A novel approach towards recovery of function of mutant proteins by slowing down translation

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Running title: Inhibition of translation improves folding

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Capsule

Background: Current strategies to alleviate protein misfolding include manipulation of chaperones, proteasome or autophagy.

Results: Mild translation inhibition disproportionally blocked production of misfolded proteins and improved mutant CFTR function.

Conclusion: Translation slowdown improves folding of newly-synthesized proteins in mammalian cells and recovers mutant protein function.

Significance: Attenuation of translation could be a novel approach towards treatment of protein misfolding disorders.

Summary

Protein homeostasis depends on a balance of translation, folding and degradation. Here we demonstrate that mild inhibition of translation results in a dramatic and disproportional reduction of production of misfolded polypeptides in mammalian cells, suggesting an improved folding of newly synthesized proteins. Indeed, inhibition of translation elongation, that slightly attenuated levels of a mutant protein copGFP, significantly enhanced its function. In contrast, inhibition of translation initiation had minimal effects on copGFP folding. On the other hand, mild suppression of either translation elongation or initiation corrected folding defects of the disease-associated mutant CFTR F508del. We propose that modulation of translation can be used as a novel approach to improve the overall proteostasis in mammalian cells, as well as functions of disease-associated mutant proteins with folding deficiencies.

Introduction

Maintenance of functional cellular proteome relies on the intricate balance of protein synthesis, folding and degradation. In the absence of stresses the newly synthesized misfolded proteins pose the main challenge to cellular protein homeostasis. Molecular chaperones, the ubiquitin-proteasome system and autophagy play an important role in handling these species (1).

Since abnormal proteins cause many life threatening diseases, there has been an ongoing interest in approaches that reduce the accumulation of misfolded species. In fact, a number of these diseases could be partially alleviated by induction of autophagy (2,3), and some inducers of autophagy have shown efficacy in animal models (4,5). Additionally, certain protein misfolding pathologies can be alleviated by overexpression of heat shock proteins (6,7), and numerous attempts have been made to develop inducers of molecular chaperones (8,9).
This approach can be used for another group of disorders associated not with gain of toxicity of misfolded polypeptides, but with insufficient function of mutant proteins. For example, the adverse effects of mutant lysosomal glucocerebrosidase (GC) in Gaucher disease (10), can be alleviated by increase in the chaperone capacity of cells (10). Misfolded molecules of the mutant GC are rapidly degraded via the ERAD pathway. However, folded species of GC escape ERAD, proceed to lysosomes, and function normally. Accordingly, induction of ER chaperones improved folding of GC and increased its levels (10). Analogous effects were seen with mutant β-hexosaminidase A, the causative agent of the Tay-Sachs disease (10). This approach may be useful in the rescue of other mutant proteins transported via the ER, such as mutant CFTR, which causes cystic fibrosis. The most prevalent disease-causing mutation in CFTR is CFTR F508del, which results in poor folding and rapid degradation of the polypeptide via the ERAD pathway (11). However, a fraction of molecules, that has acquired the correct fold, escapes ER degradation and is functional (12,13). Correcting the folding defect of mutant CFTR F508del by chemical chaperones seems to be a successful strategy in therapeutics discovery.

Here we propose that, in addition to induction of chaperones, slowing down translation may have beneficial effects on the production of functional mutant proteins. It might decrease the pool of chaperone substrates and thereby increase the capacity of the folding system. Moreover, it may improve co-translational folding (14) . This possibility is supported by the following circumstantial lines of evidence: (a) slow-translating stretches of mRNA provide for proper domain folding in vitro and in E.coli (15), (b) a mutation that reduces elongation rate of E.coli ribosomes improves folding of recombinant eukaryotic proteins which have evolved to be translated by comparatively slower eukaryotic ribosomes (16), and (c) it is a common practice to improve production of correctly folded recombinant proteins in E.coli by reducing growth temperature. However, there is no direct evidence that slowing down translation can improve folding in eukaryotic cells.

Here we demonstrate that mild inhibition of translation significantly improves overall protein folding in mammalian cells. Furthermore, slowing down translation improves folding of mutant proteins, suggesting a novel approach towards a cure for protein misfolding disorders.

Materials and Methods

Reagents and Antibodies

MG132 was purchased from Biomol (Farmingdale, NY, USA); emetine, forskolin, cycloheximide, IBMX and genistein - from SIGMA (St. Louis, MO, USA); hippuristanol was a kind gift of Dr. Pelletier; Shield1 – from CheminPharma (Framington, CT, USA), Antibodies: anti-p21 and anti-p53 were purchased from BD PharMingen (San Diego, CA, USA) and SantaCruz (Santa Cruz, CA); anti- multi-ubiquitin (FK2) – from MBL International Corporations (Woburn, MA, USA); anti-HA - and anti-β-actin  – from Cell Signaling (Danvers, MA, USA), mouse anti-GAPDH was purchased from Millipore, and anti-CFTR antibody (596), a kind gift from Dr. Jack Riordan (University of North Carolina at Chapel Hill via The Cystic Fibrosis Foundation).

Constructs

The retroviral expression construct with C-terminally tagged synphilin 1 (Syn-GFP) subcloned into pCXbsr vector was described before (17).

Copepod GFP (copGFP) was amplified from the vector pMaxGFP (Lonza, Allendale, NJ, USA) and was fused with the fk506-binding protein 12 (from the vector ppTuner, Clontech, Mountain View, CA, USA) by overlapping PCR. The product was then cloned into the lentiviral vector pTRIPz (Open Biosystems, Huntsville, AL, USA). A C-terminal hemagglutinin tag was also inserted downstream of copGFP by PCR. copGFP expression was controlled by the upstream tetracycline responsive element.
Cells cultures and Growth

HeLa (cervix carcinoma) cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, and MCF10A (human breast epithelial) cells - in DMEM/F-12 50/50 medium supplemented with 5% horse serum, 20 ng/mL epidermal growth factor, 0.5 µg/mL hydrocortisone, 10 µg/mL human insulin, and 100 ng/mL cholera toxin; all cultures were supplemented with L-glutamine as well as penicillin and streptomycin and grown at 37°C in the atmosphere of 5% CO₂.

copGFP expression was induced by the addition of 1µg/ml doxycycline and further stabilized by the addition of 5µM Shield1 (18).

Fischer Rat Thyroid (FRT) cell line stably expressing ΔF508-CFTR is a generous gift from Prof. Luis Galietta (Univ. of Genoa, Italy). Cells were grown according to (19).

For production of retroviruses HEK293T cells were co-transfected with a lentiviral plasmid and the helper plasmids expressing lentiviral proteins psPAX2 and pMD2.G. Supernatants containing the virus were collected 48 h after transfection.

Cell lysis and analysis

For analysis of levels of ubiquitination, cells were lysed with lysis buffer (40 mM Hepes, pH 7.5, 50 mM KCl, 1% Triton X-100, 2 mM DTT, 1 mM Na₂VO₄, 50 mM β-glycerophosphate, 50 mM NaF, 5 mM EDTA, 5 mM EGTA, 1 mM PMSF, 1 mM benzamidine, and 5 µg/ml each of leupeptin, pepstatin A, and aprotinin). Samples were adjusted to have equal concentration of total protein and subjected to PAAG electrophoresis followed by immunoblotting. The immunoblots represent a typical experiment repeated three times.

Microscopy: Aggresomes counting

For analysis with a fluorescence microscope cells were grown on Lab-Tek® Chambered Coverglasses (NUNC) pretreated with poly-L-lysine (Sigma). Fluorescence microscopy was performed at room temperature with Axiovert 200 (Carl Zeiss, Germany) microscope using 40x/0.75 or 100x/1.30 Oil objectives and the manufacturer’s AxioVision 4 software. GFP tagged proteins were observed with FITC filter set (AXIO. Images were gained with High Resolution Microscope Camera AxioCam MRC.

To assess the fraction of cells with a detectable aggresome the fluorescent cells were blindly counted in 10 randomly chosen fields to have more than 200 cells in total. Each counting experiment was repeated 3 times to assure reproducibility of the results. The error bars on the graphs represent standard errors.

copGFP fluorescence and levels

HeLa cell clones were plated at a density of 2x10⁴/well and allowed to adhere. copGFP expression was induced by the addition of 1µg/ml doxycycline and 5µM Shield1. Simultaneously, emetine or hippuristanol were titrated by two fold serial dilutions, as indicated. Cells were incubated for 6 hours at 37°C and then prepared for analysis by flow cytometry (FACS Calibur, Becton Dickinson). For immunoblotting, cells were plated in 12 well plates and later lysed in buffer containing 1% NP-40. Protein was quantified by BCA (Pierce, Thermo Fisher Scientific, Rockford, IL, USA) and equivalent total protein was loaded per well. copGFP expression was monitored by antibodies specific to the HA-tag (Clontech), while protein loading was monitored by β-actin levels.

FRT cell conductance assay

FRT cells were seeded at a density of ~125,000 cells per cm² onto HTS Transwell-24 well filter inserts (Corning, cat# 3378) and grown into epithelial cell monolayers as described in (19). Prior to the assay monolayers were treated on both sides with compound or vehicle (negative control) for 24 hours in a cell/tissue incubator (37°C, 5% CO₂).

After 24 hours of treatment, cell incubation media was replaced by a HEPES buffered physiological saline (HB-PS) as the serosal (bottom well) and mucosal (insert top) solution with composition: NaCl, 137 mM; KCl, 4.0 mM; CaCl₂, 1.8 mM; MgCl₂, 1 mM; HEPES, 10
mM; Glucose, 10 mM; pH adjusted to 7.4 with NaOH. The 24-well Transwell plate was then mounted onto a heated plate and transepithelial resistance was measured using a 4-channel Transepithelial Current Clamp (TECC) (EP Design, Bertem, Belgium). The assay was carried out at ~35-36°C well temperature. Resistance values were collected at ~10-15 min intervals. Four data points were measured to determine baseline resistance, three data points to determine transepithelial resistance after each addition of agonist (final concentrations: 10 µM forskolin, 100 µM 3-isobutyl-1-methylxanthine [IBMX] and 20 µM genistein) and antagonist (final concentration: 20 µM CFTRinh-172). IBMX and genistein were applied together. Agonists and antagonist were added to both the serosal and mucosal sides and pre-diluted to 10x concentrations in HB-PS.

Transepithelial conductance ($G_t$) was calculated from series resistance subtracted TECC measurements. Since CFTR mediates chloride ion passage across the epithelial monolayer, activation/inhibition of functional CFTR transport proteins results in a change in transepithelial conductance ($\Delta G_t$). The magnitude of $\Delta G_t$ therefore is a measure of functional CFTR surface expression.

To study the effect of test compounds on CFTR surface expression, the compound's dose-response characteristic was determined and compared with negative controls. $\Delta G_t$ mean values were calculated for each treatment condition and the $\Delta G_t$ ratio (cmpd/vehicle) for the CFTR specific inhibitor response was plotted for each test concentration.

**Results**

**The rate of translation influences generation of abnormal polypeptides**

In the previous work we studied formation of aggresome, an organelle that recruits small protein aggregates to the centrosome location (20,21), using GFP-tagged synphilin 1 (Syn-GFP) as a model (17). We found that this process is triggered by the buildup of newly synthesized aberrant proteins (22), which allowed assessing the levels of these species by monitoring aggresome formation. Moreover, with HeLa cells we observed that aggresome formation is extremely sensitive to inhibition of translation. To account for this unexpected effect we hypothesize that partial inhibition of translation disproportionally reduces the generation of aberrant polypeptides. We explored this possibility with another cell line MCF10A. Cells expressing Syn-GFP were incubated with the proteasome inhibitor MG132,
which led to a rapid formation of an aggresome (Fig. 1B, top panel). The translation inhibitor emetine suppressed the aggresome formation in a dose-dependent manner (Fig. 1A, B), indicating inhibition of production of misfolded polypeptides. To test whether emetine reduces generation of misfolded species preferentially, we quantitatively compared effects of emetine on aggresome formation and on protein synthesis. In the presence of the proteasome inhibitor accumulation of a de-novo synthesized short-lived protein, e.g. p53, directly reflects the rate of translation. Accordingly, to assess the effects of emetine on general translation we measured inhibition of accumulation of p53 formed (of note, accumulation of a distinct short-lived protein p21 (not shown), as well as of inducible copGFP (see below) displayed similar to p53 dose-dependence). As seen in Fig. 1A, the inhibition of the p53 synthesis was markedly less sensitive to emetine than the inhibition of aggresome formation. For example, 80 nM of emetine inhibited accumulation of p53 less than two fold, but decreased the fraction of cells forming detectable aggresomes almost ten-fold. Moreover, at low concentrations emetine markedly reduced the sizes of the remaining aggresomes (Fig. 1B). For example, diameters of aggresomes formed in the presence of 20 nM emetine were on average 3 times smaller than those seen without translation inhibition, demonstrating that even very mild inhibition of translation suppresses growth of an aggresome. Since aggresome formation depends on accumulation of abnormal newly-synthesized polypeptides (22), these data suggested that mild inhibition of translation disproportionally reduces generation of abnormal polypeptides.

To further evaluate effects of translation inhibitors on generation of abnormal polypeptides, we took advantage of the observation that the majority of proteins that are ubiquitinated and degraded by the ubiquitin-proteasome pathway are newly synthesized. Indeed, the levels of ubiquitinated species notably increased upon proteasome inhibition, but blocking translation with emetine prevented their build-up by 90-95% (Fig. 1C, D). A large fraction of newly synthesized species that are degraded by proteasome, and therefore accumulate upon its inhibition, are polypeptides that cannot fold normally. According to our hypothesis, improved protein folding due to the presence of low doses of emetine should disproportionally inhibit generation of ubiquitinated species compared to inhibition of translation. To test this possibility, MCF10A cells were incubated for 3.5 hours with MG132 and effect of various concentrations of emetine on cellular levels of ubiquitin conjugates and on the build-up of p53 were evaluated. Fig. 1C and D demonstrate that the build-up of ubiquitinated species was significantly more sensitive to emetine. This result supports our hypothesis that low concentrations of emetine specifically reduce a fraction of abnormal species among newly synthesized polypeptides. Taken together the data on aggresome formation and on the levels of ubiquitinated polypeptides indicate that inhibition of translation by about 50% almost entirely blocks generation of broad-spectrum misfolded polypeptides.

The described effect on cellular proteostasis was not specific to emetine but rather reflected mild translation inhibition. Indeed, another inhibitor cycloheximide similarly to emetine preferentially inhibited both aggresome formation and accumulation of ubiquitinated species (Fig. 2A and (23)). As most of the available ribosome inhibitors, these two reagents inhibit elongation of translation and thus slow-down growth of each polypeptide chain ribosomes.

We sought to test whether comparable reduction of the output of translation achieved without slowing-down a polypeptide growth, has similar effect on generation of abnormal species. Accordingly, we employed hippuristanol, which reduces protein synthesis by inhibiting translation initiation and thus reducing the number of active ribosomes (24). Hippuristanol elicited the same dose-responses for generation of ubiquitinated species, and for translation output (Fig. 2B). Unexpectedly, at low concentrations this inhibitor appeared to slightly increase protein synthesis resulting in the minor accumulation of both p53 and ubiquitinated species (Fig. 2B). Nonetheless, low concentrations of hippuristanol did not cause an
increase in aggresome formation which was already close to maximum. Therefore the dose-dependence curves for production of p53 and ubiquitinated species are seen shifted above the curve for aggresome formation. However, starting from 150 nM hippuristanol (at which translation output peaked) all three curves are parallel (Fig. 2B).

These data suggest that the rate of translation affects production of abnormal proteins, and in this model the rate of polypeptides growth is more important for generation of defective ribosome products than the number of translating ribosomes.

**The rate of translation affects folding of a model protein.**

To investigate effects of the rate of translation on protein folding, we utilized a recombinant green fluorescent protein from copepod *Pontellina plumata*, copGFP, as a reporter. copGFP was fused to FK506-binding protein 12 that facilitates its rapid degradation, however, the fusion protein can be stabilized by addition to the cells of the small molecule Shield-1 (this system that controls protein stability is described in (18)). Both fluorescence (which reflects folding) and protein levels of this polypeptide could be easily quantified, which makes it a useful folding reporter.

The mutant copGFP was expressed in HeLa cells under control of a tet-regulated promoter, using the retroviral expression system. Incubation with doxycycline for 6 hours in the presence of Shield-1 led to more than 10-fold induction of copGFP (Fig. 3B, compare the first two lanes). Various concentrations of emetine were added to the cells at the start of induction, and 6 hours later copGFP levels and fluorescence were measured. As expected, the build-up of copGFP, which also reflects the overall rate of translation, was reduced by emetine in a dose-dependent manner (Fig. 3A, B), similar to the inhibition of p53 accumulation (see Fig. 1). However, paradoxically, the fluorescence was affected in a very different manner, showing steady increase that peaked at about 40 nM emetine, reaching 150% of control. Importantly, at this concentration emetine already reduced the levels of copGFP by about 20% (Fig. 3A, B). Even stronger divergence between the copGFP protein levels and copGFP fluorescence was seen with 80 nM emetine (Fig. 3A). Effect of emetine on fluorescence of copGFP normalized to its levels demonstrates that slowing down translation improved folding of this protein more than four-fold (Fig. 3C).

We next investigated whether inhibition of translation initiation also improves folding of the copGFP reporter (Fig. 3D). As seen on Fig. 3 (compare C and E for dose-dependence of specific activity), the difference between the inhibitory effects of hippuristanol on copGFP levels and on its fluorescence was insignificant. Overall these experiments demonstrate that partial inhibition of translation elongation, while reducing expression, promotes folding of certain polypeptides, thus resulting in overall increase in their activity.

**Partial inhibition of translation can improve activity of mutant CFTR.**

The finding that mild inhibition of translation improves function of certain proteins suggested that this treatment may be employed to correct folding defects of disease-related mutant proteins. To test this possibility, we chose mutant CFTR F508del. Normally, CFTR functions as a chloride channel in the plasma membrane of epithelial cells, but the F508del and certain other mutations jeopardize CFTR folding and trafficking which ultimately causes Cystic Fibrosis. To study the effect of translation inhibition on functional surface expression of CFTR F508del we used Fisher rat thyroid cells (FRT) stably expressing recombinant CFTR F508del. CFTR activity was measured by two independent methods an Ussing chamber assay (25) and a conductance assay in 24-well format (26) both of which measure CFTR-dependent transepithelial Cl- transport. The Ussing chamber assay was designed to measure this transport in the presence of a chloride ion gradient. Cells were exposed to various concentrations of emetine or left untreated, and CFTR-activity was measured 24 hours after the beginning of the treatment. During the measurement, CFTR was sequentially activated...
by compounds that increase cAMP levels and stimulate CFTR transport activity: 10 μM forskolin, 100 μM IBMX and then 20 μM genistein (Fig. 4A). To confirm that the activated Cl- transport was CFTR-dependent, at the end of the measurement we added the CFTR inhibitor-172, which blocked the CFTR-dependent component of the conductance.

In line with our conjecture, we observed a dose-dependent increase in activity of the mutant CFTR in cells exposed to low concentrations of emetine (Fig. 4A). An almost 2-fold increase over vehicle in the CFTR specific inhibitor response was reached at 0.5 μM emetine (see Fig. 4A, right panel). The higher effective emetine concentrations used in this experiment compared to those used with HeLa or MCF10A cells indicates a lower susceptibility of FRT cells to emetine (not shown).

The effect of 24 hours of emetine incubation on CFTR activity at the cell surface was independently confirmed in the FRT cell conductance assay, in which CFTR-dependent transepithelial (Cl-) conductance was measured in the absence of a chloride gradient (Fig. 4B). In this experiment, cells were also pre-treated with various doses of emetine for 24 hours. During the measurements, CFTR was first activated with 10 μM forskolin and then further stimulated with the combination of 100 μM IBMX and 20 μM genistein. Inh-172 was added at the end of the experiment. Again, the dose-dependence of the CFTR specific inhibitor response peaked at 0.5 μM emetine as illustrated in Fig. 4B, right panel. At the peak concentration, the CFTR specific inhibitor response is more than 2-fold increased compared to negative control.

We further investigated effects of low doses of emetine on CFTR levels. Misfolded CFTR F508del molecules are rapidly degraded by the ubiquitin-proteasome machinery. Therefore, we predicted that a mild inhibition of translation could paradoxically lead to elevated cellular levels of CFTR F508del because folded molecules would escape degradation. Indeed, as seen in Fig. 4C, at low doses of emetine, we observed increased expression levels of CFTR represented by both mature form (C-band) and core-glycosylated form (B-band). The build-up of the core-glycosylated form of CFTR F508del, indicates that emetine enhances folding of this mutant protein in ER, where it passes a quality control and is sorted for either degradation (misfolded form) or for membrane (folded form). Therefore, the rate of translation affects folding of newly synthesized proteins not only in cytoplasm as seen with copGFP, but in ER, as well.

To assess effects of the translation initiation inhibitor hippuristanol on CFTR folding, dose response experiments were performed in FRT cell Ussing chambers. CFTR activity peaked at 0.6 μM of the inhibitor, culminating in an almost 3-fold increase in the CFTRinh-172 response over vehicle (Fig. 5A). As with emetine, the increase in activity was parallel to the increase in the CFTR protein levels, which also peaked at 0.6 μM (Fig. 5B). Therefore, with CFTR F508del both inhibition of translation elongation and initiation can improve folding.

Together the data described above indicate that modulation of translation presents a novel approach towards treatment of disorders associated with protein misfolding.

**Discussion**

A critical finding here is that mild inhibition of translation can correct folding defects of mutant disease-associated proteins. There is a wide range of diseases beside Cystic Fibrosis that may be beneficially affected by partial inhibition of translation, e.g. Gaucher, Tay-Sachs, various conditions associated with collagen mutations, and others. These diseases result from insufficient folding and function of important enzymes and structural proteins. Another class of conditions that might be improved by this approach are disorders associated with the buildup of toxic abnormal proteins, e.g. ALS, inclusion body myositis, and others. Since all these conditions are chronic, it will be necessary to develop non-toxic compounds that inhibit translation only mildly.
Another finding of this work is that the rate of translation has a significant impact on the quality of newly-synthesized proteins and the overall proteostasis. Recently, it was demonstrated that newly-synthesized polypeptides are most vulnerable to various stresses, e.g. heat shock or oxidative stress (27). These authors argued that there are normal proteins, usually multi-subunit proteins of high molecular weight, that fold slowly and often with low efficiency, and these partially unfolded species are especially sensitive to stresses. Since low doses of translation inhibitors reduce overall production of abnormal polypeptides probably via enhancing folding, we hypothesize that partial inhibition of translation may significantly protect from proteotoxic stresses.

Several mutations that reduce translation initiation and elongation in C.elegans and Drosophila have been shown to have strong anti-aging effects (28,29). Our data suggest that these anti-aging effects may be relevant to improved protein folding due to slower translation. Indeed, some of these mutations have been shown to improve the overall proteostasis (30). Therefore, there is a possibility that minor suppression of translation may provide an anti-aging effect, and be beneficial for a variety of age-related disorders.

In this work, we assessed translation rates by measuring de novo synthesis of short-lived proteins upon inhibition of their degradation. Independently, we measured accumulation of a stable reporter protein upon induction of its synthesis. The results obtained with these two distinct methods were similar. We found that slowdown of elongation of translation improves general protein folding. These effects suggest that either the rate of growth of the polypeptide chain influences the kinetics of co-translational folding (e.g. allows association with ribosome-bound chaperones prior to co-translational misfolding, or reduces the probability of improper interactions between the emerging domains) or it reduces the overall translation output thus increasing the number of available cytoplasmic chaperones (both for co-translational and post-translational folding). On the other hand, inhibitors of translation initiation do not affect growth of the polypeptides chain, but only the number of translating ribosomes. Therefore, these inhibitors cannot influence the kinetics of co-translational folding, but only the translation output.

The fact that only inhibitors of translation elongation reduced overall production of misfolded species, while hippuristanol was not effective, indicate that for the bulk of polypeptides translation influences the kinetics of co-translational folding. Indeed, in vitro studies suggested that slowing down of translation can improve co-translational folding (14,15).

On the other hand, with CFTR F508del we observed folding improvement with both emetine and hippuristanol, indicating that the overall translation output is critical for CFTR folding, and suggesting that the availability of chaperones is limiting. These findings may reflect the compartmental difference, where for cytoplasmic proteins (which represent the bulk of the translation products in HeLa cells) chaperones are not limiting, and translation mainly affects the kinetics of co-translational folding. On the contrary, for CFTR which transits through ER, ER chaperones may be limiting.

Overall, findings presented here can be considered a proof of principle that partial inhibition of translation could be developed as a novel therapeutic modality for treatment of diseases associated with inefficient folding of mutant proteins.

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Figure Legends

Fig. 1. Emetine disproportionally inhibits accumulation of ubiquitinated species and aggresome formation. (A, B) Effect of emetine on translation and aggresome formation. MCF10A cells stably expressing Syn-GFP were incubated with 5 µM MG132 and the indicated concentrations of emetine for 2 hours and the fraction of cells with an aggresome was counted under a fluorescence microscope. In parallel cell lysates were prepared after 4 hours of incubation and levels of p53 were measured by immunoblotting. A. - Quantification of the results of aggresome counting and immunoblotting. B. – Effect of low doses of emetine on aggresome appearance. Scale bar is 20 µm. (C, D) Effect of emetine on accumulation of ubiquitinated species. MCF10A cells were incubated with 5 µM MG132 and indicated concentrations of emetine, and amounts of ubiquitinated species and p53 were assessed by immunoblotting. C.- Quantification of immunoblotting. D. – Immunoblots. All experiments were reproduced three times and data are representative.

Fig. 2. Different effects of cycloheximide and hippuristanol on accumulation of ubiquitinated species and aggresome formation. (A) Effects of cycloheximide on translation, accumulation of ubiquitinated species and aggresome formation. Experiments were done as described in Fig. 1. (B) Effects of hippuristanol on accumulation of ubiquitinated species and aggresome formation. Experiments were done as described in Fig. 1. All data were reproduced several times and are representative.

Fig. 3. Inhibition of translation elongation enhances folding of mutant copGFP in HeLa cells. (A) Effects of emetine on fluorescence and levels of mutant copGFP. CopGFP was induced for 6 hours in the presence of the indicated concentrations of emetine, and its fluorescence and levels normalized by total protein were measured as described in Materials and Methods. (B) Levels of copGFP in the experiment presented in Fig. 3A assessed by immunoblotting. Levels of this inducible protein also reflect the degree of translation inhibition. (C) Effects of emetine on the ratio of fluorescence/level of copGFP. Calculation is based on data in Fig. 3A. (D) Effects of hippuristanol on fluorescence and levels of mutant copGFP. CopGFP was induced for 6 hours in the presence of the indicated concentrations of hippuristanol, and its fluorescence and levels were measured as described in Materials and Methods. (E) Effects of hippuristanol on the ratio of fluorescence/level of copGFP. Calculation is based on data in Fig. 3D. All experiments were reproduced three times and data are representative.

Fig. 4. Inhibition of translation elongation corrects folding defects of the mutant CFTR. (A) Effects of emetine on CFTR-mediated short-circuit currents in Ussing chamber assay. Cells were plated on filter supports and incubated with the indicated concentrations of emetine for 24 hours. Inserts were transferred to Ussing chambers. After acquisition of baseline current, agonists (10 µM forskolin, 100 µM 3-isobutyl-1-methylxanthine [IBMX] and 20 µM genistein) and antagonist (20µM CFTRinh-172) were added sequentially to both epithelial surfaces, and short-circuit current were recorded. To better illustrate the emetine concentration effect on the CFTR inhibitor response, the post inhibitor baseline current is subtracted from the raw current trace. Right panel: Emetine dose response. Mean value (n=2) and standard deviation (SD) of the CFTR-specific Cl current normalized to CFTRinh-172. (B) Conductance assay: Transepithelial conductance changes in response to CFTR agonists (10µM forskolin, 100µM IBMX and 20µM genistein) and antagonist (20µM CFTRinh-172) additions. Traces represent averaged records (n=3) from FRT cell monolayers treated for 24 hours with given conc. of emetine. Right panel: Emetine dose-dependence curve reflective of CFTR F508del transport activity normalized by activity in the presence of CFTR inhibitor Means and SD from three independent experiment are shown. (C) Effect of emetine on expression of CFTR F508del. Levels of CFTR F508del in cells treated as described in 3C were measured
by immunoblotting. Band C – mature form, band B – core-glycosylated form.

**Fig. 5.** Inhibition of initiation of translation alleviates folding defects of the mutant CFTR. (A) Ussing chamber assay: Hippuristanol concentration effect on CFTR-mediated short-circuit currents recorded in duplicates from FRT cell monolayers incubated for 24 hours with the inhibitor. The experiment was done as described in Fig. 4A. (B) Effect of hippuristanol on expression of CFTR F508del. Levels of CFTR F508del in cells were measured as described in Fig. 4C.
Fig. 1
Fig. 2
Fig. 3

A. Relative levels of Fluorescence and copGFP levels with varying Emetine concentrations.

B. Western blot showing bands for Nonspecific, copGFP, and Actin at 25 kDa and 50 kDa.

C. Relative specific activity of copGFP with varying Emetine concentrations.

D. Relative levels with varying Hippuristanol concentrations.

E. Relative specific activity with varying Hippuristanol concentrations.
Fig. 5
A novel approach towards recovery of function of mutant proteins by slowing down translation
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