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Full Length Article

New homocysteine consumption assay for high-throughput screening of human cystathionine-β-synthase

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ABSTRACT

Hyperhomocysteinemia is a risk factor for cardiovascular disease, neurological disorders, and bone abnormalities. The key enzyme in homocysteine metabolism, cystathionine-β-synthase (CBS) is recognized as a target for new homocysteine-lowering therapies including enzyme replacement and gene therapy. Currently, there are no pharmacotherapies available that enhance CBS activity through its allosteric mechanism. The only known allosteric activator of CBS is S-adenosyl-L-methionine (SAM), which is available as a food supplement, but its effectiveness is limited by low membrane permeability and universal involvement in methylation reactions as a substrate. The discovery of CBS activators in high-throughput screening is challenging due to a lack of dedicated assays. Available HTS-compatible activity assays for CBS rely on measuring the product hydrogen sulfide or methanethiol where the signal increases with increased CBS activity. In the case of fluorescence-based assays, it is challenging to discern activators from autofluorescent compounds.

In this study, we introduce a homocysteine consumption assay for isolated human CBS (HconCBS) based on the absorbance of Ellman's reagent. This assay leverages a decrease in signal upon CBS activation, with performance parameters exceeding the requirements for high-throughput screening. In a commercial library of 3010 compounds, the HconCBS assay identified 10 hit compounds as more active than SAM, whereas a fluorescence-based assay using 7-azido-4-methylcoumarin (AzMC) identified 141 hits. HconCBS identified 101 compounds with autoabsorbance which did not include hit compounds, while the fluorescence-based assay identified 383 autofluorescent compounds which included all hit compounds. While 4 out of 10 HconCBS hits were confirmed when purchased from a new source, the compounds affected homocysteine rather than CBS. Nevertheless, HconCBS consistently detected the CBS activator seleno-adenosyl-L-methionine (SeAM) added to 4 library plates and re-discovered the same library hits in 3 out of 4 re-screened plates.

Taken together, HconCBS was designed to enable the discovery of allosteric CBS activators with greater reliability than fluorescence-based methods. Despite identifying some compounds that acted on homocysteine rather than CBS, the assay consistently identified the CBS activators SAM and SeAM and demonstrated

Abbreviations: CBS, Cystathionine-β-synthase; HconCBS, Homocysteine consumption assay for CBS; SAM, S-adenosyl-L-methionine; SeAM, Seleno-adenosyl-L-methionine; AOAA, Aminooxyacetic acid; HTS, High-throughput screening; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid), ellman's reagent; TNB, 2-nitro-5-thiobenzoate; AzMC, 7-azido-4-methylcoumarin; AMC, 7-amino-4-methylcoumarin; DMSO, dimethyl sulfoxide.

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reproducibility across two screening rounds. These findings establish HconCBS as a valuable tool for identifying potential therapeutic candidates for hyperhomocysteinemia, addressing a key gap in the development of CBS-targeted pharmacotherapies.

1. Introduction

Homocysteine is a non-coded, intermediate sulfur amino acid and a risk factor in various diseases. Specifically, elevated blood levels of homocysteine (hyperhomocysteinemia) are associated with chronic diseases including cardiovascular disease [1–3], neurodegenerative diseases [4], and bone abnormalities [5]. Existing homocysteine-lowering interventions include supplementation of vitamins B6, B9 or B12, and folic acid [6]. However, no drug is available that directly enhances homocysteine elimination from plasma, which presents a window for innovation.

The key transsulfuration enzyme that eliminates homocysteine from plasma is Cystathionine-beta-synthase (CBS, UniProt P35520) [7] and its deficiency was linked to hyperhomocysteinemia [8]. Indeed, we observed that mice developed severe hyperhomocysteinemia when *Cbs* is knocked out in adulthood [9,10]. Approaches to reduce hyperhomocysteinemia by CBS include gene therapy using an adeno-associated virus [11] and CBS enzyme replacement therapy with pegtibatinase [12].

CBS activity can be enhanced via an allosteric activation mechanism that is present in humans but not yeast or *Drosophila* [13]. Currently, SAM is the only known endogenous activator of CBS [7]. SAM is available as a dietary supplement, but has limited cell permeability due to high polarity and is involved in transmethylation reactions as a universal methyl donor [14]. Nevertheless, allosteric activation of CBS represents an attractive target in the treatment of hyperhomocysteinemia.

An unbiased approach to discover compounds targeting CBS is high-throughput screening (HTS). HTS plays an essential role in the drug discovery process as a tool to identify initial hit compounds from libraries of small molecules with drug-like properties. The approach enables efficient and rapid screening of thousands of compounds, providing starting points for drug development programs. Hits obtained through HTS are subsequently optimized, progressing through hit-to-lead and lead optimization stages, where their activity, selectivity, and pharmacokinetic properties are refined to produce viable drug candidates.

The discovery of activators of CBS is difficult considering the challenges in measuring increased enzymatic activity and the lack of suitable assays. A standard fluorescent probe, 7-azido-4-methylcoumarin (AzMC) is useful for quantification of hydrogen sulfide produced by CBS from cysteine and homocysteine (Supplementary Fig. 1A), and was proven to be effective for discovering inhibitors [15]. In principle, the non-fluorescent azide (AzMC) is reduced to a fluorescent amine (AMC) by hydrogen sulfide [16]. As with any fluorescent probe used in an HTS setting, there is an inherent risk of discovering false positive or false negative hit compounds due to the autofluorescence of compounds in screening libraries. Autofluorescence becomes especially problematic for discovering true hits in the case of CBS activators where increased hydrogen sulfide production elevates fluorescence signal.

Another available activity assay for CBS captures hydrogen sulfide released from a reaction well in an adjacent detection well using Ellman's reagent [17]. The advantage of the tandem microwell assay is the elimination of optical interference by screened compounds. Disadvantages of the tandem assay include the release of toxic hydrogen sulfide, the requirement for custom assay plates where walls between adjacent wells are milled down to enable gas exchange, and a necessary hermetic seal which presents an additional automation challenge.

The objective of the present paper is to introduce a new activity assay for isolated human CBS (HconCBS). The assay utilizes the canonical CBS reaction between homocysteine and serine, which yields cystathionine and water (Supplementary Fig. 1B). Homocysteine, the only thiol in the reaction, is detected by Ellman's reagent (DTNB) at a standardized time point, yielding TNB and stopping the reaction. The assay eliminates problems with autofluorescence, does not produce the toxic gas hydrogen sulfide, and does not require a hermetic seal. Another advantage of the new assay is an absorbance-based readout that is inverted, i.e., color intensity decreases with increased CBS activity because the reaction substrate is measured rather than the product. We show that the assay reaction start/stop can be conveniently machine-automated, that the assay has a stable readout over time, and has robust performance parameters that are suitable for HTS.

2. Materials and methods

2.1. Production of recombinant human CBS protein

A DNA fragment containing the human CBS gene with a deletion of a sequence representing the loop residues 516–525 and an N-terminal 6x-histidine tag was subcloned into the NcoI, XhoI sites of pET-15b, replacing the original 6-his leader (BaseClear, Leiden, The Netherlands). The synthetic plasmid was transformed into *Escherichia coli* JM109 competent cells (Promega, Madison, WI, USA), amplified overnight at 37 °C in LB medium supplemented with ampicillin (100 μg/ml), and isolated using the PureYield™ Plasmid Midiprep System (Promega). Subsequently, the plasmid was transformed into *E. coli* BL21 (DE3) competent cells (New England Biolabs, Ipswich, MA, USA) for recombinant expression.

Medium (LB broth, 1 L) supplemented with pyridoxine (120 μM), 5-aminolevulinic acid (5-ALA, 300 μM), thiamine (30 μM), ferric chloride (100 μM), and 1.12 % (v/v) glycerol in a benchtop bioreactor Biostat B (Sartorius, Göttingen, Germany) was inoculated with 100 mL of overnight culture of *E. coli* BL21 (DE3) pET-15b-CBS grown in LB broth at 37 °C. CBS expression was induced with Isopropyl β-D-1-thiogalactopyranoside (IPTG, 100 μM) after the culture reached $OD_{600} = 5$. The biomass was harvested, centrifugated at 4 °C, and stored at −20 °C. Cell pellets were resuspended in Buffer A (composed of sodium phosphate buffer (50 mM, pH 7.4), NaCl (300 mM), DTT (1 mM), glycerol (10 % (v/v)) supplemented with imidazole 20 mM and 1 cOmplete protease inhibitor cocktail tablet (Roche). The suspension was cooled on ice and sonicated with Sonopuls HD3200 with a KE76 probe (Bandelin, Berlin, Germany) using 30 s/30 s on/off pulses for 15 min. The insoluble fraction was separated by centrifugation at 4 °C, 7690 g for 10 min twice and the supernatant was loaded onto two 5 mL HisTrap FF columns (Cytiva, Marlborough, MA, USA) connected in series on an Äkta AVANT 150 (GE Healthcare, Chicago, IL, USA). After washing with Buffer A supplemented with imidazole (50 mM), the columns were eluted with buffer A supplemented with 250 mM imidazole.

SDS-PAGE was carried out according to Laemmli [18] on a 12 % resolving gel and was stained using Coomassie Brilliant Blue G-250. Protein purity was determined from electrophoresis gel image by densitometry using Gel Analyzer (GelAnalyzer 23.1.1 by Istvan Lazar Jr., PhD and Istvan Lazar Sr., PhD, CSc) by quantifying the volume of gel band matching CBS protein and comparing it to total volume of gel bands present in the lane.

Purified protein was dialyzed overnight into HEPES buffer (20 mM, pH 7.4, H4034, Merck, Darmstadt, Germany) and the absorption spectrum was measured in an optical plate reader (Varioskan LUX, Thermo Fisher Scientific, Waltham, MA, USA). The Reinheitszahl (R_z), an indicator of heme incorporation and purity a ratio of the absorbance of heme

at the Soret peak (430 nm) and the absorbance of protein at 280 nm, was 0.87 and represents. The CBS protein sample was concentrated using a 15 mL Amicon centrifugation column with a 10 kDa molecular weight cutoff filter (UFC901008, Merck). Finally, glycerol was added to the sample to a final concentration of 30 % (v/v) (G9012, Merck) for prolonged storage at −20 °C in a liquid state, forming a ~30 mg/mL stock.

2.2. Homocysteine consumption assay (HconCBS)

2.2.1. Calibration of L-homocysteine and CBS

Concentration series of homocysteine were constructed by mixing 200 µL L-homocysteine (100 – 600 µM) (CSSB00000742287, ChemSpace, Riga, Latvia) with Ellman's reagent (D8130, Merck) in 20 mM HEPES buffer (pH 7.4) in a 96-well assay plate (PS, U-bottom, 650101, Greiner Bio-One, Kremsmünster, Austria). Final reaction volumes of 250 µL contained 0.25 mM Ellman's reagent. Ellman's reagent reacted with homocysteine to form yellow TNB which was quantified in an optical plate reader (Varioskan LUX) using 412 nm absorbance.

Concentration series of isolated human CBS ($hCBS^{\Delta 516–525}$, 1 – 1000 nM) were incubated in 200 µL with 3 mM L-serine (1077690010, Merck) and 0.5 mM homocysteine for 30 min at 37 °C. The reactions were stopped by adding Ellman's reagent to a final volume of 250 µL and concentration of 0.25 mM, and TNB was quantified using 412 nm absorbance.

2.2.2. Optimization of time to reaction endpoint

Isolated human CBS ($hCBS^{\Delta 516–525}$) was incubated at room temperature for 20 min with buffer control (20 mM HEPES pH 7.4) or SAM (A2408, Merck) in a 96-well assay plate. The reactions were initiated every 5 min in new triplicate wells by adding a substrate mixture containing L-homocysteine and L-serine with an automated pump inside an optical plate reader (Varioskan LUX) set at 37 °C (Fig. 1B). The resulting assay volumes of 200 µL contained 165 nM $hCBS^{\Delta 516–525}$, 30 µM SAM, 3 mM serine, and 0.5 mM homocysteine. Wells without CBS were included as controls representing unused homocysteine. After 60 min, reactions in all wells were stopped by adding Ellman's reagent to a final volume of 250 µL and 0.2 mM concentration. TNB concentrations were measured using absorbance at 412 nm. Data were collected using SkanIt Software RE 7.0 (Thermo Fisher Scientific).

2.2.3. CBS activation by concentration series of SAM

Isolated human CBS activity assays were carried out in 20 mM HEPES buffer pH 7.4.

Homocysteine consumption $hCBS^{\Delta 516–525}$ activity was measured as described above, with a modification to the pre-incubation step. Namely, $hCBS^{\Delta 516–525}$ was incubated with concentration series of SAM or negative control (inactive compound, WAY-333704). Final compound concentrations ranged from 30 nM to 30 µM.

Hydrogen sulfide production $hCBS^{\Delta 516–525}$ was incubated at room temperature for 20 min with concentration series of control (WAY-333704) or SAM in a 96-well assay plate. Reactions were initiated by adding a substrate mixture containing L-homocysteine and L-cysteine (168149, Merck) with an automated pump inside an optical plate reader set at 37 °C. The resulting assay volumes of 100 µL contained 165 nM $hCBS^{\Delta 516–525}$, 30 nM – 30 µM SAM, 3 mM cysteine, and 0.5 mM homocysteine. AzMC (10 µM, 802409, Merck) was continuously reduced by hydrogen sulfide to the fluorescent AMC and AMC concentrations were measured after 30 min using $\lambda_{ex/em}$ = 365 nm/450 nm.

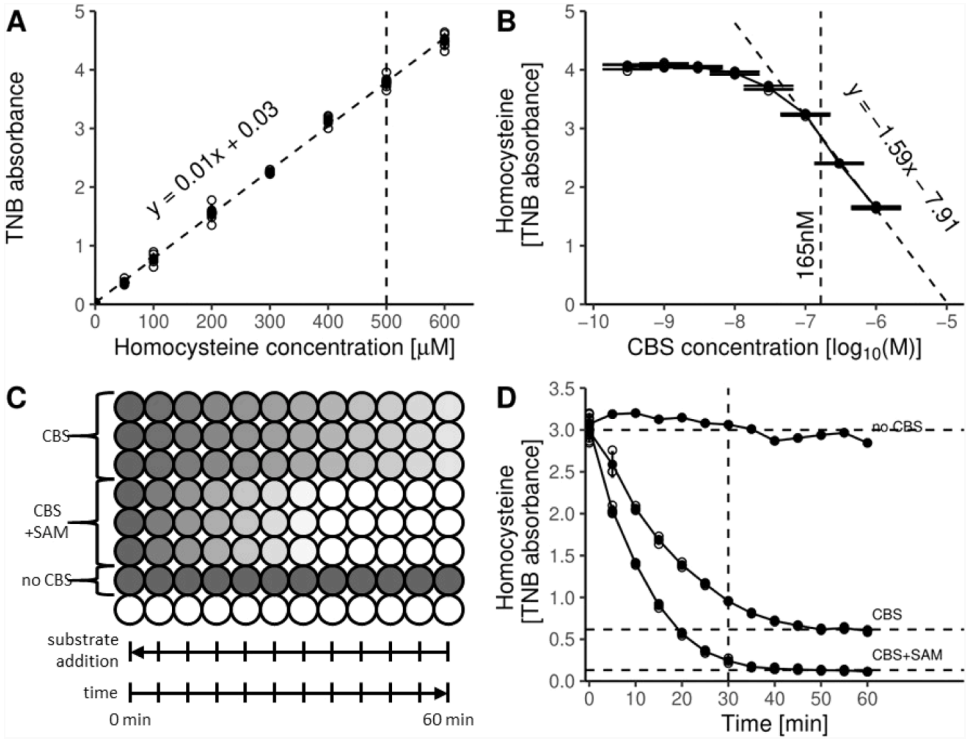

Fig. 1. Optimization of the homocysteine consumption assay for human CBS (HconCBS). A) Quantification of homocysteine by Ellman's reagent (DTNB, 0.25 mM) followed a linear trend. TNB is the product of the reaction between DTNB and homocysteine that absorbs 412 nm light. The concentration of homocysteine selected for the HconCBS assay was 500 µM (vertical dashed line). B) Titration of $hCBS^{\Delta 516–525}$ in the HconCBS assay. The 165 nM concentration (vertical dashed line) of $hCBS^{\Delta 516–525}$ was within the linear range and was selected for the HconCBS assay. C) Time series were created by pumping a substrate mixture every 5 min to new wells already containing $hCBS^{\Delta 516–525}$ using an automated pump in an optical plate reader. After 60 min, the reactions were automatically stopped by adding Ellman's reagent using a second machine pump. S-adenosyl-L-methionine (SAM, 30 µM) was included as a reference CBS activator. D) The assay plate was read after 30 min using an optical plate reader and homocysteine consumption by $hCBS^{\Delta 516–525}$ over time was quantified. Wells not containing $hCBS^{\Delta 516–525}$ represent unconsumed homocysteine. Data are expressed as mean ± SD from n = 3–6 wells (technical replicates), except for "no CBS" control (n = 1 well), with filled points representing means, bars representing SDs, and empty points representing individual replicates.

2.3. High-throughput screening

2.3.1. Optimization of HconCBS for high-throughput screening

Using an automated 96-channel pipetting system (Viaflo 96, 125 µL configuration, INTEGRA Biosciences, Tokyo, Japan), 99 µL hCBS$^{\Delta 516–525}$ was transferred from a 1-well reservoir onto a 96-well assay plate. Subsequently, 0.6 µL reference compounds were transferred from a source plate (Fig. 2, Fig. 3A) onto the reaction plate using a Viaflo 96 with 12.5 µL configuration that is suitable for accurate transfer of small volumes. The transfer consisted of 0.6 µL dispensing and six times trituration of 12.5 µL volume. Reactions were initiated by adding 99 µL substrate onto the reaction plate, resulting in 200 µL volumes containing 165 nM hCBS$^{\Delta 516–525}$, 30 µM compound, 0.5 mM L-homocysteine and 3 mM L-serine. The reactions were incubated in a 37 °C incubator and after 30 min were stopped by adding 50 µL Ellman's reagent (1.25 mM stock concentration), resulting in a final concentration of 0.25 mM Ellman's reagent in 250 µL final volume. TNB was continuously measured using absorbance at 412 nm every 10 min for 60 min, and after 10 h, in a non-heated optical plate reader. Assay performance parameters were calculated according to recommendations from the Royal Society of Chemistry [19].

2.4. Miniaturization

The HconCBS assay was miniaturized in a 384-well assay plate (3702, Corning, NY, USA). Reactions of 15 µL contained 165 nM hCBS$^{\Delta 516–525}$, 30 µM reference compound (SAM or AOAA), 3 mM serine, and 0.5 mM homocysteine. After 30 min of incubation at 37 °C, Ellman's reagent was added to the reaction to a final volume of 20 µL and concentration of 0.25 mM. TNB concentrations were measured using absorbance at 412 nm.

2.5. Pre-filtering compounds in a screening library based on optical interference

The Express-Pick Library (L3600, Selleck Chemicals, Houston, TX, USA) was provided in 96-well assay plates ($n = 38$) sealed with silicone mats. The compounds were formulated by the manufacturer in 30 µL DMSO (100 %) in 10 mM concentration. Using a Viaflo 96, 99 µL of hCBS$^{\Delta 516–525}$ was transferred from a 1-well reservoir onto a 96-well assay plate. Compounds were transferred from library plates in 0.6 µL volume onto the assay plates using another Viaflo 96. The transfer consisted of 0.6 µL dispensing and 6x trituration of 12.5 µL. The resulting 99.6 µL contained 330 nM hCBS$^{\Delta 516–525}$, 60 µM compound, or 0.6 % (v/v) DMSO control. As a pre-scan before initiating the enzymatic reactions, wells containing hCBS$^{\Delta 516–525}$ and library compounds were measured in an optical plate reader (Varioskan LUX) in a 412 nm absorbance mode, and $\lambda_{ex/em} = 365$ nm/450 nm fluorescence mode.

Since the pre-scan data were not normally distributed (Supplementary Fig. 3), compounds with optical interference were flagged using a modified Tukey's Fences approach [20]. For each measurement mode (absorbance, fluorescence), the interquartile range (IQR) was calculated as the difference between the third quartile (Q3, 75 th percentile) and the first quartile (Q1, 25 th percentile) of the data. Upper bounds for typical values were then determined using the following equation:

Equation 1 Modified Tukey's Fences approach to calculating cutoffs for autoabsorbance and autofluorescence

$$Upper\ Bound = Q3 + k \times IQR$$

where k represents the multiplier applied to the IQR, which was set to 3.0 to provide a strict criterion for identifying extreme values.

2.6. Primary high-throughput screen

Library and reference compounds (DMSO, SAM, and AOAA (C13408, Merck)) were incubated with hCBS$^{\Delta 516–525}$ at room temperature for 20 min, reactions were initiated by adding 99 µL substrate, resulting in a 198.6 µL reaction mixture consisting of 20 mM HEPES buffer (pH 7.4), 165 nM hCBS$^{\Delta 516–525}$, 30 µM library or reference compound or 0.3 % (v/v) DMSO, 3 mM serine, and 0.5 mM homocysteine. The reactions were incubated at 37 °C for 30 min. In HconCBS, 50 µL Ellman's reagent was added (0.25 mM final concentration) to stop the reaction and after additional 30 min, the reaction plates were analyzed using absorbance at 412 nm. In the hydrogen sulfide production assay, AMC fluorescence ($\lambda_{ex/em} = 365$ nm/450 nm) was measured. The final 198.6 µL reaction mixture consisted of 20 mM HEPES buffer (pH 7.4), 330 nM hCBS$^{\Delta 516–525}$, 30 µM library or reference compound or 0.3 % (v/v) DMSO, 3 mM cysteine, 0.5 mM homocysteine, and 10 µM AzMC. In each mode, the library was screened in $n = 1$ well per compound. Data were collected using SkanIt Software RE 7.0 (Thermo Fisher Scientific).

2.7. Repeated screening

Plates 10, 25, 26, 28 were re-screened using the HconCBS assay to confirm hits. Seleno-adenosyl-L-methionine (SeAM, PubChem CID 131854545), a structural analog of SAM and CBS activator, was produced in-house according to Ottink et al. [21], and was included in all re-screened plates as a positive control.

2.8. Confirmation of primary hits from a new source

The compounds WAY−224994 (CSSS00016211484), WAY−302402

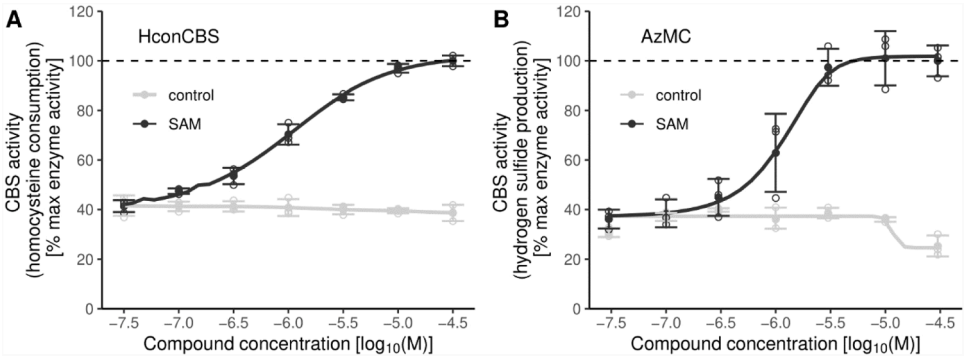

Fig. 2. The homocysteine consumption assay (HconCBS) detects activation of CBS at different concentrations of SAM. A) Activity of hCBS$^{\Delta 516–525}$ (165 nM) measured using HconCBS, B) activity of hCBS$^{\Delta 516–525}$ (165 nM) measured using the fluorescence hydrogen sulfide assay (AzMC). SAM, S-adenosyl-L-methionine is a reference CBS activator. The inactive compound WAY-333704 was included as a negative control. Data are expressed as mean ± SD from $n = 3$ wells (technical replicates), with empty points representing individual replicates and lines representing fitted curves. Horizontal dashed lines represent maximal (100 %) CBS enzyme activity.

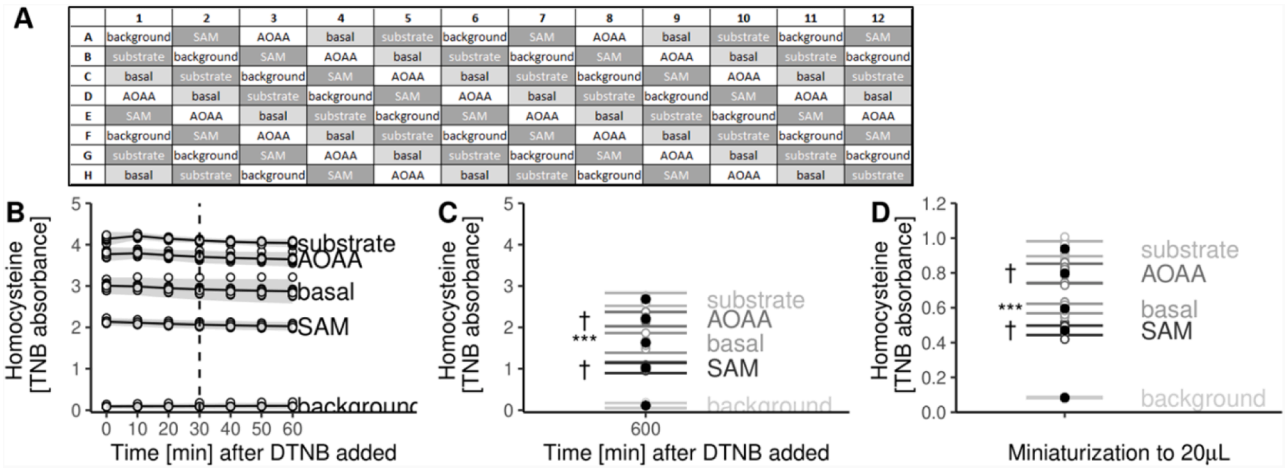

Fig. 3. Assessment of the robustness of homocysteine consumption assay. A) Checkered 96-well plate layout for assessment of spatial uniformity and readout stability. B) Readout stability of homocysteine consumption assay during 1 hr after adding Ellman's reagent (DTNB). Data are expressed as mean ± SD from n = 18–20 wells (technical replicates), with points representing means and ribbons (grey areas) representing SDs. C) Readout after 10 h. Substrate = serine and homocysteine, AOAA = aminooxyacetic acid (CBS inhibitor), basal = basal CBS activity, SAM = *S*-adenosyl-L-methionine (CBS activator), background = buffer with Ellman's reagent. D) The HconCBS assay was miniaturized to 20 µL in a 384-well plate. Data are expressed as mean ± SD from n = 16–20 wells (technical replicates), with black points representing means and error bars representing SDs. ***: $p < 0.001$ when compared to substrate, †: $p < 0.05$ when compared to basal CBS activity.

(CSSS001074441 69), WAY−306132 (CSSS00160619590), WAY−331409 (CSSS00132892223), WAY−389904 (CSSS00027938596), WAY−603891 (CSSS00028084638), WAY−611819 (CSSS00026679362), WAY−613160 (CSSS00026724576), WAY−613225 (CSSS00026083026), WAY−628153 (CSSS00028092735) were purchased from Chem-Space and tested using the HconCBS assay in 30 µM concentrations.

2.9. Data and statistical analysis

All experiments were performed once. Data were processed and analyzed using R version 4.2.2 (The R Foundation, Vienna, Austria) and the packages tidyverse, ggthemes, ggrepel, compareGroups, FSA, car, hellno, readxl, openxlsx, writexl, psych, nplr, data.table, directlabels, pdftools, imager, BiocManager, ChemmineR, and Cairo. R code was optimized using ChatGPT-4o. Means were compared using the Kruskal-Wallis test followed by Dunn's post-hoc test with adjustments for multiple comparisons. Chemical reaction schematic was created using ACD/ChemSketch (Freeware) 2022.2.3 (Advanced Chemistry Development, Inc., Toronto, Ontario, Canada). Concentration-response curves for hCBS activation by SAM were analyzed using the Hill equation [22]. In this model, E_{min} represents the response at the minimum SAM concentration, and E_{max} represents the response at the maximum SAM concentration. The Hill slope reflects the overall steepness of the curve, with higher values indicating a more sigmoidal relationship. The pAC_{50} denotes the negative $\log_{10}$ of molar concentration of SAM required to achieve a response halfway between E_{min} and E_{max}, which corresponds to the inflection point of the curve.

3. Results

3.1. Optimization of HconCBS assay in automated fluidics setting

Full-length human CBS has limited water solubility due to polymerization [23], hence we used the engineered $hCBS^{\Delta 516–525}$ dimer variant with preserved allosteric activation [7] with 84 % purity estimated by SDS-PAGE (Supplementary Fig. 2).

To demonstrate linearity of homocysteine quantification by Ellman's reagent, a 0 – 600 µM concentration range of homocysteine was measured (Fig. 1A). A homocysteine concentration of 500 µM had submaximal absorbance and was selected as the standard concentration for the HconCBS assay. Additionally, $hCBS^{\Delta 516–525}$ was titrated. Concentrations of $hCBS^{\Delta 516–525}$ spanning 100 nM, 300 nM and 1000 nM formed a linear trend after 30 min of reaction time. The 165 nM concentration was selected as the optimal concentration for the HconCBS assay, as it preserved sufficient dynamic range for detecting activators (Fig. 1B).

Homocysteine consumption by isolated $hCBS^{\Delta 516–525}$ (HconCBS) was optimized for the time of reaction endpoint. Since the start/stop nature of the reaction did not allow continuous monitoring, reactions were initiated every 5 min for 60 min in an automated optical plate reader using a programmable pump (Fig. 1C). After 60 min, the second machine pump added Ellman's reagent, which reacted with the remaining homocysteine as a stop solution (Fig. 1C). Homocysteine decreased in a time-dependent manner and reactions containing CBS reached equilibrium at t = 50 min, with some homocysteine remaining (Fig. 1D). Reactions containing CBS and SAM reached equilibrium at t = 40 min, with less homocysteine remaining (Fig. 1D). The differences in homocysteine asymptotes may be due to reduced feedback inhibition by the product L-cystathionine, since CBS activation by SAM exposes additional catalytic sites. The 30-minute time point provided sufficient residual homocysteine and was selected as the standard assay endpoint. Compared to earlier time points such as 15 min, the 30-minute mark captured the transition out of the rapid reaction phase and a larger effect of CBS activation by SAM. Importantly, the readout of the HconCBS assay is inverted, i.e. has lower readout values with increasing CBS activity (Fig. 1D).

3.2. HconCBS assay is suitable for concentration-response studies of allosteric activators of CBS

Increasing concentrations of the CBS activator SAM enhanced homocysteine consumption resulting in an S-shaped curve (Fig. 2A) suitable for quantitative enzyme pharmacology and model fitting using the Hill equation [22] (Table 1). For comparison, hydrogen sulfide production by isolated human CBS was measured with a conventional fluorescent probe (AzMC, Fig. 2B) and Hill parameters were calculated (Table 1).

In HconCBS and AzMC, basal CBS activity was similar (E_{min}, Table 1). Compared to AzMC, the slope of HconCBS was shallower, distinguishing the effects of SAM more gradually within the middle concentration range (Table 1). The potency of SAM in activating

Table 1
Calculated Hill parameters for SAM activation of $hCBS^{\Delta 516-525}$ (165 nM) in the homocysteine consumption assay (HconCBS) and in the hydrogen sulfide assay (AzMC). E_{min} = enzyme activity at minimal compound concentration, E_{max} = enzyme activity at maximal compound concentration, slope = Hill slope, pAC_{50} = negative $\log_{10}$ of molar concentration of compound at half maximal activation of CBS. Data are expressed as mean ± SD from $n = 3$ wells.

	control		SAM	
	HconCBS	AzMC	HconCBS	AzMC
E_{min} [%]	41.6 ± 4.2	37.3 ± 1.1	41.4 ± 2.4	37.2 ± 6.5
E_{max} [%]	38.7 ± 3.3	24.6 ± 4.8	100.0 ± 2.1	102.0 ± 3.4
slope	−0.03 ± 0.03	−0.12 ± 0.25	28.9 ± 3.6	52.2 ± 3.0
pAC_{50} [$-\log_{10}$(M)]			6.00 ± 0.12	5.96 ± 0.23

$hCBS^{\Delta 516-525}$ in HconCBS was not different from that of SAM measured by AzMC (pAC_{50}, Table 1). Taken together, HconCBS can distinguish subtle differences in the activation of CBS by SAM, making it suitable for concentration-response studies of future CBS activators.

3.3. HconCBS has robust parameters suitable for automated high-throughput screening

To facilitate drug discovery for human CBS using HconCBS, we verified whether the assay parameters meet the criteria for HTS, namely the signal/background ratio > 3, Z prime (Z') > 0.6, and the uniformity of the signal on the assay plate with a coefficient of variation (CV) < 10 %. Here, we tested the performance of the assay using an automated 96-channel pipetting platform (Viaflo 96, INTEGRA) and a 96-well source plate filled with reference compounds in a checkered layout (Fig. 3A). The TNB readout became stable 10 min after adding Ellman's reagent, and decreased slightly over 60 min, but maintained substantial differences in the signal between reference compounds (Fig. 3B) which at 30 min exceeded the requirement for Z' (Table 2). The signal-to-background ratio far exceeded the HTS criterion for all reference conditions, and the assay plate signal pattern remained well below 10 % (Table 2). The readout of the assay maintained discernible differences between reference compounds even when measured after 10 h (Fig. 3C).

Thus far, the HconCBS assay was showcased in 250 μL volumes in 96-well format. To demonstrate that the assay can also be applied in even higher throughput such as ultra-HTS, we miniaturized it to 20 μL volume in a 384-well plate (Fig. 3D).

3.4. Inverted colorimetric readout of HconCBS reduces the primary hit rate in high-throughput screening

The Express-Pick Library was screened against $hCBS^{\Delta 516-525}$ using HconCBS as well as the fluorescence-based AzMC assay. Compounds that exceeded the activation of CBS by SAM were considered hit activators. In HTS using AzMC, 141 compounds out of 3010 were identified as primary hits (4.7 % hit rate, Fig. 4A). HTS libraries typically contain aromatic compounds that sometimes have considerable autofluorescence which can confound the identification of true hits. To identify compounds that potentially interfered with the AMC readout, each assay plate was scanned for $\lambda_{ex/em}$ = 365 nm/450 nm fluorescence before initiating the enzymatic reaction (Fig. 4B). The autofluorescence data were not normally distributed (Supplementary Fig. 3), hence a cutoff was calculated using modified Tukey's Fences (Equation 1). This way, 383 compounds were flagged as autofluorescent, including all 141 primary hits, suggesting that they were false positives. Indeed, from 10 randomly selected primary hit compounds, none were confirmed as CBS activators (Supplementary Fig. 4).

Table 2
Assay parameters of homocysteine consumption assay (HconCBS) were calculated from $t = 30$ min after the addition of Ellman's reagent. substrate = serine and homocysteine, AOAA = aminooxyacetic acid (CBS inhibitor), basal = basal CBS activity, SAM = *S*-adenosyl-l-methionine (CBS activator), background = buffer with Ellman's reagent.

Signal to background (requirement: S/*B* > 3)		Z prime (requirement: Z' > 0.6)		Signal pattern (requirement: CV < 10 %)	
substrate/ background	42.0 ± 0.2	high: substrate, low: basal	0.72	substrate	0.55 %
basal/ background	29.9 ± 0.9			basal	2.88 %
SAM/ background	21.2 ± 0.3			SAM	1.60 %
AOAA/ background	38.0 ± 0.5			AOAA	1.36 %

Using HconCBS, 10 compounds were identified as primary hits (0.33 % hit rate, Fig. 4C, Supplementary Fig. 5). The hit compounds identified by HconCBS did not have a thiol group in their structure and were not simultaneously identified as hits by the AzMC assay (Supplementary Fig. 6). From absorbance scans (412 nm) performed before the enzymatic reaction, 101 compounds absorbed above the cutoff calculated using modified Tukey's Fences (Fig. 4D). Notably, these compounds did not affect the selection of primary hits because CBS activators decrease the absorbance readout in HconCBS. The hits identified by HconCBS were well below the autoabsorbance cutoff of $A_{412} = 0.4275$, with the highest autoabsorbance value being $A_{412} = 0.2423$ for WAY-613160. To demonstrate the reproducibility of HconCBS, a repeated screen of four library plates was carried out, and the CBS activator seleno-adenosyl-L-methionine (SeAM) was included as a positive control. The HconCBS assay indeed identified SeAM as a hit in all four plates (Fig. 4C). The four re-screened compound library plates contained 5 primary hit compounds: plate 10 - WAY-306132, plate 25 - WAY-611819, plate 26 - WAY-628153, and plate 28 - WAY-613160 and WAY-613225 (Fig. 4E). The compounds WAY-306132, WAY-611819, and WAY-628153 were rediscovered as hits upon re-screening, totaling hit confirmation in 3 out of 4 library plates.

To validate the activity of the primary hits from HconCBS, we repurchased all 10 compounds from a different vendor than Selleck which sourced the screening library. Using the HconCBS assay, the 4 library compounds WAY-224994, WAY-306132, WAY-331409, WAY-628153, and SeAM were confirmed (Fig. 4F). Concentration-response curves were constructed (Fig. 4G, CBS), showing a gradual decrease of homocysteine by WAY-224994, WAY-306132, WAY-331409, WAY-628153, SeAM, and SAM. However, protein-free controls suggested that the library compounds have homocysteine-depleting activity (Fig. 4G, no CBS).

Finally, the effects of WAY-224994, WAY-306132, WAY-331409, WAY-628153, SeAM, and SAM on CBS were assessed using AzMC as a complementary reaction (Fig. 4H). While the library compounds did not increase the amount of hydrogen sulfide produced by CBS and WAY-628153 even acted as an inhibitor, SAM and SeAM remained consistent CBS activators. In summary, including SeAM demonstrated that the HconCBS assay can robustly discover true CBS activators.

4. Discussion

In the present paper we introduce a new activity assay for human CBS based on the consumption of its substrate homocysteine. The assay was standardized for reaction and detection times and was automated using a conventional optical plate reader and pipetting robots. HconCBS was capable of quantitative pharmacology and determining the pAC_{50} of SAM. The performance parameters of HconCBS were quantified, passed the requirements for HTS, and an HTS run with evaluation of hit compounds was showcased.

Inverted readout for the discovery of activators. In contrast to the studies searching for CBS inhibitors, in fluorescence-based CBS activation assays, enhanced product formation results in rising signal

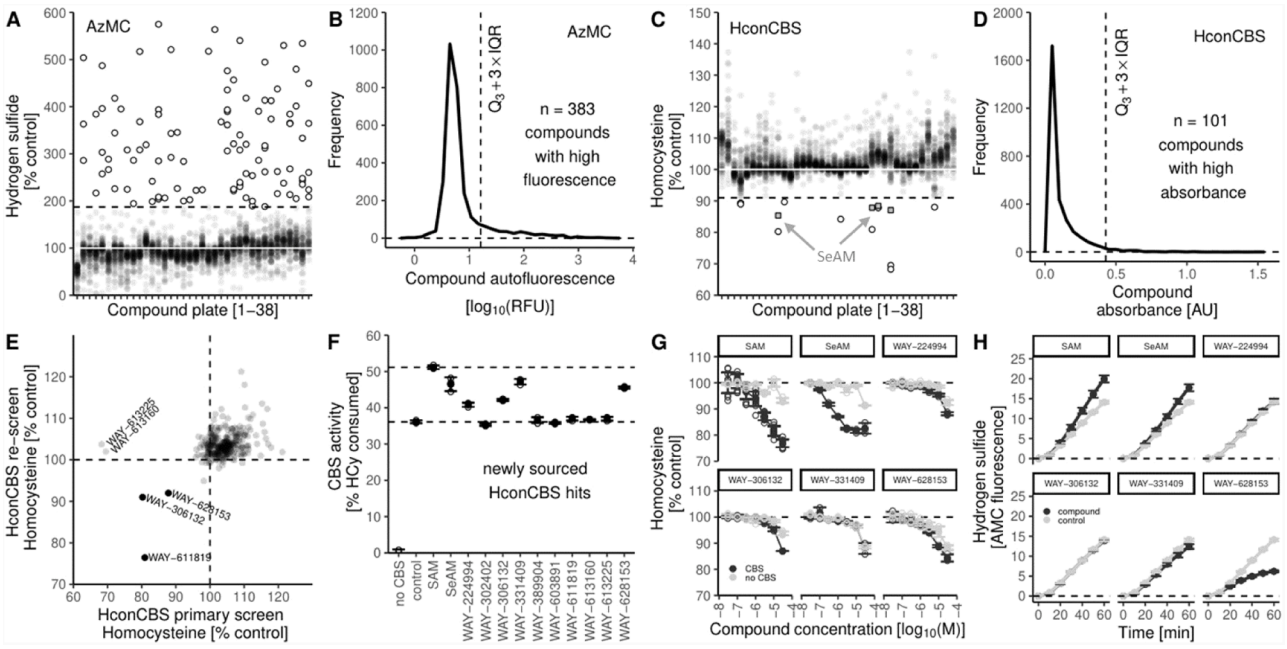

Fig. 4. High-throughput screening and hit confirmation. A) High-throughput screening of the Express-Pick Library against CBS using the AzMC assay. The white horizontal line represents 100 % control CBS activity, and the horizontal dashed line represents the mean of activation by SAM across all plates. Compounds above the SAM average are considered hits and are shown as hollow points. The y-axis was truncated to exclude extreme values. B) To filter potentially false positive hit compounds, the assay plates were scanned using $\lambda_{ex/em}$ = 365 nm/450 nm after addition of the compounds to $hCBS^{\Delta 516-525}$, when AzMC was still absent. A cutoff (vertical dashed line) for high autofluorescence was calculated using modified Tukey's Fences. C) High-throughput screening of the Express-Pick Library against CBS using the HconCBS assay. The white horizontal line represents 100 % control CBS activity, and the horizontal dashed line represents the mean of activation by SAM across all plates. Compounds below the SAM average are considered hits and are shown as hollow points. In a re-screen of four plates, seleno-adenosyl-L-methionine (SeAM, grey squares) was added to each plate as a positive control. D) To filter potentially false negative hit compounds, the assay plates were scanned using λ = 412 nm absorbance after addition of the compounds to $hCBS^{\Delta 516-525}$, when DTNB was still absent. A cutoff (vertical dashed line) for high autoabsorbance was calculated using modified Tukey's Fences. E) *Re*-screen of plates 10, 25, 26, and 28 using the HconCBS assay. The dashed lines represent 100 % control CBS activity, and black points with text labels mark re-discovered hits. F) Hits from the HconCBS (n = 10) were re-purchased from a different source and re-tested (30 μM) using the HconCBS assay. SAM was included as a reference and SeAM as positive control. The horizontal lines represent the means of control and SAM. G) The 4 confirmed HconCBS hit compounds were re-tested in concentration series using the HconCBS assay. Control reactions without CBS were included to reveal potential interference of the compound samples with the assay reagents. H) HconCBS hits and SeAM (10 μM) were tested using the AzMC assay and CBS activity was continuously measured as hydrogen sulfide production.

intensity, which may be difficult to detect if the compound has autofluorescent properties. In fact, all hit compounds identified by the fluorescence-based AzMC assay were autofluorescent. The colorimetric readout in the HconCBS assay is a solution to this as increased CBS activity consumes homocysteine and decreases the readout signal. Therefore, increased compound absorbance in HconCBS does not increase the false positive hit rate, and the hits identified by HconCBS did not have high absorbance. Moreover, the inverted readout considerably reduced the primary hit rate (10/3010 compounds) of potential CBS activators in primary screening compared to a fluorescence-based readout (141/3010 compounds).

HTS hit compounds identified by HconCBS. Upon re-purchase of the 10 primary hits from a different vendor than the screening library, 4 were confirmed by HconCBS. The non-confirmed compounds may have emerged as hits from the library due to a difference in quality of the screening compound sample. For instance, the compound WAY-611819 was re-discovered upon repeated screening, but a sample from a new source had no activity. On the other hand, the compounds WAY-306132 and WAY-628153 were re-discovered in re-screening, and were active from a new source. Unfortunately, all 4 confirmed compounds also decreased homocysteine in the absence of CBS. This effect may have resulted from impurities introduced during compound synthesis such as metals, from direct reactivity of library compounds with thiols, or from redox activity [24]. False positive hits caused by thiol reactivity are an inherent difficulty of CBS biology, as both the canonical and accessory reactions use homocysteine.

Another challenge in discovering true activators is the complexity of the allosteric mechanism of CBS, which involves the reorganization of the counterposed regulatory domains and thus exposing the catalytic sites for access to substrates [23]. It is possible that the Express-Pick Library simply does not contain allosteric activators of CBS. Considering that the drug-like chemical space was estimated to contain 10^{60} compounds [25], the 3010 screened compounds represent a small sample. We successfully miniaturized the HconCBS assay to increase screening throughput, enabling the search for CBS activators to be extended to a much larger compound library.

Nevertheless, when spiking four library plates with SeAM and performing a re-screen, the HconCBS assay consistently detected SeAM as a CBS activator. In an experiment with control reactions without CBS, SeAM had a substantially larger effect in the presence of CBS. Taken together, the repeated detection of SeAM supports the ability of HconCBS to discover true CBS activators given they are present in the screening library.

Previous high-throughput screening campaigns. To date, several HTS campaigns against CBS were concluded [15,17,26]. Zhou et al. used the more complicated tandem-microwell assay for capturing hydrogen sulfide with Ellman's reagent [17], Druzhyna et al. used the fluorescent AzMC hydrogen sulfide probe [15], whereas Niu et al. used a fluorescent 7-diethylamino-3-(4-maleimidophenyl)-4-methylcoumarin (CPM) probe to measure methanethiol released from methylcysteine [26]. However, all these studies focused on identifying CBS inhibitors, which corresponds to an inhibition in signal development, which is less affected by high compound autofluorescence.

Detection of CBS inhibitors. While designed to detect activators of

CBS, the HconCBS is also able to detect inhibitors. Notably, the effect of AOAA was robustly detectable 10 h after adding the stop solution. Since compounds with high absorbance at 412 nm may appear as false positive inhibitors of CBS, we also present an approach for library pre-scanning and flagging of potentially interfering compounds. In any case, inhibitors or activators of CBS identified by HconCBS should be verified using complementary reactions such as hydrogen sulfide production. Conversely, HconCBS may be used as a complementary secondary assay in settings where primary screening was performed using different readouts.

Use for different homocysteine- and thiol-utilizing enzymes. HconCBS may be applicable beyond CBS, for example in studies involving other enzymes that use homocysteine as substrate. Examples include methionine synthase (MS) [27] which uses methyl-THF and homocysteine as substrates to produce methionine and tetrahydrofolate, and S-adenosyl homocysteine hydrolase (SAHH)[28] which uses S-adenosyl-L-homocysteine and water to produce homocysteine and adenosine. SAHH also catalyzes the reverse reaction in which homocysteine would be a substrate measured with the proposed assay. HconCBS may also be adapted for high-throughput screening of enzymes utilizing other thiol substrates. For example, the activity of peroxiredoxin which catalyzes the oxidation of thioredoxin dithiol to thioredoxin disulfide was previously measured using Ellman's reagent [29]. Similarly, the oxidation of cysteamine to hypotaurine by cysteamine dioxygenase was also measured using Ellman's reagent [30].

Limitations. HconCBS is limited to reactions that do not produce thiols. For example, the CBS-catalyzed reaction between cysteine and homocysteine produces hydrogen sulfide; Ellman's reagent would react with all substrates and the product, generating overlapping signals and losing specificity for substrate consumption. Moreover, although the elimination of optically interfering compounds in the selection of primary hit activators is an advantage of HconCBS, it is counterbalanced by the risk of missing true activators among compounds with high absorbance (false negatives). Furthermore, there are 21 (0.7 %) thiol-containing compounds in the Express-Pick Library which might react with Ellman's reagent and increase the readout intensity, potentially becoming false negative activators or false positive inhibitors. The reactivity of compounds with Ellman's reagent will depend on the substituents surrounding the thiol group and on the tested concentration. Protein- and substrate-free reactions containing thiol compounds and Ellman's reagent should be used as controls to determine their interference with Ellman's reagent.

5. Conclusion

Taken together, we present a new activity assay for human CBS with parameters optimized for automated HTS. Utilizing the substrates serine and homocysteine, and detection by Ellman's reagent, the assay is highly cost-efficient. By eliminating interference from compound autofluorescence, the assay is expected to improve the discovery success of new compounds activating CBS which can subsequently be developed into treatment for hyperhomocysteinemia.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used ChatGPT-4o (OpenAI) in order to improve language and readability. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

CRediT authorship contribution statement

Dalibor Nakládal: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Rick Oerlemans:** Writing – review & editing, Visualization, Methodology, Investigation. **Miroslava Molitorisová:** Writing – review & editing, Investigation. **Nikola Chomaničová:** Writing – review & editing, Investigation. **Gabriel Zorkócy:** Writing – review & editing, Validation, Investigation. **Christina Yoseif:** Writing – review & editing, Validation, Investigation. **Adrianus Cornelis van der Graaf:** Writing – review & editing, Supervision, Resources, Conceptualization. **Stanislav Stuchlík:** Writing – review & editing, Resources. **Guido Krenning:** Writing – review & editing, Supervision, Conceptualization. **Matthew R. Groves:** Writing – review & editing, Validation, Supervision, Resources. **André Heeres:** Writing – review & editing, Methodology. **Zdenko Levarski:** Writing – review & editing, Validation, Supervision. **Ján Kyselovič:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Rob H. Henning:** Writing – review & editing, Validation, Supervision, Formal analysis, Conceptualization. **Leo E. Deelman:** Writing – review & editing, Validation, Supervision, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Dalibor Nakladal reports financial support was provided by Slovak Academic and Scientific Programme. Dalibor Nakladal reports financial support was provided by Slovak Research and Development Agency. Dalibor Nakladal reports financial support was provided by Ministry of Education, Research, Development and Youth of the Slovak Republic, VEGA. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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