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Emerging therapeutic approaches to cystathionine beta-synthase-deficient homocystinuria

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Abstract

Cystathionine beta-synthase (CBS)-deficient homocystinuria (HCU) is the most common inborn error of sulfur amino acid metabolism. The pyridoxine non-responsive form of the disease manifests itself by massively increasing plasma and tissue concentrations of homocysteine, a toxic intermediate of methionine metabolism that is thought to be the major cause of clinical complications including skeletal deformities, connective tissue defects, thromboembolism and cognitive impairment. The current standard of care involves significant dietary interventions that, despite being effective, often adversely affect quality of life of HCU patients leading to poor adherence to therapy and inadequate biochemical control with clinical complications. In recent years, the unmet need for better therapeutic options has resulted in development of novel enzyme and gene therapies and exploration of pharmacological approaches to rescue CBS folding defects caused by missense pathogenic mutations. Herein, we review scientific evidence and current state of affairs in development of emerging approaches to treat HCU.

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Author contribution

TM: conceptualization, visualization, writing – original draft, writing – review & editing; VK: writing – original draft, writing – review & editing; WDK: writing – original draft, writing – review & editing.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in https://www.guidetopharmacology.org/ and are permanently archived in the Concise Guide to Pharmacology 2021/22 (Alexander et al., 2021).

Conflict of interest statement

TM is inventor on patents related to pegtibatinase (e.g. US patents 9,034,318 and 9,243,239). TM provides *ad hoc* consulting services to Travere Therapeutics. VK declares that Charles University-First Faculty of Medicine received partial reimbursement from Orphan Technologies for the analysis of mouse tissues in 2014–2017. VK does not have any other conflict of interest. WDK is an inventor on a pending patent related to CBS gene therapy.

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Keywords

Cystathionine beta-synthase; homocystinuria; enzyme therapy; gene therapy; pegtibatinase; proteasome inhibitors; chaperones

1. Cystathionine beta-synthase-deficient homocystinuria

Increased urinary excretion of methionine (Met) and homocystine, an oxidized disulfide form of the non-proteinogenic amino acid homocysteine (Hcy), was first described six decades ago in individuals with cognitive impairment and marfanoid features (Carson et al., 1963). Subsequent study demonstrated a lack of cystathionine beta-synthase (CBS) activity in liver biopsies from these individuals and thus they were described as having CBS-deficient homocystinuria (HCU) (Mudd et al., 1964). CBS is a pyridoxal-5'-phosphate (PLP)-dependent heme-containing protein, which is allosterically regulated by S-adenosylmethionine (SAM) (reviewed in details in (Zuhra et al., 2020)). As illustrated in Figure 1, CBS catalyzes an irreversible step in the conversion of the essential amino acid Met to another proteinogenic sulfur amino acid cysteine (Cys) by condensing Hcy with serine forming cystathionine. In addition, CBS is one of five known enzymes producing hydrogen sulfide (H_2S) using alternative substrates (Majtan, Krijt, et al., 2018). Therefore, lack of CBS activity in HCU leads to accumulation of metabolites above the block (such as Hcy, S-adenosylhomocysteine (SAH), S-adenosylmethionine (SAM) and Met) and deficiency of metabolites below the block (such as cystathionine and Cys). The role of individual metabolic changes in the pathogenesis of HCU is mostly unknown; however, accumulation of Hcy is considered the key factor in the development of clinical complications (see below).

HCU is inherited as an autosomal recessive trait with highly varying population frequencies. The point estimates of HCU frequency worldwide range from ~1:100,000 in newborn screening (NBS) programs, ~1:122,000 in clinically ascertained cohorts and ~1:263,000 in genetic epidemiological studies (Weber Hoss et al., 2020). As of August 2022, the ClinVar database contained 949 CBS variants (out of which more than three quarters were missense variants) with 166 and 91 being classified as pathogenic and likely pathogenic, respectively (https://www.ncbi.nlm.nih.gov/clinvar/?gr=0&term=cbs%5Bgene%5D&redir=gene). Pathogenicity of variants can be inferred from their presence in clinically affected individuals, estimated from CBS variant effect map (Sun et al., 2020) or determined experimentally by assessing their activity and/or conformation after expression in heterologous systems (Kozich et al., 2010; Melenovska et al., 2015; Singh et al., 2007). The majority of missense CBS mutations cause protein misfolding, which lead to either rapid degradation of misfolded polypeptides by proteasome machinery or formation of high molecular weight aggregates suggesting that HCU is a conformational disease amenable to correction by chaperones (Figure 2) (Kopecka et al., 2011; Kozich et al., 2010; Melenovska et al., 2015; Singh et al., 2007).

Clinical consequences of pathogenic *CBS* variants are highly variable in respect to affected organs, the onset of symptoms, diagnosis delay and response to therapy. The penetrance

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of biallelic *CBS* variants is often not known and their pathogenicity is typically inferred from their observation in clinically symptomatic HCU patients. This notion is supported by the fact that homozygosity for the most common pathogenic c.833C>T (p.I278T) allele is much lower than expected based on population-based molecular epidemiological studies. The discrepancy may be explained by missed diagnosis in mildly affected patients or by much lower penetrance with no clinical phenotype (Skovby et al., 2010). One established factor underlying clinical severity is the pyridoxine responsiveness. It was originally defined as a disappearance of non-protein-bound Hcy from plasma after a loading test with the PLP precursor pyridoxine (vitamin B_6) (Barber & Spaeth, 1969), allowing the classification of HCU patients as being pyridoxine responders or non-responders. The seminal study of 629 HCU patients showed an inverse correlation of clinical severity with the level of pyridoxine responsiveness (Mudd et al., 1985). Furthermore, a recent analysis of a non-overlapping cohort of 328 HCU patients confirmed these observations and refined classification of HCU patients into four groups of pyridoxine responsiveness: non-responders, partial, full and extreme responders (Kozich et al., 2021). These four groups exhibited a gradient of clinical sequelae. In general, the non-responsive patients manifest in early childhood by combination of thromboembolia, connective tissue defects (marfanoid habitus, osteoporosis, lens dislocation) and complications associated with central nervous system (learning difficulties, cognitive impairment, behavioral changes and seizures). At the other end of phenotypic spectrum, the extreme responders manifest in (late) adulthood predominantly by thromboembolia without complications in other organs.

The pathophysiology of HCU is not completely understood and multiple mechanisms likely contribute to the disease progression and development of clinical symptoms (Grieco, 1977; Jakubowski, 2020; McCully, 2009). Hcy with its free sulfhydryl group is a highly reactive, redox active compound with potential pathological mechanisms involving formation of reactive oxygen species, protein modification, induction of unfolded protein response and endoplasmic reticulum stress (Perla-Kajan et al., 2007). These processes contribute to endothelial cell dysfunction and platelet activation eventually leading to arteriosclerosis, thrombus formation and vascular occlusion (Dayal et al., 2001; Hadi et al., 2005). Furthermore, accumulation of Hcy results in formation of excitotoxic homocysteic acid, a potent agonist of glutamatergic NMDA receptors, and Hcy thiolactone, a potent protein modifier. Homocysteic acid has also been shown to promote bone growth (Clopath et al., 1976), thus potentially contributing to marfanoid habitus typical for HCU, and to stimulate NMDA receptors (Folbergrova, 1974), thus potentially explaining mechanism of seizures in HCU patients. NMDA receptors may also be stimulated by an excess of SAH (Kožich et al., 2022). Unlike Hcy, which modifies sulfhydryl groups of Cys residues and consequently, S-homocysteinylation is a reversible event, Hcy thiolactone reacts with free amino groups of protein lysine residues causing irreversible N-homocysteinylation (Perla-Kajan et al., 2007). Protein N-homocysteinylation leads to structural changes, protein aggregation and loss of function. Specifically, modification of fibrinogen by Hcy thiolactone increases resistance to fibrinolysis and thus may contribute or explain pro-thrombotic HCU phenotype (Sauls et al., 2006). Furthermore, N-homocysteinylation impairs collagen cross-linking in mouse model of HCU thus explaining, at least in part, the underlying mechanism of skeletal and connective tissue abnormalities characteristic for HCU (Perla-Kajan et al., 2016). The role

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of imbalance in other sulfur metabolites, such as Cys, SAH or hydrogen sulfide (H_2S), in pathophysiology of HCU is even less understood than that of Hcy. Recent study observed a number of changes in sulfur metabolites in HCU patients (Kožich et al., 2022). Decreased Cys levels in plasma and tissues contribute to oxidative stress and pathophysiology of connective tissue defects in HCU (Majors & Pyeritz, 2000). Interestingly, plasma levels of H_2S in HCU patients were not decreased and in some cases were slightly elevated (Kožich et al., 2022); however, it is unclear how CBS deficiency affects intracellular H_2S concentration, protein sulfhydration and signaling. Endothelial dysfunction was found to associate with increased levels of SAH in CBS-deficient mice potentially contributing to vascular phenotype characteristic for HCU (Dayal et al., 2001). In addition, increased SAH concentrations inhibit cellular methylation reactions and contribute to cognitive impairment (James et al., 2002; Kennedy et al., 2004).

Diagnosis of HCU is based on demonstrating the typical metabolite profile (i.e. markedly elevated concentrations of plasma total Hcy (tHcy; median value 226–262 μM in the four responsiveness categories, compared to less than 15 μM in controls) and Met and decreased to low normal plasma cystathionine), decreased CBS activity in fibroblasts or plasma (Alcaide et al., 2015) and/or biallelic presence of pathogenic variants in the *CBS* gene. Newborn screening using methionine and methionine/phenylalanine ratio as primary markers has a lower sensitivity and ascertains mostly pyridoxine non-responders (Keller et al., 2019) as milder forms of HCU do not exhibit sufficiently high Met. Prenatal testing in families at risk can be performed by *CBS* gene analysis (Morris et al., 2017).

2. Current standard of care for HCU

In 2017, a comprehensive systematic review of the literature resulted in publication of guidelines for the diagnosis and management of HCU (Morris et al., 2017). For the early-diagnosed patients, therapeutic target is to avoid complications associated with HCU, while maintaining normal growth, nutrition and aiming for the minimal impact on patients' quality of life. For late-diagnosed patients, the goal is to prevent further worsening and exacerbation clinical symptoms, particularly thromboembolic events. The recommended biochemical target to prevent clinical complications is to maintain plasma tHcy below 100 μM in pyridoxine non-responders and partial responders or below 50 μM in full and extreme responders. To achieve this threshold, different strategies have been devised primarily considering the severity of the disease, which correlates with pyridoxine responsiveness (Kozich et al., 2021; Morris et al., 2017). The recommended approach for newly diagnosed patients is to correct the possible secondary deficiency of folates and vitamin B_{12} first and then to perform a standard pyridoxine responsiveness test (see below), results of which will guide further therapy. Extreme and full responders are treated by titrating down the daily dose of pyridoxine that still achieves a good biochemical control (i.e. plasma tHcy below 50 μM). Partial responders require, in addition to pyridoxine administration, Met restriction and/or betaine. Non-responders do not benefit from pyridoxine intake and the mainstay treatment for this group of HCU patients is a moderate to severe dietary Met restriction combined with betaine in cases were the 100 μM tHcy threshold cannot be achieved solely by Met/protein restriction.

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2.1 Pyridoxine

PLP, the active form of vitamin B_6 and the catalytic cofactor of CBS (Figure 1), is enzymatically produced in cells from several dietary B_6 vitamers including pyridoxine (Stach et al., 2021). The first reports on the biochemical response to pharmacological doses of pyridoxine were published in 1967 (Barber & Spaeth, 1967; Turner, 1967). Subsequent studies showed that about half of HCU patients are at least partially pyridoxine responsive (Mudd et al., 1985) and doses ranging from less than 1 mg/kg/d up to10 mg/kg/d are needed to achieve an adequate biochemical response (Kozich et al., 2021). Extreme and full pyridoxine responsiveness is defined as a decrease of plasma tHcy below 50 μM threshold on doses less and more than 1 mg/kg/d, respectively, during the standardized 6-weeks pyridoxine loading test (Kozich et al., 2021; Morris et al., 2017). In neonates, the test is shortened to 2 weeks with 100 mg/d. Partial responders decrease their plasma tHcy levels by at least 20% from the average of two measurements before the test, while non-responders exhibit minor changes in plasma tHcy.

Genetic analyses showed a consistent association of specific CBS variants with full or extreme responsiveness (e.g., p.P49L, p.A114V and p.I278T) or non-responsiveness to pyridoxine (e.g., p.R125Q, p.E176K, p.T191M, p.T262M and p.G307S). In contrast to this consistent genotype-to-pyridoxine responsiveness correlation in HCU patients, the insight gained from using *in vitro*, bacterial or eukaryotic expression systems and animal models of HCU yielded discrepant results (Chen et al., 2006; Gupta et al., 2019; Gupta et al., 2017; Majtan, Pey, Ereno-Orbea, et al., 2016). Due to the discrepancy between clinical experience and research data, pyridoxine responsiveness in HCU remains mechanistically unclear. Potential molecular mechanism may involve (1) decreased affinity of CBS variants to the PLP cofactor resulting in a sub-optimally saturated and consequently less-active enzyme or (2) conformational instability and misfolding induced by pathogenic missense mutations preventing PLP binding to CBS variants. Therefore, pyridoxine, a precursor of PLP, may actually function as a pharmacological chaperone (Figure 2) (Majtan, Pey, Ereno-Orbea, et al., 2016).

2.2. Met-/Protein-restricted diet

Met is an essential amino acid present in natural proteins (between ~0.5–2 g/100g of plant protein and 2–3 g/100 g of animal protein). The US Food and Agriculture Organization (FAO) determined the daily safe level of Met intake of 10.4 mg/kg/day in adults, which varies with age and sex.

Dietary Met restriction to limit production of Hcy is required in pyridoxine non-responsive patients and some partial responders, comprising together roughly half of HCU patients worldwide. Met restriction can be typically achieved only by decreasing substantially the amount of natural protein in the diet (to about half of the FAO age- and sex-adjusted safe protein intake with a large variability among patients) (Kozich & Stabler, 2020). Severe protein restriction does not allow maintaining nitrogen balance and, therefore, must be compensated by administering Met-free and Cys-enriched mixture of other amino acids and micronutrients.

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Dietary Met restriction is demanding as the patients or their caregivers have to limit the menu to weighed low-protein foods and maintain daily dietary records. Adherence to diet differs with the age of patients and age at diagnosis. In general, a low protein diet is more challenging to start in older children and adults who are already used to high protein foods, such as meat, eggs and dairy products. Patients and/or their caregivers also reported problems taking the amino acid mixtures due to their poor palatability (Morrison et al., 2021). Despite these problems, dietary management is an efficient standard of care for treating pyridoxine non-responsive HCU (Morris et al., 2017).

2.3. Betaine

Betaine (N,N,N-trimethylglycine) is a naturally occurring osmolyte and a donor of methyl groups for a conversion of Hcy to Met in the liver by betaine:homocysteine methyltransferase (Figure 1) (Pajares & Perez-Sala, 2006). Betaine is also produced endogenously from choline metabolism. Betaine is recommended as an adjunctive therapy to HCU patients who require Met-/protein-restriction and cannot achieve good biochemical control with diet only. The guidelines recommend two daily doses of 50 mg/kg/day and 3 g per day in children and adults, respectively (Morris et al., 2017). Recent analysis showed that the median dose in 54 pyridoxine non-responsive HCU patients was 98 mg/kg/day (range 5–316 mg/kg/day) with a good safety profile (Mutze et al., 2022). As betaine further increases already elevated plasma Met concentrations, its dose needs to be adjusted to maintain patients' plasma Met below 1,000 μM to avoid the rare occurrence of cerebral edema (Morris et al., 2017).

2.4. Other adjuvant therapies

Some additional pharmacological or dietary treatment options may be relevant for HCU patients and were reviewed elsewhere (Morris et al., 2017). These include monitoring and therapy of deficiencies of micronutrients, provision of Cys, balancing energy intake and prevention of thromboembolism during intercurrent illness, surgery and travel. Folate and vitamin B_{12} deficiency was described in patients at diagnosis, most likely due to an increased demand for these cofactors in the remethylation pathway (Figure 1). Patients with HCU should be monitored for levels of these two B-vitamins and possible deficiency should be corrected. Importance of Cys supplementation remains unclear with recent preclinical data showing no additional benefit of 3-times increased Cys intake compared to normal diet on metabolic profile and phenotype of HCU mouse models (Park, Bublil, et al., 2020).

3. Emerging therapeutic approaches to HCU

The current therapeutic approach to HCU relying on diet and nutrient supplementation can be effective in some patients, but is often plagued by poor compliance and/or insufficient metabolic control resulting in a significantly impaired quality of life. Furthermore, current treatment goals are focused on what is achievable and not necessarily what is optimal for the patients. Specifically, lowering plasma tHcy levels beyond recommended threshold values for a particular group of HCU patients stably maintained without substantial spikes has potential to further improve prospects on disease progression and clinical outcomes. In addition, there is a strong unmet need among health care providers and HCU patient

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communities for more efficacious and less burdensome therapeutic options (Morrison et al., 2021). Therefore, novel therapeutic approaches for HCU are being explored in preclinical models, with at least three currently being evaluated in human clinical trials. These emerging therapeutic approaches to HCU target one of three critical aspects related to development and progression of disease: (1) interfering with methionine uptake to decrease Hcy production, (2) increasing Hcy degradation in the bloodstream and (3) stimulating (or restoring) residual CBS activity primarily in the liver (Figure 1). Below we will discuss progress with these emerging therapeutic approaches to HCU.

3.1. Reduction of methionine uptake

As described earlier, the current mainstay strategy in treating CBS deficiency is restricting the amount of Met in the diet. The effectiveness of a low Met diet has been clearly shown in both humans and mouse models of HCU (Gupta et al., 2014; Yap & Naughten, 1998). However, since dietary control of Met without starving for total protein intake can be difficult, strategies are being developed to specifically reduce the amount of Met that is absorbed from food.

3.1.1. SYNB1353—SYNB1353 is being developed as a collaboration of Synlogic and Ginkgo Bioworks to provide a potential alternative to protein/Met-restricted diet. It is a genetically engineered probiotic, non-colonizing strain of *Escherichia coli* Nissle 1917, which is designed to efficiently metabolize Met within the gastrointestinal (GI) tract, thus preventing its absorption and conversion to Hcy. It follows the same concept as previously reported synthetic live bacterial therapeutic SYNB1618 for phenylketonuria, an inborn error of phenylalanine metabolism (Isabella et al., 2018; Puurunen et al., 2021). The development and the first pre-clinical data for SYNB1353 were presented at the 14th International Congress of Inborn Errors of Metabolism (Perreault et al., 2021). The genetic engineering of SYNB1353 involved both the modification of the Met catabolism as well as Met uptake. Degradation of Met is achieved by the modified *Streptomyces sp.* 590 Met decarboxylase (MetDC) carrying two additional missense mutations Q70D and N82H, which were selected via high throughput screening of metagenomic and protein engineered libraries. The final candidate used in SYNB1353 produced up to 4-fold more 3-methylthiopropylamine (used as a biomarker of the strain activity) and carbon dioxide from Met than the MetDC prototype. Improving Met uptake was performed via metagenomic sourcing of low affinity symporter MetP and protein engineering of the high affinity ABC transporter MetNIQ. Co-expression of MetP from *Flavobacterium segetis* with the wildtype MetDC resulted in almost 3-fold increase in Met degradation. In addition, *yjeH* gene that encodes a Met/branched chain amino acid exporter was deleted to prevent the release of Met into GI tract after its import. The genes encoding MetDC and MetP are chromosomally integrated under the control of isopropyl-β-D-1-thiogalactopyranoside (IPTG)-inducible P_{tac} promoter. For biocontainment, it is assumed that the same strategy was applied as for SYNB1618, i.e. deletion of the *dapA* gene encoding 4-hydroxy-tetrahydropicolinate synthase, which renders the engineered strain unable to divide and survive without added diaminopimelate in the environment (Puurunen et al., 2021). SYNB1353 showed time-dependent decrease in number of colony-forming units in fecal pellets similar to unmodified parent strain and dose-dependent degradation of dietary Met in wild type mice. Concomitant administration of SYNB1353 with an oral

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dose of methionine in a nonhuman primate model of acute HCU reduced plasma Met and subsequently plasma tHcy area under the curve compared to vehicle-treated controls. Based on these preclinical results, mathematical modeling predicts a doubling of natural protein intake and expects up to 58% lowering of tHcy in mild/moderate to severe HCU following three times a day dosing of 10^{12} colony forming units. In July 2022, SYNB1353 entered Phase 1 clinical trial (ClinicalTrials.gov ID# NCT05462132) carried out at a single site in the USA. The SYNB1353 clinical trial is a randomized, placebo-controlled, double-blind study using a multiple-ascending dose design to assess safety, tolerability and pharmacodynamics of SYNB1353 in heathy volunteers. The results are expected by the end of 2022.

3.1.2. CDX-6512—CDX-6512 is being developed by Codexis and, like SYNB1353, targets Met degradation in the GI tract, but uses an enzymatic as opposed to a bacterial approach. Development and the first pre-clinical data were presented at the 14th International Congress of Inborn Errors of Metabolism (Skvorak et al., 2021). CDX-6512 is a GI-stable bacterial Met gamma-lyase (MGL), which was designed to be highly resistant to both the acidic conditions of the stomach and to proteases of the upper intestines. To achieve these properties, Codexis employed their CodeEvolver protein engineering platform utilizing artificial intelligence and machine learning to deconvolute role of each amino acid change. The process of MGL optimization included 12 iterative rounds of enzyme evolution with more than 27,000 variants screened for activity in simulated gastric and intestinal challenge. Single dose of CDX-6512 demonstrated a statistically significant suppression of plasma tHcy at 4 hours post whey protein challenge in Tg-I278T $Cbs^{-/-}$ HCU mouse model (Wang et al., 2005) compared to the vehicle-treated cohort in a dose-dependent fashion with the highest efficacy achieved at the highest dose of 148 mg/kg (45% decrease in tHcy). Interestingly, plasma Met was suppressed in a similar dose-dependent manner (up to 32% reduction compared to vehicle at the highest dose), although this decrease was not statistically significant. Furthermore, a single dose of CDX-6512 significantly reduced plasma Met in a dose-dependent manner up to 70% (at the highest dose of 370 mg/kg) following a peptone challenge. CDX-6512 is currently in a pre-IND development and highlights the prospects of a GI-stable Met-degrading enzyme as a potential therapy for HCU.

3.1.3. Erymethionase—Erymethionase developed by Erytech Pharma essentially relies on the same principle as CDX-6512, but the degradation of Met occurs in bloodstream as opposed to the GI tract. Erymethionase was originally developed as a novel potential therapy for Met-dependent cancers (Gay et al., 2017). However, since Met is a precursor of Hcy, whose accumulation is responsible for clinical complications in HCU, erymethionase has been evaluated as a substrate reduction therapy in HCU. Development and the first pre-clinical data were presented at the 13th International Congress of Inborn Errors of Metabolism (Dufour et al., 2017). Delivery of recombinantly expressed and purified wild-type MGL from *Pseudomonas putida* is achieved by loading the enzyme into red blood cells using company's proprietary Erycaps technology platform by reversible hypotonic dialysis (Gay et al., 2017). Like CBS, MGL is a PLP-dependent enzyme, but has a relatively low affinity for its catalytic cofactor (Ito et al., 1976). Therefore, PLP supplementation

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increased maximal efficacy of polyethyleneglycol (PEG)-modified MGL up to 6-fold, while PEGylation decreased immunogenicity 10- to 10,000-fold depending on the extent of modification and prolonged plasma half-life up to 12-fold (Sun et al., 2003). All these insufficiencies of bacterial MGL were resolved by its encapsulation into red blood cells. Erythrocytes synthesize PLP from its precursor pyridoxine and thus represent a natural pool of PLP. Encapsulation into erythrocytes also resolved issue of immunogenicity and short half-life of unmodified MGL (Gay et al., 2017). Single dose administration of erymethionase to Tg-I278T $Cbs^{-/-}$ HCU mice resulted in more than 40% and 28% reduction of tHcy 24 h and 6 days after administration, respectively. Parenterally supplemented pyridoxine resulted in non-significant better reduction than orally provided, while oral co-administration of betaine achieved significantly better further reduction of plasma tHcy levels compared to those in mice treated only with erymethionase. Current status of the program is unknown.

3.2. Homocysteine-degrading enzymes

As buildup of Hcy in bloodstream represents a biochemical hallmark of HCU, enzymatic degradation of accumulated Hcy in the bloodstream has been explored as a new therapeutic approach to HCU.

3.2.1. Pegtibatinase—Pegtibatinase, also known as PEG-CBS, OT-58 or TVT-058, is the most advanced program of enzyme therapy for HCU. It was developed by Drs. Jan Kraus and Tomas Majtan (University of Colorado Anschutz Medical Campus) in collaboration with Orphan Technologies. In 2020, Retrophin completed acquisition of Orphan Technologies and shortly after the company rebranded globally to Travere Therapeutics, which continue with the clinical development of the program.

Pegtibatinase is a genetically engineered CBS protein that is given subcutaneously and metabolizes Hcy to cystathionine in the bloodstream. It is a recombinantly produced modified catalytic core (residues 1–413) of human CBS (hCBS) that is chemically conjugated with PEG chains (Bublil & Majtan, 2020). Use of catalytic core increases the efficiency of the enzyme by removing autoinhibitory SAM-binding domain, which in turn made the enzyme constitutively active and mostly dimeric. An additional p.C15S mutation improves these properties by preventing formation of inter-dimeric disulfide bridges and thus formation of higher order soluble multimers (Bublil et al., 2016). PEGylation with a linear 20 kDa N-hydroxysuccinimide ester-activated methoxyPEG resulted in modification of each CBS subunit by 5 PEG chains on average (Majtan, Park, et al., 2017), which improved the enzyme's half-life in circulation by about 10-fold (Bublil et al., 2016).

Pegtibatinase showed excellent efficacy during the pre-clinical testing in three different mouse models of HCU. Importantly, pegtibatinase treatment of HCU mice showed positive effects in the four key organ systems that are also affected in HCU patients: cardiovascular, skeletal, ocular and central nervous system. In the HO mouse model of HCU (Maclean et al., 2010), pegtibatinase showed linear, dose-dependent accumulation of CBS activity in plasma after single or repeated subcutaneous administration with maximal efficacy achieved with a dose as low as 8 mg/kg (Majtan, Bublil, et al., 2018). In CBS knockout (CBS

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KO) mice, which normally do not survive beyond day 29, subcutaneously administered pegtibatinase from two days of age, three times a week (7.5 mg/kg), almost completely prevented premature death of the treated CBS KO mice (Majtan, Hulkova, et al., 2017). The rescued survival of CBS KO mice by pegtibatinase was accompanied by a significant ~80% decrease of plasma tHcy and overall normalization of metabolic balance in liver, kidney and brain. In addition, administration of pegtibatinase completely prevented severe liver disease characterized by steatosis, hepatocellular necrosis, inflammation and swollen mitochondria (Majtan, Hulkova, et al., 2017). Pegtibatinase treatment entirely rescued bone mineralization and improved body composition of CBS KO as well as Tg-I278T $Cbs^{-/-}$ mice (Majtan, Park, et al., 2018). Pegtibatinase also prevented the onset or rescued already developed facial alopecia characteristic for the Tg-I278T $Cbs^{-/-}$ mouse model (Majtan, Jones, et al., 2018). In addition, pegtibatinase fully rescued the structure of ciliary zonule, which holds the lens in axis of the sight, compared to the untreated controls showing reduced density and length of the zonular fibers (Majtan, Jones, et al., 2018). Treatment with pegtibatinase also rescued endothelial dysfunction and corrected learning and cognitive deficit in Tg-I278T $Cbs^{-/-}$ mice (Majtan et al., 2019). Pegtibatinase achieved all these benefits on the background of standard mouse chow, i.e. unrestricted, normal Met intake. When evaluated against Met restriction and betaine supplementation (i.e. simulated current standard of care), pegtibatinase showed the same efficacy and actually achieved normalization of plasma tHcy on the background of 50% reduced Met intake compared to the normal control diet (Park, Hulkova, et al., 2020). Betaine showed much lower efficacy in decreasing plasma tHcy levels compared to pegtibatinase. Furthermore, pegtibatinase solely maintained the improved metabolic balance after betaine withdrawal including the periods of a simulated dietary non-compliance (by switching between high, normal and low Met diets) (Park, Hulkova, et al., 2020).

In January 2019, pegtibatinase entered Phase 1/2 clinical trial termed the COMPOSE study carried out at 7 sites in the USA (ClinicalTrials.gov ID# NCT03406611). The COMPOSE is a randomized, placebo-controlled, quadruple-blind dose escalation study evaluating the safety, tolerability, pharmacokinetics, pharmacodynamics and clinical effects of pegtibatinase in up to 40 HCU patients. Patients receiving subcutaneous doses of either pegtibatinase or placebo (randomized in 3:1 ratio) are eligible to continue in an open-label extension following the initial twelve-week blinded treatment period. In December 2021, Travere Therapeutics announced positive topline results from this ongoing trial and presented them at the 2022 annual meeting of the Society for Inherited Metabolic Disorders (Levy et al., 2022). In the cohort evaluating 1.5 mg/kg of pegtibatinase twice weekly (the highest dose cohort evaluated to date), pegtibatinase treatment resulted in a swift and sustainable reduction of plasma tHcy during the twelve-week course of treatment. Reduction of plasma tHcy by 55% in average compared to a pre-treatment levels maintained plasma tHcy below a clinically relevant 100 μM threshold (Morris et al., 2017). Pegtibatinase has been, in general, well-tolerated without any discontinuation due to treatment-related adverse events. A single case of moderate acute urticaria was reported as a serious adverse event, but resolved following a single dose interruption.

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3.2.2. Pegtarviliase—Pegtarviliase, formerly known as AGLE-177, AEB-4104 or ACN00177, developed by Aeglea Biotherapeutics is an engineered human cystathionine gamma lyase (CGL) modified to change its native substrate specificity from cystathionine to Hcy. Unlike pegtibatinase, pegtarviliase is capable to degrade *in vitro* both reduced and oxidized forms of Hcy (i.e. sulfhydryl homocysteine and disulfide homocystine) forming alpha-ketobutyrate, ammonia and either H_2S or thio-Hcy, which also serves as a substrate for pegtarviliase. The *in vivo* relevance of this unique specificity feature of pegtarviliase is unclear as over 80% of Hcy is protein-bound and only 10–15% is in a form of homocystine or Hcy-Cys mixed disulfide (Rasmussen & Moller, 2000). Therefore, various forms of Hcy must remain in a highly dynamic balance for any enzyme therapy (e.g. pegtibatinase mentioned above) targeting plasma Hcy to access the substrate and be efficacious.

Engineering of human CGL was performed by the team at the University of Texas at Austin headed by Drs. George Georgiou and Everett Stone following similar strategy as previously employed for engineering of the same enzyme to prefer Met or Cys over cystathionine for a development of biological therapies targeting a wide range of malignancies (Cramer et al., 2017; Stone et al., 2012). Therefore, pegtarviliase contains 8 missense point mutations p.E59I, p.S63L, p.L91M, p.R119D, p.K268R, p.T311G, p.E339V, p.I353S (with a resulting construct designated as CGL-ILMDRGVS), which increased the preference of the engineered construct towards Hcy by 60-fold compared to the wild type enzyme. PEGylation of CGL-ILMDRGVS with 5 kDa linear NHS-activated methoxy-PEG yielded pegtarviliase with an extended serum half-life.

Development and the first pre-clinical data on pegtarviliase were presented at the American Society for Human Genetics Annual Meeting 2020 (Daige et al., 2020). Dose levels of 1, 3 and 10 mg/kg administered subcutaneously twice per week were initially evaluated to rescue survival of CBS KO mice starting from D10 through D70. Treatment demonstrated more that 75% survival of CBS KO mice for all three tested doses compared to less than 20% of vehicle-treated controls at D30. Reduced mortality was accompanied by resolution of liver steatosis and alopecia in surviving animals. Three doses of 10 mg/kg pegtarviliase significantly reduced tHcy in plasma and brain by ~43% and ~87%, respectively, but failed to reach significance in liver. Treatment of CBS KO mice with subcutaneous dose of 10 mg/kg twice weekly from D10 to D169 resulted in a significant increase of their bone mineral density. The 4-weeks preclinical toxicology studies performed in Sprague-Dawley rats and Cynomolgus monkeys testing once weekly doses ranging from 6 to 40 mg/kg did not show any adverse findings and no observed adverse effect level was determined to be 6.67 mg/kg human equivalent dose.

In May 2021, pegtarviliase entered a multiple ascending dose Phase 1/2 clinical trial in HCU patients at one site in the USA, 2 in Australia and 5 sites in the United Kingdom (ClinicalTrials.gov ID# NCT05154890). The trial is anticipated to enroll 16–20 HCU patients aged 12 years or older. The primary endpoint is the safety and tolerability of pegtarviliase. Secondary endpoints include pharmacokinetic assessments as well as reduction in plasma tHcy levels. Patients will be dosed once weekly for four weeks (0.15 mg/kg IV or 0.15, 0.45 or 1 mg/kg SC), with four patients in each of four dosing cohorts and

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an option to include a fifth cohort as needed. The first clinical data are expected in the fourth quarter of 2022.

3.3. Strategies to stimulate or to restore residual CBS activity

As described above, current therapeutic approach of pyridoxine supplementation represents the safest and the most convenient avenue to increase residual CBS activity and subsequently reduced plasma Hcy levels. However, this approach works only for a certain subset of HCU patients. In this section, we will describe emerging strategies, which would either expand the group of responsive patients or target all HCU patients regardless of their *CBS* genetics.

3.3.1. Chemical and pharmacological chaperones—The vast majority of genetic alterations that cause CBS deficiency are missense mutations, in which a single amino acid is altered in the polypeptide sequence (Stenson et al., 2003). Missense substitutions most commonly affect underlying protein function by interfering with the process of protein folding, resulting in the protein being degraded *in vivo* by intracellular quality control mechanisms (referred to as the proteostasis network) (DePristo et al., 2005). In theory, the use of small molecules that can promote proper folding of mutant CBS polypeptides and thus rescue the enzyme activity of pathogenic missense CBS mutants, is a potential treatment for HCU. Small molecules, such as dimethylsulfoxide, ethanol, glycerol, betaine or trimethylamine-N-oxide, are chemical chaperones that typically do not interact specifically with the polypeptide, but rather act as osmolytes altering how water interacts with the protein. These interactions create a more permissive protein folding environment and allow for the protein to fold into its native-like state (Figure 2) (Bolen & Rose, 2008; Street et al., 2006). For mutant CBS proteins, exposure to chemical chaperones can increase stability, tetramer formation, and most importantly activity of several pathogenic CBS mutants (Kozich et al., 2010; Singh et al., 2007; Singh et al., 2010). In addition to their direct “chemical” action, they were also found to increase steady-state levels of molecular chaperones, such as DnaJ in a recombinant *E. coli* expression system, suggesting a possible indirect mechanism of action too (Majtan et al., 2010).

As action of chemical chaperones is often non-specific and requires high concentrations to achieve the desired effect, so-called pharmacological chaperones offer more targeted approach how to correct folding and rescue activity. Pharmacological chaperones often consist of enzyme cofactors, substrates, and other ligands that specifically bind to catalytic or allosteric cavities or other sites on the protein surface. They are though to act by stabilizing the native-like conformations and reducing degradation or aggregation of mutant CBS (Figure 2) (Bernier et al., 2004; Tran et al., 2020). It is thought that this “chaperoning” effect may be behind the phenomena of pyridoxine-responsive HCU (Barber & Spaeth, 1969; Carson & Carre, 1969; Morris et al., 2017). Heme, a non-catalytic CBS cofactor with unclear function, and its analogs were found to be essential for proper folding and maintenance of catalytic activity of wild-type CBS heterologously-expressed in *E. coli* (Majtan et al., 2008). Addition of heme arginate increased tetramer formation and activity of hCBS p.R125Q mutant recombinantly expressed in mammalian cells by impressive 600% and 900%, respectively (Melenovska et al., 2015). Lastly, as SAM, an allosteric

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activator of CBS, was found to kinetically stabilize regulatory domain and subsequently the entire polypeptide of WT and 8 studied purified missense pathogenic CBS mutants (Pey et al., 2013), SAM or its analog could act as pharmacological chaperones. Evaluation of SAM and SAH in a large set of missense pathogenic CBS mutant expressed in *E. coli* showed highly individual response by each mutant (Kozich et al., 2010). Assessment of SAM, SAH and sinefungin using purified wild-type CBS showed that SAM analogs have lower affinity and do not activate the enzyme, but substantially decreased rate of proteolysis and kinetically stabilized the enzyme (Majtan, Pey, & Kraus, 2016). Inhibitors of glycosphingolipid metabolism, such as isofagomine or migalastat, stabilize protein folding and facilitate proper trafficking of amenable mutants in Gaucher and Fabry diseases (Shayman & Larsen, 2014); therefore, competitive, reversible CBS-specific inhibitors could represent suitable pharmacological chaperones against HCU. Pharmacological inhibition of CBS activity represents a therapeutically attractive target for various cancers and Down Syndrome (Bhattacharyya et al., 2013; Panagaki et al., 2019; Szabo et al., 2013). Several screening campaigns failed to identify novel, potent CBS-specific inhibitor and therefore, aminooxyacetic acid (AOAA), despite being a non-specific inhibitor of many PLP-dependent enzymes, remains the widely used and most potent CBS inhibitor to date (Druzhyna et al., 2016; Niu et al., 2017; Thorson et al., 2013). Although AOAA was always assumed to act as an irreversible dead-end CBS inhibitor, recent report suggested that CBS substrate serine, but not H_2S-yielding alternative substrate Cys, can compete out and replace AOAA to form aminoacrylate reaction intermediate, thus allowing for progression of the CBS catalytic cycle (Petrosino et al., 2022). Although this novel observation warrants proof-of-concept study of AOAA serving as a pharmacological chaperone of amenable CBS mutants, new, truly competitive CBS-specific inhibitor lacking reactivity towards catalytic PLP cofactor would represent a more suitable approach.

Despite some promising data in heterologous expression systems, chemical/pharmacological chaperone approach has not produced any viable candidate for clinical testing so far. Perhaps, a change in research tools used so far to identify the promising candidates is necessary for this area to move forward, such as development of cell-based folding reporter assay in mammalian cells for high-throughput screening of chemical libraries or computer-aided in silico design of novel structures binding to catalytic cavity or allosteric pocket of the enzyme.

3.3.2. Proteasome inhibitors—Proteasome inhibitors (PIs) are drugs that work by inhibiting the proteasome, a molecular machine that essentially acts as the cellular garbage disposal to breakdown proteins. Bortezomib and other PIs are used in the clinic to treat humans with multiple myeloma and other cancers (Orlowski & Kuhn, 2008). Cells treated with PIs will accumulate misfolded and damaged proteins, resulting in proteotoxic stress that induces the production of molecular chaperones (heat shock proteins, e.g. HSP70) that can assist proteins in folding into their proper native state (Kim & Li, 1999; Lee & Goldberg, 1998). This was shown to be the case in *S. cerevisiae* where bortezomib was found to stimulate activity of several different mutant hCBS proteins (Singh et al., 2010). Later studies examined the effects of PIs in transgenic HCU mouse models lacking endogenous mouse CBS, but expressing various different hCBS mutant transgenes carrying

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pathogenic missense mutations derived from HCU patients. Much of the effort was focused on the Tg-I278T $Cbs^{-/-}$ model, which expresses hCBS p.I278T transgene (Wang et al., 2005). Initial work showed that a single intravenous injection of bortezomib (750 μg/kg) could increase steady-state levels of hCBS p.I278T that was associated with about 4-fold increase of CBS activity in liver compared to vehicle-injected animals (Singh et al., 2010). Follow up experiments showed that this effect could be made much more robust by delivering 490 μg/kg/day bortezomib by using subcutaneously implanted osmotic pump (Gupta et al., 2013). Specifically, median liver and kidney CBS activities in bortezomib-treated mice increased by about 13- and 57-fold, respectively; these functional consequences correlated wel with a substantial increase in the steady-state level of hCBS p.I278T protein in these tissues. More importantly, median serum tHcy dropped by almost 80% to 75 μM. In addition, orally-available PI ONX-0912 (Zhou et al., 2009) given at dose of 40 mg/kg/day orally was found similarly effective. One odd feature of these experiments was the finding of a significant mouse-to-mouse variability with treatment response. Some mice had their liver CBS activity levels similar to that of control mice expressing wild-type hCBS, while other mice showed no improvement. Global mRNA expression analysis using microarray analysis revealed that a robust response to PI treatment was associated with upregulation of SerpinA6 expression in the liver. Serpin6A encodes for a corticosteroid binding protein that is exported into the bloodstream; however, it remains unclear how SerpinA6 levels are mechanistically related to refolding of hCBS p.I278T.

Four additional missense hCBS mutant proteins have been evaluated for rescue by various PIs in transgenic HCU mice. Specifically, the Tg-S466L $Cbs^{-/-}$ model expressing hCBS p.S466L mutant treated with ONX-0912 had a 90% reduction of serum tHcy to 15 μM accompanied by a massive 52-fold increase of their liver CBS activity (Gupta et al., 2013). Similarly, Tg-R266K $Cbs^{-/-}$ mice expressing hCBS p.R266K mutant and treated with bortezomib exhibited 25-fold increase in mean liver CBS activity compared to the untreated cohort with their mean serum tHcy entirely normalized to 6.7 μM (Gupta et al., 2017). Likewise, the Tg-R336C $Cbs^{-/-}$ model expressing hCBS p.R336C mutant showed strong rescue by bortezomib with 10-fold increase of their mean liver CBS activities (Gupta et al., 2019). The one transgenic HCU mouse model that was not rescuable by PI treatment was the Tg-G307S $Cbs^{-/-}$ mice expressing hCBS p.G307S mutant, an allele primarily found in pyridoxine unresponsive HCU patients of Irish origin (Gupta et al., 2018).

The concentration of PIs used in these preclinical studies were similar to what is used in humans. For example, the mice mentioned above received the equivalent of 1.47 mg/m^2 per day for a period of 3–4 days, while a cancer patient undertaking bortezomib treatment will typically receive an infusion of 1.3 mg/m^2 twice a week. However, it should be noted that treatment with PIs such as bortezomib is clearly associated with toxicity and for that reason a specific dosing regimen (four cycles of two weeks on followed by one week off the drug with subsequent five cycles with the drug administered only once a week for two weeks followed by another two weeks resting period) has been developed and is implemented in the clinic. Peripheral neuropathy, fatigue, heart/lung/liver/GI toxicity, hypotension and thrombocytopeni/neutropeniua have been among commonly reported adverse side effects associated with PI treatment. Such serious toxicities may be acceptable for a short-term period by cancer patients with a high risk of death. However, it is highly unlikely that HCU

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patients requiring life-long treatment will tolerate the same or similar level of drug toxicity. Having said that, substantial lowering of the PI dose for chronic treatment of HCU patients compared to an acute treatment of cancer patients may represent a potential approach to overcome this issue. However, it is currently unknown whether a lower dose of PI would rescue mutant hCBS protein without serious side effects of the treatment.

3.3.3. Gene therapy—Gene therapy approaches to CBS deficiency are particularly attractive from a therapeutic perspective as they have the potential advantage of being a "one and done" treatment that directly solves the underlying cause of the disorder. The most popular and promising approach involves recombinant adeno-associated virus (AAV) (Wang et al., 2019). These single-stranded DNA viral vectors can accommodate up to 5 kb of an introduced genetic material. There are several different AAV serotypes, which differ in tissue tropism determined by variations in sequences of their capsid proteins. Upon target cell infection, the AAV DNA does not integrate, but persists as a circular, non-replicating episome.

The earliest report of an AAV-based gene therapy for HCU comes from a Korean group in 2006 (Park et al., 2006). Using an AAV-2 serotype, they reported that an hCBS cDNA driven by the human EIF1a promoter could partially suppress the neonatal lethality characteristic for CBS KO mice, increasing their lifespan by 3–7 days and decreasing plasma tHcy by about 40% at two-weeks of age. However, because the treated CBS KO mice did not survive beyond thirty days of age, no long-term follow-up could have been performed and the approach was abandoned by this group. In 2021, another AAV-CBS strategy was used in combination with the Tg-I278T $Cbs^{-/-}$ mouse model (Lee et al., 2021). In this case, an AAVrh.10-based vector expressing hCBS controlled by the liver specific CAG promoter was given in a single intravenous injection to 40 days-old Tg-I278T $Cbs^{-/-}$ mice, which were subsequently followed for up to one year of age. The data showed an increase of liver CBS activity in a dose-dependent manner, which correlated well with pattern of a decreased serum tHcy. Specifically, CBS activity in liver of the treated animals one year after the injection was similar to that of control Tg-I278T $Cbs^{+/-}$ healthy mice. The highest dose (i.e. 5.6×10^{11} genomes/mouse) administered to the mice resulted in 97% and 81% mean serum tHcy reduction one week and one year, respectively, after the injection. Phenotype associated with the Tg-I278T $Cbs^{-/-}$ mouse model, such as facial alopecia, bone mineralization or fat loss was either normalized or substantially corrected in treated mice. These results suggest that CBS gene therapy using AAVrh.10 vector is highly effective to treat CBS deficiency in mice. Current efforts are underway to license this technology to begin the initiation of human trials.

Although AAV-based gene therapies are currently the furthest along clinically, with FDA approval for Luxturna (for inherited retinal dystrophy) and Zolgensma (for spinal muscular atrophy), there is some concern about potential problems involving rare integration events, durability and immunogenicity (Kuzmin et al., 2021; Nguyen et al., 2021). Therefore, there is also a considerable interest in non-viral gene delivery systems in which nucleic acids are complexed with other molecules to deliver genetic material. For CBS deficiency, it has been demonstrated that a naked DNA-minicircle expressing hCBS from a liver-specific P3 promoter constitutes low levels of CBS activity in the livers of Tg-I278T $Cbs^{-/-}$ mice and

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lowers plasma tHcy when given by hydrodynamic tail vein injection (Lee et al., 2019). Although this form of delivery may not be feasible in humans, it demonstrates that CBS deficiency may be amenable to non-viral gene therapy in the future.

4. Conclusion

There is still a number of unmet needs in management of HCU, where availability of therapeutic options plays an integral part of this complex mosaic. A long delay in HCU diagnosis (unless achieved by a newborn screening), cost of treatment and the palatability of amino acid mixtures were also emphasized in the recent HCU patient and caregiver survey (Morrison et al., 2021). Recent progress in research and development of new therapies for HCU gives hope for immediate and long-term health benefits and better quality of life of HCU patients. It is anticipated that the increased availability of new therapies will stimulate research towards improving diagnosis of HCU early in life and increase awareness about the disease among healthcare professionals and policy makers. Although approaches like chaperone or gene therapy are highly specific for CBS-deficient HCU (or even just an amenable pathogenic CBS mutant allele), Met-reducing and Hcy-degrading strategies could successfully be applied to other indications and thus other conditions or diseases would benefit from emerging therapies for HCU. For example, Met reduction could be beneficial in treatment of various cancers. Likewise, other causes of hyperhomocysteinemia of genetic or non-genetic origin, such as remethylation defects or chronic kidney disease, could benefit from systemic Met or Hcy degradation by enzyme therapies for HCU. With several new therapies for HCU in clinical trials and other in development, we are hopeful that they may be instrumental in addressing the unmet need of HCU patients and potentially be helpful in other indications as well.

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Data availability statement:

Data sharing is not applicable to this article because no new data were created or analyzed in this study.

Abbreviations

AAV	adeno-associated virus
AOAA	aminooxyacetic acid
CBS	cystathionine beta-synthase
CGL	cystathionine gamma-lyase

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Cys	cysteine
dapA	4-hydroxy-tetrahydropicolinate synthase
GI	gastrointestinal
H_2S	hydrogen sulfide
hCBS	human CBS
HCU	homocystinuria
Hcy	homocysteine
IPTG	isopropyl-β-D-1-thiogalactopyranoside
Met	methionine
MetDC	methionine decarboxylase
MetNIQ	methionine high affinity ABC transporter
MetP	methionine low affinity symporter
MGL	methionine gamma-lyase
NMDA	N-methyl-D-aspartate
PEG	polyethylene glycol
PI	proteasome inhibitor
PLP	pyridoxal-5'-phosphate
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
tHcy	total homocysteine
yjeH	methionine/branched chain amino acid exporter

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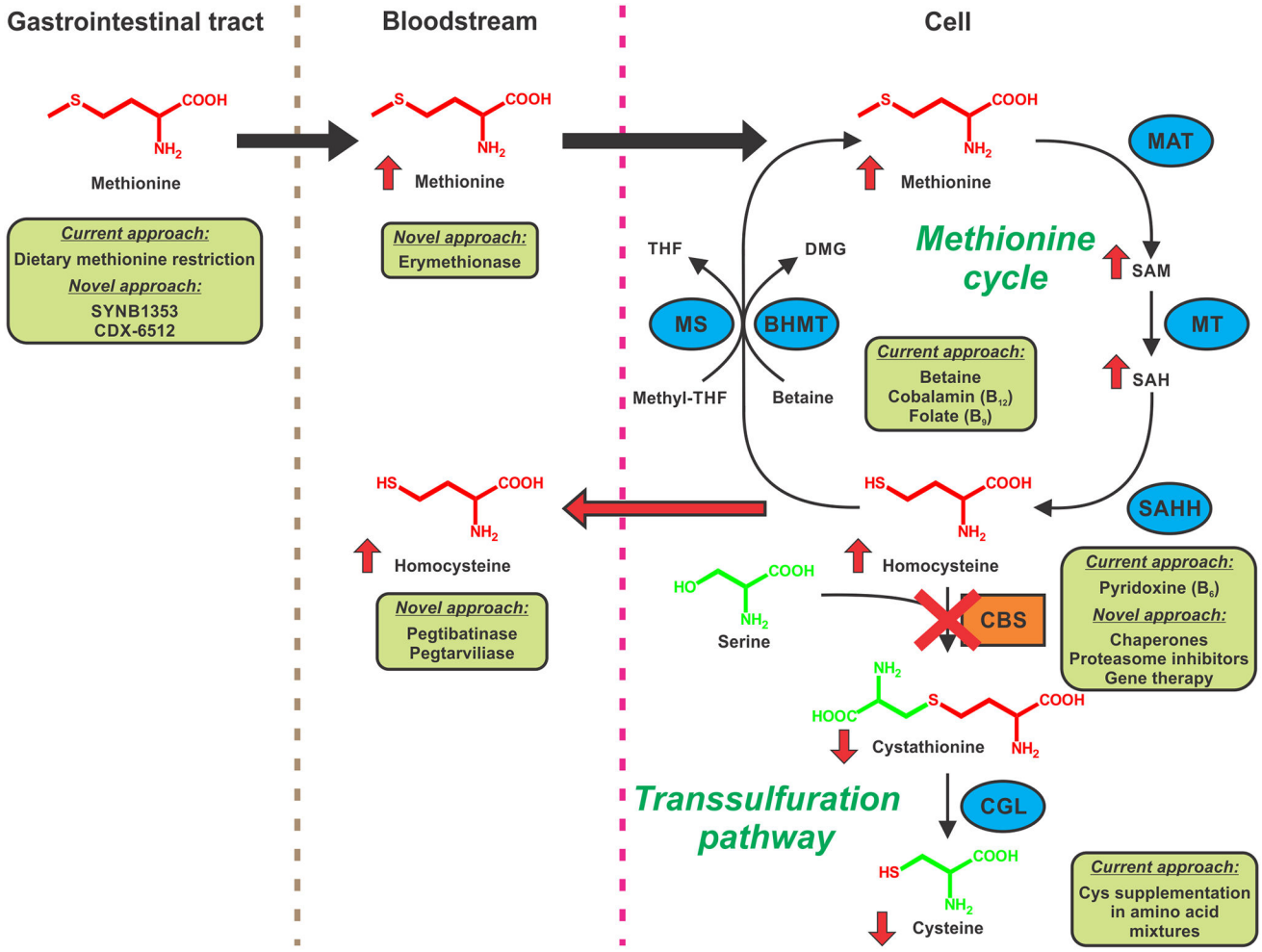

Figure 1. Sulfur amino acid metabolism and therapeutic options in HCU.

Essential amino acid Met is absorbed from the GI tract into bloodstream. After entering the cell, Met is rapidly converted by Met-adenosine transferase (MAT) to SAM, which is used as methyl donor by variety of methyltransferases (MT) yielding SAH. Subsequently, SAH is hydrolyzed by SAH hydrolase (SAHH) producing adenosine and Hcy. Toxic intermediate Hcy is recycled in the Met cycle by ubiquitous Met synthase (MS) or liver-dependent betaine-Hcy methyltransferase (BHMT). Alternatively, Hcy is irreversibly diverted by CBS towards synthesis of Cys by condensation with serine forming cystathionine, which is then hydrolyzed by CGL to yield Cys. In HCU, lack of CBS activity (red cross) results in metabolic imbalance characterized by massive elevation of Hcy and changes in other metabolites (red arrows). Green boxes denote various steps in Met/Hcy metabolism where the current and emerging therapeutic approaches for HCU act.

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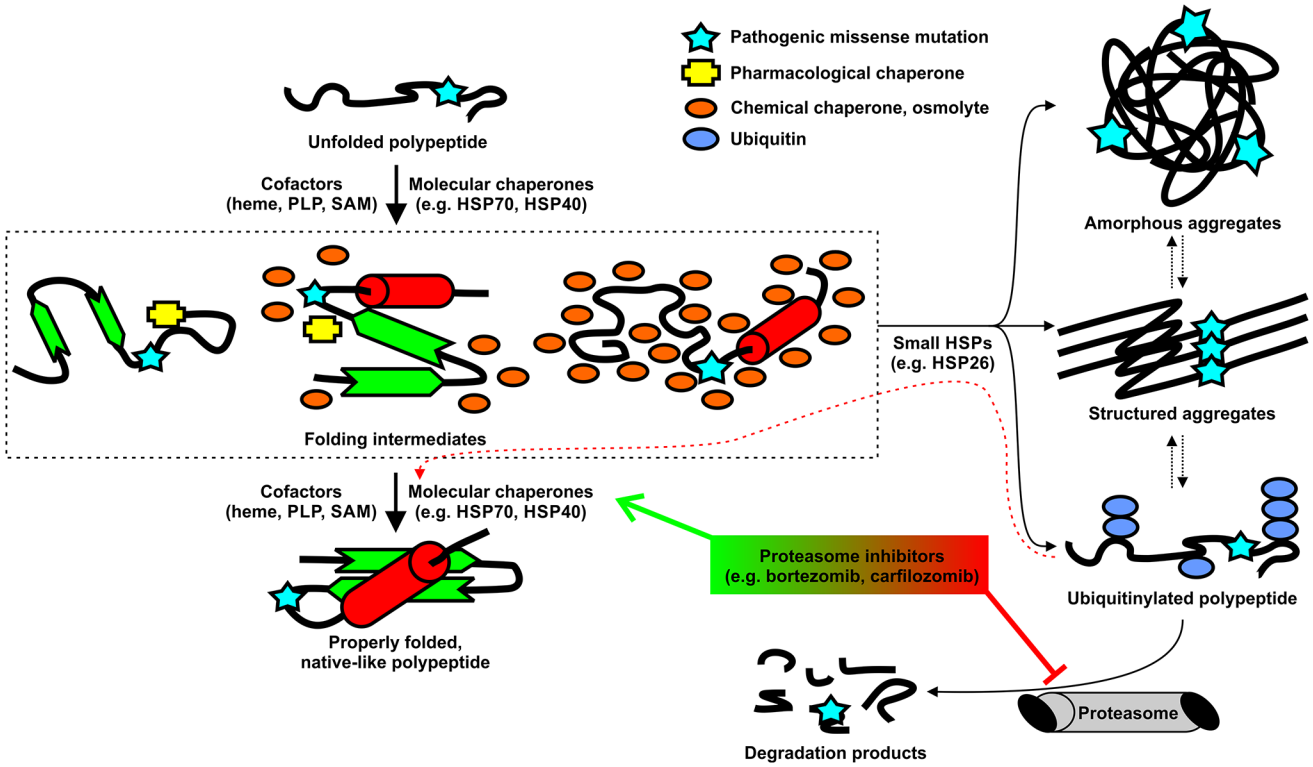

Figure 2. Potential approaches to rescue CBS folding.

Nascent CBS polypeptide carrying missense pathogenic mutation undergoes folding process in the presence of cofactors (heme, PLP, SAM) and with assistance from certain molecular chaperones, such as HSP70. Chemical chaperones/osmolytes or pharmacological chaperones modulate formation and stability of the folding intermediates on the way towards properly folded, native-like active mutant CBS. If the folding process fails, CBS polypeptide may form amorphous or structured aggregates or may be designated by small heat shock proteins (HSPs), such as HSP26, for ubiquitinylation and subsequent proteosomal degradation. Proteasome inhibitors, such as bortezomib, block degradation of misfolded CBS polypeptides and stimulate their refolding (red dashed arrow), which increases steady-state levels of CBS polypeptide and may increase residual CBS activity.