Cell Pool Selection of CHO Host and Recombinant Cell Pools by Inhibition of the Proteasome Results in Enhanced Product Yields and Cell Specific Productivity

Tanya J Knight[1], Sarah Turner[2], Colin M Jaques[2*], C Mark Smales[1*]

[1]Industrial Biotechnology Centre, School of Biosciences, University of Kent, Canterbury, Kent CT2 7NJ, U.K.

[2]Lonza Biologics, 228 Bath Road, Slough, SL1 4DX, U.K.

*Joint corresponding authors, email either
CMS; c.m.smales@kent.ac.uk
CMJ; colin.jaques@lonza.com

Abstract
Chinese hamster ovary (CHO) cells are the leading mammalian cell expression platform for biotherapeutic recombinant molecules yet some proteins remain difficult to express (DTE) in this, and other, systems. In recombinant cell lines expressing DTE proteins, cellular processes to restore proteostasis can be triggered when the folding and modification capabilities are exceeded, including the unfolded protein response and ER-associated degradation (ERAD) and proteasomal degradation. We therefore investigated whether the proteasome activity of CHO cells was linked to their ability to produce recombinant proteins. We found cell lines with diverse monoclonal antibody (mAb) productivity show different susceptibilities to inhibitors of proteasome activity. Subsequently, we applied selective pressure using proteasome inhibitors on mAb producing cells to determine the impact on cell growth and recombinant protein production, and to apply proteasome selective pressure above that of a metabolic selection marker during recombinant cell pool construction. The presence of proteasome inhibitors during cell pool construction expressing two different model molecules, including a DTE Fc-fusion protein, resulted in the generation of cell pools with enhanced productivity. The increased productivities, and ability to select for higher producing cells, has potential to improve clonal selection during upstream processes of DTE proteins.

Keywords: Proteasome inhibitors; Chinese hamster ovary cells; ERAD; recombinant protein production, difficult to express proteins.

Abbreviations: CHO, Chinese hamster ovary cells; **DMSO**, dimethyl sulfoxide; **DTE**, difficult to express; **ERAD**, endoplasmic reticulum associated degradation; **GS**, glutamine synthetase; **mAb**, monoclonal antibody; **MSX**, methionine sulfoximine; **UPR**, unfolded protein response.

1. Introduction

The Chinese hamster ovary (CHO) cell host expression system is widely employed for the commercial production of an ever-increasing number of biotherapeutic proteins. This is largely due to its ability to undertake human-like post-translational modifications, such as glycosylation, and to correctly fold, assemble and secrete multi-domain/polypeptide chain containing molecules with high yield and quality (Feary, 2017; Kunert, 2016; Mead, 2015; Povey, 2014; Walsh, 2018). One of the largest class of molecules expressed in this system is that of monoclonal antibodies (mAbs). CHO cell expression systems and associated bioprocesses have been developed through a range of approaches over the last few decades such that yields in excess of 5 g/L of mAb are now routinely achieved (Marichal-Gallardo, 2012). However, some mAbs are still difficult to express (DTE) in CHO cells (Laux, 2013; Mathias, 2020; Pybus, 2014) whilst many novel biotherapeutic molecule formats in development are DTE in CHO cells, or any other, expression system. A number of approaches have been taken to try and address this so that such DTE proteins can be produced at higher yields and quality. Approaches include: engineering of the target protein (Grote, 2012), screening of different bioprocesses, media and additives (Pybus, 2014) engineering of lipid metabolism (Budge, 2020), and manipulation of the unfolded protein response (UPR) and protein degradation (Johari, 2015; Le Fourn, 2014; Pybus, 2014). Indeed, Mathias et al have reported that DTE monoclonal antibodies have hampered secretion, and thus low secretory product yields, due to misfolding, and that as a result these molecules are degraded intracellularly by the proteasome (Mathias, 2020). This degradation does not allow the cell to recover synthesised but incorrectly folded polypeptides, reducing potential yields. There is thus an interest in furthering our understanding of the cellular properties that underpin productivity of DTE mAbs and other molecules, and whether a knowledge of these can be used to help evolve or select high producing host and recombinant CHO cell lines.

As highlighted by Mathias et al (2020), in a recombinant cell line expressing a DTE protein, particular cellular processes can be triggered within the cell when the folding and modification capabilities of the enzymes and chaperones within the ER are exceeded, to restore proteostasis. Two of the processes initiated to deal with misfolded or unassembled polypeptides during protein synthesis are the induction of the unfolded protein response (UPR) during ER stress and the activation of ER-associated degradation (ERAD) and proteasomal degradation. Key responses activated by these processes include up-regulation of ER chaperones, a reduction in global protein synthesis and thus the amount of new polypeptides entering the ER and ultimately, if the stress persists, apoptosis (Chakrabarti, 2011; Schroder, 2005). Indeed, it is proposed that up to 30% of newly translated polypeptides are targeted for degradation, possibly as a result of misfolding (Du, 2013; Schubert, 2000; Yewdell, 2006). It is thought that this cellular activity not only helps maintain protein quality, but that proteasomal degradation of polypeptides allows for amino acid recycling so that these are available to support further protein synthesis (Bröer, 2017; Suraweera, 2012).

The two cellular processes of the UPR and ERAD are coordinated, with the induction of a UPR response leading to an increased ERAD capacity. ERAD requires that polypeptides/proteins in the ER destined for destruction are transported back out of the ER to the cytosol where they are degraded by the proteasome (Olzmann, 2013). If homeostasis cannot be restored then the processes activated by the UPR can ultimately lead to apoptosis. The UPR provides the cell with the capability to adjust ER capacity during periods of high demand and an element of UPR induction is proposed to be beneficial to recombinant protein production (Prashad, 2015). However, excessive and long-term activation can be detrimental to the cell, and therefore the activation and tuning of the UPR and ERAD are thought to be related to the productivity and quality of the recombinant proteins generated. When a cell has the burden of recombinant polypeptides entering the ER, the ER can be placed under additional stress resulting in the UPR being induced (Hussain, 2014). It follows that concomitant with this, increased ERAD activity would be observed to maintain protein quality, reduce ER stress and meet the demands of high levels of recombinant protein production. Indeed, during

the differentiation of B cells into antibody producing plasma cells there is a programmed and coordinated expansion of the ER, ER chaperones, energy metabolism and ERAD to meet the demands of antibody synthesis (Aragon, 2012; Ma, 2010; Shaffer, 2004). Further, as described above, Mathias et al (2020) reported that a DTE mAb was misfolded, and degraded intracellularly by the proteasome.

We therefore explored whether the capability of CHO cells to undertake protein turnover via the proteasome is linked to their ability to produce a model mAb and DTE Fc-fusion protein. We hypothesized that those cells with a greater capacity for protein turnover would have higher recombinant protein productivity and quality attributes and that cells could be selected for, or evolved, that had improved recombinant protein production attributes using specific inhibitors of proteasomal activity. To investigate this, we established whether cells of different mAb productivity showed different susceptibilities to inhibitors of proteasomal activity. Subsequently, we applied selective pressure using proteasome inhibitors on mAb producing cells to determine any impact on cell growth and recombinant protein production. Inhibitors were then also used to apply proteasome selective pressure above that of a metabolic selection marker during a recombinant cell pool construction process and the resulting cell pools that emerged analyzed for growth and recombinant protein productivity. Finally, we undertook analysis of proteasome inhibitor evolved cells using qPCR arrays to identify changes in gene expression between control and inhibitor treated cells.

2. Methods and Materials

2.1 Cell lines and cell culture The Lonza CHOK1SV GS-KO® host cell line was cultured in CD (chemically defined)-CHO media (Invitrogen) supplemented with 6 mM glutamine; stably producing recombinant cell pools (used to generated the proteasome inhibition susceptibility correlation data) were cultured in CD-CHO media supplemented with 25 μM L-methionine sulfoximine (MSX), under batch culture conditions at 140 rpm, 36.5ºC with 5% (v/v) CO_2. Cells were passaged approximately every 3 to 4 days by performing a cell count using a ViCell XR (Beckman Coulter) instrument to determine viable cell concentrations, and resuspending the required volume to achieve seeding at 0.2 x 10^6 viable cells/ml in fresh media.

2.2 Inhibitor kill curves in 96 DWP format 96 deep well plates (Nunc) were seeded at 4 x 10^6 viable cells/ml/well for analysis of proteasome inhibitor effects. **Triplicate wells** were prepared for each inhibitor, for each panel of cell lines. 1 μl of each inhibitor stock was added to each well to give a final concentration of 0.05 μM Epoxomicin or 0.5 μM MG-132. Plates were then incubated at 36.5ºC, 5% CO_2. Cell concentration and culture viability was measured using a ViCell instrument. The remaining culture was centrifuged at 1000 rpm for 5 minutes and the supernatant and pellet stored at -20ºC prior to further analysis.

2.3 Polyclonal stable pool generation Linearization of the appropriate Lonza proprietary plasmid DNA was first performed with the PvuI restriction enzyme (New England Biolabs). Successful digests (assessed on a 1% agarose gel) were then concentrated and cleaned using a commercially available PCR clean up kit (Qiagen) and ethanol precipitation. The final DNA pellet was resolubilized in 0.2 μm filter sterilized 1 x TE buffer (Qiagen) in an appropriate volume required for the subsequent transfections and left at room temperature for approximately 1 hour, before determining the final DNA concentration of the linearized plasmid using a Nanodrop instrument. Subsequent transfection of the linearized plasmid DNA was then performed by electroporation using a GenePulser Xcell electroporator (Bio-Rad) into the Lonza CHOK1SV GS-KO® suspension cell line. Cells were taken from culture in mid exponential phase to achieve a concentration of 1 x 10^7 viable cells and combined with an appropriate quantity of linearized plasmid DNA in a BioRad electroporation cuvette. The DNA/cell mix was electroporated at 300 V and 900 uF in a cuvette with a 0.4 cm gap. For the cell

pool construction experiment, multiple electroporations were pooled together to give the required final volume. This electroporation pool was thoroughly mixed and then distributed in 10 ml volumes between T75 tissue culture flasks and incubated statically at 36.5ºC with 5% CO_2. 24 hours post-transfection, the glutamine synthetase inhibitor MSX was added at final concentrations of either 25, 37.5 or 50 μM. The addition of the proteasome inhibitors was then performed at the appropriate time-point following MSX selection as determined by the design of experiments layout detailed in Supplementary Figure 1. 0.2 μm filter sterilized DMSO was added as a control. Cultures were kept statically at 36.5ºC and 5% CO_2 until a viable cell concentration of at least 0.2 x 10^6 cells/ml was achieved, following which the cultures were transferred into 20 ml shake flask cultures at 36.5ºC and 5% CO_2 with shaking at 140 rpm. Routine sub-culture was then performed every 3 to 4 days in CD-CHO media with the addition of the appropriate MSX and proteasome inhibitor concentrations to the fresh cultures.

2.4 Batch culture assessment of cell pools generated from the proteasome inhibitor containing cell pool construction process For this purpose, cells were seeded at a concentration of 0.2 x 10^6 viable cells/ml **in triplicate flasks**, without the addition of the proteasome inhibitors, and incubated at 37ºC with shaking at 140 rpm in a Kuhner incubator and sampled every 48 to 72 hours for the determination of cell concentration and culture viability on a ViCell instrument.

2.5 Protein A high performance liquid chromatography to determine antibody concentration HPLC was performed with a Protein A column using Chemstation software and Agilent 1260 instrument following Lonza standard operating procedures.

2.6 Octet analysis to determine antibody concentration Recombinant molecule concentrations in cell culture supernatant samples were determined using the ForteBIO Octet® QK^e system and Dip and Read™ Protein A Biosensors (18-5013). Appropriate standard curves were prepared by dilution of a known concentration of the recombinant molecule being assessed. Specific productivity (Qp) values were determined from three time points of culture where product concentration and viable cell concentration using the ViCell instrument (Beckman Coulter) had been assessed. Qp was determined from the gradient of the line when average integral of viable cells (IVC) was plotted versus the average product concentration.

2.7 Glycan profiling Was undertaken internally at Lonza Biologics Analytical Research and Technology group. In brief, *N*-linked glycans were removed with peptide N-glycosidase F, labelled with 2-aminobenzamide and analysed by hydrophilic interaction chromatography with fluorescent detection.

2.8 RT^2 Profiler PCR array for CHO cell unfolded protein response Cell pools were revived and passaged four times in CD-CHO media supplemented with 37.5 μM MSX and incubated at 36.5ºC with 5% CO_2 at 140 rpm. Cell pellets of 2 x 10^6 viable cells were taken on days 3, 5 and 6 of culture and resuspended in 350 μl RLT buffer (Qiagen), before storage at -80ºC. The recombinant mAb protein expression profile of the cell pools was confirmed by western blot to be similar to the profile achieved immediately after the original cell pool construction. RNA extraction was performed on cell pellets using the Qiagen RNeasy mini kit as per the manufacturer's instructions, with the use of QiaShredder columns to lyse the cells. An on column DNase I treatment (Qiagen) was also performed on all samples and extracted RNA stored at 80ºC prior to array analysis. RNA concentration was determined using a Nanodrop 1000 spectrophotometer. A denaturing formaldehyde gel was run in MOPS buffer to assess RNA samples for additional degradation products or genomic DNA. The RT^2 Profiler PCR Array manual was followed for analysis with the initial genomic DNA elimination performed with 1 μg RNA followed by reverse transcription and RT-PCR with the RT^2 SYBR Green mastermix using a Eppendorf® Mastercycler ep realplex model 4S instrument in a 96 well plate format. Data analysis was performed using the $\Delta\Delta C_T$ method as

available on the PCR Array Data Analysis Web portal at www.SABiosciences.com/pcrarraydataanalysis.php.

2.9 Statistical analysis Correlation analysis was undertaken using linear regression and Pearson's correlation analysis. Analysis was performed in SigmaPlot 12.5 using linear regression analysis and Pearson Product Moment Correlation. The number of replicates in experiments is outlined in the methods section.

3. Results

3.1 The susceptibility of a panel of recombinant mAb producing CHO cell lines to proteasome inhibition correlates to mAb yield and mAb cell specific production

Initially we evaluated the impact on cell growth and culture viability of a panel of mAb producing cell lines of different productivities in the presence of different concentrations of proteasome inhibitors. These industrially relevant cell lines have been previously described (Porter, 2010a; Porter, 2010b). The proteasome inhibitors epoxomicin, a naturally occurring irreversible inhibitor, and MG-132, a reversible inhibitor, were added to the culture media of cells. Both compounds are well studied, selective and potent inhibitors of the proteasome (Hofmeister-Brix, 2013; Meng, 1999).

We first performed experiments using one model recombinant CHO mAb producing cell line to determine appropriate concentrations of each inhibitor for the assessment of the full panel. The concentrations analyzed were based on those concentrations previously reported in the literature; 0.04 to 0.08 μM epoxomicin has been shown to inhibit chymotrypsin activity of the proteasome (Meng, 1999); 1.5 μM MG-132 has been shown to induce apoptosis (Meriin, 1998). Both inhibitors were formulated in DMSO and DMSO alone was used as a negative control. We used these as starting concentrations to identify concentrations of inhibitors that gave some selection pressure (e.g. not all cells survived) on CHO cell lines but did not result in complete cell death, setting a criteria of addition of inhibitor concentrations giving a culture viability of between 30-60% 48 h after addition. Based on the observed culture viability and viable cell concentration up to 168 hours after exposure to the two inhibitors during batch culture, the concentrations deemed most appropriate for further studies were those around 0.5 μM for MG-132 and above 0.05 μM for epoxomicin. At these concentrations, an impact on viable cell concentrations and culture viability was observed with an approximate 30 to 60% reduction compared to control samples (Figure 1). At higher concentrations there was more cell death or the cultures were killed completely whilst at lower concentrations there was little or no impact on viable cell concentrations or culture viability (Figure 1). We note that the control DMSO experiments for MG-132 and epoxomicin gave different profiles that related to the final concentration of DMSO present in the culture (1.25 v 2.8% v/v respectively, Figure 1). The concentrations selected for further study were thus a compromise between the impact on the cell numbers that survived the presence of the inhibitor, and therefore potentially on selection of cells, and complete cell death.

The impact of the two inhibitors at the established concentrations were then investigated on a panel of CHO mAb producing cell lines, all derived from the Lonza CHOK1SV® host cell line and that were expressing the model IgG4 molecule at different yields. The viable cell concentration and culture viability were assessed after addition of the inhibitors (and control DMSO alone) in a 96 deep well plate format, with daily cell concentration being estimated using a ViCell instrument. The viable cell concentration and culture viability of the cell lines on different days post-inhibitor addition were then related to the productivity of the cell lines to establish if there was any correlation between cell line mAb productivity and susceptibility to the proteasome inhibitor concentrations investigated. Historical productivity data from shake flask fed batch and bioreactors were used to perform linear regression and Pearson's correlation analysis between viable cell concentration and culture viability in the 96 DWP format with product titre and cell specific productivity (Figure 2). The 48 hours post-addition

time-point was used to perform correlation analyses as at this time point the majority of inhibitor and inhibitor concentration combination cultures had culture viabilities above 30%. Statistically significant correlations ($p < 0.05$) were observed between antibody concentration at harvest and susceptibility (viable cell concentration and culture viability) of the cell line to the presence of the proteasome inhibitors (Figures 2B & 2D). This was also the case for cell specific productivity (Figures 2A, 2C, 2E-F), suggesting a link between susceptibility of a cell line to proteasome inhibitors, culture viability and the ability to produce mAb.

3.2 Proteasome selection pressure during cell pool construction results in the isolation of cell pools with enhanced recombinant mAb expression compared to those generated in the absence of proteasome inhibitors

Due to the correlation observed between cell line productivity and susceptibility of cell lines to proteasome inhibitors (Figure 2), we next investigated whether proteasome inhibitor presence could be used to select for or isolate higher mAb producing recombinant cell pools during a cell pool construction process. A cell pool construction process was therefore designed to generate Lonza CHOK1SV GS-KO® recombinant mAb producing cell pools using the model cB72.3 antibody. This was achieved by including the addition of the proteasome inhibitors as an additional selection pressure to Lonza's proprietary glutamine synthetase (GS®) metabolic selection system using a proprietary vector with the GS® gene in addition to the heavy and light chain mAb gene and addition of methionine sulphoximine (MSX) during recovery of transfected cells (Fan, 2012). The cell pool construction process design is described in Supplementary Figure 1 and was developed using a design of experiments (DoE) approach. Cells were transfected and then MSX added after 24 hours in glutamine free medium to aid metabolic selection for those cells that had GS® expression whilst the proteasome inhibitors were added 24, 96 or 168 hours post addition of MSX to allow selection based on plasmid uptake to occur prior to inhibition of the proteasome. The staggered addition of MSX and proteasome inhibitors was also undertaken to limit the impact of applying two inhibitors onto cells at the same time. Addition of DMSO alone was again used as a negative control as both proteasome inhibitors tested were prepared in this solvent (one or both being insoluble in other alternatives such as ethanol).

When undertaking the cell pool construction process, the concentrations of MG-132 used were lower than those used previously to generate the data presented in Figure 2. The concentrations previously investigated with the panel of recombinant mAb cell lines resulted in poor viable cell concentrations following electroporation and during the cell pool construction process, likely due to the impact of electroporation and MSX challenge on top of MG-132. As a result, the concentration of MG-132 used during cell pool construction was decreased and either 0.0625 or 0.125 μM were used in experiments whilst 0.025 or 0.05 μM epoxomicin were used. Combinations of the two proteasome inhibitors were also investigated (Supplementary Figure 1), however this combination approach or the use of higher concentrations of inhibitor did not appear to be tolerated by the cells following the transfection process.

Eight out of the thirty cell pool construction processes evaluated survived the cell pool construction process (Figure 3). These were the four controls treated with different MSX concentrations and with DMSO alone (i.e. no proteasome inhibitor was added) and four where the process contained the lower concentration of either MG-132 (0.0625 μM) or epoxomicin (0.025 μM) after addition of MSX. These cell populations were cultured and expanded in the presence of both MSX and the appropriate concentration of the proteasome inhibitors before being assessed in a suspension batch culture for growth and antibody productivity characteristics. In general, the growth characteristics were similar between all the cell populations (Figure 3). One of the cell pool constructions undertaken in the presence of 0.025 μM epoxomicin and 37.5 μM MSX grew slower than the other cultures and did not achieve as high a maximum viable cell concentration, obtaining a maximum viable cell

concentration of 5.33 x 10^6 cells/ml in comparison to 8 to 10 x 10^6 cells/ml for the other cell pool construction populations (Figure 3A). One of the two cell pool populations generated in the presence of MSX and DMSO alone declined in culture viability at an earlier time point compared to the other populations, to a viability of 5.8% at 192 hours of culture (Figure 3B).

Analysis of the antibody concentration in the supernatant demonstrated a large difference in the concentration between pools generated using the different cell pool construction processes. The antibody concentration was determined from supernatant taken at 192 hours of batch culture using Protein A HPLC (Figure 3C). All cell pools isolated in the presence of the proteasome inhibitors showed an increase in antibody concentration over that observed from the MSX and DMSO generated pools alone. Indeed, those pools generated with 0.0625 μM MG-132 and 37.5 μM MSX showed an approximate 3-fold increase in the amount of antibody present in the cell culture supernatant, whereas those generated with 0.025 μM epoxomicin and 37.5 μM MSX also displayed at least a 3-fold increase, and in one case an approximate 6-fold increase in antibody concentration over the different control pools generated with MSX and DMSO alone (Figure 3C). These data suggest that the use of the proteasome inhibitors alongside MSX selection results in the generation of cell pools that have higher recombinant monoclonal antibody productivity than those generated using MSX selection alone, being at least 3-fold, although the exact magnitude of the increase showed some variability and in some cases was larger.

3.3 The presence of proteasome inhibitors during cell pool development does not negatively impact critical quality product attributes such as *N*-glycosylation

We next investigated whether using inhibitors of proteasome degradation of mis-assembled proteins had an impact on the quality of the final recombinant product produced, specifically the *N*-glycosylation profile. To determine whether the presence of the inhibitors had any impact on the *N*-glycan profile of the model mAb, UPLC analysis was performed to assess the major *N*-glycoforms present in control and proteasome inhibitor derived cell pools from mAb samples collected after 192 hours of batch culture. The major *N*-glycan structures observed were G0F, G1Fa/b and G2F species, showing the presence of complex oligosaccharides with fucosylation, with minimal levels of G0 and Man5 observed (Figure 4C). In general the *N*-glycan profiles observed in the presence of the proteasome inhibitors were very similar to those in the control 37.5 μM MSX and DMSO control. For the majority of the N-glycan species detected, the percentage of those present were within 2-3% of each other and all were within 10% of the controls (Figure 4C). Using this criteria, collectively the *N*-glycan data shows that cell pools generated in the presence of MSX and a proteasome inhibitor were not adversely impacted in terms of the ability of these cells to undertake the desired complex *N*-glycosylation of the model recombinant mAb molecule.

3.4 Elevated mAb production from cell pools generated in the presence of proteasome inhibitors is maintained when these are later removed

In order to determine if the improved productivity of cell pools generated in the presence of the proteasome inhibitors was maintained following further passaging of the pools, and if the continued presence of the proteasome inhibitors was required, cell pools generated from the cell pool constructions (Figure 3) were revived and sub-cultured without the inhibitors present. The higher productivity (compared to the controls) as a result of the presence of the inhibitors during cell pool development was maintained following routine subculture of the cells over a number of passages, although the exact magnitude of the increase between cultures did vary (Figure 5A). This suggests that the effects on titre are due to a higher producing population of cells being selected for during the cell line construction process and not due to a direct effect of the presence of the proteasome inhibitors on the cells influencing recombinant protein production.

To demonstrate the reproducibility of the process, we undertook a further cell pool construction process using a wider range of proteasome inhibitor concentrations. Once again, this resulted in

increased productivity in those cell pools where the proteasome inhibitors epoxomicin and MG-132 were present at various concentrations (Figure 5B-C). The increase in titre observed was similar when mAb yields were determined either immediately after culture with the inhibitors present or following subculture in the absence of the inhibitors. This provides further evidence that the presence of the proteasome inhibitors following transfection results in the emergence of a higher producing population of cells and that this population can then be maintained by subculture in the absence of the inhibitors.

3.5 Culturing of host cells with proteasome inhibitors before recombinant cell pool construction does not result in the generation of cell pools with enhanced productivity

We next investigated whether it was possible to evolve the host cell line in the presence of the proteasome inhibitors such that heritable properties that gave enhanced recombinant product yields were realized in the host cell as a result of pressure from the inhibitors. We hypothesized that populations within the host cell pool with a propensity to produce higher yields of recombinant protein may be evolved and selected for by culturing in the presence of the proteasome inhibitors prior to construction of recombinant mAb expressing cell pools. If this were realized, it would negate the need for addition of the inhibitors during the cell line construction process. The Lonza host cell line was therefore cultured in the same concentrations of the proteasome inhibitors (62.5 nM and 15.6 nM MG-132 and 25 nM and 6.25 nM epoxomicin) for 5 to 7 passages. After this time, the resulting cell pools were then transfected with the cB72.3 construct either immediately after culture in the presence of the proteasome inhibitors, or following passage of the evolved host cell pools without the inhibitors present. The transient expression of cB72.3 from these cell pools was then evaluated and these did not show increased productivity over that shown when cells were evolved with DMSO alone (Figure 6). Although the impact of proteasome inhibitor evolution may give a different impact on transient expression of recombinant mAb compared to that of stable pools, the results suggest that the additional pressure of the recombinant protein production on the cells is likely to be required alongside the presence of the proteasome inhibitors to elicit the selective effects of the inhibitors.

3.6 Application of proteasome selection pressure during cell pool construction of a difficult to express recombinant Fc-fusion protein results in the isolation of cell pools with enhanced productivity

To determine if the beneficial effects on productivity of cell pool construction in the presence of proteasome inhibitors was product specific, a further cell pool construction was performed with a model Fc-fusion protein (FcFP) that was considered difficult to express (Budge et al., 2020). MG-132 was added at 15.6, 31.25 and 62.5 nM concentrations and epoxomicin at 12.5 and 25 nM in addition to 37.5 μM MSX. The transfections to which 25 nM epoxomicin was added did not result in the emergence of any colonies, however all other conditions resulted in pools emerging. These pools were expanded and batch culture assessment of growth and productivity undertaken with samples taken for assessment at 48, 96 and 168 hours for viable cell concentration, culture viability and FcFP titre (Figure 7). Lower viable cell concentrations were achieved with 31.25 and 62.5 nM MG-132 over 96 hours of culture compared to the controls (in the absence of proteasome inhibitors), however these cultures did show an extended batch-culture lifetime. Viable cell concentration and culture viability for those cultures from 12.5 nM epoxomicin and 15.6 nM MG-132 were comparable to those treated with DMSO. FcFP titre and specific productivity were enhanced in those pools generated in the presence of 15.6 and 31.25 nM MG-132 in addition to the 37.5 μM MSX. The magnitude of the increase in product concentration was approximately 50% for the best conditions.

3.7 Those cell pools generated in the presence of proteasome inhibitors with enhanced mAb product yields show changes in gene expression that reflect decreased ERAD and UPR activity

To investigate whether the impact of the presence of the proteasome inhibitors was related to

changes in the cellular folding capacity, ERAD or proteasome activity of the pools, commercially available RT^2 profiler PCR arrays for the unfolded protein response were used to monitor the expression of key genes in these processes (Figure 8). The array allows the monitoring of a range of genes involved in pathways ranging from unfolded protein binding, ER protein folding and quality control, and translation to ERAD, ubiquitination and protein folding. Samples were taken from revived representative cell pools described in Figure 3 on days 3 and 6 of batch culture, the RNA extracted and then analyzed according to the manufacturer's instructions using normalization with the *Hprt1* gene as this was determined to have the most stable expression levels.

Comparison of day 3 samples between those treated with 25 nM epoxomicin and the control cell pool treated with DMSO only showed a decrease in the expression of genes involved in unfolded protein binding, ERAD and ubiquitination, as well as ER protein folding quality control and protein folding (Supplementary Table 1 and Supplementary Figures 2-7; (Kanehisa, 2000)). There were fewer differences between the cell pools treated with 62.5 nM MG-132 and DMSO treated pools at day 3, with only 2.74- and 2.19-fold decreases in *Derlin-1* and *Chac1* detected respectively, these genes being involved in ERAD and maintenance of protein folding (Kadowaki, 2015; Nomura, 2016; Ye, 2004) and demonstrating a similar trend to that observed with the epoxomicin treatment. Further, the beneficial impact of the MG-132 treatment was less marked than with epoxomicin in these cultures expressing the model antibody. Fewer differences were observed when comparing day 6 DMSO treated control and epoxomicin treated pools. Approximate 2-fold increases were observed in *Mapk9* and *Ppil4* expression, these being involved in apoptosis and unfolded protein binding/protein folding respectively. Conversely, approximately 2-fold decreases were again observed in *Derlin-1* and *Edem1* expression, once again suggesting a decrease in the expression of genes involved in the ERAD process. No differences were observed when making a comparison of the 62.5 nM MG-132 treated pool with the DMSO treated cell pool for the Day 6 samples.

Comparison within the treatment groups (e.g. within a culture with the same addition of inhibitor or control and how gene expression varied between day 3 and 6 of culture) revealed a greater change in the levels of gene expression. Comparison of the day 3 and 6 control DMSO treated samples showed an upregulation in genes involved in ERAD, ubiquitination, UPR protein folding and apoptosis (e.g. *Derlin-1, Herpud1, Sel1l, Calr3, Chac1* and *Ddit3*) on day 6. Upregulation was also observed in samples taken from cultures treated with 25 nM epoxomicin on day 6 when compared to samples from the same cultures taken on day 3. This upregulation involved the same genes that were observed to be upregulate between day 3 and day 6 in the control cultures but the extent of upregulation was greater. Upregulation of genes between day 3 and day 6 in the 62.5 nM MG-132 treated cell pool was to an intermediate extent when compared to the control and epoxomicin treated cell pools.

4. Discussion

We have investigated whether inhibition of proteasome activity during recombinant cell pool construction impacts on the subsequent product yields and quality from these cell pools compared to those generated in the absence of such inhibitors. There have been enormous advances in the ability of CHO cells to produce large amounts of high quality recombinant therapeutic proteins, particularly mAbs in recent years. However, some molecules remain DTE in CHO cells or any other system whilst many novel format molecules in development are expressed at lower yields than mAbs and are thus considered DTE (Budge, 2020; Mathias, 2020). Recent reports suggest that DTE antibodies can be incorrectly folded and degraded by the proteasome via ERAD (Mathias, 2020).

Our initial proteasome inhibitor screening showed that the susceptibility of a panel of industrially relevant established recombinant CHO cells lines expressing a model monoclonal antibody to MG-132 and epoxomicin differed. There was a positive correlation between culture viability or viable cell

concentration and mAb productivity when the cells were cultured in the presence of the proteasome inhibitors at the concentrations investigated. There are at least three possible explanations for this. First, that this is the result of the lower producing cell lines being less equipped in terms of the cellular machinery required to support high levels of recombinant protein synthesis and assembly, resulting in increased ERAD activity and an increased susceptibility to the presence of the inhibitors. Under this scenario, the inhibition of proteasome activity is more detrimental to these low producing cell lines. Second, that in high producing cell lines, the ERAD pathway may be less active or not be as over-loaded, due to enhanced folding and assembly capacity in these cell lines. Under this scenario, the negative impact of inhibiting the proteasome on cell fitness is reduced. This second hypothesis is in contradiction to the initial hypothesis that high levels of protein production in cells would be associated with high ER stress and conversely, high levels of ERAD activity (Travers, 2000). Third, that higher producing cell lines have enhanced and 'spare' ERAD capacity and hence are less susceptible to ERAD inhibitors than low producing cell lines.

From the data reported here, it is not possible to distinguish between these hypotheses. Previously a correlation between proteasome inhibition and productivity has been reported in myeloma cells, whereby those cells with relatively high IgG synthesis were more sensitive to proteasome inhibition than those with relatively low IgG synthesis, potentially due to accumulation of unfolded proteins (Meister, 2007). It is unclear why the results in this study show a different relationship between IgG expression and susceptibility to proteasome inhibitors. Our correlation analysis suggests that high producing recombinant CHO cell lines (higher cell specific productivity) are less sensitive to the presence of proteasome inhibition than low producer cell lines (as determine by correlation with the viable cell number maintained, Figure 2), suggesting that in high producing cell lines less protein degradation is required. This is likely a result of less misfolded protein being present in the ER and more protein being correctly processed in the ER leading to higher yields. High producing cell lines have previously been associated with elevated amounts of chaperones in recombinant protein expressing mammalian cell lines, and hence the fidelity of folding may be improved in these cells, allowing them to better facilitate high loads on the cellular machinery required to support enhanced recombinant protein production (Dinnis, 2006; Smales, 2004).

We used this information to subsequently investigate if the presence of proteasome inhibitors during cell pool construction could be used as a selection pressure related to the amount of recombinant protein product. This novel selective pressure was applied in addition to a metabolic selection marker, in this case glutamine synthetase and its inhibitor MSX (Fan, 2012; Feary, 2017; Noh, 2018). The majority of combinations of MSX and either epoxomicin or MG-132 when applied to the selection process of cell pool construction post-transfection did not survive the selection process and subsequent culture. However, those populations that did emerge in the presence of either 62.5 nM MG-132 or 25 nM epoxomicin alongside 37.5 μM MSX gave enhanced cB72.3 antibody titres after 192 hours of batch culture when compared to those cultured with MSX alone. Importantly, the *N*-glycosylation patterns were similar between mAb from the inhibitor and control pools suggesting there was no negative impact on glycosylation. Calculation of the specific cell productivity (Qp) of the cell pools confirmed that those pools generated in the presence of the proteasome inhibitors had an improved specific productivity compared to those cell populations generated with MSX selection alone. Again, these data suggest that the presence of proteasome inhibitors during cell pool construction selected for a higher producing population of cells. Importantly, we showed that after the initial cell selection process the continued presence of the inhibitors was not required to maintain the productivity of the pool and that the productivity traits were heritable. However, the emergence of improved productivity in the presence of the inhibitors did appear to require the recombinant product load to also be placed on the cell as directed evolution in the absence of the recombinant protein load with the inhibitors did not give enhanced transient expression. We suggest the increased pressure on the degradation and recycling system of the cell during recombinant protein production

allows the selection pressure of the proteasome inhibition to be successful.

Finally, profiling of UPR and ERAD genes revealed that when samples from control cultures and those treated with proteasome inhibitors were compared on the same day of batch culture there was generally lower expression of UPR and ERAD genes in samples from cultures treated with proteasome inhibitors than in samples from the control cultures. When samples taken on two days of batch culture were compared (day 3 and 6) in control and proteasome inhibitor generated cell pools, there was an increased in the expression of the various genes regardless of treatment. This is likely a reflection of the difference in gene expression between different days of batch culture and increased recombinant protein production load on the cell and reduced growth, at the later culture day. The pool generated in the presence of 25 nm epoxomicin showed increased expression of the largest number of genes between day 3 and 6. Addition of proteasome inhibitors may therefore select for cells with an inherent folding capacity that matches recombinant protein demands, reducing the throughput into the ERAD pathway and material targeted for degradation.

In conclusion, we have shown that the presence of proteasome inhibitors during the construction of cell pools expressing two different model molecules, including a difficult to express Fc-fusion protein, results in the generation of cell pools with enhanced productivity. The increased productivities generated, and the ability to select for higher producing cells, has the potential to improve the clonal selection during upstream processes in an industrial setting of difficult to express proteins in particular. Further elucidation of the mechanisms underpinning the increased productivity may also open up new cell engineering approaches to generate new host cells with an enhanced ability to produce such DTE proteins.

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6. Conflict of Interest Statement

ST and CMJ are employed by Lonza Biologics, who developed and license the GS Gene Expression System®. Lonza is the assigned owner of, and TJK, CMJ and CMS are named inventors on, the filed patent ‘Inhibition of protein degradation for improved production WO2017118726A1’, patent number WO2017191165A1.

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Figure Legends

Figure 1. Assessment of proteasome inhibitor impact on model recombinant IgG producing CHO cell line growth parameters. 96 deep well plate experiments with a Lonza CHOK1SV® derived model IgG producing cell line were performed to monitor growth and culture viability in the presence of different concentrations of either epoxomicin (A,B) or MG-132 (C, D) proteasome inhibitors. Plates were left for 48 hours after seeding in an incubator under Lonza proprietary conditions before addition of the compounds at a range of concentrations. Samples were taken every 24 hours up to 168 hours after addition of the drug and counted on a ViCell instrument to determine viable cell concentration (A, C) and culture viability (B, D). Note: the final DMSO concentration in epoxomicin and epoxomicin control experiments was 2.8% (v/v) whilst in MG-132 treated cells and controls it was 1.25% (v/v).

Figure 2. Correlation analysis between susceptibility to proteasome and ERAD inhibitors, determined by viable cell concentration, and productivity data from fed-batch and bioreactor cultures for a panel of CHO cell lines producing a model monoclonal antibody. Analysis was performed in SigmaPlot 12.5 using linear regression analysis and Pearson Product Moment Correlation. Correlations found to be statistically significant ($p<0.05$) are shown. CHO mAb producing cell lines were cultured in the presence of the proteasome inhibitors in a 96DWP format and daily cell counts performed after addition of inhibitors (0.05 µM Epoxomicin and 0.5 µM MG-132). Data from the 48 hour time point was used for the correlation analysis with historical productivity data (production concentration at harvest and specific productivity) generated at Lonza under fed batch and bioreactor culture conditions. **A** Correlation assessing cell concentration in the presence of 0.05 µM epoxomicin with cell specific production rates generated from fed batch culture. **B** Correlation assessing cell concentration in the presence of 0.05 µM epoxomicin with product concentration values generated from fed batch culture. **C** Correlation assessing cell concentration in the presence of 0.05 µM epoxomicin with cell specific production rate values generated from bioreactor culture. **D** Correlation assessing cell concentration in the presence of 0.05 µM epoxomicin versus product concentration values generated from bioreactor culture. **E** Correlation assessing cell concentration in the presence of 0.5 µM MG-132 with cell specific production rate values generated from fed batch culture. **F** Correlation assessing culture viability in the presence of 0.5 µM MG-132 with specific production rate values generated from fed batch culture.

Figure 3. A-B Growth characteristics of cell populations generated using a cell pool construction process with MSX and varying proteasome inhibitors and concentrations. The Lonza CHOK1SV GS-KO® cell pools expressing the model cB72.3 monoclonal antibody were cultured in duplicate at 37ºC with 5% CO_2 and shaking at 140 rpm and cell counts were performed every 48-72 hours following cell pool construction with proteasome inhibitors in addition to MSX. **C: Antibody concentrations from cell populations generated using a cell pool construction process with MSX and varying proteasome inhibitors and concentrations**. Lonza CHOK1SV GS-KO® cell pools expressing cB72.3 were cultured and a Protein A HPLC analysis performed with supernatant collected after 192 hours of culture following cell pool construction with proteasome inhibitors in addition to MSX selection pressure. ** denotes a statistical significance ($p<0.01$) determined by one way ANOVA analysis with Tukey grouping using Minitab 17 software.

Figure 4. *N*-Glycan analysis to assess product characteristics of cell populations generated using a cell pool construction process with MSX and varying proteasome inhibitors and concentrations. Lonza CHOK1SV GS-KO® cell pools expressing cB72.3 were cultured and glycan analysis performed accordingly to Lonza internal protocols with supernatant collected after 192 hours of culture. Results from analysis of mAb material from duplicate cultures are shown.

Figure 5. (A) Estimated Qmab values for cell populations generated using a cell pool construction process with MSX and varying proteasome inhibitors and concentrations during routine subculture. Lonza CHOK1SV GS-KO® cell pools expressing cB72.3 were cultured and Protein A Octet analysis performed on supernatant collected during routine subculture following cell

pool construction with proteasome inhibitors in addition to MSX selection pressure. Estimated Qmab values were determined by dividing the titre (µg/ml) by the cell count (x10^6 cells/ml). **Specific productivities for the cell populations generated using MSX and varying proteasome inhibitor concentrations immediately after presence of the inhibitor (B) and following routine subculture (C)**. Protein A Octet analysis was performed on supernatant collected at 48, 96 and 168 hours of batch culture of Lonza CHOK1SV GS-KO® cell pools grown in triplicate and expressing cB72.3 following cell pool construction with proteasome inhibitors in addition to MSX selection pressure. Specific productivity values were calculated for each cell pool both immediately after culture in the presence of the inhibitors and following subculture where the inhibitors had been removed.

Figure 6. Specific productivities from transient transfection with cB72.3 of Lonza CHOK1SV GS-KO® host cells after directed evolution by culturing in the presence of proteasome inhibitors. Lonza CHOK1SV GS-KO® host cells were cultured in the presence of proteasome inhibitors for 5-7 passages and then transiently transfected with cB72.3. Samples were taken at 48, 96 and 168 hours post transfection. Inhibitors were also removed from the GSKO® host cultures (previously cultured in the presence of the inhibitors) for three routine subcultures before the transient transfection was repeated and samples collected at 48, 96 and 168 hours post transfection, to determine if the continued presence of the inhibitor was required. Protein A Octet analysis was performed with supernatant collected and specific productivities calculated.

Figure 7. (A & B) Growth characteristics of cell populations expressing a model Fc-fusion protein generated using a cell pool construction process with MSX and varying proteasome inhibitors and concentrations. Lonza CHOK1SV GS-KO® cell pools expressing a model Fc-fusion protein were cultured in duplicate at 37ºC with 5% CO_2 and shaking at 140 rpm and cell counts performed every 48-72 hours following cell pool construction with proteasome inhibitors in addition to MSX. (**C) Product concentrations achieved from cell populations generated using a cell pool construction process with MSX and varying proteasome inhibitors and concentrations**. Lonza CHOK1SV GS-KO® cell pools expressing a model Fc-fusion protein were cultured and Octet Protein A assay performed on supernatant collected after 48, 96 and 168 hours of culture following cell pool construction with proteasome inhibitors in addition to MSX selection pressure. Averages were derived from duplicate cultures under each condition. **(D) Calculated specific productivities achieved from cell populations generated using a cell pool construction process with MSX and varying proteasome inhibitors and concentrations**. Specific productivities were derived from the acquired growth and titre data for the above time-course. Averages were derived from individual specific productivities from duplicate cultures under each condition.

Figure 8: Scatterplots comparing the normalised expression of each gene on the array between two conditions. Qiagen Unfolded Protein Response RT2 Profiler PCR Array was performed on total RNA extracted for cell pools generated using proteasome selection during cell pool construction. Cells were cultured at 37ºC with 5% CO_2 and shaking at 140 rpm. **A** shows represents duplicate samples of day 3 culture with 25 nM Epoxomicin and 37.5 µM MSX treatment versus duplicate samples of day 3 culture with DMSO and 37.5 µM MSX treatment; **B** shows duplicate samples of day 3 culture with 62.5 nM MG-132 and 37.5 µM MSX treatment versus duplicate samples of day 3 culture with DMSO and 37.5 µM MSX treatment; **C** shows duplicate samples of day 6 culture with 25 nM Epoxomicin and 37.5 µM MSX treatment versus duplicate samples of day 6 culture with DMSO and 37.5 µM MSX treatment; **D** shows duplicate samples of day 6 culture with 62.5 nM MG-132 and 37.5 µM MSX treatment versus duplicate samples of day 6 culture with DMSO and 37.5 µM MSX treatment; **E** shows duplicate samples of day 6 cultures with DMSO and 37.5 µM MSX versus duplicate samples of day 3 culture with DMSO and 37.5 µM MSX treatment; **F** shows duplicate samples of day 6 culture with 25 nM Epoxomicin and 37.5 µM MSX treatment versus represents duplicate samples of day 3 culture with 25 nM Epoxomicin and 37.5 µM MSX treatment; and **G** shows duplicate samples of day 6 culture with 62.5 nM MG-132 and 37.5 µM

MSX treatment versus duplicate samples of day 3 culture with 62.5 nM MG-132 and 37.5 µM MSX treatment, all using auto HKG normalisation in the analysis software. Spots outside the dotted 95% confidence interval lines highlight genes whose expression was significantly different between sample sets (upregulated shown by yellow spots above upper line, downregulation shown by blue spots below lower line).