Small Molecule Induction of the Heat Shock Response: Cytoprotective Effects and Potential Clinical Applications of Celastrol

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Abstract

The cellular response to heat and other stress has been widely studied in recent years, due to its ability to protect the cell from these stresses and aid in the treatment of a number of disorders, such as arthritis, Alzheimer's disease, and cancer. "Heat shock proteins" (Hsp) are the driving force behind these properties of the heat shock response, functioning as protein chaperonins and providing general protection from conformational damage of any sort. Due to these broad protective properties, the ability to easily induce the transcription of Hsps on demand with a small, readily acquirable drug would be extremely useful. This study demonstrates that the compound celastrol is capable of not only inducing *hsp70* transcription with magnitude greater than its native induction from heat shock, both *in vivo* and *in vitro*, but also of providing cellular protection against otherwise lethal heat shock. These results suggest that celastrol offers great promise as a clinically viable activator of the heat shock response and a potent treatment of a variety of medical conditions.

Introduction

Living cells must be able to react defensively to outside stress, or they will die. In recent years, the intensification of efforts in cancer research has led to a surge of interest in the small family of molecules known as heat shock proteins (Hsp) for their cytoprotective effects and postulated promotion of wound healing. Hsps are thought to play a role as protein chaperones, protecting newly formed proteins while they fold into their proper conformation and re-folding mis-folded proteins or shepherding them to larger chaperonin molecules. The clinical applications of these proteins appear very promising and are actively under research.

The transcriptional activation of the 70 kDa heat shock protein gene *hsp70* (Hsp70 for the protein) has been extensively investigated in cancer therapy [1, 2], burn therapy [3, 4], and laser ablation [5]. Extremely high levels of Hsp70 transcriptional activity have been reported in response to an assortment of stresses: heat shocks of varying magnitudes and durations [1-5], glucose deprivation [6], inflammation [7], and injury [8]. Indeed, Hsp70 appears to be more a 'general stress indicator' than a hallmark of any specific stress.

Several methods have been developed to monitor Hsp expression. Of these, the simplest and least invasive uses reporter genes to directly detect *hsp* transcription as it occurs in real time. Reporter genes are used to study the activity of promoter regions of specific target genes by introducing into the cell or organism a gene encoding a readily detectable protein, driven by the promoter of choice. This extra gene construct does not replace the normal gene but is added to the genome elsewhere. The detection of the reporter protein indicates the active transcription of the gene being studied. Reporter genes are introduced into both cells and entire animals. This study utilized stably transfected cell lines and transgenic mice expressing an Hsp70-luciferase

reporter gene, such that *hsp70* transcription was coupled to proportional transcription of a modified firefly luciferase. By exposing the cells to luciferin, a substrate that reacts with luciferase to produce light, Hsp70 transcription was monitored directly, and extremely accurately. The accuracy of this reporting system has been investigated multiple times and is generally concluded to be indicative of the level of coupled gene expression with great precision [5].

Hsp70 has also been demonstrated to protect against many of the stresses that induce it. Protective effects have been shown in the literature against heat [9], inflammation [7], and various other sources. Further, Kovalchin et al. have demonstrated that direct injection of Hsp70 into wound sites can accelerate wound healing by up to 60%, an effect they attribute at least in part to the action of macrophages rather than intra-cellular causes [10]. This research is very promising and seems to offer the potential for a number of clinical applications, but direct injection of Hsp offers its own set of problems despite its promise. First, it is difficult to mimic the full range of the heat shock response with injection, since this response includes dozens of proteins with differing levels of induction and cytoprotective response. This means that direct introduction of Hsp70 may be giving up a large portion of its potential protection. Second, Hsps are quickly degraded *in vivo* and thus direct injection of protein may lead to a relatively short-lived response, requiring repeated treatment. Third, a relatively large number of Hsp proteins are required for *in vivo* use, which may be difficult to prepare, harvest, or synthesize. Therefore, stable compounds that induce Hsp expression and/or other stress responses would be of great advantage in cellular protection.

Celastrol (Figure 1), a small molecule extracted from the Chinese "Thunder of God" vine (*Tripterygium wilfordii*), may present a solution. Used in Chinese herbal medicine for centuries

as a remedy for inflammation, celastrol is thought to induce Hsps in a manner very similar to heat shock [11]. As a small molecule that activates the transcriptional pathway itself, celastrol offers the possibility of cheap, easy introduction and relatively long-term effects in any part of the body, important traits for clinical wound-healing applications.

Evaluations of celastrol applications have been performed on treatments for Alzheimer's disease [12] and tumor growth [15]. These studies have met with some success, and have concluded that celastrol possesses anti-oxidant and proteasome-inhibition properties in addition or as a result of Hsp70 induction. However, potential applications in the pre-warming of wounds and protection against surgical thermal damage have not been investigated.

This study aimed to investigate the cytoprotective influences of celastrol *in vitro* and *in vivo* and its potential clinical applications. It was predicted that that small quantities of celastrol would induce cellular responses that mimic the Hsp-transcription patterns of heat shock and that cells pretreated with celastrol would effectively resist subsequent stress. Such findings should encourage further investigation into celastrol as an accelerator of wound healing and other related applications.

Materials and Methods

Cell Culture

NIH3T3 fibroblast cell lines with a stably integrated Hsp70-luc reporter were cultured at 37° C and 5% CO_2 in Dulbecco's Modified Eagle Medium (GIBCO, Invitrogen Ltd., Paisley, UK) containing 10% fetal bovine serum (PAA Laboratories Gmbh, Pasching, Austria), penicillin (100 U/mL), streptomycin (100 μg/mL), and glutamine (2 mM). Heat Shock was induced by incubating 24-well plates with approximately 50,000 cells per well and 1 ml of media in a

separate incubator pre-warmed to the given temperature for the given time. Celastrol shock was induced by aspirating the media from each well and replacing it with celastrol solution, diluted to the given concentration. The cells were allowed to incubate in this solution for one hour, and then the celastrol was removed and replaced with normal media. Control samples were given an equivalent dilution of DMSO, which was used as a solvent for the celastrol solution, to isolate celastrol's effects. However, experiments revealed that Hsp70 induction from exposure to DMSO alone (data not shown) was miniscule if present at all. Time measurements were taken using the end of the shock in question as a starting point.

In vivo and In vitro Bioluminescence Imaging

Hsp70 expression was measured indirectly by measuring photon flux using an IVIS 50 imaging system (Xenogen Corporation, Alameda, CA). For each time point, 10 µl of luciferin was added to each well in a single column, and the entire plate was imaged by the IVIS for between 1 and 5 minutes depending on the expected intensity of the photon flux. These images were analyzed using Living Image® software (Xenogen Corporation), and combined to produce composite images such as that in Figure 2 and obtain quantitative measurements of well photon flux.

Primary Heat Shock

Two 24-well plates were seeded with 50,000 cells in each well. Approximately 24 hours later both plates were placed in an incubator pre-warmed to 45° C for 20 minutes, then removed and immediately returned to an incubator with standard conditions. Both plates were imaged at 1, 2, 3, 5, and 7 hours post-heat shock.

Primary Celastrol Shock

Four 24-well plates were seeded with 50,000 cells in each well. Approximately 24 hours later the cell media was aspirated from all wells. Each plate then received 1 ml per well of one of the following celastrol concentrations: 1 μM, 5 μM, 7 μM, 10 μM. The cells were allowed to incubate for 1 hour, then the celastrol was replaced with media and the cells returned to standard conditions. The plates were imaged at 3, 4, 6, 8, and 24 hours after the end of celastrol shock.

In vivo Celastrol Shock

A line of transgenic mice that stably express the Hsp70-luc reporter allowed us to test the *in vivo* effects of celastrol treatment. One mouse received a sham injection of phosphate buffered solution (PBS). Two other mice were injected with 3 mg/kg of body mass celastrol solution. A final mouse received 6 mg/kg of body mass. The mice each received subsequent injections of 100 μl of 30 mg/ml luciferin 6, 24, and 48 hours after celastrol treatment, and were immediately imaged with an IVIS100 camera. The mice were not euthanized for this project.

Heat and Celastrol Pretreatment

6 24-well plates were seeded with 2.0×10^5 cells/well. After a period of 3 hours to allow for cell adhesion, one plate was heat shocked at 45° C for 20 minutes, one placed in a separate 37° C incubator for 20 minutes, and one celastrol-shocked for 1 hour with 5 μM celastrol. All plates were then allowed to incubate under standard conditions overnight. Approximately 14 hours later (well after peak expected Hsp70 expression), each of the untouched plates underwent one of the same treatments: 37° C heat shock, 45° C heat shock, or 5 μM celastrol shock. 4 hours later (18 hours after first set of shocks, 4 hours after second set of shocks), all plates were subjected to a 50° C heat shock for 20 minutes. The plates were monitored for 8 hours post-shock and imaged periodically. 8 hours post-shock, the media in 3 sample wells from each plate was aspirated off, and the cells removed with 100 μl of trypsin. Then, the trypsin was inactivated

with media and trypan blue was added in a 1-1 volume ratio. A Nexcelom Cellometer™ was used to perform live-dead assay and cell counting on the resulting solution.

Results

The results of this study indicate that celastrol is capable of inducing Hsp70 transcription at levels even greater than those in its native induction in response to heat shock (Figure 3). A number of celastrol concentrations were tested, and it was determined that maximum induction occurred at exposures to concentrations around 5 μM for one hour. Similarly, it was found that heat shock produced maximum induction around a shock of 42-45° C for 20 minutes. Heat shocks higher than this level (for example, 50° C for 20 minutes), generally result in cell death and reduced Hsp70 transcription due to damage to protein production pathways [5]. Similarly, celastrol concentrations higher than 5 μM exhibit decreased transcription levels and eventual cell death by apoptosis. Celastrol and heat shock appear to have extremely similar curves of Hsp70 transcription activity in this cell line, but maximum celastrol induction appears to be nearly a factor of 2 greater than its heat shock counterpart. However, both treatments appear to induce maximum induction peaks at around 6 hours, although other similar tests (not shown) placed peak induction around 4-5 hours post shock.

Celastrol was administered by intraperitoneal injection to transgenic mice stably expressing the Hsp70-luciferase reporter gene in concentrations of 3 mg/kg of body mass and 6 mg/kg of body mass. Hsp70 expression was monitored over a period of two days after injection with bioluminescence imaging (Figure 4, last time point not shown).

Up-regulated luciferase expression was noted in injected mice, in unexpected locations. In particular, there appear to be strong signals in the spleen at 6 hours. Since macrophages

activated in the peritoneal cavity are known to quickly travel to the spleen, this may suggest increased induction in macrophages and other immune cells, consistent with Kovalchin et al.'s hypothesis of Hsp's role in macrophage-mediated wound healing [10]. Peak expression was noted to be some time after 24 hours (data not shown), significantly later than *in vitro*, due to the slower delivery through the circulatory system. It is clear that celastrol is effective at inducing Hsp70 expression *in vivo* as well as *in vitro*, with a time frame reasonable for clinical treatments. A more localized delivery method may be desirable, however, perhaps achieved through subcutaneous injection directly onto the location of injury.

To test for the cytoprotective effects of celastrol, the Hsp70 induction patterns and cell death ratios of 5 μM celastrol-exposed cells were compared to those of cells shocked at 45° C for 20 minutes. Heat or celastrol shock was administered 4 and 18 hours before secondary heat shock. It was found that pre-shock with celastrol 4 hours before secondary shock resulted in an exponential increase in photon flux (Figure 5), while all other treatments did not produce any expression patterns above the background signal. Trypan blue live/dead analysis reported that approximately 30% of cells pre-treated with celastrol 4 hours survived 6 hours after secondary shock, compared to 10% or less in untreated cells, cells treated the day before, or cells pretreated with 45° C shock. However, the peak photon flux of these 4-hour celastrol pretreated cells appeared to be later than that of normal heat shock – the data indicates that it occurred some time after 8 hours, while standard heat shock expects a peak expression time of 4-6 hours. These protective effects of 4-hour celastrol pretreatment were observed approximately half a dozen times in duplicate experiments.

To test whether this observed protective effect was caused by the presence of Hsp70 within the cell or from some sort of more permanent change in transcriptional mechanisms, both

heat and celastrol pretreatment were examined 18 hours before secondary shock, well after the peak expression. Our previous data indicated that only lingering levels of Hsp70 (or at least *hsp70* transcription) remain 18 hours after treatment. Therefore, Hsp70 transcriptional activity after the second shock might indicate a heightened cellular response indicative of strong, lasting, cytoprotective effects that persist even after the initial Hsp70 proteins have been degraded. However, we observed the opposite result: prior stress reduced Hsp70 expression levels after secondary stress 18 hours later, both for heat and celastrol pretreatments (Figures 6 and 7). This effect was slightly more pronounced in celastrol pretreatment, reducing *hsp70* transcription by over a factor of 2.

Discussion

These studies present celastrol as a potent alternative to other methods of heat shock response manipulation. Celastrol exhibits induction kinetics very similar to those of heat shock, but with a much stronger response, and more effective cytoprotection. The reason for celastrol's greater induction is unclear, but is most likely due to a more direct means of inducing transcriptional activity. Other heat shock response mimickers are known to work through proteosome inhibition or inactivation of chaperones which participate in feedback inhibition of the HSF1 transcriptional response [11], but this study reveals that celastrol functions mainly by direct activation of Hsp70 transcription rather than any of these methods.

Celastrol is a member of a family of compounds known as triterpenoids, which are known to possess anti-inflammatory and anti-tumor properties [11, 12, 13]. Triterpenoids act through inhibition of DNA polymerase α and β and DNA topoisomerase I and II, molecules that catalyze the replication, repair, breaking, and rejoining of DNA. These compounds are essential to cancer growth, and their inhibition is an active ingredient of anti-cancer drugs. This could

account for the anti-tumor properties of celastrol. Triterpenoids also have been implicated in altering signaling faculties, for instance the down-regulation of NF-κB, which has been implicated heavily in the heat shock response [11, 14]. This may explain celastrol's Hsp70 induction.

One interesting result is the observation that peak expression time in response to various stresses can vary widely based on the type of stress and the condition of the cells. While it may be premature to discuss the molecular basis of such a mechanism, it is still worthy of consideration. A simple explanation may be that higher levels of expression correspond to longer durations of expression and later peak times, perhaps due in part to saturation of the proteasomes that would otherwise degrade Hsp70 and luciferase, or saturation of the transcriptional facilities of the cell. Perhaps direct proteasome-inhibition plays some sort of role. However, more focused research is needed to determine a more detailed explanation for this phenomenon.

Another interesting aspect of our experiment lies in the fact that a heat shock or celastrol treatment seems to weaken the cells' Hsp70 induction pathway, leaving the cells more susceptible to stress after the first wave of Hsp proteins have been degraded. Pretreatment with heat or celastrol was unable to protect cells from subsequent lethal shock 18 or more hours later and caused a decreased induction response to non-lethal heat shock after the same time period. This suggests that cells may be unable to respond to repeated stresses due to attenuation of the *hsp70* transcriptional pathway. This result is in apparent contradiction with studies by Kimoto et al. [16] and Merwald et al. [17]; they have both published results demonstrating increased cytoprotection from repeated heat shock, the first against ischemia in rat livers and the second against ultraviolet damage to human epidermal keratinocytes. We postulate that this apparent

conflict stems from a disparity in the length of time elapsed between treatments between our results and theirs – they most likely evaluated subsequent shocks occurring before the Hsp induction levels had returned to their baseline state. Kimoto et al. subjected rats to a series of heat shocks 2 days apart, and noted that Hsp72 induction levels 48 hours after the final shock were higher in rats subjected to multiple shocks compared to those who had experienced only a single heat shock. However, this is not in contradiction with the current study since Hsp induction levels peak between 24-72 hours after treatment in rats and mice [18]. Therefore, there were significant lingering levels of Hsp72 still in the subjects' bodies, perhaps leading to increased induction via positive feedback. Indeed, their observations may be explained simply by postulating overlapping, non-interacting expression peaks due to multiple heat-shocks: there were most likely still high levels of Hsp72 within the rats' bodies from heat shocks four days previous. The studies of Merwald et al. present a case slightly more similar to this study: they shocked cells at 42° C for 4 hours every 24 hours, and found no decrease in levels of Hsp72 induction or in cellular ability to resist ultraviolet radiation (UVB). They consistently reported Hsp72 expression peaks at 4 hours after heat shock, consistent with our data, so it does not appear that they experienced much larger or longer peaks. However, they evaluated pretreatments with durations of 4 hours, far beyond the range of our study. This may affect the transcriptional response in unpredictable ways. Perhaps, for instance, the cell becomes acclimated to continual stress and continues *hsp70* transcription even after that stress has vanished. Perhaps the stress response becomes so great in magnitude that it takes much longer to dissipate, despite a similar peak time. In any case, the current study consistently demonstrated decreased Hsp70 production in response to stress in cells exposed to previous stress 18 or more hours in advance; the apparent discrepancies with the literature can only be remedied with

further investigation.

Our data also demonstrates a strong protective effect of pretreatment with celastrol and heating if the second shock occurs during the Hsp70 induction of the first. This effect was demonstrated to be significantly stronger than that induced by pre-warming (another treatment currently being researched) both in the magnitude of Hsp70 expression and the ability to protect from lethal heat shock. While we did not test whether this effect could be stronger than direct injection of Hsp70, celastrol possesses many advantages over direct injection, even should the latter prove to produce a stronger induction response. Celastrol, as a stable small molecule rather than a complex and easily degradable protein, is much easier to synthesize, store, and administer. Moreover, celastrol obviously acts much earlier in the Hsp70 production pathway than simple injection of the final product and thus amplifies the initial treatment concentration via an activation cascade. This implies that a smaller amount of the drug will be required to produce the same dose response. Finally, since celastrol activates *hsp70* transcription directly, it seems likely that the duration of response will be greater than simply injecting a given quantity of Hsp70 protein. This allows a more gradual increase of Hsp70 concentration, and also a more gradual decline, leading to a less harsh and longer lasting treatment pattern. Therefore, celastrol seems to hold great potential for many applications in a clinical setting.

While much of the experiment was rigidly controlled, several possible sources of error remain. First, the incubation method of heat shock utilized generally led to slightly uneven heating of our 24-well plates. Since a time course was determined by measuring luciferin expression of separate wells at different times, uneven heating between wells could lead to inconsistent results. This effect was minimized by not using wells on the outsides of the plate (where uneven heating was most often observed) and distributing these outside wells evenly

among time points to preserve comparison. Further, experimentation revealed that the level of expression caused by increased heating of outer wells became negligible compared to the disparity between time points. Future experiments may wish to use a more even heating method, however. Second, since several hours are required for complete cell adhesion, cell-plates were seeded approximately 24 hours in advance of their exposure to heat or celastrol to allow for complete adhesion to the bottom of the plate. It was therefore difficult to accurately control cell number, since small differences in initial cell concentration led to larger ones in concentrations during the experiment. Also, NIH3T3 cells have a doubling time of approximately 20 hours, and therefore some cell growth is expected to have occurred during the course of any given experiment. The data presented is not normalized to cell number, which would partially alleviate this problem. However, it may not be reasonable to expect that cell division preserves the molecular mechanisms of up-regulated Hsp70 transcription. Therefore, strict normalization to cell number may not be the answer; further investigation is necessary.

It remains to be seen whether celastrol exposure can truly be extended to clinical treatment. Some groups have investigated its use in Alzheimer's disease [12] and cancer treatment [15], and, indeed, the Chinese have used it in impure form as an herbal remedy for centuries. However, its application as a wound-healing accelerator or thermal protection measure, perhaps by pre-treating a surgical site before a mechanical or laser-facilitated surgical operation, has not been studied. The viability and efficacy of such a method must be more thoroughly investigated. A simple start would be an *in vivo* extension of our celastrol pre-treatment work. It may be difficult to ascertain a heat shock dosage under which the celastrol protects the animal from heat-induced death outright, but there are certainly other assays to take the place of the simple live/dead count performed *in vitro*. For instance, Hsp70 expression could

be monitored as we have demonstrated, with a higher Hsp70 concentration assumed to imply increased cytoprotection. As a next step, the experimental setup of Kovalchin et al. [10] could be duplicated with regular celastrol treatment substituted for direct Hsp70 injection. A duplication of their wound healing results – in which Hsp70 was shown to accelerate wound-closure rates by up to 60% – would be a powerful demonstration of celastrol as a viable clinical treatment.

Further work must also be done to investigate the cellular processes triggered by celastrol. It is known to stimulate heat shock transcription factor 1 (HSF1) in a manner similar to heat shock [11], but it has also been attributed with broad-scale proteasome-inhibition properties [15]. While the latter is not likely to be solely responsible for the Hsp70 induction and cytoprotective effects we have demonstrated, it and other unknown properties of celastrol may prove responsible for the differences in celastrol and heat shock expression patterns shown in this study.

Conclusion

It is clear that celastrol treatment is able to mimic the cellular response to extended heat shock both *in vitro* and *in vivo*. Further, our data reveals that, under certain conditions, celastrol can act as a potent cytoprotector against thermally-induced apoptosis through the induction of Hsp70. However, after the concentration of Hsp70 from the initial challenge is dissipated, the cell becomes somehow weakened against further stress. Nonetheless, the induction of Hsp70 provides a crucial and powerful window in which the cell becomes highly resistant to stress, and in a controlled clinical environment this should be more than sufficient.

Celastrol treatment may one day prove a potent weapon against many different problems. Its ease of use, rapidity of action, and effectiveness in a wide range of applications – from treatment of simple wounds to Alzheimer's disease to cancer – suggest that clinical treatments

may be inexpensive and effective. As a small, stable molecule, celastrol represents a cheap and easily storable alternative to heat shock and direct injection of Hsp that is capable of inducing Hsp70 expression with twice the efficacy of heat. This study provides a crucial step towards the realization of celastrol as a viable clinical treatment, providing the experimental framework upon which to rest practical therapy. As such, our research may one day play a role in improving the health of individuals worldwide.

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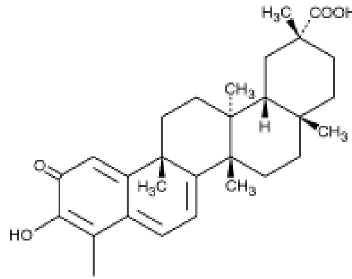
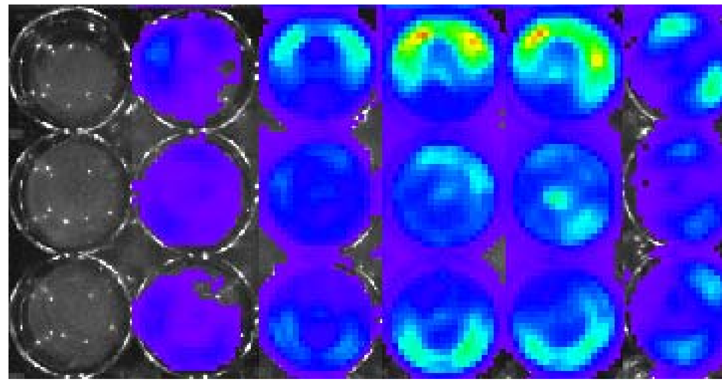


Figure 1: Celastrol is a triterpene extracted from the Chinese “Thunder of God” vine.



15 m 1 hr 2 hr 3 hr 5 hr 7 hr

Figure 2: Time courses of Hsp70 production were produced by exposing a 24-well plate of cells at approximately 80% confluence to uniform stress and imaging a single column with luciferin at each timepoint. The relevant images are spliced together in this sample graphic for convenience.

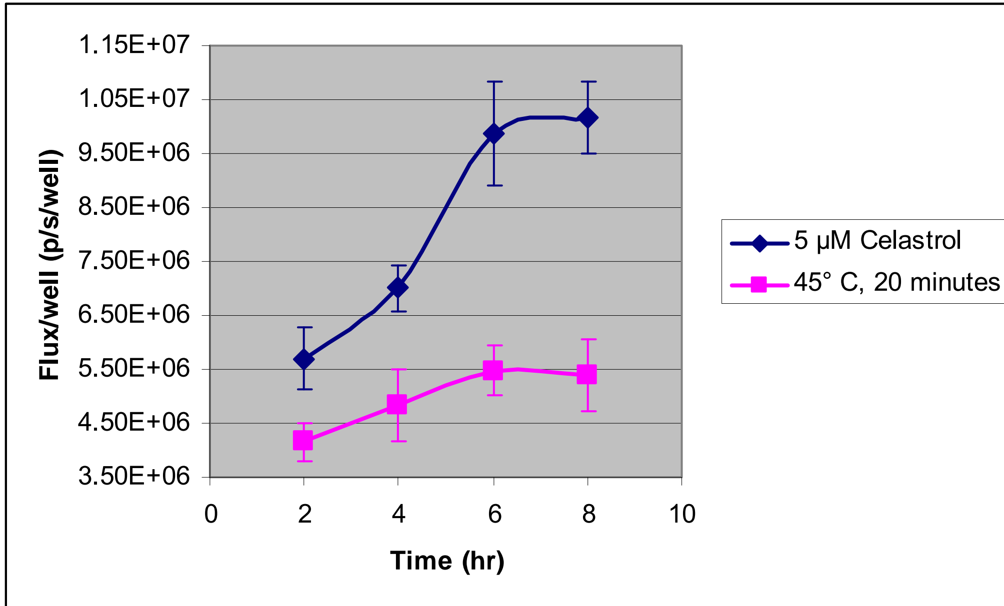


Figure 3: In a direct comparison of the induction curves of 5 μM Celastrol and 45º C heat shock, we notice that celastrol appears to possess nearly twice the inductive, and thus the protective, properties.

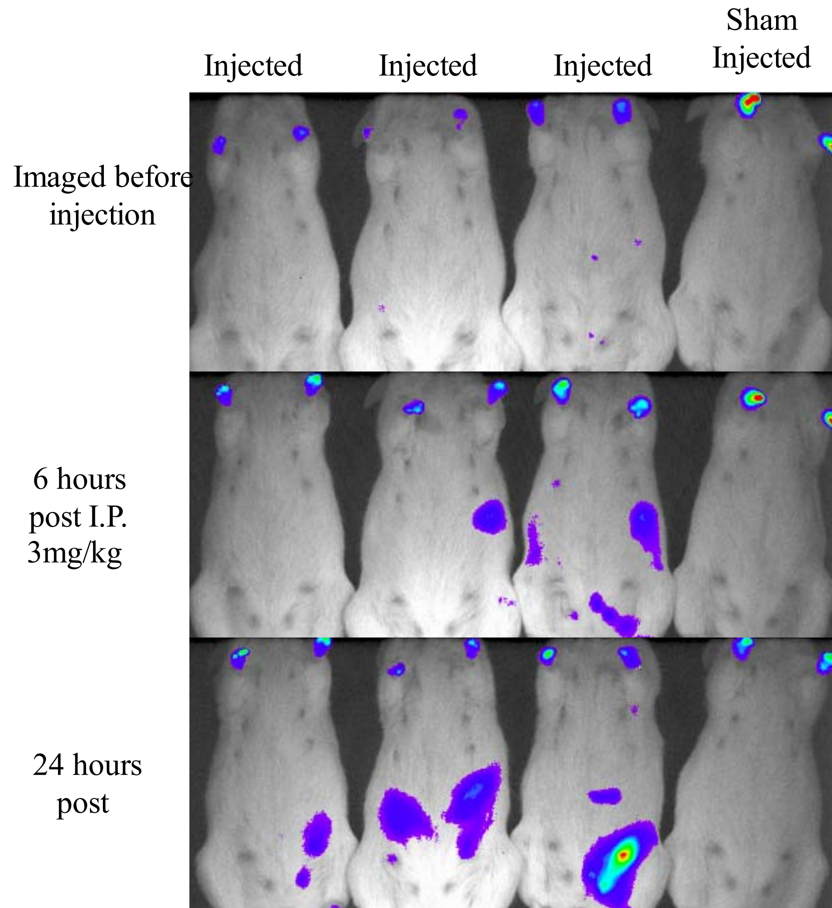


Figure 4: These images show that celastrol is capable of inducing Hsp70 *in vivo*. Note the strong signal from the spleen at 6 hours post-injection and from the intraperitoneal injection site at 24.

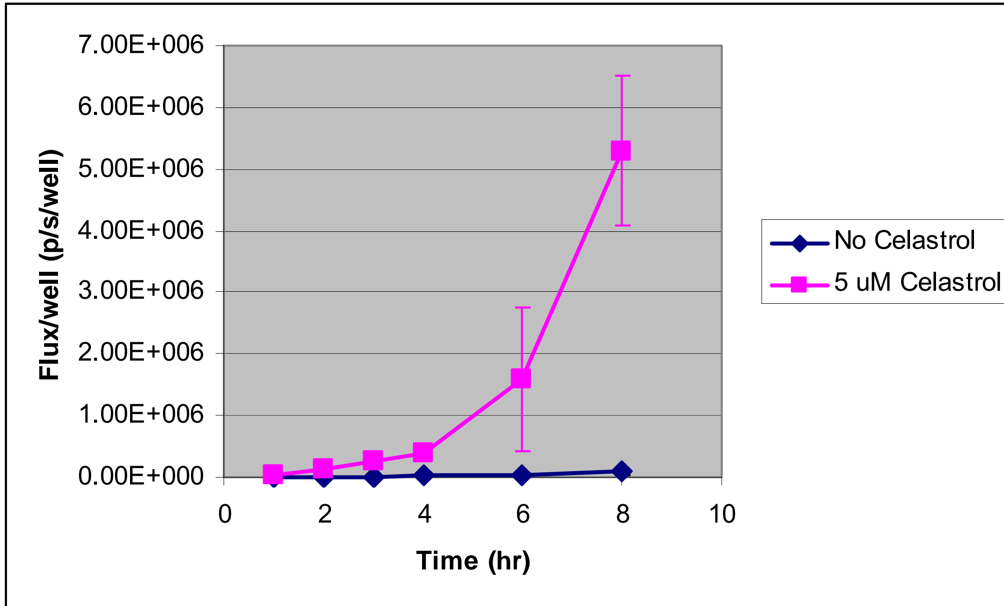


Figure 5: Celastrol pretreatment is able to protect cells from subsequent lethal shock at 50° C, 20 minutes. Subsequent Hsp70 production appears to take longer than normal 5-6 hours to reach maximum.

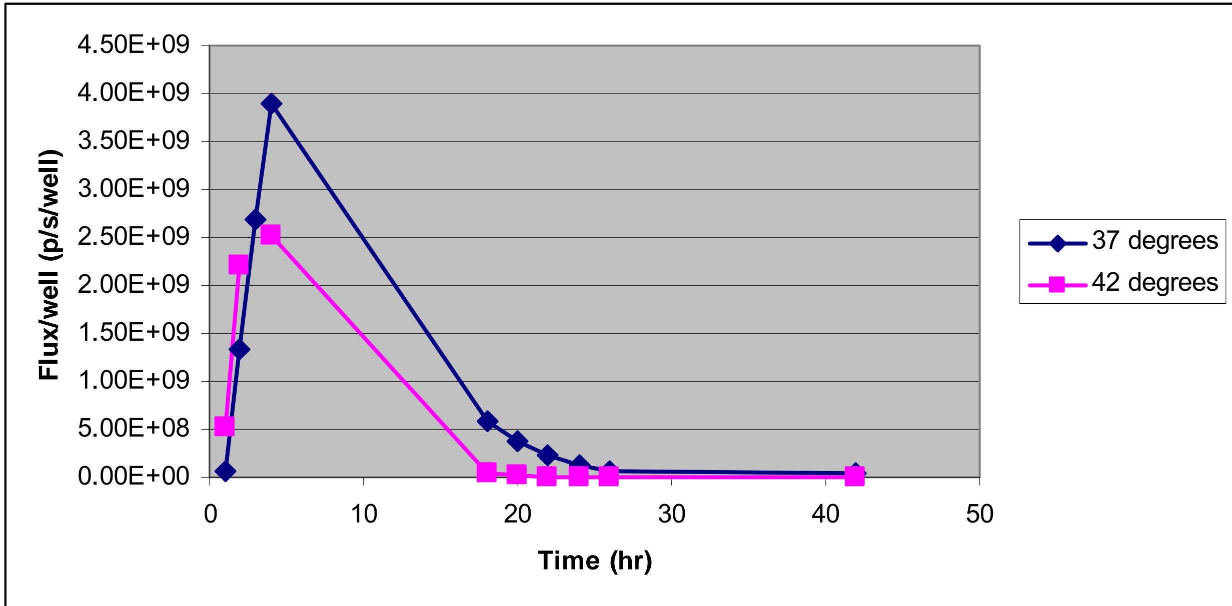


Figure 6: Prior non-lethal heat shock at 42 degrees appears to weaken subsequent response to 45º C, 20 minute heat shock after initial Hsp70 concentration has dissipated (18 hours later).

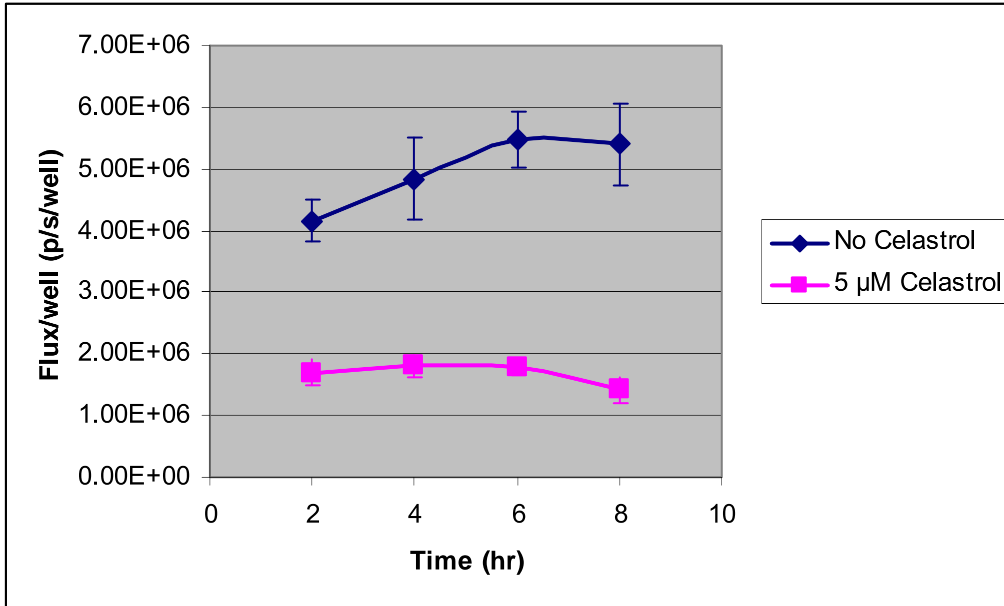


Figure 7: Prior non-lethal celastrol shock at 5 µM, 1 hour appears to weaken subsequent response to 45º C, 20 minute heat shock after initial Hsp70 concentration has dissipated (18 hours later).