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Original article

Design, synthesis and investigation on the structure–activity relationships of N-substituted 2-aminothiazole derivatives as antitubercular agents

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ABSTRACT

Tuberculosis (TB) is one of the deadliest infectious diseases of all times, and its recent resurgence is a supreme matter of concern. Co-infection with HIV and, in particular, the continuous isolation of new resistant strains, makes the discovery of novel anti-TB agents a strategic priority. The research of novel agents should be driven by the accessibility of the synthetic procedure and, in particular, by the lack of cross-resistance with the drugs already marketed. Moreover, in order to shorten the duration of the therapy, and therefore decrease the rate of resistance, these molecules should be active also against the nonreplicating persistent form (NRP-TB) of the infection. The availability of an in-house small library of compounds prompted us to investigate their anti-TB activity. Two compounds, embodying a 2-aminothiazole scaffold, were found to possess a certain inhibitory activity toward *Mycobacterium tuberculosis* $H_{37}Rv$, and therefore a medicinal chemistry campaign was initiated in order to increase the activity of the hit compounds and, especially, construct a plausible body of structure–activity relationships. The potency of the hit compound was successfully improved, and, much more importantly, some of the molecules synthesized were found to be active toward the persistent phenotype, and, also, toward a panel of resistant strains. These findings encourage further investigations around this interesting antitubercular chemotype.

1. Introduction

Tuberculosis (TB[a]), a lung infection mostly caused by *Mycobacterium tuberculosis* (*Mtb*), is considered one of the most threatening diseases for public health [1]. Despite the information about TB and *Mtb* has become more thorough over the years, the most recent reports disclosed by the World Health Organization (WHO) describe a scenario that is still frightful: in 2011, there were an estimated 8.7 million new cases of TB (13% of which co-infected with HIV) and 1.4 million people died from the disease; moreover, nearly one-third of the world's population is estimated to be latently infected by *Mtb* [2]. The majority of new cases and deaths occur in Asia and Africa: in particular, India and China together account for almost 40% of the world's TB new cases, whereas in the African regions there are the highest rates of cases and deaths per capita [2]. The current recommended therapeutic strategy, termed DOTS (Directly Observed Therapy, Short-course) [3], is based on the co-administration of isoniazid (INH), rifampin (RMP), ethambutol (EMB), and pyrazinamide (PZA) for the first two months, followed by a prolonged treatment with INH and RMP for additional 4–7 months [4,5]. The reason for this exceptionally long period of therapy resides in the peculiar ability of *Mtb* to modify his metabolism in such a way to slow down replication and survive in a dormant state (NRP-TB, nonreplicating persistent TB) [6], therefore hiding from the chemotherapeutics. The overall duration of the treatment, coupled to the undesired side effects, often leads to poor patient compliance which, in turn, contributes to the emergence of multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) [7–9]. By definition, MDR-TB is resistant to at least two of the first-line drugs, principally INH and RMP, whereas XDR-TB additionally fails to respond to any fluoroquinolone and at least

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[a] *Abbreviations:* CAP, capreomycin; DMF, *N*,*N*-dimethyl formamide; DOTS, directly observed therapy short-course; EDG, electron-donating group; EWG, electron-withdrawing group; INH, isoniazid; LORA, low oxygen recovery assay; MABA, microplate Alamar Blue assay; MIC, minimum inhibitory concentration; MDR-TB, multidrug-resistant tuberculosis; MOX, moxifloxacin; *Mtb*, *Mycobacterium tuberculosis*; NRP-TB, nonreplicating persistent tuberculosis; R-TB, replicating tuberculosis; RMP, rifampin; SDR-TB, single drug resistant tuberculosis; SI, supporting information; SM, streptomycin; TB, tuberculosis; XDR-TB, extensively drug-resistant tuberculosis.

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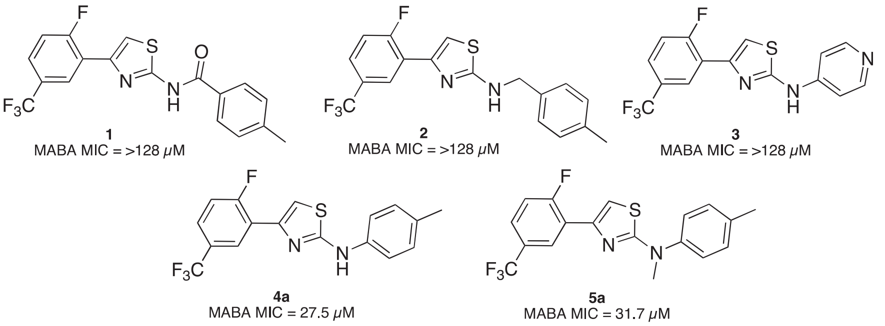


Fig. 1. 2-aminothiazole derivatives preliminary tested as anti-TB agents.

one of three injectable second line drugs (amikacin, capreomycin or kanamycin) [8]. The treatment of resistant strains requires a prolongation of the therapy, needs more toxic drugs, and increases the financial burden, thus making TB a vicious cycle; moreover, the recent isolation of a pan-resistant *Mtb* strain (TDR-TB, totally drug resistant tuberculosis) threatens the rise of a virtually incurable pathogen [10]. These facts highlight the need for novel anti-TB agents, for which the ability of treating *Mtb* in a dormant state and the activity toward resistant strains, coupled to a certain degree of chemical feasibility, should represent the most important features [11–13]. Although a considerable number of anti-TB agents has been reported over the years, to which, among the others, some of us has given his personal contribution [14–21], only a handful of compounds have reached the clinical trials, and bedaquiline (Sirturo®), former TMC207 [22], is the only new chemical entity developed for the treatment of tuberculosis that has reached the market since the introduction of RMP (1967) [23]. With regard to anti-TB agents, and to antibacterials more in general, the whole cell phenotypic assay has demonstrated to be the most fruitful method to produce lead candidates for further development, especially if compared to the target-based approaches. For instance, bedaquiline, mentioned above, and PA-824 [24], an antimycobacterial nitrofuran in phase III clinical trials, were discovered following a cell phenotypic approach. The availability of an in-house chemical library prompted us to carry out a phenotypic screening against *Mtb* in order to obtain novel anti-TB chemotypes. Within the small set taken under consideration, a number of molecules embodying a 2-aminothiazole scaffold were found to have a certain activity, with derivative **4a** being considered the hit compound (Fig. 1). The feasibility of the synthesis, and the very preliminary structure–activity relationships (SAR) stemmed from the analysis of these few derivatives, encouraged us to perform the synthesis of a number of structurally related derivatives, to thereby elucidate the anti-TB potential of these aminothiazole-based compounds. Although the inhibitory activity toward *Mtb* of some aminothiazoles and benzothiazoles has already been reported [25], up to now there has been no attempt to establish a reliable SAR for aminothiazole analogs as anti-TB agents. Hence, keeping intact the aminothiazole core, which is suggested to act as the pharmacophore, we investigated whether the appropriate substitution at the aromatic rings of the hit compound **4a** could lead to an improvement in the anti-TB activity and, also, in a reduction of the cytotoxicity (Fig. 2). The functional groups used as substituents were chosen based on their size, lipophilicity, and physico-chemical properties, and attached at various positions of the aromatic ring. Once assessed which substituents afforded the best activity, attempts to constrain the structure were made, in order to assess whether a more rigid structure could better fit the binding site of the target, that is as yet unknown. Finally, a number of substitutions at the amino moiety and the C-5 of the 2-aminothiazole were explored so as to delineate a more detailed SAR. For the most promising compounds additional assays were carried out: in particular, some of the derivatives were tested against single drug resistant strains (SDR-TB) and, in addition, the inhibitory activity of these compounds toward a model of NRP-TB was evaluated, as will be described below.

1.1. *Chemistry*

The majority of the target compounds (**4a–n**, **6–11**, **15**, **22a**, **6**, **11–14**, **17–19h**, and **17k**) were synthetized in a range of variable yields by employing an established Hantzsch protocol [26], refluxing the appropriate bromoacetophenone with an equimolar amount of thiourea in absolute ethanol (Schemes 1–4). Bromoacetophenones **4**, **6–15**, **17–19**, were readily prepared from the corresponding ketones after treatment with Br_2 at room temperature in CH_2Cl_2; thioureas **a–m**, when not commercially available, were prepared by reacting the corresponding anilines with ammonium thiocyanate in 1 N HCl at reflux. Only cyclohexylthiourea **n** was obtained starting from cyclohexyl isothiocyanate, by reaction with a solution of 0.5 M ammonia in dioxane at room temperature, as reported before [27] (Scheme 3). Compound **16a** was obtained, although in poor yield, by treating **6a** with an excess of pyridinium chloride, while reaction of **4a** with methyl iodide and **4h** with benzyl bromide using NaH as the base afforded compound **5a** and **20h**, respectively. In these experimental conditions, the alkylation occurs at 2-amino moiety, and not at the endocyclic nitrogen, as demonstrated by NOESY experiments (see Supporting information). The synthesis of compounds **4o–r** was accomplished starting from the common precursor 2-bromo-4-phenylthiazole **23**, obtained in a two-steps procedure from the corresponding bromoacetophenone **4** by treatment with ammonium thiocyanate in ethanol at reflux, followed by cyclization promoted by a 40% acetic solution of HBr in CH_2Cl_2 (Scheme 5). Displacement of the bromine atom with piperidine (**4p**) or *N*-acetylpiperazine (**4q**) was carried out in a microwave reactor at 100 °C using ethanol as the solvent and Et_3N as the base. When the reaction was carried out using DMF as the solvent, compound **4o** was obtained in 47% yield. The synthesis of the indole derivative **4r** was conveniently accomplished by employing a Buchwald–Hartwig amination protocol in a microwave reactor, using $Pd(Ac)_2$ as the

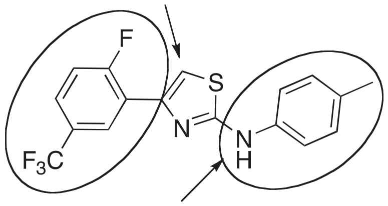
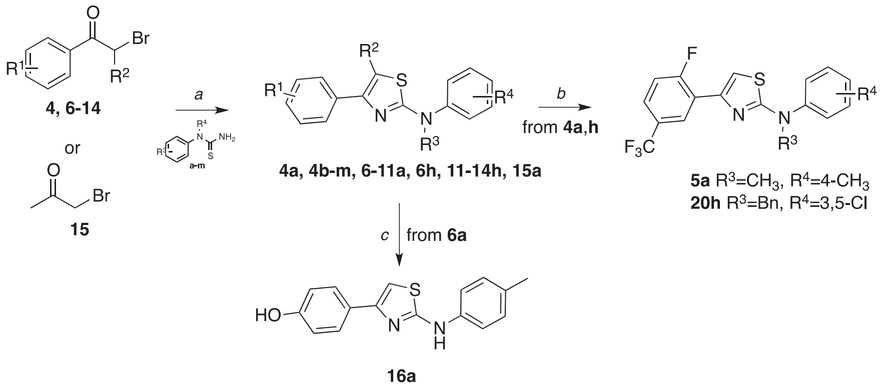
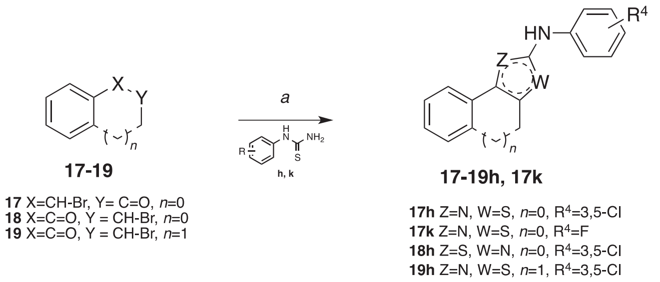


Fig. 2. Sites for modifications of 2-aminothiazole derivatives.



Scheme 1. Reagents and conditions: (a) anhydrous ethanol, 70 °C, 10–18 h, (26–98%); (b) NaH, anhydrous DMF, MeI (**5a**) or benzyl bromide (**20h**), 0 °C → rt, 18 h, (68–77%); (c) pyridine hydrochloride, MW, 5 min, 190 °C, 46%. For complete structures, see Table 1.

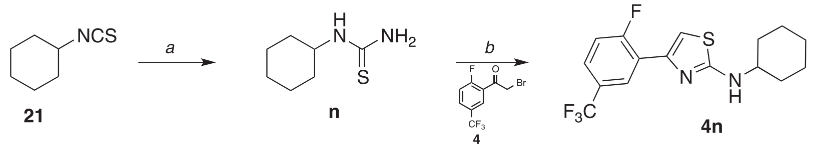


Scheme 2. Reagents and conditions: (a) anhydrous ethanol, 70 °C, 10–18 h, (6–64%). For complete structures, see Table 1.

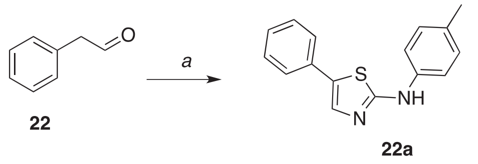
catalyst, BINAP as the ligand and Cs_2CO_3 as the base [28], whereas general Ullman-type conditions led only to several inseparable decomposition products.

2. Results and discussion

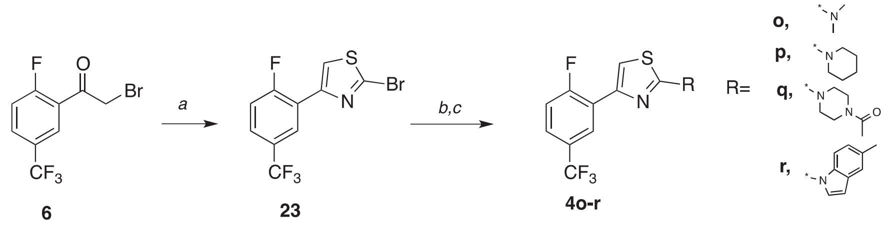
A total of 38 compounds were synthesized and tested for their ability to inhibit the growth of R-TB strain $H_{37}Rv$ in a microplate Alamar Blue assay (MABA) [29]. Compounds showing an encouraging MIC in MABA were also tested in a low oxygen recovery assay (LORA) [30], that is an *in vitro* model for the preliminary assessment of the activity against the persistent *Mtb* phenotype. Some of the most representative compounds were also tested toward Vero cells to ascertain the cytotoxicity profile. To further analyze the biological profile of these compounds, and rule out the possibility of cross-resistance with the commonly used chemotherapeutics, assessment of activity toward SDR-TB strains was carried out as well. 16 compounds resulted inactive at 128 μM concentration, whereas 21 derivatives were found to inhibit the growth of R-TB in the MABA assay at variable concentrations. These modifications led to a variable range of activities and allowed us to construct a plausible SAR as will be described below.



Scheme 3. Reagents and conditions: (a) NH_3 (0.5M in dioxane), rt, 24 h (53%); (b) anhydrous ethanol, 70 °C, 10–18 h, (3%).

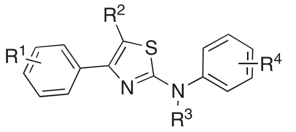


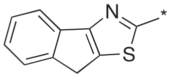
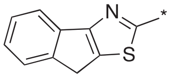
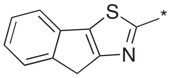
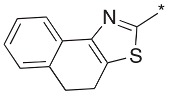
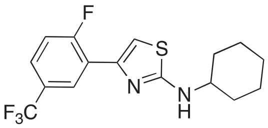
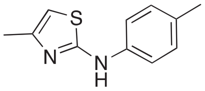
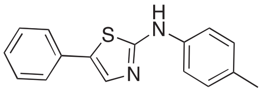
Scheme 4. Reagents and conditions: (a) anhydrous ethanol, 70 °C, 10–18 h, (3%).



Scheme 5. Reagents and conditions: (a) 1. NH_4SCN, anhydrous ethanol, 75 °C, 1 h; 2. 40% HBr in acetic acid, anhydrous CH_2Cl_2, rt, 18 h. (b) for **4o**, DMF, MW, 120 °C, 25 min (40%); **4p**–**q**, piperidine or *N*-acethylpiperazine, absolute ethanol, MW, 120 °C, 25 min (57–96%); (c) **4r**, 5-methyl-1*H*-indole, anhydrous ethanol, BINAP, $Pd(OAc)_2$, Cs_2CO_3, MW, 125 °C, 25 min (51%).

Table 1
Anti-TB activity of compounds **4a–n**, **5–11**, **15**, **16**, **22a**, **6**, **11–14**, **17–20h** and **17k** against *M. Tuberculosis* strain H_{37}Rv.



Compd	R^1	R^2	R^3	R^4	MIC (μM)		IC_{50}[a] (μM)
					MABA	LORA	
4a	2-F, 5-CF_3	H	H	4-CH_3	27.5		92.4
5a	2-F, 5-CF_3	H	CH_3	4-CH_3	31.7		>128
4b	2-F, 5-CF_3	H	H	3-CH_3	29.8		
4c	2-F, 5-CF_3	H	H	2-CH_3	35.1		
4d	2-F, 5-CF_3	H	H	4-Cl	19.2	31.5	46.6
4e	2-F, 5-CF_3	H	H	3-Cl	30.1		
4f	2-F, 5-CF_3	H	H	2-Cl	>128		
4g	2-F, 5-CF_3	H	H	2,4-Cl	>128		
4h	2-F, 5-CF_3	H	H	3,5-Cl	15.0	15.2	50
4i	2-F, 5-CF_3	H	H	3,5-CH_3	30.1		
4j	2-F, 5-CF_3	H	H	2-Cl, 4-CH_3	>128		
4k	2-F, 5-CF_3	H	H	4-F	59.5		
4l	2-F, 5-CF_3	H	H	2,4-CH_3	30.8		
4m	2-F, 5-CF_3	H	-CH_2-CH_3	3,5-Cl	>128		
6a	4-OCH_3	H	H	4-CH_3	13.0	31.8	>128
6h	4-OCH_3	H	H	3,5-Cl	15.6		
7a	4-CF_3	H	H	4-CH_3	>128		
8a	4-Cl	H	H	4-CH_3	>128		
9a	2-Cl	H	H	4-CH_3	>128		
10a	4-CH_3	H	H	4-CH_3	>128		
11a	–	H	H	4-CH_3	63.8		
16a	4-OH	H	H	4-CH_3	>128		
11h	–	H	H	3,5-Cl	28.6		
12h	–	CH_3	H	3,5-Cl	28.9		
13h	–	Ph	H	3,5-Cl	>128		
14h	–	Bn	H	3,5-Cl	20.0		82.7
20h	2-F, 5-CF_3	H	Bn	3,5-Cl	>128		
17h			H	3,5-Cl	>128		
17k			H	4-F	>128		
18h			H	3,5-Cl	>128		
19h			H	3,5-Cl	43.3		
4n					30.3		
15a					>128		
22a					>128		
INH					0.4		
RMP					0.1		
CAP					1.9		
SM					0.3		
PA824					0.8		

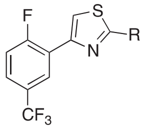
*Indicates the point of attachment.

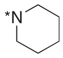
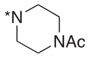
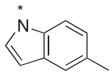
[a] Cytotoxicity to Vero cells; MIC values determined by MABA are the mean of replicated experiments (SD < 15%); LORA MIC values represent single measurements.

2.1. Substitutions at the aromatic rings: the aniline portion

The first round of modifications aimed at improving the activity was made at the aniline site. Therefore, keeping intact the 4-(2-fluoro-5-(trifluoromethyl)phenyl)thiazol-2-amine scaffold, small functional groups were attached at different positions of this aromatic ring. We reasoned that these substituents could affect the hydrogen-bond donor/acceptor ability of the amino moiety, thereby playing a role in the interactions with the target binding site. First we investigated the role of the methyl group and its position on the aniline ring. Moving the methyl from the *para* to the *meta* and the *ortho* positions (**4b**, MIC = 29.8 μM, **4c**, MIC = 35.1 μM, respectively) did not lead to any considerable variation in potency compared with the hit compound **4a** (MIC = 27.5 μM) as shown in Table 1. Next, at the same positions, we investigated the effect of the substitution with an electron-withdrawing group as the chlorine. Introduction of the chlorine atom at the *para* position of the aniline ring, led to a slight improvement of the activity compared to the *para* methyl substitution (**4d**, MIC = 19.2 μM *vs* **4a**, MIC = 27.5 μM). Switching from the *para* to the *meta* position, an almost 2-fold reduction in activity is noticed (**4d**, MIC = 19.2 μM *vs* **4e**, MIC = 30.1 μM), whereas the activity is completely lost when the chlorine is introduced in the *ortho* position (**4f**, MIC = >128 μM). A plausible explanation of this result might encompass the steric hindrance produced by the chlorine atom on the amino group of the thiazole, preventing it from establishing interactions with the surrounding amino acids at the target binding site. This notion is to some extent further corroborated by the lack of activity of **4g** and **4j** (MIC = >128 μM), in which a characteristic deemed favorable for the activity such as the methyl or the chlorine in the *para* position, was coupled to the detrimental chlorine atom in the *ortho* position. Likely, this effect is not observed with the methyl group because of its smaller size. Indeed, compound **4l** (MIC = 30.8 μM), bearing an *ortho*, *para* dimethyl substitution, showed an activity comparable to that of the hit compound **4a**. Interestingly, the introduction of a fluorine atom in the *para* position of the aniline led to a 2-fold decrease in activity compared to the *para*-methyl analog (**4a**, MIC = 27.5 μM *vs* **4k**, MIC = 59.5 μM) and 3-fold compared to the *para*-chlorine (**4d**, MIC = 19.2 μM *vs* **4k**, MIC = 59.5 μM). These results are consistent with the notion that at this position the activity is somehow dependent on the size of the substituent and its lipophilicity. In particular, the bigger and more lipophilic is the substituent, the higher is the activity (**4d** > **4a** > **4k**). This may be rationalized by the presence of a hydrophobic pocket at the target biding site, located within the vicinity of the aniline, able to accommodate a variety of hydrophobic groups. Further insights on the characteristics of this pocket are given by the activity of **4n**, which is on the same range as for the hit compound **4a** (**4n**, MIC = 30.3 μM and **4a**, MIC = 27.5 μM). Moreover, the complete lack of activity of compound **3** (Fig. 1), bearing a 4-pyridine attached to the amino group of the 2-aminothiazole, might serve as an additional evidence for this assumption. Then we investigated the disubstitution at the aniline ring in positions other than the *ortho*, both with an electron-donating and an electron-withdrawing group. While the disubstitution in the meta positions with two methyl groups did not affect the activity compare to the mono-substitution (**4i**, MIC = 30.1 μM *vs* **4b**, MIC = 29.8 μM), we were pleased to notice that two chlorines at the same positions yielded a compound about 2-fold more active than the mono-substituted counterpart (**4h**, MIC = 15.0 μM *vs* **4e**, MIC = 30.1 μM). While it is somehow surprising the capability of the methyl groups not to affect the potency of the molecules, it is reasonable to assume that the introduction of an additional chlorine atom in **4e** leads to an increase of the hydrophobicity, a characteristic likely to be important for the molecule in order to better penetrate the thick mycobacterial cell wall. Reassuming, with regard to this portion of the molecule, small lipophilic substituents are well tolerated, especially in the *para* and *meta* positions, whereas in the *ortho* position substituents like the chlorine led to loss of activity. Disubstitution at the *meta* positions, in particular with chlorine, led to lower MIC values, likely for the improvement of hydrophobicity that they confer. All these data lead to hypothesize the presence of a quite large hydrophobic pocket at the target binding site.

Table 2
Anti-TB activity of compounds **4o**–**r** against *M. Tuberculosis* strain $H_{37}Rv$.



Comp	R	MABA MIC (μM)
4o	$^{*}N\text{-}(CH_3)_2$	>128
4p		56.5
4q		117.8
4r		>128
INH		0.4
RMP		0.1
CAP		1.9
SM		0.3
PA824		0.8

*Indicates the point of attachment.
MIC values determined by MABA are the mean of replicated experiments (SD < 15%); LORA MIC values represent single measurements.

2.2. Substitutions at the aromatic rings: the 4-phenyl ring

The phenyl ring at the C-4 of the 2-aminothiazole is crucial for activity, as both its removal and its shift to the C-5 position led to loss of activity (**15a** [31], **22a**, MICs = >128 μM). We wanted then to investigate which pattern of functional groups could confer the best activity. Removal of both the substituents present in the hit compound led to a roughly 2-fold decrease in activity with respect to the substituted analogs (**11a**, MIC = 63.8 μM *vs* **4a**, MIC = 27.5 μM and **11h**, MIC = 28.6 μM *vs* **4h**, MIC = 15.0 μM). Then, as for the aniline ring, a number of different electron-donating and electron-withdrawing groups were attached at various position of the phenyl ring keeping intact, for comparative purpose, the *para*-methyl substitution at the aniline ring. Quite surprisingly, small electron-donating groups such as the methyl and the hydroxyl, or electron-withdrawing groups such as the chlorine and the trifluoromethyl, failed to show any activity (**7a**, **8a** [32], **9a**, **10a**, **16a**, MIC = >128 μM). On the other hand, compound **6a** [33], bearing a 4-methoxy substitution, was found to be the most active compound of the series (MIC = 13.0 μM). So we tried to couple the more favorable substitution pattern at the aniline ring (the 3,5 dichloro substitution) to that at the phenyl ring (the 4-methoxy group), but, although still one of the most active compounds of the series, we did not notice any evident improvement in the activity (**6h**, MIC 15.6 μM). Although the small set of compounds synthesized, we might assume that at the position *para* of the phenyl ring, slightly more polar substituents guarantee a more favorable interaction with the target binding site, likely due to the presence of polar amino acids in the close proximity. On the other hand, the lack of activity for the 4-hydroxy derivative **16a** might be due to the excessive polar character of this functional group that leads to penetration issues as already reported elsewhere. In conclusion, the phenyl ring at the C-4 of the 2-aminothiazole is important for the activity, and a polar substituent at the *para* position of this ring confers the best activity.

2.3. Substitution at the 2-amino moiety

It is known that in the majority of cases the polar portions of the molecule allow for the most specific interactions, and therefore are seldom modified. We wanted to explore whether the hydrogen of the 2-aminothiazole was important for the activity. Compound **5a** (MIC = 31.7 μM) still maintains an activity comparable to that of the hit compound **4a** (MIC = 27.5 μM), thereby indicating that the capability of being a hydrogen-bond donor is not crucial for the activity. This prompted us to gain further insights into what degree of hindrance was tolerated in regard to the substitution at this position; unfortunately, slightly larger group, such as an ethyl (**4m**, MIC = >128 μM) or more hindered substituent, such as the benzyl group (**20h**, MIC = >128 μM) failed to yield active compounds. Encouraged by the activity of compounds such **5a** and **4n**, we also investigated if the amino moiety could be embodied into a cycloaliphatic ring. The piperidine derivative **4p** had a MIC 2-fold higher than that of the hit compound **4a** (**4p**, MIC = 56.5 μM *vs* **4a**, MIC = 27.5 μM), but the lack of a lipophilic appendage, as for **4o** (MIC = >128 μM), or the introduction of a more polar moiety, as in the case of the *N*-acetylpiperazine derivative **4q** (MIC = 117.6 μM), impaired the anti-TB activity (Table 2). Briefly, we can state that a small group such as the methyl can substitute the hydrogen of the 2-aminothiazole, but larger moieties lead to loss of activity.

2.4. Constrained structures

Our investigation was also directed to explore whether a more rigid structure could affect the activity. As a matter of fact, it is known that if the less flexible structure mimics the bound conformation at the target binding site, an improvement of activity might be observed. We therefore synthesized a small set of derivatives in which the 2-aminothiazole lays on the same plan of the phenyl ring attached at C-4 via either a methylene (**17h**, MIC = >128 μM, **17k**, MIC = >128 μM) or an ethylene bridge (**19h**, MIC = 43.3 μM). Interestingly, compound **19h** had an MIC value only slightly higher than that of the counterpart (**19h**, MIC = 43.3 μM, *vs* **11h**, MIC = 28.6 μM). These results bring over further insights about the interaction between the molecule and the target: *a*) likely, we may suppose that the thiazole and the phenyl ring, at the target binding site, are coplanar; *b*) however, the phenyl ring must be positioned at a suitable distance with respect to the thiazole. Derivative **18h**, that can be considered the constrained analog of **22a**, is inactive as well. Following a similar investigational approach on the other portion of the molecule, and taking into consideration the fact that the 2-amino group of the thiazole can be also tri-substituted (see **5a**, MIC = 31.7 μM), the indole derivative **4r** (MIC = >128 μM) was synthesized, resulting inactive. Apparently, flexibility of the aromatic ring attached at the 2-amino moiety is required to confer the desired anti-TB activity.

Table 3
Activity of compounds **4d**, **h** against SDR *M. Tuberculosis* strains.

Comp	MABA MIC (μM)			
	r-RMP[a]	r-INH[b]	r-MOX[c]	r-CAP[d]
4d	10.9	10.4	15.5	17.4
4h	9.7	7.1	12.0	12.0
RMP	>4	0.06	0.14	0.05
INH	0.85	>8	3.60	0.81
CAP	1.92	3.21	3.68	>16
PA-824	0.51	0.43	0.88	1.26

[a] Rifampin (RMP) resistant strain.
[b] Isoniazid (INH) resistant strain.
[c] Moxifloxacin (SM) resistant strain.
[d] Capreomycin (KN) resistant strain.

2.5. Substitution at the C-5 of the 2-aminothiazole

We finally wanted to investigate whether substitutions at the C-5 of the 2-aminothiazole were tolerated, and, if so, what characteristics should these substituents possess. Moreover, it is known that 2-aminothiazoles without blocking substituents at C-5 can undergo oxidation in vivo to generate potentially toxic reactive epoxide metabolites, therefore the presence of a bulky substituent at the C-5 might be useful in terms of improving some pharmacokinetic characteristics [34]. A small group such as the methyl doesn't affect the activity compared to the counterpart (**12h**, MIC = 28.9 μM, *vs* **11h**, MIC = 28.6 μM), whereas the activity is abolished when a phenyl group is introduced (**13h**, MIC = >128 μM). Surprisingly, the activity is restored when a benzyl moiety is used as the substituent (**14h**, MIC = 20.0 μM). Assessment of the collocation of these data within the SAR is difficult, but we might speculate that the benzyl ring, by virtue of its higher flexibility compared to the phenyl ring, can accommodate into the target binding site in a more favorable manner that is, presumably, distant from the phenyl ring at the C-4 of the 2-aminothiazole. To reassume, several substituents, from the small methyl to the larger benzyl ring, but not the phenyl, can be introduced at the C-5 position of the 2-aminothiazole.

2.6. Activity toward resistant strains and NRP-TB

Some of the compounds were also tested in LORA, a plausible surrogate for NRP-TB. In the majority of the cases, the LORA MIC values are reported to be several fold higher than those of MABA MIC [14–16,19–21], so it was very remarkable to notice that compound **4h** had exactly the same activity in LORA as toward the actively replicating strain (**4h**, MABA MIC = 15.0 μM *vs* LORA MIC = 15.2 μM). Compound **4d** and **6a** still retained appreciable activity in the LORA assay, although the LORA MIC value being almost 2-fold higher than that of the MABA MIC (**4d**, MABA MIC = 19.2 μM *and* LORA MIC = 31.5 μM, **6a**, MABA MIC = 13.0 μM *and* LORA MIC = 31.8 μM), following the trend noticed in our previously reported works. Among the current TB drugs, only RMP and PZA have been reported to show good activity toward the dormant bacteria, and it is generally accepted that targeting the NRP-TB plays a crucial role in shortening the TB treatment. These two compounds were finally evaluated against *Mtb* strains that are resistant to two first-line and 2 s-line TB drugs (Table 3). Both **4d** and **4h** were found to maintain their anti-TB activity against RMP, INH, MOX, and CAP resistant strains, suggesting a different mode of action and indicating that this compound class holds promise for the treatment of resistant TB as well. Although it is a matter of concern the fact that RMP and INH are likely more active than our best derivatives against the respective resistant strains, however, in a preliminary study such as our work is supposed to be, the lack of cross resistance toward a biological target always represents an encouraging starting point, from which further improvements are expected.

2.7. Cytotoxicity

Some of the derivatives synthesized were tested to assess their apparent cytotoxicity toward Vero cells. In general, the selectivity index, that in this case is the ratio between IC$_{50}$ toward Vero cells and the MABA MIC toward *Mtb*, for a compound to be considered a valuable lead has usually to be >10. Therefore, we wanted to investigate whether the toxicological profile of derivative **4a** ($IC_{50} = 92.4$ μM, SI 3.4) could be improved in order to yield a better lead compound. Unfortunately, modifications leading to an enhancement of activity, also resulted in a counterproductive improvement of cytotoxicity (**4d**, $IC_{50} = 46.6$ μM, SI 2.4, **4h** $IC_{50} = 50.0$ μM, SI 3.3). Then we tried to modify the 2-aminothiazole scaffold through the introduction of a methyl group at the 2-amino moiety; we were pleased to notice that the compound was apparently not toxic (**5a**, $IC_{50} > 128$ μM), while maintaining an activity comparable to that of the hit compound. Unfortunately, as reported above, other attempts to substitute the 2-amino moiety with bulkier groups led to a drop of the activity (**4m**, MIC = >128 μM; **20h**, MIC = >128 μM). However, the substitution of the 4-phenyl ring with a more polar EDG such as the methoxy group at the *para* position, gave the best compromise between activity and lack of cytotoxicity (**6a**, MIC = 13.0 μM, $IC_{50} > 128$ μM). Summarizing, the extended investigation around the SAR for these antitubercular 2-aminothiazoles, led to the synthesis of more active compounds, moreover devoid of apparent cytotoxicity.

3. Conclusion

A total of 38 2-aminothiazole derivatives were synthesized in this work, allowing to build a reliable SAR with regards to their anti-TB activity. Most notable compounds had MICs ranging in the low micromolar values, and, more importantly, selected compounds were found to be active not only toward the actively replicating mycobacterial strain, but also toward the nonreplicating persistent phenotype in low oxygen conditions. Moreover, when tested against a panel of single-drug resistant *Mtb* strains, derivatives **4d** and **4h** maintained the same activity as for the wild type, indicating that these derivatives act with a mechanism of action different from those of the currently used drugs, although as yet unknown. The various modifications explored gave a number of valuable hints for the design of additional analogs. In particular, at the amino group of the 2-aminothiazole scaffold, an aromatic ring, preferably adorned with bulky and lipophilic functional groups such as chlorine, is better tolerated; on the other side of the molecule, attached at the C-4 position of the 2-aminothiazole, an aromatic ring is required to confer the desired activity. Lipophilic substituents of various sizes are tolerated in positions other than the *para*, where a more polar group (such as the methoxy moiety) seems to be necessary in order to maintain a certain anti-TB activity, likely for the presence of polar amino acids in the vicinity of the pocket accommodating the molecule. All of these findings made possible a preliminary perception of the shape and stereoelectronic characteristics of the target binding site. Compound **6a**, in which an encouraging improvement of activity is coupled to a lack of apparent cytotoxicity, also by virtue of its preserved activity toward NRP-TB, can be considered as a lead chemotype worth of further investigations toward the synthesis of novel antitubercular 2-aminothiazoles. Studies aimed at improving the activity of these compounds, and establishing preliminary ADMET properties, are therefore underway in our research groups.

4. Experimental section

4.1. Chemistry

Microwave assisted synthesis was performed using a CEM Microwave Synthesizer – Discover Model™. All products were characterized by ^{1}H NMR and ^{13}C NMR. The ^{1}H NMR spectra were recorded on a Bruker 300 Avance spectrometer (300 MHz), on a Bruker 400 Avance spectrometer (400 MHz) and an an Agilent 600 Advance spectrometer (600 MHz); ^{13}C NMR spectra were recorded on a Bruker spectrometer at 100 MHz. Chemical shifts (δ scale) are reported in parts per million (ppm) relative to the central peak of the solvent. ^{1}H NMR Spectra are reported in order: multiplicity and number of protons; signals were characterized as s (singlet), dd (doublet of doublet), t (triplet), m (multiplet), br s (broad signal). HRMS experiments were performed using a LTQ ORBITRAP XL Thermo by Thermo-scientific instrument coupled to HPLC endowed with a column Alltima C18 5 μ 150 mm*4.6 mm, Alltech Italia Srl. Reactions were monitored by TLC, on Kieselgel 60 F 254 (DC-Alufolien, Merck). All the final compounds were more than 95% pure by analytical HPLC.

4.2. Biology

The MICs were determined using *Mtb* $H_{37}Rv$ ATCC 27294 in MABA and LORA assays according to published procedures [29,30]. The reported MICs are an average value from 2 to 3 individual experiments. Similarly, cytotoxicities were determined on Vero cells according to a published procedure. For a brief description of the biological assays see the Supporting information.

4.3. General procedure for the synthesis of compounds **4a–n**, **6–11**, **15a**, **6**, **11–14**, **17–19h** (Hantzsch synthesis)

The properly substituted bromoacetophenone **4**, **6–15**, **17–19** (1 equiv) and thioureas **a–n** (1 equiv) were solubilized in dry ethanol (5 mL $mmol^{-1}$ of bromoacetophenone) and reacted at 70 °C until consumption of the starting materials as indicated by TLC. After cooling, the solvent was evaporated and the residue obtained was washed with diethyl ether (3 × 10 mL) to give the title compounds as a powder in good overall yields. In some cases, the crude material was purified by flash column chromatography. Yields, purification methods and purity are reported in details in the SI. Analytical data for compounds **n**, **6a**, **8a**, **15a**, matched the data published previously [27,33,32,31].

4.4. Cyclohexylthiourea (**n**) [27]

Cyclohexylisocyanate **21** (1 g, 7.12 mmol) was solubilized in a 0.5 M solution of NH_3 in dioxane (22.3 mL) and the mixture was stirred for 24 h at rt. The solvent was then evaporated and the solid obtained was washed with petroleum ether (40 mL) to give a white powder (584 mg, 53%) that was used in the next reaction step without further purification. The analytical data obtained matched those reported in literature [27].

4.5. 4-(4-Hydroxyphenyl)-N-(4-methylphenyl)thiazol-2-amine (**16a**)

4-(4-Methoxyyphenyl)-*N*-(4-methylphenyl)thiazol-2-amine **6a** [33] (50 mg, 0.17 mmol) and pyridine hydrochloride (97 mg, 0.84 mmol) were reacted for 5 min in a microwave reactor (190 °C, 215 W) under neat conditions. The crude material was solubilized in CH_2Cl_2 and washed with water (3 × 10 mL), brine and dried (Na_2SO_4). After filtration, the solvent was removed *in vacuo* and the dark red solid was purified by flash chromatography (CH_2Cl_2–MeOH 95:5) to give **16a** as a light yellow powder (22 mg, 46%): ^{1}H NMR (400 MHz, MeOD): $\delta = 2.31$ (s, 3H), 6.76 (s, 1H), 6.83 (d, $J = 8.6$ Hz, 2H), 7.14 (d, $J = 8.3$ Hz, 2H), 7.52 (d, $J = 8.3$ Hz, 2H), 7.72 ppm (d, $J = 8.61$ Hz, 2H); ^{13}C NMR (100 MHz, MeOD): $\delta = 19.41$, 98.7, 114.8, 117.42, 127.01, 129.10, 131.1, 129.1, 138.9, 151.1, 156.9, 165.1 ppm; HRMS (ESI) calculated for $C_{16}H_{14}N_2OS$ $[M + H]^+$ 283.0827, found: 283.0903.

*4.6. 4-(2-Fluoro-5-trifluoromethylphenyl)-N-methyl-N-(4-methylphenyl)thiazol-2-amine (**5a**)*

4a (35 mg, 0.10 mmol) was added to a suspension of NaH (5 mg, 0.10 mmol) in dry DMF (2 mL) at 0 °C. After stirring for 15 min, methyl iodide (22 mg, 0.13 mmol) was added and the reaction mixture was stirred at rt overnight. The mixture was then cautiously poured into ice water (10 mL), extracted with EtOAc (3 × 10 mL) and the organic layers were washed with brine and dried (Na_2SO_4). After filtration, the solvent was removed *in vacuo* and the yellow oil obtained was purified by flash column chromatography (EtOAc–petroleum ether 5:95) to give **5a** as a white solid (33 mg, 77%): ^1H NMR (600 MHz, $CDCl_3$): $\delta = 2.38$ (s, 3H), 3.59 (s, 3H), 7.02 (s, 1H), 7.18 (t, $J = 3.2$ Hz, 1H), 7.24 (d, $J = 8.8$ Hz, 2H), 7.30 (d, $J = 8.8$ Hz, 2H), 7.49–7.53 (m, 1H), 8.52 (d, $J = 3.2$ Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 15.8$, 35.1, 103.0, 111.2, 118.3, 119.9, 120.4 ($J = 3$ Hz), 121.2 ($J = 272$ Hz), 122.4, 125.2, 131.5, 138.4, 155.8, 157.4, 164.0.

Following a similar procedure, but using benzyl bromide in place of methyl iodide, and starting from compound **4h**, compound **20h** was prepared in 68% yield.

*4.7. 5-Phenyl-N-(4-methylphenyl)thiazol-2-amine (**22a**)*

To a solution of phenylacetaldehyde **22** (500 mg, 4.16 mmol) in CH_2Cl_2 (5 mL), a solution of Br_2 (697 mg, 4.36 mmol) in CH_2Cl_2 (4 mL) was added dropwise. After consumption of the starting material according to TLC, the solvent was removed *in vacuo* having care not to heat the bath more than 20 °C, so as to prevent the evaporation of the brominated compound. Without further purification, the crude red oil obtained was quickly reacted with (*p*-tolyl)thiourea a (831 mg, 5.01 mmol) in absolute ethanol (5 mL) at reflux for 2 h. The solvent was evaporated and the slurry residue was washed with water. The aqueous layer was extracted with EtOAc (3 × 7 mL) and the combined organic layers were washed with brine, dried (Na_2SO_4) and concentrated *in vacuo*. Purification by flash column chromatography (CH_2Cl_2–MeOH 98:2) gave the title compound (30 mg, 3% overall yield) as a white powder. ^1H NMR (400 MHz, $CDCl_3$): $\delta = 2.38$ (s, 3H), 7.18–7.40 (m, 7H), 7.47–7.50 ppm (m, 3H), 8.30 (bp, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 20.8$, 119.1, 125.6, 127.0, 127.2, 128.9, 130.1, 132.1, 132.3, 134.0, 137.8, 165.5 ppm; HRMS (ESI) calculated for $C_{16}H_{14}N_2S$: 267.0878 $[M + H]^+$, found: 267.0947.

*4.8. 2-Bromo-4-(2-fluoro-5-trifluoromethylphenyl)thiazole (**23**)*

Ammonium thiocyanate (38 mg, 0.52 mmol) was solubilized in absolute ethanol (5 mL) and the solution was heated at 75 °C. 2-bromo-1-(2-fluoro-5-(trifluoromethyl)acetophenone **4** (100 mg, 0.35 mmol) was added portion wise and the reaction mixture was stirred for 1 h at the same temperature. The solvent was removed *in vacuo* and the yellow solid obtained was washed with water (3 × 10 mL) and dried. The compound obtained was solubilized in dry CH_2Cl_2 (3 mL) and a solution of 40% HBr in acetic acid (100 μL, 1.65 mmol) was added dropwise at 0 °C, and the mixture was stirred at rt overnight. After 18 h, additional 40% HBr in acetic acid (100 μL, 1.65 mmol) was added and the reaction mixture was stirred at the same temperature until consumption of the limiting reagent. The organic layer was then washed with water (3 × 10 mL), sat. $NaHCO_3$ solution (1 × 10 mL), brine and dried over Na_2SO_4. Purification by flash column chromatography (EtOAc–petroleum ether 10:90) gave the desired compound **23** (63 mg, 56% overall yield) as a white solid. ^1H NMR (400 MHz, $CDCl_3$): $\delta = 7.25$–7.30 (m, 1H), 7.57–7.63 (m, 1H), 7.78 (d, $J = 1.9$ Hz, 1H), 8.52 (dd, $J_1 = 2.2$ Hz, $J_2 = 7.0$ Hz, 1H); HRMS calculated for $C_{10}H_4BrF_4NS$: m/z 325.92 (100.0%), 327.92 (97.4%), $[M + H]^+$, found 325.9262, 327.9237.

*4.9. 4-(2-Fluoro-5-trifluoromethylphenyl)-N,N-dimethylthiazol-2-amine (**4o**)*

Compound **23** (100 mg, 0.31 mmol) and Et_3N (91.1 mg, 0.93 mmol) were solubilized in dry DMF (2 mL) and the solution was reacted for 25 min in a microwave reactor (120 °C, 250 W). The solution was then poured into ice-water, the aqueous layer was extracted with EtOAc and the combined organic extracts were washed with brine and dried over Na_2SO_4. After filtration, the solvent was removed *in vacuo* and the white solid obtained was purified by flash column chromatography (EtOAc–petroleum ether 5:95) to give **4o** as a gray solid (35 mg, 47%): ^1H NMR (400 MHz, DMSO-d_6): $\delta = 3.20$ (s, 6H), 7.11 (d, $J = 2.3$ Hz, 1H), 7.21 (app t, $J = 9.8$ Hz, 1H), 7.49–7.53 (m, 1H), 8.51 ppm (d, $J = 5.3$ Hz, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): $\delta = 40.3$, 107.4, 107.5, 116.2, 116.4, 123.4 ($J = 231$ Hz), 125.2 ($J = 2.2$ Hz), 127.7 ($J = 4.2$ Hz), 144.1, 160.6, 163.1, 169.8 ppm. HRMS (ESI) calculated for $C_{12}H_{10}F_4N_2S$: 291.0501 $[M + H]^+$, found: 291.0579.

*4.10. 4-(2-Fluoro-5-trifluoromethylphenyl)-2-(piperidin-1-yl)thiazole (**4p**)*

A solution of **23** (62 mg, 0.19 mmol), piperidine (39 mg, 0.46 mmol) and Et_3N (102.0 mg, 1.01 mmol) in dry ethanol (3 mL) was heated for 30 min in a microwave reactor (120 °C, 200 W). The solvent was evaporated and the residue was solubilized with EtOAc and washed with water (15 mL). The organic layers were washed with brine, dried over Na_2SO_4, filtered and concentrated *in vacuo* to give a residue that is purified through flash column chromatography (EtOAc–petroleum ether 5:95) to afford **4p** as a yellow wax (60 mg, 96%); ^1H NMR (400 MHz, DMSO-d_6): $\delta = 1.70$–1.76 (m, 6H), 3.53–3.58 (m, 4H), 7.13 (d, $J = 2.5$ Hz, 1H), 7.18–7.23 (m, 1H), 7.49–7.52 (m, 1H), 8.50 ppm (dd, $J_1 = 2.2$ Hz, $J_2 = 7.0$ Hz, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): $\delta = 24.0$, 25.1, 49.5, 108.9, 117.6, 123.0, 123.5 ($J = 12$ Hz), 126.3 ($J = 2.8$ Hz), 126.9 ($J = 3.2$ Hz), 143.0, 160.5, 163.1, 170.2. HRMS (ESI) calculated for $C_{15}H_{14}F_4N_2S$ $[M + H]^+$ 331.0814, found: 331.0892.

Following a similar procedure, but using *N*-acetylpiperazine in place of piperidine, compound **4q** was prepared in 57% yield.

*4.11. 4-(2-Fluoro-5-trifluoromethylphenyl)-2-(5-methyl-1H-indol-1-yl)thiazole (**4r**)*

Compound **23** (75 mg, 0.23 mmol) and 5-methyl-1*H*-indole (32.8 mg, 0.25 mmol) were solubilized in anhydrous toluene (2 mL), and Cs_2CO_3 (91.2 mg, 0.28 mmol), BINAP (3.2 mg, 0.014 mmol) and $Pd(OAc)_2$ (8.7 mg, 0.014 mmol) were added, and the reaction mixture was heated for 25 min in a microwave reactor (125 °C, 200 W). The solvent was evaporated and the residue was washed with water. The aqueous layer was then extracted with EtOAc (3 × 7 mL), and the combined organic extracts were washed with brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by flash column chromatography (EtOAc–petroleum ether 5:95) to obtain the title compound as a white solid (44 mg, 51%). ^1H NMR (400 MHz, DMSO-d_6): $\delta = 2.51$ (s, 3H), 6.70 (dd, $J_1 = 0.6$ Hz, $J_2 = 3.5$ Hz, 1H), 7.26–7.33 (m, 2H), 7.47 (s, 1H), 7.57 (d, $J = 2.2$ Hz, 1H), 7.59–7.64 (m, 1H), 7.72 (d, $J = 3.5$ Hz, 1H), 8.24 (d, $J = 8.5$ Hz, 1H), 8.63 ppm (dd, $J_1 = 2.2$ Hz, $J_2 = 7.0$ Hz, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): $\delta = 21.3$, 107.2, 112.1, 112.8, 116.5, 116.8, 121.5, 123.6 ($J = 270$ Hz), 125.7, 126.2, 127.2 ($J = 32$ Hz), 127.6 ($J = 3.2$ Hz), 130.5, 131.9, 133.2, 144.4, 159.4, 160.6, 163.1. HRMS

(ESI) calculated for $C_{19}H_{12}F_4N_2S$ $[M + H]^+$ 377.0657, found: 377.0765.

Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.11.007.

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