

Use of a Small Molecule Cell Cycle Inhibitor to Control Cell Growth and Improve Specific Productivity and Product Quality of Recombinant Proteins in CHO Cell Cultures

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ABSTRACT: The continued need to improve therapeutic recombinant protein productivity has led to ongoing assessment of appropriate strategies in the biopharmaceutical industry to establish robust processes with optimized critical variables, that is, viable cell density (VCD) and specific productivity (product per cell, qP). Even though high VCD is a positive factor for titer, uncontrolled proliferation beyond a certain cell mass is also undesirable. To enable efficient process development to achieve consistent and predictable growth arrest while maintaining VCD, as well as improving qP, without negative impacts on product quality from clone to clone, we identified an approach that directly targets the cell cycle G1-checkpoint by selectively inhibiting the function of cyclin dependent kinases (CDK) 4/6 with a small molecule compound. Results from studies on multiple recombinant Chinese hamster ovary (CHO) cell lines demonstrate that the selective inhibitor can mediate a complete and sustained G0/G1 arrest without impacting G2/M phase. Cell proliferation is consistently and rapidly controlled in all recombinant cell lines at one concentration of this inhibitor throughout the production processes with specific productivities increased up to 110 pg/cell/day. Additionally, the product quality attributes of the mAb, with regard to high molecular weight (HMW) and glycan profile, are not negatively impacted. In fact, high mannose is decreased after treatment, which is in contrast to other established growth control methods such as reducing culture temperature. Microarray analysis showed

major differences in expression of regulatory genes of the glycosylation and cell cycle signaling pathways between these different growth control methods. Overall, our observations showed that cell cycle arrest by directly targeting CDK4/6 using selective inhibitor compound can be utilized consistently and rapidly to optimize process parameters, such as cell growth, qP, and glycosylation profile in recombinant antibody production cultures.

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Introduction

Recombinant protein productivity is proportional to viable cell density (VCD) and specific productivity (product per cell, qP). Even though achieving and maintaining high VCD is important for productivity, a high VCD beyond an optimal number will decrease yield due to the reduction of the harvestable production volume and possible challenges to the harvest operation. In addition, a very high VCD can have excessive nutrient and gas exchange demands that can be challenging to meet. For these reasons, it is important to control cell growth after an optimum VCD has been obtained during production. With VCD being controlled, increasing qP then becomes essential for protein productivity.

Cell cycle inhibition-related approaches have been widely used and tested previously to increase qP in recombinant cell cultures, including nutrient limitation, decreasing cultivation temperature, chemical additives such as butyrate, cell

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engineering by overexpression of endogenous cyclin-dependent kinase inhibitors (CKIs), or anti-apoptotic proteins such as Bcl-2 family members (Fomina-Yadlin et al., 2013; Kantardjieff et al., 2010; Kumar et al., 2007; O'Reilly et al., 1996; Sampathkumar et al., 2006; Simpson et al., 1999; Tey and Al-Rubeai, 2005; Yee et al., 2008). Recently the potential use of miRNAs to control cell cycle has also been studied in CHO production culture (Barron et al., 2011; Bueno et al., 2008; Doolan et al., 2013; Hackl et al., 2012; Jadhav et al., 2013; Johnson et al., 2011; Sanchez et al., 2013; Strotbek et al., 2013). While these approaches have been shown to be effective in improving qP, their effects under different circumstances, such as different expression vector design, host cell type, production medium, protein sequence, and process set points, can be variable. A common feature of all these approaches is that the cell cycle checkpoint regulators, cyclin-dependent kinases (CDKs) are not the exclusive target. Almost all these approaches have multiple cellular targets other than cell cycle, leading to varying degrees of pleiotropic effects. It is therefore not surprising to find inconsistencies from clone to clone and between experiments using these methods during production processes, presumably due to the complex signaling networks centered by different activation events that each of these approaches stimulate. Hence, the cross-talk among the different signaling pathways, such as cell cycle, apoptosis, and metabolism, will generate different cellular contexts, which then influence cell fate.

More specifically, nutrient limitation is one of commonly used approach in growth control, which can suppress cell cycle progression through the amino acid deprivation response (AAR)-associated pathways, including EF1 α -ATF4 and EF1 α -PERK pathways, which decrease intracellular levels of cyclins (Dey et al., 2010; Fomina-Yadlin et al., 2013; Hamanaka et al., 2005; Harding et al., 1999, 2000; Sonenberg et al., 2000; Shang et al., 2007; Wek et al., 2006). However, these pathways can also decrease a number of other proteins, including housekeeping genes that maintain essential metabolic and cellular function (Harding et al., 2003; Shang et al., 2007). This pathway also exhibits cross-talk to other stress pathways and is able to induce apoptosis (Ameri and Harris, 2008; Baird and Wek, 2012; Dey et al., 2010; Fomina-Yadlin et al., 2013; Harding et al., 2003; Kilberg et al., 2009). For these reasons, the effect of nutrient limitation on both proliferation inhibition and increasing recombinant protein secretion can be mild and variable. Decreasing cultivation temperature is another commonly used approach in growth control and increasing qP. The mechanism whereby cells at lower temperatures improve productivity and undergo growth arrest is still poorly understood. It is suggested that lower temperatures decrease the global transcription/translation rate, result in decreased protein levels of cyclins and lead to cell cycle arrest indirectly [Reviewed in Kumar et al. (2007)]. Reducing temperature also reduces the metabolic rate, but this may still be able to increase recombinant protein expression by invoking a coordinated response involving the cell cycle, transcription and translational machinery, and the arrangement of the cytoskeleton (Al-Fageeh et al., 2006;

Chuppa et al., 1997; Furukawa and Ohsuye, 1999; Hendrick et al., 2001; Jorjani and Ozturk, 1999; Moore et al., 1997; Yoon et al., 2003a). This sophisticated regulatory network can also lead to various phenotypes in different cell lines, resulting in variable responses.

These complex signaling networks will mediate different context-dependent effects, impacting not only mAb productivity but also product quality attributes (PQA), such as glycosylation. mAbs are glycosylated at N297 on CH2 domain. One key feature of N-linked glycosylation of mAbs is heterogeneity due to the incomplete processing of the N-linked Fc glycans, reflected by the presence or absence of different terminal sugar residues. Immature glycoforms of CHO-derived IgGs, such as the high-mannose (HM) glycoforms (Man5-9, as variable as 2–35%), are major concerns due to the higher plasma clearance rate of mAbs containing HM compared to more complex glycan linked mAbs, which may lead to a potential impact on efficacy (Goetze et al., 2011; Jones et al., 2007). It has been shown that growth control approaches, such as temperature shift or chemical additives such as butyrate, extend to impact glycan processing (Nam et al., 2008; Sajan et al., 2010; Sampathkumar et al., 2006; Trummer et al., 2006) which could compromise the employment of these established approaches into the production process if consistent PQA is required for the product, such as commercial product comparability and biosimilar development.

To directly achieve growth arrest with minimal cross talk to other pathways including glycan processing, it is necessary to identify an approach to arrest cell cycle selectively by directly targeting regulators of cell cycle checkpoints. The cell cycle consists of four phases, G0/G1, S, G2, and M, which are tightly controlled by cell cycle checkpoints. The G1 checkpoint is controlled by the cyclin D/CDK4/6 complex, which phosphorylates and deactivates Rb [reviewed in Sherr and Roberts (1999)]. Phosphorylated Rb dissociates from E2F, which then activates gene transcription of S phase cyclins to initiate DNA replication. Due to the critical role of CDK4/6-Rb in cell cycle progression, multiple cellular mechanisms are involved in control of their activities, especially CKIs. Most attempts at arresting cell cycle have focused on overexpression of CKIs, in particular p16, p21, and p27. It has been shown that over-expression of CKIs results in increasing qP (Bi et al., 2004; Fussenegger et al., 1997, 1998; Ibarra et al., 2003; Kaufmann et al., 1999, 2001; Mazur et al., 1998; Watanabe et al., 2002). However, the impact of overexpression of CKIs on Amgen mAb-expressing cell line was minimal (unpublished data) and was not sufficient to incorporate into our production processes. In addition, tight gene regulation control methods, such as inducible systems, to suppress CKI overexpression during growth phase to achieve optimum VCD and rapid activation at later production phase poses another layer of technical challenge.

To develop a robust method for both growth control and specific productivity improvement by avoiding pleiotropic cellular responses and complicated operational procedures, we attempted to identify an approach that results in exclusive

G0/G1 arrest by directly targeting the cell cycle G1 checkpoint with small molecule compound. In this approach, the goal is to specifically deactivate CDK4/6 function in Rb phosphorylation since the catalytic activity of these kinases regulates the checkpoint for the G1/S transition and the commitment to cell division [reviewed in Bloom and Cross (2007), Ekholm and Reed (2000), Hochegger et al. (2008), Morgan (1997)]. CDKs are a sub-class of serine/threonine kinases, which catalyze the phosphorylation of protein or peptide substrates via transfer of the γ -phosphate from ATP to the hydroxyl of a serine or threonine residue. We therefore screened pyridopyrimidine-type molecules, a series of ATP analogues, to block CDK4/6 kinase phosphorylation function (Du et al., 2014). One of these compounds has been used as a therapeutic drug against breast cancer due to its anti-proliferation effects (Fry et al., 2004). The selectivity of this compound has been widely studied, demonstrating that it is a highly selective inhibitor of CDK4 and CDK6 inhibiting these enzymes potently ($IC_{50} \sim 0.01 \mu\text{M}$) with minimal inhibition of at least 36 other kinases from various kinase families (Barvian et al., 2000; Ekholm and Reed, 2000; Fry et al., 2001, 2004; Toogood, 2001). We surmised that the selective inhibition of CDK4/6 by this small molecule might lead to a complete cell cycle arrest without the concomitant activation of other cellular responses seen in the less specific approaches to arrest the cell cycle described above. These experiments thus provide an opportunity to test whether an exclusive G0/G1 arrest is sufficient to induce sustained growth arrest and also increase qP. It will also be useful to examine the potential link between cell cycle, qP and glycosylation in this study. The results showed that this compound can be used as a small molecule additive to CHO recombinant protein production processes and can simultaneously control growth and increase qP with improved glycan processing. The mechanism was explored at the transcriptome level and compared with temperature shift and nutrient limitation in production cultures. The results from these studies are discussed to shed light on the differences in cell phenotypes between these three cell-cycle arrest methods.

Materials and Methods

Kinase Assays and Selectivity Profiling

CCI was tested at multiple concentrations (0.001–10 μM) against selected kinases in kinase activity assays to determine IC_{50} . The assays were performed in 96-well filter plates with a final volume of 100 μL , containing 25 μM ATP, 1 μCi [^{33}P]-ATP, CCI compound, recombinant kinase, and the corresponding substrate, that is, 1 μg Rb, 25 ng CDK4/cyclin D, in kinase reaction buffer. After 1 h incubation at room temperature, the reaction was stopped with 20% trichloroacetic acid (TCA). Wells were washed with 10% TCA, let dry, and processed for scintillation counting with TopCount (PerkinElmer, Waltham, MA).

Cell Culture

The cell lines studied herein were recombinant cell lines expressing different antibodies. The common CHO host cell line is a clone derived from the serum-free, IGF-1 dependent CHO cells described by Rasmussen et al. (1998). For the initial dosage and time course study, cells were cultured in Amgen in-house subculture medium in 24-deep well plate (DWP) (Corning, NY) for 5 days with the seeding density as 5×10^5 c/mL. CCI was added at the first day of the culture. For 24-DWP production assay, cells were seeded as high as 1×10^7 c/mL with a daily supplement of fresh Amgen in-house production medium. The media were completely exchanged by centrifugation each day for 5 day. The measurements of growth, viability, titer, osmolality, glucose and lactate were collected daily. Each experimental group was analyzed in triplicate, and the experiment was repeated twice. Bench scale perfused production bioreactors were operated by using Amgen in-house process conditions with Amgen in-house perfusion medium. VCD and culture viability were measured using a Vi-Cell counter (Beckman-Coulter, Indianapolis, IN). Glucose and lactate concentrations were determined using a PolyChem analyzer (Innovatis, Bielefeld, Germany).

Cell Cycle Analysis

Cells were harvested and fixed with 70% ethanol in PBS. The cell pellet was then resuspended in 0.5 mL PBS containing propidium iodide (50 $\mu\text{g/mL}$) and DNase-free RNase (100 $\mu\text{g/mL}$). Cell cycle analysis was performed by using a FACSCaliber (Becton Dickinson, San Jose, CA).

mAb Titer and Product Quality Analysis

The secreted mAb concentration (titer) was measured via Protein A affinity HPLC. Antibody aggregate and HMW were measured by size exclusion chromatography (SEC). N-Glycans were analyzed by 2-AA hydrophilic interaction liquid chromatography (HILIC). Chromatograms were analyzed for species percentages.

Western Blotting Analysis

Whole-cell extract preparation and Western blotting were performed as described in reference (Du et al., 2013). Membrane were probed with phospho-specific Rb (Ser⁷⁹⁵), Phospho-specific ERK (T²⁰²/Y²⁰⁴), Phospho-specific S6 (S^{235/236}), Rab 11 primary antibodies (Cell Signaling Technology, Denver, MA), followed by AlexaFluor[®] 680-conjugated secondary antibodies (Invitrogen, Carlsbad, CA). Images were acquired using the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE).

DNA Microarray and Bioinformatic Analyses

DNA microarray and bioinformatic analyses were carried out as described (Fomina-Yadlin et al., 2013).

Results

Complete and Sustained Cell Cycle G1 Arrest Using a Selective CDK4/6 Small Molecule Inhibitor

To induce a complete and selective cell cycle G0/G1 arrest without potentiating other cellular pathways, we used a small molecule cell cycle inhibitor (CCI) to selectively inhibit the kinase activity of CDK4/6 (Fig. 1A). While potently inhibiting CDK4/6, the compound was highly selective showing little activity when screened at 1 μ M compound concentration against the vast majority of >440 other kinases, including CDK2, CDK3, CDK5, and multiple cyclin-dependent kinase-like (CDKL) kinases, confirming and expanding previous reports (Barvian et al., 2000; Ekholm and Reed, 2000; Fry et al., 2001, 2004; Toogood, 2001) (data not shown). In addition, the inhibitory effect of CCI on different selected kinases, including CDK4/6, was tested over a large concentration range (0.001–10 μ M) of CCI in the kinase assays to obtain IC₅₀ values. The results demonstrate the potency

and selectivity of CCI against CDK4/6 compared to inhibition against various other kinases (Table I). The specific inhibitory effect on Rb phosphorylation was further confirmed from by Western blotting (Fig. 1B). Both ERK and S6 kinase signaling networks are involved in cell proliferation and protein expression. As shown in Figure 1B, phosphorylation of ERK and S6 kinases were not affected, suggesting that the CCI has no effect on these two signaling pathways. To test its effect on recombinant CHO cell growth, different dosages of the compound were added to the cultures of two different mAb-expressing cell lines at day 0 with seeding density at 0.5×10^5 c/mL, while VCD and viability were measured daily for 5 days. Compared to the control, in which cell growth reached 3–5 million cells per mL, cell growth after CCI treatment was significantly inhibited, and the maximum effect on growth arrest was attained between 5–10 μ M at 24 h of treatment (Fig. 2A and B). Despite the growth arrest, no significant cytotoxicity was observed at these concentrations (5–10 μ M) of the compound as deduced from ending viabilities compared to the control (Fig. 2C and D) for both cell lines. Similar results were observed with an additional 10 different Amgen recombinant cell lines with different productivity, growth rate and product quality profiles using the same dosages of CCI, suggesting a broad treatment effect (data not shown).

A selective Cdk4/6 inhibitor should cause a specific accumulation of cells in G1 but has no effect on other phases of the cell cycle in which cells should continue to progress and eventually decline in number. Indeed, cell cycle profiling indicated that 96% of the CCI-treated cells were arrested in G0/G1, compared to 49.8% of control cells at 24 h (Fig. 3A and B). S phase was diminished at 24 h post treatment, with a concomitant decline in G2/M phase of the cell cycle (Fig. 3B). The cell cycle inhibitory effect was sustained for at least 4 days without further addition (Fig. 3E). In comparison, Asn limitation and reducing culture temperature are less effective in G0/G1 arrest (Fig. 3C and D). Taken together, these data indicate that selectively blocking CDK4/6 activity can induce a maximum level of G0/G1 enrichment and growth arrest within 24 h without causing cell death with the same dosage for all recombinant CHO cells tested.

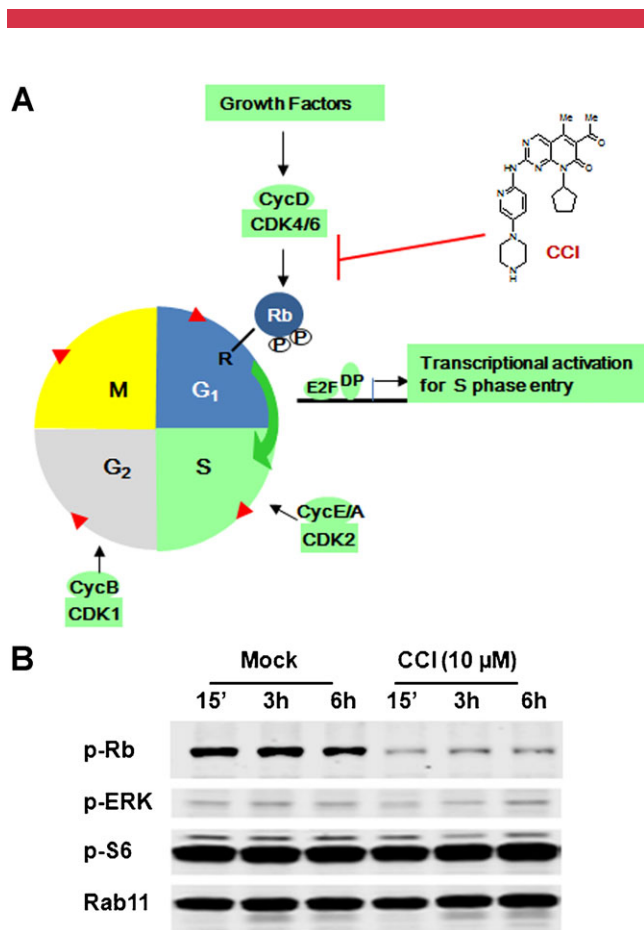


Figure 1. Selective CDK4/6 inhibition by small molecule compound. (A) Schematic diagram of the cell cycle and function of a selective CDK4/6 specific inhibitor. E2F, E2 transcription factor; DP, E2F dimerization partner; R, restriction checkpoint. (B) Whole cell extracts with or without CCI treatment were assayed for protein levels of phospho-Rb (p-Rb), phospho-ERK (p-ERK), phospho-S6 (p-S6) by Western blot. Rab11 was used as a loading control.

The Selective CDK4/6 Inhibitor Has Consistent Effect on Increasing Specific Productivity With Multiple Recombinant CHO Cell Lines

We then assessed whether the specific and complete cell cycle G0/G1 arrest observed also increases specific productivity (qP). As shown in Figure 4, a recombinant mAb-expressing CHO cell line was treated with the inhibitor using a plate-based production format, which includes complete daily medium-exchange. In this way, the cells were continuously exposed to the indicated level of CCI, which was supplied with daily medium exchange. The control cells grew continuously for 4 days from 1×10^7 /mL to 2.8×10^7 /mL in production medium, whereas addition of CCI compound arrested cell growth and maintained VCD under 1.4×10^7 /mL

Table I. Average IC₅₀ values in $\mu\text{mol/L}$ for CCI activity against a panel of Ser/Thr kinases in vitro.

Gene symbol	Description	IC ₅₀ (μM)
CDK4	Cyclin-dependent kinase 4	0.0027
CDK6	Cyclin-dependent kinase 6	0.0063
Mps1	TTK protein kinase	0.5
PIM1	Pim-1 oncogene	1.6
FLT3	Fms-related tyrosine kinase 3	1.7
CDK9	Cyclin-dependent kinase 9	1.8
PKR	Eukaryotic translation initiation factor 2-alpha kinase 2	2.1
Stk33	Serine/threonine kinase 33	2.1
JAK3	Janus kinase 3	2.5
ALK	Anaplastic lymphoma receptor tyrosine kinase	2.6
mTOR	Mechanistic target of rapamycin (serine/threonine kinase)	3.9
Tyk-2	Tyrosine kinase 2	6.1
PI3K alpha	Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic sub-unit alpha	8.1
CDK2	Cyclin-dependent kinase 2	9.9
JAK2	Janus kinase 2	11.7
JAK1	Janus kinase 1	>10
TBK1	TANK-binding kinase 1	>10
IKK (3)	Inhibitor of kappa light polypeptide gene enhancer in G cells, kinase beta	>10
IKKE	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon	>10
PKA alpha	Protein kinase, cAMP-dependent, catalytic, alpha	>10
IGF-1R	Insulin-like growth factor 1 receptor	>10
Src	v-Src avian sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog	>10

at day 4 (Fig. 4A). Cell viabilities remained above 80% throughout the 5-day production with the treatment of 5–10 μM of CCI (Fig. 4B), which is consistent with our previous subculture results (Fig. 2B and D). The qP from CCI treatment was increased more than two fold (Fig. 4C). In addition, CCI treatment showed no impact on mAb aggregation, one of PQA shown by HMW species (Fig. 4D).

Similar results had been observed with a panel of recombinant CHO cell lines expressing different recombinant antibodies, using the same concentration of CCI (10 μM) (Table II). All cell lines can be arrested in cell growth and qP consistently increased between 2 and 3 fold with the same dosage of CCI. For the high producing cell line (cell line A), the qP reached 110 pg/cell/day (Table II). Taken together,

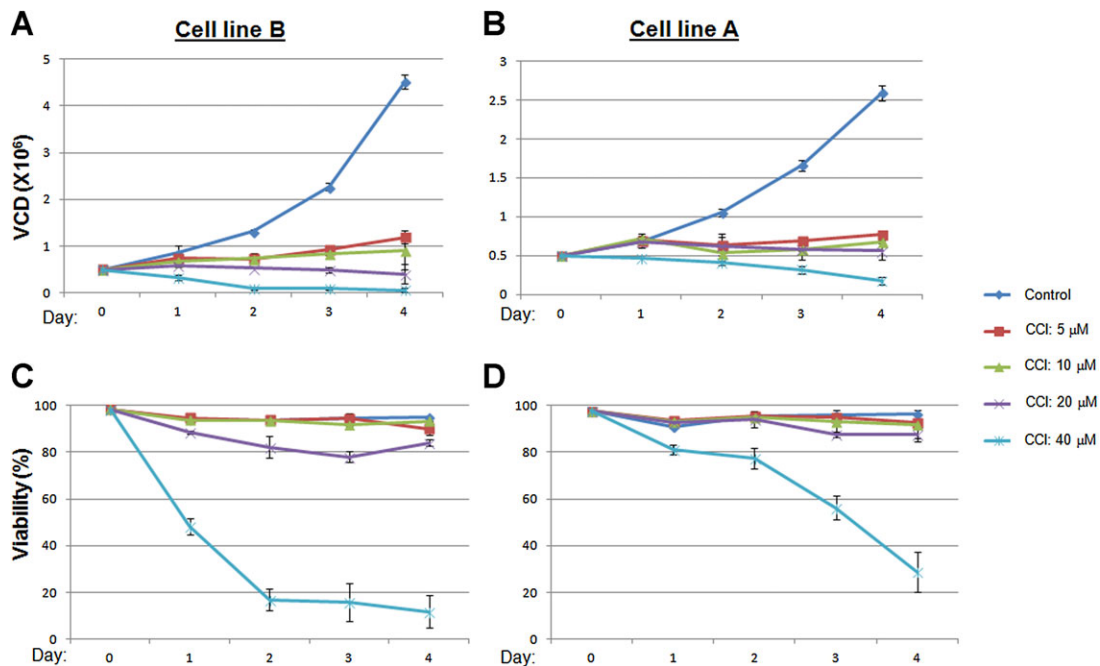


Figure 2. CDK4/6 inhibitor leads to a complete cell growth arrest. Two representative recombinant CHO cell lines were cultured and treated with the indicated dosage of inhibitor. VCD (A and B) and viability (C and D) were measured daily. Data represent the average \pm SD of triplicate samples.

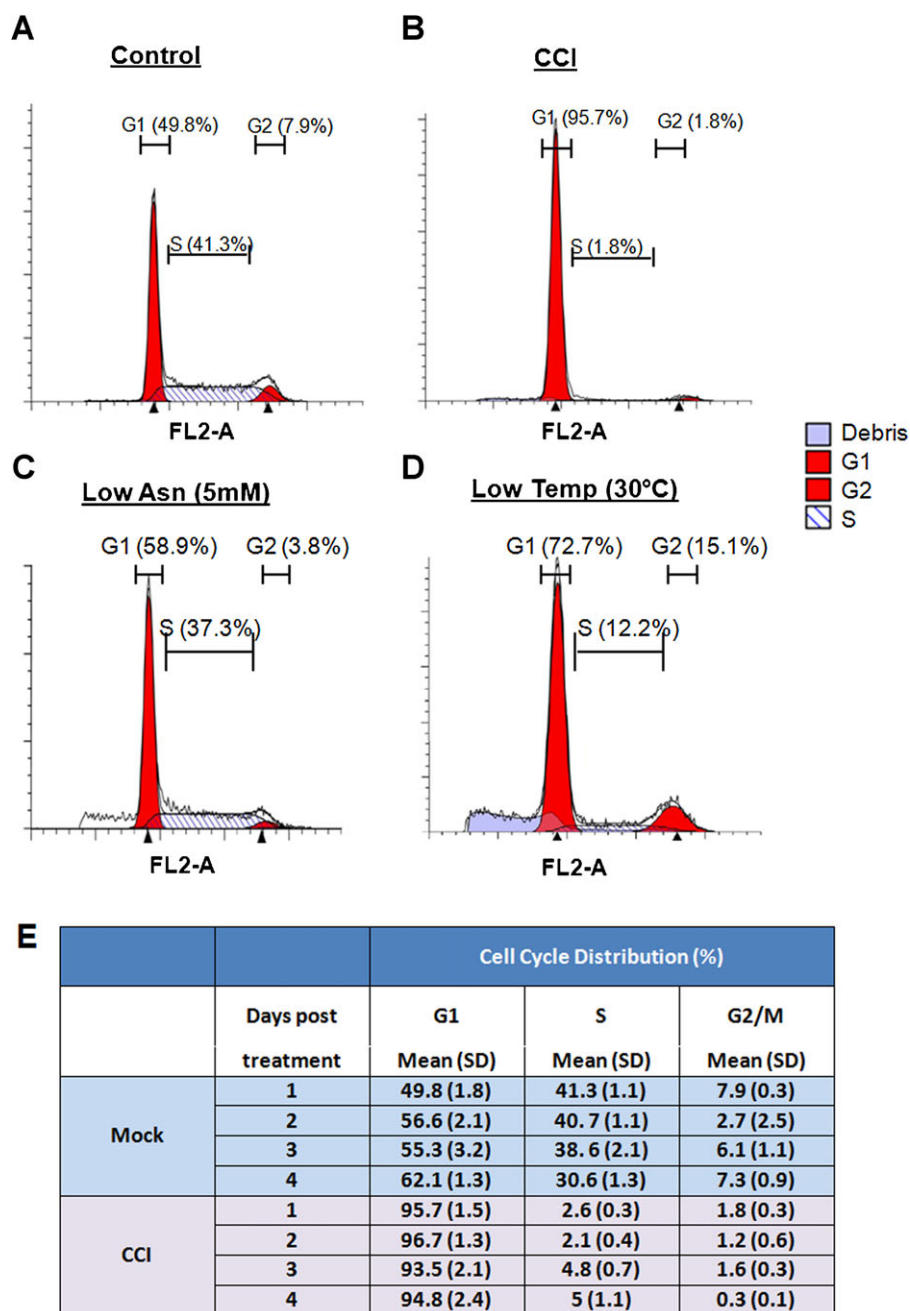


Figure 3. Cell cycle profile of CHO recombinant cells treated with CDK4/6 inhibitor. A recombinant CHO cell line was cultured in different growth control conditions. Cells were cultured in batch medium with mock (A), with 10 μ M of CCI for 24 h at 36°C (B), low Asn (C), or reducing culture temperature (D) conditions. Cell cycle profiles were obtained by PI staining followed by flow cytometry analysis. The red fill in the first and second peaks indicate the range estimate of the size of the G1 and G2 peaks, respectively. The black area indicates S-Phase estimate. (E) The cell cycle distribution of mAb-expressing cell line with or without CCI treatment. Data represent the mean \pm SD of two independent replicate samples.

these results confirmed that CDK4/6 inhibitor is able to control cell growth even at a high cell density (seeding density at 1×10^7 /mL at day 0). The effect is rapid (within 24 h) and sustained for at least 5 days. Most importantly, the data indicate that a complete and exclusive G0/G1 arrest is sufficient to consistently increase qP more than two fold in all CHO cell lines regardless of their basal qP, suggesting the

potential for broad application of this small molecule additive to various cell culture processes.

The Effects of a Complete G0/G1 Arrest on N-Linked Glycosylation Maturation

We next investigated the impact of a sustained G0/G1 arrest on N-linked glycosylation of mAb. As with productivity,

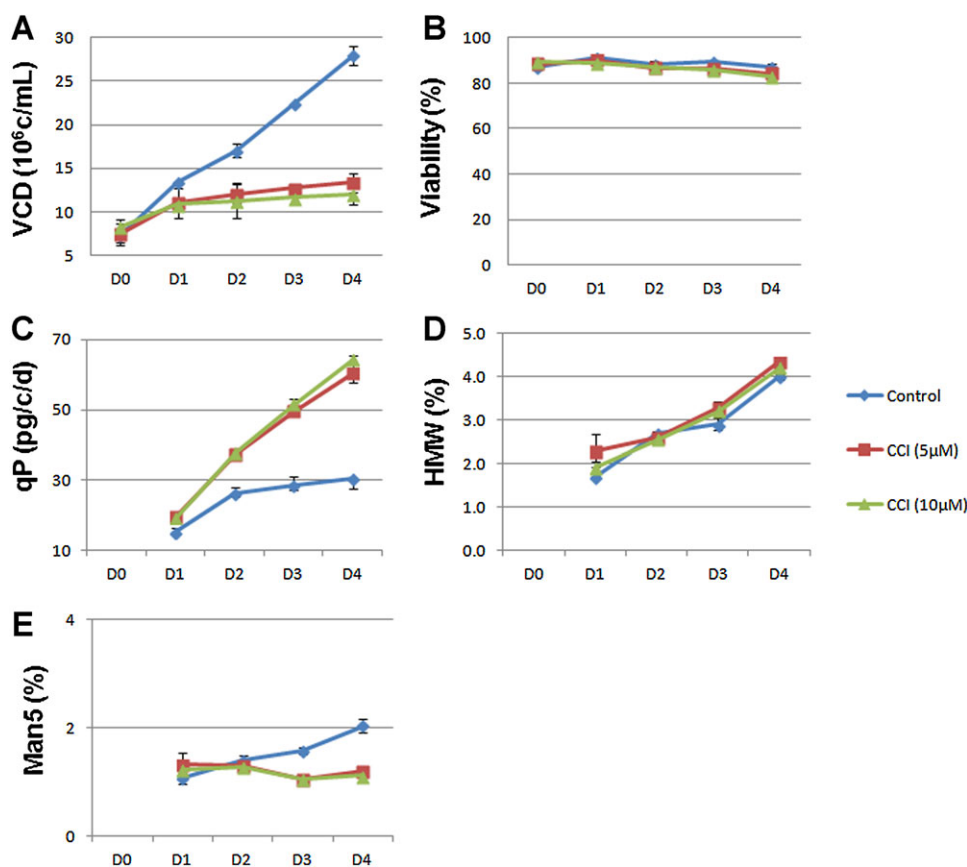


Figure 4. The effects of CKD4/6 inhibitor treatment in CHO recombinant cell production cultures. A recombinant CHO cell line was subjected to a 5-day 24DWP production assay, using batch medium with or without the indicated amount of inhibitor. VCD (A), viability (B), qP (C), HMW (D), and high mannose (E) were measured daily as described in the Materials and Methods section. Data represent the average \pm SD of three biological replicates.

PQA, in particular, mAb glycosylation profiles have become more important due to the increasing need to demonstrate product quality comparability with both commercial products comparability and biosimilar initiatives. Since G0/G1 arrest is linked to cell differentiation, it is reasonable to hypothesize that related cellular pathways, such as the secretion pathway, are highly activated, which will affect

not only protein expression but also glycosylation. As shown in Figure 4E, CCI treatment during production decreased high mannose level (Man5) suggesting G0/G1 arrest might improve glycan processing. To further investigate this effect, multiple recombinant cell lines which produce mAbs with different glycan profiles were selected and tested for glycan profile with and without CCI treatment. The result showed

Table II. The effects of CCI treatment on cell culture performance of different recombinant cell lines.

Recombinant cell line	VCD (10 ⁶ /mL)		Viability (%)		qP (pg/cell/day)	
	Control	CCI	Control	CCI	Control	CCI
Cell line A	26.50	13.20	95.20	90.20	53.96	110.56
Cell line B	34.30	10.00	94.50	91.10	31.87	72.41
Cell line C	57.00	20.60	91.61	87.17	19.98	50.11
Cell line D	28.00	11.90	87.07	84.33	30.41	64.19
Cell line E	43.66	12.16	97.56	92.37	17.73	35.87
Cell line F	55.00	14.60	90.28	85.70	16.37	39.49
Cell line G	27.20	11.80	89.84	83.40	35.23	78.68
Cell line H	44.10	17.60	91.67	87.88	22.74	47.98
Cell line I	37.50	16.30	90.75	88.60	33.35	88.13

that all high mannose glycans were significantly decreased for all tested mAbs compared to the control (Fig. 5A). Meanwhile, the fraction of complex glycans that are core fucosylated and terminally galactosylated (G1F and G2F) were increased after CCI treatment (Fig. 5B). The optimal dosage of CCI in these glycan changes appeared to be slightly different from clone to clone between 5 and 10 μM (Fig. 5A and B). The impact of CCI treatment on glycan structures and distribution are shown in Figure 5C. Results showed decreases in the levels of a variety of HM glycans (M5, M6, M7, and M8) and the hybrid structures containing HM (G0FM5, G1FM5, G0M5), suggesting improved processing of high mannose structures (Fig. 5C). This was accompanied by increases in the levels of fucosylated and galactosylated bi-antennary glycan structures (G1F and G2F), the two major mature complex structures (Fig. 5C). Core fucosylation reactions are efficient in the tested cell lines, therefore unfucosylated bi-antennary structures, such as G0 and G1, were low (<4%), and G2 was not detected (Fig. 5C). In addition, the levels of these minor glycan structures, such as G1, together with the major ungalactosylated glycan structure, G0F, were decreased after CCI treatment (Fig. 5C). Since these structures are intermediates in the pathway to synthesis of G1F and G2F structures, the decrease likely reflects the maturation of G1 to G1F by addition of core fucose, and the maturation of G0F to G1F by addition of galactose. Similar changes in glycan distribution were found in products from other recombinant clones (data not shown). Taken together, these data suggest that the efficiency of N-linked glycan processing is different with CCI treatment.

Comparison of Different Growth Arrest Methods on Cell Performance and Product Quality in Bioreactor Productions

As mentioned earlier, decreasing culture temperature and nutrient-limitation are the two major methods that have been used to control cell growth and also improve qP during perfused bioreactor production processes. Here we compared the similarity and the difference between these approaches with CDK4/6 inhibitor treatment. Recombinant cells were seeded below $1 \times 10^6/\text{mL}$. When VCD reached approximately $4 \times 10^7/\text{mL}$ (day 8), the bioreactors were treated either by CCI compound addition or a culture temperature shift from 36°C to 30°C (TS). For the nutrient-limiting condition, low Asn (5 mM) medium was used since this method has been effective in our hands for many cell lines (Fomina-Yadlin et al., 2013). As shown in Figure 6, cells from the control bioreactor reached $1.2 \times 10^8/\text{mL}$ at day 15. On the other hand, cell growth is well controlled by CCI treatment and a 30°C -temperature shift ($<7 \times 10^7/\text{mL}$) without causing cell death (Fig. 6A and B). Both approaches increased qP from 20 to 40–45 pg/cell/day at day 15 (Fig. 6C). Low Asn had a similar but less pronounced effect on both growth control and qP (Fig. 6A and C). With increased qP, the production titers were therefore not impacted compared to control, even

though VCDs were decreased in CCI, temperature shift and low Asn conditions (Fig. 6D). Differences were found in mAb PQA between these methods. Antibody produced from 30°C -temperature shift condition showed an obvious decrease in HMW compared to control, suggesting lower cultivation temperature may improve mAb assembly (Fig. 6E). However, decreasing the culture temperature to 30°C induced an increase of mAb HM level, with a concomitant decrease in the level of bi-antennary galactosylated glycans (G1F and G2F) (Fig. 6F and G). In comparison, CCI-treatment reduced HM and increased G1F/G2F levels (Fig. 6E and F), which are consistent with the 24-DWP production assay for this cell line (Figs. 4E and 5B, cell line D).

To determine if the phenotypic changes observed in production reactors and product quality correlated with changes in gene expression, DNA microarray analysis was performed. The analysis showed that the mRNA levels of many enzymes and transporters involved in the N-linked glycosylation pathway were significantly decreased in the temperature shift condition but increased in CCI-treated cells (Fig. 7). These genes are key processing enzymes in glycan trimming and maturation, including GlcNAc beta 1,4 (1,3)-galactosyltransferase (B3galt, B4galt), beta-galactosidase alpha-2,3-sialyltransferase 1 (St3gal), UDP-galactose transporter (Slc35a2), mannoside acetylglucosaminyltransferase 1 (Mgat1), UDP-N-acetylglucosamine:polypeptide-N-acetylglucosaminyl transferase (B3galnt), and fucosyltransferase 8 (Fut8) (Fig. 7). Genes that are involved in glyco-protein quality control, such as calnexin, were also downregulated in mRNA levels in the temperature shift condition (Fig. 7). Epigenetic suppressors, such as Sirtuin 1, were upregulated in the temperature shift condition, providing a possible link between metabolism homeostasis and modulation of glycosylation pathway genes. The expression profiles of genes involved in the cell cycle pathway were also compared. Interestingly, CKIs, such as p21 (Cdkn1a) and p27 (Cdkn1b), were significantly upregulated with temperature shift, at 24 h, but not as the result of CCI treatment (Fig. 8), suggesting that CKIs are involved in temperature shift-, but not CCI-, mediated cellular effects. The low Asn condition induced fewer changes in the cell cycle pathway compared to CCI or temperature shift with the exception of a slight increase in p15 (Cdkn2b) expression but not other CKIs, suggesting a different growth arrest mechanism. The result from CCI-treatment is consistent with previous studies (Barvian et al., 2000; Ekholm and Reed, 2000; Fry et al., 2001, 2004; Toogood, 2001) since all E2F target genes, which include CKIs, CDK2, CCNA, CCNB, and CCNE, are all down-regulated, due to deactivation of CDK4/6 function (Fig. 8). Thus, the CCI-mediated G0/G1 arrest is different from CKI-dependent approaches, which could partly explain the difference in phenotypes. Taken together these data support a model in which a selective and complete cell cycle G0/G1 arrest, mediated by directly blocking CDK4/6 functions, can both inhibit cell proliferation and simultaneously induce a program that favors recombinant protein secretion, assembly and glycosylation modification.

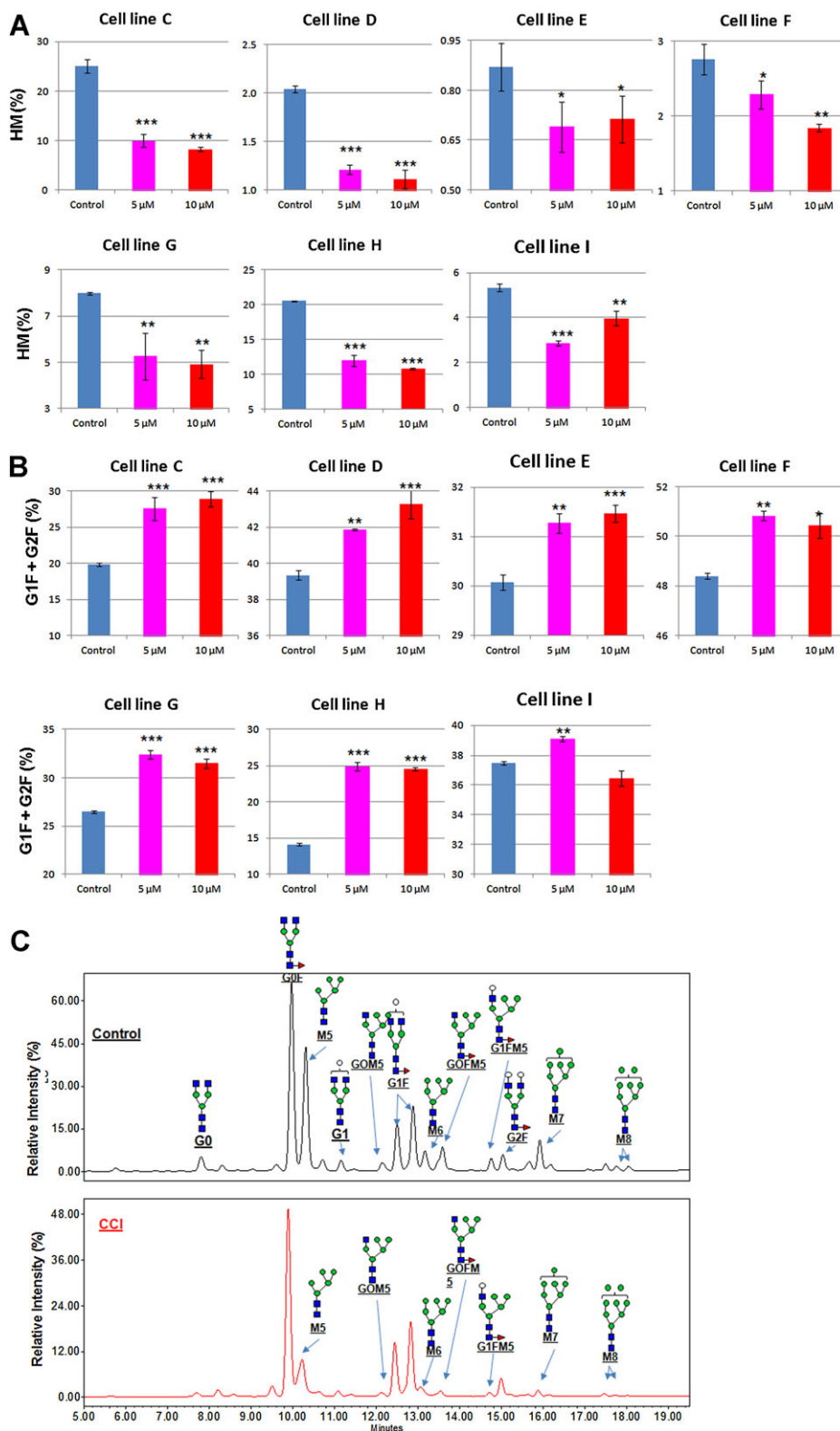


Figure 5. G0/G1 arrest improves glycan processing of mAb. The indicated recombinant CHO cell lines expressing different monoclonal antibodies were subjected to 5-day plate-based production culture with or without CCI treatment. Day 5 production supernatants were collected and protein A-purified and the glycan profiles of the mAbs were analyzed using HILIC method to determine the structures and relative levels of different glycan structures. Average percentage of High mannose (HM) (A) and G1F + G2F (B) from the indicated cell lines are shown. Data represent the average \pm SD of triplicate samples. Details of glycan profile and structures of the N-linked glycan species detected on mAb from cell line C was analyzed by HILIC assay (C). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ represent statistically significant differences between untreated controls and different CCI treated conditions.

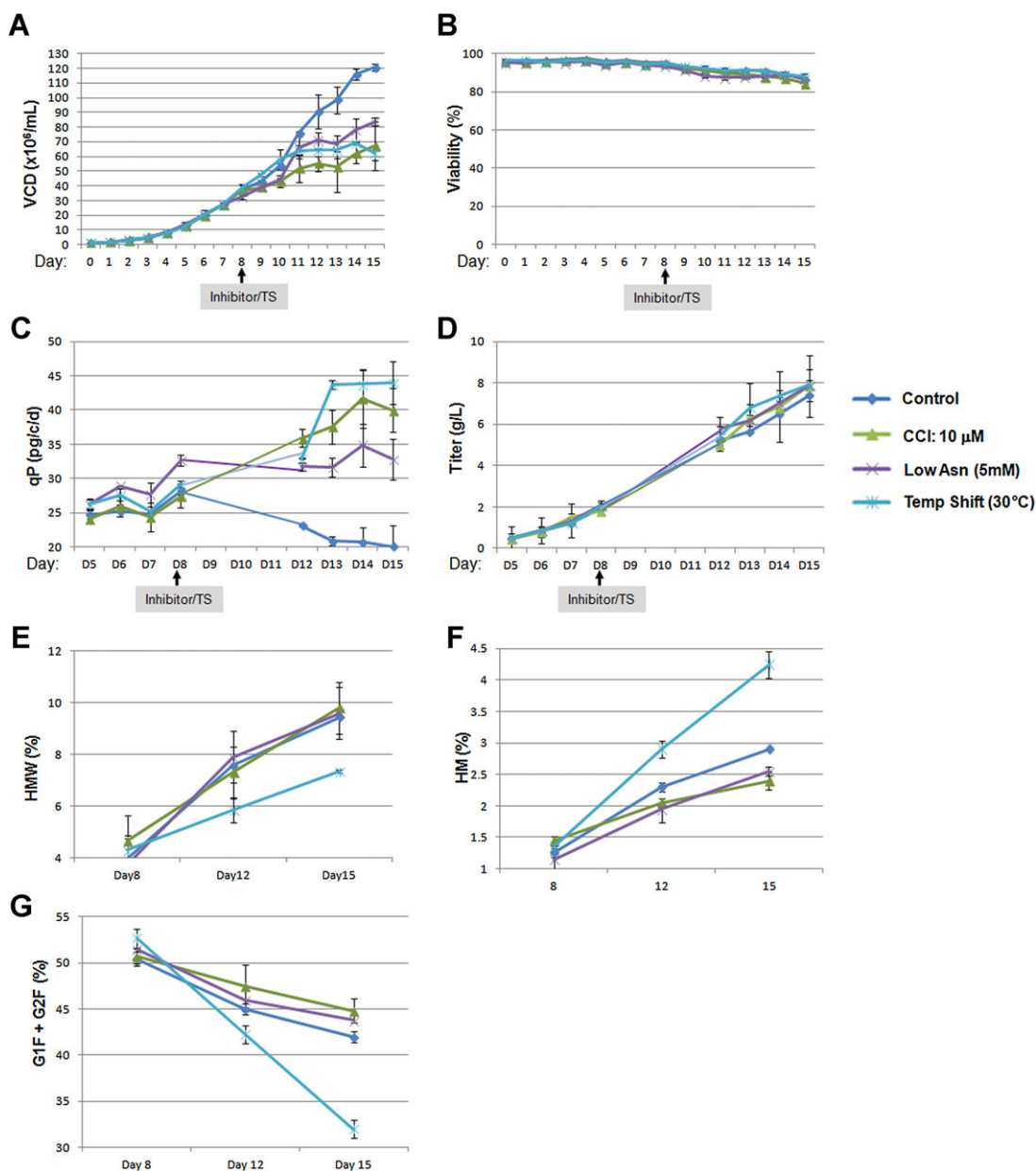


Figure 6. The comparison of different growth control approaches in bioreactors. A recombinant CHO cell line was cultured in production bioreactors as described in the Materials and Methods section. 10 μ M of inhibitor was added to the indicated bioreactor at day 8. For the temperature shift condition, the culture temperature was decreased from 36 to 30°C at day 8. For the Low Asn condition, perfusion medium with 5 mM Asn was substituted for the standard 17.3 mM for the indicated bioreactor. VCD (A) and viability (B) were measured daily. Production supernatants were collected and protein A-purified. qP (C), titer (D), HMW (E), high mannose (HM) (F), and terminal galactose (G) were analyzed as described in the Materials and Methods section. Each point represents the average and standard deviation of duplicate measurements from two independent bioreactor runs.

Discussion

In this study, we identified a novel approach to inducing complete G0/G1 arrest by directly and selectively inhibiting CDK4/6, specific CDKs involved in G1, with a small molecule CCI which, to date, has not been used in the therapeutic mAb production field. Our data demonstrate consistent and broad effects of this approach on growth control and improvement of specific productivity, without

negative impact on product quality, with all high-producing cell lines tested, including the ones that have not responded to other approaches (unpublished data). While the tested cell lines already produce 18–53 pg/cell/day mAb, that is, within the range estimated for professional secretory cells in vivo (Kantardjieff et al., 2010), CCI-treatment alone can further increase mAb secretion 2 to 3 fold up to 110 pg/cell/day. The cell cycle analysis indicated that this CCI-mediated cell cycle G0/G1 arrest is more complete (>96%) than both

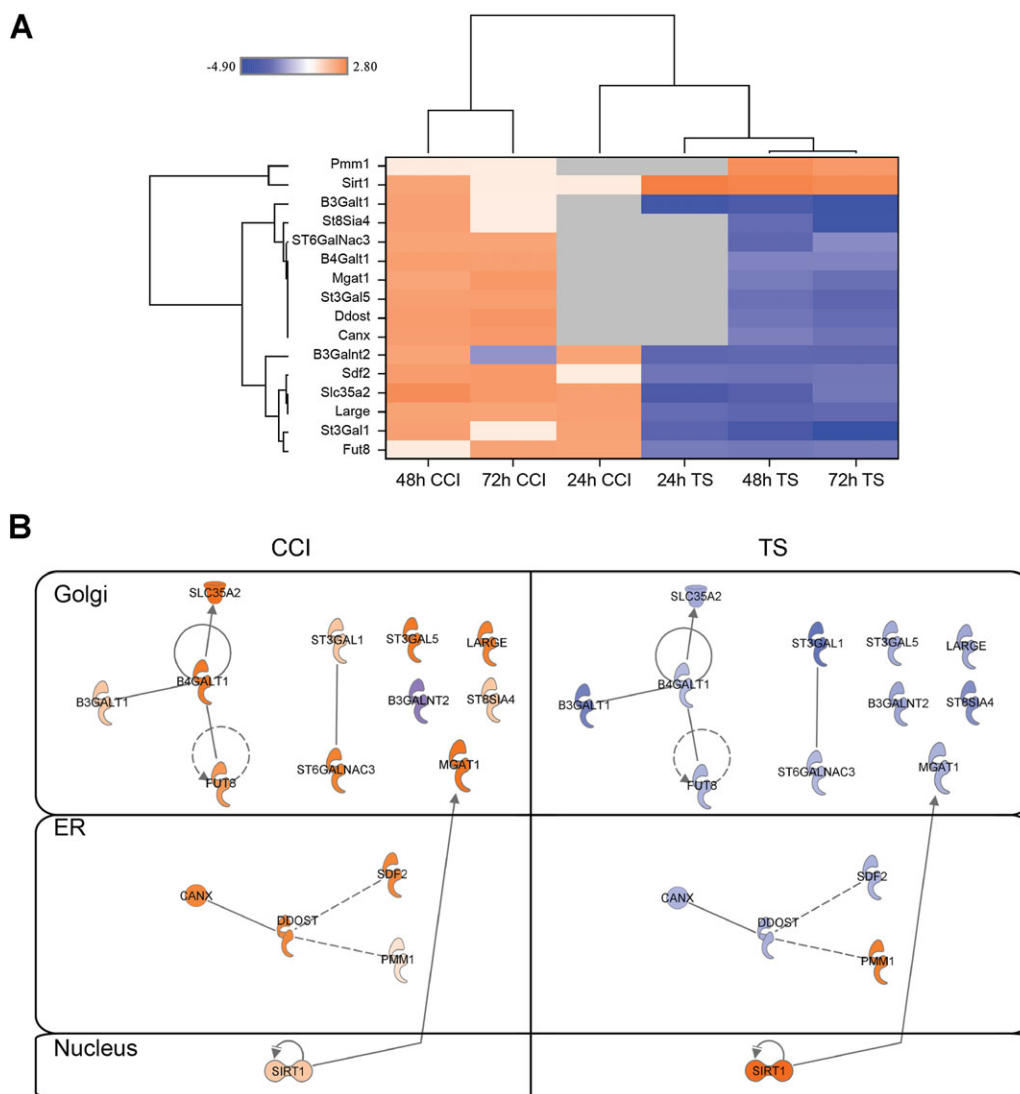


Figure 7. The changes of genes involved in N-linked glycosylation at the mRNA level. Cells were collected from the production bioreactor cultures at each indicated time point (24, 48, and 72 h). Total RNA was isolated and subjected to microarray analysis as described in Materials and Methods section. All measurements are relative to control at each time point. Genes were selected for this analysis if their expression levels deviated from the control by at least a fold change of ± 2 . Designated *P*-value cutoffs were used to compile lists of significantly changed genes used for downstream pathway analysis. The color scale ranges from saturated blue for log₂ ratios -4.0 and below to saturated orange for log ratios 2.80 and above. Each gene is represented by a single row of colored boxes; each time point is represented by a single column. **(A)** The sequence-verified named genes in these clusters involved in N-linked glycosylation. **(B)** A comparison of interactions and cellular localizations of key differentially regulated N-linked glycosylation proteins. Direct interactions are shown with solid edges while indirect interactions are represented with dashed edges.

nutrient-limitation and temperature shift ($<80\%$) (Fig. 3). The continuously decreasing number of cells in G2/M phase with the CCI suggests that the inhibitory effect is specific to G1 CDKs (Fig. 3E). This is consistent with the minimal effect on cell viability observed by CCI treatment since elevated G2/M is usually associated with enhanced apoptosis (Agarwal et al., 1995; Lian et al., 1998; Plaumann et al., 1996; Shao et al., 1998; Vikhanskaya et al., 1998; Wahl et al., 1996; Xia et al., 2000). This phenomenon distinguishes CCI-treatment from CKI-overexpression approaches since CKIs, especially p21, can induce a significant increase in G2/M arrest (Agarwal et al., 1995; Fussenegger et al., 1998; Lian et al., 1998; Shao et al., 1998; Yang et al., 2003). Our

DNA microarray data also suggests that CKIs are not the major players in the CCI-mediated effect, since mRNA levels of p21, p27, and p19 (Cdkn2d) were unchanged in the CCI treated cells compared to other cell cycle arrest conditions such as temperature shift (Fig. 8) where they were increased. p15 was slightly increased at 48 h of CCI treatment but started to decrease at 72 h, which cannot explain the rapid onset and sustained cell arrest and increasing qP observed in the CCI treated cultures (Figs. 3 and 4). Our results raise the possibility that this CCI-mediated cell cycle arrest is CKI-independent, or the involvement of other non-canonical CKIs that are not within our detectable range.

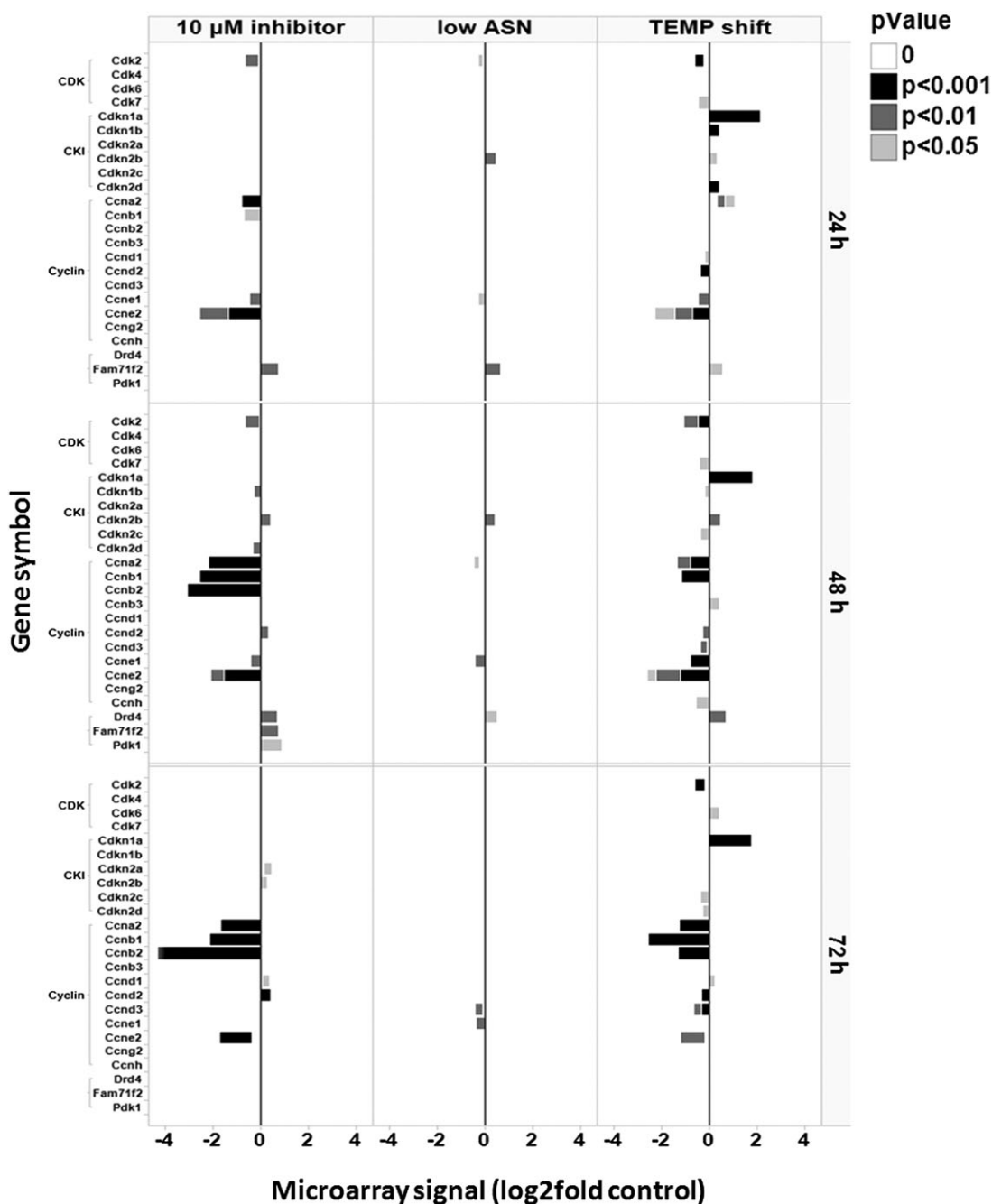


Figure 8. Expression changes of genes involved in the cell cycle pathway at the mRNA level. Cells with the treatment of CCI, low Asn, and 33°C-temperature shift were collected from the production bioreactors at each indicated time point (24, 48, and 72 h). Total RNA was isolated and subjected to microarray analysis as described in Materials and Methods section. Genes involved in the cell cycle pathway are shown. Values represent the fold changes in expression levels. Different color shades indicate significant ($P < 0.001$, $P < 0.01$, $P < 0.05$) changes in all conditions represented.

Decreasing cultivation temperature is one of the most commonly used approaches in recombinant protein expression since it can be accurately controlled during the bioreactor process (Al-Fageeh et al., 2006; Baik et al., 2006; Chuppa et al., 1997; Furukawa and Ohsuye, 1999; Kantardjieff et al., 2010; Kaufmann et al., 1999; Kumar et al., 2007; Moore et al., 1997; Nishiyama et al., 1997b; Sajan et al., 2010;

Yoon et al., 2003b). Lower temperature decreases the global transcription/translation rate but still results in increased recombinant protein expression though this mechanism is not completely understood. Cold shock proteins, such as CIRP and RBM3, have been suggested to be involved in the modulation of transcription and translation by functioning as RNA chaperones, increasing mRNA levels of the

recombinant DNA (Danno et al., 2000; Dresios et al., 2005; Nishiyama et al., 1997a,b; Sonna et al., 2002). However, reducing temperature can also induce significant changes in N-linked glycosylation (Furukawa and Ohsuye, 1999; Nam et al., 2008; Trummer et al., 2006; Yoon et al., 2003b, 2005). Our data shows that reducing cultivation temperature to 30°C impacted glycan processing. This included increased high mannose glycoforms and a decrease in terminal galactose addition to form mature G1F and G2F glycoforms (Fig. 6). The DNA microarray data supports this finding, showing that multiple genes involved in the N-linked glycosylation pathway are significantly down-regulated (Fig. 7), likely leading to the observed changes in mAb glycosylation as monitored by HILIC assay (Fig. 6). In comparison, CCI-treatment improves glycan processing by decreasing HM and increasing G1F and G2F glycoforms, in contrast to the temperature shift condition. Therefore, this additional feature makes this approach more attractive especially for commercial mAb production and biosimilar process development.

In theory, sustained cell cycle arrest at G0/G1 phase is coupled with cell differentiation, the stage at which the cell reaches its maximum level of biogenesis and protein expression (Li and Vaessin, 2000; Myster and Duronio, 2000; Steinman et al., 1994; Tang et al., 1999). Through a complete G0/G1 enrichment by CCI-treatment, we have shown that both specific productivity and secretion-related pathways, such as protein assembly and N-linked glycosylation can be increased. Mechanisms describing how these two events are coordinated in CHO cells and are being investigated. Optimization of key production process parameters, such as increasing seeding density and production culture duration, together with CCI-treatment to maximize the ultimate recombinant protein yield in a general platform process are still in development.

Overall, the data presented here indicate that complete G0/G1 arrest by directly blocking CDK4/6 kinase activity is a robust and consistent approach that is applicable to mAb production. This study provides a foothold from which to gain further insight into the interactions and importance of cell cycle G0/G1 arrest on recombinant protein secretion and the related signaling pathways. These additional insights will provide a basis for the rational design of both cellular and process engineering strategies to advance recombinant protein production processes.

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