The elusive pathways behind montelukast's repurposing for Alzheimer's disease

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Abstract

Background and purpose: Montelukast, an antagonist of the cysteinyl leukotrienes receptor 1, has been proposed for repurposing for the treatment of neurodegenerative disorders, including Alzheimer's disease. Clinical trials are ongoing but the mechanisms supporting this repurposing are still poorly understood. Experimental Approach: Taking advantage of proteomics datasets deposited in public repositories, data from mouse brain and a neuronal chicken model exposed to the drug were reinterpreted in view of the repurposing proposal. Key Results: Montelukast increases the levels of presenilin 1, nicastrin, neprilysin, and insulin-degrading enzyme, all of which are involved in the amyloid aggregation and clearance processes. Hexokinase 1, malate and isocitrate dehydrogenate enzymes, from central metabolism pathways, are also affected. Conclusions and Implications: The data suggest that montelukast is a modulator of the amyloid clearance process, favouring the removal of aggregates and counterbalancing the overall amyloidogenic process. Montelukast also acts on energy supply pathways, compensating the ageing-associated decrease of the basal cell metabolism. Taken together, these actions of montelukast clearly support its repurposing as a candidate for Alzheimer's disease management.

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Conclusions and Implications: The data suggest that montelukast is a modulator of the amyloid clearance process, favouring the removal of aggregates and counterbalancing the overall amyloidogenic process. Montelukast also acts on energy supply pathways, compensating the ageing-associated decrease of the basal cell metabolism. Taken together, these actions of montelukast clearly support its repurposing as a candidate for Alzheimer's disease management.

Keywords

Montelukast, repurposing, Alzheimer's disease, proteomics.

Bullet point summary

What is already known

Montelukast is an antagonist of the cysteinyl leukotrienes receptor 1, widely used in asthma management.

Montelukast has been identified as an inhibitor of new targets involved in neurodegenerative disorders.

What this study adds

Montelukast modulates the amyloid clearance pathway.

Central catabolic pathways are also modulated by montelukast.

Clinical significance

The identified pathways affected by montelukast support the drug's repurposing for Alzheimer's disease management.

Introduction

Montelukast (MTK, 1-([(1(*R*)-(3-(2-(7-chloro-2-quinolinyl)-(*E*)-ethenyl)phenyl)-3-(2-(1-hydroxy-1-methylethyl)phenyl)propyl)thio]methyl) cyclopropylacetic acid) is an antagonist of the cysteinyl leukotrienes receptor 1 ($CysLTR_1$), widely used by patients with asthma and allergic rhinitis to manage their symptoms. In addition to $CysLTR_1$ inhibition, MTK has been identified as an inhibitor of new targets, including 5-lipoxygenase (5-LOX) (Ramires*et al.* , 2004), as well as of the $CysLTR_2$, $P2Y_{12}$ (Trinh *et al.* , 2019), and GPR17 (Marschallinger *et al.* , 2015) receptors, suggesting that MTK can be exploited in other pathologies. In fact, MTK has been proposed for repurposing in a number of other diseases, particularly neurodegenerative disorders, including Alzheimer's disease (AD) (Lai *et al.* , 2014a; Lai *et al.* , 2014b; Marschallinger*et al.*

, 2015), Parkinson's disease (Jang *et al.* , 2017; Mansour *et al.* , 2018; Nagarajan and Marathe, 2018; Wallin and Svenningsson, 2021; Zhang *et al.* , 2014) and Huntington's disease (Kalonia *et al.* , 2010). Recent studies have identified MTK as being able to improve cognitive and neurological functions due to its modulation role in the inflammatory and apoptotic cascades involved in neurodegenerative features, particularly those where TNF-α, NF-κB, caspase-3, Bcl-2, MAPK, and IL-1β participate. Moreover, MTK appears to lead to a decrease in α-synuclein load and in $A\beta_{1\text{-}42}$induced neurotoxicity, as well as to modulate the oxidative stress associated with a dysregulation of the GSH/GSSG balance or of superoxide dismutase activity - two key factors in the maintenance of redox homeostasis (Grinde *et al.* , 2021; Jang *et al.* , 2017; Kalonia *et al.* , 2010; Lai *et al.* , 2014a; Lai *et al.* , 2014b; Mansour *et al.* , 2018; Marschallinger *et al.* , 2020; Marschallinger *et al.* , 2015; Michael *et al.* , 2021; Nagarajan and Marathe, 2018; Zhang *et al.* , 2016).

Clinical trials are ongoing to assess the effects of MTK in neurodegenerative disorders, including Alzheimer's disease. However, the mechanisms responsible for such effects are still poorly explored.

This work aims to interrogate proteomics data in order to identify biological pathways affected by montelukast that may support its repurposing for AD management. Toward this end, a reinterpretation strategy based on proteomics datasets originated form our previous work (Marques *et al.* , 2022d) and deposited in public repositories (Marques *et al.* , 2022a; Marques *et al.* , 2022b) was followed. Thus, raw mass spectrometry-based proteomics data from mice and from chicken neuron cultures treated with MTK were reinterpreted in order to identify potential mechanisms supporting MTK's repurposing for neurodegenerative diseases. One of the major features of omics studies consists of the generation of big data sets; however, the huge information contained in these data is systematically under-analysed. A data-reinterpretation strategy not only allows extracting further information from the same data sets, but also favours data dissemination among the scientific community, reducing the costs associated with research projects, as well as the laboratory resources and waste. Besides, this strategy also contributes to the 3Rs principles of animal research (Russell and Burch, 1959) since no additional animals were employed.

Experimental procedures

Mouse proteomics assays are described in detail in Marques *et al.*(2022d). Briefly, a group of healthy male C57BL/6J mice was treated with 1 mg kg^{-1} MTK by oral gavage, once a day, for a week. Brain, hippocampus, and prefrontal cortex were collected and used for protein extraction. Samples were analysed by ultra-performance liquid chromatography coupled to high resolution mass spectrometry with electrospray ionization UPLC-HRMS-ESI and the raw data were deposited in public data repositories (Marques *et al.* , 2022b). The well-characterized C57BL/6J mouse strain is the strain more commonly used for safety and toxicity assessment, and is also refractory to the development of many tumours, making it an ideal strain for metabolic and omics studies on non-tumorigenic toxic effects (Kalueff and Nguyen, 2014; Mouse Genome Sequencing Consortium, 2002; Sarsani *et al.* , 2019).

A detailed description of neuron proteomic assays is also available in Marques *et al.* (2022d). A chicken neuron-enriched cell model was exposed to MTK and cells were collected for protein extraction. Samples were analysed by UPLC-HRMS-ESI and raw data deposited in public data repositories (Marques *et al.* , 2022a). Brain-expressed chicken amyloid-β has a higher homology to the human form than that of mouse. Since amyloid-β is one the proteins involved in Alzheimer's disease, for which the repurposing of MTK is being considered, we developed an optimised chicken-based neuron model (Carrodeguas *et al.* , 2005) for assessment of MTK-driven proteomics alterations.

Data processing

Raw data were re-processed according to the parameters presented in Marques *et al.* (2022d). Mouse and chicken proteomics data were analysed on MaxQuant software v2.0.3.0 (Cox and Mann, 2008; Tyanova*et al.* , 2016a) using the internal search engine Andromeda (Cox*et al.* , 2011), and Uniprot (UniProt Consortium,

2020) databases restricted to specific groups of proteins from *Mus musculus*(Proteome UP000000589; keywords "*ADRs (anxiety + stress + sleep + depression + suicide) + apoptosis + inflammation + leukotrienes + metabolism + microglia + neurons + repurposing (amyloid, synuclein, tau, APP, BACE, Psen1, Psen2, nicastrin, neprilysin, adam 17)" + tissue name* ") or *Gallus gallus* (Proteome UP000000539, keywords "*ADRs (anxiety + stress + sleep) + inflammation + leukotrienes + neurons + repurposing (amyloid, synuclein, tau, APP, BACE, Psen1, Psen2, nicastrin, neprilysin, adam 17*)"). MaxQuant output was processed using Perseus v1.6.15.0, with default settings (Tyanova *et al.* , 2016b). Significantly altered proteins (|fold change| [?] 1.5 and $p < 0.05$) were annotated using PANTHER v16.0 and*Mus musculus* or *Gallus gallus* reference lists (Mi *et al.* , 2021).

Results

The present work reanalyses proteomics data available in public repositories, focusing on elucidating the biological mechanisms that support MTK's repurposing for AD management. With that purpose, 1) data obtained from different tissues (brain, hippocampus, and prefrontal cortex) collected from mice treated orally with daily doses of 1 mg kg^{-1} MTK for one week (Marques *et al.* , 2022b), and 2) data obtained from chicken embryo neurons exposed to 1 and 5 μM MTK for 48 h (Marques *et al.* , 2022a) were thoroughly revisited.

Brain proteomics from MTK-treated mice

Proteomics data from mouse brain tissue (Figure 1A) show that MTK is able to up-regulate the neutral cholesterol ester hydrolase 1 (Nceh1, 3.33-fold higher in MTK-treated mice) and the transcription factor HIVEP3 (3.32-fold higher in MTK-treated mice).

FIGURE 1 HERE

Figure 1. Altered proteins with an important role in Alzheimer's disease identified in mouse tissues. This figure illustrates the heatmap of identified altered proteins ($p < 0.05$ and at least 1.5-fold change) in the (A) brain, (B) hippocampus, and (C) prefrontal cortex of mice treated daily with 1 mg kg^{-1} MTK for one week. The LFQ intensity scale represents the normalised relative quantification across all samples. Adapted from Marques *et al.* (2022d), with permission from Elsevier.

Hippocampus proteomics of MTK-treated mice

Regarding the hippocampus (Figure 1B), some identified proteins could be interesting from a repurposing perspective, particularly presenilin 1 (Psen1). This protein, one of the components of the γ-secretase complex that is involved in the final cleavage of the β-C-terminal fragment (amyloidogenic pathway), originating the $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ peptides (Chen *et al.* , 2017; De Strooper*et al.* , 2010), was found to be up-regulated in the hippocampus (2.28-fold higher) of MTK treated mice. High levels of protrudin, a membrane protein that regulates polarized vesicular trafficking in neurons (3.93-fold higher in MTK-treated mice), were also found in hippocampus tissue. This protein has been associated with beneficial effects on the development of axon growth, particularly in regeneration (Petrova *et al.* , 2020). Finally, polyunsaturated fatty acid 5-lipoxygenase (5-LOX), a protein involved in the production of leukotrienes, was found to be down-regulated in the hippocampus of treated animals (1.81-fold lower in MTK treated mice).

Prefrontal cortex proteomics of MTK-treated mice

Prefrontal cortex (PFC) proteomics (Figure 1C) also provided interesting data regarding relevant proteins in Alzheimer's disease. The 14-3-3 protein family is among the most abundant proteins expressed in the brain, binding specific phosphoserine- and phosphothreonine-containing motifs from kinases, phosphatases, and transcription factors. These protein-protein interactions regulate cellular processes such as cell cycle,

transcription, intracellular trafficking, apoptosis, and autophagy (Gu *et al.* , 2020); in neurons, these proteins are involved in differentiation, migration, survival, neurite growth, and ion channel regulation (Gu *et al.* , 2020). From the seven 14-3-3 isoforms identified in the human frontal cortex, five [η (4.72-fold), γ (5.68-fold), ε (6.43-fold), ζ/δ (7.38-fold), and β/α (4.45-fold)] were found to be up-regulated in the PFC of treated mice, indicating an up-regulation of this family of proteins upon MTK treatment.

Another significantly altered protein is hexokinase 1 (HK1, 7.17-fold higher in MTK-treated mice). The brain is the organ that consumes the greatest amount of energy, and neurons require large amounts of energy to maintain their normal activity (Yan *et al.* , 2020). Thus, HK1 is fundamental for glucose conversion to glucose-6-phosphate, the first step in glycolysis. Moreover, the tricarboxylic acid cycle enzymes malate dehydrogenase 1 (5.41-fold higher), and isocitrate dehydrogenase subunit α (3.92-fold higher) were also found to be up-regulated, likely due to the feed-forward glycolytic stimulation.

Regarding proteins involved in Aβ processing, the reticulon-3 protein, a negative regulator of BACE1 (Deng *et al.* , 2013; Kume *et al.* , 2009), was found to be 5.62-fold up-regulated in the PFC of MTK-treated mice, similarly to the insulin-degrading enzyme (3.78-fold higher in MTK-treated mice) and neprilysin (3.43-fold higher in MTK-treated mice). Conversely, the subunit of the γ-secretase, nicastrin, was found to be decreased in MTK-treated mice (1.60-fold lower).

The reinterpretation of proteomics results from brain, hippocampus and PFC of MTK-treated mice shows that energy generating pathways and amyloid clearance processes are the mechanisms whose modulation in the brain would most likely be affected by MTK, providing a rationale for a possible impact of MTK on the management of neurodegenerative disorders.

Altered proteins in chicken embryo neurons exposed to MTK

To further explore MTK's effect on neuronal viability, mature isolated embryonic chicken neurons treated with MTK were collected and their proteome was analysed as before.

The data from mature neurons treated with 1 μM MTK for 48 h suggested that MTK interferes with the α-adrenergic signalling pathway (Marques*et al.* , 2022d). This pathway involves various G-protein coupled receptors that are targeted by catecholamines such as adrenaline and noradrenaline, modulating the synaptic transmission, as well as learning and memory (Perez, 2020); however, no biological processes specifically associated to the etiology or progression of AD were identified as being dysregulated.

By contrast, when mature neurons were exposed to 5 μM MTK for 48 h (Marques *et al.* , 2022d), we were able to follow a pathway-enrichment strategy, using the altered proteins (**Figure 2**). The data indicate that MTK interferes with microtubule polymerization and depolymerisation, as well as neurogenesis and the development of the nervous system (not shown). Furthermore, pathway enrichment also points to alterations in the AD presenilin-dependent pathway. Five altered proteins were found in this enriched pathway: amyloid-β A4 (APP, 2.04-fold higher), Wnt-11 (1.84-fold higher), Wnt5a (1.66-fold higher), histone acetyltransferase (KAT7) (1.56-fold higher), and tumour necrosis factor α converting enzyme (a subunit of the α-secretase complex known as ADAM17, 1.72-fold lower).

The peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α), a regulator of liver gluconeogenesis, was also altered in treated neurons (1.90-fold higher in mature neurons exposed to 5 μM MTK). The glucagon-like peptide 1 receptor, involved in energy supply (Athauda and Foltynie, 2016; Li *et al.* , 2010), was also found to be up-regulated in treated neurons (2.86-fold times higher).

FIGURE 2 HERE

Figure 2. Altered proteins with an important role in Alzheimer's disease identified in chicken embryo neurons. This figure illustrates the heatmap of identified altered proteins ($p < 0.05$ and at least 1.5-fold change) in mature isolated embryonic-chicken neurons exposed to 5 μM MTK. The LFQ intensity scale represents the

normalised relative quantification across all samples. Image adapted from Marques*et al.* (2022d), with permission from Elsevier.

To conclude, a wide range of proteins were altered in both model systems (mouse and chicken embryo neurons) and some of these have a direct contribution to the processes involved in neurodegenerative disorders, mainly in the aggregation of proteins associated with Alzheimer's and Parkinson's diseases.

Discussion and conclusions

Montelukast has been associated with some neuropsychiatric side effects, especially among children. Our previous work suggests that these adverse effects may be associated with a dysregulation of the hypothalamic-pituitary-adrenal axis and an increase of oxidative stress in children's brain (Marques *et al.* , 2022d). Since older people have a mature brain, they should be less susceptible to these effects, which could explain the decreased incidence of adverse drug reactions (ADRs) in adults. Moreover, specific montelukast effects in the older brain may underlie the proposed, yet mechanistically elusive, repurposing of the drug for the management of neurodegenerative disorders. In the current work, the same proteomics dataset generated in our previous work was interrogated from a repurposing perspective, to identify altered proteins known to be involved in the pathophysiology of Alzheimer's disease.

The up-regulation of Nceh1 and HIVEP3 in the mouse brain suggests that MTK contributes to AD management. Indeed, while Nceh1 has been associated with a protective role against α-synuclein toxicity (Zhang*et al.* , 2017) and has been found to have decreased levels in the blood of AD patients (Mandas *et al.* , 2012), HIVEP3 is underexpressed in a double transgenic APP/PS1 mouse model of early-onset AD (Islam *et al.* , 2021). Likewise, the hippocampus up-regulation of Psen1 mimics the effect observed upon γ-secretase inhibition (Campanari *et al.* , 2014; Sogorb-Esteve *et al.* , 2018). This is in agreement with the observed up-regulation, in the PFC, of the reticulon-3 protein, a known inhibitor of the anterograde transport of the BACE1 subunit of β-secretase, thus contributing to an improved clearance of amyloid deposits (Deng *et al.* , 2013; Kume *et al.* , 2009).

The insulin-degrading enzyme, up-regulated in mouse PFC, is involved in the degradation of α-synuclein aggregates, and its dysregulation has been related to the occurrence of type 2 diabetes mellitus, whereby the patients have a high risk of developing AD or PD (Sousa *et al.* , 2021). Neprilysin was also found to be up-regulated in the PFC of MTK-treated mice. It has been proposed that the up-regulation of these two enzymes could be a promising therapeutic strategy for neurodegenerative disorders (Nalivaeva and Turner, 2019). It is noteworthy that the glucagon-like peptide 1 receptor, which has been associated with improved memory and learning in mice (During *et al.* , 2003), was also overexpressed in MTK-treated mature chicken embryo neurons supporting the hypothesis of an improvement of the amyloid clearance process caused by MTK.

The lower levels of nicastrin, coupled to the altered levels of Psen1 (another component of the γ-secretase complex) in the PFC of MTK-treated mice, also indicate that MTK may be involved in the inhibition of γ-secretase, leading to a decrease of amyloid production. Factoring in the altered AD presenilin-dependent pathway, dominated by an up-regulation of the Wnt pathways, observed in treated neurons, MTK appears to have the potential to overcome the synaptic degeneration and cognitive deficits characteristic of AD. It is noteworthy that Wnt proteins are systematically found to be down-regulated in AD patients, and these lower levels are associated to activation of the amyloidogenic pathway (Palomer *et al.* , 2019). Thus, the proteomic shifts associated with MTK have the potential to counterbalance a number of pro-amyloidogenic mechanisms involved in AD's etiology.

5-LOX is involved in the synthesis of cysteinyl leukotrienes, which have a pro-inflammatory effect antagonized by MTK (Marques *et al.* , 2022c). Interestingly, while 5-LOX levels are found to be higher in AD patients (Li *et al.* , 2017), the observed lower 5-LOX levels in the mouse hippocampus also suggest that MTK is able to, at least partially, restore the altered phenotype of AD. This is further corroborated by the observed

up-regulation of the 14-3-3 protein family in the prefrontal cortex of treated mice. While decreased levels of the 14-3-3 proteins are associated with several AD and PD hallmarks (Gu*et al.* , 2020), their MTK-driven overexpression is quite promising, since the modulation of these isoforms has been described as a potential target for neurodegenerative disorders (Pair and Yacoubian, 2021).

From a central metabolism standpoint, the ageing process is characterized by a lowering of cell basal metabolic rate, particularly in the case of neurons (Yan *et al.* , 2020). HK1 levels are consistently found to be decreased in AD patients (Yan *et al.* , 2020), whereby the observed increase of HK1 levels caused by MTK could play a neuroprotective role in the brain. This HK1 increase was observed in the PFC of treated mice, together with the up-regulation of the tricarboxylic acid (TCA) cycle enzymes malate dehydrogenase and isocitrate dehydrogenase. From a metabolic perspective, the observed overexpression of the glucagon-like peptide 1 receptor in chicken embryo neurons is a further link between the brain energy pathways and AD, as this protein is also involved in the clearance of amyloid and synuclein aggregates. Notably, the glucagon-like peptide 1 receptor has been proposed as a therapeutic target for neurodegenerative disorders (Athauda and Foltynie, 2016; Li *et al.* , 2010). The MTK-induced up-regulation of this receptor observed in our work, and its mechanistic implications, may thus provide, at least in part, the missing rationale for the repurposing of MTK in these diseases. It is also noteworthy that the proteomic alterations of TCA cycle enzymes reported herein are in agreement with the TCA changes we observed previously at a metabolomics level (Marques *et al.* , 2022d).

Taken together, the proteomics data reanalysed herein clearly indicate that montelukast is associated with a shift towards amyloid clearance, coupled to an up-regulation of specific enzymes involved in energy production (**Figure 3**). These are two fundamental biological processes that sustain MTK's repurposing towards AD management, which is currently being assessed in various clinical trials (for example, ClinicalTrials.gov Identifiers NCT03991988 and NCT03402503). This wide anti-AD activity of montelukast is reminiscent of reports from studies on valproic acid, which has been shown to increase neprilysin expression and activity in hypoxic rats and in model mice, and is speculated to enhance amyloid clearance in AD patients (Nalivaeva *et al.* , 2012; Nalivaeva and Turner, 2019; Wang *et al.* , 2014). Overall, central nervous system-acting drugs of different classes offer new potential strategies for the management of AD and other neurological diseases. To be fulfilled, these promising approaches require further investigation of the biochemical mechanisms underlying such beneficial side effects.

FIGURE 3 HERE

Figure 3. Pathways identified that support montelukast repurposing. **A.** Molecular basis of Alzheimer's disease. Amyloid precursor protein (APP) can be processed through a non-amyloidogenic pathway, where it is cleaved by α- and γ-secretases, or through an amyloidogenic pathway, where it is cleaved by β- and γ-secretases, leading to the formation of Aβ peptides (Chen*et al.* , 2017; De Strooper *et al.* , 2010). Aβ peptides can then undergo clearance (or degradation) processes within the brain or after transport from the brain to the periphery (liver and kidney). These processes include proteolytic pathways that depend on neprilysin (NE), insulin-degrading enzyme (IDE), matrix metalloproteinases (MMPs), angiotensin-converting enzyme (ACE), endothelin-converting enzyme (ECE), plasmin, the activity of the ubiquitin-proteosome system, the autophagy-lysosome system, or microglial phagocytosis (Nalivaeva and Turner, 2019; Xin *et al.* , 2018). Image adapted from Marques*et al.* (2022c). **B.** Energy producing glycolytic pathway and tricarboxylic acid cycle (Yan *et al.* , 2020). Enzymes marked in red represent the MTK-altered proteins. Created with BioRender.com.

Author contributions

C.F.M. conducted the experiments, analysed and interpreted the data; M.M.M. and G.C.J. supervised the project; C.F.M. wrote the original draft; M.M.M. and G.C.J. reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

All authors declare no conflict of interest.

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Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design and Analysis, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

Data availability statement

Data supporting the findings of this study are described in Marques*et al.* (2022d) and available through public data repositories (Marques *et al.* , 2022a; Marques *et al.* , 2022b).

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