

## *In-vitro* characterisation of a novel antimicrobial agent, TNP-2092, against *Helicobacter pylori* clinical isolates

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### Summary

**BACKGROUND AND OBJECTIVES:** TNP-2092 is a novel dual-action lead compound consisting of rifamycin SV and 4H-4-oxo-quinolizine pharmacophores, with a broad spectrum of antibacterial activities. This compound is currently in the early stage of clinical development for *Helicobacter pylori* infection. The aim of the present study was to determine the antibacterial activity of TNP-2092 against *H. pylori* isolated from primary patients.

**METHODS:** A total of 100 *H. pylori* clinical isolates from primary patients were selected. The minimum inhibitory concentrations (MICs) for clarithromycin, levofloxacin, rifampin and TNP-2092 were determined using an agar dilution method. A time-kill study was performed with different concentrations of TNP-2092 relevant to MIC against *H. pylori* ATCC strain 43504 for up to 24 hours. The time-kill study with drug concentrations of 0–4 × MIC was also used to determine the antibacterial activity of TNP-2092 against *H. pylori* under different pH conditions (pH 4–7).

**RESULTS:** The primary resistance percentages to clarithromycin, levofloxacin, rifampin and TNP-2092 were 13, 18, 1 and 1%, respectively. TNP-2092 killing kinetics were both concentration and time dependent. The effectiveness of TNP-2092 against *H. pylori* was gradually reduced with a decrease in pH.

**CONCLUSIONS:** TNP-2092 is highly active against *H. pylori* and against strains resistant to clarithromycin or levofloxacin. Its antibacterial activity is both concentration- and time-dependent. The antibacterial activity of TNP-2092 appears to be pH-dependent and is more active under neutral pH. TNP-2092 represents a promising new therapy for the treatment of *H. pylori* infection in primary patients.

**Keywords:** *Helicobacter pylori*, drug resistant, TNP-2092, *in vitro* antibacterial activity

### Author contributions

Ben Wang, Qiaoyun Zhao and Wenzhu Yin contributed equally to this work.

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### Introduction

*Helicobacter pylori* is one of the most common bacterial pathogens in humans, and is closely associated with a number of prevalent gastrointestinal diseases, such as gastric cancer, chronic gastritis, peptic ulcer diseases, and mucosa-associated lymphoid tissue (MALT) lymphoma [1]. Eradication of *H. pylori* can prevent the recurrence of peptic ulcers and reduce the risk of developing gastric cancer [2, 3]. Anti-*H. pylori* therapies consisting of an acid-suppressive drug and/or a bismuth component with two or more antibiotics have been recommended by several consensus reports [3, 4]. However, elevated antimicrobial resistance has limited the efficiency of many once highly successful eradication therapies [5–7]. Therefore, the development of new drugs or drug combinations has been a focus in the field.

TNP-2092 is a novel rifampin-quinolone hybrid antibiotic consisting of covalently conjugated rifamycin SV and 4H-4-oxo-quinolizine pharmacophores; it is currently in the early stage of clinical development for the eradication of *H. pylori* [8]. TNP-2092 exhibits multitargeting activity against bacterial RNA polymerase as well as DNA gyrase and DNA topoisomerase IV [8–11]. A previous study indicated that TNP-2092 might have potential utility in the treatment of persistent *Staphylococcus aureus* infections [9]. This study sought to assess the *in vitro* antibacterial activity of TNP-2092 against *H. pylori* strains isolated from primary patients treated at the First Affiliated Hospital of Nanchang University.

### Materials and methods

#### Patients and bacterial culture

*H. pylori* isolates were obtained from patients diagnosed with duodenal ulcers who visited the First Affiliated Hospital of Nanchang University from 2013 to 2014 and underwent upper digestive tract endoscopy for the evaluation of dyspepsia. The study was approved by the ethics committee of First Affiliated Hospital of Nanchang University.

None of the patients had previous exposure to any eradication therapy for *H. pylori* infection.

*H. pylori* was cultured on Columbia agar plates with 5% sheep blood and an *H. pylori*-selective antibiotic supplement (Oxoid, Basingstoke, UK) containing vancomycin (10 mg/l), cefsulodin (5 mg/l), trimethoprim (5 mg/l) and amphotericin B (5 mg/l). The plates were incubated for up to 2 days at 37°C in microaerophilic conditions (GENbag, Biomerieux). *H. pylori* was identified from colony and microscopic morphology, as well as positive urease, catalase and oxidase tests.

### Susceptibility tests

The minimum inhibitory concentrations (MICs) for clarithromycin (Sigma-Aldrich), rifampin (Sigma-Aldrich), levofloxacin (Sigma-Aldrich) and TNP-2092 (TenNor Therapeutics) were determined using an agar dilution method according to the National Committee for Clinical Laboratory Standards Institute guidelines. *H. pylori* strains were grown on Mueller-Hinton agar plates. Briefly, *H. pylori* cells were suspended in physiological saline and adjusted to a turbidity of a McFarland No.2 standard (approximately  $10^7$ – $10^8$  cfu/ml) and inoculated directly onto different concentrations (128–0.0008 µg/ml) of antimicrobial agent-containing agar dilution plates. All plates were incubated as previously described for 3 days. The MIC was determined as the lowest concentration of antimicrobial agent preventing visible growth. For the *H. pylori* ATCC 43504 strain, the MIC of clarithromycin and levofloxacin should be in the range of 0.016–0.125 µg/ml and 0.016–0.25 µg/ml, respectively following the CLSI guidelines. The clarithromycin resistance was defined, according to the EUCAST-approved breakpoint, as  $\geq 0.5$  µg/ml. Isolates were defined as resistant to rifampin, TNP-2092, or levofloxacin, when MICs were  $>1$  µg/ml [12].

### Determination of the killing kinetics of TNP-2092

Time-kill curves were measured by exposing *H. pylori* ATCC43504 to a range of concentrations (0–64 × MIC) to determine the time required for TNP-2092 to reduce the colony forming units (CFUs) of *H. pylori* cells. Briefly, bacterial culture at the growth phase was diluted to an OD<sub>600</sub> of 0.2 and 20 µl of the diluted sample was seeded into 4 ml of Bromfield medium with 10% fetal bovine serum and TNP-2092. After 0, 0.5, 1, 3, 5, 7, 9 and 24 hours of incubation in a shaking microaerobic environment, the samples from different concentrations of TNP-2092 were collected, submitted to 10-fold serial dilutions and plated on Karmali's Campylobacter medium plates. The plates were incubated for 72 hours under 5% CO<sub>2</sub>, and the CFUs were counted.

### Impact of pH changes on the *in vitro* activity of TNP-2092 against *H. pylori*

The antibacterial activity of TNP-2092 against *H. pylori* under different pH conditions was also determined in the time-kill study by applying 0–4 × MIC of the drug against strain 43504 for up to 24 hours at different pH levels. Briefly, bacterial cultures were diluted to an OD<sub>600</sub> of 0.2 and 20 µl of the diluted sample was seeded into 4 ml Bromfield medium with 10% fetal bovine serum and TNP-2092 (concentrations 0, 0.25, 0.5 and 1 µg/ml) under different pH conditions (pH 7, 6, 5, 4). After 0, 2, 4, 6, 8, and 24 hours of incubation the samples were collected, diluted and plated on Karmali's Campylobacter medium plates and incubated for 72 hours under 5% CO<sub>2</sub>, when CFUs were counted and the log<sup>10</sup> value of CFUs was calculated.

## Results

### MIC and prevalence of antibiotic resistance

The MIC values for clarithromycin, levofloxacin, rifampin and TNP-2092 ranged from 0.008 to 16 µg/ml, 0.016 to  $>128$  µg/ml, 0.008 to  $>128$  µg/ml and 0.032 to  $>128$  µg/ml, respectively. The MIC<sub>50</sub> of clarithromycin (0.008 µg/ml) was lower than that of levofloxacin (0.25 µg/ml), rifampin (0.125 µg/ml) and TNP-2092 (0.125 µg/ml). However, the MIC<sub>90</sub> of TNP-2092 (0.5 µg/ml) and rifampin (0.5 µg/ml) was lower than that of clarithromycin (2 µg/ml) and levofloxacin (8 µg/ml).

Overall resistance percentages among the 100 *H. pylori* clinical isolates are shown in table 1. The primary resistance percentages to clarithromycin, levofloxacin, rifampin and TNP-2092 were 13% (13/100), 18% (18/100), 1% (1/100) and 1% (1/100), respectively.

Among the 100 *H. pylori* isolates, 24 isolates were single-drug resistant to either levofloxacin or clarithromycin (table 1). Double resistance was detected in seven *H. pylori* isolates (7%) (table 2). All seven of these isolates were resistant to both clarithromycin and levofloxacin. Triple resistance to levofloxacin, rifampin and TNP-2092 was found in one *H. pylori* isolate (1%) (table 2). No rifampin or TNP-2092 single resistant isolate was detected (0/100).

### Killing kinetics of TNP-2092 against *H. pylori*

The data shown in figure 1 indicate that TNP-2092 is a bactericidal agent. Figure 1 also shows that the slopes of the time-kill curves increased with the increase in drug concentration when the concentration of TNP-2092 was equal to or higher than the MIC (0.25 µg/ml). The higher the concentration of TNP-2092 was, the faster the CFUs were reduced (but not for the concentrations of 2 and 4 µg/ml). In parallel, CFUs also gradually decreased over time. When the concentration of TNP-2092 was lower than that of MIC (0.25 µg/ml), bacterial growth was observed.

**Table 1:** Resistance percentages and minimum inhibitory concentrations (MICs) of four antibiotics against *H. pylori* strains.

Antibiotic	Strains (n)	Range of MIC (µg/ml)	Resistant strains (%)
Clarithromycin	100	0.008 to 16	13
Levofloxacin	100	0.016 to $>128$	18
Rifampin	100	0.008 to $>128$	1
TNP-2092	100	0.032 to $>128$	1

### Determination of the antibacterial activity of TNP-2092 against *H. pylori* under different pH conditions

As is shown in figure 2, when the concentration of TNP-2092 was 0 µg/ml, *H. pylori* could not survive at pH 4.0 and not replicate well at pH 5.0. When the concentration of TNP-2092 was 0.25 µg/ml, the antibacterial activity of TNP-2092 against *H. pylori* seemed to be reduced at pH 5.0 and pH 6.0 compared with activity at pH 7. Similar results were obtained when the concentration of TNP-2092 was 0.5 µg/ml and 1 µg/ml.

### Discussion

With the increasing emergence of bacterial resistance to antibiotics, there is an urgent need to develop new regimens and agents for *H. pylori* infections. TNP-2092, consisting of a rifamycin and a quinolizine pharmacophore, was reported to be highly active against *S. aureus* and other Gram-positive bacterial species. Since quinolones and rifampin have been used as alternative antibiotics to eradicate *H. pylori*, it was expected that TNP-2092 may also be active against *H. pylori*. The results from the present study revealed that TNP-2092 was highly active and bactericidal against *H. pylori*. Our results also showed that TNP-2092

retained activity against prevalent quinolone-resistant isolates, which indicated that TNP-2092 could be a promising drug when the resistance rate to levofloxacin in the field is high.

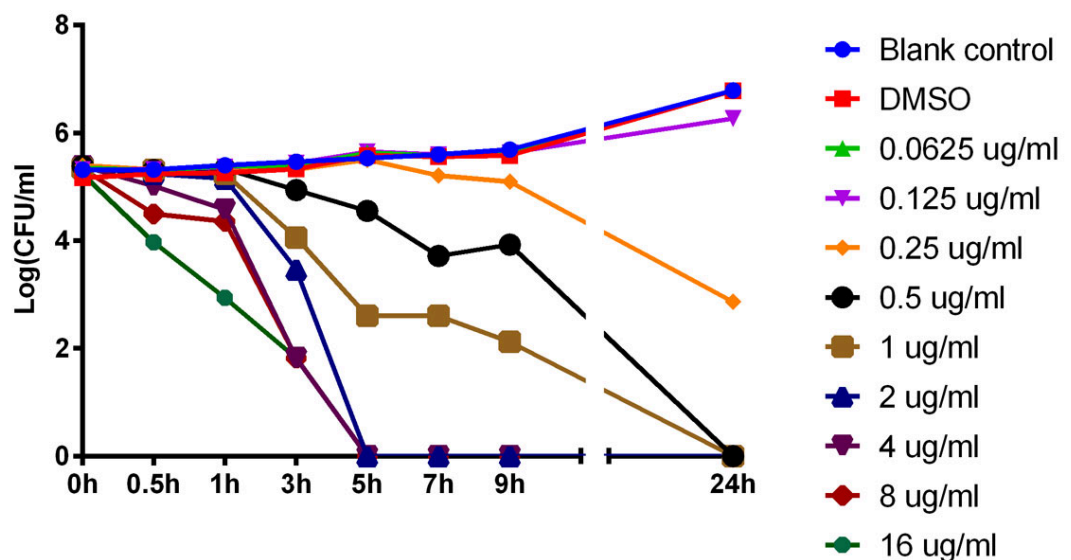
Among the 100 *H. pylori* isolates tested, only one strain was resistant to TNP-2092. The resistance mechanism of this strain is currently under investigation. The dual mechanisms of action of TNP-2092 from both portions of the conjugated pharmacophores appeared to dramatically reduce the resistance rate and were active against the single-drug resistant strains; that is, the compound was active against all 18 quinolone resistant and 13 macrolide resistant isolates.

This study showed that the clarithromycin resistance rate is 13%, which is slightly lower than that reported in some previous studies in China [13, 14]. This discrepancy may have resulted from geographic differences. Clarithromycin belongs to a macrolide family that inhibits protein synthesis at the ribosomal level [15]. Clarithromycin resistance is thought to be closely related to its reduction of the eradication rate as a first-line therapy [16]. Point mutations in the 23 s rRNA gene were described as the main mechanism for clarithromycin resistance in *H. pylori* [17].

**Table 2:** Double and multidrug resistance of *H. pylori* strains.

Antibiotic	Resistant strains (%)
Clarithromycin + levofloxacin	7
Clarithromycin + rifampin	0
Rifampin + levofloxacin	0
Clarithromycin + TNP-2092	0
levofloxacin+ TNP-2092	0
Rifampin + TNP-2092	0
Clarithromycin + levofloxacin+ rifampin	0
levofloxacin+ TNP-2092+ rifampin	1
Clarithromycin + levofloxacin+ rifampin+ TNP-2092	0

**Figure 1:** Time-kill kinetics of TNP-2092. The higher the concentration of TNP-2092 was, the faster colony forming units (CFUs) were reduced (but not for concentrations of 2 and 4 µg/ml). In parallel, CFUs were also gradually decreased over time. When the concentrations of TNP was lower than the minimum inhibitory concentration (0.25 µg/ml) bacterial growth was observed.



Levofloxacin is a broad spectrum fluoroquinolone inhibiting DNA gyrase, a type II topoisomerase, and topoisomerase IV [18, 19]. Levofloxacin-based therapies have been recommended as a second-line against *H. pylori* infection by the Maastricht/Florence IV consensus [3]. They showed high efficiency in eradicating *H. pylori* in many studies [20]. However, the resistance rate of *H. pylori* to levofloxacin appears to be rising [14, 21, 22] because of its increased use for airway or urinary tract infections [23]. Levofloxacin resistance was linked to the point mutations in the *gyrA* gene [24]. This present study showed that the levofloxacin resistance rate is 18%, which is consistent with that from a recent multicentre study conducted in China [13]. We also found that 53% of clarithromycin-resistant isolates were also resistant to levofloxacin. As a high resistance rate to levofloxacin may jeopardise the efficacy of clarithromycin, levofloxacin-based therapies may be not good choices as empirical therapies in the clinic. This finding also indicated that levofloxacin should not be administered as a second-line agent to patients in whom clarithromycin-based therapy was not successful, and susceptibility testing is recommended before its administration.

Rifampin is an antibiotic that inhibits bacterial DNA-dependent RNA polymerase, and has been primarily used in the treatment of tuberculosis [25]. In this study, the resistance rate of *H. pylori* to rifampin only (1%) was much lower than clarithromycin and levofloxacin, which is consistent with other studies conducted in Germany and Japan [26, 27]. Rifampin also retained activity against clarithromycin- and levofloxacin-resistant isolates. Further investigation is needed to explore the mechanism of resis-

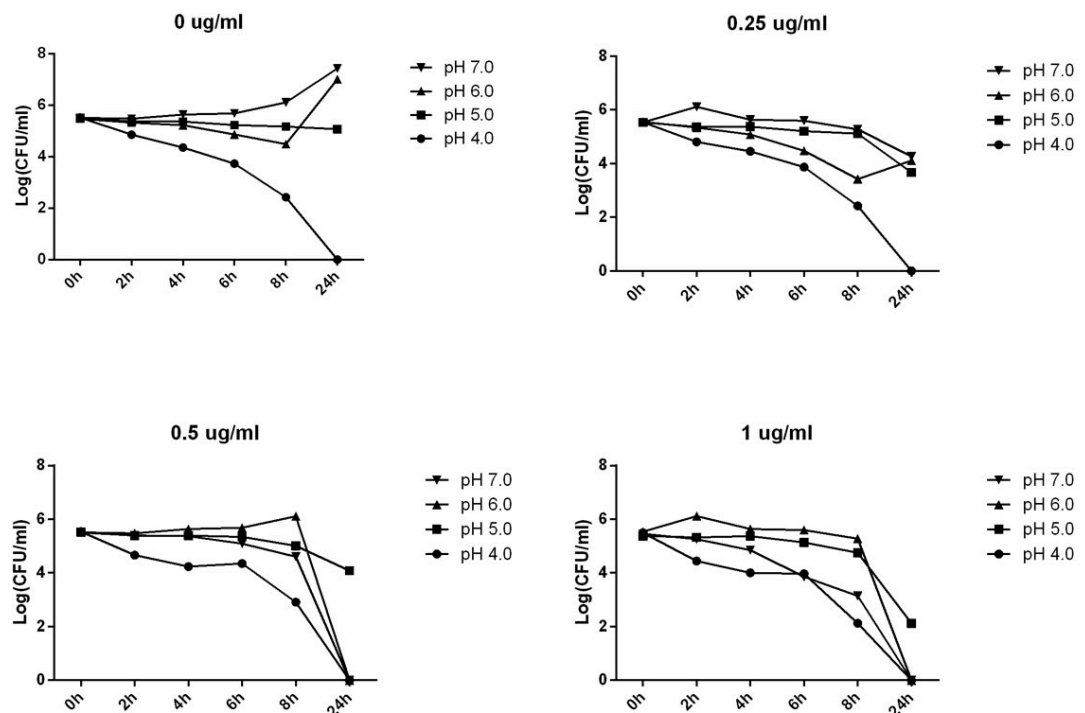
tance in this one isolate with resistance to levofloxacin, rifampin and TNP-2092. Rifabutin, a structurally related analogue of rifampin, has been shown to yield acceptable eradication rates as a third-line therapy against *H. pylori*. However, rifabutin is not available in many areas and is also expensive. Thus, rifampin-based therapies have been considered as a rescue regimen based on its high *in-vitro* activity against *H. pylori*.

The pharmacokinetic features of TNP-2092 in figure 1 show that both concentration- and time-dependent activities may serve as references to determine the dosage and the duration of drug administration in animal model studies and human trials.

The data in figure 2 show that the activity of TNP-2092 against *H. pylori* was reduced at pH 5.0, which might be attributed to several factors such as intrinsic tolerance of the bacterial cells in a nonoptimal growing state, protonation of TNP-2092, which prevents its penetration into bacterial cells and/or the instability of TNP-2092. Further investigation may be needed to address this mechanism. However, the data may suggest coadministration of a proton pump inhibitor to create the optimal local environment for the optimal antibacterial activity of this compound.

Moreover, good compliance with simplicity of drug administration is a vital factor of a successful *H. pylori* eradication programme [28]. Compliance issues are closely associated with treatment failure in patients with antibiotic-susceptible strains and the development of antibiotic resistance [29]. TNP-2092 may achieve better compliance with its dual-activity feature from both the rifamycin and quinolones pharmacophores, which could decrease the

**Figure 2:** Determination of TNP-2092 *in-vitro* activity under different pH conditions. When the concentration of TNP-2092 was 0 µg/ml, *H. pylori* could not survive at pH 4 and could not replicate well at pH 5. When the concentration of TNP-2092 was 0.25 µg/ml, its antibacterial activity against *H. pylori* seemed to be reduced at pH 5 and 6 compared with pH 7. Similar results were obtained when the concentration of TNP-2092 was 0.5 and 1.0 µg/ml.





number of drugs administered and simplify the treatment regimen. Moreover, TNP-2092 could make it difficult for *H. pylori* to develop resistance [8].

Nevertheless, rifampin is an essential antibiotic against *Mycobacterium tuberculosis*. Furthermore, the wide use of rifampin or rifabutin may increase the resistance rate of *M. tuberculosis* to this drug class. Thus, rifampin or rifabutin should not be used as a first-line therapy for *H. pylori* eradication and should be reserved for rescue treatment even though the compounds possess the characteristics of a first-line therapy. However, further *in-vitro* and *in-vivo* studies are needed to determine whether it is sufficient to prevent rifamycin resistance in *M. tuberculosis*.

In conclusion, TNP-2092 is a promising drug candidate for the treatment of diseases associated with *H. pylori* infection. Clinical studies are needed to further elucidate its efficacy and safety in patients. The antibacterial activity of TNP-2092 appears to be both concentration- and time-dependent, and it is reduced under acidic pH. TNP-2092 has good potential to reduce the development of drug resistance and maintains activity against existing infections caused by drug-resistant *H. pylori* bacterial cells.

#### Financial disclosure

Supported by: the National Key Research and Development programme of China (No: 2016YFC1302201); National Natural Science Foundation of China (No: 81460115); and Science and Technology Projects of Jiangxi Province (No: 2014BBG70019).

#### Potential competing interests

No potential conflict of interest relevant to this article was reported.

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