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RESEARCH ARTICLE

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Resveratrol addition to CHO cell culture media: The effect on cell growth, monoclonal antibody synthesis and its chemical modification.

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Abstract

The effect of the addition of resveratrol to cell culture media during the production of monoclonal antibodies was investigated. Treatments of Chinese hamster ovary (CHO) cells expressing immunoglobulin G (IgG) with 25 and 50 μM resveratrol showed that resveratrol was capable of slowing cell growth whilst almost doubling cell specific productivity to 4.7 ± 0.6 pg IgG/cell·day, resulting in up to a 1.37-fold increase of the final IgG titre. A resveratrol concentration of 50 μM slowed the progression through the cell cycle temporarily by trapping cells in the S-phase. Cation exchange chromatography showed no significant difference in the composition of acidic or basic IgG species and size exclusion chromatography indicated no change in fragmentation or aggregation of the recombinant IgG in the treatment groups. Resveratrol could be used as a chemical additive to CHO media where it would enhance IgG productivity and provide a degree of protection against hydroxyl and superoxide free radicals, expanding the range of options for process improvement available to monoclonal antibody manufacturers.

Keywords: antioxidant, polyphenol, stilbene, IgG, Mab

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Introduction

The main challenge for the biopharmaceutical industry is to produce therapeutic proteins with consistent quality, with good yields and at relatively low cost. One approach was to focus on the development of more reliable production cell lines.[1] Another strategy used chemical additives to cell culture media to improve the therapeutic protein yeilds. These additives, sometimes referred to as "chemical chaperones" are not nutrients but improve product yields by either modulating cell growth, stabilising the product or reducing chemical modification. Sodium butyrate, [2, 3] zinc [4] and valeric acid [5, 6] are examples of chemicals that led to an improvement of the recombinant protein production. Identification of further additives is desirable in order to increase the number of options cell culture process development. The introduction of a new media component to a cell culture reactor has a series of risks that need to be mitigated before it can go into commercial use. In the case of addition of a chemical like resveratrol to cell culture media, the following potential effects of the chemical need to be assessed, (A) its cell toxicity, (B) its impact on protein synthesis and post translational modification, (C) any reaction with the therapeutic protein, and (D) its potential to catalyse chemical modification of the therapeutic protein.

Resveratrol (3, 5, 4'-trihydroxystilbene) is a stilbene found in a variety of fruits and vegetables. It is a widely studied antioxidant, partially due to its presence in red wine and its reputed health benefits. Its structure consists of two phenolic groups joined by an ethene, enabling electron delocalisation across the molecule (Figure 1). Resveratrol can be radicalised at all three hydroxyl groups with 4' being the most stable [7] and spread the energised electron over the entire molecule providing radical stabilisation. Resveratrol's ability to scavenge free radicals, including superoxide and hydroxyl radicals, protects against peroxidation of membrane lipids and DNA.[8] Resveratrol is also believed to be an activator of SIRT1, which enhances mitochondrial oxidative phosphorylation in mice.[9,10]

Research on resveratrol's effect on CHO cells is limited, but indicates that resveratrol could have a protective effect. Resveratrol can provide protection from H_2O_2 induced DNA damage if added 3 hours prior to the addition of H_2O_2.[11,12] It was also shown that it can restore redox balance from induced oxidative stress, by inhibiting the mitochondrial proteasome.[13] Resveratrol seems to regulate CHO cells by upregulating 5'ADP-activated protein kinase (AMPK) and its metabolic target acetyl-CoA

carboxylase for concentrations up to 50 µM.[14] Resveratrol can be used as a protective agent when CHO cells are under stress conditions induced by beauvericin, a strong cytotoxic oxidative agent.[15] Resveratrol is a bioactive chemical that seems to have complex effects on cells. Only one study in the past has investigated the use of this molecule in biopharmaceutical production. Resveratrol was not able to improve recombinant production by inhibiting autophagy at the single concentration tested.[16] A recent screening study identified the potential of resveratrol (and series of other antioxidants) as possible media additives as they were found to slow CHO cell growth and increase cell specific IgG production at small-scale.[17]

The aim of the current study is to further examine the effect of resveratrol on the CHO cell culture over a longer time period and study its effect on quality of IgG synthesised in its presence. This was done in order to determine whether this chemical is suitable as an additive in cell culture media during the production of recombinant protein products where its antioxidant protperties may be beneficial.

Materials and Methods

Cell Line and Reagents

The reagents: dimethyl sulfoxide (DMSO), phosphate buffered saline (PBS), phenyl vinyl sulfone, RNAse I, propidium iodide, Tris(2-carboxyethyl) phosphine hydrochloride (TCEP), laemmli buffer, glycine, iodoacetic acid (IAA); were supplied by Sigma-Aldrich (St. Louis, Missouri, USA). OptiCHO™ free serum media, L-glutamine were supplied by Thermo Fisher Scientific (Ulm, Germany). Hygromicin B was supplied by Invitrogen (Carlsbad, California, USA). Tris was supplied by SLS (Nottingham, UK).

The cell line used was a stable recombinant DG44 CHO cell line that expressed an IgG monoclonal antibody, provided by Fujifilm Diosynth Biotechnologies (Billingham, UK). The cell line was grown in suspension in an OptiCHO™ free serum media supplemented with 8mM L-glutamine and 100 mg/L hygromicin B. An initial culture was maintained in a shaking incubator with 5% CO_2 modified atmosphere at 37°C. Cells were then seeded into new media at $0.2x10^6$ viable cells/ml every 3-4 days.

Cell Cycle Analysis

Treatments were carried out in 1 ml cultures in a 24-well plate platform with an initial viable cell density (VCD) of $0.2x10^6$ cells/ml. Wells were then treated at time 0 with different concentrations of

resveratrol diluted in 5µl of DMSO to a final concentrations of 0, 10, 25, 50, 75 and 100 µM. Cells were grown for a period of 3 days under static conditions. VCD was measured using a Beckman-Coulter Vi-cell™ XR cell viability analyser (Indianapolis, Indiana, USA). In parallel, samples were centrifuged for 10 minutes at 400 g, the supernatant collected, and the IgG concentration measured using bio-layer interferometry with a Pall FortéBio Octet® QK^e (Fremont, California, USA). Cultures were collected at 0, 8, 16, 24, 32, 40, 48 and 72 hours. A modified propidium iodide staining protocol was used to measure cell cycle distribution.[18] Finally, samples were measured with an Attune NxT Flow Cytometer & autosampler (Thermo Fisher Scientific) and analysed with FlowJo™ v10 (San Carlos, California, USA).

33 ml Erlenmeyer flask CHO Cell Growth and IgG Expression

Flasks with an initial concentration of 0.2x10^6 cells/ml in 33ml culture volume were grown for 10 days under a batch feeding regime with the same conditions specified in the cell culture section.

Flasks were treated at days 0, 3 and 6 with 0, 10, 25 and 50 µM final concentrations of resveratrol. A read-out of the viable cell density, viability and the IgG concentration was performed every 24 hours. This experiment was done in two independent triplicates.

VCD, viability and IgG concentration was measured as specified before. Specific protein production (q_p) was determined by dividing the total protein produced after ten days incubation by the integral of the VCD curve over the same duration.[19]

$$q_p(pg/cell \cdot day) = \frac{Final\ Recombinant\ Concentration(\mu g/mL)}{\int_0^{10} VCD(10^6\ viable\ cells/mL)}$$

IgG Characterization

To study the effects of the addition of resveratrol in flask cultures, a replicate experiment of the flask experiment as described above was used with only addition of resveratrol on day 3 at 10, 25, 50 and 100 µM concentrations. A read-out of VCD and viability was performed every 48 hours and the IgG concentration was measured on day 10. This was done in 4 independent replicates.

Cell culture was centrifuged and the supernatant was filtered and purified with an ÄKTA™ pure (GE Healthcare) with the use of a 1x5 ml HiTrap™ MabSelect Sure™ protein A column (GE Healthcare) and then stored at -80^{o}C until further analysis by size exclusion chromatography (HPLC SEC), cation exchange chromatography (HPLC CEX) and liquid chromatography tandem mass spectrometry (HPLC-

MS/MS). Experimental details of HPLC SEC, HPLC CEX and HPLC-MS/MS analysis can be found in supplementary materials.

Statistical analysis

Experimental results were expressed as mean ± 1 SD for those results generated from at least 3 independent cultures. Results were evaluated for statistical significance through one-way ANOVA tests, p-values were considered significant below 0.05.

Results

1mL Multiwell Study with Cell Cycle Analysis

The time course study undertaken in 24-well plates (Figure 2) showed that resveratrol inhibited CHO cell growth in a concentration-dependant manner. While 10 µM treatment did not cause marked changes in the growth behaviour of the CHO cell culture. There was a minor increase in cell diameter around 16 hours after inoculation but the culture recovered by 24 hours and subsequently behaved like the untreated control. The cell cycle analysis showed no observable difference in the ratio of G_1/G_0, S and G_2/M between the 10mM resveratrol treatment and the untreated control.

Treatment of the CHO cell culture with 25 µM resveratrol had an observable effect on growth (Figure 2). The VCD was reduced for the first 48 hours after inoculation before the culture started to grow at a rate similar to the untreated control. The viability dropped below 95% 24 and 32 hours after inoculation which coincided with an increase in cell diameter, before both viability and cell diameter returned to levels in the untreated control. The cell cycle analysis (Figure 3) showed an observable difference between the 25 mM resveratrol treatment group and the untreated control. 24 hours after inoculation there was a decline in the proportion of S phase cells and a rise in cells in the G_1/G_0 and G_2/M phase which was temporary but the culture took 48 hours to stabilise to the proportions seen in the untreated control.

50mM resveratrol treatment had a marked effect on CHO cells (Figure 2). The VCD was checked for the first 48 hours after inoculation before starting to recover. The cell viability declined after inoculation dropping below 90% at 48 hours. The cells also had a steady increase in diameter for the first 48 hours before starting to return to the levels in the untreated control. The cell cycle analysis

(Figure 3) differed markedly from the untreated control with a dramatic decline in G_1/G_0 phase cells and equally dramatic rise in S phase cells 32 hours after inoculation.

The 1 mL multiwell study demonstrated resveratrol's capacity to impede CHO cell growth in a concentration dependant manner. The effect was observed to be temporary with cell growth returning around 48 hours after the treatment starts. Addition of the resveratrol at the same time as inoculation may have exacerbated the check on growth as the cells are adjusting to the change in environment during the inoculation process. The following experiment was designed to determine whether the time of resveratrol addition effected its ability to check cell growth and determine what effect resveratrol had on IgG synthesis and quality.

33 mL Erlenmeyer Flask CHO Cell Growth and IgG Expression

The growth in a 33 mL Erlenmeyer flask enables CHO cell growth to be studied over a longer time span than the 1 mL multiwell plate. It also enables IgG synthesis to be studied and the cell specific productivity to be calculated.

CHO cells were grown in 33 ml volumes Erlenmeyer shake flasks for a period of 10 days and the IgG concentration measured. Resveratrol was added to the cultures on days 0, 3 and 6 after inoculation and VCD, viability and IgG concentration measure (Figure 4).

The addition of 25 μM resveratrol on day 0 delayed the rise in VCD by approximately 1 to 2 days as observed in the 24-well plate experiment. The decline in viable cells at the end of the culture was also delayed presumably as the nutrients were not spent as they would have been in the untreated control. The expression of IgG was also delayed but reached similar levels to the untreated control while the specific protein productivity (q_p) was increased from 2.76 +/- 0.16 to 3.24 +/- 0.17. Addition of 25 μM resveratrol on day 3 and 6 had little impact on CHO cell growth with marginal impact on VCD and viability. There was a marginal increase in IgG concentration associated with the day 6 addition of 25 μM resveratrol from 177+/-2 to 203+/-4 μg/ml and specific protein productivity from 2.76 to 3.38 pg/cell·day (Figure 5).

The addition of 50 μM resveratrol in 33 ml volumes Erlenmeyer shake flasks on day 0 delayed the rise in VCD (as observed in the 1 mL experiment), a temporary drop in viability and a delay in IgG synthesis which never reached the concentration observed in the untreated control. The effect on cell growth and IgG synthesis of 50 μM resveratrol addition was dependant on the time of addition (Figure 4). The

calculated q_p increased by 65% (4.55 pg/cell·day) when the resveratrol was added on Day 0. Addition of 50 mM resveratrol 3 days after inoculation caused a temporary pause (around 24 hour) in cell growth, a bearly detectable dip in viability and delay in IgG synthesis. The VCD never reached the concentration observed in the untreated control but the IgG synthesis was very similar (by day 10). The calculated q_p, however was 51% greater (4.17 pg/cell·day). Treatment with 50 mM resveratrol at day 6 did not cause changes in the viability curve. It was associated with a increased synthesis of IgG over the 10 day experiment. The increase of the calculated q_p was, however, not significant probably due to the late addition of the resveratrol. The q_p values for each treatment are shown in Figure 5.

IgG Characterization (HPLC CEX, HPLC SEC and HPLC-MS/MS)

The 33 mL Erlenmeyer flask containing CHO cell cultures were treated at day 3 with 10, 25 and 50 µM resveratrol and the quality of the IgG assessed by HPLC SEC, HPLC CEX and HPLC-MS/MS analysis. The growth curves were similar to the ones previously described.

HPLC SEC showed that the IgG purified using protein A chromatography had at least 95% in the monomeric form. No peaks were identified prior to the monomeric peak at 13.67 min, suggesting that aggregate formation was not a problem for this process. Aggregates in the reactor that do not bind to protein A resin were not measured in this experiment. The fraction of lower molecular weight fragments was also uneffected by the presence of resveratrol. Peaks at 16.24 and 17.34 min of the IgG were consistent across all resveratrol treatments, showing no difference with respect to the control (Figure A).

HPLC CEX showed that the main isoform peak accounted for over 54% of the molecules, with acidic species representing 28% and basic species 18%. There were no significant changes in any of the peaks or in the total acidic or basic species relative quantities across the different resveratrol treatments (Figure 6 and 7).

Finally, the use of HPLC-MS/MS showed an absence of IgG modification patterns that could be linked to resveratrol addition (see Supplmentary Information). The average sequence coverage for the 20 analysed samples was 97% for the light chain (LC) and 77% for the heavy chain (HC). Identification score for both HC and LC was 323.31, which is the highest score that MaxQuant is able to provide.

These values were consistent across treatments. Quantitative comparison of dependent peptide searches and targeted searches for IgG modifications provided no significant results.

Discussion

The results from this study suggest that resveratrol acts on CHO cells by slowing the cell growth and increasing the cell specific protein productivity without changing the characteristics of the IgG produced. Previous studies failed to show this because they only used one concentration of resveratrol (10μM).[16] Treatment with 10 μM resveratrol does not have a marked effect on CHO cell cultures and does not differ much from the control in terms of viability, VCD and protein production.[17]

Cytostaticity and toxicity mechanisms

Resveratrol (25 and 50 μM) had an effect on CHO cell flask cultures which was dependant on the time of addition of the resveratrol. Addition of resveratrol to the flask cultures at the same time as inoculation was effective slowing the cells adaption to the new media and subsequent cell growth. The stationary stage lengthened as resveratrol concentration increased. The build-up of CHO cells in the S-phase at 16 hours post inoculation for 25 μM resveratrol addition and at 32 hours post inoculation for 50 mM resveratrol addition followed by rapid decrease in S-phase cells is indicative of resveratrol slowing the DNA synthesis which is overcome by the cell causing imbalance in the proportion of cells in the different phases which then stabilises. Resveratrol is known to interfere with DNA duplication, repair and segregation [20,21] accumulating cells in the S phase through direct and indirect mechanisms late in the culture.[22-25] This has been observed previously; DNA damage occurred at high concentrations with the subsequent possibility of apoptotic events,[20,26] although that was not always the case.[27] One of the mechanisms by which resveratrol could be causing toxicity in CHO cell dividing cultures may be linked to its interaction with topoisomerases during the S phase.[20,28,29]

Cell growth and specific productivity

The addition of resveratrol to flask cultures at the time of inoculation slowed cell growth by lengthening the stationary phase and caused underproduction of the final titre, although q_P was improved. The treatment of the cell culture at a late stage of the exponential phase caused an increase in the final titre due to the reduced capacity of resveratrol to cause toxic effects in a stationary culture

while improving the specific protein production. Characterization of the IgG after treatment showed no main changes, indicating that this mechanism of treatment does not cause any undesired changes to the product of interest.

The inverse relationship between cell growth and specific productivity is not unique to resveratrol; other systems with similar effects on CHO cells have been used to accomplish this biphasic effect in order to improve the recombinant protein production. Two well studied ways to achieve this goal are, shifting to lower temperatures and adding sodium butyrate (NaBu). The mechanism of action of low temperature is not clearly understood but it is linked to cell cycle arrest in the G_1 phase.[30] Low temperatures have the capacity to slow down growth, increase cell size and prolong viability; all these factors cause an increase in the q_p [31] as well as final titre to up to 5 times.[32,33] This improvement is subjected to specific conditions and changes in media composition, recombinant product or producer cell line could result in no significant improvement of recombinant production.[34,35] NaBu is a well-studied chemical that is able to improve recombinant IgG production. This is done by sustaining high nutrient consumption and increasing the biosynthetic activity especially during the stationary phase, which translates in an increase of q_p.[36] Another mechanism of action was described where NaBu helped proper assembly of IgGs.[37] NaBu can result in a 2-fold increase of the q_p and the final concentration, and was attributed to improving gene accessibility. The benefits of NaBu were variable between clone lines.[3] Further studies successfully combined low temperatures and addition of NaBu to further improve yields of IgGs.[38]

IgG characterization

Characterization of the IgG in our study showed that resveratrol caused no changes in aggregation, fragmentation or charged variants. Chemical additives with the property to shift the compositions of antibodies charge variants are not unknown. The antioxidant flavanol, EGCG was found to cause reduction of acidic species [39] and in some cases the chemical additives such as NaBu, causes non desired changes.[37] The HPLC-MS/MS analyses did not identify any modifications that would shift of charge variants as seen by cation exchange chromatograms.

Applications

The capacity of resveratrol to enhance recombinant production is not unique. Some other small molecules have been proven capable of enhancing protein production while slowing down growth; examples include betaine, 4-phenylbutyric acid, valproic acid and valeric acid.[2,5,40-43] Betaine is a chemical used in combination with high osmotic conditions in order to enhance productivity in difficult to express proteins although its effectiveness varies between cell and clone lines.[2,44,45] Regarding 4-phenylbutyric acid, it is a chemical chaperone used in cases where it is difficult to express proteins.[43] Although the other chemicals have been proven to be successful in improving specific production, little is known about their mechanism of action. Each chemical may prove to be advantageous, depending on the cell growth media, the cell line, recombinant protein or specific difficulty encountered. Because of this, it is important to widen the number of additives that can be used to engineer the production process.

Resveratrol is a successful candidate in improving cell specific productivity which effects the charge species of the recombinant protein produced. Although more studies are required in order to further understand the mechanism of action of resveratrol, this flavonoid seems to have a unique complex effect in improving recombinant IgG production, which seems to differ from the other media additives studied so far. Resveratrol's free radical scavenging properties make it an attractive option where chemical stability of the recombinant protein is an issue.

Conclusions

Resveratrol is toxic to CHO cells at 100 mM concentration,[17] but at sub-toxic concentrations caused cell growth arrest in a concentration-dependent-manner while increasing the cell specific productivity of the recombinant monoclonal antibody. There was no detectable impact of 25 mM or 50 mM resveratrol on post translational modification. Similarly there was no chemical reaction with the therapeutic protein or evidence of increased chemical modification of the IgG in the presence of sub-toxic concentrations of resveratrol.

The use of resveratrol during the production of a stable monoclonal antibody (as conducted in this paper) gives a significant increase in q_p due to slight increase in IgG concentration and a reduction in cell density. While this observation is interesting, where resveratrol could be of real benefit is in the production of proteins which are vulnerable to chemical modification. Resveratrol has known capability to protect against free radicals at 25 and 50 mM concentrations[46] and has the potential to

be used with therapeutic proteins. Based on this study, we suggest that resveratrol should be considered as an additive to cell culture media. 25 mM resveratrol had minimal impact of CHO cell growth and could be used in a cell culture reactor. If 50 mM resveratrol is used, its negative effect on cell viability is reduced if it is added to actively growing cells and addition of resveratrol should be avoided in the initial media.

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Figure Titles

Figure 1. The chemical structure of resveratrol

Figure 2. Effect of resveratrol (Resv) on viable cell density (A), viability (B) and cell diameter (C) at 0 (red triangle) 10 µM (yellow diamond), 25 µM (green square) and 50 µM (blue circle) treatments for CHO cells grown in 24-well plates. Each data point represents the average from a triplicate, and the error bars correspond to ±SD. These experiments were repeated independently.

Figure 3. Profile of resveratrol (Resv) treatment effect at 0, 10, 25 and 50 µM over the relative amount of cells in different phases of the cell cycle G_1/G_0 (red triangle), S (green squared) and G_2/M

(blue circle) for a period of 3 days for CHO cells grown in 24-well plates. Each data point represents the average from a triplicate, and the error bars correspond to ±SD. These experiments were repeated independently.

Figure 4. Profiles comparison of viable cell densities (VCD) (A), viability (B) and IgG concentration (C) for 33 ml batch cultures run 10 days in a shaking humidified incubator and treated with resveratrol (Resv) concentrations of 25 µM (Left) and 50 µM (Right) at days 0, 3 or 6 as specified in the legend. Each data point represent the average from a triplicate, and the error bars correspond to ±SD. This experiments was repeated independently.

Figure 5. Relative protein production (q_p) of 33 ml batch recombinant CHO cell cultures in 125 ml Erlenmeyer flasks after a period of 10 days and treated with resveratrol (Resv) at 25 and 50 µM on days 0, 3 or 6. q_p was calculated by dividing final protein production by the integral of the VCD curve. Each data point represent the average from a triplicate, and the error bars correspond to ±SD. This experiments was repeated independently. One way ANOVA statistical test was perform for the treatment compared to the control for p-values: (*) < 0.05, (**) < 0.01 and (***) < 0.001.

Figure 6. Size exclusion (A) and cation exchange (B) chromatograms for purified IgG produce by 33 ml batch cultures run 10 days in a shaking humidified incubator after a period of 10 days and treated with resveratrol (Resv) at 0, 10, 25, 50 and 100 µM on day 3.

Figure 7. Relative quantification (%) of charge species for purified IgG. Data from cation exchange chromatography. The acidic (left), main (centre) and basic (right) species were assessed under resveratrol (Resv) culture additions at different concentrations. Recombinant CHO cells were grown for 10 days in flask cultures and treated at day 3 The IgG was then collected and purified. The error bars displayed here correlate to ± SD. The experiment was run 4 times independently. One way ANOVA statistical test was perform for the treatment compared to the control for p-values: (*)< 0.05, (**) < 0.01 and (***) < 0.001.