Protein–Protein Complex Inhibition

DOI: 10.1002/anie.200900929

Molecular Mechanism of Inhibition of the Human Protein Complex Hsp90–Cdc37, a Kinome Chaperone–Cochaperone, by Triterpene Celastrol**

*Sridhar Sreeramulu, Santosh Lakshmi Gande, Michael Göbel, and Harald Schwalbe**

Inhibition of the ATPase activity of the kinome chaperone Hsp90 (kinome = set of all protein kinases in an organism) has long been known as molecular target for anticancer therapy. Cdc37, a cochaperone of Hsp90 in mammalian cells, targets protein kinases and is upregulated in various cancers.[1,2] The protein–protein complex forms with a K_D value of 1.2 μM and is considered to mediate carcinogenesis by stabilizing a variety of different oncogenic kinases in malignant cells. However, Cdc37 as well as Hsp90 can also act alone, at least in yeast. Low-molecular-weight molecules that interfere with Cdc37 or Hsp90 or disrupt the Hsp90–Cdc37 complex have recently been proposed as a new class of anticancer agents.[3,4] Gene-based expression studies have identified the triterpene celastrol, which represents a new class of non ATP-competitive inhibitors of Hsp90.[5] Immunoprecipitation in a pancreatic cell line and docking experiments suggested that celastrol exerts its antiproliferative activity by binding to the N-terminal domain of Hsp90 ($Hsp90_N$), thereby disrupting the complex between $Hsp90_N$ and Cdc37.[6] In vivo, celastrol showed significant inhibition of tumor growth in nude mice with prostate or pancreatic cancer.[6,7]

Herein, we describe in detail how celastrol disrupts the human Hsp90–Cdc37 complex. ^1H,^{15}N-HSQC NMR experiments detect ligand binding to a target through chemical-shift perturbations (CSPs).[8–10] We investigated the effects of celastrol on the complex of ^1H,^{15}N-labeled $Hsp90_N$ (23 kDa) with unlabeled full-length Cdc37 (45 kDa) (Figure 1 A). As a result of the increased transverse relaxation rates of the protein in complex (approx. 70 kDa), the HSQC spectrum of the complex resulted in broadening of most of the cross peaks, indicating complex formation. Addition of celastrol dissociated the complex and restored the HSQC spectrum of free $Hsp90_N$, revealing that celastrol disrupted the complex by a mechanism other than binding to $Hsp90_N$. Changes in chemical shifts indicative of binding upon the addition of celastrol to free $Hsp90_N$ could not be observed (see Supporting Information 1 (SI 1)).

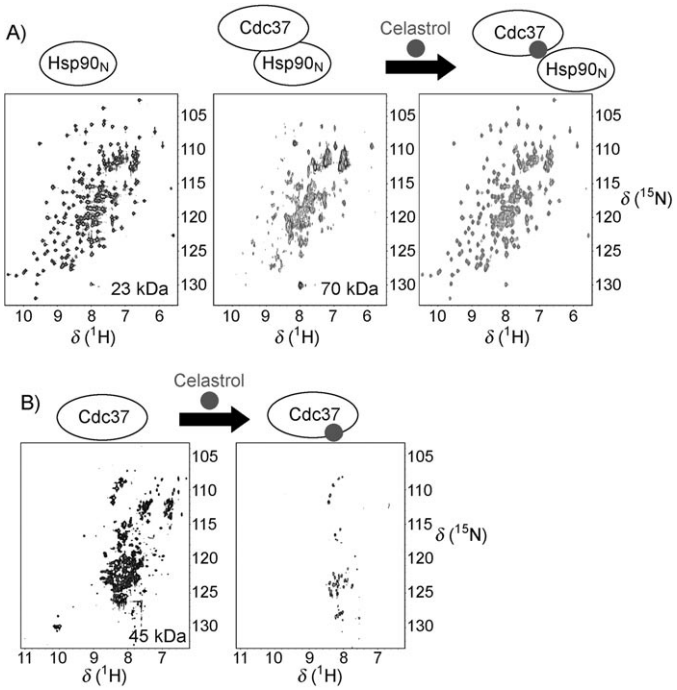


Figure 1. Binding of celastrol to Cdc37. A) ^1H,^{15}N HSQC spectra of $Hsp90_N$ (left) and of the $Hsp90_N$–Cdc37 complex before (middle) and after addition of celastrol (right; 1:7 mixture of $Hsp90_N$–Cdc37/celastrol). B) ^1H,^{15}N HSQC spectra of full-length Cdc37 (45 kDa) before (left) and after addition of celastrol (right; 1:7 mixture of Cdc37/celastrol).

We then studied the interaction between Cdc37 and celastrol. For this purpose, we recorded a ^1H,^{15}N-HSQC spectrum of ^{15}N-labeled Cdc37 (Figure 1 B, left). Upon addition of celastrol to Cdc37, most of the cross peaks in the HSQC spectrum disappeared, indicating interaction of the protein with the ligand (Figure 1 B, right). The loss of resonance signals in the HSQC spectrum could be a result of protein aggregation induced by the ligand. Celastrol has an orange color and becomes colorless upon addition to Cdc37,

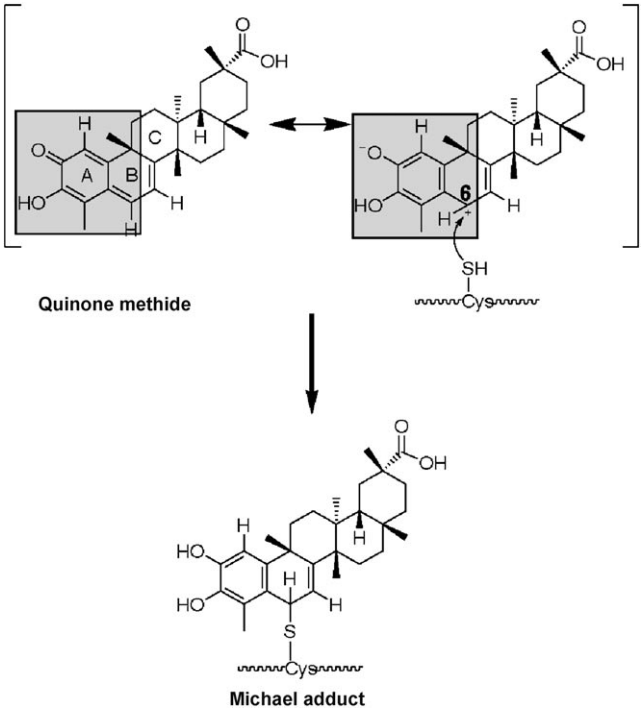
[*] S. Sreeramulu, Dr. S. L. Gande, Prof. Dr. M. Göbel, Prof. Dr. H. Schwalbe
Johann Wolfgang Goethe-University, Frankfurt am Main
Institute for Organic Chemistry and Chemical Biology
Center for Biomolecular Magnetic Resonance (BMRZ)
Max-von-Laue-Strasse 7, 60438 Frankfurt am Main (Germany)
Fax: (+49) 69-798-29515
E-mail: schwalbe@nmr.uni-frankfurt.de
Homepage: http://schwalbe.org.chemie.uni-frankfurt.de/

[**] Cdc37: Cell division cycle protein 37, Hsp90: Heat shock protein 90. We thank Frédéric Tournay at the Jardin Botanique de l'Université Louis Pasteur, France, for kindly providing the photograph of the plant *Tripterygium wilfordii*. This work was supported by the SPINEII project of the European Commission and by the Cluster of Excellence: Macromolecular Complex (DFG)

Supporting information (including experimental details) for this article is available on the WWW under http://dx.doi.org/10.1002/anie.200900929.

which suggests a loss of its chromophore as quantified by UV spectroscopy[11] (see SI 2).

Celastrol possesses electrophilic sites within the A and B rings (Scheme 1), where nucleophilic groups of amino acid residues react to form covalent Michael adducts.[7,12,13] In fact,



Scheme 1. Molecular mechanism for the addition of celastrol to the thiol group of Cdc37. The quinone methide region of celastrol is highlighted in a light gray box in the structure. The thiol group of Cdc37 reacts with the quinone methide of celastrol through a Michael addition, resulting in the formation of an adduct at C6.

the mass spectrum of Cdc37 in the presence of celastrol exhibited a mass increase of 450 Da, which corresponds to the molecular weight of celastrol (see SI 3).

To confirm that celastrol modifies the cysteine residue(s) in Cdc37, we blocked all nine cysteine residues of full-length Cdc37 with *N*-ethylmaleimide (NEM). NEM-labeled Cdc37 no longer reacted with celastrol, and the mixture did not show any change in the UV spectrum, indicating that cysteine(s) do indeed undergo(es) chemical reactions with celastrol upon binding (see SI 4). Circular dichroism (CD) spectra for the wild-type Cdc37 and Cdc37_NEM showed a decrease in secondary structure, indicating that covalent modification of the free cysteines alters the conformation of the protein (see SI 5). Further, the HSQC spectrum of $Hsp90_N$ remained unchanged upon addition of Cdc37_NEM, indicating that modification of thiols is sufficient to inhibit the interaction between $Hsp90_N$ and Cdc37. Free cysteine can undergo a covalent addition reaction with celastrol, as the lipophilic celastrol becomes water soluble after addition of cysteine at a pH of 7.4, loses its absorption at 440 nm, and displays chemical shift changes in its 1D ^{1}H NMR spectrum, characteristic for the Michael adduct at C6 in ring B of the triterpene (see SI 6).

The formation of Michael adducts in which cysteine thiol groups serve as the nucleophiles is highly dependent on pH. Under physiological conditions, the reaction is fast and reversible, thereby the ligand escapes from the high glutathionone concentration present in living cells (0.5–10 mM) and reaches the target protein.[14]

Cdc37 is composed of three domains, the N-terminal kinase-binding domain ($Cdc37_N$, residues 1–126) with three free cysteine residues, the middle, $Hsp90_N$-binding domain ($Cdc37_M$, residues 147–276) with four free cysteine residues, and the C-terminal domain ($Cdc37_C$, residues 282–376) of unknown function with two free cysteine residues (see SI 7). In order to delineate which of these domains is responsible for binding celastrol, individual domain constructs were expressed. $Cdc37_N$ reduced celastrol and resulted in an inactivated form, as observed in the ^{1}H,^{15}N-HSQC spectrum (Figure 2). $Cdc37_M$ alone precipitated upon addition of

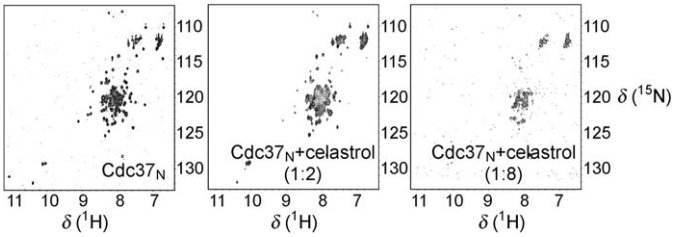


Figure 2. Binding of celastrol to $Cdc37_N$ monitored by ^{1}H,^{15}N HSQC spectroscopy. Spectra of free $Cdc37_N$ (left), after addition of celastrol (middle; 1:2 mixture of celastrol/$Cdc37_N$), and after further addition of celastrol (right; 1:8 mixture of celastrol/$Cdc37_N$).

celastrol but did not reduce the compound, while the construct with the middle and the C-terminal domain ($Cdc37_{MC}$) reacted with celastrol but also precipitated. This observation suggests that the N-terminal domain is mainly responsible for reducing celastrol and binding. Additionally, when the cysteines were blocked using NEM, the $Cdc37_N$ no longer interacted with celastrol (see SI 8).

Further, in order to pinpoint which of the three cysteine residues of $Cdc37_N$ are involved in the interaction with celastrol, we made double-cysteine mutants retaining one cysteine residue ($Cdc37_N$-C57S-C64S, $Cdc37_N$-C54S-C64S, $Cdc37_N$-C54S-C57S). CD spectra for the wild-type and mutant $Cdc37_N$ are mostly similar, indicating that there are no changes in secondary structure (see SI 5). We monitored the decrease in absorbance at 440 nm after the addition of various constructs of Cdc37 to celastrol. $Cdc37_N$ and all the three mutants reduced celastrol and were comparable to the wild-type (see SI 4), indicating they are all reactive.

In conclusion, we report that celastrol binds to Cdc37 but not to $Hsp90_N$ as previously described. This study is important as it will shift efforts in medicinal chemistry towards the correct target within the protein–protein complex. Celastrol inactivates Cdc37 by covalently binding to it or by forming either an intra- or intermolecular protein disulfide (Figure 3). Reactivities of individual cysteine residues vary slightly (as seen from the UV results); depending on accessibility and the

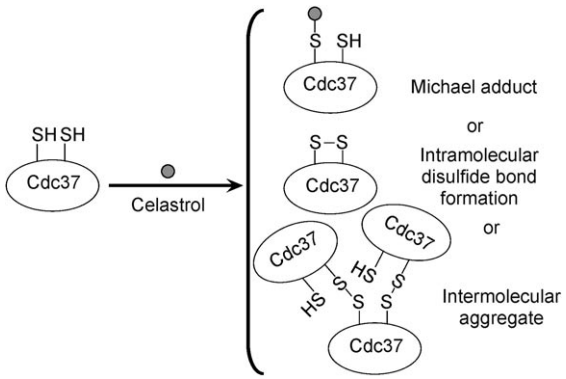


Figure 3. Possible mechanisms of inactivation of Cdc37 by celastrol.

stabilization interaction of the adjacent amino acids, celastrol binds to one of the cysteine residues. The Cdc37–Hsp90 complex also involves a large hydrophobic core at the interface.[15] Adjacent to the hydrophobic core is C203 from $Cdc37_M$, which is exposed to the surface. Celastrol harbors a hydrophobic structure apart from the quinone methide moiety. Hence, celastrol could be involved disrupting this hydrophobic core. The binding of celastrol induces large changes in conformation of the N-terminal kinase-binding domain and also the middle $Hsp90_N$-binding domain of Cdc37, thereby disrupting the Cdc37–$Hsp90_N$ complex which is crucial for stabilizing oncogenic kinases in various cancers.[3] Our results indicates that the N-terminal kinase-binding and middle $Hsp90_N$-binding domain of Cdc37 are the molecular target for the triterpene celastrol. In light of the considerable potential of Cdc37 as a drug target and the binding of celastrol to it, these results can now be used to further understand the molecular basis for inhibiting this key Cdc37–Hsp90 complex in cancer cells.

Received: February 17, 2009
Published online: July 7, 2009

Keywords: Cdc37 · chaperones · Hsp90 · inhibitors · NMR spectroscopy

[1] P. Lee, J. Rao, A. Fliss, E. Yang, S. Garrett, A. J. Caplan, *J. Cell Biol.* **2002**, *159*, 1051.
[2] L. H. Pearl, *Curr. Opin. Genet. Dev.* **2005**, *15*, 55.
[3] P. J. Gray, Jr., T. Prince, J. Cheng, M. A. Stevenson, S. K. Calderwood, *Nat. Rev. Cancer* **2008**, *8*, 491.
[4] J. R. Smith, P. A. Clarke, E. de Billy, P. Workman, *Oncogene* **2009**, *28*, 157.
[5] H. Hieronymus, J. Lamb, K. N. Ross, X. P. Peng, C. Clement, A. Rodina, M. Nieto, J. Du, K. Stegmaier, S. M. Raj, K. N. Maloney, J. Clardy, W. C. Hahn, G. Chiosis, T. R. Golub, *Cancer Cell* **2006**, *10*, 321.
[6] T. Zhang, A. Hamza, X. Cao, B. Wang, S. Yu, C. G. Zhan, D. Sun, *Mol. Cancer Ther.* **2008**, *7*, 162.
[7] H. Yang, D. Chen, C. C. Qiuzhi, X. Yuan, Q. P. Dou, *Cancer Res.* **2006**, *66*, 4758.
[8] L. D′Silva, P. Ozdowy, M. Krajewski, U. Rothweiler, M. Singh, T. A. Holak, *J. Am. Chem. Soc.* **2005**, *127*, 13220.
[9] C. A. Lepre, J. M. Moore, J. W. Peng, *Chem. Rev.* **2004**, *104*, 3641.
[10] B. J. Stockman, C. Dalvit, *Prog. Nucl. Magn. Reson. Spectrosc.* **2002**, *41*, 187.
[11] K. T. Liby, M. M. Yore, M. B. Sporn, *Nat. Rev. Cancer* **2007**, *7*, 357.
[12] A. Trott, J. D. West, L. Klaić, S. D. Westerheide, R. B. Silverman, R. I. Morimoto, K. A. Morano, *Mol. Biol. Cell* **2008**, *19*, 1104.
[13] J. H. Lee, T. H. Koo, H. Yoon, H. S. Jung, H. Z. Jin, K. Lee, Y. S. Hong, J. J. Lee, *Biochem. Pharmacol.* **2006**, *72*, 1311.
[14] T. J. Schmidt, G. Lyss, H. L. Pahl, I. Merfort, *Bioorg. Med. Chem.* **1999**, *7*, 2849.
[15] S. Sreeramulu, H. R. Jonker, T. Langer, C. Richter, C. R. Lancaster, H. Schwalbe, *J. Biol. Chem.* **2009**, *284*, 3885.

