Identification of KasA as the cellular target of an anti-tubercular scaffold

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Phenotypic screens for bactericidal compounds are starting to yield promising hits against tuberculosis. In this regard, whole-genome sequencing of spontaneous resistant mutants generated against an indazole sulfonamide (GSK3011724A) identifies several specific single-nucleotide polymorphisms in the essential Mycobacterium tuberculosis β-ketoacyl synthase (kas) A gene. Here, this genomic-based target assignment is confirmed by biochemical assays, chemical proteomics and structural resolution of a KasA-GSK3011724A complex by X-ray crystallography. Finally, M. tuberculosis GSK3011724A-resistant mutants increase the in vitro minimum inhibitory concentration and the in vivo 99% effective dose in mice, establishing in vitro and in vivo target engagement. Surprisingly, the lack of target engagement of the related β-ketoacyl synthases (FabH and KasB) suggests a different mode of inhibition when compared with other Kas inhibitors of fatty acid biosynthesis in bacteria. These results clearly identify KasA as the biological target of GSK3011724A and validate this enzyme for further drug discovery efforts against tuberculosis.

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After many years of relatively little attention, *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB), has re-emerged as a priority in the area of neglected diseases. The standard 6-month treatment for TB has remained essentially unchanged over several decades. Unfortunately, poor patient compliance and other factors have led to an increasing prevalence of drug resistance. In 2013, the World Health Organization (WHO) recorded ~480,000 new cases of multidrug-resistant (MDR)-TB, resistant to both the front-line drugs, isoniazid (INH) and rifampicin (RIF). Without these two front-line drugs, MDR-TB generally requires 24 months of treatment with a variety of second-line antibiotics, which are poorly tolerated. Due to this continued spread of MDR and extensively-drug-resistant forms of *M. tuberculosis*, the WHO has declared TB as a world health emergency. Therefore, new drugs that can complement existing front-line treatment regimens are urgently required.

In 2013 and updated in 2015, GlaxoSmithKline published a set of 228 small molecule hits from two phenotypic screening campaigns against *M. tuberculosis*. Of those 228 compounds, a number have been explored via medicinal chemistry for potential optimization to drug leads and clinical candidates. Phenotypic screening also offers the opportunity to identify novel biological targets by molecular mode of action (MoA) studies, which is generally achieved through a variety of omics-based technologies. GSK3011724A is a small molecule inhibitor from the aforementioned 228 phenotypic screening hits. In this study, we provide an initial profiling of GSK3011724A as a suitable starting point for ‘hit to lead’ drug development based on its *in vitro* and *in vivo* characteristics. In addition, through MoA studies, we identify the *M. tuberculosis* cellular target of GSK3011724A to be KasA, a β-ketoacyl synthase. Target assignment reveals specificity of GSK3011724A for KasA with a binding site distinct from other known Kas inhibitors. This different MoA creates new potential for this recognized target in future TB drug discovery efforts.

**Results**

**In vitro profiling of GSK3011724A.** From the outset, GSK3011724A, an indazole sulfonamide (Fig. 1a), represented an attractive compound for early stage drug discovery based on its anti-mycobacterial potency, small size and moderate lipophilicity (Table 1). Further profiling *in vitro* using standard assays, such as the hERG ion channel and cytochrome P450 isoforms supported this view (Table 1). Sensitivity testing of 18 Gram-positive and Gram-negative bacterial species with GSK3011724A demonstrated selectivity of this compound for *M. tuberculosis*. In addition, GSK3011724A showed negligible activity against a panel of unrelated proteins (Supplementary Table 1). These desirable characteristics demonstrated the clear potential of GSK3011724A as a starting point for medicinal chemistry optimization. A description of this optimization effort along with a thorough discussion of the drug development potential and liabilities of this series will be the subject of a future publication.

**In vivo profiling of GSK3011724A.** GSK3011724A was progressed into pharmacokinetic (PK) and efficacy experiments to generate an *in vivo* profile. Although doses above 400 mg kg⁻¹ were not well tolerated by mice, multiple days of dosing up to 300 mg kg⁻¹ once daily proceeded with no weight loss or other adverse effects. The *in vivo* PK of GSK3011724A in mice was disproportional based on dosing. At low doses, clearance close to the liver blood flow rate was observed (in agreement with the *in vitro* clearance (Cli) in mouse microsomes), but as the doses were increased, the maximum concentration (Cmax) and area under the curve values grew disproportionally (Table 2). This observation suggests a saturation of the mechanism of clearance, and may help to explain the reduction in tolerability between 300 and 400 mg kg⁻¹ dosing.

The first stage of efficacy testing involved an acute model of infection. This murine model involved infecting mice with a high inoculum (10⁵ colony-forming units (c.f.u.)) of *M. tuberculosis* H37Rv wild type and beginning treatment the next day. GSK3011724A (and INH as a positive control) were administered once daily for 8 days, while the bacilli were in the exponential growth phase. Despite exhibiting a bacteriostatic effect *in vitro* (Supplementary Fig. 1), GSK3011724A demonstrated a significant cidal effect in this murine model with a ~3.5 log c.f.u. reduction relative to untreated controls at 200 mg kg⁻¹ (Fig. 1b). The effects of GSK3011724A are similar to linezolid, which has been shown to be bacteriostatic *in vitro*, but exhibits cidal activity *in vivo*. Importantly, a clear dose–response was observed, with the ED₉₀ (dose required to reduce bacterial load by 99%) determined as 38 mg kg⁻¹. This level of ED₉₀ is only moderately higher than most of the gold standard TB drugs. GSK3011724A (and INH as a positive control) was also evaluated in a chronic infection assay in which mice were infected with a lower inoculum of *M. tuberculosis* H37Rv (10² c.f.u.) and left untreated for 6 weeks, allowing the bacilli to reach a steady state. GSK3011724A was then dosed daily for 2 months. The dose–response curve in this chronic assay was shifted to higher doses than in the acute model, but the targeted >2 log c.f.u. reduction was achieved at 100 mg kg⁻¹ for GSK3011724A, producing a 2.4 log c.f.u. reduction (Fig. 1c). These data clearly establish GSK3011724A as an active compound in both the acute and chronic *in vivo* murine models of *M. tuberculosis* infection. Importantly, if GSK3011724A were demonstrated to inhibit an unexploited potential antimicrobial target, these data would provide a significant level of validation and confidence for further exploration of that target for TB drug discovery.

**GSK3011724A target identification.** A fundamental strategy in drug discovery is establishing the MoA of inhibitory compounds. Following the identification and validation of the molecular target, target-specific optimization of the compound can be pursued to improve efficacy and reduce toxicity. Due to the recent successes of utilizing whole-genome sequencing (WGS) of spontaneous resistant mutants as a primary step in the elucidation of the target of phenotypic hits, this methodology was used to establish the target of GSK3011724A.

The minimum inhibitory concentration (MIC) of GSK3011724A in *Mycobacterium bovis* Bacillus Calmette–Guérin (BCG) was determined to be 0.5 μM. Spontaneous resistant mutants were initially generated using *M. bovis* BCG at 5 ×, 10 × and 20 × the MIC of GSK3011724A, with frequencies of resistance (FoR) of 12 × 10⁻⁸, 3 × 10⁻⁸ and 2 × 10⁻⁸, respectively. The FoR was subsequently determined against *M. tuberculosis* at 2.5 μM (10 × MIC of GSK3011724A on solid media, 0.25 μM) giving a frequency of 9.5 × 10⁻⁷. This FoR is slightly higher than normally desired but is lower than that of INH.

From the total of 17 *M. bovis* BCG spontaneous resistant mutants generated, eight were selected for WGS. Six of the eight *M. bovis* BCG mutants were found to possess a number of high-quality (statistically relevant) single-nucleotide polymorphisms (SNP) compared with the sequenced wild-type reference strain (Genbank accession number NC_008769.1), all of which located to the gene annotated kasA, encoding an essential β-ketoacyl synthase involved in mycolic acid biosynthesis (Table 3). The data offered the first evidence that KasA is the target of...
GSK3011724A, providing a starting point for validation studies. Subsequently, instead of using WGS, the kasA gene from 12 isolated M. tuberculosis resistant mutants was specifically sequenced following amplification by PCR. The data confirmed the results from M. bovis BCG, with eleven of the twelve mutants showing SNPs in kasA (Table 3). The WGS results of the two remaining M. bovis BCG mutants did not reveal SNPs of a high frequency; no gene contained a SNP of greater than 50% frequency, corresponding to the percentage of SNP in the cell population from which the genomic DNA was prepared.

**KasA target validation.** Following the target identification of KasA by WGS, a variety of techniques were utilized to confirm its role as the biological target of GSK3011724A. Firstly, the inhibition of mycolic acid biosynthesis by GSK3011724A was investigated. M. bovis BCG was labelled using [14C]-acetate and treated with increasing concentrations of GSK3011724A, with

| Assay                              | Value   |
|------------------------------------|---------|
| MIC H37Rv                          | 0.8 μM  |
| HepG2 IC50                         | >100 μM |
| ChromLogD7.4                       | 3.8     |
| Kinetic Solubility                 | >550 μM |
| hERG IC50                          | >50 μM  |
| CYP3A4 IC50                        | >40 μM  |
| CYP1A2 IC50                        | >50 μM  |
| CYP2C9 IC50                        | 12.6 μM |
| CYP2D6 IC50                        | >50 μM  |
| 18 Gram-positive and Gram-negative species | >128 μg ml^{-1} |
| Plasma protein binding (mouse)     | 76.9%   |
| Plasma protein binding (human)     | 91.8%   |
| Cli (mouse)                        | 6.1 ml min^{-1} g^{-1} |
| Cli (human)                        | 0.95 ml min^{-1} g^{-1} |

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INH and the KasA inhibitor thiolaclomycin (TLM) used as positive controls. Following drug treatment, the total fatty acid composition when apolar lipids and polar phospholipids were quantified by scintillation counting (Table 4), GSK3011724A at 44464495778541 respectively. In contrast, quantification of the levels of FAMEs inhibition and FAMEs accumulation mirrors the known biosynthetic pathways, and is specific for KasA.

To further corroborate that GSK3011724A inhibits mycolic acid biosynthesis and, more specifically, targets KasA, the impact of additional cell wall-associated targets, the total cellular lipids (and fractionated apolar lipids and polar phospholipids) were extracted and analysed following drug treatment. Firstly, a decrease in trehalose dimycolate (TDM), trehalose monomycolate (TMM) and glycerol monomycolate was observed with GSK3011724A (and TLM and INH), consistent with inhibition of mycolic acid biosynthesis (Fig. 2b; Table 4 and Supplementary Fig. 2a). Secondly, no significant differences were observed in lipid composition when apolar lipids and polar phospholipids were analysed by autoradiography–TLC (Supplementary Fig. 2a,b), suggesting that GSK3011724A does not target other lipid biosynthetic pathways, and is specific for KasA.

### Table 2 | In vivo pharmacokinetic profile of GSK3011724A.

| Target dose (mg kg⁻¹) | Cmax (ng ml⁻¹) | Cmax/Dose ng ml⁻¹ (mg kg⁻¹) | tmax (h) | AUC₂⁻⁴ (ng h ml⁻¹) | DNAUC ng h ml⁻¹ (mg kg⁻¹) |
|-----------------------|----------------|-----------------------------|---------|-------------------|-----------------------------|
| 10*                   | 917 ± 279      | 84.7 ± 21.5                 | 0.25–0.75 | 1099 ± 152        | 104 ± 19.1                   |
| 25                    | 1392 ± 424     | 63.9 ± 19.4                 | 0.5      | 2279 ± 581        | 107 ± 27.3                   |
| 50                    | 12500 ± 153    | 250 ± 23.1                  | 0.25–1.0 | 32549 ± 3009      | 667 ± 64.2                   |
| 100                   | 10570 ± 572    | 120 ± 6.49                  | 0.75–1.0 | 57179 ± 7559      | 667 ± 57.2                   |
| 200                   | 33000 ± 7017   | 165 ± 35.1                  | 2.0      | 251437 ± 37054    | 1272 ± 188                   |

AUC, area under the curve; Cmax, maximum concentration measured; DNAUC, dose-normalized area under the curve.

Pharmacokinetic parameters estimated for GSK3011724A after a single oral gavage administration to female C57BL/6J mice (n = 3) at different doses. Mice dosed above 400 mg kg⁻¹ were withdrawn after the second administration of GSK3011724A due to poor clinical status. Standard deviation (s.d.) of the mean is also included.

*Mean and s.d. values calculated from n = 3 mice.

### Table 3 | Sequencing of spontaneous resistant mutants identifies SNPs in kasA.

| Organism | Mutant | Frequency of SNP | Genome position of SNP | Base change | Amino-acid substitution |
|----------|--------|-----------------|------------------------|-------------|-------------------------|
| M. bovis BCG | 1      | 75%             | 2497100                | aCc/aGc     | T114S                   |
|          | 2      | 2.44%           | 2497376                | cCc/cTc     | P206L                   |
|          | 3      | 100%            | 2497142                | cTg/Ccg     | L128P                   |
|          | 4      | 100%            | 2497373                | cTg/Ccg     | L205P                   |
|          | 5      | 100%            | 2497376                | cCc/cTc     | P206L                   |
|          | 6      | 100%            | 2497195                | Atg/Gtg     | M146V                   |
|          | 7      | 100%            | 2497589                | aTg/aCg     | M277T                   |
| M. tuberculosis | 1      | —               | 2518524                | gTg/gCc     | V137A                   |
|          | 2      | —               | 2518524                | gTg/gYg     | V137A/V                 |
|          | 3      | —               | 2518524                | gTg/gCc     | V137A                   |
|          | 4      | —               | 2518715                | Ccc/Tcc     | P201S                   |
|          | 5      | —               | 2518715                | Ccc/Tcc     | P201S                   |
|          | 6      | —               | 2518715                | Ccc/Tcc     | P201S                   |
|          | 7      | —               | 2518715                | Ccc/Tcc     | P201S                   |
|          | 8      | —               | 2518715                | Ccc/Tcc     | P201S                   |
|          | 9      | —               | 2518943                | Atg/Gtg     | M277V                   |
|          | 10     | —               | 2518944                | aTg/aCg     | M277T                   |
|          | 11     | —               | 2519327                | Ggc/Agc     | G405S                   |

SNP, single-nucleotide polymorphism.

WGS and sequencing of kasA from GSK3011724A-resistant mutants of M. bovis BCG and M. tuberculosis, respectively, revealed kasA-specific mutations. For the WGS of M. bovis BCG mutants, the frequency of SNP is shown. This corresponds to the percentage of SNP in the cell population from which the genomic DNA was extracted. The kasA SNP frequency in the M. tuberculosis mutants cannot be deduced due to the method of analysis used. The genomic location of the mutations is stated. The missense mutations are represented in codons, where the capital letter identifies the base change. The ‘Y’ indicates a mixed population of ‘C’ or ‘T’ bases. The resulting amino-acid substitutions are listed.
Table 4 | Quantification of inhibition of mycolate containing products by GSK3011724A.

| Inhibitor concentration (µM) | FAMEs | α-MAMEs | k-MAMEs | TDM | TMM |
|-----------------------------|-------|---------|---------|-----|-----|
|                             | c.p.m. % Relative to control | c.p.m. % Relative to control | c.p.m. % Relative to control | c.p.m. % Relative to control | c.p.m. % Relative to control |
| Control                     | 0     | 9,796 100 | 4,029 100 | 7,641 100 | 3,149 100 | 1,425 100 |
| GSK3011724A                 | 0.25  | 16,207 165 | 3,438 85  | 7,147 94  | ND ND | ND ND |
|                             | 0.5   | 17,780 182 | 3,026 75  | 6,651 87  | 864 27 | 911 64 |
|                             | 1     | 16,478 168 | 2,488 62  | 4,925 64  | 874 28 | 893 63 |
|                             | 2     | 18,268 186 | 1,806 45  | 4,407 58  | ND ND | 652 21 |
|                             | 4     | ND ND | ND ND | ND ND | ND ND | 951 67 |
| Control                     | 0     | 10,231 100 | 4,156 100 | 6,722 100 | ND ND | ND ND |
| INH                         | 1.5   | 15,334 150 | 2,721 65  | 3,727 55  | 817 26 | 704 49 |
|                             | 4.0   | 16,926 165 | 1,426 34  | 1,808 27  | 642 20 | 552 39 |

α-MAMEs, α-mycolate acid methyl esters; FAMEs, fatty acid methyl esters; INH, isoniazid; k-MAMEs, keto-mycolic acid methyl esters; ND, not determined; TDM, trehalose dimycolate; TMM, trehalose monomycolate.

Quantification of [14C]-labelled products were determined by excising spots directly from TLC plates (Fig. 2a, left and middle panels, and Fig. 2b) and subjected them to scintillation counting using 10 ml of EcoScintA.

*Control values as reported above.
was also apparent for the pMV261 constructs containing \textit{kasB}, \textit{inhA}, \textit{fabH}, \textit{hadABC} and \textit{mabA} (Fig. 2c). However, ample growth of the KasA overexpressor strain was observed at 4 \textmu M, indicating an increase in resistance, and a MIC shift of >8 \times (Fig. 2c), providing further evidence to support KasA as the cellular target of GSK3011724A.

Previous studies have reported KasA activity in an \textit{in vitro} condensing assay using purified recombinant proteins\textsuperscript{18}. This assay was replicated to demonstrate the specific inhibition of KasA by GSK3011724A. Initially, FabD was used to generate $^{[14C]}$-malonyl-AcpM from holo-AcpM and $^{[14C]}$-malonyl-CoA. KasA, in the absence or presence of GSK3011724A, elongated C\textsubscript{16}-AcpM in a condensation reaction with $^{[14C]}$-malonyl-AcpM. Treatment with 1 \textmu M of GSK3011724A provided a 58.5\% inhibition of KasA activity (based on triplicate data, Supplementary Fig. 3).

To investigate whether GSK3011724A exerts its effect by directly binding to KasA, we employed a chemoproteomics strategy\textsuperscript{19,20}. A tagged analogue (1) with an MIC of 49 \textmu M was generated, which was covalently linked to Sepharose beads (Fig. 3a). The tagged analogue-(1)-derivatized beads were incubated with \textit{M. bovis} BCG extracts, in the absence or presence of an excess of unbound GSK3011724A. In this competition-binding format, target proteins would be expected to bind to the beads predominantly in the absence of excess competing compound. After washing of the beads, bound proteins were digested with trypsin and subjected to quantitative mass spectrometry. Among >2,000 proteins identified, only three proteins were efficiently competed off the beads by excess GSK3011724A: KasA, and, to a lesser degree, the non-essential polyketide synthases Pks10 and Pks11 (refs 21,22; Fig. 3a; Supplementary Data 1 and 2). The experiment was repeated in a concentration-dependent format to determine half-maximal inhibition (IC\textsubscript{50}) values that refer to the concentration of compound (GSK3011724A) required to competitively block 50\% of the target (that is, KasA) from binding to the beads (Fig. 3b; Supplementary Data 3 and 4). These IC\textsubscript{50} values are a measure of target affinity, but are also affected by the affinity of the target for

Figure 3 | Chemoproteomics profiling of GSK3011724A. (a) A propylamine-tagged derivative of GSK3011724A (1, inset) was synthesized and covalently immobilized to NHS-activated sepharose. Beads were incubated with \textit{M. bovis} BCG extract either in the presence of vehicle (DMSO) or GSK3011724A (10 \textmu M, 40 \textmu M). Proteins captured by the beads in both conditions were quantified by LC–MS/MS analysis. KasA, Pks10 and Pks11 were identified as potential targets of GSK3011724A by virtue of their reduced capturing in the presence of excess GSK3011724A. (b) Generation of IC\textsubscript{50} values for KasA, Pks10 and Pks11. The chemoproteomic experiment was performed as in a but over a range of concentrations of the competing ‘free’ inhibitor GSK3011724A (2–0.003 \textmu M for KasA, 40–0.16 \textmu M for Pks10 and Pks11) and a structurally related inactive analogue, 2 (40–0.16 \textmu M). Apparent dissociation constants for GSK3011724A were determined from two independent experiments.
the bead-immobilized ligand. The latter effect can be deduced by measuring the depletion of the target proteins (KasA, Pks10 and Pks11) by the beads. Thus, apparent dissociation constants (K_{d}^{app}) can be determined, which are largely independent from the bead ligand.\textsuperscript{19,23} The K_{d}^{app} value was determined as 9 nM for KasA, suggesting a high level of affinity for the enzyme. In excellent agreement with these chemoproteomic affinities, direct binding of GSK3011724A to purified KasA was also determined using surface plasmon resonance (SPR) and found to be in the 10–20 nM range (Supplementary Fig. 4). A much weaker apparent dissociation constant was observed for both Pks10 and Pks11 (K_{d}^{app} 1.4 μM). In addition, we compared the results of the active compound GSK3011724A with a structurally related inactive analogue 2 (MIC > 125 μM). The K_{d}^{app} for this inactive molecule was determined to be 4.5 μM for KasA, which corresponds to a ~490-fold window for KasA between active and inactive compound. There was no binding observed for Pks10 and Pks11 by the inactive analogue 2 (Fig. 3b; Supplementary Data 5).

To evaluate the potential of GSK3011724A for interaction with human proteins, which could represent off-targets relevant for drug safety, we performed a similar set of chemoproteomics experiments with a human protein extract, generated by combining detergent (NP40) lysates from HEK293 cells, K562 cells and placenta tissue (Supplementary Data 6). Only a single protein showed moderate competition by GSK3011724A, NQO2, and inactive compound. There was no binding observed for Pks10 and Pks11 by the inactive analogue 2 (Fig. 3b; Supplementary Data 5).

Analysis of the KasA-GSK3011724A co-crystal structure.

Curious to understand the molecular details of GSK3011724A binding to KasA, a 2.13 Å co-crystal structure of the dimeric KasA-GSK3011724A complex was solved (Table 5). All literature inhibitors to date, such as TLM, reside in the malonyl-binding to KasA, a 2.13 Å co-crystal structure of the dimeric KasA-GSK3011724A, to place the butyl chain into an orthogonal narrow

lipophilic channel lined by residues such as Ile347, Ile202 and Phe239.

The resistance-conferring mutations observed in Table 3 can be readily rationalized by the inhibitor binding site and MoA of GSK3011724A (Fig. 5a). Pro201 and Pro206 both lie within the acyl site and are in direct contact with the ligand (Fig. 5b). Exchange of proline for a hydrophilic serine residue (Pro201Ser) disfavours interactions with the aromatic indazole ring, whereas the Pro206Leu substitution introduces steric crowding of the ligand. The remaining mutations are found away from the inhibitor site, in regions likely to influence the conformational equilibrium and ease of transition between the closed and open state required for GSK3011724A inhibition. The α5–α6 helix-turn-helix (HTH) arm of one KasA monomer, comprising of residues 115–145, must slide past its dimer counterpart (α5’–α6’) and the α2 helix as the acyl cavity opens. Mutations Leu128Pro, Val137Ala, Met146Val and Leu205Pro are either within the HTH or the acyl site and are in direct contact with the ligand (Fig. 5c). Pro201 and Pro206 both lie within the acyl site and are in direct contact with the ligand (Fig. 5b).

### Table 5 | Data collection and refinement statistics.

| Data collection | KasA/GSK3011724A |
|-----------------|-----------------|
| X-ray diffraction data |                  |
| Space group     | P3₁             |
| Cell dimensions | a, b, c (Å)     |
| Resolution (Å)  | 66.98–213 (23.8–213) |
| Rmerge          | 0.053 (0.474)   |
| Completeness (%)| 98.6 (97.3)     |
| Wilson B-factor | 34.16           |
| No. reflections | 235866 (60834)  |
| Rfree/Rwork    | 0.162/0.186     |
| No. atoms       | 6470            |
| Protein         | 6041            |
| Ligand/ion      | 36/28           |
| Water           | 365             |
| B-factors       |                 |
| Protein         | 39.70           |
| Ligand/ion      | 39.90/48.61     |
| Water           | 43.92           |
| R.m.s.d.        |                 |
| Bond lengths (Å)| 0.0044          |
| Bond angles (°) | 0.8045          |
| Twin fraction   | 0.39            |

Values in parentheses are for the highest-resolution shell. Collection based on a single crystal.
In summary, these crystallographic insights complete a consistent picture of the molecular MoA of this inhibitor and provide a platform for rational optimization of the GSK3011724A scaffold as well as de novo structure-based drug design. Excitingly, this offers a fresh opportunity to target KasA at a site distinct from previous inhibitors and one that is able to achieve selectivity over other related β-ketoacyl synthases (FabH and KasB) involved in fatty acid biosynthesis, as key residues required for affinity are not conserved (Fig. 5f,g).

**In vivo target engagement of GSK3011724A and KasA.** Although the evidence presented above provided a high level of confidence that KasA was the true biological target of GSK3011724A, there was still a need to confirm that target engagement was responsible for the potent in vivo activity. A selection of the *M. tuberculosis* spontaneous resistant mutants isolated against GSK3011724A were tested for their growth profile in mice using the acute model11 (Fig. 1d). While the two mutants showed an attenuated growth rate in untreated animals, their response to INH treatment remained essentially unchanged. Given the close relationship between KasA and InhA, the INH data were particularly significant, confirming that the observed resistance in vivo was not merely an artefact of the limited growth rate. In contrast with INH, the response with GSK3011724A against both strains with mutations mapping to kasA showed clear signs of resistance (Fig. 1d). Mutation Met277Thr appeared to impart complete resistance up to the maximum dose tested (140 mg kg\(^{-1}\)), while mutation Pro201Ser gave a lesser response at 100 mg kg\(^{-1}\). These results support the MIC shifts observed in vitro and furthermore provide a critical link between the target identification and in vivo validation of KasA.

**Discussion**

On-going efforts to combat drug-resistant TB have taken many forms including the re-purposing of broad spectrum antibacterials, target-based programs on mycobacterial enzymes, efforts to optimize or re-invent known TB drugs (like INH and RIF), and phenotypic screening approaches, which have all been widely reported4,27–32. In this work, through a variety of in vitro and in vivo experiments, a new chemical scaffold, exemplified by GSK3011724A, has been identified to specifically target an integral component of mycopic acid biosynthesis, KasA (ref. 18). Mycolic acids are unique and fundamental components of the mycobacterial cell wall and KasA is essential in *M. tuberculosis*13.

Mycolic acid biosynthesis involves two distinct fatty acid synthesis pathways. The FAS-I system is required for de novo fatty acid synthesis, where a single, multifunctional polypeptide generates short chain fatty acyl-CoA esters. FabH, a β-ketoacylACP synthase, forms a pivotal link between FAS-I and FAS-II, condensing C 14 -CoA (generated by FAS-I) and malonyl-AcpM producing C 16 -AcpM (ref. 15). This product is channelled to KasA of the FAS-II system33. The FAS-II system is comprised of four enzymes acting in a consecutive cycle: KasA and KasB, condensing enzymes18, MabA, a keto-reductase34; HadBC, a dehydratase35; and InhA, an enoyl-reductase36. The FAS-II system enables fatty acid elongation leading to meromycolic acids (C 56 ), which are then condensed with C 26 -CoA (from FAS-I) by the polyketide synthase Pks13 (refs 37,38), followed by reduction, culminating in the production of mature mycolic acids39. The lipidomics experiments showing accumulation of FAMEs and depletion of MAMEs confirms that GSK3011724A specifically inhibits FAS-II and mycopic acid biosynthesis (Fig. 2a,b; Table 4; Supplementary Fig. 2).

Inhibitors of KasA have been reported in the literature14,40,41. Most notable among these is TLM, which is known to inhibit all three mycobacterial Kas enzymes: KasA, KasB and FabH14,15. Interestingly, GSK3011724A, unlike TLM and other Kas inhibitors, whether against *M. tuberculosis* or other bacteria (via FabH, FabB and FabF), displayed unique specificity, targeting only KasA (Fig. 2c). An explanation of this finding comes from close inspection of the GSK3011724A-KasA complex crystal structure, where specific changes in the acyl pockets of the related enzymes disfavour the unique binding mode of GSK3011724A (Figs 4 and 5). For example, the Gly200Arg and Pro201Thr changes from KasA to KasB no longer allow the indazole ring to sit favourably in the acyl pocket of KasB (Fig. 5f). This offers an explanation as to why the WGS data did not result in the identification of SNPs in
KasB and why the resistance mutations in KasA often map to points of variation amongst the Kas enzymes.

Importantly, GSK3011724A, despite representing an un-optimised screening hit, gives at least one order of magnitude greater potency relative to the most studied KasA inhibitor, TLM, and its favourable PK properties allowed for the critical in vivo experiments described in this work. The data obtained from murine assays (both acute and chronic infection) exemplify the credible potential of KasA inhibitors to give significant efficacy and, to our knowledge, offer the first in vivo validation of KasA as
as a drug target (Fig. 1d). Together with the promising drug-like profile of GSK3011724A, these data provide significant confidence for the future exploration of KasA as a drug target, inhibitors of which could become key players in the development of new anti-tubercular drugs and will be explored in a future publication.

Methods
Synthetic and characterization details for the compounds and the SPR experiment described herein can be found in Supplementary Methods. All animal studies were ethically approved and carried out in accordance with European Directive 2010/63/EU and the GSK Policy on the Care, Welfare and Treatment of Animals. The human biological samples were sourced ethically and their research use was in accordance with the terms of informed consent.

Assessment of acute and chronic efficacy in murine TB models. INH was purchased from Sigma-Aldrich and prepared freshly in distilled water. GSK3011724A was prepared freshly in 1% aqueous methocellose. The assessment of the chronic and acute efficacy murine TB models were performed using specific pathogen-free, 8–10-week-old female C57BL/6 mice purchased from Harlan Laboratories and allowed to acclimate for 1 week and kept under controlled conditions in a P3 high-security facility with unlimited sterile food and water. In the acute model1, mice were intratracheally infected with M. tuberculosis H37Rv wild-type (H37Rv WT) 100,000 c.f.u. for each mouse, and lungs harvested on day 9. GSK3011724A and INH were administered daily for 8 consecutive days, starting on day 1 after infection. In the chronic model2, mice (n = 2 mice at each dose level) were intratracheally infected with 100 c.f.u. for each mouse; INH or GSK3011724A was administered daily for 8 consecutive weeks, starting 6 weeks after infection. Lungs were harvested 24 h after the last administration in both assays. All lung lobes were aseptically removed, homogenized and frozen. Homogenates were unfiltered and plated in 10% OADC-7H11 medium supplemented with activated charcoal (0.4%) and grown for 18–25 days at 37°C. Non-linear fitting was performed with the dose–response data (log c.f.u. versus dose) and the dose in mg/kg10 that reduced lung bacterial burden by 99% with respect to untreated mice was estimated (ED90). Mice were supervised everyday under a protocol paying attention to weight loss, apparent good health (bristled hair and wounded skin) and behaviour (signs of aggressiveness or isolation). Animals were euthanized by CO2 inhalation.

MIC determination and resistant mutant generation. M. bovis BCG strain Pasteur and derivatives were cultured at 37°C and 3% CO2 in static liquid or solid medium. Liquid medium contained Middlebrook 7H9 (Difco) supplemented with 0.05% Tween-80, 10% (v/v) Middlebrook ADC and 0.25% (v/v) glycerol. Solid medium contained Middlebrook 7H11 agar (Difco) with 10% (v/v) Middlebrook OADC and 0.5% (v/v) glycerol. Where applicable, 25 µg ml−1 Kanamycin was added to the liquid or solid media to select for mycobacterial plasmids. The constructs pMV261, pMV261-Mt-kasA, pMV261-Mt-skasB, pMV261-Mt-skasC, pMV261-Mt- hadABC, pMV261-Mt-skasA and pMV261-Mt-mabA were electroimpaled into M. bovis BCG14-17. Wild-type M. bovis BCG electrocompetent cells were prepared by pelleting a mid-log culture and washing with decreasing volumes of ice-cold (0.5%) (v/v) glycerol. The cells were incubated on ice with 1 µg plasmid DNA before being transferred to a 0.1 cm electrode-gap electroporation cuvette and subjected to a single pulse of 1.8 kV. Cells were recovered in liquid media overnight at 37°C and selected on solid medium containing the appropriate antibiotic. The MIC of GSK3011724A was determined by plating 105, 104, 103 and 102 cells from a mid-log culture of M. bovis BCG on solid medium containing increasing concentrations of compound in a dose–response format. The MIC was defined as the concentration of compound that caused complete inhibition of bacterial growth. M. bovis BCG and M. tuberculosis spontaneous resistant mutants were generated by plating 104 cells from a mid-log phase culture on solid media containing either 5, 10 or 20 × MIC of GSK3011724A. Potentially resistant colonies were inculated into liquid media, cultured to mid-log growth phase, and selected on solid media containing 5 × MIC of GSK3011724A to confirm phenotypic resistance. The MIC of the resistant M. tuberculosis mutant strains against GSK3011724A was determined either using the MABA resazurin assay43 or by serial dilution and agar plating.

Sequencing of resistant mutants. Wild-type M. bovis BCG and the M. bovis BCG GSK3011724A-resistant mutants were characterized by WGS5,6, Briefly, purified genomic DNA was prepared for sequencing using the Nextera DNA Sample Preparation Kit (Illumina). The DNA library was purified and quantified using Agilent AMPure XP beads (Beckman Coulter Genomics) and QuantiFast PicoGreen dsDNA kit (Life Technologies), respectively. Fragment sizes were determined using an Agilent Technologies 2100 Bioanalyzer with a High Sensitivity DNA chip. Following the MiSeq preparation guide, the libraries were sequenced on a MiSeq Benchtop Sequencer using the MiSeq reagent Kit v2 × 300 cycles. Reads were aligned to the reference genome M. bovis BCG Pasteur 1173P2 (accession: NC_008769.1).

The kasA gene (Rv2245) from M. tuberculosis GSK3011724A-resistant mutants was sequenced specifically. The gene was amplified by PCR using 0.5 µM of the flanking primers 5’-ggagcaaggtggtccgaacggtt (forward) and 5’-gacgagcttctgtagtgag (reverse). The amplification was performed with 0.02 U of KOD Xpert Hot Start DNA Polymerase (Merk Millipore) and 0.4 mM each of dNTP. PCR conditions were: 2 min at 94°C, followed by 30 cycles of 98°C for 10 s, 66°C for 60 s and 68°C for 10 s. The PCR product was purified from the band of a 0.8% agarose gel (Lonkenm GTG Agarose G) and eluted with GeneJET PCR DNA Gel Band Purification Kit, GE Healthcare. The purified product was used for kasA automated sequencing (using the primers: Forward 1, cgaagtagctgggagca; Reverse 1, ctctcatagctggcgtcactcc; Forward 2, gtaagtagctgggagca; Reverse 2, cagatgtagctccgctagcct; Forward 3, gtctccgtagctcagcctcgct; Reverse 3, ctcctccgtagctcagcctcgctctc; Forward 4, tgcctccgtagctcagcctcgct; Reverse 5, gatcctccgtagctcagcctcgct). DNA sequencing reactions were performed with a BigDye terminator V3.1 cycle sequencing kit (Applied Biosystems).

Synthesis of FAMES and MAMES. The whole-cell effect of GSK3011724A was studied by treating M. bovis BCG cultures (10 ml) at an OD600nm of 0.4–0.6 with a dose-dependent increase in drug for 20 h before labelling using 1 µCi ml−1 [1,14C]sodium acetate (37 MBq, PerkinElmer) for a further 24 h at 37°C. The total FAMES and MAMES were extracted4,44,45. Briefly, cells were pelleted and incubated overnight in 2 ml of 5% tetrabutylammonium hydroxide at 100°C. The following day, 4 ml of dichloromethane was added with 300 µl of methylated methanol and 2 ml of water and mixed for 30 min. The reaction was centrifuged and the upper aqueous layer discarded. Water (3 ml) was added to the lower organic layer, mixed and centrifuged as before and repeated once more. The organic layer was evaporated to dryness and the methyl esters re-dissolved in diethyl-ether (4 ml) and transferred to a fresh tube. The diethyl-ether was evaporated and 200 µl of dichloromethane used to re-dissolve the extracted methyl esters. The total FAMES and MAMES were analysed using TLC (using equal Rf TLC and derivatized with Fast Blue R salt). Quantiﬁcation of labelled FAMES and MAMES was determined by excising spots directly from the TLC plates and subjecting them to scintillation counting using 10 ml of EcoScintA.

Extraction of cell wall-bound MAMES and lipids. M. bovis BCG was drug-treated and labelled as described above. The cell wall-bound MAMES, apolar and polar phospholipids were extracted and analysed46. Briefly, the cell pellet was extracted four times using 10 ml of chloroform:methanol:water (10:16:3, v/v/v).
retaining the delipidated cell pellet (for analysis of cell wall-bound mycoidic acids) and collecting the solvent extract sequentially, which was combined and dried. To the 2-propanol extract was added chloroform:methanol:water (2:3:4, v/v/v), followed by chloroform (1.75 ml) and water (0.75 ml), and the entire mixture centrifuged and the lower organic layer recovered. The lower organic layer was washed twice using chloroform:methanol:water (2:3:4, v/v/v) and an aliquot subjected to scintillation counting using 10 ml of EcoScintA. The total lipid extract was further partitioned between the phases arising from methanol:0.3% NaCl (2 ml, 100:10, v/v) and 2 ml of petroleum-ether (60–80°C). The entire contents were mixed on a blood rotor, centrifuged and the upper layer collected. The lower layer was re-extracted using 2 ml of petroleum-ether (60–80°C). The combined petroleum-ether layers were evaporated to afford the crude apolar lipids. To the lower organic layer, 2.3 ml of chloroform:methanol:0.3% NaCl (50:100:40, v/v/v) was added, followed by 750 µl of chloroform:methanol:0.3% NaCl (50:100:40) and a further 1.3 ml of chloroform and 1.3 ml 0.3% NaCl. The entire contents were mixed, centrifuged and the lower layer recovered and dried to afford the crude polar lipids. The apolar and polar lipids were resolved in chloroform:methanol (2:1, v/v) and an aliquot subjected to scintillation counting using 10 ml of EcoScintA. The total lipid extracts and apolar/polar lipids were analysed by TLC in the following solvent systems using equal counts (as stated) before being exposed to I2 vapours for detection.

Apolar lipids were resolved on three solvent systems A, first direction, petroleum-ether (60–80°C); ethyl acetate (98.2, v/v, thric); second direction, petroleum-ether (60–80°C); acetone (98.2, v/v, thric), second direction, toluene:acetone (100:3, v/v), second direction, toluene:acetone (95:5, v/v). Solvent system C: first direction, chloroform:methanol:water (65:25:5, v/v). The total lipid extract was analysed using chloroform:methanol:concentrated ammonium hydroxide (80:20:2, v/v) to reveal TDM and TMM. The recovered delipidated cells were used to analyse cell wall-bound mycoidic acids following release using 5% tetrabutylammonium hydroxide:water (30:70, v/v) as described above for total FAMEs and MAMEs. The recovered cell-wall bound MAMEs were analysed by TLC, using an equal aliquot (5%) and exposed to Kodak X-Omat film.

KasA activity assay. Recombinant FabD, holo-AcpM and C0-AcpM were overexpressed in C41 (DE3) Escherichia coli cells from pET28a-fabd and pET28a-acpm and purified45. Briefly, cells were resuspended in buffer (50 mM potassium phosphate, pH 7.5, 0.5 M NaCl and 10 mM (Acpm)/25 mM (FabD) imidazole) containing DNAase, Complete protease inhibitor-cocktail tablets (Roche) and 0.1% Triton X-100. Cells were disrupted by 6 passages through a French Press and the clarified lysate was loaded onto a pre-equilibrated (with buffer) Ni2+–charged 1 ml His-Trap column. A step gradient of imidazole (50–1000 mM) was used to wash and elute the recombinant protein. Recombinant AcpM was dialysed into 0.1 M Tris pH 7.5, 500 mM NaCl and loaded onto a column containing 1 ml of Ni-NTA (50 mg/ml) bound to Sepharose 6B in the flow through. Holo-AcpM was eluted with an increasing concentration of β-mercaptoethanol (5–100 mM). All purified enzymes were dialysed freshly against the same buffer without EDTA.

β-mercaptoethanol to a final volume of 89 µl was added and the reaction was incubated at 37°C for 2.5 h. The reaction was quenched with 2 ml of freshly prepared reducing solution: 5 mg ml–1 NaBH4 in 0.1 M K2HPO4, 0.4 M KCI, 30% (v/v) tetrahydrofuran. The reaction was incubated overnight at 37°C. The reduced β-ketocaproic product was extracted twice using 2 ml of water-saturated tolune, and the combined organic phase washed thrice using 2 ml of toluene-saturated water. The organic layer was transferred to a scintillation vial and dried. The radiolabelled product was quantified by liquid scintillation counting using 10 ml of EcoScintA.

Chemoproteomics. The chemoproteomic inhibition binding experiments were performed as previously described19,20. Briefly, sepharose beads were derivatized with 1, the GSK3011724A-tagged analogue, at 2 mM compound concentration. Beads were washed and equilibrated in lysis buffer (50 mM Tris-HCl pH 7.4, 0.4% Igepal-CA630, 1.5 mM MgCl2, 5% glycerol, 150 mM NaCl, 25 mM Na3VO4, 1 mM dithiothreitol (DTT) and one Complete EDTA-free protease inhibitor tablet (Roche). The beads were mixed with the protein sample and loaded onto a column in lysis buffer at 4°C for 1 h either with 0.1 ml (0.3 mg) M. bovis BCG extract or with 1 ml (5 mg) mixed HEK293/K562/Placenta extract, which was pre-incubated with compound or DMSO (vehicle control). Beads were transferred either to Filter plates (DuraDip (PVD membrane, Merck Millipore)) or to disposable columns (MoBio), washed extensively with lysis buffer and eluted with SDS sample buffer. Beads were alkylated, separated on 4–12% Bis-Tris NuPAGE (Life technologies) and stained with colloidal Coomassie. Gel lanes were cut into three slices and subjected to in-gel digest using LysC for 2 h and trypsin overnigh19. Digestion, labelling with TMT isobaric mass tags, peptide fractionation and mass spectrometric analyses were performed by the Marine Science Research Institute proteomics and lipid chromatography–tandem mass spectrometry (LC-MS/MS). The proteins.fasta file for M. bovis BCG was downloaded (11th May 2011) from http://genome.tdbdb.org/annotation/genome/tdbdb/MultiDownloads.html and supplemented with the sequences of bovine serum albumin, porcine trypsin and mouse trypsinogen. Decay versions of all proteins were created and added. The search database contained a total of 11,492 protein sequences, 50% forward, 50% reverse. Protein identification and quantification was performed37. Proteins identified with >1 unique peptide matches were considered for further data analysis. Apparent dissociation constants were determined by taking into account the protein depletion at the beads19. Raw data tables for all chemoproteomics experiments can be found in the Supplementary Data 1–6.

The M. bovis BCG extracts were prepared as follows: M. bovis BCG was cultured in 7H medium without glycerol and supplemented with 2% (w/v) glucose and 0.025% (v/v) glycerol at 37°C for 8–10 days to reach an OD600nm of 0.8–1.0. The culture was centrifuged and the bacterial cell wall was washed with PBS, 0.5% (w/v) glycerol at 4°C and the OD600nm reached 2.4. The culture was incubated for 20 h before harvesting the cells. The cell pellet was resuspended in 5 ml 1% (w/v) buffer in Buffer A (50 mM Tris, 500 mM NaCl, 10% (v/v) glycerol, 2 mM EDTA, pH 8.5, with 1 mg/ml -lysozyme, Protease inhibitor-cocktail set III (Sigma) and 10 µl Benzonase). The sample was lysed by sonication on ice for 10 min (10 x, 10 s off). The lysate was centrifuged at 18,000 rpm, 4°C for 30 min. The supernatant was loaded onto a pre-equilibrated Ni2+–charged 10 ml HiTrap-Trap column. The column was washed back to baseline with Buffer A and the protein was eluted using a linear gradient over 20 column volumes using Buffer B (Buffer A containing 300 mM imidazole). The protein was further purified by gel filtration in Buffer A using a Superdex 200 column.

KasA protein production and structure determination. The overexpression plasmid, pET28a-kasA, was transformed into E. coli BL21 (DE3) cells. A single colony was inoculated to inoculate 10 ml of broth containing 50 mg/ml ampicillin, 250 µg/ml kanamycin and 1% (v/v) glycerol. Cells were cultured overnight at 30°C, 240 r.p.m. A 201 µl Biolafite fermenter containing 151 of Overnight Express Instant TB (Merck), 1% (v/v) glycerol, 50 µg/ml - Kanamycin and 20 ml of antifoam (DC1520) was inoculated with the overnight culture to 2% (v/v). The culture was grown at 37°C, expressed for 21 min – 1 air exchange. The reduced kasA was purified by Ni2+–charged 10 ml HiTrap-Trap column. The column was washed back to baseline with Buffer A and the protein was eluted using a linear gradient over 20 column volumes using Buffer B (Buffer A containing 300 mM imidazole). The protein was further purified by gel filtration in Buffer A using a Superdex 200 column.

KasA was co-crystallized with GS30011724A protein using 10.66 mg/ml 1 and ligand at a nominal concentration of 30 mM in 100–150 ml sitting drops at 20°C. The well solution was 8% (w/w) isopropanol, 0.2 NaCl, 10 mM tris(2-carboxyethyl)phosphine (TCEP). Crystals were cryoprotected using 30% (v/v) glycerol before flash freezing in liquid nitrogen. Data from a single crystal was collected at the Diamond Synchrotron Radiation Facility (iod) and processed in P31; to 2.13 Å using XDS (within AUTOPROC (Global Phasing Limited) and AIMLESS). A molecular replacement solution was determined with a previously icoohouse of 40 using Phaser40. The P31 cell (x = 90°, y = 90°, z = 120°) was a = b = 77.338 Å, c = 147.675 Å) has two molecules in the asymmetric unit that form a dimer. Model building and refinement of the KasA structures was carried out using alternating rounds of COOT for manual model building and REFMAC for maximum likelihood refinement via CCP4 (ref. 33). As the data was measured in detector space, TWIN refinement was used with the refined twin fraction being 39%. A clear difference in density for GS30011724A, and also for a PEG-like molecule was present in both chains in the dimer.
PEG was not explicitly added to the wells, this was present in adjacent crystallization conditions and we believe there may be trace PEG present. Alternatively, the linear molecule may be residual lipid present in the protein, although apo-structures do not contain this lipid and the protein is crystalized in the closed conformation even in PEG conditions. A stereo diagram is displayed in Supplementary Fig. 6. Statistics for the data collection and refined co-ordinates are given in Table 5.

Data availability. The atomic co-ordinates and structure factors reported in this paper have been deposited in the Protein Data Bank with the code 5DL8. Data is available upon request from the corresponding authors.

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Author contributions

Conceived and designed the experiments: K.A.A., S.G-D., C.C., J.R., M.J.R-L., J.L., M.B., L.A.B., M.C.I., N.C., A.M., M.S.M-M., C.S., G.D., L.B., D.B., R.H.B. Performed the experiments: K.A.A., S.G-D., J.A.G.C., M.N., A.S., A.A., P.H., C.S., M.A., S.S.G., R.C., C.C., S.G.D., L.K., M.J.R-L., J.R., R.H.B. Analysed the data: K.A.A., C.C., J.R., M.J.R-L., M.B., N.J.L., S.G-D., M.S.M-M., E.J-N., A.M., M.C.I., N.C., D.B., L.B., G.S.B., R.H.B. Wrote the paper: K.A.A., L.B., S.G-D., G.D., J.A.G.C., P.J.M., C.C., G.S.B., R.H.B.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

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