**APPLICATION FORM for a target-directed screening programme**

Template application form for a screening Programme funded by the IMI-European Lead Factory project.

Please use this form to prepare your application. The review committee will judge each proposal using the criteria described in the Guidance for applicants. Please include all available information that relates to these topics and include literature references and details of unpublished results to support statements made.

In case of any questions, please contact the Programme Office at [Programme@europeanleadfactory.eu](mailto:Programme@europeanleadfactory.eu)

PROGRAMMME TITLE

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| Under-explored Antibiotic target Drug Discovery: New Hits for the Mur Ligases – Multi-targeting to Evade Resistance |

ACCEPTATION OF TERMS AND CONDITIONS

*Please make sure to involve your Technology Transfer Office, Legal and/or IP Department in this.*

x My institute or organization is eligible for receiving IMI JU funding.

(see point 3 on page 3 of the [IMI Rules for Participation Document](https://www.imi.europa.eu/sites/default/files/uploads/documents/About-IMI/imi-funding-model/IMI2_provisions_for_participating_in_IMI2_actions.pdf))

x I have attached a signed Statement of Interest for a screening programme proposal within the framework of the European Lead Factory.

x I agree that all relevant Background and Third Party limitations are indicated under part 8 below.

1. **PRINCIPAL INVESTIGATOR**

**Name** : Professor Matthew Todd

**Position** : Chair of Drug Discovery

**Institution** : University College London

**Department** : School of Pharmacy

**Address** : 29-39 Brunswick Square, London WC1N 1AX

**Phone** : +44 207 753 5568

**E-Mail** : matthew.todd@ucl.ac.uk

**Website** : https://www.ucl.ac.uk/pharmacy/people/professor-matthew-todd

*Credentials*

**Please provide evidence of your ability as a researcher in this field.** *(Max. of 2500 characters)*

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| Todd’s research focuses on the development of new methods for the synthesis of bioactive small molecules, particularly for the treatment of infectious diseases.  He has pioneered the application of open source concepts to these fields, in which all data and ideas are freely shared, anyone may participate and there is no patent protection of outputs. Todd brings extensive experience in how to create the organic molecules central to this proposal, how to structure a team to ensure timely delivery of scientific results, and how to operate using open source principles to ensure successful leveraging of the global scientific community.  The first open source project (with WHO, 2009–11) coordinated a distributed network of scientists from academia and industry in the successful discovery of a new route to the active enantiomer of praziquantel, the world’s most widely used anthelmintic (PMID 21941234). For this achievement he was awarded the 2011 New South Wales Scientist of the Year award in the Emerging Research category. Todd since founded, and now leads, the Open Source Malaria (OSM) consortium that carries out hit-to-lead drug discovery and has received contributions from around 300 scientists worldwide (PMID 27800551). In 2013 OSM was awarded a $30K Wellcome Trust/Google/PLoS Accelerating Science Award, from more than 200 entrants. He has convened and chaired several conferences on the economics and practicality of open source drug discovery (WHO, Geneva, 2013; Bellagio, Italy, 2014; Marburg, Germany, 2015; Paris, 2019) which led to the first outline of how an open source pharmaceutical industry can work (PMID 28419094). These activities led to the 2015 award from Tata Trusts of $3M to MHT and four colleagues to start the Open Source Pharma Foundation that will provide in-kind support for any open source pharmaceutical project (first clinical trial commenced: CTRI/2018/01/011176). In 2020 he co-founded the UK company M4ID that will adopt a new business model to take public domain, patent-free, infectious disease drug candidates through to market.  *Last 5 years*: research funding support ca £4M, publications: 25. Current h index 33. |

**Please indicate what expertise you or your organisation has in drug discovery and/or drug development, and/or whether you intend to collaborate with other organizations (please specify) on developing hit compounds.***(Max. of 500 characters)*

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| There is extensive expertise in drug discovery and development at UCL, with basic research activities across Pharmacy, Bioscience, Chemistry, Pharmacology, the Wolfson Institute for Biomedical Research, the Alzheimer’s Drug Discovery Initiative and the Crick Institute. Extensive clinical drug research takes place throughout UCL and its associated hospitals. Underpinning this research expertise is support from the Translational Research Office, which maintains extensive contacts with local industry. |

**How did you learn about the opportunities offered by the European Lead Factory?** *(Max. of 500 characters)*

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| Multiple, previous interactions with the ELF team (e.g. 2021 Jon de Vlieger, 2014-15 Dimitrios Tzalis). |

1. COLLABORATOR(S)

*(Not required, but fill out if the programme is a collaborative application)*

*Collaborator 1*

**Name** : Professor Christopher Dowson

**Position** : Professor of Microbiology

**Institution** : University of Warwick

**Department** : School of Life Sciences

**Address** : Gibbet Hill Rd

**Phone** : 02476523534

**E-Mail** : c.g.dowson@warwick.ac.uk

**Website** : https://warwick.ac.uk/fac/sci/lifesci/people/cdowson/

*Collaborator 2*

**Name** : Professor Frank von Delft

**Position** : Professor of Structural Chemical Biology

**Institution** : University of Oxford

**Department** : Nuffield Department of Medicine

**Address** : Old Road Campus Research Building, Old Road Campus, Headington, Oxford OX3 7BN

**Phone** : 01235778997

**E-Mail** : frank.vondelft@cmd.ox.ac.uk

**Website** : https://www.ndm.ox.ac.uk/team/frank-von-delft

**Please provide the reason for collaboration regarding this proposal and the expertise each collaborator will bring.** *(Max. of 500 characters)*

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| This proposal is being submitted by a consortium of researchers, Open Source Antibiotics, that includes the three named applicants above as well as additional expertise. Todd oversees the project and guides the synthetic chemistry, Dowson provides microbiology expertise and enzymatic assays, von Delft provides expertise from the Diamond Light Source in target-based drug discovery and crystallography. Additional team members bring additional expertise in cheminformatics and Mur ligase biology. |

1. **SCREENING PROGRAMME**

**Programme title / purpose**:

Under-explored Antibiotic Drug Discovery: New Hits for the Mur Ligases.

**Programme abstract** *(Max. of 750 characters)*

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| The hits from this ELF screen will lead to a new programme in antibiotic drug discovery targeted at inhibition of the essential bacterial Mur ligases. This group of five enzymes assembles the pentapeptide that is cross-linked to form the cell wall. We have already begun a project, driven by open science, to explore currently available starting points derived from i) a fragment screen, ii) a “parked” industry project and iii) a *pro bono* industry contribution. We now seek new starting points from unexplored libraries. Our team is particularly well placed to prosecute lead optimisation chemistry and biology. Our open source approach has already solicited many contributions, and we have established a novel business plan for translation. |

*Target protein and type of hit*

**Please specify the target protein according to the uniform identifier (**www.uniprot.org**) and the gene ID**

Target name and abbreviation : UDP-N-acetylmuramoyl-alanine: D-glutamate ligase, MurD

UDP-N-acetylmuramoyl-L-alanyl-D-glutamate-: 2,6-diaminopimelate ligase, MurE

*E.coli* MurD/E. (back up P. aeruginosa MurD/E)

UniProt IDs : P14900

UniProtKB - A0A061YL42 (A0A061YL42\_ECOLX)

(and UniProtKB - Q9HVZ9 (MURD\_PSEAE) MurD

UniProtKB - A0A072ZPH5 (A0A072ZPH5\_PSEAI) murE

Gene ID : murD, murE

**Please specify the requested type of hit (agonist/activator, antagonist/inhibitor, allosteric modulator), only one can be defined** : antagonist/inhibitor

*Major Disease areas*

**The target is involved in the following disease areas:**

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| --- | --- | --- |
|  | **YES** | **NO** |
| Oncology |  | X |
| Inflammation & Immunology |  | X |
| Respiratory Diseases | X |  |
| CNS & Neurology |  | X |
| Infectious Diseases | X |  |
| Cardiovascular Diseases and Hematology |  | X |
| Metabolic Diseases |  | X |
| Gastrointestinal diseases |  | X |

Others *(Please specify)* :

Neglected Tropical Diseases *(Please specify)* :

Orphan Diseases *(Please specify)* :

*Scientific background*

**Please describe the scientific background of the screening programme, including:**

* Function and relevance of the target
* Scientific challenges and innovative approach
* Disease area and the medical need the programme will address

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| Peptidoglycan synthesis depends upon the generation of a pentapeptide component generated by the combined activities of a series of mechanistically and structurally related ATP-dependent ligases. These enzymes are MurC, D, E and F and respectively append L-alanine, D-glutamate, L-Lysine (or DL diaminopimelic acid) and D-alanyl-D-alanine to UDP-N-acetyl-muramic acid to form the cytoplasmic peptidoglycan precursor, UDP-MurNAc pentapeptide. The ATP binding sites of these enzymes as revealed by X-ray crystallography are essentially super-imposable and as such, strongly suggest the feasibility of design of molecules that could bring about inhibition of peptidoglycan synthesis through **inhibition of multiple essential Mur ligases** via targeting their ATP-binding sites. Achievement of this goal would vastly reduce the potential for resistance to an antibiotic with this capability. We have recently built a consortium with broad expertise in the biology relevant to these targets and have commenced several chemical series with potential for delivery of novel antibiotics. We seek from the ELF novel hit classes that can be incorporated into the consortium, derived from unexplored chemical matter. Progression of hits within the open source community will yield a selection of inhibitors that target early peptidoglycan synthesis of WHO critical priority pathogens Enterobacteriaceae (including E. coli and Klebsiella pneumoniae), Pseudomonas aeruginosa and Acinetobacter baumanii. This will then provide the scientific basis for applications for further funding in order progress these molecules further. Downstream work could then address the related Mur ligases found in *Mycobacterium tuberculosis*, extending our therapeutic range to respiratory disease (potential collaborating PIs include the Perdih (*Journal of Computer-Aided Molecular Design* **2015,** *29*, 541–560) and Zega (*Journal of Enzyme Inhibition and Medicinal Chemistry* **2019,** *34*, 1010–1017) groups in Slovenia and the Blanot group in France (*Biochimie* **2010,** *92*, 1793–1800).  As the success rate of the drug development pipeline remains low, there is an urgent need for novel antibiotics to circumvent the threat from AMR, and our ability to treat common infections. Of most significance are medicines acting via novel mechanisms of action, such as through inhibition of the Mur ligases. Yet, despite recent creative interventions towards push and pull incentives such as the AMR Action Fund and the PASTEUR Initiative, there is a persistent economic challenge to developing new antibiotics in the private sector that has led to many major companies leaving the area entirely, or the bankruptcy of smaller companies that were selling approved medicines; this has led many to question whether a traditional market for antibiotics exists at all. Such challenges stimulated us to adopt an entirely different approach, one based on open research, through a consortium we have founded in 2020, Open Source Antibiotics. The research and development takes place in a way that allows for a large-scale unrestricted public collaboration, with a financial model underpinning later development of compounds (see below). |

*Target validation*

**Please describe the validation of the target and the screening approach in the disease area of interest.**

The attached guidance document indicates the type of questions the reviewers would like answers to, including:

* How has the target been validated?
* Where is the target expressed?
* Is the genotype-to-phenotype relationship understood for the target?
* Are supportive data available in predictive preclinical models or humans?
* Which target related side effects can be expected?

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| The peptidoglycan biosynthesis pathway represents a universal, well validated target for antibacterial drug discovery [Williams, K.J. and Bax R.P. (2009) Curr. Op. Invest. Drugs 10:157-163; Zoeiby, A., Sanschagrin and, F. and Levesque, R.C. (2003). Mol. Microbiol. 47, 1–12.]. The cytoplasmic phase of this pathway involves the assembly of UDP-MurNAc-pentapeptide within the bacterial cytoplasm, prior to export across the cytoplasmic membrane. MurD and E are essential ATP dependant UDP-MurNAc amino acid ligases (UDP-MurNAcLAla – D-Glu ligase and UDP-MurNAcLAlaDGlu – DAP ligase, involved in the construction of the di and tri peptide prior to the final addition of D-Ala-D-Ala by MurF to form the final UDP-MurNAc pentapeptide required for export and peptidoglycan synthesis. These enzymes presents themselves as an important unaltered target class in multiply resistant organisms.  **It is clear from the literature that Mur enzymes are essential when inhibited in bacteria**. There are a number of synthetic inhibitors targeting the steps catalysed by Mur ligases that have antibacterial potency against Gram positive and negative organisms. |

*Chemical tractability*

**Please describe the drugability of the target, the likelihood of finding useful hits, and the existing chemical matter.**

The attached guidance document indicates the type of questions the reviewers would like answers to. Note that the experiments for hit characterisation and the hit-to-lead strategy can be detailed in part 5 and 6 respectively.

* Have any hits with proven activity for this target been found before?
* What kind of hits/modulators are already reported? Are these patented?
* Is there any bio-structural information available?
* Does the bio-structural information indicate the presence of ligandable pockets?
* What is known about drugability of protein family members or other related proteins?
* What is required to achieve selectivity?

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| In organisms, such as MRSA, mycobacteria, and *P. aeruginosa, N-acyl hydrozones, thioxothiazolidinylidenes, which multitarget MurD and MurE have MIC values vs MRSA as low as 10 ug.ml-1,* GSK847920A, a compound in the GSK screening library has a 1 uM MIC against *M. bovis* where targeting of MurC *in vivo* was demonstrated by genomic analysis of resistance mutations, andpyrazolopyrimidines which are potent (nM) inhibitors of *Ps. aeruginosa* and *E. coli* MurC, have the presence of colisitin, MIC values as low as *3* uM, and have been shown to disrupt peptidoglycan synthesis and to target MurC through analysis of resistance mutations and the ablation of antibacterial effect by MurC over-expression*.* Furthermore, it is apparent that there are a number of natural products such as the *Streptomyces* antibiotic feglymycin which target multiple steps in peptidoglycan synthesis including MurC, and additionally, there are a number of other natural products, such as the aporphine alkaloids from the plant *Ocotea macrophylla*, with 4 ug.ml-1 MICs against *M. tuberculosis* and are M.tb MurE inhibitors with IC50 values of 57 uM. Other natural products targeting Mur ligases are recorded in a review by Sangshetti et al. (10.2174/1381612823666170214115048)  **Therefore, it is abundantly clear that Mur ligases are targeted by natural and synthetic products *in vitro* and in the organism from which the enzymes originate, and that this targeting results in cell death, and where studied this can be attributed to cessation of peptidoglycan synthesis.**  Potential drug target can be found not only in the peptidoglycan precursor binding site, which is unique to bacteria, but also in a highly conserved ATP binding site, which makes these attractive targets with potentially good therapeutic windows and the potential to enable multi targeting of enzymes to mitigate against the emergence of antibiotic resistance. ATP binding sites have been the target of many drug discovery efforts, as listed within “A comprehensive view of protein kinase inhibitors for cancer therapy” [Expert Rev Anticancer Ther. 2018 Dec; 18(12): 1249–1270.](https://www.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&retmode=ref&cmd=prlinks&id=30259761) Gleevec for example is a competitive inhibitor of ATP binding to the active site of BCR-ABL1 kinase. This blockbuster (>>$Bn) inhibitor is used widely in the treatment of cancer. Specific targeting of ATP binding sites within the human kinome is clearly possible. Targeting enabling a therapeutic window between bacterial and human ATP binding sites is therefore also possible, especially when targeting a bacterial biochemical pathway, peptidoglycan biosynthesis, for which there are no near human homologues.  Bacterial Mur ligase ATP binding sites are structurally very highly conserved and there are clear structural differences between Mur ligases and the most related human kinases. Indeed, the original AstraZeneca compounds targeting MurC were found to have a clean human kinase panel. |

*Level of innovation*

**Please describe the novelty of the target or the screening approach.**

The attached guidance document indicates the type of questions the reviewers would like answers to, including:

* Is the target an emerging drug target for a defined disease or disease area?
* Does the target require a novel mode of action to improve upon current therapies?
* Are there any marketed products or clinical candidates available?
* Is it a novel assay for a well-known target that aims to result in new chemical matter?
* Is the approach described dependent on background knowledge generated by the submitter or collaborators?

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| The Mur ligases, though of great promise for the development of new antibiotics, are under-explored. Though potent inhibitors were found by AstraZeneca the compounds were not progressed since they lacked activity against wild type bacteria. With recent advances in our understanding of how molecules can be made to accumulate in bacteria, and additional advances in our understanding of the molecular biology of the interactions between the Mur ligases in cells, the time is ripe to re-engage with this enzyme class, given their essentiality for bacterial survival. There are no antibiotics currently available or in development that have Mur ligase inhibition as the mechanism of action.  The level of innovation is high given that we are proposing a means to combat AMR: multi-targeting. The similarities between the Mur ligases presents us with the opportunity to develop molecules that hit more than one target, greatly reducing the ability of the bacterium to develop resistance.  Screens in the late 1990's were constructed with extracted MurA through MurF proteins, the initial substrates and cofactors run as ‘‘one pot’’ reactions with a low sensitivity, one enzyme providing substrate for the next. In such assays either no, or low affinity inhibitors (substituted D-amino acids) were obtained. Pfizer, Astra Zeneca and GSK have examined MurC as a target. |

*Differentiation*

**Please describe the difference compared to existing treatments.**

The attached guidance document indicates the type of questions the reviewers would like answers to, including:

* What is the current standard of care for the proposed indication?
* Why are different compounds needed relative to the ones discovered so far?
* What is the competitive advantage or patient benefit of this proposal?
* Are predictive preclinical models available to test this?

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| Bacterial resistance to existing antibiotic compounds is a serious and unmet health issue of international importance and notoriety. New broad or narrow spectrum therapeutics, particularly those targeting Gram-negative infections, resistant to existing antibiotics, are desperately needed across all sectors.    This requirement cuts across a number of different therapeutic areas including urinary and respiratory tract infections as well as septicemia for example. Since antibiotic treatment also underpins many areas of medicine, to prevent infection following invasive surgery in particular, new antibiotic discovery and development is imperative to support existing and future healthcare. Inhibition of the Mur ligases in Mycobacterium tuberculosis holds promise for the treatment of respiratory infections. |

*Exploitation potential*

**Please describe the likelihood that hits will reach patients.**

The attached guidance document indicates the type of questions the reviewers would like answers to, including:

* What is the intended patient population?
* Is the trajectory from in vitro models and in vivo models towards IND application well-known or does this need to be developed?
* Is there a biomarker available that can be used in clinical trials?
* Are there any predictable safety issues to consider?

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| 1. UTI, RTI, CNS, skin, soft tissue and bone, infection Infections caused by ESKAPE and WHO listed AMR pathogens. Primary focus *Pseudomonas aeruginosa*, carbapenem-resistant *Enterobacteriaceae*, carbapenem-resistant, ESBL-producing.   Openly available, unpatented molecules can be taken on further using public/NGO funding (10.1371/journal.pmed.1002276) *e.g.,* *via* a sponsor such as GARD-P. An alternative that we are keen to explore in a project derived from ELF hits is to take early stage discoveries to lead optimisation and preclinical development using a for-profit company owned by a non-profit trust where the clinically approved asset would be protected from generic competition by existing regulatory data exclusivities that allow investors to recoup costs yet while ensuring a commitment to open science and affordable medicines.( 10.12688/wellcomeopenres.14947.1) A suitable company, M4ID Pharma, was founded in 2020 by the Lead Applicant (<https://www.m4idpharma.com/faqs>).  The applicant team understands that, following the ELF screen, there will be a period of time during which the hits are not publicly available, to allow for a negotiation between the applicants and ELF partners. At the conclusion of this period, should no industrial partners express an interest in a commercial development route, the hits can be made publicly available, and it is at this point that the open source collaboration would commence. |

1. THE ASSAY

**Please describe the general aspects and development stage of your screening assay.** *(Max. of 500 characters)*

* What is the origin and current status of your assay?
* Was it developed in your own lab / co-development with another partner?
* What is the validated assay format? Is a 1536-well format available / in development? (technical details to be filled in below)
* Is it a common assay, or unique for this specific target?
* Has it already been used to perform (small-scale) screening? Please indicate platform/equipment, plate size, unusual requirements etc.

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| Having worked previously with AZ HTS assay development as part of a 200,000 compound screen we have been well versed in HTS methods, assay design and optimization : David Murray and Mark Wigglesworth, Chapter 1:HTS Methods: Assay Design and Optimisation , in *High Throughput Screening Methods: Evolution and Refinement*, 2016, pp. 1-15 DOI: [10.1039/9781782626770-00001](https://doi.org/10.1039/9781782626770-00001)  We have a series of validated highly sensitised assays for the Mur ligases, both absorbance and fluorescent format that run in 384 well plate format from 50 ml to <10 ml (and have for another target miniaturised a similar coupled assay designed at Warwick with Amplex Red to 2.5 ul in 1536 plates with a Z’ in excess of 0.75, transferred the technology to AZ and in collaboration with Mark Wigglesworth and Helen Plant at AZ Alderley Park. The Mur ligase substrates are not commercially available but we are already set up to make these at commercial scale and purity in excess of 95% and have been doing so for two decades. The current Mur ligase assays have been employed as continuously monitored or stopped fluorometric or spectrophotometric – amplex red coupled methods and have been executed as such on instrumentation such as the Thermo-Fisher Varioskan flash or BMG Clariostar plate readers in 384 well plates.    We anticipate the final format as easily meeting requirements for 384 but potentially 1536 (assay optimisation is ongoing within the laboratory, funded by a Warwick-Wellcome Trust Innovation Award). |

*Please note: You, or your collaborator, should be able to transfer the assay into our screening facilities. It’s important that someone in your group has hands-on experience with the assay in case issues come up during the transfer.*

**Please describe the successive steps of the protocol of your screening assay.** *(Max. of 1 A4 including figures)*

* Describe the complete detailed protocol (including Cat # of reagents)
* Describe reagents/components that will be provided by you (for instance recombinant protein/stable cell line)
* Describe whether a pharmacological tool compound is available
* Include information about S/B and Z-prime

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| The assay is performed in 384 well format in Grenier black clear bottomed plates in a Thermo Varioskan Flash reader or BMG LabTech Clariostar reader. The substrate, UDP MurNAc-Ala-Glu Supplied by the applicants) and ATP (Roche, 11140965001), both at 0.1 mM, and MurE (< 64 nM) are pre-incubated with or without the potential inhibitor in the presence of the 50 mM amplex red (A12222 Invitrogen) coupling enzymes purine nucleoside phosphorylase, xanthine oxidase and horse radish peroxidase (Merck N2145, X2252 and P8375 respectively) for 5 minutes in a volume of 6 ml at 37°C. At this point, the assay is initiated by addition of the missing substrate, 2 ml of 16.4 mM diaminopimelic acid (Merck D1377), or water in a control and shaken at 300 rpm for 20 seconds.. After 15 minutes, the assay is supplemented by 2 ml of a cocktail of 5 mM inosine (Merck I4125) and 5 mM b-g-methylene adenosine triphosphate (Merck M7510, an effective tool compound) ATP with a methylene in place of an oxygen between the b and g phosphorous atoms of the nucleotide)., the latter being an inhibitor of MurE with an IC50 of 2.2 mM.  Phosphate released from ATP by MurE is used by purine nucleoside phosphorylase to cleave inosine to hypoxanthine which is then oxidised by xanthine oxidase concomitant with the generation of two equivalents of hydrogen peroxide. Horse radish peroxidase consumes the latter in the presence of 50 mM amplex red to generate a fluorometric signal (excitation 545 nm, emission 585 nm) or a spectrophotometric output at 555 nm.The signal is monitored until it reaches completion (up to 30 min) and read at that point. The assay can also be run continuously at the same volume, where inosine is added at time zero, and the fluorescence is monitored from time zero without the application of ADPCP to stop the reaction.  We anticipate that hits identified through the work described in this application will be validated at Warwick by an orthogonal luminescent assay such as ATP-Glo.  The UDPMurNAc substrates, and recombinant MurE proteins (see  doi: 10.1074/jbc.M708105200)are (and will be) synthesised and purified by the applicants to greater than 95% purity and electrophoretic homogeneity respectively. These reagents would include target recombinant enzyme for primary screen, reagents, including coupled reaction (all subject to identifying additional funding (e.g. through a request to GARD-P or Antibiotic Research Uk (ANTRUK), with support from ELF). Additional reagents provided for secondary screen in the same format (second mur ligase to identify multi ligase binding).  We have previous experience of developing HTS assays to meet industry standard (we have previously run four HTS campaigns for other targets, from 20 -200,000 compounds, using our own in-house developed assays with MRCT, AZ, McMaster University and Medicines Discovery Catapult all with Z’ in excess of 0.7 when using Amplex Red/ Resourufin Fluorescence, the output being considered here. With respect to MurE assay performance; the Z’ of the assay is dependent upon time of incubation and MurE concentration and these parameters have been investigated: Under optimal conditions, the Z’ values 0.91, 0.66, 0.77 at MurE assay concentrations of 4.02, 8.02 and 64.5 nM were recorded. The corresponding signal to noise ratios (High:Low control values) are 4.4,18.0 and 12.3. |

Please justify whether your assay meets the minimum requirements.

In order to qualify for a Public Programme, your assay has to meet the minimum technical requirements as listed below. Please indicate the compliance in the table and support it with your data below the table.

Please note that:

* Assays in ELISA format are not eligible for submission, unless the assays can be converted to a homogenous format.
* In case the assay does not fully meet the requirements, please indicate in the next section what is needed in terms of resources and assistance to bring the assay up to the specified requirements.
* In case one or more assay reagents are carcinogenic or mutagenic, search for alternatives in line with the European legal framework.
* Kinetic measurements on the HTS system have limitations; alternatives are recommended.

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| *Characteristic* | *Requirement* | *Compliant? Comments?* |
| **Assay format** | Demonstrated in 384-well format, scalable to 1536-well format | 384-1536 |
| **Homogenous assay** | No wash steps | No wash steps |
| **Characterised reference** | Available from partner or commercially available | Partner |
| **Read out technology** | Compatible with mix-and-measure and homogenous formats, e.g.: •    Absorbance •    Luminescence •    Fluorescence intensity •    Fluorescence polarization •    Fluorescence resonance energy      transfer (FRET) •    Time resolved Fluorescence (TRF) •    Homogeneous Time Resolved      Fluorescence (HTREF /TR-FRET)  Or alternatives that give highly specific readouts (e.g. Alpha technology, fluorescence lifetime, fragment complementation) | Fluorescence intensity |
| **Signal / Background (S/B) in 384-well plate format**  **Max assay end volume 30 µl Sample end concentration 10E-5 M** | > 3 preferably higher | 4.4 to 18 in a 15 minute incubation depending upon [target] |
| **Z’ (Z prime) in 384-well plate format**  **Max assay end volume 30 µl Sample end concentration 10E-5 M** | > 0.6 | 0.66-0.91 depending upon [target] in a 15 minute assay |
| **DMSO tolerance** | Minimal tolerance 1.0 % DMSO | Yes, tolerance up to 10% DMSO is observed |
| **Stability of each reagent** | Stable for at least 8 hours  For proteins: proven freeze-thaw cycle stability | yes |
| **Cell lines** | Certified mycoplasma free  Stable cell lines available for transfer | N/A |
| **Minimal signal pattern on plates** | CV <10% across plate filled with reference compounds | tbd |
| **Protein** | Recombinant protein at least 80 % pure  Provided by you or feasible to produce on milligram scale  (construct and protocol available for transfer) | yes |
| **Incubation times** | Up to 4 hours | yes |
| **Readout stability** | For at least 1 hour | yes |
| **Experience** | Hands on experience in running the assay | yes |
| **Experimental data** | Experimental data available | Yes – see below |

*Experimental data*

**Please include the experimental data showing that the technical specifications are met.**

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| The figure left shows the result of scanning the impact of incubation time and [MurE] on assay performance. The data clearly show time dependence of accumulation of measurable fluorescence, and that the ability of the assay to distinguish between the presence and absence of a standard inhibitor is dependent upon the [MurE] in the assay. This is to be expected because the slower product accumulation in the presence of ADPCP will approach the value achieved in the absence of the inhibitor indicating the assay window for this (or any) stopped assay is likely to be time-dependent: |

If you are unable to meet all of these requirements, please explain what needs to be done to meet the requirements.

* This will assist the committee to estimate the efforts needed for further assay development and provide the applicant with feedback to improve the assay.
* What kind of resources or assistance is needed to meet the requirements?

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| We anticipate having these validated and in place by winter 2021/2 and have staff experienced in developing our previous HTS assays already in place and working on these. We still have to complete studies that assess inter and intra plate variability, although we anticipate this would be achievable in the next two months. |

Please summarise the current status of protein / reagents / cell lines etc.

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| Available but will require funding for scale up or support by ELF in region of £35,000 assuming full miniaturisation for 200,000 compounds. With an ELF screen offered, we anticipate being able to raise such funding from other sources. |

1. HIT CHARACTERISATION

During screening, a list of active compounds in the primary assay will be defined. These active compounds need to be further filtered using follow up assays, to identify the most promising hits and to be able to deliver you a qualified hit list. We will experimentally filter the active compounds using the following screening cascade:

Primary assay - Active confirmation - Deselection or orthogonal assay - Dose response curves - and if possible selectivity profile and/or on target binding activity

**Please provide potential deselection assays (negative selection).**

This (high throughput) assay will test for unspecific interactions, for example using an empty cell line. Please include protocols, literature references or other assay details as much as possible.

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| Deselection assays include   1. targeting only a single mur ligase 2. Counter screen, panel of human kinases (if inhibitors target mur ligase ATP binding site) 3. Low target activity tba |

*Please note that only very limited amount of compound is available for deselection and hit confirmation (5.5uL at 10mM) and that the assay needs to be transferable to the labs of the European Lead Factory.*

**Please provide potential orthogonal assays (positive selection).**

This (high throughput) assay will test for hit confirmation, for example using another readout technology. Please include protocols, literature references or other assay details as much as possible.

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| ATP-Glo (luminescence) assay (e.g. ATP-Glo™ Bioluminometric Cell Viability Assay | Biotium) following ATP depletion by loss of fire fly luciferase which was published by Zawadzke and Pfizer: Zawadzke , L.E., Norcia, M., Desbonnet, C.R., Wang, H., Freeman-Cook, K. and Dougherty, T.J. (2008). Identification of an inhibitor of the MurC enzyme, which catalyzes an essential step in the peptidoglycan precursor synthesis pathway. Assay Drug Development Technologies. 6(1), 95-103 would be a completely orthogonal approach.  Beyond that, rapid fire MS of UDP products and their fragments (minus the UDP group) are also a possibility, there is already a medium throughput MS assay published for MurC: Deng G, Gu RF, Marmor S, Fisher SL, Jahic H, Sanyal G. *Development of an LC-MS based enzyme activity assay for MurC: application to evaluation of inhibitors and kinetic analysis*. J Pharm Biomed Anal. 2004 Jun 29;35(4):817-28. doi: 10.1016/j.jpba.2004.02.029. PMID: 15193726. |

*Please note that only very limited amount of compound is available for deselection and hit confirmation (5.5uL at 10mM) and that the assay needs to be transferable to the labs of the European Lead Factory.*

**Please provide suggestions to test the selectivity profile of the compounds and other secondary assays.**

* What are the key selectivity targets (e.g. protein family members)? Which assays are available for them?
* Which biophysical assays have been generated or are known (with protocols available)?
* Which cell-based assays are available?
* Which in vivo models are available?

*Please include protocol details or literature references for all assays mentioned.*

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| Human kinase panel (for ATP binding site targeting inhibitors) industry standard assays available  Eukaryotic cell toxicity assays  Bacterial whole cell activity and HPLC assays to examine accumulation of intracellular pathway intermediates, including permeation enabled whole cells (WT plus chemically or knock out engineered to enhance uptake/ accumulation e.g. delta tolC). |

1. HIT-TO-LEAD STRATEGY

**Please describe your hit-to-lead strategy if the Programme successfully delivers a Qualified Hit List.** *(Max. 1250 characters)*

e.g. access to medicinal chemistry team, knowledge exchange, patents, spin-outs, outreach, partnering etc.

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| Existing resources to explore the hits obtained can be deployed to progress compounds towards a final data package that should include (a series of) lead compounds identified through initial HT screening which:   * Are selective inhibitors of at least 2 mur ligases of at least 1 relevant WHO-listed pathogen, with no activity on human orthologues (**pilot data already shows evidence of early stage compounds targeting more than one mur ligase**) * Have been characterised structurally and physicochemically through co-crystallisation, SPR and biochemical assays * Demonstrate potent antimicrobial activity against the selected pathogens, as well as resistant and clinical strains * Demonstrate lower propensity for resistance development, compared to single-target mur ligase inhibitors * Exhibit drug-like properties & safety (cell permeability, *in vitro* ADME, cell health assays)   Proof of concept *in vivo* (murine thigh infection model or other).  Synthetic chemistry resources include: i) Yuhang Wang, PhD student in Todd’s laboratory (2020-2022) who is available to work full-time on any resulting hits, ii) laboratory personnel at Northeastern University, via existing Open Source Antibiotics collaborators Lori Ferrins and Joe Eyermann, iii) further X-ray crystallography resources contributed through the Seattle Structural Genomics Center for Infectious Disease (PI: Bart Staker). Todd is the Head of Chemical Networks for the Structural Genomics Consortium, and it would be possible to install any hits into that program in order to elicit synthetic chemistry inputs from the broader chemical community. |

**Please describe what funding streams are envisaged to further develop the hits generated and what information is likely to be required for success in accessing these funding streams.** *(Max. 1250 characters)*

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| Success in the ELF screen will make follow-on research support significantly more likely because we will have obtained a key piece of new data: *a molecule inhibiting more than one mur ligase and which possesses activity against wild type bacteria*. We will seek research funding to extend our program from **LifeARC**, the **Wellcome Trust**, and **CARB-X**. We will also seek industrial funding for the project *via* **M4ID Pharma** through **InnovateUK**, with in-kind support from the **Medicines Discovery Catapult** (collaborating PI John Overington). We have also engaged in discussions about project inputs with the AlphaFold team at **Google** (PI Andrew Senior) who are interested in supporting Open Source Antibiotics *via* the predictions of protein structures for all Mur enzymes across all species, to assist with the prediction of new small molecules that would maintain activities *vs.* multiple enzymes |

1. ETHICAL SCREENING

In order to identify the policy/regulation to govern the undertaking of the project (e.g. European Lead Factory´s code of Practice for Research on Human Participants, Animals, Medicines for Human Use (Clinical Trials) following EU Regulations), please answer the following questions. If any of the questions are answered with YES in this section, the project will require a full ethical review before it is approved for selection.

|  |  |  |
| --- | --- | --- |
|  | **YES** | **NO** |
| Does the programme involve testing, observing, or collecting personal data from humans? |  | x |
| Does the programme involve the investigation of the safety or efficacy of a medicines, foodstuff or placebo in humans? |  | x |
| Does the programme involve patients, their carers, or staff of a health or a social care organisation? |  | x |
| Does the programme involve the collection or use of donor identifiable human biological samples? |  | x |
| Does the programme involve access to anonymised collections of patient data? |  | x |
| Does the programme involve human biological samples obtained from a tissue bank? |  | x |
| Does the programme involve human stem cells or materials derived from human stem cells? |  | x |
| Does the programme involve the collection or use of animal biological samples? |  | x |
| Does the programme give rise to issues in the sphere of bio-safety, bio-security or dual use? |  | x |
| Does the programme have a direct military application or the potential for terrorist abuse? |  | x |
| Has this programme already been reviewed by any local Ethics Committee? |  | x |

**In case one or more questions are answered with ‘Yes’, please explain in more detail** *(Max. 750 characters)*

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1. THIRD PARTY LIMITATIONS

Please indicate in the table below all Background not solely owned or controlled and that is necessary for carrying out the screening Programme, and any Third Party limitations to the granting of Access Rights and any other restriction that might substantially affect the granting of Access Rights to such Background.

|  |  |  |  |
| --- | --- | --- | --- |
| **Programme IP \*** | **Type of Programme IP** | **Owner of Programme IP**  **(+ details on license if the applicant or its organisation is not the owner)** | **Is there any legal or pre-existing contractual third party restriction to the use of the Programme IP, for performance of the Programme Plan, for Research Use or for Direct Exploitation** |
| *e.g. protein X* | *e.g. Material* |  | *describe third party limitations on use or other restrictions imposed by applicable law*  *e.g. Gene X was produced via use of plasmid Y. Plasmid Y can only be used for non-commercial purposes* |
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*\* any Intellectual Property Rights relating to the proposed Programme and pre-existing to the date of this proposal or developed independently from this proposal and which are Necessary to implement the Programme Plan or to exploit the Results of the Project.*

1. OTHER CONTACTS

**Please provide the contact details of your Technology Transfer Officer and/or Legal Representative.**

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