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Targeting the Hsp90 Molecular Chaperone with Novel Macrolactams. Synthesis, Structural, Binding, and Cellular Studies

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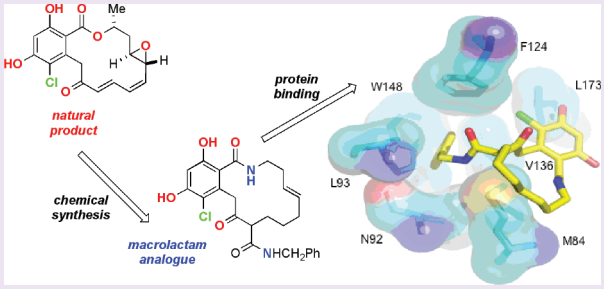
Supporting Information

ABSTRACT: A series of resorcylic acid macrolactams, nitrogen analogues of the naturally occurring macrolactone radicicol, have been prepared by chemical synthesis and evaluated as inhibitors of heat shock protein 90 (Hsp90), an emerging attractive target for novel cancer therapeutic agents. The synthesis involves, as key steps, ring opening of an isocoumarin intermediate, followed by a ring-closing metathesis reaction to form the macrocycle. Subsequent manipulation of the ester group into a range of amides allows access to a range of new macrolactams following deprotection of the two phenolic groups. These new resorcylic acid lactams exhibit metabolic stability greater than that of related lactone counterparts, while co-crystallization of three macrolactams with the *N*-terminal domain ATP site of Hsp90 confirms that they bind in a similar way to the natural product radicicol and to our previous synthetic lactone analogues. Interestingly, however, in the case of the *N*-benzylamide, additional binding to a hydrophobic pocket of the protein was observed. In biological assays, the new macrocyclic lactams exhibit a biological profile equivalent or superior to that of the related lactones and show the established molecular signature of Hsp90 inhibitors in human colon cancer cells.



The resorcylic acid lactones (RALs) form a large class of natural products, many of which exhibit potent biological activity.[1−3] Prominent among the RALs is radicicol **1** (also known as monorden), originally isolated from the fungus *Monocillium nordinii*[4] and subsequently from both *Nectria radicicola*[5] and from the plant-associated fungus *Chaetomium chiversii*.[6] Some years ago, radicicol **1** was discovered to be a potent inhibitor of the molecular chaperone heat shock protein 90 (Hsp90), binding to the ATP-binding domain in the *N*-terminal region of the protein.[7−9] Radicicol remains one of the most potent Hsp90 inhibitors *in vitro*,[10] although it has little or no activity *in vivo*.[11,12] The lack of *in vivo* activity of radicicol is presumably a result of its reactive epoxide and dienone moieties coupled with the likely hydrolytic instability of the lactone, and in an attempt to circumvent these potential problems, Danishefsky and co-workers developed a novel series of inhibitors based on cycloproparadicicol **2**.[13−16] Other RALs such as the pochonins have been extensively investigated by Winssinger and co-workers, resulting in the identification of pochoxime A **3** as a potent inhibitor of Hsp90.[17,18] However, despite these advances, the early promise of radicicol has yet to translate into a clinical candidate based on the molecular framework of the natural product, although at least two compounds based on the resorcinol pharmacophore, NVP-AUY922 **4**[19,20] and AT-13387 **5**[21,22] (Figure 1), are in clinical trial. Given the likely metabolic instability of the radicicol lactone, we considered its replacement by a more hydrolytically stable lactam unit. Although such a tactic has been used in the successful development of the macrolactam ixabepilone (Ixempra) from the corresponding macrolactone epothilone A,[23] it has not previously been exploited in the RAL arena and therefore represents a novel approach. We now report the implementation of this strategy, with the synthesis of a completely new family of radicicol analogues based on the 14-membered macrolactam framework **6**, together with a study of their metabolic stability, their binding to the protein by X-ray crystallography, and their detailed biological evaluation.

Hsp90, one of the most abundant proteins in eukaryotic cells, is an ATP-dependent chaperone and has been described as the master regulator of the stabilization, activation, and degradation of a range of overexpressed or mutant oncogenic proteins such as

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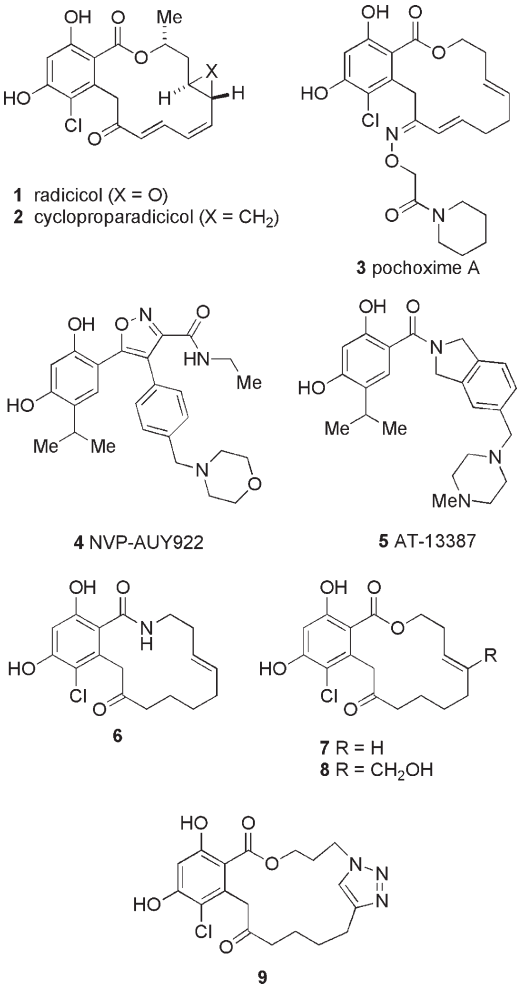


Figure 1. Structures of the naturally occurring Hsp90 inhibitor radicicol **1**, its cyclopropane analogue **2**, pochoxime A **3**, synthetic resorcinols NVP-AUY922 **4** and AT-13387 **5**, the generalized resorcylic acid macrolactam structure **6**, and radicicol analogues **7**−**9**.

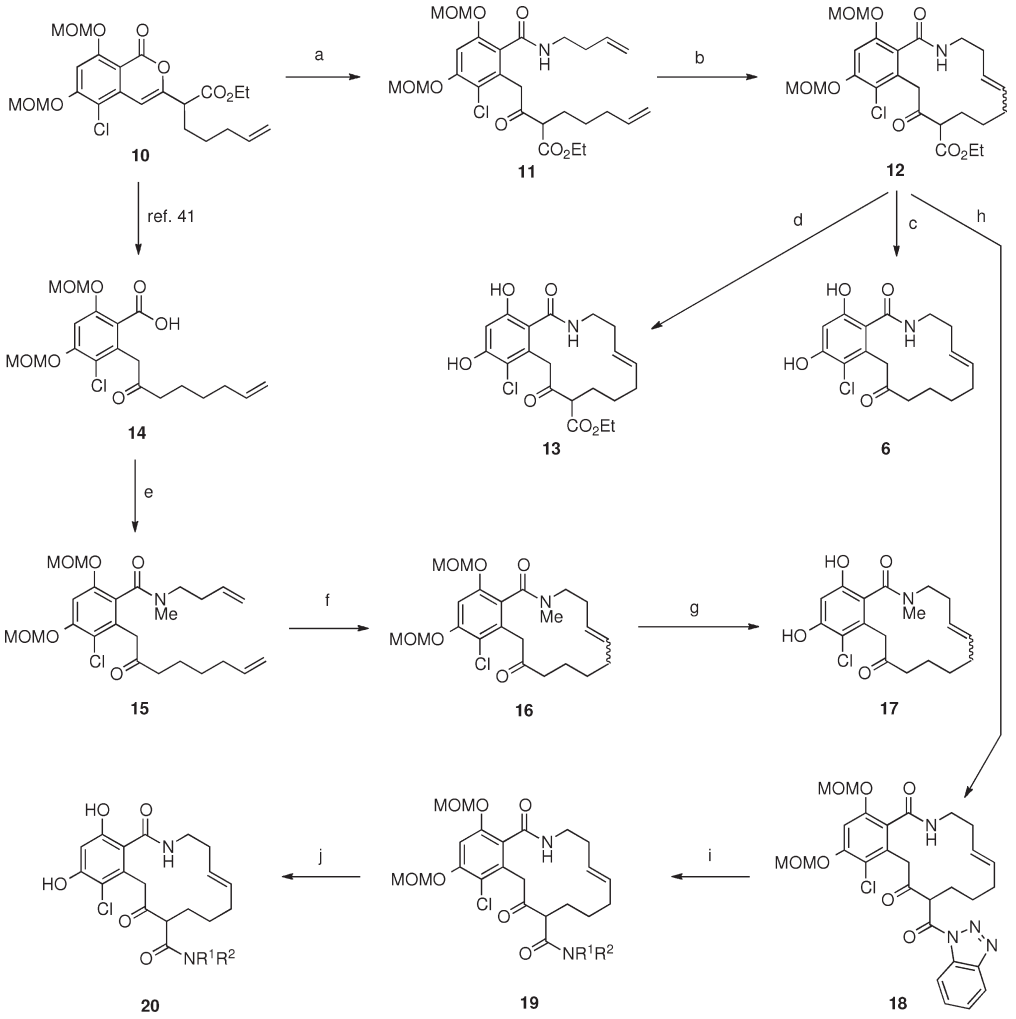
C-RAF, B-RAF, ERBB2, AKT, telomerase, and p53.[24] As a result, Hsp90 has emerged as a very attractive target for novel molecular cancer therapeutic agents. Its inhibition can cause client proteins to adopt misfolded conformations that are ubiquinated and subject to proteasomal degradation, thereby simultaneously disrupting multiple cancer-causing pathways.[25−34] The validation of Hsp90 as potential therapeutic target was greatly facilitated by natural products such as radicicol and geldanamycin, and a derivative of the latter compound, 17-allylamino-17-demethoxygeldanamycin (17-AAG, tanespimycin), was the first Hsp90 inhibitor to enter the clinic. 17-AAG has shown clinical promise, especially the recently reported proof of concept phase II activity in HER2/ERRB2-positive trastuzumab-refractory breast cancer,[35] but its development has been discontinued as the result of a combination of factors, including cumbersome formulation and hepatoxicity that is likely quinone-related. Nevertheless, Hsp90 remains a very attractive molecular target, and there are now more than 15 Hsp90 inhibitors in clinical trial, with accumulating evidence of anticancer activity.[31,34,36−38]

Our own work in this area has involved the synthesis and biological evaluation of a series of novel resorcylic acid macrolactones of varying ring size and conformation.[39] One of these analogues, NP261 **7** (Figure 1), exhibited potent binding to the *N*-terminal ATP site of Hsp90, with an IC_{50} = ∼40 nM (fluorescence polarization binding assay) and also showed the established molecular signature of Hsp90 inhibitors, *i.e.*, depletion of client proteins with upregulation of Hsp70 in treated cancer cells. X-ray crystallography studies revealed that **7** also was bound to the *N*-terminal ATP site of yeast Hsp90 in a way comparable to the structurally more complex natural product,[39] although it lacks the interaction with the ε-amino side chain of Lys44, present through the epoxide oxygen in the natural product. In an attempt to restore this potentially useful H-bonding interaction, we synthesized further resorcylic acid macrolactones **8** and **9** incorporating heteroatoms, with the potential to bind to the side chain of Lys44,[40,41] Although protein crystallography established that macrolactone **8** did indeed bind in the ATP site of Hsp90 as expected with the additional H-bond to Lys44,[41] an unexpected change in the overall conformation of the macrocyclic ring resulted in poorer binding and hence biological activity. In considering the design and synthesis of novel analogues we were struck by the fact that so little work on resorcylic acid *macrolactams* has been reported, notwithstanding the fact that replacing a potentially labile lactone with a more hydrolytically stable lactam is an established tactic in medicinal chemistry (*vide supra*). Thus, there are but two reports of the 14-membered lactam framework **6** (plus two claims in patents[42,43]) in Danishefsky and co-workers' attempts to prepare the lactam analogue of cyclopropradicicol[14] and in Winssinger and co-workers' synthesis of the lactam analogue of pochoxime A.[18] Therefore we initiated a more detailed study of the synthesis of novel resorcylic acid macrolactams and their binding to Hsp90, the results of which are reported herein. Our new macrocyclic lactams exhibit a biological profile equivalent or superior to that of the related lactones, possess greater metabolic stability, and show the established molecular signature of Hsp90 inhibition in human colon cancer cells. Of particular interest, and unlike previous radicicol-based derivatives, the *N*-benzylamide lactam analogue exhibited additional binding to a hydrophobic pocket of the protein, which likely explains the improved activity of this compound compared to that of our other synthetic macrolactones and macrolactams, including NP261 **7**.

RESULTS AND DISCUSSION

Chemistry. Many recent syntheses of macrocyclic compounds, including our own routes to resorcylic acid macrolactones,[39−41,44] rely on a ring-closing metathesis (RCM) reaction to form the macrocyclic ring,[45,46] and given the robustness of this approach it seemed equally applicable to the synthesis of the desired macrolactam framework. The key starting material was the isocoumarin **10**, prepared as previously described.[40,44] Ring opening of the isocoumarin ring with 1-aminobut-3-ene gave the RCM precursor **11** in a modest 35% yield respectively, treatment of which with Grubbs' second-generation catalyst {benzylidene[1,3-bis-(2,4,6,-trimethylphenyl)-2-imidazolidinylidene]dichloro(tricyclohexylphosphine)ruthenium} gave the macrolactam **12** in good yield but as an inseparable mixture (72:28) of *E*/*Z*-double bond isomers (Scheme 1). Hydrolysis and decarboxylation of the β-ketoester **12**, followed by deprotection of the methoxymethyl ethers using trifluoroacetic acid (TFA) in dichloromethane, gave the desired macrolactam **6**, isolated after purification as the *E*-isomer in 20% yield over the three steps. Alternatively the ester group could be retained and treatment of macrolactam **12** with

Scheme 1[a]



[a] Compounds **19** and **20**: **a**, R^1 = H, R^2 = CH_2Ph; **b**, R^1 = H, R^2 = Et; **c**, R^1 = H, R^2 = $MeOCH_2CH_2$; **d**, R^1 = H, R^2 = Ph; **e**, R^1R^2 = $(CH_2)_2O(CH_2)_2$; **f**, R^1R^2 = $(CH_2)_4$. Reagents and conditions: (a) $H_2C{=}CHCH_2CH_2NH_2 \cdot HCl$, Et_3N, THF, reflux, 18 h (35%); (b) Grubbs' II, CH_2Cl_2, reflux, 2 h (69%); (c) (i) LiOH, THF, MeOH, water, RT 16 h, (ii) toluene, reflux, 4 h, (iii) TFA, CH_2Cl_2, 2 h (20% over 3 steps); (d) TFA, CH_2Cl_2, 0.5 h (65%); (e) $H_2C{=}CHCH_2CH_2NHMe \cdot HCl$, HATU, *N*-methylmorpholine, DMF, RT, 18 h (47%); (f) Grubbs' II, CH_2Cl_2, reflux, 2 h (87%); (g) TFA, CH_2Cl_2, 0.5 h (71%); (h) (i) LiOH, THF, MeOH, water, RT 18 h, (ii) $SOCl_2$, benzotriazole, CH_2Cl_2, RT, 2 h (22%); (i) amine, THF [**19a**, 61%; **19b**, 79%; **19c**, 50%; **19d**, 55%; **19e**, 63%; **19f**, 77%]; (j) TFA, CH_2Cl_2, 0.5 h [**20a**, 47%; **20b**, 56%; **20c**, 17%; **20d**, 40%; **20e**, 15%; **20f**, 40%].

TFA gave the corresponding deprotected compound **13**, again isolated as a single *E*-isomer after purification in good yield. The corresponding *Z*-isomer was not isolated. The *N*-methyl lactam **17** was prepared slightly differently, by initial ring opening of the isocoumarin with aqueous lithium hydroxide as previously described,[40,44] followed by amide coupling of the resulting acid **14** to give the RCM precursor **15**. Thereafter treatment with Grubbs' II catalyst resulted in macrocyclization to give lactam **16** in excellent yield as a mixture (77:23) of *E*/*Z*-double bond isomers, deprotection of which gave the desired macrolactam **17** (Scheme 1). Macrolactam **17** was initially formed as a mixture of *E*/*Z*-isomers (ca. 70:30,) but a sample of the pure *E*-isomer could be obtained by crystallization, and the structure was confirmed by X-ray crystallography (see Supporting Information).

In order to provide additional functionality on the macrolactam ring, the ester group was converted into a range of amides by hydrolysis to the carboxylic acid and coupling to the relevant amine. A number of standard amide coupling protocols, including HATU and CDI, were evaluated, but none were satisfactory, and the only method that reliably gave good yields of the desired amides employed the acyl benzotriazole **18** as the key intermediate.[47] Thus reaction of benzotriazole **18** with benzylamine gave the corresponding amide **19a** (61%), isolated after purification as the pure, fully characterized *E*-isomer. Deprotection of the methoxymethyl ethers with TFA gave the pure macrolactam **20a** in modest yield. Given the biological activity of the *N*-benzylamide **20a** (see later), a range of other amides **20b**−**20f** was synthesized by a similar coupling protocol (Scheme 1).

***In Vitro* Metabolism of Macrolactones and -lactams.** A key rationale for this project was the replacement of a potentially labile lactone with a more hydrolytically stable lactam, and hence it was important to establish experimentally the relative metabolic stability of macrolactones and -lactams. Therefore the *in vitro* metabolism of macrolactone **7** was compared with representative macrolactams (compounds **17** and **20b**) using an LC−MS

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Table 1. *In Vitro* Metabolism of Macrolactones and -lactams in Human Liver Microsomes (HLM) in the Presence or Absence of NADPH/NADH Showing Percentage of Compound Metabolized in 15 and 30 min

	HLM 15 min		HLM 30 min	
compound	+ cofactors	microsomes only	+ cofactors	microsomes only
radicicol **1**	83.7 ± 1.1	11.3 ± 5.1	83.4 ± 13.7	32.7 ± 25.9
lactone **7**	97.0 ± 1.4	8.0 ± 6.8	90.0 ± 3.2	10.5 ± 3.9
lactam **17**	42.5 ± 2.5	5.9 ± 3.1	66.5 ± 1.0	5.9 ± 3.1
lactam **20b**	36.9 ± 5.3	2.8 ± 5.7	46.9 ± 5.4	8.5 ± 5.1

assay following incubation in human liver microsomes (HLM).[48,49] For comparison, radicicol itself was also studied, with all experiments being conducted in the presence or absence of the NADPH/NADH cofactors needed for phase I metabolism. The results (Table 1) clearly establish that both macrolactams **17** and **20b** are reproducibly metabolized to a lesser extent than either radicicol **1** or macrolactone **7** at both 15 and 30 min time points. Unsurprisingly, much less metabolism occurs in the absence of cofactors, although radicicol is somewhat less stable than macrolactone **7** (*ca.* 30% metabolized after 30 min). These data support our original contention that replacement of the potentially labile macrolactone with a lactam unit would result in compounds that showed greater metabolic stability.

Protein Crystallography. In order to delineate the structural details of the binding of our novel resorcylic acid lactams to Hsp90, selected representative macrolactams **6**, **13**, and **20a** were successfully co-crystallized with the *N*-terminal domain of the yeast protein, and the structures of the resulting complexes were solved by molecular replacement. Comparison with our previously determined structure of Hsp90-bound NP261 **7**[39] showed that although the macrolactone ring of NP261 **7** superimposes with the macrolactone ring of radicicol, the macrolactam rings of **6**, **13**, and **20a** adopt a slightly different conformation (Figure 2). We have previously reported this alternative conformation for a 14-membered macrolactone compound,[41] although its electron density from carbons 6 to 9 of the macrolactone ring was very weak. The equivalent electron density for the present macrolactam rings was stronger, thus showing the full details of the molecular interactions with Hsp90.

Our new macrolactams essentially bind in a similar way, making the usual contacts in the *N*-terminal Hsp90 ATP site (Leu34, Asn37, Ala41, Lys44, Asp79, Ile82, Gly83, Met84, Asn92, Leu93, Phe124, Thr171, Leu173) that are seen with radicicol **1** and NP261 **7** (Figure 3). The additional ethoxycarbonyl group of **13** makes contacts that the unsubstituted macrolactam **6** cannot (Figure 3a and b). In particular, the carboxyl oxygen makes hydrogen bond interactions *via* water molecules to the carboxyl group of Asn37 and Asp40, the main-chain carbonyl of Gly121 and the main-chain amide of Phe124. The ester oxygen of the ethoxycarbonyl group also makes a direct hydrogen bond to the carbonyl side chain of Asn92. In contrast, the *N*-benzyl group of amide **20a** binds very differently to the ethoxycarbonyl group (Figure 3c and d). Its carbonyl group makes only one hydrogen bond to the protein, *via* a water molecule, to the carbonyl of Gly121. In addition, hydrogen bonds with Asn37, Asp40, and Phe124 are disrupted, relative to compound **13**. Interestingly, the loss of these interactions is compensated by the hydrophobic interactions that result from the benzyl group binding to a

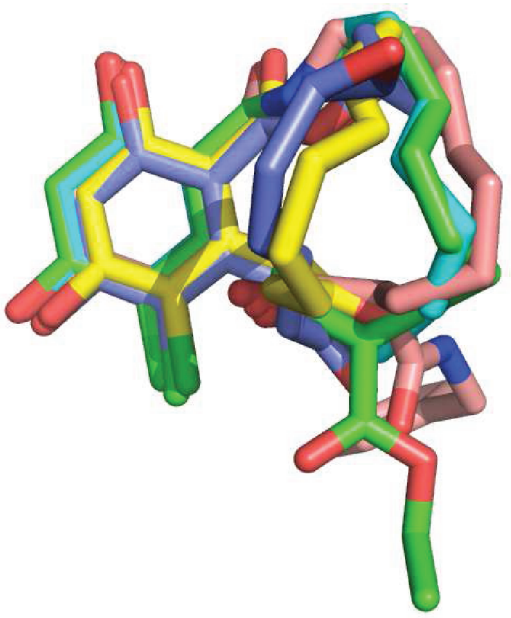

Figure 2. Binding of macrolactams to the *N*-terminal domain ATP site of yeast Hsp90 as determined by X-ray crystallography. PyMOL cartoon showing the binding conformations of radicicol **1** (blue), the macrolactone NP261 **7** (yellow), and the macrolactams **6** (cyan), **13** (green), and **20a** (salmon) in the *N*-terminal domain of yeast Hsp90.

hydrophobic pocket lined by amino acid residues Met84, Asn92, Leu93, Phe124, Trp148, and Leu173 (Figure 4). In order for the benzyl group to bind this pocket, it displaces the amino acid loop formed by residues Leu93−Lys98 in response to a steric clash with the side chain of Leu93 (Figure 3c). To our knowledge, Hsp90 radicicol-based inhibitors have not been previously seen to bind in this hydrophobic pocket, although the purine analogues PU3 (PDB 1UY6)[50] and PU-H71 (PDB 2FWZ)[51] do provide precedent for interaction with this pocket (Figure 4). Despite some minor side chain movements being seen with the residues lining the pocket in the yeast Hsp90, this pocket is essentially the same as that for human Hsp90α, except that Leu173 in yeast is Val186 in human Hsp90α (Figure 4c). However, in the Hsp90α-PU3 and H71 complexes the equivalent leucine residue of Hsp90α (Leu107) was seen to pack against the purine analogues. In contrast, we find here that the loop of the yeast Hsp90, represented by residues Leu93 to Lys98, becomes disordered when bound with macrolactam **20a**. The reason for this difference appears to be that the displaced Leu93 does not avoid the steric clash with macrolactam **20a** when it adopts the same conformation as Leu107 of Hsp90α due to the structural differences between the purine and macrolactam derivatives. Interestingly, although Leu93 fails to pack against macrolactam **20a**, Asn92 packs against the benzyl group instead (Figure 4c). Consequently, the equivalent loop in the Hsp90α-PU3 and -H71 complexes is structured as a helix rather than disordered. Mobility of this loop, Ile104−Ser113 in Hsp90α, has also been seen with other of Hsp90α-inhibitor complexes. For example, the complex with a pyrrolopyrimidine class of inhibitor (PDB 3RLR)[52] shows a backbone conformation that is open, whereas a more compact state is seen in the Hsp90α-CCT018159 complex (PDB 2CCS; Figure 4d).[53] Thus, the loop restructured as a helix, as seen in the Hsp90α-purine complexes (PU3 and H71), represents an intermediate conformation between

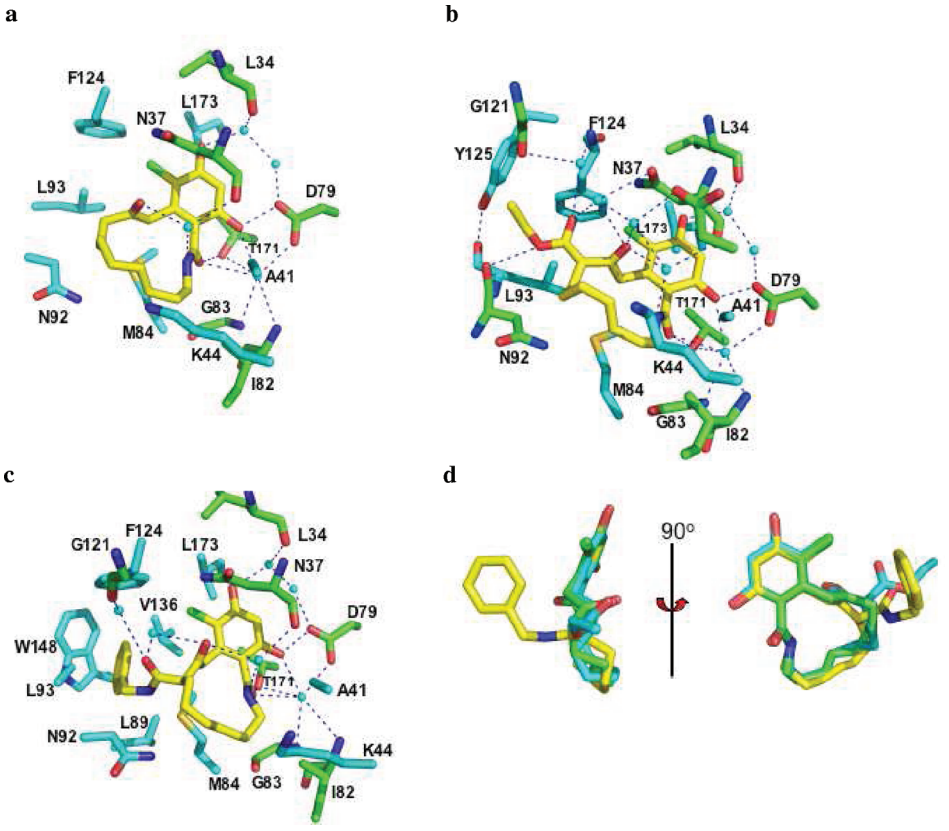

Figure 3. Binding of macrolactams **6**, **13**, and **20a** to the *N*-terminal domain ATP site of yeast Hsp90 determined by X-ray crystallography. PyMOL diagram showing binding interactions of (a) macrolactam **6**, (b) macrolactam **13**, and (c) macrolactam **20a**. Dotted blue lines, hydrogen bonds and green, amino acid residues involved; cyan-colored spheres, water molecules; cyan residues, residues solely in van der Waals contact. The structures for **6**, **13**, and **20a** were obtained at 2.2, 2.2, and 2.0 Å resolution, respectively. For atomic coordinates and structure factors, see PDB codes 2XX2, 2XX4, and 2XX5, respectively. (d) Two orthogonal views of the superimposition of **6** (green), **13** (cyan), and **20a** (yellow), showing that the compounds bind with overall similarity, except for some slight variability in the absolute conformation of the macrolactam ring. The ethoxycarbonyl group of **13** and the benzyl group of **20a** show very different binding conformations.

the open and compact states. However, for the macrolactam compound **20a**, it appears that apart from the disordered loop, the structure of the base of this loop (Ile90−Gly94) is most similar to the complex with a pyrrolopyrimidine class of inhibitor.[52] For example, Asn92 and Leu93 (yeast) and Asn106 and Leu107 (human) are in similar positions. Furthermore, the open state of the mobile loop in yeast is generally seen with ADP (PDB 1AM1), with the macrolactone class of inhibitors (PDB 2IWU), with geldanamycin (PDB 1AH4 and 2WEQ), and with macbecin (PDB 2VWC), and the compact state has been observed for some pyrazole (PDB 2BRC and 2BRE) and macrolactone (PDB 2CGF and 2IWX) class of inhibitors. However, for the open state in the examples examined herein there is no obvious steric clash with Leu93 and no groups bound to the hydrophobic pocket, which consequently remains closed.

Biology. The macrocyclic lactams **6**, **13**, **17**, and **20** were evaluated for binding to the ATP site of the *N*-terminal domain of human Hsp90β in two biochemical assays: a fluorescence polarization (FP) method[54,55] and a time-resolved fluorescence resonance energy transfer-based assay (TR-FRET).[56] Their growth inhibitory activity against a human colon cancer cell line (HCT116) was also measured by the colorimetric sulforhodamine B (SRB) assay, as used in our previous studies with Hsp90 inhibitors.[39−41,57,58] As can be seen in Table 2, the unsubstituted macrolactam **6** has a biological profile very similar to that of the equivalent lactone **7** (Entries 2 and 3). For example, IC_{50} values for Hsp90 binding in the sensitive FP assay were 50 and 40 nM, respectively. Importantly, this indicates that there is little difference in binding to Hsp90 upon changing from a lactone to lactam ring, in contrast to an earlier report that suggested a similar change in the pochoxime series of compounds was detrimental.[18] On the other hand, the corresponding *N*-methyl macrolactam **17** shows a significant reduction in binding (~35-fold in the FP assay) as compared to the NH compound **6** (entries 3 and 5). The ester-substituted compound **13** also exhibits strong binding to Hsp90, with an IC_{50} value in the FP assay of 80 nM. Interestingly, this compound also exhibited a 7- to 10-fold increase in antiproliferative activity potency against HCT116 human colon cancer cells as compared to compounds **6** and **7**.

Of special note, however, is that the benzyl amide **20a** (entry 6) exhibited an IC_{50} value in the FP assay of 200 nM compared to 40 nM for the simple lactone NP261 **7** and, surprisingly, was actually 4-fold more potent than **7** in the TR-FRET assay. In addition, **20a** was 10-fold more potent in antiproliferative activity against HCT116 colon cancer cells compared to **7**, which was the most active compound in this respect in our previous series of synthetic macrolactones, albeit that its cellular potency remained considerably less than that of radicicol. In view of the activity of compound **20a**, a range of other 10-carboxamido compounds **20b**−**20f** was investigated. Within the family of amides **20b**−**20f**,

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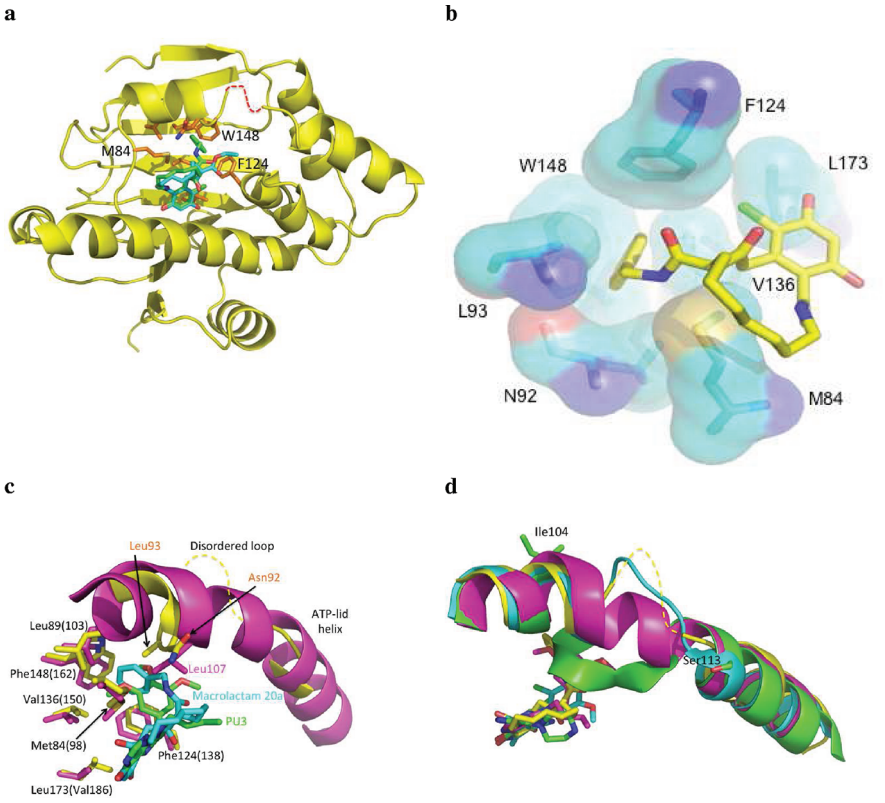

Figure 4. Binding of **20a** to the *N*-terminal domain of yeast Hsp90 determined by X-ray crystallography. (a) PyMOL cartoon showing **13** (cyan) and **20a** (green) bound to the *N*-terminal domain ATP site. Some key residues involved in the interaction with the benzyl group of **20a** are shown for orientation. The dotted red line indicates the disordered loop (amino acid residues Leu93 to Lys98). (b) PyMOL surface representation of the hydrophobic pocket bound by the benzyl group of **20a**. The benzyl group displaces and disorders the amino acid loop Leu93-Lys98 and binds in a hydrophobic pocket lined by residues Met84, Asn92, Leu93, Phe124, Val136, Trp148, and Leu173. The side chain of Leu93 beyond the Cβ carbon is disordered and not shown; **c**, PyMol diagram showing the superimposition of the residues forming the hydrophobic pocket and the ATP-lid helix of the Hsp90-macrolactam **20a** (yellow and cyan, PDB 2XX5) and -PU3 structures (magenta and green, PDB 1UY6). The dotted yellow line represents the disordered loop of the yeast complex; residues are labeled for the yeast protein, while those in parentheses are for the human Hsp90α. (d) PyMol diagram showing the alternative conformations of the mobile loop region (Ile104−Ser113) of Human Hsp90α. Yeast Hsp90-macrolactam **20a** (yellow, the dotted yellow line represents the disordered region.), Hsp90α-pyrrolopyrimidine class of inhibitor (cyan and representing the open state), Hsp90α-PU3 (magenta, helix conformation), and Hsp90α-CCT018159 (green, compact conformation).

the morpholinyl derivative **20e** (entry 10) clearly stands out as being essentially inactive compared with the others, whereas the ethyl, methoxyethyl, phenyl and pyrrolidinyl compounds **20b**−**20d** and **20f** (entries 7−9 and 11) exhibited somewhat similar biological properties, although with less potency than **6** and **7**. Hence it would appear that the advantageous properties of the *N*-benzylamide **20a** may indeed be a reflection of the additional and unusual binding to the Hsp90 protein afforded by the aromatic ring of the benzyl amide substituent.

To confirm that the observed antiproliferative effects against HCT116 human colon cancer cells were due to the intended chaperone-targeted mechanism, representative compounds **13**, **20a**, and **20f** were assessed for their effect on the established molecular signature of Hsp90 inhibition.[58−60] Macrolactam **20a** that is potent in all of the above biological assays clearly shows the depletion of the representative client proteins C-RAF, ERBB2, and CDK4, together with upregulation of Hsp72 (Figure 5). The observed upregulation of Hsp72 is a mechanism-based HSF1-mediated cytoprotection effect that is seen with all of the *N*-terminal ATP-site Hsp90 inhibitors and reduces their effectiveness; in the future this could be overcome by the use of inhibitors of Hsp72 and Hsc70.[61] This specific and diagnostic molecular signature confirms that **20a** was acting as an Hsp90 inhibitor in the HCT116 colon cancer cells studied. Likewise macrolactam **20f**, although less potent than **20a**, exhibited the same Hsp90 inhibitory molecular profile at equivalent cancer cell growth inhibitory concentrations, which were correspondingly higher in absolute terms. Interestingly, lactam **13**, which was quite potent on the Hsp90 target and on cancer cell growth, exhibited some induction of Hsp72 but did not cause depletion of client proteins, suggesting off-target effects for this compound (Figure 5). The triazole macrolactone analogue **9** prepared in our earlier study[40] is essentially inactive (IC_{50} > 100 μM) and was included as an inactive control; as expected, this compound showed no effect on Hsp90 biomarkers (Figure 5). 17-AAG was included as a positive control and exhibited the expected molecular signature. On the basis of these molecular biomarker studies, we conclude that the potent resorcylic acid macrolactam **20a** behaves *in vitro* like the clinically evaluated agent 17-AAG in inhibiting cancer cell growth through inhibition of the Hsp90 molecular chaperone.

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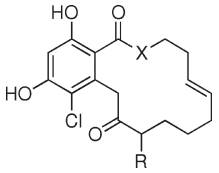
Table 2. Biological Activities of the Macrocyclic Lactams 6, 13, 17, and 20 Compared to Radicicol 1 and NP261 7[a]



entry	compound	X	R	TR-FRET IC_{50} (μM)	FP IC_{50} (μM)	HCT116 SRB GI_{50} (μM)
1	radicicol **1**			0.011	0.0043	0.00061
2	NP261 **7**	O	H	0.35	0.04	7.6
3	**6**	NH	H	0.22	0.05	9.6
4	**13**	NH	CO_2Et	0.15	0.08	1.0
5	**17**	NMe	H	5.3	1.80	33.6
6	**20a**	NH	$CONHCH_2Ph$	0.08	0.20	0.6
7	**20b**	NH	CONHEt	0.70	1.00	0.9
8	**20c**	NH	$CONH(CH_2)_2OMe$	0.60	1.10	5.6
9	**20d**	NH	CONHPh	1.60	0.66	1.7
10	**20e**	NH	CO(morpholinyl)	>100	17.3	>50
11	**20f**	NH	CO(pyrrolidinyl)	0.90	1.5	4.1

[a] TR-FRET and FP assays measure the binding of compounds to human Hsp90β, and the SRB assay measures antiproliferative activity against HCT116 human colon cancer cells.

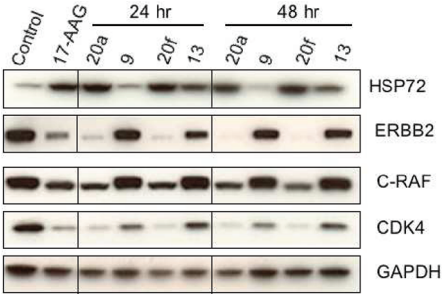


Figure 5. Western blot analysis for the expression of the Hsp90 client proteins ERBB2, C-RAF, and CDK4 and Hsp72 after treatment with novel macrolactams. HCT116 cells were treated with equivalent cell growth inhibitory concentrations corresponding to 5 × GI_{50} of compound. The triazole macrolactone analogue **9**, which is essentially inactive (GI_{50} > 100 μM), was tested at 100 μM. 17-AAG (250 nM) was included as a positive control and exhibited the expected molecular signature of Hsp90 inhibition, comprising depletion of the client proteins and induction of Hsp72. This signature was also exhibited by growth inhibitory concentrations of **20a** and **20f**. Compound **13** caused only modest induction of Hsp72 and had no effect on client proteins. GAPDH was used as a loading control.

Conclusions. Hsp90 is an important and clinically relevant new drug target for molecular cancer therapeutics and is inhibited at the *N*-terminal ATP-binding site by a range of compounds based on the resorcylic acid lactone framework, including the natural product radicicol **1** and our previously synthesized analogue **7**. Given the likely metabolic instability of the macrolactone ring in radicicol and analogues such as compound **7**, we hypothesized that its replacement by a lactam unit would result in compounds that were more metabolically stable. We have now shown that this is indeed the case, and replacement of the lactone with a lactam produces not only compounds that are more metabolically stable but also a new series of Hsp90 inhibitors with equivalent or, in some cases, superior biological profiles. Biological studies demonstrated that growth inhibitory activity of potent compound **20a** against human colon cancer cells was due to Hsp90 inhibition, as confirmed by the specific molecular signature of chaperone client protein depletion combined with Hsp72 upregulation. Protein crystallography showed that the ATP site can readily accommodate analogues of differing substitution pattern and conformation. Interestingly, the macrolactam **20a**, containing the benzyl amide group, opened a hydrophobic pocket in the *N*-terminal ATP site by displacing the loop between Leu93 and Lys98, which may explain the enhanced biological effects seen with this macrolactam. Although this effect has previously been seen for the purine-based drugs (PU3 and H71), this is unprecedented for the radicicol-based chemical class of inhibitors. The mobility of this loop raises the possibility of developing new inhibitors that are able to form favorable interactions with the alternate conformations of this loop.

METHODS

Chemistry. The synthetic procedures and characterization data for all new compounds are described in the Supporting Information.

Protein Crystallography, Data Collection, Processing, and Refinement. The expression, purification, and crystallization of the *N*-terminal domain of yeast Hsp90 has been previously described.[62] Co-crystallizations were conducted by dissolving the inhibitor in 100% DMSO at 50 mM and adding 5 μL to 1 mL of Hsp90 *N*-terminal domain at 4 mg mL^{-1} in 20 mM Tris pH 7.5 and 1 mM EDTA. The complex was then concentrated to 200 μL (20 mg mL^{-1}) and crystallized as previously described. Single crystals appeared overnight of approximate dimensions 0.3 mm × 0.2 mm × 0.2 mm. These were flash frozen after stepwise addition of glycerol to 30%, and the data were collected on

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stations I04 (compound **6**) and I02 (compound **20a**) at Diamond Light Source and in-house on a Bruker Microstar (compound **13**). The data were integrated and scaled using the automatic Xia2[63] processing at Diamond (compounds **6** and **20a**) and the Bruker AXS Proteum2 software for compound **13**.

The complexes were initially solved by isomorphous replacement using a previously determined *N*-terminal structure (PDB 1AH6) in the usual space group, $P4_322$. The model was refined using PHENIX[64] and rebuilt using COOT.[65] The FreeR for compound **6** remained high, and so the symmetry was relaxed to $P4_3$ for this structure, whereupon the refinement proceeded smoothly. The inhibitors were built using the PRODRG server. The inhibitor molecule and the waters were added in the final stages. Various programs from the CCP4 suite were used during the refinement.[63] The crystallographic statistics are given in the Supporting Information.

Biology. *Liver Microsomal Stability.* Stability in human liver microsomes was measured as previously described.[48,49]

Hsp90 Binding. Compounds were assayed for their ability to bind to Hsp90 using two assays.

TR-FRET Assay. Binding of test compounds was measured using TR-FRET. Competition by test compounds with the binding of biotinylated geldanamycin (600 nM) to the full length human Hsp90 His-tagged protein (40 nM) was determined as described previously.[56] Briefly, the europium-labeled anti-His-tagged protein antibody (Perkin-Elmer prod. no. AD0110) was added at 1 nM and the streptavidin Surelight APC (Perkin-Elmer prod. no. AD0201) at 90 nM. Compounds were tested across a 10 point concentration range up to 10 μM, and the IC_{50} values were determined.

FP Assay. Binding of test compounds was also determined by competition with a fluorescein-labeled probe as described previously.[54,55]

Cancer Cell Growth Inhibition Assay. The colorimetric sulforhodamine B (SRB) assay was used to measure the inhibition by test compounds of the growth of human HCT116 colon cancer cells *in vitro*, as described previously.[66] The GI_{50} was calculated as the compound concentration that inhibits cell growth by 50% compared with the vehicle control (0.5% DMSO).

Western Blotting. HCT116 cells were treated with 5 × GI_{50} of selected compounds for 24 and 48 h. Cell lysates were prepared, and proteins were immunoblotted and detected by enhanced chemiluminescence.[66] Antibody to Hsp72 was from Stressgen Biotechnologies and those for C-RAF, CDK4, ERBB2, and GAPDH were from BD Biosciences, Santa Cruz, Abcam, and Chemicon, respectively.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Notes

Conflict of Interest Statement: S.Y.S., M.G.R., W.A., A.H., F.I.R., and P.W. are employees of the Institute of Cancer Research (ICR), which operates a rewards to inventors scheme. P.W. and his research team, together with L.H.P. and C.P., were involved in a funded research collaboration with Vernalis, and Hsp90 inhibitors were licensed to Novartis. P.W. has been a consultant to Novartis.

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