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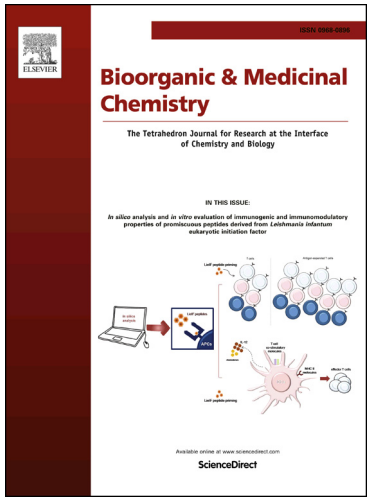

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Kiran Kumar Alluri, Rudraraju Srilakshmi Reshma, Raghuram Suraparaju, Suryanarayana Gottapu, Dharmarajan Sriram

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Synthesis and Evaluation of 4',5'-Dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran] Analogues Against Both Active and Dormant *Mycobacterium tuberculosis*

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ABSTRACT:

Need for new drugs to fight against tuberculosis (TB) is increasing day by day. In the present work we have taken a spiro compound (GSK 2200150A) reported by GSK as a lead and we modified the structure of the lead to study the antitubercular activity. For structure activity profiling twenty-one molecules have been synthesized, characterized and evaluated for their antimycobacterial potency against both active and dormant TB. Compound **06**, 1-((4-methoxyphenyl)sulfonyl)-4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran] was found to be the most potent compound (MIC : 8.23µM) in active TB and was less effective than the lead but more potent than standard first line drug ethambutol. It was also found to be more efficacious than Isoniazid and Rifampicin and equipotent as Moxifloxacin against dormant *Mycobacterium tuberculosis*(MTB). Compound **06** also showed good inhibitory potential against over expressed latent MTB enzyme lysine ε-amino transferase with an IC_{50} of 1.04 ± 0.32 µM. This compound is a good candidate for drug development owing to potential against both active and dormant stages of MTB.

Key words: Tuberculosis, *Mycobacterium tuberculosis,* Spiro compounds, Dormant TB

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1. Introduction

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* (MTB) that have evolved both as a symbiont and pathogen more than 150 million years ago.[1] The co-evolution of MTB with humans for such long periods has resulted in extensive evolutionary adaptation, which facilitated MTB survival inside the lungs and established it as a potent pathogen.[2] It earned apt sobriquets such as The Robber of youth, Captain of these men of death, The Grave yard Cough and King's evil during 19th century due to massive deaths caused by the disease.[3] MTB can remain in lungs in asymptomatic latent form for a very long time, which can get activated at any opportune time. Stringent & lipid rich cell envelope, slow metabolic rate and precise regulation of pathogenic factors make it a dreadful pathogen.[4] WHO reports showed that one third of the world population is suffering with latent TB, which has ~10 % lifetime chance to convert into active TB. In 2015, there are 10.4 million new cases reported across the world. 4,80,000 people developed multi drug resistant TB. Despite the availability of treatment regimens TB still remains among top 10 causes of death worldwide.[5] Manmade amplification of disease led to emergence of MDR and XDR forms of TB, which are resistant to most of first line and second line drugs available. The treatment options for resistant and dormant forms of TB are limited and costly, and causing more side effects. Newer drugs need to be developed which can lower duration of therapy and become more efficacious against resistant and dormant forms.[6] Various reasons are behind the lack of new medicines for the treatment of TB and other neglected diseases over the last 40 years, but most of them are generally related to a lack of critical mass in terms of funding, R&D capacity, and commercial interest.[7] Recently GlaxoSmithKilne screened around 2 million compounds phenotypically against MTB and identified and reported a total of 177 compounds with MIC_{95}< 10 µM and therapeutic index of ($HepG2IC_{50}$/MTB H37Rv MIC) >50.[8] They also reported seven chemical families with strong antitubercular actives as potential lead compounds. Among the

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reported leads; a spiro compound GSK2200150A with a cluster size of seven compounds showed MTB MIC of 0.38µM. We have taken this spiro compound as a lead compound to synthesize twenty-one compounds and evaluated biological evaluation against active and dormant H37Rv strain of MTB.

2. Results and discussion

2.1. Designing and synthesis

In this work we have taken GSK2200150A as the starting point to design a new series by shifting of sulfur atom and also studied the effect of N1 alkyl, sulfonyl and acyl derivatives (**Fig 1**) to investigate the structure activity relationship (SAR) of the lead compound. To develop series of molecules 4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran] core was retained as such and the amine was reacted with aromatic sulfonyl chlorides, acids and halides. Electron donating and withdrawing substituents are introduced on the aromatic ring in all 3 classes of sulfonamides, amides and N-alkyl derivatives to study SAR.

Designed molecules were synthesized using the protocol shown in the **Fig. 2**. 2-(thiophen-3-yl)acetic acid (**01)** was reduced with lithium aluminium hydride in THF to afford 2-(thiophen-3-yl)ethanol (**02)** which was reacted with *N*-boc protected 4-piperidone in a solvent such as dichloromethane in the presence of a suitable acid such as trifluroacetic acid gives 4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran]-1-ium (**03)** as trifluoroacetate salt which was treated with various arylsulfonyl chlorides, aryl carbonyl chlorides and aryl alkyl chlorides in presence of base such as triethyl amine in appropriate solvent such as DMF and dichlromethane at room temperature to give corresponding crude sulfonamides, amides and alkyl derivatives, which were triturated with methyl *tert* butyl ether to obtain as off-white to brown solids (**04-24**) (**Table 1**).[9-11] The purity of the synthesized compounds was checked by LC-MS and the

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structures were identified by spectral data. In the nuclear magnetic resonance spectra (^{1}H NMR), the signals of the respective protons of the prepared spiro derivatives were verified on the basis of their chemical shifts, multiplicities, and coupling constants.

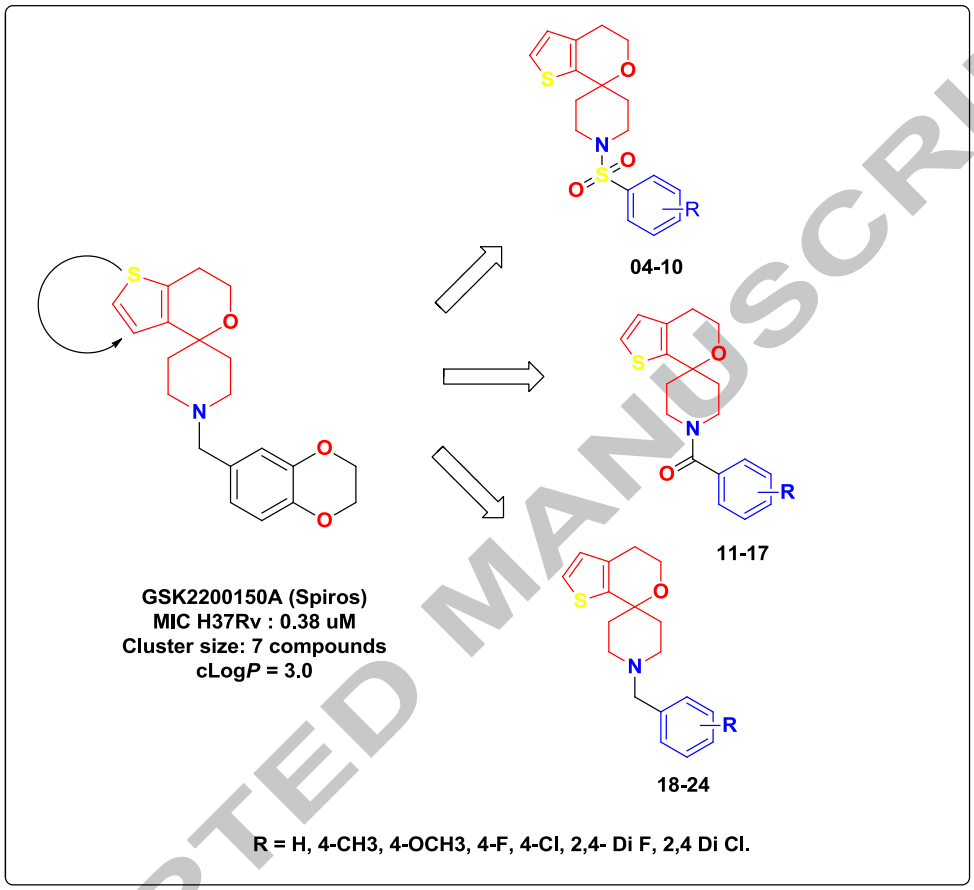

Figure 1: Modifications in lead molecule GSK2200150A by shifting of sulfur atom

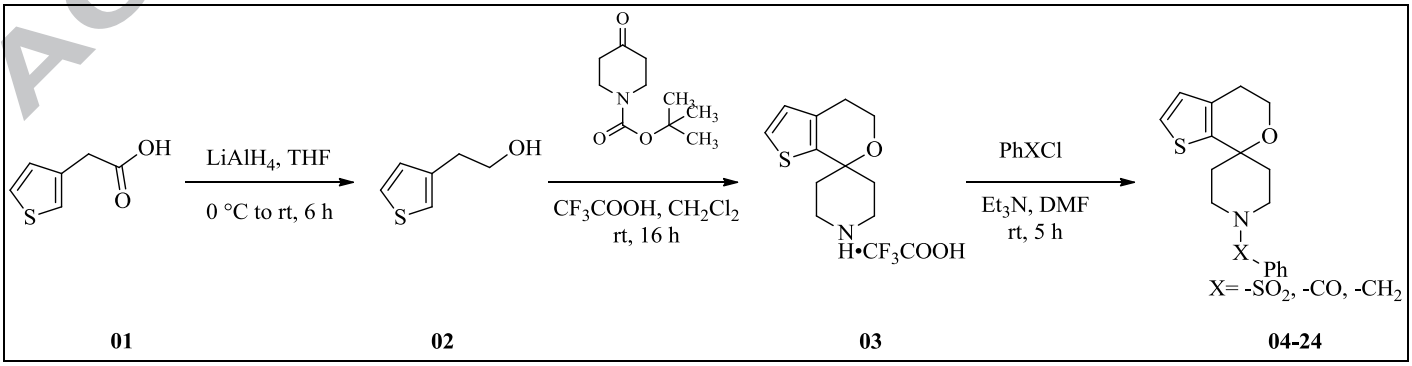

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Figure 2: Synthetic strategy employed for synthesis of final compounds **04-24**.

2.2. Biological evaluation

All the synthesized compounds (**04-24**) showed activity against replicative MTB with MIC ranging from 8.23 to 83.49 µM(**Table 1**). Five compounds (**05, 06, 13, 16** and **22**) showed MIC values less than 10 µM and were more potent than standard first line antitubercular drug Ethambutol. Compound 1-((4-methoxyphenyl)sulfonyl)-4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran] (**6**) was found to be the most potent among the synthesized derivatives with MIC of 8.23 µM and was less active than GSK lead.

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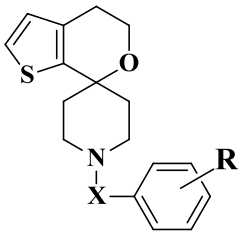
Table 1: Structures and biological activity results of synthesized compounds



04-24

COMPD	X	R	MTB MIC (µM)	Cytotoxicity (% inhib) at 50 µg/ml
04	SO_2	H	71.53	NT
05	SO_2	4-CH_3	8.59	22.16
06	SO_2	4-OCH_3	8.23	12.32
07	SO_2	4-F	34.01	NT
08	SO_2	4-Cl	65.11	NT
09	SO_2	2,4-Di F	16.21	31.08
10	SO_2	2,4-Di Cl	29.87	NT
11	CO	H	79.76	NT
12	CO	4-CH_3	19.08	23.16
13	CO	4-OCH_3	9.09	14.59
14	CO	4-F	18.85	18.98
15	CO	4-Cl	71.86	NT
16	CO	2,4-Di F	8.94	34.06
17	CO	2,4-Di Cl	32.69	NT
18	CH_2	H	83.49	NT
19	CH_2	4-CH_3	79.75	NT
20	CH_2	4-OCH_3	23.14	NT
21	CH_2	4-F	19.69	21.67
22	CH_2	4-Cl	9.35	26.54
23	CH_2	2,4-Di F	37.26	NT
24	CH_2	2,4-Di Cl	33.93	NT

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INH	0.66	NT
Ethambutol	9.84	NT
Ofloxacin	2.16	NT
GSK2200150A	0.38	NT

NT indicates not tested

With regard to structure activity relationship, we have prepared total of twenty-one compounds; seven each with sulfonyl, carbonyl, and methylene bridge (X) between spiro nucleus and aryl ring. In general, sulfonyl and carbonyl bridge showed better activity than methylene bridge. In general substituents in the aryl ring enhances activity. Among the sulfonyl bridged molecules (**04-10**); aryl ring substituents (R) alter the activity considerably. Electron donating substituents like methyl (**05**) and methoxy (**06**) groups showed very potent activity (MIC < 10 µM) than electron withdrawing substituents like chloro and fluoro groups. When compared to mono chloro or mono fluoro substituent at 4^{th} position of aryl ring; di-substitution with same group at 2,4 position enhances activity two times. Similar kind of trend is observed carbonyl bridged compounds (**11-17**) also; whereas in methylene bridged compounds mono fluoro (**21**) and mono chloro (**22**) substituted compounds showed good activity.

Further we have screened selected compounds (each one from sulfonyl, carbonyl and methylene bridged) against nutrient starved dormant TB model. Most antibiotics target biosynthetic processes that bacteria need to increase their biomass. It is not surprising that such antibiotics are more effective against replicating than non-replicating bacteria. However, a major need in global health is to eradicate persistent or non-replicating subpopulations of bacteria such as MTB. Worldwide, an estimated 1 person in 3 is infected with MTB; in about 9 of every 10 infected, MTB persist in a largely non-replicating ("dormant") state throughout the lifetime of the host.[5]

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MTB organisms subjected to stress conditions goes into dormancy phase. Three types of stress were applied hypoxia, nutrient starvation and acidic environment. J.C. Betts et al. earlier established an *in vitro* model, in which nutrient starvation caused *M. tuberculosis* to arrest growth, minimized aerobic metabolism and became resistant to existing antitubercular drugs while maintaining viability.[13] This model shows the changes associated with dormancy such as loss of Ziehl-Neelsen staining, low and continuous type of respiration, resistance to antibiotics. Genes which are found to be up regulated are involved in antibiotic production and resistance, insertion sequence elements, repeated sequences and phage, nucleotide biosynthesis and metabolism, putative enzymes and regulatory function. Nutrient starvation may therefore mimic some of the features of *M. tuberculosis* during the persistent state. In this model the MTB culture is starved in PBS for 6 weeks then treated with inhibitor at a concentration of 10 µg/ml for 7 days.

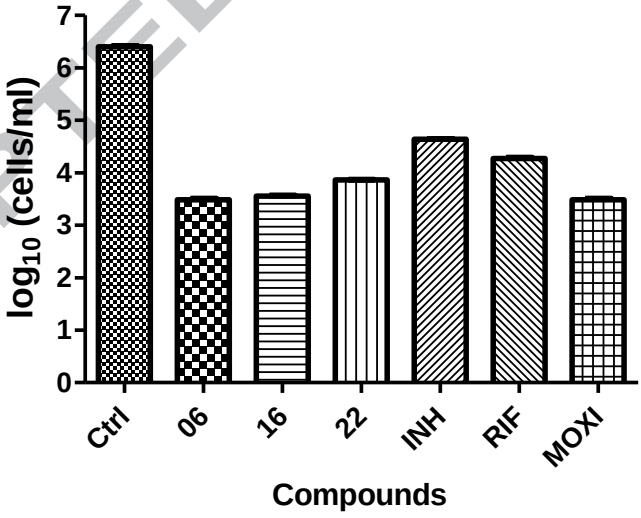

Figure 3: Biological activities of the active compounds against *M. tuberculosis* in the nutrient starvation model. Bacterial count estimation (Mean ± S.D., n = 3) for control and treated groups

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conducted by using the MPN (most probable number) assay. All compounds gave significant inhibition of growth of *M. tuberculosis* in this model as compared to the control ($p < 0.0001$, two way ANOVA using GraphPad Prism Software).

In nutrient starvation model compound **06** and **16** show 2.8 and 2.7 log fold reduction in bacterial count equipotent to standard drug Moxifloxacin and more potent than first line drugs Isoniazid (1.7 fold) and Rifampicin (2.0 fold). Compound **22** shows 2.4 log reduction which is potent than standard drugs Isoniazid and Rifampicin (**Fig 3**). These results suggest that Compound **06** is not only able to combat active stages but also dormant forms of MTB which is helpful in lowering the duration of therapy.

To get insight into mechanism of action of inhibitors the most active compounds in nutrient starvation were evaluated for inhibitory potential against lysine Ɛ-amino transferase enzyme. The reason behind selection of this enzyme is it is overexpressed 40 times in nutrient starvation model and has pivotal role in persistence and antibiotic tolerance. LAT is a PLP dependent type II aminotransferase enzyme which catalyzes reversible transamination from lysine to α-ketoglutaric acid resulting in piperidine-6-carboxylic acid and glutamate. The most active compound in nutrient starvation compound **06** shows correlating IC_{50} of 1.04 ± 0.32 µM. Compound **16** and **22** exhibits IC_{50} values of 3.48 ± 0.74 µM and 6.83 ± 0.54µM.

Docking studies of these compounds in lysine and ketoglutarate active sites of LAT (PDB code: 2CJHand 2CJD) revealed that compounds**06, 16**and **22** are targeting lysine binding site (**Fig 4**). Compound **06** has retained crucial interactions with active site by four hydrogen bonds. Dihydropyran is involved in hydrogen bonding with Lys 300, the phenyl group mediates hydrogen bond with Phe 167, sulfonamide is involved in interaction with Ala 129 and methoxy

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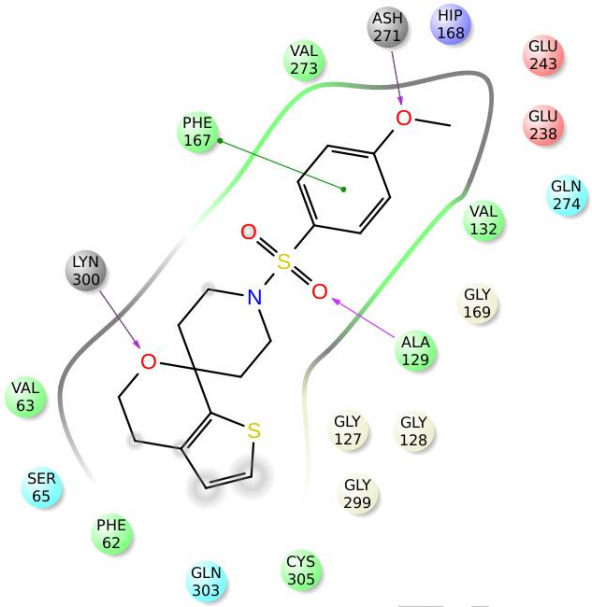
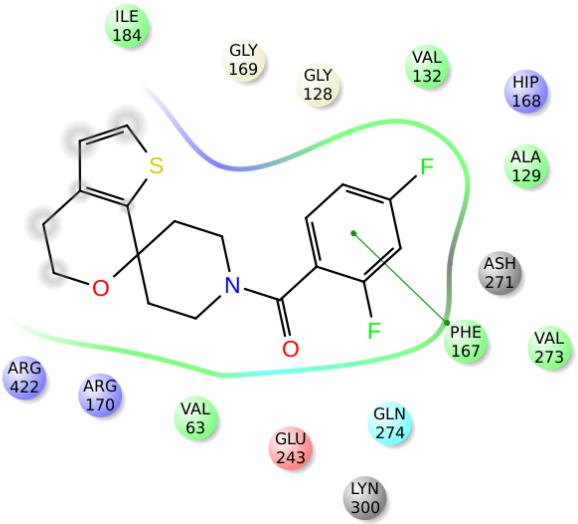
group participates in interaction with Asp 271 in compound **06**. Amide derivative **16** with difluoro substitution has only one interaction with Phe 167 but fits into active site. In case of N-alkyl derivative **22** there are 2 hydrogen interactions in active site. The dihydropyran ring participates in hydrogen bonding with Lys 300 and phenyl ring is involved in interaction with Phe 167.





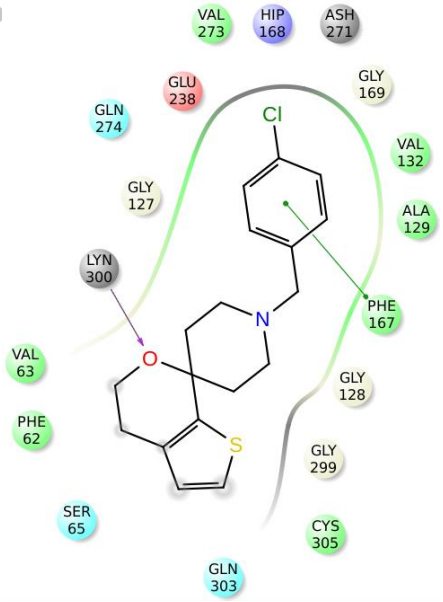


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Figure 4: Interaction profile of Compounds **06, 16** and **22** with lysine binding site of LAT

The safety profile of all the synthesized compounds was evaluated by testing there *in vitro* cytotoxicity in mouse macrophage cell line (RAW 264.7) cells at 50 µg/mL concentration using (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Since MTB reside inside the macrophage, the monocyte macrophage cell lines (RAW 264.7) were mostly used for tuberculosis research; in order to check whether the screened compounds were not toxic towards macrophages but toxic to the bacteria. Compound **06** showed 12.32 % inhibition which suggests that compound does not have cytotoxicity at highest concentration tested.

3. Conclusion

As there is growing need for new antimycobacterial agents we attempted to derivatizespiro lead reported in the literature by shifting of sulfur atom position in the spiro nucleus. Among series of twenty-one compounds, compound **06**, 1-((4-methoxyphenyl)sulfonyl)-4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran] was found to be effective than standard drug ethambutol in terms of MIC. It was also found to be more efficacious than Isoniazid and Rifampicin and equipotent as Moxifloxacin against dormant stages of Mtb. Compound **06**also shows good inhibitory potential against latent target LAT with an IC_{50} of 1.04 ± 0.32 µM. As it has minimal cytotoxicity at higher concentration it can be taken for further studies for development of new drugs in area of TB.

4. Experimental Section

4.1. Chemistry

4.1.1. General

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Reagents were purchased from commercial sources and were used as received. Proton nuclear magnetic resonance spectra were obtained on a Bruker AVANCE 400 spectrometer at 400 MHz with tetramethylsilane used as an internal reference. Carbon nuclear magnetic resonance spectra were obtained on a Bruker AVANCE 300 spectrometer at 100 MHz with the solvent peak used as the reference. Thin-layer chromatography (TLC) was performed using Whatman No.4500-101 (Diamond No MK6F silica-gel 60 A˚) plates. Visualization of TLC plates was performed using UV light (254 nm).

4.1.2. 2-(Thiophen-3-yl)ethanol (02): To a 0 °C cooled stirred suspension of lithium aluminium hydride (8.0 g, 0.21 mol) in THF (100 mL) was added 3-Thiopheneacetic acid **01**(20 g, 0.14 mol) in THF (100 mL) drop wise over 1 h. Reaction mixture was warmed to room temperature and stirred for 6 h. Reaction progress was checked by TLC for completion and then quenched with 10% NaOH solution. The mixture was filtered over celite, washed with ethylacetate. Aqueous layer was extracted with ethylacetate and the combined organic layer was washed with brine. Dried over sodium sulfate and concentrated to yield **2** as a yellow liquid (14.2 g, 78%) ^{1}H NMR (MeOD, 400 MHz): δ 5.92- 5.87 (m, 1H), 5.69- 5.66 (m, 1H), 5.57 (s, 1H) 2.90 (d, J = 14.0 Hz, 1H), 2.38 (d, J = 14 Hz, 1H), 1.55- 1.42 (m, 2H). ^{13}C NMR (DMSO-d_6, 100 MHz): 145.53, 129.13, 126.54, 121.45, 64.46, 38.85; ESI-MS *m/*z: (Calcd for C_6H_8OS: 128.03); Found: 128.9 $[(M+H]^+$.

4.1.3. 4',5'-Dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran]-1-ium trifluoroacetate (03): To a solution of **02** (50 g, 0.39 mol) in dichloromethane (500 mL) was added *N*-tert-butoxycarbonyl-4-piperidone (85.4 g, 0.43 mol) and stirred for 10 min. at room temperature. Reaction mixture was cooled to 0-5 °C then trifluoroacetic acid (133 g, 1.17 mol) was added drop wise over 30 min. Reaction mixture was warmed to room temperature and stirred for 16 h. Reaction progress

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was monitored by TLC. When TLC showed complete consumption of starting material, reaction mixture was concentrated under reduced pressure to yield crude compound and it was triturated with methyl tert-butyl ether, filtered and washed to obtain 4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran]-1-ium trifluoroacetate**03** as beige solid (115 g, 91%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 9.73 (br s, 1H), 9.24 (br s, 1H), 7.20 (d, *J* = 4.8 Hz 1H), 6.79 (d, *J* = 5.2 Hz, 1H), 3.90 – 3.93 (m, 2H), 3.26 – 3.36 (m, 4H), 2.70 – 2.73 (m, 2H), 2.12 – 2.27 (m, 4H); ^{13}C NMR (DMSO-d_6, 100 MHz): 203.03, 158.89, 139.53, 133.54, 127.13, 123.54, 70.54, 58.98, 42.21, 38.85, 37.30, 34.20, 25.76; ESI-MS *m/*z: (Calcd for $C_{13}H_{16}F_3NO_3S$: 323.08); Found: 210.1 [(M –CF_3COOH) +H] $^{+}$.

4.1.4. General procedure for the synthesis of sulfonamides (04-10): To a solution of 4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran]-1-ium trifluoroacetate (**03**) (200 mg, 0.62 mmol) in DMF (5 mL) was added triethylamine (187 mg, 1.86 mmol) followed by benzene sulfonylchloride (0.685 mmol) at ambient temperature. The reaction mixture was stirred for 5 h at room temperature. When TLC showed complete consumption of starting material, the reaction mixture was poured on ice-cold water under stirring and then filtered, washed with water, dried under vacuum at 60 °C for 2 h. The crude product was triturated with *tert*-butylmethyl ether to obtain pure corresponding substituted 1-(phenylsulfonyl)-4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran] **(04-10)** in good yield.

4.1.4.1. 1-(Phenylsulfonyl)-4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran] (04): The compound was synthesized according to the general procedure using 4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran]-1-ium trifluoroacetate (**03**) (200 mg, 0.62 mmol) and benzenesulfonyl chloride (120 mg, 0.685 mmol) to obtain **04** as off white solid (190 mg, 88%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.81 – 7.78 (m, 2 H), 7.64 – 7.52 (m, 3H), 7.14 (d, *J* =

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4.8 Hz, 1H), 6.74 (d, *J* = 5.1 Hz, 1H), 3.78 (t, *J* = 5.4 Hz, 2H), 3.72 – 3.68 (m, 2H), 2.75 – 2.52 (m, 4H), 2.07 – 1.95 (m, 4H) ppm; ^{13}C NMR (DMSO-d_6, 100 MHz): 141.62, 138.36, 131.54, 127.13, 125.61, 124.13, 123.64, 122.25, 121.85, 120.39, 69.54, 55.98, 52.23, 43.21, 41.10, 35.85, 30.20, 23.74; ESI-MS *m/z*: (Calcd for $C_{17}H_{19}NO_3S_2$: 349.08); Found: 349.8 $[M + H]^+$.

4.1.4.2. 1-Tosyl-4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran] (05): The compound was synthesized according to the general procedure using 4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran]-1-ium trifluoroacetate (**03**) (200 mg, 0.62 mmol) and 4-methylbenzene-1-sulfonyl chloride (130 mg, 0.685 mmol) to obtain **05** as light yellow solid (123 mg, 55%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.67 (d, *J* = 7.8 Hz, 2H), 7.34 (d, *J* = 7.8 Hz, 2H), 7.13 (d, *J* = 4.5 Hz, 1H), 6.73 (d, *J* = 4.5 Hz, 1H), 3.78 (t, *J* = 4.8 Hz, 2H), 3.69 – 3.66 (m, 2H), 2.78 – 2.61 (m, 4H), 2.45 (s, 3H), 2.12 – 1.95 (m, 4H) ppm;^{13}C NMR (DMSO-d_6, 100 MHz): 141.62, 140.23, 139.53, 138.36, 131.54, 127.13, 122.25, 114.61, 70.54, 55.98, 42.21, 41.10, 36.85, 31.20, 28.54, 24.74; ESI-MS *m/z*: (Calcd for $C_{18}H_{21}NO_3S_2$: 363.1); Found: 363.9 $[M + H]^+$; HRMS *m/z* $[M + H]^+$ Calcd for $C_{18}H_{22}O_3NS_2$: 364.1035, found: 364.1036.

4.1.4.3. 1-((4-Methoxyphenyl)sulfonyl)-4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran] (06): The compound was synthesized according to the general procedure using 4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran]-1-ium trifluoroacetate (**03**) (200 mg, 0.62 mmol) and 4-methoxybenzene-1-sulfonyl chloride (141 mg, 0.685 mmol) to obtain **06** as a light yellow solid (168 mg, 72%). ^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.70 (d, *J* = 8.4 Hz, 2H), 7.38 (d, *J* = 5.1 Hz, 1H), 7.17 (d, *J* = 8.4 Hz, 2H), 6.81 (d, *J* = 4.8 Hz, 1H), 3.87 (s, 3H), 3.71 (t, *J* = 4.8 Hz, 2H), 3.54 – 3.50 (m, 2H), 2.57 – 2.56 (m, 4H), 2.02 – 1.97 (m, 2H), 1.84 – 1.74 (m, 2H) ppm;^{13}C NMR (DMSO-d_6, 100 MHz): 160.88, 141.62, 138.36, 131.54, 127.13, 122.25, 121.85, 114.61, 69.54, 56.98, 53.23, 44.21, 41.10, 36.85, 31.20, 23.74; ESI-MS *m/z*: (Calcd for

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$C_{18}H_{21}NO_4S_2$: 379.09); Found: 379.9 $[M + H]^+$; HRMS *m/z* $[M + H]^+$ Calcd for $C_{18}H_{22}O_4NS_2$: 380.0984, found: 380.0987.

4.1.4.4. 1-((4-Fluorophenyl)sulfonyl)-4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran] (07): The compound was synthesized according to the general procedure using 4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran]-1-ium trifluoroacetate (**03**) (200 mg, 0.62 mmol) and 4-fluorobenzene-1-sulfonyl chloride (133 mg, 0.685 mmol) to obtain **07** as a light brown solid (94 mg, 41%). ^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.88 – 7.84 (dd, *J* = 8.4 Hz, 5.4 Hz, 2H), 7.52 (t, *J* = 9.0 Hz, 2H), 7.39 (d, *J* = 5.1 Hz, 1H), 6.81 (d, *J* = 5.1 Hz, 1H), 3.74 (t, *J* = 5.1 Hz, 2H), 3.58 – 3.54 (m, 2H), 2.58 – 2.54 (m, 4H), 2.08 – 1.98 (m, 2H), 1.84 – 1.75 (m, 2H) ppm; ^{13}C NMR (DMSO-d_6, 100 MHz): 159.89, 141.65, 139.53, 138.36, 131.54, 127.13, 122.25, 114.61, 69.54, 57.98, 42.21, 40.10, 38.85, 34.20, 24.76; ESI-MS *m/z*: (Calcd for $C_{17}H_{18}FNO_3S_2$: 367.07); Found: 367.8 $[M + H]^+$.

4.1.4.5. 1-((4-Chlorophenyl)sulfonyl)-4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran] (08): The compound was synthesized according to the general procedure using 4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran]-1-ium trifluoroacetate (**03**) (200 mg, 0.62 mmol) and 4-chlorobenzene-1-sulfonyl chloride (144 mg, 0.685 mmol) to obtain **08** as light yellow solid (110 mg, 47%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.41 – 7.22 (m, 4H), 7.16 (d, *J* = 5.1 Hz, 1 H), 6.76 (d, *J* = 4.8 Hz, 1H), 3.86 (t, *J* = 5.4 Hz, 2H), 3.76 – 3.72 (m, 2H), 3.19 – 3.12 (m, 2H), 2.68 (t, *J* = 5.4 Hz, 2H), 2.07 – 1.95 (m, 4H) ppm; ^{13}C NMR (DMSO-d_6, 100 MHz): 152.89, 141.65, 139.53, 138.36, 135.23, 131.54, 127.13, 122.25, 72.52, 56.96, 41.21, 40.25, 38.85, 34.20, 24.76; ESI-MS *m/z*: (Calcd for $C_{17}H_{18}ClNO_3S_2$: 383.04); Found: 383.9 $[M+H]^+$.

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4.1.4.6. 1-((2,4-Difluorophenyl)sulfonyl)-4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran] (09): The compound was synthesized according to the general procedure using 4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran]-1-ium trifluoroacetate (**03**) (200 mg, 0.62 mmol) and 2,4-difluorobenzene-1-sulfonyl chloride (145 mg, 0.685 mmol) to obtain **09**as offwhite solid (69 mg, 29%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.95 – 7.84 (m, 1H), 7.16 (d, *J* = 5.1 Hz, 1 H), 7.04 – 6.95 (m, 2H), 6.76 (d, *J* = 4.8 Hz, 1H), 3.84 (t, *J* = 5.4 Hz, 2H), 3.77 – 3.73 (m, 2H), 2.98 – 2.91 (m, 2H), 2.67 (t, *J* = 5.4 Hz, 2H), 2.09 – 1.94 (m, 4H) ppm; ^{13}C NMR (DMSO-d_6, 100 MHz): 160.88, 155.65, 141.62, 139.53, 138.36, 131.54, 127.13, 122.25, 121.85, 114.61, 69.54, 56.98, 43.21, 41.10, 37.85, 31.20, 24.74; ESI-MS *m/z*: (Calcd for $C_{17}H_{17}F_2NO_3S_2$: 385.06); Found: 385.8$[M+H]^+$.

4.1.4.7. 1-((2,4-Dichlorophenyl)sulfonyl)-4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran] (10): The compound was synthesized according to the general procedure using 4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran]-1-ium trifluoroacetate**(03**) (200 mg, 0.62 mmol) and 2,4-dichlorobenzene sulfonylchloride (0.685 mmol) to obtain **10**as off white solid (167 mg, 67%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.92 (s, 1H), 7.78 (d, *J* = 4.8 Hz, 1 H), 7.54 – 7.48 (dd, *J* = 5.1 Hz, *J* = 3.2 Hz, 1 H), 7.14 (d, *J* = 5.1 Hz, 1 H), 6.71 (d, *J* = 4.8 Hz, 1H), 3.82 (t, *J* = 5.4 Hz, 2H), 3.71 – 3.68 (m, 2H), 3.15 – 3.10 (m, 2H), 2.64 (t, *J* = 5.4 Hz, 2H), 2.01 – 1.92 (m, 4H) ppm; ^{13}C NMR (DMSO-d_6, 100 MHz): 158.89, 142.65, 139.53, 138.36, 135.23, 133.54, 131.54, 129.11, 127.13, 122.25, 70.54, 58.98, 42.21, 40.10, 38.85, 34.20, 25.76; ESI-MS *m/z*: (Calcd for $C_{17}H_{17}Cl_2NO_3S_2$: 417.00); Found: 417.9 $[M+H]^+$.

4.1.5. General procedure for the synthesis of amide derivatives (11-17): To a solution of 4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran]-1-ium trifluoroacetate (**03**) (200 mg, 0.62 mmol) in DMF (5 mL) was added triethylamine (187 mg, 1.86 mmol) followed by various

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benzoyl chlorides at ambient temperature. The reaction mixture was stirred for 5 h at room temperature. When TLC showed complete consumption of starting material, the reaction mixture was poured on ice-cold water under stirring and then filtered, washed with water, dried under vacuum at 60 °C for 1 h. The crude product was triturated with tert-butylmethyl ether to obtain corresponding substituted (4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran]-1-yl)(phenyl)methanone **(11-17)** in good yield.

4.1.5.1. (4',5'-Dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran]-1-yl)(phenyl)methanone (**11**): The compound was synthesized according to the general procedure using 4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran]-1-ium trifluoroacetate (**03**) (200 mg, 0.62 mmol) and benzoyl chloride (96 mg, 0.685 mmol) to obtain **11**as off white solid (146 mg, 76%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.17 – 8.09 (m, 2H), 7.48 – 7.17 (m, 3 H), 7.16 (d, *J* = 5.1 Hz, 1H), 6.77 (d, *J* = 5.1 Hz, 1H), 3.95 – 3.94 (m, 2H), 3.62 – 3.18 (m, 4 H), 2.72 (t, *J* = 5.2 Hz, 2H), 2.16 – 1.79 (m, 4H) ppm; ^{13}C NMR (DMSO-d_6, 100 MHz): 168.36, 142.62, 140.36, 131.65, 128.25, 125.61, 124.23, 123.64, 122.25, 120.85, 120.39, 69.54, 55.98, 52.23, 43.21, 41.10, 35.85, 30.20, 23;ESI-MS *m/z*: (Calcd for $C_{18}H_{19}NO_2S$: 313.11); Found: 313.9 $[M + H]^+$.

4.1.5.2. (4',5'-Dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran]-1-yl)(p-tolyl)methanone (**12**): The compound was synthesized according to the general procedure using 4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran]-1-ium trifluoroacetate (**03**) (200 mg, 0.62 mmol) and 4-methylbenzoyl chloride (105 mg, 0.685 mmol) to obtain **12** as light yellow solid (162 mg, 80%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.35 (d, *J* = 8.1 Hz, 2H), 7.21 (d, *J* = 8.1 Hz, 1H), 7.15 (d, *J* = 5.1 Hz, 2H), 6.77 (d, *J* = 5.1 Hz, 1H), 3.96 – 3.92 (m, 2H), 3.67 – 3.30 (m, 2H), 2.73 – 2.69 (m, 2H), 2.37 (s, 3H), 2.16 – 1.66 (m, 6H) ppm; ^{13}C NMR (DMSO-d_6, 100 MHz): 169.64, 141.62, 138.36, 135.62, 131.54, 127.13, 124.13, 123.64, 122.25, 69.54, 55.98, 42.21,

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41.10, 35.85, 30.20, 25.74, 21.63; ESI-MS *m/z*: (Calcd for $C_{19}H_{21}NO_2S$: 327.13); Found: 327.9 $[M + H]^+$.

4.1.5.3.(4',5'-Dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran]-1-yl)(4-methoxyphenyl)methanone (**13**): The compound was synthesized according to the general procedure using 4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran]-1-ium trifluoroacetate (**03**) (200 mg, 0.62 mmol) and 4-methoxybenzoyl chloride (116 mg, 0.685 mmol) to obtain **13**as off white solid (174 mg, 82%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.72 (d, *J* = 8.5 Hz, 2H), 7.14 (d, *J* = 5.1 Hz, 1H), 6.99 (d, *J* = 8.4 Hz, 2H), 6.74 (d, *J* = 5.1 Hz, 1H), 3.89 (s, 3H), 3.81 – 3.77 (m, 2H), 3.77 – 3.68 (m, 2H), 2.71 – 2.53 (m, 4H), 2.04 – 2.00 (m, 4H) ppm; ^{13}C NMR (DMSO-d_6, 100 MHz): 171.56, 160.88, 140.62, 137.36, 131.54, 128.13, 123.25, 121.85, 115.61, 69.54, 56.98, 53.42, 45.21, 41.10, 36.85, 31.20, 23.74; ESI-MS *m/z*: (Calcd for $C_{19}H_{21}NO_3S$: 343.12); Found: 343.9 $[M + H]^+$; HRMS *m/z* $[M + H]^+$ Calcd for $C_{19}H_{22}O_3NS$: 344.1314, found: 344.1308.

4.1.5.4. (4',5'-Dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran]-1-yl)(4-fluorophenyl)methanone (14): The compound was synthesized according to the general procedure using 4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran]-1-ium trifluoroacetate (**03**) (200 mg, 0.62 mmol) and 4-fluorobenzoyl chloride (158 mg, 0.685 mmol) to obtain **14** as off white solid (115 mg, 56%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.85 (dd, *J* = 4.8 Hz, 1.2 Hz, 2H), 7.35 (dd, *J* = 4.4 Hz, 1.2 Hz, 2H), 7.15 (d, *J* = 5.1 Hz, 1H), 6.74 (d, *J* = 5.1 Hz, 1H), 3.88 (t, *J* = 5.4 Hz , 2H), 2.71 – 2.65 (m, 4H), 2.46 – 2.38 (m, 2H), 2.06 – 2.01 (m, 4H) ppm; ; ^{13}C NMR (DMSO-d_6, 100 MHz): 169.54, 157.89, 141.65, 138.53, 138.36, 131.54, 126.13, 122.25, 114.61, 69.54, 57.98, 42.21, 40.10, 38.85, 34.20, 24.76; ESI-MS *m/z*: (Calcd for $C_{18}H_{18}FNO_2S$: 331.10); Found: 331.9 $[M + H]^+$.

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4.1.5.5. (4-Chlorophenyl)(4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran]-1-yl)methanone(**15**): The compound was synthesized according to the general procedure using 4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran]-1-ium trifluoroacetate (**03**) (200 mg, 0.62 mmol) and 4-chlorobenzoyl chloride (120 mg, 0.685 mmol) to obtain **15** as light yellow solid (174 mg, 81%).^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.32 – 7.30 (m, 4H), 7.13 (d, *J* = 5.1 Hz, 1H), 6.75 (d, *J* = 5.1 Hz, 1H), 3.91 (t, *J* = 5.4 Hz , 2H), 2.73 – 2.67 (m, 4H), 2.49 – 2.40 (m, 2H), 2.01 – 1.93 (m, 4H) ppm; ^{13}C NMR (DMSO-d_6, 100 MHz): 168.63, 155.89, 142.65, 139.53, 137.36, 135.23, 131.54, 127.13, 122.25, 72.52, 56.96, 41.21, 40.25, 38.85, 34.20, 24.76; ESI-MS *m/*z: (Calcd for $C_{18}H_{18}ClNO_2S$: 347.07); Found: 347.9 $[M + H]^+$.

4.1.5.6. (2,4-Difluorophenyl)(4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran]-1-yl)methanone (16): The compound was synthesized according to the general procedure using 4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran]-1-ium trifluoroacetate (03) (200 mg, 0.62 mmol) and 2,4-difluorobenzoyl chloride (120 mg, 0.685 mmol) to obtain **16** as a brown solid (85 mg, 39%). ^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.56 (bs, 1H), 7.42 – 7.35 (m, 2H), 7.21 – 7.17 (td, *J* = 2.0 Hz, 8.4 Hz, 1H), 6.85 (d, *J* = 4.2 Hz, 1H), 4.43 (d, *J* = 13.2 Hz, 1H), 3.94 – 3.88 (m, 2H), 3.43 – 3.26 (m, 2H), 3.10 – 3.04 (m, 1H), 2.89 – 2.87 (m, 1H), 2.66 – 2.63 (m, 2H), 2.09 – 1.71 (m, 3H) ppm; ^{13}C NMR (DMSO-d_6, 100 MHz): 163.12, 140.37, 133.41, 133.20, 130.39, 127.08, 123.40, 123.26, 120.79, 112.30, 104.34, 72.33, 58.75, 42.69, 38.01, 37.38, 37.23, 25.91; ESI-MS *m/*z: (Calcd for $C_{18}H_{17}F_2NO_2S$: 383.04); Found: 349.9 $[M + H]^+$.

4.1.5.7. (2,4-Dichlorophenyl)(4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran]-1-yl)methanone (**17**): The compound was synthesized according to the general procedure using 4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran]-1-ium trifluoroacetate (**03**) (200 mg, 0.62 mmol) and 2,4-dichlorobenzoyl chloride (143 mg, 0.685 mmol) to obtain **17** as off white solid

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(180 mg, 80%) ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.53 (d, *J* = 2.1 Hz, 1H), 7.35 – 7.24 (m, 2 H), 7.20 (d, *J* = 8.1 Hz, 1H), 6.64 (d, *J* = 5.1 Hz, 1H),), 3.80 – 3.76 (m, 2H), 3.74 – 3.63 (m, 2H), 2.68 – 2.49 (m, 4H), 2.09 – 2.02 (m, 4H) ppm; ^{13}C NMR (DMSO-d_6, 100 MHz): 170.24, 158.89, 142.65, 139.53, 138.36, 135.23, 133.54, 131.54, 129.11, 127.13, 122.25, 70.54, 58.98, 42.21, 40.10, 38.85, 34.20, 25.76; ESI-MS *m/z*: (Calcd for $C_{18}H_{17}Cl_2NO_2S$: 381.04); Found: 381.8 $[M + H]^+$.

4.1.6. General procedure for the synthesis of N-alkylated derivatives (18-24): To a solution of 4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran]-1-ium trifluoroacetate (**03**) (200 mg, 0.62 mmol) in DMF (5 mL) was added triethylamine (187 mg, 1.86 mmol) followed by various benzyl chlorides at ambient temperature. The reaction mixture was stirred for 16 h at room temperature. When TLC showed complete consumption of starting material, the reaction mixture was poured on ice-cold water and was extracted with dichloromethane for 2 times (2 × 15 mL). The combined organic layers were washed with water (10 mL) and brine (10 mL). The organic layers were dried over anhydrous sodium sulfate and concentrated under reduced pressure. The crude product was purified by column chromatography over 100-200 mesh silica-gel by using 15 % ethylacetate-n-hexane as eluant to obtain corresponding substituted 1-benzyl-4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran] **(18-24)** in good yield.

4.1.6.1. 1-Benzyl-4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran] (**18**): The compound was synthesized according to the general procedure using 4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran]-1-ium trifluoroacetate (**03**) (200 mg, 0.62 mmol) and benzyl chloride (86 mg, 0.685 mmol) to afford the crude product and it was purified by column chromatography over 100-200 mesh silica-gel by using 15 % ethylacetate-n-hexane as eluant to obtain **18**as off white solid (142 mg, 76%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.17 – 8.09 (m, 2H), 7.48 – 7.17 (m, 3 H),

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7.16 (d, *J* = 5.1 Hz, 1H), 6.77 (d, *J* = 5.1 Hz, 1H), 3.95 – 3.94 (m, 2H), 3.60 (s, 2H), 3.62 – 3.18 (m, 4 H), 2.72 (t, *J* = 5.2 Hz, 2H), 2.16 – 1.79 (m, 4H) ppm; ^{13}C NMR (DMSO-d_6, 100 MHz): 141.62, 138.36, 131.54, 127.13, 125.61, 124.13, 123.64, 122.25, 121.85, 120.39, 69.54, 55.98, 52.23, 43.21, 41.10, 35.85, 30.20, 23.74.ESI-MS *m/z*: (Calcd for $C_{18}H_{21}NOS$: 299.13); Found: 299.9 [M + H]

4.1.6.2. 1-(4-Methylbenzyl)-4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran] (19): The compound was synthesized according to the general procedure using 4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran]-1-ium trifluoroacetate (**03**) (200 mg, 0.62 mmol) and 1-(chloromethyl)-4-methylbenzene (96 mg, 0.685 mmol) to obtain the crude product and it was purified by column chromatography over 100-200 mesh silica-gel by using 12 % ethylacetate-n-hexane as eluant to obtain **19**as off white solid (171 mg, 88%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.12 (d, *J* = 5.1 Hz, 1H), 7.08 (d, *J* = 4.6 Hz, 2H), 7. 01 (d, *J* = 4.6 Hz, 1H), 6.74 (d, *J* = 4.8 Hz, 1H), 3.92 (t, *J* = 5.7 Hz, 2H), 3.64 (s, 2H), 2.80 – 2.68 (m, 4H), 2.32 (s, 3H), 2.63 – 2.44 (m, 2H), 2.00 – 1.97 (m, 4H) ppm; ^{13}C NMR (DMSO-d_6, 100 MHz): 142.62, 139.22, 138.53, 137.36, 131.54, 127.13, 125.25, 121.61, 69.54, 64.46, 54.84, 42.21, 40.10, 35.85, 32.20, 27.54, 24.74; ESI-MS *m/z*: (Calcd for $C_{19}H_{23}NOS$: 313.15); Found: 313.9 $[M + H]^+$.

4.1.6.3. 1-(4-Methoxybenzyl)-4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran] (20): The compound was synthesized according to the general procedure using 4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran]-1-ium trifluoroacetate (**03**) (200 mg, 0.62 mmol) and 1-(chloromethyl)-4-methoxybenzene (106 mg, 0.895 mmol) to afford the crude product and it was purified by column chromatography over 100-200 mesh silica-gel by using 18 % ethylacetate-n-hexane as eluant to obtain **20**as off white solid (169 mg, 84%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.25 (d, *J* = 6.8 Hz, 2H), 7.13 (d, *J* = 4.8 Hz, 1H),7.00 (d, *J* = 7.1 Hz , 2H),

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6.72 (d, *J* = 4.8 Hz, 1H), 3.91 (t, , *J* = 6.9 Hz, 2H), 3.83 (s, 3H), 3.54 (s, 2H), 2.71 – 2.65 (m, 4H), 2.42 – 2.39 (m, 2H), 2.01 – 1.95 (m, 4H) ppm; ^{13}C NMR (DMSO-d_6, 100 MHz): 158.67, 142.62, 137.36, 130.53, 127.13, 123.25, 122.85, 114.61, 69.54, 64.23, 56.98, 53.23, 44.21, 41.10, 36.85, 31.20, 23.74; ESI-MS *m/*z: (Calcd for $C_{19}H_{23}NO_2S$: 329.14); Found: 329.9 $[M + H]^+$.

4.1.6.4. 1-(4-Fluorobenzyl)-4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran] (21): The compound was synthesized according to the general procedure using 4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran]-1-ium trifluoroacetate (**03**) (200 mg, 0.62 mmol) and 1-(chloromethyl)-4-fluorobenzene (99 mg, 0.685 mmol) to afford the crude product and it was purified by column chromatography over 100-200 mesh silica-gel by using 10 % ethylacetate-n-hexane as eluant to obtain **21** as a light yellow solid (124 mg, 63%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.35 – 7.30 (dd, *J* = 8.4 Hz, *J* = 5.7 Hz, 2H), 7.12 (d, *J* = 4.8 Hz, 1H),7.00 (t, *J* = 8.7 Hz , 2H), 6.75 (d, *J* = 4.8 Hz, 1H), 3.91 (t, , *J* = 6.9 Hz, 2H), 3.54 (s, 2H), 2.73 – 2.67 (m, 4H), 2.47 – 2.39 (m, 2H), 2.01 – 1.97 (m, 4H) ppm; ^{13}C NMR (DMSO-d_6, 100 MHz): 158.71, 142.55, 138.43, 137.36, 132.54, 126.13, 122.25, 114.61, 69.54, 63.54, 56.61, 42.21, 40.10, 36.85, 34.20, 23.76; ESI-MS *m/*z: (Calcd for $C_{18}H_{20}FNOS$: 317.12); Found: 317.9 $[M + H]^+$.

4.1.6.5. 1-(4-Chlorobenzyl)-4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran] (22): The compound was synthesized according to the general procedure using 4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran]-1-ium trifluoroacetate (**03**) (200 mg, 0.62 mmol) and 1-chloro-4-(chloromethyl)benzene (110 mg, 0.685 mmol) to afford the crude product and it was purified by column chromatography over 100-200 mesh silica-gel by using 10 % ethylacetate-n-hexane as eluant to obtain **22**as off white solid (159 mg, 77%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.38 – 7.5 (m, 5H), 6.81 (d, *J* = 5.2 Hz, 1H), 3.84 (t, *J* = 5.6 Hz, 2H), 3.60 (bs,

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2H), 2.60 (t, *J* = 5.6 Hz, 4H), 2.35 – 2.32 (m, 2H), 1.95 – 1.92 (m, 2H), 1.81 – 1.78 (m, 2H) ppm; ^{13}C NMR (DMSO-d_6, 100 MHz): 148.89, 142.65, 139.22, 137.35, 134.23, 131.26, 126.13, 122.25, 72.52, 65.21, 55.96, 42.21, 40.25, 37.85, 34.20, 23.76; ESI-MS *m/z*: (Calcd for $C_{18}H_{20}ClNOS$: 333.10); Found: 333.9 $[M + H]^+$; HRMS *m/z* $[M + H]^+$ Calcd for $C_{18}H_{21}ONClS$: 334.1026, found: 334.1021.

4.1.6.6. 1-(2,4-Difluorobenzyl)-4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran] (**23**): The compound was synthesized according to the general procedure using 4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran]-1-ium trifluoroacetate (**03**) (200 mg, 0.62 mmol) and 1-(chloromethyl)-2,4-difluorobenzene (110 mg, 0.685 mmol) to afford the crude product was purified by column chromatography over 100-200 mesh silica-gel by using 15 % ethylacetate-n-hexane as eluant to obtain **23** as light yellow solid (126 mg, 60%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.43 – 7.35 (dd, *J* = 15.3 Hz, *J* = 6.9 Hz, 1H), 7.12 (d, *J* = 4.8 Hz, 1H), 6.88 – 6.76 (m, 2H), 6.75 (d, *J* = 2.1 Hz, 1H), 3.90 (t, *J* = 6.9 Hz, 2H), 3.60 (s, 2H), 2.79 – 2.67 (m, 4H), 2.53 – 2.44 (m, 2H), 2.04 – 1.93 (m, 4H) ppm; ^{13}C NMR (DMSO-d_6, 100 MHz): 158.88, 153.65, 140.62, 139.53, 138.36, 130.54, 128.13, 123.25, 121.85, 114.61, 72.54, 65.21, 59.62, 42.21, 40.10, 36.83, 30.20, 23.74; ESI-MS *m/z*: (Calcd for $C_{18}H_{19}F_2NOS$: 335.12); Found: 335.9 $[M + H]^+$.

4.1.6.7. 1-(2,4-Dichlorobenzyl)-4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran] (24): The compound was synthesized according to the general procedure using 4',5'-dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran]-1-ium trifluoroacetate (**03**) (200 mg, 0.62 mmol) and 2,4-dichloro-1-(chloromethyl)benzene (133 mg, 0.685 mmol) to afford the crude product and it was purified by column chromatography over 100-200 mesh silica-gel by using 12 % ethyl acetate-n-hexane as eluant to obtain **24** as a brown solid (145 mg, 64%). ^{1}H NMR

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($CDCl_3$, 400 MHz): δ 7.50 (d, *J* = 8.4 Hz, 1H), 7.37 (d, *J* = 2.1 Hz, 1H), 7.26 – 7.22 (dd, *J* = 5.4 Hz, *J* = 3.6 Hz 1H), 7.13 (d, *J* = 5.1 Hz, 1H), 6.76 (d, *J* = 4.8 Hz, 1H), 3.92 (t, , *J* = 5.7 Hz, 2H), 3.64 (s, 2H), 2.80 – 2.68 (m, 4H), 2.63 – 2.44 (m, 2H), 2.00 – 1.97 (m, 4H) ppm; ^{13}C NMR (DMSO-d_6, 100 MHz): 154.89, 141.65, 138.45, 137.12, 135.23, 132.44, 131.54, 128.11, 127.13, 121.25, 70.54, 64.24, 58.98, 42.21, 40.10, 38.85, 34.20, 25.76; ESI-MS *m/z*: (Calcd for $C_{18}H_{19}Cl_2NOS$: 367.06); Found: 367.9 $[M + H]^+$.

4.2. In vitro active MTB model

Briefly, the inoculum was prepared from fresh LJ medium re-suspended in 7H9-S medium (7H9 broth, 0.1% casitone, 0.5% glycerol, supplemented oleic acid, albumin, dextrose, and catalase [OADC]), adjusted to a McFarland tube No. 1, and diluted 1:20; 100 µl was used as inoculum. Each drug stock solution was thawed and diluted in 7H9-S at four-fold the final highest concentration tested. Serial two-fold dilutions of each drug were prepared directly in a sterile 96-well microtiter plate using 100 µl 7H9-S. A growth control containing no antibiotic and a sterile control were also prepared on each plate. Sterile water was added to all perimeter wells to avoid evaporation during the incubation. The plate was covered, sealed in plastic bags and incubated at 37^{o}C in normal atmosphere. After 7 days incubation, 50 µl of alamar blue solution was added to each well, and the plate was re-incubated overnight. A change in colour from blue (oxidized state) to pink (reduced) indicated the growth of bacteria, and the MIC was defined as the lowest concentration of drug that prevented this change in colour.[1]

4.3. In vitro dormant MTB model

A culture of *M. tuberculosis* H37Rv (O.D. of 0.8 - 1.0) grown in Middlebrook 7H9 medium supplemented with OADC was pelleted and washed twice with PBS. The pellet was re-

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suspended in PBS in sealed bottles and incubated at 37 °C for 6 weeks. Aliquots of these cultures were then treated with standard drugs like INH, Rif and Moxifloxacin (Mox) and the lead compounds for 7 days at a concentration of 10 μg/ml. The frequency of persistors was enumerated by MPN assay.[13]

4.4. LAT enzymatic assay

MTB LAT enzymatic assay was performed in 100 μl volume containing 200 mM phosphate buffer (pH 7.2), 1.5 mM L-lysine, 1.5 mMα-ketoglutarate, 15 μM pyridoxal 5-phosphate with MTB LAT for 1hr at 37°C. The compounds were added to the plates with different concentrations from 50 μM to 1 μM. Reactions were terminated by adding 10% trichloroacetic acid in ethanol. The MTB LAT activity was monitored and the end product piperidine-6-carboxylic acid and glutamate was detected at absorbance of 465 nm and 280 nm. Reactions were carried out in a heat-controlled Perkin Elmer Victor X3 spectrophotometer. Further the IC_{50} values were calculated using Graph Pad Prism analysis software. The error values in IC_{50}s were derived using non-linear regression mode in Graph pad prism software.[14-15]

4.5.MTT assay

The synthesized compounds were further examined for its effects on metabolism in mouse macrophage cell line (RAW 264.7) at 50μg/ml concentration. After 48 h of exposure, viability was assessed on the basis of cellular conversion of MTT into a formazan product using the Promega Cell Titer 96 non-radioactive cell proliferation assay. The compounds are tested at 50 μg/ml concentration for 48 hrs. [16]

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Synthesis and Evaluation of 4',5'-Dihydrospiro[piperidine-4,7'-thieno[2,3-c]pyran] Analogues Against Both Active and Dormant *Mycobacterium tuberculosis*

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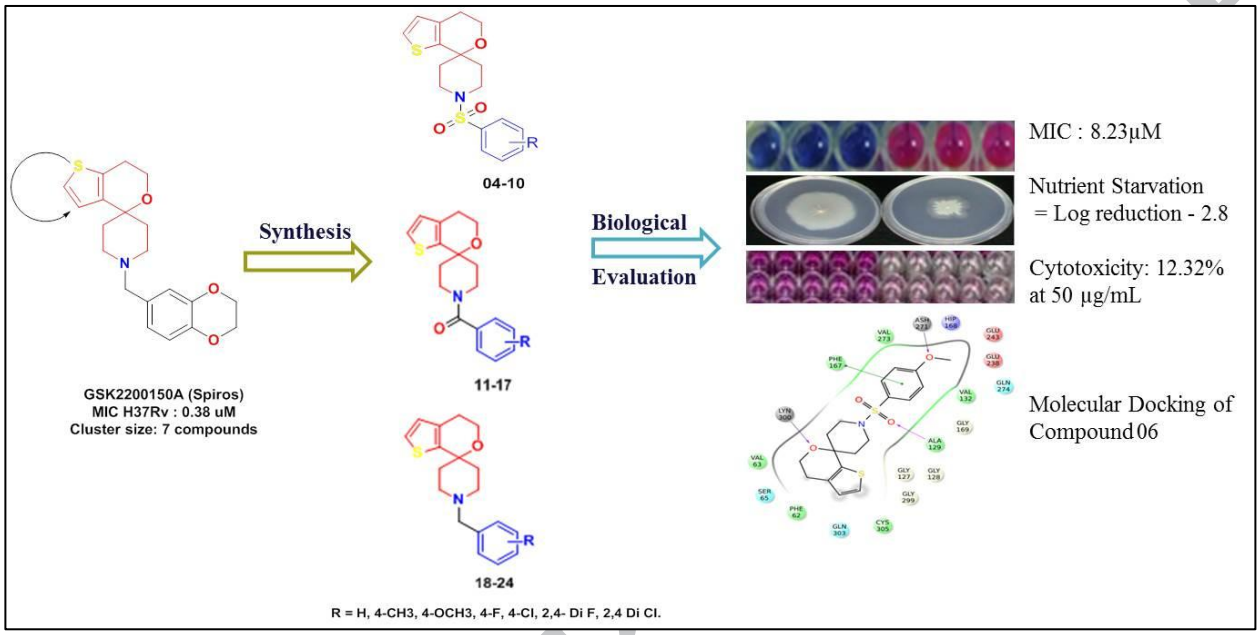

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