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A pilot study of re-purposing drugs to treat koalas with chlamydia

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The koala (*Phascolarctos cinereus*) is an endangered species in parts of Australia, in part due to chlamydial infections. Treatment is difficult due to the hepatic metabolism of the koala, and the critical reliance on a gut microbiota for survival. This study aimed to identify new compounds for treatment of *Chlamydia* infections by screening a drug re-purposing library. Screening was conducted using an in vitro cell culture model prior to in vivo mouse infection model testing of two candidates identified from the in vitro screen. One lead, bisoprolol fumarate, showed an impact on chlamydial infection and burden in vitro and in vivo. Whilst the mechanism of action may not support progressing this lead further, the approach to screening the library and list of candidates may enable identification of other new koala treatments. This study demonstrates the potential to apply drug re-purposing to koala treatment and presents a list of candidates that could be explored further.

Keywords Antibiotic, Lipid, Fatty acids, Infection

Abbreviations

h PI	Hours post infection
MOI	Multiplicity of infection
IFU	Inclusion forming units

Australia's iconic native marsupial species, the koala (*Phascolarctos cinereus*), is declining in population in large regions due to habitat loss, habitat fragmentation, and disease[1]. The koala population is now estimated to be approximately 330 000, from an estimated peak population of millions[1]. Koala were added to the Australian Environmental Protection Biodiversity Conservation Act in 2012[2]for populations in the northern half of Australia. Koala population stability is a priority for both preservation of the species but also the economic value of the associated tourism industry, estimated at up to $3.2 billion Australian dollars[3]. One contributing factor to the decline is chlamydiosis caused by infection with the obligate intracellular bacterial pathogen, Chlamydia[4]. Chlamydia are obligate intracellular Gram-negative bacterial pathogens. The organism undergoes a bi-phasic developmental cycle, consisting of extracellular infectious forms that are non-replicative (elementary bodies). A second intracellular, replicative forms (reticulate bodies, found inside specialized intracellular vacuoles) that undergo multiple rounds of replication and eventually re-differentiate into the infectious form[5,6]. *Chlamydia* (*C.*) *pecorum* has been reported in many mainland koala populations[4]. There have been previous reports of *Chlamydia* (C.) *pneumoniae*infections in koala but these seem to be isolated events (reviewed[7],). *C. pecorum*infections in koala share many hallmarks of the biology and disease presentation that have been reported for chlamydial diseases in other animal hosts, notably ocular and urogenital diseases (reviewed[8],). In the case of ocular disease, infection is within the epithelia of the conjunctiva of the eye, which leads to chronic conjunctivitis and keratoconjunctivitis, which can result in corneal scarring and may progress to blindness[9]. Reproductive tract infection and symptomatic presentations have been detected throughout the male and female reproductive tract with inflammation, scarring and infertility resulting from the tissue damage[9]. Urinary disease in the koala can impact the urethra, bladder, ureters and kidneys resulting in urethritis, cystitis, urethritis, or pyelonephritis. These are painful and can lead to urinary incontinence. This incontinence results in visible urine staining of the fur in the rump region, referred to as 'wet-bottom' [9]. The infection and disease prevalence varies by population[7].

Koalas are specialized Eucalyptus folivores who exhibit unique physiological, reproductive and dietary characteristics, including the ability to ingest and metabolize toxic plant metabolites such as phenolic compounds and terpenes[10]. The unusual metabolism results in limited efficacy of antibiotic treatment. As their

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efficient hepatic metabolism has been proposed to increase the rate of elimination of some therapeutic drugs[11]. A particular concern with the treatment of the koala is that antibiotic-induced dysbiosis of the gut microbiome can be fatal. This makes antibiotic approaches to treat infected koalas difficult to test. Further, some antibiotic treatments can be ineffective in clearing chlamydial infections of the lower genital tract[12]. Currently the most effective and commonly used antibiotic therapy for koalas with urinary-genital tract infections is systemically administered chloramphenicol (chloramphenicol 150; Delvet, Seven Hills, NSW, Australia)[13]. This antibiotic has been observed clinically in koalas to have fewer detrimental effects on their specialized gut microbiota, compared to other medications[13]. However, this treatment does not always effectively clear infections, even after lengthy durations of treatment. Whilst chloramphenicol is the most commonly used antibiotic, with a 60 mg/kg dosage for 14–28 days working well (95% successful treatment[14] on a subset of koalas with a positive prognosis, it has severe side effects including caeco-colic dysbiosis (gut microbiome impacts), and bone marrow depression[14]. Enrofloxacin has proven ineffective with treatment failure observed in animals, and laboratory minimum inhibitory concentration (MIC) testing, establishing susceptibility is higher than the conventional dosage regimen[15,16]. Doxycycline, florfenicol, and penicillin G have all also been tested in vivo or in vitro with little evidence to support proceeding with these antibiotics (reviewed, [7]). Vaccination has been explored[17,18] and shows promising indications. The vaccine has not yet been widely implemented. Overall, additional therapeutic approaches are needed to treat chlamydia infections in koala. Here a drug re-purposing library was screened to identify new possible treatments for koalas with chlamydial infection[19].

Methods

Study design and in vitro screening model

An in vitro screening study was conducted to examine a drug re-purposing library (Sellekchem, Cat. No L1300) for compounds effective against *C. pecorum*. Leads identified during the in vitro screening were progressed to an in vivo model. A selection process was followed as outlined in Fig. 1A. The first step was to conduct a single dose high throughput screen of the library of 3065 compounds. This selection round used a stringent threshold of 0% infection (no detectable inclusions after drug treatment) to shortlist only the most promising candidates to progress to the next stage. This screen was conducted in vitro on McCoy B (source ATCC: CRL-1696) cell cultures infected with *C. pecorum* at a multiplicity of infection of 1.0 (MOI), with the drugs added at 4 h post infection (h PI) at 200 µM. Cultures were fixed and screened for visible inclusions at 44 h PI, consistent with past protocols[20,21]. The strain previously referred to as *C. pecorum* G was used in this study[22]. The fixed cell cultures were screened in 96 well plates using the INCEL high content analyser (GE Healthcare). Immunocytochemistry for HtrA, alpha-tubulin, with DAPI to stain the nucleus was conducted to enable detection of inclusions[20,21]. Analysis and counting of the inclusions present with visible host cells (controls to ensure the monolayer was not impacted) was conducted using an imageJ algorithm in FIJI[23]. 43 candidates were selected in this first screening round with the basis that no inclusions but clear host cell monolayer consistent with the untreated wells was maintained. These candidates were then screened with a dose series. The dose series experiment consisted of a cell culture infection model, with the candidate drugs added at 4 h PI, and visible inclusions determined at 44 h PI. The dose series used was 12.5, 25, 50, 100, 150, 200 µM. Candidates that resulted in a dose responsive reduction in inclusions and were effective at lower doses were selected. 24 candidates were shortlisted from the second stage of the screen, these are listed in Supplementary Table S1 according to the selection criteria listed below. Combined, this screening and selection process resulted in six candidates any of which could have potentially progressed to further investigation, as listed in Supplementary Table S1. Two candidates were selected to be examined further using the in vivo model. The in vivo model is conducted with the mouse strain of chlamydia (*C. muridarum*). To confirm activity against this strain the in vitro protocol (as outlined above) was repeated on the selected candidates with *C. muridarum* culture. *C. muridarum* (strain: Weiss) infections to McCoy B cells were treated with a dose series of the two lead compounds and monitored for impact on the inclusions. This in vitro method was as outlined above.

Selection criteria

To determine which candidates to test using the in vivo model a set of criteria was determined by the authorship team. The criteria included eliminating established antibiotics or antimicrobials as these are likely to risks of gut microbiome dysbiosis severe to koala. Hormone based drugs were eliminated from further consideration for the following reasons. Hormonal therapies have a range of impacts on chlamydial biology[24,25]. These impacts are transient in nature, could involve induction of chlamydia persistence[24,25]. This means that hormones are unlikely to effectively clear the bacterial infection. In the context of chlamydia, persistence is a third form of the developmental cycle that arises under unfavourable conditions, as it is morphologically distinct, and is a non-culturable (e.g. not infectious) but is viable and can restore to an infectious form[26]. Previously reported adverse toxicity or severe side effects identified in past literature or product information sheets was also used to rule out drugs from further analysis (Supplementary materials Table S2). Such side effects may be tolerable for treatments for serious and potentially fatal diseases like cancer, but the authors agree they would not be appropriate to treat chlamydial infections in koalas. The shortlisting of candidates from each step considered all of these selection criteria. Combined, these criteria with the in vitro data showing dose dependent reduction in inclusion vacuoles was used to determine candidates which all could have progressed for further investigation in vivo. Candidates representing distinct mechanisms of action and both had what the authors considered to be the least documented adverse impacts were selected to progress.

In vivo model

The in vivo study was conducted as previously described on 6-week-old progesterone synchronised female mice, BALBc, sourced from the Animal Resource Centre Australia[20,21]. The study design was a case-control study to

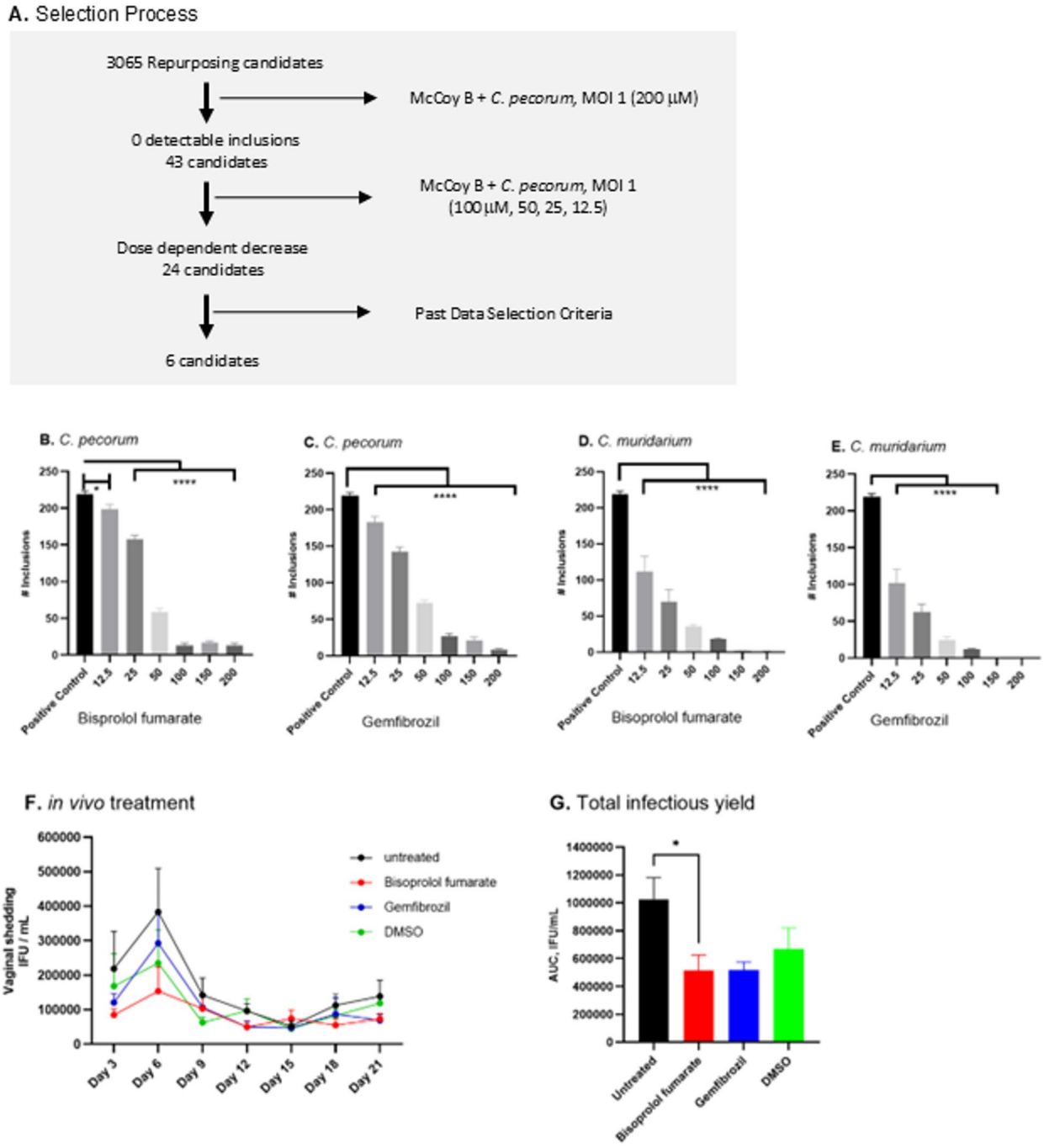

Fig. 1. **A**. Selection protocol to identify potential candidates in the drug repurposing library. The figure shows a summary of the selection protocol that was followed to identify candidates from the re-purposing drug library. **B-E:** Anti-chlamydial in vitro efficacy of the candidates (bisoprolol fumarate and gemfibrozil, x axis dose series in µM). Relative to positive controls that were untreated and 20% DMSO: Saline controls. *C. pecorum* and *C. muridarum* was tested in McCoy B cell culture and % inclusions per field of view assessed. **F.** in vivo model showing vaginal shedding of infectious chlamydia with intraperitoneal injections of the candidates. Relative to positive controls that were untreated and 20% DMSO: Saline controls. **G.** Assessment of total infectious yield for statistical significance (Area under the curve (AUC), y axis), and treatment conditions indicated on the x axis.

monitor response to treatment. The sample size was estimated based on previous published experiments with 10 mice included per group[27,28]. There was no randomisation or blinding in the study, all investigators were aware of the different treatments at use. Treatment and vehicle control animals were monitored and animal welfare checks were routinely implemented. Purified *C. muridarum* (strain: Weiss) was administered (5×10^4 IFU/20 µL of SPG) into the vaginal vault to initiate the infection. Intraperitoneal injections of bisoprolol fumarate

or gemfibrozil (Sigma Aldrich) were administered daily (5 mg/kg/day; for day 2–10 of the mouse model). Intravaginal lavage samples were collected in SPG and analysed for shedding of viable chlamydia, as previously reported[20] (Fig. 1F, G). Statistical analysis was performed using the Graph-Pad Prism 9 software (Graph-Pad, CA, USA). The total infectious units shed from the animals were analysed by converting the data in Fig. 1F to an 'area under the curve' as has been previously reported for this model[29]. The total area under the curve for each treatment condition was compared using ordinary one-way ANOVA and Bonferroni's multiple comparisons test relative to the untreated control (Fig. 1G). The in vitro experimental data was also tested using this software, dose responses were compared using ordinary one-way ANOVA with Bonferroni's multiple comparisons test to compare each dose to control (Fig. 1B and E). All animal experiments were conducted with the approval from the University of Technology Sydney Animal Care and Ethics Committee (ACEC ETH22-7172) and in accordance with the guidelines described by the Australian National Health and Medical Research council code of conduct for animals. The study was conducted and is reported in accordance with the ARRIVE guidelines (https://arriveguidelines.org/).

Results

The screening protocol step described in the Materials and Methods (outlined Fig. 1A) resulted in a shortlist of 24 candidates that showed a high anti-chlamydial impact at 200 µM. These candidates also showed a dose responsive reduction in inclusion vacuoles during an experiment to determine the impact of the drug in a a dose series in cell culture (Supplemental Material, Table S1). The candidates identified included many different compound types. The screening identified known antibiotics, and hormone-based products, both of which are expected to impact chlamydial growth. Six candidates were progressed from this list for further investigation in this study based on the selection criteria outlined in the Materials and Methods section (Supplemental Material, Table S1). These candidates were bisoprolol fumarate, carvedilol, gemfibrozil, ivacaftor (VX-770), tiagabine, and zafirlukast. These candidates, outlined in Supplementary Table S1, had a range of primary treatment applications and drug mechanisms. 2 candidates were selected to test using the in vivo model (gemfibrozil and bisoprolol fumarate), as outlined in the selection criteria in the materials and methods section.

The in vivo mouse model uses *C. muridarum*, so we confirmed in vitro activity against *C. pecorum* and *C. muridarum* (Fig. 1B-E). Both candidates showed a greater impact on reduction of inclusions for *C. muridarum* in McCoy B cell culture than C. *pecorum*, with dose dependent reductions in chlamydial inclusions apparent.

The mouse model of in vivo intravaginal infection with *C. muridarum* was conducted using intraperitoneal treatment with both drugs. The in vivo study identified that bisoprolol fumarate (shown in Fig. 1G) significantly reduced the burden of viable chlamydial infection in the mouse model. The clearance observed was not as effective as that reported with antibiotic therapy such as azithromycin[30]. Gemfibrozil did not have a notable effect on the infection in this in vivo model.

Discussion

Here we screened a drug re-purposing library in vitro and identified a range of expected and new compounds that impacted the formation of visible inclusions during a chlamydial cell culture infection model. We have provided shortlisted candidates from each stage of the screening process as Supplementary Table S1, so other investigators can progress any compounds of interest. The identification of known antibiotics in the screening process validates that the approach used was effective. The study is intended only as a pilot study to demonstrate the process and provide a list of candidates that could be explored further. Nonetheless, the screening protocol we implemented was effective to identify new candidates that could be further explored to treat koalas with chlamydial infections.

It is worth noting a comprehensive approach using a series of computational and experimental approaches has been recently published to identify novel candidates for chlamydial treatment[31]. This protocol used a computational approach to screen an electronic library of compounds, whereas here we used an existing library of compounds specifically available for re-purposing studies. The recently published protocol used a somewhat similar approach of screening for visible inclusions whilst checking for host cells to be maintained, although it used a high throughput approach and multiple doses were tested rather than the protocol here of shortlisting with a high threshold for single dose to select candidates to complete a dose series[31]. A further unique feature of the recently published work is the in vitro comparison of the drug efficacy against multiple chlamydial species. This protocol is an exciting contribution to the field, and interestingly a fatty acid synthesis protein was one of the discoveries of the possible target candidates for future drug development in this comprehensive process[31].

One of the limitations in our study was to progress only two compounds to the mouse model. It may be that other candidates in the short list will have higher efficacy against chlamydia in the mouse and other in vivo contexts, but only two compounds were elected to be screened for the purpose of this study. Another limitation of the study is the selection of which two candidates to proceed. The authors shortlisted based on the range of criteria described in the methods, but it is certainly possible that any of the final six candidates were worthy of in vivo investigation. There is no laboratory animal model for *C. pecorum* infection and treatment studies that adequately mimic the biology of the koala. The use of *C. muridarum* and the mouse model can only provide limited insights into the likely efficacy of these drugs against *C. pecorum* and the koala and this should be considered a limitation. The use of multiple species and host cell lines in the recently presented larger study presents possible approaches to address such limitations in future[31].

To select candidates to pursue further, an analysis of available data relating to each candidate was conducted. This included reviewing the literature, product information sheets where available, and product reports available in the public domain. The scope of this review was to understand possible mechanisms and to rule out those with previously reported adverse toxicity, side effects, hormonal therapies and established antibiotics or antimicrobials.

Hormonal therapies were excluded because hormone concentration changes are known to impact chlamydial growth in vitro, but this is likely to be transient or through the induction of persistence[26], so we elected to exclude these from further investigation for this study. We did not feel there was value in identification of known antibiotics or antimicrobials, given the potential for fatal impacts on the gut microbiome. Severe toxicity or adverse impacts that may be considered acceptable in some contexts would not be appropriate in the application to koala treatment for infection.

The two candidates are well known and long used human therapeutics. Bisprolol fumerate is a widely used beta-blocker (beta 1 adrenic receptor blocker) to treat hypertension and used in heart failure[32]. As McCoy B cell lines are a unique cell derived from a fibroblast cell type, we do not expect that this cell type would express beta-adrenoceptor, the target of bisoprolol fumarate. Thus, one explanation for this compound impacting chlamydial growth might be previously reported off target impacts on membrane fluidity that have been detected for other beta blockers[33]. The mechanism relating to the membrane fluidity is not fully elucidated but could well impact the chlamydial inclusion membrane and hence chlamydial infectivity or growth progress. Gemfibrozil was not significantly impactful on chlamydia infection during the in vivo study. This drug impacts on cellular lipid metabolism, specifically depleting intracellular fatty acid stores, via PPAR-alpha receptors[34], a mechanism anticipated to directly impact on McCoy B cells as PPAR isoforms are expressed on fibroblasts. It is well established that the pathogen uses host cell fatty acids and lipids, so the identification of gemfibrozil could be considered to be consistent with literature[35] relating to biological resources required by chlamydia. There are no prior reports to our knowledge of either of these two drugs having an impact on chlamydial growth. Genomic analysis does show differences between *C. trachomatis* (where much of the biological work has been conducted) and *C. pecorum*[36]. There are no biological or pathogenesis studies yet publish that explore fatty acid and lipid sequestering from host cells for *C. pecorum*. Therefore, it is unknown if the same host cell dependencies for lipids and fatty acids exist between the two species. The work here included only in vitro analysis on *C. pecorum*. There is no established koala cell line available for chlamydia culture, so it was also in a mouse model cell line commonly used in the chlamydia field. This needs to be considered in light of the data as the drugs could be acting indirectly via a host cell target. Additionally, the in vivo model study was conducted using *C. muridarum*. This is an important consideration as phylogenetically *C. muridarum* and *C. trachomatis* are more closely related than *C. pecorum* is to either of these two species[37].

One approach to future applications of the compounds identified here could be combination therapies to increase the efficacy[38]. This could include combinations of novel leads, or combinations with lower doses of known antibiotics, to reduce the broad impact of the antibiotics on the koala gut microbiota, whilst still reducing the chlamydial infection. This might be worth further investigations in future. Overall, this pilot study demonstrates the potential for re-purposed therapies to treat koala chlamydial infections.

Data availability

All data and materials are within the manuscript and Supplementary Table S1.

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Author contributions

WMH, VM, JW, JT, CF, HN Conception or design of the work; CF, HN, SLQ, BF acquisition, analysis, and interpretation of data; all authors drafted, revised and reviewed the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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