Methods used in preclinical assessment of anti-Buruli ulcer agents: a global perspective

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Abstract

Buruli ulcer (BU) caused by *Mycobacterium ulcerans* is the third most common chronic mycobacterial infection in humans. Approximately 5000 cases are reported annually from at least 33 countries around the globe, especially in rural African communities. Even though anti-mycobacterial therapy is often effective for early nodular or ulcerative lesions, surgery is sometimes employed for aiding wound healing and correction of deformities. The usefulness of the antibiotherapy nonetheless is challenged by huge restrictive factors such as high cost, surgical scars and loss of income due to loss of man-hours, and in some instances employment. For these reasons, more effective and safer drugs are urgently needed, and research programs into alternative therapeutics including investigation of natural products should be encouraged. There is the need for appropriate susceptibility testing methods for the evaluation of potency. A number of biological assay methodologies are in current use, ranging from the classical agar and broth dilution assay formats, to radiorespirometric, dye-based, and fluorescent/luminescence reporter assays. Mice, rats, armadillo, guinea pigs, monkeys, grass cutters and lizards have been suggested as animal models for Buruli ulcer. This review presents an overview of *in vitro* and *in vivo* susceptibility testing methods developed for the determination of anti-Buruli ulcer activity of natural products and derivatives.

Keywords: Methods, Preclinical, Buruli ulcer, Assays, Animal models

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1. Background

Buruli ulcer (BU) is the third commonest mycobacterial disease, after tuberculosis and leprosy; the causative organism is *Mycobacterium ulcerans* (WHO, 2011). It largely affects inhabitants of remote rural areas in West and Central Africa, usually along wetlands with limited access to health facilities and is an important cause of human suffering. At least 33 countries within the tropical, subtropical and temperate region have reported BU, with 5000–6000 cases reported annually in 15 of the 33 countries. Most cases occur in tropical and subtropical regions except in Australia, China and Japan. In West Africa, Benin, Côte d’Ivoire and Ghana reported most cases with Côte d’Ivoire reporting almost half of the global

cases (WHO, 2013). The exact mode of transmission to humans remains unknown, but in contrast to tuberculosis and leprosy, the infection is largely believed to be acquired directly or indirectly from the environment and not through contact with other patients (Stinear et al., 2008).

BU is among the so-called neglected tropical diseases (NTDs). According to a WHO report (2009), at least 1 billion people, representing about one sixth of the world's population suffer from one or more NTDs. They represent about 17% of the global burden of parasitic and infectious diseases and are endemic in rural communities of Sub-Saharan Africa and poor urban areas in low-income countries of Asia and Latin America. It is estimated that about 534,000 people worldwide die from a NTD each year (WHO, 2009).

There is no vaccine against BU and the main control strategy is early case detection and treatment to reduce the suffering associated with the disease. Although recent experience indicates that combination chemotherapy with streptomycin and rifampin improves cure rates, the utility of this regimen is limited by the 2-months duration of the treatment, potential toxicity and a required parenteral administration of streptomycin, and drug-drug interactions caused by rifampin (Chauty et al., 2007; Johnson, 2010; Zhang et al., 2013). Surgery is, however, necessary for some severe forms of the disease (large ulcerated forms, disseminated forms, and osteomyelitis) (Kibadi et al., 2010). This surgical treatment can only be used in a few medical centres with proper and adequate equipment and is neither affordable nor accessible to a large part of the population (Johnson et al., 2004).

In spite of this problem, there is inadequate emphasis on the development of new anti-mycobacterial drugs. The urgent need to alleviate the burden related to this disease led the scientific community to investigate various resources, including plant-derived biologically active metabolites against the causative agent (*M. ulcerans*). Discovery and development of new drugs for BU treatment is greatly hampered by the slow-growing rate of *M. ulcerans*, requiring even up to 6 months of incubation on solid media to produce visible colonies.

In an attempt to provide guidance on quality assurance for anti-mycobacterial screening, this review focuses on analytical methods involving primary *in vitro* approaches and *in vivo* animal models for anti-*M. ulcerans* studies. We focused our literature search on publications on Medline PubMed (NCBI) database dealing with the evaluation of activities of natural products against *M. ulcerans* and limited it to papers published in English or in French. Key words used were “antimycobacterial screening”, “Buruli ulcer”, “*Mycobacterium ulcerans*”, “drugs susceptibility test”, and “mycobacteria”. All retrieved titles and abstracts were analysed for relevant studies that are discussed here.

2. Requirements for products and growth conditions

2.1. Product management

Water, dimethyl sulfoxide (DMSO), tween 80, methanol, and ethanol are the most frequently used solvents to prepare test products solutions. Given their physico-chemical properties, methanol and ethanol present the challenge of rapid evaporation, which may alter the

concentrations of the stock solutions, and as well inhibit mycobacteria growth. Products in 100% DMSO have become the standard (Gad, 2005). Added advantages of stock solutions in 100% DMSO are: (1) elimination of microbial contamination, thereby reducing the need for sterilization by autoclaving which can affect the quality of the test product, and (2) good compatibility with test automation and integrated screening platforms, assuring, for example, good solubility during the serial dilution procedures (Cos et al., 2006). It is however noteworthy that DMSO is potentially cytotoxic to many microorganisms including *M. ulcerans*. It is therefore advisable to keep the in-test concentration of DMSO less than 0.625% to avoid any interference with the biological test systems. This requires intermediate dilution step in water. Because of the variability of individual compounds, there are no general storage conditions that guarantee sample integrity (Verkman, 2004).

2.2. Growth conditions

Mycobacterium ulcerans differs from most other pathogenic mycobacteria in that it grows best at 30–33°C and not at 37°C (Eddyani & Portaels, 2007). The generation time (36 h) of *M. ulcerans* is longer compared to that of other slow growing mycobacterial species such as *M. smegmatis* (Pattyn, 1965). Using the radiometric BACTEC 460 system, a generation time of 23 h was determined for *M. ulcerans* (Portaels et al., 2001). The media commonly used to culture slowly growing mycobacteria (e.g. Löwenstein-Jensen, Ogawa and Middlebrook media) are also suitable for *M. ulcerans*. Löwenstein-Jensen (LJ) is a conventional culture medium for *M. ulcerans*. Middlebrook 7H11 agar, Middlebrook 7H9, BacT/Alert medium, BACTEC medium and Middlebrook 7H12 liquid media are also convenient for culturing *M. ulcerans* in vitro. The incubation period varies according to the type of culture medium used and is generally longer on solid medium. Cultures are read within 2 to 3 weeks (with Middlebrook 7H12), 4 and 8 week (with Middlebrook 7H11 agar), or 6 to 8 weeks or more (with LJ) while 8 to 14 days are required when Middlebrook 7H9 broth is used (Scherr et al., 2012; Yemoa et al., 2011).

2.3. Bacterial load

The bacterial load to be used for susceptibility testing is critical for accuracy and reproducibility of results. In fact, inoculum concentration can have a profound influence on the antibacterial potency of a sample, endorsing the need for standardization of inoculates (Anon, 2003; Gautam et al., 2007). For dilution methods, Hadacek & Greger, (2000) recommend an inoculum size of about 10^5 CFU/ml for most bacteria. A false-positive activity can be obtain with a much lower inoculum size (e.g. 10^2 CFU/ml), whereas the risks for false-negatives is increased and endpoint readings hampered with a much higher inoculum size (e.g. 10^7 CFU/ml). Bacterial inoculates can be prepared from cultures or from existing biofrozen stocks. To prevent the selection of an atypical variant, sample should be collected from culture during the exponential phase and should consist of four or five colonies of a pure culture on agar (Anon., 2003). The bacterial load to be considered also depends mainly on the growth rate of the bacteria under consideration. The inoculum size of fast growing species should be less than that of slow growers.

For the preparation of *M. ulcerans* inoculum, many strategies have been used so far. The *M. ulcerans* isolates are conventionally subcultured on Lowenstein-Jensen slants at 32°C,

incubated for eight weeks and examined during that period for growth and purity. A loopful of culture is then added to sterile glass beads and distilled water in a screw-cap tube and homogenised with a vortex to fragment the colonies. The turbidity of the suspension is then adjusted to that of a No. 1 McFarland and serially diluted 10-fold to yield 10^{-2} and 10^{-4} suspensions for the susceptibility testing. Preparation of inoculum is usually done on ice to prevent aggregation of *M. ulcerans* due to the high lipid content, which allows them to stick together at room temperature. Smears of the resultant *M. ulcerans* suspensions should be stained with Ziehl Neelsen (ZN) stain to reveal acid-fast bacilli (AFB) and to check for microbiological purity (Addo et al., 2007). Indeed, for the preparation of *M. ulcerans* inoculum, bacteria can be diluted in phosphate buffered saline (PBS) instead of water.

In addition to the turbidity determination, the inoculum size can also be determined using microscopy such as the ZN staining according to the method described by Shepard and McRae (1968). Even though it is the most rapid way to verify the presence of mycobacteria, the acid-fast staining techniques are difficult to accurately quantify mycobacteria by smearing cells onto a glass slide (Treuer & Haydel, 2011). The most reliable procedure is deemed through the enumeration of bacteria colony forming unit (CFU) after culture, which is a very slow process due to its slow growing nature. Thus this approach for inoculum size determination is difficult to obtain and usually not practicable. Treuer & Haydel, (2011) proposed the use of a modified Kinyoun acid-fast staining method adapted for use with a Petroff-Hausser sperm and bacteria cell counting chamber by using a liquid suspension staining technique. The result achieved by this method accurately correlates the viable cell counts by agar plate counting.

The use of weight to determine the inoculum size has also been proposed. Portaels et al., (1998), proposed that fresh colonies of *M. ulcerans* are collected from the LJ medium and suspended either in distilled water or culture medium. The turbidity of the resulting suspension is then adjusted with distilled water to be equivalent to that of a standard 1 mg/mL suspension of *M. bovis* BCG (containing approximately 10^8 CFU per mL), from which further dilution are performed to 10^{-1} and 10^{-2} mg/mL. The inocula are 0.1 mL of diluted solutions (Yemoa et al., 2011).

Therefore in preparing bacteria suspension for susceptibility testing, *M. ulcerans* growth patterns such as slow growing and a low optimum growth temperature between 28 and 32°C; as well as ability to aggregate in broth, should be considered in designing a suitable testing system. The medium supplemented with tween 80 or in BacT/Alert culture bottles supplemented with enrichment medium can be effectively used to prevent aggregation in liquid medium and reduce incubation time. Usually, *M. ulcerans* is grown for 6 to 8 weeks at 30°C until the log phase is reached. The test inoculum is prepared by diluting the culture with BacT/Alert medium to a final optical density at 600 nm (OD₆₀₀) of about 0.02 (Scherr et al., 2012).

3. *In vitro* susceptibility testing methods

Different methods have been used to measure susceptibility of *M. ulcerans* to agents, but the outcomes are greatly affected by the sensitivity of the method used as different principle may be applied.

3.1. Dilution methods

Antimicrobial susceptibility testing of the slow-growing mycobacteria such as *M. ulcerans* can be performed using either solid (agar) or liquid (broth) media. One of the major advantages of the dilution methods is the defined concentration of test samples in medium and their suitability for activity quantification (Chand et al., 1994; Rios et al., 1988). Generally, polar and non-polar agents can be adequately assessed by dilution methods and also allow a proper determination of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values. This allows the assessment of bactericidal or bacteriostatic effects of samples at a given concentration.

3.1.1. Agar dilution

In the 1950s, Canetti et al. first described the agar dilution method for *M. tuberculosis*, where a range of drug concentrations against *M. tuberculosis* complex was prepared in LJ medium. The slant of drug-containing medium was inoculated with the test bacterial suspension and the growth inhibition read after incubation. Even though MIC was obtained using this approach, it could not boost its use for a very long time (Canetti et al., 1963; Van-Klinger, 2007). The agar dilution drug susceptibility testing method mainly involves determining the proportion of microorganisms susceptible to a specific concentration of a given drug, by comparing the number of colony forming unit in a drug-containing and drug-free control media. The method is inexpensive and highly standardized for testing susceptibility to agents. Although this method allows for activity quantification, it is labour intensive and requires a careful quality control and high concentrations of test samples. It is also noteworthy that the longer incubation period required for the assay may lead to potential exposure to contaminants it is one of the most used methods for drug susceptibility assays against *M. ulcerans*. In addition to LJ medium, 7H9 agar, 7H10 medium, or 7H11 agar supplemented with oleic acid, albumin, dextrose, and catalase, can be used as culturing medium. Additional drawback of this methods is that it requires at least 28 days to detect any visible colonies growth (Portaels et al., 1998).

3.1.2. Broth dilution

In the broth dilution method, different concentrations of the test drug are mixed with equal amount of liquid medium either in tubes or microtiter plates and inoculated with the same load of test microorganism. After incubation, the susceptibility of the microorganism is quantified and the minimal inhibitory concentration (MIC) determined as the lowest concentration of test samples that inhibit bacterial growth measured by visual turbidity or more accurately by optical density or fluorescence. Redox-indicators such as tetrazolium salts (De-Logu et al., 2003) and Alamar Blue or resazurin has been found to be useful for non-fluorometric readouts (Martin et al., 2003, 2007; Palomino et al., 2002; Caviedes et al., 2002 ; Foongladda et al., 2002; Abate et al., 1998). Alamar Blue reduction is the most frequently used endpoint for microplate assays, with a fluorometric (absorption at 530 nm and emission at 590 nm) and an absorbance (at 570 nm) or visual readout after 14 days of incubation

(Franzblau et al., 2012; Donkeng et al., 2014). Furthermore, an alamar Blue-based growth inhibition microplate assay has been adapted for the thermosensitive and slow-growing pathogen *M. ulcerans* within 10 days. The standardized test procedure is suitable for medium-throughput screening of compound libraries (Scherr et al., 2012).

The micro-dilution approach is simple, accurate, rapid, cheaper and requires smaller volumes of reagents. When coupled to spectrophotometric or fluorometric plate readers it hugely favours high throughput of results (Sánchez & Kouznetsov, 2010; Hadacek & Greger, 2000).

3.2. Radiometric/Radiorespirometry method

The radiometric BACTEC method is a more rapid method combining antimicrobial agents, the mycobacterium, and a C14-labeled substrate in a broth medium. Activity is determined by the rate and amount of labeled CO₂ produced, which is directly proportional to the rate and amount of growth that occurs in the broth medium (Wallace et al., 1997; Palomino et al., 1998). This method has been used for susceptibility of *M. ulcerans* to antibiotics and substances (Marsollier et al., 2007). However, these assays require significant, heavy very expensive equipment and isotopic disposal in some settings (Chung et al., 1995; Sánchez & Kouznetsov, 2010) and may be complicated due to the mycobacterial growth features. The BACTEC 460 system requires microaerobic conditions (2.5 to 5% oxygen) to promote the growth of *M. ulcerans*. Besides, BACTEC 460 system is not suited for the evaluation of large numbers of compounds (Chung et al., 1995; Rajab et al., 1998). Meanwhile, it can contribute significantly toward the development of an acceptable, standardized, and reproducible technique for providing rapid results of the *in vitro* drug susceptibilities of *M. ulcerans*.

3.3. Bioluminescent methods

The genes that encode for luciferase enzymes have been successfully cloned from many species of bacteria, firefly, beetle, crustacean, and the sea pansy (New et al., 2003). Fluorescent proteins such as the red fluorescent protein and green fluorescent proteins have been used, following their introduction in mycobacteria on plasmids, allowing the quick assessment of bacterial viability by assessing the expression of a hosted fluorescent or luminescent protein (Pauli et al., 2005; Arain et al., 1996). The use of reporter protein makes the use of microtiter plate format more suitable for high throughput detection and allows the development of screens that exploits common equipment and procedures to detect activity (New et al., 2003). An *M. ulcerans* strain that expresses luciferase enzymes has been shown to be useful as a real-time biomarker of mycobacterial viability for high-throughput or drug susceptibility screening of antibiotics (Zhang et al., 2010; 2013). Zang et al., (2013) engineered a recombinant *M. ulcerans* strain to express luxAB and showed that the drug activity against *M. ulcerans* can be followed up in real time after 2 days of exposure and that the relative light unit count measured in real time is consistent with the standard CFU counts available 3 months later. This method has been shown to be useful for rapid assessment of drug effectiveness and distinguishing of bacteriostatic or bactericidal effects (Zang et al., 2013).

As this assay is just one amongst many others, and as the commercialisation of this engineer organism is often restricted by patents, very few laboratories are using this approach in preliminary screening of natural products (New et al., 2003).

3.4. Criteria for quantifying activity of in-vitro assays

Numeric values are usually used to express antimicrobial activity of extracts or compounds such as inhibitory concentrations (IC₅₀, IC₉₀) and MIC. An appropriate understanding of the expressed activity requires a good knowledge of both the model and the protocol used (Cos et al., 2006). Relevant activity relates to IC₅₀ values below 100 µg/mL for plant extracts (128 µg/mL; if the Clinical and Laboratory Standards Institute MIC range is used), 0.25% v/v for essential oils and below 25 µM for pure compounds (Gibbons, 2004; Pavan et al., 2009). Yemoa et al., (2011) assumed that a plant extract which inhibits growth of *M. ulcerans* at 250 µg/mL is considered active. For antibacterial activity of compounds on a whole-cell, an optimal value of 1 µg/mL is needed (Enna, 2001). A concentration of 200 µg/mL was considered by Tosun et al., (2004) as a cut off for promising activity, but some authors reports that “antibacterial” compounds with MIC values greater than 100 µg/mL are poorly active and their clinical perspective has little significance in reality. To be considered by pharmaceutical industry for drug development, compounds should have an MIC of less than 10 µg/mL, and ideally less than 2 µg/mL with a selectivity index (SI = IC₅₀ Vero cells/MIC) of >10 (Gibbons, 2008; Orme, 2001).

4. Animal models: *In vivo* susceptibility testing methods

Since the 18th century, animal modelling has simplified and improved the multidimensional elucidation of many diseases (Migaki & Capen, 1984). WHO (2001) recommended the use of animal models for improved research in disease areas such as BU, which includes pathogenesis, transmission, diagnosis, treatment, management and general control of the disease. Mice, rats, armadillo, guinea pigs, monkeys, grasscutters and lizards have been used as animal models for BU, each with varying degrees of suitability.

4.1. Guinea pig model

Even though, the guinea pig model most closely mimics the human disease (Kreig et al., 1974), intradermal inoculation of live *M. ulcerans* into guinea pigs resulted in tissue necrosis around extracellular mycobacteria and a poor inflammatory response at the site of infection. The intradermal injection of mycolactone into guinea pigs, led to necrotic lesions like those induce by injection of live organisms (George et al., 1999; Kreig et al., 1974); while the phagocytosis of an isogenic toxin-negative mutant of *M. ulcerans* by macrophages induce a typical mycobacterial inflammatory response, with granuloma development (Coutanceau et al., 2005); it is not clear from these experiments whether mycolactone causes local immunosuppression in addition to its tissue destructive effects (Millay et al., 2006).

Cope et al., (2004) proposed a Crl:IAF(HA)-hrBR hairless guinea-pig model of BU disease. Guinea-pigs were subjected to daily UV topical treatment for three consecutive days after which they received intradermal injection of *M. ulcerans* and monitored on daily basis. This

experiment showed that topical exposure to UVR-UCA (UV radiation + urocanic acid) promotes *M. ulcerans* infection in infected guinea-pigs.

[Williamson et al., \(2014\)](#) also developed a skin abrasion guinea pig infection model based on the consideration that, an abrasion is a superficial lesion above the dermis susceptible to bacterial infection. Such abrasions are common among children especially in rural communities and the *M. ulcerans* injected within the dermis could lead to BU infection ([Adusumilli et al., 2005](#); [George et al., 1999](#)). This experiment showed that the model can be infected by passive inoculation of *M. ulcerans* on superficial skin abrasions as it was possible to find *M. ulcerans* at intra-dermal injection sites while topical application failed to do so.

4.2. Armadillo model

Experimentation with armadillos showed that nine-banded armadillos are susceptible to *M. ulcerans* and can develop cutaneous lesions that closely mimic BU in human suggesting that it could be a useful model to investigate the pathogenesis of *M. ulcerans* ([Walsh et al., 1999](#)) and to provide further insight into the assessment of new treatments for BU.

4.3. Grasscutter model

[Addo et al., \(2006\)](#) developed the grasscutter, a hystricomorph rodent as a model for BU. After subcutaneous inoculation of *M. ulcerans*, grasscutters developed progressive skin, muscular and bone lesions namely: erythema, papule, nodule, superficial ulcer, oedema, hydrocele, undermined ulcer, contracture and osteomyelitis. These lesions resulted in histopathological changes, such as coagulative necrosis, mixed inflammatory reactions, myolysis, perineuritis, neurogenic muscular atrophy and osteomyelitis, which were all similar to clinical and histopathological features observed in humans; thus making the grasscutter a potential BU animal model. Besides, grasscutters are commonly found in Sub-Saharan Africa, where the prevalence of BU is higher. Moreover they are established laboratory animals in Ghana and can easily be maintained ([Asibey & Addo, 2000](#); [Addo, 1997](#)) in any conventional animal facility. Though their large body size enables a good assessment of BU lesions ([Addo et al., 2006](#)), they may not be suitable for drug susceptibility test as they will require large quantity of the product to be tested; in addition to the long incubation period needed for manifestation of the undermined ulcer (14-16 months post-inoculation), which is the hallmark of BU. Thus, this model is more suitable for elucidation of the pathogenesis of the disease rather than drug discovery ([Addo et al., 2006](#)).

4.4. The mouse footpad model

The mouse footpad model was developed by Australian virologist Frank Fenner in the 1950s ([Fenner, 1956](#)). *M. ulcerans* bacilli are injected just under the surface of the skin into a relatively cool area of the mouse body, mimicking the peripheral location of most human lesions. Gross pathology is progressive swelling of the footpad that often leads to ulceration and loss of the foot and leg if left untreated, with no or limited pain, and is easily monitored for both worsening infection and response to antibiotic treatment. A reduction in swelling is

observed with effective curative therapy (Ji et al., 2008; Dega et al., 2002). Prevention of swelling can be seen in mice treated early after infection (Almeida et al., 2011). Although the inoculum is probably higher than that occurring in human infection, histopathological analysis of both experimentally infected footpads and clinical lesions typically results in the observation of larger numbers of acid-fast bacilli. The acid-fast bacilli degenerate and become beaded in appearance in response to treatment in both mice and human (Converse et al., 2011; Addo et al., 2005) and in humans (Ruf et al., 2011; Schütte et al., 2007).

Unlike human or guinea pig skin, there is relatively little subcutaneous fat in mice. Therefore, the action of the mycolactone toxin on adipocytes is not as marked in mice (Converse et al., 2011). Microbiological analysis of footpads allows for culture of the entire lesion and the evaluation of bactericidal and bacteriostatic drug activity. Disseminated disease and oedematous lesions occur in humans. Dissemination of *M. ulcerans* infection, with contralateral foot involvement is rarely observed in mice. Immunocompetent mice may lose feet after ulceration of lesions in the absence of treatment (Converse et al., 2011). Immunodeficient mice reveal dissemination to the thigh and the blood as determined by both culture and histopathology (Converse et al., 2011). The mouse footpad model is a useful tool for the evaluation of drugs that are active against *M. ulcerans* *in vivo*. Mice do not always metabolize drugs in the same manner as humans. A dose that shows activity in humans may not be effective in mice. Although rifampin and streptomycin are identical or very similar, the clarithromycin dose is quite different and therefore requires different dosing regimens in mice to avoid interference with the activity of rifampin. The equipotent dose has to be determined for any new potential drug (Converse et al., 2011). Although the mouse footpad model has some resemblance to human lesions, there are forms of the human disease (e.g., papule, plaque, undermined ulcer) that are not reproduced. Remarkably, rifampin -streptomycin combination treatment appears to be effective in most presentations of disease in humans (Converse et al., 2011).

Addo et al., proposed a model to assess the infectivity of *M. ulcerans* isolates in Ghana and as well analyzed the dose-response pattern in BALB/c mice. The findings suggested that the progression and final outcome of an *M. ulcerans* infection may be dose related. The mouse presented a plethora of BU lesions, confirming its usefulness as a BU animal model. However, its manifestation of ulcer, the hallmark of BU, was not classical, thus subtracting from its suitability. Firstly, the mouse was too small to facilitate proper clinical evaluation of the ulcers. Secondly, the ulcers were short-lived and progressed rapidly to necrosis, resulting in the destruction of the limb (Addo et al., 2005), as also observed by Converse et al., (2011). These findings give support to the assertion that better BU animal models need to be found.

Case: autoluminescent *M. ulcerans* expressing luxCDABE operon

Zhang et al., (2013) engineered a virulent reporter strain of *M. ulcerans* with intrinsic bioluminescence to enable serial, real-time, non-invasive *in vivo* monitoring of viable bacterial counts and demonstrated its utility for high-throughput *in vitro* and *in vivo* screening of antibiotic efficacy using a panel of anti-mycobacterial drugs with various mechanisms of

action. They showed that a drug's *in vivo* activity against *M. ulcerans* is detectable in as little as one week through rapid, serial, non-invasive assessment in live mice performed with a bench-top luminometer. This method promises dramatic reductions in time and effort as well as requirements for animals, compounds and other supplies. It was asserted that such a strain will be capable of transforming drug discovery and development efforts for BU. Surrogate markers for evaluating antimicrobial activity in real-time which can be measured serially and non-invasively in infected footpads of live mice would accelerate pre-clinical evaluation of new drugs to treat BU. Previously, bioluminescent *M. ulcerans* strains were developed, demonstrating proof of concept for measuring luminescence as a surrogate marker for viable *M. ulcerans* *in vitro* and *in vivo*. However, the requirement of exogenous substrate limited the utility of such strains, especially for *in vivo* experiments. For this study, they engineered *M. ulcerans* strains that express the entire luxCDABE operon and therefore are autoluminescent due to endogenous substrate production. The selected reporter strain displayed a growth rate and virulence similar to the wild-type parent strain and enabled rapid, real-time monitoring of *in vitro* and *in vivo* drug activity, including serial, non-invasive assessments in live mice, producing results which correlated closely with colony-forming unit (CFU) counts for a panel of drugs with various mechanisms of action. Their results indicated that autoluminescent reporter strains of *M. ulcerans* are exceptional tools for pre-clinical evaluation of new drugs to treat BU due to their potential to drastically reduce the time, effort, animals, compound, and costs required to evaluate drug activity. Meanwhile the construction and evaluation of only a single autoluminescent *M. ulcerans* strain is a clear limitation of this approach.

4.4. Rat model

[Singh et al., \(1984\)](#) proposed a rat model: Multimammate rats (*Mastomys natalensis*) that rapidly develop systemic infections. However the infected rat model are short-lived after systemic infection and die suddenly.

4.5. The cynomolgus monkey model: a non- human primate

Laboratory rats and mice develop skin lesions after intradermal injection of *M. ulcerans* suspension, but lack the widespread ulceration usually found in human disease. Multimammate rats (*Mastomys natalensis*) rapidly develop systemic infections and die ([Singh et al., 1984](#)). In the mouse footpad, *M. ulcerans* multiply, but necrosis without ulceration destroys the limb and causes death ([Addo et al., 2005](#); [Read et al., 1974](#); [Pattyn and Royackers, 1965](#)). Guinea pigs develop inflammatory lesions at the sites of inoculation that habitually resolve without formation of ulcer ([Read et al., 1974](#); [Kreig et al., 1974](#)). The nine-banded armadillo is susceptible to *M. ulcerans* and develops cutaneous lesions after experimental intradermal infection, approximating those of the human disease, but is phylogenetically distant ([Walsh et al., 1999](#)). The cynomolgus monkey has shown to be susceptible to *M. tuberculosis*, and to some degree, *M. leprae*, and it is usually used in pre-clinical drug and vaccine studies ([Walsh et al., 1996](#)). For the first time, [Walsh et al., \(2007\)](#) reported infection of a non-human primate experimentally with *M. ulcerans*. The cynomolgus monkeys showed to be mildly prone to infection and present some clinical and histologic characteristics close to that of BU in human. Meanwhile, further assessment of *M. ulcerans*

strains from various geographical locations as well as different size of inoculum are required for a good definition of the cynomolgus model (Walsh et al., 2007).

4.6. Anole lizards (*Anolis carolinensis*)

To test whether herpetofauna could be a laboratory model for *M. ulcerans* infection, Marcus et al., (1974) inoculated 21 anole lizards subcutaneously with viable *M. ulcerans*, 21 with autoclaved organisms, and 14 with an aqueous solution of 0.01% Tween 80. *M. ulcerans* was recovered in culture from the slowly progressive lesions which developed at the inoculation site in lizards receiving the viable bacteria. Progressive lesions did not occur in the two control groups. Three patterns of inflammatory response to viable *M. ulcerans* were observed: 14 lizards developed a diffuse, granulomatous reaction in which acid fast bacilli (AFB) were predominantly intracellular; 1 developed focal, encapsulated granulomas; 5 developed a diffuse, necrotizing granulomatous response in which most AFB were extracellular-similar to the characteristic lesion found in human infections (Marcus et al., 1974). This uncommon model mimics the natural occurring features of Buruli ulcer disease and may be suitable to study the pathogenesis or susceptibility of new drug.

Though many BU animal models namely mice, rats, guinea pigs, grasscutters, armadillos, lizards and monkeys have been proposed, they fail to reproduce the full spectrum of BU features presented in humans (Addo et al., 2006; Kreig et al., 1974; Read et al., 1974; Pattyn and Royackers, 1965; Dodge, 1964; Clancey, 1964; MacCallum et al., 1948); consequently, the search for an appropriate BU animal model needs to continue.

Conclusion and Perspectives

The discovery and development of new drugs against *M. ulcerans* are severely impeded by its very slow growth. In the present review, several methods described to test anti-BU activity *in vitro* and *in vivo* have been presented and their limitations and advantages discussed. Short and long term *in vitro* screening systems are available but their use as drug susceptibility test are subjected to many intrinsic or extrinsic considerations that vary according to the environment such as the sensitivity, reproducibility and simplicity of testing systems, their automatability in either medium or high throughput format, the required facility, cost and expertise to operate each systems. As well, a variety of BU animal models especially rodents have been developed for evaluation of anti-BU products. Although most of them use simple protocols, they all require expertise, well trained personnel and laboratories with good facilities to keep the animals and expensive equipment used in routine work. The sensitivity, repeatability and range of reaction to common anti-BU drugs should be considered in the selection of the BU model. Besides the rate of administration, technical complexity, available amount of the tested substance and the cost of each test are other practical requirements that should be considered in addition to the natural resistance and immune competence of the host that can impact the effectiveness of test products in different models. Meanwhile, all available BU models cannot be entirely foretelling for humans. High priority should be given to new assays that facilitate and accelerate the accurate assessment of the drug response, and

the development of novel anti-Buruli ulcer compounds. The relative ease, with which some of the new *in vitro* drug-sensitivity assays can be used, together with their ready availability, could lead to a renewed interest in this essential research field. Given the limited resources for Buruli ulcer control measures and drug development, the considerate use of available resources is essential. The newest generation of drug-sensitivity assays might provide one step in this direction.

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