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Critical Review

The Structural and Molecular Biology of Type I Galactosemia: Enzymology of Galactose 1-phosphate Uridylyltransferase

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Summary

Reduced galactose 1-phosphate uridylyltransferase (GALT) activity is associated with the genetic disease type I galactosemia. This results in an increase in the cellular concentration of galactose 1-phosphate. The accumulation of this toxic metabolite, combined with aberrant glycoprotein and glycolipid biosynthesis, is likely to be the major factor in molecular pathology. The mechanism of GALT was established through classical enzymological methods to be a substituted enzyme in which the reaction with UDP-glucose results in the formation of a covalent, UMP-histidine adduct in the active site. The uridylated enzyme can then react with galactose 1-phosphate to form UDP-galactose. The structure of the enzyme from *Escherichia coli* reveals a homodimer containing one zinc (II) and one iron (II) ion per subunit. This enzymological and structural knowledge provides the basis for understanding the biochemistry of this critical step in the Leloir pathway. However, a high-resolution crystal structure of human GALT is required to assist greater understanding of the effects of disease-associated mutations.

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Keywords galactose metabolism; disease-associated mutation; GALT; galactose 1-phosphate uridylyltransferase; histidine triad transferase; UMP-histidine.

Abbreviations DEPC, diethyl pyrocarbonate; GALE, UDP-galactose 4′-epimerase; GALK1, galactokinase; GALK2, *N*-acetylgalactosamine kinase; GALT, galactose 1-phosphate uridylyltransferase.

INTRODUCTION: TYPE I GALACTOSEMIA

Galactosemia results from the failure to metabolise galactose and its derivatives. The first cause of this failure to be identified was mutation of the gene encoding the enzyme galactose 1-phosphate uridylyltransferase (GALT, EC 2.7.7.12) in the Leloir pathway (*1*). This form of galactosemia is most common and is known as type I galactosemia (OMIM #230400). The frequency of galactosemia varies considerably between populations. Values of 1/23,000–1/50,000 have been estimated for European and North American populations (*2, 3*). Lower frequencies (1/400,000–1/1,000,000) have been measured in Chinese, Japanese, and Korean populations (*4–6*). However, much higher frequencies have been observed in some reproductively isolated populations; for example, the frequency among Irish travellers is 1/480 (*2*). Two other variants of the disease are recognized. Deficiency of galactokinase (GALK1; EC 2.7.1.6) results in type II galactosemia (OMIM #230200), the mildest form of the disease (*7*). The third variant, type III galactosemia (OMIM #230350) is caused by mutations in the gene encoding UDP-galactose 4′-epimerase (GALE; EC 5.1.3.2)) (*8*). Recent work has established that type III galactosemia is a continuum disease with variable pathology depending on the specific mutation present and the patient's environmental circumstances (*9, 10*).

The pathology of type I galactosemia is varied. Approximately 10% of patients suffer from cataracts in early infancy—a sign of disease shared with type II and III galactosemia (*11*). Cataracts can be resolved by surgery and are not considered a major problem. However, type I galactosemia is also associated with severe, irreversible damage to a variety of organs including the liver, kidneys, brain, and ovaries (*12*). Consequently, patients often suffer from developmental and cognitive defects in childhood and reproductive problems in adulthood. Severe, untreated forms of the disease can lead to death in childhood. All of these signs of disease can be reduced and slowed (but not reversed) by the exclusion of galactose from the diet (*12*). However, it is difficult to exclude this sugar completely as it occurs widely in common foodstuffs. In particular, it is a component of the disaccharide lactose, which is found in milk products. Galactose is also found in trace amounts in many meat products and some fruits. Furthermore, a fraction of patients develop severe pathology even if dietary restriction is started in the first few days of life.

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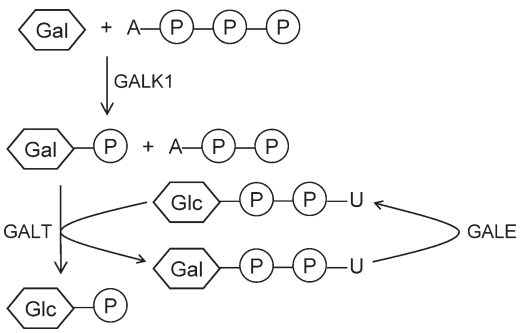

Figure 1. The Leloir pathway. The net result of this pathway is to transform galactose into glucose1-phosphate at the expense of one molecule of ATP. A, adenosine; U, uridine; Gal, galactose; Glc, glucose; P, phosphate.

Consequently, there is a pressing need to develop alternative therapies for the disease.

THE LELOIR PATHWAY

The Leloir pathway (*13*) is generally considered to describe the conversion of α-D-galactose to α-D-glucose1-phosphate—a process that requires GALK1, GALT, and GALE (Fig. 1). As cellular galactose in aqueous solution exists as a mixture of α- and β-anomers, another enzyme, galactose mutarotase (EC 5.1.3.3), is required to catalyze the interconversion of these anomers (*14*). The product of the Leloir pathway, α-D-glucose1-phosphate, is isomerized to the glycolytic intermediate D-glucose 6-phosphate by the action of phosphoglucomutase (EC 5.4.2.2).

GALT: STRUCTURE AND ENZYMOLOGY

GALT catalyzes the transfer of a UMP group from UDP-glucose to α-D-galactose 1-phosphate. The enzyme has a strict requirement for UDP-glucose or UDP-galactose as a substrate. GDP-glucose, ADP-glucose, TDP-glucose, CDP-glucose, UDP-xylose, and UDP-mannose are all unable to support activity (*15*). There is very little data available on the inhibition of GALT by nonsubstrate or product molecules. Uracil is an inhibitor of mammalian GALT along with many of its derivatives, including UTP, UDP, UMP, uridine, UDP-xylose, UDP-glucuronic acid, and UDP-mannose (*16–18*). Some other nucleotides and nucleotide sugars also inhibit, including ATP, ADP, TDP-glucose, GDP-glucose, ADP-glucose, and CDP-glucose (*16*). A report that the reduced level of GALT activity in patients with chronic lymphocytic leukemia may be due to an (unidentified) inhibitor does not appear to have been pursued (*19*).

GALT belongs to the histidine triad family of transferases (*20*). These enzymes have substituted enzyme (or ping-pong) mechanisms. In the case of GALT, a UMP group is transferred from UDP-glucose to a histidine residue (His-186 in human GALT) in the active site of the enzyme. The first product, glucose1-phosphate, then diffuses away and is replaced by the second substrate, galactose 1-phosphate. The UMP moiety is then transferred to the galactose 1-phosphate molecule, forming the second product, UDP-galactose. This regenerates the enzyme that can then participate in another round of catalysis (Fig. 2) (*21*).

The evidence for this mechanism comes from a variety of studies. Enzyme kinetic studies on *Escherichia coli* GALT in which one substrate concentration was varied and the other held constant produced parallel lines in Lineweaver–Burk (double reciprocal) plots, a classic diagnostic feature of the substituted enzyme mechanism (*22*). Inhibition patterns also provided evidence for this mechanism: UDP-galactose, a product of the forward reaction, was a competitive inhibitor with respect to UDP-glucose and a mixed inhibitor with respect to galactose 1-phosphate. Galactose 1-phosphate showed competitive substrate inhibition in the forward reaction (*22*). Similar kinetic data suggest that the mechanism is identical in the human enzyme (*23*).

A discrete, covalent uridylated enzyme intermediate could be isolated. Early experiments using radio-labelled UDP-glucose and *E. coli* GALT demonstrated that the label is transferred to the protein. This only occurred if the label was present in the UDP moiety and not in the glucose. The amount of radioactivity in the enzyme fractions could be reduced by the addition of the second substrate, galactose 1-phosphate (*24*). The stoichiometry of the uridylation reaction was shown to be 1.7 UMP moieties per enzyme dimer, strongly suggesting that both active sites in the dimer can participate in the reaction (*25*). The bond between the UMP group and the enzyme was stable to mild alkali and moderate temperatures but can be broken by overnight treatment in acid (*25*). That this link occurs through a histidine residue was suggested by experiments which showed that the treatment of *E. coli* GALT with diethyl pyrocarbonate (DEPC, a covalent modifier of histidine side chains) inactivated the enzyme, an effect that could be reversed by hydroxylamine. No effect on the reaction was seen with the amine-specific reagents pyridoxal phosphate and 5-nitrosalicylaldehyde. UDP-glucose protected GALT from modification with DEPC, presumably because it uridylates a histidine residue in the active site and, thus, prevents it from reacting with this reagent (*25*). Similar results were obtained with human GALT (*26*). Unambiguous evidence for the involvement of a histidine adduct was provided by uridylation of *E. coli* GALT with radioactive UDP-glucose, removal of the uridine moiety by sodium *meta*-periodate cleavage followed by alkaline hydrolysis of the protein and analysis of the resulting amino acids. This analysis showed the major radioactive product was N^3-phosphohistidine (*27*). Site-directed mutagenesis studies showed that the alteration of either His-164 or His-166 in *E. coli* GALT resulted in loss of activity. Both the residues are well conserved in GALT enzymes from other species. Thus, these experiments demonstrate that one of these residues is likely to be the uridylated active site histidine but were unable to distinguish which one (*28*).

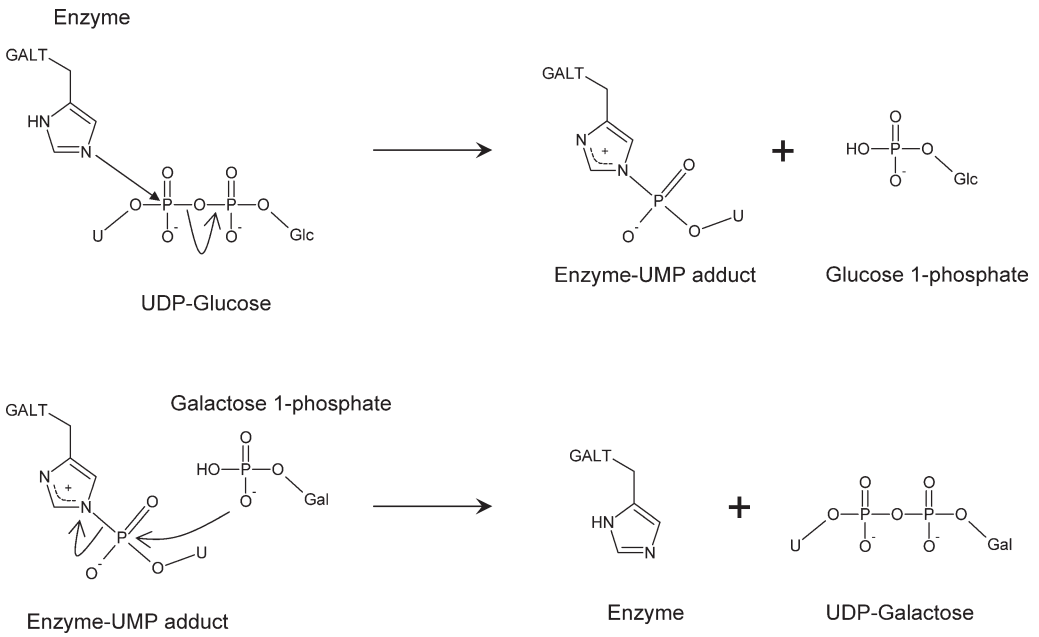

Figure 2. The catalytic mechanism of GALT. In the first step of the ping-pong (or substituted enzyme) mechanism, a lone pair of electrons on the ring nitrogen of a histidine residue (His-166 is *E. coli* and His-186 in humans) in the active site of GALT attacks the α-phosphate of UDP-glucose. The result of this reaction is a covalent UMP-enzyme adduct and glucose1-phosphate, which is released. In the second stage of the reaction, the UMP group is displaced from the enzyme by galactose 1-phosphate (the product of the first reaction of the Leloir pathway). This regenerates the active site of the enzyme and produces the second product, UDP-galactose. U represents the uridine moiety of UDP-glucose and UDP-galactose, Glc, a glucose moiety and Gal a galactose moiety.

Further, and convincing, evidence for the substituted enzyme mechanism of GALT came from detailed X-ray crystallographic studies. Although the structure of human GALT has not been solved, the *E. coli* enzyme has been (PDB IDs: 1HXQ, 1HXP, 1GUP, and 1GUQ) (*29–31*). In addition, structures of a GALT-like protein from thale cress (*Arabidopsis thaliana*) have been deposited in the Protein Data Bank (PDB IDs 2Q4H, 2Q4L, 1ZWJ, and 1Z84); however, this is likely to be an ADP-glucose phosphorylase (*32*). The lack of a human structure may result from the physical properties of the enzyme. The purified, recombinant enzyme has a tendency to aggregate and precipitate (TJM and DJT, unpublished data), and it may be difficult to obtain the high concentrations normally required for crystallization.

Like the human enzyme, *E. coli* GALT is a homodimer with a functional active site in each subunit (Fig. 3). Each subunit contains one zinc (II) ion and one iron (II) ion (Fig. 4a) (*33*). Although the zinc ion is believed to be essential for activity, the iron ion is thought to play a role in stabilizing the structure (*34*). This iron ion is coordinated by three histidine residues and a glutamate residue in a hydrophobic region close to the subunit interface (Fig. 4b) (*29*). Removal of the ion would probably result in destabilization of this region and, consequently, disrupt the dimer. The zinc ion is coordinated by two cysteine and two histidine residues and helps to orientate residues in, and close to, the active site of the enzyme (Fig. 4c) (*29*). However, it has been noted that the mammalian enzyme is different from *E. coli* GALT in that the zinc co-orientating residues are not conserved and that, therefore, it may only bind one metal ion per monomer (*29*). This active site includes residues from

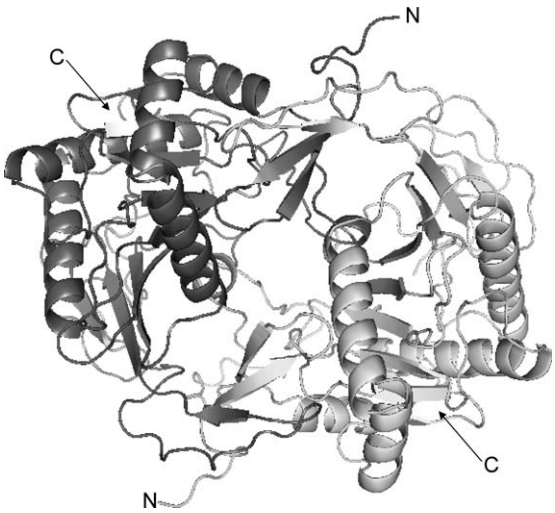

Figure 3. The overall structure of *E. coli* GALT. The enzyme is a homodimer. In this figure, the two subunits are colored dark and light gray. The figure was created in PyMol (www.pymol.org) using PDB file 1HXP (*29*).

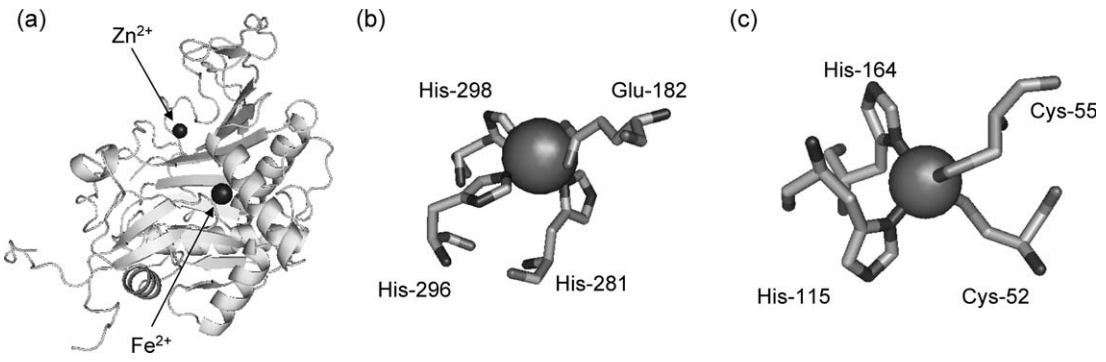

Figure 4. The metal ion binding sites in *E. coli* GALT. (a) One subunit of the homodimer with the zinc and iron ions as dark gray spheres. The second subunit would be situated to the upper left of the monomer shown here. (b) A close up of the distorted square pyramidial iron-binding site, and (c) a close up of the tetrahedral zinc binding site. The figures were created in PyMol using PDB file 1HXP (*29*).

both subunits of the dimer and the histidine residue, which is covalently modified in the catalytic cycle, has been identified unambiguously as His-166 in the *E. coli* enzyme (Fig. 5) (*30, 31*). The equivalent residue in the human enzyme is His-186. This histidine is the final residue in a well-conserved tripeptide, His-Pro-His. Mutation of the active site histidine in the *E. coli* protein results in an inactive enzyme (*31*). The central proline residue in this tripeptide is also important. Mutation of this residue in the human enzyme to any other residue resulted in substantially reduced, or zero, activity. Many of these alterations also decreased the stability of the protein, suggesting that it has an important structural role (*35*). In the *E. coli* structure, the equivalent proline restrains the flexibility of the polypeptide backbone, orientating the two histidine residues so that they can hydrogen bond directly to each other (*30*). This arrangement positions the nucleophilic histidine such that it can react with the UDP-sugar and helps to stabilize the positive charge on the histidine moiety following this reaction (*30*).

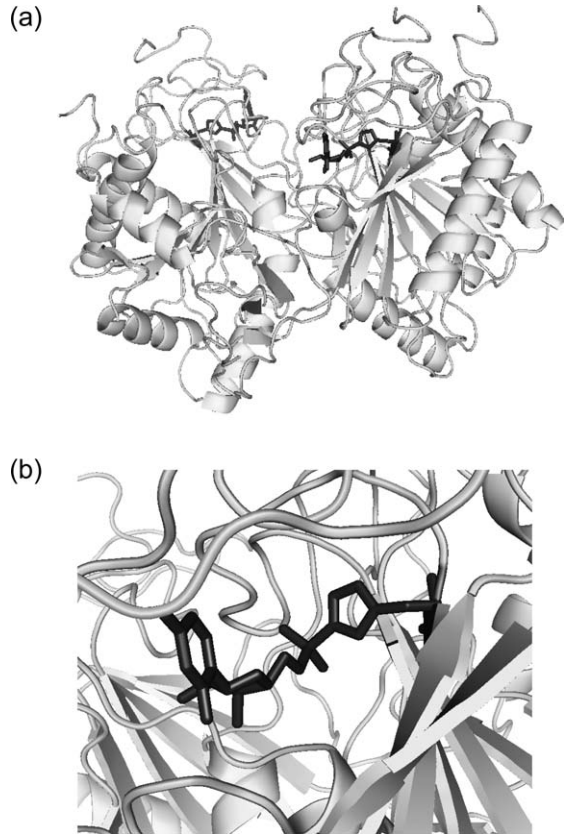

Figure 5. The uridylated active site of *E. coli* GALT. In the structure both active sites were covalently modified at His-166 with a uridyl group (a). The uridyl-histidine adducts are shown in dark gray towards the top of the dimer. A close up of one adduct is shown in (b). The figures were created in PyMol using PDB file 1HXQ (*30*).

MOLECULAR PATHOLOGY: WHY DOES DISRUPTION OF THE LELOIR PATHWAY HAVE SUCH PROFOUND CONSEQUENCES?

A reduction in the rate of GALT-catalyzed utilization of galactose 1-phosphate results in a build up of this compound. This compound is widely accepted to be toxic to cells and to be the primary cause of many of the disease indications observed in patients. However, the mechanism of galactose 1-phosphate's toxicity is not clear, and several hypotheses have been proposed. The main hypotheses include the depletion of cellular phosphate pools (leading to decreased production of ATP and other nucleotide phosphates); this may be further exacerbated by the stimulation of ATPases in brain cells by galactose and its metabolites (*36*). However, HepG2 hepatocytes when grown on media containing galactose as the sole carbon source demonstrated slow growth kinetics but no reduction in ATP levels (*37*). This suggests that the responses of different cell types to excess galactose may differ. In addition to effects on nucleotide phosphate levels, several intracellular targets for galactose 1-phosphate have been proposed. These include inositol monophosphatase, UDP-glucose pyrophosphorylase, UDP-*N*-acetyl-

glucosamine pyrophosphorylase, and aplysia ras homolog I (*38–41*). In fibroblasts derived from patients with type I galactosemia, an endoplasmic reticulum stress response was observed, suggesting that the accumulation of galactose metabolites causes the accumulation of unfolded proteins (*40*). In addition to these intracellular effects, the composition of glycolipids and glycoproteins on the surfaces of cells in galactosemic patients is altered, with reductions in the fraction of galactose and *N*-acetylgalactosamine moieties (*42–44*). These abnormalities of glycosylation are likely to result from the role of Leloir pathway enzymes in maintaining the pools of UDP-hexoses. However, no study has conclusively demonstrated reduced levels of UDP-galactose in the cells of patients with type I galactosemia. Taken together, these various studies point to a complex and, at best, partially understood molecular pathology of type I galactosemia.

FUTURE PERSPECTIVES: THERAPEUTIC INTERVENTIONS IN TYPE I GALACTOSEMIA

It was proposed nearly 10 years ago that inhibition of galactokinase could be a potential therapy for type I galactosemia (*45*). The logic of this approach is that inhibition of the Leloir pathway at the first committed step would reduce the build up of toxic galactose 1-phosphate. In effect, the treatment would convert the severe, debilitating and potentially fatal type I galactosemia into the much milder type II form. However, until 2008, the only known inhibitors of galactokinase were galactose 1-phosphate and related compounds. Recently, several promising lead compounds have been identified (Fig. 6) (*46, 47*). The best of these compounds have IC_{50} values in the low micromolar range and are able to reduce the production of galactose 1-phosphate in cells derived from patients with type I galactosemia (*47*).

However, for further development, it is important to remember that each patient will probably have a lifetime need to take the drug. Thus, the compound will need to have extremely low toxicity, be well tolerated, have negligible long term effects, and be relatively cheap to manufacture. There are a number of potential targets with high structural similarity to GALK1, including *N*-acetylgalactosamine kinase (GALK2). It will be important to ensure that GALK1 inhibitors have no activity toward GALK2. In addition, to inform the development of this therapy, further medium- and long-term monitoring of patients with type II galactosemia would be helpful. Such work would provide data on any likely complications resulting from decades of reduced GALK1 activity. It should also be noted that little, or no, work has been carried out on higher organisms lacking both GALK1 and GALT. Here, the use of model multicellular eukaryotes will be valuable.

The Leloir pathway is not the only possible means by which eukaryotes can oxidize galactose. Metabolism of galactose by patients with severe forms of type I galactosemia and mice deleted for both copies of their *GALT* gene has been observed (*48, 49*). These patients are able to metabolize galactose to

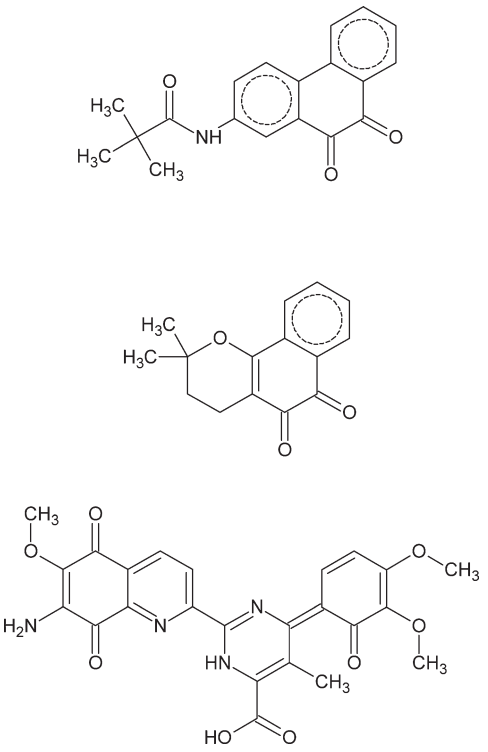

Figure 6. Inhibitors of human GALK1. These three compounds are the most promising GALK1 inhibitors thus far discovered. In the original work (*47*), these were numbered as compounds 1, 4, and 24.

UDP-glucose, although the pathway by which they achieve this is unclear but may involve the action of UDP-galactose pyrophosphorylase (*50*). Further investigation of these pathways and an assessment of their significance are required to understand the molecular pathology of type I galactosemia and also to assist the rational design of therapies. Despite these challenges, if an effective drug can be developed, the effects for patients could be comparable with the development of insulin therapies for diabetics.

CONCLUSIONS

Knowledge of the enzymology and structure of GALT is important for the understanding of type I galactosemia. Nevertheless, a high-resolution structure of human GALT is a significant research need in this field.

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