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SHORT COMMUNICATION

Inhibition of human pyridoxal kinase by 2-acetyl-4-((1*R*,2*S*,3*R*)-1,2,3,4-tetrahydroxybutyl)imidazole (THI)

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Abstract

2-Acetyl-4-((1*R*,2*S*,3*R*)-1,2,3,4-tetrahydroxybutyl)imidazole (THI) is observed as a minor contaminant in caramel food colourings (E 150c). Feeding experiments with rodents have revealed a significant lymphopenic effect that has been linked to the presence of THI in these food colourings. Pyridoxal kinase inhibition by THI has been suggested, but not demonstrated, as a mode of action as it leads to lowered levels of pyridoxal-5'-phosphate, which are known to cause lymphopenia. Recently, THI was also shown to inhibit sphingosine-1-phosphate lyase causing comparable immunosuppressive effects and derivatives of THI are being developed for the treatment of rheumatoid arthritis in humans. Interestingly, sphingosine-1-phosphate lyase activity depends on pyridoxal-5'-phosphate, which in turn is provided by pyridoxal kinase. This report shows that THI does inhibit pyridoxal kinase with competitive and mixed-type non-competitive behaviour towards its two substrates, pyridoxal and ATP, respectively. The corresponding inhibition constants are in the low millimolar range.

Keywords

Caramel food colourings, pyridoxal kinase, sphingosine-1-phosphate lyase, THI

History

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Introduction

Aside of its pleasant taste, the colouring properties of caramel make it a widely used food additive. Depending on their manufacturing, caramel food colourings are divided into four classes, i.e. caramel colourings of classes I, II, III and IV¹. Among these, caramel of colour classes III (E 150c) and IV (E 150d) are prepared by heating carbohydrates in the presence of ammonium compounds (III) and, additionally, sulfite (IV). During caramelization, these ammonium compounds serve as a source of nitrogen in the formation of a number of food contaminants including a range of imidazoles, one of them being 2-acetyl-4-((1*R*,2*S*,3*R*)-1,2,3,4-tetrahydroxybutyl)imidazole (THI, Figure 1). Interestingly, although ammonia is added during preparation of both classes III and IV caramel food colourings, THI is only observed in class III products². Such caramel colourings are used in a variety of food preparations, e.g. sauces, soups, bakery or meat products, with an estimated daily mean exposure of 300 mg/kg bodyweight³. Today, caramel food colourings (classes I–IV) account for >80% of all colorants added to foods and their annual global consumption exceeds 200 000 tons^{4,5}.

Obviously, caramel food colourings are a chemically rather ill-defined group of food additives as they are mixtures of various caramelization products. Observations of hysteria and convulsions made when feeding livestock feeds supplemented by ammoniated molasses also raised concerns about the safety of caramel food

colourings, especially of those of class III⁶. Following a 1970 initiative by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), caramel food colourings have therefore been subject of numerous toxicological investigations⁷. Noteworthy, rat feeding experiments carried out with high concentrations of caramel class III food colourings revealed a reduction in circulating lymphocyte counts^{6,8}. This lymphopenic effect has since been the focus of extensive toxicity studies and was shown to be linked to vitamin B₆ metabolism. In 1985, THI was identified as the imidazole-derived contaminant of caramel class III food colourings triggering the observed lymphopenia^{9–11}.

Yet, until today the molecular mechanism linking THI exposure to lymphopenia has not been revealed. Taking into account that lymphopenia can occur as a result of vitamin B₆ deficiency, inhibition of human pyridoxal kinase (EC 2.7.1.35), which catalyzes the random Bi Bi ATP-dependent phosphorylation of pyridoxal to pyridoxal-5'-phosphate (the active form of vitamin B₆), has been considered as a possible mechanism of action (Figure 1)¹¹. Human pyridoxal kinase has been shown to be inhibited by a range of substances of natural and synthetic origin, especially by small heterocyclic compounds. Some well-known drugs inhibit human pyridoxal kinase, causing a number of unwanted neurotoxic side effects^{12–15}.

Some reports, however, question that the pyridoxal kinase inhibiting properties of class III caramel colourings actually originate from THI¹⁶. Recently, it was discovered that THI inhibits sphingosine-1-phosphate lyase leading to increased intracellular levels of sphingosine-1-phosphate, which in turn may also cause lymphopenia¹⁷. Details about the mode of interaction have not yet been defined, however, a binary interaction between sphingosine-1-phosphate lyase and THI has

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been ruled out, while a more complex interplay has been suggested. Interestingly, sphingosine-1-phosphate lyase requires pyridoxal-5'-phosphate as a cofactor to catalyze the irreversible cleavage of sphingosine-1-phosphate into hexadecanal and phosphoethanolamine. Hence, THI might affect sphingosine-1-phosphate lyase activity in two different ways, i.e. by direct inhibition and by lowering the levels of the required cofactor through pyridoxal kinase inhibition. Here, we report the results of a detailed investigation on pyridoxal kinase inhibition by THI.

Materials and methods

Chemicals

THI was prepared as reported previously at ELFI Analytik GbR (Neufahrn, Germany)². The purity of the THI sample used for the experiments was assessed by LC-MS and combustion analysis. LC-MS: purity 99%. MS (ESI+): *m/z* calcd for C₉H₁₄N₂O₅ 230.1, found 231.4 [M+H]⁺, 253.0 [M+Na]⁺. Anal. Calcd for C₉H₁₄N₂O₅: C, 47.0; H, 6.1; N, 12.2. Found: C, 46.7; H, 6.2; N, 12.1. All other reagents were provided by Sigma-Aldrich (Milano, Italy) and were used without further purification.

Enzyme inhibition studies

A 0.5 M solution of THI in water was prepared by addition of an equimolar amount of HCl and used in this form for the assays. Wild-type human pyridoxal kinase was expressed and purified as previously published¹⁸, except for final dialysis that was carried out overnight against 100 mM sodium *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonate (BES) buffer, pH 7.3. *In vitro* activity of pyridoxal kinase can easily be assessed using a conventional UV spectrophotometer taking advantage of the significant bathochromic shift that pyridoxal experiences upon phosphorylation (320–388 nm). This shift occurs as the intramolecular hemiacetal form of pyridoxal, which is predominant in aqueous solutions, cannot form because of the phosphorylation of the 5'-hydroxy group^{19,20}. Briefly, initial velocity studies for the conversion of pyridoxal to pyridoxal-5'-phosphate were followed at 388 nm in an Agilent 8454 UV/Vis diode array spectrophotometer in 100 mM sodium

BES buffer, pH 7.3; the final enzyme concentration was 4 μM. All assays were performed at 37 °C in a 1 cm thermostated cuvette as previously described²¹. Detailed kinetic studies for the determination of the inhibition mechanism were carried out in duplicate or triplicate at different THI concentrations (4.2, 7.5, 15 and 20 mM) under two experimental conditions: (i) at a constant MgATP concentration of 1.67 mM while varying the pyridoxal concentration between 20 and 300 μM and (ii) at a constant pyridoxal concentration of 300 μM while varying the MgATP concentration between 40 μM and 1.67 mM. The kinetic parameters *K_i* and *K_{ii}* were determined by Lineweaver-Burk analysis and corresponding secondary plots of their slopes and intercepts versus THI concentrations, following the kinetic treatment proposed by Bisswanger as highlighted in the next paragraph²².

Briefly, the random Bi Bi, two-substrate rapid-equilibrium mechanism proposed for human pyridoxal kinase follows the general pathway illustrated in Figure 2, in which *A* and *B* represent the two substrates. In case of a competitive inhibitor towards substrate *A* (pyridoxal in our case), inhibitor *I* (THI) would participate at every step in the kinetic mechanism in which the substrate *A* normally binds. Thus, the following interactions of the inhibitor with the enzyme are expected (ATP representing substrate *B*):

When deriving the velocity equation for the effect of the competitive inhibitor on substrate *A*, the following rate relationship is obtained:

$$\frac{1}{V} = \frac{1}{V_{\max}} + \frac{K_{a'}}{V_{\max}[A]} \left(1 + \frac{[I]}{K_{ii}} \right) + \frac{K_{b'}}{V_{\max}[B]} + \frac{K_a K_{b'}}{V_{\max}[A][B]} \left(1 + \frac{[I]}{K_i} \right) \quad (1)$$

When double reciprocal plots of $\frac{1}{V}$ as a function of $\frac{1}{[A]}$ are prepared at different fixed concentrations of the inhibitor, only the slope term of the rate expression is altered, i.e.:

$$\text{slope} = \frac{K_{a'}}{V_{\max}} \left(1 + \frac{[I]}{K_{ii}} \right) + \frac{K_a K_{b'}}{V_{\max}[B]} \left(1 + \frac{[I]}{K_i} \right) \quad (2)$$

while the ordinate intercept is constant, being

$$\text{ordinate intercept} = \frac{1}{V_{\max}} + \frac{K_{b'}}{V_{\max}[B]}$$

When analyzing the secondary plot of slope values against [I] (middle graph of Figure 3), the abscissa intercept will result, from Equation (2):

$$\frac{K_{a'}}{V_{\max}} \left(1 + \frac{[I]}{K_{ii}} \right) + \frac{K_a K_{b'}}{V_{\max}[B]} \left(1 + \frac{[I]}{K_i} \right) = 0$$

and rearranging

$$[I] = - (K_{a'}[B] + K_a K_{b'}) \frac{K_{ii} K_i}{K_{a'} K_i [B] + K_a K_{b'} K_{ii}}$$

At quasi-saturating concentrations of substrate *B*, for which $[B] \gg K_{b'}$ the following simplification can be made:

$$[I] = - \frac{K_{a'} [B] K_{ii} K_i}{K_{a'} K_i [B]}$$

and, consequently

$$[I] \cong -K_{ii}$$

Figure 1. Molecular structures of THI (1, 2-acetyl-4-((1*R*,2*S*,3*R*)-1,2,3,4-tetrahydroxybutyl)imidazole), pyridoxal-5'-phosphate (2) and ATP (3).

Figure 2. Schematic representation of the pathways describing the interaction of pyridoxal kinase (E) with its two substrates pyridoxal (A) and ATP (B) and their corresponding products pyridoxal-5'-phosphate (P) and ADP (Q).

On the other hand, when B is the variable substrate, double reciprocal plots at different fixed concentrations of the inhibitor show increases in both slopes and ordinate intercepts, i.e.:

$$\text{slope} = \frac{1}{V_{\max}} \left(K_{b'} + \frac{K_a K_{b'}}{[A]} \left(1 + \frac{[I]}{K_i} \right) \right)$$

$$\text{ordinate intercept} = \frac{1}{V_{\max}} \left(1 + \frac{K_{a'}}{[A]} \left(1 + \frac{[I]}{K_{ii}} \right) \right)$$

From secondary plots of slopes and ordinate intercepts versus [I] (Figure 4, middle and bottom, respectively), the values of K_i and K_{ii} can be determined. Furthermore, Equation (1) predicts that following a random Bi Bi mechanism a dead end competitive inhibitor for substrate A must be a non-competitive inhibitor of substrate B, and vice versa²².

Results and discussion

Pyridoxal-5'-phosphate represents the active form of vitamin B₆, a term that refers to the three vitamers pyridoxal, pyridoxine and pyridoxamine, as well as to their phosphorylated forms. As pyridoxal-5'-phosphate, it acts as a cofactor in more than 140 vital enzyme reactions and consequently a deficiency in vitamin B₆ may cause a variety of neurological and non-neurological disorders, e.g. seizures, headache, agitation, tremors or hallucination²³. Therefore, intracellular pyridoxal-5'-phosphate levels are constantly replenished by the catalytic action of either pyridoxal kinase or pyridoxine-5'-phosphate oxidase. While the latter catalyzes the FMN-dependent oxidation of pyridoxine or pyridoxamine phosphate, pyridoxal kinase leads to the ATP-dependent phosphorylation of the pyridoxal 5'-hydroxymethyl group. In this respect, pyridoxal kinase is of paramount importance as it is responsible for pyridoxal phosphorylation once pyridoxal is imported into the cell²⁴.

Interestingly, human pyridoxal kinase is inhibited by a range of substances of natural and synthetic origin, especially by small heterocyclic compounds, e.g. ginkgotoxin or theophylline^{12–15}. Hence, inhibition of pyridoxal kinase by THI does not appear unlikely and we considered a detailed investigation worthwhile. As every other kinase, also pyridoxal kinase relies on a nucleotide cofactor supplying the phosphate moiety to be transferred, i.e. ATP. Pyridoxal phosphorylation by pyridoxal kinase further depends on the presence of catalytic magnesium ions, which is why MgATP is generally used in corresponding enzyme assays, although human pyridoxal kinase activity may also be regulated by monovalent cations^{18,25}. While the intracellular ATP concentration in human cells is typically in the 1–10 mM range²⁶, the concentration of free pyridoxal-5'-phosphate in eukaryotic cells is maintained rather low, ~1 μM, as it is a very reactive molecule²³. By now, no data on the concentration of free pyridoxal in eukaryotic cells has been reported. In *E. coli* cells, the concentration of pyridoxal is ~15% of pyridoxal-5'-phosphate and pyridoxal is mainly bound to proteins²⁷. The reported catalytic

parameters of human pyridoxal kinase²¹ are K_m (MgATP) = 420 μM, K_m (pyridoxal) = 30 μM and k_{cat} = 45 min⁻¹.

In a first series of experiments, inhibition of pyridoxal kinase with respect to the substrate pyridoxal was investigated at a constant, quasi-saturating MgATP concentration. Pyridoxal-5'-phosphate accumulation was followed spectrophotometrically in the presence of increasing THI concentrations and initial velocities of product formation were obtained by linear regression. Lineweaver–Burk analysis revealed a series of straight lines intersecting in one point on the ordinate, as it is the case with a competitive mode of inhibition indicating that THI and pyridoxal (PL) bind to the same pocket on the enzyme active site (Figure 3, top). To corroborate this observation, secondary plots were prepared relating the slopes and intercepts derived from linear regression analysis of the Lineweaver–Burk data to their corresponding inhibitor concentrations²². Hereby, the nominal abscissa intercept of a line passing through the slope values reveals an apparent inhibition constant (K_{ii}) of 11.2 mM, while the secondary graph of intercepts confirms that THI competes with pyridoxal for binding to the enzyme active site (Figure 3, middle/bottom). Similar experiments were carried out at a quasi-saturating pyridoxal concentration to evaluate the inhibitory effect of THI with respect to MgATP. This time, the Lineweaver–Burk analysis produced a series of straight lines intersecting in the second quadrant to the left of the ordinate, indicating a mixed-type non-competitive mode of inhibition (Figure 4, top). A corresponding set of secondary plots confirmed this finding, as slope and intercept plots provided apparent inhibition constants of 5.3 mM (K_i) and 23.0 mM (K_{ii}), respectively (Figure 4, middle/bottom).

This inhibition pattern is consistent with a random Bi Bi rapid equilibrium system, in which THI is a competitive inhibitor of pyridoxal and binds with different affinities to either free enzyme or the enzyme–ATP complex. Figure 5 provides a schematic overview of the equilibria underlying pyridoxal kinase inhibition by THI. The white side of the cube represents the two pathways leading to a productive ternary complex (E–ATP–PL), where the free enzyme may bind both substrates following random sequential kinetics as it has been shown to be the case for pyridoxal kinase^{28–32}. As THI and pyridoxal share the same binding site, a competitive inhibition is observed for the equilibria describing the front face of the cube (dark grey), which is why no ternary E–PL–THI complex exists. Non-competitive inhibition however characterizes the interaction of pyridoxal kinase with respect to THI and ATP that is depicted on the top face of the cube (light grey). Here, a ternary E–ATP–THI complex can be formed following two pathways, as it is the case with the productive equilibria. Following the notation described by Fromm³³, the equilibrium of THI binding to the binary E–ATP complex is thus governed by K_{ii} , whereas binding of THI to the free enzyme is described by K_i (Figure 5). According to the inhibition pattern of a random Bi Bi rapid equilibrium system, it may be assumed that the inhibition constant obtained from the secondary plot of the slopes at constant and quasi-saturating ATP concentrations (Figure 3,

Figure 3. Kinetic analysis of pyridoxal kinase inhibition by THI with respect to the substrate pyridoxal (PL) shows that THI acts as a competitive inhibitor. Lineweaver-Burk (top) and secondary plots (middle, bottom) reveal a K_{ii} inhibition constant of 11.2 mM. Mean values with standard errors are shown from duplicate or triplicate measurements; THI concentrations: $\bullet$ 0 mM, $\blacksquare$ 4.2 mM, $\blacktriangle$ 7.5 mM, $\blacktriangledown$ 15.0 mM and $\blacklozenge$ 20.0 mM.

middle) approximately corresponds to K_{ii} , since under these conditions most of the enzyme is present in complex with ATP. The inhibition constant found from the secondary plot of the ordinate intercepts in the mixed-type inhibition (Figure 4, bottom) also corresponds to the binding of THI to the enzyme-ATP complex. This is in good agreement with the experimental values of 11.2 and 23.0 mM, which are of the same magnitude. On the other hand, the inhibition constant derived from the secondary plot of the slopes in the mixed-type inhibition (5.3 mM; Figure 4, middle) corresponds to K_i , i.e. to the binding of the inhibitor to free enzyme. These results also suggest that THI binds better to the free enzyme than to the enzyme-ATP complex (K_i is smaller than K_{ii}).

Figure 4. Kinetic analysis of pyridoxal kinase inhibition by THI with respect to the cosubstrate ATP shows that THI acts as a non-competitive inhibitor. Lineweaver-Burk (top) and secondary plots (middle, bottom) reveal the inhibition constants $K_i = 5.3$ mM and $K_{ii} = 23.0$ mM. Mean values with standard errors are shown from duplicate or triplicate measurements; THI concentrations: $\bullet$ 0 mM, $\blacksquare$ 4.2 mM, $\blacktriangle$ 7.5 mM, $\blacktriangledown$ 15.0 mM and $\blacklozenge$ 20.0 mM.

Conclusions

In summary, THI acts as an inhibitor of pyridoxal kinase being competitive with respect to pyridoxal with low to moderate millimolar affinity. In view of maximum allowed THI levels of 10 mg/kg in caramel food colourings and a daily mean exposure of 300 mg/kg bodyweight, no relevant pyridoxal kinase inhibition is expected as at 80 kg bodyweight a maximum daily uptake of 240 μ g THI results. Assuming a mean body water content of 60% and a complete resorption of THI with even distribution throughout the whole body, this would provide a local THI concentration of ~ 20 nM.

However, research investigating the effects of THI derivatives on sphingosine-1-phosphate lyase activity and related

Figure 5. Schematic overview of competing equilibria describing the inhibition of pyridoxal kinase (E) by THI in the presence of both substrates pyridoxal (PL) and ATP (adapted from Asante-Appiah and Chan³⁴). K_i and K_{ii} refer to the inhibition constants describing a random Bi Bi rapid equilibrium system in which PL and THI compete for the same enzyme binding site.

lymphopenia outcome should still consider pyridoxal kinase inhibition as a possible cause. In fact, the very delicate equilibrium of pyridoxal-5'-phosphate cellular availability may be perturbed even by a moderate inhibition of pyridoxal kinase, which can lead to a significant lowering of pyridoxal-5'-phosphate levels. Furthermore, when considering pyridoxal-5'-phosphate homeostasis one has to bear in mind that a local imbalance of active vitamin B₆ concentrations as a result of varying local THI distribution is of uttermost importance.

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Declaration of interest

The authors declare no conflict of interest.

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