



S-Sulfocysteine – Investigation of cellular uptake in CHO cells

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ABSTRACT

For the generation of therapeutic proteins in cell culture, high producing clones are used. These clones have a high demand in amino acids to support cell growth and productivity. L-cysteine (Cys) is critical in highly concentrated feeds due to low stability of Cys and low solubility of the oxidation product cystine at neutral pH. S-sulfocysteine (SSC) was developed to substitute the Cys source and fed-batch experiments using SSC showed good cellular performance regarding viable cell density and titer, indicating uptake and metabolization of SSC by Chinese hamster ovary cells. However, the responsible transporter allowing cellular uptake remains unclear and was studied in this work. Due to the structure similarity of SSC with cystine and glutamate, it was proposed that the cystine/glutamate antiporter (x_c^-) allows cellular uptake of SSC. The uptake was assessed via transporter inhibition using sulfasalazine and transporter overexpression using either sulforaphane or sulforaphane-N-acetylcysteine during fed-batch experiments. Following daily addition of 50 μ M and 100 μ M sulfasalazine, the extracellular SSC concentration was increased by 65 % and 177 % respectively, suggesting a reduced uptake due to x_c^- inhibition. In contrast, enhanced transporter activity through 15 μ M sulforaphane and sulforaphane-N-acetylcysteine treatment, induced a 60 % and 52 % reduced extracellular SSC concentration, respectively. These inverse uptake results strongly suggest that x_c^- is facilitating the transport of SSC.

1. Introduction

L-Cysteine (Cys) is a critical amino acid required for the cultivation of mammalian cells such as Chinese hamster ovary (CHO) cells. The reactivity of the thiol group in Cys leads to several key functions e.g. maintaining protein conformation via disulfide bridges, ensuring catalytic activity in enzymes and redox sensitivity in peptides or proteins like thioredoxin. Glutathione (GSH), a Cys-containing tripeptide (Glu-Cys-Gly), is an intracellular antioxidant which protects cells by scavenging highly destructive reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Aquilano et al., 2014). The monomer GSH is oxidized by ROS/RNS to its disulfide-linked dimer GSSG and reduced back to GSH by glutathione reductase and its cofactor NADPH, to restore the antioxidant pool (Lu, 2013). The overall cellular GSH/GSSG ratio is commonly detected in a range from 30:1 to 100:1 and is reported as main redox

couple known to maintain the cellular redox status (Hwang et al., 1992; Schafer and Buettner, 2001). The crucial requirement for GSH re-enforces the importance of Cys supply as Cys availability determines GSH synthesis. Limitation of Cys reduces the ability to protect cells from oxidative damage via ROS/RNS, leading to reduced viability and productivity (Ali et al., 2018) whereby lethal cellular responses are reported after Cys starvation (Chung et al., 2005; Conrad and Pratt, 2019; Dixon et al., 2014).

Highly concentrated feeds are used in fed-batch production of therapeutic proteins. Production and storage of these feeds are limited by Cys, due to low stability of Cys (autooxidation) and low solubility of cystine (CysS) at neutral pH. Therefore, S-sulfocysteine (SSC) was developed to substitute Cys in highly concentrated feeds (Hecklau et al., 2016). Fed-batch experiments with CHO cells using feeds containing equimolar amounts of SSC in place of Cys showed good cellular

Abbreviations: ARE, antioxidant response element; CHO, Chinese hamster ovary; Cys, cysteine; CysS, cystine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSH, glutathione (reduced); GSSG, glutathione disulfide (oxidized); IVCD, integral of viable cell density; KEAP1, Kelch-like ECH-associated protein 1; mAbs, monoclonal antibodies; Nrf2, NF-E2-related factor 2; ROS, reactive oxygen species; RNS, reactive nitrogen species; SAS, sulfasalazine; SEM, standard error of the mean; SFN, sulforaphane; SFN-NAC, sulforaphane-N-acetyl-L-cysteine; SSC, S-sulfocysteine; UPLC-MS, ultra-performance liquid chromatography coupled to a mass spectrometer.

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performance regarding viable cell density and titer. SSC as replacement of Cys was suggested to release Cys intracellularly, which requires SSC uptake by the cells (Hecklau et al., 2016). Due to structure similarity of SSC to both L-glutamate (Glu) and CysS, the CysS/Glu antiporter (x_c^-) was suggested to allow cellular uptake of SSC (Fig. 1). Patel et al. investigated SSC, homocysteate and L-serine-O-sulphate, which all share the SO_3 moiety, as potential x_c^- inhibitors. They determined that SSC was not an inhibitor but did not investigate whether SSC may be a substrate for the antiporter. Experimental evidence suggested that homocysteate and L-serine-O-sulphate acted as substrate inhibitors (Bannai, 1986; Patel et al., 2004).

The functional antiporter complex (x_c^-) consists of two subunits connected via disulfide bridge (Bassi et al., 2001; Sato et al., 1999). The heavy chain also called 4F2hc subunit (gene: *slc3a2*), is required for proper localization of the antiporter in the membrane and the light chain called xCT subunit (gene: *slc7a11*), confers substrate specificity and is known for the 1:1 counter-transport of Glu and CysS (Koppula et al., 2018; Verrey et al., 2000). Under physiological conditions, influx of CysS into cells is enhanced by rapid intracellular CysS reduction to Cys and counter-transport of Glu out of the cells (Bannai, 1986; Geoghegan,

2015; Lewerenz et al., 2013). A high intracellular Glu concentration required for the counter-transport is maintained by additional amino acid transport systems such as X_{AG}^- (Geoghegan, 2015). Excessive extracellular Glu concentration results in competitive inhibition of the x_c^- transporter (Lewerenz et al., 2013). Beside the x_c^- self-regulation, the compounds erastin and sulfasalazine (SAS; see Fig. 1 for chemical structure) were shown to be potent inhibitors of the transporter (Chung et al., 2005; Dixon et al., 2014; Geoghegan et al., 2018; Gout et al., 2001; Zheng et al., 2018).

When cells experience oxidative stress, they can induce the transcription factor NF-E2-related factor 2 (Nrf2), which regulates several redox related protective target genes including the *slc7a11* gene, which encodes the x_c^- antiporter (Taguchi et al., 2011; Tonelli et al., 2018). In general, Nrf2 activity is regulated by association with the Kelch-like ECH-associated protein 1 (Keap1). In unstressed conditions, i.e. low ROS concentrations, Nrf2 is bound to Keap1 which inhibits translocation of Nrf2 to the nucleus. Nrf2 is further regulated by ubiquitination, which leads to Keap1 dissociation and Nrf2 degradation. In comparison, high ROS concentrations lead to Keap1 inactivation which results in Nrf2 translocation to the nucleus (Taguchi et al., 2011; Tonelli et al., 2018).

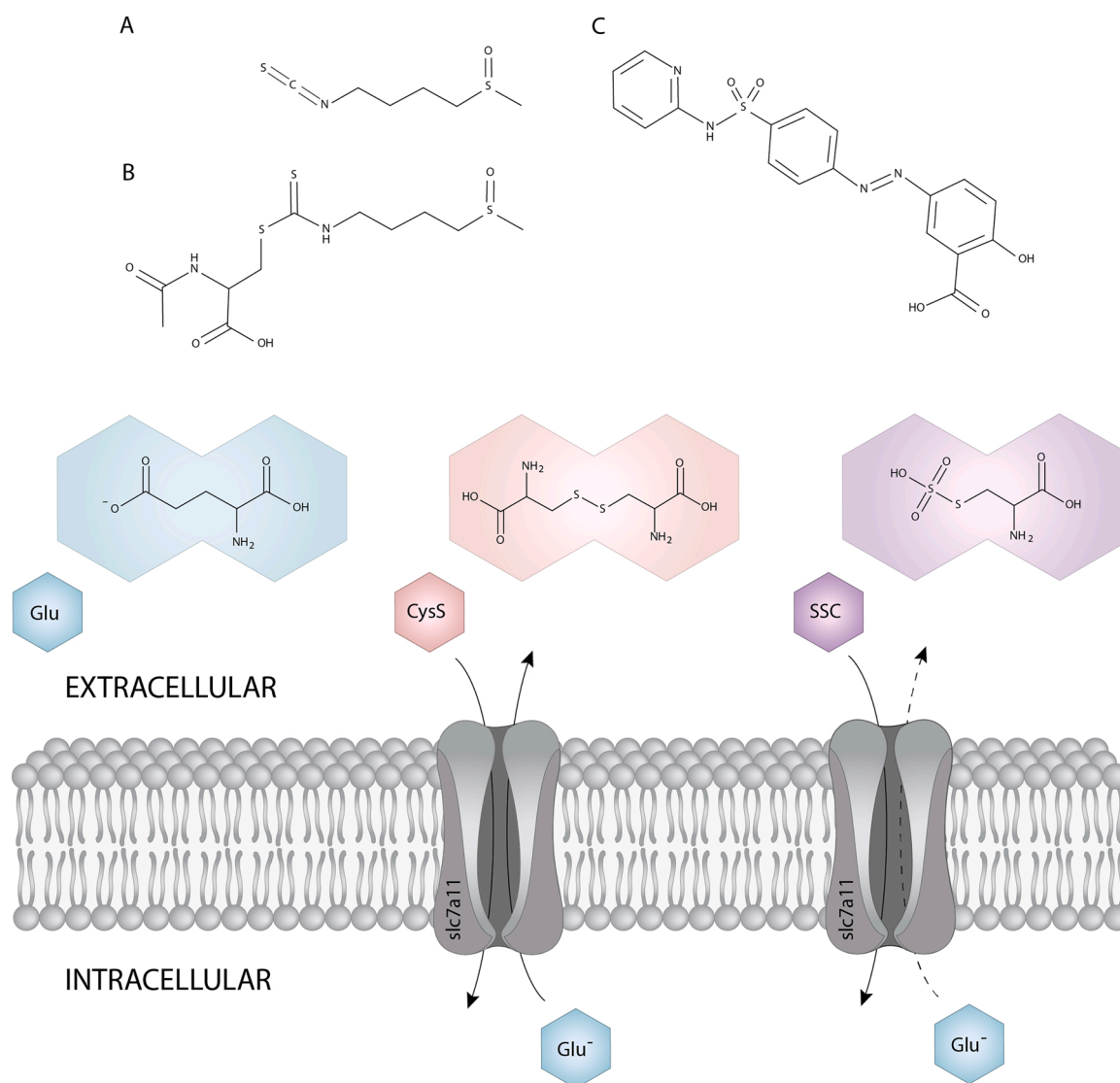


Fig. 1. Chemical structures of supplements and known x_c^- antiporter activity as well as proposed SSC uptake.

Top: A. sulforaphane (SFN), B. sulforaphane-N-acetyl-cysteine (SFN-NAC) and C. sulfasalazine (SAS). Bottom: The left antiporter (gene *SLC7A11*) visualizes the known cystine (CysS) import and glutamate (Glu) export activity with arrows. Due to structure similarity of S-sulfocysteine (SSC; purple) to CysS (pink) and Glu (blue), cellular uptake of SSC was proposed via x_c^- as visualized on the right. Potential cotransport of Glu is indicated as dashed arrow.

Nrf2 translocation can also be induced by compounds with an isothiocyanate group like sulforaphane (SFN), which in turn induce the overexpression of the CysS/Glu antiporter (Jeong et al., 2012; Zheng et al., 2018). Like ROS, SFN reacts with Keap1 thiols, leading to Nrf2 dissociation (Ahn et al., 2010; Dinkova-Kostova et al., 2017; Hu et al., 2011). Additionally, the SFN metabolite SFN-*N*-acetyl-cysteine (SFN-NAC) was shown to have similar cytoprotective effects as SFN treatment, which suggests that SFN-NAC may also induce x_c^- expression (Liu et al., 2018).

In order for SSC to function as Cys replacement in cell culture, it needs to enter the cell, which is dependent on the existence of transporters which can utilize SSC as a substrate. Previous studies showed that SSC is not an inhibitor of antiporter x_c^- but did not verify whether SSC is transported. This study aims to determine whether SSC acts as substrate for x_c^- , through the overexpression of x_c^- transporter using SFN or SFN-NAC, and inhibition of the transporter using SAS (see Fig. 1 for chemical structures). Fed-batch experiments, using either Cys or SSC as Cys source, were performed with CHO cells and side effects of SFN treatment were investigated via *in vitro* experiments.

2. Materials and methods

2.1. Reagents and cell line

D,L-sulforaphane (CAS: 4478-93-7), sulfasalazine (CAS: 599-79-1) and S-sulfocysteine (CAS: 1637-71-4) were purchased from Sigma. Sulforaphane-*N*-acetyl-cysteine (CAS: 334829-66-2) was purchased from Cayman Chemical. A CHO cell line producing a recombinant IgG1 was used in this study.

2.2. Cell culture media and process conditions

Cells were cultivated in Cellvento® 4CHO, a chemically defined CHO cell culture medium that contains all essential components for optimal cell growth and protein production. Cellvento® Feed220, a highly concentrated feed, was used as feeding medium, providing essential components for extended culture duration and productivity (except cysteine). Either 15 mM cysteine in combination with 50 mM ketoglutaric acid (Kuschelewski et al., 2017) or 15 mM S-sulfocysteine with 50 mM ketoglutaric acid were added to the feed during reconstitution. The pH of all feeds was adjusted to neutral (pH 7.0 ± 0.2). Osmolality was measured by a cryoscopic osmometer (Osmomat 3000, GonoTec, Berlin, Germany) and both cell culture media and feeds were sterile-filtered (0.22 µm) and stored at 4 °C, protected from light until usage. The fed-batch process was performed in 50 mL spin tubes with vented cap (TPP, Trasadingen, Switzerland) at 37 °C, 5 % CO₂, 80 % humidity and a rotation speed of 320 rpm. The inoculation density was 2 · 10⁵ cells/mL in 30 mL starting volume. 3 % feed (v/v) was added on day 3, 5 and 10 and 6 % (v/v) was added on day 7. The glucose level was maintained by adding a specific amount of a 400 g/L glucose stock solution on demand to up to 6 g/L during the week and up to 12 g/L over the weekend. Cell counts and viability of at least four biological replicates were measured with a Vi-CELL™ XR 2.04 cell counter (Beckman Coulter, Fullerton, CA, USA). Spent media analysis including glucose and IgG was performed with the bioprocess analyzer CEDEX Bio HT (Roche, Mannheim, Germany) after centrifugation of the sample for 5 min at 4500 rpm (2287 g). Further supernatant samples were stored at -20 °C for extracellular amino acid analysis and cells for quantification of *slc7a11* mRNA levels were stored at -80 °C until analysis.

2.3. Amino acid analysis via RP-UPLC

Extracellular amino acid concentrations of cell culture experiments were analyzed by reverse phase ultra performance liquid chromatography (RP-UPLC) using a C18 column. Samples were alkylated with 20 mM iodoacetamide and derivatized with AccQ-Tag Ultra® reagent

(Waters, Milford, US) for 10 min at 55 °C. After separation via RP-UPLC, amino acids were detected by UV using the standard Waters procedure. For data comparison, the integral of measured amino acid concentrations over the time course of the fed-batch experiment was calculated and normalized to the control condition using a Cys feed (exemplarily shown for Arg in Eq. 1).

$$\text{Normalized integral [Arg]} (\%) = \frac{\int_{\text{day } 0}^{\text{day } x} ([\text{Arg}]_{\text{treated}})}{\int_{\text{day } 0}^{\text{day } x} ([\text{Arg}]_{\text{control}})} \cdot 100 \quad (1)$$

Subsequently, the decrease in amino acid consumption resulting from the reduced cell growth (or difference in performance) was estimated per condition by calculating the average normalized integral of the concentration from the 17 amino acids that are not predicted as transported through the CysS/Glu antiporter. The normalized control condition was subtracted from this value (Eq. 2).

$$\text{Amino acid difference due to reduced growth (\%)} = \text{Average (Normalized integral [17 amino acids]_{\text{treated}})} - \text{Average (Normalized integral [17 amino acids]_{\text{control}})} \quad (2)$$

* 17 amino acids: His, Ser, Gln, Arg, Gly, Thr, Pro, Lys, Met, Val, Ile, Leu, Phe, Trp, Tyr, Asn, Ala

To take into account this growth-related effect, the normalized integral of the concentration for cystine, glutamate, aspartate and SSC (hypothesized to be transported by the CysS/Glu antiporter) were corrected (Eq. 3). The same correction was also applied to all amino acids as shown in supplementary Figs. 2 and 3, demonstrating that the calculation is correcting performance related effects.

$$\text{Corrected amino acid consumption (\%)} = \text{Normalized integral [amino acid]} - \text{Amino acid difference due to reduced growth} \quad (3)$$

In case the treatment with SAS inhibits specifically the uptake of CysS or SSC, a value above 100 is expected after subtraction of the correction factor as reduced uptake increases the extracellular concentration compared to the control. Values of corrected amino acid consumption as well as original measured values are visualized as stacked grey bar in Figs. 4 and 7 and in supplementary Figs. 2 and 3.

2.4. Antiporter mRNA analysis

The relative mRNA expression level of *slc7a11* and *slc3a2* were investigated using the Taqman technology. At least 12 · 10⁶ viable cells were used to isolate RNA with RNeasy mini/midi kits according to standard procedures (Qiagen, Hilden, Germany). RNA was converted into cDNA using the RNase-Free DNase Set in combination with Taqman reverse transcriptase. The relative RNA expression levels were obtained by quantitative PCR on a 7500 real-time PCR system (Applied Biosystems, Darmstadt, Germany) with QuantStudio 3 (Applied Biosystems, Foster City, CA, USA). Data were normalized against endogenous Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels. The fold change compared to the control was calculated by applying the 2^{-ΔΔCt} method according to Livak et al. (Livak and Schmittgen, 2001). Genes with at least two-fold increased RNA levels were considered as differentially expressed.

2.5. In vitro analysis of SFN interaction

A 15 mM Cys stock solution in water was stabilized with 50 mM ketoglutaric acid (KG) and the same KG concentration was added in the 15 mM SSC stock solution. 50 µM and 100 µM SFN or SFN-NAC was incubated with either 1.5 mM Cys or 1.5 mM SSC in water. To reflect cell cultivation, a Cys-depleted cell culture medium was added in an additional setup. All mixtures were analyzed directly after mixing and after incubation for 1.5 h at 37 °C. To stop further reactions, samples were placed on ice until analyzed via RP-UPLC. Samples without SFN or SFN-

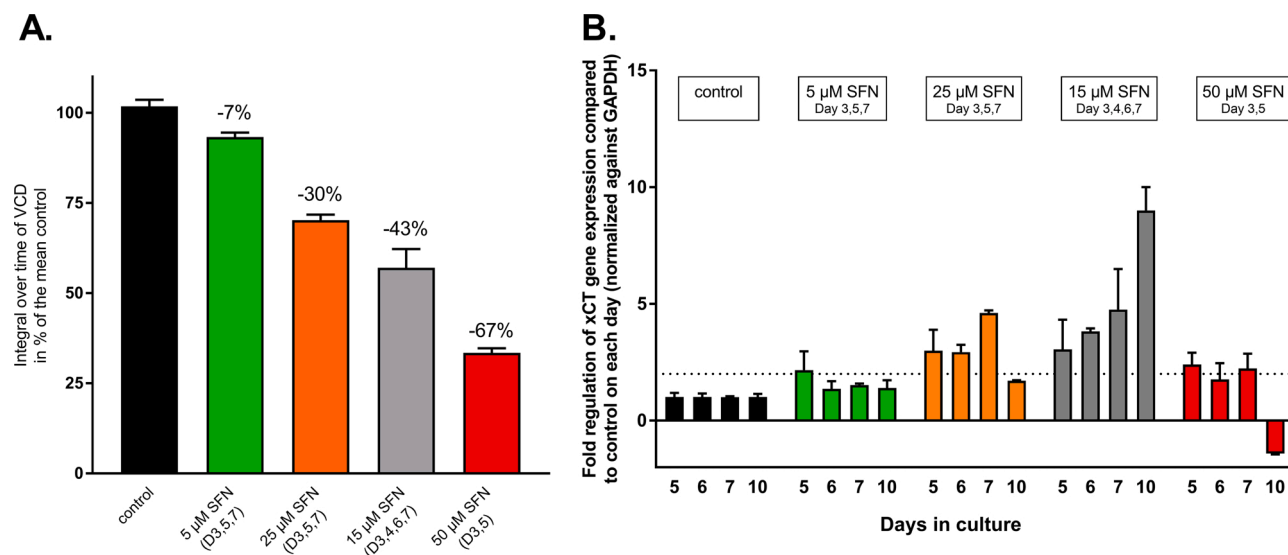


Fig. 2. Dose response of sulforaphane on CHO cells. Suspension CHO cells were seeded at 2×10^5 cells/mL, incubated at 37°C , 5% CO_2 , 80% humidity and agitated at 320 rpm.

Feed was added on day 3, 5 and 10 (3%; v/v) and day 7 (6%; v/v) whereas 5, 25 or 50 μM sulforaphane (SFN) was added on day 3, 5, 7 and 15 μM SFN was added on Day 3, 4, 6 and 7. A. Integral of viable cell density (IVCD) was calculated. Bars represent normalized values to the untreated Cys control condition ($n = 4$) and the absolute difference compared to the control is shown above the histogram. B. CysS/Glu antiporter (gene *SLC7A11*) mRNA expression as fold change relative to the untreated control on each day and normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) with $n = 2$. Two-fold increased mRNA levels were considered as differentially expressed.

NAC, Cys or SSC and also the Cys-depleted media were used as control.

2.6. Statistical analysis

Data are expressed as means \pm Standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software Inc). More precisely, for cell culture experiments, the integral of the signal over time of each biological replicate was calculated and statistical differences between groups were assessed by using one-way ANOVA and subsequent Dunn's multiple comparisons test. The comparison of two conditions was realized by the Mann-Whitney test. p -values smaller than 0.05 were considered as significant.

3. Results

3.1. Addition of sulforaphane increases the antiporter gene expression level

A sulforaphane (SFN) dose-response experiment was performed with CHO cells to define a concentration suitable to increase the CysS/Glu antiporter expression, while maintaining cell performance. Exploration of the antiporter mRNA levels (gene *slc7a11*) was used to assess antiporter upregulation as detection of antiporter expression via Western blot or flow cytometry was not successful in our hands due to a lack of specificity of commercial antibodies, which is supported by independent studies (Van Liefveringe et al., 2016). During a fed-batch process using a Cys containing feed, concentrations of 5 or 25 μM SFN, respectively were spiked to the cultures on day 3, 5 and 7 and 50 μM SFN was spiked on day 3 and 5. To compare the impact of different treatments on cell growth, the integral of viable cell density (IVCD) was calculated. Whereas 5 μM SFN had no significant impact on growth, reduced IVCD of 30% and 67% were observed for 25 μM and 50 μM SFN, respectively (Fig. 2A). 5 μM SFN treatment induced a 2.2-fold increased *SLC7A11* mRNA level on day 5, while 25 μM SFN resulted in a max upregulation of 4.6-fold on day 7 (Fig. 2B), indicating that 25 μM was more suitable than 5 μM SFN to induce antiporter expression. In both cases, the heterodimeric subunit *SLC3A2* was not upregulated (Supplementary Fig. 1). Since the *SLC7A11* gene expression was only transitory with 5 μM SFN

and the IVCD was impacted by the 25 μM SFN treatment, an intermediate concentration of 15 μM SFN and a more frequent addition during the growth phase (day 3, 4, 6 and 7) was tested to minimize the impact on IVCD while maintaining a significant increased mRNA level. Using 15 μM SFN, a reduced IVCD of about 40% was observed (Fig. 2A), but treatment yielded a 9.0-fold upregulated *SLC7A11* mRNA level on day 10 compared to the control (Fig. 2B). Although the RNA level was efficiently upregulated, the highly impacted cellular growth was suggested to influence the amino acid consumption significantly, so that a reduced CysS uptake rate might not be distinguishable from lowered consumption. Thus, treatment was not yet satisfactory and required further adaptation.

3.2. Addition of sulforaphane-*N*-acetylcysteine circumvents reduced IVCD

Reduced IVCD caused by SFN treatment might be linked to a detrimental reduction of the intracellular GSH concentration, since SFN is known to interact with GSH (Liu et al., 2018). To circumvent this reduced GSH level potentially leading to the reduced IVCD, sulforaphane-*N*-acetyl-cysteine (SFN-NAC), the interaction product of SFN and GSH, was tested in an additional experiment. To compare both treatments, the impact of equimolar concentrations of SFN and SFN-NAC (15 μM) on CHO cell growth, as well as *SLC7A11* mRNA expression level, was explored. Results obtained using the Cys feed indicate that the SFN treatment resulted in approximately 43% reduced IVCD compared to the control, while SFN-NAC reduced IVCD only by 10% (Fig. 3A), thus confirming the hypothesized lower toxicity of the interaction product SFN-NAC on CHO cells. However, SFN treatment increased the mRNA levels of the antiporter by up to 6.1-fold at day 4 and 4.8-fold at day 7, whereas SFN-NAC treatment increased the mRNA level only 4.0-fold on day 4 and 2.8 fold on day 7 (Fig. 3B), indicating that SFN-NAC is a slightly less effective inducer of the *SLC7A11* expression than SFN. Thus, both treatments were used in the next experiment to be able to study the potential impact of the reduced IVCD following SFN treatment and the potential limited overexpression of the receptor mRNA following SFN-NAC treatment, on the CysS and SSC uptake.

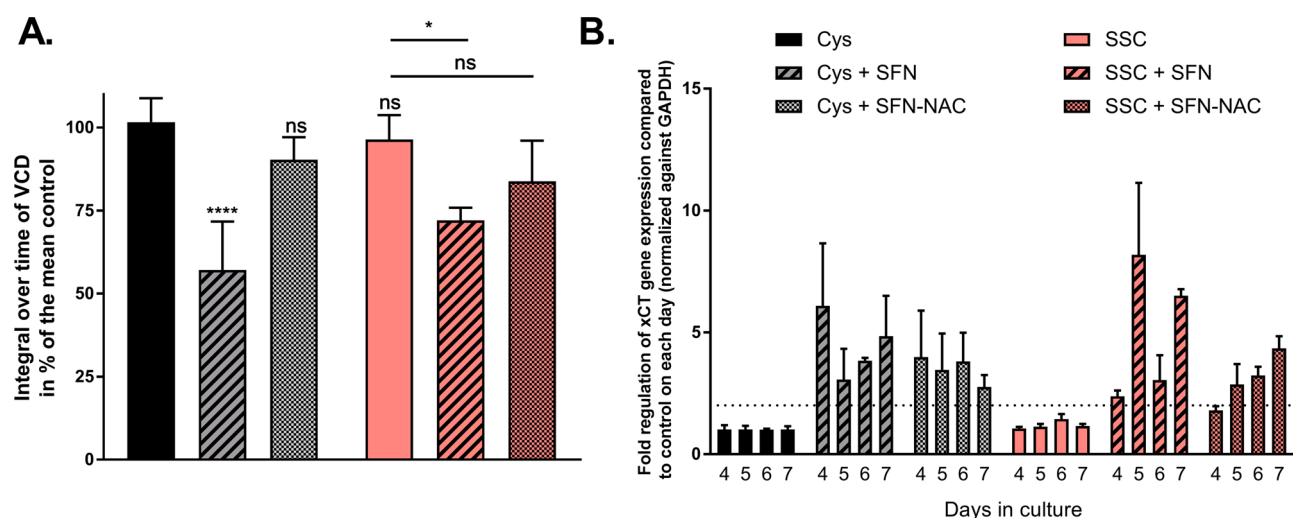


Fig. 3. Impact of SFN and SFN-NAC on CHO cells during a fed-batch.

Suspension CHO cells were seeded at 2×10^5 cells/mL, incubated at 37°C , 5% CO_2 , 80% humidity and agitated at 320 rpm. Feed containing either Cysteine (Cys) or S-Sulfolcysteine (SSC) was added on day 3, 5, 10 and 12 (3%; v/v) and day 7 (6%; v/v) whereas 15 μM SFN or SFN-NAC were added on day 3, 4, 6 and 7. A. Integral of viable cell density (IVCD) was calculated. Bars represent normalized values to the untreated Cys control condition of two independent experiments ($n = 8$). B. Fold regulation of the CysS/Glu-antiporter (*SLC7A11*) gene expression relative to the control on each day and normalized to GAPDH ($n = 2$). Data are expressed as mean values \pm standard error of the mean (SEM). Statistical analysis of the IVCD was performed with one-way ANOVA and subsequent Dunn's multiple comparison, whereas p -values indicate the following: * $p < 0.05$, **** $p < 0.0001$, ns = not significant.

3.3. SFN and SFN-NAC treatment facilitate Cys uptake

Since the increase of the *SLC7A11* mRNA does not necessarily correlate with an enhanced transporter activity, extracellular amino acid analysis was performed on supernatants from fed-batch experiments to confirm increased transporter activity after SFN or SFN-NAC treatment. The upregulation of the transporter is expected to yield an increased CysS consumption compared to the control.

To differentiate between the impact of reduced growth (due to SFN or SFN-NAC addition) and the changes in amino acid consumption due to the modulation of the antiporter activity, the impact of the treatment was determined on all amino acids contained in the medium. Since x_c^- is reported to have a high substrate specificity for cystine, glutamate and aspartate (Bassi et al., 2001), other amino acids whose transport is not affected by the antiporter (17 amino acids in total) were used to evaluate the decrease in consumption resulting from the reduced cell growth. As an example, the concentration of arginine (Arg), known to be transported by system y^+ (*Slc7a3*) via facilitated diffusion (Kyriakopoulos et al., 2013), was increased about 24% due to SFN treatment and 18% due to SFN-NAC (Fig. 4A). After subtraction of the amino acid concentration differences due to growth, the concentration of Arg varied less than 2% following both SFN treatments, confirming that the correction is needed to focus only on the amino acid changes that are related to the x_c^- transporter. Thus, the mean amino acid concentration difference due to the lower IVCD was subtracted from all the measured values and are visualized as stacked, grey bar in supplementary Fig. 2. When considering growth corrected amino acid values, extracellular CysS concentrations were significantly reduced by SFN and SFN-NAC treatment by 31% and 30%, respectively (Fig. 4B), whereby no significant change of extracellular Glu was observed with -2% and -5% for SFN and SFN-NAC, respectively (Fig. 4C), most probably suggesting the involvement of another Glu transporter. Altogether, data support elevated antiporter activity, which qualified 15 μM SFN and SFN-NAC treatment on days 3, 4, 6 and 7 to study SSC uptake.

3.4. Elevated CysS/Glu antiporter expression facilitates SSC uptake

SFN and SFN-NAC addition were applied in a fed-batch experiment using SSC-containing feed as sole Cys source. As in previous experi-

ments, different treatments were compared on basis of IVCD, mRNA levels and extracellular amino acid analysis. Data sets were normalized to untreated Cys feeding to focus on differences regarding the Cys source as well as SFN and SFN-NAC treatment. Feeding with SSC in place of Cys resulted in a non-significant reduction of the IVCD of about 3%, whereas the combination of SSC feeding with SFN and SFN-NAC treatment resulted in a reduced IVCD of 27% and 16%, respectively (compared to the SSC control Fig. 3A). The feeding with SSC had no effect on the *SLC7A11* mRNA expression, whereas the combination with SFN and SFN-NAC treatment led to 6.5 and 4.3-fold increased mRNA level after 7 days, respectively (Fig. 3B). Since SSC feeding alone did not affect the mRNA levels of the antiporter, both SFN and SFN-NAC treatments may be used to study the cellular uptake of SSC in cells with enhanced x_c^- antiporter activity. In presence of SSC, an averaged increase of about 10% extracellular amino acid concentration was detected for both SFN and SFN-NAC treatments as a result from the reduced IVCD as exemplarily shown for Arg (Fig. 4A). After subtracting the 10% to correct the data, SFN treatment caused a reduction of extracellular CysS of about 29% ($p = 0.005$) and SFN-NAC treatment about 28% ($p = 0.014$), indicating a similar enhanced transporter activity in presence of both treatments (Fig. 4B). Furthermore, significantly reduced extracellular SSC concentrations of about 60% ($p = 0.0022$) and 52% ($p = 0.0022$) were observed after correcting the data when cells were treated with either SFN or SFN-NAC, respectively (Fig. 4D). Considering that the SSC feed alone did not influence the mRNA expression levels of the antiporter, the amino acid quantification after SFN and SFN-NAC treatment suggests that enhanced SSC uptake is mediated by elevated antiporter activity and thus support the hypothesis of SSC uptake via x_c^- antiporter.

3.5. SSC does not interact with SFN or SFN-NAC

Experiments with SFN and SFN-NAC treatment indicate that increased x_c^- transporter activity can promote the uptake of extracellular CysS and SSC. Since SFN is known to react with Cys residues in proteins such as KEAP1 (Hu et al., 2011), it might as well react with Cys or SSC in the supernatant. *In vitro* experiments were performed to ensure that reduced extracellular CysS and SSC concentrations following SFN or SFN-NAC treatment were not caused by a chemical interaction. During previous fed-batch experiments, four additions of either 15 μM SFN or

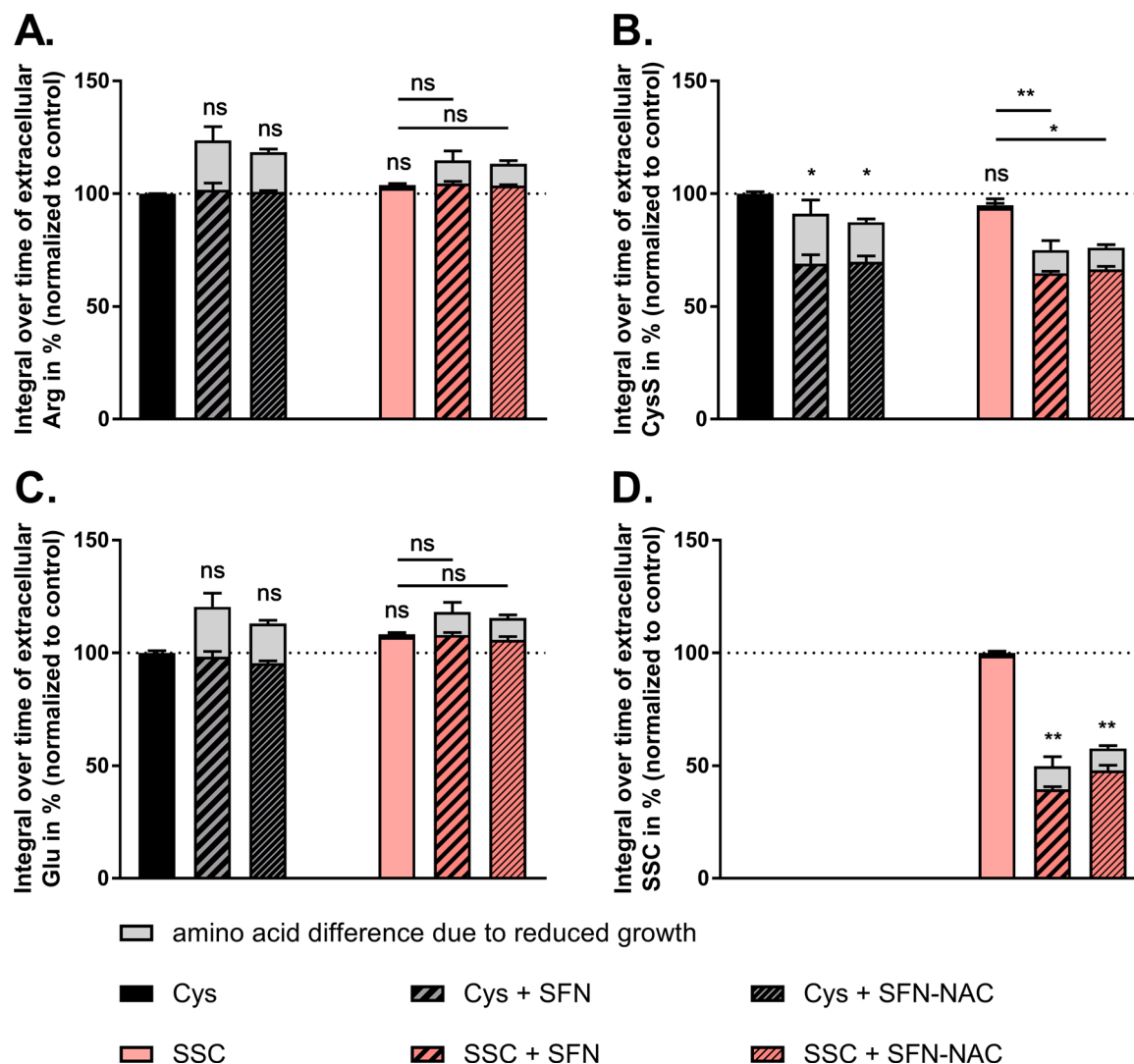


Fig. 4. Extracellular amino acids during two independent fed-batch experiments with feed containing either *L*-Cysteine (Cys) or *S*-Sulfofocysteine (SSC). With the intention to increase the expression of the CysS/Glu antiporter, cells were treated with either SFN or SFN-NAC. Integral over time of extracellular A. Arg, B. CysS, C. Glu and D. SSC was measured via RP-UPLC after iodoacetamide treatment and AccQ-Tag derivatization and was normalized to Cys containing feed. Effects due to different growth are calculated as average difference of antiporter unrelated amino acids to the control and are visualized as stacked bar in grey. Data are expressed as mean values \pm SEM ($n = 6$). Statistical analysis of the amino acids was performed either with one-way ANOVA and subsequent Dunn's multiple comparison or in case of SSC by the Mann-Whitney test, whereas p -values are indicated as followed: * $p < 0.05$, ** $p < 0.01$ and ns = not significant.

SFN-NAC were applied (total 60 μ M). To work in a similar range of concentration in the *in vitro* experiments, 50 μ M and 100 μ M SFN or SFN-NAC were incubated with either 1.5 mM Cys or 1.5 mM SSC in water to explore the possibility of a chemical reaction. Data were normalized to the respective amino acid in water alone after 0 min. When mixed in water with increasing concentrations of SFN or SFN-NAC, neither Cys (Fig. 5A) nor SSC (Fig. 5B) concentrations were decreased after incubation for 1.5 h at 37 $^{\circ}$ C, indicating that both SFN and SFN-NAC did not react with Cys or SSC.

To reflect the actual cultivation matrix (including trace elements as possible catalyzers), a similar experiment was performed by mixing 1.5 mM Cys and SSC with increasing SFN and SFN-NAC concentrations in Cys-depleted medium. In this experimental setup, a reduced Cys concentration was expected through enhanced autoxidation to CysS in presence of e.g. copper (Kachur et al., 1999) (independent of SFN) and overall interaction of Cys with other media components. Indeed, CysS was detected when Cys was incubated with medium. In the control condition, about 112 μ M CysS was detected after 1.5 h incubation at 37 $^{\circ}$ C. As CysS is the dimer of Cys, the equivalent Cys concentration is 224 μ M, which represents 15 % of the initially applied 1500 μ M. Since 17 %

of applied Cys was retained and 15 % was detected in form of CysS (Fig. 5C, whereby CysS is visualized as stacked grey bar), the remaining 68 % were likely interacting with other cell culture media components. Additional treatment did not change the Cys level. For example, about 19 % and 17 % Cys was observed for 100 μ M SFN and SFN-NAC, respectively, indicating that SFN and SFN-NAC do not interact with Cys in the cell culture media matrix. A reduction of the SSC concentration in presence of medium alone was not expected, since previous studies demonstrated that SSC was more stable compared to Cys (Hecklau et al., 2016). Indeed, about 95 % of the applied SSC concentration was recovered without treatment and 96 % and 93 % was detected in presence of 50 μ M SFN and SFN-NAC, respectively (Fig. 5D). Furthermore, no dose dependent effect was observed, as 97 % SSC was detected in presence of 100 μ M SFN and 96 % was detected in presence of SFN-NAC. These data confirm that the decrease in the extracellular SSC concentration observed in the transporter overexpressing cell experiment (reduction > 60 %) cannot be caused by an extracellular chemical interaction of SFN and SSC.

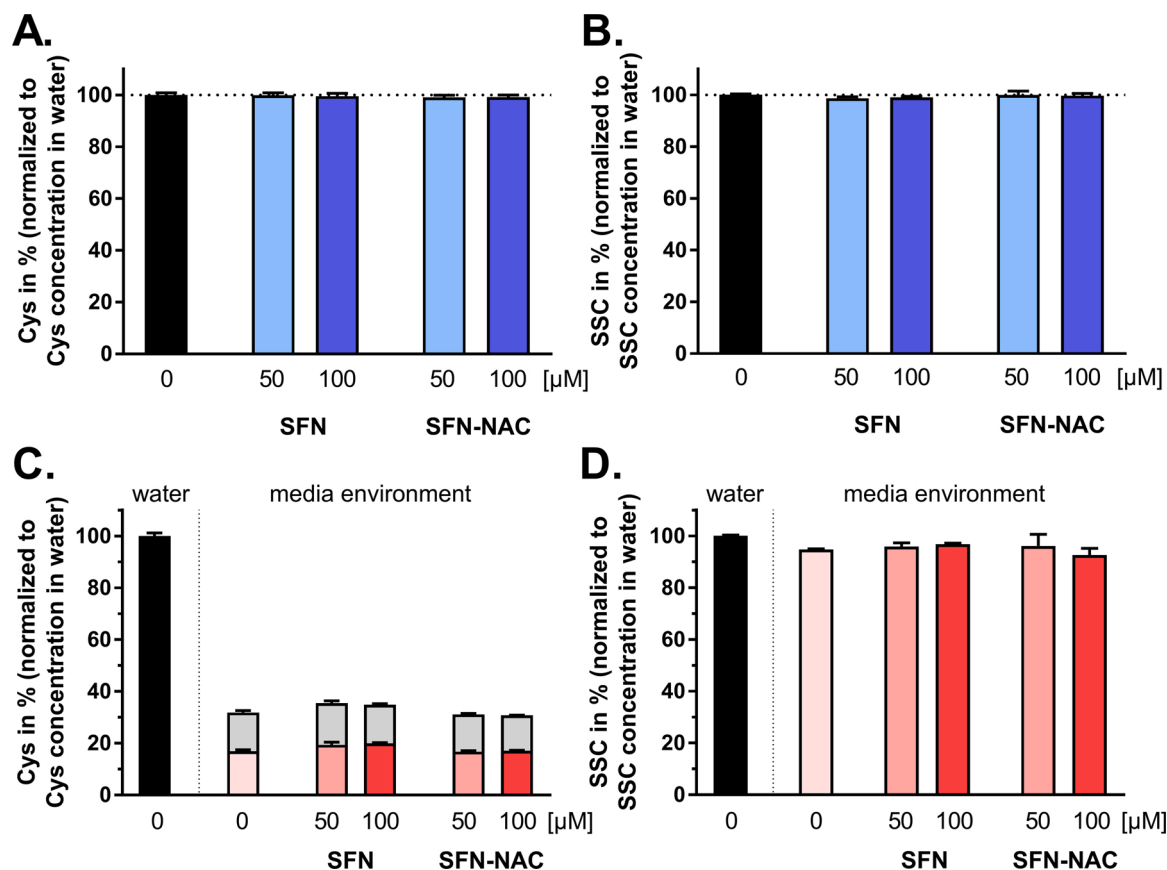


Fig. 5. Interaction of SSC or Cys with SFN or SFN-NAC in water and media. 50 μM and 100 μM SFN or SFN-NAC was incubated with either A. 1.5 mM Cys or B. 1.5 mM SSC in water (blue). To reflect cell cultivation, a Cys-depleted cell culture medium was added to C. 1.5 mM Cys or D. 1.5 mM SSC in an additional setup (red). All mixtures were analyzed directly after mixing and after incubation for 1.5 h at 37 °C. SSC, Cys and CysS were measured via RP-UPLC after iodoacetamide treatment and AccQ-Tag derivatization and were normalized to the water control condition without SFN or SFN-NAC (n = 2). The amount of Cys detected in form of CysS was visualized as stacked grey bar (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

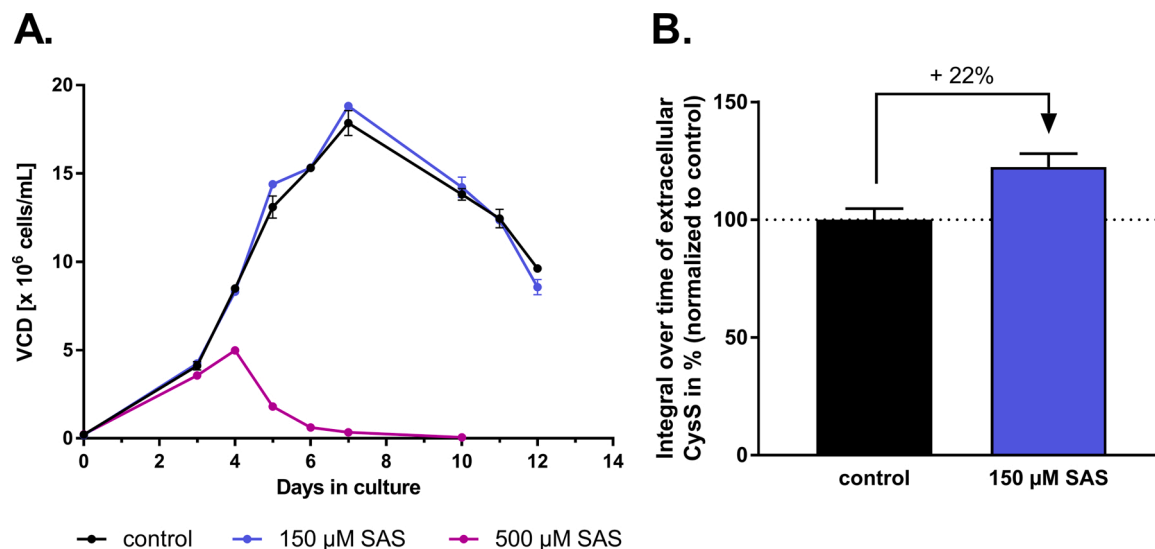


Fig. 6. Dose response of sulfasalazine on CHO cells during a fed-batch experiment. Suspension CHO cells were seeded at 2×10^5 cells/mL, incubated at 37 °C, 5 % CO₂, 80 % humidity and agitated at 320 rpm. Feed containing Cys was added on day 3, 5, 10 and 12 (3 %; v/v) and day 7 (6 %; v/v). 50 μM sulfasalazine (SAS) was added on day 3, 5 and 7 leading to 150 μM SAS in total or 500 μM SAS was added on day 3. A. Viable cell density expressed as mean values ± SEM (n = 4). B. Extracellular cystine (CysS) was measured via RP-UPLC after iodoacetamide treatment and AccQ-Tag derivatization and the integral over time of the CysS concentration was normalized to the control without SAS treatment. Data are expressed as mean values ± SEM (n = 2).

3.6. Sulfasalazine leads to reduced IVCD and an inhibition of the x_c^- antiporter activity

In contrast to increased SSC uptake via enhanced x_c^- activity, reduced cellular uptake of SSC as a consequence of x_c^- transporter inhibition was intended to be accomplished via Sulfasalazine (SAS) treatment (Gout et al., 2001). In a first test, suitable SAS concentrations were evaluated to reduce x_c^- transporter activity in CHO cells. Gout et al. reported single additions of 100 μM up to 300 μM SAS as effective concentrations inhibiting growth by limited Cys supply using a Nb2 lymphoma culture (Gout et al., 2001). Based on this publication, SAS additions of either 50 μM or 500 μM on day 3, 5 and 7 during a fed-batch using a Cys-feed (stabilized with ketoglutaric acid) were investigated. The goal of this experiment was to find suitable concentrations leading to reduced CysS uptake without being toxic for CHO cells. The impact of different treatments was evaluated by monitoring the viable cell density and by daily measurement of extracellular amino acids. The IVCD or the integral over time of the extracellular amino acid concentration was calculated and compared to an untreated condition. A lethal response was observed after addition of 500 μM SAS on day 3 (Fig. 6A). In contrast, repetitive addition of 50 μM SAS (150 μM SAS condition) showed no apparent effect on the IVCD of cultivated cells, but extracellular CysS was only increased 22 % compared to the untreated control (Fig. 6B) which was considered insufficient to study SSC uptake.

A second experiment was designed to find a SAS concentration balancing reduced IVCD and increased CysS concentration in the supernatant by increasing the addition frequency from three additions to daily treatment. Reduced IVCD of 8 % and 25 % was observed at daily addition of either 50 μM or 100 μM SAS, respectively (calculated as % difference of the IVCD visualized in Fig. 7A). Even though a reduced IVCD is known to impact the amino acid consumption, amino acids were quantified extracellularly in these conditions, hoping that the impact of the treatment would be more important than the effect of the decreased growth. Results indicate that the extracellularly concentration of CysS was increased with increasing SAS treatment (Fig. 7B) but an increase was also observed for all the amino acids, as represented exemplarily for Arg in Fig. 7C (all the other amino acid data are presented in Supplementary Fig. 2), confirming that reduction of IVCD is resulting in a lower amino acid consumption from the medium. To be able to focus on only the increase in amino acid concentration resulting from the transporter inhibition, the amino acid values were corrected to take into account the bias introduced by the decreased IVCD. Therefore, the mean increase of all extracellular amino acids (not transported by the x_c^- system) was calculated in response to SAS treatment. In response to 50 μM SAS, a minor increase of 1 % of the extracellular amino acid concentrations was calculated, whereas 20 % higher extracellular amino acid concentrations were detected after 100 μM SAS treatment. This increase did not result from the x_c^- mediated transport and was thus subtracted from all extracellular amino acid values and is visualized as stacked grey bar on the graphs of Fig. 7. As an example, Arg was increased about 24 % via 100 μM SAS treatment and after subtraction of the amino acid concentration that was not consumed due to the lower IVCD, the concentration of Arg was only increased about 4 % after 100 μM SAS treatment (Fig. 7C). In contrast, the increased CysS concentration observed in the supernatant after either 50 μM or 100 μM SAS treatment was increased by 18 % and 65 % and was still apparent after subtraction of the amino acid concentration that was not consumed due to the lower IVCD. Daily addition of 100 μM SAS resulted in 46 % higher extracellular CysS concentration (Fig. 7B), indicating that independently of the reduction in IVCD, less CysS was transported through the x_c^- transporter in presence of SAS. Extracellular Glu concentration was assessed to link reduced CysS uptake with Glu transport, since x_c^- functions as antiporter. After subtraction of the relative amino acid differences due to growth, 6 % and 4 % increased extracellular Glu were observed for 50 μM and 100 μM SAS, respectively (Fig. 7D). These data indicate no

significant change of the Glu concentration at increasing inhibitor concentrations, which might be explained by the availability of several Glu transporters in CHO cells allowing a constant Glu level independently of x_c^- inhibition. In a next step, extracellular Cys was evaluated and data showed a 7 % and 31 % reduced concentration through treatment with 50 μM and 100 μM SAS, respectively (Fig. 7E). These data suggest that in response to reduced CysS uptake, cells may promote Cys import via SAS independent transporters, e.g. EAAT3 to counteract intracellular Cys depletion. Overall, inhibition of the x_c^- antiporter through SAS showed a dose-dependent increase in extracellular CysS concentration suggesting reduced CysS uptake. Due to the low impact of the 50 μM daily addition on the CysS uptake in CHO cells and the high impact of 100 μM SAS on IVCD, both treatments were selected for further experiments.

3.7. Inhibition of x_c^- antiporter using sulfasalazine leads to reduced SSC uptake

Previous studies suggested cellular Cys supply of SSC fed cells is achieved via intracellular metabolism of SSC (Hecklau et al., 2016). To demonstrate that cellular uptake of SSC is mainly accomplished via x_c^- antiporter, the transporter activity was inhibited through daily SAS treatment (50 and 100 μM) during a fed batch experiment. Cultivation of CHO cells in presence of SAS and SSC as sole Cys source in the feed was suggested to result in increased extracellular SSC concentrations and reduced cell performance, if SAS treatment disables SSC uptake via x_c^- . As in the previous experiment, extracellular amino acids were measured daily and the integral over time was normalized to Cys fed cells to allow comparison of both feeds (different Cys-sources) and the respective effect of SAS treatment. If SSC uptake is facilitated by the x_c^- transporter, the transporter inhibition was hypothesized to result in increased extracellular SSC concentrations, as was previously observed for CysS with Cys feeding.

In presence of SSC, daily addition of either 50 μM or 100 μM SAS resulted in 14 % and 30 % reduced IVCD, respectively, when compared to the Cys containing feed (Fig. 7A). The increased extracellular amino acid concentration caused by the lower IVCD was calculated analogous to Cys fed cells. In presence of SSC, a mean concentration difference of 9 % was calculated in response to 50 μM and 31 % in response to 100 μM SAS and these values were subtracted from all extracellular amino acid values (visualized as stacked grey bar). Resulting extracellular SSC concentrations were assessed in response to transporter inhibition. A 65 % increased extracellular SSC concentration was observed after 50 μM SAS treatment and a 177 % higher extracellular SSC concentration was observed after 100 μM SAS treatment demonstrating that subtraction of the amino acid concentration differences due to growth did not disturb the significant cellular response (Fig. 7F). Concluding, SSC uptake was reduced in response to x_c^- inhibition through SAS treatment.

To investigate whether reduced SSC uptake triggered Cys uptake as previously observed with Cys feeding, extracellular Cys was evaluated. In untreated SSC fed conditions, extracellular Cys was 37 % reduced compared to the control (Fig. 7E). This striking difference of the Cys and SSC control condition is due to the fact that the SSC feed does not contain any Cys, so that the overall applied Cys concentration is lower compared to Cys fed conditions, which cannot be compensated by the calculation. Therefore, SAS treated conditions were compared to the SSC control condition. With 50 μM and 100 μM SAS, the extracellular Cys concentration was 11 % and 32 % reduced, respectively (Fig. 7E) suggesting that cells promote Cys import via SAS independent transporter to compensate for the reduced SSC uptake.

Overall, lower extracellular CysS and SSC concentrations were observed in response to increased x_c^- antiporter activity through SFN or SFN-NAC treatment suggesting an enhanced uptake. In contrast, inhibition of the x_c^- antiporter through SAS showed a dose-dependent increase in extracellular CysS and SSC concentration suggesting a reduced

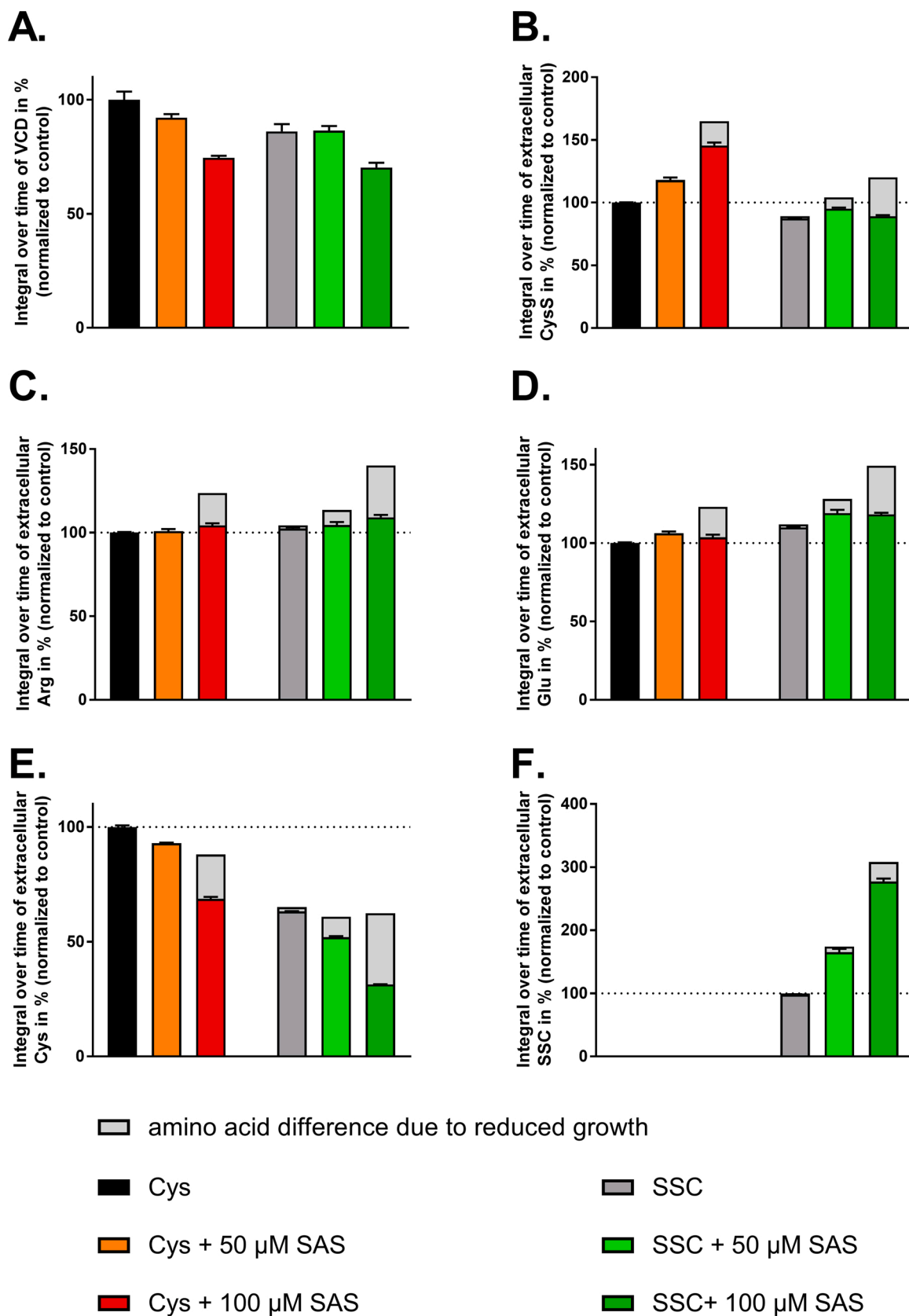


Fig. 7. Extracellular amino acids during a fed-batch experiment intended to inhibit the CysS/Glu antiporter. CHO cells were treated with 50 μM or 100 μM SAS and feed containing either Cys or SSC. The integral over time of A. Integral of viable cell density (IVCD), B. extracellular cystine (CysS), C. arginine (Arg), D. glutamate (Glu), E. Cys and F. S-Sulfocysteine (SSC) were measured via RP-UPLC after iodoacetamide treatment and AccQ-Tag derivatization and were normalized to Cys containing feed. Effects due to different growth are calculated as average difference of x_c^- antiporter non-transported amino acids compared to the control and are visualized as stacked bar in grey. Data are expressed as mean values ± SEM (n = 2).

uptake.

4. Discussion

Within this study, activity of the CysS/Glu-antiporter (x_c^-) in CHO cells was upregulated by SFN or SFN-NAC treatment, whereas antiporter inhibition was obtained by SAS treatment. Both treatments have been reported to influence the antiporter functions for various cell lines (Choi et al., 2008; Chung et al., 2005; Corssac et al., 2018; Fernandes et al., 2015; Geng et al., 2017; Gout et al., 2001; Lewinska et al., 2017; Moniruzzaman et al., 2018; Pham et al., 2004; Singh et al., 2004), whereby the response of CHO cells was not reported so far.

The initial hypothesis of SSC uptake via the CysS/Glu-antiporter is strongly supported by the results of this study, showing increased SSC uptake for cells with enhanced x_c^- transporter activity and reduced SSC uptake via transporter inhibition. Thus, it appears that SSC might be a substrate of the x_c^- transporter. Whereas most amino acid transporters have a broad substrate specificity, it is generally known that x_c^- has a high specificity for CysS and Glu (Verrey et al., 2000). However, cystathionine, an intermediate in Cys synthesis, was demonstrated to act as x_c^- substrate using mouse embryonic fibroblasts (Kobayashi et al., 2015) and L-alanosine was also suggested to be actively transported via x_c^- when analyzing human cancer cell lines (Huang et al., 2005). These studies indicate that the transporter specificity is not as restricted as expected. To verify the transport of SSC via x_c^- , the use of immature eggs (oocytes) co-expressing xCT and 4F2hc seems to be most suitable as the number of endogenous transport systems within oocytes is low and the cells readily express foreign mRNA (Pike et al., 2019). In addition, SLC7A11 knockout cells might be useful to analyze whether SSC is mainly transported via x_c^- or whether SSC is able to use additional transporter like system L, which is known to transport mixed disulfides (Ishii et al., 1981). However, due to the significant higher SSC concentrations detected in the supernatant of the culture in response to x_c^- inhibition, SSC uptake via transporters other than x_c^- is thought to be marginal.

Even though Glu is known to be exported by the x_c^- antiporter, neither transporter inhibition nor transporter upregulation affected the extracellular Glu concentrations in presence of Cys or SSC, significantly. The lack of an impacted Glu transport might be explained by the availability of several Glu transporters in CHO cells e.g. EAAT (Kanai et al., 2013), allowing a constant intracellular Glu level independently of x_c^- activity. In contrast to the results presented herein, both SFN and SAS treatments were successfully correlated with Glu transport by Zheng et al. through analyzing the cell culture media of treated and untreated fibroblasts over 60 min. More precisely, fibroblasts with a low transporter expression level were treated with SFN (24 h before the experiment), leading to an increased Glu release over time, whereas fibroblasts with a high transporter expression were inhibited with SAS, leading to a reduced Glu release (Zheng et al., 2018). This snapshot however might not represent the dynamic response of cells, when cultivated long-time during a fed-batch experiment. To assess whether co-transport of Glu occurs in response to SSC uptake, the previously described oocytes co-expressing xCT and 4F2hc might be useful to prove or not the exchange of SSC against Glu.

As side effect of SFN treatment, a significantly decreased IVCD was observed at increasing concentrations, whereby lower SFN concentrations restricted the desired antiporter mRNA upregulation within this study. The diverse cellular response of various cell lines to SFN was highly dose dependent. For example, cell viability of cardiac myoblasts was increased within a study using low SFN concentrations compared to untreated cells. This effect was linked to the upregulation of several detoxifying enzymes, protecting cells against oxidative stress, hence demonstrating that SFN treatment induced several Nrf2 regulated target genes (Fernandes et al., 2015). On the other hand, SFN was reported to cause cell cycle arrest at G2/M through inhibition of cell cycle

regulatory genes e.g. in articular chondrocytes (Jeong et al., 2012; Singh et al., 2004). Finally, an apoptotic response to SFN was reported for several cancer cells, which was linked to various mechanisms like DNA hypomethylation or mitochondria-mediated apoptotic pathway leading to increased caspase activity (Choi et al., 2008; Geng et al., 2017; Lewinska et al., 2017; Pham et al., 2004).

For studies, where an adverse effect of SFN treatment was undesired, lower SFN concentrations or shorter incubation time were used to circumvent the detrimental effects. For example, Corssac et al. tested the effects of SFN within cardiomyocytes by incubating the cells for maximum 24 h with two different SFN concentrations. No adverse effect was observed at the lower concentration, whereas a 2-fold higher concentration decreased the cellular viability significantly within that rather short incubation time (compared to fed-batch experiments). In comparison to the observed response in this study, the desired response, which was cellular protection against ROS, was already detectable at the lower SFN concentration (Corssac et al., 2018). In another study, using primary lung fibroblasts, SFN containing medium was exchanged after 4 h to allow further cultivation for 24 h, whereby the 4 h treatment was sufficient to induce SLC7A11 mRNA upregulation (Zheng et al., 2018). In the present study, however, a media exchange was undesired, as the accumulated extracellular amino acids were the most interesting functional readouts to prove that the transporter activity was modulated by the treatment. To overcome this, SFN-NAC was tested, which showed a lower impact on the cell viability compared to SFN. A lower toxicity of SFN-NAC was also reported by Liu et al. using a colorimetric assay to detect the cell viability of human umbilical vein endothelial cells (HUVECs) in response to SFN and SFN-NAC treatment after 24 h. More precisely, both treatments reduced the viability in a dose-dependent manner, whereby SFN showed a stronger cytotoxicity compared to SFN-NAC (Liu et al., 2018).

Concluding, future experiments to upregulate the transporter may focus on optimizing SFN-NAC treatment as this showed less side effects, while being able to induce transporter upregulation in CHO. Beside SFN-NAC, further intermediates originating from SFN and GSH interaction like the cysteine-conjugate (SFN-Cys) were demonstrated to exhibit similar protective effects but were less toxic compared to SFN and are therefore interesting for future studies (Liu et al., 2018). Beyond that, additional chemicals, which are reported to induce Nrf2 activity might be screened for their ability to trigger antiporter overexpression in CHO, while having less side-effects. For example, Takaya et al. described that tert-butylhydroquinone, diethylmaleate and dimethylfumarate target the same thiol as SFN within KEAP1 and were successful at inducing Nrf2 activity using embryonic fibroblasts (Takaya et al., 2012). Dimethylfumarate and diethylmaleate are oxidative stressor and were reported in independent studies to induce antiporter expression (Lo et al., 2008; Shin et al., 2017), suggesting that these chemicals might be good candidates for a screening with CHO. Furthermore, genetically engineered CHO cells, overexpressing the antiporter, might allow cross-validation of the data.

In response to SAS, the observed growth reduction of CHO cells was not surprising, as this compound is commonly used as potent tumor growth suppressor, by limiting x_c^- mediated CysS uptake (Gout et al., 2001). Further studies demonstrated that reduced CysS uptake causes subsequent GSH depletion, so that cellular protection against ROS is attenuated after SAS treatment (Chung et al., 2005; Moniruzzaman et al., 2018). Although a reduced uptake rate through x_c^- was the intended goal of the SAS treatment and not a side effect, supplementation of antioxidants such as taurine (uptake via SLC6A6 (Geoghegan et al., 2018)) might be advantageous for the cells to scavenge ROS and thereby diminish the negative response to SAS treatment. In contrast, GSH supplementation is not suitable as it was demonstrated to readily interact with SSC and might be degraded, extracellularly (Nagane et al., 2018). Still, nonspecific effects of SAS treatment cannot be ruled out and may impact the presented results. Another strategy to cross-validate the obtained data might be the use of noncoding siRNAs to inhibit target

SLC7A11 mRNA translation, leading to reduced transporter expression (Yang et al., 2018).

5. Conclusion

Within this study, cellular uptake of SSC, a modified amino acid used to replace Cys in highly concentrated feed solutions was analyzed and results suggest that the CysS/Glu antiporter (x_c^-) facilitates SSC uptake. As this transporter is commonly expressed in CHO cells for Cys supply, SSC is applicable as Cys replacement for various cell lines. The use of SSC is advantageous as it enables a stable and more concentrated feed solution and drives the development of a next generation production process.

CRedit authorship contribution statement

Martina Zimmermann: Conceptualization, Methodology, Investigation, Validation, Visualization, Writing.

Harald Kolmar: Validation, Writing; **Aline Zimmer:** Conceptualization, Supervision, Validation, Writing.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.jbiotec.2021.06.003>.

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