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# Synthesis and preliminary evaluation of neuroprotection of celastrol analogues in PC12 cells

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ABSTRACT

A series of celastrol analogues were synthesized, and their neuroprotective effect against *t*-BHP-induced cytotoxicity was investigated in neuronal PC12 cells. Their effects on Hsp70 protein expression were quantified by Western blot analysis. The study found that compound **CL12** is more effective than the parent celastrol against *t*-BHP-induced cytotoxicity. **CL12** up-regulates Hsp70 protein expression dose-dependently. These results suggest that **CL12** is a potential candidate for the intervention of neurodegenerative diseases.

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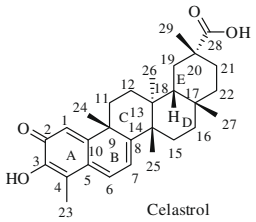
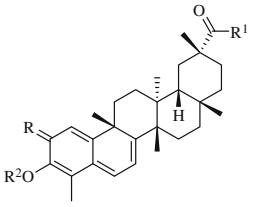
Heat shock proteins (Hsp) are a family of functionally related proteins, and their expressions are responsive to elevated temperatures and other stresses.[1] Hsp70 is a member of the Hsp family of proteins, and helps to protect cells from stresses including heat and oxidants.[2,3] Increasing evidence suggests that Hsp70 may play an important role in a number of neurodegenerative diseases and its up-regulation is neuroprotective.[4] Thus, extensive efforts are undertaken to discover agents that can protect cells or tissues through the up-regulation of Hsp70 proteins.

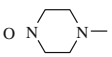
A large-scale screen has identified the natural product celastrol (Fig. 1), a triterpenoid compound isolated from the plant family *Celastraceae*, as a potential neuroprotective agent that may be beneficial in treating neurological manifestations including Huntington's disease (HD) and amyotrophic lateral sclerosis (ALS).[5,6] Recently, it is reported that celastrol is effective in attenuating the loss of dopaminergic neurons (DNs) and reduction of brain dopamine levels in a *Drosophila* DJ-IA model of Parkinson's disease (PD).[7] It could improve memory and learning in psychomotor activity tests (PMA) in a rat model of Alzheimer's (AD).[8] Celastrol reduced MPTP-induced depletion of striatal dopamine levels and decreased the striatal lesion volume induced by 3-nitropropionic acid, a neurotoxin used to model HD in rats by inducing Hsp70.[9] Thus, celastrol is a potential useful weaponry for combating various delineating neurodegenerative diseases.[10]

Celastrol at nanomolar concentrations (20–200 nM) inhibits expression of inflammatory genes, including IL-1β, TNF-α, prostaglandin E2 and inducible NOS (iNOS)[8,11] with no obvious cytotoxic effect.[12] However, celastrol at high concentrations ($IC_{50}$: 2.5 μM) induce apoptosis and exhibit antitumour activity.[13] For these reasons, it is imperative to modify the structure of celastrol to make it more neuroprotective but less toxic to cells.

Although celastrol has important pharmacological activities, its structure–activity relationship (SAR) has not been well



| | R | $R^1$ | $R^2$ |
|---|---|---|---|
| **CL1** | O | $OCH_3$ | H |
| **CL2** | O | $OCH_2CH_3$ | H |
| **CL3** | O | $OCH_2CH_2CH_3$ | H |
| **CL4** | O | $OCH_2CH_2CH_2CH_3$ | H |
| **CL5** | O | $OCH_2C_6H_5$ | H |
| **CL6** | O |  | H |
| **CL7** | O | OH | $OCH_3$ |
| **CL8** | O | OH | $OCH_2CH_3$ |

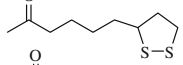
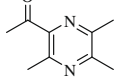
| | R | $R^1$ | $R^2$ |
|---|---|---|---|
| **CL9** | O | OH | $OCCH_2CH_2CH_3$ |
| **CL10** | O | OH | $OCCH(CH_3)CH_3$ |
| **CL11** | O | OH |  |
| **CL12** | O | OH |  |
| **CL13** | NOH | OH | H |

**Figure 1.** Chemical structures of celastrol and related analogues.

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understood. Some analogues like pristimerin and dihydrocelastrol (Fig. 2) are reported to be inducers of the heat shock response and cytoprotective agents against lethal stress in HeLa cells and SH-SY5Y neuronal cells.[14] Abbas et al. modified the carboxylic acid group of celastrol to make amides or esters with the quinone methide functional group intact.[15] It was found that the celastrol's acidic carboxylate group was not required for its apoptotic activity, but its quinone methide moiety was crucial for its cytotoxic activity in several cancer cell lines.[15]

Based on current understanding of celastrol's SAR, we designed and synthesized new celastrol analogues to identify compounds that possess an improved pharmacologic profile and low toxicity compared with the parent compound. We synthesized ester and amide analogues by the reactions at the C-28 carboxylic group to make compounds **CL1–6** following a reported procedure.[15] Abbas et al. synthesized compounds **CL1–6** and studied their antitumor activity in vitro, but the authors did not report the compounds' neuroprotective effect.[15]

We then acylated the C-3 hydroxyl group to produce esters **CL7–12** (Scheme 1). Celastrol was reacted with the appropriate acid chloride or acid anhydride under basic conditions to afford ethers **CL7–10**. Compounds **CL11** and **CL12** were obtained by coupling celastrol with either lipoic acid or tetramethylpyrazine catalyzed by DCC, DMAP. Alpha-lipoic acid (LA) is a powerful antioxidant, which directly terminates free radicals, chelates transition metal ions (iron and copper), and increases cytosolic glutathione and vitamin C levels.[16–18] These diverse actions suggest that LA acts by multiple mechanisms both physiologically and pharmacologically.[17] Because of the powerful antioxidative effect and other mechanisms of action, LA was conjugated to celastrol to make **CL11**. In China, *Ligusticum wallichii* Franchat (Chuan Xiong) and its main active ingredient 2,3,5,6-tetramethylpyrazine (TMP) have been used for treatment of neurological diseases for many years.[19] Although the exact mechanism(s) of action has/have not been completely understood, a variety of mechanisms has been attributed to TMP's beneficial effects. TMP was found to inhibit platelet aggregation,[20,21] lyse blood clots,[21] block calcium entry[22,23] and scavenge reactive oxygen species (ROS).[24,25] TMP is coupled to celastrol (**CL12**) to provide an additional neuroprotective effects by mechanisms other than increasing Hsp70 expression.

Previous studies suggest that celastrol's quinone methide moiety is not responsible for its chemical chaperone activity, but is crucial for its cytotoxic activity in cancer cell lines.[14,15] In the studies conducted by Westerheide et al. dihydrocelastrol was found to be active as a heat shock promoter.[14] However, dihydrocelastrol was inactive in the cellular assays reported by Abbas et al.[15] We therefore synthesized compound **CL13** (Scheme 2) to find if the intact quinone methide moiety is required for celastrol's neuroprotective effect.

In order to assess the neuroprotective activity of the new compounds, we established an in vitro model of *tert*-butylhydroperoxide (*t*-BHP)-induced oxidative stress cell damage. BHP is an organic hydroperoxidant which has been suggested a useful in vitro model for investigation of the cytoprotective activity.[26,27] In PC12 cells, *t*-BHP decreased cell viability dose-dependently. Approximately 50% of cells were damaged after 24 h exposure to 200 μM *t*-BHP. We found that most of the new compounds exhibited moderate to good protection against *t*-BHP-induced cell damage (Fig. 3).

Celastrol exhibited potent antioxidative effect at concentrations from 0.1–0.4 μM, with the best effect at 0.1 μM, improving cell viability to 65 ± 3.2%. However, celastrol's protective effect appeared to decrease as the drug concentration increased, and in fact it offered no protection at concentrations higher than 0.8 μM. In the absence of *t*-BHP, we found that the $IC_{50}$ value of celastrol in PC12 cells was 3.15 ± 0.43 μM (data not show), and only about 67% cells survived at 1.6 μM. These cytotoxicity data were in agreement with what have been reported by others for celastrol in PC12 cells.[28]

Among compounds coupled from the C-28 carboxyl group, **CL1–3** didn't show any protective effects against *t*-BHP-induced cytotoxicity up to 1.6 μM. Celastrol butyl (**CL4**) and benzyl (**CL5**) esters was active at certain concentrations. The amide (**CL6**) displayed strong protective effect at 0.8–1.6 μM, and this effect was concentration-dependent. At 1.6 μM, its protective effect was equal to or even better than that of celastrol (Cel). It is interesting to note that **CL1** and **CL5** had strong antitumor activity with $EC_{50}$ values of <0.1 and 0.3 μM, respectively, against SW1 cells.[15]

Among compounds coupled from the C-3 hydroxyl group, **CL7**, **CL9** and **CL12** provided good cytoprotection. Notably, **CL12**, the TMP ester, showed the strongest protective effect among all compounds including its parent celastrol. **CL12** protected 76 ± 3.6% cells from damage at 0.4 μM, better than that of celastrol at 65 ± 3.2%. **CL11**, the LA ester, had no protective effect at the concentrations tested. It is apparent that coupling LA to celastrol did not generate any synergistic effect for the new compound. LA's protective effects are usually seen at much higher concentrations. For example, we have found that LA was protective to RIN-m cells from $H_2O_2$ damage at concentrations higher than 1.0 μM.[29]

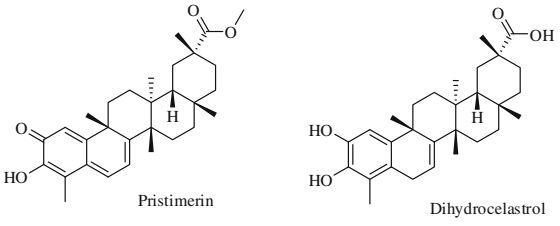
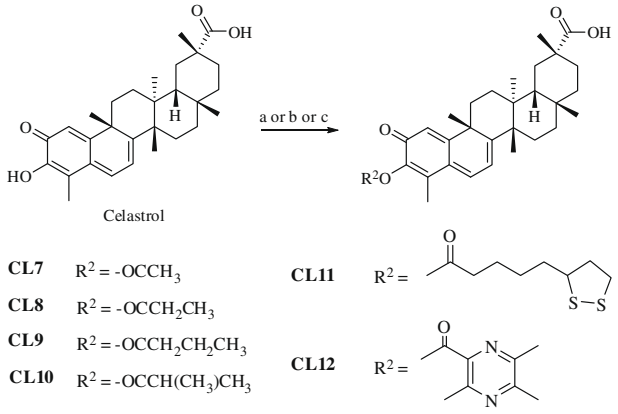
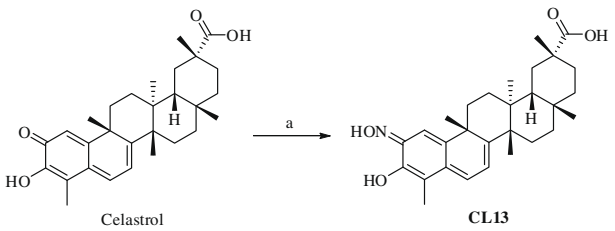



**Figure 2.** Chemical structures of pristimerin and dihydrocelastrol.




**Scheme 1.** Reagents and conditions: (a) **CL7**: acetyl chloride, TEA, $CH_2Cl_2$, 0 °C; (b) **CL8–CL10**: appropriate anhydride, TEA, DMAP, $CH_2Cl_2$, 0 °C; (c) **CL11** and **CL12**: lipoic acid or 3,5,6-tetramethylpyrazine-2-carboxylic acid, TMP acid, DCC, DMAP, DMF, room temperature.




**Scheme 2.** Reagents and conditions: (a) **CL13**: hydroxylamine hydrochloride, pyridine, 70 °C.

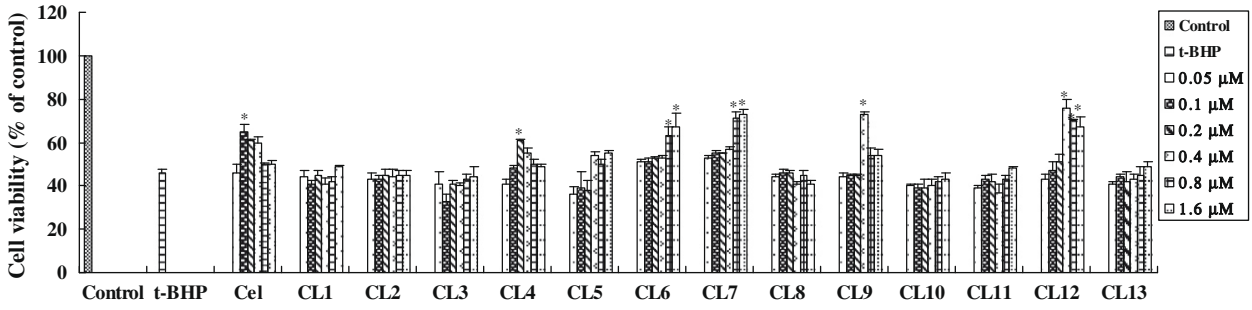



**Figure 3.** Protective effects against *t*-BHP-induced damages in PC12 cells. Cells were pretreated with various concentrations of compounds for 1 h, and then were treated with *t*-BHP for 24 h. The results are expressed as the percentage of that of the untreated cells. Data were processed statistically by a single-tail Student's *t*-test. $^{*}P$ <0.05 compared to *t*-BHP group.

**CL13**, lack of a quinone methide moiety, didn't exhibit any cytoprotective action even at 1.6 μM. It looks like that the quinone methide moiety is essential for cytoprotective activity.

To understand the neuroprotective mechanism of action, we determined the expression of Hsp70 proteins in PC12 cells. Cells treated with celastrol and **CL12** were subjected to Western blot analysis, and Hsp70 expression was presented in Figure 4.

In our experiments, treatment of cells with *t*-BHP (200 μM) alone significantly suppressed Hsp70 expression by as much as 60%. Pretreatment of cells with both celastrol and **CL12** dose-dependently increased Hsp70 protein levels. Interestingly, celastrol at 0.1 μM, at which concentration it provided the most protection to cells from damage by *t*-BHP (Fig. 3), did not increase Hsp70 expression. Celastrol significantly increased Hsp70 expression at 0.4 and 1.6 μM. **CL12** increased Hsp70 expression at a concentration as low as 0.1 μM, but its neuroprotective effect was not obvious until its concentration reached 0.4 μM. These results suggest that the protective effect and the Hsp70 protein expression were not well-correlated for both celastrol and **CL12**. It remains to be uncovered if other mechanisms of action were in play for their neuroprotective effects.

In conclusion, we synthesized a series of celastrol analogues, evaluated their cytoprotective effect against *t*-BHP-induced cell damage and determined Hsp70 protein expression induced by celastrol and **CL12**. The present findings demonstrate that **CL6**, **CL7** and **CL12** blocked *t*-BHP-induced PC12 cell damage. Importantly, the new analogue, **CL12**, was more effective in protecting cells from *t*-BHP-induced cell damage than its parent celastrol. We found that both cealstrol and **CL12** up-regulated the chaperone protein Hsp70 at the tested concentrations. These results suggest that Hsp70 up-regulation probably helps strengthen the cellular capability to resist oxidative stress, and eventually promote cell survival. Further studies are needed to understand the mechanism(s) of action, and help for design and synthesis of more effective agents to combat various stress-induced neuro-injury.

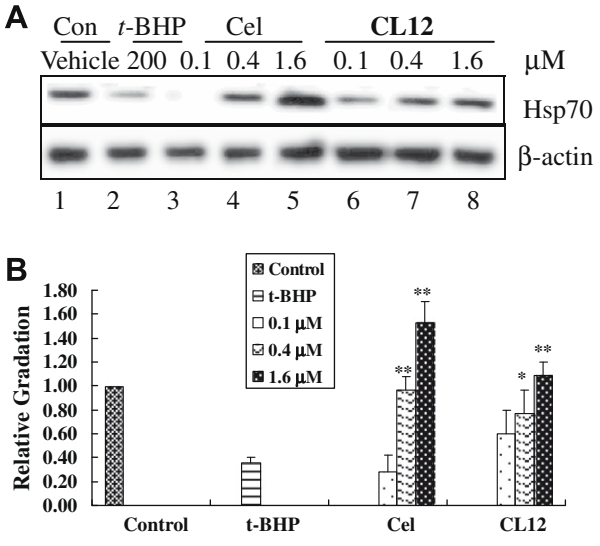



**Figure 4.** Effects of celastrol and **CL12** on Hsp70 expression in PC12 cells. Cells were treated with celastrol and **CL12** for 1 h, and were then exposed to *t*-BHP (200 μM) for 24 h. (A) Protein expression was assessed by Western blot analysis. Control (lane 1): no *t*-BHP; *t*-BHP (lane 2) alone; Celastrol (lanes 3–5): celastrol and *t*-BHP; **CL12** (lanes 6–8): **CL12** and *t*-BHP. (B) $^{*}P$ <0.05 compared to *t*-BHP group, $^{**}P$ <0.01 compared to the control group.

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.05.066.

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