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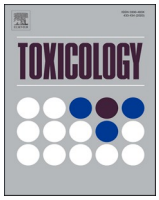

A comprehensive review of the proline mimic azetidine-2-carboxylic acid (A2C)

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A R T I C L E I N F O

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A B S T R A C T

The imino acid azetidine-2-carboxylic acid (A2C), a proline homologue, was first identified in liliaceous plants in 1955. Its ability to exchange for proline in protein synthesis is responsible for its teratogenic effects and has made it a very useful tool for generating non-native proteins to study proteotoxic stress and ER stress. The tRNA synthetases from some A2C-producing plants can discriminate between proline and A2C, but for most plants and for mammalian cells, A2C is mistakenly used in protein synthesis in place of proline and can avoid cell proof-reading mechanisms. Human exposure to A2C would be very limited had it not been for the development of sugar beets as an alternative source of dietary sucrose to sugar cane, and the widespread use of the plentiful byproducts as livestock fodder. Fodder beets, a very high yielding forage crop, are also used as livestock fodder particularly for lactating cows. It is therefore possible for A2C to enter the human food chain and impact human health. It was hypothesised that its ability to replace proline in protein synthesis generates immunogenic neo-epitopes in myelin basic protein and could therefore be a causative factor for multiple sclerosis. In this review we discuss the distribution of A2C in nature, what is known about its toxicity, and the impact of the proline to A2C exchange on protein structure and function and in particular the proteins collagen and myelin basic protein. We summarise analytical approaches that can be used to quantify A2C in complex biological samples and the adaptations made by some organisms to avoid its toxic effects. We summarise the evidence for human exposure to A2C and the geographical and temporal links to higher incidences of MS. Finally, we highlight gaps in our knowledge that require addressing before we can determine if this non-protein amino acid is a threat to human health.

1. Objectives and scope

Following its discovery in 1955 in lily-of-the-valley, azetidine-2-carboxylic acid (A2C) was primarily of interest to plant chemists. When it was later discovered that it could replace proline in protein synthesis it became of general interest to protein chemists and toxicologists. More recently due to hypothetical links to multiple sclerosis (MS) it has attracted the attention of neuroscientists and epidemiologists. In this review we provide a comprehensive overview of almost 50 years of literature on A2C for a broad scientific readership. We discuss its discovery in plants and investigations into its possible role as an allelochemical. We summarise the wide range of *in vitro* and *in vivo* toxicity studies that have been performed and have demonstrated its teratogen and proteotoxic effects. We summarise the analytical approaches used to identify and quantify A2C in complex biological samples and examine the evidence for human exposure to A2C as well as the geographical and temporal links to higher incidences of MS worldwide. Finally, we identify gaps in our knowledge that need to be addressed before we can determine if this non-protein amino acid is a threat to human health. A2C is sometimes referred to in the literature as AZE but since azetidine is also a naturally occurring molecule we feel that the abbreviated term A2C is less ambiguous.

2. The discovery of azetidine-2-carboxylic acid and its distribution in nature

Azetidine-2-carboxylic acid (A2C) was first identified in a 70 % ethanol extract of fresh leaves of *Convallaria majalis* (Lily-of-the valley) using two-dimensional paper chromatography (Fowden, 1955). It was characterised chemically by comparison to synthetic material (Fowden, 1955, 1956). A2C is a four-membered ring analogue of the protein amino acid proline which has a five-carbon ring (Kubyshkin and Rubini,

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2024) (Fig. 1). Both amino acids have a secondary amine since the nitrogen bonded to the alpha-carbon also forms part of the ring. A2C is unstable to treatment with mineral acids, and exposure to concentrated HCl produces 4 products one of which is homoserine (Fowden, 1956). Soon after Fowden's discovery, an identical substance was isolated from another liliaceous species *Polygonatum multiflorum* (Solomon's seal) (Virtanen and Linko, 1955). A2C contained more than 50 % of the total nitrogen of the *Polygonatum* rhizome (Fowden, 1972). A comprehensive survey of liliaceous and related plants (Agavaceae and Amaryllidaceae) then found A2C in twenty three of the ninety species examined. It could occur in all parts of a plant; leaf, stem, root and seed (Fowden and Steward, 1957). Analysis of the total amino acid content of Liliaceae and Agavaceae revealed that A2C was on average 85.5-fold more abundant than proline in leaves of the 9 plants examined and was on average 12-fold more abundant than proline in seeds of the 6 plants examined (Fowden, 1956). Amounts of A2C equivalent to 3–6 % of the dry weight may accumulate in tissues of *Convallaria majalis* (lily-of-the-valley) or *Polygonatum multiflorum* (Fowden, 1959; Fowden et al. 1967). Although A2C initially appeared to be confined to members of Liliaceae, and a few species of Agavaceae (Fowden and Steward, 1957) it was later characterised as a constituent of species of unrelated groups of plants, such as the legumes *Delonix regia* and *Peltophorum pterocarpum* (Sung and Fowden, 1969) and *Bussea massaienis* and *Parkinsonia aculeata* from the subfamily Caesalpinioideae (Watson and Fowden, 1973). Although A2C was not detectable in sugar beets (*Beta vulgaris,* Amaranthaceae) using the standard paper chromatographic procedures utilised in the previous studies it was found in a nitrogenous fraction that was a by-product of large-scale sugar extraction (Fowden, 1972). It was later shown that proline was present in a 150-fold excess over A2C in sugar beet pulp (Rubenstein et al. 2009). A2C was also detected in one of the lower plants; analysis of the amino acid profiles of 18 macroscopic marine algae showed that *Lophocladia lallemandi* was unique and had high concentrations of proline and A2C (Watson and Fowden, 1973). The distribution of A2C in nature has not been comprehensively examined and it has been described as having 'an apparent haphazard taxonomic distribution' (Adeyeye and Blum, 1989). Until a more thorough analysis of the plant content of A2C is carried out the potential for human exposure cannot be fully determined.

3. Biosynthesis of azetidine-2-carboxylic acid

Despite having a simple chemical structure, the biosynthetic pathway for A2C is not completely understood (Pang et al. 2023). When it was discovered in 1956 the authors proposed a pathway in which aspartic-β-semialdehyde underwent reductive cyclisation to generate A2C (Fowden, 1956). They suggested that aspartic-β-semialdehyde could be generated from either aspartic acid, homoserine or 2,4-diaminobutyric acid (2,4-DAB) (Fowden, 1956). Isotope labelling experiments over the next 20 years, in which a range of precursors were supplied to *Convallaria majalis* and *Delonix regia* demonstrated that many amino acids could be involved in the synthesis of A2C including 2,4-diaminobutanoic acid (Sung and Fowden, 1969), methionine (Leete et al. 1986) and homoserine (Leete et al. 1986). Methionine appeared to be the most efficient precursor in *Convallaris majalis* through use of radiolabelled methionine, 2,4-diaminobutanoic acid and 1-aminocyclopropane-1-carboxylic acid (Leete et al. 1986). This study also suggested that 2,4-diaminobutanoic acid is transaminated at the 4-amino group to aspartic-β-semialdehyde followed by reduction to homoserine, returning it as a methionine precursor (Leete et al. 1986) (Fig. 2). The biosynthetic enzymes involved in the synthesis of A2C in plants have yet to be identified.

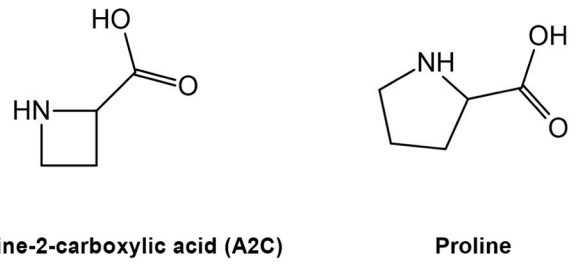


Fig. 1. Comparison of the chemical structures of the protein amino acid proline and the non-protein amino acid azetidine-2-carboxylic acid (A2C).

Beyond its synthesis in plants, A2C is also produced by several bacteria. *Pseudomonas aeruginosa* are capable of synthesising A2C via an S-adenosylmethionine enzymatic process, which feeds into a quorum-sensing regulated non-ribosomal peptide synthetase (NRPS) pathway to form azetidine-containing alkaloids called azetidomonamides such as azabicyclene (Hong et al. 2019; Patteson et al. 2019). *Cystobacter violaceous* was also shown to be able to synthesise A2C through the class 1 methyltransferase VioH catalysing the cyclisation of S-adenosyl-L-methionine (Yan and Muller, 2019).

4. Analysis of azetidine-2-carboxylic acid in biological samples

Accurate identification and quantification A2C are essential for elucidating its distribution in nature and its potential relevance to human health. It requires analytical methods that are selective, sensitive and suitable for a range of biological matrices. A2C was first identified and characterised using two-dimensional (2D) paper chromatography (Fowden, 1956). Further characterisation of A2C and related compounds employed more modern structural elucidation techniques including nuclear magnetic resonance (NMR) and infrared (IR) spectroscopy (Phillips and Cromwell, 1973; Futamura et al. 2005). Plant tissue is the most widely analysed biological matrix containing A2C. Extraction usually starts with maceration and lyophilisation of the plant material which has the advantage of allowing the determination of plant dry weight for normalisation. Water is a suitable extraction solvent due to the polar nature of A2C. Heating or sonication can further enhance extraction efficiency by increasing cell lysis and A2C solubility, although the conditions have not been fully optimised (Rubenstein et al. 2009; Ranjha et al. 2021). A post-extraction sample cleanup is usually performed due to the diversity of compounds extracted from plant matrices. Strong cation exchange SPE (Rubenstein et al. 2006) can be used to remove interfering compounds as they can reduce the sensitivity of many analytical techniques including LC-MS (Trufelli et al. 2011).

Quantitative analysis of A2C commonly employs liquid chromatographic (LC) separation of analytes coupled to mass spectrometers (MS) for detection. Early analytical efforts utilising amino acid analysers that relied on principles of ion exchange chromatography (Lane et al. 1971; Troxler and Brown, 1974; Trasko et al. 1976) have now been superseded by LC-MS. Being a small, polar molecule, A2C has poor retention on reverse phase chromatographic columns which is the most widely used LC separation method. Derivatising agents to modify A2C could enhance its column retention but this has only been reported in the literature for gas chromatographic applications. The use of hydrophobic interaction liquid chromatography (HILIC) is often the separation method of choice for small polar molecules (Kim et al. 2015). Techniques such as UV spectroscopy were initially used to detect and quantify A2C (Kim et al. 2015), but the use of tandem mass spectrometry (MS/MS) offers greater selectivity due to the fragmentation of ions for compounds with a similar mass-to-charge ratio (m/z). One consideration for the analysis of A2C on some LC-MS/MS platforms utilising electrospray ionisation (ESI) is the reported ability of the protein amino acid threonine to undergo the neutral loss of water in-source, resulting in a mass shift to 102 m/z thus requiring careful chromatographic separation from A2C for accurate determination and quantification (Rubenstein et al. 2009). The use of gas chromatography has been reported in the literature however it is less common and requires derivatisation, usually by silylation of the amino acids prior to analysis by (Kamerling et al. 1983; Baek et al. 2012).

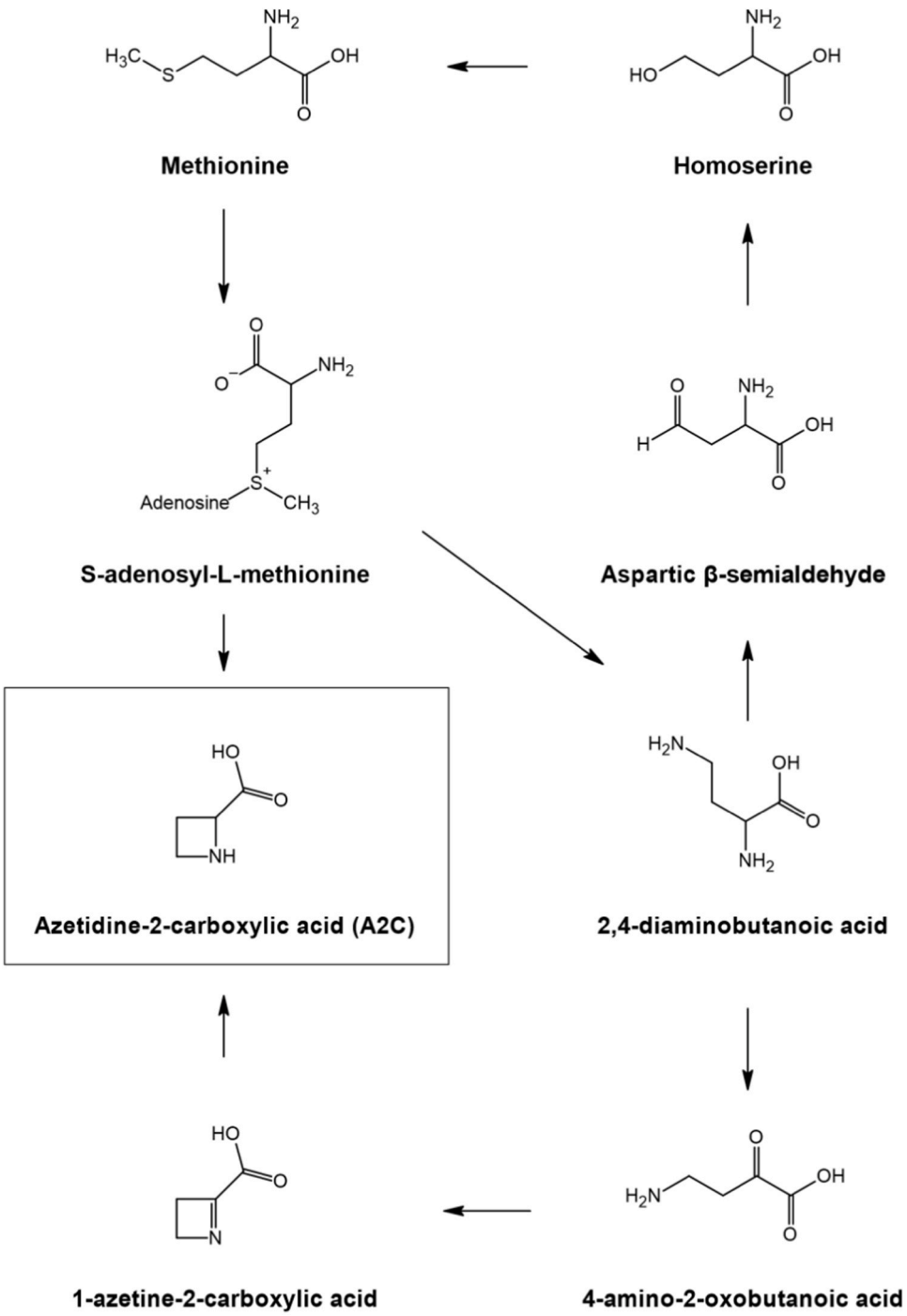


Fig. 2. Proposed biosynthetic pathways for azetidine-2-carboxylic acid (A2C).

5. Toxicity of azetidine-2-carboxylic acid to other plant species

Early A2C toxicity studies examined its allelopathic properties and demonstrated a potent inhibitory effect on the growth of carrot phloem explants (*Daucus carota*) (Steward et al. 1958). A2C was also lethal to mung bean seedlings (*Phaseolus aureus*) at low concentrations (Fowden, 1962) and in both carrot and mung bean the protein amino acid proline was shown to be protective. The growth inhibitory effect of A2C was examined in other plants that were non-producers and it was found to have a powerful inhibitory effect on the growth of peanut and cucumber seedlings and to a lesser extent on barley seedlings (Fowden, 1962). Supplementation with proline was again protective, leading the author to suggest that growth inhibition was associated with the lethal synthesis of anomalous (A2C-containing) protein molecules, and the differing degrees of growth inhibition produced in plant species was related to the levels of endogenous proline (Fowden, 1962). Susceptibility to the toxic effects of A2C however was subsequently shown to be dependent on differences in the substrate specificity of the prolyl-tRNA synthetases, with the extent of growth inhibition correlating to the extent of replacement of proline residues in newly synthesised proteins with A2C (Norris and Fowden, 1972). Endogenous proline concentrations however could also affect toxicity due to direct competition between proline and A2C for tRNA charging.

6. Toxicity studies on azetidine-2-carboxylic acid in bacteria and viruses

The first A2C toxicity study beyond plant species was carried out using *Escherichia coli* (*E. coli*); two naturally occurring proline homologues (A2C and L-pipecolic acid), and three synthetic derivatives were examined for their growth inhibitory effects (Fowden and Richmond, 1963). Only A2C was growth inhibitory in *E. coli,* and equal concentrations of DL-proline were fully protective. Alkaline hydrolysis allowed the recovery of A2C from the *E. coli* proteins suggesting that it was present in the polypeptide chain (Fowden and Richmond, 1963). The effect of A2C on the conversion of glutamic acid to pyrroline-5-carboxylic acid (PC) was also evaluated in *E. coli* as this step was crucial for regulating proline synthesis in the *E. coli* strain used (Tristram and Thurston, 1966). When A2C was introduced, it mimicked proline in the PC production pathway, acting as an end-product inhibitor and consequently deterring cell growth (Tristram and Thurston, 1966; Baich and Smith, 1968). Using electron microscopy, it was shown that the presence of A2C during bacteriophage growth gave rise to substructure aberrations (polytail tubes) (Cummings et al. 1967). Overall, these early studies provided evidence for A2C successfully competing with proline in protein synthesis and in metabolic pathways.

7. Insertion of azetidine-2-carboxylic acid into proteins

A2C is a non-protein amino (or imino) acid (NPAA) and a close structural analogue of the protein amino acid proline (Kubyshkin and Rubini, 2024). The chemistry of A2C and proline are discussed in an excellent review by Kubyshkin and Rubini (Kubyshkin and Rubini, 2024). When *E. coli* cultures were treated with 1 mM A2C it was estimated that around 50 % of proline residues in the newly synthesised proteins had been replaced by A2C (Fowden and Richmond, 1963). Similarly, analysis of mung bean radicles from seeds that had been germinated in water containing A2C identified A2C in hydrolysed proteins (Fowden and Richmond, 1963). At the highest A2C concentrations examined (3 mg/50 seeds) it was estimated that 31 % of proline was replaced by A2C in the radicle proteins (Fowden and Richmond, 1963). Protein hydrolysis in these studies was carried out using 5 N $Ba(OH)_2$ since A2C is unstable to 6 N HCl, the most commonly used reagent for protein hydrolysis. At enzyme saturating conditions, A2C could be activated by prolyl-tRNA synthetase at rates estimated to be almost half that measured for proline in non-producer plants (Norris and Fowden, 1972). The ability of A2C to replace proline in protein synthesis has subsequently been demonstrated in a diverse range of experimental systems. For example, radiolabelled (^{14}C) A2C was incorporated into haemoglobin from rabbit reticulocytes *in vitro* (Baum et al. 1975) and was incorporated into collagen from cells isolated from embryonic chick tendon (Uitto and Prockop, 1977). The unicellular alga, *Cyanidium caldarium* incorporated A2C into the protein pigment phycocyanin (Troxler and Brown, 1974). A2C was also incorporated into glutathione S-transferase (GST) synthesised by murine breast cancer cells (4T1) following treatment with A2C for 24 hours (Li et al. 2022). Analysis by mass spectrometry identified nine subtypes of the GST protein created by A2C exchange with proline (Li et al. 2022)

Since A2C can mimic proline in protein synthesis it belongs to a subset of NPAAs known as the proteinogenic NPAAs (Rodgers et al. 2002) some of which have been shown to negatively impact human health (Rodgers, 2014). When the impact of A2C on myelin basic protein (MBP) was modelled in *E. coli* overexpressing MBP, the MBP synthesised was shown to have A2C incorporated in place of proline and although the degree of proline to A2C substitution was relatively low (a maximum of 3 of 11 potential sites), molecular modelling confirmed that it could have a severe effect on the conformation of the protein (Bessonov et al. 2010). A his-tagged over-expression system in human HEK-293 cells demonstrated the misincorporation of AZE across most proline sites in maltose binding protein (Song et al. 2017) supporting the hypothesis of Rubenstein that A2C has the potential to generate multiple non-native proteins some of which might be immunogenic (Rubenstein, 2008).

8. Selectivity of prolyl-tRNA synthetases

Aminoacyl-tRNA synthetases (aaRSs) play a critical role in protein synthesis by pairing amino acids with their corresponding tRNAs according to the genetic code (Rubio Gomez and Ibba, 2020). However, due to the chemical and structural similarities of certain amino acids, aaRSs can ligate tRNAs with the wrong amino acid (Rubio Gomez and Ibba, 2020). Prolyl-tRNA synthetase (ProRS) purified from *Delonix regia* and *Parkinsonia aculeata* failed to activate A2C significantly (Norris and Fowden, 1972) suggesting that these plants had evolved a more advanced synthetase capable of discriminating against A2C. Generally, the ability of an analogue to act as a substrate for the parent aaRS does not extend to the species producing the analogue. For example, several fine fescue species (Festuca spp.) release the tyrosine derivative *meta*--tyrosine into the rhizosphere (Bertin et al. 2007). This allelochemical is mistakenly incorporated into proteins and inhibits root growth of other plants but not the producers (Bertin et al. 2007). Discrimination at the level of amino acid activation is a common mechanism whereby plants producing analogues resist possible antimetabolic effects (Rodgers, 2014). The partially purified ProRS from other plants known to be producers such as *Beta vulgaris* were found to activate A2C, leading the authors to suggested that these plants had no need to evolve a ProRS capable of discriminating against A2C because A2C never reaches a concentration in the plant cell which would lead to deleterious effects (Norris and Fowden, 1972). More recent studies have identified isoforms of ProRS in plant species (Lee et al. 2016). *Arabidopsis thaliana* which is sensitive to A2C and has two ProRS isozymes with similar affinities for proline (Lee et al. 2016); the organellar version however has a very low affinity for A2C whereas the cytosolic version shows similar affinities for A2C as proline (Lee et al. 2016). Molecular modelling studies suggest that this difference is due to different modes of substrate binding by the isoenzymes (Lee et al. 2016).

Selective tRNA charging is just one of several checkpoints where the incorporation of non-cognate amino acids into the protein can be prevented. Studies using isolated synthetases have demonstrated species differences in the capacity for editing mischarged tRNAs (Beuning and Musier-Forsyth, 2001). In mammalian cells, A2C was shown to be a 'double mimic' and was activated by both human ProRS and AlaRS however only ProRS was able to complete the charging event (Song et al. 2017). The pre-transfer editing system in AlaRS was able to clear the mischarged tRNA but the ProRS editing system was not (Song et al. 2017). Two editing systems exist, a pre-transfer editing where the incorrectly formed aminoacyladenylate is hydrolysed and post-transfer editing where the mischarged tRNA is deacylated (Beuning and Musier-Forsyth, 2001). *E. Coli* ProRS was shown to lack significant pretransfer editing capability for A2C (Beuning and Musier-Forsyth, 2001). ProRS commonly misactivates Ala, and the human trans-editing enzyme Hs ProXp-ala prevents Pro to Ala mutations by hydrolysing misacylated Ala-$tRNA^{Pro}$ (Vargas-Rodriguez et al. 2020). It was recently shown, using the isolated enzymes, that Hs ProXp-ala also hydrolyses A2C-$tRNA^{Pro}$ and this reaction is only 3.4-fold less efficient than the hydrolysis of Ala-$tRNA^{Pro}$ (Vargas-Rodriguez et al. 2020). These data would indicate that post-editing factors might significantly reduce the incorporation of A2C into human proteins.

9. Changes in protein structure following an azetidine-2-carboxylic acid to proline exchange

Despite having no functional groups, proline has a major impact on protein backbone and architecture. Generally, in a peptide bond the hydrogen atom of the amino group of one amino acid (AA) and the oxygen atom of the carboxyl group of the other AA occupy opposite positions (trans) in the plane of the bond. Since the amino group of

proline is covalently locked within a 5-member ring its mobility in peptide bond formation is constrained, so unlike other amino acids, which prefer the trans configuration, the proline molecule isomerizes by swinging its entire ring into the cis position, a conformational change that realigns the local peptide sequence, resulting in folding of the protein. Proline residues tend to be excluded from alpha helices and beta sheets but sequences of three consecutive prolines can fold into poly-proline helices; structures that join alpha helices and beta sheets as architectural motifs in protein configuration (Morgan and Rubenstein, 2013). Tri-proline sequences might fold into right-handed or left-handed helices inducing sharp turns in the local geometry and are important in protein-protein interactions and in signal transduction (Morgan and Rubenstein, 2013). 4.4 % of protein residues are in found in trimers or longer spans (Morgan and Rubenstein, 2013). Spans of 6 or more prolines are associated with DNA/RNA processing and developmental processes. A2C is identical to proline except that its ring contains 4 members instead of 5 (Fig. 1) so when A2C replaces proline in a protein, it stereochemically alters torsion angles across polypeptides to become 15° smaller, changing the angles of turns and cis-trans isomerisation within proteins (Fowden and Richmond, 1963; Baeza et al. 2008). This was consistent with studies that used conformational energy computations to demonstrate that peptides and polymers containing A2C were more flexible than corresponding peptides containing proline (Zagari et al. 1990b, 1990a; Zagari et al. 1994). Because of their hydrophobicity, proline residues tend to be located within the interior of a protein, and it has been shown that replacement of an interior (non-solvent exposed) amino acid with a NPAA has a greater impact on protein folding and 3-dimensional structure than replacement of external solvent-exposed residues (Ozawa et al. 2005). Thus, the substitution of A2C for proline will significantly impact protein structure and function.

10. Impact of azetidine-2-carboxylic acid on collagen synthesis and structure

While proteinogenic amino acids such as A2C can be randomly incorporated into any newly synthesised proteins, proteins rich in the 'parent' amino acid are likely to be more heavily impacted by the amino acid exchange. Early mammalian studies on A2C toxicity therefore focussed on its effect on the proline rich protein collagen (~10 % proline). Collagen is the most abundant protein in mammals and is laid down mostly in embryonic and early stages in life (Holwerda and van Loon, 2022) thus is heavily involved in the morphological development of embryonic organs (Hay, 1981; Butler et al., 2018). Studies using chick embryonic cartilage demonstrated that A2C inhibited the incorporation of ^{14}C proline into collagen and was itself incorporated into newly synthesised collagen resulting in abnormal procollagen (Takeuchi and Prockop, 1969). The abnormal collagen polypeptides were retained intracellularly but since A2C did not prevent the extrusion of previously accumulated and normal hydroxylated protocollagen, its retention in the cell was directly related to the incorporation of A2C (Takeuchi et al. 1969). Subsequent studies examined the effects of A2C on collagen produced by isolated limb buds from mouse embryos (Aydelotte and Kochhar, 1972) and in the presence of A2C, growth of the entire limb bud was inhibited, chondrogenesis was delayed, and the synthesised collagen was malformed (Aydelotte and Kochhar, 1972). These deleterious effects were not seen with the D-isomer of A2C and could be rescued with proline, providing further evidence for the biosynthetic incorporation of this NPAA into collagen *in vivo* (Aydelotte and Kochhar, 1972). Exposing isolated cells from the tendons of chick embryos to A2C resulted in a decreased production of extracellular procollagen leading the authors to hypothesise that the presence of A2C in the polypeptide chains was preventing them from assuming a normal triple-helical conformation, resulting in their degradation (Uitto and Prockop, 1974). A similar observation was made when human skin fibroblasts were exposed to A2C and the newly synthesised collagen polypeptides were unable to form a stable triple-helical conformation (Tan et al. 1983). As observed previously in the studies using chick tendons (Uitto and Prockop, 1974), the nonhelical polypeptides were highly susceptible to proteolysis (Tan et al. 1983). In a mouse mammary gland organ culture model, collagen synthesis stimulated by insulin, cortisol, and prolactin was inhibited by 75 % with A2C (Wakimoto and Oka, 1983). A similar effect was reported on the synthesis of casein and α-lactalbumin, and in all cases inhibition was reversed with the addition of exogenous proline so although the impact of A2C on proteins was not confined to collagen its incorporation into collagen had a major impact on foetal development (Wakimoto and Oka, 1983).

11. Teratogenic effects of azetidine-2-carboxylic acid

Studies on A2C toxicity have also examined its direct impact on development during foetal and neonatal stages of life. A2C had an influence on skeletal development when administered in the early period of organogenesis. Pregnant rats that received intraperitoneal A2C injections for 3 days, produced offspring with abnormalities, including club foot, cervical ribs and incomplete ossification (Nagai et al. 1978). Similar results were reported in hamsters where subcutaneous haemorrhage and cleft palate were the most frequently occurring externally visible defects but the major teratological effect of A2C was retardation of skeletal development (Joneja, 1981). Other studies have reported systemically inhibited mast-cell-mediated angiogenesis in A2C-treated rats (Norrby, 1993). When A2C was administered to chicken eggs it caused an inhibition of collagen development in the chicks, and growth retardation (Hall, 1978). Overall, these *in vivo* studies demonstrated in multiple animal models that the incorporation of A2C into collagen during foetal development, resulted in toxicity that manifested in stunts and retardations in growth.

12. Myelin basic protein and neurotoxicity

Multiple sclerosis (MS) is a chronic demyelinating disease with an unknown mechanism of pathogenesis characterised by inflammation of the CNS and an immune attack against the myelin sheath (Compston and Coles 2008). The major protein in the myelin sheath is myelin basic protein (MBP) and its dysfunction has been implicated as a cause of this and other demyelinating diseases (Martinsen and Kursula, 2022). Anti-MBP antibodies have been implicated as a possible cause of MS (Warren and Catz, 1992) and MBP has been extensively researched as a source of autoantigenic epitopes (Martinsen and Kursula, 2022). A key feature of MBP is its tri-proline segment (residues 99–101) which is essential to its structure and function (Ridsdale et al. 1997) and has the potential to be significantly altered by A2C misincorporation. A hypothesis linking human consumption of A2C to MS was proposed by Rubenstein in 2008 (Rubenstein, 2008). This hypothesis was built around some circumstantial evidence. For example, there appears to be a close link between the worldwide prevalence of MS and the geography of beet agriculture with some centres of beet agriculture having high-prevalence clusters of MS cases (Rubenstein, 2008). This pattern of high MS incidence in synchrony with sugar beet producing regions has been observed in Canada, Japan, Sardinia, and the Orkney Islands (Poskanzer et al. 1980; Pugliatti et al. 2001; Beck et al. 2005; Houzen et al. 2008)

Another piece of evidence presented was that the first description of MS made by Charcot in Paris in 1835 closely corresponded with the increase in sugar beet cultivation as a replacement for sugar cane as a sucrose source in France (Rubenstein, 2008). Since central nervous system myelination in humans occurs principally in late gestation and during childhood the A2C hypothesis proposed that the misincorporation of A2C into MBP reaches a critical level and triggers inflammation and activation of an autoimmune response. The hypothesis also fits the observation that studies of immigrants from a wide range of countries correlate the risk of multiple sclerosis with the place of residence in childhood (Martinsen and Kursula, 2022). Exposure to A2C during

nervous system development could generate immunogenic epitopes in the newly synthesised MBP.

13. Assessing the cytotoxicity of azetidine-2-carboxylic acid *in vitro*

As previously discussed, most early investigations into A2C toxicity focussed on its impact on the two proline-rich proteins MBP and collagen. However, since A2C can be readily incorporated into any newly synthesised proteins encoded for proline it can have a global impact on mammalian cell function as has been shown for other proteinogenic NPAAs (Rodgers et al. 2004; Giannopoulos et al. 2019; Quinn et al. 2021). When A2C was investigated in HeLa cells, high levels of A2C were required to produce significant levels of toxicity (1 mM) (Song et al. 2017). Proline was fully protective when supplied with A2C (Song et al. 2017). The accumulation of misfolded proteins in the endoplasmic reticulum (ER) activates the unfolded protein response; a program to restore ER homeostasis that includes protein refolding, degradation, and in some cases a temporary decrease in protein synthesis (Dabsan et al. 2024). Since A2C is readily incorporated into proteins generating non-native proteins it is a useful tool for mechanistic studies on ER stress (Gu et al. 2004; Roest et al. 2018; Piper et al. 2023). Using HeLa cells, it was shown that endogenously generated A2C-containing proteins triggered 2 of the 3 arms that initiate the unfolded protein response (Roest et al. 2018).

A time course, employing continuous live cell imaging demonstrated that it took 12 hours for toxicity to develop in 2 mM A2C-treated human neuroblastoma cells, consistent with a delayed cytotoxic response due to the synthesis and accumulation of non-native proteins (Samardzic and Rodgers, 2019). In addition, there was an expansion in the acidic (lysosomal/late endosomal) compartments of A2C-treated cells characteristic of the delivery of aggregates not degradable by the ubiquitin-proteasomal system to lysosomes (Rodgers et al. 2002). Aggregate accumulation was accompanied by an increase in apoptotic cell death and changes to mitochondrial numbers, area, and interconnectivity (Samardzic and Rodgers, 2019). Proteins synthesised in the mitochondria might be more susceptible to A2C incorporation due to inherent differences in their proof-reading machinery (Konovalova et al. 2015) Mitochondrial function was significantly impacted following A2C treatment and basal and maximal respiration were significantly decreased as was ATP production (Samardzic and Rodgers, 2019). The observation that A2C causes mitochondrial dysfunction is important given the link between mitochondrial dysfunction, apoptosis, and neurodegenerative diseases such as MS (Mahad et al., 2008; Mahad et al., 2009). In addition to reducing viability and increasing apoptotic cell death in microglial cells, 1 mM A2C induced pro-inflammatory responses in these macrophage-like cells (Piper et al. 2022). One common thread connecting these *in vitro* studies was the observation that the impact of A2C on cell function was preventable by co-administration with proline and therefore attributable to the global generation of non-native proteins. The observation that mitochondrial function was impacted is important since mitochondrial proofreading mechanisms might be less stringent that the cytosolic equivalent. In addition, since mitochondria play a central role in health and disease the potential for A2C to cause mitochondrial dysfunction could have important implications for human health (Casanova et al. 2023). A summary of *in vitro* studies investigating A2C toxicity is presented in Table 1.

14. Assessing the neurotoxic effects of azetidine-2-carboxylic acid *in vivo*

The neurotoxic effects of A2C have been directly assessed in some *in vivo* studies. A2C exposure in CD1 mice was examined using multiple experimental designs in which A2C was administered either by daily oral gavage, daily intraperitoneal (IP) injection or by IP injections for 4 weeks with doses ranging from 300 mg/kg to 600 mg/kg (Sobel et al.

Table 1
Summary of *in vitro* studies examining A2C toxicity.

Reference	Cell type	A2C Treatment	Results
Fowden & Richmond (1963)	E. coli	0.1 mM A2C for 2 hours.	Inhibition of growth was detected immediately after A2C treatment; prevented by coadministration of proline.
Tristram & Thurston (1966)	E. coli.	0.8 mM A2C.	A2C treatment resulted in a 20–28 % decrease in the growth rate of E. coli.
Cummings et al. (1967)	T-Even Bacteriophages.	0.45 mM A2C for 2.5 hours.	A2C treatment gave rise to aberrant substructures in bacteriophages.
Baich & Smith (1968)	E. coli.	0.1 – 0.8 mM A2C for 4 hours.	A2C inhibited the growth rate of E. coli by 70–80 %.
Takeuchi & Prockop (1969)	Chick embryonic cartilage.	0.1 – 3 mM A2C for 4 hours.	Incorporation of A2C into collagen, resulting in abnormalities in growth.
Aydelotte & Kochhar (1972)	Mouse limb buds.	0.5 – 2 mM A2C and 0.75 mM A2C with 1.3 mM proline for up to 5 days.	Limb deformities detected with 0.75 mM A2C; proline counteracted this.
Alescio (1973)	Mouse embryonic lung rudiments.	0.4 – 0.8 mM A2C for 2 days.	Lung rudiments produced fewer terminal buds and decreased branching with A2C treatment.
Uitto & Prockop (1974)	Isolated cells from embryonic tendon of chick embryos.	0.25 – 1 μM A2C for 3 hours.	A2C interferes with the helix formation of collagen and decreased its synthesis.
Grant et al. (1975)	E. coli.	0.2 mM A2C for 3–6 hours.	Resulted in a decrease in growth response and generation time.
Baum et al. (1975)	Reticulocytes from anaemic rats.	1 – 10 mM A2C or 0.8 mM A2C with 0.145 mM proline.	A2C incorporated into haemoglobin, proline addition reduced this by 92 %.
Oikarinen et al. (1976)	Chick embryo tendon cells.	2 mM A2C for 1 hour.	A2C inhibited collagen triple-helix formation.
Trasko et al. (1976)	Haemoglobin S in sickle erythrocytes.	0.1 mM A2C for 1 hour.	A2C incorporated into haemoglobin S.
Hall (1978)	Stem cells from chicken embryos.	1 mM A2C or 1 mM A2C with 2.6 mM proline for 1–4 days.	Sequential exposure of A2C inhibited chondrogenesis which was prevented with the addition of proline.
Wakimoto & Oka (1983)	Mouse mammary gland in organ culture.	0.2 – 0.8 μM A2C for 3 days.	A2C inhibits collagen and milk protein synthesis in mammary gland cultures, reversible by proline.
Tan et al. (1983)	Human skin fibroblasts	10 – 1000 μM A2C for 6 days.	A2C inhibits fibroblast proliferation and reduces collagen deposition.
Blankenship & Benson (1984)	Sea urchin micromeres.	3 – 5 M A2C for up to 7 days.	A2C significantly disrupted collagen metabolism and decreased spicule formation in micromeres.
Mizzen & Welch (1988)	Hamster kidney cells.	5 mM A2C for 12 hours.	A2C resulted in the synthesis of "non-functional" stress proteins.

(*continued on next page*)

Table 1 (*continued*)

Reference	Cell type	A2C Treatment	Results
Yokota et al. (2000)	HeLa cells.	10 mM A2C for 2–12 hours.	A2C treatment resulted in chemical stress in HeLa cells.
Nguyen et al. (2004)	FR3T3 fibroblasts	10 mM A2C for up to 2 hours.	A2C proved to be a potent activator of ER stress.
Bessonov et al. (2010)	E. coli BL21-CodonPlus (DE3)-RIP overexpressing rmMBP.	0.25 – 1 mM A2C for 24 hours.	Dose-dependent reduction in growth, with the most reduction seen in 1 mM A2C treatment.
Song et al. (2017)	HeLa cells.	5 mM A2C or 5 mM A2C with either 1 mM alanine, proline, valine, or threonine for 24 hours.	A2C toxicity from 5 mM was rescued by the addition of 1 mM proline only.
Roest et al. (2018)	HeLa cells.	1 – 25 mM A2C for 72 hours.	A2C is an ER stress inducer at 5 mM.
Samardzic & Rodgers (2019)	Neuroblastoma SHSY−5Y cells.	0.125 – 2 mM A2C for 24 hours.	2 mM A2C resulted in decreased cell viability, irreversible cell membrane damage and increased cellular stress.
Piper et al. (2022)	BV2 microglial cells.	0.5 – 2 mM A2C for 24 hours.	1 mM A2C triggered significant pro-inflammatory and pro-apoptotic responses.
Piper et al. (2023)	BV2 microglial cells.	1 mM A2C or 1 mM A2C with 0.05 mM proline for 6 or 24 hours.	Toxicity of A2C was significantly suppressed with the co-administration of proline.

2022). The highest dose resulted in a high death rate (up to 60 %), with surviving mice showing hind limb ataxia, a tremulous phenotype, and substantial liver damage (Sobel et al. 2022). Histological analysis of the brains and spinal cords of A2C-exposed mice identified alterations in oligodendrocytes (OLs), such as nucleomegaly, nucleoplasm clearing, dilated endoplasmic reticulum, cytoplasmic vacuolation, abnormal mitochondria, and dose-dependent apoptosis (Sobel et al. 2022). Myelin appeared to be mostly intact except for blister-like swellings in the inner myelin layers in the white matter of the A2C-treated mice. Despite the clear impact on OL morphology, no abnormalities in the neuron cell bodies or in other CNS cells were observed (Sobel et al. 2022). Additionally, immunohistochemistry detected myelin blistering and nuclear translocation of UPR-related and proinflammatory molecules, MHC I expression, and MBP cytoplasmic aggregates in OLs mirroring some features of the white matter of MS patients (Sobel et al. 2022). In the same set of studies, pregnant CD1 mice and their pups were administered A2C orally 3 times a week until 23 days after birth. When apoptotic cells in the spinal cord and white matter of the mice were quantified, a 2–3-fold increase in apoptotic cells were present in neonatal pups compared to their equally dosed dams which could suggest an increased susceptibility to A2C during myelination (Sobel et al. 2022). A summary of the *in vivo* studies investigating A2C toxicity is presented in Table 2.

15. Strategies to avoid azetidine-2-carboxylic acid toxicity

Allelochemistry is the production and release of toxic chemicals by one species that can adversely affect a susceptible species (Weir et al. 2004). Allelochemicals can be released into the soil by invasive plants such as fine fescue grasses which release the NPAA *meta*-tyrosine into the soil inhibiting the growth of a wide range of plant species (Bertin et al. 2007). Although A2C has not been reported in soil, an *Agrobacterium sp*. isolated from a soil extract was able to utilise A2C as its sole source of nitrogen for growth (Dunnill and Fowden, 1965). The

Table 2
Summary of *in vivo* studies examining A2C toxicity.

Reference	Sample type	A2C Treatment	Results
Lane et al. (1971)	Chick embryos.	500 μg A2C for 5 days.	A2C treatment significantly decreased collagen fibrils in tendons and skin.
Hall (1978)	Embryonated chicken eggs.	0.085 mM A2C per egg.	A2C inhibited collagen growth.
Nagai et al. (1978)	Pregnant rats.	i.p., 180 – 550 mM A2C daily within up to 3 days (0.1–0.3 g/kg).	A2C treatment delayed the ossification of the foetal skeleton and caused abnormalities.
Joneja (1981)	Pregnant hamsters.	i.p., 90 – 550 mM A2C daily within up to 6 days (0.1–0.6 g/kg).	Severe defects and mortality rates seen in doses above 0.3 g/kg A2C.
Ratzenhofer et al. (1984)	3-week-old mice.	o.g. 9.89 mM A2C.	A2C consumption resulted in collagen crosslinking disturbances.
Adamson & King (1987)	Foetal rats.	i.p., 200 mg/kg daily injections to pregnant rats for 2 days.	A2C decreased lung development and DNA synthesis. Alveolar walls showed little matrix or fibrillar collagen.
Norrby (1993)	Rats.	s.c., 0.1 g/kg A2C.	A2C significantly reduced neovascular branching.
Lee et al. (1996)	Soybean seedlings.	5 mM A2C.	Induced a heat shock like-response from disrupted protein synthesis.
Song et al. (2017)	Zebrafish embryos.	1 mM A2C or 1 mM A2C with 0.25 mM proline, valine, or threonine.	1 mM A2C induced localised cell death; only proline rescued toxicity at 0.25 mM.
Sobel et al. (2022)	Adult CD1 Mice.	i.p. or o.g., 0.3 or 0.6 g/kg A2C daily injections for 4 weeks.	0.6 g/kg A2C resulted in significant alterations in the MBP of OLs.

i.p. – intraperitoneal; s.c. – subcutaneous; o.g. - oral gavage

Agrobacterium has a pathway for A2C degradation to non-toxic metabolites and as a result no trace of A2C could be found in cell proteins (Dunnill and Fowden, 1965). Enzymatic modification of A2C by an acetyl transferase has been reported in strains of *Saccharomyces cerevisiae* (Takagi et al. 2000; Shichiri et al. 2001). In the fungal species *Aspergillus nidulans,* A2C is assimilated as a nitrogen source through GABA catabolism and the action of the enzyme AzhA hydrolase (Biratsi et al. 2021) Some species of Enterobacter also utilise A2C as a nitrogen source and as a result are resistant to its toxicity (Yeung et al. 1998). This evolutionary adaptation is likely due to their abundant presence in soil that is proximal to A2C-releasing plants.

The insecticidal properties of A2C were first reported in 1976; the liliacae species *Urginea maritima* had a lethal effect on the cotton leafworm (*Spodoptera littoralis*) larvae due to the high concentration of A2C in the foliage (Hassid et al. 1976). Retardation of larval growth, larval mortality and grossly deformed pupae due to A2C feeding was also demonstrated experimentally for the corn earworm *Heliothis zea* (Boddie) (Adeyeye and Blum, 1989). In some cases, predators have evolved advanced tRNA synthetases capable of discriminating between the NPAA and the parent amino acid preventing its mistaken incorporation into proteins. The most striking example being the bruchid beetle (*Caryedes brasiliensis*) which has an advanced tRNA synthetase that cannot be charged with the arginine analogue canavanine allowing it to feed on canavanine rich plants such as the legume *Dioclea megacarpa* (Rosenthal, 1983). To our knowledge no predators with a prolyl-tRNA synthetase that can exclude A2C have been reported. Another protective strategy that can be employed is upregulation of the pathways

leading to the biosynthesis of the parent amino acid to outcompete the analogue. Overproduction of amino acids is a common feature of microbial resistance to amino acid analogues (Riccardi et al. 1981). The cyanobacteria *Spirulina platensis* is resistant to A2C toxicity due to its excessive production of proline, which is also a feature of the *Daucus carota* plant species (Riccardi et al. 1981; Takagi et al. 2000). A summary of the studies investigating resistance to A2C is presented in Table 3.

16. Human exposure to azetidine-2-carboxylic acid

High concentrations of A2C have been reported in the liliaceous plants *Convallaria majalis* and *Polygonatum multiflorum* (Fowden, 1959), and although both are available as teas, human exposure to A2C from these plants would be very limited. A more likely exposure route is through sugar beet byproducts as proposed by Rubenstein (Rubenstein et al. 2009). During the Napoleonic wars of the early 1800s the British blocked trade routes with the Caribbean depriving Europe of sugar cane sucrose (Crouzet, 1964). It was known at this time however that both white and red beets, which could be grown in temperate climates, contained sucrose. The initial discovery of sugar in beets was made in 1747 by German chemist Andreas Marggraf (Francis, 2006). The crystallized sugar he obtained by alcoholic extraction from the root was only around 1.6 % of the roots' fresh weight. Marggraf's student Franz Achard continued this work and constructed the world's first beet sugar factory in 1801 using beets with a sugar content of about 4 % of the roots' fresh weight (Francis, 2006). Napoleon funded commercial sugar beet production and factories were established in Northern France which up until 1875 was the biggest beet sugar producer in Europe (Francis, 2006). MS was first reported by Jean Martin Charcot in Paris in 1968 (Talley, 2005) so the emergence of dietary A2C preceded in time and corresponded geographically to the first description of MS that was considered to be a new disease (Rubenstein, 2013). The geographic distribution of MS thereafter coincided with sugar beet agriculture which now accounts for nearly 35 % of the world's supply of sucrose (Rubenstein, 2013). As early as 1810 it was discovered that beets were potentially a high value crop since, in addition to the extraction of sucrose, byproducts such as the green leafy tops, beet pulp and molasses could be used as cattle feed (Francis, 2006). Sugar beet byproducts are plentiful and are still widely used as dietary supplements for livestock.

Another entry route of A2C into the human food chain is through feeding intact fodder beets (green tops and roots) to livestock. Fodder beets are a very high yielding forage crop making them attractive to mitigate seasonal herbage defects and to improve postpartum energy balance of early lactation dairy cows (Fleming et al. 2021). Cattle can eat up to 18 kg of fodder beet per day so could be exposed to significant amounts of A2C which might transfer to milk or meat (Henry, 2010; Rubenstein, 2013).

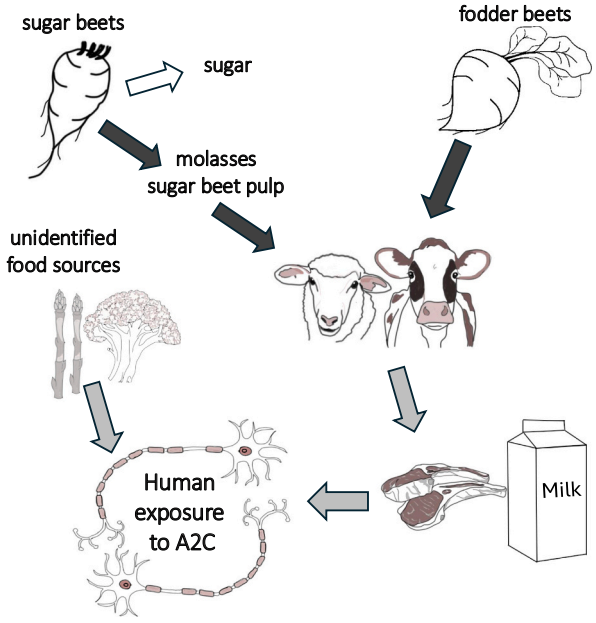
Based on dietary data from the USA, a link between MS and the consumption of cow's milk was proposed (Agranoff and Goldberg, 1974) and later supported by a study carried out in New Zealand (Butcher, 1986). More recently, a significant worldwide correlation was reported between local bovine geographic density and MS prevalence, but no correlation could be found for ovine, caprine, porcine, horse, poultry, cats and dogs (Malosse and Perron, 1993). There was a similar correlation for cow milk production (Malosse and Perron, 1993) but the strongest correlation was found for MS prevalence and fresh milk consumption ($p < 0001$) (Malosse et al. 1992). Since body growth, milk intake and myelination are all at their highest levels during infancy and through to adolescence modification of MBP from the insertion of A2C in place of proline could generate antigenic epitopes and potentially contribute to MS. While this remains a possibility a recent review outlined several alternative potential links between cow's milk and MS (Morin et al. 2024). Potential routes of human exposure to A2C are presented in Schematic 1.

Table 3
Summary of studies examining A2C resistance in organisms.

Reference	Sample type	A2C treatment	Results
Riccardi et al. (1981)	Spirulina platensis.	0.05 mM A2C for 30 days.	The Spirulina platensis is resistant to A2C toxicity due to its excessive production of proline.
Yeung et al. (1998)	Enterobacter agglomerans and Enterobacter amnigenus.	1 mM A2C	Both species showed the ability to use A2C as their sole source of nitrogen.
Takagi et al. (2000)	Saccharomyces cerevisiae.	3 mM A2C for 3 days.	MPR1 and MPR2 genes confer resistance to A2C in Saccharomyces cerevisiae strains.
Shichiri et al. (2001)	Saccharomyces cerevisiae.	50 mM A2C for 90 minutes.	An enzyme in Saccharomyces cerevisiae detoxifies A2C.
Nomura et al. (2003)	Schizosaccharomyces pombe.	1 mM A2C for 3 days.	The ppr1+ gene confers resistance to A2C in S. pombe strains.
Gross et al. (2008)	Pseudomonas sp. and Escherichia coli.	5 mM A2C.	A2C-resistance gene in Pseudomonas sp. was inserted and effective in E. coli.
Berg et al. (2020)	Saccharomyces cerevisiae.	3 mM A2C for 24 hours.	A2C disrupts protein quality control and actin organisation, with specific genes either exacerbating or suppressing its toxicity.
Biratsi et al. (2021)	Aspergillus nidulans.	5–20 mM A2C for 16–30 hours.	Aspergillus nidulans can resist A2C toxicity, using it as a nitrogen source.

17. Gaps in our knowledge/Conclusions

While it is established that A2C exchanges for proline in protein



Schematic 1. Potential routes of A2C entry of into the human food chain. Black arrows represent known A2C transfer pathways, grey arrows represent potential A2C transfer pathways, and white arrow indicates where there is no known A2C transfer.

synthesis *in vivo,* impacting proline-rich proteins such as collagen, there is currently no evidence for A2C in proteins from human tissues. In addition, there is now evidence that post translational editing mechanisms can reduce mistranslation errors involving A2C charging to $tRNA^{Pro}$(Kuzmishin Nagy et al. 2020). Post-mortem analysis of tissues from experimental animals exposed to dietary A2C would provide important data on its presence and distribution in mammalian tissues. Its close structural similarity to proline however would allow it to be widely distributed through the human body if significant exposure did occur.

It has been proposed that low levels of A2C exposure during myelination could result in the synthesis of antigenic MBP; evidence for antibodies directed against MBP neoepitopes containing A2C is required to support this hypothesis. In principle however this would appear to be possible since A2C increased tumour antigenicity in mice through its ability to generate A2C-containing proteins that were neoantigens and promoted antitumour immune responses (Li et al. 2022). It is possible that the generation of neoepitopes in MBP could occur at concentrations of A2C generally considered to be significantly below toxic levels.

To determine potential human exposure to A2C a better understanding of its distribution in nature is required. Based on our current knowledge, the most likely routes of human exposure are through feeding livestock sugar beet byproducts or intact fodder beets. The ability of A2C to transfer to meat or milk and enter the food chain however has not been demonstrated. Fodder beets developed through breeding programs and genetic manipulation are currently used as livestock feed worldwide and levels of A2C in these beets should be quantified and its presence in meat and milk investigated (Schematic 1). The key factor that determines potential harm from A2C-containing foods however is the ratio of A2C to proline in the diet which would generally be expected to have a significant excess of proline over A2C thus would limit A2C incorporation into proteins.

CRediT authorship contribution statement

James Kabalan: Writing – review & editing, Writing – original draft, Investigation. **Kenneth John Rodgers:** Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **Connor R. Phillips:** Writing – review & editing, Writing – original draft, Investigation.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Kenneth John Rodgers reports financial support and administrative support were provided by University of Technology Sydney. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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