

Article

Proteasome Inhibition Amplifies Endoplasmic Reticulum (ER) Stress Responses: Comparative Proteomics of Chinese Hamster Ovary Cell Lines

Christiana-Kondylo Sideri ^{1,2} , David Ryan ^{1,2}, Michael Henry ¹ , Esen Efeoglu ¹  and Paula Meleady ^{1,2,3,*} 

¹ Life Sciences Institute, Dublin City University, D09 K20V Dublin, Ireland;

christianakondylo.sideri3@mail.dcu.ie (C.-K.S.); michael.henry@dcu.ie (M.H.); esen.efeoglu@tudublin.ie (E.E.).

² School of Biotechnology, Dublin City University, D09 K20V Dublin, Ireland

³ SSPC Research Ireland Centre for Pharmaceuticals, V94 T9PX Limerick, Ireland

* Correspondence: paula.meleady@dcu.ie

Abstract

Chinese hamster ovary (CHO) cells are widely utilised in the biopharmaceutical industry to produce therapeutic proteins. Understanding the mechanisms of endoplasmic reticulum (ER) stress and its interplay with protein degradation pathways remains pivotal for improving production efficiency and product quality. In this study, we investigated the proteomic responses of CHO-K1 (non-producer), CHO DP-12 (IgG-producer), and NISTCHO (IgG-producer) cell lines under ER stress induced by a combination of the proteasome inhibitor MG132 and the glycosylation inhibitor tunicamycin. Viability, cell growth, and IgG titre were measured for 24 h, 48 h, and 72 h of treatment and the 48 h timepoint was used for the comparative analysis of the proteomic data across the three cell lines. Proteasome inhibition with MG132 intensified ER stress and altered ER-associated protein degradation (ERAD). Combined tunicamycin + MG132 treatment was associated with cell line-specific proteomic changes: NISTCHO upregulated ER translocation and glycoprotein quality control proteins (SSR4, SEC24C, UGGT1), CHO DP-12 activated redox/disulfide regulators (DNAJC10, CAPN1), while CHO-K1 showed broad proteome shifts, suggesting differences in baseline stress handling. These findings provide mechanistic insights into ER stress and protein quality control in CHO cells, offering a foundation for strategies to enhance cell line robustness and optimise biopharmaceutical production.

Keywords: Chinese hamster ovary cells; ER stress; tunicamycin; proteasome inhibitor; proteomics; LC-MS/MS



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1. Introduction

Chinese hamster ovary (CHO) cells remain the dominant host system for recombinant protein production, serving as the workhorse for the biopharmaceutical industry for producing the majority of approved therapeutic proteins and monoclonal antibodies (mAbs) [1]. While ongoing research focuses on increasing productivity through genetic engineering and process optimisation approaches [2–4], maximising yields remains a significant challenge due to the cellular stress imposed by high-titre production of mAbs and other difficult-to-express (DTE) therapeutic proteins that often exceeds the CHO cell's natural folding and processing capacity synthesis [5–9].

Endoplasmic reticulum (ER) stress is a central factor influencing CHO cell performance. The high folding burden imposed by recombinant protein synthesis can activate

the unfolded protein response (UPR), which initially promotes cell survival by enhancing the folding capacity and reducing the translational load [10,11]. However, when stress is prolonged or excessive, the UPR can drive growth arrest and apoptosis, ultimately reducing protein yields [12]. Understanding how ER stress shapes productivity is therefore critical for rational cell line and process design. In parallel, the ubiquitin–proteasome system (UPS) is a key component of protein quality control, eliminating misfolded proteins via ER-associated degradation (ERAD) [13,14]. Understanding the interplay between these pathways is critical; for example, CHO cell pools developed under proteasome inhibitor selection pressure exhibited higher specific productivity, suggesting that proteasomal regulation directly influences production phenotypes [15]. Proteasome inhibition can disrupt protein degradation and exacerbate ER stress by driving the accumulation of misfolded proteins [16,17]. The reversible inhibitor MG132 has been extensively used to probe these mechanisms [18–22].

Mass spectrometry-based proteomics provides a powerful approach to investigate CHO cell responses to recombinant protein production and ER stress [8,23–31]. In this study, we aimed to better understand how CHO cells balance protein folding, degradation, and secretion under stress conditions relevant to biopharmaceutical production. Building on our group’s previous characterisation of the CHO DP-12 ubiquitinated proteome [32], we aim to expand this analysis to a global proteomic comparison across multiple CHO cell lines. To achieve this, we used a dual-inhibition strategy using tunicamycin (an N-glycosylation inhibitor) to induce ER stress and MG132 (a reversible inhibitor of the 26S proteasome) to block ERAD. Tunicamycin is an analogue of UDP-N-acetylglucosamine (UDP-GlcNAc), which serves as a substrate for glycosyltransferases involved in the synthesis of N-glycans during N-linked glycosylation [33]. In the presence of tunicamycin, this process is inhibited, blocking the biosynthesis of glycoproteins in the ER and inducing the UPR [34,35]. MG132 inhibits the activity of the 26S-proteasome, thereby halting protein degradation via the UPS and promoting the accumulation of unfolded/misfolded proteins in the ER [36]. This combination prevents the degradation of misfolded proteins, effectively ‘trapping’ the cellular response and enabling the detection of proteins critical for mapping ER stress [37].

In this study, we carried out a comparative analysis across three distinct CHO cell lines with varying phenotypes: CHO-K1 (a non-producer), CHO DP-12 (low-producing recombinant mAb clone), and NISTCHO (a high-producing, clonal CHO line expressing cNISTmAb) [38,39]. This design enables the comparison of ER stress-related processes and producer-specific proteomic patterns, providing an overview of protein quality control networks relevant to bioprocessing. The inclusion of NISTCHO was particularly important, as it is a publicly available, well-characterised reference cell line recently developed by NIST for the expression of the NISTmAb. Recent publications highlight its value as a standardised model system for benchmarking, method development, and cross-laboratory comparisons in CHO biology and biomanufacturing research [39,40]. Using label-free LC–MS/MS proteomics and gene ontology analysis, we systematically evaluated the cellular responses under the combined tunicamycin and MG132 conditions in the three CHO cell lines. This study provides new insights into how ER stress and proteasome inhibition shape protein quality control in CHO cells, with implications for improving recombinant protein yields and guiding bioprocess optimisation.

2. Materials and Methods

2.1. Cell Culture

CHO-K1 (non-producer, ATCC CCL-61) and CHO DP-12 (a recombinant human anti-IL8-IgG1 producer, ATCC CRL-12445 clone #1934) were grown in BalanCD Growth A (Fuji-Film Irvine Scientific, Tilburg, The Netherlands), supplemented with 4 mM L-glutamine

(Sigma-Aldrich/Merck Ireland Ltd., Arklow, Ireland) at 37 °C under 5% CO₂ and 80% humidity, with continuous shaking at 170 rpm using a Climo-Shaker ISF1-XC (Kuhner Shaker GmbH, Herzogenrath, Germany). CHO DP-12 cells were pulsed every 2–3 weeks with 400 nM methotrexate (Sigma-Aldrich/Merck M8407) to ensure only producing cells remained viable in culture. NISTCHO cells [38] were cultured in EX-CELL[®] CD CHO Fusion (Sigma Aldrich/Merck, 14365C) supplemented with 6 mM L-glutamine (Sigma Aldrich/Merck, G7513). The cells were subcultured approximately every 3 days. Regular testing confirmed that the cells were free of *Mycoplasma* contamination throughout the study.

2.2. Treatment with Tunicamycin and MG132

Cells were seeded at 8×10^5 cells/mL in 50 mL TPP TubeSpin[®] Bioreactor (Sigma Aldrich/Merck, IE) at a working volume of 5 mL. Before treatment with tunicamycin and MG132, the cells were incubated for 24 h and viable cell density and viability were determined with trypan blue exclusion using the Countess II Automated Cell Counter (Thermo Fischer Scientific, Dublin, Ireland). This counts as the zero-hour time point result, the time just before the treatment. Tunicamycin was used at a concentration of 10 µg/mL [32,41,42] and MG132 at 0.1 µM (Sigma Aldrich/Merck) [15,32]. The concentration of choice would induce ER stress with minimal cell death over the 72 h of exposure (optimisation experiments can be found in Supplementary Material—Figures S1 and S2). Following the exposure, viable cell density and cellular viability were monitored at 24, 48, and 72 h. Afterwards, the cells were pelleted by centrifugation, washed with PBS, and snap-frozen at −80 °C for subsequent Western blotting and label-free quantitative analysis by LC-MS/MS. Media were also collected from the producer cell lines CHO DP-12 and NISTCHO for IgG ELISA analysis. All experiments were performed in biological triplicates.

2.3. Western Blotting Analysis

Thawed cell pellets were resuspended in 200 µL of lysis buffer containing 7 M Urea, 2 M Thiourea, 4% CHAPS, 30 mM Tris, pH 8.5, 1 × HALT protease inhibitors (Thermo Fischer Scientific), homogenised by carefully passing the samples through a 20-gauge needle 5 times. To achieve complete lysis, the samples were incubated at room temperature for 1 h on a rotator. Sample lysates were centrifuged at $14,000 \times g$ for 15 min, and the supernatant was assayed for protein concentration using the Pierce 660 nm Protein Assay (Thermo Fischer Scientific) as per manufacturer's instructions. Protein samples (30 µg) were mixed with 2× Laemmli buffer (Sigma Aldrich/Merck) at 1:1 ratio, heated at 70 °C for 10 min, and then allowed to cool at room temperature. Protein samples were then loaded onto SDS polyacrylamide gels (Invitrogen[™] Bolt[™] 4 to 12% Bis-Tris 1.0 mm Mini Protein Gel 12-well, Thermo Fischer Scientific) and 1 µL of the Chameleon Duo Pre-Stained Protein Ladder (LI-COR Biosciences, Cambridge, UK) was used per gel. The gels were then transferred onto a nitrocellulose membrane using the Invitrogen[™] Power Blotter Select Transfer Stacks (Thermo Fischer Scientific). Membranes were blocked in Pierce[™] Clear Milk Blocking Buffer (1×) (Thermo Fischer Scientific) for 1 h at room temperature with slight agitation and probed overnight at 4 °C with primary antibodies for BiP, ATF6, pan-ubiquitin, or β-actin (all antibodies, Cell Signalling Technology Europe B.V., Leiden, The Netherlands) used at 1:1000 *v/v*. Blots were then incubated with the relevant secondary antibodies (LI-COR Biosciences, UK) at 1:15,000 dilution and rinsed with 1× TBS Tween (Thermo Fischer Scientific). For imaging, the Odyssey Scanner (LI-COR Biosciences, UK) was used. Western blot original images can be found in Supplementary Materials.

2.4. IgG Enzyme-Linked Immunosorbent Assay

Media samples were collected by centrifugation from the cell cultures and stored at -20°C . Antibody titre was measured from the IgG-producing cell lines CHO DP-12 and NISTCHO using the human IgG ELISA kit (RAB0001, Sigma-Aldrich/Merck, and ELH-IGG, RayBiotech; supplied by Generon Ireland, Dublin, Ireland). Following the manufacturer's instructions for the Sandwich Assay Procedure, 100 μL of standards/unknown samples was added to the corresponding wells; the plate was slightly mixed and incubated overnight at 4°C . After washing the wells, samples were sequentially incubated with detection antibody, streptavidin, and substrate, with wash steps between each incubation. All incubations were performed at room temperature with agitation. The reaction was stopped with stop solution, and absorbance was measured immediately at 450 nm using a Multiskan FC microplate photometer (Thermo Fischer Scientific). Standards and samples were run in technical duplicates.

2.5. Sample Preparation and Proteomic Analysis Using Label-Free Quantitative Differential LC-MS/MS Analysis

Protein samples (100 μg each) were prepared using the PreOmics iST kit (PreOmics GmbH, Planegg, Germany) according to the manufacturer's instructions and as previously described [43]. Total protein per cell was assessed using BCA assays normalised to cell number to evaluate whether ER stress treatments altered overall protein content. No significant differences between treatments were observed. Briefly, cell pellets were lysed at 95°C for 10 min with agitation, followed by proteolytic digestion with Trypsin/LysC at 37°C for 3 h. Peptides were then washed, filtered through cartridges, dried at 45°C under vacuum, and stored at -80°C until analysis.

Peptide samples were resuspended in 50 μL with LC-LOAD buffer (supplied with the Preomics iST kit) and 2 μL from each sample was analysed using an UltiMate 3000 nanoRSLC system (Thermo Scientific, Hemel Hempstead, UK) interfaced with an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific). Peptides were loaded onto a C18 trapping column (Acclaim PepMap100, 300 $\mu\text{m} \times 5\text{ mm}$, Thermo Scientific) at a flow rate of 25 $\mu\text{L}/\text{min}$ using 2% (*v/v*) acetonitrile (ACN) and 0.1% (*v/v*) trifluoroacetic acid (TFA) for 3 min for desalting and concentration. Separation was performed on an analytical column (Acclaim PepMap100, 75 $\mu\text{m} \times 50\text{ cm}$, 3 μm particle size, Thermo Scientific) using a binary gradient of solvent A (0.1% formic acid in LC-MS grade water) and solvent B (80% ACN, 0.08% formic acid in LC-MS grade water). The gradient consisted of 2–27.5% solvent B over 100 min, 27.5–90% over 10 min, followed by a 5 min hold at 90% B, at a flow rate of 300 nL/min.

Peptides were ionised using nano-electrospray ionisation at 2.0 kV and 320°C . Full MS scans were acquired in the Orbitrap at a resolution of 120,000 (at m/z 200), with an automatic gain control (AGC) target of 4×10^5 and a maximum injection time of 50 ms. Data-dependent acquisition was employed with a scan range of 380–1500 m/z and a top-speed MS/MS acquisition algorithm. Precursor ions were selected with charge states from 2+ to 7+ and dynamically excluded for 60 s after initial selection. Fragmentation was performed using higher-energy collisional dissociation (HCD) with a collision energy of 28%. MS/MS spectra were acquired in the linear ion trap with an AGC target of 2×10^5 and a maximum injection time of 32 ms.

Label-free quantitative analysis was performed and raw LC-MS/MS data were processed using Progenesis QI for Proteomics Version 2.0.5556.29015 (NonLinear Dynamics, Waters, Newcastle upon Tyne, UK). Spectra were imported and converted into peptide ion features, which were manually and automatically aligned across the samples compared (for example, 3 replicates control vs. 3 replicates of treatment) using the software's

retention-time alignment workflow. A ‘between-subjects’ experimental design was applied, and peptide feature abundances were normalised using Progenesis’s built-in global scaling procedure. The normalised peptide abundance values were arcsinh-transformed prior to statistical analysis to better meet the assumptions of one-way ANOVA. Peptide features showing significant differences between experimental groups (Progenesis ANOVA, $p < 0.05$) were exported for peptide identification [44].

Statistically significant peptide features were identified using Proteome Discoverer 2.2 (Thermo Scientific). Searches were performed with the SEQUEST HT algorithm against a *Cricetulus griseus* UniProt database (downloaded in May 2024). Search parameters included a precursor mass tolerance of 10 ppm, fragment mass tolerance of 0.6 Da, fixed carbamidomethylation of cysteine, variable oxidation of methionine, and trypsin specificity allowing up to two missed cleavages. Peptide–spectrum matches (PSM) were filtered for high-confidence identification using Percolator at FDR < 1%, together with additional acceptance criteria: unambiguous peptide-to-protein assignment and XCorr ≥ 1.90 . Identified peptides were then re-imported into Progenesis for protein-level quantification.

Protein quantification was performed using the Hi-3 method, in which protein abundance is estimated from the mean intensity of the three most intense unique peptides [45]. Proteins were considered differentially expressed based on the following criteria: (i) protein level one-way ANOVA $p < 0.05$, (ii) identification by at least two unique peptides, and (iii) a fold-change of $\geq \pm 1.5$ between treatment groups [46–48].

The datasets have been deposited to the ProteomeXchange Consortium via the PRIDE [49] partner repository with the dataset identifier PXD069782 and 10.6019/PXD069782.

2.6. KEGG Pathway and STRING Association Analysis

Differentially expressed proteins from whole-cell lysates from control and treated samples were analysed using STRING version 12.0 (<https://www.string-db.org>, accessed on 14 April 2025) [50] to explore protein–protein interaction networks and identify functional groupings. KEGG pathway enrichment analysis was performed via STRING using annotations from the Kyoto Encyclopedia of Genes and Genomes (<https://www.genome.jp/kegg>), accessed on 14 April 2025 [51].

2.7. Statistical Analysis of the Data

Statistical analysis was performed using two-way ANOVA followed by Dunnett’s multiple comparisons test in GraphPad Prism (v10.4.1 for Windows; GraphPad Software, Boston, MA, USA; www.graphpad.com, accessed on 14 April 2025). The null hypothesis was tested at a significance level of 0.05. Graphs were generated using GraphPad Prism, ProteoRE platform (<https://www.proteore.org>, accessed on 14 April 2025), and Venny [52,53]. Error bars represent the standard deviation of the arithmetic mean from three independent experiments.

3. Results and Discussion

3.1. ER Stress-Induced Changes in Viability and Bioproduktivty of CHO Cells

In this study, the impact of ER stress and proteasome inhibition on three CHO cell lines, the non-producing CHO-K1, and two IgG-producing lines, CHO DP-12 and NISTCHO, was evaluated. Cells were exposed to tunicamycin and MG132 individually and in combination. As outlined in Figure 1, cells were cultured under four experimental conditions: untreated control, tunicamycin-treated, MG132-treated, and combination-treated (tunicamycin + MG132), and assessed at 24, 48, and 72 h post-treatment. In all three CHO cell lines, tunicamycin treatment showed minimal effects after 24 h, with viability comparable to the untreated control and only a slight reduction in cell growth. With prolonged exposure (48 and 72 h),

both viability and viable cell density (VCD) decreased, indicating increased ER stress and potential cytotoxicity (Figure 1). MG132 treatment had minimal impact on viability across all three CHO cell lines, with cell growth reduced at 48 and 72 h except in the NISTCHO cell line. It should be noted that comparisons between CHO-K1/CHO DP-12 and NISTCHO may be influenced by differences in culture media (BalanCD and EX-CELL, respectively), which could affect cellular physiology and protein expression. The combined treatment further decreased viability and growth in CHO-K1 cells, while the two IgG-producer cell lines showed responses similar to tunicamycin alone, suggesting that the combined treatment amplifies the impact of ER stress by further impairing protein degradation.

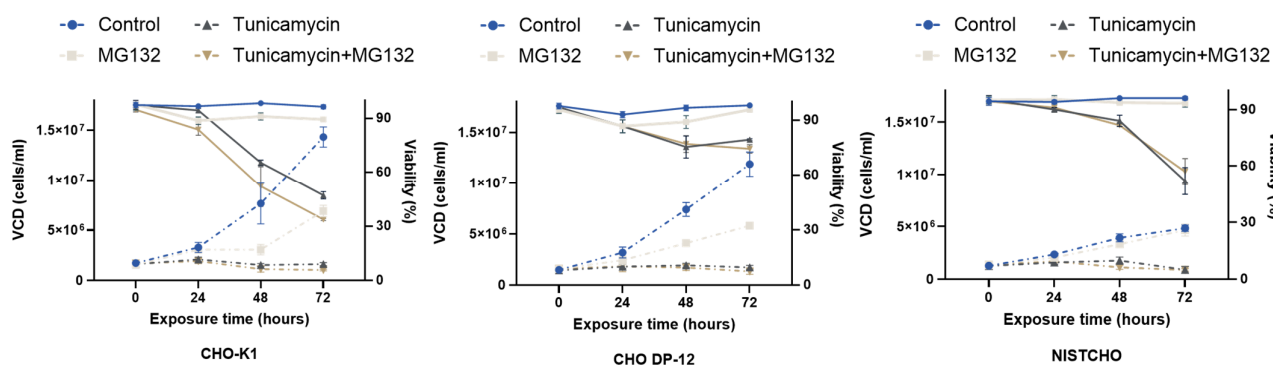


Figure 1. Viable cell density (VCD) and cell viability of CHO K-1, CHO DP-12, and NISTCHO in response to tunicamycin and MG132 treatment. CHO cells were cultured under four conditions: untreated control, tunicamycin to induce ER stress, MG132 to inhibit proteasomal degradation, and combined tunicamycin + MG132 treatment. VCD (dashed lines) and viability (solid lines) were measured at 24, 48, and 72 h post-treatment. Data represent mean $\pm$ SD from three independent experiments.

ER stress did not affect the titre but increased the specific productivity of NISTCHO cells (58 pg/cell/day) compared to the control (Figure 2). While titre remained stable across treatments, VCD declined, indicating an inverse relationship between cell proliferation and antibody production, consistent with previous findings [54]. CHO DP-12, a lower-producing line (1.09 pg/cell/day), showed no increase in specific productivity under ER stress but remains a valuable model for comparative analysis and understanding ER stress mechanisms. The two IgG-producing cell lines used in this study are expressing different antibodies. While our study aimed to compare cellular responses under the conditions tested, some of the observed proteomic differences may reflect antibody-specific effects rather than intrinsic cell line characteristics.

Western blotting using antibodies against ER stress markers BiP and ATF6 was performed to confirm ER stress induction [55,56]. ER-stress induction was confirmed qualitatively by Western blotting, consistent with our quantitative label-free proteomics data, in which BiP was upregulated 12.75-fold in the Tunicamycin + MG132 condition compared with Control (Supplementary Figure S4). As shown in Figure 3, all three CHO cell lines exhibited increased expression of BiP and ATF6 following tunicamycin treatment, with β -actin used as a loading control. Densitometry quantification on BiP and ATF6 western blots can be found in Supplementary Figure S3. Consistent β -actin levels were also confirmed by differential expression proteomic analysis, outlining the reasoning for choosing β -actin as a loading control for Western blot analysis [57]. Western blotting was also carried out on all three CHO cell lines using a pan-ubiquitin antibody, showing the increased levels of ubiquitinated proteins per condition over time, 24, 48, and 72 h post-treatment, demonstrating proteasomal inhibition following treatment with MG132.

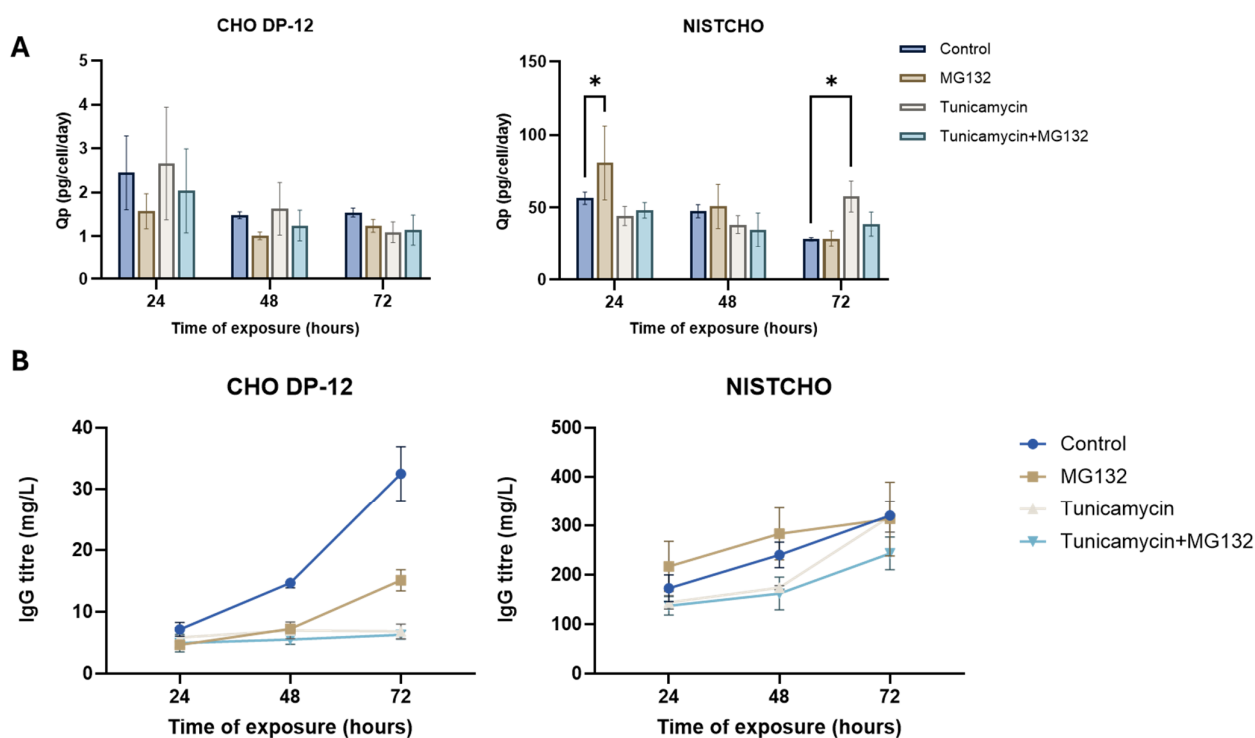


Figure 2. Effect of tunicamycin and MG132 treatment on IgG production in CHO cell lines. (A) Specific productivity (Qp; pg/cell/day) and (B) IgG titre (mg/L) were measured in the two IgG-producing cell lines, following treatment with tunicamycin, MG132, or the combination of both. Data were collected at 24, 48, and 72 h post-treatment. Two-way ANOVA was used to assess the effects of treatment on IgG expression compared to the untreated control. Bars represent mean $\pm$ SD of three independent experiments. Statistical significance is indicated as * $p < 0.05$.

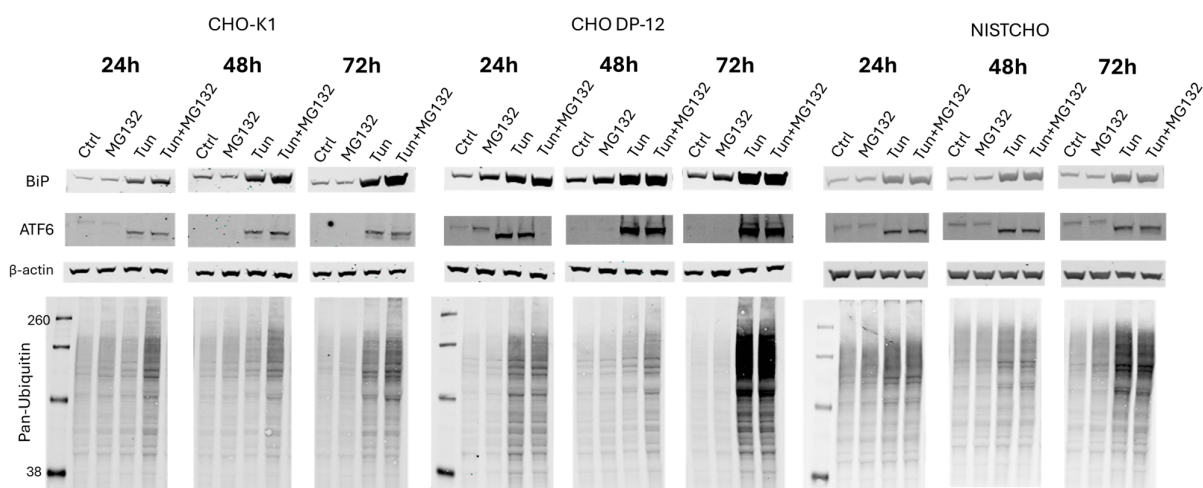


Figure 3. Western blotting analysis of the known ER stressors BiP/GRP78 and ATF6, and pan-ubiquitin on CHO K-1, CHO DP-12, and NISTCHO. Original images of Western blotting can be found in Supplementary Materials. Western blotting analysis of the pan anti-ubiquitin antibody showing the ubiquitination levels of CHO cell lines upon induction of ER stress and proteasome inhibition. Original images of Western Blotting can be found in Supplementary Materials.

3.2. Integrated Proteomic Analysis of Producer and Non-Producer CHO Cells Under ER Stress and Proteasome Inhibition

For the comparative proteomic analysis across the three CHO cell lines and four treatment conditions over time, multiple comparison sets were used. Table 1 outlines the number of up- and downregulated proteins in each condition relative to the control.

Consistent with the viability and Western blot results, MG132 alone showed minimal changes compared to the control, while tunicamycin treatment resulted in a significantly higher number of differentially expressed (DE) proteins across all cell lines. To assess DE protein patterns across all conditions, a cross-condition comparison was performed at the 48 h time point; this time point was selected because ER stress was clearly induced without a significant reduction in viability, allowing the proteome to reflect stress-associated changes (the full lists of DE proteins for each comparison at 48 h time point are shown in the Supplementary Material; CHO-K1, CHO DP-12, and NISTCHO_DE_lists).

Table 1. Overview of the number of differentially expressed (DE) proteins in three CHO cell lines under tunicamycin and MG132 treatment across time points.

Exposure Time		24 h		48 h		72 h	
Cell Line/Expression Level		UpR	DownR	UpR	DownR	UpR	DownR
CHO-K1	vs. MG132	72	92	392	164	79	219
	vs. Tunicamycin	280	326	497	419	384	460
	vs. Tunicamycin + MG132	395	374	492	424	520	559
CHO DP-12	vs. MG132	80	127	95	64	82	62
	vs. Tunicamycin	183	244	289	314	457	521
	vs. Tunicamycin + MG132	313	180	346	339	434	535
NISTCHO	vs. MG132	-	-	9	2	28	17
	vs. Tunicamycin	478	138	437	236	268	343
	vs. Tunicamycin + MG132	405	107	479	354	374	257

It is important to note that our sampling strategy focused on 24, 48, and 72 h, which primarily capture the later consequences of ER stress rather than the early 2–8 h window when key UPR signalling events occur [58]. As a result, the proteomic differences observed here may reflect downstream or cumulative effects, and total protein abundance cannot distinguish between regulated functional changes and non-functional protein accumulation. We did not detect any significant differences in total protein per cell across treatments, indicating that the observed proteomic changes reflect relative abundance patterns rather than shifts in global protein content. These factors should be considered when interpreting the proteomic patterns described below.

The datasets from the 48 h comparison were also used for STRING gene ontology and KEGG pathway analysis. Significant shared enrichments were observed in pathways including ‘Carbon metabolism’ (cge01200), ‘Protein processing in endoplasmic reticulum’ (cge04141), ‘Citrate cycle (TCA cycle)’ (cge00020), ‘RNA transport’ (cge03013), ‘Aminoacyl-tRNA biosynthesis’ (cge00970), ‘Proteasome’ (cge03050), and ‘Biosynthesis of amino acids’ (cge01230) (Figure 4 and Table 2).

Looking closely at the proteomic investigation of the mechanisms involved in ER stress, the pathway ‘Protein processing in ER’ (cge04141) along with the biological process and the molecular function were further investigated. As shown in the Venn diagrams (Figure 5), CHO-K1 exhibited the largest number of unique differentially expressed proteins across all treatments, whereas CHO DP-12 and NISTCHO showed greater overlap with one another, indicating that the non-producing parental line employs different stress response mechanisms compared to the producer cell lines. The increased similarity between CHO DP-12 and NISTCHO may be related to their common role as antibody-producing cell lines, which could contribute to more similar proteomic profiles associated with ER stress, compared to the non-producing CHO-K1. However, under the combined tunicamycin + MG132 treatment, each producer cell line exhibited unique proteins mapping to the pathways ‘Protein processing in ER’ (cge04141) and ‘Proteasome’ (cge03050). For NISTCHO, these included DNAJA1, SKP1, SEC24C, SEC31A, UGGT1, CANX, HSPA8, LMAN1, RPN1,

and SSR4. For CHO DP-12, the unique proteins were DNAJB1, DNAJC10, CAPN1, CKAP4, LOC100760218, and UBE2D4. While our study aimed to compare cellular responses under the conditions tested, some of the observed proteomic differences may reflect antibody-specific effects rather than intrinsic cell line characteristics. A core set of ER chaperones and folding enzymes that were differentially expressed (including CALR, HSP90B1, DNAJB11, GRP78, ERP29, EIF2S1, HSP90AA1, HYOU1, P4HB, PDIA4, and PDIA6) was shared across all three cell lines, indicating a shared pattern of ER stress-associated proteomic changes. These proteins may reflect the activation of the UPR, translational control (EIF2S1), and reinforcement of ER folding and redox homeostasis [59,60]. In both producer lines (CHO DP-12 and NISTCHO), the increased enrichment of PDIA3 suggests a greater involvement of glycoprotein folding via the calnexin/calreticulin cycle, which may reflect demands associated with recombinant protein production [61].

Table 2. Enriched KEGG pathways common in CHO-K1, CHO DP-12, and NISTCHO at 48 h treatment.

Enriched Pathways	CHO-K1			CHO DP-12			NIST CHO		
	Count	Strength	FDR	Count	Strength	FDR	Count	Strength	FDR
Protein processing in endoplasmic reticulum (cge04141)	36	0.61	2.89×10^{-9}	25	0.67	6.32×10^{-8}	33	0.77	3.37×10^{-12}
Citrate cycle (TCA cycle) (cge00020)	18	0.98	3.98×10^{-9}	19	1.23	3.79×10^{-13}	18	1.18	7.12×10^{-12}
Aminoacyl-tRNA biosynthesis (cge00970)	14	0.75	2.78×10^{-5}	16	1.03	5.61×10^{-9}	11	0.84	3.42×10^{-5}
Biosynthesis of amino acids (cge01230)	25	0.73	1.61×10^{-8}	19	0.83	3.65×10^{-8}	18	0.79	3.70×10^{-7}
Proteasome (cge03050)	23	0.95	5.13×10^{-11}	10	0.81	1.80×10^{-4}	14	0.94	4.14×10^{-7}
RNA transport (cge03013)	56	0.78	7.21×10^{-20}	27	0.68	1.44×10^{-8}	24	0.61	1.44×10^{-6}
Carbon metabolism (cge01200)	47	0.81	2.77×10^{-18}	36	0.92	2.71×10^{-17}	40	0.94	7.16×10^{-20}
Spliceosome (cge03040)	51	0.81	1.97×10^{-19}	24	0.7	3.98×10^{-8}	22	0.64	1.44×10^{-6}
Lysosome (cge04142)	22	0.5	1.30×10^{-4}	18	0.63	1.98×10^{-5}	19	0.63	8.73×10^{-6}
Fatty acid degradation (cge00071)	12	0.68	3.40×10^{-4}	13	0.94	8.28×10^{-7}	13	0.92	1.59×10^{-6}
Valine, leucine and isoleucine degradation (cge00280)	16	0.74	8.15×10^{-6}	17	0.99	5.61×10^{-9}	18	0.99	1.41×10^{-9}

To help interpret the data, we refer to known UPR and ERAD components, but these links should be viewed cautiously. Changes in protein abundance do not necessarily indicate functional pathway activation, especially following treatment with combined tunicamycin + MG132, where non-functional or accumulated proteins may be present. Overall, the patterns we report reflect proteomic signatures rather than confirmed mechanistic responses. Under combined tunicamycin + MG132 stress, the proteome of the two producer lines diverged. NISTCHO showed increased abundance of proteins involved in ER-to-Golgi trafficking and glycoprotein quality control (e.g., SEC24C, SEC31A, CANX, UGGT1, LMAN1), which may indicate a potential role for enhanced trafficking under combined stress conditions. In CHO DP-12 cells there was an increased abundance of ERdj5 (DNAJC10) [62] and CAPN1, pointing to redox-regulated disulfide bond reshuffling and alternative degradation strategies. Together, these findings suggest that the two producer CHO lines share core proteostasis components but exhibit distinct proteomic profiles under stress. For NISTCHO, increased abundance of proteins involved in secretory pathway quality control was shown, while CHO DP-12 displayed higher levels of proteins linked to redox and disulfide bond regulation. The functional implications of these differences remain to be validated.

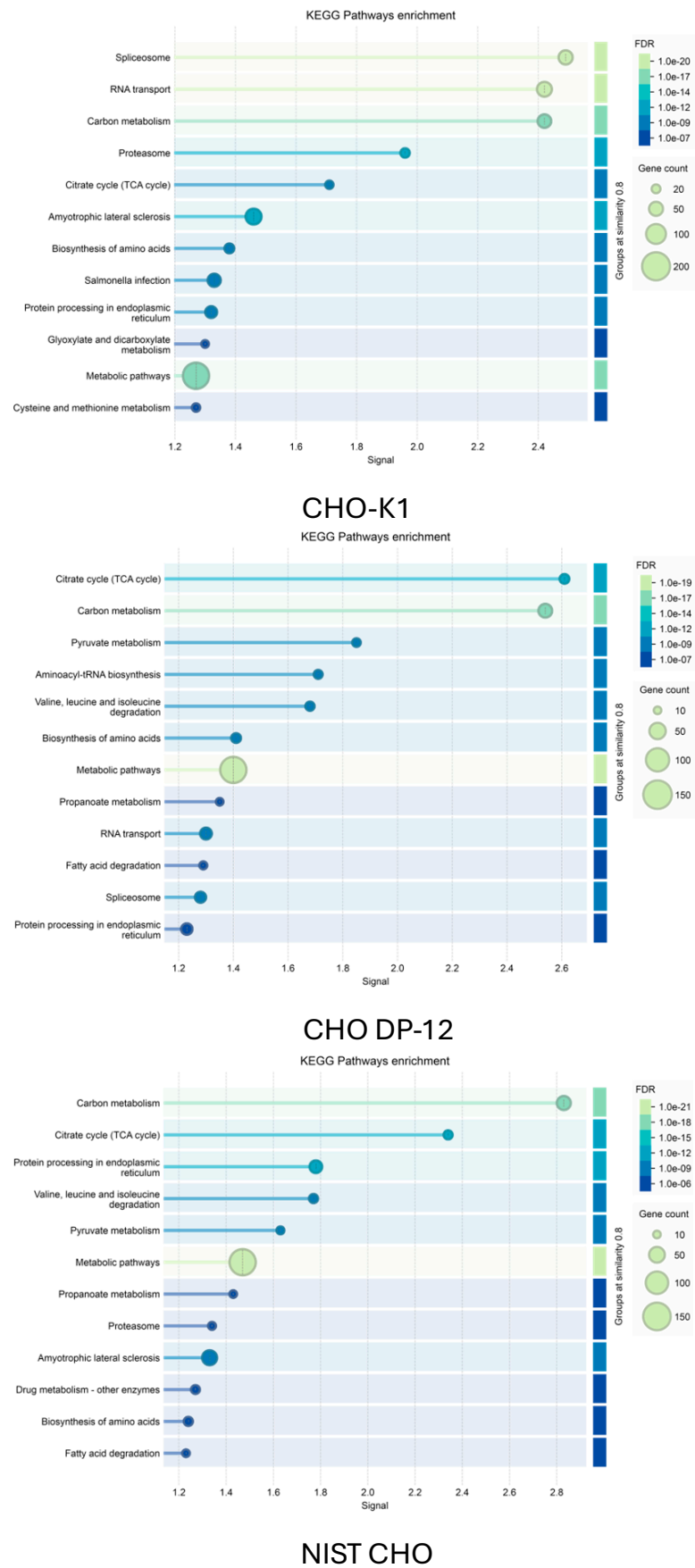


Figure 4. Top 12 enriched KEGG pathways in CHO-K1, CHO DP-12, and NISTCHO at 48 h treatment.

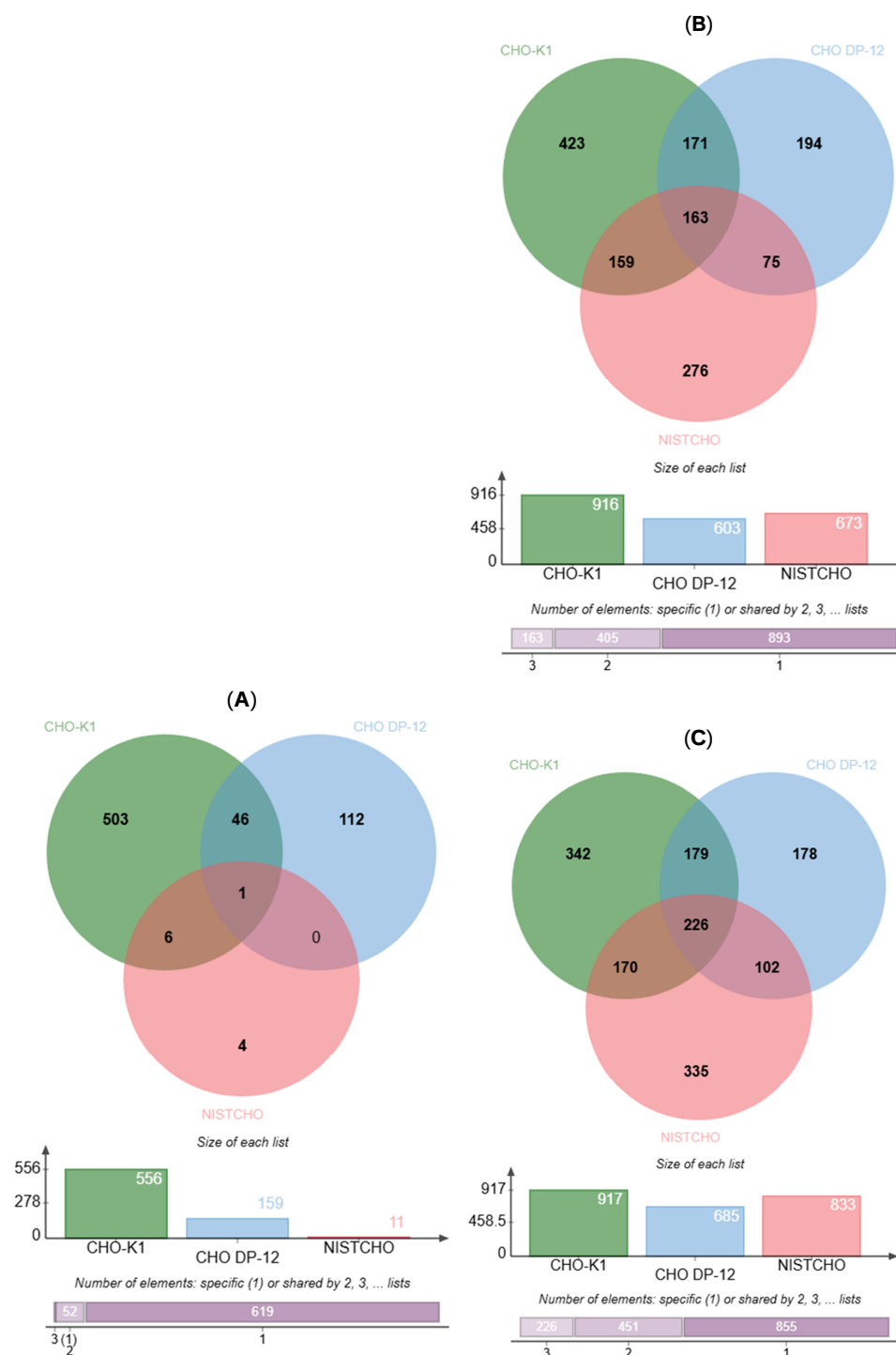


Figure 5. Venn diagrams of differentially expressed proteins in CHO cell lines under ER stress illustrate the overlap of significantly altered proteins ($FDR < 0.05$) across CHO-K1, CHO DP-12, and NISTCHO cells following (A) MG132 treatment, (B) tunicamycin treatment, and (C) combined tunicamycin + MG132 treatment, compared with untreated controls. Numbers indicate the count of unique or shared differentially expressed proteins. Shared regions highlight conserved proteomic responses among cell lines, while unique sectors reflect cell line-specific responses. Venn diagrams were generated using the ProteoRE platform (<https://www.proteore.org>, accessed on 14 April 2025).

GANAB and UGGT1 were upregulated in NISTCHO under the combined tunicamycin + MG132 treatment. Tunicamycin directly disrupts N-linked glycosylation, leading to the accumulation of misglycosylated proteins that directly engage the calnexin/calreticulin glycoprotein quality control cycle; UGGT1 and GANAB function centrally in this cycle

(reglycosylation and glucosidase trimming) [63,64]. The addition of proteasome inhibition in this study likely exacerbates the accumulation of misfolded glycoproteins [60] and further promotes the induction of glycoprotein-specific QC factors in producer lines with high secretory loads like NISTCHO [65].

3.3. Proteasome Inhibition Amplifies the Effects of ER Stress

Proteasome inhibition in recombinant CHO cell lines has been previously linked to enhanced productivity [15,61]. In this study, to assess the specific effect of proteasome inhibition, we performed three comparative analyses: untreated control vs. MG132, tunicamycin vs. tunicamycin + MG132, and untreated control vs. tunicamycin compared with untreated control vs. tunicamycin + MG132. It is important to consider that differences in MG132 uptake or proteasome inhibition efficiency between cell lines could influence the observed proteomic changes in this study. Variability in drug permeability, efflux mechanisms, or proteasome sensitivity may result in unequal levels of inhibition, potentially confounding the interpretation of stress-related responses. While our analysis assumes comparable inhibition across cell lines, this factor cannot be excluded and should be considered when interpreting cell line-specific differences [66,67].

3.3.1. Control vs. MG132

Table 1 shows the number of differentially expressed proteins, both upregulated and downregulated, at each time point for the three CHO cell lines following MG132 treatment compared to controls. At 48 h, 556 DE proteins were identified in CHO-K1, 159 in CHO DP-12, and only 11 in NISTCHO. CHO-K1, a non-producer cell line, exhibited the most pronounced response to proteasome inhibition, whereas NISTCHO, a high IgG-producing line, showed minimal changes. CHO DP-12, a low IgG-producer, displayed an intermediate response, highlighting differential sensitivities to MG132 among the CHO lines. The limited effect observed in NISTCHO could reflect reduced proteasome inhibition efficiency, differential drug uptake, higher baseline proteasome expression, or the cells may rely more heavily on alternative protein degradation pathways, such as lysosomal/autophagy pathways and ERAD-associated proteases. These findings are consistent with previous observations by Hausmann et al., who reported that, following methotrexate (MTX) selection, low IgG-producing CHO lines exhibited more extensive proteomic changes than high producers [68]. Specifically, low producers were more sensitive to selection-induced stress, whereas high producers appeared more resilient to these conditions.

3.3.2. Tunicamycin vs. Tunicamycin + MG132

Proteasome activity in CHO cells plays a key role in determining their capacity to produce recombinant proteins. Knight et al. previously demonstrated that treatment with the proteasome inhibitor MG132 enhanced mAb production by approximately threefold relative to untreated controls [15]. To further investigate the impact of proteasome inhibition on the cellular proteome, we compared 48 h treatment conditions across the three CHO cell lines: tunicamycin alone versus tunicamycin in combination with MG132. We focused on the 48 h condition, as it captures sustained ER stress while maintaining sufficient viability for meaningful proteomic comparison; earlier (24 h) and later (72 h) time points were less suitable. A total of 253 proteins were identified as differentially expressed in CHO-K1 (125 upregulated, 128 downregulated), 85 in CHO DP-12 (52 upregulated, 33 downregulated), and 41 in NISTCHO (10 upregulated, 31 downregulated) (Figure 6A). To assess the extent of proteome remodelling induced by MG132, fold-change distributions were compared among the cell lines. CHO-K1 exhibited the broadest distribution, indicating a more extensive proteomic response to proteasome inhibition. In contrast, CHO DP-12 and NISTCHO

displayed narrower fold-change distributions, consistent with a more limited proteomic response to MG132 treatment (Figure 6B).

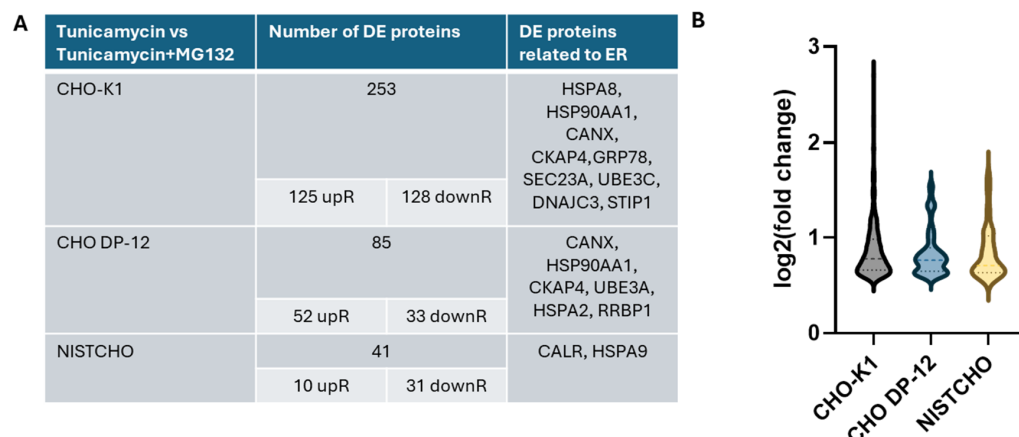


Figure 6. Impact of proteasome inhibition on the CHO cell proteome under ER stress. Comparison of tunicamycin versus tunicamycin + MG132 treatment for 48 h across three CHO cell lines (CHO-K1, CHO DP-12, and NISTCHO). (A) The table summarises the total number of differentially expressed (DE) proteins identified under each condition, along with the number of upregulated (upR) and downregulated (downR) proteins and the subset of DE proteins associated with the endoplasmic reticulum (ER). (B) Violin plots depict the distribution of protein fold changes, illustrating the magnitude of proteome remodelling across cell lines. CHO-K1 exhibited the broadest fold-change distribution, indicative of a stronger proteomic response to MG132 treatment compared with CHO DP-12 and NISTCHO.

3.3.3. Untreated Control vs. Tunicamycin-Treated Compared to Untreated Control vs. Tunicamycin + MG132

To investigate the impact of proteasome inhibition on the ER stress response, we compared differentially expressed protein profiles obtained from tunicamycin-treated cells and from cells subjected to the combined tunicamycin + MG132 treatment in each CHO cell line after 48 h. The comparison revealed 433 proteins uniquely regulated under the combined treatment and 362 proteins unique to tunicamycin alone (Figure 7A). KEGG pathway enrichment of the proteins unique to tunicamycin + MG132 indicated significant activation of the ‘Protein processing in endoplasmic reticulum’ (cge04141) and ‘Proteasome’ (cge03050) pathways (Figure 7B). The identified proteins, including HSPA1A, HSPA8, DNAJB1, and HSP90AA1, reflected an intensified chaperone response, while ubiquitin-related enzymes such as CUL1, SKP1, UBE2D4, UBE4B, and ATXN3 suggested the reinforcement of the ERAD machinery. ERAD is a protective mechanism that facilitates the removal of misfolded and unfolded proteins from the ER through proteasomal degradation [69]. During ER stress, the UPR and ERAD operate in a coordinated manner to restore protein homeostasis, although ERAD can also function independently to alleviate proteotoxic stress and maintain cellular performance [70]. Tang et al. demonstrated that the targeted regulation of the ERAD pathway in CHO cells, through either genetic or environmental approaches, can enhance recombinant protein yield [71].

Among the upregulated proteins, DnaJ homologue subfamily B member 1 (DNAJB1) showed a 1.8-fold increase in CHO DP-12 under ER stress and proteasome inhibition, consistent with its known role in recognising and transferring retrotranslocated ERAD substrates [72]. Heat shock protein 90- α (HSP90AA1) was upregulated in CHO-K1 and CHO DP-12, but 2.1-fold downregulated in NISTCHO, suggesting cell line-specific regulation of chaperone networks under stress. Similarly, ubiquitin-conjugating enzyme E2 D4 (UBE2D4) was 2.33-fold downregulated in CHO DP-12, consistent with previous

observations of reduced UBE2D4 expression in CHO cells exposed to ER stress [30]. Ataxin-3 (ATXN3), a deubiquitinating enzyme that trims polyubiquitin chains on ERAD substrates to regulate proteasomal targeting efficiency [73], was found downregulated in NISTCHO but was unchanged in CHO-K1 and CHO DP-12. NISTCHO also displayed contrasting expression patterns for CUL1 and HSP90AA1, both of which were downregulated, while these proteins were upregulated in CHO-K1 and CHO DP-12, reflecting a distinct stress response profile. In contrast, heat shock cognate 71 kDa protein (HSPA8) was upregulated in CHO-K1 but downregulated in CHO DP-12 and NISTCHO, potentially indicating differences in basal chaperone capacity and proteostasis regulation across the non-producer and IgG-producer cell lines. Translocon-associated protein subunit delta (SSR4) was 3.3-fold upregulated in NISTCHO, implying an enhanced translocation and glycoprotein processing capacity under combined ER stress and proteasome inhibition.

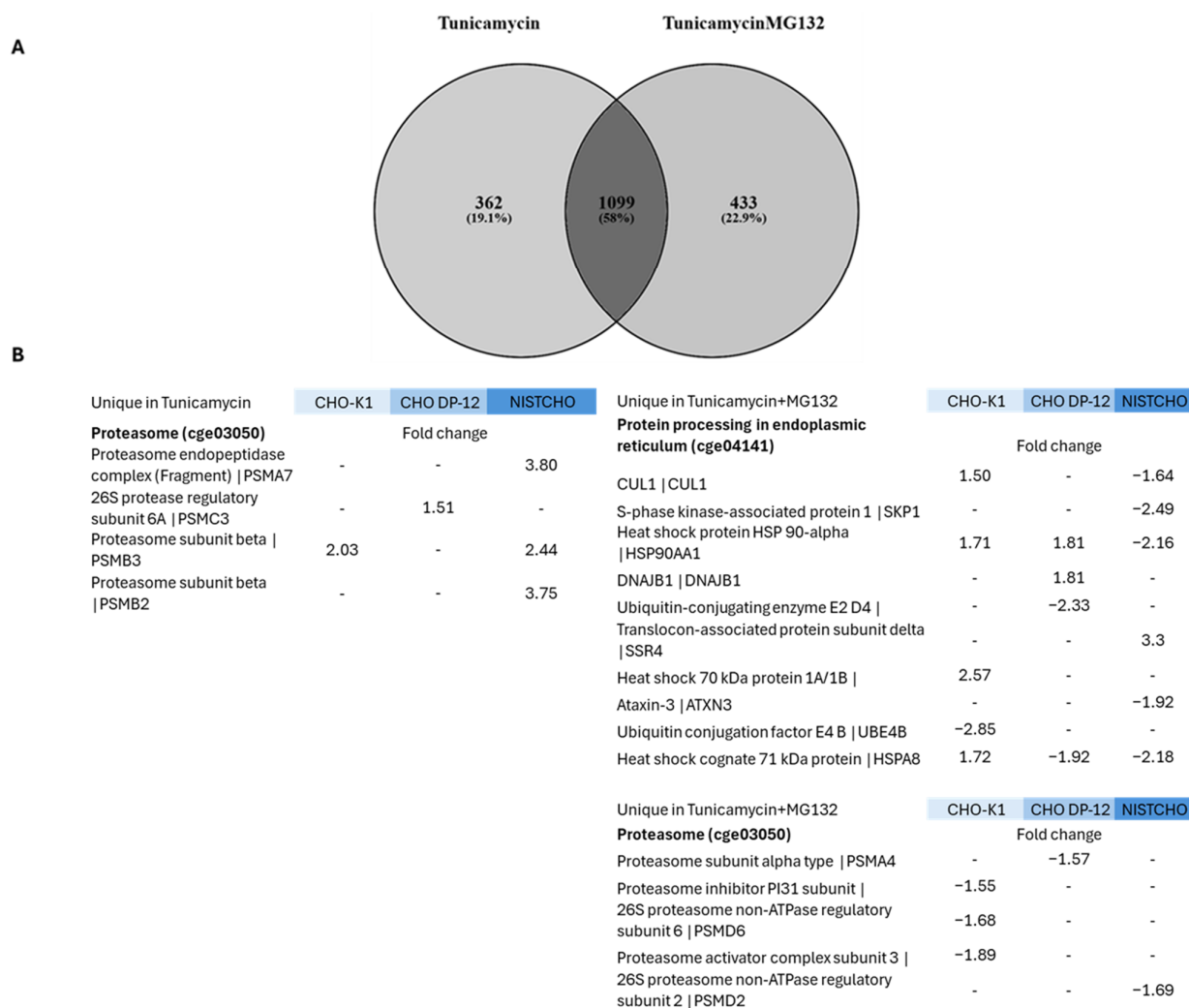


Figure 7. (A) Venn diagram DE proteins in all tunicamycin and tunicamycin + MG132 treatments (48 h). (B) Proteins unique in tunicamycin + MG132 and in tunicamycin contributing to the enrichment of KEGG pathways.

The proteins contributing to the enrichment of the ‘Proteasome’ (cge03050) pathway under the combined tunicamycin + MG132 treatment, including proteasome subunit alpha type-4 (PSMA4), proteasome inhibitor PI31 subunit (PMSF1), 26S proteasome non-ATPase regulatory subunit 6 (PSMD6), proteasome activator complex subunit 3 (PSME3), and 26S proteasome non-ATPase regulatory subunit 2 (PSMD2), were all downregulated. This enrichment reflects the significant representation of proteasome-related components among

the differentially expressed proteins, rather than increased proteasomal activity. MG132 directly blocks the catalytic activity of the 26S proteasome, leading to proteotoxic stress and likely triggering regulatory responses that reduce proteasome subunit synthesis [74]. Moreover, the combined ER stress and proteasome inhibition are known to activate the PERK-eIF2 α branch of the UPR, which can attenuate global translation, including that of proteasome components. Under such conditions, cells may also shift toward compensatory autophagy pathways, further influencing proteasome biogenesis. While these interpretations are consistent with known stress response mechanisms, they remain hypothetical in the absence of transcriptomic or functional activity assays. From the comparative proteomic analysis, the observed enrichment of the proteasome pathway may reflect changes in the abundance of multiple subunits following proteasome inhibition.

KEGG analysis of the protein list unique to the tunicamycin treatment identified the enrichment of the 'Proteasome' (cge03050) pathway driven by coordinated increases in multiple proteasome components, including proteasome subunit alpha type-7 (PSMA7), 26S proteasome regulatory ATPase subunit 3 (PSMC3), and the catalytic β subunits PSMB2 and PSMB3. These changes may reflect heightened demands on degradation pathways following tunicamycin-induced ER stress, where the inhibition of N-glycosylation elevates the burden of misfolded secretory/membrane proteins, which in turn promotes ERAD engagement and a transcriptional programme to enhance ubiquitin–proteasome capacity. The concurrent upregulation of both 20S core (PSMA7, PSMB2/PSMB3) and 19S regulatory (PSMC3) subunits suggests a broader shift in proteasome-associated protein levels during stress. Such patterns are compatible with known UPR- and stress-responsive pathways (e.g., ATF6/XBP1 and NRF1-mediated pathways) that influence the expression of proteostasis-related genes, although functional consequences were not assessed in this study [75,76]. By contrast, combined tunicamycin + MG132 treatment functionally inhibits the proteasome and elicits a different cellular response, which explains why proteasome components were downregulated in the presence of MG132. Together, these data indicate that tunicamycin treatment alone was associated with an increased abundance of proteasome-related proteins whereas pharmacologic proteasome inhibition reduced these levels. These observations suggest a shift in proteasome composition under ER stress that could be validated with functional analysis in the future.

4. Conclusions

This study provides a detailed proteomic perspective on how combined ER stress and proteasome inhibition shape cellular protein quality control networks in CHO cells. The integration of tunicamycin and MG132 treatments revealed distinct and overlapping stress response signatures across three CHO cell lines with different production capacities. While tunicamycin alone primarily activated classical UPR components, the addition of MG132 intensified proteotoxic stress and induced a stronger chaperone response, together with the modulation of the ubiquitin–proteasome system components.

Proteasome inhibition with MG132 amplified ER stress, resulting in the accumulation of misfolded proteins, downregulation of several proteasome subunits, and differential modulation of ERAD-associated components. Under combined tunicamycin + MG132 treatment, the three CHO cell lines showed distinct proteomic patterns. NISTCHO preferentially upregulated proteins involved in ER translocation and glycoprotein quality control (e.g., SSR4, SEC24C, UGGT1), while CHO DP-12 showed a higher abundance of proteins associated with redox and disulfide regulation (DNAJC10, CAPN1). CHO-K1, the non-producing line, exhibited broader proteome-wide changes, suggesting greater sensitivity to proteotoxic stress. Meanwhile, the comparatively limited changes observed in NISTCHO may reflect its higher basal proteostasis capacity as a producer cell line. These

interpretations are based on proteomic abundance patterns and do not imply the functional activation of specific pathways.

While ER stress responses in CHO cells have been previously examined using transcriptomic and proteomic approaches, most studies have focused on single CHO cell lines and single ER stressors, limiting their ability to resolve how production phenotype influences cellular stress-management mechanisms. Our results offer a new perspective on CHO cell resilience by demonstrating that the ER stress response is not uniform across multiple cell lines but appears to be contingent on the cell line's existing adaptation to protein production. This warrants further investigation with more industrial-relevant cell lines and CHO cell lines producing DTE monoclonal antibodies and complex antibody-based modalities. Specifically, the unique upregulation of ER translocation and glycoprotein quality control machinery (UGGT1, SEC24C) in the higher-producing NISTCHO cell line under combined stress suggests a more specialised 'production-ready' stress response that is absent in the non-producing CHO-K1 cell line. These insights into producer-specific adaptive strategies have the potential to provide novel targets for cell line engineering.

Overall, our results provide mechanistic insights into how ER stress and proteasome inhibition shape proteostasis in CHO cells. These findings have practical implications for rational cell line engineering and bioprocess optimisation, suggesting that the modulation of ER stress pathways, chaperone networks, and ERAD components can be leveraged to improve recombinant protein yield, maintain cellular robustness, and guide the selection of high-performing CHO clones. The cell line-specific proteomic signatures identified in this study offer several targets for CHO cell engineering and bioprocess optimisation, for example, the selective upregulation of ER translocation and glycoprotein quality control machinery, specifically SSR4, SEC24C, and UGGT1, in the higher-producing NISTCHO line. Rational genetic engineering strategies aimed at overexpressing these proteins may enhance the robustness of less resilient or lower-producing CHO cell lines to improve productivity. From a process optimisation perspective, our findings potentially indicate that the controlled modulation of ER stress and proteasome activity can differentially influence CHO production phenotypes. The observation that proteasome inhibition amplifies ER stress responses while selectively engaging quality control pathways suggests that mild stress conditions, such as temperature shifts, feed formulation changes, or chemical chaperone supplementation, could be strategically used to bias cells toward more productive phenotypes, though this needs to be further investigated. More broadly, this study demonstrates how comparative proteomic analysis under defined stress conditions can be used as a phenotyping tool to identify productive stress-adaptation strategies in CHO cells. Such knowledge can guide the rational design of next-generation host cell lines, support clone selection decisions, and enable more predictive, mechanism-based bioprocess development.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/biom16020277/s1>, Figure S1: Fold change Viability, Apoptosis and Cell death of different concentrations of Tunicamycin in 24 h exposure on CHO-K1 cells. Control cells have been set to 1, and fold changes relative control cells have been calculated accordingly; Figure S2: Fold change in viability of CHO DP-12 cells treated with 10 µg/mL of tunicamycin for 24, 48, and 72 h compared to untreated cells; Figure S3: Densitometry quantification on western blots from Figure 3; Figure S4: BiP average normalised abundances in CHO-K1 control and treated at 48 h exposure. Data generated using Proteogenis QI for Proteomics software. The full lists of DE proteins from the label-free LC-MS/MS quantification for each comparison at the 48 h time point and for each cell line are provided in the three Excel files: CHOK1_DE_lists, CHODP12_DE_lists, and NISTCHO_DE_lists. Original images can be found in the Supplementary Materials.

Author Contributions: Conceptualization, P.M. and C.-K.S.; methodology, C.-K.S., D.R. and M.H.; software, C.-K.S. and M.H.; validation, C.-K.S., D.R. and E.E.; formal analysis, P.M.; investigation, C.-K.S., E.E. and P.M.; resources, P.M.; data curation, C.-K.S., E.E. and M.H.; writing—original draft preparation, C.-K.S.; writing—review and editing, C.-K.S. and P.M.; visualisation, C.-K.S.; supervision, P.M. and E.E.; project administration, P.M.; funding acquisition, P.M. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

The following abbreviations are used in this manuscript:

CHO	Chinese hamster ovary
ER	Endoplasmic reticulum
UPR	Unfolded protein response
mAb	Monoclonal antibody
ERAD	Endoplasmic reticulum-associated degradation

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