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# **PROTEOSTASIS REGULATORS AS POTENTIAL RESCUERS OF PMM2 ACTIVITY**

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## ABSTRACT

Phosphomannomutase 2 deficiency (PMM2-CDG) is the most common N-glycosylation disorder. To date there is no treatment. Following the identification of a number of destabilizing pathogenic variants, our group suggested PMM2-CDG to be a conformational disease. The aim of the present study was to evaluate the possible use of proteostasis network regulators to increase the stability, and subsequently the enzymatic activity, of misfolded PMM2 mutant proteins. Patient-derived fibroblasts transduced with their own PMM2 folding or oligomerization variants were treated with different concentrations of the proteostasis regulators celastrol or MG132. Celastrol treatment led to a significant increase in mutant PMM2 protein concentration and activity, while MG132 had a small effect on protein concentration only. The increase in enzymatic activity with celastrol correlated with an increase in the transcriptional and proteome levels of the heat shock proteins Hsp90 and Hsp70. The use of specific Hsp70 or Hsp90 inhibitors showed the positive effect of celastrol on PMM2 stability and activity to occur through Hsp90-driven modulation of the proteostasis network. The synergistic effect of celastrol and a previously described pharmacological chaperone was also examined, and a mutation-dependent synergistic effect on PMM2 activity was noted. These results provide proof-of-concept regarding the potential treatment of PMM2-CDG by proteostasis regulators, either alone or in combination with pharmacological chaperones.

## **Keywords**

Congenital disorders of glycosylation, molecular chaperones, pharmacological chaperones, PMM2-CDG, proteostasis regulators.

## **Abbreviations**

17-AAG, 17-(Allylamino)-17-demethoxygeldanamycin; CDG, congenital defects of glycosylation; CVIII, 1-(3-chlorophenyl)-3,3-bis(pyridin-2-yl)urea; HSF1, Heat Shock Factor 1; Hsps, heat shock proteins; HSR, heat shock response; NEFs, nucleotide exchange factors; PCs, pharmacological chaperones; PFT- $\mu$ , pifithrin- $\mu$ ; PMM2, phosphomannomutase 2; PMM2-CDG, phosphomannomutase 2 deficiency; PN, proteostasis network; PR, proteostasis regulators.

## **Introduction**

Following their translation at the ribosomes, proteins must fold into the appropriate three-dimensional shape if they are to become functional. A complex quality control mechanism involving molecular chaperones and the proteasome and autophagy systems **are involved in** this folding. Collectively referred to as the proteostasis network (PN), these systems also help in the maintenance of the correct folded state [1-4]. When the PN surveillance fails, or is unable to cope with a specific protein misfolding, unstable mutants are formed with a greater tendency to be degraded or to form toxic aggregates. This can give rise to loss-of-function or gain-of-function effects respectively, and therefore, conformational disease [5, 6] .

The composition of the PN is highly dynamic. Different endogenous and exogenous conditions may disturb it, activating different cellular stress responses in different compartments and boosting cellular reactions designed to restore cellular homeostasis. This dynamism led to the idea of pharmacologically enhancing the PN via the use of proteostasis regulators (PRs) to maintain mutant proteins in a functional, non-aggregated state [7], and thus combat conformational disease.

In recent years, PRs have been shown to modulate protein function in different conformational diseases by stabilizing defective, misfolded proteins [5]. Enhancing intracellular proteostasis capacity by PRs could be used to treat different disorders with the same underlying pathogenic mechanism. Different compounds for treating conformational diseases and neurodegenerative disorders are currently in the pre-clinical and clinical stages of development [7, 8].

PMM2 is a homodimeric cytoplasmic protein involved in the conversion of mannose-6-phosphate to mannose-1-phosphate, one of the first steps in the N-glycosylation pathway [13]. It has recently been suggested that the congenital disorder of glycosylation (CDG) due to phosphomannomutase 2 deficiency (PMM2-CDG, the most common N-CDG) is a conformational disease. Patients present multisystem clinical problems, including involvement of the nervous system. Mortality is as high as 20 % in the first years of life. Lamentably, only symptomatic treatment is available for these patients [9, 10]. The scientific community is putting its efforts in the development of a successful therapeutic strategy for this disabling disease. Mannose treatment has triggered N-glycosylation recovery in cell cultures and animals, although so far, mannose administration has not had any clinical or biochemical improvement in

patients [11]. New mannose delivery strategies, which include liposomes, nanoparticles and prodrugs, are under development and some of them are being considered for preclinical and clinical validation [12-15]. An alternative to the development of novel therapies, an expensive and time-consuming process, is drug repurposing. Some commercially available drugs have successfully been tested for the treatment of PMM2-CDG in animal models [16] and in a clinical trial [17]. Other approaches rely on the use of computational tools to search for small molecules that bind PMM2 mutants with the aim to revert their pathogenic effect [18].

Several of the missense pathogenic variants that have been reported are associated with lowered PMM2 concentrations and reduced enzymatic activity [19, 20]. The inherent instability described for most of the PMM2 mutants associated with PMM2-CDG led to the idea of testing compounds capable of improving protein folding in the cytoplasm (where PMM2 is localized). Pharmacological chaperones (PCs) are interesting candidates for this [21] [22]. These small molecules bind specifically to proteins, stabilize them, and prevent their degradation and/or aggregation by promoting their correct folding. Another therapeutic possibility is the activation of the transcription factor heat shock factor 1 (HSF1), the master regulator of the heat shock response (HSR). The activation of HSF1 induces the expression of the heat shock proteins (Hsps)[23]. These molecular chaperones are classified by their molecular weight (Hsp40, Hsp60, Hsp70, Hsp90, Hsp100 and small Hsp) and have multiple functions associated with proteostasis. The Hsp70 and Hsp90 proteins have a central role in the cytosolic chaperone network. Their functional properties, such as protein folding, refolding and removal, are strongly influenced and regulated by their

interaction with multiple co-chaperones [1]. The Hsp70 family members, for example, require the presence of co-chaperones, Hsp40 family members, and nucleotide exchange factors (NEFs) [24, 25]. During the folding process, Hsp40 proteins present substrate polypeptides to the Hsp70 proteins, which interact with the peptides to induce their proper folding. If this does not occur, unfolded proteins are transferred downstream to the specialized chaperonin system or Hsp90 chaperone, to re-attempt protein folding. Alternatively, they are degraded [1, 3, 25].

Hsp90 is highly abundant in the cytosol of eukaryotic cells [26] . Its client specificity and function are regulated by more than 20 different co-chaperones [7]. Indeed, over 300 proteins have been described as Hsp90 clients, including kinases, transcription factors and proto-oncogene proteins, as well as proteins destabilized by mutations or post-translational modifications [27-29]. Hsp90 substrate recognition is not yet well understood, but it has recently been suggested that Hsp90 binds to proteins with exposed hydrophobic residues [26].

The aim of the present work was to examine the potential of proteostasis activators to increase the concentration of **some** mutant PMM2 proteins and subsequently rescue their activity. The misfolding missense mutations p.Asp65Tyr, p.Arg162Trp and p.Thr237Met, and the p.Phe119Leu variant affecting dimer formation (the second most common variant seen in patients with PMM2-CDG in Europe) were chosen to be examined.

## Results

### *Celastrol increases the activity and concentration of different PMM2 mutants in a cellular model of PMM2-CDG*

Patient-derived fibroblasts overexpressing their own PMM2 folding mutation, i.e., p.Asp65Tyr, p.Arg162Trp, p.Thr237Met, or the p.Phe119Leu mutation affecting PMM2 dimerization [20, 30], were treated with celastrol or MG132, molecules reported to be PRs [31]. Cells were incubated for 48 h with different concentrations of these agents, and activity assays performed using cell extracts. ~~The overexpression of PMM2 mutant proteins in the patient-derived fibroblasts was successfully used to measure the rescue of enzymatic activity using pharmacological chaperones in order to increase the PMM2 residual activity~~ detect changes in the PMM enzymatic activity due to the treatment [21].

A significant increase in PMM2 activity (1.2-2.8 times baseline) was observed for all four cell lines after incubation with celastrol at 0.25, 0.4 or 0.6  $\mu$ M (Figure 1A). For most of the mutants, the greatest activity increase was achieved with 0.4  $\mu$ M celastrol. MG132 treatment did not increase the enzymatic activity of any of these PMM2 mutants at the concentrations and incubation times tested (Figure 1A).

Western blots were performed with soluble extracts from the different cell lines incubated with celastrol at 0.4  $\mu$ M and MG132 at 0.1  $\mu$ M (the highest concentration tested in activity assays) for 48 h. Increases in concentration were observed after celastrol treatment for all four mutants. MG132 also slightly increased the concentration of the p.Asp65Tyr, p.Arg162Trp and p.Thr237Met mutants (Figure



1B). In the case of celastrol, these increases in mutant protein concentration seemed to be linked to a significant and reproducible boost in enzymatic activity (Figure 1A).

#### *Celastrol increases the levels of molecular chaperones*

Celastrol is reported to induce HSR, enhancing the proteostasis capacity of the cytosol and inhibiting the proteasome system [31, 32]. To study the induction of the stress response triggered by the PR in the studied cells, the expression of several molecular chaperones at the mRNA and protein levels, as well as the inhibition of the proteasome, was examined. A general increase in Hsp27, Hsp40, Hsp70 and Hsp90 proteins was seen after celastrol treatment (0.4  $\mu$ M) in all four mutant cell lines. The increase in chaperones was highest at 24 h of treatment, especially for the Hsp70 protein (Figure 2A and B). An overall increase in the transcription of the corresponding inducible genes, *HSPB1* (Hsp27), *DNAJB1* (Hsp40), *HSPA1A* and *HSPA1B* (Hsp70) and *HSP90AA1* (Hsp90), was also observed after 16 h of celastrol treatment with the exception of *DNAJB1* (Hsp40) in P2 (p.Phe119Leu) and P3 (p.Arg162Trp). This was greater in the cells overexpressing p.Asp65Tyr (Figure 2C), suggesting the response is mutation-dependent.

Further experiments to study the effect of celastrol on the proteasome system were performed with the p.Arg162Trp-overexpressing cell line (P3), in which the greatest celastrol-induced increases in protein concentration and activity were obtained (Figure 1A). Western blots of ubiquitinated proteins were performed after 24 and 48 h of celastrol (0.4  $\mu$ M) and MG132 (proteasome inhibitor used as positive control; 0.1  $\mu$ M) treatment. As expected, an accumulation of ubiquitinated proteins was observed after both treatments compared to untreated cells, confirming the

inhibitory effect of these agents on the proteasome system. The greater inhibitory effect was recorded for MG132, suggesting that the inhibition of the proteasome degradation system is insufficient for PMM2 stabilization (Figure 2D).

*Hsp70 and Hsp90 are increased after celastrol treatment, despite HSF1 downregulation*

To further examine the correlation between HSR activation and the effect of celastrol on PMM2 mutant stabilization, the HSR master regulator HSF1 was partially silenced using a small interfering RNA (siRNA). p.Arg162Trp-overexpressing cells were pre-treated with the *HSF1* siRNA or a non-targeting control siRNA (siC) for 24 h followed by 48 h of DMSO or celastrol (0.4  $\mu$ M) treatment. *HSF1* downregulation was verified by quantitative RT-PCR and Western blotting (Figure S1).

The Western blots showed that the amount of p.Arg162Trp mutant protein increased after celastrol treatment and HSF1 downregulation (Figure 3A), suggesting an HSF1-independent effect of celastrol or, alternatively, that the partial inhibition of HSF1 was not enough to repress the activation of HSR by celastrol. To distinguish between these possibilities, the mRNA expression levels of the molecular chaperones were analyzed. The result showed an increase in chaperone mRNA (Hsp27, Hsp40, Hsp70 and Hsp90) after the co-application of the *HSF1* siRNA and celastrol (Figure 3B).

Despite the partial silencing of HSF1, celastrol was still able to significantly increase the expression of *HSP1A/B* (Hsp70) and *HSP90AA1* (Hsp90), although for *HSP90AA1* (Hsp90) the increase was smaller than that seen for the negative control (siC) (Figure 3B).

*Hsp90 may be a key molecular chaperone in PMM2 folding*

To elucidate the effect of Hsp70 and Hsp90 on PMM2 protein and activity rescue, the four cell lines were treated with pifithrin- $\mu$  (4  $\mu$ M) and 17-AAG (80 nM) (Figure 4A). Pifithrin- $\mu$  is a Hsp70 inhibitor that disrupts the association between Hsp70 and several of its co-chaperones and substrate proteins [33], whereas 17-AAG binds to the ATP binding pocket of Hsp90, inhibiting its folding capacity [34]. Treatment with the Hsp90 inhibitor tended to reduce PMM2 protein levels in most of the selected mutants. Inhibition of Hsp70 triggered different effects on PMM2 protein levels depending on the mutant. The p.Aps65Tyr and p.Phe119Leu protein concentrations increased after pifithrin- $\mu$  treatment (Figure 4B and C). This might be the result of the accumulation of the mutant protein due to the inhibition of Hsp70, a chaperone that plays an essential role in protein degradation [35, 36].

The cells were next treated with celastrol (0.4  $\mu$ M) in combination with the Hsp70 or Hsp90 inhibitors. The co-application of celastrol and pifithrin- $\mu$  (an Hsp70 inhibitor) had no greater (or lesser) effect than treatment with celastrol alone. However, for all mutants except p.Phe119Leu, a significant 50-60 % reduction in protein concentration was observed following the co-application of celastrol and 17-AAG (Hsp90 inhibitor) compared to celastrol alone. These results show that Hsp90 is responsible, at least in part, for the effect of celastrol on PMM2 stabilization (Figure 4B and C).

#### *P23 silencing partially phenocopies the stabilizing effect of celastrol*

Since the preceding results suggest the possible involvement of the Hsp90 chaperone in the stabilization of the PMM2 mutant proteins, the molecular mechanism involved was explored.

In addition to inducing the HSR, and consequently increasing Hsp90 levels, celastrol can also affect Hsp90 activity indirectly via the fibrilization of the co-chaperone p23, inhibiting its proper function [37]. To test the effect of celastrol on the fibrilization of the p23 co-chaperone, p.Arg162Trp-overexpressing cells were treated with 0.4  $\mu$ M celastrol for 48 h. The soluble and insoluble fractions were then subjected to Western blotting to detect p23 protein. No differences in the soluble fraction were seen with respect to controls (Figure 5A) but an increase in p23 protein was detected in the insoluble fraction (Figure 5B), suggesting the aggregation of p23 protein following celastrol treatment. Hsp70 and PMM2 were also increased in the insoluble fraction after treatment with celastrol and MG132 (Figure 5B).

To explore the role of p23 in the effect of celastrol on PMM2 mutant stabilization, the expression of *PTGES3*, which codes for p23 protein, was subjected to siRNA gene silencing for 96 h. *PTGES3* downregulation was verified by quantitative RT-PCR and Western blotting (Figure S2). A significant increase in the mutant p.Arg162Trp protein - double the amount recorded for the control siRNA transfection - was observed by Western blotting after downregulating *PTGES3* (Figure 5C and D), suggesting that the inhibitory effect of celastrol on the Hsp90 co-chaperone p23 is, at least partially, responsible for the stabilizing effect on the PMM2 mutant.

#### *Mutant-dependent response to the co-application of a PC for PMM2 and celastrol on enzymatic activity*

The synergistic effect of PRs and PCs has already been studied in the context of other conformational diseases [31]. Our group has described four potential PCs, obtained via high-throughput screening, that enhance the stability and activity of different unstable

PMM2 mutant proteins, including three of the four mutants studied in the present work (p.Asp65Tyr, p.Arg162Trp and p.Thr237Met). Only one of the compounds, 1-(3-chlorophenyl)-3,3-bis(pyridin-2-yl)urea (referred to as compound VIII or CVIII), passed all the computational filters tested while showing no inhibitory effect on PMM2 activity [21]. To examine the possible synergistic effect of CVIII and celastrol, patient-derived fibroblasts overexpressing the p.Asp65Tyr, p.Phe119Leu, p.Arg162Trp or p.Thr237Met mutations were treated for 96 h with 0.4  $\mu$ M celastrol alone, or 0.4  $\mu$ M celastrol for 48 h followed by 0.4  $\mu$ M celastrol plus 10  $\mu$ M CVIII (i.e., the first 48 h of celastrol to activate the proteostasis network, and the next 48 h with both compounds), and the activity assay was then performed. The combination of the PC and celastrol only had a slightly greater effect on the enzymatic activity of the p.Asp65Tyr mutant than celastrol alone. The co-application of CVIII and celastrol reduced the enzymatic activity of the p.Phe119Leu mutant to half that recorded for the celastrol treatment (Figure 1A). No differences between the two treatments were observed for the other two mutants (Figure 6), suggesting the measured response to be mutant-dependent.

## Discussion

There is no treatment for PMM2-CDG, the most common N-glycosylation disorder [38]. However, the misfolded nature of most of the disease-causing mutants suggests that stabilizing molecules such as PCs and PRs might be valuable therapeutic

agents [5, 20, 39]. Our group recently reported the possibility of rescuing some unstable, disease-causing mutants via the use of specific PCs [21].

The present work focuses on the identification of agents that can stabilize PMM2 mutants by modulating the PN, and helps throw light on the quality control machinery involved in PMM2 folding. This knowledge could assist in the development of therapeutic options for individual patients. After treatment with celastrol, a significant increase in PMM2 p.Asp65Tyr, p.Phe119Leu, p.Arg162Trp, and p.Thr237Met mutant protein levels was seen, along with significant increases in enzymatic activity. Further examination confirmed celastrol to act as a PR by triggering the HSR (i.e., it increased the expression of Hsp27, Hsp40, Hsp70 and Hsp90) and by inhibiting the proteasome system.

To examine the role of HSR activation in PMM2 stabilization by celastrol, the expression of the master regulator of this response, *HSF1* [40, 41], was partially silenced. Surprisingly, *HSF1* downregulation did not prevent p.Arg162Trp stabilization, nor did it abolish the upregulation of *HSP1A/B* (Hsp70) and *HSP90AA1* (Hsp90) induced by celastrol treatment. Maybe some other form(s) of chaperone regulation may also be at work. However, it is noteworthy that total silencing of *HSF1* could not be achieved using siRNA technology, ~~and to confirm the results probably~~ *HSF1* knockout experiments by CRISPR/Cas9 should be done to confirm the results. Further, attempts to distinguish between the possible effects of the two main molecular chaperones through the use of Hsp70 and Hsp90 inhibitors revealed the importance of Hsp90 on celastrol-induced PMM2 stabilization. Compared to PMM2 protein levels after celastrol treatment alone, a reduction of ~~some 50-60 %~~ in PMM2 was detected for

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three of the four studied mutants after the co-application of celastrol and the Hsp90 inhibitor 17-AAG. No effect was seen when the inhibitor of Hsp70, pifithrin- $\mu$ , was used in combination with celastrol. The use of Hsp70 and Hsp90 inhibitors alone, suggests that the two chaperones are involved in p.Arg162Trp folding. Nevertheless, Hsp90 seems to be more important than Hsp70 for improving the folding of the p.Asp65Tyr and p.Thr237Met variants of PMM2. In addition, the inhibition of Hsp70 with pifithrin- $\mu$  caused an increase in the p.Asp65Tyr and p.Phe119Leu mutant proteins. These different responses of the mutants to the modulation of the PN suggests different cellular strategies exist for dealing with misfolded proteins.

In addition to the induction of the HSR, and therefore of Hsp90, celastrol may have other direct and indirect effects on the structural conformation and activity of Hsp90. Celastrol directly binds to the C-terminal domain of Hsp90, inducing its oligomerization. However, this binding affects neither its ability to bind the tetra- $\alpha$ -helix repeat in co-chaperones, nor its chaperone activity [42]. Additionally, celastrol induces the fibrilization of the Hsp90 co-chaperone p23, impairing its function [43]. Further, the reduction of the expression of *PTGES3* (which encodes p23 protein) partially phenocopies the effect of celastrol, since it rescues the levels of the p.Arg162Trp mutant protein, even though the siRNA knockdown experiment only reduced the amount of p23 to a 20 %. These results suggest that the inactivation of p23 by celastrol seems to be, at least in part, responsible for the PMM2 mutant's stabilization in this system.

PRs have been reported to have a synergistic effect in combination with PCs in different lysosomal storage diseases. Indeed, the combination of PRs and PCs has

already shown promising results for Gaucher's and Tay-Sachs diseases [31]. PRs could create a larger pool of mutant proteins which the PCs could bind to, and subsequently stabilize. Our results have revealed a mutation-specific effect after the co-application of celastrol and CVIII [20]: a synergistic effect increasing the activity of the p.Asp65Tyr mutant protein was seen, but no further effect was detected on the activity of p.Arg162Trp or p.Thr237Met PMM2 mutants. Indeed, a significant reduction in activity was recorded for the mutant p.Phe119Leu compared to celastrol treatment, indicating that CVIII is somehow hindering celastrol effect in this mutant.

Celastrol has been studied as a treatment for cancer and a number of inflammatory and neurodegenerative diseases [44]. In addition to being a proteostasis regulator, it is a modulator of cell death and proliferation, inflammation and antioxidant defenses [31, 45, 46]. Nevertheless, the narrow therapeutic window available for the use of this drug, and its many reported side effects [44, 47, 48], limit its potential as a therapeutic option. However, the present work provides the proof-of-concept required for seeking safer PRs.

In summary, the present results confirm that PMM2-CDG is a conformational disease, and highlight the potential use of PCs and/or PRs as options for its treatment. Future screening for drugs that can increase Hsp90 levels with fewer side effects should be conducted.

## **Materials and Methods**

### *Cell culture*



Patient-derived fibroblasts carrying the p.Arg141His/p.Asp65Tyr (P1), p.Arg141His/p.Phe119Leu (P2), p.Arg141His/p.Arg162Trp (P3) or c.640-9T>G/p.Thr237Met (P4) mutations were grown from skin biopsies (taken with informed consent) under standard conditions in minimal essential medium (MEM) supplemented with 1 % glutamine, 10 % fetal calf serum (FCS), and antibiotics. Except for the p.Arg141His/p.Phe119Leu line, these cells were immortalized using pBABE-puro containing SV40 DNA sequences. These cell lines were then transduced with their own mutations as described elsewhere [20]. Cells were synchronized by FCS deprivation (MEM\_1 % glutamine, 0.5 % FCS and antibiotics) for 48 hours, followed by 24 hours of standard medium recovery before treatment.

**Comentado [AVL1]:** Queda indicado cómo se sincronizan las células antes de cada tratamiento.

#### *Reagents*

Cells were treated with different reagents, including celastrol (Cayman Chemical, Ann Arbor, MI, USA), MG132 (Cayman), pifithrin- $\mu$  (Calbiochem, Darmstadt, Germany), 17-AAG and 1-(3-chlorophenyl)-3,3-bis(pyridin-2-yl)urea (Sigma-Aldrich, St. Louis, MO, USA), a compound described as a PC for PMM2 [21].

#### *PMM activity assay*

PMM enzymatic activity was assayed in the cellular extract of patient-derived fibroblasts treated during different hours with the selected potential PRs, as well as with a combination of celastrol and PCs. The assay was performed using the method of Van Schaftingen and Jaeken [49] as modified by de Koning *et al.* [50], with some further changes [20].

#### *Western blot analysis*

Patient-derived cells were harvested with trypsin and resuspended in lysis buffer (1 % triton, 10 % glycerol, 150 mM NaCl, 10 mM trisHCl, pH 7.5) containing Complete Mini EDTA-free Protease Inhibitor Cocktail (Roche Applied Sciences, Mannheim, Germany). After 20 min of shaking at 4°C, the cell extracts were centrifuged for 5 min at 14,000 rpm and the soluble and insoluble fractions separated. Protein in the soluble fractions was quantified using the Bradford assay (BioRad, Hercules, CA, USA). Samples were prepared in NuPage®LDS 4x sample buffer (Invitrogen, Carlsbad, CA, USA) and dithiothreitol (DTT), and were subjected to electrophoresis in 10 % or 4-12 % NuPAGE Novex Bis-Tris mini gels (Invitrogen). ProSieve Color Protein Marker (Lonza, Basel, Switzerland) and Novex Sharp Pre-stained Protein Standard (ThermoFisher Scientific, Wilmington, MA, USA) were used as molecular weight markers. Proteins were transferred to a nitrocellulose membrane using the iBlot Dry Blotting System (Invitrogen). Membranes were blocked for at least 1 h with 0.05 % PBS-Tween and 5 % low-fat milk. Immunodetections were performed using PMM2 (H00005373-A0, Abnova, Heidelberg, Germany), Hsp27 (ADI-SPA-800, Enzo Life Sciences, Farmingdale, NY, USA) Hsp40 (ADI-SPA-400, Enzo Life Sciences), Hsp70 (C92F3A-5, Novus Biologicals, Littleton, CO, USA), Hsp90 (ADI-SPA-830, Enzo Life Sciences), Ubiquitin (sc8017, Santa Cruz Biotechnology, Santa Cruz, CA, USA), HSF1 (ADI-SPA-901, Enzo Life Sciences), p23 (NB300-576, Novus Biologicals), GAPDH (ab8245, Abcam, Cambridge, UK), tubulin (T9026, Sigma-Aldrich) and  $\beta$ -actin antibodies (TA811000S, Origene, Rockville, MD, USA). Conjugated goat anti-rabbit, goat anti-mouse or anti-rabbit immunoglobulin G horseradish peroxidase (Santa Cruz Biotechnology) were used as secondary antibodies.

The Enhanced Chemiluminescence System (GE Healthcare, Buckinghamshire, UK) was used as the detection method.

The insoluble fraction of the cell extracts was resuspended in the same lysis buffer with Complete Mini EDTA-free Protease Inhibitor Cocktail and subjected to electrophoresis under the same conditions as the soluble fractions.

#### *Quantitative RT-PCR*

Total RNA was isolated using Tripure Isolation Reagent (Invitrogen) following the manufacturer's recommendations. Samples were quantified using a Nanodrop ND-1000 fluorescence detector (ThermoFisher Scientific). cDNA was synthesized by RT-PCR using the NZY First-Strand cDNA Synthesis Kit (Nzytech, Lisbon, Portugal), amplified with PerfeCTa SYBR Green FastMix (Quanta Biosciences, Beverly, MA, USA) and a LightCycler®480 device (Roche Applied Sciences, Mannheim, Germany) following the latter manufacturer's instructions and using appropriate primers (see Table S1). Data were analyzed using Lightcycler® software (Roche Applied Sciences), correlating initial template concentrations with the cycle threshold (Ct) to obtain relative quantities (RQ) of RNA. The RQ is defined as  $RQ = 2^{-\Delta\Delta Ct}$ , where  $\Delta\Delta Ct$  is the  $\Delta Ct$  of the patient cell line minus the  $\Delta Ct$  of the control cell line, and  $\Delta Ct$  is the Ct of the target gene minus the Ct of the housekeeping gene (*GAPDH*) [51].

#### *siRNA transfection*

200,000 cells per well were seeded in 6-well plates. On the following day, the cells were transfected with 25 nM (final concentration) of ON-TARGETplus Human HSF1 (3297) siRNA (J-012109-05, GE Dharmacon, Lafayette, CO, USA), 50 nM (final concentration) of ON-TARGETplus Human PTGES3 (10728) siRNA (J-004496-09, GE

Dharmacon), or SIGENOME Non-targeting siRNA (D-001210-01, GE Dharmacon) as a control, using the DharmaFECT 1 Transfection Reagent (GE Dharmacon) following the manufacturer's instructions. Knockdown efficiency was assessed after a 24 h siRNA pre-treatment followed by 48 h incubation with DMSO for *HSF1* downregulation and after 96 h siRNA treatment for *PTGES3* downregulation.

#### *Statistical analysis*

Statistical analyses were performed using GraphPad Prism 8 software for Windows. Two-way ANOVA followed by a Dunnett's or Tukey's *post hoc* test was used for multiple comparisons of treatments with different agents, concentrations or time (e.g. PMM enzymatic activity in different cell lines treated with different concentrations of PRs). One-way ANOVA followed by Dunnett's *post hoc* test was used for multiple comparisons of more than two factors (e.g. PMM2 protein levels after several treatments). Multiple t test analysis contemplating the false discovery rate (FDR), and using the Two-Stage Step-Up method of Benjamini, Krieger and Yekutieli (Q = 5 %), was employed for multiple comparisons of all pairs (e.g. PMM enzymatic activity after treatment with celastrol or the combination of celastrol and a PC in different cell lines). The Shapiro-Wilk test was used to check the normality of distributions (performed using IBM SPSS Statistics v.21 software for Windows). In cases of non-normal distribution, the data were log2 transformed before analysis. The 2-tailed Student t test was used for non-multiple comparisons, e.g., of p23 and PMM2 protein concentrations after p23 downregulation. Data are reported as means  $\pm$  SDs.

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## Figure legends

**Figure 1.** Effect of the PRs celastrol and MG132 on PMM2 activity and the concentration of different PMM2 mutant proteins in a cellular model of PMM2-CDG.

**A)** Relative PMM enzymatic activity of the cellular extracts. Baseline enzyme activity (-) was defined as '1' in each cell line. The data represent the mean  $\pm$  SD of at least three independent experiments (\*\* $p < 0.001$ ; \*\* $p < 0.01$ , \* $p < 0.05$ ). **B)** Representative Western blot of PMM2 protein. LC: loading control (tubulin for P1, P3 and P4; GAPDH for P2).

**Figure 2.** Effect of the PR celastrol (Cel) on the proteostasis network. **A)** Relative concentrations of Hsp27, Hsp40, Hsp70 and Hsp90 after treatment with celastrol (0.4  $\mu$ M) for 24 or 48 h. **B)** Representative Western blot of the p.Asp65Tyr cell line. ACT: Actin was used as loading control. **C)** Relative mRNA expression of *HSPB1* (Hsp27),

*DNAJB1* (Hsp40), *HSPA1A/B* (Hsp70) and *HSP90AA1* (Hsp90) after treatment with celastrol (0.4  $\mu$ M) for 16 h. The data represent the mean  $\pm$  SD of at least three independent experiments (\*\**p* < 0.001; \*\**p* < 0.01; \**p* < 0.05). **D)** Representative Western blot of ubiquitin (Ub). Equal amounts of protein from the soluble fractions of the p.Arg162Trp cells treated with celastrol (0.4  $\mu$ M) or MG132 (0.1  $\mu$ M) for 24 or 48 h were loaded onto SDS-PAGE gels. TUB: tubulin was used as loading control.

**Figure 3.** Role of HSF1 in the stabilizing effect of celastrol on the Arg162Trp PMM2 mutant. **A)** Representative Western blot of HSF1 and PMM2 proteins. Equal amounts of protein from the soluble fraction of the p.Arg162Trp-overexpressing cells pre-treated with the *HSF1* siRNA or non-targeting control siRNA (siC) for 24 h followed by 48 h of DMSO (-) or 0.4  $\mu$ M celastrol treatment (Cel), were loaded onto the SDS-PAGE gel. TUB: Tubulin was used as loading control. **B)** Relative mRNA expression of *HSPB1* (Hsp27), *DNAJB1* (Hsp40), *HSPA1A/B* (Hsp70) and *HSP90AA1* (Hsp90) in the p.Arg162Trp-overexpressing cells pretreated with the *HSF1* siRNA or non-targeting control siRNA (siC) for 24 h followed by 16 h of DMSO or 0.4  $\mu$ M celastrol treatment. The data represent the mean  $\pm$  SD of at least three independent experiments (\*\**p* < 0.001; \*\**p* < 0.01; \**p* < 0.05).

**Figure 4.** Role of Hsp70 and Hsp90 in the stabilization of PMM2 mutants. **A)** Diagram showing protein remodeling by Hsp70 and Hsp90 and their specific inhibitors. Created with BioRender. **B)** Relative PMM2 concentration in untreated cells (-), cells treated with pifithrin- $\mu$  (4  $\mu$ M) (PFT- $\mu$ ), 17-AAG (80 nM), celastrol (0.4  $\mu$ M) (Cel) or celastrol plus pifithrin- $\mu$  (Cel + PFT- $\mu$ ), or celastrol and 17-AAG (Cel + 17-AAG). **C)** Representative Western blot of PMM2. Equal amounts of protein from the soluble fractions of the

cells were loaded onto SDS-PAGE gels. ACT: Actin was used as a loading control. The data represent the mean  $\pm$  SD of at least three independent experiments (\*\* $p < 0.001$ ; \* $p < 0.01$ ).

**Figure 5.** Role of the Hsp90 co-chaperone p23 in the stabilization of PMM2 mutants. **A)** Western blot of p23. Equal amounts of protein from the soluble fraction of cells were loaded onto the SDS-PAGE gel. **B)** Western blot of p23, Hsp70 and PMM2. Equal amounts of protein from the insoluble fraction of cells were loaded onto the SDS-PAGE gel. **C)** Western blot of p23 and PMM2. Equal amounts of protein obtained from the cells treated with the *PTGES3* siRNA or non-targeting control siRNA (siC) for 96 h were loaded onto the SDS-PAGE gel. **D)** Relative PMM2 and p23 protein levels quantified from C). Data represent the mean  $\pm$  SD of at least three independent experiments (\*\* $p < 0.01$ ; \* $p < 0.05$ ). TUB: Tubulin was used as a loading control.

**Figure 6.** Effect of the co-application of the PR celastrol and a PC for PMM2. Relative PMM enzymatic activity of the cellular extracts after 48 h of celastrol treatment (0.4  $\mu$ M) followed by another 48 h celastrol treatment (Cel-Cel), or celastrol and the PC CVIII (10  $\mu$ M, Cel-CVIII). The enzymatic activity of cells treated with celastrol alone for 96 h (Cel-Cel) was considered to be '1' in each cell line. Data represent the mean  $\pm$  SD of at least three independent experiments (\*\* $p < 0.001$ ).

**Figure S1.** *HSF1* interference. **A)** Relative mRNA expression of *HSF1* in p.Arg162Trp-overexpressing cells treated with the *HSF1* siRNA or non-targeting control siRNA (siC) for 24 h. Data represent the mean  $\pm$  SD of at least three independent experiments. **B)** Representative Western blot of HSF1. Equal amounts of protein obtained from the

soluble fraction of the p.Arg162Trp cells treated with the *HSF1* siRNA or non-targeting control siRNA (siC) for 24 h.

**Figure S2.** *PTGES3* downregulation. **A)** Relative mRNA expression of *PTGES3* in p.Arg162Trp-overexpressing cells treated with the *PTGES3* siRNA or non-targeting control siRNA (siC) for 96 h. Data represent the mean  $\pm$  SD of at least three independent experiments **B)** Representative Western blot of the p23 protein. **Equal amounts of protein** obtained from the soluble fraction of p.Arg162Trp cells treated with the *PTGES3* siRNA or non-targeting control siRNA (siC) for 96 h.

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