Discovery of (2,4-Dihydroxy-5-isopropylphenyl)-[5-(4-methylpiperazin-1-ylmethyl)-1,3-dihydroisoindol-2-yl]methanone (AT13387), a Novel Inhibitor of the Molecular Chaperone Hsp90 by Fragment Based Drug Design[†]

Andrew J. Woodhead,*^{,*} Hayley Angove,[§] Maria G. Carr,[‡] Gianni Chessari,^{II} Miles Congreve,[‡] Joseph E. Coyle,[§] Jose Cosme,[⊥] Brent Graham,[#] Philip J. Day,[∞] Robert Downham,[‡] Lynsey Fazal,[⊥] Ruth Feltell,[#] Eva Figueroa,[‡] Martyn Frederickson,[‡] Jonathan Lewis,[⊥] Rachel McMenamin,[#] Christopher W. Murray,^{II} M. Alistair O'Brien,[‡] Lina Parra,[⊥] Sahil Patel,[∞] Theresa Phillips,[‡] David C. Rees,[‡] Sharna Rich,[#] Donna-Michelle Smith,[⊥] Gary Trewartha,[‡] Mladen Vinkovic,[∞] Brian Williams,[‡] and Alison J.-A. Woolford[‡]

^{*}Medicinal Chemistry, [§]Biophysics, [¶]Computational Chemistry and Informatics, [⊥]DMPK, [#]Biology, and [∞]Structural Biology, Astex Therapeutics, Ltd., 436 Cambridge Science Park, Milton Road, Cambridge CB4 0QA, U.K.

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Inhibitors of the molecular chaperone heat shock protein 90 (Hsp90) are currently generating significant interest in clinical development as potential treatments for cancer. In a preceding publication (DOI: 10.1021/jm100059d) we describe Astex's approach to screening fragments against Hsp90 and the subsequent optimization of two hits into leads with inhibitory activities in the low nanomolar range. This paper describes the structure guided optimization of the 2,4-dihydroxybenzamide lead molecule **1** and details some of the drug discovery strategies employed in the identification of AT13387 (**35**), which has progressed through preclinical development and is currently being tested in man.

Introduction

Hsp90 is one of a family of molecular chaperones that are produced in response to a range of cellular stresses.¹ It is involved in the activation and maintenance of a large number of regulatory and signaling proteins by acting as a scaffold to allow these proteins to fold into their correct functional states. As well as their roles in normal cellular function, many of these client proteins are also directly implicated in cancer progression such that inhibition of Hsp90 may simultaneously target many oncogenic pathways.^{2–4}

The N-terminal domain of Hsp90 contains a nucleotide binding region able to accommodate both adenosine triphosphate (ATP^{*a*}) and adenosine diphosphate (ADP), and this is the target of the most advanced clinical compounds.⁵ The binding and subsequent hydrolysis of ATP through its AT-Pase activity is a key step in the activation process of Hsp90.⁶

and is sequestered at higher concentrations within tumors in xenograft mouse models. This has subsequently been confirmed by its clinical activity.9 Some evidence also exists that the client protein bound to the Hsp90 cochaperone complex is more susceptible to ubiquitination and targeting to the proteasome for degradation.^{10,11} These data when taken together indicate the potential utility of Hsp90 inhibitors; consequently many biotechnology and pharmaceutical companies have active Hsp90 programs. At the time of submission at least 10 Hsp90 inhibitors were in active clinical development, according to the United States National Institutes of Health Web site (www.clinicaltrials. gov). These compounds range from natural products $^{12-16}$ to compounds derived from structure-based drug design.¹⁷⁻²² A number of clinical compounds have been disclosed in recent publications, and there are now a number of reviews describing Hsp90 inhibitors.^{23–26} In the preceding paper²⁷ (DOI: 10.1021/jm100059d) we

describe the identification of a potent lead compound **1** (Table 1) ($K_d = 0.54$ nM; HCT116 cell IC₅₀ = 31 nM) by fragment screening and subsequent structure-based drug design.^{27,28} In this publication we describe the biological profile of **1** and how these data influenced our optimization strategy, ultimately concluding in the discovery of the clinical compound **35** (AT13387).

Because of its ubiquitous expression, concerns have

been expressed about the specificity of Hsp90 inhibitors

for tumor Hsp90 over that expressed in normal cells.⁷

However, in vitro experiments with the ansamycin antibio-

tic geldanamycin have shown that it possesses a higher

binding affinity for Hsp90 derived from tumor rather than

normal cell lines.⁸ Additionally geldanamycin shows more

potent cytotoxic activity in tumor cells than normal cells

[†]Coordinates of Hsp90 complexes with compounds **1**, **2**, **4**, **35**, and ADP have been deposited in the Protein Data Bank under accession codes 2xab, 2xjg, 2xjj, 2xjx, and 2xkt, respectively, together with the corresponding structure factor files.

^{*}To whom correspondence should be addressed. Phone: +44 (0)1223 226287. Fax: +44 (0)1223 226201. E-mail: a.woodhead@astex-therapeutics. com.

^{*a*}Abbreviations: ADP, adenosine diphosphate; aq, aqueous; ATP, adenosine triphosphate; CDK, cyclin dependent kinase; DCE, 1,2-dichlor-oethane; DMAW, dichloromethane, methanol, acetic acid, water; DMF, *N*,*N*-dimethylformamide; EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; equiv, equivalents; hERG, human Ether-a-go-go related gene; HP β CD, hydroxypropyl- β -cyclodextrin; HOBt, 1-hydroxybenzotriazole hydrate; HOAt, 1-hydroxy-7-azabenzotriazole; Hsp, heat shock protein; ip, intraperitoneal; ITC, isothermal calorimetry; iv, intravenous; MMFF, Merck molecular force field; morph.morpholine; NMP, 1-methyl-2-pyrrolidinone; PD, pharmacodynamic; PK, pharmacokinetic; T/C, mean tumor volume of treated animals divided by the mean control tumor volume; TFA, trifluoroacetic acid; THF, tetrahydrofuran.

Table 1. Structure-activity Relationships (SAR) for the 2,4-Dihydroxybenzamide Core

Compound	Structure	Hsp90 ^b K _d (µM)	HCT116 ^a IC ₅₀ (μM)	Compound	Structure	Hsp90 ^b K _d (µM)	HCT116 ^a IC ₅₀ (μM)
1		0.00054	0.031	7		0.003	0.08
2		0.015	2.5	8		0.001	0.29
3		1.3	0% at 10 µM	9		0.004	3.5
4		0.31	25	10		~	30
5		0.002	0.23				
6		0.004	0.18	11	HO F OH	~	61% at 100 µM
	OH OH			36°	17-DMAG	0.21	0.05

^{*a*} Compounds were assayed two or more times. ^{*b*} Compounds were assayed n = 1. The cell assay was used as the primary screening assay. ^{*c*} K_d and cell data for compound **36** were generated in house and compare well with values quoted in the literature. ³²

Results and Discussion

Description of the ATPase Site and Its Use in Structure-Based Design. The apo structure of the N-terminus of Hsp90 is characterized by a network of highly ordered water molecules at the base of the adenine binding pocket (Figure 1a).²⁹ On binding of ADP, the endogenous ligand forms hydrogen bonds with three of these conserved waters and one hydrogen bond with the protein residue Asp93 (Figure 1b).³⁰ Compound 1 is a potent inhibitor of Hsp90 which binds in the ATPase binding site (Figure 1c). The 2-hydroxyl group forms hydrogen bonds with the side chain carboxylate of Asp93 and a water molecule (mediating to Asp93). The 4-hydroxyl group displaces one of the conserved water molecules observed in the apo and ATP bound structures and forms a hydrogen bond with one of the remaining conserved water molecules. The tertiary amide induces an important twist in the conformation of the molecule, forcing the carbonyl out of the plane of the phenyl ring. This allows the carbonyl to efficiently form a bidentate interaction to a conserved water and the side chain hydroxyl of Thr184. The low energy twisted conformation of the tertiary amide also allows the isoindoline group to form positive hydrophobic interactions with the backbones of Asp54 and Ala55.

Analysis of this structure suggested that substitution at the 4, 5, 6, and 7 positions of the isoindoline ought to be well tolerated. The isopropyl group at the 5-position of the central phenyl ring efficiently fills a proximal hydrophobic cavity, bounded by the residues Leu107, Phe138, Val150, and Val186, with the potential for further growth toward where the ribose and phosphate groups of ADP bind.

Profile of Lead Compound 1. Compound 1 is an attractive lead molecule, having a high binding affinity for the ATPase domain of Hsp90 ($K_d = 0.54$ nM, as determined by isothermal calorimetry)³¹ and potent antiproliferative activity in HCT116 (human colon cancer) cells of 31 nM (<100 nM antiproliferative activity in a range of other cancer cell lines). In HCT116 cells, 1 exhibits the expected cellular mode of action for a specific Hsp90 inhibitor. This includes knock down of the oncogenic client protein cyclin dependent kinase 4 (CDK4) (see preceding paper (DOI: 10.1021/jm100059d)²⁷)³² and an increase in heat shock protein 70 (Hsp70) levels. Increased expression of Hsp70 occurs as a cellular response to Hsp90 inhibitors due to the cytoprotective nature of Hsp70.^{55,56} However, the clinical efficacy displayed by



Figure 1. (a) Apo structure of the N-terminal 212 residues of Hsp90. Of particular note is the network of highly ordered water molecules at the base of the adenine binding pocket. (b, c) Hsp90 cocrystal structures of ADP (b) and compound **1** (c). Key: red spheres, water molecules; purple dashed lines, protein–ligand and water–ligand hydrogen bonds.

prototype Hsp90 inhibitors suggests this may not be limiting. 5,57,58

When administered to mice by the intravenous (iv) route, 1 exhibited a high plasma clearance of 115 (mL/min)/kg (Table 3), above mouse liver blood flow (approximately 90 (mL/min)/kg), and consequently 1 had a relatively short plasma half-life (<1 h). We postulated that this was predominantly due to phase II metabolism of the dihydroxyphenyl moiety.³³ This theory was tested by incubating 1 in the presence of mouse S9 fractions. The compound was shown to be rapidly eliminated from reaction media, with LC-MS/MS analysis showing the appearance of directly glucuronidated and sulfated species over time. These species were also subsequently detected in the in vivo plasma samples. Despite showing high plasma clearance, 1 was dosed to mice bearing HCT116 tumor xenografts to determine the distribution of compound into tumor tissue and whether a biomarker response could be observed. A single dose of 1 was administered to mice by the intraperitoneal (ip) route at 50 mg/kg, and tumor samples were collected for pharmacokinetic (PK) and pharmacodynamic (PD) analysis up to 24 h postdose. Reasonably high levels of compound were measured in all tumor samples such that the concentration was around 1 μ M at 24 h postdose (Table 3), approximately 50-fold over the cellular IC₅₀ in the HCT116 cell line. This corresponded to a markedly longer retention of compound 1 in tumor tissue compared with plasma, where only trace levels were detected from 6 h postdose. Yet despite reasonably high tumor levels a modest biomarker response was observed. Increased levels of the molecular chaperone protein Hsp70 were detected; however, no effect was observed on levels of oncogenic client proteins such as CDK4 and Raf-1. In an attempt to determine whether this limited indication of a PK/PD response was a potential indicator of in vivo efficacy, 1 was evaluated for antitumor activity in a mouse xenograft model. 1 was dosed ip b.i.d. (twice daily) at 10, 20, and 40 mg/kg to nude mice bearing early stage HCT116 human colon carcinoma xenografts for 10 days. All dose levels were well tolerated; however, compound 1 had a modest effect on tumor volume at the highest dose tested (see Supporting Information).⁶⁰ This modest effect in an efficacy model is perhaps consistent with the PK/PD response and directed the project team toward identifying compounds with a more significant effect on oncogenic client protein levels.

Lead Optimization. 2,4-Dihydroxybenzamide Optimization. From the outset of the lead optimization phase we felt that 1 had sufficient potency, given its good cell-based activity (when compared to other clinical Hsp90 compounds)⁵ and that optimization should focus on the physiocochemical and PK properties of the molecule. Two separate strategies were employed. The first involved directly blocking known sites of metabolism by capping, steric crowding, or modification of the electronics of the 2,4-dihydroxybenzamide.^{34,35,63} The second was to alter the overall properties of the molecule in an attempt to change its distribution characteristics.^{61,62} Optimization of 1 initially focused on attempting to reduce the observed secondary metabolism effects. Capping the 4-hydroxyl group by methylation (2) in an attempt to block one site of glucuronidation resulted in a 30-fold drop in enzyme activity ($K_d = 15 \text{ nM}$) but had a more significant impact on cell activity, resulting in a 80-fold reduction (IC₅₀ = $2.5 \,\mu$ M). The Hsp90 X-ray cocrystal structure of 2 shows the molecule maintaining a similar binding mode to 1; however, a highly conserved water molecule has been displaced by the methyl

Article

group, causing a disruption in the network of stabilizing hydrogen bonds (see Figure 2a and Figure 2b). In an attempt to offset the penalty associated with this water displacement the methyl group was tethered back into a cyclic ether. Disappointingly, the 2,3-dihydrobenzofuran **3** was significantly less active than **2** ($K_d = 1.3 \mu$ M) and it proved difficult to verify its binding mode because of the inability to obtain a crystal structure. This suggested that the bicyclic ether was unable to hydrogen-bond effectively to the conserved water and that a hydrogen bond donor was required. The analogous cyclic amine **4** was synthesized, and this recapitulates the binding mode of **1**, including forming seemingly favorable interactions with both of the key waters in the active site. This however did not translate into potent enzyme affinity ($K_d = 0.31 \mu$ M).

It proved possible to replace the isopropyl group of **1** with small aliphatic groups, such as ethyl and cyclopropyl (**5**, **6**), or bulkier groups including *tert*-butyl and *sec*-butyl (**7**, **8**) without losing significant enzyme affinity. None of these compounds displayed improved cellular activity over **1**, and despite the introduction of steric crowding, no improvement in PK properties was observed (data not shown).

An alternative optimization strategy for the dihydroxybenzamide core was to modify the electronics of the two hydroxyl groups. Compound 1 has measured phenolic pK_a values of 8.6 and 10.9. Introduction of a chlorine at the 5-position of the benzamide to give 9 had little effect on enzyme affinity $(K_d = 4 \text{ nM})$ but did reduce cellular potency $(IC_{50} = 3.5 \,\mu\text{M})$. No effect on PK was observed. Despite lowering the calculated pK_a of the 4-phenolic group by 2 log units, 9 had high plasma clearance (data not shown). Structural data suggested that there was little room for the introduction of further substituents; however, it was possible that a small substituent such as a fluorine may be tolerated. Fluorination of 1 resulted in the readily separable mixture of compounds 10 and 11. Compound 10 displayed improved PK properties with a low intrinsic clearance of just 4 $(\mu L/min)/mg$ protein in mouse S9 fractions. While both compounds 10 and 11 showed an indication of weak cellular activity (30 μ M and 60% at $100 \,\mu$ M, respectively) we were unable to determine the binding affinities for Hsp90 by ITC or to obtain protein ligand crystal structures presumably because they bind so weakly. There are two possible explanations for this drop in affinity. The first is the electronic impact that a fluorine atom has on the two phenolic groups and the carbonyl of the tertiary amide, as all of these groups form crucial hydrogen bonding interaction either with the protein directly or through conserved water molecules. The other possibility, particularly for compound 10, is a steric effect. If the compound bound in an analogous fashion to 1 (Figure 1c), the electronegative fluorine atom would be within van der Waals contact of the negatively charged carboxylate of Asp93, an electronically unfavorable interaction.

Modifications made to the dihydroxybenzamide core, with the exception of compound **10** (which loses activity), had little impact on PK. This region of the molecule is important for providing high binding affinity, and as such, conservative modifications reduce activity. As an alternative strategy, optimization changed focus to the isoindoine ring system.

Isoindoline Optimization. Further optimization of 1 turned toward the bicyclic isoindoline ring. Substitution here might provide two benefits. First introduction of a polar positively charged group should alter the distribution properties of this



Figure 2. (a) Hsp90 cocrystal structure of **2**. A conserved water molecule at the base of the adenine pocket has been displaced by the 4-methoxy group, which can be observed more readily in (b) when overlaid with the cocrystal structures of **1**. (c) Hsp90 cocrystal structure of **4**. The water mediated H-bonding network has been restored; however, a significant improvement in binding affinity is not observed. Key: red/cyan spheres, water molecules; dashed lines, protein–ligand and water–ligand hydrogen bonds.

Table 2. Isoindoline Structure-Activity Relationships



Compound	R	Hsp90 ^b K _d (nM)	HCT116 ^a IC ₅₀ (nM)	Amine pKa ^c
1	Н	0.54	31	-
12	5-Cl	2.4	75	-
13	4-F	4.6	90	-
14	5-F	0.31	25	-
15	4,7-di-F	1.3	200	-
16	5 N	2.7	200	4.3
17	5 N	0.9	32	8.6
18	5 O I	0.5	21	7.85
19	50	0.51	21	-
20	5- N 0	1.2	50	6.9

^{*a*} Compounds were assayed two or more times. ^{*b*} Compounds were assayed n = 1. The cell assay was used as the primary screening assay. ^{*c*} pK_a values were measured by Pharmorphix, Ltd., Cambridge, U.K.

series of molecules. A common feature of basic drugs such as the calcium channel blocker amlodipine is high tissue affinity, as characterized by high apparent volumes of distribution.^{61,62} Second substitution of the isoindoline ring may block potential sites of oxidative metabolism (although oxidation of the isoindoline ring system had not previously been observed for this series).³⁶ A feature of benzylic systems is the potential for cytochrome P450 mediated hydroxylation at the para position; consequently blocking the 5 or 6 position of the isoindoline ring should prevent this.⁶³ Structural information suggested that modification of the four unsubstituted positions of the six-membered ring ought to be tolerated, with growth vectors toward the solvent exposed surface of the protein. Substitution at the 1 and 3 positions of the isoindoline, although possible, seemed less attractive for two reasons. First, the protein architecture is very close, and second, a chiral center would be introduced into the molecule, which would complicate the synthesis. Simple modifications such as introduction of halogens at the 4, 5, and 7 positions of the isoindoline (compounds 12, 13, 14, and 15) were tolerated, maintaining both enzyme and cell activity (Table 2). These



Figure 3. Tumor profiles of compounds 1, 17 and 18 following ip administration in mouse.

Table 3.	Mouse F	PK for	Compounds	s 1.	17.	and	18
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compd	iv dose (mg/kg)	plasma clearance ((mL/min)/kg)	ip dose (mg/kg)	HCT116 tumor concn 24 h after ip dose (µM)
1	1	115	50	1
17	10	130	40^{a}	6.5
18	10	170	80^a	11

^{*a*} The doses of **17** and **18** used for tumor distribution experiments represent efficacious doses.

simple modifications perhaps not surprisingly had little effect on the in vitro PK of the series; for example, **12** and **15** had high intrinsic clearance following incubation in mouse S9 fractions (62 and 77 (μ L/min)/mg protein).

A basic moiety was introduced into the molecule to evaluate its effect on compound distribution in vivo.^{36,61,62} As compound 14 indicates, substitution at the 5-position of the isoindoline was well tolerated. We consequently synthesized a range of groups at this position focusing on basic and other solubilizing moieties. Introduction of morpholine was well tolerated (16, $K_d = 2.7$ nM); however, a 10-fold drop in cell activity was observed compared to the parent compound 1. Substitution with other solubilizing groups such as *N*-methylpiperazine (17), *N*,*N*-dimethylethanolamine (18), 2-methoxy-ethoxy (19), and 4-morpholinylmethyl (20) resulted in both good enzyme and cell activity. Compounds with basic functionality such as 17 and 18 were observed to have increased tumor retention when dosed in mice, compared to 1 (Figure 3).

For compound 18 this resulted in tumor concentrations of approximately 11 μ M at 24 h (Table 3) following a dose of 80 mg/kg by the ip route, which was approximately 500-fold greater than the cellular IC_{50} . Encouragingly this tumor concentration translated to a positive biomarker response, including knockdown of the client proteins CDK4 and Raf-1 and an increase in levels of Hsp70 (see Figure 5 for the cellular mode of action for compound 18). Compounds 17 and 18 were evaluated for their in vivo antitumor activity in nude BALB/c mice bearing early stage HCT116 human colon carcinoma xenografts (mean starting volume of approximately 100 mm³). In this study the hydrochloride salts, dissolved in 0.9% saline were administered by the ip route once daily for 8 consecutive days. Tumor growth inhibition for the 80 mg/kg dose group at the end of the experiment was 81% for 17 (% T/C = 19, measured on day 10) and 63% for 18 (% T/C = 37, measured on day 12), with compound 17 showing a cytoreductive effect (see Supporting Information).⁶⁰ This corresponded to a tumor growth delay of 10.5 and 11.4 days for compounds 17 and 18, respectively.59



Figure 4. Computational analysis of side chain energy minima for basic compounds **17**, **18**, **20**, **21**, **24**, **25**, **27–30**, **32–35**. A Monte Carlo conformational search was performed on each molecule using the MMFF force field as implemented in the Spartan '04 modeling package. The global minimum of each molecule was overlaid with the others using only the resorcinol and the isoindolines atoms. The colored spheres indicate where the basic centers of each of the compounds **17**, **18**, **20**, **21**, **24**, **25**, **27–30**, **32–35** is positioned. Key, approximated hERG IC₅₀: red spheres, $< 3 \mu$ M; orange spheres, $3-30 \mu$ M; green spheres, $\geq 30 \mu$ M.



Figure 5. Cellular biomarkers effects of compounds **18** and **35**. Immunoblot of HCT116 cell lysates treated for 18 h with the indicated compound doses of **18** (a) or **35** (b). Client proteins: Raf-1 and CDK4. Cochaperone: Hsp70. Loading control: glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Late Stage Optimization. Although compounds 17 and 18 were synthesized early in the project, they were promising advanced lead molecules. Both compounds possessed good mechanism based cell activity, potent in vivo efficacy, minimal binding to the major cytochrome P450 isoforms, and selectivity over a small in-house panel of enzymes. Compounds 17 and 18 both showed weak inhibition of hERG potassium channel current at $3 \,\mu$ M (41% and 45%, respectively) in a patch clamp assay. We felt it unlikely that this level of activity posed a developability risk, particularly when considered alongside high cellular potency and low plasma exposure at an efficacious dose.

As part of a preclinical candidate selection phase, a number of closely related analogues of **17** and **18** were

prepared. Properties that were evaluated during this phase of the project included enzyme and cell potency, in vivo PK, physicochemical properties (pK_a , log $D_{7.4}$, and aqueous solubility), and off target effects such as P450 and hERG binding. This program of optimization looked at varying a number of parameters that included changing the point of substitution to the isoindoline, altering the length and flexibility of linker between the aromatic system and the basic group, sterically crowding the basic group and modifying its pK_a , and modifying the lipophilicity of the system. On the basis of the properties of the initial compounds, attaching the correct polar positively charged solubilizing group to the isoindoline template could give us an excellent balance of properties for a clinical development candidate.

Evaluating alternative isomers with essentially the same physicochemical properties was an attractive starting point. Consequently compounds substituted at the 4-position of the isoindoline were synthesized, including the direct analogues of 17 and 18, compounds 22 and 24. These analogues had high Hsp90 affinity (< 10 nM), potent cell activity (< 100 nM), limited P450 binding (CYP3A4 < 50% inhibition at 10 μ M), and similar hERG affinity to 17 and 18. Replacing the tertiary amine of previous compounds with a primary amine gave compounds such as 25 and 26, with increased basicity and reduced lipophilicity. This change was well tolerated (Hsp90 $K_d < 5 \text{ nM}$) and caused a reduction in hERG activity, although these compounds had increased P450 binding (< 10 nM against the 2C9 and 2C19 isoforms). Sterically crowding compound 26 with bulky lipophilic groups (27 and 28), although potent against the enzyme and in cells, led to increased hERG inhibiton (97% and 92% at 30 μ M, respectively) compared to 17 and 18, possibly caused by a significant increase in lipophilicity (cLogP > 4).

More rigid compounds such as piperazine amide **29** and tertiary alcohol **30** showed potent activity in the cell based assay (< 100 nM), low P450 binding, and low activity in the hERG patch clamp assay. These compounds are significantly more polar than previous analogues (log $D_{7.4} = 2.1$ and 1.7, respectively), indicating that side chain flexibility and polarity are perhaps a contributing factor to hERG activity.

Replacing the amide linker of **29** with an amine (**31**) was also well tolerated. This compound maintained polarity $(\log D_{7.4} = 1.7)$ while introducing more conformational flexibility. A similar P450 and hERG profile is observed to 17 and 18 (CYP3A4 34% inhibition at 10 μ M; hERG = 72% inhibition at 30 μ M). The des-methyl analogue, cyclic secondary amine 32, is one of the most polar compounds we synthesized in this series (log $D_{7.4} = 0.7$), has good enzyme and cell activity, very low activity against hERG (20% at 30 μ M), and limited activity against CYP2C9 and 2C19 (approximately 1 μ M against each). To test what effect the introduction of other cyclic secondary amines would have, the des-methyl analogue of 17 was prepared. Compound 33 had subnanomolar binding affinity to Hsp90 and potent cellular activity and displayed similar hERG inhibitory activity to 17 (hERG = 72% at 30 μ M).

Analogues of the methylene linked compound **20** were also synthesized. These compounds were designed to increase the pK_a of the basic center and reduce lipophilicity. As can be seen from Table 4, the *N*,*N*-dimethyl analogue **34** and the *N*-methylpiperazine analogue **35** possess good activity in the enzyme (< 5 nM) and cell based assays (< 100 nM), reduced lipophilicity (log $D_{7.4} = 2.1$ and 2.7, respectively), and increased basicity ($pK_a = 9.2$ and 7.7, respectively). The

Table 4. Substituted Isoindoline SAR



Cmpd	R	Hsp90 ^b K _d (nM)	HCT116 ^a IC ₅₀ (nM)	logD ^c pH 7.4	Amine pKa ^c	hERG % Inhibition ^d (µM)
1	Н	0.54	31	3.7	-	25% at 3 51% at 30
17	5 N	0.9	32	2.98	7.3	41% at 3 87% at 30
18	5ON	0.5	21	2.4	8.4	45% at 3 87% at 30
20	5- N 0	1.2	50	3.3	6.9	25% at 3 48% at 30
21	5 - O(CH ₂) ₃ Morph	0.68	28	3.3	6.9	51% at 3 95% at 30
22	4 N	7.3	93	-	-	41% at 3 87% at 30
23	4 - O(CH ₂) ₃ Morph	7.9	84	3.55	7.0	23% at 3 83% at 30
24	4O	1.4	16	-	-	43% at 3 93% at 30
25	5 - O-(CH ₂) ₂ NH ₂	4.0	300	-	-	11% at 3 64% at 30
26	5 - O-(CH ₂) ₃ NH ₂	0.36	100	0.96	10.2	8% at 3 42% at 30
27	5 - O(CH ₂) ₂ NH-c- Pentyl	0.3	21	-	-	60% at 3 97% at 30
28	5 - O(CH ₂) ₂ NH- <i>neo</i> - Pentyl	0.5	30	-	-	53% at 3 92% at 30
29	5- 0 N N N	2.3	79	2.1	6.8	21% at 3 49% at 30
30	HO 5-	0.95	96	1.7	9.4	15% at 3 34% at 30
31	5-	0.9	47	1.7	9.05	25% at 3 72% at 30
32	5- H NH	1.3	130	0.7	10.2	10% at 3 20% at 30
33	5 NH	0.43	68	1.6	8.4	13% at 3 70% at 30
34	5- N	1.3	80	2.1	9.2	5% at 3 57% at 30
35	5- N N	0.71	48	2.7	7.7	7% at 3 42% at 30

^{*a*}Compounds were assayed two or more times. ^{*b*}Compounds were assayed n = 1. The cell assay was used as the primary screening assay. ^{*c*} The log *D* and p K_a values were measured by Pharmorphix, Ltd., Cambridge, U.K. ^{*d*} The hERG patch clamp assay was carried out by Quiniltes, Ltd., U.K., using stably transfected HEK293 cells and PatchXpress 7000A. Data are supplied as n = 1.

Table 5. Mouse PK of Compound 35

1	1	half-life (h)					
clearance ^a	distribution ^a						
(mL/min)/kg)	(L/kg)	plasma ^a	blood ^b	muscle ^b	tumor ^b		
113-200	11-13	0.9-3.2	4.7	3.0	65		

^a The iv dose was 2–10 mg/kg. ^b Following an ip dose of 60 mg/kg.

P450 profiles for both these analogues were favorable. Compound **34** caused approximately 50% inhibition at 10 μ M against 2C9 and 2C19, while **35** was < 50% against all the major P450 isoforms.

From this program of optimization we identified a number of compounds including 29, 30, 31, 33, 34, and 35 possessing a good balance of properties, including moderate to low human plasma protein binding (ranging from 64% to 93%), acceptable risk profiles against the major CYP450 isoforms, and the hERG ion channel, which were suitable candidates for more detailed evaluation alongside the original compounds 17 and 18. Compounds 29, 30, 31, 33, 34, and 35 were profiled initially for tolerability and then efficacy in nude BALB/c mice bearing early stage HCT116 human colon carcinoma xenografts (mean starting volume of approximately 100 mm³). Despite having good in vitro properties including low plasma protein binding (71% in mouse plasma), 29 produced a reduced effect in an efficacy study, when compared to leads 17 and 18. Compounds 30, 31, 33, 34, and 35, however, demonstrated comparable efficacy at tolerated doses to 17 and 18, with a corresponding PK/PD response confirming an Hsp90 driven mechanism of action. As previously described, a common feature of this series of compounds was their high plasma clearance (Tables 3 and 5). Compounds 17, 18, and 35 all showed glucuronide and sulfate conjugates when incubated in S9 fractions (intrinsic clearance of 41, 34, and 46 (μ L/ min)/mg of enzyme, respectively). This characteristic was observed for all these late stage compounds, meaning that they could not easily be differentiated using plasma kinetics. Instead, these seven compounds were further assessed for developability, with particular emphasis on dosing via iv infusion. Key criteria that were considered during this phase of the project included solubility of the free base and hydrochloride salt in aqueous buffers, stability of a range of pharmaceutically acceptable salts, and synthetic tractability (see Supporting Information for details).

Compound **35** proved to have the best overall properties; the hydrochloride, methanesulfonate, and L-lactate salts all displayed high solubility in a number of aqueous buffers which spanned a range of pH values (solubility of > 30 mg/mL in 0.1 M sodium acetate buffer at pH 5.5). Exposure to a range of forcing conditions (including pH 1 and 14 at 40 °C) during a 1 week stability study showed no degradation of **35**. The compound was readily scalable using the original lead optimization route. A number of other routes were also developed, which will be detailed in a future publication.

We carried out an analysis of the hERG data for this series of compounds, and there appear to be a number of subtle factors that are interlinked and contribute to hERG affinity. It is noted that the activity range is small and most of these compounds have weak binding affinities. The data suggest that important factors for reducing hERG affinity appear to be the length of linker to the basic group, where two or five atoms seemed to be optimal (**34** and **35**), and a minimum amount of conformational flexibility (**29** and **30**). Figure 4 shows where the basic center is positioned for each minimized structure. The compounds with the least hERG activity appear to be clustered relatively closely together. Little correlation could be drawn between the pK_a values of active and inactive compounds. The data for compounds **27** and **28** suggested that high lipophilicity gave rise to greater hERG affinity, whereas compounds **26** and **32** with log $D_{7.4} < 1$ suggested that increased polarity helps to minimize hERG affinity.³⁷⁻⁴¹

Characterization of Compound 35. Cell-Based Activity. Compound **35** is a potent inhibitor of HCT116 cell proliferation (used as a primary screen during lead optimization).⁴² Following 72 h of exposure, **35** potently inhibited the proliferation of a panel of human tumor cell lines (well over 100 cell lines have been tested), showing $IC_{50} < 100$ nM against more than 50 (data not shown).



Figure 6. In vivo mouse efficacy study for **35** using HCT116, human colon cancer xenografts (60 mg/kg ip 3 days on and 3 days off for four cycles). Key: blue triangles, compound dosing timepoints; blue squares, **35** at 60 mg/kg; red diamonds, vehicle controls.

Scheme 1^a

The mechanism of action of **35** in cells was investigated by monitoring the expression levels of a range of client proteins, following treatment with **35** for 18 h. These studies indicated that the expression of the oncogenic client proteins Raf-1 and CDK4 is inhibited around 30 nM in the HCT116 cell line, which is similar to the IC₅₀ for proliferation (48 nM). The molecular chaperone Hsp70 is up-regulated at 10 nM and is a more sensitive cellular response to the inhibition of Hsp90. This effect is widely utilized as a biomarker readout for Hsp90 inhibition in both cellular and in vivo experiments.^{5,56}

PK Study. As summarized in Table 5, the plasma clearance of 35 in female BALB/c mice after iv dosing ranged from 113 to 200 (mL/min)/kg with a half-life of between 0.9 and 3.2 h. Volume of distribution was 11-13 L/kg. Compound 35 was found to partition preferentially into red blood cells, both in vitro and in vivo (blood/plasma ratio up to 3:1), which may, at least partially, account for the higher than liver blood flow clearance measured in plasma. The tissue distribution of compound 35 was investigated following an ip dose of 60 mg/kg and was shown to have a half-life in HCT116 tumors of 65 h and a tumor concentration of 9.3 μ M after 24 h. This appeared to be a selective effect for tumor, as the half-life in muscle (taken as a surrogate normal tissue) was just 3 h, similar to plasma. Encouragingly 35 showed some oral bioavailability in mouse (31%) despite the high plasma clearance (113-200 (mL/min)/kg). Protein binding in mouse plasma was 91%.

In Vivo Antitumor Activity. Compound 35 was evaluated for its in vivo antitumor activity in nude BALB/c mice bearing early stage HCT116 human colon carcinoma xenografts with a mean starting volume of approximately 100 mm³ (Figure 6). In this study the L-lactate salt of 35, dissolved in 17.5% hydroxypropyl- β -cyclodextrin was administered by the ip route using a repeated cycle of dosing of once per day for 3 days, no dose for 3 days, for four dosing cycles. Tumor growth inhibition at the end of the dosing phase (day 21)



^{*a*} Reagents and conditions: (a) substituted isoindoline, EDC, HOBt, DMF; (b) 10% Pd/C, EtOH, H₂; (c) SnCl₂·2H₂O, EtOH; (d) EtN^{*i*}Pr₂, ^{*n*}Bu₄NI, NMP, [Cl(CH₂)₂]X; (e) (CH₃)₂SO₄, K₂CO₃, CH₃CN; (f) SelectFluor, CH₃CN.





^{*a*} Reagents and conditions: (a) SOCl₂, MeOH; (b) K_2CO_3 , BrCH₂C(CH₃)=CH₂, CH₃CN; (c) "Bu₃SnCl, NaBH₃CN, AIBN, C₆H₆; (d) NaOH, MeOH, H₂O; (e) (COCl₂, DMF, DCM; (f) isoindoline, NEt₃, DCM; (g) LiOH, THF, MeOH, H₂O; (h) isoindoline, EDC, HOBt, NEt₃, THF, DCM; (i) Cs₂CO₃, BrCH₂C(CH₃)=CH₂, DMF; (j) Pd(OAc)₂, HCO₂Na, NEt₃, "Bu₄NCl, DMF; (k) BBr₃, DCM.

Scheme 3^a



^{*a*} Reagents and conditions: (a) K₂CO₃, BnBr, DMF; (b) KOH, MeOH, H₂O; (c) isoindoline, EDC, HOBt, DCM; (d) boronate, Pd(dppf)Cl₂, Cs₂CO₃, THF, H₂O; (e) 10% Pd/C, EtOH, H₂; (f) BBr₃, DCM.

was 78% for the 60 mg/kg dose level (% T/C = 22). Animals were monitored for tumor regrowth after cessation of dosing, and a tumor growth delay of 17 days was observed for this dosing schedule.^{59,60} A more extensive biological characterization of compound **35** including additional in vivo efficacy studies using Hsp90 sensitive cell lines and a more detailed evaluation of PK/PD effects will be published separately.

Chemistry. Compound **1** was prepared by coupling the key intermediate 2,4-bis-benzyloxy-5-isopropenylbenzoic acid **36** with isoindoline **37a** using standard EDC/HOBt coupling conditions (Scheme 1). Catalytic hydrogenation simultaneously removed the benzyl protecting groups and reduced the isopropenyl group to the isopropyl, as described in the preceding paper (DOI: 10.1021/jm100059d).²⁷ Compounds **12–15**, **20**, **23**, **24**, **30**, and **34** were prepared in an analogous manner starting from the appropriate isoindoline. **2** was prepared by direct methylation of **1** under basic conditions, followed by chromatographic separation of the two

regioisomers. The bicyclic ether 3 was prepared by the tin mediated free radical cyclization of the allylic ether 41 followed by saponification of the ester and coupling to isoindoline (Scheme 2). The indoline analogue 4 was prepared by a similar route starting from the aniline 43. Protection of the phenol was required, as the methyl ether and cyclization were achieved using palladium catalysis. Compounds 5, 6, and 7 were prepared by Suzuki cross coupling of the bis-benzyl protected aryl bromide 49a with the appropriate activated boron species followed by catalytic hydrogenation to remove the benzyl protecting groups (Scheme 3). Compounds 8 and 9 were prepared in an analogous manner to 1 starting from the appropriate 5-substituted benzoic acid derivative (46b or 46c). Compounds 10 and 11 were prepared directly from 1 by electrophilic aromatic fluororination using SelectFluor (Scheme 1). This produced a mixture of the 3 and 6 fluorinated compounds which were readily separable by flash column chromatography. 16 and 17 were

Scheme 4^a

(d) (b), (c) (a) BnC HC BnC HC ÓBn ÒΒn 50 51а-е 18 n=1. R' = NMe 27 R" = cyclopentyl 28 R" = neo-pentyl 19 n=1, R' = OMe 21 n=2, R' = 4-morpholinyl 25 n=1, R' = NH₂ 26 n=2, R = NH₂

^a Reagents and conditions: (a) K₂CO₃, R-X, DMF; (b) 10% Pd/C, EtOH, H₂; (c) saturated HCl/EtOAc; (d) RR'C=O, NaBH(OAc)₃, AcOH, DCE.

Scheme 5^{*a*}



^{*a*}Reagents and conditions: (a) K₂CO₃, BnBr, DMF; (b) 10% Pd/C, EtOH, H₂; (c) NaOH, MeOH, H₂O; (d) *N*-methylpiperazine or HN-(OMe)Me+HCl, EDC, HOBt, NEt₃, DMF; (e) LiAlH₄, THF; (f) *N*-methylpiperazine, NaBH(OAc)₃, AcOH, DCE.

prepared by coupling 5-nitroisoindoline to 36 followed by tin reduction to aniline 39. Microwave assisted cyclization using the appropriate bis-alkylating agent under basic conditions followed by catalytic hydrogenation gave the morpholine 16 and N-methylpiperazine 17. Compounds 18 and 19 were prepared by alkylation of 50 with 2-dimethylaminoethyl chloride and 2-chloroethyl methyl ether, respectively, followed by removal of the benzyl protecting groups (Scheme 4). Compounds 21, 25, and 26 were prepared in an analogous manner, with 25 and 26 requiring a final BOC deprotection under acidic conditions. 27 and 28 were prepared directly from 25 by reductive amination, using sodium triacetoxyborohydride and cyclopentanone or 3,3-dimethylbutyraldehyde, respectively. Compounds 22 and 31-33 were prepared via palladium catalyzed Buchwald chemistry from the relevant 4- or 5-bromoisoindoline intermediate (52 and 53) followed by deprotection (Scheme 5). Coupling of methyl isoindoline-5-carboxylate to 36 gave intermediate 54. Saponification of the methyl ester followed by carbodiimide mediated coupling with N-methylpiperazine, and debenzylation gave compound 29. It proved difficult to convert 29 to 35, and a number of conditions were attempted to reduce the amide, but none of which were successful, even as the protected precursor. An alternative route from the Weinreb amide 56 was used. Reduction of 56 to the aldehyde using lithium aluminum hydride in THF followed by reductive amination using N-methylpiperazine and sodium triacetoxyborohydride and final deprotection gave 35.

Conclusions

Using fragment screening and subsequent structure guided design, we have identified a series of 2,4-dihydroxybenzamides with very high affinity for the ATPase site of Hsp90 and potent Hsp90 driven cell-based activity. Two approaches were taken in an effort to optimize compound 1. The first was directed at blocking proposed sites of metabolism by capping, sterically crowding, or modifying the electronics of the 2,4dihydroxybenzamide. This approach, while generally maintaining activity against Hsp90, did not significantly modulate the PK profile of 1. The second approach was to alter the overall physical properties of the molecule in an attempt to change its distribution characteristics. Substitution of the isoindoline ring system with a basic group gave compounds such as 17 and 18, which, although still having high plasma clearance, exhibited high tumor levels, potent biomarker modulation, and efficacy in a mouse cancer model. A number of closely related basic analogues of compounds 17 and 18 were synthesized, and seven compounds were evaluated in a preclinical candidate selection phase. Key parameters that were investigated during this phase of the project were in vivo target modulation, predicted human dose, selectivity/off-target effects, solubility, stability, formulation, and synthesis. Following this process, compound 35 was selected as a preclinical development candidate. 35 has potent affinity for Hsp90 ($K_d =$ 0.5 nM) by binding in the ATPase site at the N-terminus of Hsp90 (Figure 7). This enzyme affinity translates into good



Figure 7. Protein—ligand crystal structure of compound **35** bound in the ATPase site of Hsp90. The key binding features are identical to those of lead compound **1** (Figure 1). The pendent *N*-methylpiperazine group binds in a highly solvent exposed region and consequently has little impact on enzyme affinity. It does, however, have a marked effect on the physicochemical and pharmacokinetic properties of the molecule, resulting in elevated tumor levels, biomarker modulation, and good efficacy in a mouse cancer model.

activity against a range of human tumor cell lines and statistically significant cytostatic activity in a mouse anticancer model. **35** has a clean profile against the major cytochrome P450 isoforms (\leq 40% inhibition at 10 µM for 1A2, 2D6, 3A4, 2C9, 2C19), low hERG affinity, and low protein binding (85% in human plasma) and has high thermodynamic aqueous solubility as the methanesulfonic, hydrochloride, or L-lactate salt (\geq 30 mg/mL in 0.1 M acetate buffer). Its synthetic tractability makes it readily amenable to large scale synthesis. On the basis of this and further data (to be published in a later paper), compound **35** (AT13387) as the L-lactate salt was selected as a clinical candidate and has subsequently entered clinical development.

Experimental Section

Chemistry. Reagents and solvents were obtained from commercial suppliers and used without further purification. Thin layer chromatography (TLC) analytical separations were conducted with E. Merck silica gel F-254 plates of 0.25 mm thickness and were visualized with UV light (254 nM) and/or stained with iodine, potassium permanganate, or phosphomolybdic acid solutions followed by heating. Standard silica gel chromatography was employed as a method of purification using the indicated solvent mixtures. Compound purification was also carried out using a Biotage SP4 system using prepacked disposable SiO₂ cartridges (4, 8, 19, 38, 40, and 90 g sizes) with stepped or gradient elution at 5-40 mL/min of the indicated solvent mixture. Proton nuclear magnetic resonance (¹H NMR) spectra were recorded in the deuterated solvents specified on a Bruker Avance 400 spectrometer operating at 400 MHz. Chemical shifts are reported in parts per million (δ) from the tetramethylsilane internal standard. Data are reported as follows: chemical shift, multiplicity (br = broad, s = singlet, d =doublet, t = triplet, sp = septet, m = multiplet), integration. Compound purity and mass spectra were determined by a Waters Fractionlynx/Micromass ZQ LC/MS platform using the positive electrospray ionization technique (+ES) or an Agilent 1200SL-6140 LC/MS system using positive-negative switching, both using a mobile phase of CH_3CN /water with 0.1% formic acid. The purity is \geq 95% unless otherwise explicitly stated.

2,4-Bis-benzyloxy-5-isopropenylbenzoic acid was prepared as described previously in WO 2006/109075 A2. Experimental details for noncommercially available starting materials (for example, the substituted isoindolines) are available in the Supporting Information.

(1,3-Dihydroisoindol-2-yl)-(2,4-dihydroxy-5-isopropylphenyl)methanone (1). A mixture of 2,4-bis-benzyloxy-5-isopropenylbenzoic acid 36 (187 mg, 0.5 mmol), EDC (116 mg, 1.2 equiv), HOBt (81 mg, 1.2 equiv), and isoindoline (90 mg, 1.5 equiv) in CH_2Cl_2 (10 mL) was stirred at room temperature overnight. The organic layer was washed successively with 2 M HCl and 2 M NaOH, then dried (MgSO₄), filtered, and evaporated in vacuo. The crude product 38a (220 mg) was dissolved in MeOH (8 mL), treated with 10% Pd/C (20 mg), then subjected to atmospheric hydrogenation for 16 h. The mixture was filtered through Celite and washed with MeOH. The combined filtrates were concentrated in vacuo. The residue was triturated with Et₂O, filtered, and then dried under vacuum to give the title compound as an off-white solid (50 mg, 33%). ¹H NMR (DMSO- d_6) δ 10.03 (s, 1H), 9.63 (s, 1H), 7.43–7.15 (m, 4H), 7.03 (s, 1H), 6.40 (s, 1H), 4.77 (br s, 4H), 3.15–3.09 (m, 1H), 1.14 (d, 6H). MS: [M + H]⁺ 298. HPLC purity, >99%.

Methyl-2,3-dihydro-1*H*-isoindole-5-carboxylate Trifluoroacetate (37n). To an ice bath cooled solution of 3,4-dimethylbenzoic acid (10 g, 67 mmol) in MeOH (100 mL) was added dropwise thionyl chloride (5.84 mL, 81 mmol). The reaction mixture was warmed to room temperature and then stirred for a further 24 h. The reaction mixture was concentrated in vacuo and then azeotroped in vacuo with toluene (×3) to give 3,4-dimethylbenzoic acid methyl ester as a brown oil (10.9 g, 99%). ¹H NMR (DMSO- d_6) δ 7.74 (d, 1H), 7.69 (dd, 1H), 7.28 (d, 1H), 3.82 (s, 3H), 2.29 (s, 3H), 2.28 (s, 3H).

A solution of methyl 3,4-dimethylbenzoate (10.9 g, 66 mmol), *N*-bromosuccinimide (24.0 g, 133 mmol), and benzoyl peroxide (500 mg, 2 mmol) in CCl₄ (75 mL) was heated at reflux for 24 h. Further *N*-bromosuccinimde (12 g, 67 mmol) and benzoyl peroxide (250 mg, 1 mmol) were added, and the reaction mixture was heated at reflux for a further 4 h. The reaction mixture was filtered and then the mother liquour concentrated in vacuo to give 3,4-bis-bromomethylbenzoic acid methyl ester as a paleorange oil (26.7 g, contains 20% of two trisubstituted bromo compounds). The product was taken crude to the next step.

To a vigorously stirred solution of crude 3,4-bis-bromethylbenzoic acid methyl ester (26.7 g, assume 66 mmol) and Na₂CO₃ (55.2 g, 521 mmol) in acetone (480 mL) and water (80 mL) was added dropwise a solution of 4-methoxybenzylamine (10.4 mL, 80 mmol) in acetone (80 mL). The reaction mixture was stirred vigorously for a further 6 h. Acetone was evaporated in vacuo. The residue was partitioned between EtOAc and HCl (2 N). The organic layer was again washed with HCl (2 N). The aqueous layers were combined, washed with EtOAc, and basified to $\sim pH 10$ with solid NaHCO₃. The product was extracted with CH_2Cl_2 (×2). The combined organic layers were combined, dried (MgSO₄), and evaporated in vacuo. To the residue was added HCl in dioxane (4 M, 10 mL). The material was concentrated in vacuo and then azeotroped twice with toluene/MeOH (2:1) to give predominately 2-(4-methoxybenzyl)-2,3-dihydro-1H-isoindole-5-carboxylic acid methyl ester as a lightgreen solid hydrochloride salt (1.22 g, 6% over two steps). ¹H NMR (DMSO-d₆) δ 11.74 (s, 1H), 8.01 (d, 1H), 7.96 (dd, 1H), 7.59 (dd, 2H), 7.55 (d, 1H), 7.04 (dd, 2H), 4.73-4.49 (m, 4H), 3.87 (s, 3H), 3.80 (s 3H).

A solution of 2-(4-methoxybenzyl)-2,3-dihydro-1*H*-isoindole-5-carboxylic acid methyl ester (215 mg, 0.65 mmol) in anisole (200μ L) and TFA (1 mL) was heated in a microwave (max 200 W) at 140 °C for 30 min. The reaction mixture was partitioned between water and CH₂Cl₂. The aqueous layer was washed with CH₂Cl₂, evaporated in vacuo, and then azeotroped twice with a mixture of MeOH and toluene (1:2) to give methyl-2,3-dihydro-1*H*-isoindole-5-carboxylate trifluoroacetate as a pale-orange so-lid (105 mg, 91%). ¹H NMR (DMSO- d_6) δ 9.70 (br. s, 2H), 8.03 (d, 1H), 7.97 (dd, 1H), 7.57 (d, 1H), 4.60 (d, 4H), 3.88 (s, 3H).

2-(2,4-Bis-benzyloxy-5-isopropylbenzoyl)-2,3-dihydro-1H-isoindole-5-carboxylic Acid Methyl Ester (54). A mixture of 2,4-bisbenzyloxy-5-isopropenylbenzoic acid 36 (3.12 g, 8.35 mmol), EDC (1.92 g, 1.2 equiv), HOBt (1.35 g, 1.2 equiv), methyl-2,3dihydro-1H-isoindole-5-carboxylate trifluoroacetate 37n (2.43 g, 8.35 mmol), and NEt₃ (6 mL, 5 equiv) in DMF (20 mL) was stirred at room temperature for 48 h and then evaporated. The residue was partitioned between EtOAc and 5% citric acid solution. The organic layer was separated and washed successively with saturated sodium bicarbonate solution and brine, then dried (MgSO₄), filtered, and evaporated in vacuo. The crude material was purified by flash column chromatography, eluting with 20%, 25%, then 33% EtOAc in 40-60 petroleum ether. Product containing fractions were combined and evaporated in vacuo to give the title compound as an off-white solid (3.2 mg, 72%). ¹H NMR (DMSO- d_6) δ 7.99–7.82 (s, 2H), 7.57–7.29 (m, 8H), 7.29-7.20 (m, 3H), 7.13 (s, 1H), 7.01 (s, 1H), 5.24 (s, 1H), 5.24 (s, 2H), 5.20 (s, 2H), 5.09 (d, 2H), 4.85 (s, 2H), 4.63 (s, 2H), 3.86 (d, 3H), 2.05 (s, 3H). MS: $[M + H]^+$ 534.

2-(2,4-Bis-benzyloxy-5-isopropylbenzoyl)-2,3-dihydro-1H-isoindole-5-carboxylic Acid Methoxymethylamide (56). A solution of 2-(2.4-bis-benzvloxy-5-isopropylbenzoyl)-2.3-dihydro-1H-isoindole-5-carboxylic acid methyl ester 54 (390 mg) in MeOH (10 mL) and 2 M NaOH (10 mL) was heated at 50 °C for 48 h and then evaporated in vacuo. The residue was acidified with 2 M HCl. The solid was collected by filtration and washed with water to give the corresponding acid as a colorless solid (255 mg). MS: $[M + H]^+$ 520. A solution of this acid (1.76 g, 3.39 mmol), EDC (0.78 g, 4.06 mmol), HOBt (0.55 g, 4.06 mmol), Et₃N (1.0 mL, 6.78 mmol), and N,O-dimethylhydroxylamine hydrochloride (0.36 g, 3.72 mmol) in DMF (20 mL) was stirred at room temperature for 48 h, then evaporated under vacuum. The crude material was dissolved in EtOAc and washed twice with saturated NaHCO₃, then brine, dried (MgSO₄), filtered, then evaporated in vacuo to give the title compound (1.84 g). MS: $[M + H]^+$ 563.

(2,4-Dihydroxy-5-isopropylphenyl)-[5-(4-methylpiperazin-1ylmethyl)-1,3-dihydroisoindol-2-yl]methanone (35). A solution of 56 (0.226 g, 0.4 mmol) in THF (5 mL) was cooled to 0 °C under N₂ and treated with LiAlH₄/THF (0.3 mL, 0.3 mmol, 1 M) and stirred for 1 h. Further LiAlH₄ (0.05 mL) was added, and the mixture was stirred for 30 min. The reaction was quenched with saturated KHSO₄ solution, and the product extracted with EtOAc. The organic layer was dried (MgSO₄), filtered, and evaporated in vacuo to give 2-(2,4-bis-benzyloxy-5-isopropylbenzoyl)-2,3-dihydro-1*H*-isoindole-5-carbaldehyde (0.2 g). MS: $[M + H]^+$ 504.

To a solution of this aldehyde (0.316 g, 0.63 mmol) and *N*methylpiperazine (63 mg, 0.63 mmol) in CH_2Cl_2 (10 mL) were added AcOH (38 mg, 0.63 mmol) and NaBH(OAc)₃ (0.28 g, 1.33 mmol). Then the mixture was stirred at ambient temperature for 5 h. The reaction was quenched with water, and the product was extracted with CH_2Cl_2 (×3). The organics were combined, washed with brine, dried (MgSO₄), filtered, and evaporated in vacuo to give (2,4-bis-benzyloxy-5-isopropylphenyl)-[5-(4-methylpiperazin-1-ylmethyl)-1,3-dihydroisoindol-2-yl]methanone (0.32 g). MS: [M + H]⁺ 588.

Deprotection was carried out in a manner analogous to **1** with the following modifications. K_2CO_3 (2 equiv) was added to the reaction, and a MeOH/H₂O (9:1) mixture was used as solvent. Upon completion, the MeOH was evaporated in vacuo and the mixture was diluted with water, neutralized using 1 M HCl, and extracted with CH₂Cl₂ (×2). The combined organic layers were dried (MgSO₄), filtered, and evaporated under vacuum. The product was purified by preparative HPLC to give the title compound (21 mg, 9%). ¹H NMR (Me- d_3 -OD) δ 7.37–7.23 (br s, 3H), 7.19 (s, 1H), 6.39 (s, 1H), 4.94–4.87 (br s, 4H), 3.57 (s, 2H), 3.27–3.16 (m, 1H), 2.67–2.39 (m, 8H), 2.31 (s, 3H), 1.23 (d, 6H). MS: [M + H]⁺ 410. HPLC purity, 95%.

Full Chemistry Experimental Procedures, in Vitro and in Vivo Biological Assays, X-ray Crystallographic Data, and DMPK, Solubility, and Stability Studies. Detailed experimental data can be found in the Supporting Information.

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Supporting Information Available: Experimental details of syntheses, biological assays, and DMPK studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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