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Selective activity against *Mycobacterium tuberculosis* of new quinoxaline 1,4-di-*N*-oxides

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ABSTRACT

New series of 3-phenylquinoxaline 1,4-di-*N*-oxide with selective activity against *Mycobacterium tuberculosis* have been prepared and evaluated. Thirty-four of the seventy tested compounds showed an MIC value less than 0.2 μg/mL, a value on the order of the MIC of rifampicin. Furthermore, 45% of the evaluated derivatives showed a good in vitro activity/toxicity ratio. The most active and selective compounds carry a fluorine atom in the quinoxaline 7-position or in the phenyl substituent *para*-position. In conclusion, the potency, low cytotoxicity and selectivity of these compounds make them valid lead compounds for synthesizing new analogues, particularly compound 7-methyl-3-(4'-fluoro)phenylquinoxaline-2-carbonitrile 1,4-di-*N*-oxide (MIC <0.2 μg/mL and SI >500).

1. Introduction

Tuberculosis (TB), an infection of *Mycobacterium tuberculosis*, still remains the leading cause of worldwide death among infectious diseases. The statistics indicate that 1.6 million people throughout the world die from Tuberculosis. In addition, the statistics showed that an estimated 8.8 million new cases emerged in 2005; 34% of these cases occurred in the South-East Asia region.[1] One-third of the population is infected with *M. Tuberculosis* and the World Health Organization (WHO) estimates that within the next 20 years approximately 30 million people will be infected with the bacillus.[2] The current frontline therapy for tuberculosis consists of administering three or more different drugs (usually isoniazid, rifampin, pyrazinamide and ethambutol) over an extended period of time.[3] Consequently, problems due to multidrug-resistant TB arise and it is necessary to develop new therapeutic agents in order to treat drug resistant forms of the disease.[4]

Quinoxalines and their mono- and di-*N*-oxide derivatives display a broad range of biological activities[5] and quinoxaline di-*N*-oxides are known to undergo bioreductions under hypoxia causing DNA damage.[6–9] Given the known activity of other classes of bioreductive agents (e.g. metronidazole, PA-824 and OPC-67683), it appeared logical to us to evaluate our group of structures against mycobacteria.[10]

The Tuberculosis Antimicrobial Acquisition and Coordinating Facility (TAACF) was established in 1994 by the National Institute of Allergy and Infectious Diseases (http://www.taacf.org) in order to provide drug testing facilities which contribute to the discovery of new antitubercular drugs. Over 87,000 compounds have been supplied to TAACF, of which only 0.9% have shown a good in vitro activity/toxicity ratio.[10] One of five lead compound series which were identified and are currently been pursued under the TAACF program is the series of quinoxaline 1,4-di-*N*-oxide derivatives. Over 500 quinoxaline derivatives from our laboratory have been tested by the TAACF program,[10] including simple substituted quinoxalines and their corresponding 1,4-di-*N*-oxides. Many of these compounds possess excellent antitubercular activity, and the range of possible substituents affords an opportunity to tailor both the pharmacokinetic and activity profiles.

As a result of the anti-tuberculosis research project, our group published several papers in which the synthesis and in vitro biological evaluation of a large amount of quinoxaline and quinoxaline 1,4-di-*N*-oxide derivatives have been described.[11–18] These studies have facilitated a wide-ranging structure-activity relationship (SAR) analysis with diverse functionality incorporated primarily at the 2-, 3-, 6-, and 7-positions (Fig. 1). In addition, we have observed that the lack of the two *N*-oxide groups generally led to the loss of the antimycobacterial activity.[14,15]

Subsequently, an extended evaluation of the in vitro and in vivo antitubercular activity of most interesting quinoxaline 1,4-di-*N*-oxide derivatives was performed.[19,20] Almost all of these derivatives displayed good inhibitory activity against resistant strains.

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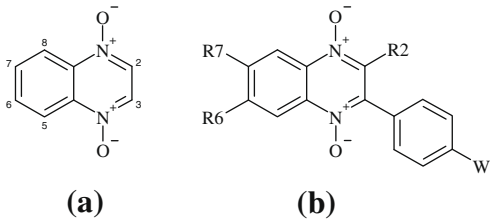

Figure 1. (a) Numbered quinoxaline 1,4-di-*N*-oxide ring; and (b) general structure for all 3-phenylquinoxaline 1,4-di-*N*-oxide derivatives in Table 1.

The susceptibilities of these strains to certain compounds was comparable to that of $H_{37}Rv$, supporting the theory that 1,4-di-*N*-oxide quinoxaline derivatives have a novel mode of action unrelated to the currently used antitubercular drugs.[19,20] In addition, specific derivatives were further evaluated in a series of in vivo assays and two of them (lead compounds **I** and **II**, Fig. 2) were found to be active in reducing CFU counts in both the lung and spleen of infected mice following oral administration. This in vivo efficacy is comparable to clinically used TB drugs, although a relatively high dose of compounds was required in order to obtain equivalent reductions in lung CFU.[19,20]

Thus, quinoxaline 1,4-di-*N*-oxides represent a new class of orally active antitubercular drugs. They are most likely bioreduced to an active metabolite and are active on PA-824 resistant *M. bovis*, thereby indicating that the pathway of bioreduction/activation was different from PA-824, a bioreducible nitroimidazole in clinical trials. In addition, all of the analogues which were tested against non-replicating bacteria (NRP) adapted to low oxygen showed very good activity, indicating that activation occurred in both growing and non-replicating bacteria leading to cell death.[19,20] If the bactericidal activity and activity on NRP bacteria in vitro translate to in vivo conditions, quinoxaline 1,4-di-*N*-oxides may lead to shortened therapy, because the presence of NRP bacteria is believed to be a major factor responsible for the prolonged nature of antitubercular therapy.

This data indicates that 1,4-di-*N*-oxide quinoxalines hold promise for the treatment of tuberculosis. Therefore, we are now reporting the antimycobacterial activity of a series of 3-phenylquinoxaline 1,4-di-*N*-oxide derivatives with different patterns of substituents at quinoxaline nucleus (**1-70**, Fig. 1). The compounds were also tested against VERO cells in order to obtain parameters of cytotoxicity and selectivity.

2. Results and discussion

Antimycobacterial activity of a series of 3-(4-phenylpiperazin-1-yl)quinoxaline-2-carbonitrile and another series of 3-methylquinoxaline-2-carboxylate 1,4-di-*N*-oxide derivatives was previously determined (Fig. 3). The first series demonstrated very good antimycobacterial activity but almost all of the tested derivatives were insoluble or nonselective.[14] On the other hand, the second series showed very interesting results (lead compounds **II** and **IV**, Figs. 2 and 3).[18,20]

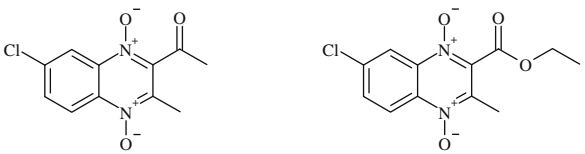

Figure 2. Structure of the *in vivo* lead compounds, **I** and **II**.

Several structural modifications were carried out on the lead compounds of both series by applying the isosteric and homologous strategies (as shown in Fig. 3), with the aim of improving their antimycobacterial activity, solubility and selectivity. The replacement of carbonitrile moiety by an ester group was performed in order to study the structural contribution of the carbonitrile group to the antimycobacterial activity. These modifications were proposed in an attempt to study the importance of the molecular volume and hybridization of C-atom (from sp to sp^2) in position 2 of the 1,4-di-*N*-oxide quinoxaline scaffold within the optimization process of previous prototypes.

Homologation of the substituents linked at C-3 of quinoxaline scaffold was obtained by elimination of the piperazinyl ring (series of carbonitrile derivatives) or by replacement of a methyl with a phenyl group (series of carboxylate derivatives). Variations of the electronic profile of group W linked to the *para* position of the phenyl moiety were carried out.

All of the modifications were conducted in order to establish the contributions of electronic and steric parameters for the optimization of the previous lead compounds.

The compounds **1–70** were prepared following the classical Beirut reaction[21] using unsubstituted, 5-substituted or 5,6-disubstituted benzofuroxans as starting compounds. The formation of isomeric quinoxaline 1,4-di-*N*-oxide was observed in the case of monosubstituted benzofuroxans. Coinciding with previous reports,[22] we have observed that 7-substituted quinoxaline 1,4-di-*N*-oxide derivatives were prevailing over the 6-isomer, or in the case of the methoxy substituent, only the 7-isomer was formed. In practice, workup and purification permitted the isolation of the 7 isomer.[23]

In general, solubility problems detected in previous derivatives[14,15] have been resolved. More specifically, quinoxaline-2-carboxylates appear to be more soluble than quinoxaline-2-carbonitrile, as demonstrated by analogues **6** and **60**.

Biological evaluation is a part of the screening assays of the Tuberculosis Antimicrobial Acquisition and Coordinating Facility, TAACF. All of the compounds, with the exception of **55**, were active in preliminary assays, with more than 90% of growth inhibition at 6.25 μg/mL. Table 1 shows the results obtained from the determination of the MIC values against $H_{37}Rv$ strain of *M. tuberculosis*, the IC_{50} in VERO cells, and the selectivity index (SI) calculated as IC_{50}/MIC.

As shown in Table 1, 60 derivatives showed an MIC value equal to or less than 1.56 μg/mL. The significance of this value depends on several factors, such as compound structure, novelty, toxicity and potential mechanism of action; in general, MIC $\leqslant$1 μg/mL in a novel compound class may be considered an excellent lead compound. Moreover, thirty-four of these sixty compounds showed an MIC value less than 0.2 μg/mL, a value on the order of MIC of rifampicin (RIF).

Some interesting structure–activity relationships can be observed from these results. Comparing the analogues, carbonitrile derivatives (**1**, **3**, **4**, **8**, **9**, **17**, **25** and **37**) with carboxylate derivatives (**57**, **58**, **59**, **63**, **64**, **65**, **67** and **69**, respectively), we concluded that quinoxaline-2-carbonitriles were more active against *M. tuberculosis* $H_{37}Rv$ strain but that they also displayed lower IC_{50} values in VERO cells than ethyl quinoxaline-2-carboxylates. Within carboxylate's series, the best derivative found was ethyl 3-(4′-fluoro)phenylquinoxaline-2-carboxylate 1,4-di-*N*-oxide, **64**, with MIC = 0.39 μg/mL and SI = 22.82 (Fig. 4).

Curiously, the electronic profile of substituent *W* does not appear to influence the antimycobacterial activity because either an electron-donating group or an electron-withdrawing group leads to compounds with the same MIC value (e.g. compounds **67** and **70**; Table 1). However, the influence of substituent W on cytotoxicity appears to be more important, with fluorinated derivatives (**9–16** and **64**) being the least cytotoxic and the most selective compounds.

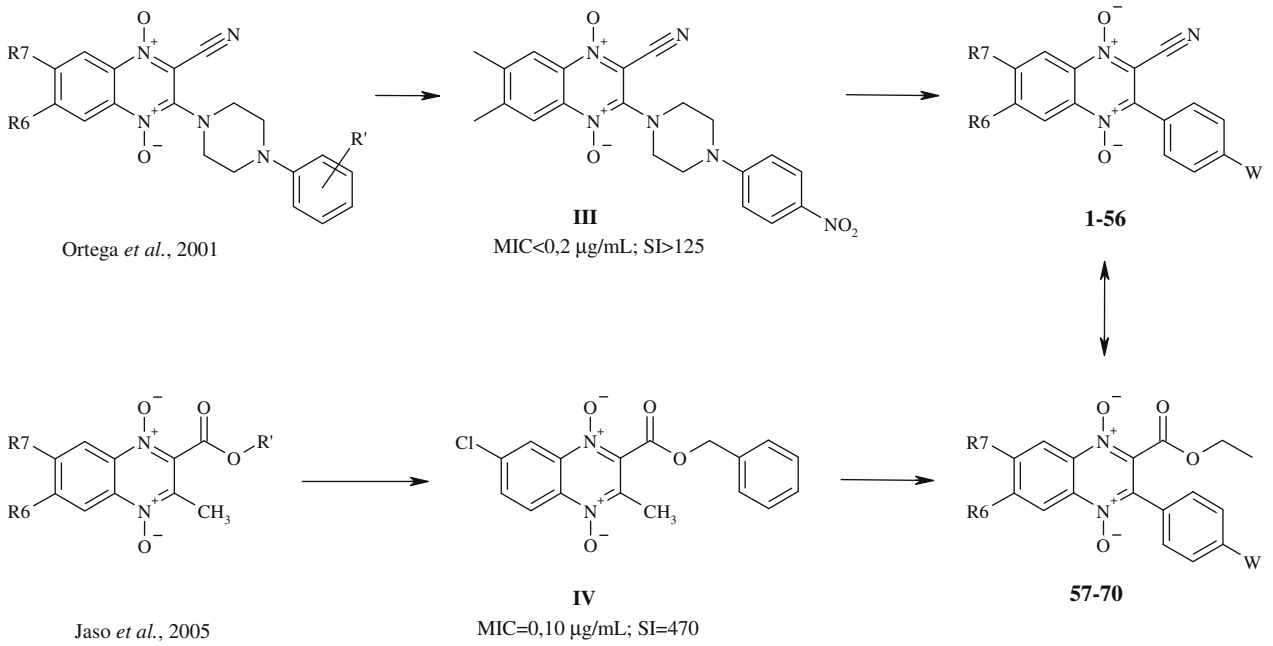

Figure 3. Design of new 3-phenylquinoxaline 1,4-di-*N*-oxide derivatives, **1–70**, as antitubercular drugs from structural modifications from the previous *in vitro* lead compounds, **III** and **IV**.

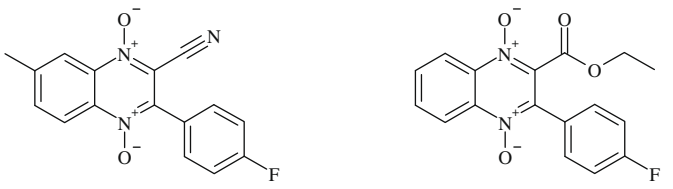

Figure 4. Lead compounds of the new series of quinoxaline 1,4-di-*N*-oxide derivatives, **12** and **64**.

A similar behaviour is found regarding R6 and R7 positions. At these positions, the substituents do not influence the antimycobacterial activity but they do influence the cytotoxicity. The least cytotoxic compounds are those with a fluorine atom at R7 position (**2**, **10**, **18**, **26** and **50**) or those substituted by a methyl group in both positions (**8**, **16**, **24**, **32** and **56**).

Considering that a SI value >10 is required for a compound to be selected for further testing, the results indicate that very interesting anti-TB prototype agents were identified. Twenty-two of the 49 evaluated compounds in VERO cells have demonstrated selectivity indexes greater than the established cut-off; this means 45% of the derivatives showed a good in vitro activity/toxicity ratio. Outstanding compounds are **10**, **11**, **14**, **18** and **50** (all of them carbonitrile derivatives bearing, at least, a fluorine atom), with MIC <0.2 μg/mL and SI >100, and the new lead compound, **12** (Fig. 4), with MIC <0.2 μg/mL and SI >500. These derivatives are quite promising due to their low MIC value and, moreover, due to their great selectivity which improves in vitro results of our best candidates (**see** Figs. 2 and 3).[19,20]

3. Conclusions

Proposed modifications on previous lead-compounds give rise to a new series of 3-phenylquinoxaline-2-carbonitrile 1,4-di-*N*-oxide derivatives with greatly improved activities and selectivities and also to a new series of ethyl 3-phenylquinoxaline-2-carboxylate 1,4-di-*N*-oxide derivatives with maintained biological properties.

In conclusion, the potency, low cytotoxicity and selectivity of these compounds make them valid lead-compounds for synthesizing new analogues that possess better activity, particularly compound 7-methyl-3-(4'-fluoro)phenylquinoxaline-2-carbonitrile 1,4-di-*N*-oxide (**12**, MIC <0.2 μg/mL and SI >500), as it improves the in vitro results of our best candidates.

4. Experimental

4.1. General procedure for the synthesis of compounds

The aforementioned compounds, **1–70**, were prepared according to previously reported synthetic processes.[24–26] The functionalized carbonitrile-derivatives **1–56** were obtained from the appropriate benzofuroxan and the corresponding arylacetonitrile, which were dissolved in dry dichloromethane in the presence of triethylamine as the catalyst.[24,26] The synthesis of carboxylate-derivatives **57–70** was carried out exploring the condensation of the fitting benzofuroxan with ethyl functionalized benzoylacetate in the presence of potassium carbonate, using acetone as the solvent.[25] The structural properties of the compounds were confirmed by nuclear magnetic resonance, mass spectrometry and infrared; purity was established by elemental analysis.[24–26]

4.2. In vitro evaluation of antituberculosis activity

In vitro evaluation of the antituberculosis activity was carried out at the GWL Hansen's Disease Center within the Tuberculosis Antimicrobial Acquisition & Coordinating Facility (TAACF) screening program for the discovery of novel drugs for the treatment of tuberculosis. Under the direction of the US National Institute of Allergy and Infectious Disease (NIAID), the Southern Research Institute coordinates the overall program. The purpose of the screening program is to provide a resource whereby new experimental compounds can be tested for their capability to inhibit the growth of virulent *M. tuberculosis*.[10]

Table 1
Antimycobacterial activity against *M. tuberculosis* (H_{37}Rv strain), cytotoxicity in VERO cells and selectivity for new quinoxaline 1,4-di-*N*-oxide derivatives, **1–70**[a].

ID	R2	W	R6	R7	MIC[b] (μg/mL)	IC_{50}[c] (μg/mL)	SI[d]
1	CN	H	H	H	0.20	1.9	9.5
2	CN	H	H	F	<0.2	17.60	>88.03
3	CN	H	H	Cl	0.39	0.36	0.92
4	CN	H	H	CH_3	0.39	3.6	9.2
5	CN	H	H	CF_3	<0.2	3.78	>18.89
6	CN	H	H	OCH_3	3.13	Insoluble	
7	CN	H	Cl	Cl	0.20	Insoluble	
8	CN	H	CH_3	CH_3	0.39	3.9	10
9	CN	F	H	H	<0.2	3.83	>19.16
10	CN	F	H	F	<0.2	24.93	>124.67
11	CN	F	H	Cl	<0.2	51.73	>258.67
12	CN	F	H	CH_3	<0.2	>100	>500
13	CN	F	H	CF_3	<0.2	0.99	>4.94
14	CN	F	H	OCH_3	<0.2	95.11	>475.55
15	CN	F	Cl	Cl	<0.2	7.41	>37.06
16	CN	F	CH_3	CH_3	1.69	>100	>59.07
17	CN	Cl	H	H	0.39	0.36	0.92
18	CN	Cl	H	F	<0.2	47.94	>239.69
19	CN	Cl	H	Cl	0.39	Insoluble	
20	CN	Cl	H	CH_3	0.39	0.45	1.15
21	CN	Cl	H	CF_3	<0.2	1.34	>6.68
22	CN	Cl	H	OCH_3	0.78	1.00	1.30
23	CN	Cl	Cl	Cl	0.20	Insoluble	
24	CN	Cl	CH_3	CH_3	0.78	>10	>12.8
25	CN	CH_3	H	H	0.39	0.68	1.70
26	CN	CH_3	H	F	<0.2	14.64	>73.19
27	CN	CH_3	H	Cl	6.25	0.63	0.10
28	CN	CH_3	H	CH_3	0.78	Insoluble	
29	CN	CH_3	H	CF_3	<0.2	0.28	>1.42
30	CN	CH_3	H	OCH_3	1.56	3.80	2.40
31	CN	CH_3	Cl	Cl	3.13	0.72	0.23
32	CN	CH_3	CH_3	CH_3	1.56	>62.5	>40.0
33	CN	OCH_3	H	H	<0.2	ND[e]	ND
34	CN	OCH_3	H	F	<0.2	13.03	>65.15
35	CN	OCH_3	H	Cl	<0.2	ND	ND
36	CN	OCH_3	H	CH_3	<0.2	ND	ND
37	CN	OCH_3	H	CF_3	<0.2	0.90	>4.53
38	CN	OCH_3	H	OCH_3	<0.2	ND	ND
39	CN	OCH_3	Cl	Cl	<0.2	ND	ND
40	CN	OCH_3	CH_3	CH_3	<0.2	ND	ND
41	CN	OCF_3	H	H	<0.2	ND	ND
42	CN	OCF_3	H	F	<0.2	ND	ND
43	CN	OCF_3	H	Cl	<0.2	ND	ND
44	CN	OCF_3	H	CH_3	<0.2	ND	ND
45	CN	OCF_3	H	CF_3	<0.2	ND	ND
46	CN	OCF_3	H	OCH_3	<0.2	ND	ND
47	CN	OCF_3	Cl	Cl	<0.2	ND	ND
48	CN	OCF_3	CH_3	CH_3	0.42	ND	ND
49	CN	$COOCH_3$	H	H	0.78	4.80	6.20
50	CN	$COOCH_3$	H	F	<0.2	57.49	>258.67
51	CN	$COOCH_3$	H	Cl	3.13	20.87	6.70
52	CN	$COOCH_3$	H	CH_3	0.39	3.20	8.20
53	CN	$COOCH_3$	H	CF_3	<0.2	1.93	>9.64
54	CN	$COOCH_3$	H	OCH_3	3.13	3.90	1.20
55	CN	$COOCH_3$	Cl	Cl	>6.25	ND	ND
56	CN	$COOCH_3$	CH_3	CH_3	6.25	9.66	1.50
57	$COOC_2H_5$	H	H	H	1.56	24.76	15.87
58	$COOC_2H_5$	H	H	Cl	1.56	3.33	2.13
59	$COOC_2H_5$	H	H	CH_3	1.56	18.61	11.93
60	$COOC_2H_5$	H	H	OCH_3	6.25	17.78	2.84
61	$COOC_2H_5$	H	F	F	1.56	0.38	0.24
62	$COOC_2H_5$	H	Cl	Cl	<0.2	ND	ND
63	$COOC_2H_5$	H	CH_3	CH_3	6.25	>10	>1.6
64	$COOC_2H_5$	F	H	H	0.39	8.90	22.82
65	$COOC_2H_5$	Cl	H	H	0.39	4.80	12.31
66	$COOC_2H_5$	Br	H	H	0.39	6.12	15.69
67	$COOC_2H_5$	CH_3	H	H	0.78	11.29	14.47
68	$COOC_2H_5$	CF_3	H	H	1.56	2.24	1.44
69	$COOC_2H_5$	OCH_3	H	H	1.56	7.65	4.90
70	$COOC_2H_5$	NO_2	H	H	0.78	2.26	2.9
RIF[f]					0.125	>100	>800

[a] For a general structure and definition of W, R2, R6 and R7, see Figure 1.
[b] Minimum inhibitory concentration against H_{37}Rv strain of *M. tuberculosis* (μg/mL).
[c] Measurement of cytotoxicity in VERO cells: 50% inhibitory concentrations (μg/mL).
[d] Selectivity index (in vitro): IC_{50} in VERO cells/MIC against *M. tuberculosis*.
[e] Not determined.
[f] Rifampin.

4.3. Determination of growth inhibition (GI) percentage via MABA

A primary screen was conducted at 6.25 μg/mL against *M. tuberculosis* $H_{37}Rv$ (ATCC 27294) in BACTEC 12B medium, using the Microplate Alamar Blue Assay (MABA).[27] Compounds exhibiting fluorescence were tested in the BACTEC 460-radiometric system. Compounds effecting <90% inhibition in the primary screen (MIC >6.25 μg/mL) were not further evaluated.

4.4. Determination of minimum inhibitory concentration (MIC) via MABA

Compounds demonstrating at least 90% inhibition in the primary screen were re-tested against *M. tuberculosis* $H_{37}Rv$ at lower concentrations in order to determine the actual minimum inhibitory concentration (MIC) in the MABA. The MIC was defined as the lowest concentration effecting a reduction in fluorescence of 90% relative to controls. Rifampicin (RIF) was used as the reference compound (RIF MIC = 0.015–0.125 μg/mL).

4.5. Determination of 50% inhibitory concentrations (IC_{50}) in VERO cells

Concurrent with the determination of MIC's, compounds were tested for cytotoxicity (IC_{50}) in VERO cells at concentrations less than or equal to 62.5 μg/mL or 10 times the MIC for *M. tuberculosis* $H_{37}Rv$. After 72 h of exposure, viability was assessed on the basis of cellular conversion of MTT into a formazan product using the Promega CellTiter 96 Non-radioactive Cell Proliferation Assay. The Selectivity Index (SI = IC_{50}/MIC) was also determined; it was considered significant when SI >10 (RIF IC_{50} >100 μg/mL, SI >800).

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