Development of an *In Vitro* Model to Identify *Mycobacterium tuberculosis* DNA Gyrase Inhibitor Compounds

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Abstract

According to the World Health Organization (WHO), tuberculosis (TB) causes more than 1.4 million deaths a year. Tuberculosis drugs resistance constitutes a first magnitude public health problem, which threatens the success of the strategy to detect and cure TB recommended by WHO and constitutes an obstacle to the fight against disease worldwide. The search for new antibiotics to overcome the increasing spread of multidrug-resistant tuberculosis (MDR-TB), can be approached from several directions: identification and validation of novel targets, exploration of new chemical series or modification of existing drug classes, to eliminate possible cross-resistances. The development of *in vitro* models that allow us to identify and characterize DNA gyrase inhibitors in mycobacteria, is particularly interesting in the context of antimicrobial therapy with particular interest for those compounds whose DNA gyrase binding site is different from corresponding to moxifloxacin (Phase III clinical trials) and therefore can be expected to be active against fluoroquinolone-resistant strains. In this work we will address the assay development and validation of an *in vitro* model that allows the identification of new inhibitors of *Mycobacterium tuberculosis* DNA gyrase. We propose to test a set of 177 compounds from GSK collection, built up from the results of a phenotypic screening campaign against *Mycobacterium bovis* BCG and subsequently successfully confirmed in *Mycobacterium tuberculosis* H37Rv. These compounds were identified and made available to the scientific community to maximize the potential impact thereof as tools for target validation, while at the same time provides possible starting points for new synthesis routes for lead generation.

Key Words: *Mycobacterium tuberculosis*, DNA Gyrase, Inhibitor, Electrophoresis, Probe.

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Introduction

Tuberculosis (TB) is an infectious disease caused by the bacillus *Mycobacterium tuberculosis* (*Mtb*) and causes the death of 1.4 million people each year. This disease typically affects the lungs (pulmonary TB) and is spread from person to person through the air by coughing, sneezing, or even talking. It is estimated that about one third of the world population is infected by *Mtb* [1].

Mtb's unique cell wall, which has a waxy coating primarily composed of mycolic acids, allows the bacillus to lie dormant for many years. The body's immune system may restrain the disease, but it does not destroy it. While some people with this latent infection will never develop active TB, five to 10 percent of carriers will become sick in their lifetime [2]

People living with HIV are around 21-34 times more likely to develop TB than persons without HIV, since one accelerates the progress of the other. The risk of TB is also higher in people whose immune system is compromised due to other causes such as malnutrition, diabetes or smoking.

More than 95% of the cases and deaths caused by TB occur in developing countries. Without an adequate treatment up to two-thirds of people with the disease of tuberculosis will die. The current antitubercular medicines inhibit cell processes. These agents can be classified in: Protein synthesis inhibitors, Nucleic acid synthesis inhibitors and Energy production inhibitors [3].

These are long and difficult to administer treatments besides they vary depending on the seriousness of the disease. There are various treatment options, depending on the type of tuberculosis (Table 1) [4].

Type	Description	Treatment	Problems
SAT	Sensible Active Tuberculosis.	6 months duration. Four antimicrobial agents: Isoniazid, rifampicin, streptomycin y pyrazinamide.	Adherence to the treatment Resistances.
MDR-TB	Resistance to isoniazid and rifampicin.	8-10 combination drug therapies lasting 18-24 months.	Prolonged, expensive and toxic.
XDR-TB	Resistant to all fluoroquinolones and at least one or the three injectable second-line drugs (capreomycin, kanamycin y amikacin).	Very limited treatment options and difficult to acquire in resource poor environments.	Side effects, such as nephrotoxicity and ototoxicity.
LTBI	Asymptomatic and non-infectious, immune system restricts the growth of *Mtb*.	9 months of isoniazid preventive therapy.	Adherence to the treatment Toxicity.

Table 1. Antitubercular treatments. **SAT** (Sensitive Active Tuberculosis), **MDR-TB** (Multi-drug-resistant tuberculosis), **XDR-TB** (Extensively-drug-resistant tuberculosis), **LTBI** (Latent tuberculosis infection).

The current situation immediately requires the identification of new medicines which can solve the problems of current treatments and be effective and safe. This search can be focused towards two different directions: identification and validation of novel targets and exploration of new chemical series or modification of existing drugs.

DNA gyrase as a pharmacological target

DNA gyrase is a type II topoisomerase found in all bacteria, but it is not present in both humans and animals. It plays an important role in DNA replication. This makes it a good target for antibacterial chemotherapy.

DNA gyrase generates negative supercoils for the entire bacterial chromosome. This relaxes the positive supercoils that transcription generates ahead of the translocating RNA polymerase, which results in a condensed chromosome for proper partitioning during cell division. It is a tetramer composed of two A subunits, where the DNA binding domain is located, and two B subunits with ATPase activity, which catalyze reactions that transiently cleave two DNA strands by a process dependent on ATP hydrolysis (Figure 1).

The mechanism [5] starts when the DNA fragment (G-segment) binds to the A subunit. Then, two ATP molecules bind to the active site of B subunit, which causes a conformational change that allows the entry of a second fragment of DNA (T-segment). After that, the G-segment is cleaved and subsequently binds to the enzyme by a covalent bond. T segment then passes through the resultant split, generating a supercoil. Finally, the enzyme reseals both ends that were generated after cleavage of G-segment for subsequent release of the T and G fragments.

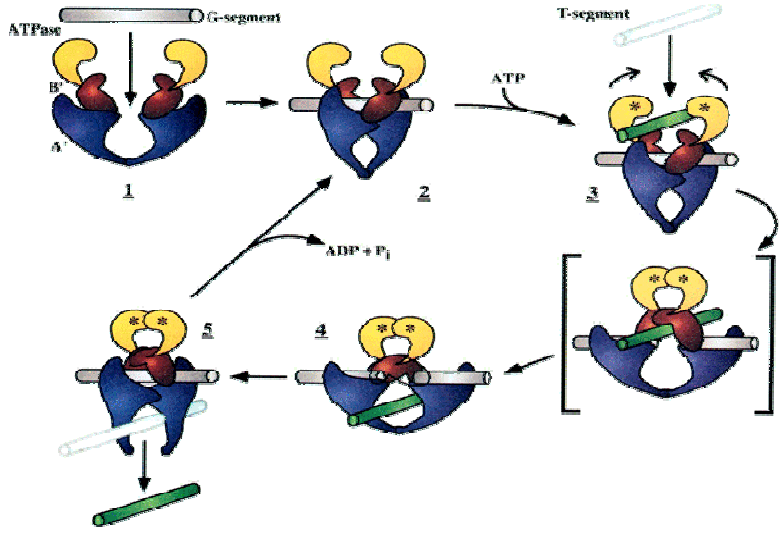


Fig.1. DNA Gyrase Mechanism of action [6].

Most drugs that target DNA gyrase such as quinolones [7], which also includes fluoroquinolones, either inhibit the DNA gyrase activity stabilizing the complex formed between the enzyme and DNA when cleavage occurs or blocking the active ATP binding site. DNA gyrase inhibitors that have a mechanism of action different from existing drugs would be effective in both sensitive tuberculosis and drug-resistant tuberculosis.

Mutations in DNA gyrase have been associated with antibiotic resistances [8]. These mutations cause a decrease of permeability of cell wall for drugs, drug sequestration and inactivation of the active compounds of the drug. Hence, it is very important to find and characterize novel *Mtb* inhibitors in order to ensure the success of future inhibitors.

Hypothesis and objectives

We intend to develop an in vitro model to detect *Mtb* DNA gyrase inhibitor compounds to facilitate the identification and characterization of new antitubercular drugs. The main objectives are the set-up of the commercial kit "*Mycobacterium tuberculosis* Gyrase Supercoiling Assay" and validate this kit by using known inhibitors, as well as the identification of selective inhibitors of *Mtb* DNA gyrase from a set of 177 compounds from GSK compound collection. This set was built based on the results of phenotypic screening campaign against *M.bovis* BCG and subsequently confirmed successfully against *M.tuberculosis* H37Rv.

Materials and Methods

To carry out the supercoiling assays, we used the commercial kit *"Mycobacterium tuberculosis Gyrase Supercoiling Assay kit"* from Inspiralis (MTS001 y MTS002). This kit contains *Mtb* DNA gyrase, assay buffer (5X) and dilution buffer. The substrate we add to the kit for the reaction to occur, is the relaxed plasmid pBR322 (R5003) from Inspiralis with 4361 base pairs. The reaction consists of 1 U of *Mtb* DNA gyrase which will supercoil 0.5 µg of relaxed DNA pBR322, in a reaction volume of 30 µl at 37º C for 1 h in the assay buffer.

DNA supercoiling

The supercoiling capacity of *Mtb*DNA gyrase is measured by using 1% (w/v) agarose gels (100 mL of TAE for 1g of agarose LE) without Ethidium bromide. In our case, 20 µl of reaction with *Mtb* DNA gyrase was mixed with 5 µl of stop solution [50% (v / v) glycerol containing 0.25% (v / v) bromophenol blue]. Previously prepared samples were resolved on a 1% agarose gel using electrophoresis equipment (Pharmacia LKB Power GPS-200/400) at 20V for 16h. Thereafter, we stained with a probe for 30 minutes. Then proceed to the image and analyze the gel using the BioRad ChemiDoc ™ MP System that is an equipment specifically designed for documentation and analysis of banding patterns. The conditions used to capture the image are: epi long wave UV, 1.38 MPixel (1x1 bin) with EtBr/uv filter.

We will discuss the set-up of the assay in the results section, which will include the selection of the quantity of enzyme and plasmid, assay volume optimization, the voltage needed and selection of the best probe for processing.

Test of the GSK set of compounds

This platform is set-up by measuring the inhibitory potential of a group of 177 compounds from the GSK compound collection over the *Mtb* DNA gyrase, which have been identified as H37Rv inhibitors and non-cytotoxic compounds [9].

Data analysis

The data obtained from the experiments, were statistically analyzed using Microsoft Excel, Grafit and Image Lab programs. These data were normalized with respect to the minimum and maximum activity controls included in this experiment (Equations 1 and 2) [10].

$$\% \text{ Inhibition} = 100 - 100 * \left[\frac{\text{data} - \text{control 2}}{\text{control 1} - \text{control 2}}\right]$$

Equation 1. Inhibition percentage equation. **Control 1**: Maximum enzyme activity. **Control 2**: Absence of enzyme.

$$y = \frac{Range}{1 + \left(\frac{x}{IC_{50}}\right)^{s}} + Background$$

Equation 2. IC_{50} equation (four parameters)

Results

Set-up and validation of the commercial kit "*Mycobacterium tuberculosis* Gyrase Supercoiling Assay"

Selection of the enzyme and plasmid concentration

First of all, to optimize the amount of enzyme the amount of substrate was kept constant at 0.5µg. We performed a serial dilution of the enzyme (starting at 2U/mL, eight points and dilution 1/2). In the results we can see that the lanes 5 and 6 (Figure 2) are quite similar to Control 1 (maximum enzyme activity), so 0.1 U/µL of enzyme are sufficient for the reaction is completed correctly, which is three times higher than the concentration recommended by the supplier.

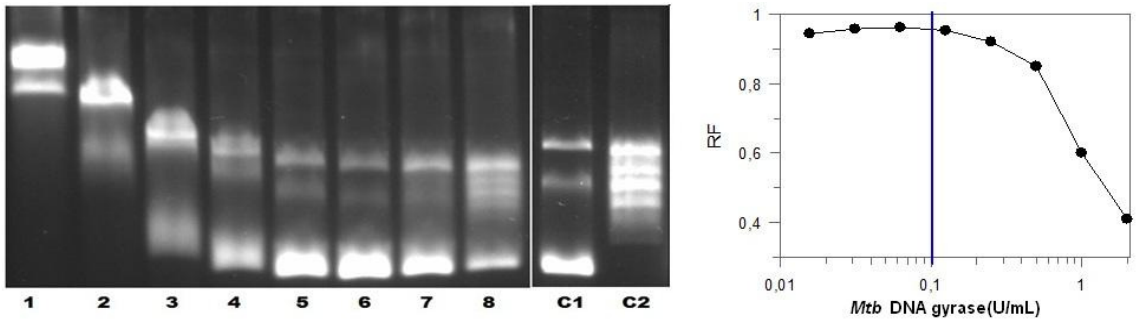


Fig 2. Enzyme dose response. RF= Relative Front

Thereafter, we optimized the amount of plasmid by keeping constant the amount of enzyme at 0.1 U/µL, we carried out a dose response of the relaxed plasmid pBR322 (from 0.08 µg/µL, seven points and dilution 1/2). Likewise it is observed that the lanes 2 and 3 (Figure 3) are similar to the control 1, so we conclude that 0.03 µg/µL of relaxed plasmid will be sufficient to allow the reaction, which means a plasmid concentration two times higher than the commercial kit supplier recommendation.

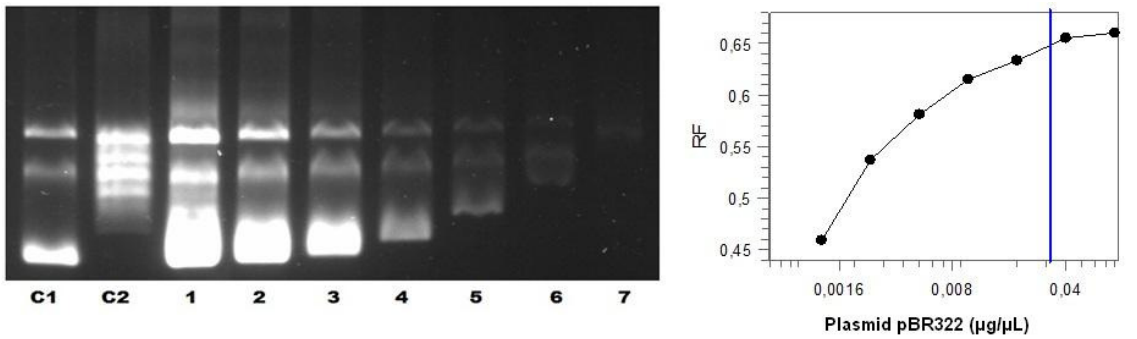


Fig 3. Plasmid dose response. RF= Relative Front

Optimization of the reaction volume

To successfully complete the *Mtb*DNA gyrase reaction , we should take into account the ratio among the various components of the enzyme solution and substrate solution, since some of these components present in the assay buffers, as the cation Mg^{2+} or DTT, may be necessary for the course of the reaction.

To solve this issue, we performed three experiments at different conditions of addition. We kept constant the enzyme solution concentration at 0.1U /µL and the substrate solution concentration at 0.03 µg /µL in the final reaction volume. In the first assay, were added 10µL of enzyme solution plus 10 µL of substrate solution, in the second case were added 2.5 µl of the enzyme solution plus 22.5 µL of substrate solution and in the third case 2.5 µl of the enzyme solution plus 17.5 uL substrate solution.

From the obtained results, it is concluded that the reaction elapses best using 2.5 µL of enzyme solution plus 17.5 µL of substrate solution. The same proportions recommended by the supplier but with a 30% reduction in the assay volume.

Agarose gel electrophoresis set-up

To obtain well-defined bands we optimized both the electrophoresis voltage and fluorescent probes for DNA quantification.

Voltage optimization for electrophoresis of DNA gels.

To select the voltage at which DNA gel electrophoresis is carried out, four experiments at reaction conditions described above were carried out using different voltages: 100, 75, 50 and 25V respectively. In this way we see that we obtain the best band definition and resolution after an electrophoresis at 25V for 12 h, which facilitates the subsequent image analysis of the band patterns.

Optimization of the gel stain.

Ethidium bromide (EtBr) is the marker commonly used in molecular biology laboratories but it is a substance with mutagenic effects and because it is a DNA intercalator, we decided to try 6 commercial stains in order to work with the safer one (Table 2).

Sonda	Hazardous/ Environmentally-Friendly	Sensitivity	λ EXCITATION	λ EMISION
Ethidium Bromide	YES/YES	1ng	300/520 nm	600 nm
SYBR® Safe	Low/NO	3ng	280/502nm	546 nm
SYBR® Gold	Low/NO	>25 pg	300/495nm	537nm
SYBR® Green I	Low/NO	>60 pg	290/380nm	520nm
GelGreen)	NO/NO	-----	500nm	540nm
GelRed	NO/NO	-----	280nm	590nm
Diamond™	NO/NO	> EtBR	494nm	558nm

Table 2. Comparative table of the different probes tested to reveal agarose gels [11].

Six gels were made under the same reaction conditions set forth in the materials and methods section. Each of these was stained with one of the 6 different probes, 40 µL of probe (stock 10^4x) in 400 mL of TAE buffer for 30 minutes. According to the results we can appreciate that the probe that provides the best image of the banding pattern is SYBR® Safe (Figure 4B).

Respective reading protocols were used for each probe based on its own properties and additionally another reading protocol was used for all of them: Alexa 546 [12] (Figure 4A).

The SYBR® Safe probe reading protocol excites at 300nm, at this wavelength the probe has an excitation peak, but lower than the one presented at 502nm. For this reason, the reading protocol Alexa 546 provide us a better defined image of the banding pattern, since it excites at λ = 502 nm and collects emission at λ = 546 nm.

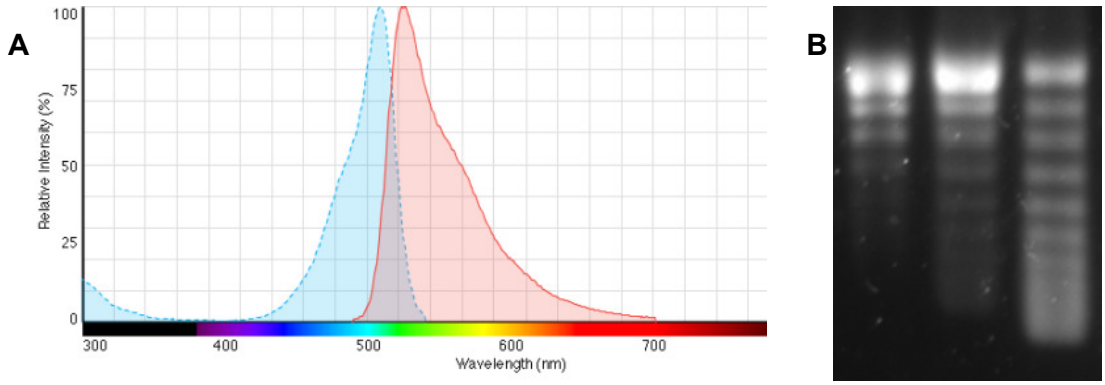


Figure 4. (A): Emission/excitation spectrum of SYBR® Safe DNA Gel Stain [13]. (B): Image of the agarose gel stained with SYBR® Safe DNA Gel Stain and reading protocol Alexa 546.

Assay validation

The next step is to validate the assay using a known *Mtb* DNA gyrase inhibitor, Novobiocin, which has been shown to be a competitive inhibitor that binds to the ATP pocket of GyrB [14]. The compound was tested using a serial dilution in DMSO (10 points, with a dilution factor 1/3) starting at 100 µM, with 0.03µg/mL of relaxed plasmid pBR322 and 0.1U/uL of *Mtb* DNA gyrase in a total reaction volume of

20 µL. Control 1(maximum enzymatic activity) and control 2 (absence of enzyme) were included. After that, the reaction was incubated at 37 °C, and samples were resolved on an agarose gel.

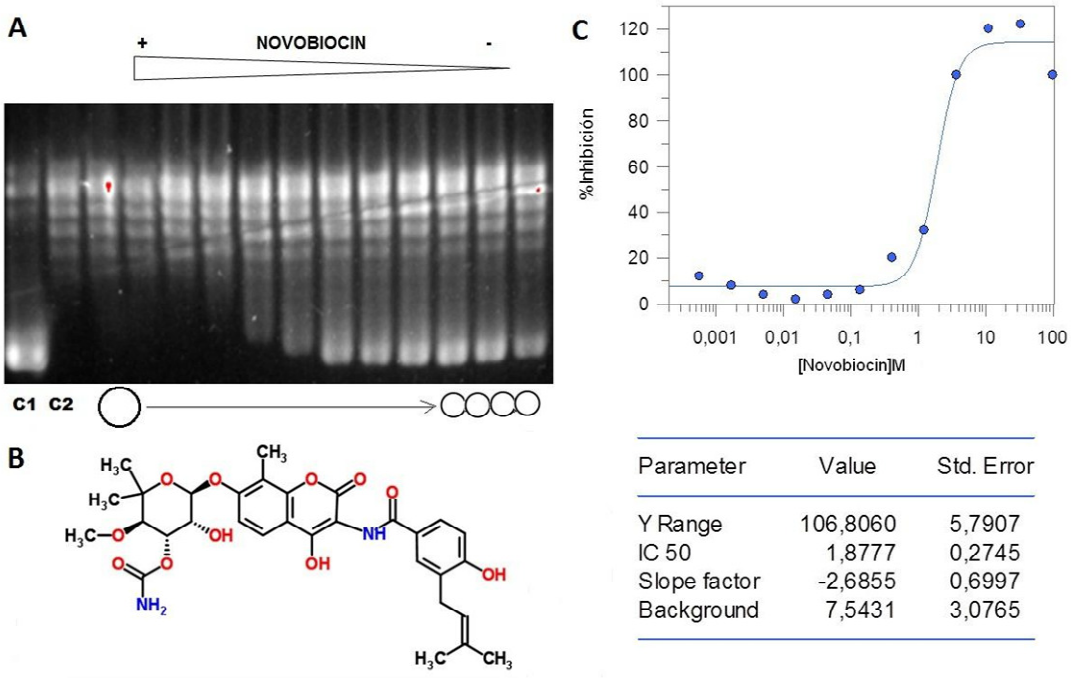


Figure 5. (A).Bioimage of the agarose gel. C1(control 1, maximum enzyme activity). C2 (control 2: absence of enzyme activity) and a serial dilution of Novobiocin. (B) Novobiocin structure [15]. (C) Novobiocin IC50 based on the gel quantification.

Then, the gel was stained with SYBR® Safe DNA Gel Stain for 30 minutes to reveal the bands. Bands were quantified using the Image Lab program. Finally, the data obtained were normalized with respect to control 1 and control 2, using the Equation1, to quantify the degree of supercoiling in the presence of different concentrations of inhibitor. Once we had the normalized data, we applied the equation 2 to obtain the value of IC50 of Novobiocin (Figure 5).

Platform

Set-up of the platform for the identification of *Mtb* DNA gyrase inhibitor compounds by using the TB set.

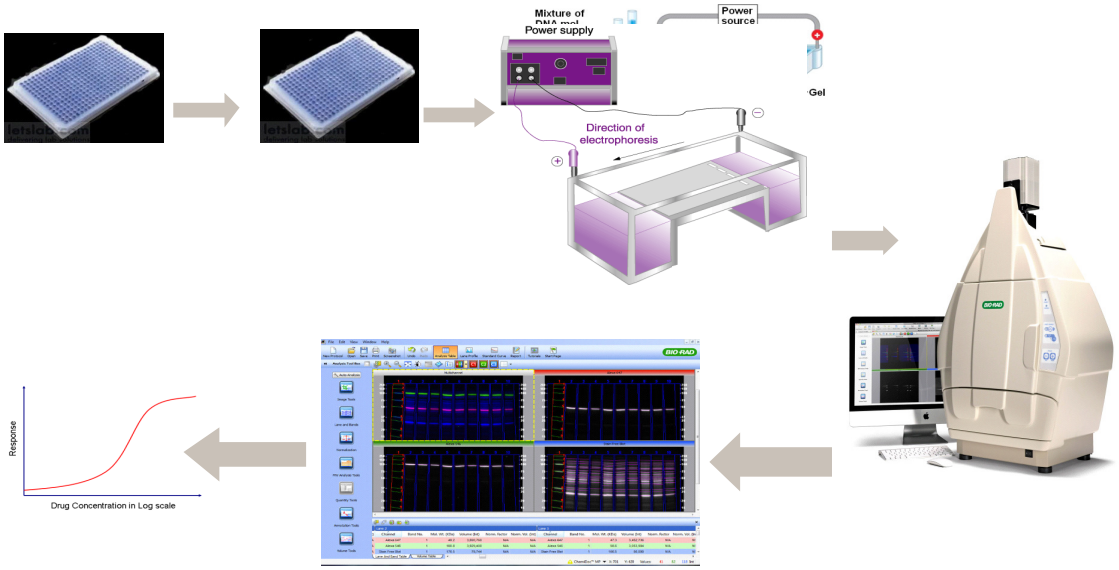


Figure 6. Platform

To be able to measure the activity of *Mtb* DNA gyrase in a large number of compounds, we developed a semi-automatic platform to measure DNA supercoiling using pipettors (Multidrop-Combi and 16-channel multichannel pipetts), as well as use of barcode on the 384 multiwell plates and on the agarose gels to maintain the data integrity, besides an automated data processing (Figure 6).

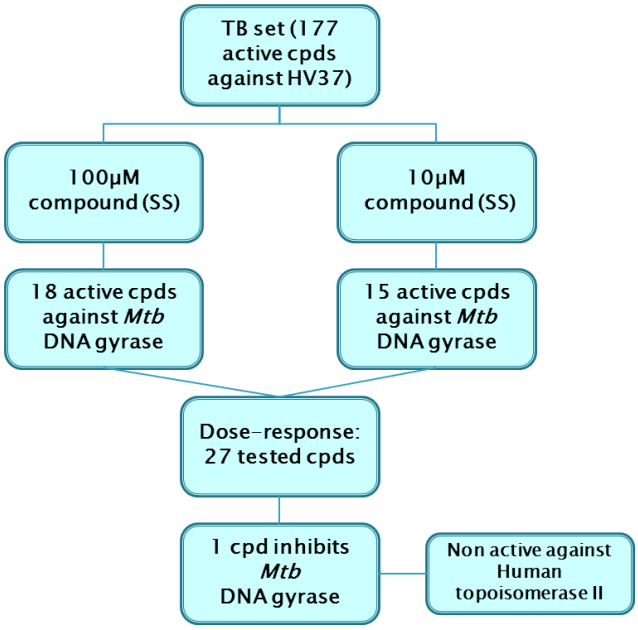
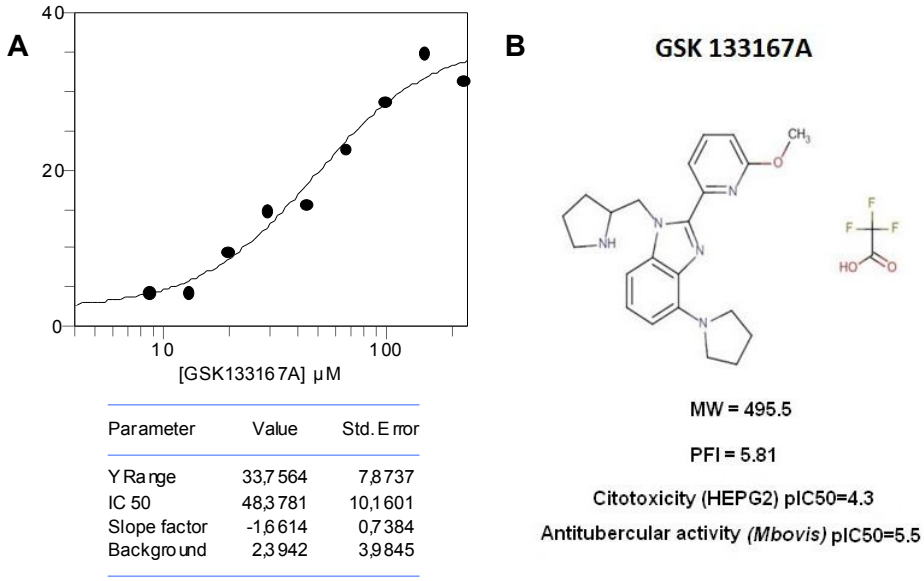


Figure 7.TB set assay. SS: Single shot. Cpds: compounds

Compounds were tested in separate experiments at 100 µM and at 10 µM. First 2.5µL of enzyme solution (0.1U/µL of *Mtb* DNA gyrase) was added to each well that containing 200nL of a 10mM GSK compound solution in DMSO. Then 17.5 µL of substrate solution (0.03 µg/ µL pBR322) was added. It was incubated at 37ºC for 1h and finally the reaction was stopped with 5 µL of stop solution to subsequently apply the samples to the gel. The electrophoresis was carried out at 25V for 12h and after that the gel was stained with SYBR® Safe for 30 minutes. Reading was performed in ChemiDoc™ MP System using Alexa 546 protocol and the banding pattern obtained was analyzed with the Image Lab software. We obtained a total of 27 compounds (100 µM and/or 10 µM) that inhibited assay.



Parameter	Value	Std. Error
Y Range	33,7564	7,8737
IC 50	48,3781	10,1601
Slope factor	-1,6614	0,7384
Background	2,3942	3,9845

Figure 8 .(A) Dose of GSK 133167A compound, enzyme inhibitor.(B) Properties of GSK 133167A compound.

Thereafter, to confirm the 27 compounds identified in the previous experiments (Figure 7), an additional assay was performed to determine the dose response of each compound, using serial dilutions of each compound in DMSO (starting at 500µM, 11 points, dilution factor of 1/2), and the reaction conditions previously described. After processing the gels, we obtained the result that only one of our potential candidates dose-dependently inhibits the *Mtb* DNA gyrase (Figure 8).

Conclusions

Due to mortality rates of tuberculosis and the emergence of new drug resistance, the discovery of new drugs for the treatment of this disease is necessary. The challenge in developing drugs against tuberculosis is to shorten, simplify and make these treatments highly effective. Therefore, focusing on different ways to find new drugs, we believe that inhibitors of *Mtb* DNA gyrase with a mechanism of

action different from existing treatments could be effective against both sensitive tuberculosis as resistant tuberculosis drugs.

In this work, we have developed an assay that allows us to identify *Mtb* DNA gyrase inhibitors. We have set-up a commercial kit that measures the supercoiling caused by this enzyme. For this, we have determined the optimum reaction conditions, voltage and type of probe needed for the proper course of the experiment, and then we have validated this assay with a known inhibitor.

We have developed a semi-automatic platform for measuring DNA supercoiling using pipettors and barcodes that allow us to maintain better data integrity, besides an automated data processing. This platform allows us to significantly increase the number of compounds to test in agarose gels, both for this test as for any other, with a capability to test tens to thousands of compounds in a single week.

This platform has been set-up using a set of selected compounds from GSK collection, called the TB set (177 compounds with antitubercular activity), which previously have been identified in a phenotipic screening campaign. We have detected a Mtb DNA gyrase inhibitor (GSK133167A). In future, this assay can be a part of strategy to prioritize compounds based on their inhibitory potency and their mechanism of action. Experiments with mutant strains will be required to confirm that the proposed mechanism of action is correct.

As future work, it is intended to test a set of a larger number of compounds, called TB-box (11124 compounds with antitubercular activity) , and those resulting positives will be tested for their inhibitory activity over human topoisomerase II, in order to determine if they are selective against *Mycobacterium tuberculosis.*These compounds could be useful as starting points to develop future drugs for the treatment of tuberculosis.

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References

1. Global tuberculosis report. **2013**. In WHO, World Health Organization. http://www.who.int/tb/publications/global_report/en/
2. The TB pandemic. **2014**. In Global Alliance for TB Drug Development. http://www.tballiance.org/why/the-tb-pandemic.php
3. Huang Y. Y., Deng J. Y., et al. **2006**. The key DNA-binding residues in the C-terminal domain of Mycobacterium tuberculosis DNA gyrase A subunit (GyrA). Nucleic Acids Res. 34 (19): 5650-9.
4. Wai Yew, W., Cynamon, M. and Zhang, Y. **2011**. Emerging drugs for the treatment of tuberculosis. *Review Expert Opinion. Emerging Drugs*. 16 (1): 1-21.
5. Mdluli K., Ma Z. **2007**. Mycobacterium tuberculosis DNA gyrase as a target for drug discovery. *Infect. Disord. Drug Targets*. 7 (2): 159-68.
6. James C. Wang. **2009**. DNA entanglement and the action of the DNA Topoisomerases, *Cold pring Harbor Laboratory Press, Cold Spring Harbor*, NY. 245.
7. Aubry, A. et al. **2004**. *Mycobacterium tuberculosis* DNA Gyrase: Interaction with Quinolones and Correlation with antimycobacterial drug activity. *Antimicrobial Agents and Chemotherapy.* 48(4): 1281-88
8. Ginsburg, A. S., Grosset, J. H. and Bishai, W. R. **2003**. Fluoroquinolones, tuberculosis and resistance. *The Lancet infectious Diseases*. 3: 432-42.
9. Ballell, L., et al. **2013**. Fueling Open- Source Drug Discovery: 177 Small-Molecule Leads against Tuberculosis. *Chem. Med. Chem*. 8: 313-21.
10. Zhang, J. H., Chung T. D. & Oldenburg K. R. **1999**. A simple statistical parameter for use in evaluation and validation of high throughput screening assays .*Biomol Screen.J*. 4: 67-73.
11. DNA Stains. **2014**. In Life Technoligies. http://www.lifetechnologies.com/es/en/home/life-science/dna-rna-purification-analysis/nucleic-acid-gel-electrophoresis/dna-stains.html
12. The Alexa Fluor Dye Series-Note 1.1. **2014**. In Life Technologies. http://www.lifetechnologies.com/es/en/home/references/molecular-probes-the-handbook/technical-notes-and-product-highlights/the-alexa-fluor-dye-series.html
13. SYBR® Safe DNA Gel Stain.2014. In Life Technologies. http://www.lifetechnologies.com/order/catalog/product/S33102

14. Pravin S., Shahul H. **2012**. Nonfluoroquinolone-Based Inhibitors of Mycobacterial Type II Topoisomerase as Potential Therapeutic Agents for TB. Annual Reports in Medicinal Chemistry. 47: 319-28.
15. Novobiocin. **2014**. In Pubchem. http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=23710502