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ARTICLE

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Chemical inhibitors of hexokinase-2 enzyme reduce lactate accumulation, alter glycosylation processing, and produce altered glycoforms in CHO cell cultures

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Abstract

Chinese hamster ovary (CHO) cells, predominant hosts for recombinant biotherapeutics production, generate lactate as a major glycolysis by-product. High lactate levels adversely impact cell growth and productivity. The goal of this study was to reduce lactate in CHO cell cultures by adding chemical inhibitors to hexokinase-2 (HK2), the enzyme catalyzing the conversion of glucose to glucose 6-phosphate, and examine their impact on lactate accumulation, cell growth, protein titers, and *N*-glycosylation. Five inhibitors of HK2 enzyme at different concentrations were evaluated, of which 2-deoxy-D-glucose (2DG) and 5-thio-D-glucose (5TG) successfully reduced lactate accumulation with only limited impacts on CHO cell growth. Individual 2DG and 5TG supplementation led to a 35%–45% decrease in peak lactate, while their combined supplementation resulted in a 60% decrease in peak lactate. Inhibitor supplementation led to at least 50% decrease in moles of lactate produced per mol of glucose consumed. Recombinant EPO-Fc titers peaked earlier relative to the end of culture duration in supplemented cultures leading to at least 11% and as high as 32% increase in final EPO-Fc titers. Asparagine, pyruvate, and serine consumption rates also increased in the exponential growth phase in 2DG and 5TG treated cultures, thus, rewiring central carbon metabolism due to low glycolytic fluxes. *N*-glycan analysis of EPO-Fc revealed an increase in high mannose glycans from 5% in control cultures to 25% and 37% in 2DG and 5TG-supplemented cultures, respectively. Inhibitor supplementation also led to a decrease in bi-, tri-, and tetra-antennary structures and up to 50% lower EPO-Fc sialylation. Interestingly, addition of 2DG led to the incorporation of 2-deoxy-hexose (2DH) on EPO-Fc *N*-glycans and addition of 5TG resulted in the first-ever observed *N*-glycan incorporation of 5-thio-hexose (5TH). Six percent to 23% of *N*-glycans included 5TH moieties, most likely 5-thio-mannose and/or 5-thio-galactose and/or possibly 5-thio-*N*-acetylglucosamine, and 14%–33% of *N*-glycans

included 2DH moieties, most likely 2-deoxy-mannose and/or 2-deoxy-galactose, for cultures treated with different concentrations of 5TG and 2DG, respectively. Our study is the first to evaluate the impact of these glucose analogs on CHO cell growth, protein production, cell metabolism, *N*-glycosylation processing, and formation of alternative glycoforms.

KEYWORDS

bioprocessing, Chinese hamster ovary (CHO) cells, glucose analogs, glycolysis, glycosylation, lactate

1 | INTRODUCTION

Recombinant protein therapeutics are used for the treatment of numerous diseases including cancer, autoimmune disorders, and hematologic diseases. Since 2002, more than 300 biotherapeutics including monoclonal antibodies (mAbs), vaccines, hormones, growth factors, and fusion proteins have been approved by the FDA (U.S. Food and Drug Administration). This number is growing rapidly and mAb sales alone are expected to reach a market value of US$200 billion by 2023 (Dhara et al., 2018; Grilo & Mantalaris, 2019). While a number of mammalian cell lines are used for the production of recombinant proteins biotherapeutics, Chinese hamster ovary (CHO) cells are the predominant production hosts due to their robustness in high cell density, large-scale suspension cell culture processes and capacity to produce posttranslational modifications including glycosylation compatible with humans (Kim et al., 2012).

Major goals in cell culture bioprocessing include achieving high productivity as well as desired product quality characteristics, however, both of these attributes can be affected by the cellular metabolism of CHO cells (Naik et al., 2018; Templeton et al., 2013). Early in a batch or fed-batch culture, CHO cells typically proliferate rapidly. This exponential growth phase is often accompanied by high glucose consumption, active glycolysis, and high levels of lactate production, even in the presence of oxygen (Coulet et al., 2022; Kirsch et al., 2022). A large fraction of the glucose and pyruvate are converted to lactate with limited amounts feeding into the tricarboxylic acid cycle (TCA) cycle and pentose phosphate pathway. Some amount of lactate is also produced simultaneously via glutaminolysis (Ahn & Antoniewicz, 2011; Sengupta et al., 2011). Following the accumulation of lactate in the growth phase, a stationary phase occurs which is characterized by a higher fraction of energy generation through oxidative phosphorylation, increased TCA fluxes, reduced fluxes through glycolysis, lactate consumption, and high product titers (Ahn & Antoniewicz, 2011; Martínez et al., 2013; Templeton et al., 2013). The switch from net lactate production to net lactate consumption has been linked to several factors including activities of the proteins—lactate dehydrogenase (LDH) and monocarboxylate transporter (MCT), redox balancing, and regulation of mitochondrial activity (Hartley et al., 2018; Zagari et al., 2013). Lactate accumulation during the exponential phase acidifies the cell culture, leading to the need for base addition to control the pH which concomitantly increases the osmolarity of the culture. Indeed, lactate accumulation by itself and increased osmolarity have been shown to adversely affect cell growth and productivity (Cruz et al., 2000; Le et al., 2012; Li et al., 2012; Pacis et al., 2011). Hence, it could potentially be advantageous from a bioprocess performance perspective if methods were put in place to limit the lactogenic behavior of CHO cells.

Given the impact of lactate production on bioprocess performance, previous studies have employed various strategies including controlling process parameters, redesigning basal medium and feed, and metabolic engineering of CHO cell lines, to limit its accumulation in CHO cell cultures. Approaches to control lactate accumulation in CHO cell cultures through process modifications include high-end pH-controlled delivery of glucose (HIPDOG) by Gagnon et al. (2011) and control of temperature and pH set points by Yoon et al. (Yoon et al., 2003, 2006; Zalai et al., 2015). Changing these process parameters can also influence cell growth, productivity, and glycosylation and hence, must be evaluated on a case-by-case basis depending on the cell line (Ivarsson et al., 2014).

Redesigning cell culture medium such as replacement of glucose with different sugars such as galactose, fructose, mannose, and maltose as well as substitution of glutamine with glutamate, TCA cycle intermediates and L-alanyl-L-glutamine dipeptide has been shown to lower lactate accumulation (Hossler et al., 2014; Zhang et al., 2020). However, replacing glucose and glutamine, two major carbon sources for CHO cells can lead to broader impacts on specific cell growth rate and protein quality characteristics including glycosylation (Altamirano et al., 2004; Berrios et al., 2011; Ha & Lee, 2014; Hossler et al., 2014; Imamoto et al., 2013; J. Liu et al., 2015). Changes in the levels of trace metals including copper has been shown to alter lactate accumulation and impact other cellular phenomenon as well (Nargund et al., 2015). In one study, Nargund et al. applied ^{13}C-metabolic flux analysis (MFA) to demonstrate that copper deficiency in CHO cell cultures leads to lactate accumulation and energy production via aerobic glycolysis, with negative impacts on the redox balance, ATP levels, and electron transport due to reduced oxidative phosphorylation (Yuk et al., 2015). Metabolic engineering has also been employed to reduce lactate accumulation by either targeting enzymes in the glycolysis pathway or by improving

the poor connectivity between the mitochondrial and cytosolic metabolic systems. These efforts include reducing the expression of enzymes such as pyruvate kinase muscle (PKM), lactate dehydrogenase A (LDHA), and pyruvate dehydrogenase kinases (PDHks) as well as overexpressing enzymes such as recombinant yeast pyruvate carboxylase (PYC2) enzyme and malate dehydrogenase-2 (MDH-2) (Chong et al., 2010; Gupta et al., 2017; Tang et al., 2021; Toussaint et al., 2016; Zhou et al., 2011). In these studies, reduction in lactate levels were observed along with a redirection of more pyruvate into the TCA cycle for oxidation in the mitochondria, higher specific productivities, and in some cases improved product quality (Chong et al., 2010; Gupta et al., 2017; Tang et al., 2021; Toussaint et al., 2016; Zhou et al., 2011).

An alternative strategy to genetic engineering, utilization of alternative substrates, and changes in the metal content, to potentially alter lactate accumulation is the addition of small molecules to alter enzymatic activity in the glycolysis pathway. Buchsteiner et al. observed that PDHk activity can be inhibited by using 5 mM dichloroacetate (DCA), resulting in reduced glycolytic fluxes, less pyruvate being available for lactate formation, increased peak viable cell density (VCD), higher antibody titer, and no change in TCA fluxes (Buchsteiner et al., 2018). In another study, Montégut et al. showed that supplementing CHO cell cultures with a combination of drugs used for metabolic therapy of cancer, namely, α-lipoic acid, a pyruvate dehydrogenase activator and methylene blue, a mitochondrial electron transfer carrier, stimulated TCA cycle activity and mitochondrial respiration, reduced lactate accumulation, and increased antibody titer (Montégut et al., 2020). These previous strategies to mitigate lactate accumulation in CHO cells have targeted enzymes far downstream in the glycolysis pathway and at the glycolysis-TCA interface. However, small molecule inhibitors for enzymes in the early stages of the glycolysis pathway such as hexokinase-2 (HK2), glucose-6-phosphate isomerase (GPI), 6-phosphofructokinase-1 (PFK1), and 6-phosphofructokinase-2 (PFK2) (Figure 1) (Joost et al., 2002; Kanehisa, 2000; Yu et al., 2020) have not been considered extensively as targets for altering lactate accumulation and other performance properties in CHO cell cultures. Small molecule inhibitors of the HK2 enzyme including 3-bromopyruvate (3BP), 2-deoxy-D-glucose (2DG), 5-thio-D-glucose (5TG), Lonidamine, and D-mannoheptulose (MH) have been previously investigated in cancer cells to inhibit enzymatic activity and alter glycolysis activity (Scatena et al., 2008). Due to metabolic similarities that exist between CHO cells and cancer cells, we decided to elucidate the impact of these molecules on CHO cell growth, metabolism, productivity, and product quality. Therefore, in this study, we supplemented the small molecule inhibitors of the HK2 enzyme including 3BP, 2DG, 5TG, Lonidamine, and MH to CHO cell cultures and then examined the impact on lactate accumulation, glucose-to-lactate flux, central carbon metabolism, and product quality.

The small molecule inhibitors were initially supplemented one at a time in CHO cell batch-cultures at different concentrations to

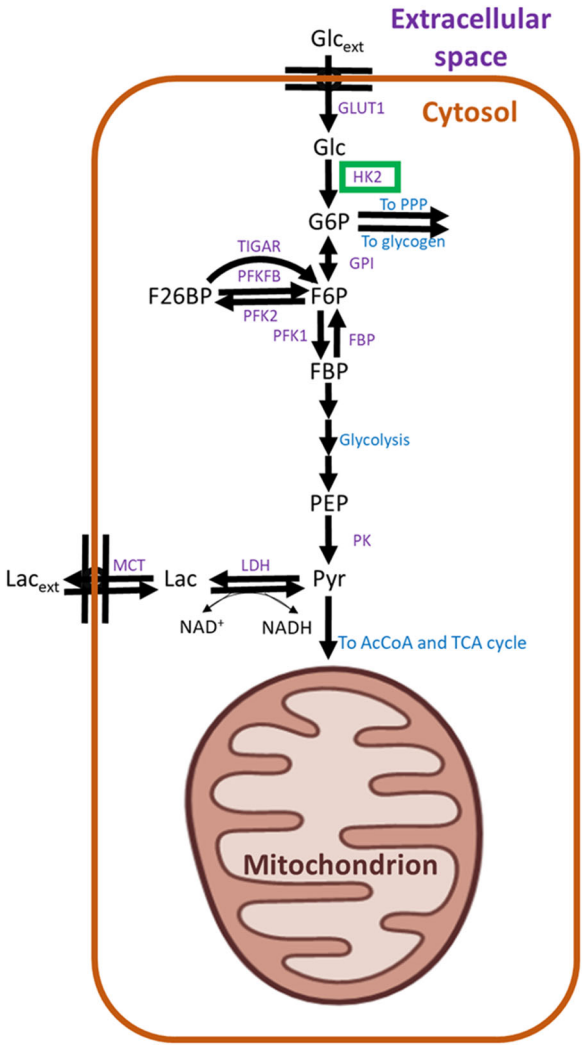


FIGURE 1 Glycolysis is the major pathway in Chinese hamster ovary (CHO) cells through which imported glucose is oxidized into pyruvate in the cytosol. Some of the pyruvate is converted into lactate which can be transported into the extracellular environment. Pyruvate can also enter into the mitochondria and be oxidized in the TCA cycle. Another branch of glycolysis is the PPP which is responsible for the production of NADPH and supply of ribose for subsequent biosynthetic pathways. The major enzymes and metabolites involved in the glycolysis pathway are shown in the figure above. AcCoA, acetyl-coenzyme A; FBP (enzyme), fructose-1,6-bisphosphatase isoenzyme 2; FBP (metabolite), fructose 1,6-bisphosphate; F26BP, fructose 2,6-bisphosphate; F6P, fructose 6-phosphate; Glc, intracellular glucose; Glc_{ext}, extracellular glucose; GLUT1, glucose transporter-1; GPI, glucose-6-phosphate isomerase; G6P, glucose 6-phosphate; HK2, hexokinase-2; Lac, intracellular lactate; Lac_{ext}, extracellular lactate; LDH, L-lactate dehydrogenase; MCT, Monocarboxylate transporter; PEP, Intracellular phosphoenolpyruvate; PFKFB, 6-phosphofructo-2-kinase//fructose-2,6-biphosphatase 1; PK, pyruvate kinase; Pyr, pyruvate; PPP, pentose phosphate pathway; PFK1, 6-phosphofructokinase 1; PFK2, 6-phosphofructo-2-kinase; TCA, tricarboxylic acid cycle; TIGAR, fructose-2,6-bisphosphatase.

determine their impact on cell growth, glucose-to-lactate flux, and productivity. Of these, 5TG and 2DG were observed to reduce lactate accumulation and promote lower lactate-to-glucose ratios in CHO cell cultures when supplemented individually and, when applied together, the two compounds often combined for even larger reductions in lactate accumulation with only moderate impacts on the growth rate. Interestingly, recombinant protein production also often peaked earlier in the overall growth phase for the 5TG and 2DG treated cultures. Supplementation with 2DG and 5TG also led to increased consumption rates of asparagine, serine, and pyruvate earlier in the culture to drive central carbon metabolism in the presence of low glycolytic fluxes. We also determined the impact of supplementing 5TG and 2DG alone and in combination on *N*-glycosylation using MALDI-TOF MS-based released *N*-glycan analysis. Supplementation of 5TG and 2DG in CHO cell cultures increased the fraction of high mannose glycans, reduced sialylation, and resulted in the incorporation of nonnatural sugars for the *N*-glycans structures attached to the recombinant EPO-Fc product. This study will help elucidate the effectiveness of using small molecule inhibitors of the HK2 enzyme to control glucose fluxes, alter accumulation of lactate, and further examine its impact on bioprocess performance parameters including titers and glycosylation processing.

2 | MATERIAL AND METHODS

2.1 | Cell culture

CHO-GS cell line (CHOZN® GS-/- ZFN-modified CHO cell line) producing EPO-Fc and chemically defined cell culture medium, Immediate Advantage® (Cat. No. 87093C) were obtained from MilliporeSigma. 3BP (Cat. No. 16490; MilliporeSigma), 2DG (Cat. No. D6134), 5TG (Cat. No. 88635; MilliporeSigma), Lonidamine (Cat. No. L4900; MilliporeSigma), and MH (Cat. No. 97318; MilliporeSigma) were all purchased from MilliporeSigma. All compounds were dissolved in water to prepare stock solutions of different concentrations, filter-sterilized, and then added to cell culture medium one at a time on Day 0 at different concentrations. Cells were thawed and subcultured every 3 days for two passages at a seeding density of 0.4×10^6 cells/mL. At the end of the second passage, batch cultures were initiated. CHO cells were seeded at 0.4×10^6 cells/mL in 30 mL medium and cultured in 125 mL shaker flasks (Corning® Erlenmeyer cell culture flasks, Cat. No. CLS431143; Sigma-Aldrich) in incubators operating at 37°C, 125 rpm and 5% CO_2 level. 500uL of cell culture sample was taken every 24 h. VCD and viability (%) were calculated using cell counts based on the trypan blue dye exclusion method and a hemocytometer. The samples were then centrifuged, supernatant transferred and stored at −80°C for extracellular measurements. At the end of culture duration, cell cultures were harvested, filtered using a 0.22-µm-pore-size membrane and stored at −80°C for subsequent downstream analysis. Glucose and lactate were measured using a YSI 2950D biochemical analyzer (Yellow Spring Instrument).

Different cell culture performance parameters were calculated as follows:

(i) Specific glucose consumption rate:

$$qGlucose = \frac{(Glucose)n - (Glucose)n+1}{\Delta IVCDn \to n+1},$$

where, n represents a particular day of cell culture.

(ii) Specific lactate production rates:

$$qLactate = \frac{(Lactate)n+1 - (Lactate)n}{\Delta IVCDn \to n+1},$$

where, n represents a particular day of cell culture.

(iii) Cell-specific productivity (qP) (Adams et al., 2007; Jarusintanakorn et al., 2021):

$$q_p = \frac{1}{X}\frac{d[EPO - Fc]}{dt},$$

$$\int_0^{[EPO-Fc]_{tf}} d[EPO - Fc] = \int_0^{tf} q_p X dt,$$

Assuming constant qP,

$$q_p = \frac{[EPO - Fc]_{tf}}{\int_0^{tf} X dt} = \frac{[EPO - Fc]_{tf}}{IVCD}.$$

where, t_f represents last day of cell culture.

(iv) % Change in growth rate with respect to control

$$= \frac{100 \times (\text{(growth rate with inhibitor)} - \text{(growth rate for control)})}{\text{(growth rate for control)}}.$$

(v) % Change in peak viable cell density (VCD) with respect to control

$$= \frac{100 \times (\text{(peak VCD with inhibitor)} - \text{(peak VCD for control)})}{\text{(peak VCD for control)}}.$$

(vi) % Change in ratio of moles of lactate produced (L) for every mole of glucose consumed (G) with respect to control = $\frac{100 \times ((L/G\text{with inhibitor}) - (L/G\text{for control}))}{(L/G\text{for control})}$.

(vii) % Change in peak lactate produced with respect to control

$$= \frac{100 \times (\text{(peak lactate produced with inhibitor)} - \text{(peak lactate produced for control)})}{\text{(peak lactate produced for control)}}.$$

Note that growth rate was calculated for the exponential phase of cell culture and L/G ratio was calculated till the day of peak lactate accumulation. Growth rate was determined as the slope of ln (VCD) versus time plot and L/G ratio was determined as the slope of lactate produced versus glucose consumed plot. Statistics (p values) were

determined using the two-tailed *t* test against the control. The following convention was used: $p > 0.1$ indicated by not significant (NS), $0.1 > p > 0.05$ indicated by *, $0.05 > p > 0.01$ indicated by **, $0.01 > p$ value indicated by ***.

2.2 | Anti-EPO enzyme-linked immunosorbent assay (ELISA)

EPO-Fc titers were measured using an anti-EPO ELISA as previously described by Wang, Chung, et al. (2019). Briefly, 5 μL of supernatants from each sample were loaded in a 96-well plate to test biological triplicates and then coated with carbonate/bicarbonate buffer overnight at 4°C. Next day, 5% BSA blocking solution, chicken anti-EPO antibody (Thermo Fisher Scientific), and goat anti-chicken IgY antibody linked with HRP (Abcam) were added to each well to capture and detect antibodies. 3,3′,5,5′-Tetramethylbenzidine (TMB, 100 μL, Thermo Fisher Scientific) was used as a chromogenic substrate for HRP. After the plate was incubated at room temperature for 5 min, sulfuric acid was added at 2 M to stop the reaction. Absorbance OD values were measured immediately at 450 nm. Statistics (*p* values) were determined using the two-tailed *t* test against the control. The following convention was used: $p > 0.1$ indicated by NS, $0.1 > p > 0.05$ indicated by *, $0.05 > p > 0.01$ indicated by **, $0.01 > p$ value indicated by ***.

2.3 | Extracellular and intracellular metabolite profiling

Cell culture samples collected every 24 h from batch experiments were centrifuged to obtain the supernatant containing spent media and separate the cell pellet. Spent media collected were subjected to metabolite profiling using GC-MS (Agilent 7890 GC with 5977 MSD). Extracellular concentrations of pyruvate, alanine, glycine, serine, aspartate, asparagine, and glutamate were measured using a GC-MS-based method used in previous studies (Dhara et al., 2023; Long & Antoniewicz, 2019; Y. Chen et al., 2019). Estimation of cell-specific consumption rates (q_s) were calculated using the following equation (Pan et al., 2017): $C_s(t) - C_s(0) = q_s IVCD$, where $C_s(t)$ is the concentration of substrate S at time t, $C_s(0)$ is the concentration of substrate *S* at the start of the culture. IVCD is the integral of VCD from the start of the culture to time *t*. Based on the notation that we used, a negative sign or negative value indicated production and a positive sign or a positive value indicates consumption (Figure 7). For each condition, spent media from two biological replicates and four-time points (Days 0, 1, 2, and 3) were analyzed ($n = 8$).

Relative intracellular metabolite levels were also determined by GC-MS analysis of CHO cell extracts. Briefly, CHO cell pellets were washed with cold saline solution. Intracellular metabolites were then extracted using the methanol/chloroform/water extraction method, followed by MOX-TBDMS derivatization, and GC-MS analysis as described previously (Oates & Antoniewicz, 2023). To determine relative metabolite levels, the total ion counts for each metabolite were compared against control experiments without 2DG and 5TG. For each condition, cell extracts from two biological replicates and three-time points (Days 1, 2, and 3) were analyzed ($n = 6$).

2.4 | Protein purification

Cell culture harvests were purified using affinity chromatography and gravity flow as previously described by Wang, Yang, et al. (2019). Protein A agarose bead slurry (Vector Lab) was added to cell culture supernatants and these mixtures were incubated overnight at 4°C on a rotator. Next day, the mixtures were loaded onto a 15 mL polypropylene column (QIAGEN) to retain the agarose beads followed by three washes with phosphate-buffered saline (PBS) buffer. The bound EPO-Fc was eluted using 0.1 M glycine, pH 2.7 elution buffer. 1 M Tris-HCl, pH 9.0 neutralization buffer was immediately added to neutralize the acidic pH of the eluate. The eluates were subsequently dialyzed several times against PBS using Amicon Ultra Centrifugal Filters (MilliporeSigma) at 5000 g for 20 min at 10°C using a filter with 10 kDa molecular weight cut-off. The final concentration of purified EPO-Fc was measured by bicinchoninic acid (BCA) assay (Thermo Fisher Scientific), and purity was assessed on 10% SDS-PAGE gels (Bio-Rad) followed by Coomassie blue staining. Gels were de-stained in water overnight and clear single bands were visible for EPO-Fc the next day.

2.5 | Glycosylation profiling

Glycosylation profiles of the purified EPO-Fc protein samples were determined using the glycoprotein immobilization for glycan extraction (GIG) method (Q. Wang et al., 2021; S. Yang et al., 2013). In the first step, the purified EPO-Fc protein was conjugated to a solid support using reductive amination and the unconjugated entities were washed away. 400 μg of purified EPO-Fc was added to a 500 μL solution mixture containing binding buffer (pH 10, 40 mM sodium citrate and 20 mM sodium carbonate) and denaturing buffer (B1704S; New England BioLabs) and incubated at 100°C for 10 min. All chemicals were purchased from MilliporeSigma unless otherwise noted. Aminolink resin (Thermo Fisher Scientific) was washed twice with binding buffer and added to a snap-cap spin column (SCSC; Thermo Fisher Scientific) along with denatured protein sample followed by overnight incubation at room temperature. Next day, 50 mM sodium cyanoborohydride ($NaCNBH_3$) was added to the sample for further reduction on the solids support by mixing for 4 h at room temperature. The resin was washed twice using 1× PBS and 50 mM sodium cyanoborohydride ($NaCNBH_3$) was added again to block any unreacted aldehyde active sites by mixing for 4 h at room temperature. In the second step, sialic acid on the *N*-glycans of immobilized EPO-Fc was modified using chemical treatment. To protect the sialic acid, immobilized *N*-glycans were incubated with a 465 uL mixture (pH 4–6) containing 400 μL p-toluidine, 25 μL

36%–38% HCl, and 40 μL EDC (*N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide; 5.6 M) for 4 h at room temperature. Chemicals were removed by washing the samples sequentially with 10% formic acid, 10% acetonitrile, 1 M NaCl and H_2O, each for three times. In the third and final step, *N*-glycans were released by adding 3 μL of PNGase F (New England BioLabs) in NEB Glycobuffer 2 to the samples followed by overnight incubation at 37°C. Released *N*-glycans were purified using Carbograph column (S. J. Yang & Zhang, 2012). *N*-glycans were analyzed by Axima MALDI Resonance mass spectrometer (Shimadzu) using 4 μL DMA in 200 μL DHB (100 μg/μL in 50% acetonitrile, 0.1 mM NaCl) as MALDI matrix. Laser power was set to 200 for two shots each in 100 locations per spot. GlycoWorkBench software was used to predict glycan structures. The p-toluidine modified Neu5Ac and Neu5Gc had a mass shift of +89.135 Da.

3 | RESULTS

3.1 | Preliminary screening of HK2 inhibitors in CHO cell batch cultures for prospective candidates

Five HK2 inhibitors, Lonidamine, 3BP, MH, 2DG, and 5TG, were added at different concentrations (Table 1) on Day 0 to CHO cell batch cultures. The concentrations tested were selected based on previous studies in other mammalian cells, principally cancer cells (Al-Ziaydi et al., 2020; Floridi et al., 1981; Hervé et al., 1992; Ihrlund et al., 2008; Pantic et al., 2021; Sadowska-Bartosz et al., 2014; Zhao et al., 2019). For the control cultures, no inhibitors were added.

The effectiveness of each inhibitor was evaluated in terms of four different parameters, % change in growth rate, % change in peak VCD, % change in ratio of moles of lactate produced (L) per mole of glucose consumed (G), and % change in peak lactate, all with respect to the control (Figure 2 and see Section 2.1 for calculations):

Due to the large number of candidates and concentrations, each condition was tested initially in a single-pass screen. Lonidamine was added at three different concentrations—0.04, 0.08, and 0.12 mM (see Table 1). Addition of Lonidamine at 0.04 mM led to a decrease in growth rate and peak VCD by 36% and 62%, respectively, compared with control. This concentration of Lonidamine also resulted in a slight increase in peak lactate and the L/G ratio. However, no cell growth was observed when Lonidamine was added at 0.08 and 0.12 mM. Thus, Lonidamine was considered ineffective in reducing lactate accumulation without significantly impacting cell growth. 3BP was considered as a second inhibitor at three different concentrations—0.001, 0.002, and 0.003 mM. At 0.001, 0.002, and 0.003 mM, 3BP led to a decrease in peak lactate by 5%–10% and a reduction in L/G ratio by 10%–20%. However, this could be attributed to the decrease in growth rate also observed. Thus, the reduction of lactate accumulation occurring with 3BP may have occurred at the expense of poor cell growth. Similarly, addition of 0.1 mM MH, led to a decrease in peak lactate produced and the L/G ratio but was also accompanied by a 25% reduction in peak VCD. No cell growth was observed at 1 mM MH. Thus, addition of lonidamine, 3BP and MH either did not support cell growth or reduced lactate accumulation but at the expense of reduced cell growth and lower peak VCDs. Hence, these three inhibitors were not selected for further study.

In contrast, addition of 2DG at 4 and 8 mM reduced peak lactate accumulation by more than 20% and 30%, respectively, with a reduction of L/G above 60% in both cases. There was a modest reduction of 6%–12% in growth rate and 6%–8% in peak VCD in both cases. Thus, 2DG was effective in reducing lactate accumulation and controlling glucose to lactate flux with a lower impact on growth rate. At a higher concentration of 20 mM, 2DG addition resulted in a greater than 55% reduction in peak VCD and a 25% decline in growth rate with a much lower reduction in lactate accumulation (6%). When 5TG was added at 10 mM, peak lactate and L/G ratio was reduced by more than 70% each along with a 20% decrease in peak VCD and no observed change in growth rate. At higher concentrations of 5TG (20 and 30 mM), a more than 80% reduction in peak lactate and L/G ratio was observed. However, this was accompanied by a 40%–50% reduction in peak VCD. Thus, initial first-pass screening experiments indicated that 2DG and 5TG supplementation to CHO cell batch cultures at less than or equal to 10 mM concentrations is capable of inhibiting lactate accumulation with limited negative impacts on cell growth and peak viable cell densities compared with the other inhibitors tested. Therefore, our next goal was to test 2DG and 5TG more in-depth in CHO cell batch cultures and determine the impact of these inhibitors on CHO cell productivity and product quality.

TABLE 1 HK2 inhibitors and their different concentrations (in mM) supplemented individually to CHO cell batch cultures for preliminary screening of prospective candidates.

HK2 inhibitor tested	Concentrations tested (mM)
Control (no HK2 inhibitor added)	–
Lonidamine	0.04, 0.08, 0.12
3-bromopyruvate (3BP)	0.001, 0.002, 0.003
D-mannoheptulose (MH)	0.1, 1
2-deoxy-D-glucose (2DG)	4, 8, 20
5-thio-D-glucose (5TG)	10, 20, 30

3.2 | Extensive evaluation of 2DG, 5TG, and their combination on growth and glucose to lactate flux

2DG and 5TG were supplemented in CHO cell batch cultures individually and in combination at concentrations lower than 10 mM. Multiple conditions as shown in Table 2 were tested in triplicates.

As shown in Figure 3, supplementation of 5TG at 7 and 10 mM led to a decrease in growth rate by 6%–10% compared with the control while cultures supplemented with 2DG had a growth rate

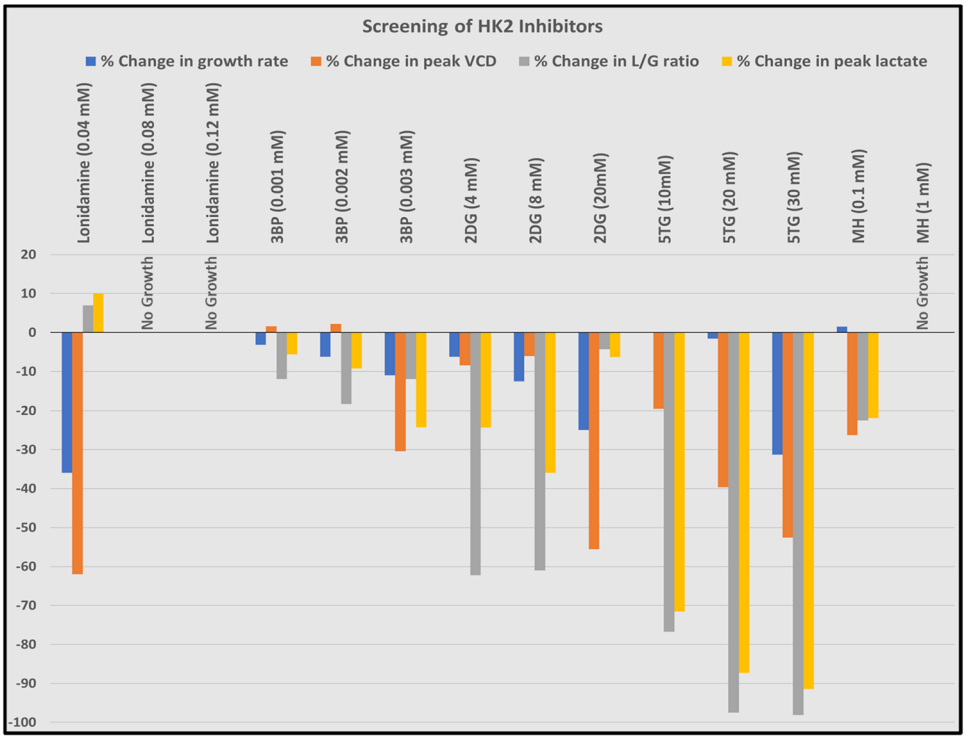


FIGURE 2 Preliminary first-pass screening and evaluation of hexokinase-2 (HK2) inhibitors-Lonidamine, 3-bromopyruvate (3BP), 2-deoxy-D-glucose (2DG), 5-thio-D-glucose (5TG), and D-mannoheptulose (MH), when supplemented at different concentrations in CHO cell batch cultures. Each condition was compared against the control using four different parameters: % change in growth rate, % change in peak viable cell density (VCD), % change in moles of lactate produced (L) per mole of glucose consumed (G), and % change in peak lactate produced in culture.

TABLE 2 Different concentrations and combinations of 5TG and 2DG supplemented to CHO cell batch cultures for in-depth evaluation of their impact on cell culture performance.

5TG and/or 2DG	Concentrations tested (mM)
Control (without 5TG and 2DG)	–
5TG	7, 10
2DG	7, 10
2DG + 5TG	5 + 5, 5 + 7, 7 + 5, 7 + 7

reduced by 3%–6% compared with the control. Due to a slower growth rate earlier in the cultures, 5TG and 2DG-supplemented cultures exhibited a growth period 1–2 days and 3–4 days longer, respectively, compared with controls (Figure 4a). Cell viability was above 90% on every day for all the cultures up until Day 7, when viabilities declined likely because of exhaustion of glucose and/or other nutrients (Figure 4b,c). There was a minor reduction in peak VCD in which 5TG and 2DG-supplemented cultures peaked at a VCD of 10–11.7 million cells/mL while the control cultures reached a peak VCD of 13.3 million cells/mL (Figure 4a). However, the reduction in peak lactate produced was 30%–35% in 5TG-supplemented cultures and 29%–45% in 2DG-supplemented cultures at the two different concentrations (Figures 3 and 4d). Indeed, increasing concentrations of 5TG and 2DG supplemented from 7 to 10 mM led to even greater reductions in peak lactate albeit with lower growth rates and slightly lower peak VCD. In addition, cell-specific lactate production rates of the control cultures exceeded the 2DG and 5TG-supplemented cultures by nearly fourfold for the first 2 days of the cultures (Figure 4e). Indeed, the moles of lactate produced per mole of glucose (L/G ratio) declined by more than 50% for cultures supplemented with 5TG and by more than 60% for 2DG-supplemented cultures (Figure 3).

We also examined the effect of adding 5TG and 2DG in combination to CHO cell cultures. Cultures supplemented with 5TG and 2DG together exhibited even higher reductions in lactate accumulation with comparable or lower peak VCD declines (Figures 3 and 5a) at most concentrations when compared with individual supplementation cultures. The growth rate reduction was less than 10% for all combination cultures except 2DG 7 mM + 5TG 7 mM where the growth rate was lowered by 15% compared with the nonsupplemented control. Combination cultures lasted 2–4 days longer than the control due to slower initial growth rates (Figure 5a) and exhibited viabilities greater than 85% on all days except the last day of culture corresponding to glucose exhaustion. A 46%–63% reduction in peak lactate was observed in combination cultures compared with control (Figures 3 and 5d) and cell-specific lactate production rate for the combination cultures was lower than all but one singly supplemented culture (Figures 4e and 5e). Most importantly, the

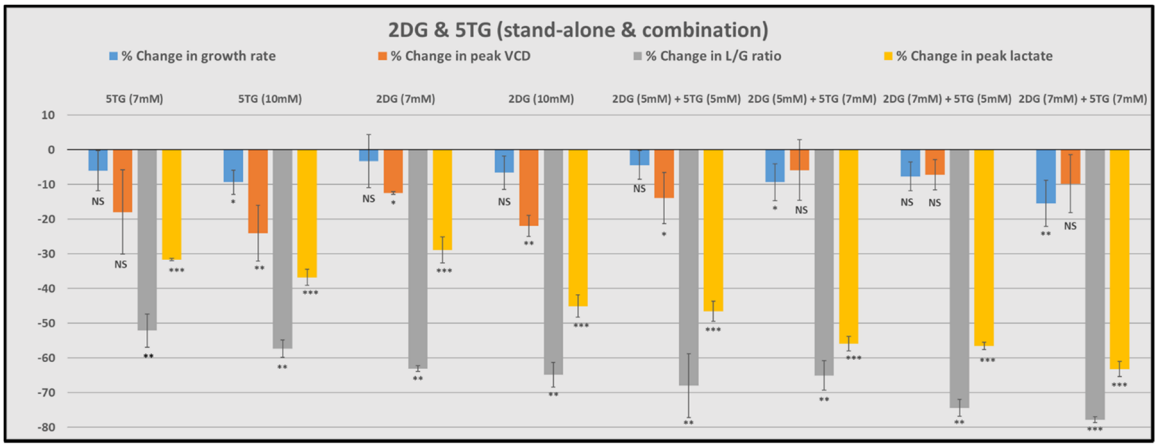


FIGURE 3 Extensive evaluation of hexokinase-2 (HK2) inhibitors 2-deoxy-D-glucose (2DG), and 5-thio-D-glucose (5TG) when supplemented in CHO cell batch cultures. Both 2DG and 5TG were supplemented individually at 7 and 10 mM. In combination, both 2DG and 5TG were supplemented at (i) 5 mM each (ii) 2DG 5 mM + 5TG 7 mM (iii) 2DG 7 mM + 5TG 5 mM, and (iv) 7 mm each. Each condition was cultured in triplicates and compared against the control using four different parameters: % change in growth rate, % change in peak viable cell density (VCD), % change in moles of lactate produced (L) per mole of glucose consumed (G), and % change in peak lactate produced in culture. Error bars, mean ± standard deviation measurement in the cell culture experiments ($n = 3$). Statistics (p values) were determined using the two-tailed t test against the control. The following convention was used: $p > 0.1$ indicated by not significant (NS), $0.1 > p > 0.05$ indicated by *, $0.05 > p > 0.01$ indicated by **, $0.01 > p$ value indicated by ***.

reduction in L/G compared with control was above 50% for all combinations of the two inhibitor molecules and exceeded 70% at the highest concentrations of the two inhibitors.

3.3 | Impact of 2DG and 5TG on EPO-Fc titers

These CHO cells were previously engineered to produce EPO-Fc as a target recombinant protein of interest and we also wanted to examine the impact of these inhibitors on the final titers and product yields. Thus, levels of recombinant EPO-Fc produced by CHO cells under different culture conditions were measured using an ELISA (see Section 2.2) on Day N-1 or Day N-2, where Day N is the end of the culture. At these time points, cells were either at or near peak VCD and viability was above 85% in all cases. As indicated in Figure 6, control cultures produced 1.9 mg/L (N-2) and 2.5 mg/L (N-1) of EPO-Fc. Cultures supplemented with 5TG produced higher levels of EPO-Fc including more than 40% higher than control on Day N-2. Alternatively, cultures treated with 2DG at both concentrations (7 and 10 mM) produced EPO-Fc levels that were more than 60% higher at N-2 and 32% higher on Day N-1. Cultures supplemented with both 5TG and 2DG also produced higher levels of EPO-Fc especially at Day N-2 although the increase was not as great as with 2DG alone. Thus, supplementing CHO cell cultures with 2DG and 5TG, individually or in combination led to an increase in EPO-Fc titers by at least 35% on Day N-2 and 11% on Day N-1 and as high as 70% on Day N-2 and 32% on Day N-1. It is interesting that the % increase in EPO-Fc titer on Day N-1 was not as significant especially for 5TG-treated cultures compared with Day N-2.

3.4 | Impact of 2DG and 5TG on central carbon metabolism of CHO cells

Cell-specific consumption rates of key nutrients including glucose, pyruvate, alanine, glycine, serine, asparagine, aspartate, and glutamate feeding into the glycolysis and TCA cycle pathways as well as intracellular levels of lactate and TCA cycle metabolites were measured using GC-MS for the first 3 days of the culture when cells are in exponential growth phase (Figure 7, Supporting Information: Figures 1 and 2). Supplementation of 2DG and 5TG individually and in combination at 7 mM each to CHO cell batch cultures reduced cell-specific glucose consumption rates due to inhibition of the HK2 enzyme compared with control (Supporting Information: Figure 1). Due to reduced glucose uptake rates, less glucose was available to drive glycolysis and the subsequent TCA cycle pathway earlier in the culture in 2DG and 5TG-supplemented cultures when compared with control. This in turn resulted in a reduction in intracellular levels of lactate by greater than twofold in the cells following the treatment with 2DG and 5TG (Supporting Information: Figure 2). Interestingly, we observed an increase in serine consumption rate by 43%–91% in cultures supplemented with 2DG, 5TG, and 2DG in combination with 5TG when compared with control (Figure 7e). Subsequently, glycine production rates increased by greater than twofold in all 2DG and 5TG-supplemented cultures when compared with cultures (Figure 7f). Reduction in glycolytic fluxes by 2DG and 5TG could have resulted in reduced 3-phosphoglycerate (3PG) levels required for serine production from glucose by CHO cells, thus, leading to an increase in extracellular uptake rates of serine available from cell culture media. In CHO cells, serine's β-carbon is transferred to tetrahydrofolate (THF) via serine hydroxymethyltransferase to

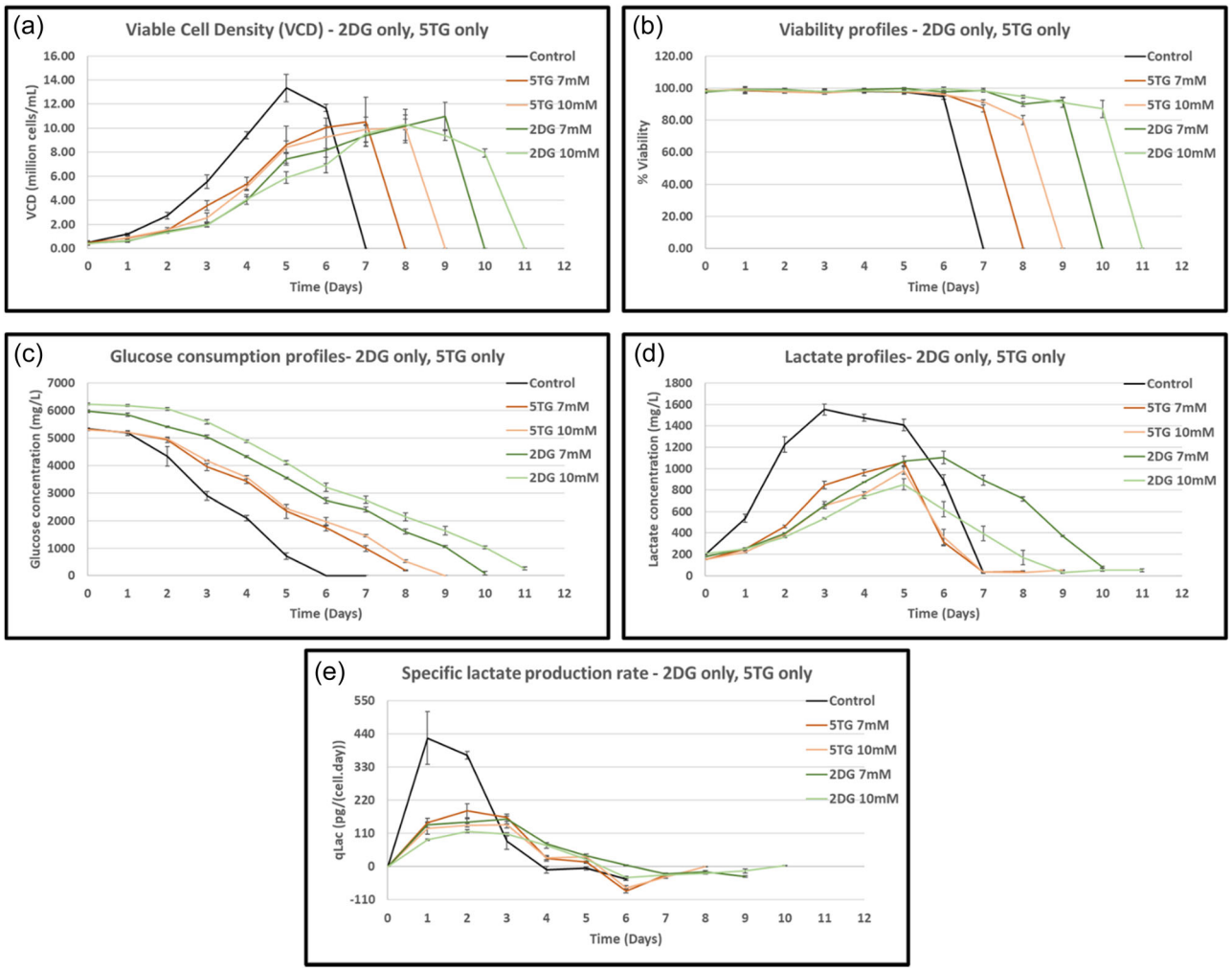


FIGURE 4 Cell culture performance of CHO cells under different concentrations of hexokinase-2 (HK2) inhibitors, 2-deoxy-D-glucose (2DG), and 5-thio-D-glucose (5TG), when supplemented individually at 7 and 10 mM each in batch cultures. (a) Average viable cell density (VCD), in million cells/mL. (b) Average cell viability (%) versus cell culture duration in days. (c) Glucose concentration, in mg/L. (d) Lactate concentration, in mg/L. (e) Cell-specific lactate production (qLac), in pg/(cell-day). Each condition was cultured in triplicates. Error bars, mean ± standard deviation measurement in the cell culture experiments ($n = 3$).

produce glycine (Hansen & Emborg, 1994; Hefzi et al., 2016). Hence, it is not surprising to see an increased glycine production rate. As shown in Figure 7a, individual supplementation of 2DG and 5TG also led to a reduction in pyruvate production rates by at least 40% when compared with control. Combined supplementation of 2DG and 5TG even led to pyruvate consumption while significantly reducing lactate production rate, thus, indicating that suppressing glycolytic fluxes can reduce pyruvate formation through glycolysis causing cells to channel pyruvate from the extracellular medium either into the TCA cycle or into alanine production and away from lactate production. We did not observe any significant changes in alanine production rates compared with control (Figure 7g). In the case of 2DG we observe a reduction in intracellular levels of citrate, succinate, and malate by at least 25% and in the case of 5TG we observe a reduction in intracellular levels of citrate by at least 25%. When 2DG and 5TG are added in combination, we observe a reduction in intracellular citrate and fumarate by at least 25%. Decrease in intracellular concentrations of TCA cycle metabolites has been correlated to decreased TCA cycle fluxes in the literature (Savizi et al., 2022). Hence, indicating a reduction in TCA cycle fluxes, potentially, due to reduced preceding glycolytic fluxes. We observed an increase in asparagine consumption rate by 20%–22% when 2DG and 5TG were supplemented individually at 7 mM and by 48% when 2DG and 5TG were supplemented in combination at 7 mM compared with control (Figure 7b). Indeed, previous studies have reported that in the absence of glucose or in the presence of low glycolytic fluxes, amino acids contribute more toward TCA cycle fluxes (C. Yang et al., 2014; Shiratori et al., 2019; Zhou et al., 2011). In concert, we observed an increase in production rate of glutamate and aspartate in 2DG and 5TG-supplemented cultures, perhaps due in part to the increased consumption of asparagine in the treated cells (Figure 7c,d). Similarly, Kirsch et al., detected increased ^{13}C labeling in aspartate and glutamate when cultures were fed with ^{13}C labeled asparagine (Kirsch et al., 2022). Thus, supplementing CHO cell cultures with HK2

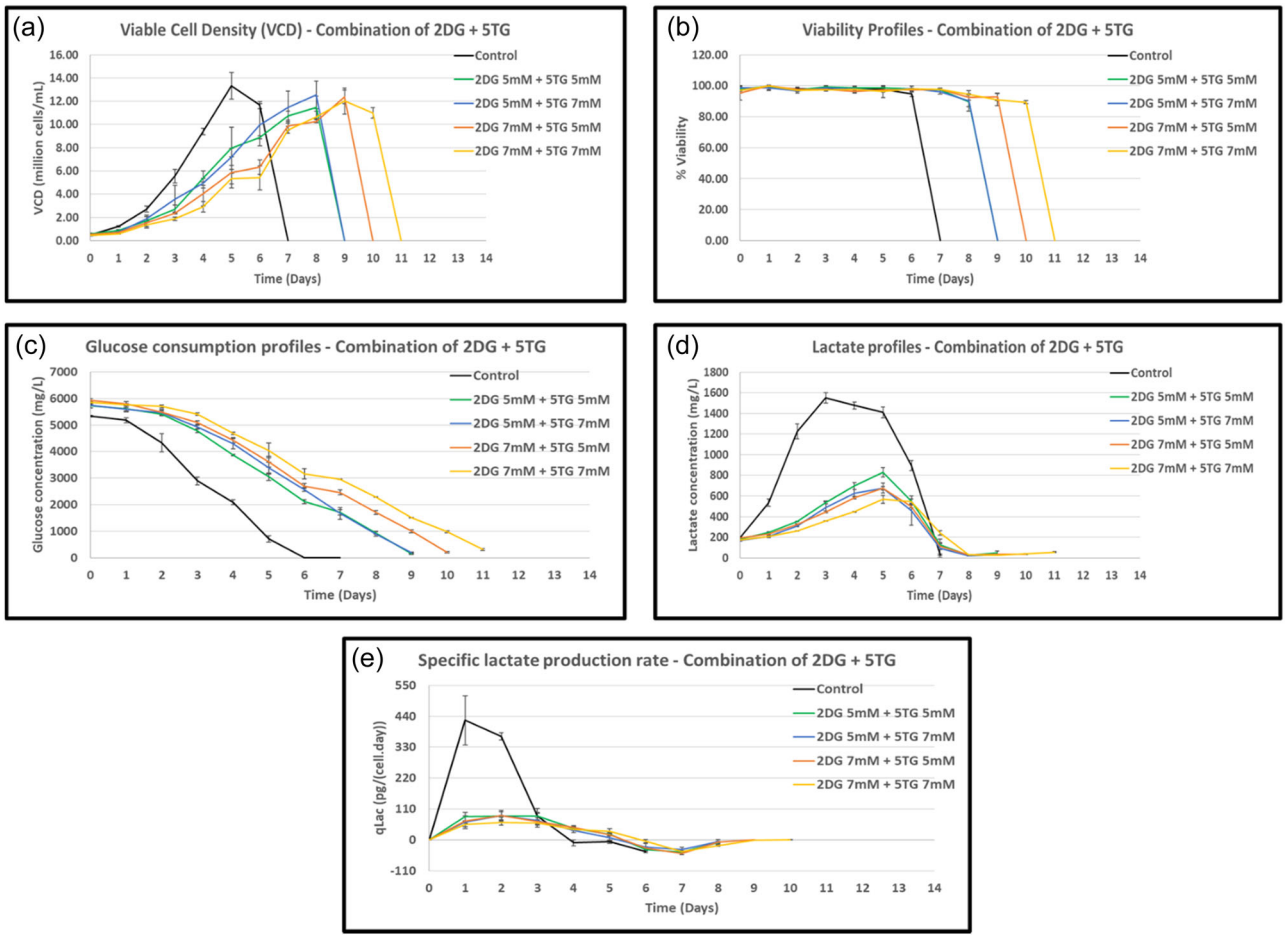


FIGURE 5 Cell culture performance of CHO cells under different combinations of the two hexokinase-2 (HK2) inhibitors, 2-deoxy-D-glucose (2DG), and 5-thio-D-glucose (5TG), supplemented at the following concentrations: (i) 5 mM each, (ii) 2DG 5 mM + 5TG 7 mM, (iii) 2DG 7 mM + 5TG 5 mM, and (iv) 7 mm each. (a) Average viable cell density (VCD), in million cells/mL. (b) Average cell viability (%) versus cell culture duration in days. (c) Glucose concentration, in mg/L. (d) Lactate concentration, in mg/L. (e) Cell-specific lactate production (qLac), in pg/(cell-day). Each condition was cultured in triplicates. Error bars, mean ± standard deviation measurement in the cell culture experiments ($n = 3$).

inhibitors, 2DG and 5TG, not only led to a decrease in lactate production but also rewired metabolism of key amino acids involved in central carbon metabolism.

3.5 | Impact of 2DG and 5TG on EPO-Fc glycosylation

To examine product quality attributes, recombinant EPO-Fc was harvested from supernatants of multiple batch cell cultures containing 2DG and/or 5TG at several different concentrations (Table 3) and purified using Protein A-based affinity chromatography (see Section 2.4).

N-glycans were released from the purified recombinant EPO-Fc following PNGase F digestion, and then analyzed by MALDI-TOF (see Section 2.5). The categories of *N*-glycans detected and percentages are summarized in Supporting Information: Table 1. The native EPO-Fc protein has three EPO *N*-glycosylation sites and one Fc *N*-glycosylation site per chain of the 2-chain EPO-Fc fusion molecule. While the EPO region presented bi-, tri- and tetra-branched *N*-glycan structures, the Fc *N*-glycan displayed solely bi-antennary structures, consistent with our previous studies that the antibody Fc region had *N*-glycans with a predominantly bi-antennary structure (Wang, Yang, et al., 2019).

N-glycan antennary analysis revealed that supplementation with 2DG, 5TG, or both led to an increase in high mannose glycans and a corresponding decrease in bi-, tri-, and tetra-antennary glycans of EPO-Fc when compared with EPO-Fc from control cultures (Figure 8a). Hybrid structures also increased in 2DG-treated cultures as well. Only high mannose *N*-glycans and no hybrid *N*-glycans were observed in 5TG-treated cultures. In addition, the supplementation of 10 mM 2DG or 10 mM 5TG resulted in a 40% decrease in total sialylation content of EPO-Fc as seen in Figure 8b. Based on the data in Figure 8b, addition of 2DG led to a decrease in trisialylated glycans on EPO-Fc whereas addition of 5TG led mostly to a decrease in monosialylated glycans. A 23% drop in total sialylation content was observed for cultures treated with 5 mM 2DG in combination with 5 mM 5TG whereas a 50% drop in total sialylation content was

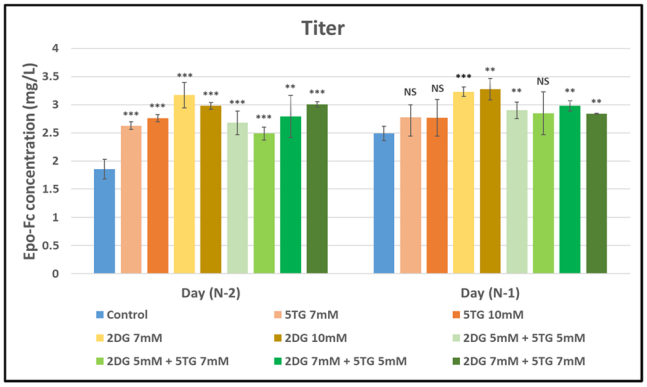


FIGURE 6 Impact of supplementing 2DG and 5TG in CHO cell batch cultures on EPO-Fc titers at different days before the end of the culture (*N*) for that condition. Both 2DG and 5TG were supplemented individually at 7 and 10 mM, and, in combination at (i) 5 mM each, (ii) 2DG 5 mM + 5TG 7 mM, (iii) 2DG 7 mM + 5TG 5 mM, and (iv) 7 mm each. Samples were collected from all cell cultures on Day (*N*-2) and Day (*N*-1), where, Day *N* is the end of culture, to measure levels of recombinant EPO-Fc produced by CHO cells. EPO-Fc titers, in mg/L, were measured using an anti-EPO enzyme-linked immunosorbent assay (ELISA). Each condition was cultured in triplicates. Error bars, mean ± standard deviation measurement in the cell culture experiments ($n = 3$). Statistics (p values) were determined using the two-tailed t test against the control. The following convention was used: $p > 0.1$ indicated by not significant (NS), $0.1 > p > 0.05$ indicated by *, $0.05 > p > 0.01$ indicated by **, $0.01 > p$ value indicated by ***.

observed for cultures treated with 7 mM 2DG in combination with 7 mM 5TG. This data suggest a dose-dependent drop in total EPO-Fc sialylation content with increasing concentrations of 2DG and 5TG. Finally, no change in total EPO-Fc fucosylation was observed in cultures supplemented with 10 mM 2DG (Figure 8c). However, when cultures were supplemented with 5TG with or without 2DG, total fucosylation content of EPO-Fc decreased. A 40% decrease in fucosylation was observed when cultures were supplemented with 10 mM 5TG or with 7 mM of 2DG together with 7 mm of 5TG. These results indicate that levels of fucosylation can be affected by addition of 5TG and the overall percentage of sialylation is consistently lowered by the addition of either 2DG or 5TG or both in combination across four different inhibitor treatment scenarios. Part of the reason for the reduced sialic acid content may be understood by evaluating the *N*-glycan antennary profiles as shown in Figure 8a. While the combined levels of high mannose and hybrid structures were observed to be less than 8% for the control *N*-glycan profile, treatment with inhibitors enhanced these totals to between 31% and 40% for the treated cultures, indicating a clear impact on *N*-glycan processing. This enhanced high mannose and hybrid *N*-glycan formation led to drops in the bi-, tri-, and tetra-antennary structures available for additional processing including sialylation in the treated cultures with the largest impact for 5TG treated cultures.

Interestingly, we observed a shift in the molecular masses of the final *N*-glycans when fed with either 2DG, 5TG or both. Specifically, we observed the presence of *N*-glycans containing a shift in size consistent with the substitution of a 2-deoxy-hexose (2DH) for a hexose residue for the EPO-Fc cultures structures treated with 2DG (Supporting Information: Tables 1 and 2), where the hexose could be mannose and/or galactose. For the case of 2DG (164.16 g/mol), the replacement of oxygen with a 2-hydroxyl group of glucose (180.16 g/mol) (Figure 9), causes a mass decrease of about 16 Da which, when converted to 2DH gets incorporated into the final *N*-glycan structures instead of the hexose, thus, accounting for lowered m/z value detected by MALDI-TOF MS for 2DG treated-cultures. Conversely, in the case of 5TG (196.22 g/mol)-treated cultures, replacement of the ring oxygen on glucose by sulfur (Figure 9) causes a mass increase of about 16 Da which, when converted to 5-thio-hexose (5TH), where the hexose could be mannose and/or galactose and/or possibly *N*-acetylglucosamine, gets incorporated into the final structure, and, is consistent with the increase observed on the resulting *N*-glycans attached to EPO-Fc (Supporting Information: Tables 1 and 2). For the 5TG treated cultures, 6.5%, 8%, and 23% of total *N*-glycans included 5TH moieties as the concentration of 5TG increased from 5 to 7 mM, to 10 mM in the cultures. (Figure 10a). Previously, Zimmermann et al. supplemented CHO cell cultures with 5-thio-L-fucose (5TF), which also resulted in a dose-dependent decrease in total fucosylation and a dose-dependent increase in thiofucosylation in place of core-fucosylation on rituximab (Zimmermann et al., 2021). The authors also noted that the exchange of the oxygen in the fucose ring (164.0684 g/mol) with sulfur to make 5-thio-L-fucose (180.0455 g/mol) causes a mass shift of about +16 Da, consistent with our findings with 5TH. Similarly, the levels of *N*-glycans containing modified residues increased in a dose-dependent manner for 2DG such that 14%, 18%, and 33% of total *N*-glycans included 2DH for cultures supplemented with 2DG at 5, 7, and 10 mM, respectively (Figure 10b). Unlike the 5TG-supplemented cultures which only contained one modified residue, we observed one and two 2DHs on the *N*-glycan moieties with 5 mM and 7 mM 2DG. Furthermore, with 10 mM 2DG, we observed as many as three and four 2DHs on some *N*-glycans. Thus, addition of 2DG not only increased the total fraction of EPO-Fc *N*-glycans containing 2DH but also the number of 2DHs on a single glycan moiety in a dose-dependent manner. Thus, the supplementation of CHO cell cultures with the HK2 inhibitors, 2DG and 5DG, caused changes not only in the lactate production patterns but also manifested in modifications to the branching and extension of the *N*-glycans generated as well as in the specific chemical nature of the *N*-glycan structures produced.

4 | DISCUSSION

Based on the initial first-pass screening experiments, Lonidamine, 3BP, and MH did not reduce lactate accumulation without impacting cell growth. Either these small molecules do not efficiently target the machinery of CHO cell HK2 to inhibit its activity or they are involved in broader mechanisms hindering cell growth. Furthermore, these molecules could also be tested at lower concentrations in future studies to determine their impact on cell growth and lactate

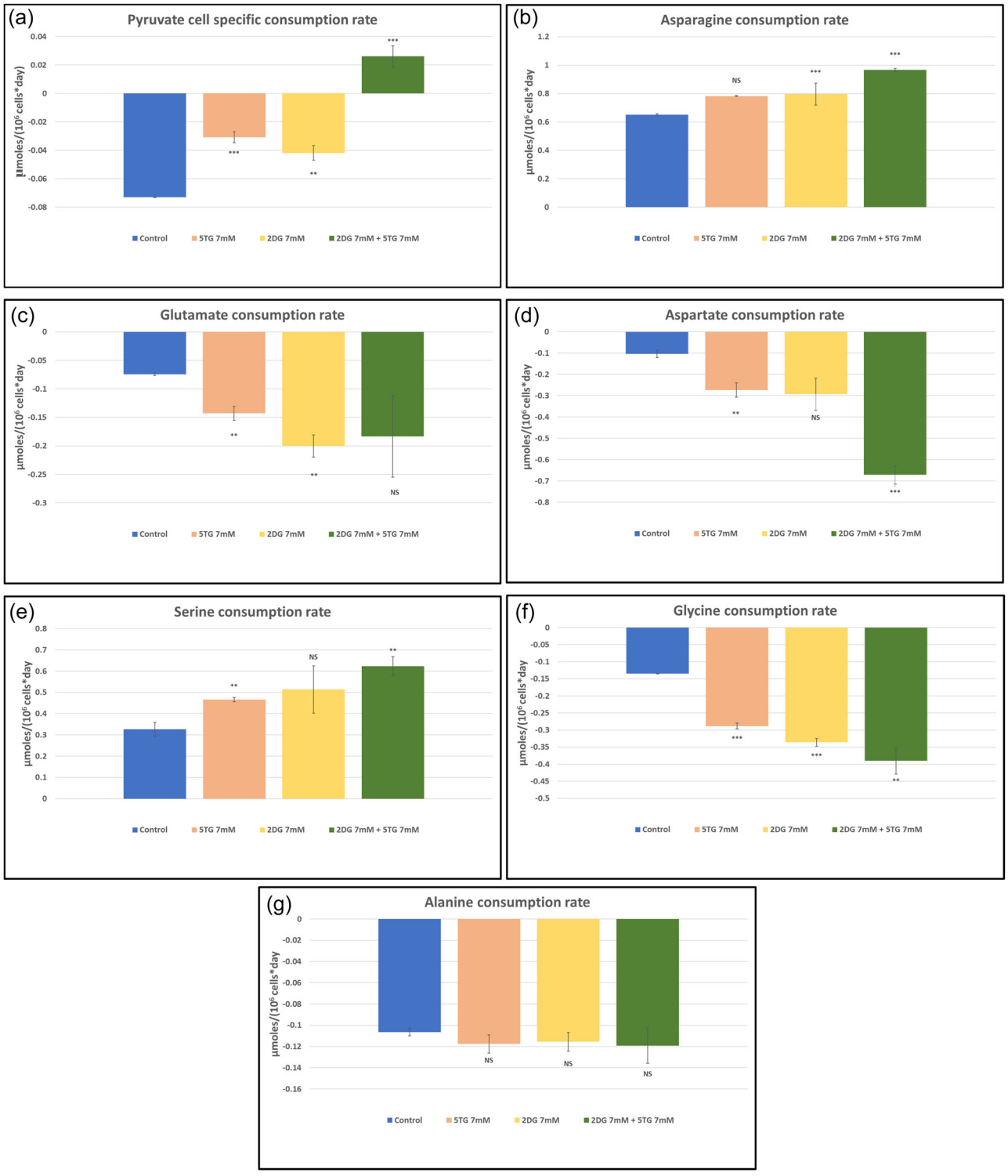


FIGURE 7 Cell specific uptake rates (µm/(10^6 cells × day)) of metabolites driving glycolysis and TCA cycle pathways over the culture duration in CHO cells compared between the following conditions—control, 5TG 7 mM, 2DG 7 mM, and combination of 2DG 7 mM and 5TG 7 mM. Cell-specific uptake rates (mM/(10^6cells/day)) of (a) Pyruvate (b) Asparagine (c) Glutamate (d) Aspartate (e) Serine (f) Glycine (g) Alanine. Each condition was cultured in duplicates. Error bars, mean ± standard deviation measurement in the cell culture experiments (n = 8). Statistics (p values) were determined using the two-tailed t test against the control. The following convention was used: $p > 0.1$ indicated by not significant (NS), $0.1 > p > 0.05$ indicated by *, $0.05 > p > 0.01$ indicated by **, $0.01 > p$ value indicated by ***.

TABLE 3 Different concentrations and combinations of 5TG and 2DG supplemented to CHO cell batch cultures to evaluate their impact on protein *N*-glycosylation.

Cell culture condition	Concentrations tested (mM)
Control (without 2DG or 5TG)	–
2DG	10
5TG	10
2DG + 5TG	5 + 5, 7 + 7

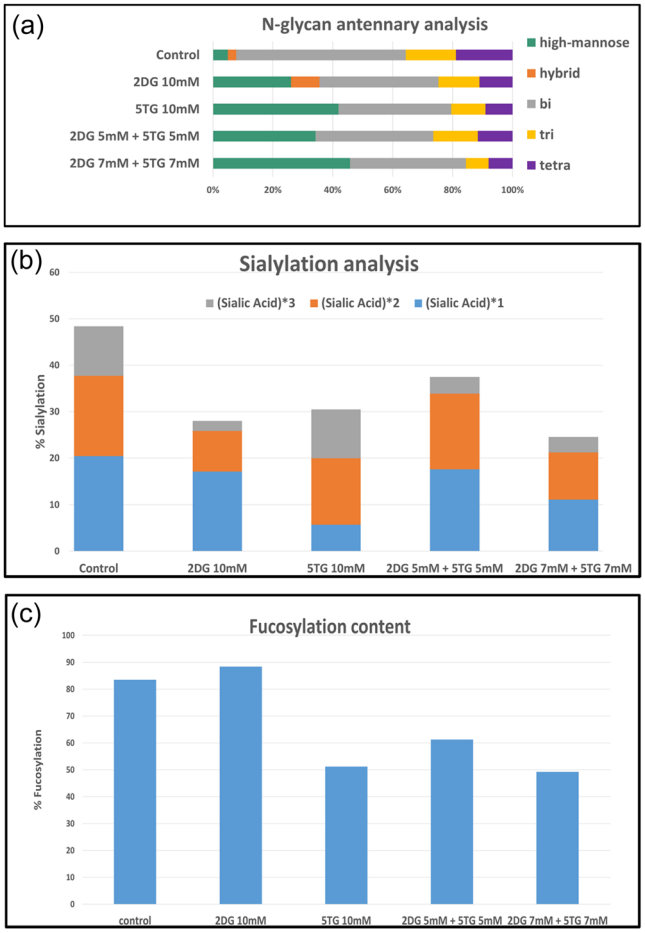
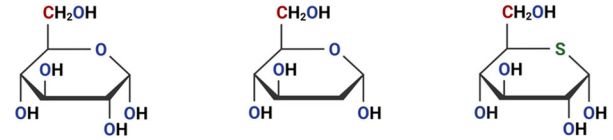


FIGURE 8 Impact of supplementing 2DG and 5TG in CHO cell cultures on EPO-Fc *N*-glycosylation. Both 2DG and 5TG were supplemented individually at 10 mM. In combination experiments, both 2DG and 5TG were supplemented at 5 mM each and 7 mm each. (a) *N*-glycan antennary analysis and branch distribution of EPO-Fc protein. (b) *N*-glycan sialylation analysis on EPO-Fc protein. (c) *N*-glycan fucosylation analysis of EPO-Fc protein. EPO, erythropoietin.



(a) D-Glucose (b) 2-Deoxy-D-glucose (c) 5-Thio-D-glucose

Analog	Molecular weight (g/mol)	Difference in molecular weight with respect to D-Glucose (g/mol)
D-Glucose	180.16	-
2-Deoxy-D-glucose	164.16	-16
5-Thio-D-glucose	196.22	+16.06

FIGURE 9 Structural formulae and molecular weight of 5TH (a) D-Glucose (b) 2-deoxy-D-glucose (2DG) (c) 5-thio-glucose (5TG). 2DG is a glucose analog in which the 2-hyrdoxyl group is replaced by a hydrogen and has a molecular weight which is 16 g/mol lower than that of glucose. 5TG is a thiosugar in which the oxygen in the cyclic ring is replaced by a sulfur atom and has a molecular weight which is 16 g/mol higher than that of glucose.

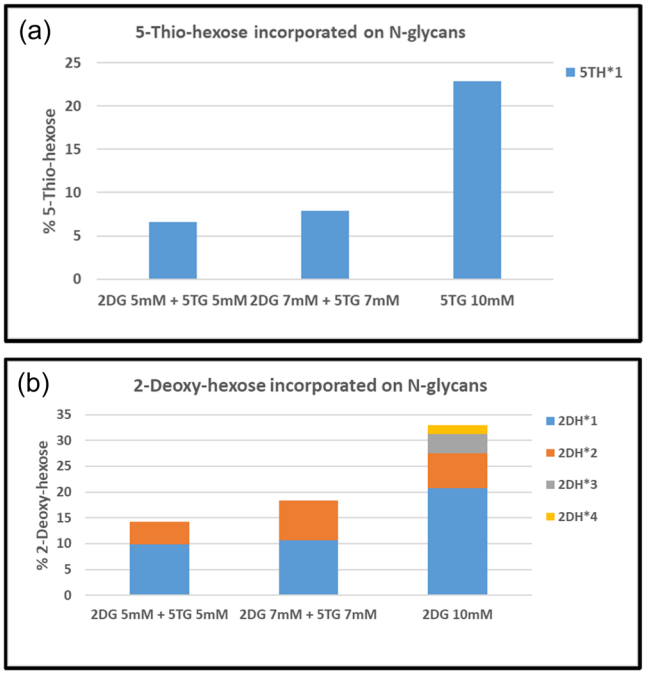


FIGURE 10 Incorporation of nonnatural glycans on EPO-Fc produced from CHO cell cultures supplemented with 2DG and 5TG (a) Dose-dependent incorporation of a single 5-thio-hexose (5TH) on EPO-Fc *N*-glycans, where the hexose could be mannose, galactose or *N*-acetylglucosamine respectively on the *N*-glycans (b) Dose-dependent incorporation of multiple 2-deoxy-hexose (2DH) on EPO-Fc *N*-glycans, where the hexose could be mannose or galactose.

accumulation. On the contrary, the addition of inhibitors 2DG and 5TG resulted in a reduction in peak lactate accumulation between 30% and 45% and a decline in the L/G ratio of at least 50% with only modest reductions in growth rate of less than 10%. Furthermore, combining 5TG and 2DG resulted in an even greater reduction in peak lactate accumulation and moles of lactate produced per mole of glucose was even lower in cultures having both compounds to suggest complementary activities. Especially surprising was that the peak VCD was often not reduced as much as when each inhibitor was added individually. 2DG is a glucose analog in which the 2-hyrdoxyl group is replaced by a hydrogen (Figure 9b). Pusapati et al. reported that in cancer cells, 2DG downregulated GLUT1 expression leading to

decreased glucose uptake in response to 2DG (Pusapati et al., 2016). Furthermore, 2DG and 5TG may compete with glucose for transport into the cells. The maximum cell-specific glucose consumption rate was indeed lower for cultures supplemented with 2DG. Urakami et al. demonstrated that after entering the cell, 2DG is phosphorylated to 2-deoxy-glucose-6-phosphate (2DG6P) by HK2. Unlike G6P, 2DG6P is neither isomerized to 2-deoxy-F6P nor metabolized further by the GPI enzyme since 2DG6P is missing the 2-OH group. Hence, 2DG6P acts as a noncompetitive inhibitor of HK2 enzyme and as a competitive inhibitor of GPI enzyme (Pajak et al., 2019; Urakami et al., 2013; W. Chen & Guéron, 1992). Alternatively, 5TG is a thiosugar in which the oxygen in the cyclic ring is replaced by a sulfur atom (Figure 9c). Machado de Domenech and Sols demonstrated that 5TG is very poorly phosphorylated by yeast HK2. The presence of a sulfur atom in 5TG in place of oxygen atom in the glucopyranose ring leads to a loss of some amount of HK2 affinity and phosphorylation rate (Machado de Domenech & Sols, 1980) and, as a result, 5TG acts as a competitive inhibitor of HK2 (Board et al., 1995; Wilson & Chung, 1989). The fact that 2DG serves as a noncompetitive inhibitor of HK2 and 5TG is a competitive inhibitor may help to explain the cumulative effects on lactate accumulation when both are added together. Noncompetitive inhibition of HK2 by 2DG and competitive inhibition of HK2 by 5TG possibly slowed down the availability of G6P needed for processing the subsequent steps downstream in the glycolysis pathway and in turn, led to lower lactate production. It is interesting that the % increase in EPO-Fc titer on Day *N*-1 was not as significant, especially for 5TG-treated cultures as observed on Day *N*-2. This suggests that accumulation of EPO-Fc in 2DG and 5TG treated cultures may have begun earlier (relative to the end of culture, Day *N*) than control cultures. Perhaps the control cultures were allocating more resources to growth while the treated cells were channeling more of their resources to recombinant protein production potentially due to rewiring of glycolysis metabolism.

Indeed, the measurements of intracellular metabolites and changes in extracellular amino acid consumption rates were indicative of changes to the intracellular metabolism. The effect on glycolysis was evident from a reduction in both intracellular and extracellular lactate accumulation following addition of the HK2 inhibitors. A change in glycolytic metabolism was also supported by increased demands for extracellular serine along with a complementary increase in glycine secretion as serine is converted glycine as part of the THF and purine synthesis pathway. Further support for a decline in the channeling of glucose through glycolysis was indicated by a switch from pyruvate secretion in control cells to modest levels of pyruvate consumption in the combined presence of 2DG and 5TG. This slowdown in glucose-channeling through glycolysis was also evident in low intracellular levels of the glycolytic intermediate phosphoenolpyruvate following combined inhibitor treatment. The impact of glucose restriction likely impacted TCA metabolism which was manifested in a consistent reduction in intracellular citrate, the entry point of the TCA cycle, following addition of individual or combined HK2 inhibitors. Increased consumption of asparagine may be required to provide sufficient nitrogen to the cells as well as to facilitate TCA activity if there is a restriction in the glucose channeling through the pathway. Interestingly aspartate and glutamate secretion increased suggesting the impact of transaminase reactions driving the production or consumption of TCA cycle intermediates such as oxaloacetate and alpha-ketoglutarate. Clearly, the restriction of glucose utilization through addition of HK2 inhibitors exhibited rippling effects that were felt down the glycolysis pathway and likely into the TCA cycle resulting in changes in the consumption or production of other nutrients that can ultimately impact cell growth and protein production. Indeed, previous studies reported that suppression of glycolytic fluxes earlier in the culture can potentially result in channeling more pyruvate into the TCA cycle, rewiring intracellular metabolism toward mitochondrial oxidative phosphorylation, and resulting in more ATP generation, slower growth, and higher protein production (J. Liu et al., 2015; Shiratori et al., 2019).

In addition to impacting lactate accumulation, cell metabolism, and product yields, the addition of 2DG and 5TG also altered glycosylation patterns of the recombinant EPO-Fc. Indeed, we observed lower sialylation levels in EPO-Fc glycans in the presence of 5TG and 2DG. Furthermore, supplementation with 2DG and 5TG individually and together led to high mannose or hybrid forms unlike the control cell cultures which generated mature glycans that include higher levels of complex bi-antennary, galactosylated, and sialylated glycans. Consistent with our findings, Ahadova et al. reported a significant increase in mannose incorporation onto cellular glycoproteins when cancer cell cultures were treated with 2DG. Their studies indicated that this phenomenon was not caused by mannosidase inhibition but rather by 2DG exerting its effect on monosaccharide incorporation in the phase of glycoprotein synthesis (Ahadova et al., 2015). 2DG is synthesized from a D-glucose molecule by the elimination of the hydroxyl group at C-2 in D-glucose. However, eliminating the same hydroxyl group at C-2 in a D-mannose molecule also yields the same 2DG compound. Thus, 2DG not only acts as a glucose mimetic molecule but also as a mannose mimetic molecule, thereby, interfering with both D-glucose and D-mannose metabolism. 2DG likely not only competes with mannose metabolism but may even be converted to Guanosine diphosphate (GDP)-2-Deoxy-mannose and further be incorporated into dolichol-phosphate (lipid)–linked oligosaccharides, the precursors for N-linked glycosylation (Kurtoglu et al., 2007). Furthermore, as a glucose mimetic molecule, 2DG likely also competes with glucose metabolism leading to the formation of 2-Deoxy-glucose-1-phosphate and the subsequent nucleotide sugars – Uridine diphosphate (UDP)-2-deoxy-glucose and UDP-2-deoxy-galactose. Indeed, Urakami et al. detected the presence of 2-deoxy-glucose-1-phosphate in 2DG treated cancer cells (Urakami et al., 2013). However, we did not observe the presence of glucose residues on any of our *N*-glycan structures in 2DG treated cultures. Further, most secreted mammalian glycoprotein lacks Glc residues. Additionally, since 2DG lacks a hydroxyl group, it cannot be isomerized by the GPI enzyme from 2DG6P to 2-deoxy-F6P, thus, making it impossible to produce UDP-2-deoxy-glucosamine and UDP-2-deoxy-galactosamine from 2DG. Hence, the 2-deoxy-hexose we observed on *N*-glycans of EPO-Fc in

2-DG treated cultures is most likely 2-deoxy-mannose (2DM) and/or 2-deoxy-galactose (2DGal) and not 2-deoxy-analogs of glucose, glucosamine, and galactosamine. Thus, 2DG potentially affects monosaccharide incorporation during oligosaccharide synthesis and not through inhibition of glycoprotein degradation enzymes (although we cannot exclude an effect on these activities as well) (Ahadova et al., 2015). This incorporation into nucleotide sugars and lipid-linked oligosaccharides can also explain why we observed incorporation of 2DM and/or 2DGal on final glycan structures (Figure 10b).

5TG could follow a similar fate as 2DG in the glycosylation pathway but no such studies have been reported in the literature for processing of 5TG in mammalian cells. 5TG could serve as a glucose analog or alternatively, 5TG could also be converted to 5-Thio-mannose (5TM) and 5-Thio-galactose (5TGal) and be subsequently incorporated on the final glycan structures (Figure 10a). Tsuruta et al. reported in their in vitro studies that GDP-5-thio-mannose was a donor substrate for α(1,2)mannosyltransferase leading to the formation of a 5-thio-mannose-containing disaccharides (Tsuruta et al., 2003). In a similar fashion, UDP-5-thio-galactose and UDP-*N*-acetyl-5-thio-galactosamine are functional substrates for galactosyltransferase (Tsuruta et al., 1997). We do not observe any galactosamine residues on our EPO-Fc *N*-glycans which is not uncommon in glycoproteins produced from CHO cells. Hence, the 5TH that we observe in our *N*-glycans is most likely 5-thio-mannose and/or 5-thio-galactose, however, we cannot rule out the possibility of the 5TH to be 5-thio-*N*-acetylglucosamine. For the high mannose *N*-glycans identified without any galactose residue, the 5TH is most likely to be 5-thio-mannose and/or possibly 5-thio-*N*-acetylglucosamine. For hybrid, bi-, tri-, and tetra-antennary *N*-glycans, the 5TH is most likely to be 5-thio-mannose and/or 5-thio-galactose and/or possibly 5-thio-*N*-acetylglucosamine. The presence of 5-thio-glucose structures is also theoretically possible but glucose residues are not common on high mannose *N*-glycans with a hexose number above nine. We also did not observe any glucose residues on our *N*-glycans in 5TG treated cultures. Other in vitro studies have also observed that 5-thio-glycosides, the ring sulfur analogs of native glycosides, are resistant to glycosidases (García Fernández et al., 1998; Mehta et al., 1995; Yuasa et al., 1992). This resistance to glycosidases could explain in part the high mannose forms that we observed in 5TG-treated cultures compared with control and 2DG-treated cultures. Interestingly, our study is the first to evaluate the impact of a thioglucose on cell growth, protein production, and protein *N*-glycosylation processing, including the formation of alternative glycoforms.

The presence of high mannose structures indicates the generation of truncated or improper oligosaccharides that are unable to be extended to form the structures appropriate for subsequent *N*-glycan modifications. This eventuality is also consistent with the incorporation of 5TG and 2DG derivatives, which may have a direct impact on *N*-glycan branching and extension reactions. The lack of robust *N*-glycan branching and extension may also indirectly be due to the presence of these alternative sugars perhaps through a decrease in the relative activity of *N*-Acetylglucosamine transferases (GNT1 and GNT2), which adds the *N*-Acetylglucosamine transferases (GlcNAc) residues onto the biantennary structures. This decreased activity may be due to low levels of the proper UDP-GlcNAc substrates present in the cells as a result of feeding glucose analogs since GlcNAc and UDP-GlcNAc as well as most other sugars are derived principally from glucose. Alternatively, GNT1 and GNT2 activities may be reduced due to the presence of the nonnatural nucleotide sugar analogs. It is interesting that 2DG resulted in the accumulation of some hybrid structures along with high mannose forms while 5TG addition resulted in the elevation of the high mannose forms with no observed hybrid structures. Perhaps 5TG is a more potent inhibitor of the formation of the proper mannosylated precursors along the *N*-glycan processing pathways or it has a larger negative impact on the enzymatic addition of GlcNAc driven by GNT1. Furthermore, the absence of proper acceptor substrates also likely drives a reduction in the accumulation of tetra-antennary *N*-glycans along with fewer tri-antennary structures in most cases for the inhibitor-treated samples. As these branching enzymes also depend on the UDP-GlcNAc and GlcNAc transferases (GNT4 and GNT5) activities, insufficient levels of proper nucleotides or enzymatic inhibition may play a role. Likewise, the reduction in sialylation and galactosylation observed for all the treated samples can be due to the lack of sufficient branches available for generating tri-sialylated structures but we cannot exclude insufficient nucleotide sugar levels and inhibition of these transferases as well. Previous reports have suggested an inversion relationship between cell-specific productivity (qP) and glycan processing with increased qP leading to insufficient glycan processing (Fan et al., 2015). However, in our study, p-values calculated using the two-tailed t-test showed statistically insignificant differences between qP values of control, 2DG treated cultures, and 5TG treated cultures (see Supplementary Figure 3). This suggests that, at least in our study, there is no inverse correlation evident between qP and glycan processing. Indeed, it is the feeding of 2DG and 5TG that leads to less processed glycans. In addition, we also observed that EPO-Fc produced from 5TG-supplemented cultures had reduced fucosylation. Indeed, if 5TG inhibited fucosyltransferase or GDP-fucose generation, this would help to explain the reduction of fucosylation found in the CHO cells containing HK2 inhibitor molecules. Levels of intracellular nucleotide sugars and enzyme activity levels in the presence of these two analogs can be investigated further to fully delineate how these glucose analogs are processed in the *N*-glycosylation pathways.

The presence of these monosaccharide substitutions may potentially have varied impacts on the activities of the produced recombinant proteins such as EPO, EPO-Fc, or other antibody targets. The amount and nature of glycan structures can affect multiple protein properties including effector functions and cytotoxicity. High mannose glycans and reduced terminal sialylation can decrease serum half-life of glycoproteins such as EPO, leading to more rapid clearance from the human body. High mannose glycans can also lower C1q binding and subsequent CDC activity. Alternatively, reduced terminal sialylation and presence of high mannose and

afucosylated glycans have been reported to enhance FcγRIIIa binding and ADCC activity of glycoproteins (Stanley, 1992). Interestingly, Zimmermann et al. reported that thiofucosylated rituximab exhibited an enhanced FcγRIIIa binding, ADCC efficacy, potency, and ADCC activity compared with fucosylated rituximab (Zimmermann et al., 2021). It would be worthwhile to investigate if the presence of these modified hexoses has an impact on the biological properties of EPO-Fc and other recombinant proteins. In some cases, the presence of chemical modifications on glycans can be advantageous for targeted delivery. However, the presence of a modified chemical structure can also have implications on whether such a structure is accepted by regulatory authorities. In case these modified residues are not desired in the final product, additional purification steps may be required to eliminate them from the final product. These modified residues may also be candidates for additional chemical modifications of the altered *N*-glycan residues. For example, ManNAc has been modified with azides as part of click chemistry incorporation of toxins that can then be targeted to specific tissues for directed cancer therapy (H. Wang et al., 2019; Saxon & Bertozzi, 2000). Another example includes the replacement of natural sialic acid groups in leukemic cells with unnatural analogs using *N*-propionylmannosamine for cancer immunotherapy (T. Liu et al., 2000). Alternatively, glycan analogs can be used to tether additional structures such as BSA to enhance serum circulatory lifetime (Kontermann, 2011). Indeed, these altered mannose or other residues arising from 2DG and 5TG or other closely related analogs could be investigated for their capacity to be modified by linking additional chemical agents postproduction to provide novel glycoconjugates with applications in targeted delivery. The modified residues could serve as the scaffold for additional chemical modifications of *N*-glycans that can impact either clearance or functions in antibody drug conjugates. Either way, these glucose analogs are highly effective modulators of CHO cell behavior through their capacity to alter lactate generation, glucose to lactate ratios, cell growth, protein production, and glycosylation processing in ways that can be used to modify the bioprocessing performance of CHO cells in culture.

AUTHOR CONTRIBUTIONS

Harnish Mukesh Naik: Conceptualization, methodology, investigation, formal analysis, validation, visualization, writing—original draft, writing—review and editing. **Swetha Kumar, Jayanth Venkatarama Reddy, Jacqueline E. Gonzalez, Brian O. McConnell, Venkata Gayatri Dhara, Tiexin Wang**: Investigation, formal analysis, validation, visualization. **Marcella Yu**: Conceptualization, methodology, project administration; **Maciek R. Antoniewicz**: Conceptualization, methodology, investigation, supervision, project administration, funding acquisition. **Michael J. Betenbaugh**: Conceptualization, methodology, investigation, writing- original draft, writing—review and editing, supervision, project administration, funding acquisition.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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