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Spirocyclic sulfonamides with carbonic anhydrase inhibitory and anti-neuropathic pain activity

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ABSTRACT

A novel series of 4-oxo-spirochromane bearing primary sulfonamide group were synthetized as Carbonic Anhydrase inhibitors (CAIs) and tested for their management of neuropathic pain. Indeed, CAs have been recently validated as novel therapeutic targets in neuropathic pain. All compounds, here reported, showed strong activity against hCA II and hCA VII with K_I values in the low or sub-nanomolar range. Two compounds (**6d** and **6l**) showed good neuropathic pain attenuating effects and longer duration than drug reference acetazolamide in an animal model of oxaliplatin induced neuropathy.

1. Introduction

Spirocycle containing moieties are well represented in Nature and within the Medicinal Chemistry field [1,2,4–7] with the first report dealing with such an interesting scaffold dated over 60 years ago [3]. The major advantages offered by spirocyclic scaffolds are mainly related to its inherent three-dimensional feature and concomitant ability to project chemical functionalities all over the three dimensions [4]. This makes the spirocycles the ideal platform for designing ligands, able to bind towards biological targets, far more easier and clever when compared to planar systems [4].

In the last years spirocycle derivatives were attributed with several biological proprieties such as antibiotics, antitumor agents, enzyme inhibitors, receptor modulators, protein–protein interaction inhibitors and transporters [6]. Quite recently spirocycles emerged as promising neuropathic pain (NP) relievers [7,8], and the present study follows this direction. NP affects approximately 6% of the adult population worldwide [9] and despite several therapeutic options are clinically used its treatment and/or control still remains an unmet challenge [10]. Important contributions on NP were recently introduced by means of biochemical evidences that accounted for the γ-aminobutyric acid (GABA) receptor functionality being directly dependent from the HCO_3^- anion concentration [9,10]. Thus modulation of HCO_3^--dependent depolarization of $GABA_A$ receptors, when the function of KCC2 is compromised after nerve injury, may be a new potential strategy to treat NP [7,11–14]. In this context the inhibition of the Carbonic Anhydrase (CA; EC 4.2.1.1) enzymes acts as the ideal biological targets since such enzymes are mainly involved in producing HCO_3^- and H^+ ions as by-products of the CO_2 hydration reaction catalysis [7,11–14]. As extension of our previous contribution based on merging chemical moieties well known for possessing anticonvulsant effects, such as the spirocycle scaffold (i.e. gaba-pentin) with the CA inhibitor (CAI) of the benzenesulfonamide type [7], we report the 4-oxo-spirochromanes **6a-v** and we investigated any possible effects both *in vitro* and *in vivo* when substituents were introduced at positions 6 and 7.

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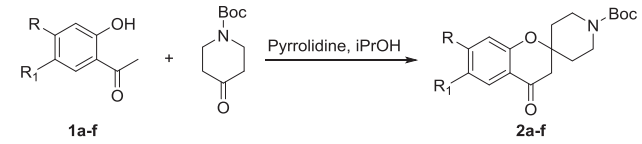


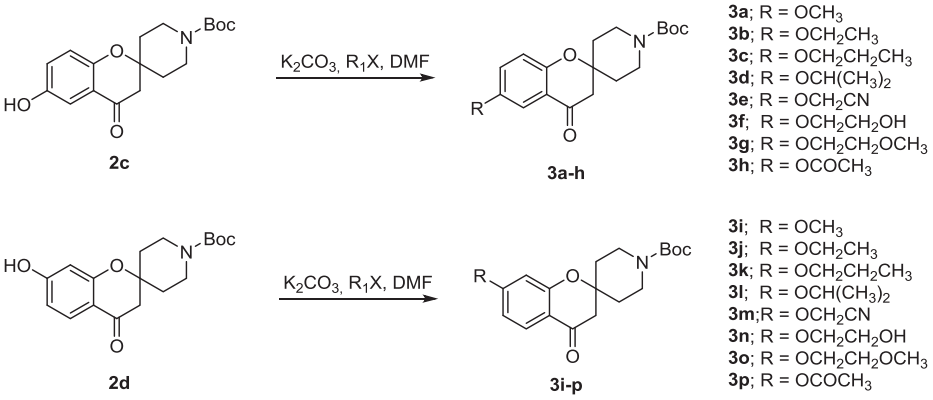
1a; R = H R_1 = H
1b; R = H R_1 = Me
1c; R = H R_1 = OH
1d; R = OH R_1 = H
1e; R = H R_1 = Br
1f : R = Br R_1 = H

2a; R = H R_1 = H
2b; R = H R_1 = Me
2c; R = H R_1 = OH
2d; R = OH R_1 = H
2e; R = H R_1 = Br
2f : R = Br R_1 = H

Scheme 1. General procedure to obtain derivatives **2a-f.**

primary sulfonamide group at *para* position (Schemes 1–4). The planned synthesis of the compounds **6a-v** involved the coupling of 2-hydroxy acetophenone (**1a-f**) with *N*-protected-4-piperidinone and pyrrolidine as a base in isopropanol to give the spiro intermediates **2a–f** as reported in Scheme 1 [15–19].

Treatment of free phenol **2c** and **2d** with commercially available alkyl halides using K_2CO_3 as base in DMF afforded the spiroalkyl derivatives **3a–p** (Scheme 2) [15,20,21].



3a; R = OCH_3
3b; R = OCH_2CH_3
3c; R = $OCH_2CH_2CH_3$
3d; R = $OCH(CH_3)_2$
3e; R = OCH_2CN
3f; R = OCH_2CH_2OH
3g; R = $OCH_2CH_2OCH_3$
3h; R = $OCOCH_3$

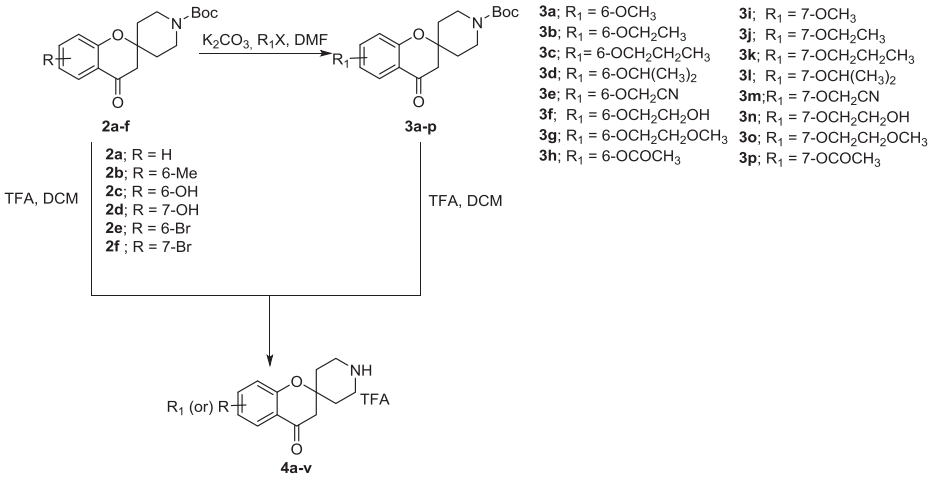
3i; R = OCH_3
3j; R = OCH_2CH_3
3k; R = $OCH_2CH_2CH_3$
3l; R = $OCH(CH_3)_2$
3m;R = OCH_2CN
3n; R = OCH_2CH_2OH
3o; R = $OCH_2CH_2OCH_3$
3p; R = $OCOCH_3$

Scheme 2. General procedure to synthetized compounds **3a-p.**

2. Chemistry

We designed two series of compounds with different groups at 6 and 7 positions of novel highly flexible 4-oxo-spirochromanes bearing the

Boc-deprotection of **2a–f** and **3a–p** with trifluoro acetic acid (TFA) afforded the spiroamines **4a-f** and **4g-u** respectively as outlined in Scheme 3.



3a; R_1 = 6-OCH_3
3b; R_1 = 6-OCH_2CH_3
3c; R_1= 6-$OCH_2CH_2CH_3$
3d; R_1 = 6-$OCH(CH_3)_2$
3e; R_1 = 6-OCH_2CN
3f; R_1 = 6-OCH_2CH_2OH
3g; R_1 = 6-$OCH_2CH_2OCH_3$
3h; R_1 = 6-$OCOCH_3$

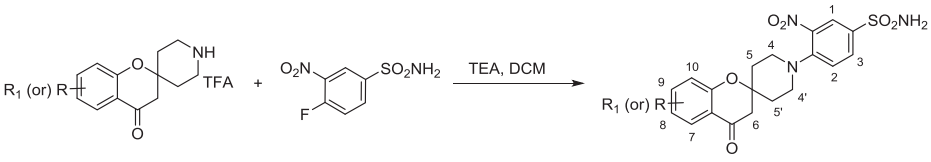
3i; R_1 = 7-OCH_3
3j; R_1 = 7-OCH_2CH_3
3k; R_1 = 7-$OCH_2CH_2CH_3$
3l; R_1 = 7-$OCH(CH_3)_2$
3m;R_1 = 7-OCH_2CN
3n; R_1 = 7-OCH_2CH_2OH
3o; R_1 = 7-$OCH_2CH_2OCH_3$
3p; R_1 = 7-$OCOCH_3$

4a; R = H
4b; R = 6-Me
4c; R = 6-OH
4d; R = 7-OH
4e; R = 6-Br
4f ; R = 7-Br
4g; R_1 = 6-OCH_3
4h; R_1 = 6-OCH_2CH_3
4i; R_1 = 6-$OCH_2CH_2CH_3$
4j; R_1 = 6-$OCH(CH_3)_2$
4k; R_1 = 6-OCH_2CN

4l; R_1 = 6-OCH_2CH_2OH
4m; R_1 = 6-$OCH_2CH_2OCH_3$
4n; R_1 = 6-$OCOCH_3$
4o; R_1 = 7-OCH_3
4p; R_1 = 7-OCH_2CH_3
4q; R_1 = 7-$OCH_2CH_2CH_3$
4r; R_1 = 7-$OCH(CH_3)_2$
4s; R_1 = 7-OCH_2CN
4t; R_1 = 7-OCH_2CH_2OH
4u; R_1 = 7-$OCH_2CH_2OCH_3$
4v; R_1 = 7-$OCOCH_3$

Scheme 3. General procedure to obtain spiroamines **4a-v.**

Finally the desired compounds **6a-v** were obtained by SNAr reaction of amines **4a–u** with 4-fluoro-3-nitrobenzenesulfonamide (**5**) as reported in Scheme 4.



4a; R = H
4b; R = 6-Me
4c; R = 6-OH
4d; R = 7-OH
4e; R = 6-Br
4f ; R = 7-Br
4g; R_1 = 6-OCH_3
4h; R_1 = 6-OCH_2CH_3
4i; R_1 = 6-$OCH_2CH_2CH_3$
4j; R_1 = 6-$OCH(CH_3)_2$
4k; R_1 = 6-OCH_2CN
4l; R_1 = 6-OCH_2CH_2OH
4m; R_1 = 6-$OCH_2CH_2OCH_3$
4n; R_1 = 6-$OCOCH_3$
4o; R_1 = 7-OCH_3
4p; R_1 = 7-OCH_2CH_3
4q; R_1 = 7-$OCH_2CH_2CH_3$
4r; R_1 = 7-$OCH(CH_3)_2$
4s; R_1 = 7-OCH_2CN
4t; R_1 = 7-OCH_2CH_2OH
4u; R_1 = 7-$OCH_2CH_2OCH_3$
4v; R_1 = 7-$OCOCH_3$

6a; R = H
6b; R = 6-Me
6c; R = 6-OH
6d; R_1 = 6-OCH_3
6e; R_1 = 6-OCH_2CH_3
6f; R_1 = 6-$OCH_2CH_2CH_3$
6g; R_1 = 6-$OCH(CH_3)_2$
6h; R_1 = 6-OCH_2CN
6i; R_1 = 6-OCH_2CH_2OH
6j; R_1 = 6-$OCH_2CH_2OCH_3$
6k; R_1 = 6-$OCOCH_3$
6l; R = 7-OH
6m; R_1 = 7-OCH_3
6n; R_1 = 7-OCH_2CH_3
6o; R_1 = 7-$OCH_2CH_2CH_3$
6p; R_1 = 7-$OCH(CH_3)_2$
6q; R_1 = 7-OCH_2CN
6r; R_1 = 7-OCH_2CH_2OH
6s; R_1 = 7-$OCH_2CH_2OCH_3$
6t; R_1 = 7-$OCOCH_3$
6u; R = 7-Br
6v; R = 6-Br

Scheme 4. General procedure to obtain 3-nitrobenzenesulfonamide derivatives **6a-v.**

All compounds were purified by aqueous workup and trituration using ethyl acetate to afford desired products with high purity and yields between 50 and 90%. Degradation of compounds **6c**, **6h**, **6q** and **6t** was observed during work-up and attempt of further purification failed. The analytical and spectroscopic data (^{1}H NMR and ^{13}C NMR chemical shifts) of the purified compounds are in agreement with the purposed structures (see experimental section for details).

3. Carbonic anhydrase inhibition

All compounds here reported were tested *in vitro* for their inhibitory activity against the physiologically relevant hCA isoforms I, II, VII and IX by means of the stopped-flow carbon dioxide hydration assay [22]. Their activities were compared to the standard CA inhibitor acetazolamide (**AAZ**).

We have investigated the novel series of 4-oxo-spirochromane derivatives bearing primary sulfonamide group for their interaction with four hCAs of pharmacologic interest, using a period of 15 min of incubation of the enzyme and inhibitor solutions [23–26]. The following structure-activity-relationship (SAR) may be noted regarding the inhibition data of Table 1:

(i) The cytosolic hCA I was weakly inhibited by almost of compounds here reported in micromolar range. On the other hand, only three compounds showed K_I values in the nanomolar range (6k, 6p, 6r with K_I of 91.1, 81.5 and 78.5, respectively). The same substituents

Table 1
Inhibition data of human CA isoforms I, II, VII and IX with compounds **6a, 6b, 6d-g, 6i-p, 6r, 6s, 6u, 6v** and acetazolamide (**AAZ)** as standard drug by a stopped-flow CO_2 hydrase assay [22].

K_I (nM)*				
Cmp	**hCA I**	**hCA II**	**hCA VII**	**hCA IX**
6a	5500.0	462.7	37.0	229.3
6b	790.9	430.0	54.5	3550.5
6d	7520.4	44.6	2.8	1557.0
6e	7841.0	410.1	41.1	2836.5
6f	8694.1	290.5	75.8	1242.0
6g	8940.5	461.3	68.3	3295.5
6i	8051.3	51.8	1.5	3745.0
6j	8332.4	406.8	65.0	4338.0
6k	91.1	63.7	0.96	4075.0
6l	250.0	3.8	0.28	4406.5
6m	6392.6	5.1	0.39	2750.0
6n	8008.6	53.7	5.8	2653.5
6o	7124.1	5.9	2.3	4152.5
6p	81.5	3.1	0.35	34.6
6r	78.5	4.2	0.47	46.5
6s	2849.7	8.8	15.9	45.4
6u	7240.6	188.1	61.2	1470.0
6v	4261.9	6.1	14.4	199.3
AAZ	250	12.1	2.5	25.8

*Mean from 3 different assays, by a stopped flow technique (errors were in the range of ± 5–10% of the reported values).

in position 6 or 7 of 4-oxo-spirochromane scaffold did not effectively influence the inhibition profile against this isoform.

(ii) The second dominant cytosolic human isoform, hCA II, on the other hand, was effectively inhibited by these compounds. In particular, this time, substituents in 7 position of 4-oxo-spirochromane scaffold showed a better constant inhibition values than derivatives with substituents in 6 position leading to low nanomolar range inhibition such as 6l, 6m, 6o, 6p, 6r and 6s with K_I that spanning among 3.1–8.8 nM. One exception was showed by compounds 6u and 6v where bromine atom increased the potency when located in 6 position of 4-oxo-spirochromane scaffold (K_I 6.1 nM than 188.1 nM of 6v).

(iii) The last cytosolic isoform here investigated, hCA VII, was strongly inhibited by all compounds here reported showing inhibition constants in low nanomolar range and reaching for several of them such as 6k-m subnamolar range (K_I 0.28–0.96 nM). These compounds even if more active against this isoform showed the same features of hCA II where substituents in 7 position of 4-oxo-spirochromane scaffold prove to be more efficaciously than 6 position except for bromine atom of derivatives 6u and 6v.

(iv) The transmembrane tumor-associate isoform hCA IX, contrarily to the other cytosolic human isoforms, was weakly inhibited by these derivatives with constant of inhibition values spanning in the micromolar range except for compounds 6p, 6r and 6s where the potency increase drastically reaching to medium nanomolar range of inhibition (K_I 34.6–46.5 nM). For this isoform, anyway, the position of substituents did not play an important role for the inhibition activity.

4. Biological assays

Kipping in mind the inhibition pattern of the compounds here reported and the not a precise knowledge which CA isoforms are involved in the neuropathic pain response. We focused the attention on inhibition of physiologically cytosolic relevant CA isoforms hCA II where it is expressed in the central/peripheral nervous system and hCA VII predominantly expressed in the brain and being absent in most other tissues. In this context, we investigated 4 different 4-oxo-spirochromane derivatives (**6d, 6i, 6l** and **6m**) and compared with acetazolamide (**AAZ**) as possible pain relievers in a mice model of neuropathic pain induced by oxaliplatin (Fig. 1).

Compounds **6d** and **6l** showed a dose dependent pain-relieving effect (30 and 100 mg kg^{-1}). Moreover, all derivatives, here tested, lead the maximum of the pain threshold at 30 min of administration and their effects finished at 60 min. In particular, **6d** and **6l** at 100 mg kg^{-1} reaching the same maximum pain-relieving effect when the same model was treated with acetazolamide (**AAZ**). The position of OH group in 4-oxo-spirochromane scaffold did not play a crucial role for the maximum of the efficacy, indeed, derivatives **6d** and **6l** showed the same peak at 30 min. On the other hand, hydroxyl group in 7 position (**6l**) increased the potency at 30 mg kg^{-1}. Different substituents from OH group such as for compounds **6i** or **6m** lead to decrease on the potency of pain-relieving effects.

5. Conclusions

In conclusion, we have synthetized a novel series of 4-oxo-spirochromanes bearing the primary sulfonamide group as CAIs and potentially useful for the management of neuropathic pain. All compounds here reported, showed strong activity against the hCA II and hCA VII isoforms with K_I values in the low or sub-nanomolar range. Compounds **6d** and **6l** showed comparable pain threshold level to **AAZ** in an *in vivo* model of the disease. Quite interestingly **6d** and **6l** showed the maximum effects at 30 min after administration and lasted up to 60 min. Taken together, these data strongly suggest the translational utility of these inhibitors as novel pain relievers.

6. Experimental part

6.1. General

All reactions were carried out in an oven-dried glassware under inert atmosphere (N_2). Solvents and all the reagents were purchased from Sigma- Aldrich (India), Alfa Aesar (India) and TCI (India). Analytical thin-layer chromatography (TLC) was carried out on silica gel F-254 plates (Merck). Nuclear magnetic resonance spectra (^{1}H NMR: 400 MHz and ^{13}C NMR: 100 MHz) were recorded in $CDCl_3$ or DMSO-d_6 using an Avance III 400 MHz spectrometer (Bruker). Chemical shifts are reported in parts per million (ppm) and the coupling constants (J) are expressed in Hertz (Hz). Splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; q, quadruplet; m, multiplet; br s, broad singlet; dd, double of doublets. The LC mass spectra were obtained using a 1200 l triple quadrupole system (Varian, Palo Alto, CA) equipped by electron spray source (ESI) operating in both positive and negative ions and the compounds resulted > 95% pure. Melting points (m.p.) were carried out in open capillary tubes on a Büchi Melting Point B-540 apparatus and were uncorrected. Elemental composition of compounds was calculated on the basis of their measured accurate masses, accepting only results with an attribution error less than 5 ppm and not integer RDB (double bond/ring equivalents). Stock solutions of analytes were prepared using acetone (1.0 mg mL^{-1}) and stored at 4 °C. Then working solutions of each analyte were prepared by dilution of the stock solutions using mQ H_2O/CAN 1/1(v/v) up to a concentration of 1.0 μg mL^{-1}.

6.2. General procedure for the synthesis of compounds 2a to 2f

To a stirred solution of 2-hydroxyacetophenone derivative (1 eq) and Boc-4-piperidone (1.5 eq) in Isopropanol (10 mL for 1 mmol of substrate), pyrrolidine (2 eq) was added drop wise at RT and stirred for 15 min. Reaction mass heated to reflux for 24 h. TLC showed completion of starting material. The reaction mixture was cooled to RT and concentrated under reduced pressure. The crude compound was diluted with water, pH adjusted to ~6 using 1 N HCl and extracted with ethyl acetate. Combined organic layer washed with brine, dried over Na_2SO_4, filtered and concentrated to get crude compound. The crude compound was purified by flash column chromatography using 10–30% (v/v) ethyl acetate in pet ether as eluent to get desired compound **2**.

Tert-butyl 4-oxospiro[chroman-2,4′-piperidine]-1′-carboxylate (**2a**) [15,16]:

Following the above general procedure using compound **1a** and N-Boc-4-piperidone yielded compound **2a** in 85% yield as a white solid. M.p: 217–219 °C; TLC: R_f: 0.4 (30% EtOAc in pet ether). ^{1}H NMR in $CDCl_3$: δ 7.85 (dd, J = 2 Hz, 8.4 Hz, 1H), 7.51–7.46 (m, 1H), 7.02–6.96 (m, 2H), 3.86 (m, 2H), 3.21 (br t, 2H), 2.71 (s, 2H), 2.02 (d, J = 8 Hz, 2H), 1.64–1.55 (m, 2H) and 1.46 (s, 9H). LCMS (ESI positive) m/z = 218.2 $[M+H\text{-}100]^+$ and m/z = 262.1 $[M+H\text{-}56$ (*tert*-butyl)$]^+$.

*Tert-butyl 6-methyl-4-oxospiro[chroman-2,4′-piperidine]-1′-carboxylate (**2b**)* [17]:

Following the above general procedure using compound **1b** and N-Boc-4-piperidone yielded compound **2b** in 70% yield as a white solid. M.p: 218–220 °C; TLC: R_f: 0.3 (30% EtOAc in pet ether). ^{1}H NMR in $CDCl_3$: δ 7.65 (d, J = 2 Hz, 1H), 7.29 (dd, J = 2 Hz, 8 Hz, 1H), 6.68 (d, J = 8 Hz, 1H), 3.85 (m, 2H), 3.23–3.20 (m, 2H), 2.68 (s, 2H), 2.29 (s, 3H), 2.01 (d, J = 8 Hz, 2H), 1.58–1.55 (m, 2H) and 1.46 (s, 9H). LCMS (ESI positive) m/z = 232.2 $[M+H\text{-}100]^+$ and m/z = 276.1 $[M+H\text{-}56$ (*tert*-butyl)$]^+$.

Tert-butyl 6-hydroxy-4-oxospiro[chroman-2,4′-piperidine]-1′-carboxylate (**2c**) [15]:

Following the above general procedure using compound **1c** and N-Boc-4-piperidone yielded compound **2c** in 72% yield as an off white solid. M.p: 222–224 °C; TLC: R_f: 0.2 (40% EtOAc in pet ether). ^{1}H NMR in $CDCl_3$: δ 7.29 (d, J = 3.2 Hz, 1H), 7.07 (dd, J = 3.2 Hz, 8.8 Hz, 1H),

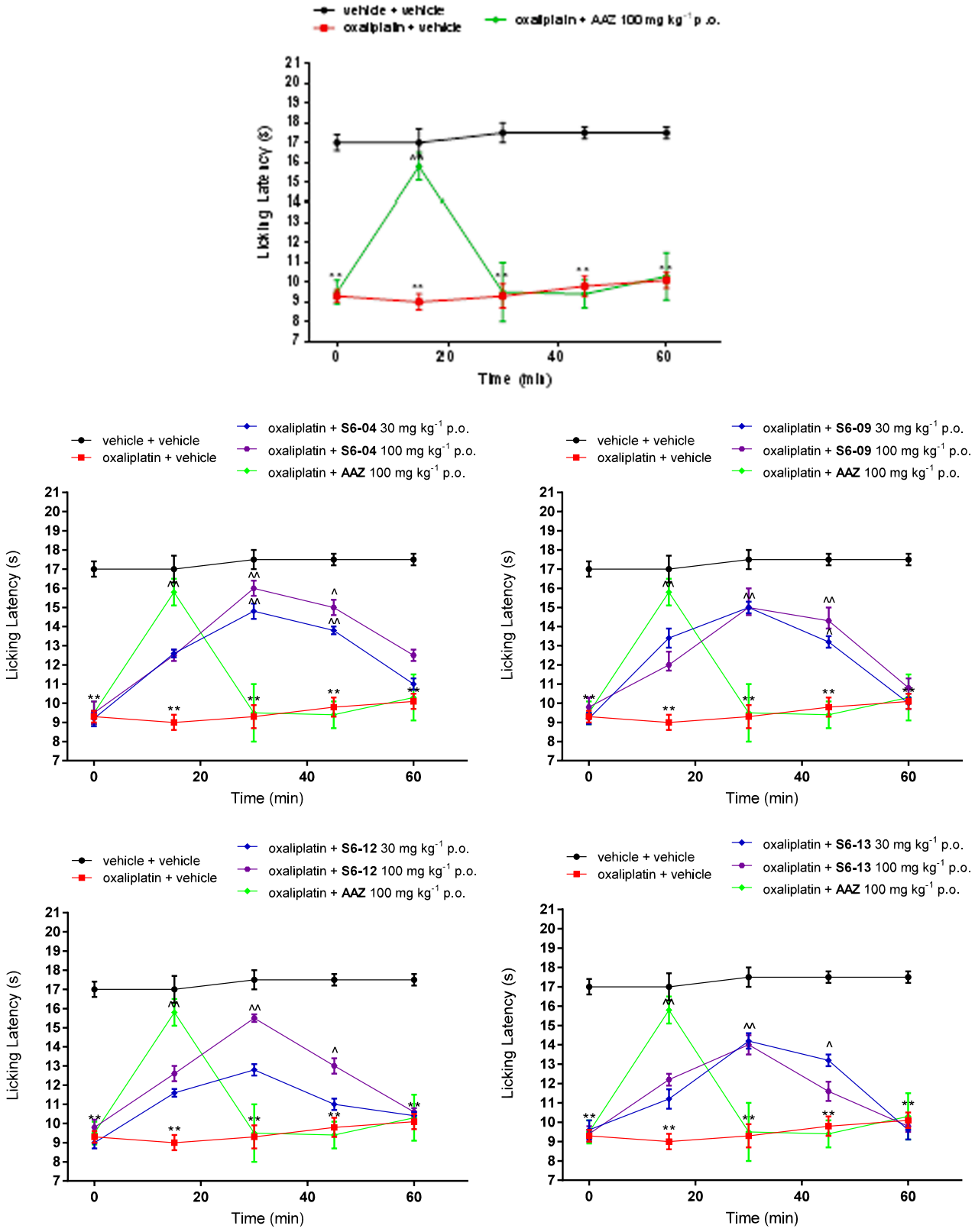


Fig. 1. Effect of acute administration of carbonic anhydrase inhibitors **6d, 6i, 6l** and **6m** and **AAZ** on oxaliplatin induced neuropathic pain in the mouse Cold plate test **P < 0.01 vs vehicle + vehicle treated animals; ^P < 0.05 and ^^P < 0.01 vs oxaliplatin + vehicle treated animals. Each value represents the mean ± S.E.M. of 10 mice performed in 2 different experimental sets.

6.87 (d, J = 8.8 Hz, 1H), 6.03 (s, 1H), 3.85 (s, 2H), 3.21–3.19 (m, 2H), 2.67 (s, 2H), 2.00 (d, J = 8 Hz, 2H), 1.61–1.54 (m, 2H) and 1.46 (s, 9H). LCMS (ESI positive) m/z = 234 $[M+H\text{-}100]^+$ and m/z = 278 $[M+H\text{-}56$ (tert-butyl)$]^+$.

Tert-butyl 7-hydroxy-4-oxospiro[chroman-2,4′-piperidine]-1′-carboxylate (**2d**) [15]

Following the above general procedure using compound **1d** and N-Boc-4-piperidone yielded compound **2d** in 60% yield as an off white solid. M.p: 221–223 °C; TLC: R_f: 0.22 (40% EtOAc in pet ether). ^{1}H NMR in $CDCl_3$: δ 8.16 (brs, 1H, –OH), 7.77 (d, J = 8.8 Hz, 1H), 6.52 (d d, J = 2.4 Hz, 8.8 Hz, 1H), 6.36 (d, J = 2.4 Hz, 1H), 3.90–3.87 (m, 2H), 3.20 (t, 2H), 2.65 (s, 2H), 2.03 (d, J = 8 Hz, 2H), 1.63–1.55 (m, 2H) and 1.47 (s, 9H). LCMS (ESI positive) m/z = 234.1 $[M+H\text{-}100]^+$ and m/z = 278 $[M+H\text{-}56$ (*tert*-butyl)$]^+$.

*Tert-butyl 6-bromo-4-oxospiro[chroman-2,4′-piperidine]-1′-carboxylate (**2e**)* [18]:

Following the above general procedure using compound **1e** and N-Boc-4-piperidone yielded compound **2e** in 83% yield as a white solid. M.p: 220–222 °C; TLC: R_f: 0.4 (30% EtOAc in pet ether). ^{1}H NMR in $CDCl_3$: δ 7.97 (s, 1H), 7.55 (d, J = 8.8 Hz, 1H), 6.82 (d, J = 8 Hz, 1H), 3.85 (m, 2H), 3.2 (t, 2H), 2.7 (s, 2H), 2.00 (d, J = 8 Hz, 2H), 1.62–1.58 (m, 2H) and 1.44 (s, 9H). LCMS (ESI positive) m/z = 296, 298 $[M+H$-100, Bromo pattern$]^+$ and m/z = 339.8, 341.9 $[M+H$-56 (*tert*-butyl, bromo pattern)$]^+$.

*Tert-butyl 7-bromo-4-oxospiro[chroman-2,4′-piperidine]-1′-carboxylate (**2f**)* [17,19]:

Following the above general procedure using compound **1f** and N-Boc-4-piperidone yielded compound **2f** in 80% yield as a white solid. M.p: 220–222 °C; TLC: R_f: 0.4 (30% EtOAc in pet ether). ^{1}H NMR in $CDCl_3$: δ 7.71 (d, J = 8.8 Hz, 1H), 7.20 (d, J = 2 Hz, 1H), 7.15 (d d, J = 2 Hz, 8.4 Hz, 1H), 3.96–3.86 (m, 2H), 3.23–3.19 (m, 2H), 2.70 (s, 2H), 2.04–1.95 (m, 4H) and 1.46 (s, 9H). LCMS (ESI positive) m/z = 296.1, 298.1 $[M+H$-100, Bromo pattern$]^+$ and m/z = 339.8, 341.9 $[M+H$-56 (*tert*-butyl, bromo pattern)$]^+$

6.3. General procedure for the synthesis of compounds 3a to 3p

To a stirred solution of compound **2** (**c** and **d**) (1 eq) in anhydrous DMF (5 mL for 1 mmol of substrate), K_2CO_3 (3 eq) was added and stirred for 15 min. Appropriate alkyl halide (1.5 eq) was added dropwise at RT and stirred for 20 h. TLC showed completion of reaction. The reaction mixture was quenched in ice cold water and extracted with diethyl ether. The combined organic layer was washed with brine, dried over Na_2SO_4, filtered and concentrated to get crude compound. The crude compound was purified by flash column chromatography using 10–30% EtOAc in pet ether as eluent to get desired compound **3**.

*Tert-butyl 6-methoxy-4-oxospiro[chroman-2,4′-piperidine]-1′-carboxylate (**3a**)* [15]:

Following the above general procedure using compound **2c** and methyl iodide yielded compound **3a** in 83% yield as an off white solid. M.p: 218–220 °C; TLC: R_f: 0.3 (15% EtOAc in pet ether). ^{1}H NMR in $CDCl_3$: δ 7.29 (d, J = 2.4 Hz, 1H), 7.09 (d d, J = 2 Hz, 8.4 Hz, 1H), 6.91 (d, J = 8 Hz, 1H), 3.85–3.79 (m, 2H), 3.78 (s, 3H), 3.22–3.16 (m, 2H), 2.68 (s, 2H), 2.02–1.99 (m, 2H), 1.63–1.54 (m, 2H) and 1.45 (s, 9H). ^{13}C NMR in DMSO-d_6: δ 190.9, 159.6, 152.4, 152.1, 120.2, 119.3, 115.3, 113.5, 79.8, 68.7, 55.8, 44.5, 43.0, 34.2, 28.4. LCMS (ESI positive) m/z = 248.1 $[M+H\text{-}100]^+$ and m/z = 292.1 $[M+H$-56 (*tert*. butyl)$]^+$.

*Tert-butyl 6-ethoxy-4-oxospiro[chroman-2,4′-piperidine]-1′-carboxylate (**3b**)* [15]:

Following the above general procedure using compound **2c** and ethyl bromide yielded compound **3b** in 80% yield as an off white solid. M.p: 219–221 °C; TLC: R_f: 0.3 (15% EtOAc in pet ether). ^{1}H NMR in $CDCl_3$: δ 7.28 (d, J = 3.2 Hz, 1H), 7.10 (dd, J = 3.2 Hz, 8.8 Hz, 1H), 6.90 (d, J = 8.8 Hz, 1H), 4.00 (q, 2H), 3.85 (s, 2H), 3.19 (t, 2H), 2.68 (s, 2H), 2.01 (d, J = 8 Hz, 2H), 1.6–1.57 (m, 2H), 1.45 (s, 9H) and 1.39 (t, J = 3 Hz, 3H). ^{13}C NMR in DMSO-d_6: δ 190.8, 159.7, 156.0, 151.7, 119.8, 119.4, 114.9, 113.6, 79.7, 68.8, 64.6, 44.4, 34.2, 28.4, 14.8. LCMS (ESI positive) m/z = 262.2 $[M+H\text{-}100]^+$ and m/z = 306.1 $[M+H$-56 (*tert*. butyl)$]^+$.

Tert-butyl 4-oxo-6-propoxyspiro[chroman-2,4′-piperidine]-1′-carboxylate (**3c**):

Following the above general procedure using compound **2c** and *n*-propyl iodide yielded compound **3c** in 80% yield as an off white solid. M.p: 222–224 °C; TLC: R_f: 0.3 (15% EtOAc in pet ether). ^{1}H NMR in $CDCl_3$: δ 7.28 (d, J = 3.2 Hz, 1H), 7.10 (d d, J = 3.2 Hz, 8.8 Hz, 1H), 6.90 (d, J = 8.8 Hz, 1H), 3.89 (t, 2H), 3.85 (s, 2H), 3.19 (t, 2H), 2.68 (s, 2H), 2.01 (d, 2H), 1.81–1.74 (m, 2H), 1.61–1.53 (m, 2H), 1.45 (s, 9H) and 1.01 (t, J = 3 Hz 3H). ^{13}C NMR in DMSO-d_6: δ 190.9, 159.6, 156.0, 151.7, 119.8, 119.4, 114.8, 113.5, 79.8, 69.3, 68.7, 44.5, 34.2, 28.4, 22.6, 10.4. LCMS (ESI positive) m/z = 276.1 $[M+H\text{-}100]^+$ and m/z = 320.1 $[M+H$-56 (*tert*. butyl)$]^+$.

*Tert-butyl 6-isopropoxy-4-oxospiro[chroman-2,4′-piperidine]-1′-carboxylate (**3d**)* [15]:

Following the above general procedure using compound **2c** and isopropyl iodide yielded compound **3d** in 82% yield as an off white solid. M.p: 220–222 °C; TLC: R_f: 0.3 (15% EtOAc in pet ether). ^{1}H NMR in $CDCl_3$: δ 7.29 (d, J = 3.2 Hz, 1H), 7.08 (dd, J = 3.2 Hz, 9.2 Hz, 1H), 6.89 (d, J = 8.8 Hz, 1H), 4.52–4.46 (m, 1H), 3.86 (s, 2H), 3.20 (t, 2H), 2.68 (s, 2H), 2.01 (d, J = 8 Hz, 2H), 1.57–1.54 (m, 2H), 1.45 (s, 9H) and 1.31 (d, 6H). ^{13}C NMR in DMSO-d_6: δ 190.9, 159.6, 151.7, 151.0, 119.8, 119.4, 114.9, 113.6, 79.8, 75.8, 68.7, 44.5, 34.2, 28.4, 22.0. LCMS (ESI positive) m/z = 276.1 $[M+H\text{-}100]^+$ and m/z = 320.1 $[M+H$-56 (*tert*. butyl)$]^+$.

Tert-butyl 6-(cyanomethoxy)-4-oxospiro[chroman-2,4′-piperidine]-1′-carboxylate (**3e**):

Following the above general procedure using compound **2c** and bromo acetonitrile yielded compound **3e** in 86% yield as an off white solid. M.p: 220–222 °C; TLC: R_f: 0.22 (30% EtOAc in pet ether). ^{1}H NMR in $CDCl_3$: δ 7.40 (d, J = 3.2 Hz, 1H), 7.20 (dd, J = 3.2 Hz, 8.8 Hz, 1H), 6.99 (d, J = 9.2 Hz, 1H), 4.75 (s, 2H), 3.87 (s, 2H), 3.19 (t, 2H), 2.71 (s, 2H), 2.01 (d, J = 8 Hz, 2H), 1.64–1.56 (m, 2H) and 1.45 (s, 9H). ^{13}C NMR in DMSO-d_6: δ 190.9, 159.6, 152.4, 149.2, 120.2, 119.3, 115.7, 115.3, 113.5, 79.8, 68.7, 60.3, 43.0, 34.2, 28.4. LCMS (ESI positive) m/z = 272.9 $[M+H\text{-}100]^+$.

Tert-butyl 6-(2-hydroxyethoxy)-4-oxospiro[chroman-2,4′-piperidine]-1′-carboxylate (**3f**):

Following the above general using procedure compound **2c** and bromo ethanol yielded compound **3f** in 64% yield as an off white solid. M.p: 218–220 °C; TLC: R_f: 0.3 (40% EtOAc in pet ether). ^{1}H NMR in $CDCl_3$: δ 7.30 (d, J = 2.7 Hz, 1H), 7.14 (dd, J = 2.8 Hz, 8.8 Hz, 1H), 6.93 (d, J = 8.8 Hz, 1H), 4.07 (t, 2H), 3.96 (s, 2H), 3.86 (s, 2H), 3.18 (t, 2H), 2.69 (s, 2H), 2.01 (d, J = 8 Hz, 2H), 1.62–1.56 (m, 2H) and 1.46 (s, 9H). ^{13}C NMR in DMSO-d_6: δ 190.9, 159.6, 156.0, 151.7, 119.8, 119.4, 114.9, 113.6, 79.8, 69.5, 68.7, 60.9, 44.5, 43.0, 34.2, 28.4.LCMS (ESI positive) m/z = 278.2 $[M+H\text{-}100]^+$ and m/z = 322.1 $[M+H$-56 (*tert*. butyl)$]^{1+}$.

*Tert-butyl 6-(2-methoxyethoxy)-4-oxospiro[chroman-2,4′-piperidine]-1′-carboxylate (**3 g**)*: Following the above general using procedure compound **2c** and methoxy ethyl bromide yielded compound **3 g** in 80.5% yield as an off white solid. M.p: 220–222 °C; TLC: R_f: 0.4 (30% EtOAc in pet ether). ^{1}H NMR in $CDCl_3$: δ 7.30 (d, J = 2.7 Hz, 1H), 7.17 (dd, J = 2.9 Hz, 8.8 Hz, 1H), 6.91 (d, J = 8.8 Hz, 1H), 4.10 (t, 2H), 3.86 (s, 2H),3.74 (t, 2H), 3.44 (s, 3H), 3.20 (t, 2H), 2.69 (s, 2H), 2.01(d, J = 8 Hz, 2H), 1.60–1.57 (m, 2H) and 1.46 (s, 9H). ^{13}C NMR in DMSO-d_6: δ 190.9, 159.6, 156.0, 151.7, 119.8, 119.4, 114.9, 113.6, 79.8, 72.2, 69.0, 68.7, 59.3, 44.5, 43.0, 34.2, 28.4. LCMS (ESI positive) m/z = 292.1 $[M+H\text{-}100]^+$ and m/z = 336.1 $[M+H$-56 (*tert*. butyl)$]^+$.

Tert-butyl 6-acetoxy-4-oxospiro[chroman-2,4′-piperidine]-1′-carboxylate (**3 h**):

Following the above general procedure using compound **2c** and acetic anhydride yielded compound **3 h** in 70% yield as a white solid.

220–222 °C; TLC: R_f: 0.40 (30% EtOAc in pet ether). ^{1}H NMR in $CDCl_3$: δ 7.55 (d, J = 2.4 Hz, 1H), 7.22 (dd, J = 2 Hz, 8.8 Hz, 1H), 6.99 (d, J = 8.8 Hz, 1H), 3.88 (s, 2H), 3.18 (t, 2H), 2.7 (s, 2H), 2.29 (s, 3H), 2.02 (d, J = 8 Hz, 2H), 1.7–1.6 (m, 2H) and 1.46 (s, 9H). ^{13}C NMR in DMSO-d_6: δ 190.9, 169.0, 159.6, 156.9, 142.8, 126.6, 122.5, 119.6, 114.7, 79.8, 68.7, 44.5, 43.0, 34.2, 28.4, 20.3. LCMS (ESI positive) m/z = 276.1 $[M+H\text{-}100]^+$ and m/z = 320.1 $[M+H\text{-}56$ (*tert.* butyl)$]^+$.

*Tert-butyl 7-methoxy-4-oxospiro[chroman-2,4′-piperidine]-1′-carboxylate (**3i**)* [20,21]:

Following the above general procedure using compound **2d** and methyl iodide yielded compound **3i** in 90% yield as a white solid. M.p: 221–223 °C; TLC: R_f: 0.3 (15% EtOAc in pet ether). ^{1}H NMR in $CDCl_3$: δ 7.80 (d, J = 8.8 Hz, 1H), 6.57 (d, J = 8.8 Hz, 1H), 6.43 (s, 1H), 3.84 (s, 5H), 3.20 (t, 2H), 2.66 (s, 2H), 2.02 (d, J = 8 Hz, 2H), 1.59–1.57 (m, 2H) and 1.46 (s, 9H). ^{13}C NMR in DMSO-d_6: δ 190.9, 165.6, 162.6, 159.6, 130.4, 114.0, 105.8, 101.4, 79.8, 68.7, 55.8, 44.5, 43.0, 34.2, 28.4. LCMS (ESI positive) m/z = 248.1 $[M+H\text{-}100]^+$ and m/z = 292.1 $[M+H\text{-}56$ (*tert.* butyl)$]^+$.

*Tert-butyl 7-ethoxy-4-oxospiro[chroman-2,4′-piperidine]-1′-carboxylate (**3j**)* [20]:

Following the above general procedure using compound **2d** and ethyl bromide yielded compound **3j** in 94% yield as a white solid. M.p: 220–222 °C; TLC: R_f: 0.3 (15% EtOAc in pet ether). ^{1}H NMR in $CDCl_3$: δ 7.79 (d, J = 8.8 Hz, 1H), 6.55 (d, J = 8.8 Hz, 1H), 6.40 (s, 1H), 4.06 (q, 2H), 3.86 (s, 2H), 3.19 (t, 2H), 2.65 (s, 2H), 2.02 (d, J = 8 Hz, 2H), 1.62–1.57 (m, 2H), 1.46 (s, 9H) and 1.42 (t, 3H). ^{13}C NMR in DMSO-d_6: δ 190.9, 164.4, 162.2, 159.6, 130.0, 113.3, 105.9, 101.5, 79.8, 68.7, 64.6, 44.5, 43.0, 34.2, 28.4, 14.8. LCMS (ESI positive) m/z = 262.1 $[M+H\text{-}100]^+$ and m/z = 306.1 $[M+H\text{-}56$ (*tert.* butyl)$]^+$.

Tert-butyl 4-oxo-7-propoxyspiro[chroman-2,4′-piperidine]-1′-carboxylate (**3k**)

Following the above general procedure using compound **2d** and *n*-propyl iodide yielded compound **3k** in 92% yield as a white solid. M.p: 223–226 °C; TLC: R_f: 0.3 (15% EtOAc in pet ether). ^{1}H NMR in $CDCl_3$: δ 7.79 (d, J = 8.8 Hz, 1H), 6.56 (d, J = 8.8 Hz, 1H), 6.41 (s, 1H), 3.95 (t, 2H), 3.86 (s, 2H), 3.18 (t, 2H), 2.65 (s, 2H), 2.02 (d, J = 8 Hz, 2H), 1.84–1.79 (m, 2H), 1.59–1.57 (m, 2H), 1.46 (s, 9H) and 1.03 (t, 3H). ^{13}C NMR in DMSO-d_6: δ 190.9, 164.4, 162.2, 159.6, 130.0, 113.3, 105.9, 101.5, 79.8, 69.3, 68.7, 44.5, 43.0, 34.2, 28.4, 22.6, 10.4. LCMS (ESI positive) m/z = 276.1 $[M+H\text{-}100]^+$ and m/z = 320.1 $[M+H\text{-}56$ (*tert.* butyl)$]^+$.

Tert-butyl 7-isopropoxy-4-oxospiro[chroman-2,4′-piperidine]-1′-carboxylate (**3l**):

Following the above general procedure using compound **2d** and isopropyl iodide yielded compound **3l** in 90% yield as a white solid. M.p: 221–223 °C; TLC: R_f: 0.3 (15% EtOAc in pet ether). ^{1}H NMR in $CDCl_3$: δ 7.79 (d, J = 8.8 Hz, 1H), 6.52 (d, J = 8.8 Hz, 1H), 6.39 (s, 1H), 4.61–4.58 (m, 1H), 3.86 (s, 2H), 3.19 (t, 2H), 2.65 (s, 2H), 2.02 (d, J = 8 Hz, 2H), 1.58–1.56 (m, 2H), 1.46 (s, 9H) and 1.36 (d, 6H). ^{13}C NMR in DMSO-d_6: δ 190.9, 164.3, 162.2, 159.6, 130.0, 113.3, 105.9, 101.5, 79.8, 75.8, 68.7, 44.5, 43.0, 34.2, 28.4, 22.0. LCMS (ESI positive) m/z = 276.1 $[M+H\text{-}100]^+$ and m/z = 320.1 $[M+H\text{-}56$ (*tert.* butyl)$]^+$.

Tert-butyl 7-(cyanomethoxy)-4-oxospiro[chroman-2,4′-piperidine]-1′-carboxylate (**3 m**):

Following the above general procedure using compound **2d** and bromo acetonitrile yielded compound **3 m** in 92% yield as a white solid. M.p: 222–224 °C; TLC: R_f: 0.2 (30% EtOAc in pet ether). ^{1}H NMR in $CDCl_3$: δ 7.87 (d, J = 8.8 Hz, 1H), 6.63 (dd, J = 2.2 Hz, 8.8 Hz, 1H), 6.53 (d, J = 2.0 Hz, 1H), 4.81 (s, 2H), 3.88 (s, 2H), 3.19 (t, 2H), 2.69 (s, 2H), 2.02 (d, J = 8 Hz, 2H), 1.64–1.59 (m, 2H) and 1.46 (s, 9H). ^{13}C NMR in DMSO-d_6: δ 190.9, 163.6, 162.6, 159.6, 130.4, 115.7, 114.0, 105.8, 101.4, 79.8, 75.8, 68.7, 60.3, 44.5, 43.0, 34.2, 28.4. LCMS (ESI positive) m/z = 273.1 $[M+H\text{-}100]^+$ and m/z = 317.1 $[M+H\text{-}56$ (*tert.* butyl)$]^+$.

Tert-butyl 7-(2-hydroxyethoxy)-4-oxospiro[chroman-2,4′-piperidine]-1′-carboxylate (**3n**):

Following the above general procedure using compound **2d** and bromo ethanol yielded compound **3n** in 80% yield as a white solid. M.p: 220–222 °C; TLC: R_f: 0.3 (30% EtOAc in pet ether). ^{1}H NMR in $CDCl_3$: δ 7.81 (d, J = 8.8 Hz, 1H), 6.59 (d, J = 7.2 Hz, 1H), 6.45 (s, 1H), 4.12–4.11 (m, 2H), 3.99–3.95 (m, 2H), 3.87 (s, 2H), 3.19 (t, 2H), 2.66 (s, 2H), 2.01 (d, J = 8 Hz, 2H), 1.60–1.57 (m, 2H) and 1.46 (s, 9H). ^{13}C NMR in DMSO-d_6: δ 190.9, 164.4, 162.2, 159.6, 130.0, 113.3, 105.9, 101.5, 79.8, 69.5, 68.7, 60.9, 44.5, 43.0, 34.2, 28.4. LCMS (ESI positive) m/z = 278.2 $[M+H\text{-}100]^+$ and m/z = 322.1 $[M+H\text{-}56$ (*tert.* butyl)$]^+$.

Tert-butyl 7-(2-methoxyethoxy)-4-oxospiro[chroman-2,4′-piperidine]-1′-carboxylate (**3o**):

Following the above general procedure using compound **2d** and methoxy ethyl bromide yielded compound **3o** in 83% yield as a white solid. M.p: 221–223 °C; TLC: R_f: 0.4 (30% EtOAc in pet ether). ^{1}H NMR in $CDCl_3$: δ 7.80 (d, J = 8.8 Hz, 1H), 6.60 (d, J = 8 Hz, 1H), 6.45 (s, 1H), 4.16–4.15 (m, 2H), 3.86 (s, 2H), 3.77 (s, 2H), 3.45 (s, 3H), 3.19 (t, 2H), 2.66 (s, 2H), 2.01 (d, J = 8 Hz, 2H), 1.64–1.57 (m, 2H) and 1.46 (s, 9H). ^{13}C NMR in DMSO-d_6: δ 190.9, 164.4, 162.2, 159.6, 130.0, 113.3, 105.9, 101.5, 79.8, 72.2, 69.0, 68.7, 59.3, 44.5, 43.0, 34.2, 28.4. LCMS (ESI positive) m/z = 292.2 $[M+H\text{-}100]^+$ and m/z = 337.1 $[M+H\text{-}56$ (*tert.* butyl)$]^+$.

Tert-butyl 7-acetoxy-4-oxospiro[chroman-2,4′-piperidine]-1′-carboxylate (**3p**):

Following the above general procedure using compound **2d** and acetic anhydride yielded compound **3p** in 93% yield as a white solid. M.p: 220–222 °C; TLC: R_f: 0.41 (30% EtOAc in pet ether). ^{1}H NMR in $CDCl_3$: δ 7.88 (d, J = 8.8 Hz, 1H), 6.78 (s, 1H), 6.76 (d, J = 8.0 Hz, 1H), 3.87 (s, 2H), 3.19 (t, 2H), 2.70 (s, 2H), 2.31 (s,3H), 2.02 (d, J = 8 Hz, 2H), 1.61–1.57 (m, 2H) and 1.46 (s, 9H). ^{13}C NMR in DMSO-d_6: δ 190.9, 170.5, 169.0, 162.0, 159.6, 129.8, 118.5, 113.1, 110.1, 79.8, 68.7, 44.5, 43.0, 34.2, 28.4, 20.3. LCMS (ESI positive) m/z = 276.2 $[M+H\text{-}100]^+$ and m/z = 320.1 $[M+H\text{-}56$ (*tert.* butyl)$]^+$.

6.4. General procedure for the synthesis of compounds 4a to 4b

To an ice cold stirred solution of compound **2a-2f** or **3a-3p** (1 eq) in anhydrous DCM (5 mL for 1 mmol of substrate), Trifluoroacetic acid (0.5 mL for 1 mmol of substrate) was added drop wise under N_2 atmosphere and stirred for 30 min. The reaction mixture slowly warmed to RT and stirred for 16 h. TLC showed completion of reaction. The reaction mixture was concentrated under reduced pressure, co-distilled with DCM (2 × 5 mL) and dried under high vacuum to get desired compound **4a to 4u** as thick brown liquid. This was used in next step without any purification.

6.5. General procedure for the synthesis of compounds 6a-v

To an ice cold stirred solution of compound **4a-4u** (1 eq) in anhydrous DCM (10 mL for 1 mmol of substrate), TEA (3 eq) was added drop wise and stirred for 10 min. Compound **5** (1 eq) was added slowly and stirred at RT for 16 h (precipitation was observed during the reaction progress). TLC showed completion of reaction. The reaction mixture was concentrated under reduced pressure. Water was added to the crude material and stirred for 30 min. Obtained solid was filtered, washed with water and dried under vacuum. The solid was triturated with ethyl acetate, filtered and dried under high vacuum to afford compound **6a-v.**

3-nitro-4-(4-oxospiro[chroman-2,4′-piperidin]-1′-yl)benzenesulfonamide (**6a**):

48% yield as a yellow solid. TLC: R_f: 0.25 (30% Acetone in pet ether). m.p. 125 °C; ^{1}H NMR in DMSO-d_6: δ 8.22 (d, J = 2.4 Hz, 1H, H-1), 7.88 (dd, J = 2 Hz, 8.8 Hz, 1H, H-3), 7.74 (dd, J = 1.6 Hz, 8.8 Hz, 1H, H-7), 7.61–7.57 (m, 1H-H-8),7.48 (d, J = 8.8 Hz, 1H, H-10), 7.41 (s, 2H, SO_2NH_2), 7.11–7.04 (m, 2H, H-2,8), 3.30–3.27 (m, 2H, H-4),

3.22–3.18 (m, 2H, H-4′), 2.9 (s, 2H, H-6), 2.02 (m, 2H, H-5) and 1.90–1.83 (m, 2H, H-5′). ^{13}C NMR in DMSO-d_6: δ 191.8, 159.0, 147.8, 139.0, 136.9, 135.0, 131.2, 126.3, 124.9, 121.7, 121.6, 120.9, 118.8, 77.9, 47.2, 46.6 and 33.7. LCMS (ESI positive) m/z = 418.1 $[M+H]^+$; Elemental Analysis Calculated C, 54.67; H, 4.59; N, 10.07; S, 7.68. Found C, 54.69; H, 4.58; N, 10.06; S, 7.70.

4-(6-methyl-4-oxospiro[chroman-2,4′-piperidin]-1′-yl)-3-nitrobenzenesulfonamide (**6b**):

65% yield as a yellow solid. TLC: R_f: 0.25 (30% Acetone in pet ether). m.p. 112 °C. ^{1}H NMR in DMSO-d_6: δ 8.20 (s, 1H, H-1), 7.87 (d, J = 8.8 Hz, 1H, H-3), 7.52 (s, 1H, H-7), 7.46 (d, J = 8.8 Hz , 1H, H-9), 7.39 (m, 1H, H-2), 6.99 (d, J = 8.4 Hz, 1H, H-10), 3.30–3.25 (m, 2H, H-4), 3.19–3.13 (m, 2H, H-4′), 2.85 (s, 2H, H-6), 2.26 (s, 3H), 1.99 (m, 2H, H-5) and 1.84–1.81 (m, 2H, H-5′). ^{13}C NMR in DMSO-d_6: δ 192.1, 156.5, 147.3, 138.4, 137.3, 134.4, 130.7, 130.1, 125.4, 124.3, 121.2, 120.0, 118.2, 77.1, 46.8, 46.0, 33.1 and 19.8. LCMS (ESI positive) m/z = 432.2 $[M+H]^+$; Elemental Analysis Calculated C, 55.68; H, 4.91; N, 9.74; S, 7.43. Found C, 55.69; H, 4.89; N, 9.73; S, 7.41.

4-(6-hydroxy-4-oxospiro[chroman-2,4′-piperidin]-1′-yl)-3-nitrobenzenesulfonamide (**6c**):

80% yield as an orange solid. TLC: R_f: 0.1 (30% Acetone in pet ether). m.p. 130 °C. ^{1}H NMR in DMSO-d_6: δ 9.36 (s, 1H, –OH), 8.20 (s, 1H, H-1), 7.86 (d, J = 8 Hz, 1H, H-3), 7.46 (d, J = 8 Hz, 1H, H-9), 7.40 (brs, 2H, SO_2NH_2), 7.06–7.00 (m, 2H. H-2, H-7), 6.94 (m, 1H, H-10), 3.31–3.27 (m, 2H, H-4), 3.18–3.16 (m, 2H, H-4′), 2.81 (s, 2H-H-6), 2.01 (m, 2H, H-5) and 1.84–1.81 (m, 2H, H-5′). ^{13}C NMR in DMSO-d_6: δ 191.45, 151.57, 151.34, 147.3, 138.39, 134.38, 130.74, 124.6, 124.37, 121.19, 120.58, 119.3, 109.65, 76.82, 46.86, 46.10 and 33.17. LCMS (ESI positive) m/z = 434.3 $[M+H]^+$; Elemental Analysis Calculated C, 52.65; H, 4.42; N, 9.69; S, 7.40. Found C, 52.67; H, 4.40; N, 9.70; S, 7.38.

4-(6-methoxy-4-oxospiro[chroman-2,4′-piperidin]-1′-yl)-3-nitrobenzenesulfonamide (**6d**):

67% yield as a yellow solid. TLC: R_f: 0.15 (30% Acetone in pet ether). m.p. 142 °C. ^{1}H NMR in DMSO-d_6: δ 8.21 (s, 1H, H-1), 7.87 (d, J = 8 Hz, 1H, H-3), 7.46 (d, J = 8 Hz , 1H, H-9), 7.41 (brs, 2H, SO_2NH_2),7.21–7.17 (m, 2H, H-7, H-10), 7.03 (d, J = 8 Hz, 1H, H-2), 3.74 (s, 3H), 3.28–3.25 (m, 2H, H-4), 3.19–3.16 (m, 2H,H-4′), 2.86 (s, 2H, H-6), 1.99 (m, 2H, H-5) and 1.86–1.83 (m, 2H, H-5′). ^{13}C NMR in DMSO-d_6: δ 191.3, 153.4, 152.8, 147.3, 138.4, 134.4, 130.7, 124.6, 124.4, 121.2, 120.3, 119.7, 107.1, 77.1, 55.5, 46.7, 46.1 and 33.1. LCMS (ESI positive) m/z = 448.2 $[M+H]^+$; Elemental Analysis Calculated C, 53.69; H, 4.73; N, 9.39; S, 7.16. Found C, 53.72; H, 4.72; N, 9.38; S, 7.14.

4-(6-ethoxy-4-oxospiro[chroman-2,4′-piperidin]-1′-yl)-3-nitrobenzenesulfonamide (**6e**):

75% yield as a yellow solid. TLC: R_f: 0.15 (30% Acetone in pet ether). m.p. 138 °C. ^{1}H NMR in DMSO-d_6: δ 8.21 (s, 1H, H-1), 7.85 (d, J = 8 Hz, 1H, H-3), 7.46 (d, J = 8 Hz , 1H, H-9), 7.41 (br s, 2H, SO_2NH_2), 7.15 (m, 2H, H-7, H-10), 7.04 (d, J = 8.4 Hz, 1H, H-2), 3.98 (brs, 2H), 3.32–3.28 (m, 2H, H-4), 3.19 (m, 2H, H-4′), 2.86 (s, 2H, H-6), 2.00 (m, 2H, H-5), 1.83 (m, 2H, H-5′) and 1.29 (brs, 3H). ^{13}C NMR in DMSO-d_6: δ 191.3, 152.7, 152.6, 147.3, 138.4, 134.4, 130.7, 125.0, 124.4, 121.2, 120.3, 119.7, 107.8, 77.1, 63.5, 46.7, 46.1, 33.1 and 14.5. LCMS (ESI positive) m/z = 462.2 $[M+H]^+$; Elemental Analysis Calculated C, 54.66; H, 5.02; N, 9.11; S, 6.95. Found C, 54.64; H, 5.00; N, 9.13; S, 6.97.

3-nitro-4-(4-oxo-6-propoxyspiro[chroman-2,4′-piperidin]-1′-yl)benzenesulfonamide (**6f**):

72% yield as a yellow solid. TLC: R_f: 0.16 (30% Acetone in pet ether). m.p. 145 °C. ^{1}H NMR in DMSO-d_6: δ 8.20 (s, 1H, H-1), 7.87 (d, J = 8.4 Hz, 1H, H-3), 7.46 (d, J = 8.8 Hz , 1H, H-9), 7.40 (br s, 2H, SO_2NH_2), 7.19–7.16 (m, 2H, H-7, H-10), 7.03 (d, J = 8.4 Hz, 1H, H-2), 3.89 (brs, 2H), 3.32–3.28 (m, 2H, H-4), 3.19 (m, 2H, H-4′), 2.86 (s, 2H, H-6), 2.00 (d, J = 8 Hz, 2H), 1.84–1.83 (m, 2H, H-5), 1.70–1.69 (m, 2H, H-5′) and 0.96 (t, J = 6 Hz, 3H). ^{13}C NMR in DMSO-d_6: δ 191.2, 152.7, 147.2, 138.4, 134.4, 130.7, 124.9, 124.3, 121.2, 120.3, 119.6, 107.9, 77.1, 69.4, 46.7, 46.0, 33.1 and 21.9. LCMS (ESI positive) m/z = 476.3 $[M+H]^+$: Elemental Analysis Calculated, 55.57; H, 5.30; N, 8.84; S, 6.74. Found, 55.58; H, 5.32; N, 8.82; S, 6.77.

4-(6-isopropoxy-4-oxospiro[chroman-2,4′-piperidin]-1′-yl)-3-nitrobenzenesulfonamide (**6 g**):

76% yield as a yellow solid. TLC: R_f: 0.18 (30% Acetone in pet ether). m.p. 98 °C ^{1}H NMR in DMSO-d_6: δ 8.20 (s, 1H, H-1), 7.87 (d, J = 8.8 Hz, 1H, H-3), 7.46 (d, J = 8.8 Hz , 1H, H-9), 7.41 (br s, 2H, SO_2NH_2), 7.19–7.15 (m, 2H, H-7, H-10), 7.02 (d, J = 8.4 Hz, 1H, H-2), 4.52 (m, 1H), 3.31–3.28 (m, 2H, H-4), 3.19–3.16 (m, 2H, H-4′), 2.85 (s, 2H, H-6), 2.01–1.98 (m, 2H, H-5), 1.84–1.83 (m, 2H, H-5′) and 1.23 (d, J = 8 Hz, 6H). ^{13}C NMR in DMSO-d_6: δ 191.3, 152.7, 151.4, 147.3, 138.4, 134.4, 130.7, 126.1, 124.4, 121.2, 120.4, 119.7, 109.7, 77.1, 70.0, 46.7, 46.1, 33.1 and 21.70. LCMS (ESI positive) m/z = 476.2 $[M+H]^+$; Elemental Analysis Calculated, C, 55.57; H, 5.30; N, 8.84; S, 6.74. Found, C, 55.59; H, 5.31; N, 8.83; S, 6.75.

4-(6-(2-hydroxyethoxy)-4-oxospiro[chroman-2,4′-piperidin]-1′-yl)-3-nitrobenzenesulfonamide (**6i**):

57% yield as a yellow solid. TLC: R_f: 0.15 (40% Acetone in pet ether). m.p. 137 °C ^{1}H NMR in DMSO-d_6: δ 8.20 (s, 1H, H-1), 7.87 (d, J = 8 Hz, 1H, H-3), 7.46 (d, J = 8.8 Hz , 1H, H-9), 7.40 (br s, 2H, SO_2NH_2), 7.24–7.18 (m, 2H, H-7, H-10), 7.04 (d, J = 8 Hz, 1H, H-2), 3.96 (brs, 2H), 3.68 (brs, 2H), 3.30–3.27 (m, 2H, H-4), 3.19–3.13 (m, 2H, H-4′), 2.86 (s, 2H, H-6), 2.00 (m, 2H, H-5) and 1.84–1.82 (m, 2H, H-5′). ^{13}C NMR in DMSO-d_6: δ 191.2, 152.8, 147.3, 138.4, 134.4, 130.7, 125.0, 124.3, 121.2, 120.3, 119.6, 108.1, 77.1, 70.1, 59.4, 46.7, 46.1 and 33.1. LCMS (ESI positive) m/z = 478.2 $[M+H]^+$; Elemental Analysis Calculated, C, 52.82; H, 4.86; N, 8.80; S, 6.71. Found, C, 52.81; H, 4.85; N, 8.82; S, 6.69.

4-(6-(2-methoxyethoxy)-4-oxospiro[chroman-2,4′-piperidin]-1′-yl)-3-nitrobenzenesulfonamide (**6j**):

76% yield as a yellow solid. TLC: R_f: 0.28 (40% Acetone in pet ether). m.p. 109 °C. ^{1}H NMR in DMSO-d_6: δ 8.20 (s, 1H, H-1), 7.87 (d, J = 8.4 Hz, 1H, H-3), 7.45 (d, J = 8.8 Hz , 1H, H-9), 7.40 (br s, 2H, SO_2NH_2), 7.25–7.18 (m, 2H, H-7, H-10), 7.04 (d, J = 8 Hz, 1H, H-2), 4.06 (brs, 2H), 3.63 (brs, 2H), 3.33–3.29 (m, 5H), 3.18–3.16 (m, 2H, H-4′), 2.86 (s, 2H, H-6), 2.01 (m, 2H, H-5) and 1.83–1.81 (m, 2H, H-5′). ^{13}C NMR in DMSO-d_6: δ 191.2, 152.9, 152.6, 147.2, 138.4, 134.4, 130.7, 125.0, 124.3, 121.2, 120.3, 119.7, 108.0, 77.1, 70.2, 67.4, 58.0, 46.6, 46.0 and 33.1. LCMS (ESI positive) m/z = 492.3 $[M+H]^+$; Elemental Analysis Calculated, C, 53.76; H, 5.13; N, 8.55; S, 6.52. Found, C, 53.74; H, 5.11; N, 8.56; S, 6.51.

1′-(2-nitro-4-sulfamoylphenyl)-4-oxospiro[*chroman*-2,4′-*piperidin*]-6-yl acetate (**6k**):

60% yield as a yellow solid. TLC: R_f: 0.2 (40% Acetone in pet ether). m.p. 133 °C ^{1}H NMR in DMSO-d_6: δ 8.21 (s, 1H, H-1), 7.87 (d, J = 8.8 Hz, 1H, H-3), 7.50–7.35 (m, 5H), 7.14 (d, J = 8 Hz, 1H, H-2), 3.32–3.30 (m, 2H, H-4), 3.21–3.19 (m, 2H, H-4′), 2.92 (s, 2H, H-6), 2.24 (s, 3H), 2.05 (m, 2H, H-5) and 1.87–1.85 (m, 2H, H-5′). ^{13}C NMR in DMSO-d_6: δ 190.9, 169.3, 156.0, 147.2, 144.1, 138.4, 134.5, 130.7, 130.2, 124.3, 121.2, 120.4, 119.5, 118.1, 77.7, 46.4, 46.0, 33.1 and 20.7. LCMS (ESI positive) m/z = 476.2 $[M+H]^+$; Elemental Analysis Calculated, C, 53.05; H, 4.45; N, 8.84; S, 6.74. Found, C, 53.03; H, 4.46; N, 8.85; S, 6.76.

4-(7-hydroxy-4-oxospiro[chroman-2,4′-piperidin]-1′-yl)-3-nitrobenzenesulfonamide (**6 l**):

61% yield as a yellow solid. TLC: R_f: 0.15 (40% Acetone in pet ether). m.p. 144 °C. ^{1}H NMR in DMSO-d_6: δ 10.59 (br s, 1H), 8.21 (s, 1H, H-1), 7.87 (d, J = 8.4 Hz, 1H, H-3), 7.59–7.43 (m, 4H), 6.47–6.36 (m, 2H), 3.27–3.14 (m, 4H, H-4-4′), 2.75 (s, 2H, H-6), and 1.99–1.83 (m, 4H, H5-5′). ^{13}C NMR in DMSO-d_6: δ 189.3, 164.8, 160.5, 147.3, 138.4, 134.4, 130.7, 128.0, 124.4, 121.2, 113.2, 110.2, 103.1, 77.4, 46.5, 46.1 and 33.4. LCMS (ESI positive) m/z = 434.2 $[M+H]^+$; Elemental Analysis Calculated, C, 52.65; H, 4.42; N, 9.69; S, 7.40. Found, C, 52.64; H, 4.40; N, 9.71; S, 7.38.

4-(7-methoxy-4-oxospiro[chroman-2,4′-piperidin]-1′-yl)-3-nitrobenzenesulfonamide (**6m**):

74% yield as a yellow solid. TLC: R_f: 0.15 (30% Acetone in pet ether). m.p. 110 °C. ^{1}H NMR in DMSO-d_6: δ 8.20 (s, 1H, H-1), 7.87 (d, J = 8.4 Hz, 1H, H-3), 7.66 (d, J = 8 Hz , 1H, H-7), 7.46 (d, J = 8 Hz, 1H, H-2), 7.40 (br s, 2H, SO_2NH_2), 6.61 (m, 2H, H-8, H-10), 3.81 (s, 3H), 3.30–3.28 (m, 2H, H-4), 3.20–3.18 (m, 2H, H-4′), 2.80 (s, 2H, H-6), 2.02 (m, 2H, H-5) and 1.87–1.84 (m, 2H, H-5′). ^{13}C NMR in DMSO-d_6: δ 189.6, 165.8, 160.5, 147.2, 138.3, 134.4, 130.7, 127.5, 124.3, 121.1, 114.1, 109.5, 101.5, 77.7, 55.7, 46.4, 46.1 and 33.3. LCMS (ESI positive) m/z = 448.2 $[M+H]^+$; Elemental Analysis Calculated, C, 53.69; H, 4.73; N, 9.39; S, 7.16. Found, C, 53.67; H, 4.72; N, 9.40; S, 7.18.

4-(7-ethoxy-4-oxospiro[chroman-2,4′-piperidin]-1′-yl)-3-nitrobenzenesulfonamide (**6n**):

56% yield as a yellow solid. TLC: R_f: 0.15 (30% Acetone in pet ether). m.p. 131 °C. ^{1}H NMR in DMSO-d_6: δ 8.21 (s, 1H, H-1), 7.87 (d, J = 8 Hz, 1H, H-3), 7.65 (d, J = 8 Hz, 1H, H-7), 7.46 (d, J = 8.8 Hz , 1H, H-2), 7.41 (br s, 2H, SO_2NH_2), 6.59 (m, 2H, H-8,H-10), 4.09 (q, J = 1.5 Hz, 2H), 3.29 (m, 2H, H-4), 3.20 (m, 2H, H-4′), 2.79 (s, 2H, H-6), 2.00 (m, 2H, H-5), 1.84 (m, 2H, H-5′) and 1.31 (t, J = 6 Hz, 3H). ^{13}C NMR in DMSO-d_6: δ 189.6, 165.1, 160.5, 147.3, 138.3, 134.3, 130.7, 127.6, 124.4, 121.1, 114.0, 109.8, 101.9, 77.7, 63.8, 46.5, 46.1, 33.3 and 14.3. LCMS (ESI positive) m/z = 462.2 $[M+H]^+$; Elemental Analysis Calculated, C, 54.66; H, 5.02; N, 9.11; S, 6.95. Found, C, 54.68; H, 5.01; N, 9.12; S, 6.97.

3-nitro-4-(4-oxo-7-propoxyspiro[chroman-2,4′-piperidin]-1′-yl)benzenesulfonamide (**6o**):

70% yield as a yellow solid. TLC: R_f: 0.15 (30% Acetone in pet ether). m.p. 145 °C. ^{1}H NMR in DMSO-d_6: δ 8.21 (s, 1H, H-1), 7.87 (d, J = 8.4 Hz, 1H, H-3), 7.65 (d, J = 8 Hz, 1H, H-7), 7.46 (d, J = 8.8 Hz , 1H, H-2), 7.41 (br s, 2H, SO_2NH_2), 6.60 (m, 2H, H-8,H-10), 3.99 (q, J = 2.6 Hz, 2H), 3.29 (m, 2H, H-4), 3.32 (m,2H, H-4′), 3.19 (m, 2H), 2.79 (s, 2H, H-6), 2.00 (m, 2H, H-5), 1.84 (m, 2H, H-5′), 1.71 (m, 2H) and 0.95 (t, J = 6 Hz, 3H). ^{13}C NMR in DMSO-d_6: δ 189.6, 165.3, 160.5, 147.3, 138.3, 134.4, 130.7, 127.5, 124.4, 121.1, 114.0, 109.8, 101.9, 77.7, 69.5, 46.5, 46.1, 33.3, 21.7 and 10.2. LCMS (ESI positive) m/z = 476.3 $[M+H]^+$; Elemental Analysis Calculated, C, 55.57; H, 5.30; N, 8.84; S, 6.74. Found, C, 55.56; H, 5.28; N, 8.83; S, 6.76.

4-(7-isopropoxy-4-oxospiro[chroman-2,4′-piperidin]-1′-yl)-3-nitrobenzenesulfonamide (**6p**):

87% yield as a yellow solid. TLC: R_f: 0.18 (30% Acetone in pet ether). m.p. 88 °C. ^{1}H NMR in DMSO-d_6: δ 8.21 (s, 1H, H-1), 7.87 (d, J = 8 Hz, 1H, H-3), 7.64 (d, J = 8 Hz , 1H, H-7), 7.47 (d, J = 8.8 Hz, 1H, H-2), 7.41 (br s, 2H, SO_2NH_2), 6.58–6.56 (m, 2H, H-8,H-10), 4.73–4.70 (m, 1H), 3.32–3.28 (m, 2H, H-4), 3.20–3.17 (m, 2H, H-4′), 2.78 (s, 2H, H-6), 2.01–1.98 (m, 2H, H-5), 1.86–1.83 (m, 2H, H-5′) and 1.27 (d, J = 4 Hz, 6H). ^{13}C NMR in DMSO-d_6: δ 189.5, 164.2, 160.5, 147.3, 138.3, 134.3, 130.7, 127.6, 124.4, 121.1, 113.8, 110.3, 102.5, 77.6, 70.0, 46.5, 46.1, 33.3 and 21.6. LCMS (ESI positive) m/z = 476.2 $[M+H]^+$; Elemental Analysis Calculated, C, 55.57; H, 5.30; N, 8.84; S, 6.74. Found, C, 55.55; H, 5.31; N, 8.83; S, 6.75.

4-(7-(2-hydroxyethoxy)-4-oxospiro[chroman-2,4′-piperidin]-1′-yl)-3-nitrobenzenesulfonamide (**6r**):

52% yield as a yellow solid. TLC: R_f: 0.15 (40% Acetone in pet ether). m.p. 74 °C. ^{1}H NMR in DMSO-d_6: δ 8.21 (s, 1H, H-1), 7.87 (d, J = 8 Hz, 1H, H-3), 7.65 (d, J = 8.4 Hz, 1H, H-7), 7.47 (d, J = 8.4 Hz , 1H, H-2), 7.41 (br s, 2H, SO_2NH_2), 6.61 (m, 2H, H-8,H-10), 4.9 (br s, 1H, –OH), 4.05 (t, J = 6 Hz, 2H), 3.70 (m, 2H, H-4), 3.32 (m, 2H, H-4′), 3.20 (m, 2H), 2.8 (s, 2H, H-6), 2.00 (m, 2H, H-5) and 1.84 (m, 2H, H-5′). ^{13}C NMR in DMSO-d_6: δ 189.6, 165.3, 160.5, 147.2, 138.3, 134.3, 130.7, 127.5, 124.4, 121.1, 114.1, 109.8, 102.0, 77.7, 70.1, 59.2, 46.5, 46.1 and 33.3. LCMS (ESI positive) m/z = 478.2 $[M+H]^+$; Elemental Analysis Calculated, C, 52.82; H, 4.86; N, 8.80; S, 6.71. Found, C, 52.80; H, 4.85; N, 8.81; S, 6.73.

4-(7-(2-methoxyethoxy)-4-oxospiro[chroman-2,4′-piperidin]-1′-yl)-3-nitrobenzenesulfonamide (**6 s**):

73% yield as a yellow solid. TLC: R_f: 0.28 (40% Acetone in pet ether). m.p. 105 °C. ^{1}H NMR in DMSO-d_6: δ 8.21 (s, 1H, H-1), 7.87 (d, J = 8 Hz, 1H, H-3), 7.65 (d, J = 8 Hz, 1H, H-7), 7.47 (d, J = 8 Hz , 1H, H-2), 7.41 (br s, 2H, SO_2NH_2), 6.63 (m, 2H, H-8,H-10), 4.16 (t, J = 6 Hz, 2H), 3.64 (t, J = 6 Hz, 2H), 3.31 (m, 2H, H-4), 3.29 (s, 3H), 3.20 (m, 2H, H-4′), 2.8 (s, 2H), 2.00 (m, 2H, H-5) and 1.84 (m, 2H, H-5′). ^{13}C NMR in DMSO-d_6: δ 189.6, 165.0, 160.5, 147.3, 138.3, 134.4, 130.7, 127.6, 124.4, 121.1, 114.2, 109.8, 102.0, 77.7, 70.0, 67.5, 58.1, 46.5, 46.1 and 33.3. LCMS (ESI positive) m/z = 492.2 $[M+H]^+$; Elemental Analysis Calculated C, 53.76; H, 5.13; N, 8.55; S, 6.52. Found, C, 53.75; H, 5.12; N, 8.56; S, 6.51.

4-(7-bromo-4-oxospiro[chroman-2,4′-piperidin]-1′-yl)-3-nitrobenzenesulfonamide (**6u**):

73% yield as a yellow solid. TLC: R_f: 0.3 (30% Acetone in pet ether). m.p. 99 °C. ^{1}H NMR in DMSO-d_6: δ 8.20 (s, 1H, H-1), 7.88 (d, J = 7.2 Hz, 1H, H-3), 7.65 (d, J = 8.4 Hz, 1H, H-7), 7.47 (d, J = 8.8 Hz , 1H, H-2), 7.41 (m, 3H), 7.25 (d, J = 8.4 Hz, 1H, H-8), 3.19–3.16 (m, 2H, H-4), 3.09–3.07 (m, 2H, H-4′), 2.91 (s, 2H, H-6), 2.00 (m, 2H, H-5) and 1.88–1.85 (m, 2H, H-5′). ^{13}C NMR in DMSO-d_6: δ 190.6, 159.0, 147.2, 138.5, 134.5, 130.7, 129.5, 127.6, 124.4, 124.3, 121.2, 119.5, 78.4, 46.0, 45.7 and 33.1. LCMS (ESI positive) m/z = 495.9 & 497.9 [Br pattern, $M+H]^+$; Elemental Analysis Calculated C, 45.98; H, 3.66; N, 8.47; S, 6.46. Found, C, 45.97; H, 3.65; N, 8.45; S, 6.48.

4-(6-bromo-4-oxospiro[chroman-2,4′-piperidin]-1′-yl)-3-nitrobenzenesulfonamide (**6v**):

85% yield as an orange solid. TLC: R_f: 0.3 (30% Acetone in pet ether). m.p. 103 °C. ^{1}H NMR in DMSO-d_6: δ 8.21 (s, 1H, H-1), 7.93–7.77 (m, 3H), 7.47–7.41 (m, 3H), 7.11 (s, 1H, H-10), 3.39–3.31 (m, 2H, H-4), 3.13–3.08 (m, 2H, H-4′), 2.93 (s, 2H, H-6), 2.00 (m, 2H, H-5) and 1.88–1.85 (m, 2H, H5′). ^{13}C NMR in DMSO-d_6: δ 190.3, 157.6, 147.2, 138.7, 138.4, 134.5, 130.7, 127.8, 124.3, 121.8, 121.2, 121.1, 112.8, 78.0, 47.2, 47.0 and 36.0. LCMS (ESI positive) m/z = 496.2 & 498.2 [Br pattern, $M+H]^+$; Elemental Analysis Calculated C, 45.98; H, 3.66; N, 8.47; S, 6.46. Found, C, 45.97; H, 3.64; N, 8.45; S, 6.47.

7. Carbonic anhydrase inhibition

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration activity [20]. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 20 mMHepes (pH 7.5) as buffer, and 20 mM Na_2SO_4 (for maintaining constant the ionic strength), following the initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (0.1 mM) were prepared in distilled-deionized water and dilutions up to 0.01 nM were done thereafter with the assay buffer. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E-I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3 and the Cheng–Prusoff equation, as reported earlier [23–26], and represent the mean from at least three different determinations. All CA isofoms were recombinant ones obtained in-house as reported earlier [23–26].

8. Biological assays

Animals. Male CD-1 albino mice (Envigo, Varese, Italy) weighing approximately 22–25 g at the beginning of the experimental procedure, were used. Animals were housed in Ce.S.A.l (Centro Stabulazione Animali da Laboratorio, University of Florence) and used at least

1 week after their arrival. Ten mice were housed per cage (size 26 × 41 cm). Animals were fed a standard laboratory diet and tap water ad libitum, and kept at 23 ± 1 °C with a 12 h light/dark cycle, light at 7 a.m. All animal manipulations were carried out according to the Directive 2010/63/EU of the European parliament and of the European Union council (22 September 2010) on the protection of animals used for scientific purposes. The ethical policy of the University of Florence complies with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (NIH Publication No. 85-23, revised 1996; University of Florence assurance number: A5278-01). Formal approval to conduct the experiments described was obtained from the Animal Subjects Review Board of the University of Florence. Experiments involving animals have been reported according to ARRIVE guideline. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Oxaliplatin-induced neuropathic pain model and pharmacological treatments. Mice treated with oxaliplatin (2.4 mg kg^{-1}) were administered intraperitoneally (i.p.) on days 1–2, 5–9, 12–14 (10 i.p. injections) [27]. Oxaliplatin was dissolved in 5% glucose solution. Control animals received an equivalent volume of vehicle. Behavioural tests were performed starting from day 15. **AAZ** new compounds were suspended in 1% carboxymethylcellulose sodium salt (CMC, Sigma-Aldrich, Milan, Italy) and *per os* (p.o.) acutely administered.

Cold plate test. The animals were placed in a stainless steel box (12 cm × 20 cm × 10 cm) with a cold plate as floor. The temperature of the cold plate was kept constant at 4 °C ± 1 °C. Pain-related behaviour (licking of the hind paw) was observed and the time (seconds) of the first sign was recorded. The cut-off time of the latency of paw lifting or licking was set at 60 s [28,29].

Statistical analysis. Behavioural measurements were performed on 12 mice for each treatment carried out in 2 different experimental sets. Results were expressed as mean ± S.E.M. The analysis of variance of behavioural data was performed by one way ANOVA, a Bonferroni's significant difference procedure was used as post-hoc comparison. *P* values of less than 0.05 or 0.01 were considered significant. Investigators were blind to all experimental procedures. Data were analysed using the “Origin 9” software (OriginLab, Northampton, USA).

Declaration of Competing Interest

The authors state no conflict of interests.

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