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Natural and semisynthetic azaphilones as a new scaffold for Hsp90 inhibitors

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ABSTRACT

A series of mold metabolites of Ascomycetes, structurally belonging to the class of azaphilones, were found to inhibit the heat shock protein Hsp90. In particular, bulgarialactone B was tested for its binding to Hsp90 using surface plasmon resonance and limited proteolysis assays and for its effects on Hsp90 client proteins expression in a series of human tumor cell lines. This compound showed high affinity for Hsp90, interacting with the 90–280 region of the N-terminal domain and down-regulated the Hsp90 client proteins Raf-1, survivin, Cdk4, Akt, and EGFR. Bulgarialactone B and other natural azaphilones showed antiproliferative activity in a panel of human tumor cell lines; their conversion into semisynthetic derivatives by reaction with primary amines increased the antiproliferative activity. Preliminary results indicated in vivo activity of bulgarialactone B against an ascitic ovarian carcinoma xenograft, thus supporting the therapeutic potential of this novel series of Hsp90 inhibitors.

1. Introduction

Interest in the heat shock protein 90 (Hsp90) molecular chaperone as a therapeutic target is related to its central role in correct folding and stabilization of proteins involved in malignant behavior and tumor progression.[1] Multiple signal transduction pathways implicated in the regulation of cell proliferation and survival are dependent on Hsp90.[2] Several Hsp90 client proteins are involved in critical processes including cell-cycle regulation and apoptosis. The heat shock proteins are often overexpressed in tumor cells, and this supports their ability to survive under unfavorable stress conditions (e.g., hypoxia and acidosis). The essential chaperoning function of Hsp90 is subverted during oncogenesis to make malignant transformation possible and to facilitate rapid somatic evolution. Functioning as a biochemical buffer for the numerous genetic lesions that are present within tumors, Hsp90 allows mutant proteins to retain or even gain function while permitting cancer cells to tolerate the imbalanced signaling that such oncoproteins create. Thus, targeting Hsp90 may have the potential advantage of simultaneously blocking multiple oncogenic pathways.

Hsp90 exists as a homodimer made up of three domains.[1,3] The N-terminal domain contains an ATP-binding site that binds the natural products geldanamycin and radicicol, and the analogue 17-AAG (Chart 1). The middle domain is highly charged and has high affinity for cochaperones and client proteins. A second ATP-binding site is located in the C-terminus of Hsp90. This C-terminal nucleotide binding pocket has been shown to bind not only ATP, but cisplatin, novobiocin, epigallocatechin-3-gallate (EGCG) and taxol.[4]

A number of highly specific Hsp90 inhibitors have been identified. They redirect Hsp90 chaperoning activity and decrease cellular levels of its numerous cancer-related client proteins.[1] Such inhibitors exhibit promising antitumor activity as single agents or in combination with other cytotoxic agents,[5–7] and some of them are in clinical development.[8,9]

During a target-oriented screening of natural compounds aimed at stabilizing and enhancing the function of p53, a transcription

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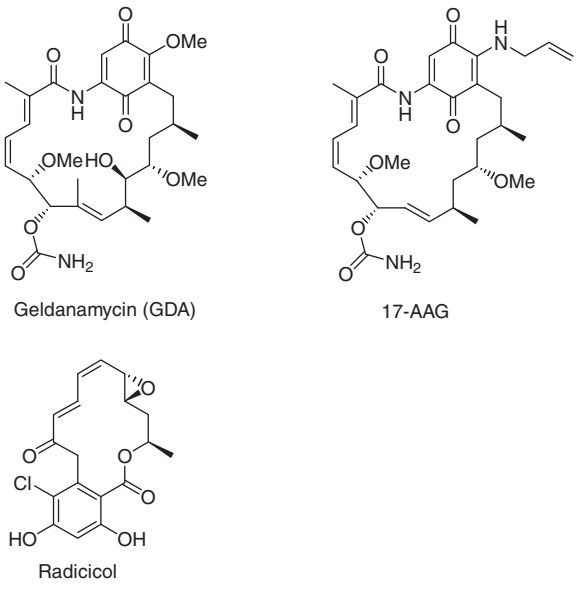


Chart 1.

factor which regulates the cell cycle and thus functions as a tumor suppressor, we found that a fungal metabolite, named bulgarialactone B (**2**),[10] did not change the wild-type p53 protein level but induced partial down-regulation of the mutant p53, which is known to be stabilized by Hsp90. This result prompted us to investigate the activity of bulgarialactone B as a potential Hsp90 inhibitor.

Structurally, bulgarialactone B features a highly oxygenated tricyclic core linked to a 13-C unsaturated chain containing a β-diketone. It is a member of a large group of fungal pigments known as azaphilones, mainly isolated from perfect and imperfect stages of Ascomycetes, such as *Aspergillus, Penicillium, Hypoxylon*, and *Monascus* spp.[11]

Azaphilones have been reported to exhibit a wide range of biological activities, including monoamine oxidase inhibition,[12] tumor promotion inhibition,[13–15] cytotoxicity,[16,17] gp120-CD4 binding inhibition,[18] and sphingosine kinase inhibition.[19]

The promising results obtained by testing compound **2** prompted us to investigate the activity of bulgarialactone B and other azaphilones as potential novel Hsp90 inhibitors.

2. Chemistry

Bulgarialactone B (**2**), together with bulgarialactone A (**1**) and bulgarialactone C (**3**), was first isolated in low yields from a strain of *Bulgaria inquinans* by Stadler et al.[10] In order to increase the production of compounds for biological tests and chemical derivatization, we cultured *B. inquinans* ICRM-184 on corn-steep agar. Under these conditions, we obtained bulgarialactones A and B in high yields and, in addition, we isolated a new azaphilone, named bulgarialactone D (**4**), whose structure was established on the basis of spectroscopic data (Chart 2).

Catalytic hydrogenation of **2** saturated the triene system of the side-chain, giving compound **5** (Scheme 1).

Among the rich class of azaphilones, the metabolites of the ascomycete *Monascus purpureus* are well known and easily available, due to the use of the mold to ferment edible rice in East Asia.[20] From a culture of this fungus on RA medium, we isolated the known ankaflavin (**6**),[21] monascin (**7**),[22] monascorubrin (**8**),[23] and rubropunctatin (**9**)[24] (Chart 3), all possessing the peculiar core of azaphilones. These compounds possess different side-chains in position 3 and 6 compared to bulgarialactone B.

Finally, we investigated a different series of azaphilones, deflectins[25] (**10–13**), that we reisolated from cultures of a strain of *Aspergillus deflectus* CBS 109.55 on RA medium (Chart 4). These compounds, differently from the *Monascus* metabolites, have an angular lactone fused to a 3,7-dimethyl-7,8-dihydroisochromen-6-one.

To gain insight into the structural requirements affecting the biological activity of these natural compounds, we exploited the known reactivity of azaphilones toward amines, consisting in an exchange of the pyranyl oxygen for nitrogen to give vinylogous γ-pyridones.[11]

Thus, we prepared derivatives of the three series with differently substituted primary amines (Schemes 2 and 3).

The reaction of bulgarialactone B and of its hexahydroderivative **5** with amines proceeded via Michael addition of the nucleophilic amines to the electrophilic C-8 carbon. This resulted in the formation of carbinolamines which underwent C–O bond cleavage to generate β-enaminones **14a–e** (Scheme 2). This behavior had previously been observed in epicocconone[26] and wortmannin.[27]

The reactions proceeded smoothly with primary amines, whereas treatment with secondary amines did not produce the desired enaminones. A possible explanation for this different reactivity could be the higher stability of enaminones **14** derived from primary amines due to hydrogen bonding between the enamine and the carbonyl at position 7. In fact, the chemical shift of the amine hydrogens, which is always in the range of 10–11 ppm, is indicative of strong hydrogen bonding[26] (Scheme 2).

When **5** was reacted with an excess of benzylamine for a prolonged time, compound **15** was obtained due to a further nucleophilic attack of the amine on the carbonyl in position 3′ (Scheme 3).

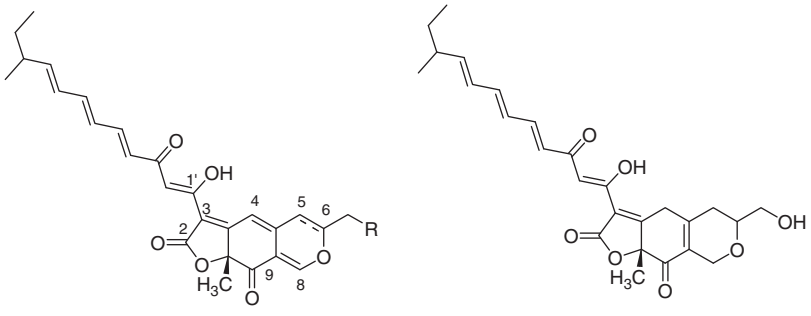
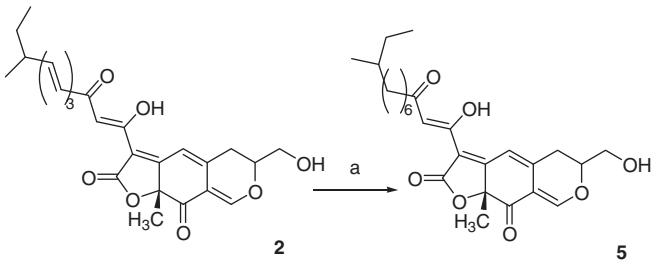
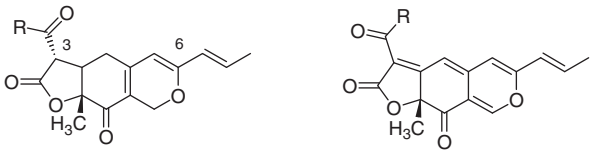


Chart 2.



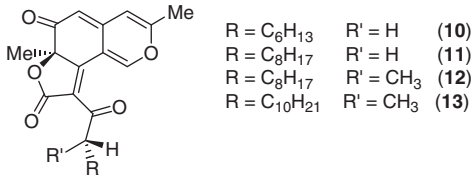
Scheme 1. Reagents and conditions: (a) H_2/Pd/C 10%, AcOEt, rt, 78%.



R = C_7H_{15} Ankaflavin (**6**)
R = C_5H_{11} Monascin (**7**)

R = C_7H_{15} Monascorubrin (**8**)
R = C_5H_{11} Rubropunctatin (**9**)

Chart 3.



R = C_6H_{13} R' = H (**10**)
R = C_8H_{17} R' = H (**11**)
R = C_8H_{17} R' = CH_3 (**12**)
R = $C_{10}H_{21}$ R' = CH_3 (**13**)

Chart 4.

Treatment of compound **14a** with sodium periodate afforded the tricyclic derivative **16** possessing the 7-nitrogen substituted core, without isolation of the intermediate aldehyde (Scheme 4).

By contrast, azaphilones containing a pyranyl ring, such as **6** and **14**, reacted with amines to give directly the corresponding cyclic derivatives **17–19**. The reaction apparently proceeds via an open-ring intermediate containing an enaminone and a carbonyl group, which immediately cyclizes to the vinylogous pyridone[11] (Schemes 5 and 6).

3. Results and discussion

As a first step of this study, we investigated the cellular and biochemical profile of compound **2**.

The antiproliferative assays were performed on a panel of human tumor cell lines following 72 h exposure. The IC_{50} values of **2**, reported in Table 1, indicated variable antiproliferative effects on the tested cell lines in a range of micromolar concentrations.

Successively, compound **2** was tested for its effects on Hsp90 client protein expression in the same cell lines used in the antiproliferative assay (Fig. 1). Following 24-h exposure to a concentration corresponding to IC_{80} (around $2 \times IC_{50}$; i.e., ca. 10 μM), the examined client proteins (Raf-1, survivin, Cdk4, Akt, and EGFR) were down-regulated. The effect was marginal in melanoma JR8 cells. In contrast, protein depletion was almost complete in the ovarian carcinoma cell line, IGROV-1. The p53 protein was partially down-regulated only in A431 cells carrying a mutant p53 protein. This effect was consistent with the stabilization of the mutant form by Hsp90.

In an effort to confirm the Hsp90/compound **2** binding and to identify the binding site, we used surface plasmon resonance (SPR) analyses and the limited proteolysis–mass spectrometry technique.[28] SPR allows to verify the affinity of compounds toward the protein, and to assess how they associate with and dissociate from the protein in real time, giving a more detailed view of their interaction with Hsp90. SPR experiments were performed using radicicol (Chart 1), a well-known Hsp90 inhibitor, as a positive control. Our data demonstrated the high affinity of **2** for Hsp90, comparable with that measured for radicicol (Table 2), both in terms of thermodynamic (K_D) and kinetic (k_d) dissociation constants.

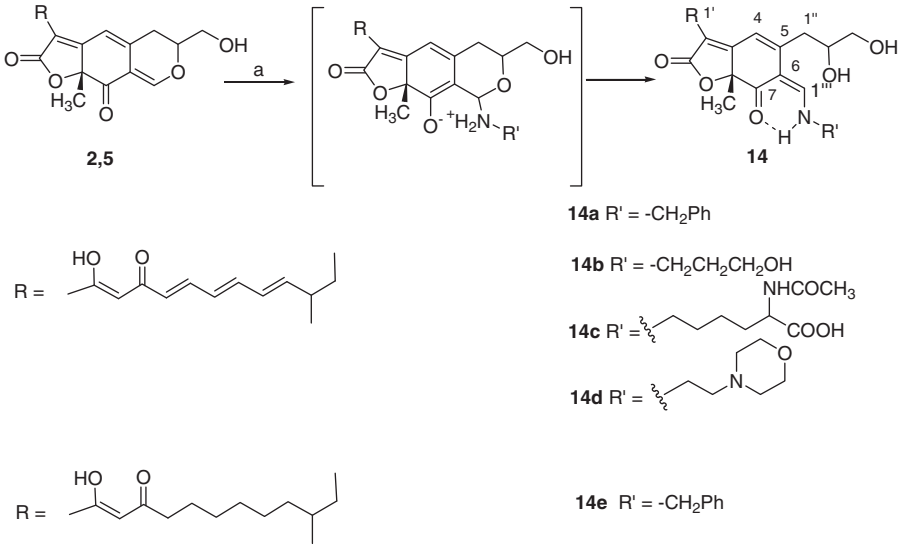
The limited proteolysis approach is based on the evidence that exposed, weakly structured, and flexible regions of a protein can be recognized by a proteolytic enzyme. Trypsin, chymotrypsin, and endoprotease V8 were selected as conformational probes in order to provide structural information on different regions of Hsp90. The fragments released from the protein were identified by MALDI-TOF/MS analyses, leading to the assignment of cleavage sites (Table 2). The differences in the proteolytic patterns were analyzed to identify the protein regions involved in the molecular interactions.[29]

In Figure 2A, the preferentially hydrolyzed positions observed on isolated Hsp90 are shown: widespread cleavage sites within the entire protein structure have been identified. Comparing our data with the predicted secondary structure organization of Hsp90,[30] it can be deduced that most of the identified cleavage sites are located in unstructured portions, thus confirming the efficiency of the approach. To further validate the method, the radicicol/Hsp90 complex was investigated, since the radicicol binding region has been deeply characterized by crystallography.[31] The results achieved in the radicicol/Hsp90 complex are shown in Figure 2B. Comparison between the proteolysis patterns observed on Hsp90 with or without radicicol indicates significant protection of the N-terminal domain from enzymatic hydrolysis. This result is in agreement with the radicicol/Hsp90 interaction involving the ATPase located in the N-terminal domain of Hsp90.[31]

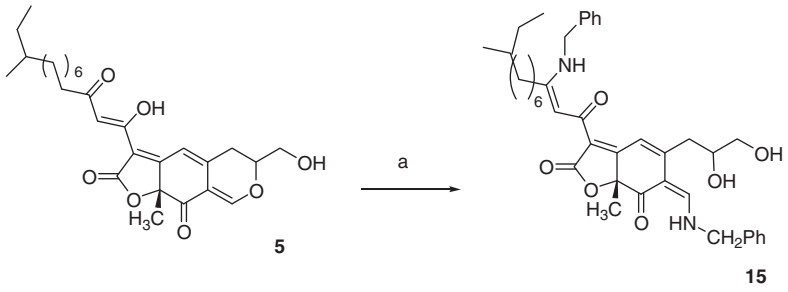
Limited proteolysis analysis of the compound **2**/Hsp90 complex produced the fragmentation pattern summarized in Figure 2C. These data demonstrate that most of the N-terminal domain sites as well as those located in the protein portion interconnecting this domain to the middle domain were protected following complex formation, thus suggesting an interaction involving the 90–280 region of Hsp90.

In contrast to the effects of ansamycin inhibitors of Hsp90 (e.g., 17AAG), compound **2** did not induce up-regulation of Hsp70. The lack of modulation of Hsp70 could reflect a different binding mode of **2** to Hsp90, which did not involve the shift of client association from Hsp90 to Hsp70 prior to proteasome-mediated degradation. In A431 cells treated with **2** for 4 h, a coimmunoprecipitation experiment performed with anti-Raf-1 antibody revealed a reduced amount of Hsp90 bound to the client protein, as could be expected from an Hsp90 inhibitor (Fig. 3A). The effect was comparable to that of 17AAG at equitoxic concentrations. However, this short treatment did not induce a shift of the binding of Raf-1 from Hsp90 to Hsp70, because the level of coprecipitated Hsp70 with Raf-1 was undetectable (Fig. 3B). The lack of up-regulation of Hsp70 in treated cells has been observed with other putative inhibitors of Hsp90 (e.g., epigallocatechin[32] and curcumin[33]). This finding would suggest a mechanism of interaction different from that of geldanamycins, that target the ATP-binding site of Hsp90. This interpretation was also supported by proteolysis experiments (Fig. 2). If interaction with other cochaperones contributes to the modulation of Hsp90 function by the novel agents remains to be explored.

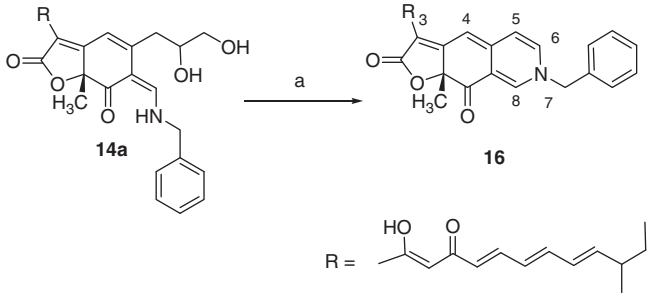
In the above experiments, aimed at a biochemical analysis of protein expression modulation (Fig. 1), the exposure time was only 24 h, because prolonged exposure resulted in the partial detachment of cells. Indeed, at 48 h, marked cleavage of poly(ADP-ribose)polymerase and activation of caspase-3 were found in cells treated with **2**, supporting apoptosis induction (Fig. 4).



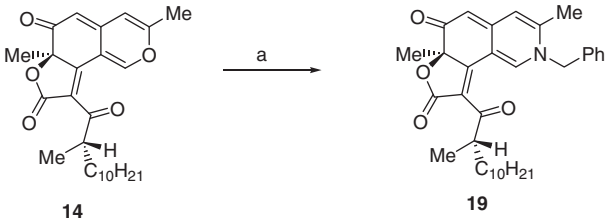
Scheme 2. Reagents and conditions: (a) $R'NH_2$, MeOH or CH_2Cl_2, 2–24 h, rt, N_2.



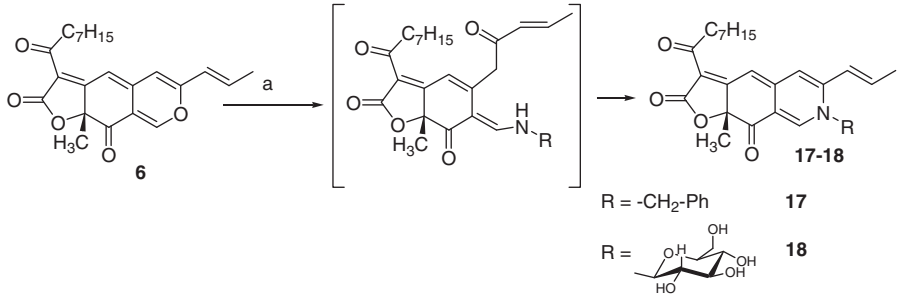
Scheme 3. Reagents and conditions: (a) $PhCH_2NH_2$ (4 mequiv), MeOH, rt, 72 h, 30%.



Scheme 4. Reagents and conditions: (a) $NaIO_4$, CH_3OH/CH_2Cl_2 1:1, H_2O, 0 °C, 45 min, then rt 72 h, 55%.



Scheme 6. Reagents and conditions: (a) $PhCH_2NH_2$, THF, rt, 10 min, 62%.



Scheme 5. Reagents and conditions: (a) for **17**: RNH_2, THF, rt, 1 h, 77%; for **18**: RNH_2, H_2O/MeOH 1:1, phosphate buffer pH 7, rt, 51%.

Table 1
Antiproliferative activity in different cell lines, ATPase activity inhibition and binding to Hsp90 (IC_{50}, μM ± SD) of azaphilones **1–13**

Compd	Antiproliferative activity (μM) Cell lines				Hsp90-ATPase activity inhibition	Binding Hsp90 (FP)
	NCI-H460	A431	JR8	IGROV-1		
1	18.7 ± 0.4	6.87 ± 0.82	11.45 ± 1.71	4.12 ± 0.53	>50	>100
2	50	5.53 ± 0.71	6.42 ± 0.87	4.65 ± 0.64	9.87 ± 0.71	61.7 ± 0.85
4	>50	17.60 ± 1.9	—	—	>50	>100
5	21.3 ± 3.1	1.20 ± 0.11	5.70 ± 0.95	0.80 ± 0.11	14.5 ± 0.93	35.4 ± 1.3
6	30 ± 0.2	7.05 ± 0.89	5.64 ± 0.79	5.08 ± 0.64	26.3 ± 3.5	>100
7	>50	17.77 ± 2.5	30.64 ± 3.9	>30	>50	>100
8	>50	130.89	130.89	102.88	22.9 ± 3.3	0.27 ± 0.01
9	>50	28.2 ± 3.4	>30	28.2 ± 3.5	9.2 ± 1.2	0.040 ± 0.001
10	>50	>28	>28	>28	>50	>100
11	—	26 ± 3.7	26 ± 3.2	26 ± 3.5	>50	>100
12	>50	>25	—	—	>50	>100
13	>50	>25	—	—	>50	>100

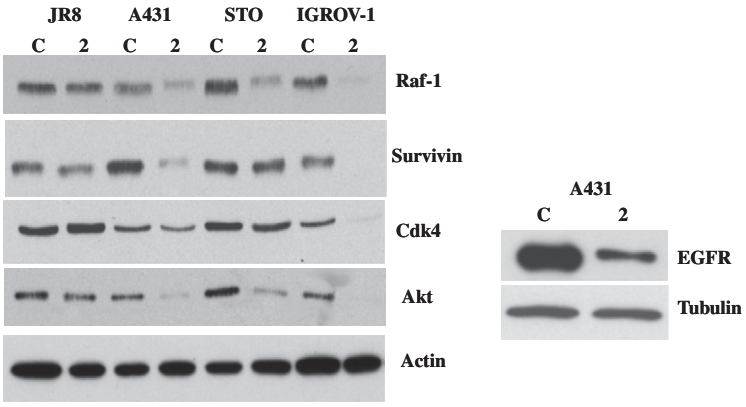


Figure 1. Analysis of Hsp90 client protein levels (survivin, Cdk4, Akt, Raf-1) in tumor cells treated with (**2**). Total cellular extracts were obtained 24 h after treatment (11 μM, IC_{80}). Actin is shown as a control for protein loading. EGFR levels were analyzed only in A431 cells and tubulin is shown as a control for protein loading.

Table 2
Study of the interaction with Hsp90

Compd	K_D[a] (nM)	k_d[a] (s^{-1})	Protected sites[b]
Radicicol	3.04 ± 0.82	0.011 ± 0.003	75, 94, 215
2	1.30 ± 0.53	0.017 ± 0.82	94, 215, 245, 277

[a] SPR data.
[b] Limited proteolysis data.

The promising results obtained by testing compound **2** prompted us to investigate the activity of other azaphilones as potential Hsp90 inhibitors. We therefore tested the natural compounds **1–13** for their cytotoxic activity against a variety of human tumor cell lines; they were also tested for their ability to inhibit Hsp90 ATPase activity and for binding affinity in the FP assay (Table 1).

The most potent compounds of this series were **1**, **2**, and **5**, characterized by IC_{50} values $\leqslant$10 μM against several cell lines. The potency of these compounds was substantially lower than that of geldanamycin or its analogue 17-AAG (IC_{50} in A431 cells, 0.2 ± 0.04 and 0.07 ± 0.01 μM, respectively). In the case of compound **2**, the reduced potency was consistent with a low affinity for Hsp90 in the competitive binding assay (IC_{50} 61.7 ± 0.85 vs

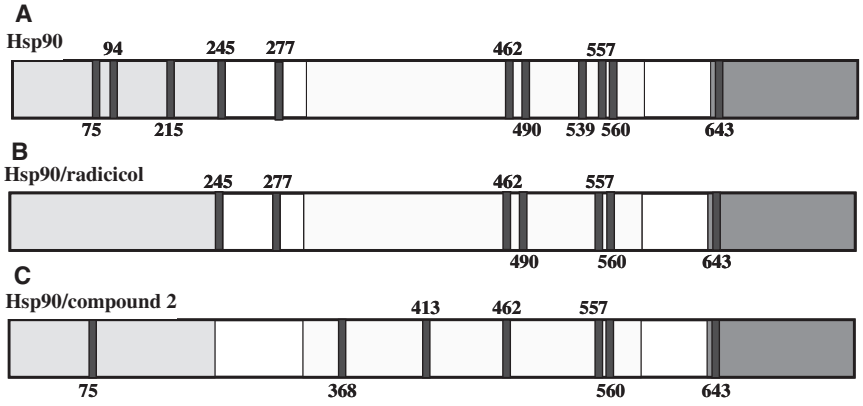


Figure 2. Schematic representation of the results obtained from limited proteolysis experiments on recombinant Hsp90. The preferential cleavage sites detected performing enzymatic digestions on recombinant Hsp90 (A), on the Hsp90/radicicol complex (B), or on the Hsp90/compound **2** complex (C), are in dark gray. The Hsp90 N-terminal domain is highlighted in light gray, the middle domain is boxed and the C-terminal domain is highlighted in gray.

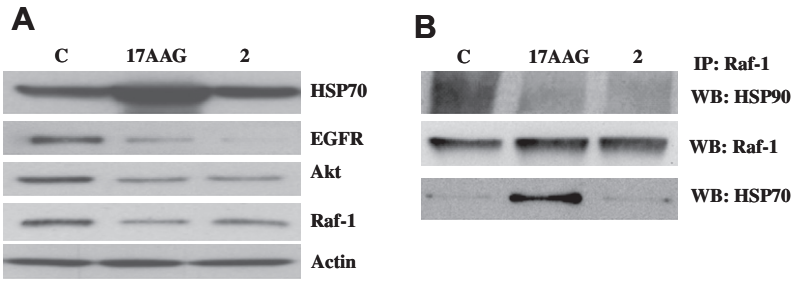


Figure 3. (A) Analysis of Hsp90 client protein levels and Hsp70 in A431 cells. Total cellular extracts were obtained 24 h after treatment (11 μM, IC_{80}). Actin is shown as a control for protein loading. EGFR levels were analyzed only in A431 cells and tubulin is shown as a control for protein loading. (B) Coimmunoprecipitation of Raf-1/Hsp90 4 h after 17-AAG or (**2**) treatment, in A431 cells. Cells were treated with equitoxic (IC_{80}) concentrations of 17AAG (0.1 μM) or **2** (11 μM). Cell lysates were than harvested and immunoprecipitated with anti-Raf-1 rabbit polyclonal antibody. Immunoprecipitates were immunoblotted for Hsp90 Blots were stripped and probed for Raf-1.

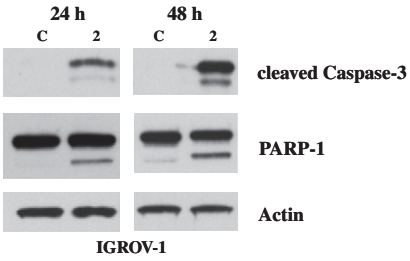


Figure 4. Biochemical analysis of apoptosis-related factors in IGROV-1 cells. Cleaved Caspase-3 and PARP-1 expression levels were analyzed by Western blot and total cellular extracts, used for this analysis, were obtained both 24 h or 48 h after treatment with **2** (11 μM).

1.095 ± 0.05 for 17AAG). Interestingly, the two pyranyl compounds **8** and **9**, with a saturated side-chain, exhibited strong binding to Hsp90. This was paralleled by the twofold increase of binding going from **2** to **5**. However, this strong binding was not reflected in the antiproliferative potency. A plausible explanation for this discrepancy could be the unfavorable cellular pharmacokinetics.

The series of natural deflectins (**10–13**), characterized by an angular lactone fused to a dihydroisochromenone ring, were found to be almost inactive.

In an attempt to identify the structural requirements involved in the Hsp90 inhibitory activity, the semisynthetic compounds **14–19** were prepared exploiting the well-known reactivity of azaphilones with amines. The IC_{50} values of compounds **14–19**, reported in Table 3, indicated that these agents were generally more potent than compounds of the previous series (**1–13**).

Among the open-ring analogues (**14a–e**), compound **14a**, containing a benzyl substituent on the nitrogen, was the most potent in the antiproliferative assay. These compounds showed moderate binding to Hsp90, comparable to that of **2**. On the contrary, compounds **16** and **17**, containing the same tricyclic system and the same benzyl substituent on N-7, exhibited increased Hsp90 binding, the highest value (IC_{50} 4 nM) being shown by the monascorubrin derivative **17**.

Figure 6 shows the analysis of client protein expression modulation. The effects on the examined proteins were variable among cell lines. Compounds **14a** and **14d** caused marked depletion of Raf-1 and Akt in IGROV-1 and JR8 cells (Fig. 5A). In contrast to the marginal effect of **2**, compounds **14a**, **14d, 15**, and **17** were very effective in causing depletion of client proteins in JR8 cells (Fig. 5A and B). Compound **14d** caused almost complete depletion of EGFR in A431 (Fig. 5A). The effects of **14a** and **16** on EGFR expression in A431 cells were less marked at equitoxic concentrations (IC_{80}) (Fig. 5A and C). For compound **14a**, coimmunoprecipitation with anti-Raf-1 antibody, performed after 4-h exposure, revealed reduced binding of Raf-1 to Hsp90 (not shown). However, the depletion was dose dependent and the variable effects on the client protein levels were likely determined by the relative sensitivity of the various cell lines (Fig. 6).

Compound **17** shows a strong binding to the enzyme (FP test), confirmed by the SPR analysis (K_D 47.8 ± 0.5 nM). However, the limited proteolysis experiment for **17** (Fig. 7) indicates a pattern similar to that of control, that is, no protection of the sensitive regions. It is therefore possible that **17** interacts with the protein in rather rigid regions (beta leaflets or, more likely, helical regions), about which the limited proteolysis approach cannot give enough information. Again, the deflectin derivative **19** showed no appreciable Hsp90 binding, like the parent natural compounds, thus confirming the detrimental effect of an angular structure.

The lack of close correlation between affinity for Hsp90, as determined in the FP assay, and cellular effects, in terms of cell growth inhibition and of client protein depletion, could reflect an unfavorable cellular pharmacokinetics or interaction with other proteins which may limit the Hsp90 binding inside the cell. Indeed, the compounds related to bulgarialactone (e.g., **9** and **17**) are characterized by a reactive conjugate system that could react with amines and thiols, present at high concentrations inside the cell (e.g., GSH).

Preliminary in vivo antitumor activity studies supported the therapeutic potential of this new series of compounds. In particular, intraperitoneal administration of **2** to athymic nude mice bearing ovarian carcinoma xenografts growing intraperitoneally as ascitic tumors resulted in a substantial increase in survival (Fig. 8).

In conclusion, the available evidence based on the biochemical, pharmacodynamic and cellular effects indicated that the compounds described in this paper may represent a novel series of Hsp90 inhibitors.

It is evident that a major limitation of this class of natural compounds is the low potency in target inhibition, which requires micromolar concentrations to inhibit cell proliferation. Also considering the chemical features of these agents, we cannot rule out the possibility of interactions with other cellular components. If this is the case, these interactions may have a detrimental effect by limiting an effective inhibition of the primary target. However, our approaches aimed at modifying the basic structure of these agents provide evidence that specific variations may improve the Hsp90 inhibitory effect. In particular, compounds **16** and **17**, containing a lipophilic chain linked to the azaphilone tricyclic ring system, brought about substantially increased binding to the enzyme, accompanied by relevant cytotoxic effects. Finally, we have documented the pharmacological interest of a representative compound of the novel series. Current efforts are directed toward optimization of the in vivo properties of selected analogues.

Table 3
Antiproliferative activity on different cell lines, ATPase activity inhibition and binding to Hsp90 (IC_{50}, μM ± SD) of compounds **14–19**

Compd	Antiproliferative activity (μM) Cell lines					Hsp90 ATPase activity inhibition	Binding Hsp90 (FP)
	NCI-H460	A431	JR8	IGROV-1	STO		
14a	4.3 ± 0.1	0.30	4.80	0.20	3.57	5.75 ± 0.75	27.7 ± 0.65
14b	11.0 ± 4.2	3.30	6.00	1.90	9.47	13.11 ± 0.87	2.29 ± 0.06
14c	17.3 ± 0.3	4.48	15.46	2.32	5.77	–	6.83 ± 0.04
14d	10.2 ± 1.7	1.54	8.59	1.72	2.06	6.10 ± 0.68	27.4 ± 0.38
14e	30 ± 7.5	4.24	9.02	2.86	4.0	19.9 ± 1.24	37.5 ± 0.59
15	21.3 ± 3.1	1.20	5.70	0.80	4.36	14.5 ± 0.93	35.4 ± 1.3
16	>20	3.98	5.16	2.92	2.92	8.9 ± 0.5	0.28 ± 0.06
17	3.4 ± 0.06	2.67	4.0	3.46	2.28	–	0.004 ± 0.0001
18	–	>18	–	>18	>10	>50	>50
19	6.7 ± 0.2	2	–	–	4.5	>50	>100

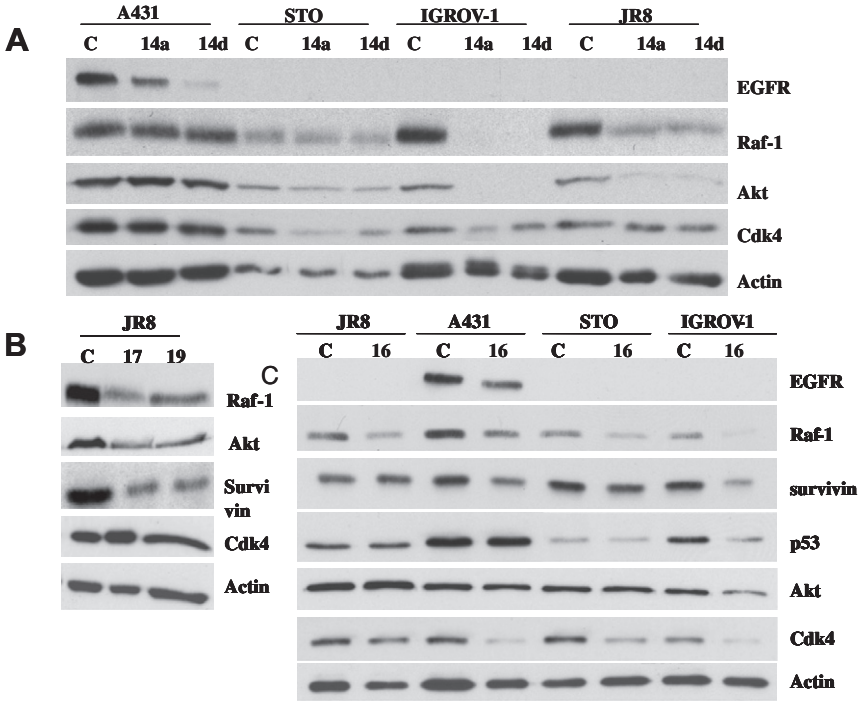


Figure 5. Comparison of biochemical effects on Hsp90 client proteins. (A) Hsp90 client expression levels after 24 h treatment with (**14a**) or (**14d**). Total cellular extracts were obtained 24 h after treatment with equitoxic (IC_{80}) doses of (**14a**, 2.7 μM for A431 cells; 10.7 μM for JR8 cells, 2.3 μM for IGROV-1 and 11.6 μM for STO cells) or (**14d**, 8.6 μM for A431; IGROV-1 and STO cells; 16 μM for JR8 cells). Actin is shown as a control for protein loading. (B) Hsp90 client expression levels after 24 h treatment with (**17**) or (**5**) in JR8 cells. Total cellular extracts were obtained 24 h after treatment with equitoxic (IC_{80}) doses of (**17**, 6.36 μM) or (**5**, 9.3 μM). Actin is shown as a control for protein loading. (C) Hsp90 client expression levels after 24 h treatment with (**16**). Total cellular extracts were obtained 24 h after treatment (**16**, 9.8 μM, IC_{80}). Actin is shown as a control for protein loading.

4. Experimental

4.1. General experimental procedures

Mass spectra were obtained with a Finnigan-MATT-TSQ 70ev, a Bruker Esquire 3000 and for HR-MS with a Bruker APEX-QZT ICR spectrometers. The ^1H NMR spectra were carried out on a Bruker DMX 500 or ARX 400 instruments at the temperature of 305 K. HPLC analyses were performed using a LiChroCARTcolumn RP-18 250-4 (Merck) on an Agilent 1100 instrument; mobile phase: H_2O/MeCN; flow rate = 0.5 mL min^{-1}. Thin and preparative layer chromatography (TLC and PLC) were performed on precoated Merck Silica Gel 60 F_{254} plates. FC (flash chromatography) was performed with Merck silica gel (0.04–0.06 mm).

4.2. Microorganisms

The Ascomycetes used in the present study, *B. inquinans* (ICRM-184, from the Collection of CNR-ICRM Institute), *M. purpureus* CBS 285.34 and *A. deflectus* CBS 109.55, were purchased from CBS collection (Centraal Bureau voor Schimmelcultures, Baarn). The master fungal strains were preserved as mycelium (*Bulgaria* and *Monascus*) or spore (*A. deflectus*) plugs, suspended in physiological solution (0.9% NaCl), at 4 °C. Stock cultures for experimental trials were grown in slants on agar medium preserved at 4 °C until the use and sub-cultured once every three weeks (*M. purpureus*) or two months (*B. inquinans* and *A. deflectus*).

4.3. Solid-state fermentation and extraction procedures

Large-scale cultures were made in Roux flasks (750 mL), containing 100 mL of productive RA (composition in g/L: rice 90, agar 20) medium or CSA (composition in g/L: corn-steep liquor 10, sucrose 100, glucose 90, yeast extract 5, K_2HPO_4 2, agar 20) medium. The flasks were inoculated with 5 mL of a mycelium or spore suspension of a preculture of fungus in slants containing 40 mL OA (Oatmeal agar Merck) medium, PDA (Potato Dextrose agar Merck) or CSA medium. One slant was used to inoculate two flasks. After

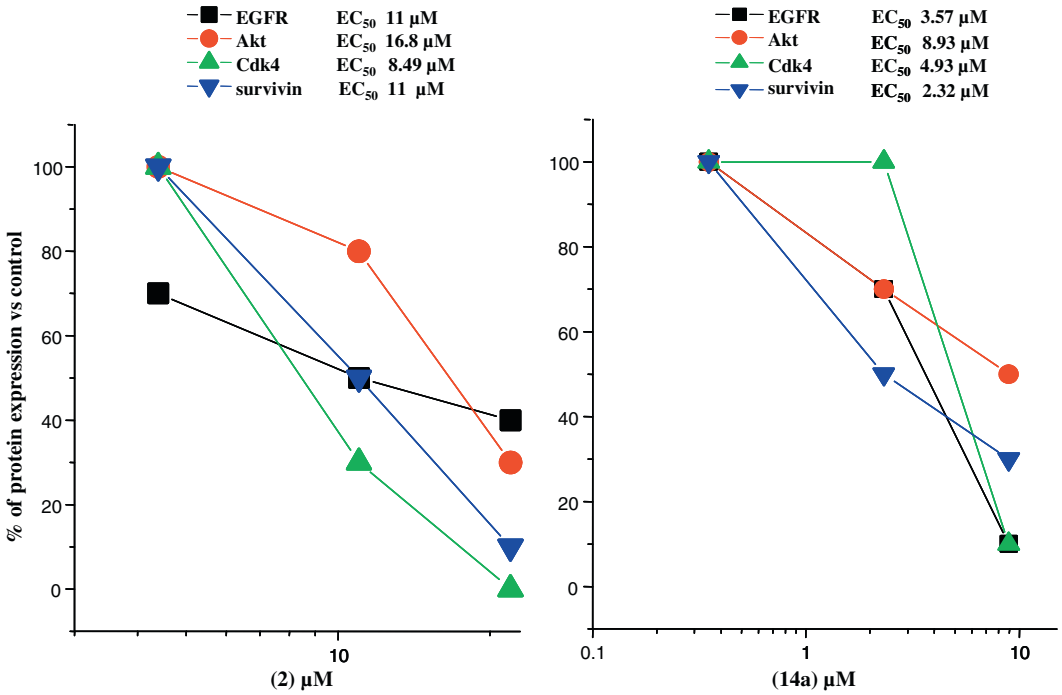


Figure 6. Dose–response of the biochemical effect of (**2**) and (**14a**) on Hsp90 client proteins in A431 cells. Total cellular extracts were obtained 24 h after treatment with (**2**) (4.4, 11, 22 µM) or (**14a**) (0.3, 2.32, 8.93 µM). Protein expression levels of EGFR, Akt, Cdk4 and survivin were analyzed by Western blot. The quantification of Western blot analyses was obtained with the Image Quant 5.2 software and results (relative expression normalized to the tubulin, as loading control) are presented as % of protein expression, as compared to the control. IC_{50} values of the down-regulation of each protein level are reported in the plots.

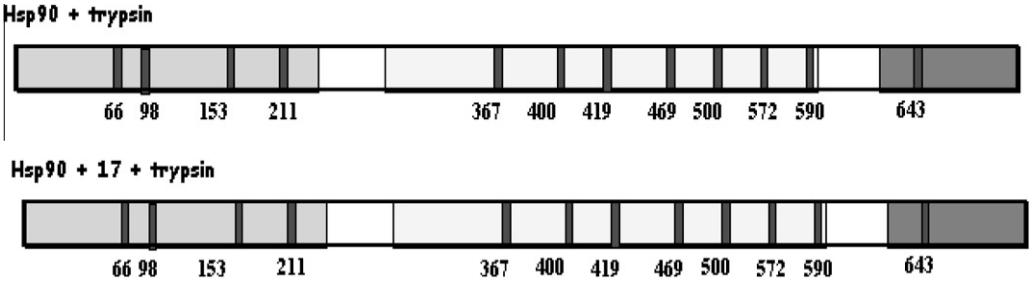


Figure 7. Effect of **2** on survival of mice bearing ip ovarian carcinoma tumor IGROV-1. (○) Control mice; (●) mice treated ip daily with **2** (25 mg/kg) for 4 days/week for 2 weeks.

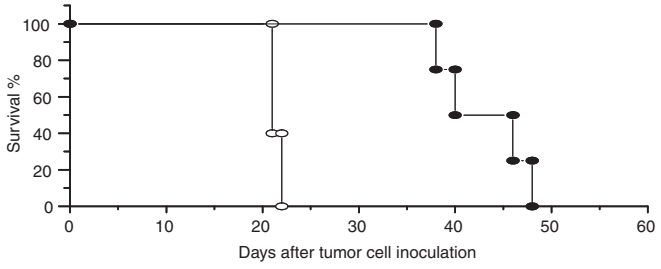


Figure 8.

incubation in thermostat at 24 °C, the cultures were extracted two times with a mixture of AcOEt/MeOH (100:1). The organic fraction was dried with Na_2SO_4 and evaporated under vacuum. Separation and purification of the metabolites were performed by column chromatography on silica gel. Further purification was performed on preparative chromatography plates (PLC), when needed. Finally, the purified compounds were checked for their identity by NMR, MS and HPLC and consistency with other spectroscopic data reported for reference compounds.

4.4. Isolation and purification of bulgarialactones A (1), B (2) and D (4) from cultures of *B. inquinans* ICRM-184

After preliminary screening on several media either in surface or submerged culture, the best results were obtained when the fungus was grown on CSA medium. *B. inquinans* large-scale cultures were made in 40 Roux flasks inoculated with 5 mL of a mycelium suspension prepared from 15 days old preculture in the same medium CSA, maintained at 24 °C in the dark and extracted three times after 30–35 days of growth. The dark oil crude extract (32 g) was partially purified by solvents: after washing with hexane, the solid residue was dissolved in CH_2Cl_2, until discoloration of the solvent. The CH_2Cl_2 fraction (22 g), containing the mixture of Bulgarialactones, was subjected first to FC in a column preconditioned with hexane/AcOEt 2:1, eluting with a mixture of hexane/AcOEt/MeOH. The fractions eluted with hexane/AcOEt 1:1 afforded bulgarialactone A (**1**, 450 mg). Elution with hexane/AcOEt from 1:2 to AcOEt 100% gave pure bulgarialactone B (**2**, 5.3 g, yield 16.5%), while elution in AcOEt/MeOH 100:1 gave bulgarialactone D (**3**) as a mixture with bulgarialactone B (4.8 g). Final purification of metabolites was reached by means of further PLC or FC.

Bulgarialactone B (**2**) was separated from bulgarialactone D (**3**) first by a FC stepwise elution with CH_2Cl_2/MeOH from 50:1 to 10:1 and then on PLC preparative plate eluting with toluene/acetone 8:2 to give 1.4 g of bulgarialactone B (yield 4.4%) and 240 mg of bulgarialactone D (yield 0.75%). The final yield of the main metabolite, bulgarialactone B, was 6.7 g (ca. 21% of crude total extract). The spectroscopic data (^{1}H NMR and MS-ESI) for bulgarialactones A and B matched with those reported in the literature.[10]

Bulgarialactone D (**4**): Brown solid; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.86 (t, J = 7.25 Hz, 3H, H-12′), 1.01 (d, J = 6.87, 3H, C10′CH_3), 1.28–1.42 (m, 2H, H-11′), 1.70 (s, 3H, C9aCH_3), 2.09–2.27 (m, 2H, H-10′ + H-5), 2.44–2.59 (m, 1H, H-5), 3.45–3.74 (m, 4H, CH_2OH + H-6 + H-4), 4.15–4.27 (m, 1H, H-4), 4.59–4.76 (m, 2H, H-8), 5.87 (dd, J = 7.6, 15.3 Hz, 1H, H-9′), 6.07 (d, J = 14.9 Hz, 1H, H-4′), 6.14 (dd, J = 10.7, 15.26 Hz, 1H, –CH═CH–), 6.27 (dd, J = 11.4, 14 Hz, 1H, –CH═CH–), 6.60 (dd, J = 10.7, 14.9 Hz, 1H, –CH═CH–), 6.72 (s, 1H, H-2′), 7.35 (dd, J = 11.4, 14.9 Hz, 1H, –CH═CH–), 15.84 (br s, 1H, OH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 189.62, 185.48, 177.05, 169.21, 167.69, 150.63, 147.54, 143.27, 143.00, 129.55, 128.74, 128.62, 126.03, 120.61, 101.19, 86.06, 73.66, 66.91, 63.84, 39.02, 32.62, 31.73, 29.63, 23.36, 19.81, 11.89. ESI-MS: m/z 455 [M+H$^+$] and 477 [M+Na]; negative ion: m/z 453 [M−H$^+$]. Anal. ($C_{26}H_{30}O_7$) C, H.

4.5. Isolation and purification of azaphilones 6–9 from cultures of *M. purpureus* CBS 285.34

A preliminary screening on small-scale (15 flasks) was made in order to isolate and characterize the azaphilones produced from the strain of *M. purpureus*. The orange pigments monascorubrin (**8**) and rubropunctatin (**9**), were isolated only from cultures grown on RA medium and at short-time incubation (max 10 days, 28 °C); the yellow pigments ankaflavin (**6**) and monascin (**7**), and the red pigments monascorubramin, and rubropunctamin were isolated from cultures of the fungus when grown on RA medium or CSA medium, but at longer incubation time (30–35 days, 28 °C). The crude extracts were separated and purified first by means of FC on silica gel with a stepwise elution with hexane/AcOEt from 8:2 to 0:1, to afford yellow and orange pigments or CH_2Cl_2/acetone from 50:1 to 1:1 for the red pigments. The pure pigments were obtained by chromatography on reverse phase (RP-18): yellow pigments ankaflavin and monascin and orange pigments monascorubrin and rubropunctatin were separated on preparative plates (PLC) using H_2O/CH_3CN 3:7 as eluent, while the separation of red pigments was performed eluting with H_2O/CH_3CN 1:1. The spectroscopic data (^{1}H NMR and MS) for compounds **6–9** were identical with those reported in lit.[14,34]

4.6. Large-scale production of monascorubrin 8 and rubropunctatin 9 from *M. purpureus*

For the preparation of semisynthetic amino-derivatives of monascorubrin **8**, the strain of *M. purpureus* was grown in batches of 40 flasks on RA medium (100 mL). Each flask was inoculated with a suspension of the fungus prepared from 7 days old cultures grown in slants on OA medium (28 °C). The crude extract fraction, soluble in CH_2Cl_2 (4.3 g), was purified by FC on silica gel eluting with hexane/AcOEt at increasing polarity to give a mixture of yellow and orange pigments. The final purification of these pigments was performed by chromatography on reverse phase (RP-18), eluting with H_2O/acetone from 1:1 to 2:8 to give 470 mg of compound **8** (11%) and 300 mg of compound **9** (7%).

4.7. Isolation and purification of deflectins 10–13 from cultures of *A. deflectus*

The strain of *A. deflectus* was maintained in slants on HA (composition in g/L: yeast extract 4, malt extract 10, glucose 4, agar 15) medium and stored as spores, at 4 °C. The fungus was inoculated in 10 Roux flasks on RA medium with a 10 days old preculture grown on PDA medium and incubated at 24 °C. After 30 days, the cultures were extracted to give a brown oil. The crude residue (5.5 g) was purified by FC on silica gel with a stepwise elution with hexane/AcOEt from 7:3 to 4:6. A further purification by reverse phase (RP-18) column chromatography, with a stepwise elution with H_2O/acetone from 4:6 to 1:9, afforded 350 mg (6.4 %), 600 mg (ca. 11%), and 75 mg (1.4 %) of deflectins **10–12**, respectively, and 550 mg (10 %) of deflectin **13**.

4.8. 6-Hydroxymethyl-3-(1-hydroxy-10-methyl-3-oxo-dodec-1-enyl)-9a-methyl-5,6-dihydro-9a*H*-furo[3,2-*g*]isochromene-2,9-dione (5)

Compound **2** (350 mg, 0.77 mmol) dissolved in AcOEt (15 mL) was hydrogenated at room temperature with 10% Pd/C (25 mg). The reaction was monitored frequently on TLC in hexane/acetone 2:1 and stopped when the orange pigment was completely converted into the yellow pigment, before the formation of more polar products. After filtration on Celite and evaporation of the solvent, the crude residue was purified by FC eluting with hexane/*i*-PrOH 3:1 to afford the title compound (350 mg, 78%). HPLC: retention time (t_R) 14.37 min, solvent CH_3CN–H_2O, 85:15; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.75–0.88 (m, 6H, H-12′, C10′CH_3), 0.99–1.16 (m, 2H, H-11′), 1.16–1.39 (m, 8H, 4CH_2), 1.51–1.68 (m, 2H, CH_2), 1.72 (s, 3H, C9aCH_3), 1.87–2.04 (m, 1H, H-10′), 2.43 (t, J = 8.0 Hz, 1H, CH_2CO), 2.73–2.96 (m, 2H, H-5), 3.83 (dd, J = 5.0 Hz, 12.2 Hz, 1H, CH_2OH), 3.96 (dd, J = 3.4, 12.2 Hz, 1H, CH_2OH),4.34–4.44 (m, 1H, H-6), 6.70 (s, 1H, H-2′), 7.05 (s, 1H, H-4), 7.84 (s, 1H, H-8), 15.60 (br s, 1H, OH). ESI-MS: m/z 459 [M+H$^+$] and 481 [M+Na$^+$]. Anal. ($C_{26}H_{34}O_7$) C, H.

4.9. 6-(Benzylaminomethylene)-5-(2,3-dihydroxypropyl)-3-(1-hydroxy-10-methyl-3-oxo-dodeca-1,4,6,8-tetraenyl)-7a-methyl-6*H*,7a*H*-benzofuran-2,7-dione (14a)

To a solution of **2** (300 mg, 0.66 mmol) in methanol (8 mL) benzylamine (72 μL, 0.66 mmol) was added under nitrogen atmosphere. The solution was stirred 24 h at room temperature, then the solvent was removed under reduced pressure. The crude was purified by gel permeation chromatography on Sephadex LH-20 using CH_2Cl_2 as eluent to obtain the title compound as a red solid (120 mg, 52%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.88 (t, J = 7.3 Hz, 3H, H-12′), 1.00 (d, J = 6.7, 3H, C10′CH_3), 1.26–1.41 (m, 2H, –H-11′), 1.70 (s, 3H, C7aCH_3), 2.06–2.21 (m, 1H, H-10′), 2.56–2.66 (m, 2H, H-1″), 3.53 (dd, J = 6.3, 11.16 Hz, 1H, H-3″), 3.71 (dd, J = 3.7, 11.2 Hz, 1H, H-3″), 3.85–3.95 (m, 1H, H-2″), 4.55 (d, J = 6.3 Hz, 2H, –CH_2Ph), 5.84 (dd, J = 7.8, 15.26 Hz, 1H, H-9′), 6.05 (d, J = 14.8 Hz, 1H, H-4′), 6.13 (dd, J = 10.8, 15.26 Hz, –CH═CH–), 6.25 (dd, J = 11.3, 14.9 Hz, 1H, –CH═CH–), 6.54 (dd, J = 10.8, 14.89 Hz, 1H, –CH═CH–), 6.74 (s, 1H, H-2′), 6.93 (s, 1H, H-4), 7.21–7.41 (m, 6H, Ph + –CH═CH–), 7.70 (d, J = 13.4 Hz, 1H, H-1‴), 10.51–10.65 (m, 1H, NH), 16.00 (br s, 1H, OH). ESI-MS: m/z 560 [M+H$^+$]; Anal. ($C_{33}H_{37}NO_7$) C, H, N.

4.10. 5-(2,3-Dihydroxypropyl)-3-(1-hydroxy-10-methyl-3-oxo-dodeca-1,4,6,8-tetraenyl)-6-[(3-hydroxypropylamino)-methylene]-7a-methyl-6*H*,7a*H*-benzofuran-2,7-dione (14b)

Under nitrogen atmosphere 3-aminopropanol (2 μL, 0.02 mmol) was added to a solution of **2** (10 mg, 0.02 mmol) in CH_2Cl_2 (1 mL). The solution was stirred for 2 h at room temperature, then the solvent was removed under reduced pressure. The crude was purified by PLC using CH_2Cl_2/CH_3OH 95:5 as eluent to

obtain the title compound as a red oil (4 mg, 38%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.85 (t, J = 7.2 Hz, 3H, H-12′), 1.00 (d, J = 6.9 Hz, 3H, C10′CH_3), 1.28–1.42 (m, 2H, H-11′), 1.66 (s, 3H, H-7a), 1.77–1.94 (m, 4H, H-4‴ + 2OH), 2.05–2.21 (m, 1H, H-10′), 2.56–2.76 (m, 2H, H-1″), 3.44–3.80 (m, 6H, H-3‴ + H-5‴ + H-3″), 3.86–4.00 (m, 1H, H-2″), 5.81 (dd, J = 7.6, 15.26 Hz, 1H, H-9′), 6.05 (d, J = 15.3 Hz, 1H, H-4′), 6.15 (dd, J = 11.4, 15.64 Hz, 1H, –C*H*=CH–), 6.25 (dd, J = 11.8, 14.50 Hz, 1H, –*CH*=CH–), 6.54 (dd, J = 10.7, 14.50 Hz, 1H, –*CH*=CH–), 6.71 (s, 1H, H-2′), 6.90 (s, 1H, H-4), 7.27 (dd, J = 11.4, 15.6 Hz, 1H, C*H*=CH), 7.74 (d, J = 14.1 Hz, 1H, H-1‴), 10.28–10.46 (m, 1H, –NH). ESI-MS: m/z 528 [M+H$^+$]; Anal. ($C_{29}H_{37}NO_8$) C, H, N.

4.11. 2-Acetylamino-6-{[5-(2,3-dihydroxypropyl)-3-(1-hydroxy-10-methyl-3-oxo-dodeca-1,4,6,8-tetraenyl)-7a-methyl-2,7-dioxo-7,7a-dihydro-2H-benzofuran-6-ylidenemethyl]-amino}-hexanoic acid (14c)

A solution of N_α-acetyl-L-lysine (124 mg, 0.66 mmol) in water (400 μL) was added to a solution of **2** (50 mg, 0.11 mmol) in CH_3OH (5 mL). The resulting mixture was stirred 2 h at room temperature. The solvent was removed under reduced pressure and the crude was washed with CH_2Cl_2/CH_3OH 95:5; the excess of N_α-acetyl-L-lysine was filtered off under vacuum and washed with CH_2Cl_2/CH_3OH 5:1. The solution was then evaporated and the crude was purified by preparative chromatography using CH_2Cl_2/CH_3OH 85:15 as eluent, to give the title compound as a red solid (28 mg, 39%). ^{1}H NMR (300 MHz, DMSO): 0.83 (t, J = 7.07 Hz, 3H, H-12′), 0.98 (d, J = 7.1 Hz, 3H, C10′CH_3), 1.19–1.41 (m, 4H, H-11′ + CH_2Lys), 1.44–1.74 (m, 4H, CH_2Lys), 1.82 (s, 3H, C7aCH_3), 2.05–2.42 (m, 1H, H-10′), 2.50 (m, 2H, CH_2Lys), 2.54–2.79 (m, 2H, H-1″), 3.30–3.50 (m, 2H, H-3″), 3.56–3.70 (m, 1H, H-2″), 3.98–4.18 (m, 1H, –*CH*COOH), 5.88 (dd, J = 7.8, 15.3 Hz, 1H, H-9), 6.08–6.30 (m, 2H, C*H*=CH), 6.31–6.47 (m, 1H, C*H*=CH), 6.63 (s, 1H, H-2′), 6.71 (dd, J = 10.4, 14.5 Hz, 1H, C*H*=CH), 6.77 (s, 1H, H-4), 7.22 (dd, J = 11.5, 14.9 Hz, 1H, C*H*=CH), 7.86–8.06 (m, 2H, H-1‴ + CONH), 10.22–10.42 (m, 1H, NH). ESI-MS: m/z 641 [M+H$^+$]. Anal. ($C_{34}H_{44}N_2O_{10}$) C, H, N.

4.12. 5-(2,3-Dihydroxypropyl)-3-(1-hydroxy-10-methyl-3-oxo-dodeca-1,4,6,8-tetraenyl)-7a-methyl-6-[(2-morpholin-4-yl-ethylamino)-methylene]-6*H*,7a*H*-benzofuran-2,7-dione (14d)

Under nitrogen atmosphere 4-(2-aminoethyl) morpholine (16 μL, 0.12 mmol) was added to a solution of **2** (56 mg, 0.12 mmol) in CH_3OH (2 mL). The solution was stirred 23 h at room temperature. The solvent was removed under reduced pressure and the crude was purified by PLC using CH_2Cl_2/CH_3OH 95:5 as eluent to obtain the title compound as a red oil. (11 mg, 15%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.88 (t, J = 7.07 Hz, 3H, H-12′), 1.03 (d, J = 6.70 Hz, 3H, C10′CH_3), 1.30–1.45 (m, 2H, H-11′), 1.70 (s, 3H, C7aCH_3), 2.07–2.25 (m, 1H, H-10′), 2.44–2.58 (m, 4H, H-1″ + NCH_2), 2.59–2.76 (m, 4H, H-4‴ + NCH_2), 3.41–3.54 (m, 2H, H-3‴), 3.59 (dd, J = 6.3, 10.79 Hz, H-3″), 3.69–3.85 (m, 5H,OCH_2-morpholine + H-3″), 3.94–4.05 (m, 1H, H-2″), 5.84 (dd, J = 8.2, 15.6 Hz, H-9′), 6.03–6.36 (m, 3H, C*H*=CH), 6.57 (dd, J = 10.8, 14.1 Hz, 1H, C*H*=CH), 6.79 (s, 1H, H-2′), 6.94 (s, 1H, H-4), 7.21–7.39 (m, 1H, C*H*=CH), 7.73 (d, J = 14.5 Hz, 1H, H-1‴), 10.38–10.55 (m, 1H, NH). ESI-MS: m/z 583 [M+H$^+$]. Anal. ($C_{32}H_{42}N_2O_8$) C, H, N.

4.13. 7-Benzyl-3-(1-hydroxy-10-methyl-3-oxo-dodeca-1,4,6,8-tetraenyl)-9a-methyl-7*H*,9a*H*-furo[3,2-*g*]isoquinoline-2,9-dione (16)

To a solution of **14a** (50 mg, 0.09 mmol) in CH_3OH/CH_2Cl_2 1:1 (2 mL) cooled at 0 °C were added water (0.4 mL) and $NaIO_4$ (25 mg, 0.12 mmol). The mixture was stirred 45 min at 0 °C and then 3 days at rt. The solvent was removed under reduced pressure and the crude was extracted with CH_2Cl_2. The combined organic fractions were washed with saturated NaCl solution, dried over Na_2SO_4, and concentrated in vacuo. The crude was purified by flash column chromatography on deactivated silica gel (110 g of SiO_2 in 400 mL of a 4% aqueous solution of KH_2PO_4 evaporated under reduced pressure and dried at 110 °C overnight) using CH_2Cl_2/acetone 19.5:0.5 as eluent, to give the title compound (25 mg, 55%).^{1}H NMR (300 MHz, $CDCl_3$): δ 0.85 (t, J = 7.5 Hz, 3H, H-12′), 1.00 (d, J = 6.5 Hz, 3H, C-10′CH_3), 1.28–1.41 (m, 2H, H-11′), 1.69 (s, 3H, H-7a), 2.05–2.20 (m, 1H, H-10′), 4.89 (s, 2H, CH_2Ph), 5.77 (dd, J = 8.1, 15.3 Hz, 1H, CH=CH), 6.05 (d, J = 14.9 Hz, 1H, CH=CH), 6.12 (dd, J = 10.4, 15.3 Hz, 1H, CH=CH), 6.25 (dd, J = 11.4, 14.9 Hz, 1H, CH=CH), 6.49 (dd, J = 10.4, 14.9 Hz, 1H, CH=CH), 6.57 (d, J = 7.5 Hz, 1H, CH=CHN), 6.70 (s, 1H, H-2′), 6.75 (s, 1H, H-4), 6.91 (d, J = 7.5, 1H, CH=CHN), 7.39–7.49 (m, 6H, CH=CH + Ph), 16.81 (br s, 1H, C1′-OH). ESI-MS: m/z 510 [M+H$^+$] and 532 [M+Na$^+$]. Anal. ($C_{32}H_{31}NO_5$) C, H, N.

4.14. 7-Benzyl-9a-methyl-3-octanoyl-6-propenyl-7*H*,9a*H*-furo-[3,2-*g*]isoquinoline-2,9-dione (17)

To a stirred solution of compound **6** (50 mg, 1 mequiv) in THF (50 mL), benzylamine (21 μL, 1.5 mequiv) was added. The solution changed from orange to red. The reaction was stirred at room temperature for 1 h. After evaporation of the solvent, the residue was taken up with water and the aqueous phase was extracted with AcOEt (100 mL). The organic phase was concentrated in vacuo to give a residue that was purified by PLC using hexane/AcOEt 1:1 as eluent, to afford compound **17** (48 mg, 77%) as a red solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.87 (t, J = 7.1 Hz, 3H, H-7′), 1.22–1.42 (m, 10H, CH_2), 1.62 (s, 3H, C9aCH_3), 1.86 (dd, 3H, J = 1.5, 6.3 Hz, C=CHCH_3), 2.74–2.80 (m, 2H, H-2′), 5.49 (s, 2H, –CH_2Ph), 6.51 (d, J = 16.0 Hz, 1H, –C*H*=CHCH_3), 6.64 (m, 1H, –CH=*CH*CH_3), 6.68 (s, 1H, H-5), 7.04 (s, 1H, H-4), 7.27–7.48 (m, 5H, Ph), 8.23 (s, 1H, H-8). ESI-MS: m/z 494 [M+Na$^+$]. Anal. ($C_{30}H_{33}NO_4$) C, H, N.

4.15. 9a-Methyl-3-octanoyl-6-propenyl-7-(3,4,5-trihydroxy-6-hydroxymethyl-tetrahydro-pyran-2-yl)-7*H*,9a*H*-furo[3,2-*g*]isoquinoline-2,9-dione (18)

This compound was synthesized following the procedure described by Lin et al.[35] The ^{1}H NMR matched with that reported for the natural product.[36]

4.16. 2-Benzyl-3,6a-dimethyl-9-(2-methyl-dodecanoyl)-2*H*,6a*H*-furo[2,3-*h*]isoquinoline-6,8-dione (19)

To a stirred solution of deflectin **13** (25 mg, 1 mequiv) in THF (5 mL) benzylamine (8 μL, 1.2 mequiv) was added. The mixture was maintained at room temperature for 10 min. After evaporation of the solvent, the residue was dissolved in CH_2Cl_2 and purified by PLC using hexane/acetone 7:3 as eluent to afford the single isomer **19** as a red solid (19 mg, 62%).^{1}H NMR (400 MHz, acetone-d_6): δ 0.88 (3H, m, Me-8′), 0.96 (3H, d, J = 7.0, Me-12), 1.1–1.5 (12H, m, H_2-2′ and H_2-7′), 1.61 (3H, s, Me-7), 1.35 and 1.77 (2H, m, H_2-1′), 2.29 (3H, s, Me-3), 3.58 (1H, tq, J = 6.5, 7.0, H-12), 4.85 (1H, s, H-5), 5.29 and 5.33 (2H, d, J = 16.3, CH_2-Ph), 6.40 (1H, s, H-4), 7.38–7.45 (5H, m, Ph), 8.73 (1H, s, H-1). ESI-MS: m/z 538 [M+Na$^+$]. Anal. ($C_{33}H_{41}NO_4$) C, H, N.

4.17. 6-(Benzylaminomethylene)-5-(2,3-dihydroxypropyl)-3-(1-hydroxy-10-methyl-3-oxo-dodec-1-enoyl)-7a-methyl-6*H*,7a*H*-benzofuran-2,7-dione (14e) and 3-(3-benzylamino-10-methyldodec-2-enoyl)-6-(benzylaminomethylene)-5-(2,3-dihydroxy-propyl)-7a-methyl-6*H*,7a*H*-benzofuran-2,7-dione (15)

A solution of compound **5** (15 mg) in MeOH (1 mL) was stirred at room temperature and benzylamine (12 μL) was added. The mono-adduct **14e** was isolated stopping the reaction after 30 min. The residue was purified by means of PLC eluting in hexane/acetone 2:1, to give 16 mg (85%) of compound **14e**. The bis-adduct **15** was obtained by reaction of **14e** (60 mg, 1 mequiv) with benzylamine (57 μL, 4 mequiv) dissolved in MeOH (10 mL). The mixture was stirred at room temperature for 72 h. After evaporation of the solvent, the residue was purified first by FC with hexane/acetone 6:4 as eluent and then by PLC eluting in CH_2Cl_2/MeOH 40:1 to afford 26 mg (30%) of compound **15**.

Compound **14e**: ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.78–0.93 (m, 6H, H-12′ + C10′CH_3), 0.97–1.18 (m, 2H, H-11′), 1.19–1.43 (m, 8H, 4CH_2), 1.50–1.67 (m, 2H, CH_2), 1.70 (s, 3H, C7aCH_3), 1.94–2.11 (m, 1H, H-10′), 2.42 (t, J = 7.2 Hz, CH_2CO), 2.54–2.70 (m, 2H, H-1″), 3.55 (dd, J = 6.2, 11.0 Hz, 1H, H-3″), 3.74 (dd, J = 3.3, 11.0 Hz, 1H, H-3″), 3.87–3.97 (m, 1H, H-2″), 4.75 (m, J = 5.5 Hz, 2H, –CH_2Ph), 6.70 (s, 1H, H-2′), 6.87 (s, 1H, H-4), 7.32–7.47 (m, 5H, Ph), 7.69 (d, J = 13.24 Hz, 1H, H-1‴), 10.53–10.71 (m, 1H, NH), 15.78 (br s, 1H, OH). ESI-MS: m/z 566 [M+H$^+$]. Anal. ($C_{33}H_{43}NO_7$) C, H, N.

Compound **15**: ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.76–0.89 (m, 6H, H-12′ + C10′CH_3), 0.98–1.15 (m, 2H, H-11′), 1.15–1.37 (m, 8H, 4CH_2), 1.46–1.57 (m, 2H, CH_2), 1.60 (s, 3H, C7aCH_3), 2.22–2.33 (m, 2H, CH_2CO), 2.45–2.63 (m, 2H, H-1″), 3.38–3.54 (m, 1H, H-3″), 3.54–3.69 (m, 1H, H-3″), 3.75–3.99 (m, 1H, H-2″), 4.41–4.56 (m, 4H, 2CH_2Ph), 6.24 (s, 1H, H-2′), 6.94 (s, 1H, H-4), 7.14–7.39 (m, 10H, 2Ph), 7.61 (d, J = 12.97 Hz, H-1‴), 10.41–10.55 (m, NH, H-2‴), 11.79–11.90 (m, NH, C3′NH). ESI-MS: m/z 655 [M+H$^+$] and 677 [M+Na$^+$]. Anal. ($C_{40}H_{50}N_2O_6$) C, H, N.

Surface plasmon resonance analyses: SPR analyses were conducted using a Biacore 3000 optical biosensor equipped with research-grade CM5 sensor chips (Biacore AB). Using this platform, two separate recombinant Hsp90 surfaces, a BSA surface and an unmodified reference surface, were prepared for simultaneous analyses. Proteins (100 μg/mL in 10 mM sodium acetate, pH 5.0) were immobilized on individual sensor chip surfaces at a flow rate of 5 μL/min using standard amine-coupling protocols[37] to obtain densities of 8–12 kRU. Compound **2** and radicicol were dissolved in 100% DMSO to obtain 4 mM solutions, and diluted 1:200 (v/v) in PBS (10 mM NaH_2PO_4, 150 mM NaCl, pH 7.4) to a final DMSO concentration of 0.5%. Compounds concentration series were prepared as twofold dilutions into running buffer: for each sample the complete binding study was performed using a six points concentration series, typically spanning 0.05–10 μM, and triplicate aliquots of each compound concentration were dispensed into single-use vials. Included in each analysis were multiple blank samples of running buffer alone.[38] Binding experiments were performed at 25 °C, using a flow rate of 50 μL/min, with 60 s monitoring of association and 200 s monitoring of dissociation. Simple interactions were adequately fit to a single-site bimolecular interaction model (A + B = AB), yielding a single K_D. Sensorgram elaborations were performed using the Biaevaluation software provided by Biacore AB.[39]

4.18. Limited proteolysis

Limited proteolysis experiments were performed at 37 °C, PBS 0.1% DMSO, at a 3 μM recombinant Hsp90 concentration using trypsin, chymotrypsin, and endoprotease V8 as proteolytic agents; 30 μL of solution were used radical experiment. Binary complexes Hsp90/inhibitor were formed by incubating the protein with a 10:1 molar excess of the individual inhibitor at 37 °C for 15 min prior to proteolytic enzyme addition. Each complex was digested using a 1:100 (w/w) enzyme-to-substrate ratio. The extent of the reactions was monitored on a time-course basis by sampling the incubation mixture after 5, 15, and 30 min of digestion. Samples were desalted by ziptip C_4 (Millipore) and the proteolytic fragments were analyzed by MALDI-TOF/MS using a MALDI-MX micro (Waters). In order to optimize sensitivity and accuracy of the mass measurements, three different m/z ranges were explored for each sample: a first range, from m/z 500 to 3500 was analyzed in reflector mode; the other two ranges, from m/z 3500 to 20,000 and from m/z 20,000 to 95,000 were analyzed in linear mode. Each m/z range was calibrated using a suitable peptide or protein mixture. Mass data were elaborated using the Masslynx software (Waters).

Preferential hydrolysis sites on Hsp90 under different conditions were identified on the basis of the fragments released during the enzymatic digestions. When comparative experiments were carried out on Hsp90 in the presence or in the absence of inhibitors, differences in the susceptibility of specific cleavage sites were detected, from which protein regions involved in the conformational changes could be inferred.[28]

4.19. Cell lines and culture conditions

The human ovarian carcinoma cell line IGROV-1, the human melanoma cell line JR8, and the human epithelial carcinoma cell line A431 were routinely grown in RPMI 1640 (Lonza, Vierviers, Belgium), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (GIBCO, Invitrogen, Paisley, UK). The human peritoneal mesothelioma cell line STO was kindly provided by Dr. N. Zaffaroni (IRCCS Istituto Nazionale Tumori, Milan) and grown in 50/50 DMEM-Ham's F12 medium (Lonza, Vierviers, Belgium), supplemented with 10% (v/v) heat-inactivated fetal bovine serum and 2 mM L-glutamine (L-Gln). All cell lines were maintained at 37 °C in a 5%/95% CO_2/air atmosphere.

4.20. Drugs and treatment conditions

All the compounds tested were freshly prepared dissolving powder in dimethylsulfoxide (DMSO) (BDH Prolabo, Milan, Italy) and following dilution in culture medium. 17-AAG was a gift from Dr. N. Zaffaroni and it was provided as a stock solution (5 mM) that we freshly diluted in culture medium before each experiment. Cells were incubated with drugs for 6, 24 or 72 h (depending on the assay), at 37 °C in culture medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum; drug concentrations used for each assay are reported in Legends to Figures.

4.21. Cell growth inhibition

Cell sensitivity to all the tested compounds was determined by a growth inhibition assay. Briefly, cells were seeded in 12-well plates (50,000 cells/well), 24 h before experiments. Cells were exposed to the drugs for 72 h to allow approximately three replications in the control cells. Adherent cells were trypsinized and counted with a cell counter (Coulter Electronics, Luton, UK). IC_{50} values, derived from dose–response curves, were defined as drug concentrations required for 50% inhibition of cell growth.

4.22. Western blot analysis

Twenty-four hour after seeding (100,000 cells/mL) on Petri dishes, cells were treated with drugs for 24 h and then processed to obtain whole-cell extracts. Cells were rinsed twice with ice-cold

PBS supplemented with 0.1 mM sodium orthovanadate and then lysed in hot sample buffer.[40] After determination of the protein concentration by BCA Protein Assay (Thermo Scientific, Rockford, IL, USA), cellular extracts (40 μg) were separated by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto nitrocellulose membranes. Immunoreactive bands were revealed by enhanced chemiluminescence detection (Amersham Biosciences, Rockford, IL, USA) using anti-Raf-1 and anti-Cdk4 (Santa Cruz Biotechnology Inc., CA, USA), anti-EGFR (Upstate Biotechnology, Millipore Corporate, Billerica, MA, USA), anti-Survivin (Abcam, Cambridge, UK), anti-Akt (Transduction Laboratories, Lexington, USA), anti-PARP-1 (Calbiochem, Merck Chemicals Ltd, Nottingham, UK), anti-Cleaved Caspase-3 (Asp175) (Cell Signaling Technology, Inc., MA, USA), anti-p53 (Dako, Glostrup, Denmark), anti-Actin and anti-Tubulin antibodies (Sigma Chemical Co., St. Louis, MO, USA).

4.23. Coimmunoprecipitation and immunoblot analysis

Following the designated treatments, cells were lysed in NET buffer [50 mmol/L Tris (pH 7.4), 150 mmol/L sodium chloride, 0.1% NP40, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L EDTA, 2.5 μg/mL leupeptin, 5 μg/mL aprotinin] first for 30 min on ice and then for 30 min at 4 °C in rotation on a wheel. Nuclear and cellular debris were cleared by centrifugation (10,000*g*, 10 min, 4 °C). Total cellular proteins were then quantified using the BCA protein assay. Cell lysates (500 μg) were incubated with the Raf-1-specific antibody (Santa Cruz Biotechnology Inc., CA, USA) for 2 h at 4 °C. Protein A-sepharose (Sigma–Aldrich, Germany) (90 μL), previously prepared in TNT solution (20 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% Triton X-100) was added to the lysates and incubated overnight at 4 °C. The immunoprecipitates (separated by centrifugation 15,000*g*, 2 min, 4 °C) were washed twice in NET buffer and twice in PBS with 1% aprotinin and 1 mmol/L PMSF. Proteins were eluted with the SDS sample loading buffer before the immunoblot analyses with specific antibodies against Raf-1, Hsp90 or Hsp70 (Santa Cruz Biotechnology Inc., CA, USA).

4.24. Determination of yeast Hsp90 ATPase activity

Inhibition of the intrinsic ATPase activity of full-length recombinant yeast Hsp82 was measured by a modified enzymatically coupled ATPase assay,[41] in which the amount of inorganic phosphate released by HSP90-dependent ATP hydrolysis is utilized by maltose phosphorylase, which phosphorylates maltose and then cleaves the disaccharide to produce glucose and glucose-1-phosphate. Glucose is a substrate for glucose oxidase, which uses atmospheric oxygen to produce gluconolactone and hydrogen peroxide. Finally, horseradish peroxidase consumes hydrogen peroxide by the oxidation of the colorless *N*-acetyl-3,7-dihydroxyphenoxazine (Amplex Red) to provide the Resorufin product, which has a unique absorption spectra at 563 nm. For every molecule of ATP, that is, hydrolyzed by yeast Hsp90, one molecule of Amplex Red is ultimately oxidized to Resorufin.

Inhibition of yeast Hsp82 ATPase activity by test compounds was tested, following 3 h incubation in the presence of 500 μM ATP, in 96-well cell culture cluster clear plates by using a commercially available kit (P_iPer™ Phosphate Assay kit; Molecular Probes, Eugene, OR), according to manufacturer's instructions. To determine IC_{50} values, several appropriate concentrations of test compounds were evaluated depending on their relative potency.

Each assay plate was run with a positive control which consisted of three wells containing the well-known Hsp90 inhibitor 17-DMAG, at a final concentration of 20 μM.

4.25. Fluorescence polarization assay

GM-FITC, supplied by Invivogen (06C23-MT, California 92192, USA), was previously dissolved in DMSO to obtain 10 mM stock solutions and kept at −20 °C until use. HSP90, purchased from Stressgen (cat. No. SPP-776, Victoria BC, Canada), was previously dissolved in assay buffer (HFB) to form 2.2 μM stock solutions and kept at −80 °C until use. The compounds were previously dissolved in DMSO to obtain stock solutions and kept at −20 °C. The day of experiment, the compounds were prepared by serial dilutions in assay buffer (HFB) containing 20 mM HEPES (K) pH 7.3, 50 mM KCl, 5 mM $MgCl_2$, 20 mM Na_2MoO_4, and 0.01% NP40. Before each use, 0.1 mg/mL bovine gamma globulin and 2 mM DTT were freshly added.

Fluorescence polarization (FP) was performed in Opti-Plate™-96F well plates (Perkin Elmer, Zaventem, Belgium) using a plate reader (Wallac Envision 2101 multilabel reader, Perkin Elmer, Zaventem, Belgium) To evaluate the binding affinity of the molecules, 50 μL of the GM-FTC solution (5 nM) were added to 30 nM of HSP90 in the presence of 5 μL of the test compounds at increasing concentrations. The plate was mixed on a shaker at 4 °C for 4 h, and the FP values in mP (millipolarization units) were recorded. The IC_{50} values were calculated as the inhibitor concentration where 50% of the tracer is displaced; each data point is the result of the average of triplicate wells, and was determined from a plot using nonlinear least-squares analysis. Curve fitting was performed using Prism GraphPad software program (GraphPad software Inc., San Diego, CA).

4.26. Yeast Hsp82 protein expression and purification

Escherichia coli strain BL21-CodonPlus(DE3)-RIL (Stratagene; La Jolla, CA) was transformed with the plasmid pET28-Hsp82/NdeI containing the full-length Hsp82 gene of *Saccharomyces cerevisiae*, with an N-terminal His6 tag for purification. Transformed cells were selected on LB agar, containing 50 μg/mL kanamycin and 40 μg/mL chloramphenicol, and a preliminary analysis on individual colonies was performed to evaluate their overexpressing ability.

A 4 L culture of the clone with the highest Hsp82 expression was grown in the presence of kanamycin until A_{600} reached 1.2, and then expression of His-tagged Hsp82 was induced by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside. Cells were then grown for 3 h and harvested by centrifugation at 7000 rpm for 30 min, at +4 °C. Harvested cells were washed in ice-cold 1× PBS, weighed and then stored at −70 °C. Frozen cells were thawed and then re-suspended and lysed in B-PER® Bacterial Protein Extraction Reagent (Pierce; Rockford, IL), containing phenylmethylsulfonyl fluoride (PMSF). The lysate was centrifuged at 27,000*g* for 15 min at +4 °C and supernatant was ultra-centrifuged at 100,000*g* for 45 min at +4 °C. Supernatant was then loaded, at a flow rate of 1 mL/min, onto a Ni–NTA column (HisTrap™ HP Columns, GE Healthcare) prepacked with NiSepharose and activated according to manufacturer's instructions. The column was washed with potassium phosphate buffer (pH 7.4) containing 30 mM imidazole. A linear gradient of imidazole in potassium phosphate buffer was finally applied to the column for Hsp82 elution. The collected fractions were pooled, then subjected to gel filtration on Superdex 200 resin (GE Healthcare) in 20 mM Tris–HCl, pH 7.5, containing 300 mM KCl and 1 mM EDTA, and finally stored at −80 °C in the presence of glycerol (10% final concentration).

4.27. Animals

Experiments were carried out using female athymic Swiss nude mice, 8–10 weeks old (Charles River, Calco, Italy). Mice were main-

tained in laminar flow rooms keeping temperature and humidity constant. Mice had free access to food and water. Experiments were approved by the Ethics Committee for Animal Experimentation of the Istituto Nazionale Tumori of Milan according to institutional guidelines.

4.28. Intraperitoneally growing tumor

The human IGROV-1 ovarian carcinoma was adapted to grow ip and maintained by serial ip passages of ascitic cells into healthy mice. Ascitic tumor cells 2.5×10^6 in 0.2 mL of saline were ip injected. Mice developed hemorrhagic ascites and diffuse peritoneal carcinomatosis and died by 15–30 days. Survival was the end point of the study and the median day of death (median survival time: MST) was calculated for each group. Antitumor activity was assessed as T/C%, that is, the ratio of MST in treated over control mice $\times$ 100. Treatment started the day after tumor cell injection, delivering **2** ip every day for four days a week for two weeks (qdx4/wx2w) at a dose of 25 mg/kg.

4.29. Statistical analysis

Curves reporting the percentage of survivors over time were estimated by the Kaplan–Meyer product limit method and compared with the log-rank test. Analyses were done with Graph Pad Prism (GraphPad Software, Inc., San Diego, CA). The test was two-sided.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.06.068. These data include MOL files and InChiKeys of the most important compounds described in this article.

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