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**Identification of Novel Benzothiopyranone Compounds against *Mycobacterium Tuberculosis* through Scaffold Morphing from Benzothiazinones**

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## Abstract

In this study, three novel series of benzoxazinone, benzothiopyranone and benzopyranone derivatives were designed and synthesized through scaffold morphing from benzothiazinones targeting DprE1. All compounds were evaluated for their *in vitro* activities against *Mycobacterium tuberculosis* (*M. tuberculosis*) and cytotoxicity against Vero cell line. Among the three series, the benzothiopyranone series displayed excellent antimycobacterial activity and low cytotoxicity. Compound **6b** exhibited potent *in vitro* activity against both drug-susceptible and drug-resistant *M. tuberculosis* clinical strains with MICs < 0.016 µg/mL. In addition, compound **6b** demonstrated excellent ADME/T and PK properties and potent *in vivo* efficacy with bactericidal activity comparable to human equivalent dose of isoniazid (INH) in an acute mouse model of TB. Compound **6b** which exhibits good inhibitory activity against DprE1 is under evaluation as a potential drug candidate for the treatment of tuberculosis. The current study provided new insight into the structural and pharmacological requirements for DprE1 inhibitors as potent antitubercular agents.

## Keywords

Benzothiopyranones; drug-resistant *M. tuberculosis*; ADME/T; DprE1 inhibitors; antitubercular agents

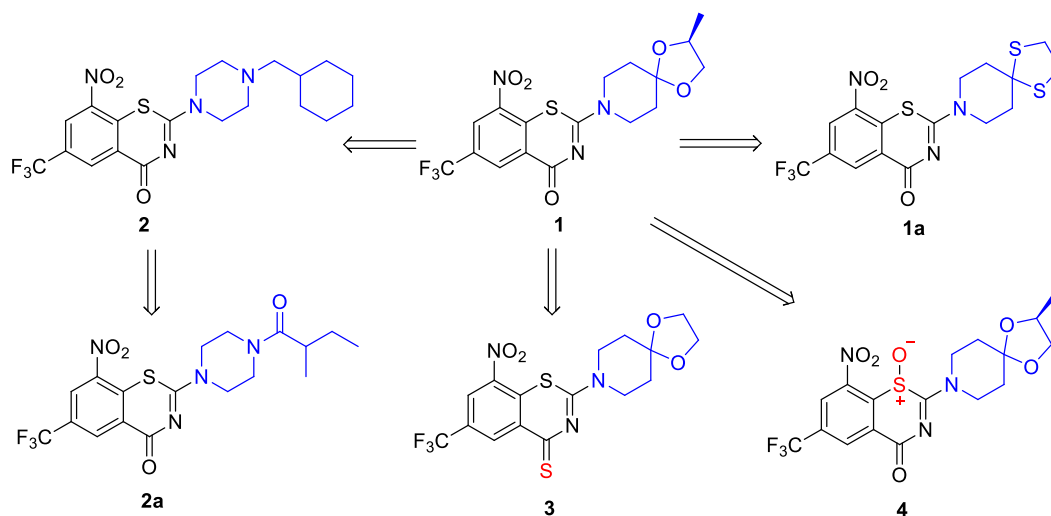
## 1. Introduction

Tuberculosis (TB) continues to be a major global health problem with millions of new cases every year. In a 2017 global TB report, TB was the ninth leading cause of death worldwide, and for the past five years (2012-2016) had been the leading cause of death from a single infectious agent, ranking above HIV/AIDS [1]. The prevalence of multidrug-resistant TB (MDR-TB) and extensively drug resistant TB (XDR-TB) has created a renewed demand for the discovery and development of novel TB drugs to improve the treatment outcomes.

Due to the high attrition rate in new drug development, the need for safer and more effective agents with novel mechanisms of action for the treatment of TB cannot be overemphasized [2].

The evaluation of novel chemical scaffolds possessing desirable pharmacological profiles against *M. tuberculosis* and the identification of novel drug targets on *M. tuberculosis* are all at the forefront of new TB drug discovery and development. Remodelling the existing antibacterial drug classes and structural modifications based on natural products with antibacterial activity are undoubtedly effective approaches to discover new antituberculosis agents [3-5]. Cell wall is a functional and protective interface between external and internal environment for every living cell. Targeting the cell wall biosynthesis has been a successful strategy in TB drug development. Indolcarboxamide is a novel series of antituberculosis agent identified recently. This series targets MmpL3, a transporter of trehalose monomycolate that is essential for mycobacterial cell wall biosynthesis [6]. Two other cell wall biosynthesis drug targets, decaprenylphosphoryl- $\beta$ -D-ribose 2'-epimerase (DprE1) and decaprenylphosphoryl-D-2-ketoerythropentose reductase (DprE2) were also identified recently which catalyze the epimerization of decaprenylphosphorylribose (DPR) to decaprenylphosphorylarabinose (DPA), a unique precursor for the synthesis of cell-wall arabinans [7]. DprE1, as a vulnerable tuberculosis drug target due to its cell wall localization and specific to mycobacteria and actinomycete, has become an attractive target for developing more effective and safer medicines for the treatment of drug-sensitive TB as well as MDR/XDR-TB [8-10]. The first DprE1 inhibitor is nitrobenzothiazinone **1** (BTZ043) (**Figure 1**) [11]. Phase I clinical trials for its next generation analogue **2** (PBTZ169) with improved efficacy and safety (**Figure 1**) have been completed [1, 12]. Compound **1** serves as a suicide substrate for the reduced form of DprE1 to irreversibly inactivate the enzyme by undergoing nitro reduction to yield a nitroso species that specifically attacks the thiol side chain of the active site cysteine residue, Cys387, to form a covalent adduct [13]. The

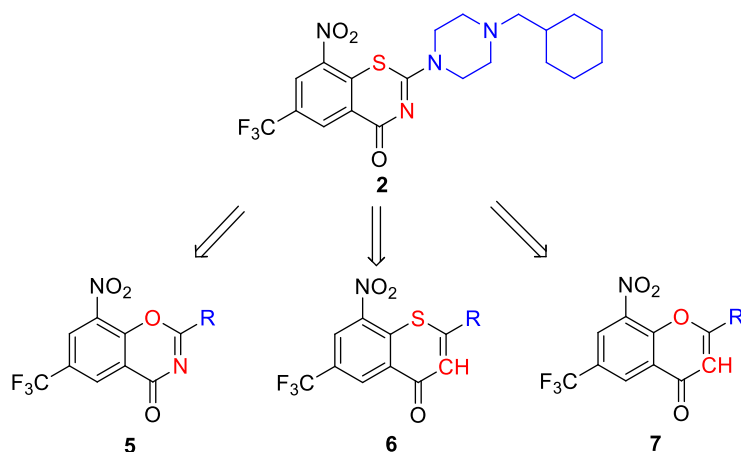
discovery of 1,3-benzothiazin-4-ones (BTZs), specifically **1** and its analogue **2**, prompted further research related to the identification of potential antituberculosis agents based on electron deficient nitroaromatic structures. A series of potential DprE1 covalent inhibitors with modification on the 2 position of BTZs represented as compounds **1a** and **2a** was reported (**Figure 1**) [14-17]. Interestingly, two compounds with relatively minor modifications to the BTZs core, benzothiazinethione (SKLB-TB1001, **3**) and sulfoxide of **1** (BTZ-SO, **4**), maintain potent antimycobacterial activities (**Figure 1**) [18, 19].



**Figure 1.** Nitrobenzothiazinone **1** and Its Representative Derivatives.

The crystal structures of the DprE1 with **1** and DprE1 with **2** complex as well as the structure-activity relationships (SARs) of this series indicated that the key constituents for potent activity are the sulfur atom and carbonyl group in the thiazinone ring, a strong electron withdrawing group (CF<sub>3</sub>, CN, NO<sub>2</sub>, etc.) in the 6 position, and more importantly a nitro group in the 8 position [11, 12]. However, we noticed that the nitrogen atom in the thiazinone ring has no direct interaction with the enzyme. Inspired by the above background information, we employed bioisosteric replacement strategy to replace the nitrogen atom in the 3 position with a carbon atom and replace the sulfur atom in the 1 position with an oxygen atom aimed to identify new DprE1 inhibitors with improved physicochemical properties and safety profiles.

Herein, we report three novel structural series, benzoxazinone **5**, benzothiopyranone **6** and benzopyranone **7**, which maintain a CF<sub>3</sub> group at the 6 position and a NO<sub>2</sub> group at the 8 position (**Figure 2**). After further evaluation, a benzothiopyranone compound **6b** as a DprE1 inhibitor was identified as a promising preclinical candidate for the treatment of drug-resistant tuberculosis.



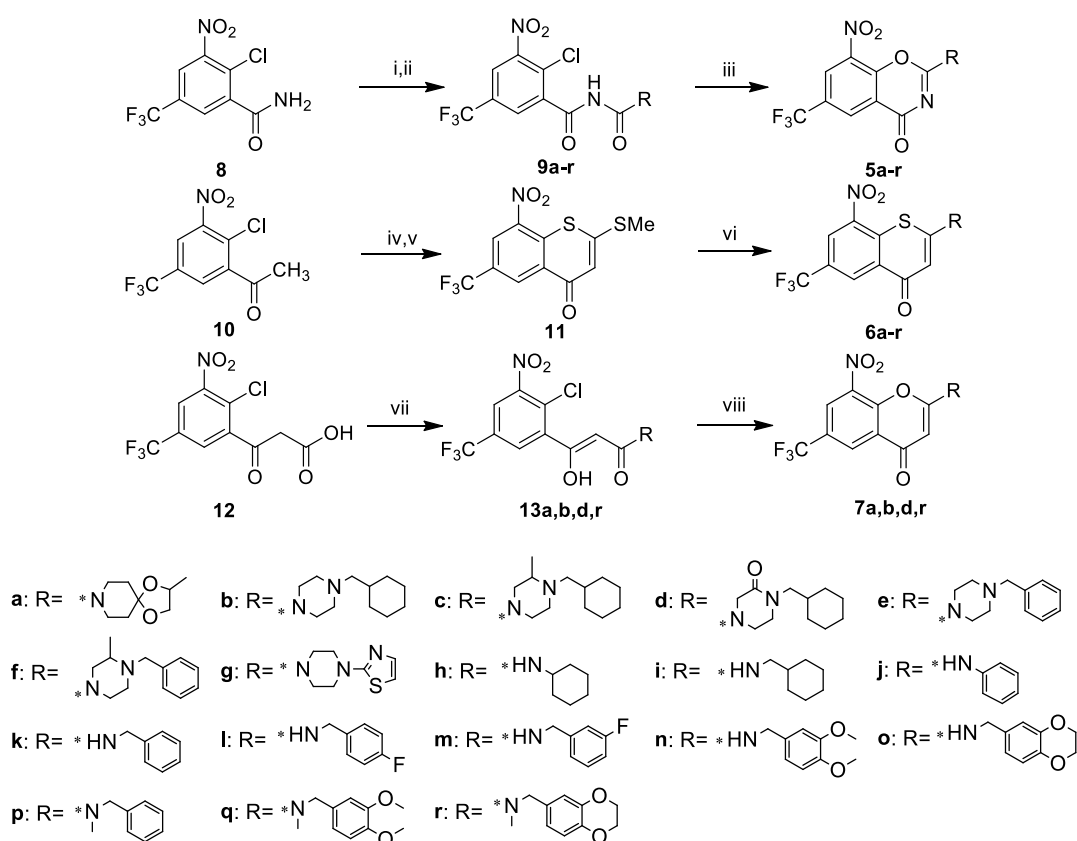
**Figure 2.** Structures of **2** and Three Target Compound Series.

## 2. Chemistry

The target compounds were synthesized following the procedure as outlined in **Scheme 1**. 2-Chloro-3-nitro-5-(trifluoromethyl)benzamide (**8**) [11] was treated with oxalyl chloride followed by an appropriate amine to afford intermediates **9a-r**. Compounds **9a-r** were heated in DMF in the presence of K<sub>2</sub>CO<sub>3</sub> to give benzoxazinones **5a-r** after purification by column chromatography [20]. Benzothiopyranones **6a-r** were prepared by a two-steps process. The key intermediate **11** was first synthesized by treating 1-(2-chloro-3-nitro-5-(trifluoromethyl)phenyl)ethan-1-one (**10**) [21, 22] with NaOH and carbon disulfide (CS<sub>2</sub>) followed by methyl iodide (MeI) in DMSO [23]. The desired compounds **6a-r** were obtained through nucleophilic substitution reaction of **11** with an appropriate amine. Due to the low reactivity of the leaving group methylthio, it was noted that the yield for the nucleophilic reaction between **11** and different amine could be improved

by extending the reaction time. For the synthesis of the benzopyranone series, the key intermediates **13a**, **13b**, **13d** and **13r** were prepared by mixing 3-(2-chloro-3-nitro-5-(trifluoromethyl) phenyl)-3-oxopropanoic acid (**12**) [24] with dicyclohexylcarbodiimide (DCC) followed by an appropriate amine at room temperature [25]. Then, the above intermediates were heated in DMF in the presence of  $K_2CO_3$  to give the target benzopyranone compounds **7a**, **7b**, **7d** and **7r** in good yields. The structures of all new target compounds were characterized by  $^1H$  NMR,  $^{13}C$  NMR and HRMS.

**Scheme 1.** Synthesis of the Target Compounds<sup>a</sup>



<sup>a</sup>Reagents and conditions: i) oxalyl chloride, 1,2-dichloroethane, reflux, 3 h; ii) the corresponding amine, acetonitrile, -25 °C, 0.5-2 h, two steps, 39-95%; iii)  $K_2CO_3$ , DMF, 110 °C, 0.3-1 h, 20-79%; iv)  $CS_2$ , NaOH, DMSO, 20 °C, 0.5 h; v) MeI, rt, 1 h, two steps, 60%; vi) the corresponding amine, isopropanol, 140 °C, 12-48 h, 27-75%; vii) the corresponding amine, DCC,  $CH_2Cl_2$ , rt, 6-12 h, 27-60%; viii)  $K_2CO_3$ , DMF, 110 °C, 1 h, 62-76%.

### 3. Results and discussion

### 3.1. Identification of benzothiopyranones as a promising lead series

All target compounds were evaluated for their antimycobacterial activities against *M. tuberculosis* H<sub>37</sub>Rv using the microplate alamar blue assay (MABA) [26]. The minimum inhibitory concentration (MIC) was defined as the lowest concentration effecting a reduction in fluorescence of  $\geq 90\%$  relative to the mean of replicate bacterium-only controls. All target compounds were further tested for their cytotoxicity against mammalian cell line (Vero cells) as measured by the concentration required for inhibiting 50% cell growth (IC<sub>50</sub>) as compared to no-treatment control. **Table 1** summarizes the biological data for 40 newly synthesized benzoxazinones, benzothiopyranones and benzopyranones. Isoniazid (INH), rifampicin (RFP), **1** and **2** were used as the reference compounds in these assays.

To explore the impact of bioisosteric replacement of the core structure, the C-2 side chains in the first batch compounds (**5a-b**, **6a-b** and **7a-b**) were kept the same as in BTZ038 (racemate of **1**) and **2** (2-spiroketal and 2-piperazino moiety, respectively). As shown in **Table 1**, three series displayed potent *in vitro* antimycobacterial activities. However, their IC<sub>50</sub>s against Vero cell line were substantially different. When R is a spiroketal group, the benzoxazinone **5a** and benzopyranone **7a** showed equivalently potent antimycobacterial activities as compared to the reference compounds **1** and **2** with MICs < 0.016  $\mu\text{g/mL}$ , while the benzothiopyranone **6a** displayed less activity with a MIC 0.03  $\mu\text{g/mL}$ . When R is a piperazino group, different level of antimycobacterial activities were observed in the following order: benzothiopyranone > benzoxazinone  $\approx$  benzopyranone (**6b** MIC < 0.016  $\mu\text{g/mL}$ , **5b** MIC 0.159  $\mu\text{g/mL}$  and **7b** MIC 0.116  $\mu\text{g/mL}$ ). It appeared that the benzoxazinones (**5a-b**) and benzopyranones (**7a-b**) showed a certain level of cytotoxicity regardless the C-2 substitution, but benzothiopyranones (**6a-b**) displayed lower cytotoxicity with IC<sub>50</sub>s > 64  $\mu\text{g/mL}$ . Considering that the IC<sub>50</sub> values of **1** and **2** are both > 64  $\mu\text{g/mL}$ , this result indicated the importance of the sulfur atom in position 1 not only on antimycobacterial



activity but also toxicity. Due to the cytotoxicity concern, benzopyranone scaffold was dropped from further consideration.

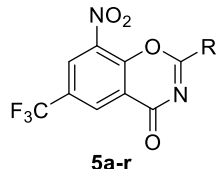
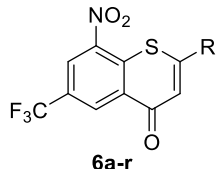
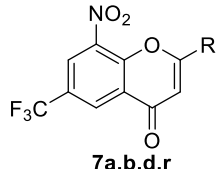
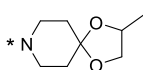
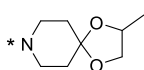
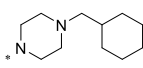
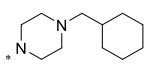
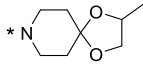
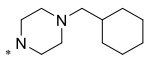
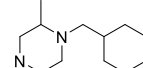
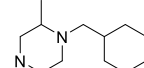
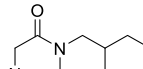
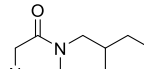
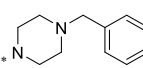
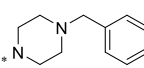
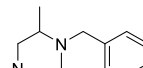
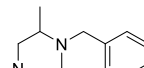
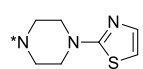
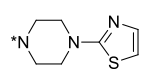
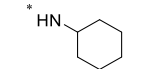
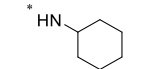
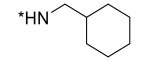
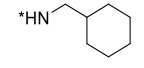
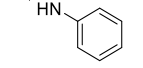
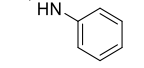
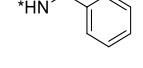
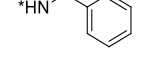
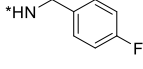
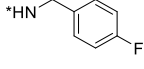
To further understand the structure-activity and structure-toxicity relationships (SARs and STRs), the R group in position 2 of the benzoxazinone and benzothiopyranone scaffolds were systematically investigated. The lipophilicity of the synthesized compounds was estimated based on their calculated  $ClogP$  values by employing ChemDraw prediction software (ChemDraw Professional 16.0, PerkinElmer Informatics, Inc.). A series of benzoxazinones (**5c-r**) and benzothiopyranones (**6c-r**) compounds was designed and synthesized. Generally, compounds in the benzothiopyranone series had slightly higher  $ClogP$  values as compared to the corresponding compounds in the benzoxazinone series ( $\Delta ClogP$  is about 1). As anticipated, most benzothiopyranones exhibited more potent antimycobacterial activities than the corresponding benzoxazinones. This observation is consistent with the general SAR knowledge for the benzothiazinone class – more lipophilicity ( $\log P$ ) correlates with better antimycobacterial activity [12]. Despite their potent antimycobacterial activities, all benzothiopyranone compounds exhibited low cytotoxicity with  $IC_{50}$ s  $> 64 \mu\text{g/mL}$ , whereas the benzoxazinone compounds displayed certain level of toxicity with  $IC_{50}$ s in the range from 3.84 to 37.85  $\mu\text{g/mL}$  with the exceptions of compounds **5l** and **5m**. To further confirm the correlation between the cytotoxicity and the oxygen atom in position 1, we decided to re-investigate the benzopyranone series by synthesizing compounds **7d** and **7r**. Although both compounds **7d** and **7r** exhibited potent antimycobacterial activities, they all appeared to be cytotoxic with  $IC_{50}$  values less than 30  $\mu\text{g/mL}$ . Our studies indicated that the benzothiopyranone series appears to have the desired antimycobacterial activities and cytotoxicity profiles.

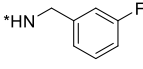
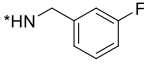
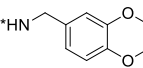
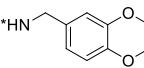
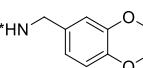
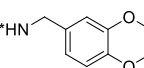
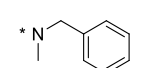
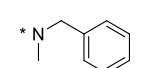
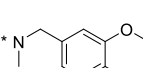
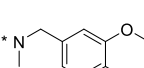
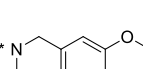
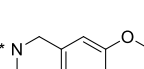
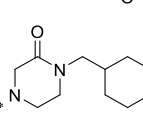
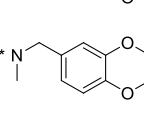
Benzothiopyranone compounds substituted with a tertiary amino R (such as **6b-g** containing a heterocyclic amino and compounds **6p-r** containing an acyclic amino group)

displayed potent antimycobacterial activities (MICs < 0.016 µg/mL) with the exceptions of compounds **6e** (MIC 0.03 µg/mL) and **6g** (MIC 0.054 µg/mL). However, the corresponding benzoxazinone compounds (**5b-g** and **5p-r**) exhibited a wide range of MICs from 6.28 µg/mL to less than 0.016 µg/mL. It appears that the tertiary amino group in position 2 is better tolerated in the benzothiopyranone series than in the benzoxazinone series. Replacement of the hydrogen atom of NH in compound **6k** with a methyl group resulted in a significant improvement of activity (**6p**, MIC < 0.016 µg/mL). In both the benzothiopyranone and benzoxazinone series, compounds with a tertiary amino R group demonstrated better activities than those with the corresponding secondary amino R group as exemplified by **6q** (MIC < 0.016 µg/mL, ClogP 4.53) vs. **6n** (MIC 0.902 µg/mL, ClogP 3.88), **6r** (MIC < 0.016 µg/mL, ClogP 4.80) vs. **6o** (MIC 0.03 µg/mL, ClogP 4.15), **5q** (MIC 0.057 µg/mL, ClogP 3.64) vs. **5n** (MIC 0.226 µg/mL, ClogP 2.98) and **5r** (MIC < 0.016 µg/mL, ClogP 3.91) vs. **5o** (MIC 0.03 µg/mL, ClogP 3.25). The volume of the substituent may also be an important factor for antimycobacterial activity, as the lipophilicity and the size of the substituent increase at the same time. *M. tuberculosis* possesses a thick, lipid rich cell wall, which becomes a permeable barrier for antimicrobial agents. It has been a challenge to balance the lipophilicity and the antimycobacterial activity in the field of new TB drug development. The goal of this study is to identify a new generation of BTZs derivatives with the right balance between its lipophilicity (and therefore potent antimycobacterial activity) and its pharmacokinetic profile (and therefore better *in vivo* efficacy). The structure of the R group in position 2 has a significant influence on the physicochemical properties of the molecule as evidenced by the improved druggability profile of compound **2** as compared to **1** [12]. However, the tertiary amino R group in position 2 in the benzothiopyranone series seems to have a minimum influence on the antimycobacterial activities of the investigated compounds. It appears that there are more opportunities to identify better compounds by optimizing the

side chain R in position 2 of the benzothiopyranone series to deliver a potential drug candidate for the treatment of TB.

**Table 1.** MIC, IC<sub>50</sub> and ClogP Values of the Target Compounds

 5a-r					 6a-r					 7a,b,d,r				
Comps	R	ClogP <sub>a</sub>	MIC <sup>b</sup> (μg/mL)	IC <sub>50</sub> (Vero) (μg/mL)	Comps	R	ClogP <sub>a</sub>	MIC <sup>b</sup> (μg/mL)	IC <sub>50</sub> (Vero) (μg/mL)					
5a		1.94	<0.016	9.21	6a		2.84	0.03	>64					
5b		4.60	0.159	52.14	6b		5.49	<0.016	>64					
7a		2.33	<0.016	18.87	7b		4.98	0.116	10.61					
5c		5.12	<0.016	10.87	6c		6.01	<0.016	>64					
5d		4.29	6.28	25.41	6d		5.18	<0.016	>64					
5e		3.79	3.93	33.50	6e		4.68	0.03	>64					
5f		4.31	<0.016	15.59	6f		5.20	<0.016	>64					
5g		2.43	4.00	ND <sup>c</sup>	6g		3.33	0.054	>64					
5h		3.91	0.118	5.39	6h		4.81	1.527	>64					
5i		4.53	0.05	29.73	6i		5.43	0.402	>64					
5j		3.71	30.65	37.85	6j		4.61	>32	>64					
5k		3.33	30.54	25.36	6k		4.22	>32	>64					
5l		3.47	7.22	>64	6l		4.37	0.206	>64					

<b>5m</b>		3.47	>32	>64	<b>6m</b>		4.37	0.118	>64
<b>5n</b>		2.98	0.226	29.22	<b>6n</b>		3.88	0.902	>64
<b>5o</b>		3.25	0.03	25.27	<b>6o</b>		4.15	0.03	>64
<b>5p</b>		3.98	0.031	3.84	<b>6p</b>		4.88	<0.016	>64
<b>5q</b>		3.64	0.057	9.46	<b>6q</b>		4.53	<0.016	>64
<b>5r</b>		3.91	<0.016	9.27	<b>6r</b>		4.80	<0.016	>64
<b>7d</b>		4.67	0.091	9.73	<b>7r</b>		4.29	<0.016	29.39
<b>1</b>			<0.016	>64	<b>2</b>			<0.016	>64
INH			0.035		RFP			0.096	

Calculated with ChemDraw Professional 16.0. <sup>a</sup>Minimum inhibitory concentration against *M. tuberculosis* H<sub>37</sub>Rv. <sup>b</sup>ND, not determined.

### 3.2. Evaluation of Compound 6b as a potential drug candidate for the treatment of TB

#### 3.2.1. Metabolic Stability and Antimycobacterial Activity against Clinical Isolates of *M. tuberculosis* of Selected Benzothiazinones

One significant challenge associated with the benzothiazinone series is its poor pharmacokinetic (PK) profile. Microsomal instability was identified as a possible liability of BTZs such as **2** showed medium clearance values when incubation with human or mouse microsomes [12]. To identify the metabolic liabilities of the novel benzothiazinone series, eight compounds (**6a-f**, **6p** and **6r**) with a variety of side chain R and potent antimycobacterial activities together with the reference compound **2** were evaluated for their stability in mouse liver microsome (MLM) and human liver microsome (HLM) (**Table 2**). As shown in **Table 2**, compounds with relative rigid cyclic amino side chain R (**6a-f**) exhibited medium to good stability in MLM or HLM with the exception of **6d** that was unstable in MLM. However, compounds **6p** and **6r** showed low microsomal stability possibly due to the

flexible acyclic amino side chain R. The side chain R could play a major role on influencing the PK profile. Further medicinal chemistry effort by increasing the size of the R group and therefore sterical hinderance could potentially further improve microsomal stability. Encouragingly, compounds **6b**, **6c**, **6e** and **6f** showed similar MLM stability as that of **2**, but better stable than **2** in HLM.

**Table 2.** Mouse and Human Liver Microsome Stability of Selected Benzothioipyranone Compounds

Compds	substrate remaining (%) <sup>a</sup>	
	MLM <sup>b</sup>	HLM <sup>c</sup>
<b>6a</b>	47.4	82.8
<b>6b</b>	61.5	66.7
<b>6c</b>	59.9	62.7
<b>6d</b>	7.38	86.6
<b>6e</b>	60.5	87.6
<b>6f</b>	54.5	76.7
<b>6p</b>	13.0	11.6
<b>6r</b>	6.46	44.9
<b>2</b>	63.9	58.7

<sup>a</sup>Substrate concentrations were determined in incubations with NADPH after 30 min and normalized to concentrations at time zero. <sup>b</sup>Mouse liver microsome. <sup>c</sup>Human liver microsome.

Based on their good liver microsome stability, compounds **6b** and **6e** were further tested against two XDR-TB clinical isolates (**Table 3**). Cytotoxicity against mammalian cells was also evaluated against HepG2 cells. Selectivity index (SI) was subsequently calculated (**Table 3**). Inspiringly, the two new compounds together with the reference control **2** retained potent activities against XDR-TB strains and also displayed very low cytotoxicity with high IC<sub>50</sub> values of > 64 µg/mL against HepG2 cells as compared to the reference control **2**. Of particular interest, compound **6b** exhibited potent activity against XDR-TB strains with MIC less than 0.002 µg/mL and excellent SI.

**Table 3.** Activity of **6b** and **6e** against the Selected Clinical Isolates of *M. tuberculosis* and HepG2 Cells

Compds	MIC ( $\mu\text{g/mL}$ )			IC <sub>50</sub> ( $\mu\text{g/mL}$ )	SI <sup>c</sup>
	H <sub>37</sub> Rv	12611 <sup>a</sup>	14231 <sup>b</sup>		
<b>6b</b>	<0.016	<0.002	<0.002	>64	>4000
<b>6e</b>	0.03	0.011	0.023	>64	>2133
<b>2</b>	<0.016	<0.002	<0.002	18.98	>1186
INH	0.035	19.469	>40		
RFP	0.096	>40	>40		

<sup>a</sup>Resistance to isoniazid (INH), rifampicin (RFP), streptomycin (SM), ethambutol (EMB), rifapentine (RFT), rifabutin (RFB) and paza-aminosalicylate (PAS). <sup>b</sup>Resistance to INH, EMB, RFP, RFB, RFT, amikacin (AMK) and capreomycin (CPM). <sup>c</sup>SI = selectivity index, IC<sub>50</sub>/MIC.

### 3.2.2. Preliminary ADME/T Studies on Compound **6b**

Based on the promising biological results described above, selected ADME/T studies were conducted on compound **6b** to investigate its drug-like properties (**Table 4**). As presented in **Table 4**, compound **6b** did not inhibit cytochrome P450 enzymes (CYP 1A2, CYP 2C9, CYP 2C19, CYP 2D6 and CYP 3A4) with IC<sub>50</sub>s > 50  $\mu\text{M}$ , suggesting a low potential for drug-drug interactions. In order to further evaluate its metabolic stability, compound **6b** along with the reference compound **2** were investigated for their hepatocyte stability in mouse and human species. To our delight, compound **6b** exhibited superior stability both in mouse and human hepatocyte with much longer  $t_{1/2}$  and lower intrinsic clearance (Cl<sub>int</sub>) compared to the reference compound **2**. Low inhibition of hERG (IC<sub>50</sub> > 30  $\mu\text{M}$ ) suggests low risk for blocking the cardiac potassium channel and causing QT prolongation. A preliminary *in vivo* tolerability study was carried out in mice with a single dose at 2 g/kg. All animals survived after oral administration followed by a 7-day observation. Thus far, all the results supported compound **6b** to be worth of further investigation.

**Table 4.** ADME/T Data for Compound **6b**

Assay	<b>6b</b>
CYP inhibition IC <sub>50</sub> (μM)	>50 (1A2, 2C9, 2C19, 2D6, 3A4)
Hepatocyte Stability T <sub>1/2</sub> (min);	462 (mouse), 224 (human);
Clint (μL/min/million cell) <sup>a</sup>	1.5 (mouse), 3.1 (human)
hERG IC <sub>50</sub> (μM)	>30
Acute Toxicity Study <sup>b</sup>	10/10 <sup>c</sup> (LD <sub>50</sub> >2 g/kg)

<sup>a</sup>Hepatocyte stability for reference compound **2**: T<sub>1/2</sub> (min) = 165 (mouse), 47.8 (human); Clint (μL/min/million cell) = 4.2 (mouse), 14.5 (human). <sup>b</sup>Single oral dose in mice. <sup>c</sup>No. of animals that survived/total no. of animals after 7 days.

### 3.2.3. Pharmacokinetic Studies on Compound **6b**

Pharmacokinetic studies on compound **6b** were performed in male Balb/c mice, following single oral and intravenous dose administration (**Table 5**). As shown in **Table 5**, except relatively low bioavailability (13.1%), compound **6b** displayed an excellent PK profile as reflected by high plasma exposure (AUC<sub>0-∞</sub> = 1695 ng·h/mL), and long elimination half-life (t<sub>1/2</sub> = 7.25 h) after oral administration. It is worth to indicate that the reference compound **2** exhibited short half-life with t<sub>1/2</sub> = 1.85 h when dosed at 25mg/kg in Balb/c mice as reported [12]. These results indicated that compound **6b** had promising PK properties to move into *in vivo* efficacy studies in animal models.

**Table 5.** Pharmacokinetic Parameters of Compound **6b**

Compd	route	dose (mg/kg)	C <sub>max</sub> (ng/mL)	t <sub>max</sub> (h)	t <sub>1/2</sub> <sup>a</sup> (h)	AUC <sub>0-24h</sub> (ng·h/mL)	AUC <sub>0,∞</sub> <sup>b</sup> (ng·h/mL)	MRT <sub>0,∞</sub> <sup>c</sup> (h)	clearance (mL/min/kg)	F <sup>d</sup> (%)
<b>6b</b>	po	25	588	0.5	7.25	1581	1695	6.59		13.1
<b>6b</b>	iv	10			6.54	4954	5184	4.91	32.3	

<sup>a</sup>Plasma elimination half-life. <sup>b</sup>Plasma exposure. <sup>c</sup>Mean residence time. <sup>d</sup>Bioavailability.

### 3.2.4. *In vivo* Efficacy Study on Compound **6b** in Mouse Model of TB

Lastly, the preliminary *in vivo* efficacy evaluation of compound **6b** was conducted in a murine model of acute infection with *M. tuberculosis* H<sub>37</sub>Rv. Compound **6b** was orally

administered at 100 mg/kg, whereas the positive control drug isoniazid (INH) was given at 25 mg/kg. The same formulation 0.5% carboxymethylcellulose (CMC) in water was used for all tested compounds. As exhibited in **Table 6**, compound **6b** appeared highly active after 3 weeks treatment, which resulted in a significant reduction of *M. tuberculosis* colony-forming unit (CFU) in lungs by 5.4 logs as compared to the untreated control group. Encouragingly, compound **6b** at 100 mg/kg demonstrated similar bactericidal activity compared to INH (25 mg/kg). Further evaluation of this promising compound is currently under way to fully assess its potential for further development for the treatment of MDR-TB and XDR-TB.

**Table 6.** Efficacy of **6b** against *M. tuberculosis* H<sub>37</sub>Rv infection after 3 weeks of treatment in Balb/c Mice

Comps	Dose (mg/kg)	Log <sub>10</sub> CFU/lung
Untreated	-	7.102±0.152
INH	25	1.929±0.213
<b>6b</b>	100	1.678±0.174

### 3.2.5. DprE1 Inhibition Assay

In order to confirm the inhibition of *M. tuberculosis* by the benzothiofuranone derivatives via DprE1 inhibition, we measured the inhibitory activities of the representative compounds **6a**, **6b**, **6e** and positive controls **1**, **2** against DprE1. The results demonstrate that the selected benzothiofuranone compounds with potent antimycobacterial activities and diversified side chains displayed potent DprE1 inhibition with IC<sub>50</sub> values from 0.63 to 4.53 μM, which exhibited the similar mechanism against *M. tuberculosis* as compared to the benzothiazinone controls **1** and **2** (**Table 7**).



**Table 7.** Inhibition on DprE1 of compounds **6a**, **6b**, **6e** and positive controls **1**, **2**

Compds	IC <sub>50</sub> DprE1 (μM) <sup>a</sup>
<b>6a</b>	0.63
<b>6b</b>	4.53
<b>6e</b>	1.33
<b>1</b>	1.03
<b>2</b>	0.20

<sup>a</sup>All *in vitro* assays were performed using *M. tuberculosis* DprE1.

#### 4. Conclusion

Antimycobacterial drug target DprE1 and its inhibitor benzothiazinone series represent the new trend in TB drug discovery. Benzothiopyranone compound **6b**, obtained by scaffold morphing from benzothiazinones, has been identified as a promising TB drug candidate with potent activity, low toxicity and acceptable PK properties. Extensive SAR studies provide insight into the structural requirements for potent antimycobacterial activity and low cytotoxicity. Benzothiopyranone compounds with a tertiary amino side chain R in the 2 position demonstrated potent activities against *M. tuberculosis* with low mammalian cell cytotoxicity. The oxygen atom in the 1 position in the benzoxazinone and benzopyranone series appeared to be associated with cytotoxicity. Further *in vitro* and *in vivo* studies indicated that the benzothiopyranone series is a promising series for further development. Particularly, compound **6b** exhibited potent *in vitro* activity against *M. tuberculosis* H<sub>37</sub>Rv as well as two XDR-TB clinical isolates. This compound demonstrated excellent efficacy *in vivo* in an acute mouse model of TB by reducing bacterial load up to 5.4 log units after 3 weeks treatment, which is equivalent to that of the first-line TB drug INH. Especially, the preliminary ADME/T data displayed that compound **6b** owned potential druggability profiles. Additionally, in a PK study in mice, compound **6b** exhibited a long half-life ( $t_{1/2} = 7.25$  h) after oral administration, therefore it has the potential to be given at a lower dose and less

frequency to improve patient compliance. With the encouraging data, compound **6b** which exhibits good inhibitory activity against DprE1 has been selected as a potential drug development candidate for the treatment of TB. Further evaluation including combination study and deep druggability of compound **6b** is under way to fully assess its potential as a new agent to treat DR-TB.

## 5. Experimental

### 5.1. Chemistry

#### 5.1.1. General experimental information

All the solvents and chemicals were obtained from commercial sources and used without further purification. TLC was performed on silica gel plates (GF254) with visualization of components by UV light (254 nm) or exposure to I<sub>2</sub>. Column chromatography was carried out on silica gel (300-400 mesh). The structural identities of the prepared compounds were confirmed by <sup>1</sup>H NMR and <sup>13</sup>C NMR spectroscopy and mass spectrometry. <sup>1</sup>H NMR spectra were obtained on Varian Mercury-400 at 400 MHz. <sup>13</sup>C NMR spectra were obtained on Varian Mercury-400 at 100 MHz. Chemical shifts ( $\delta$ ) values were referenced to the residual solvent peak and reported in ppm and all coupling constant (*J*) values were given in Hz. CDCl<sub>3</sub> or DMSO-*d*<sub>6</sub> were used as the standard NMR solvents. The following multiplicity abbreviations are used: (s) singlet, (d) doublet, (t) triplet, (q) quartet, (m) multiplet, and (brs) broad. The chemical shifts of isomer of <sup>13</sup>C NMR were given in parenthesis. ESI-HRMS data were measured on Thermo Exactive Orbitrap plus spectrometer. Melting points were determined on Yanaco MP-J3 microscope melting point apparatus.

5.1.2. General procedure for the synthesis of target compounds **5a-r**. To a magnetically stirred solution of intermediate **9** (1 mmol) in dry DMF (5 mL) was added potassium carbonate (138 mg, 1 mmol). The contents were heated at 110 °C for 0.3-1 h under an atmosphere of argon. After filtration and concentration under reduced pressure, the residue

was purified by flash column chromatography (EtOAc: PE = 1:4) to afford the target compounds **5a-r**.

*5.1.2.1. 2-(2-Methyl-1,4-dioxo-8-azaspiro[4.5]decan-8-yl)-8-nitro-6-(trifluoromethyl)-4H-benzo[e][1,3]oxazin-4-one (5a)*. Yellow solid; yield 50.3%. mp 210-212 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 8.72 (s, 1H), 8.60 (s, 1H), 4.32-4.28 (m, 1H), 4.14-4.11 (m, 1H), 4.10-3.98 (m, 4H), 3.51 (t, *J* = 8.0 Hz, 1H), 1.91-1.83 (m, 4H), 1.32 (d, *J* = 6.4 Hz, 3H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ: 163.2, 155.2, 148.7, 136.3, 131.1, 127.5 (q, <sup>2</sup>*J*<sub>F,C</sub> = 35 Hz), 127.0, 122.2 (q, <sup>1</sup>*J*<sub>F,C</sub> = 271 Hz), 120.3, 106.1, 72.6 (72.5), 70.9, 43.8 (43.7), 43.0, 36.4 (35.9), 35.2 (34.7), 18.4. HRMS (ESI): *m/z* [M+H]<sup>+</sup> calcd for C<sub>17</sub>H<sub>17</sub>F<sub>3</sub>N<sub>3</sub>O<sub>6</sub>, 416.1064; found, 416.1062.

*5.1.2.2. 2-(4-(Cyclohexylmethyl)piperazin-1-yl)-8-nitro-6-(trifluoromethyl)-4H-benzo[e][1,3]oxazin-4-one (5b)*. Yellow solid; yield 51.2%. mp 194-195 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 8.72 (s, 1H), 8.59 (s, 1H), 4.00 (brs, 4H), 2.61 (brs, 4H), 2.26 (brs, 2H), 1.82-1.68 (m, 5H), 1.27-1.16 (m, 4H), 0.93-0.90 (m, 2H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ: 163.2, 155.2, 148.6, 136.3, 131.1, 127.2 (q, <sup>2</sup>*J*<sub>F,C</sub> = 35 Hz), 127.1, 122.2 (q, <sup>1</sup>*J*<sub>F,C</sub> = 272 Hz), 120.3, 65.2, 53.1, 52.6, 45.4, 44.9, 35.0, 31.7, 26.7, 26.1. HRMS (ESI): *m/z* [M+H]<sup>+</sup> calcd for C<sub>20</sub>H<sub>24</sub>F<sub>3</sub>N<sub>4</sub>O<sub>4</sub>, 441.1744; found, 441.1733.

*5.1.2.3. 2-(4-(Cyclohexylmethyl)-3-methylpiperazin-1-yl)-8-nitro-6-(trifluoromethyl)-4H-benzo[e][1,3]oxazin-4-one (5c)*. Yellow solid; yield 48.4%. mp 142-144 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 8.72 (s, 1H), 8.59 (s, 1H), 4.28-4.04 (m, 2H), 3.69-3.57 (m, 1H), 3.47-3.22 (m, 1H), 3.01-2.89 (m, 1H), 2.62-2.32 (m, 3H), 2.09-1.99 (m, 1H), 1.89-1.85 (m, 1H), 1.73-1.71 (m, 4H), 1.45 (brs, 1H), 1.27-1.15 (m, 3H), 1.11-1.07 (m, 3H), 0.90-0.86 (m, 2H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ: 163.2, 155.2, 148.7, 136.2, 131.1, 127.1 (q, <sup>2</sup>*J*<sub>F,C</sub> = 35 Hz), 126.3, 122.2 (q, <sup>1</sup>*J*<sub>F,C</sub> = 272 Hz), 120.3, 60.3 (60.0), 55.0, 51.2 (50.9), 50.4 (49.3), 45.7 (45.1), 35.6, 32.0 (31.7), 26.8, 26.2 (26.0), 15.0 (14.3). HRMS (ESI): *m/z* [M+H]<sup>+</sup> calcd for C<sub>21</sub>H<sub>26</sub>F<sub>3</sub>N<sub>4</sub>O<sub>4</sub>, 455.1901; found, 455.1892.

5.1.2.4. 2-(4-(Cyclohexylmethyl)-3-oxopiperazin-1-yl)-8-nitro-6-(trifluoromethyl)-4H-benzo[e][1,3]oxazin-4-one (**5d**). Yellow solid; yield 70.4%. mp 236-238 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 8.74-8.72 (m, 1H), 8.63-8.61 (m, 1H), 4.53 (s, 1H), 4.50 (s, 1H), 4.16-4.12 (m, 2H), 3.59-3.57 (m, 1H), 3.53-3.51 (m, 1H), 3.34-3.31 (m, 2H), 1.73-1.65 (m, 5H), 1.26-1.20 (m, 4H), 1.01-0.99 (m, 2H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ: 163.3 (162.9), 162.4, 155.1 (154.6), 148.4 (148.3), 136.5 (136.3), 131.4 (131.0), 128.1 (q, <sup>2</sup>J<sub>F,C</sub> = 35 Hz), 127.3, 121.0 (q, <sup>1</sup>J<sub>F,C</sub> = 272 Hz), 120.3 (120.2), 53.6 (53.5), 48.1 (47.4), 46.3 (45.8), 42.4 (42.1), 36.0 (35.9), 30.8 (30.7), 26.2, 25.7. HRMS (ESI): m/z [M+H]<sup>+</sup> calcd for C<sub>20</sub>H<sub>22</sub>F<sub>3</sub>N<sub>4</sub>O<sub>5</sub>, 455.1537; found, 455.1519.

5.1.2.5. 2-(4-Benzylpiperazin-1-yl)-8-nitro-6-(trifluoromethyl)-4H-benzo[e][1,3]oxazin-4-one (**5e**). Yellow solid; yield 73.7%. mp 193-195 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 8.71 (s, 1H), 8.58 (s, 1H), 7.36-7.29 (m, 5H), 3.96-3.94 (m, 4H), 3.59 (s, 2H), 2.62-2.60 (m, 4H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ: 163.1, 155.3, 148.6, 137.1, 136.2, 131.1, 129.1, 128.5, 127.6, 127.5 (q, <sup>2</sup>J<sub>F,C</sub> = 35 Hz), 127.1, 122.2 (q, <sup>1</sup>J<sub>F,C</sub> = 271 Hz), 120.3, 62.6, 52.4 (52.0), 45.4 (44.9). HRMS (ESI): m/z [M+H]<sup>+</sup> calcd for C<sub>20</sub>H<sub>18</sub>F<sub>3</sub>N<sub>4</sub>O<sub>4</sub>, 435.1275; found, 435.1256. IR (KBr): ν 3029, 2931, 2809, 1686, 1645, 1542, 1306, 734 cm<sup>-1</sup>.

5.1.2.6. 2-(4-Benzyl-3-methylpiperazin-1-yl)-8-nitro-6-(trifluoromethyl)-4H-benzo[e][1,3]oxazin-4-one (**5f**). Off-white solid; yield 24.8%. mp 152-154 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 8.70 (s, 1H), 8.58 (d, J = 7.6 Hz, 1H), 7.33-7.29 (m, 5H), 4.39-3.98 (m, 3H), 3.63-3.24 (m, 3H), 2.90-2.64 (m, 2H), 2.31-2.29 (m, 1H), 1.25 (brs, 3H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ: 163.2, 155.2, 148.6, 137.9, 136.2, 131.1 (130.9), 129.0 (128.9), 128.5, 127.6 (q, <sup>2</sup>J<sub>F,C</sub> = 35 Hz), 127.4, 127.1, 122.2 (q, <sup>1</sup>J<sub>F,C</sub> = 271 Hz), 120.3, 57.8 (57.6), 54.8, 51.1 (50.9), 49.8 (48.7), 45.6 (45.0), 15.3 (14.5). HRMS (ESI): m/z [M+H]<sup>+</sup> calcd for C<sub>21</sub>H<sub>20</sub>F<sub>3</sub>N<sub>4</sub>O<sub>4</sub>, 449.1431; found, 449.1422.

5.1.2.7. 8-Nitro-2-(4-(thiazol-2-yl)piperazin-1-yl)-6-(trifluoromethyl)-4H-benzo[e][1,3]oxazin-4-one (**5g**). Yellow solid; yield 32.2%. mp 144-145 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 8.73 (s, 1H), 8.62 (s, 1H), 7.24 (d, *J* = 3.6 Hz, 1H), 6.67 (d, *J* = 3.6 Hz, 1H), 4.12-4.07 (m, 4H), 3.73-3.66 (m, 4H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ: 171.2, 162.9, 155.5, 148.5, 139.7, 136.3, 131.2, 127.9 (q, <sup>2</sup>*J*<sub>F,C</sub> = 35 Hz), 127.3, 122.2 (q, <sup>1</sup>*J*<sub>F,C</sub> = 272 Hz), 120.3, 108.9, 48.2 (47.9), 44.4 (44.0). HRMS (ESI): *m/z* [M+H]<sup>+</sup> calcd for C<sub>16</sub>H<sub>13</sub>F<sub>3</sub>N<sub>5</sub>O<sub>4</sub>S, 428.0635; found, 428.0632.

5.1.2.8. 2-(Cyclohexylamino)-8-nitro-6-(trifluoromethyl)-4H-benzo[e][1,3]oxazin-4-one (**5h**). Off-white solid; yield 65.2%. mp 176-178 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 8.70 (s, 1H), 8.50 (s, 1H), 6.27 (brs, 1H), 4.04-3.92 (m, 1H), 2.12-2.04 (m, 2H), 1.88-1.78 (m, 2H), 1.70-1.62 (m, 2H), 1.49-1.34 (m, 4H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ: 163.4 (162.9), 155.8 (157.2), 148.6 (148.4), 136.5, 130.9, 127.3 (q, <sup>2</sup>*J*<sub>F,C</sub> = 35 Hz), 126.9, 122.2 (q, <sup>1</sup>*J*<sub>F,C</sub> = 271 Hz), 120.2, 52.5 (51.4), 32.6 (32.5), 25.1 (25.0), 24.6 (24.8). HRMS (ESI): *m/z* [M+H]<sup>+</sup> calcd for C<sub>15</sub>H<sub>15</sub>F<sub>3</sub>N<sub>3</sub>O<sub>4</sub>, 358.1009; found, 358.0996.

5.1.2.9. 2-((Cyclohexylmethyl)amino)-8-nitro-6-(trifluoromethyl)-4H-benzo[e][1,3]oxazin-4-one (**5i**). Yellow solid; yield 70.0%. mp 218-220 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 8.71 (s, 1H), 8.61 (s, 0.5H), 8.52 (s, 0.5H), 3.49 (d, *J* = 6.8 Hz, 1H), 3.43 (t, *J* = 6.8 Hz, 1H), 1.82-1.68 (m, 6H), 1.29-1.20 (m, 3H), 1.11-1.01 (m, 2H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ: 163.2 (162.7), 158.2 (157.0), 148.6 (148.4), 136.6, 131.0 (130.8), 128.0 (127.6), 127.1 (127.0), 122.2 (q, <sup>1</sup>*J*<sub>F,C</sub> = 271 Hz), 120.6 (120.3), 48.3 (48.1), 38.3 (37.3), 30.6 (30.5), 26.2, 25.8 (25.6). HRMS (ESI): *m/z* [M+H]<sup>+</sup> calcd for C<sub>16</sub>H<sub>17</sub>F<sub>3</sub>N<sub>3</sub>O<sub>4</sub>, 372.1166; found, 372.1160. IR (KBr): ν 3096, 2930, 1661, 1641, 1550, 1304 cm<sup>-1</sup>.

5.1.2.10. 8-Nitro-2-(phenylamino)-6-(trifluoromethyl)-4H-benzo[e][1,3]oxazin-4-one (**5j**). Off-white solid; yield 66.7%. mp 255-257 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 8.92 (brs, 1H), 8.71 (s, 1H), 8.02 (s, 1H), 7.48-7.46 (m, 4H), 7.26-7.24 (m, 1H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>)

$\delta$ : 158.9, 148.7, 140.3, 137.3, 135.4, 130.9, 130.1, 129.5, 129.3 (129.2), 128.9, 128.3, 126.2 (q,  ${}^2J_{\text{F,C}} = 35$  Hz), 122.1 (q,  ${}^1J_{\text{F,C}} = 271$  Hz), 119.6. HRMS (ESI):  $m/z$   $[\text{M}+\text{H}]^+$  calcd for  $\text{C}_{15}\text{H}_9\text{F}_3\text{N}_3\text{O}_4$ , 352.0540; found, 352.0532.

*5.1.2.11. 2-(Benzylamino)-8-nitro-6-(trifluoromethyl)-4H-benzo[e][1,3]oxazin-4-one (5k).* Yellow solid; yield 20.3%. mp 170-172 °C.  ${}^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$ : 9.43 (brs, 1H), 8.69 (d,  $J = 1.6$  Hz, 1H), 8.10 (d,  $J = 2.0$  Hz, 1H), 7.27-7.25 (m, 3H), 7.02-6.99 (m, 2H), 5.29 (s, 2H).  ${}^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$ : 158.9, 150.2, 140.2, 137.1, 133.5, 130.1, 129.0, 128.7 (128.6), 128.5, 127.0, 125.9 (q,  ${}^2J_{\text{F,C}} = 36$  Hz), 122.1 (q,  ${}^1J_{\text{F,C}} = 271$  Hz), 120.5, 50.2. HRMS (ESI):  $m/z$   $[\text{M}+\text{H}]^+$  calcd for  $\text{C}_{16}\text{H}_{11}\text{F}_3\text{N}_3\text{O}_4$ , 366.0696; found, 366.0690.

*5.1.2.12. 2-((4-Fluorobenzyl)amino)-8-nitro-6-(trifluoromethyl)-4H-benzo[e][1,3]oxazin-4-one (5l).* Yellow solid; yield 54.8%. mp 174-176 °C.  ${}^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$ : 12.40 (brs, 1H), 8.57 (s, 1H), 8.48 (s, 1H), 7.23-7.22 (m, 2H), 7.12-7.08 (m, 2H), 5.00 (s, 2H).  ${}^{13}\text{C}$  NMR (100 MHz,  $\text{DMSO}-d_6$ )  $\delta$ : 161.2 ( $J = 241$  Hz), 159.6, 150.6, 139.1, 137.0, 131.3, 131.2, 127.9, 127.8, 122.8 (q,  ${}^2J_{\text{F,C}} = 35$  Hz), 122.5 (q,  ${}^1J_{\text{F,C}} = 271$  Hz), 120.8, 114.9 ( $J = 21$  Hz), 49.4. HRMS (ESI):  $m/z$   $[\text{M}+\text{H}]^+$  calcd for  $\text{C}_{16}\text{H}_{10}\text{F}_4\text{N}_3\text{O}_4$ , 384.0602; found, 384.0601.

*5.1.2.13. 2-((3-Fluorobenzyl)amino)-8-nitro-6-(trifluoromethyl)-4H-benzo[e][1,3]oxazin-4-one (5m).* Yellow solid; yield 26.1%. mp 208-210 °C.  ${}^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$ : 12.39 (brs, 1H), 8.59 (s, 1H), 8.49 (s, 1H), 7.35-7.30 (m, 1H), 7.13 (d,  $J = 10.4$  Hz, 1H), 7.09-7.04 (m, 2H), 5.00 (s, 2H).  ${}^{13}\text{C}$  NMR (100 MHz,  $\text{DMSO}-d_6$ )  $\delta$ : 162.1 ( $J = 241$  Hz), 159.6, 150.6, 139.1, 138.4, 137.1, 130.0, 127.8, 127.7, 122.7 (q,  ${}^2J_{\text{F,C}} = 35$  Hz), 122.5 (q,  ${}^1J_{\text{F,C}} = 271$  Hz), 121.6, 120.8, 113.7 ( $J = 21$  Hz), 112.7 ( $J = 23$  Hz), 49.7. HRMS (ESI):  $m/z$   $[\text{M}+\text{H}]^+$  calcd for  $\text{C}_{16}\text{H}_{10}\text{F}_4\text{N}_3\text{O}_4$ , 384.0602; found, 384.0591.

*5.1.2.14. 2-((3,4-Dimethoxybenzyl)amino)-8-nitro-6-(trifluoromethyl)-4H-benzo[e][1,3]*

*oxazin-4-one (5n)*. Yellow solid; yield 27.0%. mp 210-212 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 8.71-8.66 (m, 1H), 8.60-8.51 (m, 1H), 7.11-7.03 (m, 1H), 6.94-6.91 (m, 1H), 6.85-6.78 (m, 1H), 4.72-4.65 (m, 2H), 3.88-3.82 (m, 6H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ: 163.2 (162.4), 156.5 (157.8), 149.3 (149.2), 149.1 (148.8), 148.5 (148.3), 136.8 (136.5), 130.9 (130.6), 129.0 (128.2), 127.8 (127.7), 127.2 (127.1), 122.2 (q, <sup>1</sup>J<sub>F,C</sub> = 271 Hz), 120.9 (120.8), 120.3 (120.5), 111.8 (111.5), 111.3 (111.2), 56.0, 55.9, 46.1 (45.3). HRMS (ESI): m/z [M+H]<sup>+</sup> calcd for C<sub>18</sub>H<sub>15</sub>F<sub>3</sub>N<sub>3</sub>O<sub>6</sub>, 426.0908; found, 426.0891.

*5.1.2.15. 2-(((2,3-Dihydrobenzo[b][1,4]dioxin-6-yl)methyl)amino)-8-nitro-6-(trifluoromethyl)-4H-benzo[e][1,3]oxazin-4-one (5o)*. Yellow solid; yield 39.4%. mp 198-200 °C. <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ: 9.62 (brs, 1H), 8.74-8.71 (m, 1H), 8.40-8.38 (m, 1H), 6.91-6.89 (m, 1H), 6.86-6.79 (m, 2H), 4.40 (s, 2H), 4.20 (s, 4H). <sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>) δ: 162.9 (162.8), 157.1, 149.1 (148.8), 143.3, 142.8, 137.2 (136.9), 130.5 (130.4), 128.6 (128.8), 126.6 (126.8), 124.5 (q, <sup>2</sup>J<sub>F,C</sub> = 35 Hz), 122.7 (q, <sup>1</sup>J<sub>F,C</sub> = 271 Hz), 120.7, 120.4, 117.0 (117.1), 116.6, 64.1, 43.9. HRMS (ESI): m/z [M+H]<sup>+</sup> calcd for C<sub>18</sub>H<sub>13</sub>F<sub>3</sub>N<sub>3</sub>O<sub>6</sub>, 424.0751; found, 424.0734.

*5.1.2.16. 2-(Benzyl(methyl)amino)-8-nitro-6-(trifluoromethyl)-4H-benzo[e][1,3]oxazin-4-one (5p)*. Yellow solid; yield 79.1%. mp 154-156 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 8.75 (s, 1H), 8.59 (s, 1H), 7.39-7.34 (m, 4H), 7.31-7.29 (m, 1H), 4.93-4.91 (m, 2H), 3.29-3.27 (m, 3H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ: 163.0, 157.1, 156.3, 148.6, 136.4, 134.4, 131.1, 129.2 (129.0), 128.5, 127.8 (127.6), 127.0, 122.2 (q, <sup>1</sup>J<sub>F,C</sub> = 271 Hz), 120.3 (120.2), 53.6 (53.2), 36.5 (34.4). HRMS (ESI): m/z [M+H]<sup>+</sup> calcd for C<sub>17</sub>H<sub>13</sub>F<sub>3</sub>N<sub>3</sub>O<sub>4</sub>, 380.0853; found, 380.0842. IR (KBr): ν 3092, 2944, 1683, 1639, 1544, 1313, 736 cm<sup>-1</sup>.

*5.1.2.17. 2-((3,4-Dimethoxybenzyl)(methyl)amino)-8-nitro-6-(trifluoromethyl)-4H-benzo*

[*e*][1,3]oxazin-4-one (**5q**). Yellow solid; yield 45.7%. mp 142-144 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 8.75 (s, 1H), 8.59 (s, 1H), 6.97-6.91 (m, 1H), 6.86-6.83 (m, 2H), 4.85-4.82 (m, 2H), 3.88 (s, 3H), 3.87 (s, 3H), 3.30-3.26 (m, 3H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ: 163.1, 156.9 (156.2), 149.3, 148.6, 136.4, 131.1, 128.2, 127.1, 126.9, 122.2 (q, <sup>1</sup>J<sub>F,C</sub> = 271 Hz), 121.4, 120.3 (120.2), 111.8, 111.4, 111.1 (111.0), 56.0, 55.9, 53.5 (53.1), 36.4 (34.2). HRMS (ESI): m/z [M+H]<sup>+</sup> calcd for C<sub>19</sub>H<sub>17</sub>F<sub>3</sub>N<sub>3</sub>O<sub>6</sub>, 440.1064; found, 440.1049.

5.1.2.18. 2-(((2,3-Dihydrobenzo[*b*][1,4]dioxin-6-yl)methyl)(methylamino)-8-nitro-6-(trifluoromethyl)-4*H*-benzo[*e*][1,3]oxazin-4-one (**5r**). Yellow solid; yield 57.2%. mp 69-71 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 8.71 (s, 1H), 8.59 (s, 1H), 6.87-6.77 (m, 3H), 4.80 (s, 1H), 4.78 (s, 1H), 4.24 (s, 4H), 3.25 (d, *J* = 2.0 Hz, 3H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ: 163.1, 156.9 (156.1), 148.7, 143.8, 143.7, 136.3, 130.9, 127.6 (127.5), 127.1, 122.2 (q, <sup>1</sup>J<sub>F,C</sub> = 271 Hz), 121.6, 121.0, 120.3 (120.2), 117.8 (117.6), 117.3 (116.7), 64.3, 53.0 (52.6), 36.1 (34.2). HRMS (ESI): m/z [M+H]<sup>+</sup> calcd for C<sub>19</sub>H<sub>15</sub>F<sub>3</sub>N<sub>3</sub>O<sub>6</sub>, 438.0908; found, 438.0902. IR (KBr): ν 3067, 2986, 1706, 1636, 1545, 1311, 887 cm<sup>-1</sup>.

5.1.3. General procedure for the synthesis of target compounds **6a-r**. A 15 mL capped tube was charged with intermediate **11** (64 mg, 0.2 mmol) and corresponding amine (1.0 mmol). The degassed isopropanol (5 mL) was added. The tube was flushed with argon, capped, and heated at 140 °C for 12-48 h. After cooling to room temperature, the reaction mixture was concentrated in vacuum, and the residue was purified by column chromatography (EtOAc: PE = 1:2) to give the target compounds **6a-r**.

5.1.3.1. 2-(2-Methyl-1,4-dioxo-8-azaspiro[4.5]decan-8-yl)-8-nitro-6-(trifluoromethyl)-4*H*-thiochromen-4-one (**6a**). Yellow solid; yield 54.7%. mp 165-167 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 9.13 (s, 1H), 8.77 (s, 1H), 6.45 (s, 1H), 4.29-4.26 (m, 1H), 4.14-4.09 (m, 1H), 3.84-3.81 (m, 4H), 3.53-3.48 (m, 1H), 1.91-1.85 (m, 4H), 1.32 (d, *J* = 6.0 Hz, 3H). <sup>13</sup>C NMR



(100 MHz, CDCl<sub>3</sub>) δ: 176.9, 156.0, 144.4, 134.0, 133.5, 131.4, 128.9 (q, <sup>2</sup>J<sub>F,C</sub> = 35 Hz), 125.3, 122.7 (q, <sup>1</sup>J<sub>F,C</sub> = 267 Hz), 106.2, 100.2, 72.5, 70.9, 46.5, 36.0 (34.8), 18.4. HRMS (ESI): m/z [M+H]<sup>+</sup> calcd for C<sub>18</sub>H<sub>18</sub>F<sub>3</sub>N<sub>2</sub>O<sub>5</sub>S, 431.0890; found, 431.0879.

*5.1.3.2. 2-(4-(Cyclohexylmethyl)piperazin-1-yl)-8-nitro-6-(trifluoromethyl)-4H-thiochromen-4-one (6b)*. Orange solid; yield 69.6%. mp 184-186 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 9.12 (d, *J* = 2.0 Hz, 1H), 8.75 (d, *J* = 2.0 Hz, 1H), 6.25 (s, 1H), 3.66 (brs, 4H), 2.56 (brs, 4H), 2.20-2.19 (m, 2H), 1.80-1.68 (m, 5H), 1.51 (brs, 1H), 1.27-1.16 (m, 3H), 0.94-0.85 (m, 2H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ: 176.9, 159.6, 144.4, 134.0, 133.7, 131.5, 128.9 (q, <sup>2</sup>J<sub>F,C</sub> = 35 Hz), 125.3, 122.6 (q, <sup>1</sup>J<sub>F,C</sub> = 271 Hz), 100.2, 65.1, 52.7, 48.0, 35.0, 31.7, 26.7, 26.1. HRMS (ESI): m/z [M+H]<sup>+</sup> calcd for C<sub>21</sub>H<sub>25</sub>F<sub>3</sub>N<sub>3</sub>O<sub>3</sub>S, 456.1563; found, 456.1544. IR (KBr): ν 3033, 2926, 1638, 1595, 1548, 1296 cm<sup>-1</sup>.

*5.1.3.3. 2-(4-(Cyclohexylmethyl)-3-methylpiperazin-1-yl)-8-nitro-6-(trifluoromethyl)-4H-thiochromen-4-one (6c)*. Yellow solid; yield 58.4%. mp 162-164 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 9.12 (s, 1H), 8.74 (s, 1H), 6.24 (s, 1H), 3.80-3.77 (m, 2H), 3.47 (brs, 1H), 3.19 (brs, 1H), 2.98-2.95 (m, 1H), 2.58-2.48 (m, 2H), 2.36 (brs, 1H), 2.02-1.99 (m, 1H), 1.89-1.85 (m, 1H), 1.74-1.68 (m, 4H), 1.46 (brs, 1H), 1.25-1.11 (m, 6H), 0.91-0.88 (m, 2H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ: 176.8, 159.4, 144.4, 133.9, 133.7, 131.5, 128.9 (q, <sup>2</sup>J<sub>F,C</sub> = 35 Hz), 125.3, 122.6 (q, <sup>1</sup>J<sub>F,C</sub> = 271 Hz), 99.8, 60.2, 55.1, 54.2, 50.0, 48.2, 35.6, 32.0 (31.7), 26.7, 26.2 (26.0), 15.3. HRMS (ESI): m/z [M+H]<sup>+</sup> calcd for C<sub>22</sub>H<sub>27</sub>F<sub>3</sub>N<sub>3</sub>O<sub>3</sub>S, 470.1720; found, 470.1707.

*5.1.3.4. 1-(Cyclohexylmethyl)-4-(8-nitro-4-oxo-6-(trifluoromethyl)-4H-thiochromen-2-yl)piperazin-2-one (6d)*. Orange solid; yield 63.8%. mp 217-219 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 9.14 (s, 1H), 8.79 (s, 1H), 6.18 (s, 1H), 4.24 (brs, 2H), 3.91-3.89 (m, 2H), 3.61-3.58 (m, 2H), 3.36-3.34 (m, 2H), 1.73-1.66 (m, 6H), 1.26-1.21 (m, 3H), 1.03-0.99 (m, 2H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ: 176.5, 163.4, 157.6, 144.3, 133.6, 133.4, 131.8, 129.3 (q, <sup>2</sup>J<sub>F,C</sub> = 35 Hz), 125.5, 122.5 (q, <sup>1</sup>J<sub>F,C</sub> = 272 Hz), 100.2, 53.5, 50.7, 46.0, 44.8, 36.0, 30.8, 26.2,

25.7. HRMS (ESI):  $m/z$   $[M+H]^+$  calcd for  $C_{21}H_{23}F_3N_3O_4S$ , 470.1356; found, 470.1344. IR (KBr):  $\nu$  3084, 2923, 1652, 1637, 1590, 1548, 1446, 1297  $cm^{-1}$ .

5.1.3.5. 2-(4-Benzylpiperazin-1-yl)-8-nitro-6-(trifluoromethyl)-4H-thiochromen-4-one (**6e**). Yellow solid; yield 62.2%. mp 188-190 °C.  $^1H$  NMR (400 MHz,  $CDCl_3$ )  $\delta$ : 9.12 (s, 1H), 8.75 (s, 1H), 7.35-7.26 (m, 5H), 6.24 (s, 1H), 3.68 (brs, 4H), 3.60 (s, 2H), 2.63 (brs, 4H).  $^{13}C$  NMR (100 MHz,  $CDCl_3$ )  $\delta$ : 176.9, 159.6, 144.4, 137.0, 134.0, 133.6, 131.5, 129.1, 128.8, 128.5, 127.6, 125.4, 122.6 (q,  $^1J_{F,C} = 271$  Hz), 110.4, 62.6, 52.1, 47.9. HRMS (ESI):  $m/z$   $[M+H]^+$  calcd for  $C_{21}H_{19}F_3N_3O_3S$ , 450.1094; found, 450.1076.

5.1.3.6. 2-(4-Benzyl-3-methylpiperazin-1-yl)-8-nitro-6-(trifluoromethyl)-4H-thiochromen-4-one (**6f**). Yellow solid; yield 50.8%. mp 183-185 °C.  $^1H$  NMR (400 MHz,  $CDCl_3$ )  $\delta$ : 9.12 (s, 1H), 8.75 (s, 1H), 7.35-7.26 (m, 5H), 6.23 (s, 1H), 4.06 (d,  $J = 13.2$  Hz, 1H), 3.84 (t,  $J = 15.2$  Hz, 2H), 3.40 (t,  $J = 9.6$  Hz, 1H), 3.27-3.20 (m, 2H), 2.85 (d,  $J = 12.4$  Hz, 1H), 2.71 (brs, 1H), 2.32 (t,  $J = 9.2$  Hz, 1H), 1.26 (d,  $J = 6.0$  Hz, 3H).  $^{13}C$  NMR (100 MHz,  $CDCl_3$ )  $\delta$ : 176.8, 159.3, 144.4, 137.8, 133.9, 133.6, 131.5, 129.0, 128.9 (q,  $^2J_{F,C} = 35$  Hz), 128.5, 127.4, 125.3, 122.6 (q,  $^1J_{F,C} = 271$  Hz), 100.0, 57.7, 54.8, 54.2, 49.4, 48.1, 15.5. HRMS (ESI):  $m/z$   $[M+H]^+$  calcd for  $C_{20}H_{21}F_3N_3O_3S$ , 464.1250; found, 464.1228. IR (KBr):  $\nu$  3077, 2971, 1634, 1587, 1538, 1299, 737  $cm^{-1}$ .

5.1.3.7. 8-Nitro-2-(4-(thiazol-2-yl)piperazin-1-yl)-6-(trifluoromethyl)-4H-thiochromen-4-one (**6g**). Yellow solid; yield 38.1%. mp 243-245 °C.  $^1H$  NMR (400 MHz,  $CDCl_3$ )  $\delta$ : 9.14 (s, 1H), 8.80 (s, 1H), 7.28 (d,  $J = 3.6$  Hz, 1H), 6.70 (d,  $J = 3.6$  Hz, 1H), 6.31 (s, 1H), 3.86-3.81 (m, 8H).  $^{13}C$  NMR (100 MHz,  $CDCl_3$ )  $\delta$ : 177.1, 171.0, 159.4, 144.4, 139.4, 133.8, 133.5, 131.7, 129.2 (q,  $^2J_{F,C} = 35$  Hz), 125.5, 122.5 (q,  $^1J_{F,C} = 271$  Hz), 108.7, 101.3, 47.7, 47.2. HRMS (ESI):  $m/z$   $[M+H]^+$  calcd for  $C_{17}H_{14}F_3N_4O_3S_2$ , 443.0454; found, 443.0455.

5.1.3.8. 2-(Cyclohexylamino)-8-nitro-6-(trifluoromethyl)-4H-thiochromen-4-one (**6h**).

Yellow solid; yield 40.5%. mp 247-249 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 9.15 (d, *J* = 2.0 Hz, 1H), 8.78 (d, *J* = 2.0 Hz, 1H), 6.37 (s, 1H), 3.59 (s, 1H), 2.14-2.11 (m, 2H), 1.86-1.82 (m, 2H), 1.72-1.68 (m, 2H), 1.45-1.38 (m, 4H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ: 175.3, 158.6, 143.9, 134.2, 133.6, 131.6, 129.0, 125.2, 122.6 (q, <sup>1</sup>*J*<sub>F,C</sub> = 271 Hz), 98.2, 53.8, 32.3, 25.2, 24.6. HRMS (ESI): *m/z* [M+H]<sup>+</sup> calcd for C<sub>16</sub>H<sub>16</sub>F<sub>3</sub>N<sub>2</sub>O<sub>3</sub>S, 373.0828; found, 373.0824.

5.1.3.9. 2-((Cyclohexylmethyl)amino)-8-nitro-6-(trifluoromethyl)-4H-thiochromen-4-one (**6i**). Yellow solid; yield 59.7%. mp 220-222 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 9.14 (d, *J* = 1.6 Hz, 1H), 8.77 (d, *J* = 1.6 Hz, 1H), 6.14 (s, 1H), 5.39 (brs, 1H), 3.20 (t, *J* = 6.4 Hz, 2H), 1.84-1.68 (m, 5H), 1.30-1.21 (m, 4H), 1.05-1.01 (m, 2H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ: 176.4, 158.6, 143.8, 134.5, 134.2, 131.8, 128.7, 125.2, 122.6 (q, <sup>1</sup>*J*<sub>F,C</sub> = 271 Hz), 97.9, 50.7, 37.1, 30.9, 26.2, 25.6. HRMS (ESI): *m/z* [M+H]<sup>+</sup> calcd for C<sub>17</sub>H<sub>18</sub>F<sub>3</sub>N<sub>2</sub>O<sub>3</sub>S, 387.0985; found, 387.0974.

5.1.3.10. 8-Nitro-2-(phenylamino)-6-(trifluoromethyl)-4H-thiochromen-4-one (**6j**). Yellow solid; yield 57.3%. mp 244-246 °C. <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ: 10.27 (brs, 1H), 8.84 (s, 2H), 7.50-7.46 (m, 2H), 7.35-7.27 (m, 3H), 6.22 (s, 1H). <sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>) δ: 175.4, 157.3, 144.6, 137.6, 134.9, 133.4, 129.8, 129.6, 126.7 (q, <sup>2</sup>*J*<sub>F,C</sub> = 34 Hz), 126.2, 125.5, 123.9, 122.8 (q, <sup>1</sup>*J*<sub>F,C</sub> = 271 Hz), 98.3. HRMS (ESI): *m/z* [M+H]<sup>+</sup> calcd for C<sub>16</sub>H<sub>10</sub>F<sub>3</sub>N<sub>2</sub>O<sub>3</sub>S, 367.0359; found, 367.0371.

5.1.3.11. 2-(Benzylamino)-8-nitro-6-(trifluoromethyl)-4H-thiochromen-4-one (**6k**). Yellow solid; yield 57.9%. mp 187-189 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 9.13 (d, *J* = 2.0 Hz, 1H), 8.77 (d, *J* = 2.0 Hz, 1H), 7.40-7.34 (m, 5H), 6.17 (s, 1H), 4.50 (d, *J* = 5.2 Hz, 2H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ: 176.7, 157.7, 143.8, 135.2, 134.4, 134.0, 131.9, 129.3, 129.1, 128.7, 127.8, 125.4, 122.6 (q, <sup>1</sup>*J*<sub>F,C</sub> = 271 Hz), 98.6, 48.6. HRMS (ESI): *m/z* [M+H]<sup>+</sup> calcd for C<sub>17</sub>H<sub>12</sub>F<sub>3</sub>N<sub>2</sub>O<sub>3</sub>S, 381.0515; found, 381.0522.

5.1.3.12. 2-((4-Fluorobenzyl)amino)-8-nitro-6-(trifluoromethyl)-4H-thiochromen-4-one (**6l**). Yellow solid; yield 75.3%. mp 207-209 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 9.14 (s, 1H), 8.79 (s, 1H), 7.35 (t, *J* = 6.4 Hz, 2H), 7.10 (t, *J* = 6.8 Hz, 2H), 6.21 (s, 1H), 5.51 (brs, 1H), 4.50 (d, *J* = 2.8 Hz, 2H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ: 176.7, 162.8 (*J* = 247 Hz), 157.7, 143.9, 134.3, 133.9, 131.9, 131.0, 129.7 (*J* = 8 Hz), 129.0 (q, <sup>3</sup>*J*<sub>F-C</sub> = 35 Hz), 125.4, 122.6 (q, <sup>1</sup>*J*<sub>F-C</sub> = 271 Hz), 116.3 (*J* = 22 Hz), 98.7, 47.8. HRMS (ESI): *m/z* [M+H]<sup>+</sup> calcd for C<sub>17</sub>H<sub>11</sub>F<sub>4</sub>N<sub>2</sub>O<sub>3</sub>S, 399.0421; found, 399.0414.

5.1.3.13. 2-((3-Fluorobenzyl)amino)-8-nitro-6-(trifluoromethyl)-4H-thiochromen-4-one (**6m**). Yellow solid; yield 50.2%. mp 173-175 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 9.13 (d, *J* = 2.0 Hz, 1H), 8.80 (d, *J* = 2.0 Hz, 1H), 7.39-7.35 (m, 1H), 7.14 (d, *J* = 8.0 Hz, 1H), 7.06 (d, *J* = 8.0 Hz, 2H), 6.16 (s, 1H), 4.55-4.53 (m, 2H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ: 176.7, 163.2 (*J* = 247 Hz), 157.8, 143.9, 137.7, 134.3, 133.9, 131.9, 130.9 (*J* = 8 Hz), 129.0 (q, <sup>3</sup>*J*<sub>F-C</sub> = 35 Hz), 125.5, 123.1, 122.6 (q, <sup>1</sup>*J*<sub>F-C</sub> = 271 Hz), 115.6 (*J* = 21 Hz), 114.5 (*J* = 22 Hz), 98.9, 47.9. HRMS (ESI): *m/z* [M+H]<sup>+</sup> calcd for C<sub>17</sub>H<sub>11</sub>F<sub>4</sub>N<sub>2</sub>O<sub>3</sub>S, 399.0421; found, 399.0408.

5.1.3.14. 2-((3,4-Dimethoxybenzyl)amino)-8-nitro-6-(trifluoromethyl)-4H-thiochromen-4-one (**6n**). Yellow solid; yield 56.8%. mp 193-195 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 9.14 (d, *J* = 1.6 Hz, 1H), 8.79 (d, *J* = 2.0 Hz, 1H), 6.95 (d, *J* = 8.0 Hz, 1H), 6.91 (d, *J* = 1.6 Hz, 1H), 6.86 (d, *J* = 8.0 Hz, 1H), 6.45 (s, 1H), 4.50 (s, 2H), 3.90 (s, 3H), 3.88 (s, 3H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ: 175.2, 159.8, 149.5, 149.3, 143.9, 134.1, 133.4, 131.7, 129.1 (q, <sup>3</sup>*J*<sub>F-C</sub> = 35 Hz), 127.2, 125.4, 122.5 (q, <sup>1</sup>*J*<sub>F-C</sub> = 271 Hz), 120.7, 111.4, 111.2, 98.7, 56.0, 55.9, 48.6. HRMS (ESI): *m/z* [M+H]<sup>+</sup> calcd for C<sub>19</sub>H<sub>16</sub>F<sub>3</sub>N<sub>2</sub>O<sub>5</sub>S, 441.0727; found, 441.0743.

5.1.3.15. 2-(((2,3-Dihydrobenzo[*b*][1,4]dioxin-6-yl)methyl)amino)-8-nitro-6-(trifluoromethyl)-4H-thiochromen-4-one (**6o**). Yellow solid; yield 27.2%. mp 198-200 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 9.14 (d, *J* = 1.6 Hz, 1H), 8.79 (d, *J* = 1.6 Hz, 1H), 6.89-6.86 (m,

3H), 6.50 (s, 1H), 4.46 (s, 2H), 4.25 (s, 4H).  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$ : 144.0, 143.9, 143.8, 134.2, 133.7, 131.8, 131.7, 129.0 (q,  $^2J_{\text{F,C}} = 35$  Hz), 128.1, 125.4, 125.3, 122.6 (q,  $^1J_{\text{F,C}} = 273$  Hz), 120.9, 118.0, 116.8, 98.7, 64.3, 48.1. HRMS (ESI):  $m/z$   $[\text{M}+\text{H}]^+$  calcd for  $\text{C}_{19}\text{H}_{14}\text{F}_3\text{N}_2\text{O}_3\text{S}$ , 439.0570; found, 439.0571. IR (KBr):  $\nu$  3081, 2988, 1633, 1585, 1552, 1296, 890  $\text{cm}^{-1}$ .

*5.1.3.16. 2-(Benzyl(methyl)amino)-8-nitro-6-(trifluoromethyl)-4H-thiochromen-4-one (6p).* Yellow solid; yield 39.3%. mp 142-144 °C.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$ : 9.17 (d,  $J = 2.4$  Hz, 1H), 8.77-8.76 (m, 1H), 7.40-7.34 (m, 4H), 7.26-7.24 (m, 1H), 6.26 (s, 1H), 4.85 (s, 2H), 3.26 (s, 3H).  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$ : 176.2, 159.4, 144.3, 134.5, 133.7, 133.6, 131.6, 129.2, 128.7 (q,  $^2J_{\text{F,C}} = 35$  Hz), 128.4, 126.8, 125.3, 122.6 (q,  $^1J_{\text{F,C}} = 271$  Hz), 99.2, 56.6, 39.5. HRMS (ESI):  $m/z$   $[\text{M}+\text{H}]^+$  calcd for  $\text{C}_{18}\text{H}_{14}\text{F}_3\text{N}_2\text{O}_3\text{S}$ , 395.0672; found, 395.0671.

*5.1.3.17. 2-((3,4-Dimethoxybenzyl)(methyl)amino)-8-nitro-6-(trifluoromethyl)-4H-thiochromen-4-one (6q).* Yellow solid; yield 59.5%. mp 186-188 °C.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$ : 9.17 (s, 1H), 8.76 (s, 1H), 6.86 (d,  $J = 6.4$  Hz, 1H), 6.79 (d,  $J = 6.4$  Hz, 1H), 6.76 (s, 1H), 6.23 (s, 1H), 4.77 (s, 2H), 3.88 (s, 3H), 3.87 (s, 3H), 3.23 (s, 3H).  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$ : 176.3, 159.2, 149.6, 149.1, 144.3, 133.8, 133.7, 131.6, 129.0 (q,  $^2J_{\text{F,C}} = 35$  Hz), 126.9, 125.2, 122.6 (q,  $^1J_{\text{F,C}} = 271$  Hz), 119.4, 111.5, 110.2, 99.2, 56.3, 56.0, 55.9, 39.3. HRMS (ESI):  $m/z$   $[\text{M}+\text{H}]^+$  calcd for  $\text{C}_{20}\text{H}_{18}\text{F}_3\text{N}_2\text{O}_5\text{S}$ , 455.0883; found, 455.0878.

*5.1.3.18. 2-(((2,3-Dihydrobenzo[b][1,4]dioxin-6-yl)methyl)(methyl)amino)-8-nitro-6-(trifluoromethyl)-4H-thiochromen-4-one (6r).* Yellow solid; yield 55.3%. mp 200-202 °C.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$ : 9.16 (s, 1H), 8.76 (s, 1H), 6.86 (d,  $J = 6.4$  Hz, 1H), 6.74-6.71 (m, 2H), 6.20 (s, 1H), 4.72 (s, 2H), 4.25 (s, 4H), 3.22 (s, 3H).  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$ : 176.2, 159.2, 144.3, 144.0, 143.6, 133.8, 133.7, 131.5, 128.6 (q,  $^2J_{\text{F,C}} = 35$  Hz), 127.6, 125.2,

122.6 (q,  $^1J_{\text{F,C}} = 271$  Hz), 120.0, 118.0, 115.8, 99.2, 64.4, 64.3, 56.0, 39.3. HRMS (ESI):  $m/z$   $[\text{M}+\text{H}]^+$  calcd for  $\text{C}_{20}\text{H}_{16}\text{F}_3\text{N}_2\text{O}_3\text{S}$ , 453.0727; found, 453.0697.

*5.1.4. General procedure for the synthesis of target compounds 7a, 7b, 7d, 7r.* To a magnetically stirred solution of intermediate **13** (0.2 mmol) in dry DMF (2 mL) was added potassium carbonate (28 mg, 0.2 mmol). The contents were heated at 110 °C for 1 h under an atmosphere of argon. After filtration and concentration under reduced pressure, the residue was purified by flash column chromatography (MeOH: $\text{CH}_2\text{Cl}_2$  = 1:100) to afford the target compounds **7a**, **7b**, **7d**, **7r**.

*5.1.4.1. 2-(2-Methyl-1,4-dioxo-8-azaspiro[4.5]decan-8-yl)-8-nitro-6-(trifluoromethyl)-4H-chromen-4-one (7a).* Yellow solid; yield 72.5%. mp 197-199 °C.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$ : 8.74 (s, 1H), 8.51 (s, 1H), 5.66 (s, 1H), 4.32-4.27 (m, 1H), 4.14-4.10 (m, 1H), 3.77-3.71 (m, 4H), 3.53-3.49 (m, 1H), 1.88-1.85 (m, 4H), 1.31 (d,  $J = 6.0$  Hz, 3H).  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$ : 173.0, 161.4, 147.9, 137.2, 129.0, 126.8 (q,  $^2J_{\text{F,C}} = 35$  Hz), 126.0, 125.5, 122.5 (q,  $^1J_{\text{F,C}} = 271$  Hz), 106.1, 86.9, 72.5, 70.9, 43.8 (43.7), 35.9 (34.7), 18.4. HRMS (ESI):  $m/z$   $[\text{M}+\text{H}]^+$  calcd for  $\text{C}_{18}\text{H}_{18}\text{F}_3\text{N}_2\text{O}_6$ , 415.1112; found, 415.1114. IR (KBr):  $\nu$  3072, 2977, 1656, 1611, 1543, 1325  $\text{cm}^{-1}$ .

*5.1.4.2. 2-(4-(Cyclohexylmethyl)piperazin-1-yl)-8-nitro-6-(trifluoromethyl)-4H-chromen-4-one (7b).* Yellow solid; yield 61.5%. mp 229-231 °C.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$ : 8.74 (d,  $J = 2.0$  Hz, 1H), 8.50 (d,  $J = 2.0$  Hz, 1H), 5.59 (s, 1H), 3.65 (brs, 4H), 2.55 (brs, 4H), 2.20-2.19 (m, 2H), 1.80-1.62 (m, 5H), 1.28-1.19 (m, 4H), 0.91-0.86 (m, 2H).  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$ : 172.9, 161.7, 147.8, 137.3, 129.0, 126.6, 126.1, 125.4, 122.5 (q,  $^1J_{\text{F,C}} = 272$  Hz), 86.8, 65.2, 52.6, 45.3, 35.0, 31.7, 26.7, 26.1. HRMS (ESI):  $m/z$   $[\text{M}+\text{H}]^+$  calcd for  $\text{C}_{21}\text{H}_{23}\text{F}_3\text{N}_3\text{O}_4$ , 440.1792; found, 440.1792. IR (KBr):  $\nu$  3063, 2922, 1664, 1615, 1532, 1324  $\text{cm}^{-1}$ .

5.1.4.3. 1-(Cyclohexylmethyl)-4-(8-nitro-4-oxo-6-(trifluoromethyl)-4H-chromen-2-yl)piperazin-2-one (**7d**). Yellow solid; yield 66.1%. mp 134-136 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 8.76 (d, *J* = 2.0 Hz, 1H), 8.55 (d, *J* = 2.0 Hz, 1H), 5.54 (s, 1H), 4.14 (s, 2H), 3.98-3.96 (m, 2H), 3.58-3.56 (m, 2H), 3.34 (d, *J* = 7.2 Hz, 2H), 1.77-1.66 (m, 5H), 1.29-1.19 (m, 4H), 1.02-0.99 (m, 2H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ: 172.8, 163.1, 160.5, 147.7, 137.3, 129.3, 127.2 (q, <sup>2</sup>*J*<sub>F,C</sub> = 35 Hz), 126.0, 125.8, 122.4 (q, <sup>1</sup>*J*<sub>F,C</sub> = 271 Hz), 87.1, 53.6, 48.1, 46.2, 42.1, 36.0, 30.8, 26.2, 25.7. HRMS (ESI): *m/z* [M+H]<sup>+</sup> calcd for C<sub>21</sub>H<sub>23</sub>F<sub>3</sub>N<sub>3</sub>O<sub>5</sub>, 454.1584; found, 454.1583.

5.1.4.4. 2-(((2,3-Dihydrobenzo[*b*][1,4]dioxin-6-yl)methyl)(methyl)amino)-8-nitro-6-(trifluoromethyl)-4H-chromen-4-one (**7r**). Yellow solid; yield 76.2%. mp 159-161 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 8.76 (d, *J* = 2.4 Hz, 1H), 8.51 (d, *J* = 2.4 Hz, 1H), 6.86-6.84 (m, 1H), 6.76-6.72 (m, 2H), 5.61 (s, 1H), 4.66 (s, 2H), 4.25 (s, 4H), 3.13 (s, 3H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ: 172.6, 162.2, 147.8, 143.9, 143.6, 137.3, 129.1, 127.8, 126.9 (q, <sup>2</sup>*J*<sub>F,C</sub> = 35 Hz), 126.0, 125.4, 122.5 (q, <sup>1</sup>*J*<sub>F,C</sub> = 271 Hz), 120.5, 117.9, 116.2, 86.6, 64.4, 64.3, 53.4, 36.2. HRMS (ESI): *m/z* [M+H]<sup>+</sup> calcd for C<sub>20</sub>H<sub>16</sub>F<sub>3</sub>N<sub>2</sub>O<sub>6</sub>, 437.0955; found, 437.0946.

5.1.5. General procedure for the synthesis of intermediates **9a-r**. To a magnetically stirred solution of 2-chloro-5-(trifluoromethyl)-3-nitrobenzamide (**8**, prepared according to the reported method [11]) (1.07 g, 4.0 mmol) in dry 1,2-dichloroethane (10 mL) was added oxalyl chloride (863 mg, 6.8 mmol). The mixture was heated to reflux for 3 h under an atmosphere of argon. The solvent was evaporated under reduced pressure. The residue was dissolved in acetonitrile (15 mL) and then cooled to -25 °C. Corresponding amine (4.0 mmol) in acetonitrile (5 mL) was slowly added to the above solution keeping the reaction under -25 °C for 0.5-2 h. The precipitated solid was filtered, washed with cooled acetonitrile and dried. The crude product was purified by silica gel column chromatography (EtOAc : PE = 1:2) to obtain the intermediates **9a-r**.

5.1.5.1. *N*-(2-Chloro-3-nitro-5-(trifluoromethyl)benzoyl)-2-methyl-1,4-dioxo-8-azaspiro [4.5]decane-8-carboxamide (**9a**). Yellow solid; yield 77.2%. mp 115-117 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 8.12 (s, 1H), 7.81 (s, 1H), 4.26-4.22 (m, 1H), 4.08-4.05 (m, 1H), 3.63-3.55 (m, 4H), 3.47-3.43 (m, 1H), 1.78-1.74 (m, 4H), 1.27 (d, *J* = 6.0 Hz, 3H).

5.1.5.2. *N*-(2-Chloro-3-nitro-5-(trifluoromethyl)benzoyl)-4-(cyclohexylmethyl)piperazine-1-carboxamide (**9b**). Yellow solid; yield 61.8%. mp 105-107 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 8.14 (s, 1H), 7.84 (s, 1H), 3.55 (brs, 4H), 2.47 (brs, 4H), 2.21 (brs, 2H), 1.81-1.71 (m, 5H), 1.28-1.20 (m, 4H), 0.91-0.89 (m, 2H).

5.1.5.3. *N*-(2-Chloro-3-nitro-5-(trifluoromethyl)benzoyl)-4-(cyclohexylmethyl)-3-methylpiperazine-1-carboxamide (**9c**). Yellow solid; yield 46.9%. mp 120-122 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 9.17 (brs, 1H), 8.14 (s, 1H), 7.82 (s, 1H), 3.69-3.66 (m, 2H), 3.30 (brs, 1H), 3.00 (brs, 1H), 2.87-2.84 (m, 1H), 2.45-2.43 (m, 2H), 2.20 (brs, 1H), 1.98-1.94 (m, 1H), 1.86-1.83 (m, 1H), 1.72-1.66 (m, 5H), 1.43 (brs, 1H), 1.19-1.17 (m, 2H), 1.03-1.02 (m, 3H), 0.88-0.83 (m, 2H).

5.1.5.4. *N*-(2-Chloro-3-nitro-5-(trifluoromethyl)benzoyl)-4-(cyclohexylmethyl)-3-oxopiperazine-1-carboxamide (**9d**). Yellow solid; yield 38.7%. mp 103-105 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 9.73 (brs, 1H), 8.13 (s, 1H), 7.87 (s, 1H), 4.29 (brs, 2H), 3.75 (brs, 2H), 3.42 (brs, 2H), 3.25-3.24 (m, 2H), 1.71-1.61 (m, 5H), 1.26-1.18 (m, 4H), 0.96-0.94 (m, 2H).

5.1.5.5. 4-Benzyl-*N*-(2-chloro-3-nitro-5-(trifluoromethyl)benzoyl)piperazine-1-carboxamide (**9e**). Yellow solid; yield 69.0%. mp 157-158 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 9.27 (brs, 1H), 8.13 (s, 1H), 7.81 (s, 1H), 7.31-7.26 (m, 5H), 3.54 (brs, 6H), 2.49 (brs, 4H).

5.1.5.6. 4-Benzyl-*N*-(2-chloro-3-nitro-5-(trifluoromethyl)benzoyl)-3-methylpiperazine-1-



*carboxamide (9f)*. Yellow solid; yield 59.8%. mp 108-110 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 9.05 (brs, 1H), 8.13 (s, 1H), 7.81 (s, 1H), 7.33-7.26 (m, 5H), 4.05-4.01 (m, 1H), 3.75-3.67 (m, 2H), 3.24-3.21 (m, 2H), 3.06 (brs, 1H), 2.77-2.74 (m, 1H), 2.56 (brs, 1H), 2.18 (brs, 1H), 1.18 (brs, 3H).

5.1.5.7. *N-(2-Chloro-3-nitro-5-(trifluoromethyl)benzoyl)-4-(thiazol-2-yl)piperazine-1-carboxamide (9g)*. Yellow solid; yield 68.3%. mp 180-182 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 9.18 (brs, 1H), 8.16 (s, 1H), 7.86 (s, 1H), 7.22 (d, *J* = 3.6 Hz, 1H), 6.66 (d, *J* = 3.6 Hz, 1H), 3.70 (brs, 4H), 3.62 (brs, 4H).

5.1.5.8. *2-Chloro-N-(cyclohexylcarbamoyl)-3-nitro-5-(trifluoromethyl)benzamide (9h)*. Yellow solid; yield 65.5%. mp 168-170 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 10.98 (brs, 1H), 8.22 (d, *J* = 7.6 Hz, 1H), 8.15 (s, 1H), 7.99 (s, 1H), 3.48-3.47 (m, 1H), 1.83-1.80 (m, 2H), 1.72-1.69 (m, 2H), 1.62-1.59 (m, 1H), 1.32-1.19 (m, 5H).

5.1.5.9. *2-Chloro-N-((cyclohexylmethyl)carbamoyl)-3-nitro-5-(trifluoromethyl)benzamide (9i)*. Yellow solid; yield 88.3%. mp 158-160 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 10.96 (brs, 1H), 8.34 (brs, 1H), 8.15 (s, 1H), 8.00 (s, 1H), 3.02 (t, *J* = 6.4 Hz, 2H), 1.74-1.66 (m, 5H), 1.49-1.39 (m, 1H), 1.27-1.13 (m, 3H), 0.93-0.84 (m, 2H).

5.1.5.10. *2-Chloro-3-nitro-N-(phenylcarbamoyl)-5-(trifluoromethyl)benzamide (9j)*. Yellow solid; yield 41.2%. mp 199-201 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 10.72 (brs, 1H), 10.32 (brs, 1H), 8.21 (s, 1H), 8.02 (s, 1H), 7.30-7.27 (m, 4H), 7.19-7.15 (m, 1H).

5.1.5.11. *N-(Benzylcarbamoyl)-2-chloro-3-nitro-5-(trifluoromethyl)benzamide (9k)*. Yellow solid; yield 68.3%. mp 151-153 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 10.63 (brs, 1H), 8.64 (brs, 1H), 8.12 (s, 1H), 8.00 (s, 1H), 7.36-7.30 (m, 3H), 7.22-7.19 (m, 2H), 4.42-4.38 (m, 2H).

5.1.5.12. 2-Chloro-*N*-((4-fluorobenzyl)carbamoyl)-3-nitro-5-(trifluoromethyl)benzamide (**9l**). Yellow solid; yield 94.7%. mp 190-191 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 10.40 (brs, 1H), 8.62 (brs, 1H), 8.14 (s, 1H), 8.01 (s, 1H), 7.20 (brs, 2H), 7.02 (brs, 2H), 4.38 (s, 2H).

5.1.5.13. 2-Chloro-*N*-((3-fluorobenzyl)carbamoyl)-3-nitro-5-(trifluoromethyl)benzamide (**9m**). Yellow solid; yield 85.8%. mp 204-205 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 10.33 (brs, 1H), 8.68 (brs, 1H), 8.14 (s, 1H), 8.01 (s, 1H), 7.33-7.26 (m, 1H), 7.02-6.92 (m, 3H), 4.41 (d, *J* = 6.0 Hz, 2H).

5.1.5.14. 2-Chloro-*N*-((3,4-dimethoxybenzyl)carbamoyl)-3-nitro-5-(trifluoromethyl)benzamide (**9n**). Yellow solid; yield 82.3%. mp 61-62 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 10.47 (brs, 1H), 8.57 (brs, 1H), 8.14 (s, 1H), 8.02 (s, 1H), 6.83-6.76 (m, 3H), 4.33 (d, *J* = 5.6 Hz, 2H), 3.88 (s, 6H).

5.1.5.15. 2-Chloro-*N*-(((2,3-dihydrobenzo[*b*][1,4]dioxin-6-yl)methyl)carbamoyl)-3-nitro-5-(trifluoromethyl)benzamide (**9o**). Yellow solid; yield 48.9%. mp 155-156 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 10.97 (brs, 1H), 8.60 (brs, 1H), 8.16 (s, 1H), 8.01 (s, 1H), 6.80 (d, *J* = 8.4 Hz, 1H), 6.68-6.65 (m, 2H), 4.25 (s, 2H), 4.23 (s, 4H).

5.1.5.16. *N*-(Benzyl(methyl)carbamoyl)-2-chloro-3-nitro-5-(trifluoromethyl)benzamide (**9p**). Yellow solid; yield 90.1%. mp 129-131 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 9.30 (brs, 1H), 8.13 (s, 1H), 7.77 (s, 1H), 7.35-7.31 (m, 4H), 7.21-7.20 (m, 1H), 4.53 (s, 2H), 3.01 (s, 3H).

5.1.5.17. 2-Chloro-*N*-((3,4-dimethoxybenzyl)(methyl)carbamoyl)-3-nitro-5-(trifluoromethyl)benzamide (**9q**). Yellow solid; yield 54.3%. mp 70-72 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 8.36 (brs, 1H), 8.16 (s, 1H), 7.81 (s, 1H), 6.84 (d, *J* = 8.0 Hz, 1H), 6.79-6.76 (m, 2H), 4.45 (s, 2H), 3.89 (s, 3H), 3.87 (s, 3H), 3.02 (s, 3H).

5.1.5.18. 2-Chloro-N-(((2,3-dihydrobenzo[b][1,4]dioxin-6-yl)methyl)(methyl)carbamoyl)-3-nitro-5-(trifluoromethyl)benzamide (**9r**). Yellow solid; yield 76.0%. mp 156-158 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 8.47 (brs, 1H), 8.15 (s, 1H), 7.80 (s, 1H), 6.85 (d, *J* = 8.0 Hz, 1H), 6.73 (s, 1H), 6.69 (d, *J* = 8.0 Hz, 1H), 4.41 (s, 2H), 4.26 (s, 4H), 3.01 (s, 3H).

5.1.6. Synthesis of the 2-(methylthio)-8-nitro-6-(trifluoromethyl)-4H-thiochromen-4-one (**11**). To a magnetically stirred solution of NaOH (800 mg, 20 mmol) in DMSO (5 mL) was added CS<sub>2</sub> (2.34 g, 30 mmol) and intermediate **10** (prepared according to the reported method [21, 22]) (2.67 g, 10 mmol) in turn, keeping the reaction solution under 20 °C for 30 min. CHI (10 mmol) was then added to the reaction mixture. The contents were stirred at room temperature for 1 h. The reaction was quenched by H<sub>2</sub>O (50 mL) and the mixture was extracted with EtOAc (3 × 50 mL). The combined extracts were washed with brine, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure. The residue was purified by column chromatography (EtOAc:PE = 1:4) to obtain the target compound **11**. Yellow solid; yield 60.0%. mp 146-148 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 9.12 (d, *J* = 2.0 Hz, 1H), 8.84 (d, *J* = 2.0 Hz, 1H), 6.91 (s, 1H), 2.70 (s, 3H).

5.1.7. General procedure for the synthesis of intermediates **13a**, **13b**, **13d**, **13r**. To a magnetically stirred solution of intermediate **12** (prepared according to the reported method [24]) (312 mg, 1.0 mmol) in dry dichloromethane (5 mL) was added dicyclohexylcarbodiimide (DCC) (201 mg, 1.0 mmol). The contents were stirred at room temperature for 30 min under an atmosphere of argon. Corresponding amine (1.0 mmol) was then added to the reaction solution. The reaction mixture was stirred at room temperature for 6-12 h. After filtration, the filtrate was concentrated under reduced pressure. The residue was purified by column chromatography (EtOAc:PE = 1:10) to give the intermediates **13a**, **13b**, **13d**, **13r**.

5.1.7.1. 3-(2-Chloro-3-nitro-5-(trifluoromethyl)phenyl)-3-hydroxy-1-(2-methyl-1,4-dioxo-8-azaspiro[4.5]decan-8-yl)prop-2-en-1-one (**13a**). Yellow solid; yield 55.5%. mp 108-110 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 15.64 (brs, 1H), 8.01 (s, 1H), 8.00 (s, 1H), 5.72 (s, 1H), 4.30-4.13 (m, 1H), 4.12-4.08 (m, 1H), 3.75-3.47 (m, 5H), 1.79-1.75 (m, 4H), 1.31-1.29 (m, 3H).

5.1.7.2. 3-(2-Chloro-3-nitro-5-(trifluoromethyl)phenyl)-1-(4-(cyclohexylmethyl)piperazin-1-yl)-3-hydroxyprop-2-en-1-one (**13b**). Yellow solid; yield 57.1%. mp 105-107 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 15.62 (brs, 1H), 8.01 (s, 1H), 8.00 (s, 1H), 5.67 (s, 1H), 3.73-3.49 (m, 4H), 2.45 (m, 4H), 2.17 (brs, 2H), 1.79-1.71 (m, 5H), 1.26-1.20 (m, 4H), 0.90-0.87 (m, 2H).

5.1.7.3. 4-(3-(2-Chloro-3-nitro-5-(trifluoromethyl)phenyl)-3-hydroxyacryloyl)-1-(cyclohexylmethyl)piperazin-2-one (**13d**). Yellow solid; yield 26.5%. mp 146-148 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 15.1 (brs, 1H), 8.04 (s, 1H), 7.99 (s, 1H), 5.64 (s, 1H), 4.16-4.12 (m, 2H), 3.93-3.90 (m, 2H), 3.47-3.42 (m, 2H), 3.30-3.28 (m, 2H), 1.73-1.64 (m, 5H), 1.26-1.13 (m, 4H), 1.00-0.97 (m, 2H).

5.1.7.4. 3-(2-Chloro-3-nitro-5-(trifluoromethyl)phenyl)-N-((2,3-dihydrobenzo[*b*][1,4]dioxin-6-yl)methyl)-3-hydroxy-N-methylacrylamide (**13r**). Yellow solid; yield 60.3%. mp 150-152 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 15.57 (brs, 1H), 8.02 (s, 2H), 6.86-6.69 (m, 3H), 5.72-5.70 (m, 1H), 4.57-4.44 (m, 2H), 4.26-4.24 (m, 4H), 3.07-2.96 (m, 3H).

## 5.2. Biological evaluation

5.2.1. Minimum inhibitory concentration assay (MIC). MICs against replicating *M. tuberculosis* were determined by the microplate alamar blue assay (MABA) [26]. RFP, INH, **1** and **2** were included as positive controls. Compound stock solutions and the range of final testing concentrations were 32 to 0.5 µg/mL, respectively. For the most active compounds, the stock concentration and final testing concentration range were lowered to 3.2 µg/mL and

2 to 0.002 µg/mL, respectively. *M. tuberculosis* H<sub>37</sub>Rv or clinical isolates were grown to late log phase (70 to 100 Klett units) in Difco Middlebrook 7H9 Broth (Seebio) supplemented with 0.2% (vol/vol) glycerol, 0.05% Tween 80, and 10% (vol/vol) albumin-dextrose-catalase (Seebio) (7H9-ADC-TG). Cultures were centrifuged, washed twice, and then re-suspended in phosphate-buffered saline. Suspensions were then passed through an 8 µm-pore-size filter to remove clumps, and aliquots were frozen at -80 °C. Twofold dilutions of compounds were prepared in 7H9-ADC-TG in a volume of 100 µL in 96-well clear-bottom microplates (BD). *M. tuberculosis* (100 µL containing  $2 \times 10^5$  CFU) was added to yield a final testing volume of 200 µL. The plates were incubated at 37 °C; on day 7 of incubation, 12.5 µL of 20% Tween 80 and 20 µL of alamar blue were added to all wells. After incubation at 37 °C for 16 to 24 h, the fluorescence was read at an excitation of 530 nm and an emission of 590 nm. The MIC was defined as the lowest concentration effecting a reduction in fluorescence of  $\geq 90\%$  relative to the mean of replicate bacterium-only controls.

5.2.2. *Cytotoxicity assay.* Vero cells and HepG2 cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS). The cells were incubated in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C. Stocks of cells were cultured in 25-cm<sup>2</sup> tissue culture flasks and subcultured two to three times per week. Cytotoxicity testing was performed in a transparent 96-well microplate. Outer perimeter wells were filled with sterile water to prevent dehydration in experimental wells. The cells were incubated at 37 °C under 5% CO<sub>2</sub> until confluent and then diluted with culture medium to  $4 \times 10^5$  cells/mL. Threefold serial dilutions of the stock solutions resulted in final concentrations of 64 to 0.26 µg/mL in a final volume of 100 µL. After incubation at 37 °C for 48 h, the medium was removed, and the monolayers were washed twice with 100 µL of warm Hanks balanced salt solution (HBSS). Warm medium (100 µL) and 10 µL of freshly made methyl-thiazolyldiphenyl-tetrazolium bromide

(MTT) were added to each well, and then the plates were incubated for 4 h, after which the absorbance was determined at 492 nm.

*5.2.3. Liver microsome stability assay.* The assay was performed with liver microsomes from male CD-1 mouse (Xenotech) and pooled human (Bioreclamation). Compounds of interest were tested at 1  $\mu$ M with a final concentration of microsomal protein of 1 mg/mL. The reaction was initiated by the addition of NADPH (1 mM), and samples were incubated for up to 60 min at 37 °C in a shaking incubator. The reaction was terminated at 0, 5, 15 and 30 min by the addition of ice-cold ACN/MeOH (50:50) spiked with internal standard. An aliquot of reaction mixture was removed at 0, 5, 15, 30, and 60 min, respectively, followed by addition of ice-cold ACN/MeOH (50:50, v/v) spiked with internal standard. Samples were centrifuged at 4,000 rpm at 4 °C for 15 min and the supernatants were analysed by LC-MS/MS. The assay evaluated the metabolic stability of compounds by measuring the amount of parent remaining to test compounds with or without NADPH cofactor.

*5.2.4. In vitro CYP inhibition.* Briefly, compound **6b** was incubated with human liver microsome (0.2 mg/mL) and CYP enzyme (1A2, 2C9, 2C19, 2D6 and 3A4) for 20 min. The compound was tested at a range of concentrations (0.1-50  $\mu$ M), alongside a relevant positive control. The positive controls, which are CYP isoform-specific substrates were also incubated with human liver microsomes at a range of test compound concentrations (0.1-50  $\mu$ M). At the end of the incubation, the amount of parent remaining to each substrate is monitored by LC-MS/MS at each of the test compound concentrations. IC<sub>50</sub> was then determined based on the measurements.

*5.2.5. Hepatocyte stability assay.* The assay was performed with hepatocytes from pooled male CD-1 mouse (Bioreclamation IVT) and pooled human (Bioreclamation IVT). Compounds of interest (**6b** and **2**) were tested at 1  $\mu$ M with a final hepatocyte concentration

of 1 million cells/mL. The reaction was initiated by addition of pre-warmed hepatocyte working solution (2 million cells/mL) to the compound working solution (2  $\mu$ M). Reaction mixtures were incubated for up to 120 min at 37 °C in a CO<sub>2</sub> incubator at ~ 100 rpm. At the pre-determined time points (0, 15, 30, 60, 90 and 120 min), 30  $\mu$ L of the reaction mixtures was removed and reaction was terminated by addition of 200  $\mu$ L ice-cold ACN/MeOH (50:50) spiked with internal standard. Samples were mixed well and then were centrifuged at 4,000 rpm at 4 °C for 15 min. Supernatants were removed and were analysed by LC-MS/MS. The assay evaluated the metabolic stability of compounds in hepatocytes by measuring amount of parent remaining of the test compounds.

*5.2.6. Inhibition Evaluation on hERG K<sup>+</sup> Channel.* The electrophysiology recording of hERG channel current was carried out following the standard protocol as described previously [27]. HEK 293 cells were stably transfected with human Ether-à-go-go related gene (hERG) channel. The voltage-gated hERG potassium channel current was recorded at room temperature (25 °C) from randomly selected transfected cells under whole-cell manual patch clamp systems equipped with EPC10 USB (HEKA) or Multiclamp 700B amplifier (Molecular Devices), while electrical data was digitalized by Digidata1440A with sampling frequency at 10 kHz using Patchmaster or pClamp10 respectively. hERG current inhibition in presence of 5 concentrations, including 30, 10, 3.0, 1.0 and 0.3  $\mu$ M, was tested for IC<sub>50</sub> determination. Dofetilide was also included as a positive control to ensure the accuracy and sensitivity of the test system. All experiments were performed in duplicate for IC<sub>50</sub> determination. The compound with IC<sub>50</sub> > 30  $\mu$ M was generally considered to have a lower potential for hERG K<sup>+</sup> channel inhibition.

*5.2.7. Acute toxicity study.* Compound **6b** was screened *in vivo* with a single dose in Balb/c mouse (female) weighing 18 to 21 g with ten mice. The number of mice which survived after

an oral administration of a single dose at 2 g/kg, followed by a 7-day observation, was recorded.

*5.2.8. Pharmacokinetic studies in mice.* All animal protocols were approved by Institute Animal Care and Use Committee. The selected compound **6b** was subjected to pharmacokinetic studies in Balb/c mouse (male) weighing 22 to 23 g with three mice in oral administration group and three mice in intravenous injection group. The tested compound was formulated at a concentration of 2.5 mg/mL for a dose of 25 mg/kg given orally (p.o.) and at 2.0 mg/mL for a dose of 10 mg/kg given intravenously (i.v.). The tested compound was formulated with 0.5% carboxymethyl cellulose and 0.5% Tween 80 for p.o. administration and with 20% HP- $\beta$ -CD with 4 mol/L HCl for i.v. administration. Blood samples were collected at 5, 15, 30 min, 1, 2, 4, 7, 24 h after oral dosing and i.v. administration. Plasma was harvested and stored at -80 °C until analysed. 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 100A column (30 mm  $\times$  3 mm, 2.6  $\mu$ m) with an isocratic mobile phase of acetonitrile/water (80:20, v/v) containing 0.1% formic acid at 0.8 mL/min flow rate. Compound detection on the mass spectrometer was performed in electrospray positive ionization mode. The selected reaction monitoring transition was m/z 456.17/359.80. 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 ( $F = (AUC_{p.o.} \times dose_{i.v.}) / (AUC_{i.v.} \times dose_{p.o.})$ ).

*5.2.9. In vivo TB infection assay.* SPF Balb/c mice (female) weighing 18–20 g were used in this study. Each treated group was composed of 6 mice. Mice were infected via aerosol with a



suspension of  $5 \times 10^6$  CFU/mL *M. tuberculosis* (H<sub>3</sub>Rv) using a Glas-Col inhalation system, to deposit 50–100 bacilli into the lungs of each animal. The course of infection was followed by plating homogenates of harvested organs [n = 3] on 7H11 agar plates (7H11 plates containing 10% oleic acid-albumin-dextrose-catalase (OADC) enrichment and 50 µg/mL cycloheximide, 200 U/mL polymyxin B, 50 µg/mL carbenicillin, and 20 µg/mL trimethoprim) and determining CFU on days 3, 10, and 30 post infection. INH and compound **6b** were dissolved or suspended in 0.5% CMC and administered by oral gavage in a maximum volume of 200 µL such that a dose of 25 and 100 mg/kg body weight was achieved. The control group was received only 0.5% CMC. Mice were began to treat on days 10 post infection and 5 times per week. The treatment period was 3 weeks. Mice were sacrificed the day after the last day of treatment, lungs removed, homogenized, and serially diluted in 10-fold steps in HBSS. One hundred µL were spread on 7H11 agar in duplicate. The plates were incubated at 37 °C for 3 weeks. Data are expressed as the log<sub>10</sub> (and as log<sub>10</sub> reduction) provided by a given dose of the compound against the growth of the organism in the untreated control group. Mean log<sub>10</sub> values were calculated from bacterial burden counts. Student's *t* test was used to compare means between the test and control groups. A P value of  $\leq 0.05$  was considered significant.

*5.2.10. In vitro DprE1 inhibition assay.* DprE1 assays were performed as described previously [28]. Briefly, a 10 µL reaction was performed at 30 C in 384-well black plates in buffer containing 50 mM Hepes, pH 7.5, 100 mM NaCl, 1.5% (v/v) DMSO, 100 µM Tween-20, 2 µM FAD, and 50 µM resazurin, with variable concentrations of FPR and DprE1. Reactions were monitored by following an increase in fluorescence intensity ( $\lambda_{ex} = 530$  nm,  $\lambda_{em} = 595$  nm) associated with the formation of resorufin. For inhibition studies, DprE1 (1 µM) was measured with the resazurin assay with 1 mM FPR in the presence of different inhibitor concentrations. The IC<sub>50</sub> values were obtained by plotting the initial velocities with inhibitor concentration. The IC<sub>50</sub> values calculated using the software program GraphPad

Prism.

## Notes

The authors declare no competing financial interest.

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