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A New Beginning to the Existing Medicines; Repurposing FDA-Approved Drugs for the Neglected Re-Emerging Disease Leptospirosis

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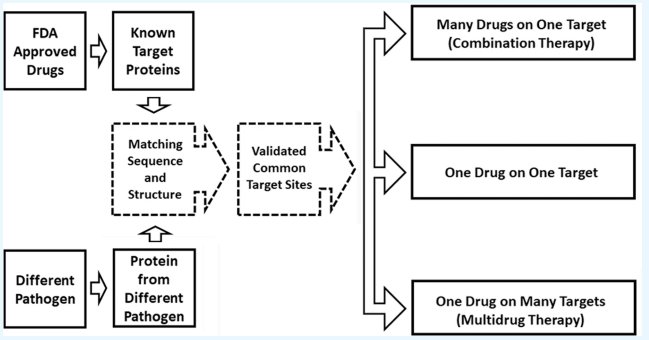
ABSTRACT: Leptospirosis is one of the re-emerging zoonotic diseases, especially in tropical regions. Many antibiotics are used to treat leptospirosis, but there are no scientific evidence-based guidelines or systematic clinical trials for using these drugs. A bioinformatics approach was made to shortlist some Food and Drug Administration (FDA) of the United States of America-approved and currently used drugs for leptospirosis. The existing drugs from the Drug Bank database, which are currently not used for leptospirosis, were selected to identify their target proteins and binding sites using bioinformatics methods. Orthologues of these target proteins were selected from the proteome database of *Leptospira*. The similar sites and their interactions with the drugs were validated and recommended for use in leptospirosis. Further, the sensitivity of recommended drugs was also validated in vitro. The sequences and structures of these proteins were compared under strictly controlled parameters and shortlisted Gatifloxacin, Imipenem, Latamoxef, Doripenem, Tigecycline, and Lactams as repurposable drugs for leptospirosis. An in vitro validation of the drugs showed significant antileptospiral activity in 12 serovars with low IC_{50} concentrations and also showed that the IC_{50} values varied across *Leptospira* serovars. Further, suitable proteins under the concept of "One Target, Many Drugs" identified DNA gyrase subunit A (Q72WD1), 30S ribosomal protein S9 (Q72U99), and 30S ribosomal protein S12 (Q72UA6), and these proteins were found across the pathogenic, saprophytic, and intermediate species of *Leptospira*. We describe a method to find repurposable drugs from the approved list that are not currently used to treat leptospirosis and validate them to be taken forward for systematic clinical trials specific to leptospirosis for recommendations in clinical use.



INTRODUCTION

Leptospirosis is a zoonotic disease caused by a corkscrew-shaped pathogenic spirochete under the genus *Leptospira* that comprises 64 species and more than 260 serovars.[1] Although several mammals have been identified as potential reservoirs, rats are the most significant.[2] Leptospirosis occurs after direct or indirect contact with the water contaminated with the urine of the reservoir as well as other host animals, including domestic animals such as cattle, dogs, pigs, and many forest animals. In rodents, the infection is asymptomatic, which results in chronic renal carriage and perpetual dissemination of the pathogen in the environment.[3] In earlier days, the disease was mainly found in villages and is presently prevalent in urban areas. Increased urbanization increased slum areas with poor drainage, hideouts for reservoir animals, and living near domestic and pet animals. Heavy rains and frequent floods due to global climate change bring out water contaminated with the urine of these animals from drainage and hideouts and make a highly contagious environment, sprouting leptospirosis outbreaks, which classified the disease under re-emerging zoonoses.[4,5]

Leptospira is estimated to infect more than a million people, with approximately 60,000 deaths annually.[6,7] The number of fatal cases is comparable to or even higher than some major neglected tropical diseases, such as dengue or visceral leishmaniasis.[6] Clinical manifestations in infected humans are incredibly variable, including flu-like symptoms, and spontaneously resolve in 90% of cases.[8,9] However, 10% of patients in this review develop severe disease forms and are designated as susceptible hosts. Hepatic dysfunctions associated with renal failure and hemorrhages constitute Weil's syndrome, a severe

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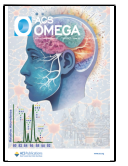

Table 1. Drugs Currently in Use for the Treatment of Leptospirosis

drug bank ID	drug name	molecular mass (g/mol)	description	chemical class
DB00430	cefpiramide	612.6	3rd generation cephalosporin family	carboxylic acids and derivatives
DB01329	cefoperazone	645.7	3rd generation cephalosporin family	lactams
DB01332	ceftizoxime	383.4	3rd generation cephalosporin family	lactams
DB01333	cefradine	349.4	1st generation cephalosporin family	carboxylic acids and derivatives
DB01415	ceftibuten	410.42	3rd generation cephalosporin family	lactams
DB00274	cefmetazole	471.5	2nd generation cephalosporin family	carboxylic acids and derivatives
DB00438	ceftazidime	546.6	3rd generation cephalosporin family	organoheterocyclic compounds
DB01327	cefazolin	454.507	1st generation cephalosporin family	lactams
DB01413	cefepime	480.6	4th generation cephalosporin family	lactams
DB01416	cefpodoxime	427.5	3rd generation cephalosporin family	lactams
DB04918	ceftobiprole	534.6	5th generation cephalosporin family	lactams
DB00671	cefixime	453.5	3rd generation cephalosporin family	lactams
DB01150	cefprozil	389.426	2nd generation cephalosporin family	lactams
DB00254	doxycycline	444.4	2nd generation tetracycline family	phenylpropanoids and polyketides

form of leptospirosis.[8] Acute kidney injury (AKI) is commonly reported as an early manifestation of acute leptospirosis and could evolve into chronic kidney disease (CKD).[10,11] Severe pulmonary hemorrhagic syndrome (SPHS) with acute respiratory distress syndrome (ARDS) can also occur and can be confused with viral pneumonitis.[8] Severe human leptospirosis is characterized by multiorgan failures and is associated with a dramatic increase in the mortality rate.[8] The most severe form of the disease, with multisystem damage, including vascular, hepatic, renal, pulmonary, and skeletal muscle injury, is known as the Weil syndrome.[12] Early diagnosis remains challenging due to a lack of clinical suspicion among physicians, its nonspecific symptoms, and limited availability of rapid point-of-care diagnostic tests, and the initial presentations are usually difficult to distinguish from other tropical infectious diseases.

Antibiotic treatment is widely used, but a Cochrane review found insufficient evidence to recommend or against using antibiotics for leptospirosis.[13] The use of antibiotics for leptospirosis may decrease the duration of clinical illness by 2−4 days, although this result was not statistically significant.[13] The efficacy of treatment with antibiotics such as penicillin, doxycycline, azithromycin, or cephalosporins, commonly found in leptospirosis treatment regimes, did not seem to impact mortality or the duration of the fever.[14,15] Therefore, it was concluded that antibiotic therapy's benefit in treating leptospirosis remains unclear, particularly for severe diseases.[16] Therefore, antibiotics may not be used for mild cases in low-risk people.

Though penicillin is the first choice in treating leptospirosis, antibiotics like azithromycin, chloramphenicol, cephalosporins, and doxycycline are also explored.[17] However, the lack of well-defined clinical trials and opacity in the exact role of antibiotics hinders the treatment of leptospirosis.[17] Many antibiotics have not undergone clinical trials, specifically for leptospirosis.[18] Doxycycline, the preferred drug for prophylaxis, is also under ambiguity regarding its effectiveness.[15,19] Attempting undesignated antibiotics to treat leptospirosis can lead to complications like the Jarisch−Herxheimer reaction.[20] The advent of bioinformatics could find drug targets, identify similar targets in other organisms, and assess the compatibility of repurposing the drug to treat a different disease. Here, we describe several antibiotics that have already been used to treat other illnesses and could be repurposed for leptospirosis (Table 1).

RESULTS

Sequence Analysis. Sequence similarity of query and target was performed using the HMMER web server program, resulting in 125 drugs linked with 42 target protein sequences that were considered for further analysis (Table 2). The drugs belonged to the class benzene and substituted derivatives (Sulfacetamide), carbohydrates and carbohydrate conjugates (Paromomycin and Framycetin), carboxylic acids and derivatives (Latamoxef and Plazomicin), cyclohexylamines (Spectinomycin), lactams (Mezlocillin, Imipenem, Doripenem, and Ertapenem), organooxygen compounds (Amikacin, Netilmicin, and Tobramycin), quinolines and derivatives (Gatifloxacin and Besifloxacin), stilbengs (Bedaquiline), and tetracyclines (Lymecycline, Tigecycline, Demeclocycline, Rolitetracycline, and Minocycline). The target proteins were 30S ribosomal proteins (S9, S10, 11, and 12), ATP synthase subunit c, DNA gyrase subunits A and B, D-transpeptidase, folic acid synthesis protein, penicillin-binding proteins 1A and 1B, and peptidoglycan D. The organisms affected were *Enterobacteriaceae bacterium*, *Escherichia coli*, *Mycobacterium tuberculosis*, *Saccharomyces cerevisiae*, *Streptococcus pneumoniae*, and *Thermus thermophilus*.

Structure Analysis. Without available crystal structures of established targets, potential drug−target interactions in the bacterial proteins were conjectured based on the alignment of functionally relevant regions. Structure analysis was considered for the query protein, which shows >70 similarities in TM-align, which resulted in 22 repurposable drugs with 12 targets (Table 2). There were three targets for each Imipenem, Doripenem, and Ertapenem and two targets for each Gatifloxacin, Latamoxef, and Tigecycline, and all others had one target each (Figure 1).

Identification of Polypharmacological Targets. Screening for drugs that can act on multiple targets was carried out from the identified drugs and their targets. The number of drugs and classes and their interactions with multiple therapeutic indications/disease targets yielded 6 drugs with polypharmacological activity. The list of drugs and their multiple targets is shown in Table 3.

Identification of Drugs That Can Act on Many Targets (One Target−Many Drugs). The screening for drugs that can act on the same target showed 11 drugs that act on three proteins, as shown in Table 4. There were two quinolines and their derivatives, 5 tetracyclines, 3 aminoglycosides, and one aminocyclitol. While quinolines and derivatives act on DNA

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Table 2. Comprehensive List of 22 Shortlisted Potential Repurposable Drugs with Their Known Targets and Predicted Targets in *Leptospira*, Along with Relevant Details (the Query Coverage of All of the Pairs of Query and the Target Protein Is More Than 70%)

si. no.	drug name (drug bank ID)	description chemical class	affected organism	UniProt ID	protein name (gene name)	organism
1	gatifloxacin (DB01044)	quinolines and derivatives	enteric bacteria and other eubacteria, *Mycobacterium, Chlamydia pneumoniae, Legionella pneumophila, Chlamydia psittaci. M. pneumoniae, Chlamydia trachomatis*	P72524	DNA gyrase subunit A (gyrA)	*S. pneumoniae* serotype 4
				P0A4L9	DNA gyrase subunit B (gyrB)	*S. pneumoniae* serotype 4 (strain ATCC BAA-334/TIGR4)
2	besifloxacin (DB06771)	quinolines and derivatives	gram negative and gram-positive bacteria, *Pseudomonas aeruginosa, S. pneumoniae, Haemophilus influenzae, Staphylococcus aureus, Aerococcus viridans, Corynebacterium sp. G, Corynebacterium pseudodiphtheriticum, Corynebacterium striatum, Moraxella catarrhalis, Moraxella lacunata, Staphylococcus epidermidis, Staphylococcus hominis, Staphylococcus warneri, Staphylococcus lugdunensis, Streptococcus mitis, Streptococcus oralis, Streptococcus salivarius.*	P72524	DNA gyrase subunit A (gyrA)	*S. pneumoniae* serotype 4
3	paromomycin (DB01421)	carbohydrates and carbohydrate conjugates	enteric bacteria and other eubacteria	Q5SHN7	30S ribosomal protein S10 (rpsJ)	*T. thermophilus* (strain HB8/ATCC 27634/DSM 579)
4	mezlocillin (DB00948)	lactams	enteric bacteria and other eubacteria	P0AD65	peptidoglycan D (mrdA)	*E. coli* (strain K12)
5	imipenem (DB01598)	lactams	enteric bacteria and other eubacteria	P0AD65	D-transpeptidase (MrdA)	*E. coli* (strain K12)
			N/A	P02918	penicillin-binding protein 1A (mrcA)	*E. coli* (strain K12)
			enteric bacteria and other eubacteria	P02919	penicillin-binding protein 1B (mrcB)	*E. coli* (strain K12)
6	latamoxef (DB04570)	carboxylic acids and derivatives	not available	P02918	penicillin-binding protein (mrcA)	*E. coli* (strain K12)
			enteric bacteria and other eubacteria	P02919	penicillin-binding protein 1B (mrcB)	*E. coli* (strain K12)
7	doripenem (DB06211)	lactams	aerobic and anaerobic microorganisms, *P. aeruginosa, S. pneumoniae, H. influenzae, E. coli, S. aureus, Enterococcus faecalis, M. catarrhalis, Acinetobacter, Enterococcus faecium, Klebsiella, Enterobacter, Streptococcus constellatus, Proteus mirabilis*	P0AD65	penicillin-binding protein 1A (mrdA)	*E. coli* (strain K12)
			enteric bacteria and other eubacteria	P02918	penicillin-binding protein 1A (mrcA)	*E. coli* (strain K12)
			enteric bacteria and other eubacteria	P02919	penicillin-binding protein 1B (mrcB)	*E. coli* (strain K12)
8	plazomicin (DB12615)	carboxylic acids and derivatives	*P. aeruginosa, E. coli*, acinetobacter, enterobacter.	L0LZJ6	30S ribosomal protein S11 (rpsK)	Enterobacteriaceae bacterium (strain FGI 57)
9	lymecycline (DB00256)	tetracyclines	enteric bacteria and other eubacteria	P0A7 × 3	30S ribosomal protein S9 (rpsI)	*E. coli* (strain K12)
10	tigecycline (DB00560)	tetracyclines	enteric bacteria and other eubacteria	P0A7 × 3	30S ribosomal protein S9 (rpsI)	*E. coli* (strain K12)
				P0A7S3	30S ribosomal protein S12 (rpsL)	*E. coli* (strain K12)
11	demeclocycline (DB00618)	tetracyclines	enteric bacteria and other eubacteria	P0A7 × 3	30S ribosomal protein S9 (rpsI)	*E. coli* (strain K12)

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Table 2. continued

si. no.	drug name (drug bank ID)	description chemical class	affected organism	UniProt ID	protein name (gene name)	organism
12	rolitetracycline (DB01301)	tetracyclines	enteric bacteria and other eubacteria	P0A7 × 3	30S ribosomal protein S9 (rpsI)	*E. coli* (strain K12)
13	minocycline (DB01017)	tetracyclines	enteric bacteria and other eubacteria	P0A7 × 3	30S ribosomal protein S9 (rpsI)	*E. coli* (strain K12)
14	ertapenem (DB00303)	lactams	enteric bacteria and other eubacteria	P02918	penicillin-binding protein (mrcA)	*E. coli* (strain K12)
				P0AD68	peptidoglycan D (ftsI)	*E. coli* (strain K12)
				P02919	penicillin-binding protein 1B (mrcB)	*E. coli* (strain K12)
15	sulfacetamide (DB00634)	benzene and substituted derivatives	enteric bacteria and other eubacteria	P53848	Folic acid synthesis protein (FOL1)	*S. cerevisiae*
16	bedaquiline (DB08903)	stilbenes	*M. tuberculosis*	P9WPS1	ATP synthase subunit C (atpE)	*M. tuberculosis* (strain ATCC 25618/H37Rv)
17	framycetin (DB00452)	carbohydrates and carbohydrate conjugates	eEnteric bacteria and other eubacteria	P0A7S3	30S ribosomal protein S12 (rpsL)	*E. coli* (strain K12)
18	amikacin (DB00479)	organooxygen compounds	enteric bacteria and other eubacteria	P0A7S3	30S ribosomal protein S12 (rpsL)	*E. coli* (strain K12)
19	spectinomycin (DB00919)	cyclohexylamines	enteric bacteria and other eubacteria	P0A7S3	30S ribosomal protein S12 (rpsL)	*E. coli* (strain K12)
20	netilmicin (DB00955)	organooxygen compounds	enteric bacteria and other eubacteria	P0A7S3	30S ribosomal protein S12 (rpsL)	*E. coli* (strain K12)
21	tobramycin (DB00684)	organooxygen compounds	enteric bacteria and other eubacteria	P0A7S3	30S ribosomal protein S12 (rpsL)	*E. coli* (strain K12)
22	lactic acid (DB04398)	N/A	N/A	P53848	folic acid synthesis (FOL1)	*S. cerevisiae* (strain ATCC 204508/S288c)

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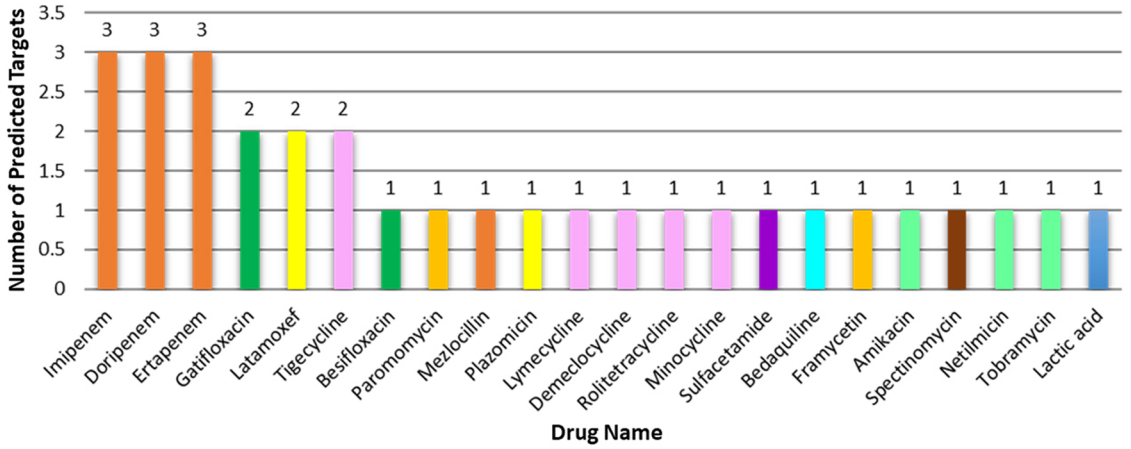

Figure 1. Predicted targets for shortlisted drugs in *Leptospira*: the bar chart shows the number of predicted targets for 22 shortlisted drugs. The bars are color-coded based on the chemical class to which the drug belongs.

Table 3. List of Potential Polypharmacological Agents Identified in the Study

si. no	chemical class	drug name	gene names of the predicted potential targets	no. of predicted targets
1	quinolines and derivatives	gatifloxacin	gyrA, gyrB	2
2	lactams	imipenem	MrdA, mrcA, mrcB	3
3	carboxylic acids and derivatives	latamoxef	mrcA, mrcB	2
4	lactams	doripenem	mrdA, mrcA, mrcB	3
5	tetracyclines	tigecycline	rpsI, rpsL	2
6	ertapenem	lactams	mrcA, ftsl, mrcB	3

gyrase subunit A and tetracyclines on 30S ribosomal protein S9, the 30S ribosomal protein S12 was affected by drugs belonging to multiple chemical classes.

Sensitivity of Repurposing Drugs. In the testing, the controls were not changed from blue to pink color, and there was a blue to pink color change in the test groups, which reveals that in the wells, antimicrobials inhibited the growth of leptospiral cells and in lower concentrations, the growth was not inhibited. The OD values showed that Doripenem, Ertapenem, Gatifloxacin, and Tigecycline can inhibit the growth of *Leptospira* at a concentration of 0.195 μg/mL except for Cynopteri with Gatifloxacin that required 1.563 μg/mL (Figure 2A–D). The Imipenem showed no inhibition of leptospiral growth until 25 μg/mL of concentration (Figure 2E). Further, though the OD range of Copenhageni was low for control and test, the serovar was inhibited by all of the drugs at 25 μg/mL of concentration, which is shown separately in Figure 2F.

IC_{50} values of repurposing drugs: the IC_{50} was calculated from the drug inhibition plot and showed a marked difference in the inhibition of various leptospiral growth. The IC_{50} values are shown as μM concentration and μg/mL in parentheses in Table 5.

Antimicrobial Activity in Various *Leptospira* Serovars: 585.65. Apart from the serovars tested for drug sensitivity at various ranges, an additional 8 serovars were tested for drug sensitivity at 0.195 μg/mL of concentration, which is the effective inhibitory concentration found in the activity analysis in 4 serovars. The results show that Bankinang, Canicola, Djasiman, Hardjo, Grippotyphosa, Pomona, Pyrogenes, and Icterohemorrhagiae are inhibited by Ertapenem, Tigecycline, Gatifloxacin, and Doripenem (Figure 3A,B) and no significant inhibition by Imipenem (Figure 3C).

Drug Targets across *Leptospira* Species. Blast analysis of multiple target proteins DNA gyrase subunit A (Q72WD1), 30S ribosomal protein S9 (Q72U99), and 30S ribosomal protein S12 (Q72UA6) was carried out within the *Leptospira* genus. It was found that most of the pathogenic leptospires were *L. interrogans*, *Leptospira borgpetersenii*, *Leptospira noguchii*, *Leptospira kirschneri*, *Leptospira weilii*, *Leptospira wolffii*, *Leptospira santarosai*, *Leptospira idonii*, *Leptospira alstonii*, *Leptospira perolatii*, *Leptocanna chinensis*, *Leptospira licerasiae*, *Leptospira langatensis*, *Leptospira ryugenii*, and *Leptospira terpstrae* had all three proteins. The Q72U99 and Q72WD1 were found in *Lewis stimsonii*, *Leptospira chreensis*, *Leptospira abararensis*, *Leptospira sarikeiensis*, *Leptospira vanthielii*, *Leptospira hartskeerlii*, *Leptospira brenneri*, *Laurentaeglyphea neocaledonica*, *Leptospira kmetyi*, *Leptospira kemamanensis*, *Leptospira tipperaryensis*, *Leptospira ainlahdjerensis*, *Leptospira bouyouniensis*, *Leptospira harrisiae*, *Louis adleri*,

Table 4. List of "One Target–Many Drugs' Associations Predicted in the Study

si. no.	UniProt ID	protein name of *Leptospira interrogans*	gene name	no. of predicted drugs	predicted drug name	chemical class
1	Q72WD1	DNA gyrase subunit A	gyrA	2	gatifloxacin besifloxacin	quinolines and derivatives
2	Q72U99	30S ribosomal protein S9	rpsI	5	lymecycline tigecycline demeclocycline minocycline rolitetracycline	tetracyclin
3	Q72UA6	30S ribosomal protein S12	rpsL	5	tigecycline	tetracyclin
					framycetin amikacin netilmicin	aminoglycoside
					spectinomycin	aminocyclitol

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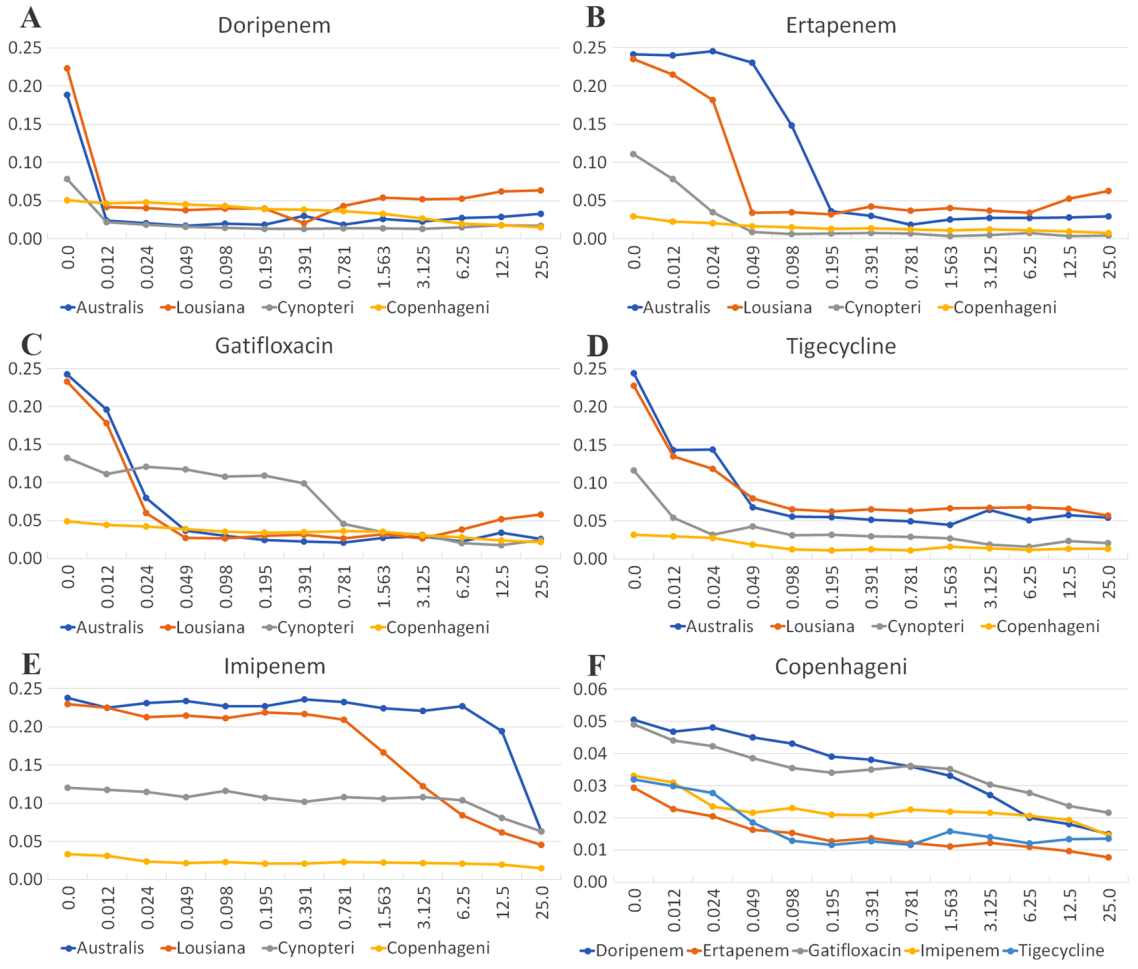

Figure 2. Antimicrobial susceptibility testing: Leptospires at an initial concentration of 2×10^6 cells/ml were grown in EMJH culture medium, and seven plus 1 day scheme was used for antibiotic susceptibility testing with or without treatment in 96-well plates. On day 4, Alamar blue was added for color development, and on the fifth day of incubation, OD at 560 nm was measured using a spectrophotometer. The line chart shows the antileptospiral activity of five drugs: (A) Doripenem, (B) Ertapenem, (C) Gatifloxacin, (D) Tigecycline, and (E) Imipenem at various concentrations (0−25 μg/mL) in Australis, Lousiana, Cynopteri, and Copenhageni. Panel (F) shows Copenhageni in higher resolution to show the slope. All of the "*X*" values are in μg/mL, and the blank-corrected OD values are in the "*Y*" axis.

Table 5. IC_{50} Value of Repurposing Drugs on *Leptospira* serovars

	doripenem μM (μg/mL)	ertapenem μM (μg/mL)	gatifloxacin μM (μg/mL)	imipenem μM (μg/mL)	tigecycline μM (μg/mL)
Australis	0.00002 (0.0069)	0.00026 (0.1244)	0.00005 (0.0196)	0.06447 (19.2987)	0.04525 (0.0265)
Lousiana	0.00002 (0.0074)	0.00007 (0.0329)	0.00005 (0.0176)	0.01409 (4.2169)	0.03074 (0.018)
Cynopteri	0.00002 (0.0084)	0.00004 (0.0180)	0.00166 (0.6219)	0.08579 (25.6817)	0.02493 (0.0146)
Copenhageni	0.01011 (4.2510)	0.00028 (0.1346)	0.04327 (16.2448)	0.06607 (19.7778)	0.13079 (0.0766)

Leptospira wolbachii, *Leptospira fainei*, *Leptospira gomenensis*, *Leptospira biflexa*, *Leptospira ainazelensis*, and *Leptospira barantonii*. The Q72UA6 and Q72WD1 were found in *Leptospira broomii*, *Leptospira inadai*, and i. Other species contained only Q72WD1. The Q72WD1 was found in all of the species of *Leptospira*.

DISCUSSION

From the 22 shortlisted repurposable drugs with their known targets and predicted targets in *Leptospira*, we identified 6 potential polypharmacological agents under the chemical class of quinolines and derivatives, lactams, carboxylic acids and derivatives, and tetracyclines. Four of the experimentally validated 5 drugs showed explicit antileptospiral activity in vitro. Further, the dosage of the drugs also varies depending on the *Leptospira* serovar. It is shown that Doripenem, Ertapenem, Gatifloxacin, and Tigecycline are effective against *Leptospira* at low concentrations under in vitro drug sensitivity testing. It was found that many of the typical tropical or subtropical fever-causing organisms are susceptible to quinolines, lactams, carboxylic acids and derivatives, and tetracycline[21,22] class of antibiotics, and the related gene is also found in *Leptospira*. Similarly, many organisms that cause zoonotic infections show symptomatic and infecting pattern similarity and are susceptible to quinoline, lactam, carboxylic acid, derivative, and tetracycline classes of antibiotics. These broad-spectrum antibiotics are also used for nonzoonotic diseases like enteric fever.[23]

A drug that "hits" multiple sensitive nodes belonging to a network of interacting targets offers the potential for higher

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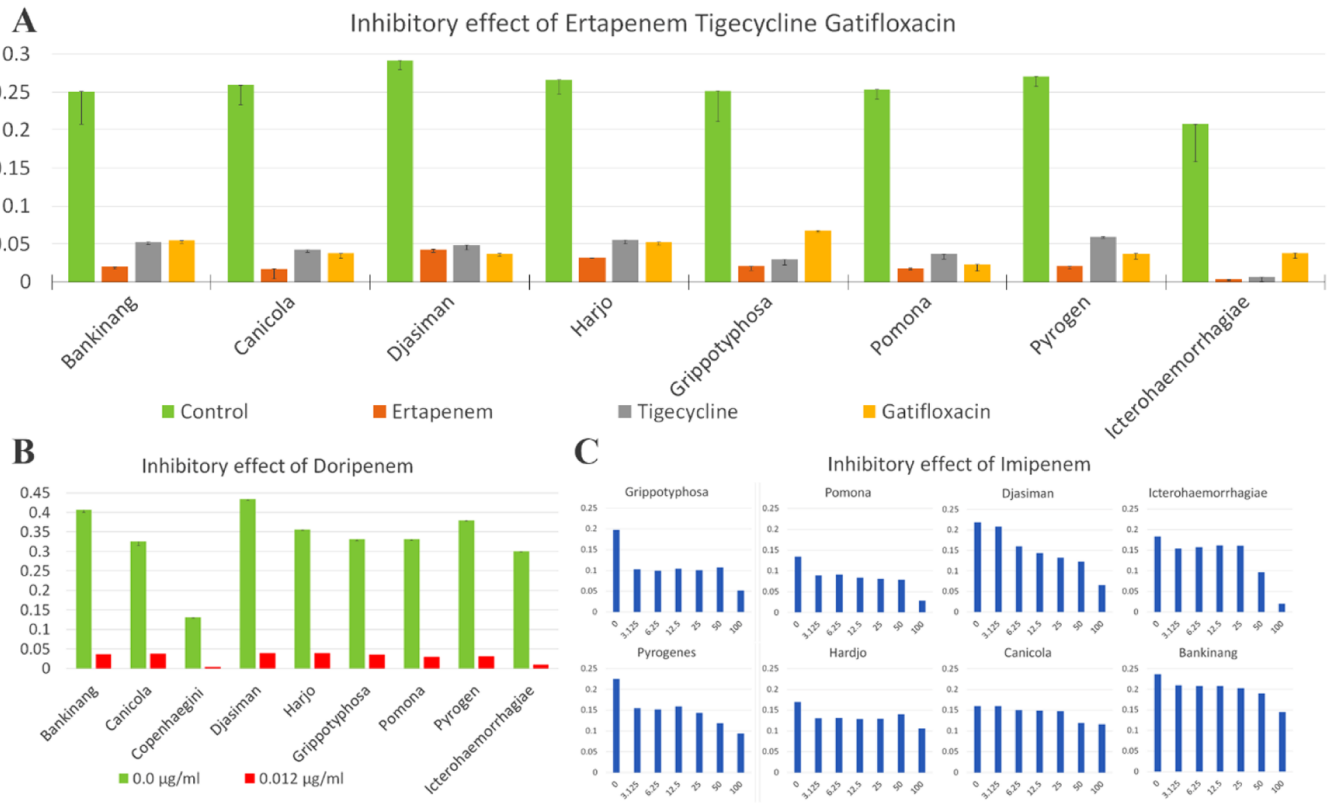

Figure 3. Effect of drugs in major *Leptospira* serovars: eight *Leptospira* serovars cultured in the EMJH medium were treated with (A) 0.195 μg/mL of concentration of Ertapenem, Tigecycline, and Gatifloxacin and (B) 0.012 μg/mL of concentration of Doripenem and (C) 0, 3.125, 6.25, 12.5, 25, 50, and 100 μg/mL of concentration of Imipenem to various serovars of *Leptospira*.

efficacy and may limit drawbacks generally arising from using a single-target drug or a combination of multiple drugs.[24] In some cases, diseases involving multiple pathogenic factors are not adequately addressed with single-target medicines. For these diseases, standard treatment often involves multidrug therapy or a "cock tail" of drugs.[25] In such cases, the same drug having multiple targets will be helpful. Some evidence shows that other zoonotic diseases like scrub typhus caused by *Orientia tsutsugamushi* and melioidosis caused by *Burkholderia pseudomallei* are coinfected with leptospirosis.[22,26,27] Imipenem is one of the antibiotics we have identified used in melioidosis treatment.[22] It is possible to use one effective antibiotic to clear the pathogens that coinfect with a similar target of antibiotic susceptibility. This gives an exciting concept for designing a drug by combination and targeting multiple pathways. In this combination approach, if one fails to hit the target, then the other drug may succeed in reaching the same. Combination therapy, using multiple drugs to improve the clinical condition, has many advantages over monotherapy.[28,29] For multiple complex diseases, combination therapy is widely advisable.[30] Trying a combination with other drugs may reduce the side effects of the drug alone or both. Identifying the multiple target proteins across the *Leptospira* species shows that the drugs will be effective irrespective of whether the *Leptospira* species is infected. Further clinical studies are required to recommend the drugs for the treatment regime of leptospirosis.

CONCLUSIONS

Apart from several computational studies, experimental investigations of exploiting the use of available drugs as possible repurposable candidates for leptospirosis have been demonstrated by numerous groups. However, we approached the question of identifying FDA-approved drugs that may be effective against leptospirosis without considering previously known information. Among the 22 finalized drugs, Doripenem, Ertapenem, Gatifloxacin, and Tigecycline show considerable inhibition on the growth of *Leptospira*. The drugs proposed in our analysis are shown to treat many other diseases, indicating that they can work against *Leptospira* and inhibit other pathogens alone or in combination with other drugs. However, thorough experimental investigations are necessary to ascertain the likelihood of these drugs being repurposable candidates.

MATERIALS AND METHODS

Data Sets. The data set I: DrugBank[31] provides information on medical or veterinary approval, investigational, withdrawn, or illicit small molecule drugs, biotech drugs, nutraceuticals, and associated targets. The current version of DrugBank 5.0.11 holds information on 2520-approved drugs and 4912-associated targets/enzymes/transporters/carriers. The information on the protein−target sequences of only those approved drugs not reported to have a known human target was retrieved for the current study from DrugBank version 5.0.10. The idea behind such a selection is to minimize the chances of any off-target effects on the host.[32]

The data set II: data set II comprises sequences of proteins encoded in the *L. interrogans* whole proteome of the *L. interrogans* serogroup Icterohemorrhagiae serovar copenhageni (strain Fiocruz L1−130), encoding 3654 proteins, was retrieved from the UniProt (http://www.uniprot.org/proteomes/) database (Proteome ID: UP000007037) in FASTA format and used for further analysis. The Universal

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Protein Resource (UniProt) is a comprehensive resource for protein sequence and annotation data that provide functional information about proteins with accuracy and consistency. The UniProt Knowledgebase (UniProtKB) is a central hub of protein knowledge that provides a unified view of protein sequence and functional information. UniProtKB is updated and distributed every 4 weeks and can be accessed online for searches or downloaded at www.uniprot.org.

Approach to Recognize Potential Drug−Target Interactions. Exclusion of antibiotic agents currently in use: the protocol adopted to identify FDA-approved drugs that can be repurposed against proteins in *L. interrogans* is similar to the protocol employed in an earlier published report on repurposing drugs against *Candida albicans*.[32] The FDA-approved drugs were initially subjected to a filter to eliminate the ones known to act on human proteins since the efficacy of a drug known to target proteins in humans is questionable in its use as a repurposed drug against a pathogen, and the exclusion of antibiotic agents currently in use (Table 1) formed the initial step of the approach. Additionally, only drugs with evidence of pharmacological validation against established targets were considered. Thus, 196 FDA-approved drugs associated with 138 protein targets were considered for subsequent analysis. The likelihood of drugs being active as potential antileptospiral agents depends on the similarity of known targets to *L. interrogans* proteins.

L. interrogans homologues of established targets of FDA-approved drugs were identified using sequence and structural analyses described in the Materials and Methods Section. If *L. interrogans* homologue of a known drug−target could be identified, and the drug concerned is an attractive possibility for antileptospiral activity.

Sequence Analysis. To recognize target-related proteins in *L. interrogans*, they were screened with an iterative sequence search program, jackhammer, availed through the HMMER3.0 web server (http://hmmer.org/).[33] The jackhammer does an iterative search of the given protein sequences with the protein sequence database jackhammer search (https://www.ebi.ac.uk/Tools/hmmer/search/jackhmmer).[33] The *E*-value cutoff was 0.0001 with five rounds of iteration. An alignment coverage cutoff of 70% or an alignment encompassing at least one functional and/or structural domain was used as an additional criterion to eliminate unreliable hits characterized by short stretches of alignment.

Structure Analysis. A comparative structural analysis was undertaken to pinpoint probable drug-binding sites in the bacterial proteins based on information on ligand-binding sites in the known targets. Structural information for target and bacterial proteins was obtained from the Protein Data Bank (PDB). Structural models were retrieved from ModBase (https://modbase.compbio.ucsf.edu/modbase-cgi/query_results.cgi?queryfile=1718003411_3483&searchmode=default&displaymode=overseqview&referer=yes&&username=Lepto&password=Lepto&) for bacterial proteins of no known structure[34] or built using MODELER v.9.14.[35] The structural models obtained were checked for reliability based on the *z*-DOPE (Discrete Optimized Protein Energy) score that was <0,[36] ModPipe Quality Score (MPQS) with a cutoff of 1.1, and a query coverage threshold of 80% or a query coverage of at least one functional and/or structural domain. Models were checked for GA341 and TSVMOD scores (Table S1). These were a comparative evaluation of binding sites across established targets and their homologues in L. interrogans. This evaluation was pursued depending on the availability of crystal structures of ligand-bound targets.

TM-align is employed as the structural alignment algorithm to assess the extent of conservation of residues in locally aligned regions between the bacterial proteins and corresponding targets.[37] A TM-score cutoff of 0.5 was used, which typically implies apparent structural similarity of aligned proteins.[37] In some instances, a reliable structural model is used for the *L. interrogans* protein or its domain could not be built, primarily due to the abundance of low-complexity regions in the protein and/or the presence of nonconserved inserts within the functional domains.

Assay of Antileptospiral Activity. Leptospira. *Leptospira* strains were obtained from the repository at the WHO Collaborating Centre for Leptospirosis at the ICMR Regional Medical Research Centre, Port Blair, Andaman and Nicobar Islands, India.

Leptospira Culture. *Leptospira* was cultured in the Ellinghausen−McCullough−Johnson−Harris (EMJH) liquid medium supplemented with 10% bovine serum albumin at 30 °C.[38,39] The growth is verified with the naked eye by swirling the container against a dark background and contamination under a dark-field microscope. The cell count was taken using a Petroff−Hausser counting chamber.

Drug Susceptibility Testing. Seven plus 1 day scheme was used for the drug susceptibility testing.[40] The test was performed in 96-well microtiter plates with positive controls (bacteria without drug) and blanks (EMJH medium for control and EMJH medium + drug at respective concentrations of each test). *Leptospira* inoculum was produced from cultures grown for 7 days at 30 °C with organism counts determined using a Petroff−Hausser counting chamber under dark-field microscopy. After adding 100 μL of inoculum containing 2×10^6 leptospires/ml was added to the antimicrobial-containing and positive control wells, the plates were incubated at 30 °C. The final volume of each well was 200 μL. Each of the five drugs was serially diluted in the EMJH medium. After 4 days of incubation, 10 μL of 10× Alamar blue stock in the EMJH was added to all wells. Alamar blue turned from dark blue to bright pink depending on the growth of organisms. Following the 4 day incubation period, on the fifth day of incubation, the color of each well was measured with a spectrophotometer at 560 nm. The OD values were plotted and analyzed using MS Excel software, and the standard deviation and Student *T*-test value were determined on blank-corrected OD values.

Calculation of IC_{50}. The blank-corrected OD values were converted into a percentage of inhibition with reference to the control. The percentage of inhibition was plotted as an *X*−*Y* scatter curve, and its linear trendline was made in MS Excel. The trendline equation, $y = mx + c$, obtained from the plot was used to get the values of *c*, the *Y* axis intercept, *m* the slope of the line, and *x* the concentration. We kept *y* as 50 and found the concentration at IC_{50} i.e., "*x*" by rewriting the formula $x = (50-c)/m$ using the *c* and *m* values from the formula of slope in the plot. The IC_{50} values obtained in μg/mL were converted to mM to represent the IC_{50} for each drug against various serovars.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c02535.

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GA341 and TSVMOD score and analysis data of structural models (Table S1) (PDF)

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Author Contributions

S.A.D.C., S.C., and N.S. conceptualized the work, S.A.D.C., S.H., and S.C. carried out the work, S.A.D.C., S.H., and M.G. analyzed the data, and S.A.D.C. and M.G. drafted the manuscript.

Notes

The authors declare no competing financial interest.

[§]The author Narayanaswamy Srinivasan passed away on 3rd September 2021 before the preparation of this manuscript.

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