In Vitro and *In Vivo* Activities of TP0480066, a Novel Topoisomerase Inhibitor, against *Neisseria gonorrhoeae*

Aiko Masuko,^a Iichiro Takata,^a Kiyoko Fujita,^a Hirotohi Okumura,^b Fumihito Ushiyama,^c Hideaki Amada,^c Hiroyuki Sugiyama^a

^aPharmacology Laboratories, Taisho Pharmaceutical Co., Ltd., Saitama, Japan

^bDrug Safety and Pharmacokinetics Laboratories, Taisho Pharmaceutical Co., Ltd., Saitama, Japan

^cChemistry Laboratories, Taisho Pharmaceutical Co., Ltd., Saitama, Japan

ABSTRACT Gonorrhea is a common, sexually transmitted disease caused by *Neisseria gonorrhoeae*. Multidrug-resistant *N. gonorrhoeae* is an urgent threat, and the development of a new antimicrobial agent that functions via a new mechanism is strongly desired. We evaluated the *in vitro* and *in vivo* activities of a DNA gyrase/topoisomerase IV inhibitor, TP0480066, which is a novel 8-(methylamino)-2-oxo-1,2-dihydroquinoline derivative. The MICs of TP0480066 were substantially lower than those of other currently or previously used antimicrobials against gonococcal strains demonstrating resistance to fluoroquinolones, macrolides, β -lactams, and aminoglycosides (MICs, $\leq 0.0005 \mu\text{g/ml}$). Additionally, no cross-resistance was observed between TP0480066 and ciprofloxacin. The frequencies of spontaneous resistance to TP0480066 for *N. gonorrhoeae* ATCC 49226 were below the detection limit ($< 2.4 \times 10^{-10}$) at concentrations equivalent to $32 \times \text{MIC}$. TP0480066 also showed potent *in vitro* bactericidal activity and *in vivo* efficacy in a mouse model of *N. gonorrhoeae* infection. These data suggest that TP0480066 is a candidate antimicrobial agent for gonococcal infections.

KEYWORDS DNA gyrase, DNA topoisomerase, MAOQ, *Neisseria gonorrhoeae*, TP0480066, antibiotic resistance, enzyme inhibitor

Gonorrhea is one of the most prevalent sexually transmitted diseases in the world (1). In 2012, the World Health Organization (WHO) estimated that 78 million adults contract gonorrhea globally every year (1). In 2009, the national rate of reported cases of gonorrhea reached a historic low in the United States, but the rate has recently increased once again. In 2016, a total of 468,514 cases of gonorrhea were reported in the United States, yielding a rate of 145.8 cases of gonorrhea per 100,000 population (2).

Drug-resistant gonorrhea is a major public issue and is now recognized by the U.S. Centers for Disease Control and Prevention (CDC) as one of the top three urgent antibiotic resistance threats (3). The WHO Global Gonococcal Antimicrobial Surveillance Program (WHO GASP) monitors trends in drug-resistant gonorrhea. WHO GASP data from 2009 to 2014 showed continued widespread resistance to penicillin, tetracycline, and ciprofloxacin, increasing resistance to azithromycin, and the emergence of decreased susceptibility and resistance to extended-spectrum cephalosporins (ESCs) (4). In Japan and several other countries, the failure of gonorrhea treatment with cefixime has been reported since the early 2000s (5, 6). Currently, in most countries, the injectable ESC ceftriaxone is the only remaining empirical monotherapy for gonorrhea. However, extensively drug-resistant gonococcal strains with high-level resistance to ceftriaxone have been reported (7–9). Therefore, new antibiotics are needed to treat gonococcal infections.

To the best of our knowledge, only a few antimicrobial agents for the treatment of gonorrhea are presently being investigated in phase III clinical trials. Solithromycin

Citation Masuko A, Takata I, Fujita K, Okumura H, Ushiyama F, Amada H, Sugiyama H. 2021. *In vitro* and *in vivo* activities of TP0480066, a novel topoisomerase inhibitor, against *Neisseria gonorrhoeae*. Antimicrob Agents Chemother 65:e02145-20. <https://doi.org/10.1128/AAC.02145-20>.

Copyright © 2021 American Society for Microbiology. All Rights Reserved.

Address correspondence to Hiroyuki Sugiyama, hio-sugiyama@taisho.co.jp.

Received 10 October 2020

Returned for modification 7 November 2020

Accepted 30 January 2021

Accepted manuscript posted online 8 February 2021

Published 18 March 2021

TABLE 1 *In vitro* antibacterial activities of TP0480066 and comparators against *N. gonorrhoeae*

Strain	MIC ($\mu\text{g/ml}$)						
	TP0480066	Ceftriaxone	Penicillin G	Ciprofloxacin	Levofloxacin	Azithromycin	Spectinomycin
ATCC 49226	0.0005	0.015	1	0.004	0.008	0.25	32
ATCC 43069	≤ 0.00012	≤ 0.002	≤ 0.015	0.004	0.008	0.12	16
ATCC BAA-1846	0.00025	0.004	0.12	0.004	0.008	0.06	32
ATCC 700717	0.0005	0.015	16	0.03	0.06	0.25	>128
ATCC 700825 (FA 1090)	0.0005	≤ 0.002	0.12	0.004	0.008	0.12	32
NCTC 13477 (WHO F)	0.0005	≤ 0.002	0.03	0.004	0.008	0.12	32
NCTC 13478 (WHO G)	≤ 0.00012	0.015	0.5	0.12	0.25	0.25	32
NCTC 13479 (WHO K)	0.0005	0.06	2	64	16	0.25	32
NCTC 13480 (WHO L)	≤ 0.00012	0.12	4	32	32	0.5	32
NCTC 13481 (WHO M)	≤ 0.00012	0.015	64	2	2	0.25	32
NCTC 13482 (WHO N)	0.00025	0.008	32	8	4	0.25	32
NCTC 13483 (WHO O)	0.00025	0.015	64	0.008	0.03	0.25	>128
NCTC 13818 (WHO V)	0.0005	0.03	>128	32	16	>128	32
NCTC 13821 (WHO Y, F89)	0.0005	1	1	16	8	0.5	32

(CEM-101) is a fluoroketolide with a high activity against *Neisseria gonorrhoeae*. However, strains with high-level azithromycin resistance appear to be resistant to solithromycin, as well (10). Zoliflodacin (ETX0914) (11) and gepotidacin (GSK2140944) (12) belong to a new class of bacterial type II topoisomerase inhibitors for the treatment of gonorrhea.

Bacterial DNA gyrase and topoisomerase IV are highly conserved enzymes that are essential for DNA replication. They each consist of two subunits (GyrA and GyrB in DNA gyrase, ParC and ParE in topoisomerase IV). GyrA/ParC serve as targets for fluoroquinolones (e.g., ciprofloxacin), whereas GyrB/ParE are targets for aminocoumarins (e.g., novobiocin). Studies of genetic mutations resulting in resistance to fluoroquinolones in gonococcal isolates have shown that mutations within the quinolone resistance-determining regions (QRDRs) of GyrA/ParC are responsible for resistance to these agents (13–15). These mechanisms are analogous to those observed in *Escherichia coli* and other bacteria (16). Although GyrB amino acid alterations are also responsible for resistance to fluoroquinolones in *E. coli*, amino acid alterations in *N. gonorrhoeae* GyrB do not appear to have a clinically significant impact on fluoroquinolone resistance (16, 17). The spread of fluoroquinolone-resistant bacteria among various bacterial species is now a clinically significant problem.

TP0480066, a novel 8-(methyamino)-2-oxo-1,2-dihydroquinoline (MAOQ) derivative, effectively inhibits not only DNA gyrase but also topoisomerase IV (18). We evaluated the potential of TP0480066 for use in the treatment of gonorrhea.

RESULTS

Antibacterial activity. The MICs of TP0480066 against *N. gonorrhoeae* strains were ≤ 0.00012 to $0.0005 \mu\text{g/ml}$. TP0480066 was more active than all the tested comparators against the tested strains (Table 1). TP0480066 demonstrated potent antibacterial activity against *N. gonorrhoeae*, including strains with decreased susceptibility or resistance to currently available antimicrobial agents. The MICs of TP0480066 against *N. gonorrhoeae* strains NCTC 13479, NCTC 13480, NCTC 13818, and NCTC 13821, which are all high-level ciprofloxacin-resistant strains (MICs, $\geq 16 \mu\text{g/ml}$), were also ≤ 0.00012 to $0.0005 \mu\text{g/ml}$. Reproducibility was confirmed by two independent experiments.

Frequency of resistance. The frequencies of resistance to TP0480066 for *N. gonorrhoeae* ATCC 49226 ranged from 8.3×10^{-7} at $2 \times \text{MIC}$ to $<2.4 \times 10^{-10}$ at 32 and $64 \times \text{MIC}$. The frequencies of resistance to ciprofloxacin ranged from 1.1×10^{-8} at $2 \times \text{MIC}$ to $<2.4 \times 10^{-10}$ at 32 and $64 \times \text{MIC}$ (Table 2). TP0480066- or ciprofloxacin-resistant isolates were obtained in frequency of resistance experiments. The first-step TP0480066-resistant isolates TP-R1, TP-R2, TP-R3, and TP-R4 were isolated on 8, 8, 4, and $2 \times \text{MIC}$ agar plates inoculated with *N. gonorrhoeae* ATCC 49226, respectively. The

TABLE 2 Frequencies of spontaneous resistance to TP0480066 and ciprofloxacin for *N. gonorrhoeae* ATCC 49226

Fold MIC	Frequency of resistance at 48 h			
	1st step		2nd step	
	TP0480066	Ciprofloxacin	TP0480066	Ciprofloxacin
2	8.3×10^{-7}	1.1×10^{-8}	1.4×10^{-4}	$>2.1 \times 10^{-3}$
4	2.2×10^{-7}	9.2×10^{-9}	9.6×10^{-6}	$<7.0 \times 10^{-8}$
8	1.8×10^{-7}	5.9×10^{-9}	$<7.1 \times 10^{-8}$	$<7.0 \times 10^{-8}$
16	2.5×10^{-8}	2.8×10^{-9}	$<7.1 \times 10^{-8}$	$<7.0 \times 10^{-8}$
32	$<2.4 \times 10^{-10}$	$<2.4 \times 10^{-10}$	$<7.1 \times 10^{-8}$	$<7.0 \times 10^{-8}$
64	$<2.4 \times 10^{-10}$	$<2.4 \times 10^{-10}$	$<7.1 \times 10^{-8}$	$<7.0 \times 10^{-8}$

first-step ciprofloxacin-resistant isolates CIP-R1 and CIP-R2 were also isolated on 16× MIC and 8× MIC agar plates inoculated with *N. gonorrhoeae* ATCC 49226, respectively.

For TP-R1, the frequencies of resistance to TP0480066 ranged from 1.4×10^{-4} at 2× MIC to $<7.1 \times 10^{-8}$ at 8, 16, 32, and 64× MIC. For CIP-R1, the frequencies of resistance to ciprofloxacin ranged from $>2.1 \times 10^{-3}$ at 2× MIC to $<7.0 \times 10^{-8}$ at 4, 8, 16, 32, and 64× MIC. The second-step TP0480066-resistant isolates TP-R1-1 to TP-R1-3 were isolated on 4× MIC agar plates inoculated with TP-R1. The experiment was performed only one time.

Characterization of TP0480066-resistant isolates. We evaluated the drug susceptibility of the above-mentioned isolates (Table 3). The MICs of TP0480066 against TP-R1 to TP-R4 were increased 8 to 16 times relative to those of the parent strain. The MICs of TP0480066 against TP-R1-1 to TP-R1-3 were increased 2 to 4 times relative to those of TP-R1. On the other hand, the MICs of ciprofloxacin and ceftriaxone were not changed in the TP0480066-resistant isolates relative to those of the parent strain.

The MICs of ciprofloxacin against CIP-R1 and CIP-R2 were increased 16 to 32 times relative to the parent strain. On the other hand, the MICs of TP0480066 for these ciprofloxacin-resistant isolates were almost unchanged relative to those of the parent strain.

DNA sequencing showed that all the TP0480066-resistant isolates contained a single amino acid Thr169Ile alteration in ParE. No additional mutations were identified in the second-step resistant isolates. CIP-R1 and CIP-R2 each had a single amino acid Asp95Asn alteration in a GyrA QRDR.

Time-kill curve analysis. TP0480066 reduced the viable *N. gonorrhoeae* ATCC 49226 counts by more than 3-log₁₀ CFU/ml (99.9%) after 6 h at 4× MIC and after 24 h at the MIC, respectively (Fig. 1A). Ciprofloxacin reduced the viable *N. gonorrhoeae*

TABLE 3 Characterization and susceptibilities of isolates obtained from *in vitro* spontaneous resistance study of TP0480066 and ciprofloxacin

Isolate	Description	MIC (μg/ml) (fold-increase in MIC)			Amino acid alteration relative to parent isolate
		TP0480066	Ciprofloxacin	Ceftriaxone	
ATCC 49226	Parent	0.0005 (–)	0.004 (–)	0.015 (–)	NA ^a
TP-R1	1st-step isolate	0.008 (16)	0.004 (–)	0.015 (–)	ParE;Thr169Ile
TP-R2	1st-step isolate	0.008 (16)	0.004 (–)	0.015 (–)	ParE;Thr169Ile
TP-R3	1st-step isolate	0.008 (16)	0.004 (–)	0.015 (–)	ParE;Thr169Ile
TP-R4	1st-step isolate	0.004 (8)	0.004 (–)	0.015 (–)	ParE;Thr169Ile
CIP-R1	1st-step isolate	0.0005 (–)	0.12 (32)	0.015 (–)	GyrA;Asp95Asn
CIP-R2	1st-step isolate	0.001 (2)	0.06 (16)	0.015 (–)	GyrA;Asp95Asn
TP-R1-1	2nd-step isolate	0.015 (32)	0.004 (–)	0.015 (–)	ParE;Thr169Ile
TP-R1-2	2nd-step isolate	0.015 (32)	0.004 (–)	0.015 (–)	ParE;Thr169Ile
TP-R1-3	2nd-step isolate	0.03 (64)	0.004 (–)	0.015 (–)	ParE;Thr169Ile

^aNA, not applicable.

FIG 1 *In vitro* time-kill curves of TP0480066, ciprofloxacin, and ceftriaxone against *N. gonorrhoeae*. Time-kill curves of TP0480066 (A), ciprofloxacin (B), and ceftriaxone (C) against *N. gonorrhoeae* ATCC 49226. Time-kill curves of TP0480066 against *N. gonorrhoeae* NCTC 13479 (D) and *N. gonorrhoeae* NCTC 13821 (E).

ATCC 49226 counts by more than 3-log_{10} CFU/ml after 2 h at $4\times$ MIC and after 6 h at the MIC (Fig. 1B). Ceftriaxone reduced the viable *N. gonorrhoeae* ATCC 49226 counts by more than 3-log_{10} CFU/ml after 6 h at $4\times$ MIC and 8 h at the MIC (Fig. 1C). TP0480066 also reduced the viable *N. gonorrhoeae* NCTC 13479 (ciprofloxacin-resistant strain) and *N. gonorrhoeae* NCTC 13821 (ciprofloxacin- and ceftriaxone-resistant strain) cell counts after 6 h at $4\times$ MIC (Fig. 1D and E). Reproducibility was confirmed by two independent experiments.

***In vivo* efficacy.** The viable cell counts of ciprofloxacin-susceptible *N. gonorrhoeae* ATCC 49226 or ciprofloxacin-resistant NCTC 13479 in control mice and in mice after the administration of the test compounds are shown in Fig. 2. The oral administration

FIG 2 *In vivo* effect of TP0480066 on vaginal infection with *N. gonorrhoeae*. Therapeutic effects of TP0480066 and ciprofloxacin against vaginal infection with *N. gonorrhoeae* ATCC 49226 (A) or *N. gonorrhoeae* NCTC 13479 (B) in mice. Detection limit: $0.699 \log_{10}$ CFU/vagina. *, $P < 0.05$; **, $P < 0.01$.

of ciprofloxacin at the two doses of 10 and 30 mg/kg led to significant decreases in the numbers of viable cell counts of the ciprofloxacin-susceptible strain. However, the viable cell count of the ciprofloxacin-resistant strain in mice treated with ciprofloxacin (30 mg/kg) was not significantly lower than that in the control group. On the other hand, dose-dependent decreases in the numbers of not only ciprofloxacin-susceptible but also ciprofloxacin-resistant strains at 24 h after a single dose of subcutaneously administered TP0480066 were observed. At doses of 30 and 100 mg/kg of TP0480066, the mean viable cell counts were significantly decreased in both strains relative to those in the vehicle control. Reproducibility was confirmed by two independent experiments. Furthermore, the pharmacokinetic profiles of TP0480066 in BALB/c mice were investigated after a single subcutaneous administration at a dose of 10 mg/kg. The maximum plasma concentration, time to maximum plasma concentration, half-life, and area under the concentration-time curve from 0 h to infinity of TP0480066 were determined to be 3,210 ng/ml, 0.333 h, 1.75 h, and 3,370 ng · h/ml, respectively.

DISCUSSION

WHO has reported that antibiotic resistance is making gonorrhea much harder to treat, and in 2016, they issued updated global treatment recommendations advising doctors to prescribe two antibiotics: ceftriaxone and azithromycin (4, 19). We have synthesized a new class of MAOQ derivative, TP0480066. TP0480066 is a novel bacterial topoisomerase inhibitor that inhibits both bacterial DNA gyrase and topoisomerase IV (18). The present study demonstrated that TP0480066 is highly effective against *N. gonorrhoeae* both *in vitro* and *in vivo*.

TP0480066 showed potent antibacterial activity against tested *N. gonorrhoeae* strains, including strains with decreased susceptibility or resistance to currently available antimicrobial agents. Moreover, the MIC of TP0480066 against the CLSI QC strain *N. gonorrhoeae* ATCC 49226 (MIC, 0.0005 µg/ml) was substantially lower than that of solithromycin (QC MIC range, 0.03 to 0.25 µg/ml) (20), zoliflodacin (MIC, 0.06 µg/ml) (21), and gepotidacin (QC MIC range, 0.25 to 1 µg/ml) (22). TP0480066 reduced viable cell counts of not only *N. gonorrhoeae* ATCC 49226, a susceptible strain, but also ciprofloxacin- and ceftriaxone-resistant *N. gonorrhoeae* strains by more than 3-log₁₀ CFU/ml. These results indicate that TP0480066 has a potent bactericidal activity against *N. gonorrhoeae* strains, including drug-resistant strains.

The MICs of TP0480066 against ciprofloxacin-resistant isolates were similar to those against the parent strain. Furthermore, the MICs of ciprofloxacin against TP0480066-resistant isolates were the same as those against the parent strain. Thus, no cross-resistance was seen between TP0480066 and ciprofloxacin. Next, we analyzed the amino acid alterations of topoisomerases in these isolates. The *gyrA*, *gyrB*, *parC*, and *parE* gene sequences of the TP0480066-resistant isolates were obtained, assembled, and mapped against the parent strain to identify genetic changes. All the TP0480066-resistant isolates had a Thr169Ile alteration in ParE. Based on the results of an amino acid alignment analysis, Thr169 in *N. gonorrhoeae* ParE is equivalent to Thr165 in *Escherichia coli* GyrB. The cocrystal structure of *E. coli* GyrB and 5'-adenylyl-β,γ-imidodiphosphate (ADPNP), a nonhydrolyzable ATP analog, suggests that Thr165 is an amino acid that constitutes an ATP binding site (23). On the other hand, ciprofloxacin-resistant isolates had an Asp95Asn alteration in GyrA QRDRs (Table 3). The *N. gonorrhoeae* ParC Asp86Asn alteration, which is associated with fluoroquinolone resistance, appeared to be associated with increased gepotidacin MICs, as well (24). Furthermore, GyrA (Ser91Phe and Asp95Asn/Gly) and ParC (Asp86Asn) alterations were observed in isolates from three microbiological failures in the gepotidacin phase II trial (25). In contrast, zoliflodacin-resistant *N. gonorrhoeae* isolates had Asp429Asn, Lys450Thr, and Ser467Asn alterations in GyrB (21). These results suggest that TP0480066 interacts with DNA gyrase or topoisomerase IV via a binding mode that is distinct from that of ciprofloxacin, zoliflodacin, and gepotidacin.

The second-step isolates obtained from a spontaneous resistance study had no

additional mutations in topoisomerase genes *gyrA*, *gyrB*, *parC*, or *parE* sequenced from PCR products. Although whole-genome sequencing was not performed, considering that the increase in the MIC of TP0480066 against second-step isolates was only 2- to 4-fold compared with that of TP-R1 (Table 3), another mechanism, such as an efflux pump mutation, might be related to resistance of second-step isolates (26–29). All TP0480066-resistant isolates had an alteration in *parE* gene but not in *gyrB*, indicating the possibility that TP0480066 acts as a single target inhibitor of ParE intracellularly which could be one of the causes of the relatively higher frequency of resistance of TP0480066. The unbalanced activity of TP0480066 observed in *E. coli* DNA gyrase and topoisomerase IV (18) could make TP0480066 a single target inhibitor since the tested concentration of TP0480066 might not be sufficient to inhibit both enzymes in bacterial cell. Although enzyme inhibitory activity of TP0480066 against topoisomerases of *N. gonorrhoeae* is not tested, unbalanced activity of TP0480066 might also be observed in *N. gonorrhoeae*.

We investigated the *in vivo* efficacy of TP0480066 in a mouse model of vaginal *N. gonorrhoeae* infection. Although *N. gonorrhoeae* is a strictly human pathogen, estradiol-treated female mice can be infected with *N. gonorrhoeae*, and murine infection mimics several aspects of human infection (30). At doses of 30 and 100 mg/kg of TP0480066, the mean viable cell counts were significantly decreased relative to those of the vehicle control in both ciprofloxacin-susceptible and ciprofloxacin-resistant *N. gonorrhoeae* models (Fig. 2).

Here, we showed that TP0480066, a novel MAOQ derivative, exhibits potent *in vitro* antibacterial and bactericidal activities against *N. gonorrhoeae*, including drug-resistant strains, a low frequency of resistance, and a potential new mode of target binding. TP0480066 was also effective when administered as a single dose in an *N. gonorrhoeae* *in vivo* model. These results support that TP0480066 could be a new option for the treatment of gonorrhea.

MATERIALS AND METHODS

Antibacterials. TP0480066 was synthesized by Taisho Pharmaceutical Co., Ltd. (Saitama, Japan) as described elsewhere (18). Ciprofloxacin was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Penicillin G, ceftriaxone, azithromycin, vancomycin, levofloxacin, spectinomycin, streptomycin, and trimethoprim were purchased from Sigma-Aldrich (St. Louis, MO).

Bacterial strains. *N. gonorrhoeae* strains ATCC 49226, ATCC 43069, ATCC BAA-1846, ATCC 700717, and ATCC 700825 were obtained from the American Type Culture Collection (ATCC). *N. gonorrhoeae* strains NCTC 13477 (WHO F), NCTC 13478 (WHO G), NCTC 13479 (WHO K), NCTC 13480 (WHO L), NCTC 13481 (WHO M), NCTC 13482 (WHO N), NCTC 13483 (WHO O), NCTC 13818 (WHO V), and NCTC 13821 (WHO Y) were obtained from the National Collection of Type Cultures (NCTC) (31, 32).

Antimicrobial susceptibility testing. The MICs of TP0480066 and the comparator agents were determined using the Clinical and Laboratory Standards Institute (CLSI) methodology, described in CLSI documents (33, 34).

Frequency of resistance determinations. The frequencies of spontaneous resistance to TP0480066 and ciprofloxacin for *N. gonorrhoeae* ATCC 49226 were measured by plating onto GC agar plates (GC agar base [Nippon Becton, Dickinson and Company, Tokyo, Japan] and 1% defined growth supplement [35, 36]) containing TP0480066 or ciprofloxacin at 2, 4, 8, 16, 32, and 64 times the MIC. Inoculated plates were incubated for 48 h at 36°C, and the numbers of colonies were counted. The frequency of spontaneous resistance to TP0480066 and ciprofloxacin was calculated as the ratio of the number of confirmed resistant colonies to the total number of inoculated viable cells. If no colonies were observed on the compound-containing agar plates, the frequency of spontaneous resistance development was expressed as less than the frequency at which one resistant colony was obtained. The frequencies of second-step spontaneous resistance to TP0480066 or ciprofloxacin for *N. gonorrhoeae* TP-R1 or CIP-R1 were obtained in the same way.

Characterization of TP0480066-resistant mutants. To identify the mutated genes of TP0480066- and ciprofloxacin-resistant mutants, *gyrA*, *gyrB*, *parC*, and *parE* were amplified using PCR and sequenced. The following PCR primers were used: Ng_gyrA_Fw1 5'-ACTCCGCCACGAAATTCCT-3' and Ng_gyrA_Rv1 5'-GCTTCGTGCGCTTATCTGA-3', Ng_gyrB_Fw1 5'-CAAGGTGCGGACTGTTTGG-3' and Ng_gyrB_Rv1 5'-CAAGCGAGCCGTAATGTCG-3', Ng_parC_Fw1 5'-CGTTCATTGCGTTTGTCCG-3' and Ng_parC_Rv1 5'-AAAGCCTGCCATTGCCATTG, and Ng_parE_Fw1 5'-GCGACGTTTATTGGAGGCG-3' and Ng_parE_Rv1 5'-GCGCAAAACCATCTTCCTG-3'. The primers were purchased from Eurofins Genomics KK (Tokyo, Japan).

A single colony of each isolate was selected from a plate, transferred to Tris-EDTA (TE) buffer (pH 8.0) in a microtube, and treated for 10 min at 95°C. An aliquot was used as the source of template DNA in a PCR using the Tks Gflex DNA polymerase kit (TaKaRa Bio Inc., Shiga, Japan) and a TaKaRa PCR Thermal

Cycler Dice. The PCR product was purified using a QIAquick PCR purification kit (Qiagen KK, Tokyo, Japan) and sequenced using an Applied Biosystems 3730 series DNA analyzer (Thermo Fisher Scientific KK, Kanagawa, Japan). The obtained sequences of *gyrA*, *gyrB*, *parC*, and *parE* were compared with those of *N. gonorrhoeae* ATCC 49226.

Time-kill curve analysis. Time-kill studies were performed for three representative *N. gonorrhoeae* strains (ATCC 49226, NCTC 13479, and NCTC 13821) by referring to previously published methods (37). Briefly, freshly prepared colonies were suspended in gonococcal broth (35, 36) containing 1% supplement C (Nippon Becton, Dickinson Co., Ltd., Tokyo, Japan) and cultured at concentrations of one-fourth the MIC (1/4× MIC), 1× MIC, and 4× MIC of TP0480066, ciprofloxacin, and ceftriaxone, respectively. The CFU count was determined by plating aliquots from serial 10-fold dilutions; the limit of detection was set at $\leq 10^3$ CFU/ml. Bactericidal activity was defined as a ≥ 3 -log₁₀ reduction in bacterial counts.

Mouse model of *N. gonorrhoeae*. A mouse genital tract infection model (38–40) was used to evaluate the *in vivo* effect of TP0480066 ($n = 5$ to 8). Female BALB/c mice were purchased from Japan SLC, Inc. (Shizuoka, Japan) at 6 weeks of age and allowed to acclimate to the animal facility for 3 days. Mice were then treated with water-soluble 17 β -estradiol (Sigma-Aldrich Japan, Tokyo, Japan) administered subcutaneously 2 days prior to inoculation and on the day of inoculation. Antibiotics (0.24 mg streptomycin and 0.4 mg vancomycin administered twice daily via intraperitoneal injection, and 0.04 g trimethoprim per 100 ml drinking water) were administered from 2 days prior to inoculation through the day of inoculation to control commensal bacteria. On day 0, the mice were vaginally inoculated with *N. gonorrhoeae* ATCC 49226 (2.6×10^7 CFU) or *N. gonorrhoeae* NCTC 13479 (1.5×10^7 CFU). TP0480066 was then administered 2 h after inoculation at a dose of 1, 3, 10, 30, or 100 mg/kg (subcutaneously). Ciprofloxacin was dosed at 3, 10, or 30 mg/kg (orally) as a positive control. At 24 h after inoculation, a vaginal mucus swab was collected and suspensions were plated onto Modified Thayer-Martin (MTMII) Agar (Nippon Becton, Dickinson and Company, Tokyo, Japan) to determine the *N. gonorrhoeae* count.

All the *in vivo* experimental protocols used in this study were approved by the guidelines of the Taisho Pharmaceutical animal care committee and met the Japanese Experimental Animal Research Association standards, as defined in the Guidelines for Animal Experiments (1987).

Statistical analyses. Statistical significance was tested using the Wilcoxon's test for two sample comparisons and the Steel's test for multiple sample comparisons. All the data were analyzed using SAS9.2 (SAS Statistical Institute, Cary, NC, USA).

Pharmacokinetic study. Seven-week-old female BALB/c mice (Japan SLC) were used for the pharmacokinetic study. TP0480066 was subcutaneously administered to mice ($n = 3$) at a dose of 10 mg/kg. Blood samples were taken from the tail vein at 0.0833, 0.25, 0.5, 1, 2, 4, and 8 h postdose and from the posterior vena cava under isoflurane anesthesia at 24 h postdose. The blood sample was placed into a tube containing ethylenediamine-*N,N,N',N'*-tetraacetic acid dipotassium salt dihydrate as an anticoagulant and centrifuged to prepare plasma sample. The quantitative analysis was performed using a liquid chromatography-tandem mass spectrometry method. The lower limit of quantification for TP0480066 was 10.0 ng/ml in plasma. The pharmacokinetic parameters of TP0480066 in plasma were calculated by a noncompartmental analysis method with pharmacokinetic analysis software Phoenix WinNonlin 6.2 (Certara, Princeton, NJ).

ACKNOWLEDGMENTS

We thank Masashi Mima at Taisho Pharmaceutical Co., Ltd., for valuable discussions. All the authors are employees of Taisho Pharmaceutical Co., Ltd. The authors have nothing else to declare.

REFERENCES

- Newman L, Rowley J, Vander Hoorn S, Wijesooriya NS, Unemo M, Low N, Stevens G, Gottlieb S, Kiarie J, Temmerman M. 2015. Global estimates of the prevalence and incidence of four curable sexually transmitted infections in 2012 based on systematic review and global reporting. *PLoS One* 10:e0143304. <https://doi.org/10.1371/journal.pone.0143304>.
- Centers for Disease Control and Prevention. 2016. Sexually Transmitted Diseases Surveillance. Centers for Disease Control and Prevention, Atlanta, GA.
- Centers for Disease Control and Prevention. 2013. Antibiotic resistance threats in the United States. Centers for Disease Control and Prevention, Atlanta, GA.
- Wi T, Lahra MM, Ndowa F, Bala M, Dillon J-AR, Ramon-Pardo P, Eremin SR, Bolan G, Unemo M. 2017. Antimicrobial resistance in *Neisseria gonorrhoeae*: global surveillance and a call for international collaborative action. *PLoS Med* 14:e1002344. <https://doi.org/10.1371/journal.pmed.1002344>.
- Yokoi S, Deguchi T, Ozawa T, Yasuda M, Ito S, Kubota Y, Tamaki M, Maeda S. 2007. Threat to cefixime treatment for gonorrhea. *Emerg Infect Dis* 13:1275–1277. <https://doi.org/10.3201/eid1308.060948>.
- Deguchi T, Yasuda M, Yokoi S, Ishida K, Ito M, Ishihara S, Minamidate K, Harada Y, Tei K, Kojima K, Tamaki M, Maeda S. 2003. Treatment of uncomplicated gonococcal urethritis by double-dosing of 200 mg cefixime at a 6-h interval. *J Infect Chemother* 9:35–39. <https://doi.org/10.1007/s10156-002-0204-8>.
- Unemo M, Golparian D, Nicholas R, Ohnishi M, Galloway A, Sednaoui P. 2012. High-level cefixime- and ceftriaxone-resistant *Neisseria gonorrhoeae* in France: novel *penA* mosaic allele in a successful international clone causes treatment failure. *Antimicrob Agents Chemother* 56:1273–1280. <https://doi.org/10.1128/AAC.05760-11>.
- Ohnishi M, Golparian D, Shimuta K, Saika T, Hoshina S, Iwasaku K, Nakayama S, Kitawaki J, Unemo M. 2011. Is *Neisseria gonorrhoeae* initiating a future era of untreatable gonorrhea? Detailed characterization of the first strain with high-level resistance to ceftriaxone. *Antimicrob Agents Chemother* 55:3538–3545. <https://doi.org/10.1128/AAC.00325-11>.
- Cámara J, Serra J, Ayats J, Bastida T, Carnicer-Pont D, Andreu A, Ardanuy C. 2012. Molecular characterization of two high-level ceftriaxone-resistant *Neisseria gonorrhoeae* isolates detected in Catalonia, Spain. *J Antimicrob Chemother* 67:1858–1860. <https://doi.org/10.1093/jac/dks162>.
- Golparian D, Fernandes P, Ohnishi M, Jensen JS, Unemo M. 2012. *In vitro* activity of the new fluoroketolide solithromycin (CEM-101) against a large collection of clinical *Neisseria gonorrhoeae* isolates and international reference strains, including those with high-level antimicrobial resistance:

- potential treatment option for gonorrhea? Antimicrob Agents Chemother 56:2739–2742. <https://doi.org/10.1128/AAC.00036-12>.
11. Kern G, Palmer T, Ehmann DE, Shapiro AB, Andrews B, Basarab GS, Doig P, Fan J, Gao N, Mills SD, Mueller J, Sriram S, Thresher J, Walkup GK. 2015. Inhibition of *Neisseria gonorrhoeae* type II topoisomerases by the novel Spiropyrimidinetrione AZD0914. J Biol Chem 290:20984–20994. <https://doi.org/10.1074/jbc.M115.663534>.
 12. Farrell DJ, Sader HS, Rhomberg PR, Scangarella-Oman NE, Flamm RK. 2017. *In vitro* activity of gepotidacin (GSK2140944) against *Neisseria gonorrhoeae*. Antimicrob Agents Chemother 61:e02047-16. <https://doi.org/10.1128/AAC.02047-16>.
 13. Belland RJ, Morrison SG, Ison C, Huang WM. 1994. *Neisseria gonorrhoeae* acquires mutations in analogous regions of *gyrA* and *parC* in fluoroquinolone-resistant isolates. Mol Microbiol 14:371–380. <https://doi.org/10.1111/j.1365-2958.1994.tb01297.x>.
 14. Deguchi T, Yasuda M, Nakano M, Asano M, Tada K, Iwata H, Komeda H, Ezaki T, Saito I, Kawada Y. 1995. DNA gyrase mutations in quinolone-resistant clinical isolates of *Neisseria gonorrhoeae*. Antimicrob Agents Chemother 39:561–563. <https://doi.org/10.1128/AAC.39.2.561>.
 15. Deguchi T, Yasuda M, Nakano M, Ozeki S, Ezaki T, Saito I, Kawada Y. 1996. Quinolone-resistant *Neisseria gonorrhoeae*: correlation of alterations in the GyrA subunit of DNA gyrase and the ParC subunit of topoisomerase IV with antimicrobial susceptibility profiles. Antimicrob Agents Chemother 40:1020–1023. <https://doi.org/10.1128/AAC.40.4.1020>.
 16. Deguchi T, Yasuda M, Nakano M, Ozeki S, Kanematsu E, Kawada Y, Ezaki T, Saito I. 1996. Uncommon occurrence of mutations in the *gyrB* gene associated with quinolone resistance in clinical isolates of *Neisseria gonorrhoeae*. Antimicrob Agents Chemother 40:2437–2438. <https://doi.org/10.1128/AAC.40.10.2437>.
 17. Llanes R, Sosa J, Guzmán D, Gutiérrez Y, Llop A, Ricardo O. 2001. *Neisseria gonorrhoeae* resistant to ciprofloxacin: first report in Cuba. Sex Transm Dis 28:82–83. <https://doi.org/10.1097/00007435-200102000-00004>.
 18. Ushiyama F, Amada H, Mihara Y, Takeuchi T, Tanaka-Yamamoto N, Mima M, Kamitani M, Wada R, Tamura Y, Endo M, Masuko A, Takata I, Hitaka K, Sugiyama H, Ohtake N. 2020. Lead optimization of 8-(methylamino)-2-oxo-1,2-dihydroquinolines as bacterial type II topoisomerase inhibitors. Bioorg Med Chem 28:115776. <https://doi.org/10.1016/j.bmc.2020.115776>.
 19. Alirol E, Wi TE, Bala M, Bazzo ML, Chen XS, Deal C, Dillon JR, Kularatne R, Heim J, Hooft van Huijsduijnen R, Hook EW, Lahra MM, Lewis DA, Ndowa F, Shafer WM, Tayler L, Workowski K, Unemo M, Balasegaram M. 2017. Multidrug-resistant gonorrhea: a research and development roadmap to discover new medicines. PLoS Med 14:e1002366. <https://doi.org/10.1371/journal.pmed.1002366>.
 20. Riedel S, Ross JE, Farrell DJ, Flamm RK, Jones RN. 2015. Determination of disk diffusion and MIC quality control guidelines for Solithromycin, a novel fluoroketolide antibacterial, against *Neisseria gonorrhoeae*. J Clin Microbiol 53:3888–3890. <https://doi.org/10.1128/JCM.02250-15>.
 21. Alm RA, Lahiri SD, Kutschke A, Otterson LG, McLaughlin RE, Whiteaker JD, Lewis LA, Su X, Huband MD, Gardner H, Mueller JP. 2015. Characterization of the novel DNA gyrase inhibitor AZD0914: low resistance potential and lack of cross-resistance in *Neisseria gonorrhoeae*. Antimicrob Agents Chemother 59:1478–1486. <https://doi.org/10.1128/AAC.04456-14>.
 22. Jones RN, Fedler KA, Scangarella-Oman NE, Ross JE, Flamm RK. 2016. Multicenter investigation of gepotidacin (GSK2140944) agar dilution quality control determinations for *Neisseria gonorrhoeae* ATCC 49226. Antimicrob Agents Chemother 60:4404–4406. <https://doi.org/10.1128/AAC.00527-16>.
 23. Brino L, Urzhumtsev A, Mousli M, Bronner C, Mitschler A, Oudet P, Moras D. 2000. Dimerization of *Escherichia coli* DNA-gyrase B provides a structural mechanism for activating the ATPase catalytic center. J Biol Chem 275:9468–9475. <https://doi.org/10.1074/jbc.275.13.9468>.
 24. Jacobsson S, Golparian D, Scangarella-Oman N, Unemo M. 2018. *In vitro* activity of the novel triazaacenaphthylene gepotidacin (GSK2140944) against MDR *Neisseria gonorrhoeae*. J Antimicrob Chemother 73:2072–2077. <https://doi.org/10.1093/jac/dky162>.
 25. Scangarella-Oman N, Hossain M, Dixon P, Ingraham I, Min S, Tiffany C, Perry C, Raychaudhuri R, Dumont E, Huang J, Hook E, III, Miller L. 2017. Microbiological analysis from a phase II study in adults evaluating single doses of gepotidacin (GSK2140944) in the treatment of uncomplicated urogenital gonorrhea caused by *Neisseria gonorrhoeae*. Antimicrob Agents Chemother 62:e01221-18.
 26. Hagman KE, Pan W, Spratt BG, Balthazar JT, Judd RC, Shafer WM. 1995. Resistance of *Neisseria gonorrhoeae* to antimicrobial hydrophobic agents is modulated by the mtrRDE efflux system. Microbiology 141:611–622. <https://doi.org/10.1099/13500872-141-3-611>.
 27. Rouquette-Loughlin CE, Balthazar JT, Shafer WM. 2005. Characterization of the MacA-MacB efflux system in *Neisseria gonorrhoeae*. J Antimicrob Chemother 56:856–860. <https://doi.org/10.1093/jac/dki333>.
 28. Lee EH, Rouquette-Loughlin C, Folster JP, Shafer WM. 2003. FarR regulates the farAB-encoded efflux pump of *Neisseria gonorrhoeae* via an MtrR regulatory mechanism. J Bacteriol 185:7145–7152. <https://doi.org/10.1128/jb.185.24.7145-7152.2003>.
 29. Rouquette-Loughlin C, Dunham SA, Kuhn M, Balthazar JT, Shafer WM. 2003. The NorM efflux pump of *Neisseria gonorrhoeae* and *Neisseria meningitidis* recognizes antimicrobial cationic compounds. J Bacteriol 185:1101–1106. <https://doi.org/10.1128/jb.185.3.1101-1106.2003>.
 30. Jerse AE. 1999. Experimental gonococcal genital tract infection and opacity protein expression in estradiol-treated mice. Infect Immun 67:5699–5708. <https://doi.org/10.1128/IAI.67.11.5699-5708.1999>.
 31. Unemo M, Fasth O, Fredlund H, Limnios A, Tapsall J. 2009. Phenotypic and genetic characterization of the 2008 WHO *Neisseria gonorrhoeae* reference strain panel intended for global quality assurance and quality control of gonococcal antimicrobial resistance surveillance for public health purposes. J Antimicrob Chemother 63:1142–1151. <https://doi.org/10.1093/jac/dkp098>.
 32. Unemo M, Golparian D, Sánchez-Busó L, Grad Y, Jacobsson S, Ohnishi M, Lahra MM, Limnios A, Sikora AE, Wi T, Harris SR. 2016. The novel 2016 WHO *Neisseria gonorrhoeae* reference strains for global quality assurance of laboratory investigations: phenotypic, genetic and reference genome characterization. J Antimicrob Chemother 71:3096–3108. <https://doi.org/10.1093/jac/dkw288>.
 33. Clinical and Laboratory Standards Institute. 2015. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard—tenth edition. CLSI document M07-A10. Clinical and Laboratory Standards Institute, Wayne, PA.
 34. Clinical and Laboratory Standards Institute. 2016. Performance standards for antimicrobial susceptibility testing; twenty-sixth informational supplement. CLSI document M100-S26. Clinical and Laboratory Standards Institute, Wayne PA.
 35. Geers TA, and, Donabedian AM. 1989. Comparison of broth microdilution and agar dilution for susceptibility testing of *Neisseria gonorrhoeae*. Antimicrob Agents Chemother 33:233–234. <https://doi.org/10.1128/aac.33.2.233>.
 36. Hafiz S, and, McEntegart MG. 1976. Prolonged survival of *Neisseria gonorrhoeae* in a new liquid medium. Br J Vener Dis 52:381–383. <https://doi.org/10.1136/sti.52.6.381>.
 37. Hauser C, Hirzberger L, Unemo M, Furrer H, Endimiani A. 2015. *In vitro* activity of fosfomycin alone and in combination with ceftriaxone or azithromycin against clinical *Neisseria gonorrhoeae* isolates. Antimicrob Agents Chemother 59:1605–1611. <https://doi.org/10.1128/AAC.04536-14>.
 38. Kita E, Matsuura H, Kashiba S. 1981. A mouse model for the study of gonococcal genital infection. J Infect Dis 143:67–70. <https://doi.org/10.1093/infdis/143.1.67>.
 39. Mathanraj P, Hong W, Sandra JV, Nikolaos M, Ann EJ, Robin RI. 2012. Protective role of toll-like receptor 4 in experimental Gonococcal infection of female mice. Mucosal Immunol 5:19–29.
 40. Savage VJ, Charrier C, Salisbury AM, Box H, Chaffer-Malam N, Huxley A, Kirk R, Noonan GM, Mohamed S, Craighead MW, Ratcliffe AJ, Best SA, Stokes NR. 2016. Efficacy of a novel tricyclic topoisomerase inhibitor in a murine model of *Neisseria gonorrhoeae* infection. Antimicrob Agents Chemother 60:5592–5594. <https://doi.org/10.1128/AAC.00913-16>.