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Celastrol binds to its target protein *via* specific noncovalent interactions and reversible covalent bonds†‡

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Celastrol is one of the most studied natural products. Our studies show for the first time that celastrol can bind to its target protein *via* specific noncovalent interactions that position celastrol next to the thiol group of the reactive cysteine for reversible covalent bond formation. Such specific noncovalent interactions confer celastrol binding specificity and demonstrate the feasibility of improving the efficacy and selectivity of celastrol for therapeutic applications.

Tripterygium wilfordii plant, the "Thunder God Vine" (*lei gong teng*), has been used traditionally in Chinese medicine for the treatment of rheumatoid arthritis, lupus and other autoimmune diseases.[1] Celastrol, viewed as one of the most promising bioactive molecules of the "Thunder God Vine", has drawn much attention. An increasing number of studies have revealed the potential of celastrol in treating diverse diseases and cancers due to its potent anti-inflammatory effect.[2–4] Multiple pathways have been proposed to be regulated by celastrol, however, the direct cellular targets for celastrol remain elusive.

Nur77 (also called TR3, NGFIB, or NR4A1), a unique member of the nuclear receptor superfamily, acts as an immediate early or stress response gene.[5,6] Nur77 has emerged as a critical regulator for the onset and progression of cancer and metabolic and inflammatory diseases.[5,7–12] The potent anti-inflammatory role of Nur77 in inflammatory diseases and cancer has received particular attention. An aberrant expression of Nur77 is observed in inflamed human synovial tissues, cancer cells, psoriasis, atherosclerotic lesions and multiple sclerosis.[13] Thus, Nur77 represents a promising target for anti-inflammatory therapy. We recently identified Nur77 as a direct cellular protein target of celastrol. We found that celastrol was a potent binder of Nur77 with a K_d of 0.29 μM and showed that Nur77 was required for the anti-inflammatory effects of celastrol *in vitro* and in inflammatory animal models. Celastrol binding to the ligand-binding domain (LBD) of Nur77 induces Nur77 mitochondrial targeting, resulting not only in the suppression of inflammation but also the induction of mitochondrial autophagy.[14] Because celastrol contains an electrophilic quinone methide moiety (Fig. 1A, red), a known Michael acceptor, it is widely speculated and assumed that celastrol acts through a covalent binding mechanism in which an adduct is formed between celastrol and specific cysteines on the target proteins.[15–17] However, few studies have conclusively demonstrated so. Furthermore, detailed characterization of how celastrol could bind to the target protein has not been reported. Given the importance of celastrol and Nur77, we set out to study the binding mechanism of celastrol action.

We first used LC-MS to investigate if celastrol bound covalently to Nur77-LBD. Fig. 1B shows that in the absence of celastrol the Nur77-LBD peak corresponded to a 29 672 Da molecule, but in the presence of celastrol, the Nur77-LBD/celastrol solution displayed a second peak at 30 122 Da, corresponding to the Nur77-LBD/celastrol complex with an increase in MW of 450 Da compared to Nur77-LBD. This observed increase in MW is equal to the expected MW of celastrol (Fig. 1B and Fig. S1A, ESI‡). These results confirm that celastrol covalently binds to Nur77. We then asked if the covalent bond formation was a result of Michael adduction between cysteines of Nur77-LBD and celastrol. There are 6 cysteines in Nur77-LBD: C465, C475, C505, C534, C551 and C566. The crystal structures of Nur77-LBD show that C475, C505 and C534 are buried (Fig. S2, ESI‡), and C465, C551 and C566 are solvent exposed and accessible for modification with celastrol. However, not all spatially accessible cysteines have the potential to act as nucleophilic thiols. To identify the cysteine(s) responsible for adduct formation, 3 individual mutations, C465A, C551A and C566A, were generated for LC-MS analyses. The results showed that mutating C551 to alanine abolished celastrol covalent modification (Fig. 1C), and C465A and C566A had the same LC-MS spectra

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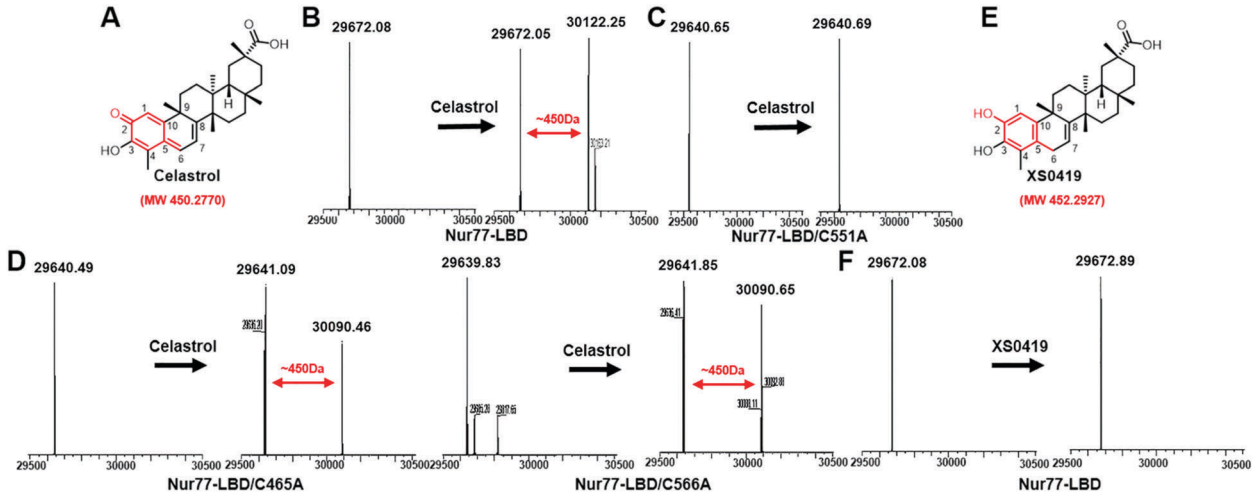

Fig. 1 C551 is the cysteine responsible for the adduct formation between Nur77-LBD and celastrol. (A) Structure of celastrol. (B) The deconvoluted mass spectra before and after celastrol binding. (C) The deconvoluted mass spectra of mutant C551A in the absence and presence of celastrol. (D) The deconvoluted mass spectra of mutants C465A and C566A, respectively, in the absence and presence of celastrol. (E) Structure of XS0419. (F) The deconvoluted mass spectra of Nur77 in the absence and presence of XS0419. Supporting LC/MS spectra are presented in the ESI.‡

distribution as the wild type protein in the presence of celastrol (Fig. 1D). Mutating the cysteine to alanine did not alter the overall structure of Nur77-LBD as shown by circular dichroism (CD) spectra and confirmed by the mutants' ability to dimerize with RXR as the wild-type Nur77 (Fig. S3, ESI‡). Taken together, these results identify C551 as the residue covalently interacting with celastrol.

Celastrol's susceptibility to reduction has been studied and it has been demonstrated that among the 3 electrophilic carbons in the quinone methide motif of celastrol, C-6 is the most reactive for nucleophilic addition with a remarkable stereospecificity.[18] To confirm whether the nucleophilic addition occurs at C-6 of celastrol, we synthesized XS0419, where the C-6 carbon is reduced and is not capable of nucleophilic addition (Fig. 1E). Fig. 1F shows that the molecular weight for Nur77 did not change in the presence of XS0419 (Fig. 1F and Fig. S1B, ESI‡), indicating the involvement of C-6 in adduct formation.

We then asked whether celastrol was an irreversible or reversible electrophile. To answer this question, we used a UV-visible spectroscopy method originally developed for the covalent reversibility studies of cyanoacrylamides as Michael acceptors.[19] We first investigated the nature of the covalent bond resulting from the adduct formation between celastrol and glutathione (GSH), a weak nucleophilic thiol. The visible absorption spectrum (with a maximum visible spectrum at 450 nm) was used to monitor the reactivity of celastrol. When celastrol solution was treated with GSH, the celastrol absorption band was reduced, indicating the occurrence of a reaction between celastrol and GSH (Fig. S4A, ESI‡). When the reaction mixture was diluted, we observed an increase in the absorption spectra at 450 nm, consistent with a reversible reaction (Fig. S4B, ESI‡). Fitting titration data generated by treating a celastrol solution with increasing concentration of GSH yielded an apparent K_d of 86 μM (Fig. S4C, ESI‡). Consistently, the adduct reaction of celastrol with the reducing agent beta-mercaptoethanol also demonstrated reversibility (Fig. S4D–F and S5, ESI‡). Satisfied that this methodology demonstrates the covalent reversibility for celastrol-cysteine modification, we applied the same method to the Nur77/celastrol system. Fig. 2A shows that the absorption peak of celastrol disappeared when it was incubated with Nur77-LBD and its absorption peak reappeared when the Nur77-LBD/celastrol mixture was denatured with SDS (Fig. 2A). These data suggest that the covalent bond between Nur77-LBD and celastrol is reversible. Furthermore, analysis of the Nur77-LBD/celastrol

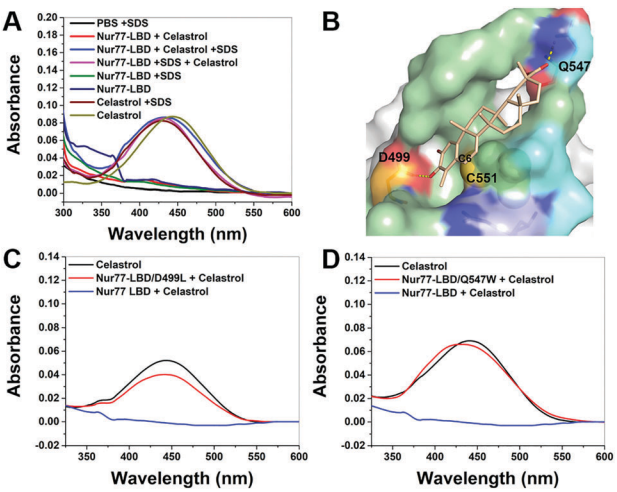

Fig. 2 The covalent and noncovalent interactions between celastrol and Nur77. (A) UV-visible spectra showing that the covalent addition of celastrol to Nur77 is reversible. (B) Model of celastrol binding to Nur77: the protein is displayed as a surface representation and celastrol is shown as wheat sticks. The contributing hydrophobic residues are colored in pale green. The polar residues Q547 and D499 are colored in blue and in orange, respectively. Docked celastrol displays a good shape complementary with Nur77 and places the C-6 atom of celastrol close to C551. (C and D) UV-visible spectra of Nur77-LBD/D499L and Nur77-LBD/Q547W mutants in the presence and absence of celastrol.

complex trypsin digestion using Orbitrap mass spectrometry showed that the derived peptide $_{537}$EHVAAVAGEPQPASCLSR$_{554}$ was the only peptide labelled by celastrol. This peptide contains C551, therefore providing independent evidence that covalent modification occurs at this cysteine (Fig. S6A–C, ESI‡).

Reversible covalent ligands require specific noncovalent interactions with the target protein at a region near the targeted nucleophilic residue.[20] The fact that celastrol acts as a reversible covalent ligand to Nur77 prompted us to further explore if celastrol binds to Nur77 in close proximity to C551 by using molecular modeling and the mutagenesis approach. The crystal structure of Nur77-LBD (PDB code: 4KZI) reveals that C551 is located at the end of a disordered loop, $_{541}$AVAGEPQPAS$_{550}$, and at the start of helix 10. Given our results, the C6 atom of celastrol should be in close proximity to C551 and the interaction between celastrol and Nur77 would require the participation of this disordered loop. The predicted binding mode for celastrol to Nur77-LBD was developed in two steps: first building the disordered loop using Schrödinger's Prime,[21–23] and then docking celastrol to a region partly composed of the constructed loop using Glide.[24–26] The binding mode (Fig. 2B) from the docking studies showed that celastrol fits well into this region with the C6 atom positioned next to the –SH group of C551: the polar groups, hydroxyl and carboxyl at the opposite ends of the celastrol molecule forming H-bonds with D499 and Q547, respectively, and the central hydrophobic portion of the celastrol molecule making extensive van der Waals interactions with the protein involving residues L496, L497, V498, A502, V539, A540, V542, A543, A549 and L552 (Fig. 2B). To validate this model, Nur77-LBD/D499L and Nur77-LBD/Q547W mutants were made, respectively, and tested for their binding to celastrol.

The involvement of D499 and Q547 in the noncovalent binding was first confirmed by binding affinity studies. In the absence of the covalent bond, celastrol bound to Nur77/C551A-LBD with a K_d of 1.17 μM, reflecting a noncovalent binding between Nur77 and celastrol (Fig. S7B, ESI‡). Mutants D449L and Q547W, respectively, displayed weaker binding to celastrol (Fig. S7D and E, ESI‡), indicating the contribution of D499 and Q547 to the binding of celastrol. UV-visible absorption studies showed that the absorption peak of celastrol was consistent with the unbound compound spectrum when incubated with either mutant Nur77-LBD/D499L (Fig. 2C) or Nur77-LBD/Q547W (Fig. 2D). These results indicate that both D499 and Q547 residues contribute to the noncovalent binding of celastrol, supporting our celastrol binding model. The observation that unfolding Nur77 releases bound celastrol is also consistent with our binding model because the stability of the Nur77/celastrol complex requires the intact folded structure of Nur77 and noncovalent interactions are required to bring celastrol close to nucleophile C551.

To explore the biological relevance of the identified binding mechanism of celastrol, we studied the roles of C551, D499 and Q547 in the Nur77-mediated activities of celastrol. Celastrol acts as an anti-inflammation agent by binding to Nur77 to prime inflamed mitochondria for autophagy through inducing Nur77 interactions with tumor necrosis factor receptor-associated factor 2 (TRAF2) and the autophagic adaptor p62/SQSTM1.[14] Co-IP assay showed that Nur77/C551A, but not Nur77/C566A, markedly reduced its interaction with TRAF2 (Fig. 3A) and p62/SQSTM1 (Fig. 3B). This impact of mutation on celastrol's function demonstrates the importance of the covalent bond in celastrol's potency and is consistent with the observed change in the binding affinity between celastrol and Nur77/C551A, and between celastrol and Nur77/C566A (Fig. S7B and C, ESI‡). The role of the covalent bond in celastrol's potency is also reflected in the biological function of XS0419 and the binding affinity of XS0419 to Nur77 (Fig. S8A–D, ESI‡). Substitution of D499 with Leu (Nur77/D499L) or Q547 with Trp (Nur77/Q547W) also reduced the ability of Nur77 to interact with p62/SQSTM1, while their simultaneous mutation (Nur77/D499L/Q547W) completely abolished the interaction (Fig. 3C), which again is consistent with the change in the binding affinity of celastrol to the wild type and mutant Nur77. Therefore, these mutagenesis studies confirm that celastrol binds to C551 in the Nur77 protein and the importance of the noncovalent interactions between celastrol and the protein.

$$E + \mathrm{I} \underset{K_1}{\overset{\text{non-covalent binding}}{\rightleftharpoons}} E\bullet\mathrm{I} \underset{K_2}{\overset{\text{covalent binding}}{\rightleftharpoons}} E-\mathrm{I} \qquad (1)$$

$$K_d = \frac{[E\bullet\mathrm{I}] + [E-\mathrm{I}]}{[E][\mathrm{I}]} = \frac{1+K_2}{K_1K_2} \qquad (2)$$

A general mechanism for the binding of a reversible covalent inhibitor consists of two steps as shown in eqn (1).[27] K_1 and K_2, respectively, are the equilibrium dissociation constants of step 1 and step 2 and the overall K_d for the reversible covalent inhibitor can be defined by K_1 and K_2 using eqn (2).[27] In the case of celastrol binding to Nur77, K_d and K_1 were determined

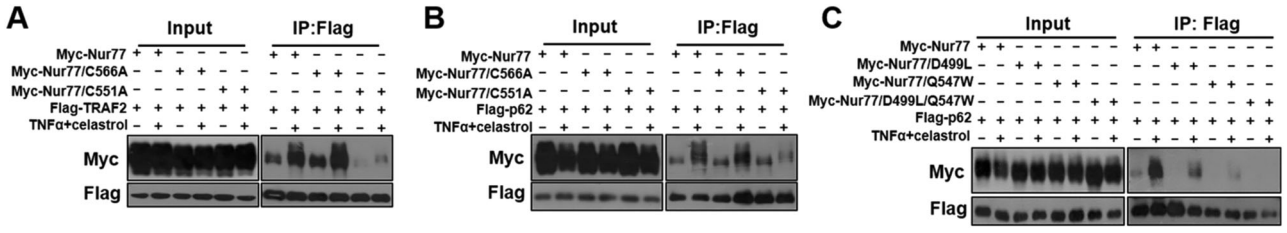

Fig. 3 Biological studies support the identified binding nature of celastrol. (A and B) Co-immunoprecipitation assays revealed the importance of C551 in mediating the effect of celastrol on inducing Nur77 interactions with TRAF2 and p62/SQSTM1. (C) Co-immunoprecipitation assay revealed that both D499 and Q547 played key roles in interacting with celastrol.

to be 0.29 μM and 1.17 μM (Fig. S7B, ESI‡) respectively, therefore, K_2 is equal to 0.23 μM according to eqn (2). This derived result showed that the reversible covalent bond formation increased the binding affinity and rendered celastrol more potent.

In conclusion, using diverse approaches, we have demonstrated and confirmed that celastrol binds to the target protein Nur77 *via* a Michael addition reaction at C551 in Nur77. Importantly, the formation of this covalent bond is reversible and requires specific noncovalent interactions with Nur77 to position celastrol in close proximity to the –SH group of C551. The results elucidate an unprecedented mechanism on how celastrol can selectively bind to an intracellular target to exert specificity, offering new strategies to improve the efficacy and selectivity of celastrol for therapeutic applications *via* modifying its electrophilicity and/or noncovalent interaction properties.

Conflicts of interest

There are no conflicts to declare.

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