CO_2 euthanasia. The mean tumor volumes of each group were calculated. The change in mean treated tumor volume was divided by the change in mean control tumor volume, multiplied by 100 and subtracted from 100% to give the tumor growth inhibition for each group. Statistical analysis was performed using the standard *t*-test and using GraphPad Prism Software.

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