Thymoquinone inhibits growth of ampicillin resistant K12 *E. coli*

Word count: 2857

Abstract:

With the rise of antibiotic resistant bacteria and the creation of superbugs, there has been an unparalleled need for new antibiotics. Thymoquinone, the active antibacterial ingredient in black cumin, has proven to be a potent molecule that is relatively harmless and targets ATP synthase—a novel antibiotic target. In this study, thymoquinone's inhibitory effects in wild type and ampicillin resistant *E. coli* were compared. Thymoquione was tested in four different concentrations and the *E. coli's* growth was measured through O.D.'s. Results showed that thymoquinone significantly inhibited growth in the wild type and was up to 16% more effective than ampicillin at inhibiting growth of the ampicillin resistant *E. coli*.

Background:

Escherichia Coli (E. coli) is a bacterium found in both the environment and the intestines of animals and humans. While most strains are harmless, a few are pathogenic, causing bloody diarrhea and diarrheal illnesses. In fact, the O157-H7 strain can cause hemolytic uremic syndrome and is the principal cause of acute kidney failure in the United States (Rowe, 1995).

After the discovery of penicillin, humans have relied on antibiotics to treat bacterial infections such as *E. coli*. With their increased usage, bacteria have adapted and transformed to develop resistance against common antibiotics. For example, beta lactamase has enabled *E. coli* to develop resistance to ampicillin by breaking the b-lactam ring of ampicillin open and rendering the antibiotic useless (Munoz-Price, 2017).. Furthermore, pharmaceutical industries have only been able to create three new classes of antibiotics within the last four decades, while bacteria develop antibiotic resistance much faster (Ventola, 2015). Figure 1 shows all the common antibiotics approved by the FDA in recent years (Antibiotic resistance, 2017.).

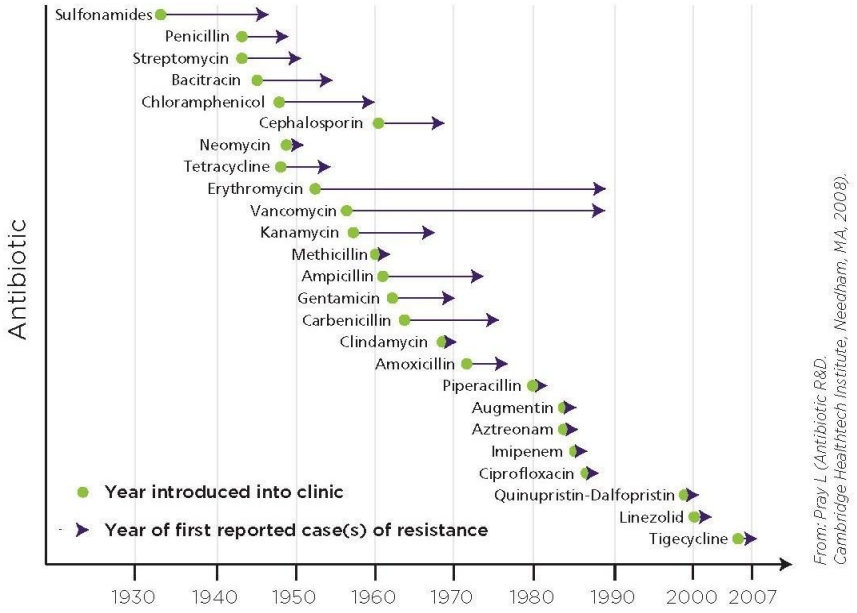

Figure 1) Timeline showing the introduction of new antibiotics and the development of antibiotic resistant strains. Notably, bacteria have developed resistance to all currently approved antibiotics.

The listed antibiotics target cell membranes, cell walls, protein synthesis, or nucleic acid synthesis. All 4 target sites are essential in all organisms, which makes them potent active sites as bacteria are unable to develop resistance by removing their cell membranes, walls, proteins, or RNA. Even with such a wide range of targets, strains such as pan-resistant CRE have emerged that are not susceptible to any conventional antibiotics (Chen et al., 2017). With the need for new antibacterial agents to kill antibiotic resistant bacteria, researchers have turned to natural remedies and traditional medicines.

The black seeds in the *Nigella sativa* plant (N. Sativa), more commonly known as black cumin, have been cultivated in Asia, Europe, and North Africa and used for medicinal purposes for centuries (A. Ahmad et al., 2013). They originate from Southeastern Asia and were also used in

ancient Egypt, Greece, Middle East and Africa (A. Ahmad et al., 2013). In Islamic countries, they are regarded as one of the greatest forms of healing medicine available(Nigella-sativa-research.com, 2010; Wikipedia, 2010).

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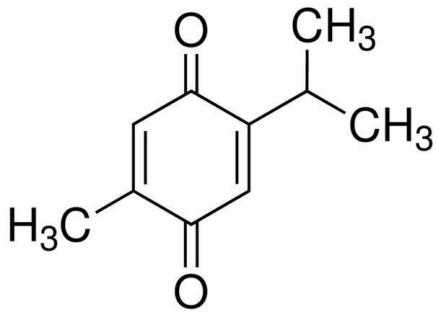

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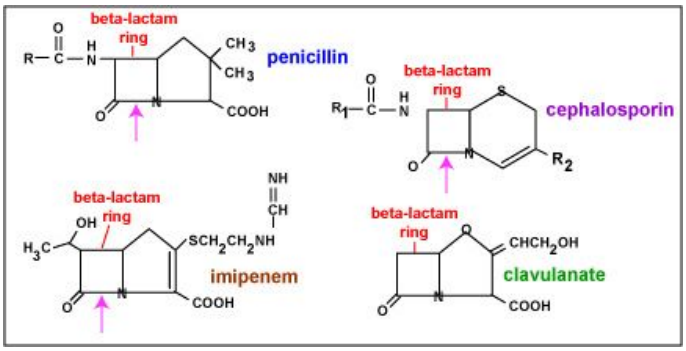

Figure 2a) Structure of Thymoquinone 2b) Structures of common beta-lactams.

They were used to treat asthma, hypertension, diabetes, inflammation, bronchitis, headaches, fever, dizziness and influenza (Khan et al., 2011). *N. sativa's* success as both a medicine and a food preservative has been explained by its active ingredient, thymoquinone(TQ) (Khan et al., 2011). Researchers have found that TQ also inhibits the growth of multiple cancer cell lines and has proven to have antioxidant, anti inflammatory, antidiabetic, antibacterial, antifungal, antitussive, and neuroprotective properties (A. Ahmad et al., 2013).

Thymoquinone has shown promise as a natural remedy in multiple studies that have proven *N.sativa* and TQ's antibacterial effect (Forouzanfar, 2014). TQ's active site is currently thought to be ATP synthase, and TQ has the ability to inhibit up to 98% of the protein's activity (Z. Ahmad, 2015). Currently, ATP synthase is a novel target. A new antibiotic, Bedaquiline, was recently approved by the FDA and is the first and only approved antibiotic that targets ATP synthase

(Lakshmanan, 2013). Bedaquiline is designed to kill multi-drug resistant tuberculosis (Lakshmanan, 2013). Most importantly, there is no known strain of bacteria that is resistant to antibiotics that target ATP synthase. While TQ has proven to inhibit growth of both gram positive and gram negative bacteria, it has shown to better inhibit growth of bacteria like *E. coli* that are more dependent on ATP synthase (Balemans, 2012). ATP synthase is the main energy generating protein in most organisms through oxidative phosphorylation or photophosphorylation (Lodish, 1970). The protein complex uses protons from an electron transport chain to convert ADP into ATP. The electron transport chain is the most efficient part of cellular respiration, and with ATP synthase's activity inhibited, energy production is significantly stunted, slowing cell processes.

Thymoquinone is a 1-4 benzoquinone (Figure 2a) (National Center for Biotechnology Information, 2017). The 6 Carbon chain is connected to two methyl groups on the 2-Carbon and one methyl group on the 5-Carbon (National Center for Biotechnology Information, 2017). A carbonyl group is attached to both the 1 Carbon and the 4 Carbon. Notably, TQ does not have a beta-lactam ring (Figure 2b) and thus should be resistant against beta-lactamase.

Purpose:

Measure TQ's growth inhibitory effect on ampicillin resistant *E. coli* in vitro.

Hypothesis:

TQ should inhibit the function of wild type *E. coli's* ATP synthase and, in turn, inhibit its growth. Because TQ's molecular structure lacks a b-lactam ring, the beta lactamase sequence in the pARA-R plasmid should not affect TQ's structure or growth inhibitory effects. Thus, TQ should equally inhibit the growth of wild type and pARA-R transformed *E. coli.*

Materials:

- 99% purity 5G Thymoquinone purchased from Caymen Chemicals
- DMSO purchased from Amazon
- Erlenmeyer flasks, Petri Dishes, LB Agar, LB broth to grow the *E. coli* colonies
- E. coli K12 beta strain
- pARA-R plasmid (80 ng/ml)
- Ampicillin obtained from Caymen Chemicals
- Kanamycin obtained from Caymen Chemicals
- Syringe and .22 micrometer filter
- Ice bath/hot water bath
- Incubator and shaker to grow bacteria
- Filter paper for zones of inhibition
- Sterile Falcon Tubes
- Spectrophotometer
- Analytical balance
- Microcentrifuge tubes

Methods:

Creation of 7.5 ml 250 mM stock TQ solution

1) 99% pure thymoquinone was bought from Cayman Chemicals and 98% pure DMSO was bought from Amazon
2) 410mg of thymoquinone was added to 2.5ml of 2% DMSO and stored at 0℃
3) The next day, 2.5 ml of 100% DMSO was added to the solution, vortexed and left in a 42℃ water bath. After 20 minutes, 2.5 more ml of 100% DMSO was added and the procedure was repeated. All crystals were dissolved and a stock 250 mM solution was made.
4) Half of the solution was kept at -18℃ and covered.
5) Half of the solution was kept at 0℃ and covered when not used.

General Care of E.coli

1) Lb Broth was made at a concentration of 25 g/L. In an erlenmeyer flask, 100 ml of DI water was added to 2.5 grams of LB broth, autoclaved, and stored up to a month. The same procedure was done for LB agar except at a concentration of 4.0 g/L.
2) A standing culture was constantly kept that started with 100 µL of wild type *E.coli* in 10 ml of LB broth and kept at 37℃. 100 µL of the standing culture was inoculated into 10 ml of broth every week to dilute the standing culture.
3) Experimental cultures were created by inoculating 100 µL of standing culture into 100 ml of LB Broth and incubated at 37℃ for 24 hours.

Transformation of E. coli

1) 3 1.5 ml sterile microcentrifuge tubes labelled + and 2 1.5ml sterile microcentrifuge tubes labelled -.
2) 100 µL of *E. coli* was inoculated into each microcentrifuge tube and 250 µL of 0.05M CaCl2 was transferred into each of the microcentrifuge tubes. All 5 tubes were then incubated on ice for 5 minutes.
3) 10 µL of pARA-R plasmid was transferred into each of the + labelled microcentrifuge tubes and returned to ice for 15 more minutes.
4) All 5 tubes were placed into a 42℃ water bath for 45 seconds and returned to the ice bath for 10 minutes immediately after.
5) 100 µL of every tube was then plated onto both an ampicillin plate and a normal LB Agar plate and the cells were spread evenly.
6) All plates were then incubated for 24 hours at 37℃
7) After 24 hours, colonies that grew on the ampicillin plate and were from a + tube were inoculated into 10 ml of LB Broth that contained 40 µL of ampicillin.

Optical Densities

1) The Optical Densities (O.D.'s) of both transformed and wild type *E. coli* were taken at 600 nm. The procedure was identical for both except 20 µL of ampicillin was added to each of the transformed *E. coli's* solutions
2) 7 Sterile 15 ml Falcon tubes were obtained and 100 µL of *E.coli* and 10 ml of broth were added to each of the tubes.

3) In separate tubes:
    a) Tube 1:Nothing was added(negative control)
    b) Tube 2: 250µL of 1 M DMSO added to create a 2% DMSO solution(the highest concentration at which DMSO has no effect on E. coli, 6.24 mM had a working DMSO concentration of 1.67%) (Wadhwani, 2008).
    c) Tube 3: 7µL 250 mM TQ added to create a .175mM solution (maximum inhibition of ATP synthase)(Z. Ahmad, 2015) .
    d) Tube 4: 65.2µL of 250 mM TQ added to create a 1.6mM solution(Middle Concentration)
    e) Tube 5: 123µL of 250 mM TQ added to create a 3.12mM solution(MIC) (Kouidhi, 2011).
    f) Tube 6: 250µL of 250 mM TQ added to create a 6.24mM solution(super concentration)
    g) Tube 7: 20 µL of ampicillin added to wild type as negative control and 20 µL of kanamycin added to transformed E. coli as negative control.

4) All 7 O.D.'s taken at 0 time, shaken at 37℃ for 24 hours and O.D's were taken a second time after 24 hours.

Zones of Inhibition

1) Zones of inhibition were created by preparing 7 stock solutions in 1.5 ml microcentrifuge tubes. Each solution was created in a total volume of 1ml. All concentrations were identical to the one's used in O.D.'s. Zones of inhibition were

not used with the transformed *E.coli* and all procedures were completed in the biosafety hood.

2) Microcentrifuge Tube 1: 0.7 μL of thymoquinone was added to 1ml of DI water to create a 0.175 mM stock solution
    a) Microcentrifuge Tube 2: 6.58 μL of thymoquinone was added to 1ml of DI water to create a 1.6 mM stock solution
    b) Microcentrifuge Tube 3: 12.5 μL of thymoquinone was added to 1ml of DI water to create a 3.12 mM stock solution
    c) Microcentrifuge Tube 4: 25 μL of thymoquinone was added to 1ml of DI water to create a 6.24 mM stock solution
    d) Microcentrifuge Tube 6: A stock solution of 2% DMSO was created as negative control.
    e) 20 μL of ampicillin was added to 1ml of DI water to create a positive control for the wild type *E. coli*.
3) 40 μL of each solution was added to 3 separate disks. The procedure was repeated for each of the solutions.
4) The disks were set out for ten minutes to allow for all the solution to be absorbed.
5) Disks were then transferred into sterile microcentrifuge tubes,covered, and stored at 0℃.
6) The following day, plates were streaked with *E. coli* to create a lawn and the disks were placed in shape of a triangle on the petri dish.

7) The plates were incubated at 37℃ for 24 hours and afterwards, the diameter of the zone was measured.

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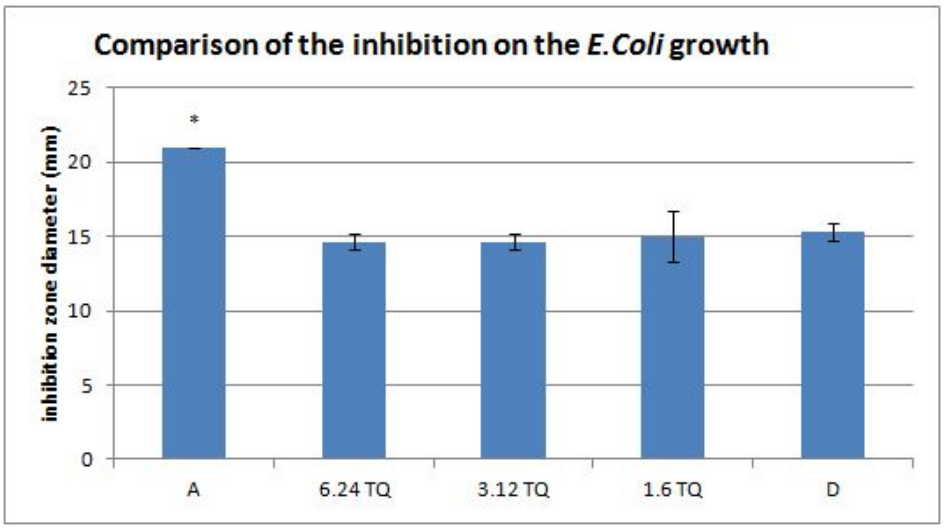

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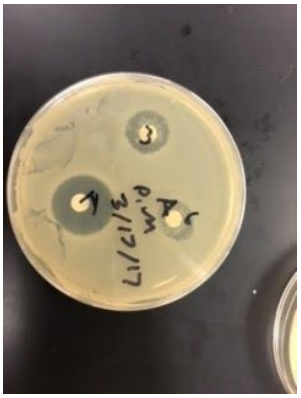

Figure 3a) The effects of different TQ concentrations on wild type *E. coli.* DMSO acted as negative control and ampicillin as positive. Growth was measured after 24 hours through zones of inhibition. 3 samples were taken (N=3) 3b) Gross appearance of one of the plates with 2 disks of TQ (3.12 and 6.24 mM) and 1 disk of Ampicillin. Satellite colonies indicated by red arrow. Statistical analysis differences between parts of means were inspected by student t-test. Means were considered significantly different at $P<0.05$

Results:

First, wild type *E. coli* growth was measured through zones of inhibition to confirm TQ's growth inhibitory properties (Figure 3a). While ampicillin significantly inhibited growth (P<.001*),

all TQ's inhibitory zones were similar to DMSO's. Thus, we were unsure if TQ or DMSO was inhibiting growth. Furthermore, it was difficult to quantify TQ's growth inhibition as colonies grew in the TQ's zones of inhibition (Figure 3b). We realized, however, that using optical densities(O.D.) to measure bacterial growth would allow us to retest DMSO and TQ's inhibitory effects using a more sensitive quantification method; O.D. would account for all growth, while zones of inhibition disregard the colonies within the zones of inhibition.

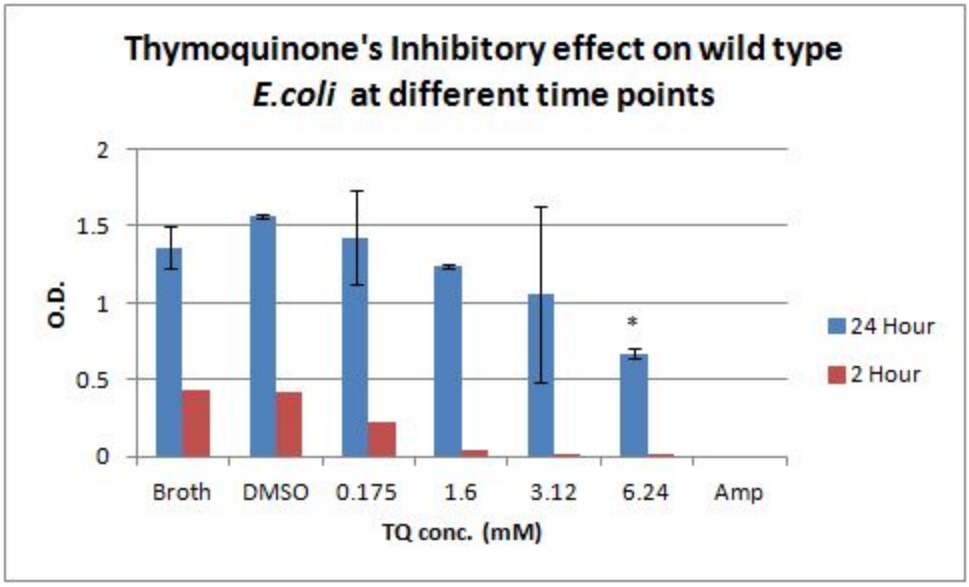

Figure 4) The effects of varying TQ concentrations on wild type *E. coli.* DMSO, and LB broth acted as negative controls and Amp as positive. Growth was measured after 2 and 24 hours through O.D.s. 24 hour experiments were done twice (N=2).

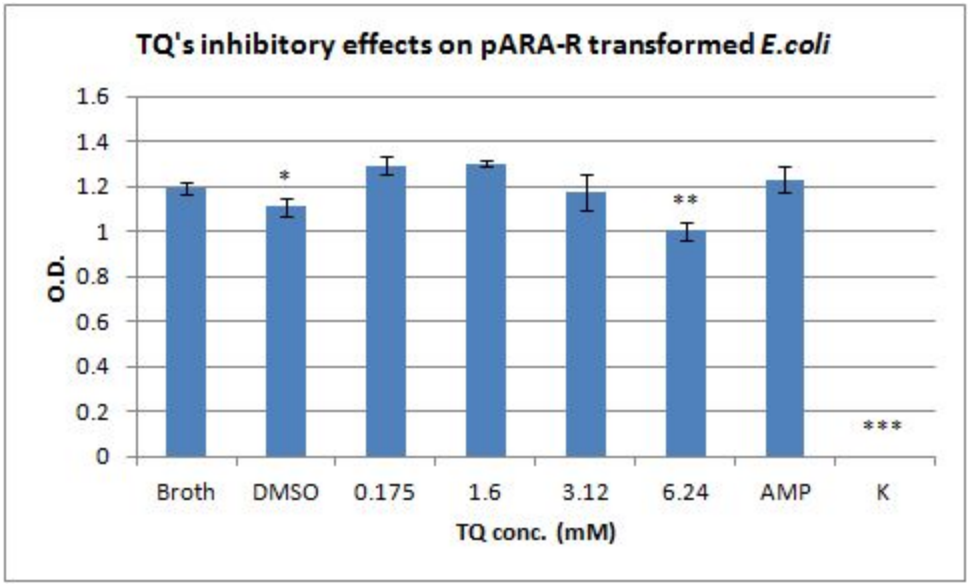

Figure 5) The effects of varying TQ concentrations on pARA-R transformed *E. coli.* DMSO and LB Broth acted as negative controls and Kanamycin served as the positive control. Ampicillin was added to all solutions for selection. Growth was measured after 24 hours through O.D.s. Experiments were repeated 2 times (N=3).

The effect of different TQ concentrations was then tested on wild type *E. coli* using O.D., and the growth followed a dose dependent trend (Figure 4). After 2 hours, all TQ concentrations showed growth inhibition; 1.6 mM, 3.12 mM, and 6.24 mM all showed similar growth inhibition. After 24 hours, the effect of different TQ concentrations became more apparent, and only the 6.24 mM showed significant growth inhibition($P<0.05$*) with a maximum inhibition of 50.7%. Ampicillin showed no growth.

Next, the experiment was repeated on *E. coli* transformed with pARA-R, a plasmid that contains beta lactamase, an enzyme that makes bacteria ampicillin resistant. Similar to the wild type *E. coli,* the transformed *E. coli* followed a dose dependent trend (Figure 5). Only DMSO and 6.24

mM showed significant growth inhibition ($P<0.05$*, $P<.01$** respectively). However, 6.24 mM showed significant growth inhibition compared to DMSO ($P<0.05$). 6.24 mM had the highest inhibition at 16.2%. Kanamycin, which works by inhibiting the bacterial ribosome, showed no growth. Furthermore, TQ seems to inhibit growth to a limit.

Discussion:

The inhibition of *E. coli* matches the results of previous research (Bakathir, 2011). In both the wild type and transformed *E. coli,* all TQ concentrations failed to fully inhibit growth. However, 6.24 mM was the minimum inhibitory concentration(MIC) at which TQ could delay growth. This challenges previous findings that 3.12 mM is TQ's MIC in *E. coli* (Kouidhi, 2011). One possible reason why TQ was unable to fully inhibit growth is that the drug is being metabolized by the bacteria. This is supported by the fact that in the wild type, even the 0.175 mM TQ solution was able to inhibit growth after 2hr, but unable to have a significant impact after 24hr. Additionally, TQ is normally an amber color in solution, but the samples that did not show growth inhibition lost that color.

Surprisingly, TQ's inhibitory effect seemed to be more effective on the wild type than the transformed *E. coli* as the 6.24 mM TQ was 313% more effective on the wild type than the transformed *E. coli.* The DMSO control, however, showed significant variability in the transformed *E. coli*, so these results might be an artifact of experimental variation. Nevertheless, the possibility of cross-resistance is not ruled out, and repeat experiments may have to be done to confirm this phenomenon.

TQ has shown its ability to combat ampicillin resistant bacteria, but its effects are solely growth inhibiting and it lacks a killing mechanism. While the 6.24 mM solution inhibited growth, we did not attempt to use anything more concentrated. Therefore, a more concentrated solution could potentially improve TQ's antibacterial effect. Additionally, pairing TQ with a bactericidal antibiotic could create a synergistic effect.

Furthermore, while it is known that ATP synthase is TQ's target it is unknown how TQ inhibits ATP synthase. This may explain why TQ seems to metabolise over time. Further research on both its active site and developed resistance could help design more effective synthetic drugs that mimic TQ.

Conclusion:

In the past decade, bacteria have developed resistance to all common antibiotics. The issue of antibiotic resistance bacteria and superbugs have increased the value of novel targets. We were able to show that TQ, through the inhibition of ATP synthase, has the potential to kill drug resistant bacterial strains. Our success suggests that ATP synthase may be a promising novel target for new antibiotics. In fact, the first antibiotic to target ATP synthase, Bedaquiline, was recently approved by the FDA. Further research into the mechanism of how TQ inhibits ATP synthase may produce a new generation of antibiotics with broad efficacy.

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