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Celastrol Analogues as Inducers of the Heat Shock Response. Design and Synthesis of Affinity Probes for the Identification of Protein Targets

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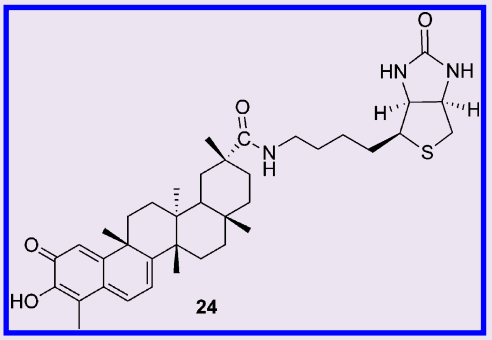
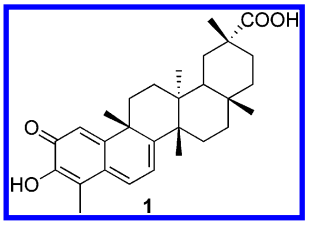
Supporting Information

ABSTRACT: The natural product celastrol (**1**) possesses numerous beneficial therapeutic properties and affects numerous cellular pathways. The mechanism of action and cellular target(s) of celastrol, however, remain unresolved. While a number of studies have proposed that the activity of celastrol is mediated through reaction with cysteine residues, these observations have been based on studies with specific proteins or by *in vitro* analysis of a small fraction of the proteome. In this study, we have investigated the spatial and structural requirements of celastrol for the design of suitable affinity probes to identify cellular binding partners of celastrol. Although celastrol has several potential sites for modification, some of these were not synthetically amenable or yielded unstable analogues. Conversion of the carboxylic acid functionality to amides and long-chain analogues, however, yielded bioactive compounds that induced the heat shock response (HSR) and antioxidant response and inhibited Hsp90 activity. This led to the synthesis of biotinylated celastrols (**23** and **24**) that were used as affinity reagents in extracts of human Panc-1 cells to identify Annexin II, eEF1A, and β-tubulin as potential targets of celastrol.



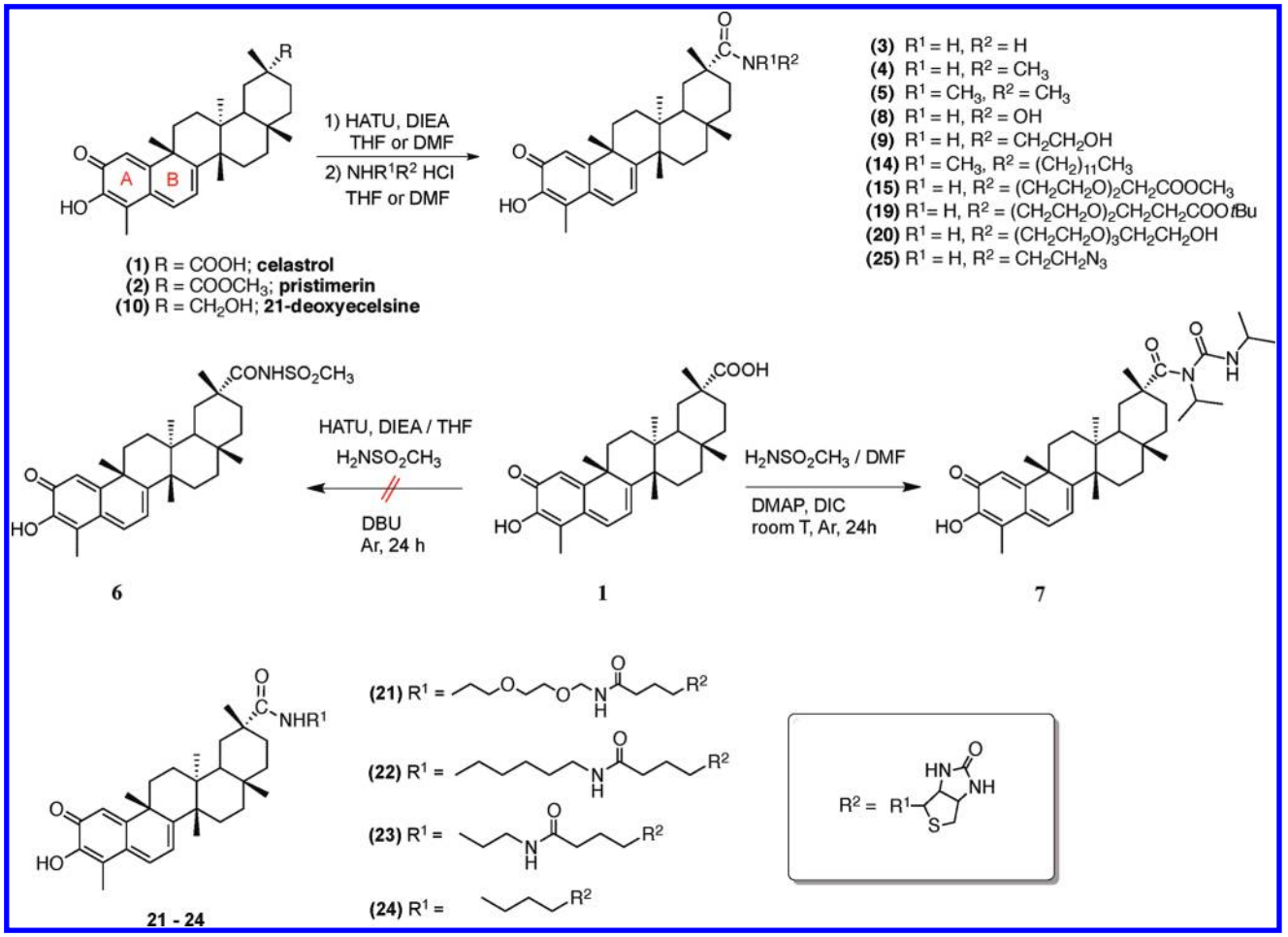
Celastrol (**1**), a quinone methide triterpene, is among the most active small molecule components of extracts of *Celastraceae* plants used in traditional Chinese medicine for the treatment of fevers, chills, joint pain, inflammation, edema, and rheumatoid arthritis.[1] Celastrol also has been shown to have diverse biological activities as an antimalarial agent;[2] an inhibitor of lipid peroxidation;[3] and a downregulator of mediators of anti-inflammatory responses, such as IL-1α,[4] TNF-α,[5] and nuclear factor κB (NF-κB).[6] It also exhibits cytotoxicity against various human cancer cell lines[7−9] and inhibits human prostate tumor growth and human glioma xenografts in mice.[10,11] Other important biological activities of celastrol include induction of the heat shock response (HSR), increased expression of cytoplasmic chaperones,[12] activation of the unfolded protein response (UPR), and induction of lumen-localized chaperones. The ability of celastrol to potently induce the heat shock response has far-reaching consequences.[12] Regulation of heat shock transcription factor 1 (HSF1)[21,22] is an evolutionarily conserved cellular mechanism, which is essential for proper cytoplasmic protein folding. Its induction results in rapid and robust synthesis of heat shock proteins, which function as molecular chaperones[23−27] and fulfill the proteostasis surveillance role.[28−30] Enhanced levels of molecular chaperones have proven benefits in the suppression of various neurodegenerative phenotypes associated with Huntington's,[31] Alzheimer's,[32] Parkinson's,[33] and amyotrophic lateral sclerosis.[34] Because of induction of the heat shock response, celastrol can ameliorate a number of protein misfolding diseases other than neurodegenerative diseases, namely, lysosomal storage diseases, such as Gaucher and Tay-Sachs diseases.[13]



Despite these diverse biological properties, our understanding of the cellular target(s) of celastrol is limited. From a chemical mechanism, we[14,15] and others[10,16−18] have proposed a mode of action involving conjugate addition by cysteine residues. Studies have shown effects on various signaling pathways; targets proposed from *in vitro* studies include Hsp90,[19] Cdc37,[16] p23,[20] IKKβ,[17] and the proteasome.[10] Whether these interactions occur in a more native environment remains unknown, and the discovery of the target(s) of celastrol is critical to explain its diverse biological activities.

Received: December 19, 2011
Accepted: March 1, 2012
Published: March 1, 2012

Scheme 1. Synthesis and Structures of Celastrol Analogues



In this study we have investigated structural modifications of celastrol to determine what part(s) of the molecule is responsible for its activity in several signaling pathways (*i.e.*, the HSR, antioxidant response, and inhibition of the Hsp90 pathway). We also searched for a site on celastrol where a biotin-containing affinity probe could be linked for affinity pull-down experiments to identify cellular binding partners.

RESULTS AND DISCUSSION

The goal of this study is to further investigate the structure–activity relationship of celastrol through chemical modification (compounds **3**–**25**, Scheme 1). Since a very small number of celastrol's synthetic analogues have been reported, any modification to its structure should be highly informative in defining the structural requirement for activation of the heat shock response and antioxidant response (Table 1). The new analogues have guided us in developing affinity-based probes for the investigation of the cellular targets of celastrol.

Structural Modifications of Celastrol and Effects on the HSR. We have previously shown that esterification of the carboxylic acid functionality of celastrol (**1**) to the methyl ester (known as pristimerin, **2**, Scheme 1) retains the celastrol signature induction of heat shock gene expression, whereas conversion to the *n*-butyl and benzyl esters results in a significant reduction or complete loss of activity. Closely related structures, such as pristimerol and pristimerol diacetate, are almost inactive.[12] In addition to various multiring compounds, such as anthraquinone and baicalein, compounds that contain the quinone methide substructure of celastrol, such as brazilein and hematein, do not induce the HSR, suggesting that a quinone methide alone is insufficient for heat shock induction.[12] However, pretreatment of celastrol with excess dithiothreitol, which reduces the quinone methide, results in a compound that is inactive toward induction of the HSR.[14] Taken together, this suggests that the general structure of celastrol (**1**) has specific chemical characteristics required for the induction of the HSR, and although the quinone methide is important, it is not sufficient.

Various modifications to the functional groups of celastrol were made to determine the importance of these groups for activity and also to identify sites on the molecule where an affinity probe could be attached for target pull-down experiments. The carboxylic acid functionality is the most reasonable position for modification because of the simplicity of transformations; however, as noted above, esterification leads to a reduction of its potential as a HSR inducer.[12] Conversion to an amide would benefit from increased *in vitro* and *in vivo* stability relative to an ester, as it would be less prone to enzymatic hydrolysis, and a secondary amide also allows for interrogation of the importance of a hydrogen-bond donor.

A successful synthesis of celastrol amides was accomplished *via* activation of the carboxylic acid functionality with HATU (Scheme 1). The same coupling conditions were subsequently applied to the preparation of secondary (**4**) and tertiary amides (**5**). All synthesized compounds were purified using reversed phase HPLC prior to testing in the HSR and antioxidant

dx.doi.org/10.1021/cb200539u | *ACS Chem. Biol.* 2012, 7, 928–937

Table 1. Activation of the Heat Shock Response (HSR) and Antioxidant Response Element (ARE) by Celastrol Analogues

compound	HSR EC_{50} (μM)	max fold induction HSR (%)	ARE EC_{50} (μM)	max fold induction ARE (%)
celastrol (**1**)	2.2 ± 0.1	100	1.6 ± 0.1	100
3	1.7 ± 0.2	120	1.6 ± 0.2	60
4	2.6 ± 0.4	98	2.3 ± 0.1	50
5	3.0 ± 0.2	87	2.6 ± 0.2	48
8	1.5 ± 0.1	110	1.1 ± 0.1	80
9	2.7 ± 0.3	87	2.9 ± 0.1	50
21-deoxyeclesine (**10**)	2.1 ± 0.2	59	1.9 ± 0.2	76
14	N/A at 100 μM		N/A at 100 μM	
15	2.5 ± 0.2	110	2.7 ± 0.3	75
18	N/A at 100 μM		N/A at 100 μM	
19	1.4 ± 0.2	45	0.6 ± 0.1	50
20	2.7 ± 0.2	110	1.0 ± 0.1	60
25	1.5 ± 0.1	50	1.8 ± 0.1	50
biotinylated				
21	N/A at 100 μM		N/A at 100 μM	
22	N/A at 100 μM		N/A at 100 μM	
23	40 + 3.6	45	19.8 + 3.6	50
24	6.6 + 0.3	90	3.1 + 0.3	40

response cell-based assays (Figure 1). To evaluate the activity of celastrol in inducing the HSR, we used a HeLa cell line containing a heat-shock-inducible reporter construct[12] that contains the human hsp70.1 promoter sequence starting at position −188 upstream of the start site of transcription fused to a luciferase reporter gene (Figure 1a). We evaluated our synthesized analogues for their ability to induce an antioxidant response using the stable ARE-driven reporter gene cell line AREc32 fused to a luciferase gene (Figure 1b).

As shown in Table 1, amides **3**−**5** induced a HSR at a level similar to that of celastrol, although primary amide **3** both exhibited a lower EC_{50} (1.7 μM) and induced a higher level of heat shock gene expression. The activity decreased with increasing methyl substitution on the amide nitrogen, which might be attributed to decreased cell permeability of **4** and **5**, steric hindrance to binding to some receptor, or the importance of a hydrogen bond donor for activation of the HSR.

To investigate the importance of the acidity of the carboxylic acid or amide proton for activity, we attempted to synthesize analogues with a lower amide pK_a, such as **6** (Scheme 1), using the same coupling methodology. The coupling of **1** to methanesulfonamide[35] at RT led to recovery of starting material and at elevated temperature to decomposition. Coupling using DIC provided **7** (Scheme 1), which stems from rearrangement of the intermediate *O*-acylisourea. In an attempt to circumvent this unforeseen rearrangement, **1** was converted to the acid fluoride *in situ* using TFFA;[36] however, coupling of methanesulfonamide with the acid fluoride also failed to provide **6**.

As an alternative to the sulfonamide, the corresponding hydroxamate (**8**) was synthesized, as described in Scheme 1. The corresponding 2-hydroxyethylamide (**9**) also was synthesized to improve solubility and bioavailability. Hydroxamate **8** exhibited a small enhancement of activation of the HSR

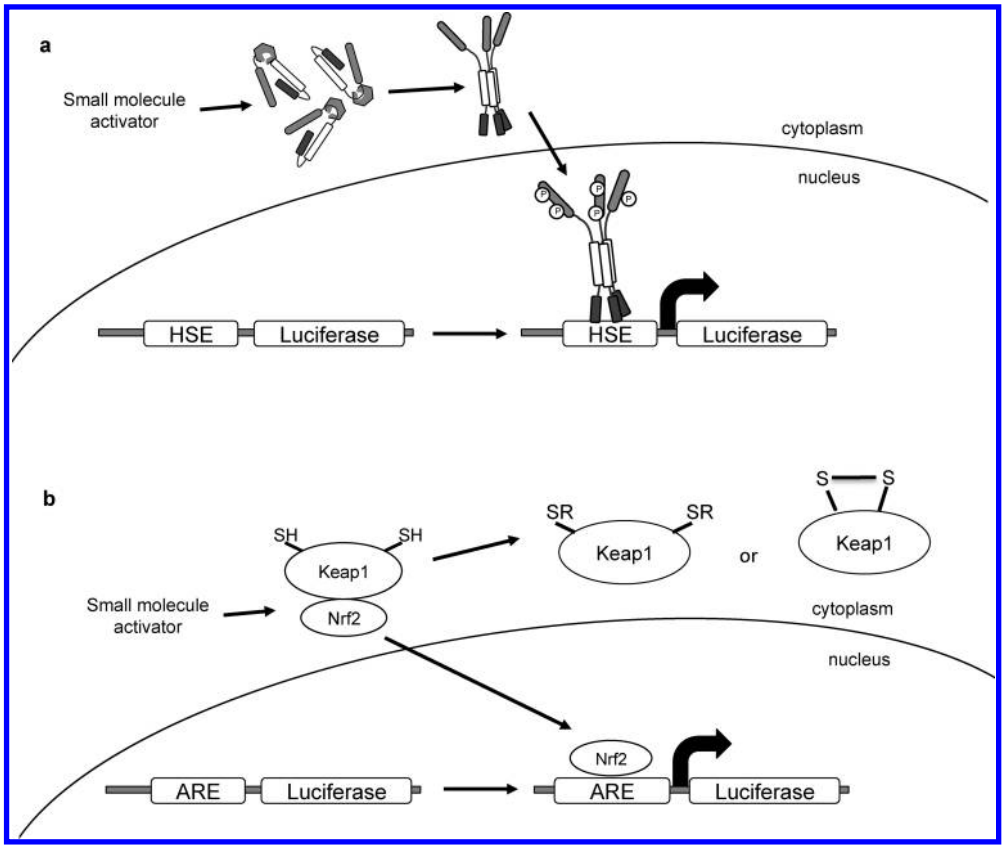


Figure 1. Schematic of the cellular assay for activation of (a) HSR and (b) antioxidant response.

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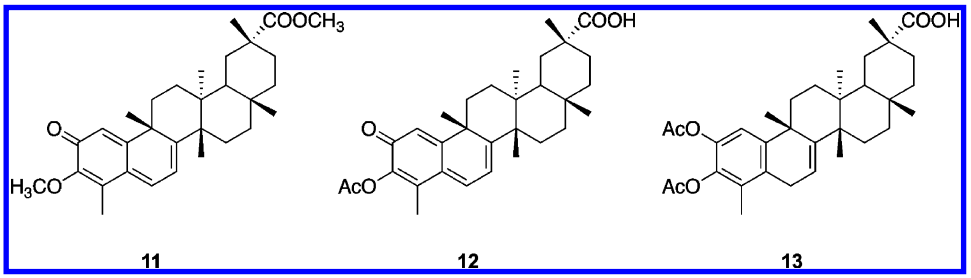

11 12 13

compared to that of celastrol (Table 1), consistent with the importance of the carboxylic acid moiety or an isostere with related properties.

To determine whether the anionic character of the carboxylate (or related moiety) and/or the hydrogen bonding properties are relevant to activity, **1** was converted to the natural product 21-deoxyecelsine (**10**, Scheme 1). Initial attempts to selectively reduce the carboxylic acid functionality of **1** by BH_3-THF resulted in recovery of starting material; instead, **10** was obtained from pristimerin (**2**) by $LiAlH_4$ reduction followed by aerobic oxidation of the hydroquinone intermediate back to quinone methide **10**.[37] The ED_{50} of alcohol **10** for activation of the HSR was unchanged from that of celastrol, although the maximum fold-induction was lower. This indicates that neither a carboxylate anion nor a carbonyl at that position is required for activity.

Attempts were made to modify the enol hydroxyl group of the A ring. This modification might elucidate the importance of the hydrogen bond donor properties of the enol hydroxyl group and the effect of intramolecular hydrogen bonding. Various methods employed to methylate this position in **1** using a range of bases and methyl iodide were unsuccessful, possibly because of the formation of dianionic species. Further attempts, starting from methyl ester **2** (to give **11**), led to either recovery of the starting material or to formation of complex mixtures. The enol hydroxyl group of **1** or **2** can be converted to an acetoxyl group using acetyl chloride and triethylamine. Isolation of **12** was complicated by its instability on silica gel and because it was prone to hydrolysis, leading to recovery of only the starting material. On the basis of these experiments, we concluded that this part of celastrol would not be suitable for modification by an affinity probe.

It has been proposed that the intracellular activity of celastrol and its derivatives results from the electrophilicity of the quinone methide substructure extending over the A and B rings. Our studies[14,15] and observations of others[16] have suggested that reactivity toward thiols is the predominant mode of action of celastrol. Inconsistent with this hypothesis are experiments with dihydrocelastrol diacetate (**13**), which lacks the quinone methide substructure and is not electrophilic, showing that **13** is similar in potency to celastrol as a heat shock activator[12] and Hsp90 inhibitor.[19,20] However, given the instability of **12**, we predicted that **13** might also be unstable and prone to hydrolysis or possibly to enzymatic degradation in cells. Indeed, when **13** was incubated under the assay conditions (DMSO, DMEM, 37 °C), and monitored by HPLC, approximately 60% of the material underwent hydrolysis and oxidation to celastrol within 4 h (Figure 2). Given that the incubation time for the cellular assay is 7 h, these data strongly suggest that the activity of **13** derives from its conversion back to celastrol and is not related to the structure of the ring system.

These structure–activity studies confirm that an amide linkage is more appropriate for tethering celastrol to a bioprobe for identification of protein target(s). The design of bioprobes requires an affinity tag sufficiently removed from the pharmacophore so that it does not affect target binding. Also, the bioprobes must be validated in cells prior to their use in the pull-down experiments to ascertain that they remain active in the HSR. To evaluate spatial requirements, we synthesized a number of long chain amides (**14**, **15**, **19**, Scheme 1) that serve as an approximation of the spacer-arm length. Long-chain amide **14** did not activate a HSR at concentrations up to 100 μM (Table 1), possibly because of limited permeability. To enhance drug permeability, an amide linked poly(ethylene glycol) (PEG), as in **15** (Supporting Information Scheme S-1) was introduced, which showed complete retention of HSR activity at 2.5 μM, comparable to that of celastrol (Table 1).

To rule out the possibility that the PEG side chain was responsible for activation of heat shock gene transcription, the acetylated side chain (**18**) was prepared (Supporting Information, Scheme S-1). This compound did not activate the hsp70.1 promoter up to 100 μM concentration, confirming that it is the celastrol that induces the observed gene transcription. Further elongation of the PEG side chain (**19** and **20**) induced heat shock at lower concentrations. In agreement with our cellular heat shock reporter model, the activity was confirmed by reverse transcription-polymerase chain reaction (RT-PCR) analysis of the endogenous Hsp70-A6 genes in HeLa cells (Supporting Information Figure S-1).

Synthesis of Activity-Based Probes and Their Activation of the HSR. To identify the proteome target(s) of celastrol, we generated a biotin-celastrol conjugate that could be used with neutravidin to pull down any proteins bound to celastrol from the cells. We were able to accomplish that with **24** (Scheme 1). Coupling of biotin-PEG_2-amine to **1**, with the intent of increasing solubility, resulted in biotin-conjugate **21**, which was difficult to purify and exhibited limited solubility. A more lipophilic amide side chain (**22**), which was successfully utilized to generate probes of other natural products,[38] was attached without much improvement. Neither of these conjugates had the ability to induce a HSR up to 100 μM concentration (Table 1). Using a computational approach (QikProp v3.2 software),[39] we compared the physicochemical properties of **21** and **22** to parent compound **1** and amide **3** (see Supporting Information Table S-1) and found that the predicted values were consistent with our experimental findings that **21** and **22** exhibited poor cell permeability.

Improved pharmacokinetic properties were predicted for **23** and **24** (Figure 1; Supporting Information Table S-1). Compound **23** was prepared from biotin ethylenediamine and was designed to avoid the additional chain linker between **1** and biotin. To achieve this, the terminal functional group of biotin was converted to the appropriate norbiotinamine[40] and coupled to **1** using standard conditions. Both **23** and **24** activated the HSR pathway (Table 1); **24** exhibited an EC_{50} of 6.6 μM and the ability to

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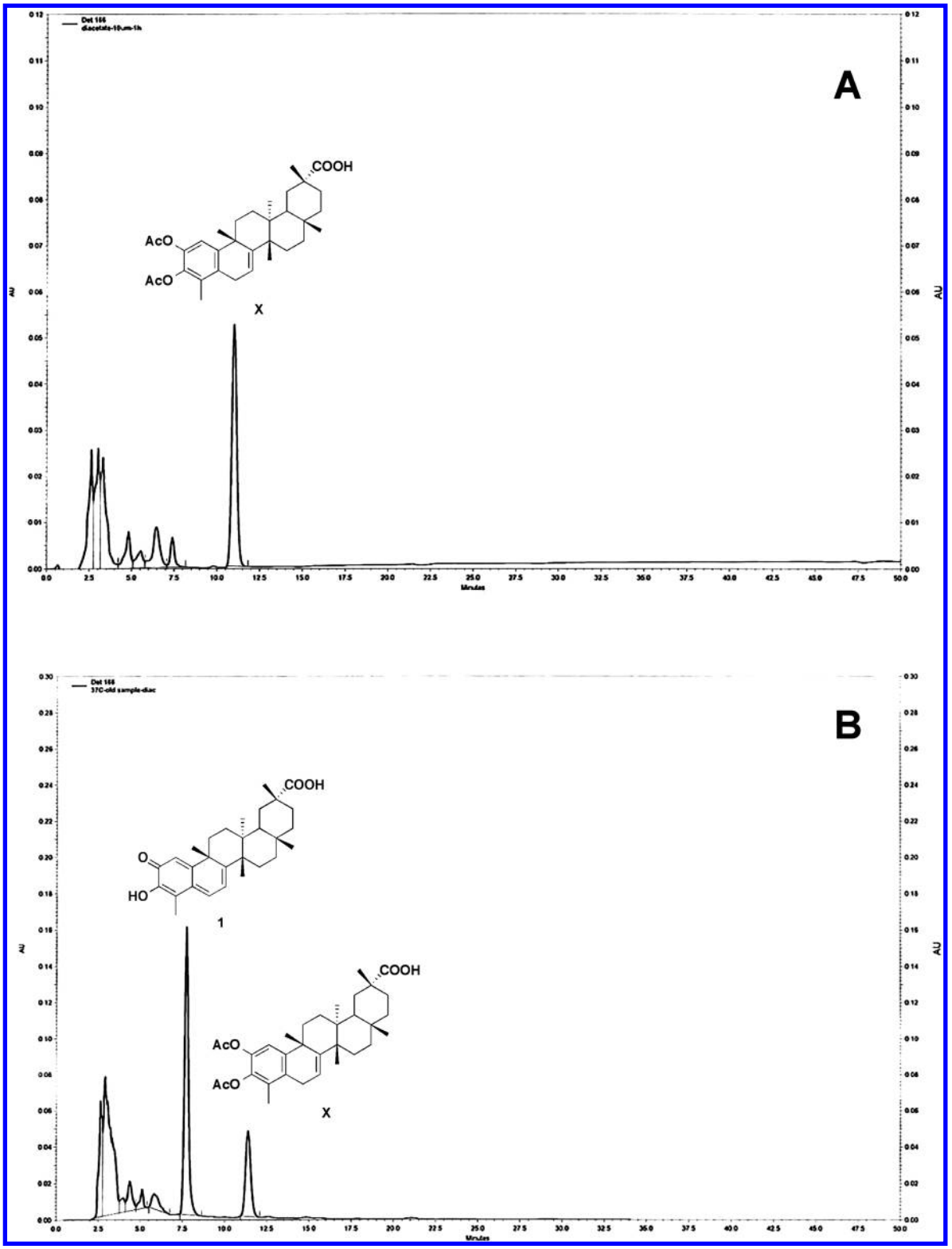

Figure 2. Instability of dihydrocelastrol diacetate (**13**). (A) A solution of **13** in DMSO was diluted in DMEM (10 μM) and (B) incubated at 37 °C for 4 h. Stability was monitored using a reversed phase HPLC analytical column with isocratic conditions (90% MeOH/10% H_2O) at a flow rate of 1 mL/min.

induce the heat shock-luciferase reporter to levels comparable to that of celastrol (**1**).

In addition to the biotinylated analogues, we also prepared biotin-free azide analogue **25** (Scheme 1), which could be used for profiling studies by coupling to biotin linkers using the Staudinger–Bertozzi reaction[41,42] or "click chemistry".[43] Analogue **25** (Supporting Information Scheme S-2) showed good activity in the heat shock assay with a low EC_{50} of 1.5 μM but somewhat lower induction of luciferase expression (Table 1).

dx.doi.org/10.1021/cb200539u | *ACS Chem. Biol.* 2012, 7, 928–937

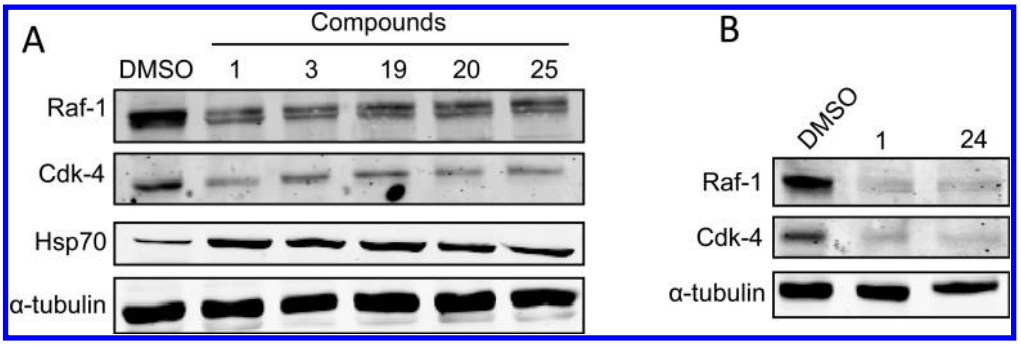

Figure 3. (A) Western blot analysis of the effect of **3**, **19**, **20**, and **25** on certain proteins in PANC-1 cancer cells after 24 h of incubation at a concentration of 5 μM. (B) Western blot analysis of biotin conjugate **24** (30 μM) in comparison to celastrol (**1**) (5 μM) .

Induction of the Antioxidant Response. We have previously demonstrated the potency of **1** to induce an antioxidant response in conjunction with a HSR in both mammalian and yeast cells.[14] This response, a major defense mechanism against carcinogens, aging, and neurodegeneration, is regulated by Nrf2 transcription factor, which is sequestered in the cytoplasm by Keap1. When activated, Nrf2 is released and translocated to the nucleus, where it binds to the antioxidant-responsive element (ARE), inducing transcription of genes for protective, antioxidant enzymes (Figure 1b).[44] Activation of ARE by celastrol is prevented by preincubation with DTT;[14,45] as Keap1 is highly cysteine rich, it is a plausible candidate for the intracellular target of celastrol. The stable ARE-driven reporter gene cell line AREc32 fused to a luciferase gene was used to evaluate the compounds (Figure 1b). As shown in Table 1, compounds that exhibited activation of the HSR are also potent inducers of the antioxidant response, confirming that modifications to the parent structure preserve induction of both responses, which implies a shared pharmacophore.

Inhibition of the Hsp90 Pathway. Celastrol (**1**) has recently been identified as inhibitor of heat shock protein 90 (Hsp90).[46] Because of enhanced expression of Hsp90 in cancer cells and its role in maturation of oncogenic proteins, the identification and development of small molecule inhibitors of Hsp90 has been pursued for anticancer therapeutics.[16,47,48] Celastrol (**1**) is of particular interest as a potential anticancer drug because it does not bind to the ATP/ADP pocket of Hsp90, which is targeted by other inhibitors including geldanamycin, 17-AAG, and radicicol. Although the end result of inhibition of the Hsp90 chaperone pathway by **1** in cells has been confirmed,[19] the conclusions about mechanism and identity of the protein(s) targeted by celastrol remain open. Although docking studies by Zhang *et al.*[49] suggested that celastrol bound to the N-terminal domain of Hsp90, *in vitro* studies using purified proteins suggested the C-terminal domain of Hsp90 as the target. Other studies propose that modification of co-chaperones Cdc37[16] or p23[20] could also be responsible for the observed inhibition. These conflicting conclusions emphasize the necessity for chemical tools, such as biotinylated conjugates, to elucidate the nature of this inhibition. To assess whether our modified celastrols had an impact on Hsp90 inhibition, selected potent analogues were evaluated for their ability to induce degradation of the well-characterized Hsp90-dependent client proteins, cyclin-dependent kinase Cdk4 and Raf protein kinase. Other Hsp90 inhibitors, such as geldanamycin and 17-AAG, also have the ability to induce client protein degradation and induce expression of Hsp70.[48,50] As shown in Figure 3, quantification by Western blot analysis demonstrated a clear reduction in kinase levels upon treatment with **3**, **19**, **20**, and **25** relative to DMSO-treated control Panc-1 cells. The marked decrease in client protein levels is accompanied by elevated levels of Hsp70 as a consequence of concomitant induction of the HSR. We have demonstrated that the carboxylic acid functionality of **1** is not required for Hsp90 inhibition and that biotinylated derivative **24** also preserves inhibitory properties, making **24** an appropriate tool for a mechanistic investigation of celastrol activity. The higher concentration of **24** was utilized to observe an effect comparable to that of unmodified compound **1** so **24** could be recognized as a tool for protein target pull down. The higher concentration used was expected, as **24** has increased hydrophobicity and lower permeability (see Supporting Information, pages S10–S11) and because higher concentrations of **24** are required for induction of both the heat shock and antioxidant responses at levels comparable to **1** (Table 1). α-Tubulin was used as a loading control in this experiment; it is known that at 5 μM celastrol the levels of α-tubulin are unaffected (see Supporting Information).

Protein Target Identification. Our previous studies[14,15] suggest that the intracellular activity of celastrol (**1**) stems from the electrophilicity of the quinone methide substructure. Studies using celastrol demonstrate its effect on various signaling pathways involving cysteine-mediated mechanisms. The activity of celastrol was probed with isolated proteins or demonstrated as the end-point effect of the drug on selected cellular pathways. To identify cellular targets of celastrol within the cellular proteome, we immobilized biotinylated **24** on neutravidin agarose beads under saturating conditions and incubated the beads with cell lysates from pancreatic carcinoma cells (PANC-1), shown to be sensitive to Hsp90 inhibition by celastrol.[49] Cell extracts were preincubated with DMSO vehicle (lanes 3–5) or free celastrol (**1**, lanes 6 and 7) (Figure 4). Nonspecifically bound proteins were removed by successive low stringent washing with reaction buffer, followed by elution of proteins in the sample buffer. The eluted proteins were run on SDS-PAGE and visualized using Coomassie Blue staining. As shown in Figure 4, although multiple proteins were found to bind nonselectively, based on the inherent chemical reactivity of celastrol, the enrichment in several bands is significant. Two prominent bands of approximate mass 35 and 50 kDa (compare lanes 3 and 4) are intriguing because they were eluted from the affinity resin by treating the sample with DTT. The reactivity of the extended conjugated substructure of celastrol with sulfhydryl nucleophiles suggests that interaction with these proteins involves a conjugate addition. Since we have observed that the formation of a Michael adduct of celastrol and the cysteine residue of GSH is reversible (data not shown), it is reasonable to assume that reactions of cysteine residues of proteins with celastrol also are reversible. Compound **23** gave

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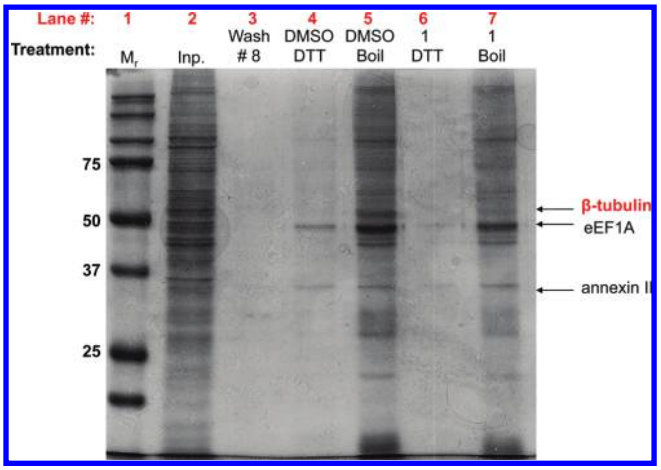

Figure 4. SDS-PAGE of protein binding using analogue **24** with PANC-1 lysate. Lane 1 is the MW ladder; lane 2 is the input, the untreated protein lysate; lane 3 corresponds to the 8th and final wash of the column; lane 4 is the proteins removed after the eight washes followed by addition of DTT (the same amount of DMSO was added that corresponds to the amount of DMSO needed to dissolve **1** in the lane 6/7 experiments); lane 5 is the proteins released by boiling of the beads after the treatment in lane 4; lane 6 is the lysate incubated with celastrol (dissolved in DMSO) and then loaded on the beads, washed 8 times, then DTT added; lane 7 is the proteins released by boiling the beads after the treatment in lane 6.

the same result as **24** (data not shown). Control experiments using acetylated norbiotin immobilized on neutravidin beads confirmed that the observed protein binding is attributed to celastrol and not to the biotin-containing side chain. The 35 kDa and 50 kDa bands were excised from the gel (lanes 4 and 5), destained, digested with trypsin, and the peptides analyzed by liquid chromatography-tandem mass spectrometry (LC−MS/MS). A eukaryotic database search identified the 35 kDa protein (actually 38 kDa, but it ran as 35 kDa on this gel) as human annexin II. This protein is a member of a family of proteins that binds acidic phospholipids in the presence of Ca^{2+} and is involved in bundling of F-actin.[51] Annexin II is an oxidant-sensitive protein, prone to glutathionylation,[52] and its activity is regulated by the oxidation state of its sulfhydryl groups. Annexin II was found to be targeted by the natural product withaferin A, a Michael acceptor like celastrol, *via* alkylation of Cys133.[53]

The more abundant 50 kDa protein band was identified as the eukaryotic translation elongation factor 1A (eEF1A), whose function is the delivery of the GTP-dependent aminoacyl-tRNA (aa-tRNA) to the A site of the ribosome during protein elongation. In addition to this role, eEF1A can bind and bundle actin filaments *in vitro*.[54] Protein eEF1A is ubiquitous and constitutes about 2% of the total protein in normal growing cells. Examination of the amino acid sequence of this protein reveals six (human) or eight (*S. cerevisiae*) cysteine residues. It is unclear from the literature whether these cysteine residues are prone to modification. However, it was shown recently that eEF1A is a potential binding partner in a protein complex with the Hsp90 client protein Akt2 and β-tubulin.[55] As shown previously, celastrol induces degradation of Akt,[49] and we identified β-tubulin as an interacting protein of celastrol (Figure 4; the band running just above the eEF1A band). This β-tubulin band was displaced by the addition of unmodified celastrol (**1**) to the cell lysate (Figure 4, lane 6) prior to affinity capture by the biotin-celastrol conjugate (Figure 4, lane 4). This suggests that β-tubulin binds to free celastrol in solution and is sequestered from the lysate. As a result of being bound to free celastrol, there is diminished capture of this protein by the celastrol attached to affinity beads (compound **24**). Interestingly, this band was not competed off by the addition of DTT to the reaction buffer, suggesting that the reaction is either irreversible or is not mediated through a cysteine residue of the protein. β-Tubulin, a major component of microtubules, might be an *in vivo* target for celastrol. Microtubules, cylindrical cytoskeletal filaments composed of α- and β-tubulin heterodimers, have an essential role in the maintenance of cellular shape and processes such as intracellular transport, cell polarity, and migration. Their role in signaling mitosis and cell division makes targeting microtubules or tubulins one of the strategies in development of cancer antiproliferation therapeutics.[56] Our data are in agreement with recent studies by Jo *et al.*,[57] who have identified celastrol as an inhibitor of microtubule-mediated processes. The observed inhibition was independent of Hsp90 or proteasome inhibition, which suggests a cellular target independent of these pathways. This study also demonstrated that celastrol inhibits the formation of tubulin oligomers both *in vitro* and in cell lysate. Furthermore, disruption of the interaction of α- and β-tubulin was demonstrated by immunoprecipitation as well as a concentration-dependent decrease in β-tubulin levels. Importantly, inhibition of tubulin oligomerization is detectable only under non-reducing conditions, which implies that celastrol might affect cysteine residues of β-tubulin, which is in agreement with our data. Tubulin heterodimer has 20 reduced and reactive cysteine residues, and binding of ligands to those residues is known to inhibit polymerization. Most of these cysteine residues are buried within the native protein but are accessible to small molecules.

Since binding of tubulin to celastrol is not sensitive to the addition of DTT and is irreversible, one hypothesis is that celastrol, apart from reacting with cysteine residues, undergoes additional side reactions, possibly with the negative charge generated upon 1,6-Michael addition, or there are hydrophobic

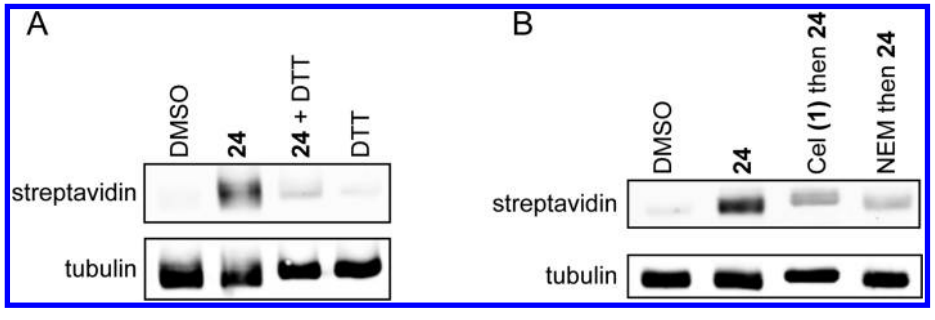

Figure 5. Western blot analysis of *in vitro* binding of tubulin and affinity probe **24**. Experimental details are described in Methods.

interactions that might induce a structural change, making tubulin subject to degradation. This would be in accord with the decreasing levels of β-tubulin observed in cellular experiments,[57] which we confirmed (see Supporting Information, Figure S-2). As shown in Figure 5, when tubulin was treated *in vitro* with biotinylated-conjugate **24**, incorporation of the molecule was observed by detection of the biotin moiety with the streptavidin-IR conjugate. In contrast, little incorporation of biotin was observed once **24** was pretreated with an excess of DTT, which quenches its reactivity toward the cysteine residues of tubulin. These data confirm that tubulin is targeted by celastrol and that the interaction goes through the cysteine residues (Figure 5A). When the cysteine residues of tubulin were first pretreated with either free celastrol or *N*-ethylmaleimide (NEM), both reactive toward cysteine residues, and then incubated with biotin-conjugate **24**, the binding was abolished (Figure 5B). Taken together these data demonstrate that celastrol modifies tubulin at cysteine residues and is consistent with the data obtained in the protein pull-down experiments (Figure 4).

Hsp90 is one of the most abundant proteins in the cell (2%), and its levels are comparable to those of tubulin, eEF1A, and annexin. Thus, if Hsp90 were targeted directly by celastrol in our experiments, we would have expected to see an Hsp90 band at levels at least comparable to the proteins identified, but we do not see an Hsp90 band. This result is consistent with other studies of celastrol and Hsp90 for which there is no experimental evidence for direct binding of celastrol to Hsp90. *In vitro* experiments with purified proteins, with the aid of molecular docking and molecular dynamic simulations, noted the disruption of the Hsp90/Cdc37 chaperone system in cells by celastrol.[49] It was then shown that celastrol does disrupt Hsp90/Cdc37 binding, and it was suggested that the mechanism involved interactions with the C-terminal domain of Hsp90.[16] However, Sreeramulu *et al.*[16] carried out NMR and mass spectral studies and concluded that celastrol does not bind to Hsp90 but instead modifies Cdc37. Another study of the effect of celastrol on Hsp90 proposed a different target. Chadli *et al.*[20] used reconstituted chaperone/co-chaperone in an *in vitro* system developed for proper chaperoning of the progesterone receptor (PR), a physiological client of Hsp90. This system uses five core components of the Hsp90 machinery (Hsp90, Hsp70, Hsp40, Hop, and p23) but does not require the presence of Cdc37. It was found that celastrol was able to inhibit Hsp90 chaperoning of PR *in vitro* by interaction with co-chaperone p23, not by binding to Hsp90. Direct binding of celastrol to Hsp90, therefore, may not be relevant to its ability to induce heat shock proteins.

Conclusions. We have designed a family of celastrol (**1**) analogues to elucidate its mechanism of action by attempting to identify its target(s) of binding. We have determined that the carbonyl of the carboxylic acid group of celastrol is required for maximal activity, and modification of the carboxylic functionality into an amide leads to improved potency. This allowed us to tether a biotin to celastrol *via* an amide linkage and to develop active biotinylated conjugates of celastrol (**23** and **24**) to perform affinity pull-down experiments with PANC-1 cell lysates to identify three potential cellular targets of celastrol (Figure 4). Annexin II and eEF1A are possibly targeted by celastrol in a reversible complex. Our studies also confirm β-tubulin, a previously suggested target, as another target of celastrol, further supported by *in vitro* binding studies with purified tubulin (Figure 5). Notably, Hsp90 was not detected as a target in the DTT-reduced or free molecule competing experiments, despite its comprising about 2% of the protein content of the cell. Given its chemical reactivity with intracellular thiols, such as glutathione, and its ability to induce the generation of reactive oxygen species,[18] it is plausible that celastrol targets Hsp90 indirectly by invoking a redox imbalance.

METHODS

Cell Culture Conditions. Human HeLa cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. The cells were maintained at 5% CO_2. Hsp70.1-luc cells are a stable cell line derived from the human HeLa cervical carcinoma cell line whose generation is described by Westerheide *et al.*[12] Briefly, a heat shock reporter construct (HSE-luc) that expresses luciferase under the control of the Hsp70.1 promoter was generated. This HSE is generated from the −188 to 150 fragment of the Hsp70.1 promoter region that contains five HSEs and the binding site for TBP and Sp1 transcription factors. HeLa cells were transfected with the reporter and selected using G418. AREc32 is a stable cell line derived from the human MCF7 breast carcinoma cell line and was provided by a generous donation from Prof. Roland Wolf at the University of Dublin. Generation of the cell line was described by Wang *et al.*[45] Both the AREc32 and HeLa-luc cells were maintained in Dulbeco's modified Eagle's medium, supplemented with 10% v/v fetal bovine serum (FBS), 1% L-glutamine, 100 U/mL penicillin/streptomycin, and 100 μg/mL of G418. The compounds were dissolved in DMSO and added to cells at indicated concentrations and for the indicated times. Control cells were left untreated or were treated with an equivalent concentration of DMSO.

Luciferase Assays. The cells were plated at 15,000 cells/well in a 96-well plate 20 h prior to compound treatment using the Biomex FX-96 fix channel head (Beckman Coulter). The indicated compounds were dissolved and serially diluted at millimolar concentrations in DMSO with the Biomex FX-Span 8 channel head (Beckman Coulter). Prior to addition to cells, each DMSO solution was additionally diluted in medium and added to the cells using the Biomex FX-96 fix channel head (Beckman Coulter). Following incubation for 7 h at 37 °C and 5% CO_2, the cells were harvested for luciferase activity using the SteadyLite HTS (Perkin-Elmer) according to the manufacturer's instructions. Luciferase activity was quantified using a multimode luminometer plate reader Analyst GT (Molecular Devices).

Western Blot Analysis. Analysis of protein expression was carried out using PANC-1 cells that were treated with the indicated compounds for 24 h. Cells were lysed in a buffer containing 20 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-X, supplemented with a protease and phosphatase inhibitor cocktail (Thermo Scientific) for 45 min on ice. Whole cell extracts (20 μg) were run on 10% SDS-PAGE gels and transferred to nitrocellulose. Primary antibody incubations were for 12 h at 4 °C in 10% BSA. The following primary antibodies were used: a mouse monoclonal Hsp70 antibody (4g4, Affinity Bioreagents Inc., Golden, CO) was used in dilution 1:5,000; a mouse monoclonal Cdk4 (DCS-31; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used in dilution 1:200. Equal protein loading was verified by anti-α-tubulin antibody (T5168; Sigma, St. Louis, MO) in 1:5,000 dilution. Goat anti-mouse Alexa Fluor 680 conjugated antibody (A-21057, Molecular Probes Inc. Eugene, OR) was used as a secondary antibody in 1;10,000 dilution or directly following incubation with **24**. Western analysis was performed with the Odyssey infrared imaging system (Li-COR Bioscience, Lincoln, NE).

Affinity Purification. Two plates of PANC-1 cells, grown to ~80% confluence, were harvested at 4 °C in PBS and centrifuged. The pellet was mixed with 500 μL of lysis buffer (20 mM HEPES, pH 7.6, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X) supplemented with Halt protease inhibitor cocktail (ThermoFisher Scientific, Rockford, IL), incubated on ice for 40 min, and centrifuged. The total cleared lysate was incubated with neutravidin beads for 1 h at 4 °C to remove endogenously biotinylated proteins and to minimize nonspecific

dx.doi.org/10.1021/cb200539u | ACS Chem. Biol. 2012, 7, 928–937

binding. Then the lysate was divided into equal portions and incubated with either DMSO or free celastrol. Affinity beads were prepared as follows: Neutravidin-functionalized beads (1 mL) were washed three times in cold lysis buffer (without protease inhibitors), gently shaken for 10 min, and spun at 1,000*g* for 1 min. Equal amounts (300 μL) of bead slurry were transferred into separate Eppendorf tubes and nutated with 10 μL of a 5 mM solution of compounds for 1 h at RT. The treated beads were centrifuged and washed four times with lysis buffer containing 1% DMSO to remove excess compound. Success of functionalization was judged by the change of color (white to orange) characteristic of celastrol.

Following the incubation, preincubated lysate portions were loaded onto the functionalized neutravidin beads, and incubation was continued for 3 h at 4 °C with gentle shaking. A separate experiment was carried out in the same way using functionalized affinity beads prepared as described above. Upon completion of the incubation, samples were spun for 1 min at 25,000 rpm, the supernatant was removed, and the beads were washed eight times with 200 μL of cold lysis buffer containing 1% DMSO. Next, the beads were washed with cold lysis buffer containing 1 mM DTT. Finally, beads were boiled with 5× the loading dye solution. Samples (24 μL with 6 μL of 5× the loading dye) were boiled and loaded on polyacrylamide gel and run at 120 V. Polyacrylamide gels were stained with Coomassie Blue dye and destained with 10% acetic acid solution. The eluted protein bands were excised from the gel, destained, and analyzed as described in Supporting Information.

Detection of *in Vitro* Adducts between Celastrol and Tubulin. Purified porcine tubulin (Cytosekeleton Inc.) was dissolved in PBS buffer at 0.5 mg/mL. Aliquots of the protein solution were incubated at RT for 60 min in the presence of 50 μM of **1**, **24**, or **24** pretreated with DTT or *N*-ethylmaleimide. Protein samples were treated with the same amount of the vehicle solvent (DMSO), which served as control. The incubations were followed by subsequent 1 h incubation with **24** depending on experiment. Equal volumes from each experiment were run on SDS-PAGE followed by the Western blot protocol as described above.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Chemical procedures and spectral data (NMR, HRMS, HPLC) for synthesized compounds; RNA isolation and PCR data; prediction of pharmacokinetic properties of biotinylated derivatives **21**−**24** and mass spectral analysis of isolated protein bands. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We are grateful to the National Institute of Neurological Disorders and Stroke (grant NS047331, jointly to R.I.M. and R.B.S.) for financial support of this research. We thank R. Wolf for a generous gift of MCF AREc32 cells. Proteomics and informatics services were provided by the CBC-UIC Research Resources Center Mass spectrometry, the Metabolomics and Proteomics Facility, which was established in part by a grant from The Searle Funds at the Chicago Community Trust to the Chicago Biomedical Consortium. Mass spectral analyses were performed by A. Schilling. The authors are grateful to S. Fernandez Dunne of Northwestern University's High Throughput Analysis Laboratory (HTA) for help with screening of compounds, R. Mishra in the Center for Molecular Innovation and Drug Discovery (CMIDD) of Northwestern University for permeability calculations, and B. Calamini and P. Trippier for helpful suggestions.

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