Identification, preclinical profile, and clinical proof of concept of a novel palindromic orally bio-available pro-drug of miridesap

Running title (<70 characters): Characterisation of an oral pro-drug of miridesap

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Abstract *(250 words)*

Background and Purpose: Miridesap depletes serum amyloid P component (SAP) and forms a component of a novel approach to remove systemic amyloid deposits; low oral bioavailability necessitates parenteral administration. We sought to identify a pro-drug that preserves the pharmacological properties of miridesap while having adequate oral bioavailability and physical stability. The pharmacology of palindromic miridesap is unique; previous pro-drug efforts did not progress to the clinic.

Experimental Approach: Using a screening cascade focused on physicochemical properties, physical and gut stability and conversion to miridesap in liver microsomes and blood, we identified GSK3039294 (GSK294). Based on a favourable preclinical profile, we tested it in healthy participants.

Key Results: GSK294 was highly soluble and stable in simulated gastric and intestinal fluids, stable in intestinal microsomes, and permeable in MDCK-II cells. GSK294 was rapidly hydrolysed to miridesap and its mono pro-drug ester in blood and liver microsomes. GSK294 showed good oral bioavailability of miridesap in rats and dogs. Following administration of GSK294 600 mg QD for 7 days in humans, pharmacodynamically active concentrations of miridesap were achieved with substantial depletion of plasma SAP. The study was terminated due to arrhythmia, the relation of which to GSK294 remains unclear.

Conclusion and Implications: Our preclinical screening cascade, identified a novel palindromic orally dosed pro-drug that preserved the unique pharmacology of parenteral miridesap. The pro-drug depleted circulating SAP with a time course and extent similar to that of parenterally-administered miridesap. This is the first drug reported to lower SAP in the clinic following oral dosing.

Key words: Pro-drug; serum amyloid P component; pharmacokinetic/pharmacodynamic profile

Abbreviations: ATCC, America type culture collection; AUC, area under the concentration time curve; CHI, Chromatographic Hydrophobicity Index; ChromlogD, chromatographic logD; CI, confidence interval; C_{max}, maximum serum concentration; CVb, between-participant variability; DCS, Developability Classification System; DMSO, dimethyl sulfoxide; FaSSIF, fasted state simulated intestinal fluid; FeSSIF, fed state simulated intestinal fluid; IV, intravenous; LC-MS/MS, liquid chromatography with tandem mass spectrometry; LLQ, lower limit of quantification; mAb, monoclonal antibody; MDCK-II, Madine Darby Canine Kidney type II cells; PD, pharmacodynamic; PK, pharmacokinetic; PO, oral administration; SAP, serum amyloid P component; SGF, simulated gastric fluid; SVT, supraventricular tachycardia; $t_{1/2}$, terminal half-lives; t_{max}, time at which the maximum concentration is observed; UPLC, ultraperformance liquid chromatography

What is already known: Miridesap is a depleter of circulating SAP in humans, but has to be given parenterally.

What this study adds: We describe discovery of a novel orally bioavailable pro-drug of miridesap and characterisation in humans.

What is the clinical significance: GSK294 was comparable to parenteral miridesap regimens used in clinical studies in systemic amyloidosis.

Introduction

Serum amyloid P component (SAP) is a non-fibrillar, plasma glycoprotein that circulates at a concentration of 20–40 mg L^{-1} and is a normal constituent of the extracellular matrix located on the microfibrillar mantle of elastic fibres throughout the body and in the *lamina rara interna* of the glomerular basement membrane. In systemic amyloidosis, a clinical disorder that is caused by a progressive accumulation of insoluble, misfolded deposits of normally soluble precursor proteins (amyloid) in the extracellular matrix of target organs, SAP universally decorates amyloid deposits and contributes to their persistence. Professor Pepys identified SAP as a drug target in systemic amyloidosis, which led to the development of miridesap (Pepys, Herbert, *et al*., 2002) and subsequently dezamizumab (anti-SAP monoclonal antibody) for clinical testing (Bodin, Ellmerich, *et al.*, 2010; Richards, Cookson, *et al.*, 2015; Richards, Cookson, *et al.*, 2018). Miridesap is a small molecule depleter of circulating SAP with a unique mechanism of action: it non-covalently crosslinks pentameric SAP molecules to form a decameric complex that is rapidly removed from the circulation (Pepys, Herbert, *et al*., 2002). In clinical studies to date it has been well tolerated with predictable pharmacokinetic/pharmacodynamic (PK/PD) characteristics (Kolstoe, Wood, 2009; Gillmore, Tennent, *et al.*, 2010; Richards, Cookson, *et al.*, 2015; Sahota, Berges, *et al.*, 2015; Richards, Cookson, *et al.*, 2018). Despite these favourable characteristics, its clinical utility may be limited by the fact that it must be given parenterally, owing to very low oral bioavailability. Miridesap is a hydrophilic diacid and we hypothesised that it was the presence of the two acid moieties that were not only essential to binding to SAP, but also responsible for severely restricted absorption following oral dosing. We therefore sought to identify a pro-drug with good oral bioavailability and, upon entering the circulation, converts quickly and completely to miridesap, thereby preserving the favourable PK/PD characteristics of miridesap. The palindromic nature of miridesap and its unique pharmacology dependent on the diacid functionality present particular challenges to the identification of a pro-drug. Indeed, previous attempts to generate a potentially orally bioavailable double acid pro-drug of miridesap (Huwyler, Jakob-Roetne, *et al.*, 2003) did not lead to a drug.

Here we describe a successful preclinical screening cascade approach that identified an orally bioavailable pro-drug molecule, GSK3039294 (GSK294), with the desired balance of physical properties and stability, leading to preservation in the clinic of the unique pharmacology of miridesap. Unexpected safety observations in the clinical study, although not thought to be related to the pharmacology of miridesap, limited preliminary evaluation in humans.

Methods

Materials

The compound GSK294 was synthesised and analysed according to the published method (Denis and Mirguet, 2015).

Control-naïve blood and plasma were ethically sourced from approved vendors or within GlaxoSmithKline (GSK), all *in vivo* studies were ethically reviewed according to local site practices under specific animal protocols in AAALAC accredited facilities in accordance with GSK's policies on the Care, Welfare and Treatment of laboratory animals in the UK; the Animals (Scientific Procedures) Act, 1986; and the Human Tissue Act, 2004. Individual *in vitro* and preclinical *in vivo* experiments were conducted with fewer than n=5 animals in accordance with the 3Rs (replacement, reduction and refinement). Han Wistar male rats (10−12 weeks old with body weight of 200−250 g) and Marshal beagle male dogs (at least 10 months old with body weight of 6−15 kg) were supplied by Charles River UK or France, or Harlan UK. These animals were selected as the intended toxicological species as required by most regulatory guidelines to provide safety and systemic exposure information using the intended clinical route of oral administration prior to dosing in the clinic. All animals were housed in standard conditions and provided with food and water *ad libitum*.

The Madine Darby Canine Kidney type II (MDCK-II) cells were obtained from America Type Culture Collection (MDCK.2 [ATCC® CRL2936™]).

Data and Statistical Analysis

The data and statistical analysis comply with the recommendations of the British Journal of Pharmacology on experimental design and analysis in pharmacology (Curtis *et al.,* 2018). Statistical analyses were not conducted on the preclinical results of this study due to low sample size (n<5). Pharmacokinetic parameters were determined by non-compartmental analyses using Phoenix WinNonlin Enterprise software (Certara, Princeton NJ). The sample size for the clinical data was primarily based on feasibility and the relative gain in precision around SAP depletion. No formal statistical analyses or hypothesis tests were performed on the clinical data.

Analytical Methods

Chromatographic logD Determination

The Chromatographic Hydrophobicity Index (CHI) (Valko, Bevan, *et al.*, 1997) values were measured using reversed phase high performance liquid chromatography column (50 x 2 mm, 3 µM Gemini NX C18, Phenomenex, UK) with fast acetonitrile gradient at starting mobile phase of pHs of 2, 7.4 and

10.5. CHI values are derived directly from the gradient retention times by using a calibration line obtained for standard compounds. The CHI value approximates to the volume % organic concentration when the compound elutes. CHI is linearly transformed into Chromatographic logD (ChromlogD) (Young, Green, *et al.*, 2011).

Solubility in Physiological Solutions

Compound sample was accurately prepared to approximately 10 mg mL^{-1} in the relevant simulated physiological fluid according to established protocols (Butler and Dressman, 2010) and mechanically agitated at ambient temperature (21–23°C) for 24 h. Samples were taken at 0.5, 4 and 24 h, solid removed by filtration, and the solution quantitatively analysed using ultraperformance liquid chromatography (UPLC), with a standard solution of the analyte as reference.

Physical Stability in Solution

The assay was designed to determine physical stability of a compound in buffer at various pHs. GSK294 was dissolved in dimethyl sulfoxide (DMSO) at 1 mg mL^{-1}. Phosphate buffer was prepared by mixing K_2HPO_4 50 mM and KH_2PO_4 50 mM solutions to obtain 4 buffers at pH 6.0, 7.0, 7.5 and 8.0.

Stability studies were conducted at room temperature (25°C) and were started by the addition of 8 µL of DMSO solution to 792 µL of Phosphate buffer 50 mM at pH 6.0, 7.0, 7.5 or 8.0 (1% DMSO) to give 10 µg mL^{-1} final solutions. 75 µL of mixture was taken at 0, 1, 2, 4 and 24 h and 225 µL acetonitrile containing internal standard was added prior to liquid chromatography with tandem mass spectrometry (LC-MS/MS); conditions are described below.

Hydrolysis of GSK294 in blood from Rat, Dog, and Human

The assay was designed to determine stability of compound in fresh blood. Compound was dissolved in DMSO at 1 mg mL^{-1}. Daughter solution was prepared in DMSO at 100 µg mL^{-1}. Fresh blood was diluted by ½ in isotonic physiological pH 7.4 buffer (n=2).

Pre-incubation consisted of warming 495 µl of blood at 37°C for 7 min. Incubations were started with the addition of 5 µL of daughter solution. 50 µl of mixture were taken at 0, 5, 15, 30 and 60 min. 50 µL of water was add to sample and then quenched with 300 µL acetonitrile containing internal standard, centrifuged (10 min at 4000 rpm) prior to analysis by LC-MS/MS.

Hydrolysis of GSK294 by Intestinal and Liver Microsomes

The assay was designed to determine stability of compound in intestinal and liver microsomal matrices (n=2). Compound was dissolved in DMSO at 1 mg mL^{-1}. Daughter solution was prepared in methanol/water (50/50) at 30.3 ng mL^{-1}. Human, rat and dog liver and intestinal microsomes (from Xenotech) were diluted at 0.625 mg protein per mL in phosphate buffer 50 mM pH 7.4 (0.5 mg mL^{-1}).

Pre-incubation consisted of warming microsomal solution 395 µL with 100 µL NADPH in $NaHCO_3$ (2%) at 37°C for 7 min. Incubation was started with the addition of 5 µL of daughter solution. 50 µL aliquots of mixture were taken at 0, 3, 6, 12 and 30 min and quenched with 150 µL acetonitrile-containing internal standard. After 10 min centrifugation at 4000 rpm, 2 µL of samples were analysed by LC-MS/MS.

Passive Diffusion Permeability of GSK294 in MDCK-II Cells at pH=7.4

An assessment of intestinal absorption was conducted using the MDCK-II cell line (ATCC), which were cultured and amplified to determine the passive permeability of GSK294. This was based on the published validated methodology (Theil-Demby, Humphreys, *et al.*, 2009) where basolateral to apical (B→A) directional transfer of GSK294 (3 µM) was tested in triplicate and incubated for 90 min at pH 7.4. Lucifer yellow was used as a paracellular marker to determine the integrity of MDCK-II monolayer and propranolol and atenolol used as positive controls. All samples were diluted 1:1 with acetonitrile, and test drug concentration was determined using LC-MS/MS analysis.

Preliminary Pharmacokinetics

Intravenous Administration of GSK294 in Male Han-Wistar Rats and Male Beagle Dogs

GSK294 in solution in DMSO/hydroxypropyl-beta-cyclodextrin 20% in phosphate buffer 60 mM pH 7 (5:95) was administered as a single intravenous bolus injection to non-fasted rats (n=2 at 10 mg kg^{-1}, 2 mL kg^{-1}) and to dogs (n=1 at 1 mg kg^{-1}, 1 mL kg^{-1}) with food given 2 h post administration.

Serial blood samples were collected *via* tail vein (rat) or jugular vein (dog) at intervals up to 24 h post administration, respectively. Aliquots of whole blood were diluted 50:50 with water and stored at approximately -20°C prior to LC-MS/MS analysis using a method based upon protein precipitation with acetonitrile and a generic internal standard.

Oral Bioavailability of GSK294 in Male Han-Wistar Rats and Male Beagle Dogs

GSK294A in HPMC K100 0.5%/Tween 80 0.1% in phosphate buffer 60 mM pH 7 was administered by oral gavage at doses ranging from 3 to 100 mg kg^{-1} (n=2 at 10 mL kg^{-1}) to non-fasted rats and to dogs (5 mg kg^{-1}, 5 mL kg^{-1}) with food given 2 h post administration. Samples were collected over 24 h and stored at -20°C prior to LC-MS/MS analysis.

Toxicokinetics

Assessment of GSK294 in Male Han-Wistar Rats and Male Beagle Dogs following oral administration

Using the same oral formulation, GSK294 was administered by oral gavage as part of the toxicological safety assessment at doses ranging from 100 to 1000 mg kg^{-1} (n=3 at 10 mL kg^{-1}) to non-fasted rats for

up to 6 months and to dogs up to 28 days (n=3 at 5 mL kg^{-1}) with food given 2 h post administration. Blood (GSK 294) and plasma (miridesap) were collected over 24 hours for PK analysis.

Serial blood samples were collected into EDTA tubes containing BNPP chilled on ice and triplicate aliquots were precipitated immediately with acetonitrile containing internal isotopic stable standard for GSK294 and centrifuged (10 min at 4000 g, at +4°C). The extracted solvent was removed from the blood pellet and stored at approximately -20°C prior to LC-MS/MS analysis for GSK294 blood concentrations. Each remaining blood sample was also immediately centrifuged (10 min at 4000 g, at +4°C) to yield plasma and aliquots precipitated with internal isotopic stable standard for miridesap and stored at -20°C prior to LC-MS/MS analysis for miridesap plasma concentrations.

Clinical Methods

This single centre (GSK Clinical Unit), open-label, non-randomised, first-in-human study of the GSK294 pro-drug was approved by the UK Medicines and Healthcare Products Regulatory Agency and the Berkshire "B" phase 1 accredited Research Ethics Committee. Part A was a single dose escalation over 3 dose levels; the two cohorts received two dose levels (200 mg and 600 mg; or 600 mg and 1200 mg respectively). Part B consisted of 7 days repeat dosing (600 mg OD) in a single cohort. Sentinel dosing was employed in both study parts.

Only healthy participants aged 18–70 years who provided informed consent were eligible for the trial. Health status was assessed at screening based on medical history, physical examination, cardiac monitoring and laboratory testing. Participants were monitored in house for 3 days in Part A and 7 days in Part B. Follow up was conducted 7–14 days post dose. Detailed safety assessments consisting of adverse event monitoring, ECGs, cardiac telemetry, vital signs, urinalysis, haematology and biochemistry were undertaken.

Blood and plasma samples for PK analysis (GSK294 and miridesap, respectively) were taken at the following time points in Part A: pre-dose, 0.25, 0.5, 0.75, 1, 2, 3, 4, 5, 6, 8, 10, 12, 16, 24 and 48 h and processed as described for preclinical toxicokinetic studies. Samples for measurement of plasma SAP were taken in Part B only at pre-dose and 2 h on Day 1, pre-dose on Days 2 and 4, pre-dose, 0.5, 1, 2, 3, 4, and 6 h post dose on Day 5, and again pre-dose on Day 7 and off-dose on Day 14.

Further repeat dose cohorts were planned (Part C, repeat dosing in patients with amyloidosis) but the study was terminated before these were conducted owing to arrhythmia findings that require further evaluation.

Sample Analysis

Samples were analysed by LC-MS/MS with an electrospray source (API5000), in positive mode and with following mass transitions: GSK294: 657 to 384; Monoester of miridesap: 499 to 226; and miridesap: 341 to 226 or 198.

For early *in vitro* (physical, physiological, blood and microsomal stability) samples and preliminary *in vivo* PK (rat and dog) sample extracts were injected on an Acquity UPLC system (Waters) and eluted on an Ascentis Express C18 column (50 × 2.1 mm id, 2.7 µm) at 0.5 mL min^{-1} at 50°C, with 0.1% formic acid in water (A) and 0.1 % formic acid in acetonitrile (B), using the following elution 2 min gradient: 5 to 95% B over 1.5 min, 95% B over 0.5 min and 0.1 min for re-equilibrate column. Controls were made to calculate percentage of disappearance of parent but also appearance of suspected metabolite (*i.e.* monoester and di-acidic form against appropriate calibration lines).

Toxicokinetic (rat and dog) and human clinical samples were quantitatively analysed using a fully validated assay using a 50 µL aliquot of sample. The lower limit of quantification was 10 ng mL^{-1} and the upper limit of quantification was 5000 ng mL^{-1} in blood for GSK294 extracted using 0.1% formic acid in acetonitrile and in plasma for miridesap extracted using 0.1% formic acid in methanol containing appropriate internal standard. Samples were injected on an Acquity UPLC system (Waters) and eluted on a BEH Phenyl C18 column (50 × 2.1 mm id, 1.7 µm) at 0.6 mL min^{-1} at 50°C, with 0.1% formic acid in water (A) and acetonitrile (B), using the following elution 2 min gradient: held at 5% B for 0.2 min, then 5 to 40% B for 0.8 min and increased to 90% B by 1.3 min and held for 0.4 min, before re-equilibrating column for 0.2 min.

Non-compartmental methods were used for PK analysis of blood or plasma concentration *versus* time data using Phoenix WinNonlin Enterprise (Cetara, Princeton NJ).

For total SAP biomarker concentration, clinical study samples from participants dosed with GSK294 were quantitatively analysed in a fully validated electrochemiluminescent immunoassay method using the MSD QuickPlex platform and software Discovery Workbench (v4.0). Plasma samples were diluted 1:1000 and a further 1:2-fold dilution when mixed with assay buffer and antibody cocktail (biotinylated capture and sulfo-tag detection), incubated and transferred to Streptavidin Gold MSD plates. Using a 70 µL diluted aliquot of sample, the lower limit of quantification was 0.065 ng mL^{-1} and the upper limit of quantification was 100 ng mL^{-1} using SAP depleted plasma for calibration curve preparation. Therefore, to obtain the actual SAP concentration in each sample a back-calculated multiplier was applied. Five levels of validation control samples with nominal values of 40,000, 4,000, 2,000, 1,000, 500 ng mL^{-1} were prepared in SAP-depleted plasma. Concentrations of the validation

controls were analyzed against the calibration curve, and the back-calculated concentrations were compared to the nominal value. To assess *intra*- and *inter*-assay precision, 2-4 sets of validation controls were analyzed in a single run over 5 days by 2 analysts. *Inter*- and *intra*-assay precision had to be ≤ 20% CV (≤ 25% at 1-2x LLOQ, ULOQ).

Plasma was depleted of SAP by passing it 4x through a column (100ml) of immobilized anti-SAP antibody to remove the SAP component. A final polishing step was performed on a similar column, 20 mL, to remove residual cross-reacting material. The depleted plasma was tested by ELISA: the SAP component levels were found to be below the Lower Limit of Quantification of the assay (0.16ng mL^{-1}), representing a depletion in excess of 99.999% of total reactive protein.

Results

This report focusses on the properties of GSK294, the final successful candidate molecule. During screening and optimisation many compounds were profiled and we observed that structurally related molecules could have very different physicochemical and pharmacokinetic properties; of those profiled, only GSK294 was considered to have the right balance of properties.

GSK294 preclinical profile

In Vitro Lipophilicity, Solution Stability, Solubility and Permeability

Physicochemical properties for GSK294 are shown in Table 1 , indicating a log D in the desired range for oral bioavailability (Lipinski, Lombardo, *et al.*, 1997), high solubility in simulated gastric and intestinal fluids and high permeability through MDCK-II cell monolayer (Thiel-Demby, Humpheys, *et al*., 2009) commensurate with a Developability Classification System (DCS) class I molecule, the class most likely to be developable as an orally administered drug based on the precedent of other molecules (Butler and Dressman, 2010; Rosenberger, Butler, *et al.*, 2018).

The solution stability of GSK294 decreases notably at pH 8, with the main route of degradation *via* hydrolysis to the monoester (intermediate 1) and miridesap (Figure 1). However, in simulated gastric fluid (SGF; pH 1.6, data not shown) and at pH6 (the pH expected in the intestine) GSK294 has a long half-life of degradation (145–179 h).

Hydrolysis of GSK294 in Blood and by Intestinal and Liver Microsomes

To gain insight to the potential for stability to intestinal enzymes during absorption and then cleavage of the pro-drug to miridesap once absorbed, the hydrolysis of GSK294 was evaluated in rat, dog and human intestinal and liver microsomes and blood (Figure 2). Hydrolysis rates by intestinal microsomes

are low, with low intrinsic clearance rates, suggesting the pro-drug might not be cleaved by this route during absorption. Conversely, in liver microsomes substantial cleavage of the pro-drug GSK294 to miridesap *via* the monoester intermediate 1 is predicted in all species, with high intrinsic clearance rates, suggesting GSK294 might be a suitable pro-drug for cleavage to miridesap once absorbed. The rate of hydrolysis was also high in rat blood, suggesting a potential additional systemic route of cleavage of pro-drug, although hydrolysis was lower in dog blood and in human blood.

These data suggested GSK294 would be absorbed *in vivo* following administration by the oral route and then cleaved to afford miridesap once systemically available. The PK profile of the pro-drug was therefore evaluated *in vivo*.

Preliminary Pharmacokinetics in Rats and Dogs

Miridesap generated by oral administration of GSK294 has a high equivalent oral bioavailability in the rat (56–80% at 3 mg kg^{-1} and 36–58% at 10 mg kg^{-1}) although only 22% equivalent bioavailability in dog at a similar dose level (5 mg kg^{-1}) (Supplementary Table 1). At these doses, the pro-drug GSK294 was not detected up to 24 h post oral administration in the 2 preclinical species, indicating a rapid *in vivo* cleavage once absorbed. The monoester intermediate 1 was detected up to 2 h post oral administration only in the dog but at a very low concentration.

Additional preliminary PK studies in rats at 10, 30 and 100 mg kg^{-1} (n=2) indicated similar release of miridesap with bioavailability of 50±21%, 37±8%, 30±6%, respectively. At doses up to 100 mg kg^{-1} in the rat, GSK294 showed a roughly dose-proportional increase in miridesap exposure and maximum plasma concentration (C_{max}). Monoester intermediate 1 was detected only at the top dose and up to 2–4 h post oral administration.

Toxicokinetic repeat dose studies (100, 300 and 1000 mg kg^{-1}; n=3) further supported consistent high exposure of miridesap in both rat and dog but in a less than dose proportional manner, indicating absorption as a potential rate-limiting factor at such doses (Figure 3 for rat and Figure 4 for dog). GSK294 and monoester intermediate were not measurable in rodent blood due to the rapid systemic hydrolysis. In the dog, GSK294 was quantifiable and exposure increased with increasing dose, albeit with an ‘intravenous-like’ profile that rapidly declined with time and with very low levels detectable up to 24 h (data not shown).

Thus, GSK294 is soluble, permeable, and stable across species in intestinal microsomes, sufficient to enable oral delivery and absorption to the systemic circulation. It is hydrolysed rapidly *in vitro* across species in blood and in liver microsomes and this is reflected in a good miridesap PK profile in both rat

and dog following oral dosing of GSK294. This data suggests the clinical PK profile would also be appropriate.

Clinical Study

All recruited participants were male, 17 in Part A (mean age 41 years) and 8 in Part B (mean age 35 years). Most participants were white; one was black and one Asian. One participant was withdrawn owing to an adverse event, all others completed the protocol as planned. The 50-year-old participant who was withdrawn experienced an atrial tachycardia 1 day after administration of a single dose of 600 mg of GSK294 and further experienced an accelerated idioventricular rhythm on the same day. Both were self-limiting and not associated with cardiovascular compromise. One further participant in Part A experienced a self-limiting period of atrial tachycardia following administration of 1200 mg GSK294. In response to these observations cardiac monitoring was increased in Part B.

One serious AE was reported in Part B. The 39-year-old male participant experienced short, recurring episodes of supraventricular tachycardia (SVT, accelerated junctional rhythm/junctional tachycardia), which became mildly symptomatic from Day 6 onwards and resolved on Day 10. Episodes occurred at rest with symptoms of mild palpitations. All episodes were brief, self-terminating and not associated with cardiovascular compromise. The participant's drug exposure and degree of SAP depletion were consistent with other participants. No clinically significant changes in ECG were reported.

The study was temporarily halted at this point. Subsequent review concluded that the design of the remainder of the study did not lend itself to adequate evaluation of the cardiac profile of GSK294 and therefore the study was formally terminated. No other pattern of adverse events or trends in clinical laboratory parameters suggestive of a safety signal was observed in this limited study (Supplementary Table 2).

Clinical Pharmacokinetics and Pharmacodynamics

Following dosing with GSK294 at single doses of 200, 600 or 1200 mg, GSK294 concentrations were mostly non-quantifiable or very low. At all dose levels, in about half of the participants it was measurable in one or two time points per participant up to 2 h post administration with maximum GSK294 concentrations of about 2–3x the lower limit of quantification of the analytical assay (LLQ 10 ng mL^{-1}). All other participants had concentrations lower than the LLQ at all time points.

As shown in Figure 5, miridesap itself, however, was detectable with maximum concentrations similar to reported concentrations following parenteral administration (Pepys, Herbert, e*t al.*, 2002; Gillmore, Tennent, *et al.*, 2010; Sahota, Berges, *et al.*, 2015), which suggests a good bioavailability of GSK294, followed by a quick and complete conversion into miridesap.

The PK parameters of miridesap following single oral doses of GSK294 are shown in Supplementary Table 3. Between-participant variability (% CVb) in PK parameters was generally low (<30%). Increases in miridesap area under the concentration time curve (AUC) were dose proportional at 200–600 mg GSK294, and slightly less than proportional at 1200 mg. C_{max} displayed slightly less than dose-proportional increases over the full dose range tested. Terminal half-lives ($t_{1/2}$) did not show any trend with dose.

Based on the single dose PK data, a dose of 600 mg day^{-1} was selected for the repeat dose part (Part B) of the study, in which plasma SAP was measured. A preliminary assessment of the effect of food was investigated by comparing miridesap concentrations on dosing Day 4 without food, following a standardised (but not high fat) breakfast on dosing Day 5.

GSK294 600 mg day^{-1} for 7 days resulted in a rapid depletion of SAP in plasma with reductions of ~85% as early as 2 h post dose. Plasma SAP levels lower than 3 mg L^{-1} were achieved from 24 h post dose onwards (see Figure 6). Although a defined target degree of SAP depletion has not been formally established, these concentrations allowed administration of anti-SAP antibodies in clinical studies in systemic amyloidosis (Richards, Cookson, *et al.*, 2015, Richards, Cookson, *et al.*, 2018).

PK parameters of miridesap following repeat dosing with GSK294 at 600 mg day^{-1} are shown in Supplementary Table 4. The median t_{max} (time at which the maximum concentration is observed) of miridesap following oral dosing with GSK294 is somewhat later in the fed state compared with the fasted state, although the ranges completely overlap. The effect of food on systemic exposure of miridesap, (C_{max} and $AUC_{(0\text{-}tau)}$), was modest, the increase was 1.5- and 1.2-fold higher, respectively, in the fed *versus* fasted state. As in Part A of the study, the majority of samples had concentrations of GSK294 that were below the LLQ.

Discussion and Conclusions

The pharmacology of miridesap is unique and it has the rare distinction of being pharmacologically active in all human subjects in whom it has been tested (Pepys, Herbert, *et al.*, 2002; Kolsto, Wood, *et al.*, 2009; Gilmore, Tennent, *et al.*, 2010; Sahota, Berges, *et al.*, 2015; Richards, Cookson, *et al.*, 2015; Richards, Cookson, *et al.*, 2018). Its physicochemical properties limit administration to parenteral routes. We sought to identify a pro-drug that rapidly and efficiently generated miridesap in the blood following oral administration. Miridesap contains two *N*-linked *R*-carboxypyrrolidine moieties (Figure 7), which use functional groups with high atom efficiency in binding to SAP such that two molecules of SAP are cross-linked for hepatic clearance. The two carboxylic acid functional groups are key to the binding affinity, but are probably responsible for poor membrane permeability and, thus, oral bioavailability. Analysis of the architecture of the miridesap binding site on SAP indicates that changing

the carboxylic acids for more membrane permeable groups while retaining SAP binding affinity would be extremely challenging; a *de novo* drug discovery campaign using an appropriate screening cascade (Figure 8) was deemed more likely to succeed. The palindromic, diacidic nature of miridesap presented the key challenge in defining a pro-drug: masking of two functionalities simultaneously is required, which is especially challenging when the physicochemical properties of the singly cleaved monoester prodrug are very different from the parent prodrug. Ensuring appropriate stability and overall properties to enable delivery, absorption and distribution whilst also enabling efficient cleavage to the active drug once absorbed is a delicate balance. Previous attempts by others were unsuccessful. We identified for the first time, a molecule that can successfully lower SAP in the clinic following oral administration.

We explored several pro-drug functionalities, including esters, amides, ketal esters and acetal esters, anticipating that, once absorbed, miridesap would be liberated through sequential cleavage of the derivatised acid groups by blood and/or liver enzymes. The head groups for these functional groups were selected to manage overall physicochemical properties; ChromlogD was used to measure lipophilicity to benchmark *in silico* predictors for the chemical series. This enabled chemistry to focus only on molecules (GSK294 ChromlogD 3.6), in drug-like lipophilicity space (Lipinski, Lombardo, *et al.*, 1997). Molecules were screened for solubility and permeability, properties key to efficient and reproducible oral absorption. To limit future developability challenges, we only further evaluated the subset of molecules that proved to be crystalline, defined qualitatively initially and subsequently confirmed by X-ray powder diffraction (XRPD) analysis (data not shown).

Having identified molecules that were crystalline and more likely to be DCS class 1 (kinetic solubility >100 $\mu g\ mL^{-1}$; permeability >100 nm s^{-1}), we tested for solution stability, including at acid pH and in SGF. GSK294 exhibited minimal hydrolysis in acid solution, including SGF. We sought to identify compounds with minimal hydrolysis by intestinal enzymes prior to absorption, so screened compounds in rat, dog and human intestinal microsomes. GSK294 was substantially intact after 1 h exposure to intestinal microsomes across species.

Once absorbed, hydrolysis at each ester of the GSK294 molecule is required to liberate miridesap (Figure 4). Esterases are prevalent in blood, intestinal wall and liver. We selected compounds based on their profile in rat, dog and human liver microsomes and blood. GSK294 showed substantial conversion to miridesap in liver microsomes from all species; conversion in dog liver microsomes was almost complete by 1 h. *In vitro* conversion of GSK294 to miridesap was less extensive in blood than in liver microsomes. Other compounds showed greater conversion to miridesap in blood *in vitro* but also

showed greater conversion in the intestinal microsome assay. We focussed on compounds that were more stable prior to systemic exposure to mitigate against developability issues such as instability during manufacture and storage. The principal concern at this stage was that monohydrolysis *in vivo* would liberate a pharmacologically inactive molecule; miridesap depends on non-covalent crosslinking for its action (Pepys, Herbert, *et al.*, 2002). We therefore profiled GSK294 in rat and dog *in vivo*. The results showed rapid and substantial exposure to miridesap following a single dose; oral bioavailability of miridesap was higher in rat than dog. Exposure to pro-drug or the mono ester intermediate could not be measured owing to rapidity of conversion. This would limit the ability of preclinical toxicology studies to assess toxicity of parent and intermediates if conversion in man proved markedly slower. The estimated bioavailability was variable but estimates in both species were potentially acceptable if observed in man.

Given the known pharmacology and favourable safety profile of miridesap (Sahota, Berges, *et al*., 2015), the initial (single) doses to be tested in man were selected to give a robust PK assessment. Assuming fast and complete oral absorption of GSK294 and fast and complete subsequent conversion of GSK294 into miridesap, both processes can be described by a single first-order process (Ka) with high bioavailability (F). To select the starting dose, Ka and F were assumed to be high: 1.3 h^{-1} and 100%. Distribution and elimination of miridesap was expected to be the same whatever the route of adminstration. The concentration-time profiles of miridesap after single dose GSK294 were simulated, and predicted, C_{max} and $AUC_{(0\text{-}inf)}$ derived. For the starting dose of 200 mg, predicted $AUC_{(0\text{-}inf)}$ was 14.5 μg.h mL^{-1} (95% CI 9.5, 22.4 μg.h mL^{-1}) and C_{max} 3.4 μg mL^{-1} (95% CI 2.1, 5.0 μg.h mL^{-1}). The predicted exposure was well within the exposure limit of $AUC_{(0\text{-}24)}$ of 250 μg.h mL^{-1} and C_{max} of 22.1 μg mL^{-1} set by the no observed adverse effect level in non-clinical toxicology studies. Observed AUC and C_{max} in man, at a dose of 200 mg, were 8.0 μg.h mL^{-1} (95% CI 6.3, 10.0 μg.h mL^{-1}) and 0.72 μg mL^{-1} (95% CI 0.59, 0.87 μg.h mL^{-1}), respectively. Consistent with the preclinical profile, exposure to GSK294 was low. A formal exposure profile was not observed with any administered dose (LLQ 10 ng mL^{-1}). The preclinical profile of GSK294 was predictive of human, with the profile in rat more representative than dog.

Repeat dosing of GSK294 600 mg day^{-1} resulted in rapid and sustained reduction in plasma SAP (~85% reduction as soon as 2 h post dose). In studies with dezamizumab, intravenous miridesap was used to reduce plasma SAP to 3 mg L^{-1} or lower before administering the antibody. Following repeat dosing of GSK294, this target was met at 24 h and maintained for the dosing period of 7 days.

The hydrolysis of GSK294 results in the liberation of formaldehyde. Formaldehyde is an *in vitro* mutagen but also a by-product of many biological reactions. Humans are routinely exposed to formaldehyde from the environment, food, and as an endogenous metabolite. There are several

marketed pro-drugs (including fosphenytoin, tenofovir disoproxil fumarate and fospropofol disodium) that generate formaldehyde *in vivo* without evidence of harm to human health. As a precaution we limited the dose to 1200 mg daily, which would be predicted to liberate 110 mg formaldehyde, an amount similar to fospropofol disodium. The other molecule generated by cleavage of the pro-drug is 4-hydroxy-tetrahydropyran (Figure 7), which is not well characterised but did not have structural features that indicated specific risks.

In Part A of the clinical study, a total of four separate arrhythmia events were reported in three participants aged 46–55 years: three episodes of SVT (at 600 and 1200 mg), all consistent with atrial tachycardia morphology and one episode of a 5-beat accelerated idioventricular rhythm (600 mg). Each event was of short duration, asymptomatic, and not associated with haemodynamic instability. SVT is more common in those aged >45 years compared with those <45 years (13.9% vs 1.1%, respectively) (Hingorani, Karnad, *et al.*, 2016). There was no association between the time of onset and concentration of GSK294 or miridesap. As a precaution, cardiac monitoring was increased in Part B. In Part B, one participant experienced junctional tachycardia necessitating prolonged inpatient monitoring and thus fulfilled the definition of a serious adverse event. There was no relationship between onset of arrhythmia and peak plasma GSK294 concentration or miridesap. Follow-up exercise testing, echocardiogram and 48 h Holter recording were normal. Miridesap has not been associated with arrhythmias in clinical studies to date, including patients with cardiac amyloidosis (Gilmore, Tennent, *et al.*, 2010; Richards, Cookson, *et al.*, 2015; Richards, Cookson, *et al.*, 2018). Preclinical profiling against a wide range of ion channels, and *in vivo* pharmacology studies did not identify a pro-arrhythmic potential for GSK294 or miridesap. The overall assessment of these events was that, while GSK294 was unlikely to be responsible, these unusual findings warranted further evaluation. The optimal design would be a placebo-controlled crossover; the current study design did not enable this approach and so the study was terminated early.

Using a preclinical screening cascade, we identified a pro-drug that showed good oral bioavailability of miridesap. Preclinical results proved predictive of the profile in humans. GSK294 had a favourable PK/PD profile in preliminary human testing indicating it could be a viable replacement for parenteral miridesap. A higher than expected incidence of supraventricular arrhythmias, although not clearly related to GSK294 curtailed the clinical study and requires further evaluation in future clinical studies.

Authors contribution

MB, JS, DR, DSH, NH, DT, GL and LL contributed to the conception and design of the study.
DF and SK contributed to the acquisition of data.
MB, JS, DR, DSH, NH, DT, GL, LL and SK contributed to data analysis and/or interpretation.

All authors were involved in development of the manuscript and approved the final version.

Data Sharing Statement

Anonymized individual participant data and study documents can be requested for further research from www.clinicalstudydatarequest.com

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Tables

Table 1. Physicochemical & physiological properties of GSK294

Lipophilicity	**Solubility (mg mL^{-1})**			**MDCK-II Passive permeability (nm s^{-1})**	
ChromlogD	SGF (pH 1.6)	FaSSIF	FeSSIF	P_{app} (nm s^{-1})	P_{exact} (nm s^{-1})
3.6	5.2, 5.0	4.4, 4.8	5.0, 3.8	133	148

ChromlogD, chromatographic logD; FaSSIF, fasted state simulated intestinal fluid; FeSSIF, fed state simulated intestinal fluid; MDCK-II, Madine Darby Canine Kidney type II cells; $P_{app/exact}$, apparent/exact permeability; SGF, simulated gastric fluid.

Figure legends

Figure 1. *In vitro* physical hydrolysis profile of GSK294 at varying pH in aqueous solution

Figure 2: *In vitro* matrix hydrolysis profile of GSK294 by intestinal and liver microsomes and blood

Figure 3. Plasma pharmacokinetic profile of miridesap after IV administration of miridesap at 10 mg kg^{-1} and after oral administration of GSK294 at a range of doses in rat

IV, intravenous; LLQ, lower limit of quantification.

Figure 4. Plasma pharmacokinetic profile of miridesap after IV administration of miridesap at 10 mg kg^{-1} and after oral administration of GSK294 at a range of doses in dog

IV, intravenous; LLQ, lower limit of quantification.

Figure 5. Miridesap concentration versus time following oral administration of GSK294 in humans

LLQ, lower limit of quantification.

Figure 6. Plasma SAP levels after oral administration of GSK294 600 mg day^{-1} for 7 days in humans

SAP, Serum amyloid P component.

Figure 7. Sequential hydrolysis of GSK294 pro-drug via its monoester derivative to generate miridesap and two molecules each of formaldehyde, carbon dioxide and tetrahydropyran-4-ol (THP-OH)

Figure 8. Cascade of assays used to identify GSK294