Letters

Discovery of Benzisoxazoles as Potent Inhibitors of Chaperone Heat Shock Protein 90

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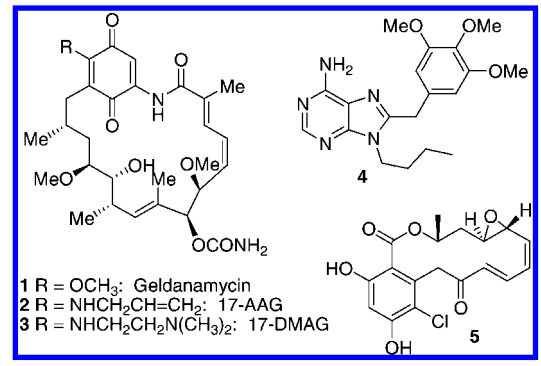
Abstract: Heat shock protein 90 (Hsp90) is a molecular chaperone that is responsible for activating many signaling proteins and is a promising target in tumor biology. We have identified small-molecule benzisoxazole derivatives as Hsp90 inhibitors. Crystallographic studies show that these compounds bind in the ATP binding pocket interacting with the Asp93. Structure based optimization led to the identification of potent analogues, such as **13**, with good biochemical profiles.

Since multiple signal transduction pathways contribute to the etiology of human cancer, considerable skepticism exists that inhibition of a single molecular target will be broadly efficacious in cancer treatment regimens. Clinical trials with signal transduction agents evolve into multiple agent affairs of increasing complexity, unpredictability, and expense. Kinase inhibitors with serendipitously defined multiple activities provide one means of target multitasking.[1] However, a more defined path to global inhibition of multiple activated pathways in tumor cells is preferable and is possible via inhibition of molecular chaperone complexes.[2–4] Chaperone function is required for folding, stabilization, and activation of many "clients" (protein kinases, steroid hormone receptors, and other diverse signaling molecules).[5–8] Loss of chaperone function leads to loss of client activity and destabilization.

Several essential molecular chaperones and cochaperones have been identified. Of these, Hsp90 has perhaps been the most extensively studied.[9–11] Its broad clientele includes proteins as structurally and functionally different as telomerase, the actin organizer N-WASP, nitric oxide synthase, a range of nuclear hormone receptors, and an ever-growing selection of protein kinases.[12] The activity of the complex depends on the Hsp90 ATPase activity, which resides in its N-terminal domain, for which 3D structures have been reported.[10,11] A number of natural products and synthetic small molecules have emerged as Hsp90 inhibitors, and most bind to the Hsp90 N-terminal domain.[13] Natural products (Chart 1) such as the benzoquinone ansamycin geldanamycin **1** and its 17-amino derivatives like 17-AAG **2** and 17-DMAG **3** are the most advanced. Purine analogues like PU3 **4**, radicicol **5**, and related resorcinol derivatives are other classes of N-terminal binders of Hsp90.

Chart 1. Examples of Hsp90 Inhibitors



Through a high-throughput screening effort followed by database mining, we identified the benzisoxazole derivative **6** (Figure 1) as an Hsp90 inhibitor. Compound **6** exhibited an IC_{50} of 0.19 μM in the florescence polarization competition assay using bodipy-geldanamycin.[14] Binding of this low molecular weight inhibitor was further evaluated by 1D NMR competition STD experiments. The compound was found to bind to the ATP binding domain as inferred from the presence of direct STD from the compound and decrease in AMP-PNP STD, indicating that it competes with AMP-PNP as well. A cocrystal structure of **6** with N-terminal domain of Hsp90 was solved at 1.6 Å resolution, clearly indicating that the compound binds in the active site (the ATP binding site).[15] However, this molecule had suboptimal aqueous solubility (4 μg/mL at pH 7.4) and probably the poor activity of this compound in the immunofluorescence-based HER2 degradation assay in the SKBR3 cell line (IC50 $\geq$ 20 μM) and in the cellular proliferation assay (IC50 $\geq$ 20 μM) in the HCT116 cell line can be attributed to low aqueous solubility. Herein, we describe our optimization efforts on this hit to obtain favorable biochemical and physical chemical profiles using structure-based design.

The benzisoxazole hit **6** and additional derivatives like **14**−**16** and **19** were prepared following the route previously described from our laboratories.[16] Derivatives designed to introduce or modify substituents to infer SAR were prepared using the appropriately substituted benzophenone intermediates **9** as shown in Scheme 1. Buchwald coupling using the iodo precursor

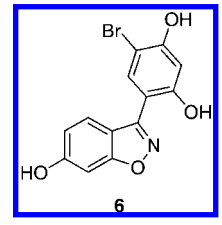


Figure 1. Hsp90 hit structure.

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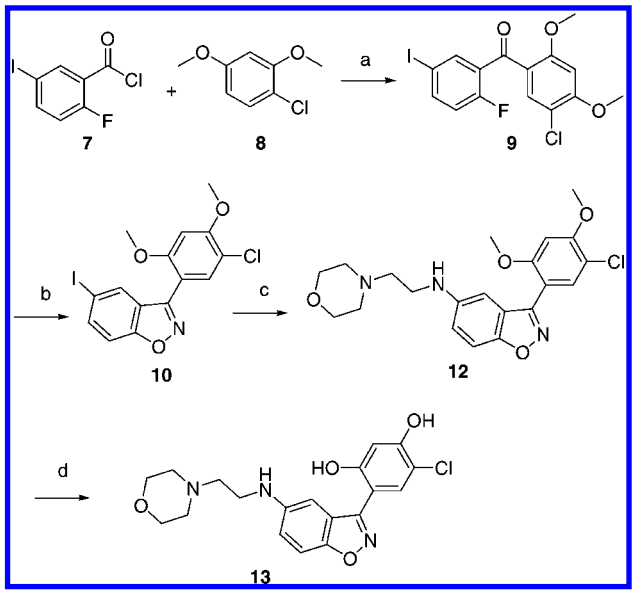
Scheme 1[a]



[a] Reagents: (a) ZnO, room temp, overnight, 50%; (b) (1) NH_2OH, pyridine, 115 °C, 16 h; (2) NaH, DMF, room temp, overnight, 90% for two steps; (c) morpholinoethylamine, $Pd_2(dba)_3$, BINAP, *t*-BuONa, toluene, 80 °C, 35%; (d) BBr_3, DCM, room temp, 8 h, 90%.

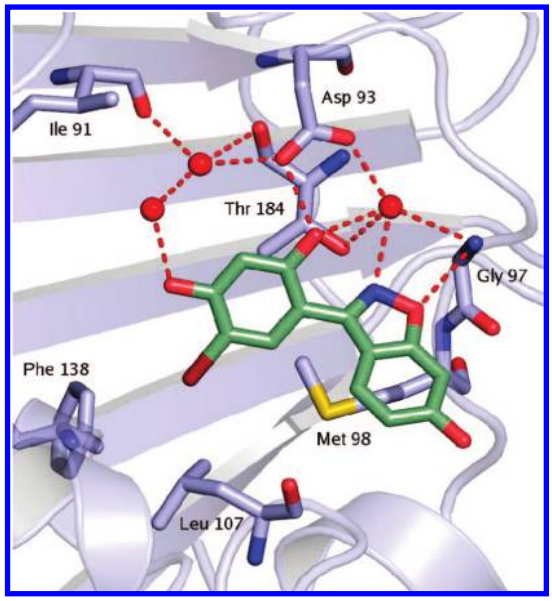
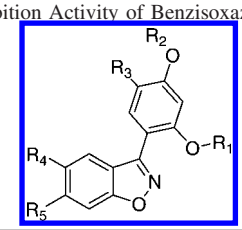


Figure 2. Atomic interactions of **6** with human Hsp90α. Polar interactions closer than 3.2 Å are shown as dashed lines.

10 introduced the water solubilizing groups on the aromatic ring. Deprotection of the methoxy group afforded the final benzisoxazole derivatives like **13**, **17**, and **18**.

The cocrystal structure of **6** with N-terminal domain of human Hsp90α (Figure 2) indicated that the bound conformation of the protein was similar to the apo structure. It is also very clear that the 2′ hydroxyl of the resorcinol moiety plays a crucial role in the ATP binding pocket interacting with Asp 93 and one of the conserved water molecules. The second hydroxyl group (4′) is involved in maintaining the network of hydrogen bonds established by the other two conserved water molecules resulting in interaction with some of the backbone residues like Leu48. This interaction is reflected in the observed SAR, wherein analogues **16** and **12** with methoxy in the place of hydroxyl groups were much less potent in the binding assay (Table 1).

Table 1. Hsp90 Inhibition Activity of Benzisoxazole Derivatives



compd	R_1	R_2	R_3	R_4	R_5	FP IC_{50}[17] (μM)	std dev
6	H	H	Br	H	OH	0.19	0.089
14	H	H	H	H	OH	15.2	(n = 1)
19	H	H	Me	H	OH	*a*	
15	H	H	Cl	H	OH	0.06	0.03
16	Me	Me	Cl	H	OMe	>20	
17	H	H	Cl	*N*-methylpiperizino-	H	0.8	0.189
18	H	H	Br	$-NHCH_2CH_2$-morpholino	H	0.04	0.001
12	Me	Me	Cl	$-NHCH_2CH_2$-morpholino	H	>20	
13	H	H	Cl	$-NHCH_2CH_2$-morpholino	H	0.03	0.012
Geldanamycin						0.02	0.009

[a] In the case of **19**, the compound fluorescence was interfering under the assay condition.

Table 2. Biochemical Profile of Analogue **13**

assay	IC_{50} (μM) (n)	std dev
Hsp90 FP binding	0.03 (5)	0.012
HCT 116 cell proliferation	0.28 (4)	0.06
SKBR3 cell proliferation	0.37 (3)	0.06
LnCap cell proliferation	0.17 (3)	0.006
DU145 cell proliferation	0.29 (3)	0.08
H157 cell proliferation	0.23 (3)	0.02
SKBR3 Her-2 degradation	0.33 (6)	0.1
LnCAP androgen receptor degradation	0.33 (4)	0.06
HCT 116 cell proliferation (geldanamycin)	0.05 (54)	0.035

In the crystal structure, the 5′ bromo group is pointing toward a small hydrophobic pocket interacting with Phe138 and Leu107. Analogue **14**, which lacks a hydrophobic group in this position, leads to a significant loss in potency, indicating the importance of this favorable interaction. Replacing the bromo group with chloro group (analogue **15**) did not dramatically change the activity. From the cocrystal structure it was also evident that the benzisoxazole scaffold by itself makes some key interactions with the binding pocket. The N of the ring interacts with a conserved water molecule, which in turn is in proximity to Asp93 carboxylate to form a hydrogen bond. However, the 5-hydroxyl group on the benzisoxazole group was well exposed to the solvent.

As mentioned earlier, although **6** showed favorable potency in the FP binding assay, its lack of potency in the cellular assays was of concern. On the basis of the structural information, it is very clear that the solvent exposed C-5 and C-6 regions of the benzisoxazole scaffold are very well suited for incorporating water-solubilizing groups. Analogue **17** with the *N*-methylpiperizino group as the water solubilizing group in the C-5 position was found to be slightly less potent in the Hsp90 binding assay but showed modest potency in the proliferation assay (IC_{50} = 9.4 μM) in the HCT-116 cell line. Probably the rigidity posed by the piperizine ring is not highly favorable for the binding. To address this, analogue **13** with a flexible linker to append the water-solubilizing group (aminoethylmorpholino group) was prepared. This compound showed improved potency (Table 2) in the proliferation assay and maintained or slightly enhanced the potency in the binding assay. The corresponding bromo analogue **18** was equipotent as well. Compound **13** was cocrystallized with the amino terminal domain of Hsp90 (1.6 Å)[15] (Figure 3) and was indeed found to bind in the same

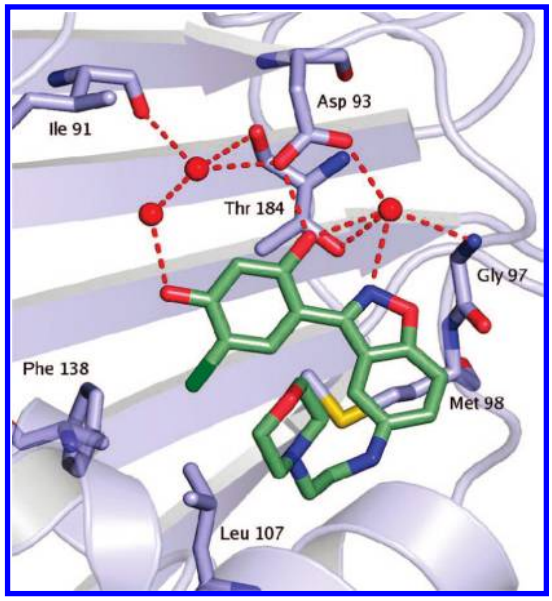


Figure 3. Cocrystal structure of **13** with human Hsp90α.

fashion, maintaining the atomic contacts with the active site Asp93 and conserved water molecules. The water-solubilizing group was readily accommodated by the rearrangement of the L2 loop, which is no longer in the closed conformation observed earlier with compound **6**.

Compound **13** was tested in a panel of cell lines for its ability to inhibit cellular proliferation. It was found to be active in a number of cell lines with submicromolar IC_{50} values. To validate that the antiproliferative effect observed with **13** is indeed related to the inhibition of Hsp90, the compound's ability to degrade the Hsp90 client proteins Her-2 and androgen receptor was determined. The compound had an IC_{50} in these assays comparable to that of cell proliferation indicative of inhibition of cellular Hsp90. These effects are not a result of nonspecific degradation because actin is unaffected by treatment with **13** and Hsp70 is up-regulated under conditions where Bcr-Abl is down-regulated in K562 cell extracts. Although the compound binds to the ATP binding pocket of Hsp90, it was found to be very selective for Hsp90 inhibition and was not active when tested against a panel of kinases like B-Raf, PKC-ϵ, PKC-θ, PI3K-α, PDK-1, MK2, IKK-2, ActRIIB, and m-TOR (IC_{50} > 20 μM).

In summary, we have identified benzisoxazole derivatives as potent and selective inhibitors of molecular chaperone Hsp90. The hit to lead optimization was guided by structure-based design facilitated by the cocrystallization efforts. Inhibitors with improved physical properties resulting in enhanced potency in the cellular systems are disclosed.

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Supporting Information Available: Experimental details and spectral data for the compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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