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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

8-cyanobenzothiazinone analogs with potent antitubercular activity

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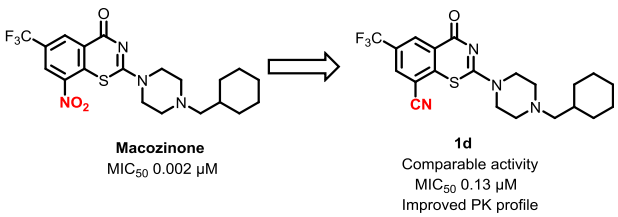
Abstract

8-Nitrobenzothiazinones (BTZs) exemplified by macozinone are a new class of antitubercular agents with exceptionally potent activity. The aryl nitro group has been considered indispensable for activity since this is bioactivated within mycobacteria by the flavoenzyme DprE1 to a reactive nitroso metabolite that covalently labels Cys387. However, the aryl nitro group is a potential liability with regards to safety, stability, and resistance. In this paper, we introduced a nitrile as a bioisosteric replacement of the nitro group, which we hypothesize can maintain a similar covalent mechanism of inhibition, but mitigate against the aforementioned concerns. 8-cyanobenzothiazinone **1d** displayed potent antitubercular activity with an MIC of 130 nM and had an improved volume of distribution in mice that increased the intrinsic half-life by twofold compared to macozinone. Analysis of the C-2 substituent of **1d** revealed similar structure–activity relationships as observed for macozinone. Overall, the results confirm the 8-nitro group of benzothiazinones can be successfully replaced with a nitrile to retain useful activity and favorable pharmacokinetic properties.

Graphical Abstract



Introduction

Tuberculosis (TB) is one of the top 10 causes of death worldwide and the leading cause from a single infectious agent [1]. Moreover, nearly one quarter of the world's population are latently infected with *Mycobacterium tuberculosis* (*Mtb*). Reactivation of latent TB occurs when the immune system is weakened through aging, HIV co-infection, malnutrition, or diabetes. The Covid-19 pandemic and continued rise of drug-resistant TB threatens the modest gains made in recent years to bring TB back under control [1]. The development of new therapeutic agents, improved diagnostics, and effective vaccines will be required to achieve the WHO End TB strategy to decrease the TB incidence rate and the number of TB deaths by 80–90% within the next decade [1].

The benzothiazinones initially reported in 2009 have attracted consider attention for their potent antimycobacterial activity against drug-susceptible and resistant strains, synergy with other TB drugs, and outstanding in vivo activity in murine TB models [2–5]. Optimization of the initial lead candidate BTZ043 led to Macozinone (PBTZ169) with improved drug disposition properties and enhanced in vitro and in vivo activity

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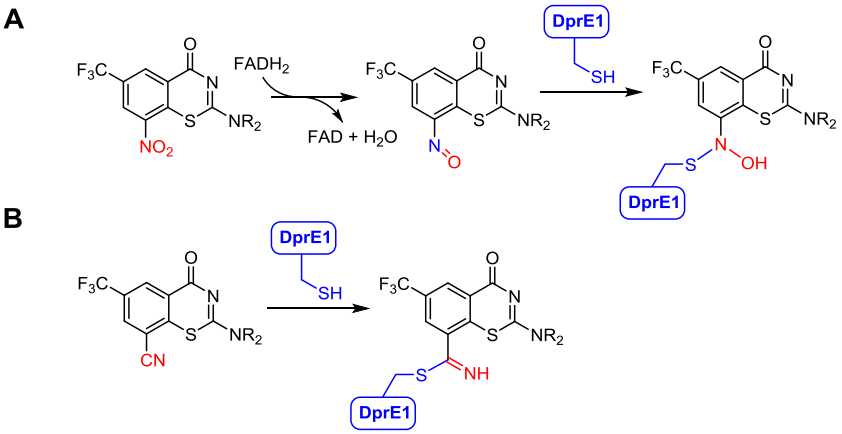
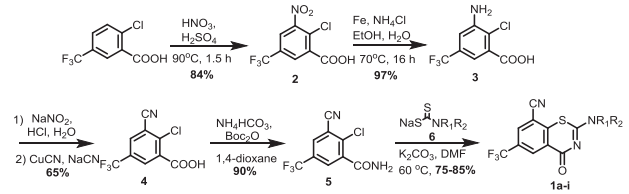


Fig. 1 Mechanism of benzothiazinone inactivation of DprE1. **a** Mechanism of 8-nitrobenzothiazinones. **b** Proposed covalent mechanism of 8-cyano benzothiazinones

[5, 6]. Macozinone successfully completed phase I clinical studies (NCT03036163 and NCT03423030); however phase 2a clinical trials to evaluate the early bactericidal activity were halted due to low enrollment (NCT03334734).

The benzothiazinones inhibit the biosynthesis of the arabinans, which form an integral part of the mycobacterial cell envelope [3, 4, 7–9]. The flavoenzyme DprE1 catalyzes the penultimate biosynthetic step of the decaprenyl-phospho-D-arabinose building block for construction of the highly branched arabinoglycan polymer, which links the inner peptidoglycan layer to the outer mycomembrane. Following binding to DprE1, the benzothiazinone 8-nitro group is reduced by the flavin cofactor of DprE1 to a nitroso metabolite, which covalently reacts with Cys387 of DprE1 to form a stable semimercaptal adduct (Fig. 1a) [10, 11]. The initially reported structure–activity relationships (SAR) demonstrated a requirement for a nitro group at C-8, a strongly electron-withdrawing group at C-6, no substituents at C-5 or C-7, a carbonyl at C-4 and sulfur at the first position [12–14]. The amino group at the C-2 position is the most tolerant to modification, consequently this position has been the most extensively explored to modulate potency and drug disposition properties [3, 6, 15–33]. However, the 8-nitro group remains a potential liability for idiosyncratic toxicity, metabolism by abundant commensal bacterial nitroreductases, reductive activation by glutathione and other biological thiolates and observed drug resistance by mutation of Cys387 to Ser387 [34–37].

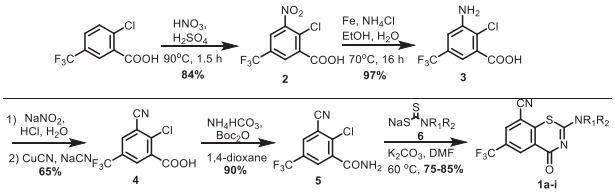
Replacement of the C-8 nitro group of the benzothiazinones with pyrrole and triazole groups, which cannot covalently react with Cys387, afforded compounds with modest micromolar activity suggesting 'the invariant C-8 nitro group' may be more tolerant to modification than originally suggested [12, 17]. We hypothesize that bioisosteric replacement of the 8-nitro group with a nitrile will maintain activity by enabling reaction with Cys387 or the Cys387Ser mutant to form a covalent (thio)imidate adduct, but mitigate some of the undesirable features of the nitro



Scheme 1 Synthesis of 8-cyano-benzothiazinones **1a–i**

group (Fig. 1b). The use of a nitrile to react with a nucleophilic serine or cysteine residue in an enzyme active site to form a reversible, but covalent, adduct has been demonstrated for Saxagliptin, a proline dipeptidase inhibitor used for type-2 diabetes and for Odanacatib, an investigator drug for osteoporosis that inhibits cathepsin K [38]. Qiao and co-workers recently disclosed the first successful bioisosteric replacement of the 8-nitro group with a nitrile [39]. Herein, we report our independent studies confirming this initial finding as well as the first SAR studies of 8-cyano derivatives.

Benzothiazinones containing an 8-cyano substituent were synthesized using an adaptation of the initial Makarov route starting from 2-chloro-5-(trifluoromethyl) benzoic acid, which was nitrated using concentrated sulfuric and nitric acid to provide known intermediate **2** (Scheme 1) [40]. Subsequent nitro reduction with iron powder in aqueous ethanol provided aniline **3** that was smoothly converted to aryl nitrile **4** through diazotization followed by Sandmeyer reaction with CuCN. Formation of the resultant benzamide derivative **5** was accomplished under mild conditions employing di-tert-butyl dicarbonate and ammonium bicarbonate [41]. Condensation of benzamide **5** with a variety of sodium carbodithioates **6a–i** in DMF through nucleophilic aromatic substitution followed by intramolecular cyclization and dehydrosulfidation yielded target compounds **1a–i** in good yields without competitive reaction with the nitrile [13, 42].



The 8-cyanobenzothiazinone derivatives and macozinone as a positive control were screened for antimycobacterial activity against *Mtb* H37Rv to determine the minimum inhibitory concentration (MIC) resulting in complete inhibition of observable mycobacterial growth (Table 1). Compound **1d** containing the optimal cyclohexylmethylpiperazine substituent found in macozinone displayed an MIC of 130 nM providing a rare example of a *des*-nitro benzothiazinone analog with potent nanomolar activity serving to validate our design principle. We next investigated the SAR of the cyclohexyl group with ring homologues **1a–c**, but only cyclopentyl methyl **1c** retained activity with an MIC of 200 nM while **1a–b** were inactive. While oxetanylmethyl **1f** was inactive consistent with the data obtained from cyclobutylmethyl **1b**, we were pleasantly surprised to observe tetrahydrofuran-3-ylmethyl **1e** incorporating a polar oxygen atom retained moderate activity showing only a 10-fold loss of potency relative to cyclopentyl methyl **1c**. Modification of the piperazine by methylation in **1g–h** or replacement with the bicyclic isostere in **1i** abolished activity. Taken together, the limited SAR of this nitrile series demonstrates the cyclohexylmethylpiperazine substituent is optimal, only very conservative modifications are tolerated in the cycloalkyl substituent, and the piperazine must be strictly maintained. The active compound **1c**, **1d**, and **1e** were further evaluated for potential cytotoxicity against Vero cells, but none demonstrated any inhibition of cellular growth at the highest concentration evaluated ($CC_{50} > 150\,\mu M$) furnishing a therapeutic index (MIC/CC_{50}) of greater than 1000 for **1d**.

The calculated and experimentally determined physicochemical properties of the analogs and macozinone are shown in Tables 2 for comparison. We calculated the overall lipophilicity as measured by the partition coefficient clogP, the lipophilic ligand efficiency (LLE, logMIC -logP), the total polar surface area (tPSA) and experimentally measured the melting points as a proxy for compound solubility [43]. Replacement of the C-8 nitro group of macozinone with a nitrile in **1d** results in a modest decrease in clogP of only 0.3 units. However, the LLE for **1d** decreases by 1.5 units relative to macozinone, due to the loss of antimycobacterial activity. The nitrile analog does significantly reduce the tPSA, which is expected to enhance membrane permeability. Among the other active nitrile analogs, compound **1e** containing a tetrahydrofuran-3-ylmethyl substituent had an improved LLE attributed to the dramatically lower clogP that offset the loss of potency.

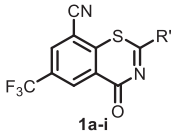
The metabolic stability of **1d** and macozinone were evaluated using mouse and human liver microsomes with and without NADPH. Macozinone was included as a positive control while samples without NADPH served as negative controls. In the absence of NADPH neither macozinone nor **1d** were metabolized. The metabolic stability of macozinone was consistent with previous reports showing a half-life of 34–43 min [30]. Compound **1d** was metabolized approximately twice as quickly in human liver microsomes, but nearly to the same extent in mouse liver microsomes compared to macozinone (Table 3).

Compound **1d** and macozinone were evaluated in male ICR mice to determine their pharmacokinetic parameters. Compound **1d** reached a maximum plasma concentration of 110 ng/mL or 240 nM, following oral dosing at 10 mg/mL and showed a 2.1-fold increase in the intrinsic half-life relative to macozinone (determined from intravenously (i.v.) dosing). The increased half-life of **1d** was driven by a corresponding 2.0-fold increase in the volume of distribution (V) since the clearance (Cl) remained nearly unchanged. The enhanced volume of distribution may in turn reflect the reduced tPSA that can enhance membrane permeability. However, the oral bioavailability of **1d** was decreased twofold relative to macozinone that may in turn potentially reflect lower solubility of **1d**, which is predicted based on the substantially higher melting point of **1d** compared to macozinone. While the increased volume of distribution is favorable, compounds **1d** was deemed to possess inadequate PK parameters due to its low oral bioavailability. To the best of our knowledge, this report also provides the first data for the oral bioavailability of macozinone (Table 4).

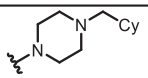
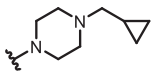
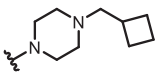
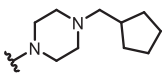
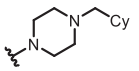
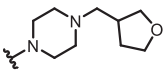
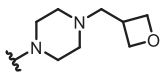
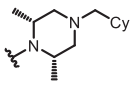
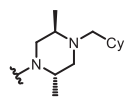
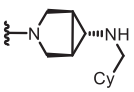
In conclusion, we successfully replaced the 8-nitro group of macozinone with an isosteric nitrile moiety to address potential concerns of safety, stability, and resistance. Compound **1d**, containing an identical C-2 substituent as found in macozinone, possessed respectable antimycobacterial activity with an MIC of 130 nM, displayed no cytotoxicity, and an improved intrinsic half-life owing to its greater volume of distribution. However, **1d** has approximately twofold lower oral bioavailability than macozinone, which we demonstrated was also low. We explored the SAR of the C-2 substituent of **1d** and showed only conservative modifications were tolerated. Further optimization at C-2 will be required to enhance oral bioavailability and exposure as well as potency before the 8-cyano benzothiazinones can be advanced.

Table 1 MIC and CC_{50} of compound 1a–i

Macozinone



1a-i

Compounds	R´	MIC (μM)[a]	CC_{50} (μM)[b]
Macozinone		0.002	>150
1a		> 2.0	nd[c]
1b		> 2.0	nd
1c		0.20	>150
1d		0.13	>150
1e		1.9	>150
1f		> 2.0	nd
1g		> 2.0	nd
1h		> 2.0	nd
1i		> 2.0	nd

[a]Minimum inhibitory concentration against *M. tuberculosis* H37Rv

[b]The concentration required to decrease cell viability of Vero cells by 50%; cnd = not determined

Methods

General

Unless otherwise noted, reagents and materials were obtained from commercial suppliers and were used without further purification. Solvents were dried by the appropriate drying agents prior to use. Anhydrous tetrahydrofuran and dichloromethane were obtained from commercial sources.

Table 2 The ClogP, LLE, and melting point of 1a–i

Compounds	ClogP[a]	LLE[b]	Mp (°C)	tPSA
Macozinone	5.1	3.6	185–187	87.7
1a	3.1	–	151–153	59.7
1b	3.7	–	162–165	59.7
1c	4.2	2.5	196–198	59.7
1d	4.8	2.1	218–221	59.7
1e	2.0	3.8	173–175	68.9
1f	1.3	–	172–174	68.9
1g	5.8	–	182–185	59.7
1h	5.8	–	180–182	59.7
1i	4.1	–	193–195	68.5

[a]ClogP and tPSA were determined by Chemdraw Professional Version 16.0

[b]Lipophilic Ligand Efficiency (LLE) was calculated from the equation: $LLE = \log_{10}MIC - Clog_{10}P$

All solvents used for routine isolation of products and for chromatography were reagent grade. Moisture- and air-sensitive reactions were carried out under an atmosphere of Argon. All reactions were monitored by thin layer chromatography (TLC) and column chromatography purification was performed using 230-400 mesh silica gel. NMR spectra were measured on Bruker AV400 spectrometer at 400 or 300 MHz for 1H spectra and at 100 or 75 MHz for ^{13}C spectra using $CDCl_3$, CD_3OD, and D_2O, and calibrated from the residual solvent signal. All final products were characterized by ^1H NMR, ^{13}C NMR, and MS analyses. Except for the known compounds, all new compounds were also characterized and confirmed by HRMS.

2-chloro-3-nitro-5-(trifluoromethyl)benzoic acid (2)

To a mixture of conc. H_2SO_4 (3.0 mL) and conc. HNO_3 (3.0 mL) was added 2-chloro-5-(trifluoromethyl)benzoic acid (0.52 g, 2.32 mmol). After stirred for 1.5 h at 90 °C, the reaction was cooled to room temperature and poured into ice water (10 mL), filtered and washed with cold water (5.0 mL), dried in vacuo to afford the title compound (0.52 g, 84%) as a white solid: ^1H NMR (400 MHz, DMSO-d_6) δ 8.72 (s, 1H), 8.41 (s, 1H); ^{13}C NMR (DMSO-d_6) δ 164.8, 150.1, 136.1, 130.2 (d, $J = 3.8$ Hz), 129.4, 129.0, 127.7, and 124.4.

3-amino-2-chloro-5-(trifluoromethyl)benzoic acid (3)

A mixture of intermediate **2** (0.5 g, 1.9 mmol, 1.0 equiv.), reductive iron powder (0.5 g, 8.7 mmol, 4.6 equiv.), NH_4Cl (0.7 g, 14 mmol, 7.4 equiv.) in 60 mL of ethanol aqueous solution (ethanol: water, 5:1) was stirred at 70 °C and monitored by TLC. After 16 h, the solvent was removed, and the

Table 3 Microsomal stability of **1d**

Compounds	Mouse		Human	
	(% remaining at 30 min)			
	With NADPH	Without NADPH	With NADPH	Without NADPH
Macozinone	50.7	102	46.8	101
1d	38.6	96.4	26.3	102

Table 4 Pharmacokinetic parameters of macozinone and **1d** in male ICR mice following oral or intravenous administration

Parameters	Units	Macozinone		1d	
		PO	iv	PO	iv
t1/2β	h	1.68	0.86	1.74	1.84
Tmax	h	0.25	0.033	0.25	0.033
Cmax	ng/ml	251	1230	110	1335
AUC(0-t)	h[a]ng/ml	354	423	174	445
Vd	L/kg	–	5.64	–	11.3
Cl	ml/min/kg	–	75.8	–	71.2
F	%	16.7	–	7.8	–

[a]The doses of oral and intravenous administration were 10 and 2 mg/kg, respectively

residue was suspended in 10 mL of ethyl acetate. The suspension was filtered, and the filtrate was washed with 1 *N* HCl (10 mL) and brine (15 mL), dried over Na_2SO_4, filtered and concentrated in vacuo to afford the title compound (0.40 g, 91%) as a brown solid: $R_f = 0.36$ (DCM:MeOH, 8:1 with a trace amount of acetic acid); ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.53 (s, 1H), 7.21 (s, 1H), 7.09 (s, 1H), 6.09 (s, 2H); ^{13}C NMR (DMSO-d_6) δ 166.9, 147.0, 134.2, 128.3 (d, J = 32.4 Hz), 125.5, 122.8, 118.4, and 112.8 (d, $J = 20.2$ Hz).

2-chloro-3-cyano-5-(trifluoromethyl)benzoic acid (4)

To a mixture of intermediate 3 (0.63 g, 2.63 mmol, 1.0 equiv.) in H_2O (5.0 mL) and *conc.* HCl (0.4 mL), was added dropwise a solution of $NaNO_2$ (0.24 g, 3.42 mmol, 1.3 equiv.) in H_2O (0.5 mL) at 0 °C. After stirred at 0–10 °C for 2 h, the solution was added to a mixture of CuCN (0.23 g, 2.63 mmol, 1.0 equiv.) and NaCN (0.19 g, 3.95 mmol, 1.5 equiv.) in H_2O (3.0 mL) at 0 °C. The mixture was stirred at 70 °C for 6 h and cooled to room temperature. The pH value was adjusted to 1 with *conc.* HCl. The crude product was extracted with ethyl acetate (3 × 10 mL), washed with 1 *N* HCl (1 mL), brine (10 mL), dried over Na_2SO_4, filtered and concentrated in vacuo. The crude product was purified by silica gel column chromatography (eluting with 100: 1 DCM: MeOH) to afford the title compound (0.43 g, 65%) as a colorless solid: $R_f = 0.47$ (DCM:MeOH, 8:1 with a trace amount of acetic acid). ^{1}H NMR (400 MHz, CD_3OD) δ 8.37 (s, 1H), 8.35 (s, 1H); ^{13}C NMR (151 MHz, CD3OD) δ 164.3, 139.1, 133.8, 133.2 (d, $J = 3.9$ Hz), 131.5 (d, $J = 3.7$ Hz), 129.8, 129.5, 116.4, and 114.0.

2-chloro-3-cyano-5-(trifluoromethyl)benzamide (5)

To a stirred solution of 2-chloro-3-cyano-5-(trifluoromethyl)-benzoic acid (0.25 g, 1.0 mmol, 1.0 equiv.), pyridine (0.05 mL, 0.62 mmol, 0.62 equiv.), and Boc_2O (0.28 g, 1.3 mmol, 1.3 equiv.) in 1,4-dioxane (2.0 mL) at room temperature was added ammonium bicarbonate (0.10 g, 1.26 mmol, 1.26 equiv.). The reaction was stirred overnight at room temperature and then partitioned between EtOAc (10.0 mL) and H2O (10.0 mL). The organic layer was separated, washed consecutively with water (10.0 mL) and 0.6 *N* aqueous HCl (10.0 mL), dried over anhydrous Mg_2SO_4, and filtered. The filtrate was concentrated under reduced pressure to afford the title compound (0.22 g, 90%) as an off-white solid: ^{1}H NMR (600 MHz, CD_3OD) δ 8.30 (d, $J = 1.0$ Hz, 1H), 8.09 (d, J = 1.0 Hz, 1H); ^{13}C NMR (151 MHz, CD_3OD) δ 168.8, 138.3, 133.2 (q, $J = 3.6$ Hz), 131.3 (q, $J = 34.4$ Hz), 130.7 (q, J = 4.0 Hz), 124.0 (q, $^1J_{C\text{-}F} = 272.5$ Hz), 116.8, and 115.4.

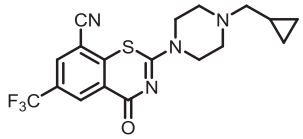
General procedure for the synthesis of compounds 6a–i

To a pre-cooled mixture of amine (2.04 mmol, 1 equiv) in EtOAc was added 30% aqueous NaOH (0.68 mL,

5.09 mmol, 2.5 equiv) followed by CS_2 (0.15 mL, 2.45 mmol, 1.2 equiv). The reaction mixture was stirred for 3 h at 0 °C, then at room temperature for another 3 h. The reaction mixture was filtered and the filter cake was washed by ethyl acetate and dried under vacuum to afford intermediates 6a-i as published in our previous papers [30].

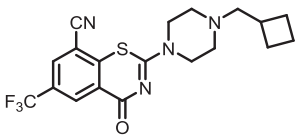
General procedure for the synthesis of compounds 1a–i

A mixture of intermediate **5** (0.16 g, 0.61 mmol, 1.2 equiv), **6a–i** (0.51 mmol, 1.0 equiv) and anhydrous K2CO3 (0.08 g, 0.56 mmol, 1.1 equiv) was stirred in anhydrous DMF (5 mL) at 60 °C. After 2 h, the reaction mixture was cooled down to room temperature and poured into ice water. The product was extracted with CH_2Cl_2 (5 × 20 mL). The organic layer was combined, washed by saturated brine (3 × 5 mL) and dried by anhydrous sodium sulfate which was removed by filtration after 20 min. The solvent CH_2Cl_2 was removed under reduced pressure. The product was purified by silica flash column chromatography with the indicated solvent system (petroleum ether/ethyl acetate 10:1–>5:1–>2:1–>1:1–>1:2–>1:10–>100% ethyl acetate).



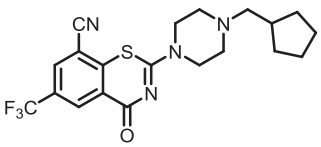
2-(4-(cyclopropylmethyl)piperazin-1-yl)-4-oxo-6-(trifluoromethyl)-4H-benzo[e][1,3]thiazine-8-carbonitrile (1a)

The title compound was prepared from sodium 4-(cyclopropylmethyl)piperazine-1-carbodithioate **6a** (0.08 g, 0.34 mmol, 1.0 equiv) and 2-chloro-3-cyano-5-(trifluoromethyl)benzamide **5** (0.10 g, 0.41 mmol, 1.2 equiv) using the general procedure for cyclization to afford compound **1a** (0.12 g, 86%) as a light yellow solid: mp 151–153 °C; $R_f = 0.20$ (1:2 Hexane-EtOAc); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.91 (d, $J = 2.0$ Hz, 1H), 8.08 (d, $J = 2.0$ Hz, 1H), 4.19 (br s, 2H), 3.83 (br s, 2H), 2.69 (br s, 4H), 2.34 (d, $J = 6.6$ Hz, 2H), 0.91–0.86 (m, 1H), 0.60–0.54 (m, 2H), 0.16-0.11 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 166.4, 159.5, 140.6, 132.9 (q, $J = 2.6$ Hz), 131.6 (q, $J = 2.6$ Hz), 130.9 (q, $J = 25.9$ Hz), 124.8, 122.5 (q, $^1J_{C\text{-}F} = 203.7$ Hz), 114.0, 111.1, 63.3, 52.5, 47.0–46.3 (m), 29.8, 8.2, and 4.1; HRMS (ESI+) m/z $[M + H]^+$ calcd for $C_{18}H_{18}F_3N_4OS$: 395.1148; found 395.1144 (error 1.0 ppm).



2-(4-(cyclobutylmethyl)piperazin-1-yl)-4-oxo-6-(trifluoromethyl)-4H-benzo[e][1,3]thiazine-8-carbonitrile (1b)

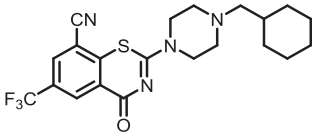
The title compound was prepared from sodium 4-(cyclobutylmethyl)piperazine-1-carbodithioate **6b** (0.09 g, 0.34 mmol, 1.0 equiv) and 2-chloro-3-cyano-5-(trifluoromethyl)benzamide **5** (0.10 g, 0.41 mmol, 1.2 equiv) using the general procedure for cyclization to afford compound **1b** (0.12 g, 85%) as light yellow solid: mp 162–165 °C; $R_f = 0.20$ (1:2 Hexane-EtOAc); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.91 (d, $J = 2.0$ Hz, 1H), 8.08 (d, $J = 2.0$ Hz, 1H), 4.13 (m, 2H), 3.77 (m, 2H), 2.55–2.46 (m, 7H), 2.10–2.04 (m, 2H), 1.98–1.82 (m, 2H), 1.75–1.67 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 166.2, 159.2, 140.5, 132.7 (q, $J = 3.6$ Hz), 131.4 (q, $J = 3.6$ Hz), 130.5 (q, $J = 34.7$ Hz), 124.6, 122.4 (q, $^1J_{C\text{-}F} = 271.4$ Hz), 113.9, 110.9, 64.5, 52.6, 47.0–46.4 (m), 33.4, 27.6, and 18.8; HRMS (ESI+) m/z $[M + H]^+$ calcd for $C_{19}H_{20}F_3N_4OS$: 409.1304; found 409.1302 (error 0.6 ppm).



2-(4-(cyclopentylmethyl)piperazin-1-yl)-4-oxo-6-(trifluoromethyl)-4H-benzo[e][1,3]thiazine-8-carbonitrile (1c)

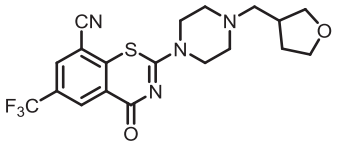
The title compound was prepared from sodium 4-(cyclopentylmethyl)piperazine-1-carbodithioate **6c** (0.09 g, 0.34 mmol, 1.0 equiv) and 2-chloro-3-cyano-5-(trifluoromethyl)benzamide **5** (0.10 g, 0.41 mmol, 1.2 equiv) using the general procedure for cyclization to afford compound **1c** (0.12 g, 86%) as light yellow solid: mp 196–198 °C; $R_f = 0.20$ (1:2 Hexane-EtOAc); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.91 (d, $J = 2.0$ Hz, 1H), 8.08 (d, $J = 2.0$ Hz, 1H), 4.15 (m, 2H), 3.78 (m, 2H), 2.58 (m, 4H), 2.33–2.30 (m, 2H), 2.13–2.03 (m, 1H), 1.77–1.75 (m, 2H), 1.61–1.55 (m, 4H), and 1.24–1.18 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 166.3, 159.3, 140.6, 132.7 (q, $J = 3.6$ Hz), 131.4 (q, $J = 3.6$ Hz), 130.6 (q, $J = 34.7$ Hz),

124.5, 122.4 (q, $^{1}J_{C\text{-}F} = 271.4$ Hz), 113.9, 110.9, 63.9, 52.7, 47.1–46.5 (m), 36.9, and 31.1, 25.1; HRMS (ESI+) m/z $[M + H]^+$ calcd for $C_{20}H_{22}F_3N_4OS$: 423.1461; found 423.1457 (error 0.9 ppm).



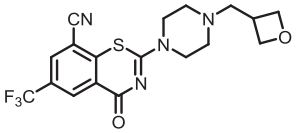
2-(4-(cyclohexylmethyl)piperazin-1-yl)-4-oxo-6-(trifluoromethyl)-4H-benzo[e][1,3]thiazine-8-carbonitrile (1d)

The title compound was prepared from sodium 4-(cyclohexylmethyl)piperazine-1-carbodithioate **6d** (0.10 g, 0.34 mmol, 1.0 equiv) and 2-chloro-3-cyano-5-(trifluoromethyl)benzamide **5** (0.10 g, 0.41 mmol, 1.2 equiv) using the general procedure for cyclization to afford compound **1d** (0.13 g, 89%) as light yellow solid: mp 218–221 °C; $R_f = 0.20$ (1:2 Hexane-EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.91 (d, $J =$ 2.0 Hz, 1H), 8.07 (d, $J = 2.0$ Hz, 1H), 4.14 (s, 2H), 3.77 (s, 2H), 2.53 (s, 4H), 2.18 (d, $J = 7.1$ Hz, 2H), 1.80–1.66 (m, 5H), 1.52–1.46 (m, 1H), 1.29–1.15 (m, 3H), 0.93–0.84 (m, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.5, 159.4, 140.7, 132.9 (q, $J = 3.7$ Hz), 131.6 (q, $J = 3.6$ Hz), 130.8 (q, $J =$ 34.9 Hz), 124.8, 122.5 (q, $^{1}J_{C\text{-}F} = 274.2$ Hz), 114.1, 111.1, 65.2, 53.1, 47.2–46.7 (m), 35.1, 31.8, and 26.8, 26.1; HRMS (ESI+) m/z $[M + H]^+$ calcd for $C_{21}H_{24}F_3N_4OS$: 437.1617; found 437.1613 (error 1.0 ppm).



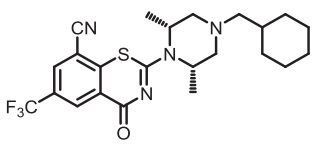
4-Oxo-2-(4-((tetrahydrofuran-3-yl)methyl)piperazin-1-yl)-6-(trifluoromethyl)-4H-benzo[e][1,3]thiazine-8-carbonitrile (1e)

The title compound was prepared from sodium 4-((tetrahydrofuran-3-yl)methyl)piperazine-1-carbodithioate **6e** (0.09 g, 0.34 mmol, 1.0 equiv) and 2-chloro-3-cyano-5-(trifluoromethyl)benzamide **5** (0.10 g, 0.41 mmol, 1.2 equiv) using the general procedure for cyclization to afford compound **1e** (0.11 g, 76%) as light yellow solid: mp 173–175 °C; $R_f = 0.20$ (1:2 Hexane-EtOAc); ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.92 (d, $J = 2.0$ Hz, 1H), 8.08 (d, $J = 2.0$ Hz, 1H), 4.16–4.11 (m, 2H), 3.91–3.72 (m, 5H), 3.59–3.54 (m, 1H), 2.62 (m, 4H), 2.50–2.43 (m, 2H), 2.10–1.99 (m, 1H), 1.68–1.58 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 166.4, 159.5, 140.6, 132.9 (q, $J = 2.7$ Hz), 131.6 (q, $J = 2.7$ Hz), 130.9 (q, $J = 26.0$ Hz), 124.8, 122.5 (q, $^{1}J_{C\text{-}F} = 203.5$ Hz), 114.0, 111.1, 72.1, 67.8, 61.5, 52.8, 47.0–46.6 (m), 36.7, 30.6, and 29.8; HRMS (ESI+) m/z $[M + H]^+$ calcd for $C_{19}H_{20}F_3N_4O_2S$: 425.1254; found 425.1249 (error 1.1 ppm).



2-(4-(oxetan-3-ylmethyl)piperazin-1-yl)-4-oxo-6-(trifluoromethyl)-4H-benzo[e][1,3]thiazine-8-carbonitrile (1f)

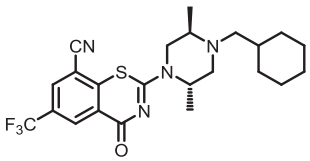
The title compound was prepared from sodium 4-(oxetan-3-ylmethyl)piperazine-1-carbodithioate **6f** (0.09 g, 0.34 mmol, 1.0 equiv) and 2-chloro-3-cyano-5-(trifluoromethyl)benzamide **5** (0.10 g, 0.41 mmol, 1.2 equiv) using the general procedure for cyclization to afford compound **1f** (0.10 g, 72%) as light yellow solid: mp 172–174 °C; $R_f = 0.20$ (1:2 Hexane-EtOAc); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.92 (d, $J = 2.0$ Hz, 1H), 8.08 (d, $J = 2.0$ Hz, 1H), 4.85–4.81 (m, 2H), 4.43–4.39 (m, 2H), 4.13 (m, 2H), 3.78 (m, 2H), 3.24 (m, 1H), 2.80–2.77 (m, 2H), 2.56 (m, 3H), and 1.57 (m, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 166.4, 159.5, 140.5, 132.9 (q, $J = 2.6$ Hz), 131.7 (q, $J = 2.7$ Hz), 130.9 (q, $J =$ 26.2 Hz), 124.7, 122.5 (q, $^{1}J_{C\text{-}F} = 203.7$ Hz), 114.0, 111.1, 61.5, 52.7, 46.5, 32.9, and 29.8; HRMS (ESI+) m/z $[M + H]^+$ calcd for $C_{18}H_{18}F_3N_4O_2S$: 411.1097; found 411.1094 (error 0.8 ppm).



2-((2*S*,6*R*)-4-(cyclohexylmethyl)-2,6-dimethylpiperazin-1-yl)-4-oxo-6-(trifluoromethyl)-4H-benzo[e][1,3]thiazine-8-carbonitrile (1g)

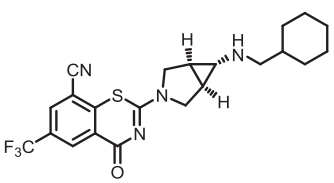
The title compound was prepared from sodium (2*S*,6*R*)-4-(cyclohexylmethyl)-2,6-dimethylpiperazine-1-carbodithioate **6i** (0.10 g, 0.34 mmol, 1.0 equiv) and

2-chloro-3-cyano-5-(trifluoromethyl)benzamide **5** (0.10 g, 0.41 mmol, 1.2 equiv) using the general procedure for cyclization to afford compound **1g** (0.13 g, 83%) as light yellow solid: mp 182–185 °C; $R_f = 0.20$ (1:2 Hexane-EtOAc); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.93 (d, $J = 2.0$ Hz, 1H), 8.07 (d, $J = 2.0$ Hz, 1H), 5.21 (m, 1H), 4.21 (m, 1H), 2.80–2.74 (m, 2H), 2.31–2.15 (m, 4H), 1.83–1.67 (m, 5H), 1.59–1.44 (m, 7H), 1.32–1.11 (m, 3H), 0.98–0.85 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 166.1, 159.1, 141.1, 132.7 (q, $J = 3.6$ Hz), 131.5 (q, $J = 3.6$ Hz), 130.5 (q, $J = 34.6$ Hz), 124.8, 122.5 (q, $J = 271.4$ Hz), 114.1, 110.8, 64.9, 58.3, 57.8, 51.4, 50.8, 35.4, 31.6, 26.8, 26.1, 20.5, and 20.1; HRMS (ESI+) m/z $[M + H]^+$ calcd for $C_{23}H_{28}F_3N_4OS$: 465.1930; found 465.1928 (error 0.5 ppm).



2-((2*S*,5*R*)-4-(cyclohexylmethyl)-2,5-dimethylpiperazin-1-yl)-4-oxo-6-(trifluoromethyl)-4H-benzo[e][1,3]thiazine-8-carbonitrile (1h)

The title compound was prepared from sodium (2*S*,5*R*)-4-(cyclohexylmethyl)-2,5-dimethylpiperazine-1-carbodithioate **6m** (0.10 g, 0.34 mmol, 1.0 equiv) and 2-chloro-3-cyano-5-(trifluoromethyl)benzamide **5** (0.10 g, 0.41 mmol, 1.2 equiv) using the general procedure for cyclization to afford compound **1h** (0.13 g, 80%) as light yellow solid: mp 180–182 °C; $R_f = 0.20$ (1:2 Hexane-EtOAc); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.92 (d, $J = 2.0$ Hz, 1H), 8.07 (d, $J = 2.0$ Hz, 1H), 5.35–3.11 (m, 3H), 3.11 (m, 1H), 2.84–2.80 (m, 1H), 2.44–2.40 (m, 1H), 2.21–2.16 (m, 2H), 1.87–1.83 (m, 1H), 1.75–1.71 (m, 4H), 1.57–1.36 (m, 4H), 1.21–1.14 (m, 3H), 0.99–0.82 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 166.4, 160.0, 140.7, 132.8 (q, $J = 3.6$ Hz), 131.5 (q, $J = 3.6$ Hz), 130.6 (q, $J = 34.6$ Hz), 124.8, 122.4 (q, $J = 271.4$ Hz), 114.0, 110.9, 61.1, 53.2, 50.8, 49.3, 47.5, 35.3, 31.6, 31.6, 26.8, 26.1, 26.0, 16.4, and 7.7; HRMS (ESI+) m/z $[M + H]^+$ calcd for $C_{23}H_{28}F_3N_4OS$: 465.1930; found 465.1930 (error 0.1 ppm).



2-((1*R*,5*S*,6*s*)-6-((cyclohexylmethyl)amino)-3-azabicyclo[3.1.0]hexan-3-yl)-4-oxo-6-(trifluoromethyl)-4H-benzo[e][1,3]thiazine-8-carbonitrile (1i)

The title compound was prepared from sodium (1*R*,5*S*,6*S*)-6-((cyclohexylmethyl)amino)-3-azabicyclo[3.1.0]hexane-3-carbodithioate **6p** (0.10 g, 0.34 mmol, 1.0 equiv) and 2-chloro-3-cyano-5-(trifluoromethyl)benzamide **5** (0.10 g, 0.41 mmol, 1.2 equiv) using the general procedure for cyclization to afford compound **1i** (0.11 g, 71%) as light yellow solid: mp 193–195 °C; $R_f = 0.20$ (1:2 Hexane-EtOAc); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.77 (d, $J = 2.1$ Hz, 1H), 8.63 (d, $J = 2.1$ Hz, 1H), 3.95–3.91 (m, 1H), 3.84–3.72 (m, 3H), 3.34 (m, 2H), 2.04 (m, 1H), 1.85–1.81 (m, 2H), 1.73–1.63 (m, 5H), 1.42 (m, 1H), 1.23–1.09 (m, 4H), 0.91–0.79 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.0, 166.5, 150.6, 143.4, 139.5, 138.1 (q, $J = 35.0$ Hz), 133.5, 124.2 (q, $J = 277.7$ Hz), 123.6, 120.0, 74.5, 64.3, 61.3, 58.7, 50.8, 45.5, 40.2, 38.5, 35.6, 34.9, 32.4, 31.5, and 28.2; HRMS (ESI+) m/z $[M + H]^+$ calcd for $C_{22}H_{24}F_3N_4OS$: 449.1617; found 449.1613 (error 1.0 ppm).

MIC determination

The test compound MICs against *Mtb* H37Rv were assessed by the microplate alamar blue assay protocol using macozinone as positive control. Compound stock solutions were prepared in DMSO at a concentration of 32 μg/mL, and the final test concentrations ranged from 3.2 to 0.002 μg/mL. Twofold dilutions of compounds were prepared in 7H9-ADC-TG in a volume of 100 μL in 96-well microplates (BD OptiluxTM, 96-well Microplates, black/clear flat bottom). The TB cultures (100 μL inoculua of 2×10^5 cfu/mL) were added to the media, yielding a final testing volume of 200 μL. The plates were incubated at 37 °C. On the seventh day of incubation, 12.5 μL of 20% Tween 80, and 20 μL of alamar blue (Invitrogen BioSource™) were added to the wells of test plate. After incubation at 37 °C for 16–24 h, fluorescence of the wells was measured at 530 nm (excitation) and 590 nm (emission). The MICs are defined as the lowest concentration effecting a reduction in fluorescence of ≥90% relative to the mean of replicate bacteria-only controls.

Microsomal stability

The selected compounds were dissolved in DMSO to make 10 mM of stock solution. Midazolam, Dextromethorphan, Diclofenac, Omeprazole, and Phenacetin are used as controls. The 5 μL of 10 mM stock solution of the target compounds was diluted to 100 μM of compound solution by adding 495 μL of DMSO. The 2.5 μL control/test compound was mixed with 197.5 μL liver microsome (mouse and

human) gently and preincubate at 37 °C for 5 min. The reaction was initiated by adding 50 µL buffer/NADPH working solution (5 mM) and mixed well. At each time point of 0 min and 30 min, 30 µL of reaction solution was taken out and quenched by adding 300 µL of internal standard (10 ng/mL). The mixture was centrifuged at 4000 rpm at 4 °C for 15 min. 100 µL of the supernatant was mixed with 100 µL distilled water and then analyzed by LC-MS/MS.

LC-MS/MS analysis was performed on a Kinetex C18 100 A column (3.0 mm × 30 mm, 2.6 µm) with a gradient mobile phase of acetonitrile/water containing 0.1% formic acid at 0.8 mL/min flow rate. LC condition: solvent A = H2O (0.1% formic acid), solvent B = acetonitrile (0.1% formic acid). Gradient elution: 0.00 min, 95.0% A; 0.50 min, 95% A; 0.80 min, 5% A; 1.50 min, 5% A; 1.51 min, 95% A; 2.00 min, 95% A. The injection volume was 5 µL.

In vivo pharmacokinetic profiles

Animal Care and Welfare Committee of Institute of Materia Medica, Chinese Academy of Medical Sciences approved all animal protocols (1 Xian nong tan Street, Xicheng District, Beijing, China; protocol #SYXK 2014-0023). All animal programs were in compliance with the Guide for the Care and Use of Laboratory Animals issued by Beijing Association on Laboratory Animal Care (BALAC). SPF male ICR mice weighing 22–23 g were divided into two groups with three mice each: one for oral administration and intravenous injection, separately. The tested compound was formulated at a concentration of 1.0 mg/mL for a dose of 10 mg/kg given orally (p.o.) and at 0.4 mg/mL for a dose of 2 mg/kg given i.v. The tested compound was formulated by 0.5% carboxymethyl cellulose and 0.5% Tween 80 for p.o. administration and with 20% HP-β-CD with 4 mol/L HCl for i.v. administration, respectively. Plasma samples were extracted with acetonitrile containing Terfenadine as an internal standard using a 20:1 extractant-to-plasma ratio. Analyte quantitation was performed by a LC/TSQ Quantum Access mass spectrometer (AB Sciex 5500). Chromatographic separation was performed on a Kinetex C18 100 A column (30 mm × 3 mm, 2.6 µm) with an isocratic mobile phase of acetonitrile/water (80:20, v/v) containing 0.1% formic acid at 0.8 mL/min flow rate. The pharmacokinetic parameters were calculated using WinNonlin software version 6.3 based on non-compartmental analysis (Pharsight Corporation, Mountain View, USA). The oral bioavailability was calculated as the ratio between the area under the curve (AUC) following intravenous administration corrected for dose ($F = (AUCp.o.\times dosei.v.)/(AUCi.v.\times dosep.o.)$).

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

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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