The antibiotic robenidine exhibits guanabenz-like cytoprotective properties by a mechanism independent of protein phosphatase PP1:PPP1R15A

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The aminoguanidine compound robenidine is widely used as an antibiotic for the control of coccidiosis, a protozoal infection in poultry and rabbits. Interestingly, robenidine is structurally similar to guanabenz (analogs), which are currently undergoing clinical trials as cytoprotective agents for the management of neurodegenerative diseases. Here we show that robenidine and guanabenz protect cells from a tunicamycin-induced unfolded protein response to a similar degree. Both compounds also reduced the tumor necrosis factor α王子-induced activation of NF-κB. The cytoprotective effects of guanabenz (analogs) have been explained previously by their ability to maintain eIF2α phosphorylation by allosterically inhibiting protein phosphatase PP1:PPP1R15A. However, using a novel split-luciferase–based protein–protein interaction assay, we demonstrate here that neither robenidine nor guanabenz disrupt the interaction between PPP1R15A and either PP1 or eIF2α in intact cells. Moreover, both drugs also inhibited the unfolded protein response in cells that expressed a nonphosphorylatable mutant (S51A) of eIF2α. Our results identify robenidine as a PP1:PPP1R15A-independent cytoprotective compound that holds potential for the management of protein misfolding–associated diseases.

The unfolded protein response (UPR) is a cellular reaction to the accumulation of unfolded proteins in the endoplasmic reticulum (ER). This stress response aims to restore homeostasis in the ER by lowering protein translation rates, augmenting the number of folding chaperones, and increasing the degradation of misfolded proteins. This is achieved through different pathways originating from three different ER-stress sensors that are activated by unfolded proteins in the ER (1). One sensor is inositol-responsive enzyme 1 (IRE1), which catalyzes splicing of transcription factor X-box–binding protein 1 (XBP1) mRNA, enabling translation of functional XBP1 and up-regulation of target genes encoding ER chaperones and components of the ER-associated protein degradation pathway (2). A second sensor is activating transcription factor 6 (ATF6), which induces various stress-related genes following its translocation to the Golgi and subsequent proteolytic activation (3). The third sensor is protein kinase R–like endoplasmic reticulum kinase, which phosphorylates the α subunit of eIF2α at Ser-51 to reduce global translation while enhancing the translation of specific stress response genes such as ATF4, CHOP, and PPP1R15A (4). The latter branch of the UPR is also part of the more general integrated stress response (ISR) pathway. Importantly, the UPR and ISR are both regulated by a negative feedback loop that involves the delayed expression of PPP1R15A, also known as growth arrest and DNA damage–induced protein 34 (GADD34) or R15A, which forms a trimmeric complex with protein phosphatase 1 (PP1) and G-actin (5, 6). This PP1 holoenzyme counteracts UPR and ISR signaling through dephosphorylation of eIF2α at Ser-51. Inhibition of eIF2α phosphatase(s) is generally considered an attractive therapeutic strategy for prolonging UPR signaling, as it gives cells more time to cope with the detrimental effects of unfolded proteins, a hallmark of several neurodegenerative diseases (7, 8).

Guanabenz is an aminoguanidine-type α2 adrenoreceptor agonist developed during the 1980s as an anti-hypertension drug but was later identified in a yeast-based screen as a compound with anti-prion activity (9). Subsequently, several groups demonstrated cytoprotective effects of guanabenz (analogs) in murine models of neurodegenerative diseases, such as amyotrophic lateral sclerosis (10), multiple sclerosis (11), and vanishing white matter disease (12). The cytoprotective effects of guanabenz (analogs) have been explained by its ability to maintain eIF2α phosphorylation through inhibition of PP1:R15A/B (13). However, the molecular mechanism underlying the cytoprotective action of guanabenz (analogs) is controversial (14–16). Indeed, the proposed effects of guanabenz (analogs) on PP1: R15 complexes include such diverse mechanisms as disruption of the PP1:R15A interaction (10, 13), interference with the recruitment of eIF2α (17), and targeting of R15B (a constitutively expressed R15 variant) for proteolytic degradation (18). Moreover, guanabenz (analogs) had no effect on the composition or activity of an in vitro reconstituted PP1:R15A:G-actin

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This article contains Figs. S1 and S2.

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§ The abbreviations used are: UPR, unfolded protein response; ER, endoplasmic reticulum; ISR, integrated stress response; TNF, tumor necrosis factor; CTRL, control; CHO, Chinese hamster ovary; ANOVA, analysis of variance.
denzyme (16). Furthermore, guanabenz retained its cytoprotective effects in cells and organisms that lacked R15A or only expressed nonphosphorylatable eIF2α (S51A) (15). Despite the uncertainties concerning their mechanism of action, guanabenz (analogos) remain attractive therapeutics. Guanabenz itself has rapidly progressed to clinical trials using a drug-repurposing approach. A phase I clinical trial to evaluate the pharmacokinetics of guanabenz in multiple sclerosis patients has been completed (NCT02423083), and a phase II clinical trial for ALS (EudraCT no. 2014-005367-32) and early-childhood onset vanishing white matter disease (EudraCT no. 2017-001438-25) are ongoing.

Robenidine (1,2-bis[(E)-(4-chlorophenyl)methylideneamino]-guanidine) is an aminoguanidine that has been used since the early 1970s to prevent coccidian infections in rabbits, chickens, and turkeys (19, 20). Often, animals are fed with robenidine-supplemented pellets (50 mg/kg of food) for their entire life, except for a washout period before slaughter. The chronic and widespread use of robenidine as a food supplement indicates that it is safe and well-tolerated, making it a suitable candidate therapeutic agent for humans. Furthermore, robenidine belongs to the aminoguanidine compound class, which represents a diverse group of bioactive compounds used for the treatment of a broad variety of diseases ranging from bacterial infections to cancer and diabetes, and many of these compounds are approved for human use (21). Despite its long history of use in animals, there have been no in-depth examinations of robenidine as a potential therapeutic agent for the treatment of human diseases. Here we show that robenidine and guanabenz show similar cytoprotective effects in stressed cells. Furthermore, both compounds reduced the expression of UPR and ISR markers in stressed cells. Finally, using mutant cell lines and split-luciferase sensors, we demonstrate that the cytoprotective effects of robenidine do not involve changes in eIF2α phosphorylation or the eIF2α phosphatase PP1:R15A. Our data identify robenidine as a novel aminoguanidine with therapeutic potential for the treatment of protein misfolding diseases.

Results and discussion

Robenidine is a structural and functional analog of guanabenz

Using the PubChem search engine, we noticed structural similarities between robenidine and guanabenz (Fig. 1A). In contrast to guanabenz, robenidine has two substituted phenyl rings attached to its central aminoguanidine scaffold. In addition, these phenyl rings are para-chloro-substituted, whereas the phenyl ring of guanabenz has two ortho-chloro substitutions. Further mining of the PubChem BioAssay database revealed that guanabenz and robenidine were both active in a high-throughput screen against the malaria-causing protozoan Plasmodium falciparum (Fig. 1B) (National Center for Biotechnology Information, PubChem BioAssay Database, AID 504834, https://pubchem.ncbi.nlm.nih.gov/bioassay/504834, accessed July 28, 2018). Several anti-malaria drugs target the apicoplast (a nonphotosynthetic plastid) through interference with its translational machinery (23). Interestingly, a similar mechanism of action was proposed for the cytoprotective properties of guanabenz in mammalian cells undergoing unfolded protein stress (24). These findings prompted us to explore whether robenidine has cytoprotective properties similar to those of guanabenz.

Cytoprotective effects of robenidine

The ER stress–induced UPR hampers cell cycle progression and proliferation. Indeed, treatment of HeLa cells with tunicamycin, an inhibitor of protein glycosylation that induces accumulation of misfolded proteins and activates the UPR, caused a dose-dependent decrease in proliferation, as derived from confluency assays with live-cell microscopy (Fig. 2A). This effect of tunicamycin can be rescued by guanabenz (13, 17). Likewise, 5–20 μM robenidine reversed the proliferation defect of tunicamycin-treated HeLa cells in a dose-dependent manner (Fig. 2B). However, at higher concentrations of robenidine, this cytoprotective effect was lost. We selected 15 μM robenidine as an optimal concentration for treatment, as it produced nearly maximal cytoprotective effects but was sufficiently separated from the higher, toxic concentrations. The cytoprotective effect of 15 μM robenidine was detected at relatively narrow tunicamycin concentrations of 400–800 ng/ml (Fig. 2C), similar to what has been reported for guanabenz (15). Guanabenz and robenidine were similarly cytoprotective at a concentration of 15 μM (Fig. 2D).

To evaluate whether drug–drug interactions exist between robenidine and guanabenz, we performed an isobologram analysis (25). For this purpose, we first generated dose–response curves to accurately determine their EC50 values (Fig. 2E). These studies indicated that, on average, guanabenz is 10–15 times more potent than robenidine. Next, the isobologram was drawn by connecting the two axis intersection points (i.e. the EC50 for each drug alone), according to the “Loewe additivity” principle. Robenidine and guanabenz drug combinations were tested at 10:1 and 15:1 ratios, and the corresponding EC50 values of these combination regimens were plotted against the isobologram (Fig. 2F). The 95% confidence interval of the combination treatments overlapped with the Loewe additivity line, indicating that there are no synergistic or antagonistic interactions between robenidine and guanabenz.

Figure 1. Structural and bioactive similarities between robenidine and guanabenz. A, chemical structure of guanabenz and robenidine. B, the inhibitory potency (EC50) of guanabenz and robenidine in a quantitative high-throughput screen against P. falciparum apicoplast development. The data were extracted from the PubChem database using the indicated BioAssay assay identification numbers.
Cytoprotective effects of robenidine

Pyrimethamine shares structural features with robenidine, as it also has a central guanidine scaffold and a para-chloro-substituted phenyl ring (Fig. S1A). Moreover, pyrimethamine also displays potent anti-malaria activity (26). However, pyrimethamine was lethal at 10 μM (data not shown) and did not promote survival of tunicamycin-treated HeLa cells at a sublethal dose of 3 μM (Fig. S1B).

Robenidine inhibits TNFα-induced NF-κB activation

Guanabenz inhibits the TNFα-induced activation of the transcription factor NF-κB (27). However, the concentration of guanabenz (200 μM) that was used in the latter study is toxic and far exceeds the concentrations that are routinely used to study its cytoprotective effects in cultured cells. Therefore, we tested whether robenidine and guanabenz affect NF-κB activation at a more optimal concentration of 15 μM (Fig. 2). To this end, HEK293 cells were transfected with an NF-κB–driven luciferase reporter plasmid and treated with TNFα concentrations of 0.1–10 ng/ml in the absence (CTRL) or presence of 15 μM robenidine (Fig. 3A). Robenidine significantly inhibited NF-κB activation by TNFα concentrations between 0.1 and 0.3 ng/ml, with a maximal effect (inhibition of ~25%) at 0.3 ng/ml TNFα. A similar level of inhibition was observed with 15 μM guanabenz (Fig. 3B). We ruled out that the observed reduction in bioluminescence was due to loss of viable cells, as detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-dipheny lurazolium bromide assays (Fig. S2).

Robenidine does not affect the interaction between R15A and PP1 or elf2α

The cytoprotective effects of guanabenz (analogs) have been explained by an inhibitory effect on the elf2α phosphatase PP1:R15A, resulting from disruption of either the PP1:R15A interaction (13) or recruitment of the substrate elf2α (17). To exam-

Figure 2. Robenidine protects HeLa cells from tunicamycin-induced toxicity. A, proliferation of HeLa cells treated with the indicated concentrations of tunicamycin for 3 days. B, dose–dependent rescue of HeLa cells treated with 400 ng/ml tunicamycin (3 days) by the indicated concentrations of robenidine. The data were analyzed with one-way ANOVA using Dunnett’s post hoc test. C, proliferation of HeLa cells treated for 4 days with or without 15 μM robenidine at the indicated concentrations of tunicamycin. Two-way ANOVA indicated that both tunicamycin (F5,24 = 197.1, p < 0.001) and robenidine (F1,24 = 130.7, p < 0.001) significantly affected proliferation of the cells. Additionally, there was an interaction of the two factors regarding their effects on proliferation (F5,24 = 13, p < 0.001). Tukey’s post hoc test was used to compare control versus robenidine for every concentration of tunicamycin. D, comparison of the cytoprotective effect of 15 μM guanabenz or robenidine in HeLa cells treated with tunicamycin for 3 days. The data were analyzed with one-way ANOVA using Tukey’s post hoc test. E, dose–response relationship of the cytoprotective effect of robenidine or guanabenz on HeLa cells treated with 400 ng/ml tunicamycin for 3 days. Curves were fitted using a four-parameter model with variable slope. For each concentration, the average of three technical repeats is presented. F, isobologram analysis of robenidine (R) and guanabenz (G) combination treatments at the indicated dose ratios. EC50 values of dose–response experiments are plotted. Black open and closed dots represent the mean with 95% confidence intervals of 15:1 and 10:1 combination treatments, respectively. The Loewe additivity line (black line) represents all theoretical robenidine–guanabenz combinations resulting in a 50% cytoprotective effect, assuming that both drugs interact in an additive manner. Datapoints to the left of the curve are synergistic combinations, whereas datapoints to the right are antagonistic. All panels except F show one of at least three independent experiments. A–D show individual datapoints of at least three technical replicates, with bar graphs indicating the mean and error bars the standard deviation (n.s., not significant, p > 0.05; *, p < 0.05; **, p < 0.01; ***, p < 0.001).
Figure 3. Robenidine inhibits NF-κB activation by TNFα. A, HEK293 cells were transfected with an NF-κB–driven luciferase reporter plasmid. After 24 h, the cells were treated for an additional 24 h with vehicle (CTRL) or 15 μM robenidine and the indicated concentrations of TNFα. NF-κB activity was derived from firefly bioluminescence assays and plotted as a percentage of the activity in cells treated with TNFα alone (CTRL). Two-way ANOVA indicated that both TNFα (F5,231 = 2.6, p = 0.024) and robenidine (F1,231 = 130.7, p < 0.001) significantly affected the activation of NF-κB. Additionally, there was an interaction of the two factors regarding their effects on NF-κB activation (F5,231 = 3.3, p = 0.0065). Tukey's post hoc test was used to compare control versus robenidine for every concentration of TNFα. B, the same as in A, but cells were treated with 0.3 ng/ml TNFα with or without the indicated concentrations of robenidine and/or guanabenz. Statistical analysis was performed using one-way ANOVA with Tukey’s post hoc test. Datapoints show all technical replicates from three independent experiments, with the means represented by bar graphs and error bars indicating the standard deviation (n.s., not significant, *p < 0.05; **p < 0.01; ***p < 0.001).

Robenidine inhibits the UPR and ISR

The cytoprotective effects of guanabenz under conditions of ER stress have been attributed to its inhibitory effect on the UPR and ISR (10). To investigate whether robenidine acts through the same mechanism, we used CHO cells containing reporters for both the ISR (CHOP::GFP) and the UPR branch that involves IRE1 (XBP1s::Turquoise) (15). Flow cytometry analysis disclosed clear induction of both genes following addition of 175 ng/ml tunicamycin for 55 h (Fig. 5A). Cotreatment of these cells with 15 μM robenidine or guanabenz decreased the expression of CHOP and XBP1s by 40–60% (Fig. 5B).

A previous study showed that guanabenz (analogs) retain their cytoprotective activity in cells that express a nonphosphorylatable eIF2α mutant (15). This finding contradicts the proposal that guanabenz’s cytoprotective effects are mediated by an increased phosphorylation of eIF2α at Ser-51 (8, 24). To examine whether inhibition of the expression of CHOP and XBP1s by robenidine and guanabenz is mediated by increased eIF2α phosphorylation, we used a variant CHO reporter cell line that expresses nonphosphorylatable eIF2α (S51A). Consistent with ISR-dependent CHOP activation, the activation of the CHOP::GFP reporter was less pronounced in the cell line expressing eIF2α S51A (compare Fig. 5, A and C). Nonetheless, both robenidine and guanabenz inhibited the induction of CHOP::GFP and XBP1s::Turquoise in the mutant cell line upon treatment with tunicamycin (Fig. 5D). These results demonstrate that robenidine, like guanabenz, does not exert its cytoprotective effect by increasing the phosphorylation of eIF2α at Ser-51.

Conclusions

Here we identified robenidine as a new aminoguanidine compound with cytoprotective properties similar to those described previously for the analogs salubrinal (28), guanabenz (13), sephin1 (10), and raphin1 (18). Indeed, robenidine reduced the antiproliferative effects of tunicamycin (Fig. 2), dampened TNFα-induced NF-κB activation (Fig. 3), and inhibited the expression of ISR and UPR markers (Fig. 5). We also found that the cytoprotective effects of robenidine and guana-
Benz could not be explained by disruption of R15A:PP1 or R15A:eIF2\(\alpha\)/H9251 and were not mediated by changes in the phosphor-
ylation state of eIF2\(\alpha\)/H9251 (Fig. 5). These results therefore demon-
strate that the phenotypic effects of these drugs cannot be
mediated by inhibition of the R15A–PP1–actin holoenzyme or
altered phosphorylation of eIF2\(\alpha\)/H9251 at Ser-51. Similar conclu-
sions were recently drawn for guanabenz and sephin1 (15, 28)
but are at variance with reports hinting at inhibitory effects of guana-
benz (analogs) on PP1:R15 assembly and its recruitment of
eIF2\(\alpha\)/H9251 as a substrate (10, 17).

Other targets of guanabenz (analogs) that have been pro-
gressed to underly their cytoprotective function are choles-
terol 25-hydroxylase (29), rRNA (30), and lipid membranes (31).
It is currently uncertain whether the antibacterial, anti-
inflammatory, and cytoprotective properties of robenidine
and guanabenz (analogs) arise from the same cellular tar-
get(s), but this seems likely, as both compounds had very
similar effects at high nanomolar to low micromolar concen-
trations. Identification of the cellular target(s) of robenidine
and guanabenz (analogs) remains an important goal for fur-
ther exploration, as it will aid the development of analogs with higher potency.

We show here, for the first time, that guanabenz and robenidine significantly inhibit the activation of NF-κB (Fig. 3). However, this effect was rather small (~25% inhibition) and therefore unlikely to contribute significantly to the cytoprotective properties of these compounds. At present, we cannot exclude that the inhibitory effects of robenidine and guanabenz in the NF-κB and CHOP/XBP1s reporter cell lines (Figs. 3 and 5) are (partially) explained by a general inhibitory effect on protein

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Figure 5. Robenidine inhibits the UPR and ISR independent of eIF2α phosphorylation. A, representative graph of the two-dimensional plot of the fluorescence signals from WT CHO reporter cells stably transduced with both a CHOP::GFP reporter (reflecting ISR activity) and a XBP1::Turquoise reporter (reflecting Ire1α activity), treated with or without tunicamycin and/or robenidine and analyzed with flow cytometry. Histograms of the distribution of the two reporter signals in the three treatment conditions are plotted on the corresponding axes. B, scatterplots of the median fluorescence signals of WT CHO reporter cells treated for 55 h with the indicated concentrations of tunicamycin, robenidine, and guanabenz and analyzed by flow cytometry. Bar graphs indicate the mean and error bars the standard deviation. C, as in Fig. 4A but with CHO reporter cells expressing eIF2α-S51A. D, as in Fig. 4B, but with CHO reporter cells expressing eIF2α-S51A. For B and D, all technical replicates from four independent experiments were plotted. Statistical analysis was performed using one-way ANOVA with Dunnett’s post hoc test (**, p < 0.01; ***, p < 0.001).
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translation, as the assays rely on the production of luciferase and fluorescent protein for signal readout.

Guanabenz, sephin1, and raphin1 have shown promising results in various murine models of neurodegenerative diseases (10, 11, 18). We speculate that robenidine has similar therapeutic potential, in particular because its widespread industrial use for nearly 50 years as a food additive and coccidostat documents its safety and tolerance, at least in animals. Pharmacokinetic studies in rabbits showed that robenidine is orally available and has a moderate half-life of around 12 h (32). Micromolar serum concentrations are reached with a single oral dose, which are the concentrations needed for cytoprotective effects in vitro (Fig. 2). Our isobolographic analysis suggests that there is no benefit of combining robenidine and guanabenz (analogs). Nonetheless, our studies indicate that robenidine is an attractive candidate for preclinical testing in animal models of neurodegenerative diseases.

Experimental procedures

Materials

The following antibodies were used: FLAG (Sigma-Aldrich, F1804), β-actin (GeneTex, GTX26276), PP1 (made in-house (22)), elf2α (Thermo Fisher Scientific, AHO0802). Secondary HRP-tagged antibodies were purchased from Agilent-Dako. Guanabenz was purchased from TCI Europe (G0456), robenidine from Acros Organics NV (459400010), tunicamycin from SanBio BV (11445), human TNFα from Sigma-Aldrich (SRP3177), coelenterazine from Carl Roth GmbH (4094.3), D-luciferin from SanBio BV (14682-50), and pyrimethamine from Acros Organics NV (461200010). Split-luciferase sensors were made using ligation-independent cloning techniques. LgBiT was fused via a 15-amino-acid flexible linker (GSSGGGGSGGGGSG) to the N terminus of full-length P1α or elf2α. SmBiT was inserted at residue 537 of R15A with a five-residue flexible linker (GSTSG) at both ends. A constitutively active NanoBiT luciferase that served as a control consisted of LgBiT fused to SmBiT via a 15-amino-acid flexible linker.

PubChem database searching

The guanabenz entry was accessed (https://pubchem.ncbi.nlm.nih.gov/) under PubChem CID 5702063. Robenidine (CID 9570438) was identified through the “related record” search function, focusing on substituted aminoguanidines. High-throughput assays that tested both compounds were identified manually under the “BioAssay results” section. Subsequently, the BioAssay assay identification numbers were used to query the obtained potency for each compound. MarvinSketch was used for drawing and exporting chemical structures (MarvinSketch, 17.21.0, ChemAxon).

Cytoprotection experiments

HeLa cells were plated at ~10% confluence in transparent 96-well plates. Three technical replicates were used per condition. Treatment with compounds started at the time of seeding. All compounds were dissolved in DMSO. The final concentration of DMSO was the same under all conditions of each experiment and never exceeded 0.5%. Proliferation was measured using an IncuCyte ZOOM system and analyzed using IncuCyte ZOOM software (Essen BioScience).

Isobologram analysis

Cytoprotection experiments were performed as described above. The EC50 isobole was generated by performing three dose–response experiments for both robenidine and guanabenz. From these data, the average EC50 values for both drugs were calculated and used as intercepts of the isobologram. Next, dose–response experiments were performed for the indicated robenidine–guanabenz combination ratios, and the calculated EC50 values were plotted individually on the isobologram. Calculation of EC50 values was performed using the four-parameter model with variable slope with GraphPad Prism V5.

NF-κB reporter assay

HEK293 cells were transfected with an NF-κB–driven firefly luciferase reporter plasmid (Addgene, 14886) for 24 h. Subsequently, the cells were seeded in white opaque 96-well plates at ~50% confluence and treated with the indicated concentrations of TNFα, guanabenz, and/or robenidine. A minimum of four technical replicates were used per condition. The final concentration of DMSO was identical under all experimental conditions and never exceeded 0.5%. One day after seeding and treatment of the cells, firefly activity was measured on a Luminoskan Ascent (Thermo Scientific) after replacement of the cell culture medium with homemade firefly assay buffer (0.5% Triton X-100, 50 μM Tris (pH 7.4), 5 mM MgCl2, 20 μM pyrophosphate, 500 μM ATP, and 150 μg/ml D-luciferin). As firefly luciferase exhibits flash-type kinetics, the signal was measured in the stable phase, which typically occurred 3–4 min after incubation with firefly assay buffer. To account for possible variations in cell proliferation between the different conditions, a parallel plate of HEK293 cells was seeded and treated identically to the firefly assay plate. At the time of luciferase measurement, cell proliferation was measured in the parallel plate using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. For this, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dissolved in Tris buffer (pH 7.4) was added at a final concentration of 0.5 mg/ml to the assay medium and incubated overnight. Formazan crystals were dissolved in DMSO and transferred to a transparent 96-well plate, and the absorbance was measured at 550 nm using a BioTek ELx808 microplate reader.

Flow cytometry with CHO reporter cell lines

CHO cells were plated in 6-well plates at ~20% confluence and treated with the indicated concentration of compounds for 55 h. Three technical replicates were included per condition. The final concentration of DMSO (~<0.5%) was the same under all conditions. Cell culture medium and compounds were refreshed halfway through the incubation period to prevent medium evaporation and stressing the cells. Subsequently, cells were washed, harvested in PBS, and used for flow cytometry. GFP and Turquoise expression were measured on a BD FACSCanto II HTS system using the following fil-
ters: GFP: excitation, 488 nm; emission, 500–560 nm; Turquoise: excitation, 405 nm; emission, 400–500 nm. At least 10,000 cells were recorded per sample. Dead cells were excluded based on the scatter profile of a calibration sample containing 50% dead and 50% live cells. Data were processed using FlowJo software (Tree Star, Inc.).

**NanoBiT split-luciferase assay**

HEK293 cells were plated at ~20% confluency and transfected with the indicated split-luciferase sensors for 48 h. To achieve sufficient sensor activity, sensors were cloned in vectors containing the cytomagalovirus promoter and not in the weaker TK promoter-driven vectors of the NanoBiT kit. Subsequently, the cells were harvested and resuspended in PBS. The luciferase substrate coelenterazine was added to the cell suspension at a final concentration of 25 μM, and cells were dispensed at ~10,000 cells/well in white 96-well plates. Luciferase activity was measured on a Luminoskan Ascent (Thermo Scientific). When the cells were treated with compounds, they were reseeded in opaque 96-well plates after the 48-h transfection period. A minimum of three technical replicates were used per condition. To correct for effects of the compounds on cell proliferation or expression of the split-luciferase sensors, a third batch of cells, expressing a constitutively active NanoBiT luciferase (see “Materials”), was seeded and treated in parallel.

**Cell culture**

HEK293 cells (ATCC) were cultured in Dulbecco’s modified Eagle’s medium with high glucose (4,500 mg/liter glucose, Sigma-Aldrich) containing 10% fetal calf serum (Sigma-Aldrich), Eagle’s medium with high glucose (4,500 mg/liter glucose). Transfections were performed with jetPRIME (Polyplus). All cell lines were mycoplasma-free.

**Biochemical techniques**

HEK293 cells were lysed with lysis buffer (50 mM Tris-HCl (pH 7.4), 0.01% saponin, 150 mM NaCl, and 10% glycerol) supplemented with protease inhibitors (0.5 mM phenylmethanesulfonyl fluoride, 0.5 mM benzamidine, and 5 μM leupeptin). Subsequently, lysates were sonicated with three 10-s cycles at high intensity and cleared by centrifugation at 10,000 × g. For FLAG traps, cleared cell lysates were incubated with 20 μl of FLAG affinity beads (Sigma, catalog no. P4333) for 2–3 h at 4 °C. The beads were then washed once with PBS and subjected to immunoblotting. SDS-PAGE gel electrophoresis was performed with 10% or 4%–12% BisTris (NuPAGE, Invitrogen). Immunoblots were visualized using ECL reagent (PerkinElmer Life Sciences) in an ImageQuant LAS4000 imaging system (GE Healthcare). The signals were quantified using ImageJ (National Institutes of Health).

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**References**

1. Walter, P., and Ron, D. (2011) The unfolded protein response: from stress pathway to homeostatic regulation. Science 334, 1081–1086 CrossRef Medline
2. Mori, K. (2000) Tripartite management of unfolded proteins in the endoplasmic reticulum. Cell 101, 451–454 CrossRef Medline
3. Shen, J., Chen, X., Hendershot, L., and Prywes, R. (2002) ER stress regulation of ATF6 localization by dissociation of Bip/GRP78 binding and unmasking of Golgi localization signals. Dev. Cell 3, 99–111 CrossRef Medline
4. Cao, S. S., and Kaufman, R. J. (2012) Unfolded protein response. Curr. Biol. 22, R622–R626 CrossRef Medline
5. Chambers, J. E., Dalton, L. E., Clarke, H. J., Malzer, E., Dominicus, C. S., Patel, V., Moorhead, G., Ron, D., and Marciniak, S. J. (2015) Actin dynamics tune the integrated stress response by regulating eukaryotic initiation factor 2alpha dephosphorylation. Elife CrossRef Medline
6. Yan, Y., Crespillo-Casado, A., Clarke, H. J., Harding, H. P., Marciniak, S. J., Read, R. J., and Ron, D. (2015) G-actin provides substrate-specificity to eukaryotic initiation factor 2α holophosphatases. Elife CrossRef Medline
7. Sundaram, J. R., Lee, I. C., and Shenolikar, S. (2017) Translating protein phosphatase research into treatments for neurodegenerative diseases. Biochem. Soc. Trans. 45, 101–112 CrossRef Medline
8. Schneider, K., and Bertolotti, A. (2015) Surviving protein quality control catastrophes: from cells to organisms. J Cell Sci. 128, 3861–3869 CrossRef Medline
9. Tribouillard-Tanvier, D., Bérinque, V., Desban, N., Gug, F., Bach, S., Voisset, C., Galons, H., Laude, H., Vilette, D., and Blondel, M. (2008) Antihypertensive drug guanabenz is active in vivo against both yeast and mammalian prions. PLoS ONE 3, e1988 CrossRef Medline
10. Das, I., Krzyzosiak, A., Schneider, K., Wrbetz, L., Antonio, M. D., Barry, N., Sigurdardottir, A., and Bertolotti, A. Preventing proteostasis diseases by selective inhibition of a phosphate regulatory subunit. Science 348, 239–242 CrossRef Medline
11. Way, S. W., Podojil, J. R., Clayton, B. L., Zaremba, A., Collins, T. L., Kunjamma, R. B., Robinson, A. P., Brugarolas, P., Miller, R. H., Miller, S. D., and Popko, B. (2015) Pharmaceutical integrated stress response enhancement protects oligodendrocytes and provides a potential multiple sclerosis therapeutic. Nat. Commun. 6, 1–13 CrossRef Medline
12. Dooves, S., Bugiani, M., Wisse, L. E., Abbink, T. E. M., van der Knaap, M. S., and Heine, V. M. (2018) Bergmann glia translocation: a new disease marker for vanishing white matter identifies therapeutic targets of guanabenz treatment. Neuropathol Appl. Neurol. Biol. 44, 391–403 Medline
13. Tsaytler, P., Harding, H. P., Ron, D., and Bertolotti, A. (2011) Selective inhibition of a regulatory subunit of protein phosphatase 1 restores proteostasis. Science 332, 91–94 CrossRef Medline
14. Peti, W., Nairn, A. C., and Page, R. (2013) Structural basis for protein phosphatase 1 regulation and specificity. FEBS J. 280, 596–611 CrossRef Medline
15. Crespillo-Casado, A., Chambers, J. E., Fischer, P. M., Marciniak, S. J., and Ron, D. (2017) PPI1R15A-mediated dephosphorylation of eIF2α is unaffected by Sephini or guanabenz. Elife 6, pii: e26109 CrossRef Medline
16. Crespillo-Casado, A., Claes, Z., Choy, M. S., Peti, W., Bollen, M., and Ron, D. (2018) A Sephin1 insensitive tripartite holophosphatase dephosphorylates translation initiation factor 2α. J. Biol. Chem. 293, 7766–7776 CrossRef Medline
17. Carrara, M., Sigurdardottir, A., and Bertolotti, A. (2017) Decoding the selectivity of eIF2α holophosphatases and PPI1R15A inhibitors. Nat. Struct. Mol. Biol. 24, 708–716 CrossRef Medline
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18. Krzyzosiak, A., Sigurdardottir, A., Luh, L., Carrara, M., Das, I., Schneider, K., and Bertolotti, A. (2018) Target-based discovery of an inhibitor of the regulatory phosphatase PPP1R15B. *Cell* **174**, 1216–1228.e19 [CrossRef Medline]

19. Kantor, S., Kennett, R. L., Jr., Waletzky, E., and Tomcuicik, A. S. (1970) 1,3-Bis(p-chlorobenzylideneamino)guanidine hydrochloride (robenzidene): new poultry anticoccidial agent. *Science* **168**, 373–374 [CrossRef Medline]

20. European Food Safety Authority (2011) Scientific opinion on safety and efficacy of Cycostat® 66G (robenidine hydrochloride) for rabbits for breeding and fattening. *EFSA J.* **9**, 2102 [CrossRef Medline]

21. Sańczewski, F., and Balewski Ł. (2013) Biological activities of guanidine compounds, 2008–2012 update. *Expert Opin. Ther. Pat.* **23**, 965–995 [CrossRef Medline]

22. Van Dessel, N., Beke, L., Görnemann, J., Minnebo, N., Beullens, M., Tanuma, N., Shima, H., Van Eynde, A., and Bollen, M. (2010) The phosphatase interactor NIPP1 regulates the occupancy of the histone methyltransferase EZH2 at Polycomb targets. *Nucleic Acids Res.* **38**, 7500–7512 [CrossRef Medline]

23. Botté, C. Y., Dubar, F., McFadden, G. I., Maréchal, E., and Biot, C. (2012) *Plasmodium falciparum* apicoplast drugs: targets or off-targets? *Chem. Rev.* **112**, 1269–1283 [CrossRef Medline]

24. Tsaytler, P., and Bertolotti, A. (2013) Exploiting the selectivity of protein phosphatase 1 for pharmacological intervention. *FEBS J.* **280**, 766–770 [CrossRef Medline]

25. Foucquier, J., and Guedj, M. (2015) Analysis of drug combinations: current methodological landscape. *Pharmacol. Res. Perspect.* **3**, e00149 [CrossRef Medline]

26. McCollum, A. M., Poe, A. C., Hamel, M., Huber, C., Zhou, Z., Shi, Y. P., Ouma, P., Vulule, J., Bioland, P., Slutsker, L., Barnwell, J. W., Udhayakumar, V., and Escalante, A. (2006) A Antifolate resistance in *Plasmodium falciparum*: multiple origins and identification of novel dhfr alleles. *J. Infect. Dis.* **194**, 189–197 [CrossRef Medline]

27. Nakajima, S., Chi, Y., Gao, K., Kono, K., and Yao, J. (2015) elf2α-independent inhibition of TNF-α–triggered NF-κB activation by salubrinal. *Biol. Pharm. Bull.* **38**, 1368–1374 [CrossRef Medline]

28. Boyce, M., Bryant, K. F., Jousse, C., Long, K., Harding, H. P., Scheuner, D., Kaufman, R. J., Ma, D., Coen, D. M., Ron, D., and Yuan, J. (2005) A selective inhibitor of elf2α dephosphorylation protects cells from ER stress. *Science* **307**, 935–939 [CrossRef Medline]

29. Perego, J., Mendes, A., Bourbon, C., Camosseto, V., Combes, A., Liu, H., Manh, T. V., Dalet, A., Chasson, L., Spinelli, L., Bardin, N., Chiche, L., Santos, M. A. S., Gatti, E., and Pierre, P. (2018) Guanabenz inhibits TLR9 signaling through a pathway that is independent of elf2α dephosphorylation by the GADD34/PP1c complex. *Sci. Signal.* **11**, eaam8104 [CrossRef Medline]

30. Nguyen, P. H., Hammoud, H., Halliez, S., Pang, Y., Evrard, J., Schmitt, M., Oumata, N., Bourguignon, J. J., Sanyal, S., Beringue, V., Blondel, M., Bihel, F., and Voisset, C. (2014) Structure-activity relationship study around guanabenz identifies two derivatives retaining antiprion activity but having lost α2-adrenergic receptor agonistic activity. *ACS Chem. Neurosci.* **5**, 1075–1082 [CrossRef Medline]

31. Ogunniyi, A. D., Khazandi, M., Stevens, A. J., Sims, S. K., Page, S. W., Garg, S., Venter, H., Powell, A., White, K., Petrovski, K. R., Laven-Law, G., Tótoli, E. G., Salgado, H. R., Pi, H., Coombs, G. W., et al. (2017) Evaluation of robenidine analog NCL195 as a novel broad-spectrum antibacterial agent. *PLoS ONE* **12**, e0183457 [CrossRef Medline]

32. Tulliez, J. E., Durand, E. F., and Bories, G. F. (1982) Metabolic fate and pharmacokinetics of tissue residues of the anticoccidial drug robenidine in the rabbit: incidence of coprophagy on its bioavailability. *J. Agric. Food Chem.* **30**, 1071–1075 [CrossRef Medline]