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Title:	**Investigating the Anti-Tubercular Potential of Novel Non-Antibiotic Agents against Drug-Resistant Mycobacterium Tuberculosis**
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Investigating the Anti-Tubercular Potential of Novel Non-Antibiotic Agents against Drug-Resistant *Mycobacterium Tuberculosis*

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ABSTRACT

Background. The ever-increasing prevalence of multidrug-resistant *Mycobacterium tuberculosis* (MDR-TB) presents an alarming challenge to existing tuberculosis (TB) treatment stratgies. Hence, the current study explores the potential of dihydroergotamine and abiraterone acetate, two non-antibiotic compounds, as innovative anti-tubercular agents.

Methods. *In silico* analyses were conducted to identify potential drug targets for dihydroergotamine and abiraterone acetate. Subsequently, these compounds were evaluated for their bactericidal efficacy against both the reference H37Rv strain and an MDR-TB strain of *M. tuberculosis in vitro.* A range of drug concentrations were tested to determine their inhibitory effects.

Results. Both dihydroergotamine and abiraterone acetate exhibited substantial inhibitory activity against *M. tuberculosis*. Dihydroergotamine demonstrated efficacy at higher concentrations (128 µg and 256 µg), while abiraterone acetate exhibited potency at lower concentrations (64 µg, 128 µg, and 256 µg). The observed dose-dependent inhibitory effect emphasizes the importance of optimizing drug concentrations in anti-tubercular therapy.

Conclusion. Both compounds act as potential anti-tubercular agents by effectively inhibiting the growth of *M. tuberculosis*, with abiraterone acetate demonstrating greater potency at lower concentrations. These findings suggest both compounds may be promising candidates for further research and development as potential treatments for tuberculosis.

Keywords: abiraterone acetate, antimicrobial resistance, dihydroergotamine, *in vitro* evaluation, *Mycobacterium tuberculosis*

Highlights

- Novel Anti-TB Agents: *In Vitro* activity of dihydroergotamine and abiraterone acetate against *M. tuberculosis* was investigated using the BACTEC MGIT 960 system and broth macrodilution method.

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- Drug Concentrations: Tested concentrations ranged from 32 µg to 0.25 µg, with the minimum inhibitory concentration (MIC) defined as the lowest concentration with GU < 100, validated against a drug-free control.
- Control Measures: Absolute growth controls were included in every assay to ensure the accurate monitoring of strain growth and assay reliability.

1. INTRODUCTION

Tuberculosis (TB) is a disease that can often be prevented and treated. Despite this fact, by 2022, TB became the second most deadly infectious disease globally, following only the coronavirus disease (COVID-19), and responsible for nearly twice the number of deaths as HIV/AIDS. Annually, TB continues to infect over 10 million individuals [1]. Additionally, infections caused by multidrug-resistant TB (MDR-TB) strains have surged significantly in various regions, such as Africa and Asia. It has been estimated that approximately 500,000 people are infected with MDR-TB annually, with less than half of the treated patients completing the lengthy treatment course necessary for complete remission, leading to high mortality rates [2]. Due to the seasonal nature of the disease, computer models have been developed to improve control over its incidence [3].

One process that truly needs to be looked into is drug repurposing as a novel means for the development of new antimicrobials. Drug repurposing is the discovery of new uses of previously established drugs beyond the scope of their original medical indications [4]. These drugs have already been tested on human beings and are considered safe, effective, and remain pharmacokinetically and toxicologically well-explored. As a result, there exists a comprehensive understanding of the general pharmacology of these drugs, potential routes of administration, and optimal dosing regimens. Repurposing allows some drugs to bypass certain clinical trials, thereby reducing costs and development time. Scientists utilize existing collections of clinical molecules to find new antimicrobial uses for old drugs [5].

Over the past 40 years, only a limited number of anti-TB compounds have received approval for clinical use [6]. Moreover, some promising drugs, such as bedaquiline (a mycobacterial ATP synthase inhibitor), are efficiently expelled by pathogens through bacterial efflux pumps. Consequently, conventional anti-TB therapies are now enhanced with efflux pump inhibitors, such as verapamil, to boost antimicrobial efficacy against pathogens [6]. The small number of molecular scaffolds with anti-infective properties discovered thus far contribute to a rapid increase in antimicrobial resistance [7]. Therefore, ongoing research is focused on identifying new molecular scaffolds with anti-infective activity. Fortunately, the repurposing of anti-infective drugs is now considered a highly promising strategy to develop new treatment options for MDR-TB and extensively drug-resistant TB (XDR-TB) strains, which are responsible for otherwise incurable infections [8]. The primary objective of drug repurposing is to expedite the initial stages of the drug development process. This approach simplifies preclinical research and obviates the need for extensive clinical safety trials, thereby reducing the time and investment required to discover new treatments [9, 10].

Drug-resistant pathogens have become a major threat to public health, posing significant challenges to healthcare

providers. Indeed, the decline in effective antibiotics has led to the emergence of complex, difficult-to-treat infections. There is an urgent need to develop new anti-TB drugs, but this process is both costly and time-consuming. Hence, this study aims to evaluate the antibacterial activity of currently commercially available non-antibiotic drugs against *Mycobacterium tuberculosis*.

2. METHODOLOGY

In this study, the efficacy of two compounds, namely dihydroergotamine and abiraterone acetate, was evaluated *in vitro*. These compounds were previously shown to exhibit inhibitory activity against *M. tuberculosis* through *in silico* analysis [11]. The invitro evaluation followed the previously published protocol [12]. The two *M. tuberculosis* strains (H37Rv and one MDR strain) collected from Provincial TB Reference Lab Peshawar were used for the evaluation of lead compounds. Decreasing concentrations of the selected compounds were prepared and inoculated with *M. tuberculosis* strains. Minimum inhibitory concentration (MIC) was determined after a 14-day incubation period.

2.1. Preparation of Drug Solution

Dihydroergotamine (CAS# 6190-39) and abiraterone acetate (CAS# 154229-18) were purchased in powder form from Macklin, Canada. The stock solution was prepared by adding 2.688 mg of the drug in 1 ml of Dimethylsulfoxide (DMSO). The solution was thoroughly mixed to ensure homogeneity. A two-fold serial dilution of both drugs was prepared in DMSO from the stock solution. Dilutions were carried out to create a range of concentrations for each drug [13] (Table 1). The respective doses of dihydroergotamine and abiraterone acetate were optimized for drug susceptibility testing (DST) [14].

Table 1. Different Concentrations of Drugs Used for *In Vitro* Evaluation

Drug (mg/ml)	Quantity (µl)	Drug Concentration in MGIT tube (µg/8.4ml)
21	100	0.25
42	100	0.5
84	100	1
168	100	2
336	100	4
672	100	8
1344	100	16
2688	100	32
5376	100	64
10752	100	128
21504	100	256

2.2. Preparation of Positive Culture Inoculum

When the MGIT960 instrument signals a positive result, the positive MGIT tube is processed for DST, with Day 0 marking the detection. To promote stronger growth, the tubes were incubated an additional day (Day 1) in a separate incubator set at 37°C. The incubated tube remains viable for DST for up to 5 days, including Day 5. If it surpasses 5 days, then subculturing and re-incubating in the instrument, until it yields a positive result again, is necessary. Processing a culture older than 5 days for DST may lead to less reliable results [15].

2.3. Determination of Minimum Inhibitory Concentrations

The *in vitro* activity of dihydroergotamine and abiraterone acetate against *M. tuberculosis* was determined using the BACTEC MGIT 960 system in conjunction with the broth macrodilution method, with growth monitored at 37°C, as

previously reported [16]. The MGIT tubes were inoculated with 0.8 ml of OADC (oleic acid, albumin, dextrose, catalase), 0.1 ml of the compound at the appropriate concentration, and 0.5 ml of strain suspension [12]. The drug concentrations ranged from 32 µg to 0.25 µg. On the day of testing, two-fold serial dilutions were performed to achieve the desired concentrations. Positive growth in a drug-containing tube (Growth Unit, GU ≥ 100) before the positivity of the proportional growth control (containing a 1/100 dilution of strain suspension) indicated that more than 1% of the bacterial population could grow in the presence of the anti-TB drug concentration, classifying the strain as resistant according to the WHO's proportion testing method [17]. The MIC was recorded as the lowest drug concentration at which GU < 100, when the drug-free 1/100 proportional control tube crossed the positivity threshold of 400 GU. An undiluted absolute growth control was included in each assay to monitor normal strain growth. Each set of measurements was repeated three times (biological triplicates) and the final MIC value was taken as the concentration with at least two concordant results from the replicates [18]. Microsoft Excel was used for data recording, organization, and the construction of graphs to visualize the inhibitory activity of the compounds against *M. tuberculosis*.

3. RESULTS

DST was performed to check the activity of the compounds against *M. tuberculosis*. The experiment was carried out in triplicates. The MGIT tube containing inoculum without any antimicrobial agent was used as growth control. Two *M. tuberculosis* isolates (H37Rv strain and MDR strain) were used for the evaluation of compounds. Drug susceptibility is determined by monitoring the growth of bacteria in the presence of the drug, as compared to growth control. The instrument interprets the results when growth in control tubes reaches a predefined threshold (such as 400 GU within 4-13 days). A low GU value (below 100) in the drug tube as compared to the control indicates susceptibility, whereas a high GU value (such as 100 or more) suggests resistance.

Compounds were tested at different concentrations. They did not show any inhibitory activity at lower concentrations. Even up to 32 µg, there was no change in the growth of *M. tuberculosis* with respect to growth control. To further validate whether these drugs have inhibitory activity or not, their concentration was increased to 256 µg. *In vitro* experiments revealed that both drugs did not inhibit the growth of *M. tuberculosis* at lower concentrations, although they inhibited growth at higher concentrations. The MGIT 960 instrument declares a tube sensitive or resistant based on the growth unit. Dihydroergotamine showed inhibitory activity at higher concentrations, that is, 128 µg and 256 µg on both strains (Reference H37Rv and MDR-TB). Whereas, abiraterone acetate showed inhibitory activity at 64 µg, 128 µg, and 256 µg and inhibited the growth of both strains (Table 2 and Figure 1).

Table 2. Inhibitory Activity of Different Concentrations of Dihydroergotamine and Abiraterone Acetate on H37Rv and MDR Strains

Concentraion of drug (ug/ml)	Dihydroergotamine		Abiraterone Acetate	
	Growth Unit		Growth Unit	
	H37Rv	MDR-TB	H37Rv	MDR-TB
0.25	400 (R)	400 (R)	400 (R)	400 (R)
0.5	400 (R)	400 (R)	400 (R)	400 (R)
1	400 (R)	400 (R)	400 (R)	400 (R)
2	400 (R)	400 (R)	400 (R)	400 (R)
4	400 (R)	400 (R)	400 (R)	400 (R)
8	400 (R)	400 (R)	400 (R)	400 (R)
16	400 (R)	400 (R)	400 (R)	400 (R)
32	400 (R)	400 (R)	400 (R)	400 (R)
64	400 (R)	400 (R)	<100 (S)	<100 (S)
128	<100 (S)	<100 (S)	<100 (S)	<100 (S)
256	<100 (S)	<100 (S)	<100 (S)	<100 (S)

S* sensitive, R* resistant, *$H_{37}Rv$** reference strain

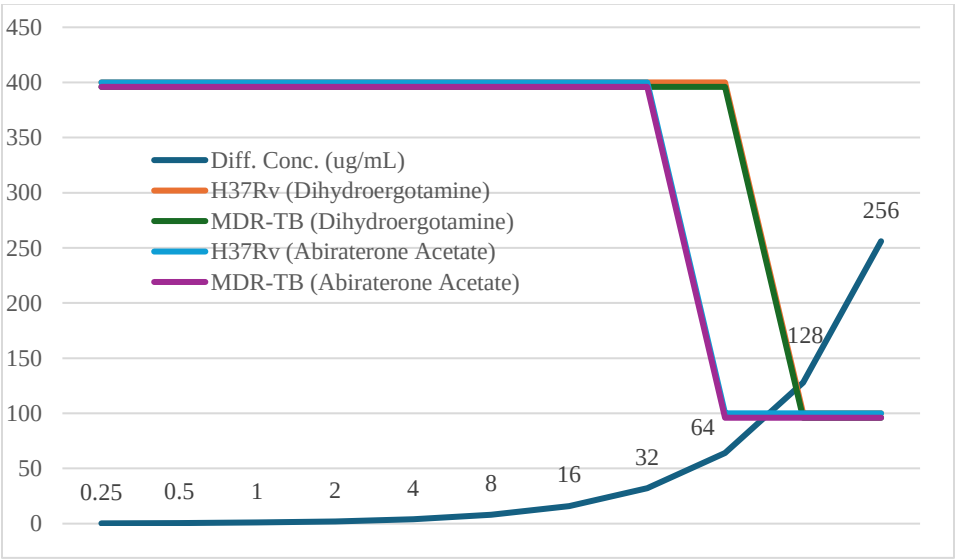

Figure 1. Inhibitory Activity of Different Concentrations of Dihydroergotamine and Abiraterone Acetate on H37Rv and MDR Strains

4. DISCUSSION

The results of this study demonstrate the potential of dihydroergotamine and abiraterone acetate as anti-tubercular agents against *M. tuberculosis*. *In silico* analysis identified potential drug targets for these two compounds [11], suggesting their potential role in the chemotherapy of MDR-TB. Both compounds demonstrated inhibitory activity against both the reference H37Rv and the MDR-TB strain, indicating their potential to combat drug-

resistant tuberculosis. Dihydroergotamine was effective at higher concentrations (128 µg and 256 µg), suggesting that it may be particularly useful in combination with other drugs. In contrast, abiraterone acetate exhibited inhibitory activity at lower concentrations (64 µg, 128 µg, and 256 µg), suggesting its potential as a more potent anti-tubercular agent. The fact that both compounds exhibited no inhibitory activity at lower concentrations but demonstrated growth inhibition at higher concentrations suggests a possible dose-dependent effect, emphasizing the importance of optimizing drug concentrations in anti-tubercular therapy.

Schmitt et al. [19] also investigated the anti-TB potential of the non-antibiotic lead compound Cyclomarin A using a similar method. Cyclomarin A exhibited antibacterial activity against replicating *M. tuberculosis* in culture broth [19]. Similarly, azole drug was tested against MDR *M. tuberculosis* clinical isolates to evaluate their efficacy for both latent tuberculosis (LTB) and active MDR/XDR-TB treatments [20]. Similar to the current study, they found that metronidazole and ipronidazole exhibited bacteriostatic activity against *M. tuberculosis* strains.

Another study was conducted on Lassomycin, a peptide compound with strong anti-TB activity. Lassomycin is effective against drug-sensitive, MDR, and XDR strains of *M. tuberculosis*, with MIC ranging from 0.41 to 1.65 µm. Lassomycin can kill both inactive and actively growing *M. tuberculosis*. In contrast, rifampicin is not effective against inactive *M. tuberculosis* [21]. Another macrocyclic peptide Ecumicin was tested for its antibacterial potential. It showed strong anti-TB activity against MDR, XDR, and sensitive *M. tuberculosis* strains, with MIC values ranging between 0.16 and 0.62 µm. The drug also effectively killed inactive *M. tuberculosis* at a minimal bactericidal concentration of 1.5 µm, suggesting that it could shorten treatment duration [22]. A nitro-dihydro-imidazooxazole derivative OPC-67683 showed highly potent activity against TB, including MDR-TB, with an exceptionally low MIC range of 0.006–0.024 µg/ml *in vitro* and high efficacy at low doses *in vivo* [23]. Since OPC-67683 was effective at low doses *in vivo,* it suggests that, like OPC-67683, the current study drugs could potentially be effective at low doses *in vivo* and meet safety standards. However, these drugs have not been tested on mice or *in vivo*. The combination of OPC-67683 with rifampicin (RFP) and pyrazinamide (PZA) removed TB bacteria from the lungs two months faster than the usual treatment with RFP, isoniazid, ethambutol (EB), and PZA [23]. This suggests that the tested drugs might work well against TB when combined with current antibiotics.

4.1. Implications

Over the years, numerous studies have investigated the antibacterial potential of non-antibiotic drugs and novel compounds to identify alternative therapeutic approaches, worldwide. However, studies such as the current one which aim to discover alternative drugs (dihydroergotamine and abiraterone acetate) through *in silico* approaches, followed by laboratory testing on bacterial cultures, are rarely published. This is primarily due to the ineffectiveness of these compounds in experimental settings. The lack of publication of such data is a significant setback for practitioners and clinicians striving to combat antimicrobial resistance, as it may lead to redundant efforts on the same compounds. Therefore, disseminating these findings, even when results are negative, is crucial to aid others

in avoiding repetition and potentially finding solutions through alternative approaches. While the current study shows promising *in vitro* results, *in vivo* testing is essential to understand the future therapeutic potential and safety of these compounds. *In vivo* studies would help to determine the pharmacokinetics, bioavailability, and potential side effects of dihydroergotamine and abiraterone acetate, providing a more comprehensive assessment of their efficacy as anti-tubercular agents. The authors propose using established animal models, such as the murine model of *M. tuberculosis* infection, to assess drug efficacy. Different dosing strategies, including dose escalation and combination therapy, can be explored to optimize pharmacokinetics, particularly absorption, distribution, metabolism, and excretion (ADME), along with monitoring tissue-specific drug concentrations in the lungs [24–26].

4.2. Conclusion

This study demonstrates the significant anti-tubercular potential of dihydroergotamine and abiraterone acetate against MDR *M. tuberculosis*. The compounds exhibited effective inhibition at varying concentrations, suggesting their potential as alternative therapeutic options. Further *in vivo* studies are warranted to evaluate their pharmacokinetics, bioavailability, and overall efficacy. Disseminating these preliminary results is crucial to advance the battle against antimicrobial resistance and for guiding future research endeavors. The repurposing of these non-antibiotic drug classes could lead to new therapeutic approaches for resistant tuberculosis, accelerating the fight against drug-resistant strains.

CONFLICT OF INTEREST

The authors of the manuscript have no financial or non-financial conflict of interest in the subject matter or materials discussed in this manuscript.

DATA AVAILABILITY STATEMENT

The data associated with this study will be provided by the corresponding author upon request.

FUNDING DETAILS

No funding has been received for this research.

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