The scientific justification for the repurposing of ambroxol for Parkinson's

From the research team at Cure Parkinson's

Lysosomal dysfunction is implicated as playing a role in many cases of Parkinson's disease (PD). Through associations with genetic risk factors and inherited diseases, as well as research in preclinical models, this cellular waste recycling process is now viewed as a potential avenue for corrective intervention. One common genetic risk locus associated with PD involves the glycosylceramidase beta (*GBA1*) gene, which encodes the lysosomal enzyme β-glucocerebrosidase (GCase). Carriers of *GBA1* variants with PD typically display faster rates of progression and a higher risk of cognitive complaints, giving rise to a sub-type of PD known as "GBA-associated PD". A reduction in levels of GCase activity is believed to bely this phenotype, leading to a search for agents that can elevate levels of this enzyme as potential therapeutics. Drug screening experiments have demonstrated that the clinically available secretolytic agent ambroxol binds to the active site of the GCase enzyme, stabilizing the protein, and transporting it to lysosomes. Numerous models of PD and recent clinical data indicate that ambroxol may have potentially beneficial properties in the context of PD. In this review, we will provide an overview of the current data supporting the justification for the repurposing of ambroxol as a possible disease modifying therapy for PD. We would welcome any thoughts from the wider research and Parkinson's communities.

INTRODUCTION

First described over 200 years ago, Parkinson's disease (PD) is now considered to be the second most common neurodegenerative condition after Alzheimer's. It is a clinical syndrome that is classically characterised by motor (akinesia, bradykinesia, and a resting tremor) and non-motor (anosmia, sleep disruption, gastrointestinal complaints, etc) features. The appearance of the motor symptoms of PD are associated with a dramatic loss of dopamine terminals in the basal ganglia and subsequent reduction in the number of dopamine neurons in the substantia nigra region of the midbrain. The condition afflicts approximately 1-2% of the population over 65 years of age, is diagnosed in males more than females (approximately 2:1), and the aetiology is yet to be determined (Poewe et al 2017 https://pubmed.ncbi.nlm.nih.gov/28332488/ ; Bloem et al 2021 https://pubmed.ncbi.nlm.nih.gov/33848468/). A fundamental feature of PD is that it is progressive, and while currently available symptomatic therapies provide temporary relief, there are currently no disease halting treatments.

Since the late 1990s, a number of genetic risk loci have been identified as inferring an increased risk of developing PD (Blauwendraat et al 2020 https://pubmed.ncbi.nlm.nih.gov/31521533/). These variants are present in approximately 20% of the PD community, and are often associated with a younger age of disease onset. The discovery of these risk factors has stimulated research into the functions of the biological pathways related to the genes located in these regions, highlighting not only potential insights into the pathogenesis of PD and routes for possible therapeutic intervention, but also presenting researchers with a promising means of patient stratification. Clinical histories and cohort studies are now being conducted on these genetic sub-types of the condition, and clinical trials are underway exploring agents targeting specific genetically defined cohorts. A good example of one such risk locus is *GBA1*.

GLYCOSYLCERAMIDASE BETA – *GBA1*

The most common genetic risk loci associated with PD is found in the glycosylceramidase beta 1 (*GBA1*; HGNC:4177; Gene ID: 2629) gene, which is located on chromosome 1 (1q21). The gene encodes a lysosomal enzyme called β-glucocerebrosidase (GCase) that is primarily engaged in the digestion of glucosylceramide (GlcCer, also known as glucocerebroside; Brady et al 1966 https://pubmed.ncbi.nlm.nih.gov/5338605/; Reiner et al 1998 https://pubmed.ncbi.nlm.nih.gov/3359914/). The *GBA1* gene consists of 11 exons and 10 introns and is approximately 7.6kb in length. There is also a highly homologous (96%) pseudogene of *GBA1* (known as GPAP) that lies 16kb downstream of GBA. In addition, there is microsomal glucosylceramidase-beta gene, *GBA2* (HGNC:18986; Gene ID: 57704) which is located on chromosome 9 (9p13.3) of the human genome. This nonlysosomal glucosylceramidase catalyzes the conversion of cytoplasmic glucosylceramide to free glucose and ceramide. GBA2 variants are associated with hereditary spastic paraplegia (Martin et al 2013 https://pubmed.ncbi.nlm.nih.gov/23332916/).
Over 300 mutations have been identified in the *GBA1* gene, but the two most common variants are N370S and L444P, point mutations located in exon 9 and 10, respectively (O'Regan et al 2017 https://pubmed.ncbi.nlm.nih.gov/28598856/ ; Sanz Muñoz et al 2021 https://pubmed.ncbi.nlm.nih.gov/33832803/).
Homozygous or compound heterozygous variants in the *GBA1* gene have long been associated with Gaucher disease (GD). Named after the French dermatologist Ernest Gaucher, who published the first case report, GD is a rare, autosomal recessive, lysosomal-storage disorder that is characterised by the accumulation of glycolipid substrates as a result of reduced GCase activity (Brady et al 1966 https://pubmed.ncbi.nlm.nih.gov/5338605/ ; Boer et al 2020 https://pubmed.ncbi.nlm.nih.gov/32182893/). The classical hallmark of GD is lipid-laden macrophages with lysosomal GlcCer deposits, referred to as Gaucher cells. There are three types of GD, with type 1 GD primarily affecting the viscera, while types 2 and 3 are also associated with neurological impairments (Stirnemann et al 2017 https://pubmed.ncbi.nlm.nih.gov/28218669/). Current treatment of GD involves enzyme replacement therapy, intravenously providing sufferers with recombinant DNA-produced analogues of GCase.

B-GLUCOCEREBROSIDASE – GCASE

GCase (also known acid β-glucosidase or D-glucosyl-N-acylsphingosine glucohydrolase) is a 495 amino acid glycoprotein with four N-linked glycans (Ginns et al 1985 https://pubmed.ncbi.nlm.nih.gov/3863141/). The protein contains three domains: an antiparallel B-sheet, a triose phosphate isomerase barrel (the active site of the protein), and an 8-stranded B-barrel. It primarily functions as a lysosomal glycoside hydrolase that cleaves GlcCer, but GCase has also been reported to cleave β-glucosides, such as glucosylsphingosine (Vanderjagt et al 1994 https://pubmed.ncbi.nlm.nih.gov/8002933/ ; Akiyama et al 2013 https://pubmed.ncbi.nlm.nih.gov/24211208/).
GCase is synthesized on endoplasmic reticulum (ER)-bound polyribosomes, translocated into the ER, glycosylated and then transported to the lysosome (Bendikov-Bar et al 2013 https://pubmed.ncbi.nlm.nih.gov/23158495/). GCase differs from other lysosomal hydrolases, however, in the mechanism by which it is sorted and transported to lysosomes. While most lysosomal hydrolases are transported to lysosomes by mannose-6-phosphate receptors, GCase is transferred via the lysosomal integral membrane protein-2 (LIMP2), which is encoded by the *SCARB2* gene (Reczek et al 2007 https://pubmed.ncbi.nlm.nih.gov/18022370/). Progranulin is believed to be a co-chaperone for GCase transportation to the lysosome,

recruiting heat shock protein 70 to the GCase/LIMP2 complex in the ER (Zhou et al 2019 https://pubmed.ncbi.nlm.nih.gov/31291241/ ; Jian et al 2016 https://pubmed.ncbi.nlm.nih.gov/27789271/). Incorrectly folded GCase protein is retained in the ER for refolding, which if unsuccessful will result in the protein being retrotranslocated from the ER to the cytoplasm, and subject to degradation via the ubiquitin proteasome system. Once within the acidic lumen of the lysosome, GCase is a membrane associated protein and only becomes active by interacting with its activator protein saposin C (Sap C) - a subunit of the precursor protein, prosaposin (PSAP; Tamargo et al 2012 https://pubmed.ncbi.nlm.nih.gov/22652185/).

The primary substrate of GCase is GlcCer, which is considered an essential molecule as it is found in three out of six kingdoms of life (animal, plant and fungus). It is a founder molecule for the synthesis of hundreds of glycosphingolipids (GSLs). Reduced GCase activity results in increased levels of un-degraded GlcCer (and its deacylated form glucosylsphingosine) building up in cells - particularly macrophages in the case of GD. While *GBA1*/GCase deficiency has generally been associated with GD, a second and closely linked condition is PD. Curiously in PD, however, while there is a reduction in levels of GCase activity (see below), a subsequent accumulation of GlcCer is not observed (Gegg et al 2015 https://pubmed.ncbi.nlm.nih.gov/26096906/ ; Lansbury 2022 https://pubmed.ncbi.nlm.nih.gov/36087026/). This has led to questions regarding the exact mechanisms of how *GBA1* variants are associated with PD. We will explore these mechanisms further in the sections below.

GBA1-ASSOCIATED PARKINSON'S

During the 1990s/early 2000s, it was noted that individuals with Gaucher disease exhibited a higher risk of developing PD (Neudorfer et al 1996 https://pubmed.ncbi.nlm.nih.gov/8917744/ ; Bembi et al 2003 https://pubmed.ncbi.nlm.nih.gov/12847165/ ; Mitsui et al 2009 https://pubmed.ncbi.nlm.nih.gov/19433656 ; Tayebi et al 2001 https://pubmed.ncbi.nlm.nih.gov/11509013/ ; DePaolo et al 2009 https://pubmed.ncbi.nlm.nih.gov/19425057/). Subsequent genetic analyses found that as many as 25% of patients with PD carry *GBA1* variants, and up to 20% of *GBA1* variant carriers will eventually develop PD by the age of 80, depending on populations being investigated (Goker-Alpan et al 2006 https://pubmed.ncbi.nlm.nih.gov/16790605/ ; Lwin et al 2004 https://pubmed.ncbi.nlm.nih.gov/14728994/ ; Sidransky et al 2009 https://pubmed.ncbi.nlm.nih.gov/19846850/ ; Anheim et al 2012 https://pubmed.ncbi.nlm.nih.gov/22282650/ ; Balestrino et al 2020 https://pubmed.ncbi.nlm.nih.gov/32767585/). These observations led to the attribution of the term '*GBA1*-associated PD' to differentiate this form of the condition from idiopathic PD and other genetic subtypes.

GBA1-associated PD is clinically indistinguishable from idiopathic PD. There is a marginally earlier age of onset and slightly higher risk of cognitive issues, but few differences are observed in pharmacological response to treatment or general pathology (Nichols et al 2009 https://pubmed.ncbi.nlm.nih.gov/18987351/ ; Alcalay et al 2012 https://pubmed.ncbi.nlm.nih.gov/22442429/ ; McNeill et al 2013 https://pubmed.ncbi.nlm.nih.gov/23935950/). The two most common *GBA1* variants associated with PD are the N370S and L444P mutations, which in some populations account for 70–80% of the total number of *GBA1* variants associated with PD (Lesage et al 2011 https://pubmed.ncbi.nlm.nih.gov/20947659/).

It has been reported that GCase protein levels and enzyme activity are reduced between the 6th and 8th decades of normal human brain aging, but this reduction is exaggerated in *GBA1*-

associated PD (Rocha et al 2015 https://pubmed.ncbi.nlm.nih.gov/25909088/). Levels of GCase activity are reduced by approximately 20% to 50% in the brains of *GBA1*-associated PD, and it is also selectively reduced in pathologically affected areas of the brain in the early stages of idiopathic PD (Gegg et al 2012 https://pubmed.ncbi.nlm.nih.gov/23034917/ ; Murphy et al 2014 https://pubmed.ncbi.nlm.nih.gov/24477431/). This reduction is associated with evidence of lysosomal dysfunction and reduced ceramide levels. Reductions in GCase activity in idiopathic cases of PD have also been reported in peripheral blood cells (Atashrazm et al 2018 https://pubmed.ncbi.nlm.nih.gov/30337601/ ; Hughes et al 2021 https://pubmed.ncbi.nlm.nih.gov/33935104/).

In preclinical cellular and animal models of GCase deficiency, the autophagy–lysosomal pathway has been shown to be impaired, leading to increased α-synuclein levels and decreased mitochondrial function (Mazzulli et al 2011 https://pubmed.ncbi.nlm.nih.gov/21700325/ ; Sardi et al 2011 https://pubmed.ncbi.nlm.nih.gov/21730160/ ; Osellame et al 2013 https://pubmed.ncbi.nlm.nih.gov/23707074/). But the exact mechanisms by which *GBA1* variants lead to increased risk of PD are not clear, particularly as there is no evidence for substrate accumulation – GlcCer, glucosylsphingosine, sphingomyelin, gangliosides, or total cholesterol - in the postmortem *GBA1*-associated PD brain (Gegg et al 2015 https://pubmed.ncbi.nlm.nih.gov/26096906/). In the case of *GBA1*-associated PD, it has been reported that the misfolded versions of GCase protein may accumulate on the outer membrane of lysosomes and disrupt delivery of chaperone-mediated autophagy substrates like α-synuclein into the interior of the lysosome (Kuo et al 2022 https://pubmed.ncbi.nlm.nih.gov/35138901/). This phenotype was observed in cells carrying different *GBA1* variants, and could explain the accumulation of α-synuclein, but not the absence of GlcCer.

While the biology underlying *GBA1*-associated PD is still being determined, reductions in levels of GCase point towards a potential therapeutic avenue for agents that can both transport GCase into the lysosome and enhance lysosomal function. And given that GCase levels are reduced in some idiopathic cases of PD, such therapies could have broader application than just *GBA1*-associated PD. This situation has resulted in an active search for agents that can increase GCase function, and thereby improve lysosomal function. One example of a molecule that fits this criteria is ambroxol.

AMBROXOL

The leaves of *Adhatoda vasica* - a small, evergreen, perennial shrub that is native to the Indian subcontinent - have been utilised in traditional Unani and Ayurvedic systems of medicine for the treatment of respiratory conditions for thousands of years (Paleari et al 2011 https://pubmed.ncbi.nlm.nih.gov/22646987/). The quinazoline alkaloid *vasicine* is the likely primary active component in these leaves, and in 1965 a chemically related molecule – bromhexine - was introduced in Europe to treat mild respiratory conditions. Ambroxol, one of the active metabolites of bromexine, was subsequently found to be pharmacokinetically superior to the parent compound and it was approved for use in 1979 (Weiser 2008 https://pubmed.ncbi.nlm.nih.gov/18482096/). It differs from bromexine by the absence of a methyl group as well as the introduction of a hydroxyl group in the para-trans position of the cyclohexyl ring. The agent is now available in different formulations and is the active ingredient in Mucoangin, Mucosolvan, Bisolvon, Ambolar, and Lasolvan.

Ambroxol Hydrochloride (also known as hydrochloric acid bromine cyclohexylamine alcohol; or trans-4-[(2-amino-3, 5-dibromo-benzyl) amino] cyclohexanol hydrochloride) is a mucoactive drug with multiple properties. It is primarily considered to be a secretolytic with secretomotoric actions, facilitating expectoration by stimulating synthesis and release of

surfactant by type II pneumocytes. Surfactants reduce both the surface tension in the alveoli and the adhesion of mucus to the bronchial wall. They also enhance the transport of mucus, in addition to offering protection against infection.
In addition to the secretolytic properties, ambroxol also displays robust anti-infection activity, suppressing the proliferation of various viruses, including influenza (Yang et al 2002 https://pubmed.ncbi.nlm.nih.gov/12030738/; Kido et al 2004 https://pubmed.ncbi.nlm.nih.gov/15576322/), and more recently SARS-CoV-2 (Alkotaji et al 2020 https://pubmed.ncbi.nlm.nih.gov/33045350/). Treatment with ambroxol has been shown to significantly reduce the incidence of acute upper respiratory diseases during winter season in humans (Nobata et al 2006 https://pubmed.ncbi.nlm.nih.gov/16820995/). A third property of ambroxol is its anti-inflammatory actions (Pfeifer et al 1997 https://pubmed.ncbi.nlm.nih.gov/9113503/; Gibbs et al 1999 https://pubmed.ncbi.nlm.nih.gov/10202994/ ; Beeh et al 2008 https://pubmed.ncbi.nlm.nih.gov/19073395/). It has been reported to inhibit proinflammatory cytokines and reduce lung inflammation in models of acute lung injury (Su et al 2004 https://pubmed.ncbi.nlm.nih.gov/14504727/), and inhibiting 50% of superoxide production of lung alveolar macrophages (Suzuki et al 1998 https://pubmed.ncbi.nlm.nih.gov/9661679/). In addition to these useful properties, ambroxol can also act as a direct scavenger of reactive oxygen metabolites (Gillissen et al 1997 https://pubmed.ncbi.nlm.nih.gov/9195551/; Nowak et al 1994 https://pubmed.ncbi.nlm.nih.gov/8005537/ ; Stetinová et al 2004 https://pubmed.ncbi.nlm.nih.gov/15599665/). It has been shown to act as a lipid peroxidation inhibitor in lung and cardiac tissue (Nowak et al 1993 https://pubmed.ncbi.nlm.nih.gov/8220663/ ; Nowak et al 1994 https://pubmed.ncbi.nlm.nih.gov/7995392/ ; Nowak et al 1995 https://pubmed.ncbi.nlm.nih.gov/8529925/).

NEUROPROTECTIVE PROPERTIES OF AMBROXOL

Ambroxol has been reported to cross the blood-brain-barrier (see below for further discussion), and recently the neuroprotective potential of this agent has been explored in preclinical models of several neurological conditions, including stroke. Administration of ambroxol facilitated neuronal survival and reduced white matter fiber bundle damage in mice with intracerebral hemorrhages (Jiang et al 2020 https://pubmed.ncbi.nlm.nih.gov/32309438/). This effect was partly due to mitigating microglial activation and reducing proinflammatory cytokine accumulation. Another report found that by upregulating GCase activity, ambroxol could promote neural stem cells differentiation into neurons (via the Wnt/β-Catenin pathway) in a model of ischemic stroke (Ge et al 2021 https://pubmed.ncbi.nlm.nih.gov/33551744/).
It has also been reported that ambroxol is able to inhibit microsomal beta-glucosidase (which is encoded by *GBA2*). Recently it has been reported that *GBA2* GCase activity is markedly elevated in the spinal cord of transgenic SOD1G86R mice - a model of familial ALS. This phenotype is evident before the onset of symptoms in these mice. Administration of ambroxol improved motor functions and preserved neuromuscular junction in these mice, significantly increasing their survival (Bouscary et al 2019 https://pubmed.ncbi.nlm.nih.gov/31447678/). In addition to this, there has been considerable preclinical work on ambroxol in models of PD.

AMBROXOL IN MODELS OF PARKINSON'S

In 2009, a screening experiment of 1040 compounds from the NINDS library identified ambroxol as a potent stabilizer of GCase and validation studies revealed it to be a pH-dependent, mixed inhibitor of the enzyme (Maegawa et al 2009

https://pubmed.ncbi.nlm.nih.gov/19578116/). The GCase inhibiting/stabilizing activity of ambroxol was highest at the neutral pH that is found in the endoplasmic reticulum (ER), but undetectable in the acidic pH of lysosomes. Three-dimensional docking models predicted that ambroxol interacted with GCase not only by hydrogen bonding, but also via hydrophobic and π-π interactions. Treatment of GD fibroblasts significantly increased the levels and activity of GCase in lysosomes in mutant biallelic N370S and F213I/L444P fibroblasts, but not in biallelic L444P fibroblasts. Subsequent research has supported the concept that ambroxol binds to the active site of the GCase enzyme in the ER, stabilizing and inhibiting the protein. Once translocated to the acidic environment of lysosomes, however, GCase is released from ambroxol, increasing both the lysosomal fraction and the enzymatic activity of several mutant GCase variants in GD fibroblasts and macrophages (Bendikov-Bar et al 2013 https://pubmed.ncbi.nlm.nih.gov/23158495/ ; Luan et al 2013 https://pubmed.ncbi.nlm.nih.gov/22682976/ ; Kopytova et al 2021 https://pubmed.ncbi.nlm.nih.gov/33609962/).

Ambroxol has been found to enhance the removal of mutant L444P-GCase from the ER, leading to a concomitant increase in enzymatic activity in some cases (Bendikov-Bar et al 2013 https://pubmed.ncbi.nlm.nih.gov/21106416/). The enhancement of GCase activity may be dependent on the variant being assessed as some studies have noted ER release and increase in steady-state mutant N370S-GCase levels following ambroxol treatment, but no subsequent increase activity (Babajani et al 2012 https://pubmed.ncbi.nlm.nih.gov/22592100/). Further replication in fibroblasts collected from healthy controls and patients with Gaucher disease or GBA1-associated PD revealed similar ambroxol-induced increases in GCase activity, that were accompanied by significant reductions in markers of oxidative stress (McNeill et al 2014 https://pubmed.ncbi.nlm.nih.gov/24574503/).

In addition to GCase, ambroxol has also been reported to increase levels of the endogenous GCase activator saposin C and the activity of cathepsin D (which cleaves saposin C from precursor prosaposin) in GBA1-associated PD fibroblasts (Ambrosi et al 2015 https://pubmed.ncbi.nlm.nih.gov/26094596/). Furthermore, Fois et al reported that ambroxol can accumulate in lysosomes, where it directly affects the H+ and Ca2+ homeostasis and stimulates exocytosis from secretory lysosomes via pH-dependent Ca2+ release (Fois et al 2015 https://pubmed.ncbi.nlm.nih.gov/26560688/). Additional *in vitro* investigations demonstrated that ambroxol treatment of GBA1-associated PD cells not only decreases α-synuclein levels but also improves mitochondrial function (via increased PGC1-α levels, Yuan et al 2017 https://pubmed.ncbi.nlm.nih.gov/28216145/ ; Magalhaes et al 2018 https://pubmed.ncbi.nlm.nih.gov/29362387/ ; Magalhaes et al 2018 https://pubmed.ncbi.nlm.nih.gov/31634558/).

Ambroxol treatment has also resulted in enhanced GCase activity *in vivo*. Parkinsonian features in drosophila carrying the mutant (N370S or L444P) human GCase are partially rescued by ambroxol treatment (Maor et al 2016 https://pubmed.ncbi.nlm.nih.gov/27162249/ ; Sanchez-Martinez et 2016 https://pubmed.ncbi.nlm.nih.gov/27539639/). Studies in rodents have demonstrated that ambroxol is able to cross the blood-brain barrier and increase brain GCase activity in wild-type animals treated with the neurotoxin 6-OHDA (Mishra et al 2018 https://pubmed.ncbi.nlm.nih.gov/30040928/ ; Mishra et al 2020 https://pubmed.ncbi.nlm.nih.gov/31654086/), and transgenic mice carrying a heterozygous L444P *GBA1* mutation or overexpressing human α-synuclein. Of interest here was the ability of ambroxol to reduce both α-synuclein and phosphorylated α-synuclein protein levels in the mice overexpressing human α-synuclein (Migdalska-Richards et al 2016 https://pubmed.ncbi.nlm.nih.gov/27859541/). In addition to rodent studies, daily oral delivery of ambroxol (100 mg/day) for 28 days has also been shown to increase brain GCase activity in nonhuman primates (Migdalska-Richards et al 2017

https://pubmed.ncbi.nlm.nih.gov/28295625/). These encouraging results have driven efforts to evaluate ambroxol in clinical trials for PD and associated conditions.

CLINICAL DATA

Since 1979, ambroxol has been widely used in clinical practice and has a well characterised safety profile. During its long history of use, it has been administered off-label to individuals with GD or GBA1 associated-PD, and real-world observational data has been collected. In a cohort of 45 patients with GD or GBA1 associated-PD that were treated with ambroxol, 25 exhibited clinical benefits (including stable or improved neurological status, increased physical activity, and reduced fatigue; Istaiti et al 2021 https://pubmed.ncbi.nlm.nih.gov/33606887/). The doses employed in this collection of cases varied greatly (from 75 to 1485 mg/day), compared to standard measures of ambroxol. High-dose ambroxol (up to 27 mg/kg/day for >2 years) has been evaluated in four patients with GD and myoclonic epilepsy (Kim et al 2020 https://pubmed.ncbi.nlm.nih.gov/31649052/). This treatment regime was found to be safe and markedly reduce seizure frequencies from the baseline levels.
Given the interaction with the GCase enzyme, ambroxol has been tested in a randomized, double-blind, placebo-controlled trial of PD dementia (Silveira et al 2019 https://pubmed.ncbi.nlm.nih.gov/30738426/). This ongoing study is employing three arms (placebo, low-dose (525 mg/day), and high dose (1050 mg/day)) and involves 75 individuals with mild to moderate PD dementia being treated for 12 months.

THE INTERNATIONAL LINKED CLINICAL TRIALS STUDY

Since 2012, the international Linked Clinical Trials committee has gathered annually to evaluated and prioritise agents that have demonstrated potential for disease modification in PD. The committee is made up of 20+ world-leading experts on PD, who are tasked with reviewing a collection of approximately 20 dossiers. Of these, five agents will be prioritized and Cure Parkinson's is then mandated to take those agents into clinical testing. This programme of work has resulted in 8 completed clinical trials, 16 ongoing studies, and further projects in development (Brundin et al 2013 https://pubmed.ncbi.nlm.nih.gov/24018336/ ; Brundin & Wyse 2019 https://pubmed.ncbi.nlm.nih.gov/30269406/ ; Stott et al 2021 https://pubmed.ncbi.nlm.nih.gov/33815053/).
In 2014, a dossier containing extensive data on ambroxol was presented to the iLCT committee and it was prioritized for clinical evaluation. This led to the development and initiation of the "Ambroxol in Disease Modification in Parkinson Disease" (or AiM-PD) study. Between January 2017 and April 2018, 17 PD patients (8 carriers of *GBA1* variants and 9 without) were recruited to this open label Phase IIa study, which evaluated 1260mg/day of ambroxol over a 6 month assessment period. The results found that the treatment accessed the brain, was safe and well tolerated, and elevated levels of GCase in cerebrospinal fluid (Mullin et al 2020 https://pubmed.ncbi.nlm.nih.gov/31930374/). A longer efficacy study in a larger cohort is now being proposed to determine if ambroxol has disease modifying potential in *GBA1*-associated PD and idiopathic PD.

THE PHARMACOKINETICS OF AMBROXOL

The pharmacokinetics of ambroxol have been extensively investigated in a range of animal species, including humans. It is rapidly absorbed after oral administration (maximum plasma levels are reached within 0.5 to 3 hours). The absolute oral bioavailability of the drug is high

in humans (70-80%), while low in other animals (Hammer et al 1978 https://pubmed.ncbi.nlm.nih.gov/581988/; Jannsen et al 1988 https://pubmed.ncbi.nlm.nih.gov/3365282/ ; Yang et al 2015 https://pubmed.ncbi.nlm.nih.gov/26770490/ ; Vergin et al 1985 https://pubmed.ncbi.nlm.nih.gov/4074420/), and it has moderate plasma protein binding (90%). The terminal half-life of ambroxol is short in mice and dogs, but moderate in humans (8–12 hours) and the plasma half-life of some ambroxol metabolites is approximately 22 hours (Ollier et al 2020 https://pubmed.ncbi.nlm.nih.gov/32372294/; Cazan et al 2018 https://pubmed.ncbi.nlm.nih.gov/30372367/).
Ambroxol is highly metabolized via multiple routes. In plasma, 3,5 dibromoanthranilic acid (DBAA) is the main circulating metabolite, but in contrast to ambroxol, the tissue distribution of DBAA is very low. About 30% of the administered oral dose is eliminated via first pass. Ambroxol is primarily metabolised in the liver by conjugation. About 90% of the administered dose is eliminated by kidneys and only 10% of renal excretion is unchanged ambroxol (https://mri.cts-mrp.eu/download/DK_H_2364_002_FinalPI.pdf).
Following oral administration, ambroxol displays good distribution. The agent has good lipophilicity (cLogP = 2.8) and low polar surface area, and has been shown to cross the blood-brain-barrier, with cerebrospinal levels of 11% of the mean blood levels (Mullin et al 2020 https://pubmed.ncbi.nlm.nih.gov/31930374/). It has been reported to be as high as 17% in one study (Luan et al 2013 https://pubmed.ncbi.nlm.nih.gov/22682976/). Once in the brain, ambroxol is believed to enter cells by unspecific, passive diffusion, based on the observation that the effects of the agent on internal lysosomal pH occurs rapidly and this cannot be explained by endocytosis.

THE SAFETY OF AMBROXOL

Since it was first marketed in 1979, ambroxol has presented a long and well characterised safety profile. Toxicity studies have been conducted in a range of animals, with ambroxol displaying a low index for acute toxicity. Repeat-dose studies have reported very high no-observed adverse effect levels (NOAEL; 150 mg/kg/day for 4 weeks in mice; 50 mg/kg/day for 78 weeks in rats; 40 mg/kg/day for 26 weeks in rabbits; and 10 mg/kg/day for 52 weeks in dogs). Overdose concentrations resulted in dyspnoea, ataxia and convulsions, but these effects were uncommon and reversible in nature (Tsunenari et al 1981 https://jglobal.jst.go.jp/en/detail?JGLOBAL_ID=200902061809579329). Further studies have found ambroxol to be devoid of any mutagenic or tumerogenic effect (Iida et al 1981 https://jglobal.jst.go.jp/en/detail?JGLOBAL_ID=200902042198997737).
In humans, no specific overdose symptoms have been reported to date. The principal adverse effects of ambroxol are gastrointestinal disturbance and there is a small risk of anaphylaxis. Common side-effects of the medication (may affect up to 1 in 10 people) typically include nausea, changed taste, dryness & numbness of the mouth or throat (hypoaesthesia). In situations of impaired renal function or severe hepatopathy, precaution should be taken with ambroxol administration . Metabolites of ambroxol generated in the liver can be expected to accumulate in cases of severe renal insufficiency. Co-administration of ambroxol and certain antibiotics can results in increased antibiotic concentrations in bronchopulmonary secretion and sputum.
At the high doses used in the AiM-PD study, previous studies have indicated that ambroxol is well tolerated. Ambroxol has been administered to a small cohort of Gaucher disease patients with severe neurological disease. These individuals were treated with 25 mg/kg/day or a maximum dose of 1300 mg/day for 6–48 months, without complication. The treatment significantly increased lymphocyte GCase activity, and was report to have permeated the

blood–brain barrier (Narita et al 2016 https://pubmed.ncbi.nlm.nih.gov/27042680/). High dose ambroxol has also been tested in individuals with acute respiratory distress syndrome. In a meta-analysis of 10 clinical trials evaluating treatment of ≥15 mg/kg (≥1000 mg/day for 1 week) ambroxol found few reported adverse effects and a significant reduction in inflammatory markers (such as serum TNF∝, Il-6 and SOD1) across the studies (Wu et al 2014 https://pubmed.ncbi.nlm.nih.gov/25174313/). These findings indicate that ambroxol is a safe and well tolerated agent, even at elevated doses.

GENETIC CONSIDERATIONS IN CLINICAL TESTING OF AMBROXOL

GBA1 variants can be classified as 'mild' or 'severe' based on their phenotype in GD. Mutations associated with non-neuropathic type I GD are considered as 'mild', while mutations that cause the neuropathic types II and III are categorised as 'severe' (Gan-Or et al 2015 https://pubmed.ncbi.nlm.nih.gov/25653295/; Menozzi & Schapira 2021 https://pubmed.ncbi.nlm.nih.gov/34248830/). The most common mild *GBA1* variant is N370S, and the most frequent severe *GBA1* variant is L444P. There is also a third class of *GBA1* mutations that are termed "risk" variants. They are not associated with GD, but nevertheless confer increased risk of PD. Both the p.E365K and p.T408M *GBA1* variants that are associated with PD but not with GD (Greuel et al 2020 https://pubmed.ncbi.nlm.nih.gov/32853481/). Like "mild" variants, the "risk" variants are generally associated with a more benign course of PD compared to the "severe" variants.
These variants have differing impacts on both GCase activity and clinical phenotypes. A longitudinal analysis of cerebrospinal fluid GCase activity found that samples from mild and severe samples were significantly lower than controls and iPD samples (Lerche et al 2021 https://pubmed.ncbi.nlm.nih.gov/33547828/). Similar results have been reported in other studies analysing blood samples (Alcalay et al 2020 https://pubmed.ncbi.nlm.nih.gov/32888397/ ; Omer et al 2021 https://pubmed.ncbi.nlm.nih.gov/34550621/). In all of these studies, there has been little difference between mild and severe variant carriers in terms of GCase activity, indicating that GCase should not be used as a biomarker measure between groups of variant carriers. Alternatively, the result may also reflect on our poor ability to measure lysosomal GCase activity in biological samples (Ysselstein et al 2021 https://pubmed.ncbi.nlm.nih.gov/34613624/), and further investigations are required.
In clinical progression of PD, however, the more severe variants are generally associated with more rapid progression of both motor and non-motor symptoms (Petrucci et al 2020 https://pubmed.ncbi.nlm.nih.gov/32658388/ ; Stoker et al 2020 https://pubmed.ncbi.nlm.nih.gov/32303560/ ; Thaler et al 2018a https://pubmed.ncbi.nlm.nih.gov/29784561/). Longitudinal studies conducted on Ashkenazi-Jewish PD cohorts have found no significant effect of either mild or severe variants on overall survival though (Thaler et al 2018b https://pubmed.ncbi.nlm.nih.gov/30288804/ ; Cilia et al 2016 https://pubmed.ncbi.nlm.nih.gov/27632223/). Thus, genetic testing and stratification of participants in a clinical trial involving individuals with *GBA1*-associated PD should be considered in order to avoid any effect of genotype.

SUMMARY/CONCLUSIONS

The evidence supporting the repurposing ambroxol for PD is strong. It has a long and well characterised history of clinical use, with an excellent safety and tolerability profile. There is substantial published data that this agent can bind to the GCase, stabilizing and inhibiting its function while transporting it to the lysosome, where the enzyme is then released and becomes

functionally available. Ambroxol has also been shown to improve overall lysosomal function and have anti-inflammatory effects. These properties have demonstrated beneficial neuroprotective effects in models of PD, including genetic models involving *GBA1*-associated PD related genetic variants. And finally, ambroxol has been found to be safe and well tolerated at high doses in a clinical trial involving people with PD. In that study, ambroxol was able to access the brain and elevate levels of GCase in the cerebrospinal fluid. Based on all of this data, we now propose that the disease modifying potential of ambroxol should be tested in a large, properly powered Phase III clinical trial involving participants with both idiopathic and *GBA1*-associated PD.