Brief Articles

Molecular Characterization of Macbecin as an Hsp90 Inhibitor[#]

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Macbecin compares favorably to geldanamycin as an Hsp90 inhibitor, being more soluble, stable, more potently inhibiting ATPase activity ($IC_{50} = 2\ \mu M$) and binding with higher affinity ($K_d = 0.24\ \mu M$). Structural studies reveal significant differences in their Hsp90 binding characteristics, and macbecin-induced tumor cell growth inhibition is accompanied by characteristic degradation of Hsp90 client proteins. Macbecin significantly reduced tumor growth rates (minimum *T*/*C*: 32%) in a DU145 murine xenograft. Macbecin thus represents an attractive lead for further optimization.

Introduction

The 90 kDa heat shock protein (Hsp90[a]) is an abundant molecular chaperone involved in the ATP-dependent folding of numerous client proteins with a broad range of functions, including steroid receptors, nonreceptor tyrosine kinases, cyclin-dependent kinases, the cystic transmembrane regulator, and nitric oxide synthase.[1–3] Furthermore, Hsp90 plays a key role in stress response and protection of the cell against the effects of mutation.[4,5] Many Hsp90 client proteins are overexpressed in cancer, often in mutated forms, and are responsible for unrestricted cancer cell proliferation and survival.[6] Recently, Hsp90 has also been identified as an important extracellular mediator for tumor invasion.[7] Inhibition of Hsp90 results in the simultaneous destabilization and degradation of multiple oncogenic client proteins leading to cell growth inhibition and apoptosis.[8] Thus, Hsp90 is considered a major therapeutic target for anticancer drug development because inhibition of a single target represents attack on all of the hallmark traits of cancer.[9]

Several chemical classes of Hsp90 inhibitors are known, including ansamycins, macrolides, purines, pyrazoles, and coumarin antibiotics.[10,11] The ansamycin polyketide geldanamycin (Figure 1, **1**)[12] was the first Hsp90 inhibitor reported.[13,14] Although too toxic to be developed as an anticancer drug,[15] its optimization by semisynthesis resulted in two promising derivatives, 17-allylamino-17-demethoxygeldanamycin (17-AAG, Figure 1, **2**)[16] and 17-(2-dimethylamino)ethylamino-17-demethoxygeldanamycin (17-DMAG, Figure 1, **3**),[17] which are currently in clinical trials. 17-AAG has reduced hepatotoxicity compared to geldanamycin and is the most advanced Hsp90 inhibitor in clinical development (phase II/III) but suffers from poor solubility and formulation difficulties.[18] A reduced version, dihydro-17-AAG (or IPI-504, Figure 1, **4**), forms water-soluble salts and is currently under clinical development.[19] 17-DMAG has greater solubility than geldanamycin or 17-AAG, but its therapeutic window is narrower than 17-AAG.[20] It is therefore desirable to find an alternative lead molecule that bears a similar "pharmacophore" to geldanamycin but exhibits different or improved molecular characteristics.

Structurally, geldanamycin (Figure 1, **3**) belongs to a class of natural products called benzenoid ansamycins. They are characterized by an aliphatic chain of varying length joined to an aromatic ring in a metacyclophane manner. In addition to geldanamycin, other ansamycins including macbecins (Figure 1, **5** and **6**),[21] herbimycins (Figure 1, **7–9**),[22] and the TAN compounds (Figure 1, **10–14**)[23] have been reported to act like Hsp90 inhibitors, although with limited experimental evidence. Herbimycin A was deemed a poor candidate for further development because of hepatotoxicity.[15] Interestingly, herbimycins share the same polyketide backbone structure as geldanamycin and the structural differences between the two are the results of post-polyketide synthase modifications.[24] Macbecin (Figure 1, **5** and **6**) on the other hand differs from the rest of the chemical series in that its polyketide backbone structure contains a methyl group instead of a methoxy group at the C6 position. It also lacks the 17-substituent found in geldanamycin and derivatives and has a methoxy substituent at the C15 position (Figure 1) which may offer different conformational flexibilities.

Macbecin has been shown to exhibit antitumor and cytocidal activities,[25] but there is a lack of direct evidence supporting these activities as the result of Hsp90 inhibition, for example, no quantitative measurements of macbecin's interaction with Hsp90 have been reported to date. The combination of distinct structural features and antitumor activities prompted us to characterize macbecin in detail as an alternative lead to geldanamycin for Hsp90 inhibition.

Macbecin exists in two forms: the quinone macbecin I (Figure 1, **5**) and the dihydroquinone macbecin II (Figure 1, **6**). Like other quinone-containing Hsp90 inhibitors, there exists a redox cycling between these in vivo; i.e., the quinone form can be
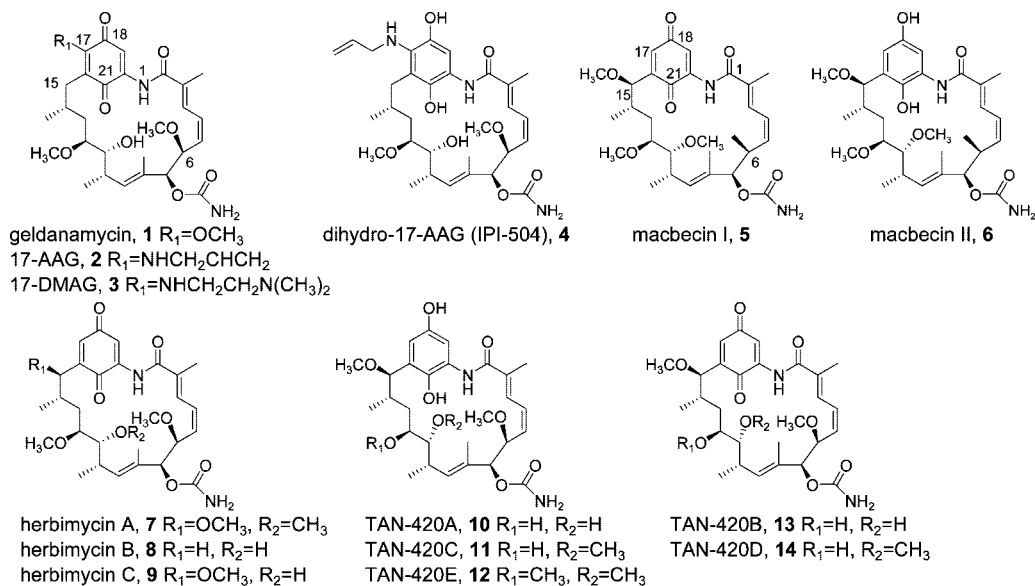

Figure 1. Chemical structures of ansamycin-class Hsp90 inhibitors.

reduced by NAD(P)H/quinone oxidoreductase 1 (NQO1 or DT-diaphorase) to the dihydroquinone form,[26,27] and the dihydroquinone form can be oxidized by molecular oxygen to the quinone form. The equilibrium between the two forms is probably responsible for part of the overall in vivo efficacy of these compounds, as both the quinone 17-AAG and the dihydroquinone of 17-AAG (IPI-504) inhibit Hsp90.[27] In this paper all data reported are for the quinone macbecin I unless noted otherwise. This is because macbecin I is chemically more stable than the dihydroquinone macbecin II, although we expect that macbecin II contributes to the overall in vivo efficacy.

Results and Discussion

Binding and Inhibition of Hsp90. Macbecin binds to Hsp90 with a slightly higher affinity than geldanamycin (Figure S1; $K_d = 0.24$ μM vs $K_d = 1.2$ μM of geldanamycin) as determined by isothermal titration calorimetry (ITC). The binding is primarily enthalpy driven ($\Delta H = -12360$ cal/mol; $\Delta S = -10.54$ cal/mol), whereas for geldanamycin ($\Delta H = -6206$ cal/mol; $\Delta S = 6.65$ cal/mol) entropy gain also contributes to the overall binding affinity. Consistent with its higher binding affinity, macbecin is more potent than geldanamycin in inhibiting the ATPase activity of Hsp90 with an IC$_{50}$ of 2 μM vs an IC$_{50}$ of 7 μM for geldanamycin. It is noted that geldanamycin has been reported as being a slow, tight-binding inhibitor of Hsp90 and that full levels of inhibition are not reached until 24 h.[28]

Examination of the X-ray crystal structure of macbecin-Hsp90 complex (Figure 2A, PDB code 2VLS) reveals that, like geldanamycin, macbecin binds to the ATP binding site at the N-terminal of Hsp90, adopting a "folded" conformation with the benzoquinone moiety located at the entrance of the pocket and the carbamate group being involved in hydrogen-bonding at the bottom of the active site with Asp79 as well as Gly83, Thr171, and Leu34 via water bridges. There are in total nine hydrogen-bonding interactions between the ligand and the protein, six of which are via water molecules (Table 1).

An overlay with the crystal structure of geldanamycin bound to Hsp90 (PDB code 1YET) shows that the positioning of the two ligands is very similar and that very little conformational change in the protein has been induced by the binding of the two ligands (Figure 2B). There is one less hydrogen-bond interaction in the geldanamycin−Hsp90 complex than the
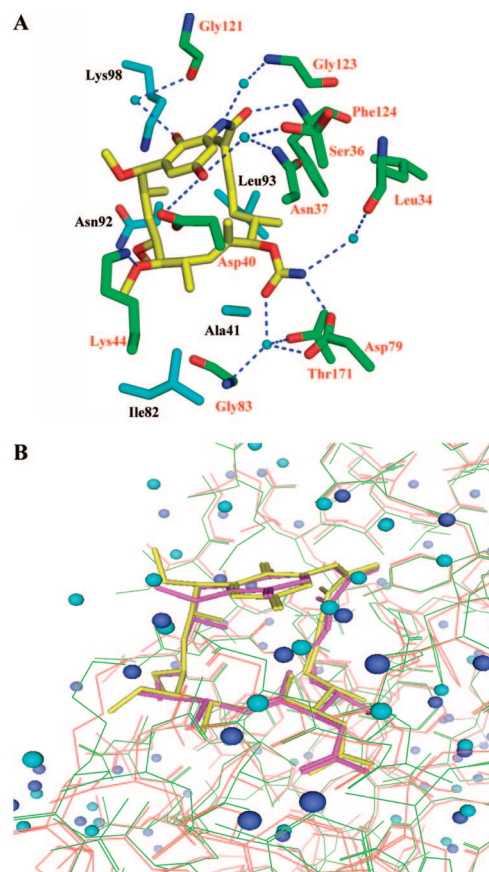

Figure 2. (A) X-ray crystal structure of macbecin (yellow) bound in the ATP-binding site of Hsp90 N-terminal. (B) Superimposition of X-ray crystal structures of Hsp90−geldanamycin (Hsp90 red, geldanamycin pink, H$_2$O blue) and Hsp90−macbecin (Hsp90 green, macbecin yellow, H$_2$O cyan) complexes.

macbecin−Hsp90 complex. Only four of geldanamycin's hydrogen bonds are bridged by water (Table 1), which may explain the difference in entropy between the two complexes, with geldanamycin displacing more water molecules in the binding pocket than macbecin. On balance, the extra hydrogen bond gained in macbecin interactions (Table 1) through, for example,

Table 1. Summary of Conserved (Black), Balanced Nonconserved (Green), and Gained (Blue) Hydrogen Bonds between the Geldanamycin and Macbecin Crystal Structures

H-Bonding Groups	Geldanamycin	Macbecin
Lys to C11 substituent	Yes	Yes
Phe to C1 carbonyl	Yes	Yes
Asp to NH off C7	Yes	Yes
H_2O to C=O off C7	Yes	Yes
H_2O to NH off C7	Yes	Yes
H_2O to N (btwn C1, C20)	Yes	Yes
Lys to dione C=O	Yes	No
H_2O to dione C=O	No	Yes
Extra H_2O to dione C=O	No	Yes
H_2O to C6 OMe	Yes	No
H_2O to C15 OMe	No	Yes

Figure 4. Western blot of protein expression in lysates of the prostate cancer cell line DU145 following treatment with macbecin.

the C15-OMe moiety may offer enough enthalpy gain to yield slightly tighter binding than geldanamycin.

Targeted Molecular Dynamics (TMD). It was reported that there was a considerable energy barrier for geldanamycin to adopt the bound conformation from its free conformation.[29] According to the report, the aromatic ring of geldanamycin flips backward in the initial stages of the targeted molecular dynamics (TMD) simulation and later flips forward, overcoming two unfavorable high-energy intermediate conformations before adopting the bound conformation. We carried out a TMD simulation of macbecin starting with a conformation initially approximated by taking the free conformation of geldanamycin, editing it structurally to the macbecin topology, and then performing energy minimization. This yielded a lower energy conformation that was used as the free conformation of macbecin for the TMD simulation. Unlike geldanamycin, the aromatic ring of macbecin did not first flip backward and then forward. Instead, the ring tilted forward gradually, simultaneously with the diene moving inward to form intermediate 1 (Figure 3) around 430 ps. Flexing of the methoxy substituted alkyl chain then occurred to form intermediate **2** (Figure 3) around 540 ps. The amide bond finally flipped trans to cis around 695 ps to form the bound conformation (Figure 3). The results of the energetic analysis of the start, finish, and intermediate conformations of macbecin are shown in Table S1.

It can be seen here that the energetic change from free to bound states is more favorable than that reported for geldanamycin with ΔG (compared with the unbound conformation) of the bound conformation being 3.83 kcal/mol compared with 8.1 kcal/mol for geldanamycin.[29]

In Vitro and in Vivo Anticancer Activity. Macbecin inhibited the growth of a panel of 38 cancer cell lines derived from
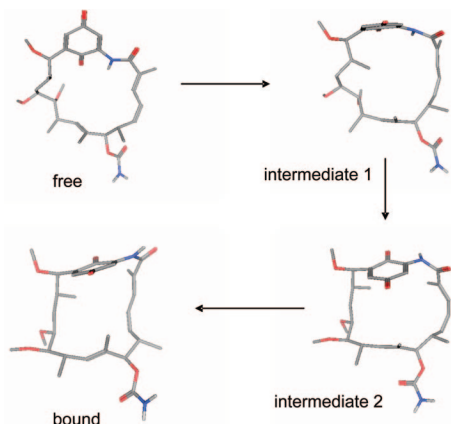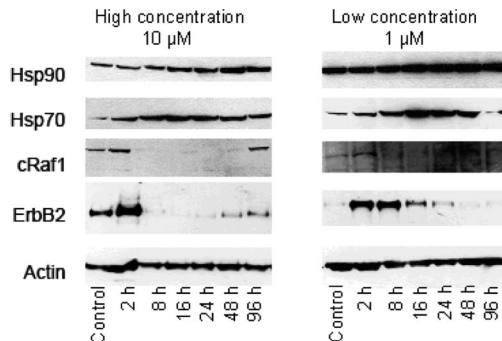
human solid tumors with mean IC_{50} and IC_{70} values of 0.4 and 3.2 μM, respectively (Figure S2). Among the 38 cell lines, 9 cell lines (24%) exhibited above average sensitivity toward macbecin (determined as individual IC_{70} values less than $^1/_3$ of the mean IC_{70}). The most sensitive cell lines (individual IC_{70} $\leq$ $^1/_{10}$ mean IC_{70}) were bladder BXF 1218L ($IC_{70} < 0.01$ μM), colon HT29 ($IC_{70} = 0.29$ μM) and SW620 ($IC_{70} < 0.01$ μM), mammary MAXF 401NL ($IC_{70} < 0.01$ μM), melanoma MEXF 394NL ($IC_{70} < 0.01$ μM), and prostate DU145 ($IC_{70} < 0.01$ μM). Interestingly, 5 out of the 6 tested lung cancer cell lines (LXF 289L, 526L, 529L, 629L, H460), 4 out of the 5 renal cancer cell lines (RXF 1781L, 393NL, 486L, UO31), and all 3 ovarian cancer cell lines (OVXF 1619L, 899L, OVCAR3) showed clearly below-average sensitivity, indicating that lung, renal, and ovarian cancers may represent resistant tumor types.

Western blot analysis of prostate cancer cell line DU145 lysate after treatment with macbecin showed clear concentration-dependent degradation of client proteins ErbB2 and cRaf1, consistent with the mechanism of Hsp90 inhibition.[30,31] As shown in Figure 4, the higher concentration of 10 μM macbecin is more effective than the lower concentration of 1 μM at which the client proteins reappeared after 48 and 96 h. Concomitantly, Hsp70 protein expression increased over time after macbecin treatment at 1 and 10 μM, again consistent with the signatory feature of Hsp90 inhibition.[31–34]

When dosed at 10 mg/kg ip to mice bearing xenografts of the human prostate carcinoma cell line DU145, macbecin significantly reduced tumor growth with the minimum *T/C* value of 32.2% ($p < 0.01$; U-test by Mann–Whitney−Wilcoxon) achieved on day 31. There was a significant growth delay of 2.3 days for tumor doubling time and 20.4 days of delay for tumor quadrupling time (Figure 5). However, two unscheduled deaths occurred on days 25 and 44 in the treatment group. Both animals had lost significant body weight prior to their deatha and were carrying mid-sized tumors only. Overall in the treatment group (six mice each bearing two tumors) there was a median body weight loss of 5.3% on day 3, but in the course of the experiment the median body weight increased again and was higher than the starting body weight on day 45 when the study was terminated (Figure S3).

Conclusion

As demonstrated in this paper, macbecin acts clearly as an Hsp90 inhibitor. It binds to the ATP-binding site of Hsp90 N-terminal with higher affinity than geldanamycin (ITC, $K_d = 0.24$ μM for macbecin vs 1.2 μM for geldanamycin). This is likely due to the presence of an extra hydrogen bond involved in the macbecin−Hsp90 interaction compared to the geldanamycin−Hsp90 interaction. Lack of a substituent at C17
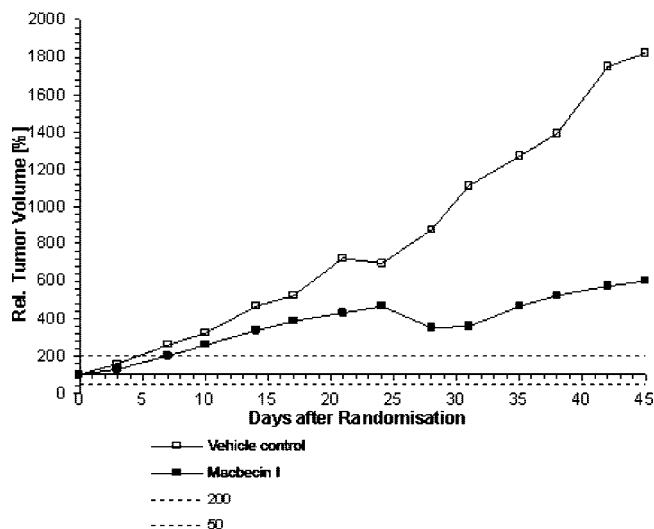

Figure 3. Intermediate conformations of macbecin from free to Hsp90-bound conformations, as identified by TMD using MOE.

Figure 5. Macbecin I therapy of DU145: in vivo antitumor activity of macbecin in DU145 tumor bearing mice. Macbecin was dosed at 10 (mg/kg)/day ip on days 0–4, 7–11, 17, 18, 21–25, 28–32, 35–39, 42–45. Values are expressed as group median relative tumor volumes over time.

probably contributed to the lower energy barrier for macbecin to adopt the bound conformation from the free conformation than geldanamycin. As demonstrated in the TMD study, macbecin does not undergo the "backward flip" followed by "forward flip" conformational change of its aromatic ring, unlike geldanamycin which has to overcome a much higher energy intermediate conformation before adopting the bound conformation.[29] Additionally, there may exist a Hsp90 binding kinetic difference between macbecin and geldanamycin, as the latter has been reported to undergo slow association/dissociation, approaching binding equilibrium at incubation times of >10 h.[28]

Consistent with its apparent higher affinity to Hsp90 than geldanamycin, macbecin is also slightly more potent in inhibiting the ATPase activity of the protein ($IC_{50} = 2$ μM vs 7 μM of geldanamycin). It inhibits cancer cell growth by causing degradation of key oncogenic client proteins of Hsp90, such as ErbB2 and cRaf1. At concentrations relevant to cell growth inhibition (1 and 10 μM), macbecin treatment caused degradation of ErbB2 and cRaf1 in the prostate cancer cell line DU145. This is accompanied by an up-regulation of the cochaperone Hsp70, a signatory feature of Hsp90 inhibitors. The anticancer potential of macbecin is further demonstrated by its in vivo efficacy in a human prostate carcinoma DU145 xenograft model. Macbecin at 10 mg/kg, ip, significantly ($p < 0.01$; U-test by Mann–Whitney–Wilcoxon) inhibited tumor growth (minimum *T/C* of 32.2) and delayed disease progression (2.3 days of delay for tumor doubling time and 20.4 days of delay for tumor quadrupling time).

Finally, macbecin is significantly more water soluble than geldanamycin with a solubility in phosphate buffer (pH 7.4) being 81 μM vs 1.7 μM of geldanamycin. Interestingly the reduced dihydroquinone form of macbecin (macbecin II) has an even higher solubility of 136 μM in PBS. The approximately 50-fold higher solubility of macbecin compared with that of geldanamycin makes it a promising starting point for further optimization.

We conclude that macbecin represents an interesting alternative lead to geldanamycin. Its differences from geldanamycin in structure and Hsp90 binding characteristics, although subtle, are attractive because they offer the opportunity to exploit different chemical space within the proven structural framework (or "pharmacophore") of an effective Hsp90 inhibitor. A soluble and fast onset analogue of macbecin would be a significant step toward a best-in-class Hsp90 inhibitor.

Experimental Section

The production of macbecin has been described previously.[35] Purified macbecin was characterization to be indistinguishable from that published previously.[36] Macbecin was assessed in a series of in vitro assays to determine solubility in PBS, inhibition of ATPase activity[37] and binding affinity to Hsp90 by isothermal titration calorimetry. The X-ray crystal structure of macbecin–Hsp90 complex was obtained and the structure deposited at Protein Structure Database (PDB code 2VLS). Targeted molecular dynamics modeling studies were carried out to simulate the energetics of binding of macbecin and geldanamycin to Hsp90. Anticancer activity of macbecin was evaluated in vitro using a 38 cell line panel of human tumor cell lines in a monolayer proliferation assay, and Western blot analysis carried out to demonstrate the effect of macbecin on Hsp90, Hsp70, and the Hsp90 client proteins cRaf1 and ErB2 of the human prostate cancer cell line DU145. The antitumor activity and side effects of macbecin were investigated in nude mice bearing xenografts of the human prostate carcinoma cell line DU145. Detailed experimental procedures can be found in the Supporting Information.

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

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