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A Rapid Method for Estimation of the Efficacy of Potential Antimicrobials in Humans and Animals by Agar Diffusion Assay

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Abstract

Drug resistance continues to challenge traditional antimicrobial drugs and limit their clinical utility. This requires us to continue our search for new drug candidates with novel mechanisms of action against infectious diseases. We now describe a simple agar diffusion assay, which can be used as a general method for the rapid detection of antimicrobial activity of drug candidates in animal or human blood plasma for the ultimate prediction of the efficacy of potential drugs prior to clinical trials. We present an example for a clinical candidate against *Mycobacterium tuberculosis*.

Graphical Abstract

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Keywords

Tuberculosis; macozinone; antimicrobial activity; drug candidate; agar diffusion assay; clinical trials

Introduction

Despite striking reductions in infectious disease burden over the past century, the search for new treatments and prevention procedures is necessary because morbidity and mortality remain strikingly high especially in developing countries.^[1] Microbial pathogens remain an enormous threat to human health, causing millions of deaths and new cases annually.^[2] The wide prevalence of drug resistant microbial pathogens and, consequently, the low

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Conflict of Interest

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effectiveness of treatment with traditional antimicrobial drugs require the continued development of new antimicrobials with novel mechanisms of action.^[3]

As a pertinent example, tuberculosis (TB) is known to cause around 1.7 million deaths and the emergence of 10 million new cases of disease each year, which is largely due to the development of multi drug resistant *Mycobacterium tuberculosis* strains and, consequently, the low effectiveness of treatment with traditional anti-TB drugs.^[4,5] Drug resistance to front- and second-line therapies continues to spread with the number of reported multi-drug resistant cases being 490,000.^[6] Most concerning are the reports of infections resistant to all known drugs. In stark contrast, the only two approved drugs in the last four decades for tuberculosis are bedaquiline (approved by the FDA in 2012) and delamanid (approved by the EMA in 2014). Paradoxically, this global health pandemic is demanding new therapies when resources and interest are flat if not waning. Academic groups are still challenged by tuberculosis also because of its complex biology and by the hurdle of gathering sufficient resources (both monetary and facility-driven due to the requirement for BSL3 labs to safely work with the infectious agent).^[7] Finding a viable clinical drug from target-driven drug discovery has also been very difficult and at the same time so have attempts to evolve a hit from high-throughput whole-cell phenotypic screens.^[3,8–10]

Macozinone (PBTZ169) is one of the most promising potential TB drugs targeting DprE1, the enzyme responsible for the transformation of decaprenylphosphoryl- β -D-ribose into decaprenylphosphorylarabinose, which is a precursor of a fundamental component of the cell wall arabinan.^[11] Macozinone is highly active against *M. tuberculosis* and *M. smegmatis* with MIC values of 2.3 nM and of 9.2 nmol, respectively^[12] and is currently in a Phase II clinical trial <https://clinicaltrials.gov/ct2/show/NCT03334734>.

Here we describe a new method for the detection of antimicrobial activity of drug candidates in animals or human plasma by an agar diffusion assay by using as the example of the antitubercular agent macozinone.^[12,13] The opportunity of using the agar diffusion assay for detection of antimicrobial activity of drugs was reported.^[14–16] The antimycobacterial activity of clinical blood plasma samples of healthy volunteers after macozinone administration by agar diffusion assay is demonstrated. This approach may serve as a simple, cost effective and convenient tool for prediction of the efficacy of potential antimicrobial drugs.

Methods and materials:

Drug.

Macozinone (PBTZ169), (2-[4-(cyclohexylmethyl)piperazine-1-yl]-8-nitro-6-(trifluoromethyl)-4*H*-1,3-benzothiazine-4-one hydrochloride).

Blood plasma samples collection.

Clinical blood plasma samples of healthy volunteers were obtained in a Phase 1 clinical trial “An open prospective non-comparative clinical study of the safety, tolerability and pharmacokinetics of the preparation PBTZ169” <https://clinicaltrials.gov/ct2/show/>

[NCT03036163](#). The time points for collecting blood plasma were 1, 3, 5, 7 hours after oral taking 160 mg of PBTZ169.

Bacteria and media.

Mycobacterium smegmatis mc²155 was used to study the antimycobacterial activity of the clinical blood plasma samples of healthy volunteers after macozinone administration. To obtain a lawn of *M. smegmatis* on Nutrient broth E agar ('Lab M', UK) bacilli from frozen stocks were grown at 37°C on an orbital shaker (200 rpm) in 100 ml flasks containing 20 ml of the Nutrient broth supplemented with 0.05% Tween-80 for 24 h, until the exponential growth phase was reached (about 10⁸ CFU/ml).

Agardiffusion assay.

10⁶ CFU of *M. smegmatis* culture were placed on individual Petri dishes, containing 15 ml of Nutrient broth agar and spread over the surface of the medium to obtain the lawn. An aliquot of each plasma sample (100 µl) was spotted into the well onto the agar surface (diameter = 9 mm) and dried for 6–8 hours, then incubated at 37°C for 36 h to let bacilli grow. After incubation at 37°C, the lysis zones were visually detected and measured. The lysis zone is a result of the diffusion of the drug from plasma samples into agar and its size correlates with antibacterial activity of plasma with a solution limit of 9 mm, which corresponds to the diameter of an empty well.

Results

Macozinone has a comparable activity *in vitro* against *M. smegmatis* mc²155 and *M. tuberculosis* H37Rv with MIC values of 4 ng/ml and of 1 ng/ml, respectively. [12,13] Consequently, *M. smegmatis* can be used as a model organism for testing antimycobacterial activity of macozinone in clinical plasma samples because of its lack of pathogenicity and short generation time. [17] As a negative control, blank human blood plasma of healthy volunteers (no drug exposure) was used. As expected, no activity against *M. smegmatis* was shown after spotting 100 µl of a blank plasma sample onto nutrient broth agar containing 10⁶ CFU bacilli (Fig. 1A). Macozinone dissolved in blank human blood plasma in the concentration range 1.6 – 200 ng/ml revealed dose-escalating antimycobacterial activity inhibiting *M. smegmatis* growth (Fig. 1B).

The dependence of the lysis zone diameter on macozinone concentration onto the *M. smegmatis* lawn was characterized by a logarithmic character that allowed us to produce a corresponding calibration curve (Fig. 2).

To elucidate whether clinical blood plasma samples of healthy volunteers possess antibacterial activity after administration of 160 mg of macozinone, the samples obtained from the Phase 1 clinical trial at various sampling times (1, 3, 5, 7 hours after taking the drug) were placed on nutrient broth agar containing 10⁶ CFU of *M. smegmatis* cells (100 µl). The human blood plasma samples from this study were found to form visually detected lysis zones on the *M. smegmatis* bacterial lawn caused by diffusion of tested compound from plasma samples into nutrient broth agar, and the size of the lysis zone clearly correlated with the sampling time (Figure 3). Interestingly, the maximum antibacterial activity in blood

plasma samples corresponded to 1 hour after administration of 160 mg of macozinone. Blood plasma samples of healthy volunteers possessed antibacterial activity against *M. smegmatis* up to 7 hours after oral administration of 160 mg of macozinone (Figure 3).

Discussion

The rapid detection of efficacy of antimicrobial drug candidates would be ideal along with saving costs on animal and humans clinical trials and pharmacokinetic studies. The direct testing of antimicrobial activity of blood plasma samples using the agar diffusion assay is based on the migration of the drug from plasma into agar and subsequent inhibition of the bacterial lawn growth. This provides an opportunity for the quick estimation of antibacterial activity of blood plasma after administration of a drug candidate in any species.

M. smegmatis known to represent a model organism for the study of *M. tuberculosis* and other mycobacterial pathogens in *in vitro* experiments due to its convenient handling and short doubling-time.^[18,19] What is more, macozinone is known to have a comparable activity *in vitro* against *M. tuberculosis* and *M. smegmatis*, this fact allows non-pathogenic fast-growing *M. smegmatis* to be a model organism in preclinical studies of this drug. We have demonstrated that exploiting the agar diffusion assay allows detection of the concentration of macozinone in blood plasma samples from healthy volunteers after oral administration of 160 mg of macozinone with limit of drug detection 12.5 ng/ml. The maximum antimycobacterial activity was detected at 1 hour after taking the drug that is in accordance with pharmacokinetic analysis data obtained in a Phase 1 clinical trial <https://clinicaltrials.gov/ct2/show/NCT03036163>.

Blood plasma samples of healthy volunteers possessed antibacterial activity against *M. smegmatis* for up to 7 hours after oral administration of 160 mg of macozinone that demonstrated its stability in human plasma. Moreover, for the first time we clearly demonstrated the activity of a volunteer's plasma samples turned over to a higher extent than was expected based on bacterial lysis zones data of macozinone titration (Fig 1). Drug candidates are known to be metabolized in living organisms with the formation of a wide variety of active and non-active metabolites, consequently, the actual efficacy of a drug candidate may be represented by the activity of the compound itself and its metabolites. Indeed, further investigation of macozinone properties showed that it is metabolized into a series of active metabolite *in vivo* that is the likely explanation of the inconsistency between bacterial zone of inhibition for titrated macozinone and plasma samples^[20]. Thus, enlarged zones of inhibition of a volunteer's plasma samples after 160 mg dosing demonstrated the additive/synergistic activity of macozinone and its active metabolites, and its high bioavailability. The results obtained in this way anticipate the therapeutic efficacy of macozinone which has recently completed a Phase II clinical trial and pharmacokinetic studies (unpublished data). Consequently, this easy and quick procedure could be used for direct testing of the activity of prospective drug candidates in blood plasma of humans/ animals for other bacteria.

Conclusions

In conclusion, we demonstrate that the antimicrobial efficacy of drug candidates in animals and humans may be rapidly detected by the agar diffusion assay using corresponding blood plasma samples. The agar diffusion assay provides an opportunity to visualize a therapeutic effect of potential antimicrobials prior to clinical trials. This simple approach could be implemented in resource limited environments to perform important preliminary work prior to more expensive animal or human studies. While we have demonstrated this approach for a drug used against *M. tuberculosis*, the same approach could also be applicable elsewhere. If we are to address the increasing drug resistance against antibiotics we should leave no stone unturned in our efforts to find new drugs and that includes using simpler approaches to testing new molecules and visualization of the biological effect.

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Figure 1.

A dose-escalating activity of macozinone dissolved in blank blood plasma of healthy volunteer against *M. smegmatis* in agar diffusion assay. A – blank plasma, B – the range of exact amount of macozinone (1.6 – 200 ng/ml) dissolved in blank plasma.

Figure 2.

Logarithmic dependence of lysis zones' diameter on macozinone concentration in blank plasma samples on to *M. smegmatis* lawn in agar diffusion assay.

Figure 3.

The lysis zones diameter of the volunteers' blood plasma samples in *M. smegmatis* lawn are dependent on the sampling time went after oral administration of 160 mg of macozinone. Antibacterial activity of plasma samples was tested using 2-fold serial dilutions from neat to 1/8. The experiments were performed in triplicates; a typical experiment was shown.