Antitubercular Polyhalogenated Phenothiazines and Phenoselenazine with Reduced Binding to CNS Receptors

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ABSTRACT

Targeting energy metabolism in *Mycobacterium tuberculosis* (Mtb) is a new paradigm in the search for innovative anti-TB drugs. NADH:menaquinone oxidoreductase is a non-proton translocating type II NADH dehydrogenase (NDH-2) that is an essential enzyme in the respiratory chain of *Mtb* and is not found in mammalian mitochondria. Phenothiazines (PTZs) represent one of the most known class of NDH-2 inhibitors, but their use as anti-TB drugs is currently limited by the wide range of potentially serious off-target effects.

In this work, we designed and synthesized a series of new PTZs by decorating the scaffold in an unconventional way, introducing various halogen atoms. By replacing the sulfur atom with selenium, a dibromophenoselenazine **20** was also synthesized. Among the synthesized poly-halogenated PTZs (HPTZs), dibromo and tetrachloro derivatives **9** and **11**, along with the phenoselenazine **20**, emerged with a better anti-TB profile than the therapeutic thioridazine (TZ). They targeted non-replicating Mtb, were bactericidal, and synergized with rifampicin and bedaquiline. Moreover, their anti-TB activity was found to be related to the NDH-2 inhibition. Most important, they showed a markedly reduced affinity to dopaminergic and serotonergic receptors respect to the TZ.

From this work emerged, for the first time, as the poly-halogenation of the PTZ core, while permitting to maintain good anti-TB profile could conceivably lead to fewer CNS side-effects risk, making more tangible the use of PTZs for this alternative therapeutic application.

Keywords: Phenothiazines, Tuberculosis, NDH-2, Phenoselenazine, Respiratory chain, Halogenated-phenothiazines.

INTRODUCTION

Phenothiazines (PTZs) are well known tricyclic compounds that show antifungal, anthelmintic, antihistaminic, antiviral, and antibacterial activities.[1] Their sole medical application is for the treatment of psychotic disorders, with chlorpromazine (CPZ) and fluphenazine included by the World Health Organization (WHO) among the Essential Medicines.[2] The very versatile structure of PTZs makes them one of the most promiscuous lead-structures of the 20th century. However, with appropriate structural changes, potent and selective molecules against a defined biological target could be generated and with this aim many analogues continue to appear in the literature.[3–5]

In recent years, a structurally specific and directional non-covalent interaction between a halogen atom and a Lewis base or an electron-rich moiety, has gained attention of the medicinal chemistry community. This so called halogen bond (X-bond)[6,7] has been found in biomolecular complexes including those with pharmaceutical relevance.[8] Intriguingly, all of the X-bonded complexes feature protein-ligand interactions.[9] A survey over the Protein Data Bank revealed that X-bonds do not exist in nucleic acid complexes, despite the favorable electrostatic properties of DNA and RNA.[10] It was argued that this is observed due to a low number of known nucleic-acid binders bearing a halogen atom, and that the role of X-bond in nucleic acid complexes remains to be clarified.

Searching for viral nucleic acid Trans-activation Response (TAR) RNA binders as innovative anti-HIV-1 agents,[11–14] we have recently explored the possibility to increase the ability of a small molecule to interact with (TAR) RNA through X-bond. Among the known TAR RNA binders, we selected PTZs both for their well-known pharmacological profiles and the synthetic feasibility. From a molecular dynamics simulations,[15] a set of mono and poly HPTZs functionalized with chlorine, bromine and/or fluorine atoms in various positions of the scaffold emerged, that was consequently synthesized and tested for their anti-HIV-1 activity. Since the compounds resulted inactive, the TAR RNA research line was not pursued.

Despite a large body of literature about PTZs, only rare examples of poly HPTZs have been reported,[16,17] prompting us to re-purpose them for an alternative therapeutic application, *i.e.* tuberculosis (TB). TB is a major public health threat worldwide. In 2018 alone 1.5 million people died from TB.[18] The discovery of new anti-TB compounds to treat and minimize the development of multi, extensively and totally drug-resistant strains (MDR, XDR, TDR) is definitely a priority.[19–21] Importantly, the PTZs inhibit *Mycobacterium tuberculosis* (Mtb) growth by targeting the type II NADH dehydrogenase (NDH-2).[22] NDH-2 is a membrane-bound bi-substrate enzyme that catalyzes the cytoplasmic oxidation of NADH to NAD^+, followed by the reduction of quinone to quinol in the membrane in the presence of flavin adenine dinucleotide (FAD) co-factor. It is the primary entry point of electrons derived from NADH into the mycobacterial respiratory chain thus playing a central role in Mtb for generating ATP through oxidative phosphorylation.[23,24] This key function along with its absence in mammalian mitochondria make it an attractive target for drug development. The entire mycobacterial respiratory chain is an important source of antimycobacterial drug targets,[25,26] as demonstrated by the FDA-approval of bedaquiline (BDQ) that targets F_1F_0-ATP synthase.[27]

Various chemical compounds have been identified as NDH-2 inhibitors although none have progressed to drug development.[28] Among the therapeutic PTZs, the most potent antituberculars CPZ and thioridazine (TZ) inhibited purified NDH-2 with an IC_{50} of 70 µM and 11 µM, respectively.[29]

Although the antipsychotic PTZs are reported to inhibit the growth of Mtb, they are definitely less potent than clinical TB drugs, but of even greater consequence they target neurological pathways with associated side effects. Nevertheless, their applicability to the treatment of TB is under consideration.[30,31] TZ was used on a compassionate basis to treat XDR-TB patients curing the majority of them.[32] In addition, CPZ synergized with rifampicin and streptomycin.[33] Thus, they have a great potential to be used in the fight of TB, but additional efforts are required to increase potency and mainly reduce the CNS effects.

With this aim, in the present study we profiled our first set of HPTZs (compounds **1-5**, Table 1) for the anti-TB activity. Based on the preliminary biological results, additional compounds were designed maintaining the best halogen substitution pattern around the scaffold while studying the *N*-10 alkyl side chain (compounds **6-19**, Table 1). In a more drastic structural modification the sulfur atom was replaced by a selenium giving the 3,7-dibromophenoselenazine **20** (Table 1).

For the best compounds, an in-depth characterization of the anti-TB profile and mechanism of action was performed along with the evaluation of their affinity towards a panel of dopaminergic and serotonergic receptors as well as metabolic stability and hepatotoxicity risk in vitro.

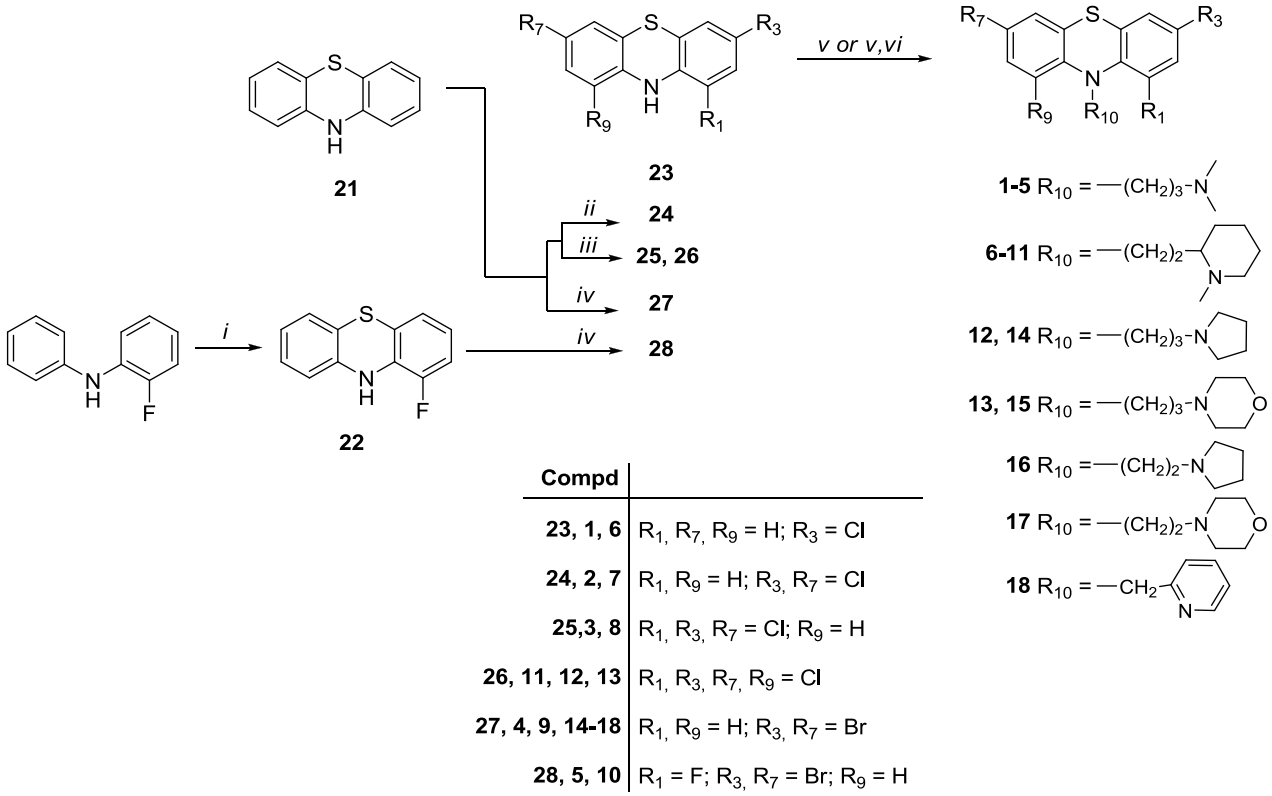
RESULTS AND DISCUSSION

Chemistry

The synthesis of most of the HPTZs was accomplished starting from the commercial 10*H*-phenothiazine **21**, which was halogenated in the planned positions under different conditions (Scheme 1). On the other hand, 1-fluoro-10*H*-phenothiazine **22**[16] was prepared by thionation of 2-fluoro-*N*-phenylaniline with sulfur and iodine at high temperature as previously described (Scheme 1). Starting from synthone **21**, the 3,7-dichloro derivative **24**[17] was prepared by reaction with *N*-chlorosuccinimide (NCS) in DMF at room temperature (rt). By using the same condition but increasing the equivalents of NCS, the synthesis of 1,3,7-trichloro derivative **25** and 1,3,7,9-tetrachloro derivative **26** was attempted, but unsuccessfully. Thus, derivatives **25** and **26** were prepared through an alternative procedure. In particular, in an attempt to prepare derivative **24** by following a synthetic procedure previously reported[34] and entailing the chlorination of **21** with 3 equivalents PCl_5 in $CHCl_3$ at reflux, derivative **25** was obtained instead of **24**. By exploiting the same chlorinating agent and increasing its amount to 5 equivalents, derivative **26** was also prepared. The synthesis of 3,7-dibromo derivative **27**[16] and 1-fluoro-3,7-dibromo derivative **28**[35] was accomplished, as previously described, by bromination of compounds **21** and **22**, respectively, by using 2.5 equivalents of Br_2 in glacial acetic acid at rt.

The successive *N*-alkylation reaction of the commercial 3-chloro-10*H*-phenothiazine **23** and synthesized halogenated synthones **24-28** with the appropriate (chloroalkyl)amine chains was performed in the presence of NaH in dry DMF at 80 °C affording target compounds as a sticky semisolid. In order to obtain them solids, the corresponding hydrochloride salts **1**,[36] **2-15**, **17** and **18** were prepared by bubbling HCl in ethereal solution of the compounds. Only for compounds **16**, obtained already as a solid, the corresponding hydrochloride salt was not prepared.

Scheme 1[a]



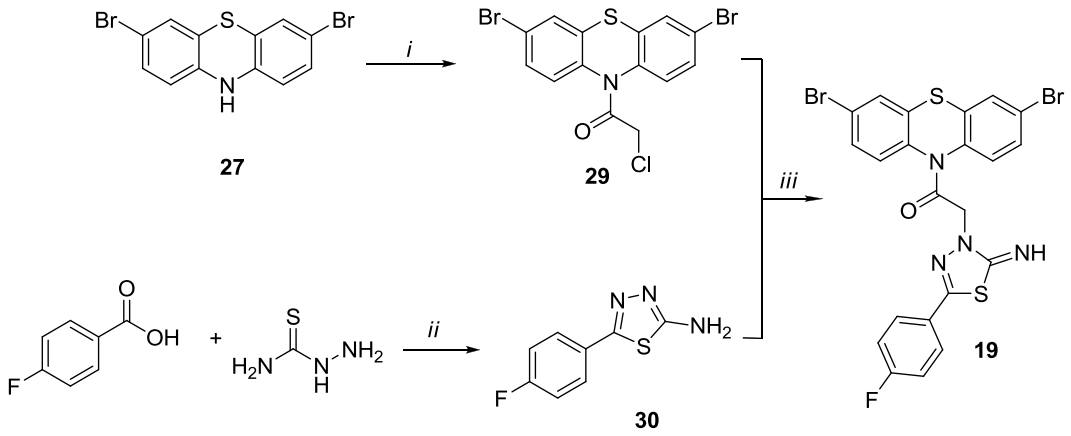
Compd	
23, 1, 6	R_1, R_7, R_9 = H; R_3 = Cl
24, 2, 7	R_1, R_9 = H; R_3, R_7 = Cl
25,3, 8	R_1, R_3, R_7 = Cl; R_9 = H
26, 11, 12, 13	R_1, R_3, R_7, R_9 = Cl
27, 4, 9, 14-18	R_1, R_9 = H; R_3, R_7 = Br
28, 5, 10	R_1 = F; R_3, R_7 = Br; R_9 = H

[a] Reagents and conditions: (i) S_8, I_2, from 150 °C to 210 °C; (ii) NCS, DMF, from 0 °C to rt; (iii) PCl_5, $CHCl_3$, reflux; (iv) Br_2, glacial AcOH, rt; (v) (chloroalkyl)amino chain hydrochloride, NaH, dry DMF, 80 °C; (vi) HCl, Et_2O, rt.

The synthesis of 3,7-dibromo derivative **19** was performed by a convergent approach entailing the preparation of the main fragments **29** and **30**[37] (Scheme 2). In particular, the 3,7-dibromo-10-(2-chloroacetyl)-10*H*-phenothiazine **29** was synthesized by reacting synthone **27** with chloroacetyl

chloride in toluene at 80 °C. The thiadiazol-2-amino derivative **30**[37] was obtained as reported in literature by treating the 4-fluorobenzoic acid with thiosemicarbazide in the presence of $POCl_3$ at 75 °C. Finally, the nucleophilic reaction between **29** and **30** was performed in ethanol at 80 °C affording target compound **19**.

Scheme 2[a]

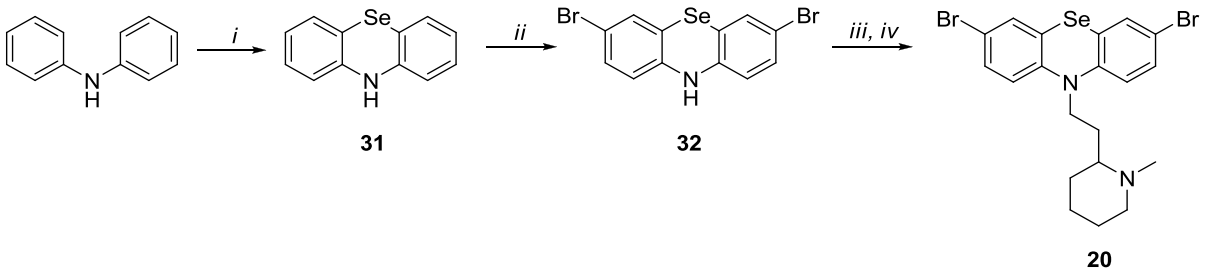


[a] Reagents and conditions: (i) 2-chloroacetyl chloride, toluene, 80 °C; (ii) $POCl_3$, 75 °C; (iii) ethanol, 80 °C.

The synthesis of phenoselenazine derivative **20** (Scheme 3) was accomplished starting from synthone **31**,[38] which was prepared as reported in literature reacting diphenylamine at 150 °C in a pressure vial, in the presence of selenium, selenium dioxide and iodine, using sulfolane as solvent. Even if the yield was very low, 16%, any modification of this procedure gave the **31** even with lower yield.

The successive bromination, *N*-alkylation and salification reactions, performed according to the phenothiazine analogue **9** (Scheme 1), gave target compound **20**, through intermediate **32**.

Scheme 3[a]



[a] Reagents and conditions: (i) Se, SeO_2, I_2, sulfolane, 150 °C, pressure vial; (ii) Br_2, glacial AcOH, rt; (iii) 2-(2-chloroethyl)-1-methylpiperidine hydrochloride, NaH, dry DMF, 80 °C; (iv) HCl, Et_2O, rt.

Structure-(anti-Mtb)Activity Relationship of HPTZs.

All the synthesized compounds were initially tested against Mtb strain H37Rv through the Microplate Alamar blue assay (MABA), and in parallel for the cytotoxicity toward mammalian Vero cells, using the PTZs TZ, CPZ, along with Rifampin (RMP), Isoniazid (INH) and Linezolid (LIZ) as reference compounds. As shown in Table 1, the first set of HPTZs (compounds **1**-**5**) displayed MIC values ranging from 2.38 to 6.59 μg/mL, with the 3,7-dichloro and 3,7-dibromo derivatives **2** and **4**, respectively, which emerged as the most active compounds with MIC values in the same order as TZ. Analogously, the cytotoxicity was in the same range as TZ and CPZ with the dibromo derivative **4** that showed the best profile. The therapeutic PTZs as well as almost all of the PTZs endowed with anti-TB activity are mono-substituted on the PTZ ring and the substituent is exclusively placed at the C-2 position. On the other hand, from this small set of compounds emerged that the presence of two halogens imparted the strongest anti-TB activity. Thus, we sought to further investigate these compounds designing and synthesizing additional analogues. Initially, the *N*,*N*-dimethylpropan-1-amine side chain at the *N*-10 position was replaced by the 2-(1-methylpiperidin-2-yl)ethyl chain characterizing the TZ, synthesizing the analogues **6**-**10**. The tetrachloro phenothiazine **11** was also prepared. Comparing the MIC values of the two series of analogues, it emerged that the presence of the 2-(1-methylpiperidin-2-yl)ethyl chain imparted a better anti-TB activity. The dibromo and tetrachloro derivatives **9** and **11** emerged with a very interesting MIC value of 1.50 μg/mL, with **11** that was the most selective.

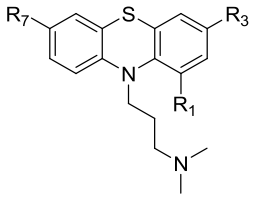
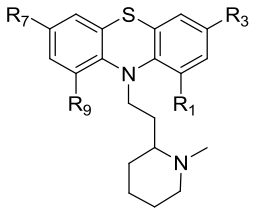
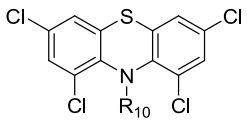
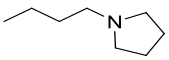
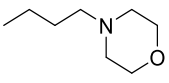
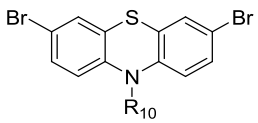
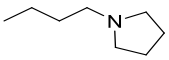
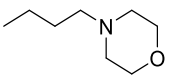
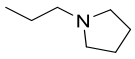
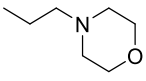
Selecting the dibromo and tetrachloro PTZ scaffolds, we further enlarged the chemical space at the *N*-10 position. In particular, *N*-propylpyrrolidine and *N*-propylmorpholine were inserted synthesizing compounds **12-15**. Shorter *N*-10 side chains were also introduced to further functionalize the dibromo scaffold (compounds **16 -18**).

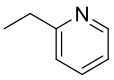
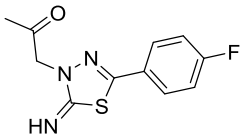
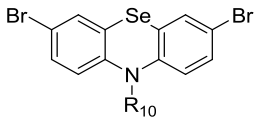
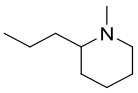
From the anti-Mtb evaluation, clear SAR insights emerged. In particular, the pyrrolidine ring on the *N*-10 side chain granted good activity (MICs of about 1.50 μg/mL) when coupled to both the PTZ scaffolds (compounds **12** and **14**). Also when linked to a shorter side chain (compound **16**), it conferred good activity although significantly increasing the cytotoxicity. On the other hand, the activity of the morpholine derivatives strongly depends on the length of the side chain as well as by the halogen substitution pattern on the PTZ scaffold. Indeed, while the dibromo derivative **15** showed a good activity coupled with the lack of cytotoxicity up to 50 μg/mL, derivatives **13** and **17** were completely inactive. The presence of a methyl-2-pyridine gave the inactive compound **18**, indicating that an aromatic group is not tolerated at this position at least when so near to the PTZ core. Exploiting a side chain previously reported in an anti-Mtb PTZ,[39] derivative **19** was also prepared but was inactive.

Finally, a more incisive structural modification entailed the replacement of the sulfur atom by a selenium synthesizing the phenoselenazine **20**. Although many aspects of selenium physiological and pathological roles need to be clarified, over the last years there has been a growing interest in the synthesis of Se-containing compounds yielding promising biological activities such as antioxidant, anticancer, anti-inflammatory, and anti-infective.[40,41] A very good result was obtained by this bioisosteric approach, indeed compound **20** showed an anti-TB profile that was superimposable to that of the sulfur analogue **9**. Therefore, phenoselenazine scaffold emerged as a new chemotype that under proper functionalization could be exploitable not only in the anti-TB but also in other therapeutic fields.

An in-depth investigation was undertaken for some selected derivatives to define the target, further investigate the anti-TB profile, evaluate the central effects, and preliminary study the pharmacokinetic profile.

Table 1. Structure, Antitubercular activity and Cytotoxicity of HPTZs.

	Compd	R_1	R_3	R_7	R_9	MABA MIC[a] (µg/mL)	IC_{50} Vero cells[b] (µg/mL)
	**1**	H	Cl	H	-	6.59	35.76
	2	H	Cl	Cl	-	2.51	16.73
	3	Cl	Cl	Cl	-	4.46	16.75
	4	H	Br	Br	-	2.38	26.03
	5	F	Br	Br	-	5.70	34.67
	**6**	H	Cl	H	H	5.48	30.78
	7	H	Cl	Cl	H	2.40	17.90
	8	Cl	Cl	Cl	H	2.67	19.20
	9	H	Br	Br	H	1.50	18.26
	10	F	Br	Br	H	2.65	17.70
	11	Cl	Cl	Cl	Cl	1.55	26.94
		R_{10}					
	**12**					1.50	17.97
	13					>50	NT[c]
	**14**					1.45	17.07
	15					5.52	>50
	16					2.57	9.42
	17					>100	>100

	18		>100	>100
	19		>50	NT[c]
	**20**		2.35	17.57
	TZ[d]		2.40	17.79
	CPZ[e]		4.41	26.52
	RMP[f]		0.01	>100
	INH[g]		0.25	NT[c]
	LIZ[h]		0.87	NT[c]

[a] Antitubercular activity of the HPTZs through Microplate Alamar Blue Assay (MABA) assay against Mtb H37Rv strain. The Minimum Inhibitory Concentration (MIC) is the minimum concentration of the compound required to complete inhibition of bacterial growth. [b] The IC_{50} value represents the compound concentration that causes a decrease of Vero cells viability of 50%. [c] NT = not tested [d] Thioridazine; [e] Chlorpromazine; [f] Rifampin; [g] Isoniazid; [h] Linezolid. All compounds are hydrochloride with the exception of **16** and **19**.

NDH-2 inhibitory activity. Both NADH oxidation and oxygen consumption assays using inverted membrane vesicles (IMVs) from wild-type *M. smegmatis* $mc^2$4517[42] were conducted to assess inhibition activities against NDH-2 of some HPTZs variously functionalized (compounds **4**, **6**, **7**, **9**, and **11**). NADH oxidation assay showed that compounds **7**, **9**, and **11** were potential NDH-2 inhibitors with IC_{50} values of 6.5, 4.7, and 3.6, (μg/mL), respectively (Table 2, Figure S1), on the same order of that reported for TZ (IC_{50} of 4.5 μg/mL).[29] Importantly, these compounds inhibited NADH-coupled respiratory activity to ~90% at 50 μM. To validate NDH-2 as the target of our HPTZs, they were tested additionally against IMVs containing overexpressed Mtb H37Rv NDH-2 (TBNDH).[42] Similar inhibitory activity was observed for **9** and **11**, of which **11** appeared the most potent with an IC_{50} of 4.5 (μg/mL). In these assays, 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO), a compound that targets the quinone-binding site of NDH-2 exhibited weak inhibitory activity.[43] Being the NDH-2 inhibitory activity proportional to the anti-TB activity, it seems

emerging as the primary target responsible for the anti-TB activity of the HPTZs. However, additional targets cannot be excluded considering that an interference with a large number of targets essential for Mtb life is reported to be responsible for the anti-TB activity of PTZs.[3,21]

Table 2. Inhibition of NDH-2 by selected HPTZs

	Wild-type				TBNDH overexpressed			
Compd	**IC_{50} (μg/mL)[a]**	**95% Confidence Interval[b]**	**R^{2}[c]**	**Reduction in oxygen consumption (% inhibition)[d]**	**IC_{50} (μg/mL)[a]**	**95% Confidence Interval[b]**	**R^{2}[c]**	**Reduction in oxygen consumption (% inhibition)[d]**
4	NC[e]	NC	NC	69.4 ± 9.4	NC	NC	NC	40.7 ± 7.2
6	NC	NC	NC	47.5 ± 2.5	NC	NC	NC	3.6 ± 12.5
7	6.5	7.6-5.6	0.97	85.4 ± 2.9	NC	NC	NC	59.3 ± 2.1
9	4.7	5.7-3.7	0.99	93.7 ± 1.7	11.2	45.2-2.8	0.95	84.8 ± 4.0
11	3.6	4.1-3.2	0.99	92.4 ± 1.9	4.5	5.1–4.0	0.99	74.2 ± 1.8
HQNO[f]	NC	NC	NC	18.1 ± 12.0	NC	NC	NC	29.2 ± 6.7

[a] The IC_{50} value represents the compound concentration that causes an inhibition of the 50% NDH-2 activity. Thioridazine showed IC_{50} = 4.5 (μg/mL) [29]; [b] The Confidence Interval is a range referred to the IC_{50} values expressed in (μg/mL); [c] Goodness of fits; [d] The inhibition % was performed at concentration of 50 μM of each compound; [e] Not calculated; [f] 2-Heptyl-4-hydroxyquinoline-***N***-oxide; All experiments were performed in triplicate.

Bactericidal activity. Many NDH-2 inhibitors are reported to produce bactericidal effect on Mtb.[44,45] Thus, we assayed compounds **9** and **11** for their bactericidal activity at 4 and 7 days through MABA assay, including rifampicin (RMP) as a reference drug. They showed minimum bactericidal concentration (MBC) of 3.1 μg/mL at both times (Table 3), that is only two-fold higher than the MICs and thus they can be considered to be bactericidal,[46] with the potential to prevent the infection reactivation.

Anti-Mtb activity under low oxygen condition. Mtb is capable of establishing persistent infection in the host, in which it adapts to depletion of available oxygen and nutrients and enters a stage of non-replicating persistence. It is widely accepted that the physiological state of non-replicating persistence subpopulation of mycobacteria is responsible for the antimycobacterial tolerance, as well as the key to shortening the long treatment regimen. Unfortunately, most of the first- and second-line drugs are inactive against the Mtb dormant species.[47] Thus, in order to fully define the real potential of HPTZs we have also determined the anti-TB activity of compounds **9** and **11** under hypoxic conditions using low oxygen recovery assay (LORA).[48] TZ, as well as the other antitubercular agents RMP, INH, LIZ, were tested in parallel. As shown in Table 3, the tested compounds maintained a good activity, which is slightly lower than those measured in replicating Mtb. Of note, both compounds are better than TZ , definitely better than INH, while comparable to LIZ.

Table 3. Minimum bactericidal concentration (MBC) and low oxygen recovery assay (LORA) for selected HTPZs.

Compd	MABA MBC (μg/mL)[a]		LORA MIC (μg/mL)[b]
	Incubation 4 days	Incubation 7 days	
9	3.1	3.1	2.96
11	3.1	3.1	2.89
TZ[d]	NT[c]	NT	5.29 – 5.70
RMP[e]	0.06	0.06	0.08
INH[f]	NT	NT	>128
LIZ[g]	NT	NT	2.46

[a] Microplate Alamar Blue Assay (MABA) MBC was performed against Mtb H37Rv after 4 and 7 days incubation; [b] The LORA MIC was defined as the lowest drug concentration that inhibited ≥ 90% of the luminescence compared to the drug-free control, determined after 10 days of anaerobic incubation and 28h of aerobic incubation (recovery). [c] Not tested; [d] Thioridazine; [e] Rifampin; [f] Isoniazid; [g] Linezolid.

Synergistic activity. Finally, we verified if our HPTZs showed synergistic effect with some first and second line anti-TB drugs, in agreement with what has been reported for the therapeutic PTZs. [49,50] To this end, a checkerboard growth-inhibition assay at various concentrations of compounds **9** and **11** was performed. As shown in Table 4, when combined with RMP and BDQ, the tested compounds showed a fractional inhibitory concentration (FIC) index ranging from 0.45 to 0.78, which being < 1 indicates a synergistic effect. The synergism with BDQ is particularly interesting showing that the inhibition of two different enzymes both involved in the ATP synthesis, could result in an efficient in vivo anti-TB activity. An additive effect was instead observed when the compounds were combined with moxifloxacin, in contrast to TZ that instead synergized with this fluoroquinolone. An antagonistic effect was finally measured for all the tested compounds when combined with INH, LIZ and Pretomanid (PRT).

Table 4. Fractional inhibitory concentration (FIC) index measurement of selected HTPZs in combination with anti-TB drugs.

Anti-TB drugs	MIC (μM)	Expected MIC (μM)			Observed MIC (μM)			FIC[a]		
		9	10	TZ	9	10	TZ	9	10	TZ
RMP[b]	0.01	3.03	2.82	8.26	1.45	2.21	3.32	0.48	0.78	0.40
INH[c]	0.72	3.35	3.15	8.58	6.27	6.03	15.27	1.87	1.92	1.78
LIZ[d]	1.80	3.90	3.68	9.12	6.73	6.22	16.55	1.72	1.69	1.82
MOX[e]	0.19	3.12	2.90	8.35	3.04	3.13	4.01	0.97	1.08	0.48
PRT[f]	0.22	3.12	2.92	8.36	11.38	5.93	15.79	3.65	2.03	1.89
BDQ[g]	0.05	3.04	2.84	8.28	1.37	1.41	1.58	0.45	0.50	0.19

[a] The FIC index was calculated as: FIC = ($MIC_{1\ combination}/MIC_{1\ alone}$) + ($MIC_{2\ combination}$ / $MIC_{2\ alone}$) where 1 and 2 were the two combined compounds; FIC <1 synergistic effect; FIC = 1 additive effect; FIC >1 antagonistic effect.[51,52] [b] Rifampin; [c] Isoniazid; [d] Linezolid; [e] Moxifloxacin; [f] Pretomanid; [g] Bedaquiline.

CNS side-effects risk. The potential use of therapeutic PTZs as alternative anti-TB drugs is definitely limited by their cognitive side effects derived from the ability to recognize various postsynaptic receptors, of which dopaminergic and serotonergic are those mainly responsible for their pharmacological profile.[53] Based on the well-delineated SAR, the PTZ core has to be functionalized with an electron withdrawing substituent at the C-2 position to achieve a central activity. Having a set of anti-TB PTZs that does not fit this SAR, it was mandatory to assay their potential CNS effects. Thus, the affinity of the most active derivatives **9** and **11** towards selected dopaminergic (D_2) and serotonergic (5-HT_{2A}, 5-HT_6 and 5-HT_7) receptors was examined in the radioligand binding assays, using cloned receptors and four suitable radioligands (Table 5). The new chemotype phenoselenazine **20** was also studied while TZ was included as reference D_2-agent with significant affinities also for the serotonin receptors (K_i < 200 nM). In these assays, potent ligands display K_i values lower than 10 nM, the value 200 nM > K_i > 1 μM indicates a mean to weak affinity, while K_i > 1μM can be considered as very low activity.

Table 5. Affinity of selected HPTZs towards dopaminergic and serotonergic receptors.

Compd	K_i (nM)[a]			
	D_2	5-HT_{2A}	5-HT_6	5-HT_7
9	688	1749	758	2149
11	639	1244	387	1003
20	367	1762	222	2340
TZ[b]	8	84	71	181

[a] Affinities tested experimentally in the radioligand binding assays. The binding affinity constant, K_i, expressed as the average of at least two independent experiments. Radioligands used: [^{3}H]-Raclopride (D_2); [^{3}H]-Ketanserin (5-HT_{2A}), [^{3}H]-LSD (5-HT_6), [^{3}H]-5-CT (5-HT_7). [b] Thioridazine.

The tested compounds showed rather low or very low affinities for the panel of central GPCRs, definitely lower than that shown by TZ. In the case of D_2 receptor, our HPTZs showed a 45-80 fold reduced affinity with respect to TZ. The reduction of the 5-HT_{2A}R affinity in 15-21 fold respect to TZ, with values that give a very low risk of interaction with this GPCR, seems to be promising as the agonistic action towards 5-HT_{2A}R is responsible for dangerous CNS-side effects, *i.e.* psychedelic and hallucinogenic ones. The relatively lowest decrease of the HPTZs' affinity compared to TZ (3-11 fold) was observed in the case of 5-HT_6R, however, taking into account the role of 5-HT_6R in CNS, this result does not worsen their pharmacological profile. Although this serotonin receptor subtype is almost exclusively distributed in CNS, lines of evidence hardly describe any undesirable or toxic effects mediated via 5-HT_6R. Paradoxically, similar and beneficial neuroprotective effects, *i.e.* antidepressive, anxiolytic and procognitive ones, have been confirmed for both agonists and antagonists of 5-HT_6R. Thus, the moderate but statistically significant affinity observed for 5-HT_6R, in particular for the selenocompound **20**, does not disqualify these HPTZs in search for new anti-TB drugs.

While from the whole anti-TB biological characterization the poly HPTZs emerged as only slightly better than TZ, their very low central receptor affinity definitely differentiate them from the commercial PTZs highlighting their superior anti-TB therapeutic potential.

Metabolic stability and hepatotoxicity. To further characterize the HPTZs, metabolic stability and a toxicity risk were evaluated for compounds **9**, **11** and **20**, in comparison to TZ. Hepatotoxicity as the probable toxicity risk for the HPTZs was selected on the basis of previous lines of evidence indicating hepatotoxic effects of TZ.[54,55] To estimate the number of metabolites and probable biotransformation ways, compounds were incubated with human liver microsomes (HLMs) for 120 min and the reaction mixture was analyzed by LC/MS, according to the previously described protocols.[56–59] Verapamil was used as metabolically instable reference compound (Figure S2). Analyzing the spectra, it clearly emerged as the HPTZs (Figure S4-S6) are more stable than TZ (Figure S3), which gave three metabolic products related to hydroxylated or *S*-oxidized metabolites (M1-M3, *m/z* = 387.25).[60] In particular, only the dibromo derivative **9** underwent to a slight metabolism with the formation of one metabolite, probably obtained after debromination (M1, *m/z* = 405.20) (Figure S4). The same reaction was not observed for the other dibromo derivative phenoselenazine **20** (Figure S6) in which the presence of a selenium atom seems to improve the stability.

For the same compounds, the hepatotoxicity was studied evaluating the viability of hepatoma HepG2 cell line after 48 h of incubation. Doxorubicin and the toxin carbonyl cyanide 3-chlorophenyl-hydrazone (CCCP, 10 μM) were used as comparators. As shown in Figure 1, either TZ or HPTZs displayed lower hepatotoxic effects than those of DOX, being non-toxic at the concentration of 1 μM. At 10 μM concentration, compound **9** showed a certain toxicity analogously to TZ, while phenoselenazine **20** was less toxic. Tetrachloroderivative **11** was the least hepatotoxic causing toxic effect only at the concentration of 50 μM.

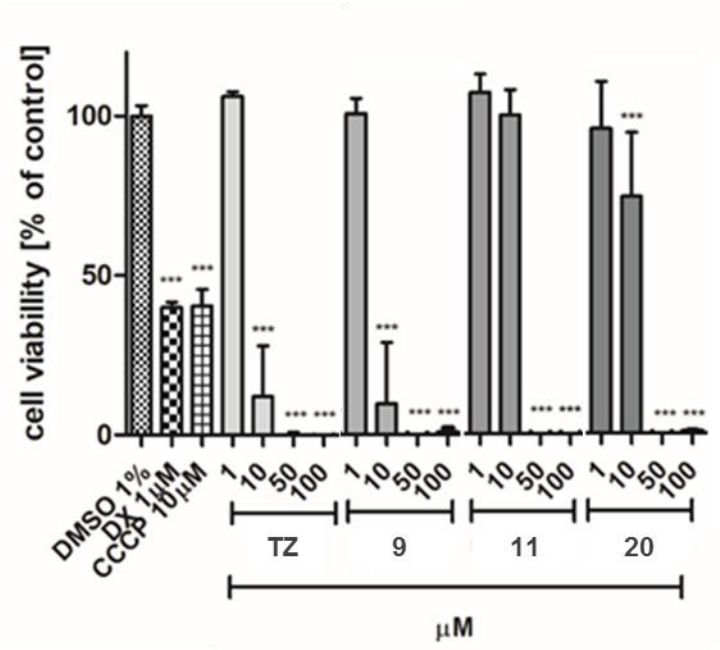


Figure 1. The effect of doxorubicin (DX, 1 µM), mitochondrial toxin carbonyl cyanide 3-chlorophenyl-hydrazone (CCCP, 10 µM) and tested compounds (**9**, **11**, and **20** and TZ) on *hepatoma* HepG2 cell line viability after 48 h of incubation.

CONCLUSIONS

PTZs show broad spectrum of activities including the anti-TB property, but the impossibility to separate the central effects from the other pharmacological activities relegated them to the treatment of psychosis.

In this work, by initially re-purposing a small set of poly HPTZs, we designed and synthesized additional analogues that led to identify compounds showing anti-TB properties superimposable or slightly better than those shown by TZ. Most importantly, they had a markedly lower affinity to CNS receptors and thus potentially decreased central effects. The PTZ poly-halogenation is a great structural novelty within this class of compounds that, independently from the biological activity, usually bear only one substituent at the C-2 position. The identification of the anti-TB phenoselenazine **20**, which is also devoid of central receptors affinity, adds another important insight to the PTZ SAR.

The best compounds, such as dibromo and tetrachloro PTZs **9** and **11**, showed MIC values against Mtb strain H37Rv in the low micromolar range coupled with positive SI values. They were

bactericidal and showed good activity also against non-replicating Mtb, with the potential to prevent the infection reactivation and shortening the long treatment regimen. In addition, they synergized with RMP and BDQ, were metabolically stable, and showed hepatotoxicity only at highest concentrations. Of note, HPTZs inhibited both oxygen consumption and NADH oxidation, two essential energetic processes in the growth and survival of mycobacteria, indicating NDH-2 as their putative target. The inhibition of elements of the oxidative phosphorylation pathway and the entire energy metabolism in mycobacteria has recently emerged as an innovative approach to treat dormant or latent mycobacteria, as shown by the approval of BDQ. In this context, the inhibition of NDH-2 could represent an alternative valid way to interrupt the mycobacterial respiratory chain. Unfortunately, the unavailability of TB structural data of NDH-2 did not permit us to elucidate the exact role of X-bonds in HPTZs mechanism of action, that could contribute to further optimize these compounds.

In conclusion, we have shown as poly-halogenation of the PTZs is a valid approach to switch off the central receptor affinity. However, to actually define their anti-TB applicability, HPTZs with higher selectivity index should be achieved while ruling out the side-effects characterizing all the clinical PTZs.

EXPERIMETAL SECTION

General Chemistry. All reactions were routinely checked by TLC on silica gel $60F_{254}$ (Merck) and visualized by using UV or iodine. Flash column chromatography (FCC) separations were carried out on Merck silica gel 60 (mesh 230-400). The purity of all final compounds was over 95% as determined by HPLC analysis monitored at 254 nm. HPLC analysis were performed on HPLC Waters LC Module I Plus, equipped with XTerra MS C18 column reversed-phase (3.5 μ spherical hybrid, 4.6 mm x 150 mm, 3.5 μm particle size) at 25 °C and were carried out at 25 °C according to the method described below. Compounds structures were confirmed by ^{1}H NMR (200 or 400MHz) and ^{13}C NMR (101 MHz) spectra recorded on Bruker Avance DPX-200 or DPX-400 spectrometers

by using chloroform (δ = 7.26) or dimethylsulfoxide (δ = 2.48) as an internal standard. Chemical shifts are given in ppm (δ) and the spectral data are consistent with the assigned structures. Data are reported as: chemical shift (multiplicity, coupling constants where applicable, number of hydrogen atoms). Abbreviations are: s (singlet), d (doublet), t (triplet), q (quartet), qui (quintuplet) dd (doublet of doublet), dt (double of triplet), tt (triplet of triplet), m (multiplet), br (broad signal). Coupling constant (*J*) quoted in Hertz (Hz) to the nearest 0.1 Hz. After extraction, organic solutions were dried over anhydrous Na_2SO_4, filtered, and concentrated with Büchi rotary evaporator at reduced pressure. Yields are of purified product and were not optimized.

Reagents and solvents were purchased from common commercial suppliers and were used without further purification. All starting materials were commercially available unless otherwise indicated.

3,7-Dichloro-10*H*-phenothiazine (24).[17] NCS (0.737 g, 5.52 mmol) was added portionwise to a solution of **21** (0.500 g, 2.50 mmol) in DMF (5.00 mL) maintained at 0 °C. The reaction mixture was stirred for 2 h at this temperature and then let to stir at rt overnight. Then, the reaction mixture was poured into ice/water and extracted with Et_2O. The organic layers were evaporated to dryness affording a residue, which was purified by FCC (Cyclohexane/EtOAc 80:20) to give **24** as green solid (0.2 g, 31% yield). ^{1}H NMR (200 MHz, DMSO-d_6): δ 6.64 (d, *J* = 9.1 Hz, 2H, aromatic CH), 7.05–7.01 (m, 4H, aromatic CH), 8.85 (s, 1H, NH).

1,3,7-Trichloro-10*H*-phenothiazine (25). PCl_5 (3.13 g, 15.06 mmol) was added to a solution of **21** (1.00 g, 5.02 mmol) in $CHCl_3$ (40 mL) and the reaction mixture was refluxed overnight. The $CHCl_3$ was eliminated by distillation under vacuum and the crude product was poured into ice/water and stirred for 20 min. The precipitate obtained was filtered, washed with EtOH and purified by FCC (cyclohexane/ EtOAc 98:2) to give **25** as a teal green solid (1.51 g, 99% yield). ^{1}H NMR (200 MHz, $CDCl_3$): δ 6.29 (bs, 1H, NH), 6.50 (d, *J* = 8.2 Hz, 1H, aromatic CH), 6.81 (dd, *J* = 2.2 and 0.4 Hz, 1H, aromatic CH), 6.91 (m, 1H, aromatic CH), 6.96 (d, *J* = 2.3 Hz, 1H, aromatic CH), 7.00 (d, *J* = 2.2 Hz, 1H, aromatic CH).

1,3,7,9-Tetrachloro-10*H*-phenothiazine (26). The title compound was synthesized according to the procedure reported for the synthesis of **25** but by using five equivalents of PCl_5. After filtration, compound **26** was obtained in 36% yield as a grey solid and it was used without further purification. ^{1}H NMR (200 MHz, $CDCl_3$): δ 6.89 (d, *J* = 2.2 Hz, 2H, aromatic CH), 7.14 (d, *J* = 2.2 Hz, 2H, aromatic CH), 11.20 (bs, 1H, NH).

General procedure for the alkylation reaction and salification (Method A). A solution of the appropriate phenothiazine or phenoselenazine (1.0 equiv) in dry DMF was slowly added at 0 °C to a suspension of NaH (5.0 equiv) in dry DMF. The reaction mixture was stirred at rt for 30 min. Then, a solution of the appropriate (chloroalkyl)amine chain (2.0 equiv) in dry DMF was added dropwise and the reaction mixture was maintained at 80 °C until no starting material was detected by TLC. After cooling, the reaction mixture was partitioned between EtOAc and water and extracted with EtOAc. The organic layers were evaporated to dryness obtaining an oil that was purified by FCC as described below. With the exception of compound **15**, each compound was dissolved in Et_2O and HCl gas was bubbled into the solution. The mixture was then filtered or evaporated to dryness depending on whether the hydrochloride salt was obtained as a solid or as an oil, respectively, furnishing target compounds.

3-(3-Chloro-10*H*-phenothiazin-10-yl)-*N*,*N*-dimethylpropan-1-amine hydrochloride (1).[36] The title compound was prepared starting from **23** following Method A (2h) and by using 3-chloro-*N*,*N*-dimethylpropan-1-amine hydrochloride. The compound was purified by FCC ($CHCl_3$/MeOH from 98:2 to 95:5) and obtained as a brown oil in 59% yield; after salification, **1** was obtained by filtration as a white solid in 66% yield. ^{1}H NMR (200 MHz, $CDCl_3$): δ 2.25-2.38 (m, 2H, CH_2*CH_2*CH_2), 2.61 (s, 6H, CH_3), 2.97-3.08 (m, 2H, CH_2CH_2*CH_2*), 4.01 (t, *J* = 5.7 Hz, 2H, *CH_2*CH_2CH_2), 6.82-6.95 (m, 4H, aromatic CH), 6.98-7.06 (m, 1H, aromatic CH), 7.12-7.21 (m, 2H, aromatic CH), 12.49 (bs, 1H, NH). ^{13}C NMR (101 MHz, $CDCl_3$): 21.66, 42.99, 44.33, 55.77, 116.37, 116.46, 123.21, 123.79, 124.64, 125.91, 127.92, 128.36, 133.71, 143.66, 146.02. Purity: 99.54% (HPLC, R_t:2.479 min; Method: solvent A, CH_3CN; solvent B, FA solution (0.1% v/v),

gradient from 60% to 20% of FA solution; 15 min). HRMS-ESI: *m/z* $(M + H)^+$ calcd for $C_{17}H_{19}ClN_2S$ 319.10357, found 319.1043.

3-(3,7-Dichloro-10*H*-phenothiazin-10-yl)-*N*,*N*-dimethylpropan-1-amine hydrochloride (2). The title compound was prepared starting from **24**[17] following Method A (1h) and by using 3-chloro-*N*,*N*-dimethylpropan-1-amine hydrochloride. The compound was purified by FCC ($CHCl_3$/MeOH 95:5) and obtained as a blue oil in 47% yield; after salification, the solution was evaporated to dryness, triturated with EtOAc, and filtered to give **2** as a light-yellow solid in 18% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.30-2.47 (m, 2H, $CH_2CH_2CH_2$), 2.66 (s, 6H, CH_3), 2.85-3.04 (m, 2H, $CH_2CH_2CH_2$), 4.04-4.12 (m, 2H, $CH_2CH_2CH_2$), 6.80 (d, *J* = 7.6 Hz, 2H, aromatic CH), 7.15-7.18 (m, 4H, aromatic CH), 12.59 (bs, 1H, NH). ^{13}C NMR (101 MHz, $CDCl_3$): 21.84, 43.15, 44.56, 55.86, 116.89, 127.19, 127.48, 127.80, 128.64, 142.97. Purity: 98.95% (HPLC, R_t:2.848 min; Method: solvent A, CH_3CN; solvent B, FA solution (0.1% v/v), gradient from 80% to 20% of FA solution; 12 min). HRMS-ESI: *m/z* $(M + H)^+$ calcd for $C_{17}H_{18}Cl_2N_2S$ 353.06460, found 353.0652.

***N*,*N*-Dimethyl-3-(1,3,7-trichloro-10*H*-phenthiazin-10-yl)-propan-1-amine hydrochloride (3).** The title compound was prepared starting from **25** following Method A (2h) and by using 3-chloro-*N*,*N*-dimethylpropan-1-amine hydrochloride. The compound was purified by FCC ($CHCl_3$/MeOH 97:3) and obtained as a brown oil in 52% yield; after salification, the solution was evaporated to dryness, triturated with EtOAc, and filtered to give **3** as a white solid in 38% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.01-2.26 (m, 2H, $CH_2CH_2CH_2$), 2.63 (s, 6H, CH_3), 2.85-3.05 (m, 2H, $CH_2CH_2CH_2$), 4.06-4.09 (m, 2H, $CH_2CH_2CH_2$), 7.00 (d, *J* = 8.5 Hz, 1H, aromatic CH), 7.08 (d, *J* = 2.2 Hz, 1H, aromatic CH), 7.14 (d, *J* = 2.0 Hz, 1H, aromatic CH), 7.20 (dd, *J* = 2.0 and 8.3 Hz, 1H, aromatic CH), 7.25 (d, *J* = 2.1 Hz, 1H, aromatic CH), 12.56 (bs, 1H, NH). ^{13}C NMR (101 MHz, $CDCl_3$): δ 23.42, 50.65, 55.86, 122.17, 126.06, 127.03, 127.59, 128.34, 129.75, 129.87, 130.16, 130.36, 134.30, 139.35, 143.28. Purity: 98.52% (HPLC, R_t:4.193 min; Method: solvent A, CH_3CN;

solvent B, FA solution (0.1% v/v), isocratic 80% of FA solution; 12 min). HRMS-ESI: *m/z* $(M + H)^+$ calcd for $C_{17}H_{17}Cl_3N_2S$ 387.02563, found 387.0262.

3-(3,7-Dibromo-10*H*-phenothiazin-10-yl)-*N*,*N*-dimethylpropan-1-amine hydrochloride (4). The title compound was prepared starting from **27**[16] following Method A (3h) and by using 3-chloro-*N*,*N*-dimethylpropan-1-amine hydrochloride. The compound was purified by FCC ($CHCl_3$/MeOH from 99:1 to 95:5) and obtained as green oil in 44% yield; after salification, **4** was obtained by filtration as a white solid in 49% yield. ^{1}H NMR (200 MHz, $CDCl_3$): δ 2.30-2.37 (m, 2H, $CH_2CH_2CH_2$), 2.64 (s, 6H, CH_3), 2.72-3.01 (m, 2H, $CH_2CH_2CH_2$), 4.00-4.10 (m, 2H, $CH_2CH_2CH_2$), 6.73 (d, *J* = 8.0 Hz, 2H, aromatic CH), 7.21-7.25 (m, 4H, aromatic CH), 10.30 (bs, 1H, NH). ^{13}C NMR (101 MHz, $CDCl_3$): δ 21.85, 43.21, 44.53, 55.89, 115.91, 117.34, 127.55, 130.27, 130.71, 143.44. Purity: 97.72% (HPLC, R_t:3.595 min; Method: solvent A, CH_3CN; solvent B, FA solution (0.1% v/v), isocratic 90% of FA solution; 15 min). HRMS-ESI: *m/z* $(M + H)^+$ calcd for $C_{17}H_{18}Br_2N_2S$ 440.96357, found 440.9637.

3-(3,7-Dibromo-1-fluoro-10*H*-phenothiazin-10-yl)-*N*,*N*-dimethylpropan-1-amine hydrochloride (5). The title compound was prepared starting from **28**[16] following Method A (1h) and by using 3-chloro-*N*,*N*-dimethylpropan-1-amine hydrochloride. The compound was purified by FCC ($CHCl_3$/MeOH 95:5) and obtained as a brown oil in 54% yield; after salification, **5** was obtained by filtration as a white solid in 57% yield. ^{1}H NMR (200 MHz, DMSO-d_6) δ 1.81-1.88 (m, 2H, $CH_2CH_2CH_2$), 2.57 (s, 6H, CH_3), 2.94 (t, *J* = 7.9 Hz, 2H, $CH_2CH_2CH_2$), 3.88-3.91 (m, 2H, $CH_2CH_2CH_2$), 7.09 (d, *J* = 8.3 Hz, 1H, aromatic H_9), 7.29-7.30 (m, 1H, aromatic CH), 7.34-7.48 (m, 3H, aromatic CH), 10.04 (bs, 1H, NH). ^{13}C NMR (101 MHz, $CDCl_3$) δ 22.86, 43.11, 48.23, 55.83, 116.18, 116.51, 119.23, 119.54, 126.10, 128.65, 130.07, 130.44, 131.09, 132.51, 144.01, 153.42. Purity: 98.87% (HPLC, R_t:2.675 min; Method: solvent A, CH_3CN; solvent B, FA solution (0.1% v/v), isocratic 60% of FA solution; 15 min). HRMS-ESI: *m/z* $(M + H)^+$ calcd for $C_{17}H_{17}Br_2FN_2S$ 458.95415, found 458.9541.

3-Chloro-10-[2-(1-methylpiperidin-2-yl)ethyl]-10*H*-phenothiazine hydrochloride (6). The title compound was prepared starting from **23** following Method A (3h) and by using 2-(2-chloroethyl)-1-methylpiperidine hydrochloride. The compound was purified by FCC ($CHCl_3$/MeOH 98:2) and obtained as an orange oil in 80% yield; after salification, **6** was obtained by filtration as a white solid in 47% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.25-1.53 (m, 1H, piperidine CH_2), 1.54-1.86 (m, 4H, piperidine CH_2), 1.86-2.13 (m, 2H, CH_2CH_2), 2.19-2.29 (m, 1H, piperidine CH_2), 2.60 (s, 3H, CH_3), 2.86-3.02 (m, 1H, piperidine CH), 3.02-3.22 (m, 2H, piperidine CH_2), 3.82-4.15 (m, 2H, CH_2CH_2), 6.96-7.00 (m, 2H, aromatic CH), 7.05-7.07 (m, 1H, aromatic CH), 7.11 (s, 1H, aromatic CH), 7.15-7.29 (m, 3H, aromatic CH), 10.75 (bs, 1H, NH). ^{13}C NMR (101 MHz, DMSO-d_6): δ 22.01, 22.77, 27.99, 28.30, 40.52, 43.42, 55.44, 63.14, 116.43, 116.93, 122.88, 123.60, 123.73, 124.41, 127.86, 128.28, 128.70, 132.99, 144.09, 146.75. Purity: 99.87% (HPLC, R_t: 3.138 min; Method: solvent A, CH_3CN; solvent B, FA solution (0.1% v/v), gradient from 80% to 20% of FA solution; 12 min).

3,7-Dichloro-10-[2-(1-methylpiperidin-2-yl)ethyl]-10*H*-phenothiazine hydrochloride (7). The title compound was prepared starting from **24**[17] following Method A (5h) and by using 2-(2-chloroethyl)-1-methylpiperidine hydrochloride. The compound was purified by FCC ($CHCl_3$/MeOH 98:2) and obtained as a blue oil in 68% yield; after salification, the solution was evaporated to dryness, triturated with a mixture of $CHCl_3$/MeOH, and filtered to give **7** as a light-yellow solid in 57% yield. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.32-1.42 (m, 1H, piperidine CH_2), 1.50-1.68 (m, 4H, piperidine CH_2), 1.84-2.06 (m, 3H, piperidine CH_2 and CH_2CH_2), 2.20-2.29 (m, 1H, piperidine CH), 2.58 (s, 3H, CH_3), 2.79-2.85 and 2.94-3.16 (m, each 1H, piperidine CH_2), 3.78-3.99 (m, 2H, NCH_2CH_2), 7.05 (d, J = 8.6 Hz, 2H, aromatic CH), 7.24-7.30 (m, 4H, aromatic CH), 10.50 (bs, 1H, NH). ^{13}C NMR (101 MHz, DMSO-d_6): δ 21.97, 22.73, 27.72, 28.13, 43.53, 55.38, 59.13, 62.97, 117.82, 126.07, 127.00, 127.16, 127.99, 143.63. Purity: 95.00% (HPLC, R_t:2.159 min; Method: solvent A, CH_3CN; solvent B, FA solution (0.1% v/v), gradient from 80% to 20% of FA solution; 12 min).

1,3,7-Trichloro-10-[2-(1-methylpiperidin-2-yl)ethyl]-10*H*-phenothiazine hydrochloride (8). The title compound was prepared starting from **25** following Method A (3h) and by using 2-(2-chloroethyl)-1-methylpiperidine hydrochloride. The compound was purified by FCC ($CHCl_3$/MeOH 98:2) and obtained as a brown oil in 91% yield; after salification, the solution was evaporated to dryness, triturated with $CHCl_3$ and filtered to give **8** as a light-yellow solid in 59% yield. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.18-1.70 (m, 7H, piperidine CH_2 and CH_2CH_2), 1.83-2.06 (m, 1H, piperidine CH), 2.62-2.80 and 2.80-2.97 (m, each 1H, piperidine CH_2), 3.30 (s, 3H, CH_3), 3.75-4.09 (m, 2H, NCH_2CH_2), 7.23 (d, J = 8.5 Hz, 1H aromatic CH), 7.30 (d, J = 8.5 Hz, 1H, aromatic CH), 7.30-7.40 (m, 2H, aromatic CH), 7.50 (d, J = 1.5 Hz, 1H, aromatic CH), 8.30 (s, 1H, NH). ^{13}C NMR (101 MHz, DMSO-d_6): δ 21.97, 22.72, 28.18, 29.43, 40.49, 50.48, 55.49, 62.54, 123.17, 126.37, 126.93, 127.18, 128.37, 128.66, 129.19, 129.57, 130.58, 134.91. Purity: 99.77% (HPLC, R_t:2.717 min; Method: solvent A, CH_3CN; solvent B, FA solution (0.1% v/v), isocratic 60% of FA solution; 12 min).

3,7-Dibromo-10-[2-(1-methylpiperidin-2-yl)ethyl]-10*H*-phenothiazine hydrochloride (9). The title compound was prepared starting from **27**[16] following Method A (3h) and by using 2-(2-chloroethyl)-1-methylpiperidine hydrochloride. The compound was purified by FCC ($CHCl_3$/MeOH from 98:2 to 97:3) and obtained as a green oil in 40% yield; after salification, **9** was obtained by filtration as a white solid in 44% yield. ^{1} H NMR (400 MHz, DMSO-d_6): δ 1.19-1.94 (m, 7H, piperidine CH_2 and CH_2CH_2), 2.19-2.37 (m, 1H, piperidine CH_2), 2.59 (s, 3H, CH_3), 2.87-2.94 (m, 1H, piperidine CH), 3.04-3.25 (m, 2H, piperidine CH_2), 3.83-4.13 (m, 2H, CH_2CH_2), 7.01 (d, J = 8.6 Hz, 2H, aromatic CH), 7.36-7.40 (m, 4H, aromatic CH), 10.64 (bs, 1H, NH). ^{13}C NMR (101 MHz, DMSO-d_6): δ 21.94, 22.79, 27.74, 28.16, 40.50, 43.46, 55.38, 63.03, 114.88, 118.27, 126.50, 129.71, 130.86, 144.03. Purity: 99.35% (HPLC, R_t:2.966 min; Method: solvent A, CH_3CN; solvent B, FA solution (0.1% v/v), isocratic 90% of FA solution; 15 min).

3,7-Dibromo-1-fluoro-10-[2-(1-methylpiperidin-2-yl)ethyl]-10*H*-phenothiazine hydrochloride (10). The title compound was prepared starting from **28**[35] following Method A (3h) and by using

2-(2-chloroethyl)-1-methylpiperidine hydrochloride. The compound was purified by FCC ($CHCl_3$/MeOH 95:5) and obtained as a yellow semisolid in 55% yield; after salification, **10** was obtained by filtration as a white solid in 70% yield. ^{1}H NMR (400 MHz, DMSO-*d*$_6$): δ 1.15-1.97 (m, 6H, piperidine CH_2 and CH_2*CH_2*), 2.12-2.35 and 2.72-2.85 (m, each 1H, piperidine CH_2), 2.99-3.15 (m, 2H, piperidine CH_2), 3.27-3.53 (m, 1H, piperidine CH), 3.90-4.25 (m, 2H, *CH_2*CH_2), 7.13 (d, *J* = 8.6 Hz, 1H, aromatic CH), 7.33 (m, 1H, aromatic CH), 7.40-7.48 (m, 3H, aromatic CH), 10.51 (bs, 1H, NH). ^{13}C NMR (101 MHz, DMSO-*d*$_6$) δ 22.01, 22.71, 28.28, 28.95, 40.29, 47.52, 55.44, 62.75, 115.43 (d, *J* = 10.2 Hz,), 115.68, 119.54 (d, *J* = 24.9 Hz), 120.56, 126.21, 128.55 (d, *J* = 12.7 Hz), 129.77, 131.11, 132.62, 144.61, 153.24 (d, *J* = 250. 5 Hz). Purity: 99.71% (HPLC, R_t:3.289 min; Method: solvent A, CH_3CN; solvent B, FA solution (0.1% v/v), isocratic 70% of FA solution; 15 min).

1,3,7,9-Tetrachloro-10-[2-(1-methylpiperidin-2-yl)ethyl]-10*H*-phenothiazine hydrochloride (11). The title compound was prepared starting from **26** following Method A (overnight) and by using 2-(2-chloroethyl)-1-methylpiperidine hydrochloride. The compound was purified by FCC ($CHCl_3$/MeOH 98:2) and obtained as an orange oil in 29% yield; after salification, the solution was evaporated to dryness, triturated with EtOAc and filtered to give **11** as a light-pink solid in 50% yield. ^{1}H NMR (400 MHz, DMSO-*d*$_6$): δ 1.50-1.76 (m, 6H, piperidine CH_2 and CH_2*CH_2*), 1.87-2.14 (m, 1H, piperidine CH_2), 2.51 (s, 3H, CH_3), 2.69-2.80 (m, 1H, piperidine CH_2), 2.94-2.99 (m, 2H, piperidine CH_2), 3.16-3.27 (m, 1H, piperidine CH), 3.64-3.91 (m, 2H, *CH_2*CH_2), 7.52 (s, 2H, aromatic CH), 7.61 (s, 2H, aromatic CH), 10.30 (bs, 1H, NH). ^{13}C NMR (101 MHz, DMSO-*d*$_6$): δ 21.98, 22.66, 28.11, 29.73, 40.13, 50.88, 55.49, 62.41, 126.44, 129.83, 130.45, 136.36, 136.45, 139.33, 139.89. Purity: 99.40% (HPLC, R_t:9.553 min; Method: solvent A, CH_3CN; solvent B, FA solution (0.1% v/v), isocratic 80% of FA solution; 20 min).

1,3,7,9-Tetrachloro-10-(3-pyrrolidin-1-ylpropyl)-10*H*-phenothiazine hydrochloride (12). The title compound was prepared starting from **26** following Method A (4h) and by using 1-(3-chloropropyl)pyrrolidine hydrochloride. The compound was purified by FCC ($CHCl_3$/MeOH 97:3)

and obtained as a brown oil in 73% yield; after salification, **12** was obtained by filtration as a brown solid in 53% yield. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.73-1.81 (m, 4H, pyrrolidine CH_2), 1.83-1.91 (m, 2H, $CH_2CH_2CH_2$), 2.75-2.84 and 2.97-3.03 (m, each 2H, pyrrolidine CH_2), 3.28-3.38 (m, 2H, $CH_2CH_2CH_2$), 3.72 (t, J = 7.6 Hz, 2H, $CH_2CH_2CH_2$), 7.51 (d, J = 2.3, 2H, aromatic CH), 7.61 (d, J = 2.2, 2H, aromatic CH), 10.21 (bs, 1H, NH). ^{13}C NMR (101 MHz, DMSO-d_6,): δ 22.92, 25.27, 51.68, 51.86, 53.29, 126.34, 129.25, 129.90, 130.46, 136.36, 139.61. Purity: 98.17% (HPLC, R_t:19.248 min; Method: solvent A, CH_3CN; solvent B, FA solution (0.1% v/v), gradient from 90% to 40% of FA solution; 25 min).

1,3,7,9-Tetrachloro-10-(3-morpholin-4-ylpropyl)-10*H*-phenothiazine hydrochloride (13). The title compound was prepared starting from **26** following Method A (5h) and by using 4-(3-chloropropyl)morpholine hydrochloride. The compound was purified by FCC (CH_2Cl_2/Cyclohexane/EtOAc 80:10:10) and obtained as a brown oil in 81% yield; after salification, **13** was obtained by filtration as a white solid in 40% yield. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.77-1.85 (m, 2H, $CH_2CH_2CH_2$), 2.83-2.91 (m, 2H, morpholine CH_2), 2.94-3.00 (m, 2H, morpholine CH_2), 3.19-3.22 (m, 2H, $CH_2CH_2CH_2$), 3.63 (t, J = 11.9 Hz, 2H, morpholine CH_2), 3.68 (t, J = 7.6 Hz, 2H, morpholine CH_2), 3.82-3.85 (m, 2H, $CH_2CH_2CH_2$), 7.50-7.51 (m, 2H, aromatic CH), 7.60-7.61 (m, 2H, aromatic CH), 10.59 (bs, 1H, NH). ^{13}C NMR (101 MHz, DMSO-d_6): δ 23.09, 51.42, 51.98, 53.79, 63.57, 126.43, 129.31, 129.97, 130.56, 136.51, 139.64. Purity: 99.58% (HPLC, R_t:18.769 min; Method: solvent A, CH_3CN; solvent B, DEA solution (0.1% v/v), gradient from 30% to 5% of DEA solution; 35 min).

3,7-Dibromo-10-(3-pyrrolidin-1ylpropyl)-10*H*-phenothiazine hydrochloride (14). The title compound was prepared starting from **27**[16] following Method A (4h) and by using 1-(3-chloropropyl)pyrrolidine. The compound was purified by FCC ($CHCl_3$/MeOH 90:10) and obtained as a brown oil in 64% yield; after salification, **14** was obtained by filtration as a grey solid in 18% yield. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.77-1.80 (m, 2H, pyrrolidine CH_2), 1.83-1.91 (m, 2H, pyrrolidine CH_2), 1.96-2.03 (m, 2H, $CH_2CH_2CH_2$), 2.85-2.92 (m, 2H, pyrrolidine CH_2), 3.10-3.15

(m, 2H, pyrrolidine CH_2), 3.42-3.44 (m, 2H, $CH_2CH_2\mathit{CH_2}$), 3.89 (t, *J* = 6.9 Hz, 2H, $\mathit{CH_2}CH_2CH_2$), 7.00 (d, *J* = 8.6 Hz, 2H, aromatic CH), 7.35 (dd, *J* = 2.2 and 8.6 Hz, 2H, aromatic CH), 7.40 (d, *J* = 2.1 Hz, 2H, aromatic CH), 10.45 (bs, 1H, NH). ^{13}C NMR (101 MHz, DMSO-d_6): δ 23.06, 40.55, 44.38, 51.81, 53.39, 114.82, 118.22, 126.20, 129.62, 130.81, 143.92. Purity: 97.63% (HPLC, R_t: 4.069 min; Method: solvent A, CH_3CN; solvent B, FA solution (0.1% v/v), gradient from 100% to 60% of FA solution; 25 min).

3,7-Dibromo-10-(3-morpholin-4-ylpropyl)-10*H*-phenothiazine hydrochloride (15). The title compound was prepared starting from **27**[16] following Method A (4h) and by using 4-(3-chloropropyl)morpholine. The compound was purified by FCC ($CHCl_3$/MeOH 98:2) and obtained as a brown oil in 69% yield; after salification, **15** was obtained by filtration as a white solid in 10% yield. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.87-1.92 (m, 2H, $CH_2\mathit{CH_2}CH_2$), 2.46-2.48 (m, 2H, morpholine CH_2), 2.92-3.00 (m, 2H, morpholine CH_2), 3.05-3.11 (m, 2H, $CH_2CH_2\mathit{CH_2}$), 3.63-3.69 (m, 2H, $\mathit{CH_2}CH_2CH_2$), 3.86- 3.90 (m, 4H, morpholine CH_2), 7.00 (d, *J* = 8.7 Hz, 2H, aromatic CH), 7.35 (dd, *J* = 2.3 and 8.7 Hz, 2H, aromatic CH) 7.38 (d, *J* = 2.0 Hz, 2H, aromatic CH), 10.48 (bs, 1H, NH). ^{13}C NMR (101 MHz, DMSO-d_6): δ 20.98, 44.44, 51.52, 54.02, 63.62, 114.84, 118.20, 126.22, 129.62, 130.82, 143.89. Purity: 96.81% (HPLC, R_t:18.236 min; Method: solvent A, CH_3CN; solvent B, FA solution (0.1% v/v), gradient from 90% to 40% of FA solution; 25 min).

3,7-Dibromo-10-(2-(pyrrolidin-1-yl)ethyl)-10*H*-phenothiazine (16). The title compound was prepared starting from **27**[16] following Method A (2h) by using 1-(2-chloroethyl)pyrrolidine and obtained after purified by FCC ($CHCl_3$/MeOH 98:2) as a white solid in 78% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.62-1.77 (m, 4H, pyrrolidine CH_2), 2.56-2.73 (m, 4H, pyrrolidine CH_2), 2.81 (t, *J* = 7.0 Hz, 2H, $CH_2\mathit{CH_2}$), 3.93 (t, *J* = 7.2 Hz, $\mathit{CH_2}CH_2$), 6.75 (d, *J* = 8.5 Hz, 2H, aromatic CH), 7.19-7.24 (m, 4H, aromatic CH). ^{13}C NMR (101 MHz, DMSO-d_6): δ 23.57, 47.18, 52.76, 54.18, 114.57, 118.00, 125.63, 129.50, 130.85, 144.10. Purity: 99.85% (HPLC, R_t: 3.287 min; Method: solvent A, CH_3CN; solvent B, FA solution (0.1% v/v), isocratic 80% of FA solution; 15 min).

4-(2-(3,7-Dibromo-10*H*-phenothiazin-10-yl)ethyl)morpholine hydrochloride (17). The title compound was prepared starting from **27**[16] following Method A (3h) and by using 4-(2-chloroethyl)morpholine. The compound was purified by FCC ($CHCl_3$/MeOH 95:5) and obtained as a brown oil in 64% yield; after salification, **17** was obtained by filtration as a dirty white solid in 34% yield. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.07-3.10 (m, 2H, morpholine CH_2), 3.33-3.34 (m, 2H, morpholine CH_2), 3.43-3.46 (m, 2H, CH_2CH_2), 3.70-3.76 (t, J = 11.9 Hz, 2H, morpholine CH_2), 3.89-3.91 (m, 2H, morpholine CH_2), 4.24-4.34 (m, 2H, CH_2CH_2), 7.13 (d, J = 8.6 Hz, 2H, aromatic CH), 7.38-7.43 (m, 4H, aromatic CH), 11.65 (bs, 1H, NH). ^{13}C NMR (101 MHz, DMSO-d_6): δ 41.68, 51.63, 52.29, 63.66, 115.42, 118.49, 126.77, 129.91, 131.06, 143.68. Purity: 99.32% (HPLC, R_t: 2.842 min; Method: solvent A, CH_3CN; solvent B, FA solution (0.1% v/v), isocratic 80% of FA solution; 15 min).

3,7-Dibromo-10-(pyridin-2-ylmethyl)-10*H*-phenothiazine hydrochloride (18). The title compound was prepared starting from **27**[16] following Method A (5h) and by using 2-(chloromethyl)pyridine. The compound was purified by FCC (Cyclohexane/EtOAc 40:60) and obtained as a brown oil in 48% yield; after salification, **18** was obtained by filtration as a yellow solid in 37% yield. ^{1}H NMR (400 MHz, DMSO-d_6): δ 5.26 (s, 2H, CH_2), 6.65 (d, J = 8.7 Hz, 2H, phenothiazine CH), 7.21 (dd, J = 1.7 and 8.7 Hz, 2H, phenothiazine CH), 7.36 (d, J = 2.1 Hz, 2H, phenothiazine CH), 7.45 (d, J = 7.9 Hz, 1H, pyridine CH), 7.51 (t, J = 5.9 Hz, pyridine CH), 7.96 (t, J = 7.6 Hz, 1H, pyridine CH), 8.75 (d, J = 5.0 Hz, 1H, pyridine CH), 12.03 (bs, 1H, NH). ^{13}C NMR (101 MHz, DMSO-d_6): δ 51.26, 115.10, 117.87, 123.66, 124.56, 124.94, 129.28, 130.72, 143.23, 154.28. Purity: 97.54% (HPLC, R_t:8.204 min; Method: solvent A, CH_3CN; solvent B, FA solution (0.1% v/v), isocratic 40% of FA solution; 20 min).

3,7-Dibromo-10-(chloroacetyl)-10*H*-phenothiazine (29). Chloroacetyl chloride (0.37 g, 3.27 mmol) was added dropwise to a suspension of **27**[16] (0.78 g, 2.18 mmol) in toluene (20 mL) at 0 °C and the reaction mixture was stirred at 80 °C for 10h. After cooling, the reaction mixture was poured into ice/water and extracted with EtOAc. The organic layers were evaporated to dryness to

give a reside that was purified by FCC (Cyclohexane/EtOAc 90:10) affording **29** as a grey solid (0.450 g, 47% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.12 (s, 2H, CH_2), 7.42 (d, *J* = 8.5 Hz, 2H, aromatic CH), 7.47 (dd, *J* = 2.0 and 8.7 Hz, 2H, aromatic CH), 7.58 (d, *J* = 2.0 Hz, 2H, aromatic CH).

3-[2-(3,7-Dibromo-10*H*-phenothiazin-10-yl)-2-oxoethyl]-5-(4-fluorophenyl)-1,3,4-thiadiazole-2(3*H*)-inine (19). A mixture of **29** (0.40 g, 0.92 mmol) and **30**[37] (0.18 g, 0.92 mmol) in ethanol (30 mL) was stirred at 80° C for 24h. After cooling, the reaction mixture was poured into ice/water, basified with a saturated solution of Na_2CO_3 until pH = 8.0 and then extracted with EtOAc. The organic layers were evaporated to dryness to give a residue that was purified by FCC ($CHCl_3$/MeOH 98:2) affording **19** as a pink solid (0.07 g, 13% yield). ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.85 (s, 2H, CH_2), 7.23–7.28 (m, 2H, aromatic CH), 7.54–7.59 (m, 6H, aromatic CH), 7.73–7.80 (m, 2H, aromatic CH). ^{13}C NMR (101 MHz, DMSO-d_6): δ 49.74, 116.67 (d, *J* = 22.0 Hz), 120.22, 127.09, 128.06 (d, *J* = 8.6 Hz) 129.03, 130.68, 130.89, 134.28, 136.91, 144.04, 158.77, 163.32 (d, *J* = 247.0 Hz), 165.90. Purity: 98.96% (HPLC, R_t: 5.302 min; Method: solvent A, CH_3CN; solvent B, DEA solution (0.1% v/v), isocratic 20% of DEA solution; 15 min).

3,7-Dibromo-10*H*-phenoselenazine (32). A solution of Br_2 (1.13 g, 7.10 mmol) was added dropwise to a solution of **31**[38] (0.70 g, 2.84 mmol) in glacial acetic acid (40 mL), and the mixture was stirred at rt for 5h. Then, a solution of 10% Na_2SO_3 was added and the mixture was stirred at rt for 1h. The reaction mixture was poured into ice/water and the precipitate obtained was filtered affording **32** as a grey solid (0.242 g, 21% yield). ^{1}H NMR (400 MHz, DMSO-d_6): δ 6.62 (d, *J* = 8.6 Hz, 2H, H_1 and H_9), 7.14 (dd, *J* = 2.3 and 8.6 Hz, 2H, H_2 and H_8), 7.31 (d, *J* = 2.1 Hz, 2H, H_4 and H_6), 8.80 (s, 1H, NH).

3,7-Dibromo-10-[2-(1-methylpiperidin-2-yl)ethyl]-10*H*-phenoselenazine hydrochloride (20). The title compound was prepared starting from **32** following Method A (3h) and by using 2-(2-chloroethyl)-1-methylpiperidine hydrochloride. The compound was purified by FCC ($CHCl_3$/MeOH 95:5) and obtained as a blue solid in 60% yield; after salification, **20** was obtained

by filtration as a grey solid in 41% yield. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.25-1.51 (m, 2H, piperidine CH_2), 1.55-1.92 (m, 5H, piperidine CH_2 and CH_2CH_2), 2.16-2.23 (m, 1H, piperidine CH), 2.56 (s, 3H, CH_3), 2.81-3.01 (m, 3H, piperidine CH_2), 3.84-4.02 (m, 2H, CH_2CH_2), 7.06 (d, *J* = 8.7 Hz, 2H, aromatic CH), 7.40-7.42 (m, 2H, aromatic CH), 7.60-7.61 (m, 2H, aromatic CH), 10.27 (bs, 1H, NH). ^{13}C NMR (101 MHz, DMSO-d_6): δ 21.94, 22.81, 28.23, 40.28, 44.62, 55.43, 63.10, 63.43, 115.46, 119.68, 124.44, 130.89, 132.33, 144.27. Purity: 99.74% (HPLC, R_t:2.723 min; Method: solvent A, CH_3CN; solvent B, FA solution (0.1% v/v), isocratic 60% of FA solution; 12 min).

Pan-assay interference compounds (PAINS) Filters. All the tested compounds were examined for known classes of PAINS[54] by using the PAINS remover filter at http://zinc15.docking.org/patterns/home/[55] and for known classes of molecular aggregators by using http://zinc15.docking.org/patterns/home/,[55] and none of them was found as a potential PAINS and aggregator.

Biological Methods

In vitro Anti-Mtb activity. MICs against replicating and non-replicating cultures of Mtb were performed using the Microplate Alamar Blue Assay (MABA) and Low Oxygen Recovery Assay (LORA), respectively.[48] MBCs were determined by inoculation of Middlebrook 7H11 agar with serially diluted MABA cultures (without addition of resazurin and Tween 80) following 7 days incubation. Colonies were enumerated after 3 weeks incubation at 37 °C and MBC was defined as the minimum concentration effecting a reduction of 99% of colony forming units (CFU) relative to the pre-incubation CFU of untreated control cultures.[61]

Cytotoxicity in mammalian cells. Cytotoxicity for Vero cells (ATCC CRL-81) was determined by resazurin reduction following exposure to test compounds for 72 hours.[61]

Combined drug action. Compounds were tested alone at their respective MICs and in combination at one-half their respective MICs. The FIC of a pairwise combination (FIC_2) was defined as

observed MIC90 of the 2-drug combination/expected MIC90 in additivity.[51] FIC_2 values of <1, ~1 and >1 were interpreted as synergistic, additive and antagonistic interactions, respectively**.**

NADH oxidation and oxygen consumption assays.[42] Inverted membrane vesicles from wild-type *Mycobacterium smegmatis* $mc^2$4517 strain or *M. smegmatis* $mc^2$4517 over-expressing *M. tuberculosis* H37Rv *ndh* gene (Rv1854) were prepared as described previously. NADH oxidation assays was performed as described previously except using a 200 µL volume in a 96-well plate, a VarioSkan Flash plate reader and IMVs containing 4 µg of protein. HPTZs and HQNO were tested at 0.3, 1, 3.2, 10, 100, 315 and 1000 µM. The rate of oxygen consumption was measured using an Oroboros O2k fluorespirometer as previously described.[25,62] IMVs containing 0.5 mg of protein were resuspended in 2 mL of 10 mM HEPES-NaOH buffer pH 7.5 containing 100 mM KCl and 5 mM $MgCl_2$, the cell was closed and oxygen consumption was initiated by the addition of 1 mM NADH (final concentration) through the injection port. Compounds were subsequently added and inhibition was expressed as a percentage of the untreated rate. Experiments were performed at 37 °C with 750 rpm stirring. HTPs and HQNO were tested at 50 µM.

Radioligand Binding Assays. Radioligand binding assays to determine affinities of the tested compounds towards selected GPCRs were carried out, using [^{3}H]-Ketanserin (53.4 Ci/mmol), [^{3}H]-LSD (83.6 Ci/mmol), [^{3}H]-5-CT (80.1 Ci/mmol) and [^{3}H]-Raclopride (76.0 Ci/mmol) for 5-HT_{2A}, 5-HT_6, 5-HT_7 and D_2 receptors, respectively. Non-specific binding was defined with 10 mM of 5-HT in 5-HT_7R binding experiments, whereas 10 µM of chlorpromazine, 10 µM of methiothepine or 10 µM of haloperidol were used in 5-HT_{2A}R, 5- HT_6R and D_2L assays, respectively. Each compound was tested in triplicate at 7–8 concentrations (10^{-11}–10^{-4} M). The inhibition constants (K_i) were calculated from the Cheng-Prusoff equation.[63] HEK293 cells with stable expression of human serotonin 5-HT_6R, 5-HT_{7b}R or dopamine D_{2L}R as well as CHO-K1 cells with stable expression of human serotonin 5-HT_{2A}R purchased from PerkinElmer BioSignal Inc were used in the assays, according to the procedures described previously.[64,65]

Metabolic stability. The metabolic stability of TZ and derivatives were estimated by using human liver microsomes (HLMs) (Sigma-Aldrich, St. Louis, MO, USA), according to the described previously protocols.[56–59] The compounds (50 μM) were incubated in the presence of HLMs (1 mg/ml) for 120 min in 10 mM Tris–HCl buffer (37°C). The reaction mixtures were analyzed next by LC/MS Waters ACQUITY™ TQD system with the TQ Detector (Waters, Milford, USA). Verapamil (VL), used as reference, was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Hepatotoxicity. The hepatotoxicity was evaluated with use of hepatoma HepG2 (ATCC® HB-8065™). Cells were grown under described previously conditions.[56–59] Compounds were incubated at 96-wells plate with cells for 48 h in the final concentration range (0.1–100 μM), whereas the references CCCP and DX at 10 μM and 1 μM, respectively. The cells' viability was determined by CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTS), which was purchased from Promega (Madison, WI, USA). The absorbance was measured using a microplate reader EnSpire (PerkinElmer, Waltham, MA USA) at 490 nm. All compounds were tested in quadruplicate. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) and doxorubicin (DX), used as the references were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Supporting information: Figure S1 showing the effect of compounds **4, 6, 9, 7, 11**, and reference HQNO on NADH oxidation; Figures S2-S6 reporting the UPLC/MS spectra of compounds **9, 11, 20**, and references TZ and verapamil after reaction with HLMs to measure their metabolic stability.

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ABBREVIATIONS

PTZ, phenothiazine; CPZ, chlorpromazine; X-bond, halogen bond; TAR, trans-activation response; HPTZ, halogenated phenothiazine; TB, tuberculosis; MDR, multi drug-resistant; XDR extensively drug-resistant; TDR totally drug-resistant; Mtb, *Mycobacterium tuberculosis*; NDH-2, type II NADH dehydrogenase; BDQ, bedaquiline; TZ, thioridazine; NCS, *N*-chlorosuccinimide; MABA, Microplate Alamar blue assay; HQNO, 2-heptyl-4-hydroxyquinoline-*N*-oxide; IMVs, inverted membrane vesicles; RMP, rifampicin; MBC, minimum bactericidal concentration; LORA, low oxygen recovery assay; INH, isoniazid; LIZ, linezolid; FIC, fractional inhibitory concentration; PRT, pretomanid; FCC, flash column chromatography.

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