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Laboratory note

A new convenient synthetic method and preliminary pharmacological characterization of triazinediones as prokineticin receptor antagonists

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ABSTRACT

A new efficient synthetic method to obtain prokineticin receptor antagonists based on the triazinedione scaffold is described. In this procedure the overall yield improves from 13% to about 54%, essentially for two factors: 1) *N*-(chlorocarbonyl) isocyanate is no more used, it represents the yield limiting step with an average yield not exceeding 30%. 2) The Mitsunobu reaction is not involved in the new synthetic scheme avoiding the use of time and solvent consuming column chromatography. All synthesized triazinediones were preliminary pharmacologically screened *in vivo* for their ability to reduce the Bv8-induced thermal hyperalgesia. In this assay all compounds displayed EC_{50} values in the picomolar −subpicomolar range, some triazinediones containing a 4-halogen substituted benzyl group in position 5 showed the best activity. The analogues containing a 4-fluorine atom (PC-7) and a 4-bromobenzyl group (PC-25) resulted 10 times more potent than the reference PC-1 that bears a 4-ethylbenzyl group. While the 4-trifluoromethylbenzyl substituted analog (PC-27) was 100 times more potent as compared to PC1.

1. Introduction

The mammalian prokineticin 1 (PK1) and prokineticin 2 (PK2), also known as Endocrine Gland-derived Vascular Endothelial Growth Factor (EG-VEGF) and Bombina Variegata 8 (Bv8) are a pair of bioactive peptides highly conserved across species [1]. PK1 and PK2 make up a new family of chemokines which activate two G-protein linked receptors (prokineticin receptor 1 and 2, PKR_1 and PKR_2). Intensive research of the prokineticin system over the past decade has revealed a dazzling array of physiological functions [2]. Since their initial identification, multiple physiological roles have been discovered including: gastrointestinal motility [3,4], regulation of circadian rhythms [5], angiogenesis [6,7], olfactory bulb neurogenesis [8], neuroexcitation [9] inflammation [10,11], and reproduction [12]. In addition, the disruption of prokineticin system has been implicated in several pathological conditions, including immunological response [13] and persistent pain [14].

Recent evidences implicated PK2 in a human disease in which different point mutations in genes encoding PK2 or its receptor (PKR_2) lead to a type of Kallmann syndrome, a disease characterized by a deficiency in hypothalamic gonadotropin-releasing hormones [15]. Furthermore PK2 is reported as an endangering mediator of cerebral ischemia injury [16].

Considering the potential involvement of the prokineticin system in different human disease, the availability of prokineticin receptor modulators represents an important task.

At the best of our knowledge, until now only two articles describing the synthesis of triazinediones endowed with a preferential PKR_1 antagonist activity have been reported [17,18]. Only one unselective antagonist endowed with a morpholine scaffold is published to date [16]. On the contrary, numerous patents were filed about the synthesis and use of such antagonists endowed with triazine and morpholine scaffolds. The two most recent patents were filed in early 2012 [19,20]. Furthermore in the late 2013, Takeda Pharmaceuticals filed a patent for new PKR antagonists characterized by a sulfonylpiperidine scaffold [21].

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In an attempt to identify one or more selective ligands for prokineticin receptors, we started the synthesis of triazinediones endowed with different substituted/unsubstituted benzyl groups at positions 1 and 5. The substitution pattern of triazine scaffold was selected taking into account the results of patent literature and the availability of the synthetic precursors. Looking at published and patented synthetic ways to triazinediones, we identified essentially two different routes clearly reported by Ralbovsky et al. and our group [18,17]. Unfortunately, each synthetic way is characterized by limiting steps which significantly lowers the overall yield. In Scheme 1 it is depicted the first of two synthetic methods published by Ralbovsky et al. [18] and also partially adopted in our previous paper [17].

The major drawback of this route is represented by the N-acylation of isothiourea **24** to give the intermediate **25**. This reaction per se is not difficult, but often it is a source of side reactions and it is quite easy to obtain considerable amounts of the disubstituted isothiourea. Indeed, in our hand this step never gave yields higher than 50%. Furthermore, if the second benzyl group is introduced by a Mitsunobu reaction, a column chromatography purification is always necessary to obtain the intermediate **27** of good purity.

The second route to triazinediones is summarized in Scheme 2. In this synthetic way, the ring closure by *N*-(chlorocarbonyl) isocyanate (step b) to give the intermediate **30** represents the yield limiting step. The average yield of this reaction does not exceed 30%.

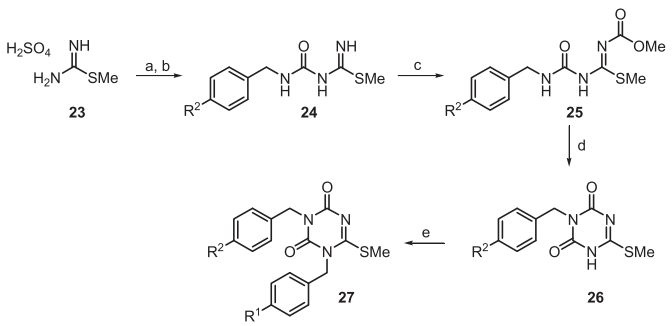
Taking into account these considerations, here we report a new synthetic method that can give easy access to high yield and purity new triazinediones as potential ligands for prokineticin receptors. All triazinediones were screened *in vivo* in mice to evaluate their ability to reduce the hyperalgesia induced by BV8, a selective agonist for the prokineticin receptors expressed on the peripheral endings of nociceptors.

2. Results and discussion

2.1. Chemistry

In Scheme 3 is depicted the new way we used for the synthesis of **PC-25** and all the other triazinediones reported in Table 1.

Coupling 4-bromobenzylurea **31**, obtained upon reaction of 4-bromobenzylamine hydrochloride with potassium cyanate [22], with the commercially available ethoxycarbonyl isothiocyanate gave the *N*-ethoxycarbonylthiourea **32** in 85% yield. Subsequently, **32** was cyclized under basic conditions to yield the triazine **33** (yield 93%), which was alkylated by methyl iodide under basic conditions to provide the key intermediate **34** in 90% yield (method A). Alternatively, cyclization and successive methylation steps can be conveniently done one pot (Method B), avoiding the isolation and purification of **33**, with a further improvement of yield up to 95%. The 4-methoxybenzyl group was introduced as a chloride in presence of potassium carbonate to give the intermediate **35**. Displacement of the thiomethyl group by ethylenediamine in toluene at 90 °C yielded **36**, which was converted into the final guanidine compound **PC-25** with pyrazole-1-carboxamidine hydrochloride.



Scheme 1. Reagents and conditions: (a) 3 N NaOH; (b) R^2-C_6H_4–CH_2–NCO, H_2O/MeOH/THF, 0 °C to rt; (c) methylchloroformate, NEt_3, CH_2Cl_2, −10 °C to rt; (d) NaOMe, MeOH; (e) R^1-C_6H_4–CH_2–OH, PPh_3, DEAD, THF (Mitsunobu reaction); or R^1-C_6H_4–CH_2–Cl, NaOMe, CH_3CN, heat.

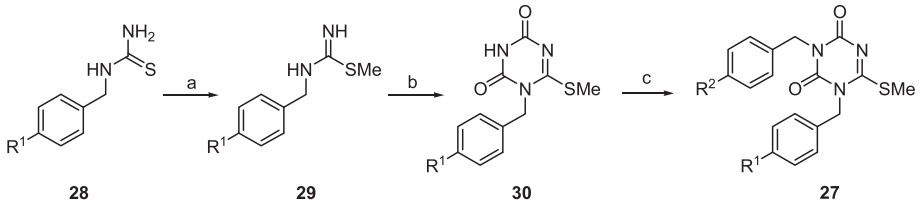
This synthetic method is endowed with two important features: high yield in each step, and the purification of intermediates never needs the use of time and solvent consuming column chromatography. In this way it is easy to obtain triazinediones in high amount and high purity for furthermore in deep pharmacological *in vitro* and *in vivo* studies.

2.2. Analgesic activity

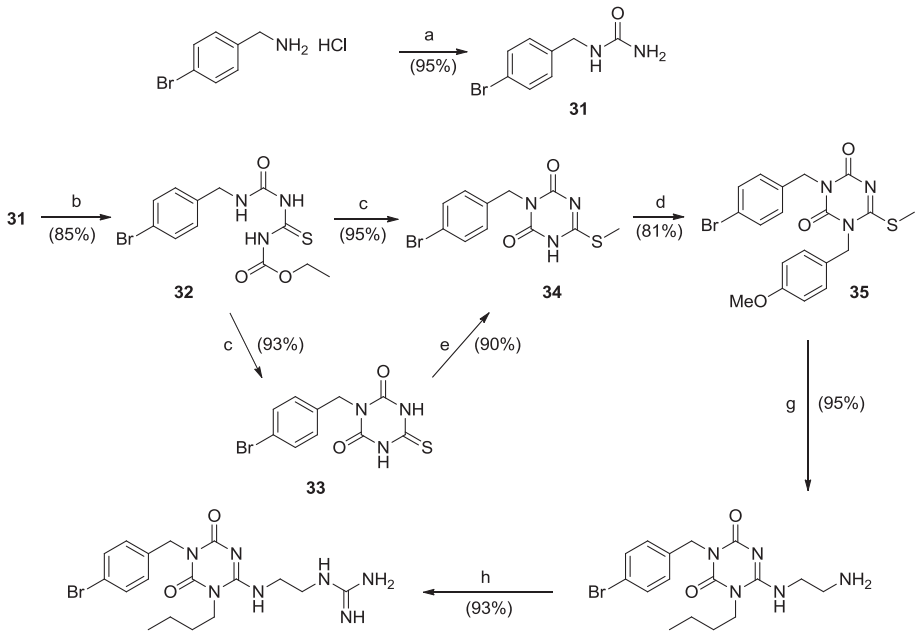
All synthesized triazinediones were screened *in vivo* in mice with a quick and simple test: by injecting them into one hind paw of mice and evaluating their ability to reduce the hyperalgesia induced by BV8, a selective agonist for the prokineticin receptors expressed on the peripheral endings of nociceptors. The compounds injected into the hind paw of mice up to doses of 120 pmol never produced changes in the basal thermal sensitivity, measured as the latency to paw withdrawal from 48 °C water, indicating they do not activate any receptor involved in nociceptor sensitization. Hence we tested their antagonistic activity looking for effectiveness in antagonizing the hyperalgesic effect of Bv8, a selective PKR-1 and PKR-2 agonist which, binding these receptors, increases the nociceptor sensitization, so reducing the paw withdrawal latency. As already demonstrated [23] intraplantar (i.pl. – subcutaneous injection in the paw) injection of 63 fmol of Bv8 induced more than 50% decrease in thermal threshold of the injected paw in 30 min. We evaluated if and at which doses the new molecules were able to reduce the Bv8-induced decrease in thermal threshold when injected by intraplantar route 5 min before Bv8 injection. The ability of different doses of each compound in antagonizing the Bv8-induced hyperalgesia was evaluated. In Fig. 1 the dose–response curves of all synthesized triazinediones are reported and the corresponding EC_{50} values are showed in Table 1. Because of the high number of compounds, the dose response curves are divided in four panels. In each panel the dose–response curve of the reference PC-1 is reported. In Panel A, the most active compounds are shown while in panel B, C and D there are the compounds as/less effective than PC-1.

In this *in vivo* assay all triazinediones showed picomolar EC_{50} values. The analogues PC-7 and PC-25 displayed about 0.3 pmolar EC_{50} values and resulted 10 times more potent than the reference PC-1, while PC-27 (EC_{50} value 0.033 pmol) was 100 times more potent. The replacement of PC-1 4-ethylbenzyl group in position 5 with a 4-fluorine atom to give PC-7 or with a 4-bromobenzyl group to give PC-25 highly improved analgesic activity. The presence in 4-position of a chorine atom (PC18) produced antagonistic activity comparable with that of PC1, while its substitution with an iodine atom or a trifluoromethyl group led to reduction in activity. The opposite effect is showed by analog PC27 bearing a 4-trifluoromethylbenzyl group in position 5 and 4-methyl group in position 1, that is the most potent. The presence of a 4-methoxylbenzyl group in position 5 generally led to analogs that were less effective than the reference PC1, except PC24, PC26, PC29 and PC31 that displayed antagonistic activity comparable with that of PC1.

Peripheral nociceptors of mice contain both PKR_1 and PKR_2, hence with this test we cannot evaluate selectivity for either



Scheme 2. Reagents: (a) MeI, MeOH; (b) ClCONCO, DIEA, CH_2Cl_2; (c) R^2-C_6H_4–CH_2–OH, PPh_3, DEAD, THF (Mitsunobu reaction).



Scheme 3. Reagents and conditions: (a) KOCN, H_2O, 60 °C, 30 min; (b) ethoxycarbonyl isothiocyanate, toluene reflux, 4 h; (c) c[I]) NaOMe, MeOH, 50 °C, 0.5 h, c[II]) MeI, rt, 3 h; (d) NaOMe, MeOH, 50 °C, 0.5 h; (e) NaOMe, MeI, MeOH, rt, 3 h; (f) 4-MeO–C_6H_4–CH_2–Cl, K_2CO_3, DMF, rt, 48 h; (g) ethylenediamine, toluene, 90 °C; (h) pyrazole-1-carboxamidine hydrochloride, DIPEA, CH_3CN.

receptor. Because thermal nociception is mainly mediated by PKR_1 activation we might predict these compounds bind the PKR_1 however we cannot exclude they bind also PKR_2. To answer this question next step of our research will be to evaluate the affinity and selectivity for PKR_1 and PKR_2 by *in vitro* binding and *in vivo* test in mice lacking the *pkr1* or the *pkr2* gene.

3. Conclusions

We have reported a new improved synthetic method to triazinediones as potential antagonists for prokineticin receptors with an overall yield of about 54%, far better than 13% reported by us in a previous paper [17]. The advantage of this new route derives essentially by two factors: 1) the reaction of *N*-(chlorocarbonyl) isocyanate to give the intermediate **30** is not used; in fact it represents the yield limiting step with an average yield not exceeding 30%. 2) The Mitsunobu reaction is not involved in the synthetic scheme avoiding the use of time and solvent consuming column chromatography.

Using the new synthetic method we easily synthesized more than 20 triazinediones that were preliminary tested *in vivo* as potential antagonists of the hyperalgesic effect induced by Bv8. All triazinediones showed picomolar EC_{50} values and three compounds (PC-7; PC-25 and PC-27) showed an efficacy better than the reference PC-1.

Finally, now we are able to provide large amounts of the reference PKR antagonist PC-1 for *in vivo* studies regarding the inflammatory and neuropathic pain [14], and of PC-7 for *in vivo* studies in animal model of multiple sclerosis (manuscript in preparation) and Parkinson disease [24].

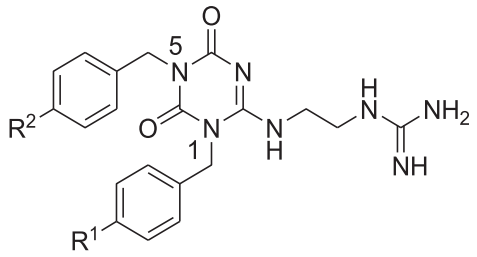
4. Experimental section

4.1. Chemistry

4.1.1. General methods

All commercially available solvents and reagents were used without further purification. ^1H and ^{13}C NMR spectra were recorded on a Varian Inova 500 spectrometer. The chemical shifts (δ) are reported in part per million downfield from tetramethylsilane (TMS), which was used as internal standard, and the spectra were recorded in DMSO-d_6. Infrared spectra were recorded on a Bruker Vector 22 spectrometer in Nujol mull. The main bands are given in cm^{-1}. Positive-ion electrospray ionization (ESI) mass spectra were recorded on a double-focusing Finnigan MAT 95 instrument with BE geometry. Melting points (mp) were determined on a Stuart Scientific Melting point SMP1 apparatus and are uncorrected. All products reported showed ^1H and ^{13}C NMR spectra in agreement with the assigned structures. The purity of tested compounds was determined by combustion elemental analyses conducted by the Microanalytical Laboratory of the Chemistry Department of the University of Ferrara with a Yanagimoto MT-5 CHN recorder elemental analyzer. All tested compounds yielded data consistent with a purity of at least 95% as compared with the theoretical

Table 1
Structures of new synthesized triazinediones and their EC_{50} analgesic activity values.



Compound	R^1	R^2	EC_{50} (pmol) [95% confidence intervals]
PC-1 (Reference)	*OMe*	*Et*	5.8 [3.6–9.1]
PC-7	*OMe*	*F*	0.31 [0.16–0.59]
PC-8	H	OMe	63 [44–90]
PC-15	OMe	Me	4.4 [2.5–7.8]
PC-17	OMe	OMe	0.053 [0.038–0.067]
PC-18	OMe	Cl	4.0 [2.9–5.7]
PC-23	OMe	CF_3	51 [27–83]
PC-24	Br	OMe	4.3 [2.7–6.8]
PC-25	*OMe*	*Br*	0.36 [0.17–0.78]
PC-26	3,4-diCl	OMe	4.5 [2.9–6.4]
PC-27	*Me*	*CF_3*	0.033 [0.018–0.061]
PC-28	3,4-diCl	Me	5.1 [3.8–6.8]
PC-29	Me	OMe	4.5 [3.4–5.6]
PC-30	F	OMe	36 [24–59]
PC-31	Cl	OMe	4.4 [3.1–5.9]
PC-32	CF_3	OMe	48 [33–70]
PC-33	OMe	3,4-methylenedioxy	39 [29–53]
PC-34	I	OMe	39 [27–52]
PC-35	OMe	I	51 [32–80]
PC-36	OMe	NO_2	48 [35––69]

values. Reaction courses and product mixtures were routinely monitored by TLC on silica gel (precoated F_{254} Merck plates), and compounds were visualized with aqueous $KMnO_4$, ninhydrin (1% ethanol, Merck) and chlorine spray reagents. R_f were determined in one or more of the following solvent systems: (A) AcOEt/Pe (1:9, v/v); (B) AcOEt/Pe/ammonia 30% in H_2O (1:1:0.3, v/v/v); (C) AcOEt/Pe (1:1, v/v); (D) AcOEt/Pe (1:2, v/v). Final crude compounds were purified by preparative reversed phase HPLC [Waters Delta Prep LC 40 mm assembly column C18 (30 cm × 4 cm, 15 μm particle size)] eluted at a flow rate of 20 mL/min with mobile phase solvent A (10% CH_3CN + 0.1% TFA in H_2O, v/v), and a linear gradient from 10% to 60% B (60% CH_3CN + 0.1% TFA in H_2O, v/v) in 25 min. Analytical HPLC analyses were performed on a Beckman System Gold (Beckman ultrasphere ODS column, 250 mm × 4.5 mm, 5 μm particle size). For analytical determinations and capacity factor (K') of then products were used solutions A and B as reported above, programmed at a flow rate of 1 mL/min with a linear gradient from 0% to 100% B in 25 min.

*4.1.2. 1-(4-Bromobenzyl)urea (**31**) was prepared according to the procedure of Carmellino et al. [22]*

Briefly, to a solution of 4-bromobenzylamine hydrochloride (4.45 g, 20 mmol) in H_2O (20 mL) potassium cyanate (1.62 g, 20 mmol) was added, and the mixture was heated at 60 °C for 30 min. After cooling at room temperature, the precipitate was filtered, rinsed with ice water and dried [25].

*4.1.3. 1-((4-Bromobenzyl)aminocarbonyl)-3-(ethoxycarbonyl) thiourea (**32**)*

A mixture of 1-(4-bromobenzyl)urea (**31**) (2.29 g, 10 mmol) and ethoxycarbonyl isothiocyanate (12 mmol, 1.2 mL) in toluene (15 mL) was refluxed for 4 h. The solvent was evaporated under vacuum, and the crude product was collected and washed with *i*-Pr_2O to give a pale-yellow solid: yield 3.06 g (85%); *Rf*(D) 0.66; HPLC K' 3.24; mp 180–182 °C; *m/z* 361 $(M+H)^+$; ^1H NMR (DMSO-d_6):

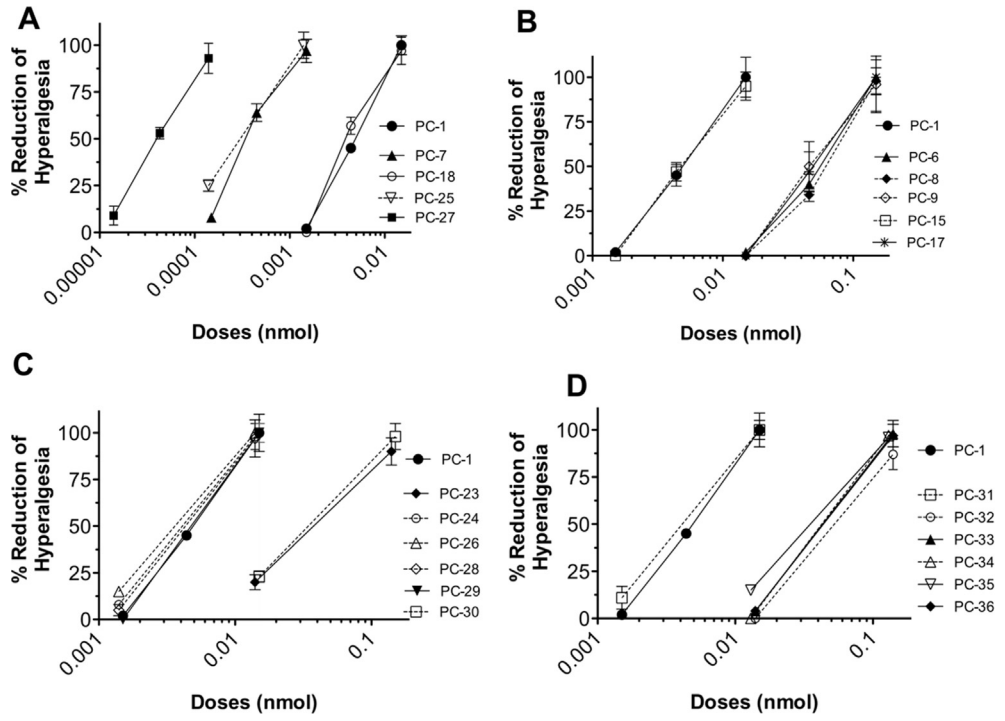


Fig. 1. Dose–response curves of all synthesized triazinediones.

δ 11.12 (s, 1H), 12.22 (s. 1H), 7.55–7.32 (m, 4H), 4.52 (d, $J = 6.0$ Hz, 2H), 4.26 (q, $J = 7.0$ Hz, 2H), 1.35 (t, $J = 7.0$ Hz, 3H); IR (nujol) 3320, 1746, 1731, 1654 cm^{-1}. Anal. Calcd. for $C_{12}H_{14}BrN_3O_3S$ (360.23): C, 40.01; H, 3.92, N, 11.66. Found: C, 40.06; H, 3.91, N, 11.64.

4.1.4. 3-(4-Bromobenzyl)-6-thioxo-1,3,5-triazine-2,4-dione (**33**)

To a solution of 1-((4-bromobenzyl)aminocarbonyl)-3-(ethoxycarbonyl)thiourea (**32**) (3.6 g, 10 mmol) in anhydrous MeOH (5 mL) 5.4 M methanolic NaOMe (1.7 mL) was added at room temperature. The reaction was heated at 50 °C for 0.5 h. The solvent was evaporated under vacuum, then H_2O (10 mL) was added and the solution was treated with acq. 10% hydrochloric acid to pH 5–6. The precipitate was filtered off, washed with H_2O (2 × 5 mL), air dried, then crystallized with EtOH to give a white solid: yield 2.92 g (93%); *Rf*(D) 0.49; HPLC *K′* 7.24; mp 236–238 °C; *m/z* 315 $(M+H)^+$; ^1H NMR (DMSO-d_6): δ 12.57 (s, 2H), 7.52–7.20 (m, 4H), 4.91 (s, 2H). IR (Nujol) 3075, 1763, 1570 cm^{-1}. Anal. Calcd. for $C_{10}H_8BrN_3O_2S$ (314,16): C, 38.23; H, 2.57, N, 13.38. Found: C, 38.20; H, 2.58, N, 13.41.

4.1.5. 3-(4-Bromobenzyl)-6-(methylthio)-1,3,5-triazine-2,4(1H,3H)-dione (**34**)

Method A) To a solution of 3-(4-bromobenzyl)-6-thioxo-1,3,5-triazine-2,4-dione (**33**) (3.14 g, 10 mmol) and 5.4 M methanolic NaOMe (1.7 mL) in anhydrous MeOH (5 mL) methyl iodide (1.9 mL, 30 mmol) was added at room temperature and the reaction mixture was stirred for 3 h. The solvent was evaporated under vacuum, then H_2O (10 mL) was added. The precipitate was filtered off, washed with H_2O (2 × 5 mL), then crystallized with 2-PrOH to give a pale-yellow solid: yield 2.95 g (90%); *Rf*(D) 0.28; HPLC *K′* 3.56; mp 166–168 °C; *m/z* 329 $(M+H)^+$; ^1H NMR (DMSO-d_6): δ 7.56–7.38 (m, 4H), 4.99 (s, 2H), 2.56 (s, 3H). IR (Nujol) 3421, 1756, 1737, 1644 cm^{-1}. Anal. Calcd. for $C_{11}H_{10}BrN_3O_2S$ (328.19): C, 40.26; H, 3.07, N, 12.80. Found: C, 40.20; H, 3.05, N, 12.83.

Method B) To a solution of 1-((4-bromobenzyl)aminocarbonyl)-3-(ethoxycarbonyl)thiourea (**32**) (3.6 g, 10 mmol) in anhydrous MeOH (5 mL) 5.4 M methanolic NaOMe (1.7 mL) was added at room temperature. The reaction was heated at 50 °C for 0.5 h. After cooling methyl iodide (0.8 mL, 12 mmol) was added at room temperature and the reaction mixture was stirred for 3 h. The title compound was isolated as described in method A: yield 3.11 g (95%).

4.1.6. 3-(4-Bromobenzyl)-1-(4-methoxybenzyl)-6-(methylthio)-1,3,5-triazine-2,4(1H,3H)-dione (**35**)

To a solution of 3-(4-bromobenzyl)-6-(methylthio)-1,3,5-triazine-2,4(1*H*,3*H*)-dione (**34**) (3.28 g, 10 mmol) in anhydrous DMF (5 mL) anhydrous potassium carbonate (2.76 g, 20 mmol) and 4-methoxybenzyl chloride (1.88 g, 12 mmol) were added. The mixture was stirred for 48 h at room temperature monitoring the reaction *via* HPLC. H_2O (20 mL) was added and the precipitate was filtered off, washed with H_2O (2 × 5 mL) and crystallized with MeCN to give a white solid: yield 3.63 g (81%); *Rf*(D) 0.53; HPLC *K′* 6.81; mp 171–173 °C; *m/z* 449 $(M+H)^+$; ^1H NMR (DMSO-d_6): δ 7.66–7.07 (m, 8H), 5.00 (s, 2H), 4.87 (s, 2H), 3.86 (s, 3H), 2.58 (s, 3H). IR (nujol) 1726, 1670, 1610 cm^{-1}. Anal. Calcd. for $C_{19}H_{18}BrN_3O_3S$ (448.33): C, 50.90; H, 4.05, N, 9.37. Found: C, 50.95; H, 4.06, N, 9.34.

4.1.7. 6-(2-Aminoethylamino)-3-(4-bromobenzyl)-1-(4-methoxybenzyl)-1,3,5-triazine-2,4(1H,3H)-dione (**36**)

To a solution of intermediate (**35**) (0.2 g, 0.45 mmol) in toluene (10 mL) at room temperature, ethylenediamine (0.16 mL, 2.41 mmol) was added. The reaction mixture was refluxed for 12 h. After solvent evaporation, the residue was dissolved in EtOAc and washed twice with H_2O (2 × 10 mL). The organic layer was dried (Na_2SO_4) and evaporated to afford (**36**) as a pale-yellow oil: yield 0.2 g (95%); *Rf*(D) 0.38; HPLC *K′* 5.41; mp 180–182 °C; *m/z* 461 $(M+H)^+$; ^1H NMR (DMSO-d_6): δ 7.53–6.88 (m, 8H), 4.98 (s, 2H), 4.87 (s, 2H), 3.73 (s, 3H), 3.28–3.25 (t, 2H, $J = 6.4$ Hz), 2.53–2.50 (t, 2H, $J = 6.4$ Hz); IR (nujol) 3337, 1702, 1570 cm^{-1} Anal. Calcd. for $C_{20}H_{22}BrN_5O_3$ (460.32): C, 52.18; H, 4.82, N, 15.21. Found: C, 52.12; H, 4.80, N, 15.24.

4.1.8. (2-(5-(4-Bromobenzyl)-1-(4-methoxybenzyl)-1,4,5,6-tetrahydro-4,6-dioxo-1,3,5-triazin-2-ylamino)ethyl)guanidine (**11**) **[PC-25]**

To a solution of intermediate (**36**) (0.17 g, 0.37 mmol) in CH_3CN (10 mL) at room temperature, DIPEA (0.13 mL, 0.75 mmol) and pirazole-1-carboxamidine hydrochloride (0.05 g, 0.37 mmol) were added. The reaction mixture was stirred for 12 h at room temperature. The white solid formed was collected by centrifugation and washed twice with ethyl ether (2 × 10 mL). The crude final compound was purified by reversed phase preparative HPLC. Yield 0.17 g (93%); *Rf*(D) 0.42; HPLC *K′* 5.52; mp 215–217 °C; *m/z* 503.37 $(M+H)^+$; ^1H NMR (DMSO-d_6): δ 7.52–6.89 (m, 8H), 4.98 (s, 2H), 4.87 (s, 2H), 3.73 (s, 3H), 3.39–3.28 (m, 4H); ^{13}C NMR (DMSO-d_6): 158.55, 156.91, 154.04, 153.27, 150.90, 136.66, 131.08 (2 carbon atoms), 129.77 (2 carbon atoms), 127.92 (2 carbon atoms), 126.99, 120.17, 113.87 (2 carbon atoms), 55.00, 44.28, 43.90, 36.98, 36.81; IR (nujol) 3396, 3149, 1724, 1666, 1578 cm^{-1}. Anal. Calcd. for $C_{25}H_{26}BrF_6N_7O_7$ (502.36) C, 50.21; H, 4.82, N, 19.52. Found: C, 50.26; H, 4.83, N, 19.55.

4.1.9. 1-((4-Fluorobenzyl)aminocarbonyl)-3-(ethoxycarbonyl) thiourea (**32-a**)

Commercially available 1-(4-fluorobenzyl)urea (**31-a**) [alternatively, it can be prepared as reported for (**31**) starting from the appropriate benzyl amine] was reacted with ethoxycarbonyl isothiocyanate as reported for (**32**): yield 2.63 g (88%); *Rf* (D) 0.55; HPLC *K′* 3.18; mp 185–187 °C; *m/z* 300 $(M+H)^+$; ^1H NMR (DMSO-d_6): δ 12.30 (s. 1H), 11.20 (s, 1H), 7.46 (m, 2H), 7.28 (m, 2H), 4.48 (d, $J = 6.0$ Hz, 2H), 4.26 (q, $J = 7.0$ Hz, 2H), 1.35 (t, $J = 7.0$ Hz, 3H); IR (nujol) 3317, 1744, 1702 cm^{-1}. Anal. Calcd. for $C_{12}H_{14}FN_3O_3S$ (299.32): C, 48.15; H, 4.71, N, 14.04. Found: C, 48.20; H, 4.69, N, 14.08.

4.1.10. 3-(4-Fluorobenzyl)-6-thioxo-1,3,5-triazine-2,4-dione (**33-a**)

1-((4-fluorobenzyl)aminocarbonyl)-3-(ethoxycarbonyl)thiourea (**32-a**) was reacted with NaOMe as reported for (**33**): yield 2.33 g (92%); *Rf*(D) 0.45; HPLC *K′* 6.84; mp 230–232 °C; *m/z* 254 $(M+H)^+$; ^1H NMR (DMSO-d_6): δ 12.84 (s, 2H), 7.48 (m, 2H), 7.26 (m, 2H), 4.91 (s, 2H); IR (nujol) 3085, 1765, 1691 cm^{-1}. Anal. Calcd. for $C_{10}H_8FN_3O_2S$ (235.25): C, 47.43; H, 3.18, N, 16.59. Found: C, 47.47; H, 3.19, N, 16.54.

4.1.11. 3-(4-Fluorobenzyl)-6-(methylthio)-1,3,5-triazine-2,4(1H,3H)-dione (**34-a**)

3-(4-Fluorobenzyl)-6-thioxo-1,3,5-triazine-2,4-dione (**33-a**) was reacted with methyl iodide as reported for (**34**): yield 2.38 g (89%); *Rf*(D) 0.31; HPLC *K′* 3.42; mp 171–173 °C; *m/z* 268 $(M+H)^+$; ^1H NMR (DMSO-d_6): δ 12.85 (brs, 1H), 7.26 (m, 2H), 7.46 (m, 2H), 4.97 (s, 2H), 2.57 (s, 3H); IR (nujol) 3136, 1733, 1658 cm^{-1}. Anal. Calcd. for $C_{11}H_{10}FN_3O_2S$ (267.28): C, 49.43; H, 3.77, N, 15.72. Found: C, 49.50; H, 3.75, N, 15.70.

4.1.12. 3-(4-Fluorobenzyl)-1-(4-methoxybenzyl)-6-(methylthio)-1,3,5-triazine-2,4(1H,3H)-dione (**35-a**)

3-(4-Fluorobenzyl)-6-(methylthio)-1,3,5-triazine-2,4(1*H*,3*H*)-dione (**34-a**) was reacted with 4-methoxybenzyl chloride as reported for (**35**): yield 3.21 g (83%); *Rf*(D) 0.49; HPLC *K′* 6.71; mp 175–177 °C; *m/z* 388 $(M+H)^+$; ^1H NMR (DMSO-d_6): δ 7.49 (m, 2H),

7.37 (d, $J = 8.4$ Hz, 2H), 7.27 (m, 2H), 7.02 (d, $J = 8.4$ Hz, 2H), 5.15 (s, 2H), 5.05 (s, 2H), 3.85 (s, 3H), 2.59 (s, 3H); IR (nujol) 1721, 1679, 1562 cm^{-1}. Anal. Calcd. for $C_{19}H_{18}FN_3O_3S$ (387.43): C, 58.90; H, 4.68, N, 10.85. Found: C, 58.85; H, 4.70, N, 10.81.

4.1.13. 6-(2-Aminoethylamino)-3-(4-fluorobenzyl)-1-(4-methoxybenzyl)-1,3,5-triazine-2,4(1H,3H)-dione (**36-a**)

The intermediate (**35-a**) was treated with ethylenediamine as reported for (**36**): yield 017 g (93%); *Rf*(D) 0.35; HPLC *K′* 5.28; mp 184–186 °C; *m/z* 400 $(M+H)^+$; ^1H NMR (DMSO-d_6): δ 7.34–6.87 (m, 8H), 4.96 (s, 2H), 4.86 (s, 2H), 3.73 (s, 3H), 3.28–3.25 (t, 2H, $J = 6.4$ Hz), 2.53–2.50 (t, 2H, $J = 6.4$ Hz); IR (nujol) 3331, 1720, 1561 cm^{-1}. Anal. Calcd. for $C_{20}H_{22}FN_5O_3$ (399.42): C, 60.14; H, 5.55, N, 17.53. Found: C, 60.10; H, 5.57, N, 17.50.

4.1.14. (2-(5-(4-Fluorobenzyl)-1-(4-methoxybenzyl)-1,4,5,6-tetrahydro-4,6-dioxo-1,3,5-triazin-2-ylamino)ethyl)guanidine (**3**) **[PC-7]**

The intermediate (**36-a**) was treated with pyrazole-1-carboxamidine hydrochloride as reported for (**11**): yield 0.15 g (92%); *Rf*(D) 0.43; HPLC *K′* 6.05; mp 221–223 °C; *m/z* 442.47 $(M+H)^+$; ^1H NMR (DMSO-d_6): δ 8.28 (br, 1H), 7.81 (s, 1H), 7.39 (m, 2H), 7.29 (d, $J = 8.4$ Hz, 2H), 7.17 (m, 2H), 6.94 (d, $J = 8.6$ Hz, 2H), 5.11 (s, 2H), 4.93 (s, 2H), 3.77 (s, 3H), 3.40 (m, 4H); ^{13}C NMR (DMSO-d_6): 163.01, 160.61, 159.10, 157.49, 154.60, 153.86, 151.48, 134.01, 130.26 (2 carbon atoms), 128.50 (2 carbon atoms), 115.39 (2 carbon atoms), 114.41 (2 carbon atoms), 55.56, 44.82, 44.31, 36.98, 36.81; IR (nujol) 3405, 3157, 1719, 1661, 1572 cm^{-1} Anal. Calcd. for $C_{25}H_{26}F_7N_7O_7$ (441.46): C, 57.13; H, 5.48, N, 22.21. Found: C, 57.10; H, 5.47, N, 22.25.

4.1.15. 1-((4-(Trifluoromethyl)benzyl)aminocarbonyl)-3-(ethoxycarbonyl)thiourea (**32-b**)

Commercially available 1-(4-(trifluoromethyl)benzyl)urea (**31-b**) (alternatively, it can be prepared as reported for (**31**) starting from the appropriate benzyl amine) [26] was reacted with ethoxycarbonyl isothiocyanate as reported for (**32**): yield 2.86 g (82%); *Rf*(D) 0.51; HPLC *K′* 3.11; mp 189–191 °C; *m/z* 350 $(M+H)^+$; ^1H NMR (DMSO-d_6): δ 12.27 (s. 1H), 11.19 (s, 1H), 7.55–7.32 (m, 4H), 4.49 (d, $J = 6.0$ Hz, 2H), 4.28 (q, $J = 7.0$ Hz, 2H), 1.33 (t, $J = 7.0$ Hz, 3H); IR (nujol) 3303, 3177, 1734, 1683 cm^{-1}. Anal. Calcd. for $C_{13}H_{14}F_3N_3O_3S$ (349.33): C, 44.70; H, 4.04, N, 12.03. Found: C, 44.64; H, 4.05, N, 12.06.

4.1.16. 3-(4-(Trifluoromethyl)benzyl)-6-thioxo-1,3,5-triazine-2,4-dione (**33-b**)

1-((4-(trifluoromethyl)benzyl)aminocarbonyl)-3-(ethoxycarbonyl)thiourea (**32-b**) was reacted with NaOMe as reported for (**33**): yield 2.76 g (91%); *Rf*(D) 0.40; HPLC *K′* 6.66; mp 233–235 °C; *m/z* 304 $(M+H)^+$; ^1H NMR (DMSO-d_6): δ 12.70 (s, 2H), 7.70–7.54 (m, 4H), 4.90 (s, 2H); IR (nujol) 3073, 1766, 1691, 1571 cm^{-1}. Anal. Calcd. for $C_{11}H_8F_3N_3O_2S$ (303.26): C, 43.57; H, 2.66, N, 13.86. Found: C, 43.62; H, 2.65, N, 13.70.

4.1.17. 3-(4-(Trifluoromethyl)benzyl)-6-(methylthio)-1,3,5-triazine-2,4(1H,3H)-dione (**34-b**)

3-(4-(trifluoromethyl)benzyl)-6-thioxo-1,3,5-triazine-2,4-dione (**33-b**) was reacted with methyl iodide as reported for (**34**): yield 2.85 g (90%); *Rf*(D) 0.28; HPLC *K′* 3.39; mp 175–177 °C; *m/z* 318.29 $(M+H)^+$; ^1H NMR (DMSO-d_6): δ 12.80 (br s, 1H), 7.65 (d, $J = 7.5$ Hz, 2H), 7.49 (d, $J = 7.5$ Hz, 2H), 4.95 (s, 2H), 2.49 (s, 3H); IR (nujol) 1757, 1644 cm^{-1}. Anal. Calcd. for $C_{12}H_{10}F_3N_3O_2S$ (317.29): C, 45.43; H, 3.18, N, 13.24. Found: C, 45.49; H, 3.17, N, 13.20.

4.1.18. 3-(4-(Trifluoromethyl)benzyl)-1-(4-methoxybenzyl)-6-(methylthio)-1,3,5-triazine-2,4(1H,3H)-dione (**35-b**)

3-(4-(trifluoromethyl)benzyl)-6-(methylthio)-1,3,5-triazine-2,4(1*H*,3*H*)-dione (**34-b**) was reacted with 4-methylbenzyl chloride as reported for (**35**): yield 3.50 g (80%); *Rf*(D) 0.45; HPLC *K′* 6.56; mp 178–180 °C; *m/z* 422,44 $(M+H)^+$; ^1H NMR (DMSO-d_6): δ 7.69–7.11 (m, 8H), 5.02 (s, 2H), 4.99 (s, 2H), 2.58 (s, 3H), 2.27 (s, 3H). 7.49 (m, 4H), 7.28 (m, 4H), 7.17 (s, 2H), 5.05 (s, 2H), 3.86 (s, 3H), 2.58 (s, 3H); IR (nujol) 1728, 1688, 1519 cm^{-1}. Anal. Calcd. for $C_{20}H_{18}F_3N_3O_2S$ (437.44): C, 54.91; H, 4.15, N, 9.61. Found: C, 54.86; H, 4.13, N, 9.58.

4.1.19. 6-(2-Aminoethylamino)-3-(4-(trifluoromethyl)benzyl)-1-(4-methylbenzyl)-1,3,5-triazine-2,4(1H,3H)-dione (**36-b**)

The intermediate (**35-b**) was treated with ethylenediamine as reported for (**36**): yield 0.19 g (92%); *Rf*(D) 0.34; HPLC *K′* 5.19; mp 187–189 °C; *m/z* 434,43 $(M+H)^+$; ^1H NMR (DMSO-d_6): δ 7.69–7.11 (m, 8H), 5.02 (s, 2H), 4.99 (s, 2H), 3.28–3.25 (t, 2H, $J = 6.4$ Hz), 2.53–2.50 (t, 2H, $J = 6.4$ Hz). 2.27 (s, 3H); IR (nujol) 3298, 1697, 1588 cm^{-1}. Anal. Calcd. for $C_{21}H_{22}F_3N_5O_2$ (433.43): C, 58.19; H, 5.12, N, 16.16. Found: C, 58.16; H, 5.13, N, 16.18.

4.1.20. (2-(5-(4-(Trifluoromethyl)benzyl)-1-(4-methylbenzyl)-1,4,5,6-tetrahydro-4,6-dioxo-1,3,5-triazin-2-ylamino)ethyl) guanidine (**13**) **[PC-27]**

The intermediate (**36-b**) was treated with pyrazole-1-carboxamidine hydrochloride as reported for (**11**): yield 0.17 g (92%); *Rf*(D) 0.52; HPLC *K′* 6.51; mp 225–227 °C; *m/z* 476,47 $(M+H)^+$; ^1H NMR (DMSO-d_6): δ 7.69–7.11 (m, 8H), 5.02 (s, 2H), 4.99 (s, 2H), 3.40–3.28 (m, 4H). 2.27 (s, 3H); ^{13}C NMR (DMSO-d_6): 156.95, 154.17, 153.30, 150.95, 142.02, 136.52, 132.07 (2 carbon atoms), 129.02 (2 carbon atoms), 128.03 (2 carbon atoms), 126.24 (2 carbon atoms), 125.14 (2 carbon atoms), 125.10, 44.61, 44.15, 36.98, 36.81, 20.53; IR (nujol) 3397, 3166, 1721, 1660, 1579 cm^{-1}. Anal. Calcd. for $C_{26}H_{26}F_9N_7O_6$ (475.45): C, 55.57; H, 5.09, N, 20.62. Found: C, 55.63; H, 5.11, N, 20.68.

4.2. Biological activity

4.2.1. Animals

Male C57Bl/6 mice weighing 25–30 g were used for behaviorals experiments. Mice were housed in plastic cages (5 for each group) and maintained under 12:12 light–dark cycle at 21 ± 1 °C and 50 ± 5% humidity with food and water *ad libitum*.

All animal experiments were conducted under protocols approved by the Animal Care and Use Committee of the Italian Ministry of Health. Animal care was in compliance with the IASP and European Community (E.C.L.358/118/12/86) guidelines on the use and protection of animals in experimental research. All efforts were made to minimize animal suffering and to reduce the number of animal used.

4.2.2. Measurement of nociceptive threshold

The nociceptive threshold to thermal stimuli was evaluated by the Paw–Immersion test.

This test was performed by dipping one mouse hind-paw into hot water (48 °C) and measuring the latencies to paw withdrawal. For measurement of the nociceptive threshold, mice were trained in paw withdrawal test during the week preceding the experiment. This adaptation protocol reduced variability in threshold measurements, giving a more stable baseline and making drug-induced changes easier to detect. On the day of the experiment, nociceptive threshold was measured for 2 h at 30 min intervals before drug injection. The mean of the last three of these threshold measurements were taken as baseline nociceptive threshold (NT_B). Nociceptive threshold was then determined three times at 15, 30, 60, 90,

120, 150, 180 min after saline or drug administration. The mean of the three readings at each time point was defined as the nociceptive threshold at that time in the presence of the test solution (NTts). The effect of the tested drug was calculated as the percentage change in nociceptive threshold from baseline threshold (% ΔNT) according to the following equation: % $\Delta NT = 100 \times (NT_{TS} - NT_B)/NT_B$. The EC_{50} values, with 95% confidence intervals, were determined using the PRISM software (GraphPad Software 5.4, San Diego, CA, U.S.A.)

4.2.3. Drug injections

Bv8 was dissolved in saline and *injected* in a volume of 20 μl by i*ntraplantar* (i.pl.) *route at the dose of 63 fmol in mice*. The antagonists were dissolved in a saline and *injected* in a volume of 20 μl by i*ntraplantar* (i.pl.) *route* 5 min before Bv8. After drugs administration the animals were observed for 3 h at the established time intervals. For each drug dose, a different group of 5 male mice was used.

4.2.4. Statistical analysis

The data are presented as mean ± SEM. Two-way ANOVA followed by Bonferroni's post tests was performed using GraphPad PRISM 5.4.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.05.030.

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