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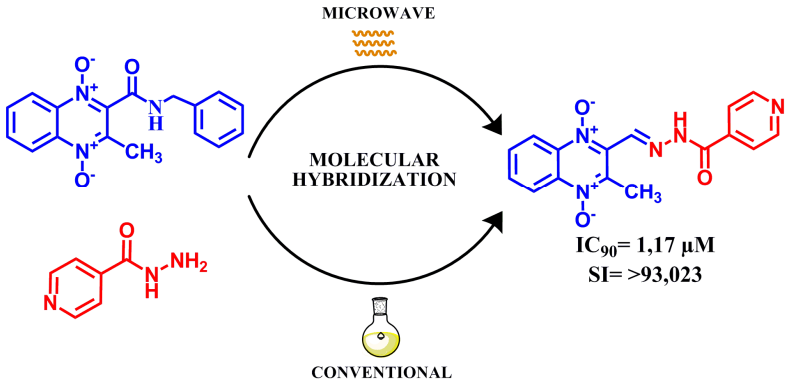

New 1,4-di-*N*-oxide-quinoxaline-2-ylmethylene isonicotinic acid hydrazide derivatives as anti-*Mycobacterieum tuberculosis* agents

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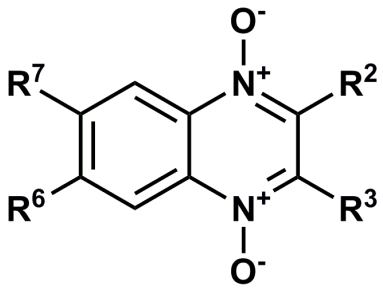
ABSTRACT

The increase in the prevalence of drug-resistant tuberculosis cases demonstrates the need of discovering new and promising compounds with antimycobacterial activity. As a continuation of our research and with the aim of identifying new antitubercular drugs candidates, a new series of quinoxaline 1,4-di-*N*-oxide derivatives containing isoniazid was synthesized and evaluated for *in vitro* anti-tuberculosis activity against *Mycobacterium tuberculosis* H37Rv strain. Moreover, various drug-like properties of new compounds were predicted. Taking into account the biological results and the promising drug-likeness profile of these compounds, make them valid leads for further experimental research.

Tuberculosis (TB), caused by *Mycobacterium tuberculosis (M. Tb)*, is a major infectious disease suffered by mankind in mostly low and middle income countries, although no region in the world remains untouched. According to World Health Organization (W.H.O.) data, every second a newly infection by tuberculosis bacillus occurs somewhere in the world; the number of infections is constantly rising and will soon affect a third of the world's population. The statistics indicate that 1.3 million people throughout the world died from TB in 2008 [1].

The control of this disease is seriously threatened mainly due to the explosive spread of the HIV epidemic, especially in Africa where two-thirds of HIV patients also carry TB. It is also due to the recent influx of immigrants from countries where TB is endemic, and to the increasing emergence of multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB). Recent reports from W.H.O. and the International Union Against Tuberculosis and Lung Disease show that the drug-resistant tuberculosis emergent epidemic is at a global-level and that the problem is most likely underestimated by many [2-6]. The increased incidence of MDR-TB, wich is defined as resistance to the first-line drugs isoniazid and rifampicin, and XDR-TB, defined as resistance not only to rifampicin and isoniazid but also fluoroquinolones and to at least one of the injectable second-line drugs demonstrates the need for further research [7-8].

Therefore, the challenge of chemotherapy development in the future is to discover drugs with new targets and new mechanisms of action for treating patients infected by strains of MDR-TB and XDR-TB, as well as to demonstrate lower levels of toxicity and to shorten treatment periods [9-10]. Despite the efforts and resources involved in anti-TB therapy, no new TB drugs have been introduced in therapy during the past 40 years. Many classes of organic compounds have been tested to achieve this aim, whith special attention being paid to nitrogen heterocycles, five and six membered rings [11-13]. With this idea, our group has been working for several years in the synthesis and biological evaluation of new structures derived from quinoxaline heterocycle (**Figure 1**), with promising results. Quinoxaline derivatives show interesting biological properties such as antibacterial, antiviral, anticancer, antifungal, antihelmintic and insecticidal.



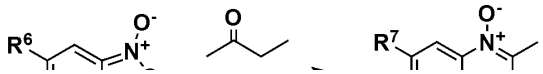


Figure 1. General structure of quinoxaline-1,4-di-*N*-oxide.

As a result of our anti-tuberculosis research project, several papers have been published, in which both synthesis and biological activity assessments have been described for a large number of quinoxaline and quinoxaline 1,4-di-*N*-oxide derivatives with a variety of substituents in positions 2, 3, 6 and 7 (**Figure 1**) of the quinoxaline ring. Some of them have shown growth inhibition values of 99% and 100%. In addition, we observed that the absence of the two *N*-oxide groups generally led to the loss of the antimycobacterial activity [14-22] .

Also, different papers indicate that many isonicotinic acid hydrazide (Isoniazid) derivatives have shown interesting anti-TB activities [23-32]. Isoniazid (INH) possesing the highest activity (MIC<0.05 μg/ml) against actively dividing *M. tuberculosis* [33-34]. INH is a pro-drug activated by catalase-peroxidase hemoprotein, KatG, once inside *M. Tb*. In the activation reaction the hydrazine group is removed and two different active intracellular bacterial active metabolites have been proposed (Scior), a isonicotinyl radical form that is added to NAD (ref:nueva public) or isonicotinic acid, a stable oxidative end product (Scior). The mechanism of action is not well known yet.

The new compounds were designed based on the fusion between the INH and a quinoxaline 1,4-di-*N*-oxide as shown in **Figure 2**. This design is based on the molecular hybridization, defined by Viegas-Junior et al. as a strategy of rational design based on the combination of pharmacophoric moieties of different bioactive substances to produce a new hybrid compound with improved affinity and efficacy when compared to the parent drugs [35]. In addition, this strategy can result in compounds presenting a modified selectivity profile, different and/or dual modes of action and reduced undesired side effects [36-38].

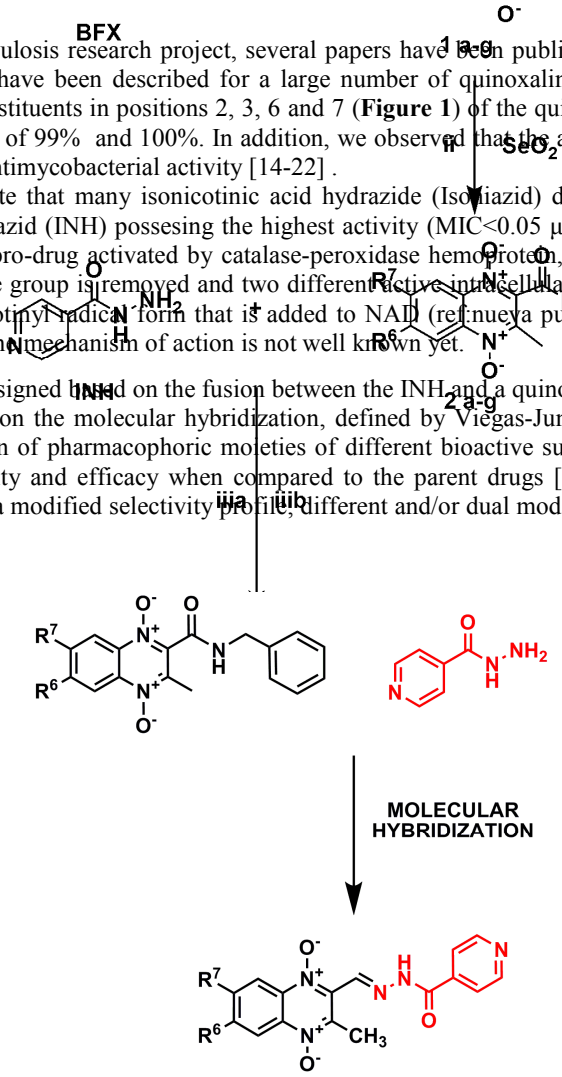


Figure 2. Design concept of the new synthesized compounds.

As a continuation of our research in quinoxalines 1,4-di-*N*-oxide, and with the aim of identifying new antitubercular drug candidates, we have synthesized and evaluated seven new 1,4-di-*N*-oxide-quinoxaline-2-ylmethylene isonicotinic acid hydrazide derivatives possessing different substituents in positions 6 and/or 7 (Comp. **3a-g**, scheme 1). Moreover, we calculated a number of physicochemical parameters to quantifying drug-likeness and the "lipinsky rule of 5", which provides a method for assessing the likelihood that a given molecule could be orally bioavailable based on a series of physicochemical requirements.

The seven new 1,4-di-*N*-oxide-quinoxaline-2-ylmethylene hydrazide isonicotinic acid derivatives presented in this paper were prepared through the synthetic route illustrated in **Scheme 1**.

R^6/R^7	
H/H	a
H/Cl	b
H/CH_3O	c
H/CH_3	d
CH_3/CH_3	e
H/F	f
H/CF_3	g

Scheme 1. General synthesis of 1,4-dioxy-quinoxaline-2-ylmethylene hydrazide isonicotinic acid derivatives. Reagents and conditions: i) triethylamine, pyrrolidine, (51-60%); ii) ethyl acetate, reflux; iiia) ethanol, water, r.t., (29-42%); iiib) tetrahydrofuran, sodium metabisulfite, microwave assisted synthesis, (35-44%). R_6, R_7= H, CH_3O, CH_3, Cl, F, CF_3.

The synthesis sequence was carried out in three stages; first, using a variation of the Beirut reaction, the 2,3-dimethylquinoxaline 1,4-di-*N*-oxide intermediates (**1a-1g**) were synthesized, in wich the appropriate benzofuroxane (BFXs) react with butanone in presence of triethylamine and pyrrolidine. The starting compounds, 5-substituted benzofuroxane or 5,6-disubstituted benzofuroxane were obtained by previously described methods [15, 17, 22, 39-45]. We then proceeded to the oxidation of methyl group in position 2 of quinoxaline ring, using SeO_2 as the oxidant agent, in order to obtain the carboxaldehyde derivatives (**2a-2g**). The oxidation was carried out by microwave assisted synthesis. Finally, by carrying out a variation of a previously described method [25, 32], the new 1,4-dioxy-quinoxaline-2-ylmethylene hydrazide isonicotinic acid derivatives (**3a-3g**) were obtained through the reaction of the corresponding aldehyde derivative (**2a-2g**) with isoniazid (INH). Two different methods were used to obtain the **3a-3g** derivatives. One being conventional method with water and ethanol as solvents at room temperature and the other being, a microwave assisted method using tetrahydrofuran as solvent and $Na_2S_2O_5$ as catalyst.

This synthesis strategy used allowed us to conduct a comparative study between the conventional method and the microwave assisted method. The most significant observations made when comparing the two methods were that the optimized microwave-assisted method dramatically shortened reaction times and that the yields obtained were similar to when the conventional method was used. Furthermore, the amount of solvent used with the microwave-assisted method was less than that used with the conventional method. As a result, a minimum of solvent waste is generated, making this a more efficient and environmentally sustainable chemical process.

New derivatives were unsubstituted or substituted in positions 6 and 7 by methyl or methoxy moiety as electron-releasing groups and by chloro, fluoro or trifluoromethyl moiety as electron-withdrawing groups. Formation of isomeric quinoxaline 1,4-di-*N*-oxides was observed in the case of monosubstituted benzofuroxanes. According to the previous reports [46-47], we have observed that 7-substituted quinoxaline 1,4-di- N-oxides prevailed over the 6-isomer, or only the 7- isomer formed in the case of methoxy substituent. In practice, the workup and purification allow isolation of the major isomer.

In vitro anti-tuberculosis activity evaluation of compounds **3a-3g** was carried out within the Tuberculosis Antimicrobial Acquisition & Coordinating Facility (TAACF) screening program for the discovery of novel drugs for the treatment of tuberculosis [48].

The results of the *in vitro* evaluation of antituberculosis activity are reported in Table 1. Table 1 shows IC_{50} and IC_{90} data against *M. tb.* H37Rv strain, CC_{50} values in VERO cells and SI values obtained for the seven new 1,4-dioxy-quinoxaline-2-ylmethylene hydrazide isonicotinic acid derivatives and the reported 2-carboxamide-1,4-di-*N*-oxyde quinoxaline derivatives as a parent compounds (**P1-P7**) (ref.Saioa). Six out of seven new compounds were considered active in the primary screening (Concentration-Response) with IC_{90} values ranging between 1,16 µM and 23,05 µM. The compounds which passed the primary screening with IC_{90} values lower than 10 µg/mL and were considered for the

cytotoxicity assay in VERO cells. Only compound **3c**, with a methoxy group in seven position of quinoxaline ring, didn't pass on to the secondary screening. Four out of six compounds (**3a**, **3b**, **3d** and **3g**) passed the cut-off established by the TAACF, showing SI>10. Compounds **3a**, **3b** and **3d** showed the higher selective indexes, identifying the **3a** compound as the most promising.

Table 1. Biological results of the first and second antituberculous screening.

			Anti-tubercular activity ($H_{37}Rv$)		Cytotoxicity	
Compound	R^6	R^7	IC_{50}[a] (μM)	IC_{90}[b] (μM)	CC_{50}[c] (μM)	SI[d] (CC_{50}/IC_{90})
P1	H	H	4,91	8,42	□323,28	□38,39
P2	H	Cl	1,10	1,25	□290,90	□230,94
P3	H	OCH_3	18,87	36,38	N.T.	N.T.
P4	H	CH_3	8,35	13,01	□309,26	□23,77
P5	CH_3	CH_3	90,84	296,41	N.T.	N.T.
P6	H	F	2,83	4,65	□305,52	□65,70
P7	H	CF_3	<0,81	1,07	□265,02	□246,30
3a	H	H	1,17	1,32	>123,72	>93,02
3b	H	Cl	1,04	1,16	55,81	48,10
3c	H	OCH_3	<0,59	50,60	N.T.[e]	N.T.[e]
3e	CH_3	CH_3	1,22	2,57	>123,72	>43,47
3d	H	CH_3	1,33	18,18	52,06	2,86
3f	H	F	<0,58	23,05	76,46	3,31
3g	H	CF_3	1,500	3,50	70,01	19,99
INH			-	0,21	>500	>20,000

a IC_{50} against M.tb H37Rv $x10^{-6}$.
b IC_{90} against M.tb H37Rv $x10^{-6}$.
c Cytotoxicity in VERO cells.
d Selectivity index.
e Not tested.

Currently, there are many approaches that assess a compound's druglikeness partially based on topological descriptors, fingerprints of molecular druglikeness structure keys or other properties as clogP and molecular weights. In this work we calculated various physicochemical parameters using Osiris Property Explorer [49-50].

We subjected the seven new derivatives (**3a-3g**) and the reference compounds to the analysis of Lipinski's rule of five which indicates if a chemical compound could be an orally active drug in humans [51]. We calculated theoretical clogP using Osiris Property Explorer, molecular weight (MW) and number of hydrogen bond donors and acceptors. Observing the results in Table 2 it can be said that compounds **3a-3g** satisfied the physicochemical parameters range established by the Lipinski's rule of 5.

Table 2. ClogP, molecular weight (MW), number of H bond donors, H bond acceptors, druglikeness and drug-score values for compounds (**3a**-**3g**).

Compound	clogP[a]	H bond donors	H bond acceptors	Molecular weight	Drug-likeness[a]
3a	1.66	1	6	323.31	4.41
3b	2.27	1	6	353.33	3.34
3c	1.56	1	6	337.33	4.84
3d	2.28	1	6	351.36	4.46
3e	1.98	1	6	357.75	4.54
3f	1.72	1	6	341.30	3.13
3g	2.42	1	6	391.30	-2.29

[a]Theoretical calculated values using Osiris program.

Fragment based druglikeness was predicted for the new derivatives (**3a-3g**). This drug property indicates if the compound predominantly contains fragments which are frequently present in commercial drugs. As can be observed in Table 2**,** our theoretical data showed that compounds **3a**-**3f** presented positive values and only compound 3g presented a negative druglikeness. The majority of marketed drugs show values between 0 and 4. This indicates that druglikeness values of our new compounds were comparable to those of the majority of the commercial drugs [52-55].

Moreover, we used the Osiris program to predict the overall toxicity of the derivatives **3a**-**3g** and indicated a low toxicity risk profile.

In conclusion, a new class of quinoxaline 1,4-di-*N*-oxide derivatives containing isonicotinic acid hydrazide pharmacophore has been synthesized using a new optimized microwave assisted method. Microwave method allowed us to greatly reduce reaction times keeping or even improving reaction yields. The new compounds were evaluated against M.Tb. H_{37}Rv strain; six were active in the primary screening, showing an $IC_{90} \leq 10$ μg/mL, and then moved on to the secondary screening level. Four of the compounds were active at this level, showing a SI ≥10. The promising biological results obtained, along with the good drug-likeness predictors that were calculated, make these compounds valid leads for further studies in anti-TB therapies and for synthesizing new compounds that possess better activity.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://www.sciencedirect.com

Acknowledgments

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