

## Brief Articles

### Triazine Compounds as Antagonists at Bv8-Prokineticin Receptors

Gianfranco Balboni,<sup>\*,†,||</sup> Ilaria Lazzari,<sup>†</sup> Claudio Trapella,<sup>†</sup> Lucia Negri,<sup>‡</sup> Roberta Lattanzi,<sup>‡</sup> Elisa Giannini,<sup>‡</sup> Annalisa Nicotra,<sup>‡</sup> Pietro Melchiorri,<sup>‡</sup> Sergio Visentin,<sup>§</sup> Chiara De Nuccio,<sup>§</sup> and Severo Salvadori<sup>†</sup>

Department of Pharmaceutical Sciences, University of Ferrara, I-44100 Ferrara, Italy, Department of Physiology and Pharmacology "Vittorio Erspamer", University "La Sapienza", I-00185 Rome, Italy, Department of Cell Biology and Neuroscience, Istituto Superiore di Sanità, Rome, Italy, Department of Toxicology, University of Cagliari, I-09124, Cagliari, Italy

Received July 11, 2008

On the basis of a Janssen's patent, we approached a new synthesis of some 1,3,5-triazin-4,6-diones as potential non peptidic prokineticin receptor antagonists, containing the following substitutions: (*N*<sup>1</sup> and *N*<sup>5</sup> link a 4-methoxybenzyl and a 4-ethylbenzyl, respectively; *C*<sup>2</sup> can link an amino-ethyl-guanidine (reference compound **1**) or an ethyldiamine (**2**) or an amino-ethyl-amino-2-imidazoline (**3**). New compounds were assessed for PKR1 and PKR2 affinity. Antagonist properties were evaluated as inhibition of 1 nM Bv8-induced intracellular Ca<sup>2+</sup> mobilization.

#### Introduction

A small protein, named Bv8 to indicate its origin from the skin secretion of *Bombina variegata* and its molecular weight (8 kDa), is the first amphibian member of the Bv8-Prokineticin family.<sup>1</sup> Homologues of Bv8 are present in skin secretion of other *Bombina* species, in the venom of the snake black mamba (mamba intestinal toxin, MIT-1<sup>a</sup>), in lizards, and in fishes. Striking characterization of these proteins are their identical amino terminal sequence, AVITG, and the presence of 10 cysteines with identical spacing that define a five disulphide-bridged motif called a colipase fold.<sup>2</sup> The high degree of identity between amphibian Bv8 peptides, fish peptides, and mamba MIT-1 (58%) suggested that similar peptides could also be present in other species, including mammals. In the mouse, rat, cattle, monkey, and man, cDNA cloning identified orthologues of Bv8. The two mammalian proteins were named prokineticin 1 (PK1, or EG-VEGF) that is 80% homologous to MIT, and prokineticin 2 (PK2 or mBv8) that is an orthologue of amphibian Bv8. The name prokineticin refers to the ability of these peptides to contract guinea pig ileum (GPI), a property shared with amphibian Bv8.<sup>3</sup> The two G-protein-coupled receptors for Bv8-PKs, prokineticin receptor 1 (PKR1) and prokineticin receptor 2

(PKR2), encoded within distinct chromosomes in both mouse and human, share about 85% amino acid identity, with most differences at the N-terminal. Their sequences are almost identical in the transmembrane domains. Affinity of Bv8 and PKs for their receptors is similar, with Bv8 showing a 10 times higher affinity for either receptor, while MIT-1 is a PKR2 preferring ligand. PKRs have been reported to couple either to G<sub>i</sub> or to G<sub>q/o</sub> proteins.<sup>4–6</sup> Intensive research over the past few years has shown that the biological activities of Bv8/PK proteins range from angiogenesis and involvement in reproduction and cancer, to neuronal survival and neurogenesis, hypothalamic hormone secretions, circadian rhythm control, and modulation of complex behaviours such as feeding and drinking. The high expression level of human Bv8/PK2 in bone marrow, lymphoid organs, and leukocytes suggested an involvement of these peptides in hematopoiesis and in inflammatory and immunomodulatory processes. Moreover, the dramatic reduction in the pain threshold produced by Bv8 indicates that Bv8/PKs and their receptors may act as mediators of inflammatory and neuropathic pain.<sup>7–11</sup> PKRs are present in DRG, in the outer layers of the dorsal horns of the spinal cord, and in peripheral terminals of nociceptor axons. Activation of nociceptor PKRs by Bv8 in rats and mice produces sensitization to thermal and mechanical stimuli. A physiological role of Bv8/PKs as peripheral and central pain modulators is supported by the observation that mice lacking the PKRs or PK2 are less sensitive to noxious stimuli than wild type mice. PKR1-null mice also exhibited impaired development of hyperalgesia after tissue injury. PK2 released by inflammatory cells can bind and activate PKRs on the primary sensitive neurons, contributing to inflammatory pain.<sup>11,12</sup> Hence the PKRs are potential targets for novel analgesic drugs that block the nociceptive information before it reaches the brain. Identifying of the structural determinants required for receptor binding and hyperalgesic activity of Bv8-PKs is thus mandatory for the design of PKR antagonists. The highly conserved amino terminal sequence AVITGA and the tryptophan (Trp) residue in position 24 in all members of the Bv8/PK family

\* To whom correspondence should be addressed. Phone: (+39)-70-675-8625. Fax: (+39)-70-675-8612. E-mail: gbalboni@unica.it; bbg@unife.it.

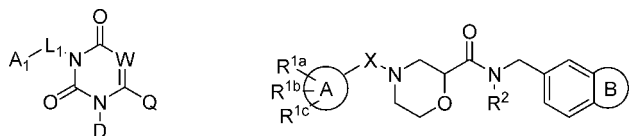
<sup>†</sup> Department of Pharmaceutical Sciences, University of Ferrara.

<sup>||</sup> Department of Toxicology, University of Cagliari.

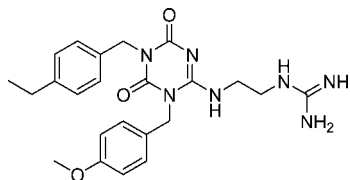
<sup>‡</sup> Department of Physiology and Pharmacology "Vittorio Erspamer", University "La Sapienza".

<sup>§</sup> Department of Cell Biology and Neuroscience, Istituto Superiore di Sanità.

<sup>a</sup> Abbreviations. In addition to the IUPAC-IUB Commission on Biochemical Nomenclature (*J. Biol. Chem.* **1985**, *260*, 14–42), this paper uses the following additional symbols and abbreviations: AcOEt, ethyl acetate; Boc, *tert*-butyloxycarbonyl; CDCl<sub>3</sub>, deuteriochloroform; DCM, dichloromethane; DEAD, diethyl azodicarboxylate; DIPEA, *N,N*-diisopropylethylamine; DMF, *N,N*-dimethylformamide; DMSO-*d*<sub>6</sub>, hexadeuteriodimethyl sulfoxide; ESI, electrospray ionization; Et<sub>2</sub>O, diethyl ether; HPLC, high performance liquid chromatography; MIT, mamba intestinal toxin; Pe, petroleum ether; PKR1, prokineticin receptor 1; PKR2, prokineticin receptor 2; PPh<sub>3</sub>, triphenylphosphine; TEA, triethylamine; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TLC, thin-layer chromatography; Y, yield.



**Figure 1.** General structures of patented non-peptide prokineticin antagonists.



**Figure 2.** Structure of patented reference 1.

**Table 1.** Effect of Triazine Compounds 1–3 on 1 nM Bv8 Induced Intracellular  $\text{Ca}^{2+}$  Mobilization

treatment	PKR1% active cells	PKR2 % active cells
BV8 1 nM	93 ± 8.6	95 ± 7.8
+(1) 100 nM	52 ± 4.8	48 ± 3.7
+(1) 300 nM	4 ± 0.7	6 ± 0.9
+(2) 10 $\mu\text{M}$	90 ± 9.3	89 ± 9.2
+(2) 1 mM	0.3 ± 0.7	2 ± 1
+(3) 100 $\mu\text{M}$	91 ± 12	92 ± 8.8

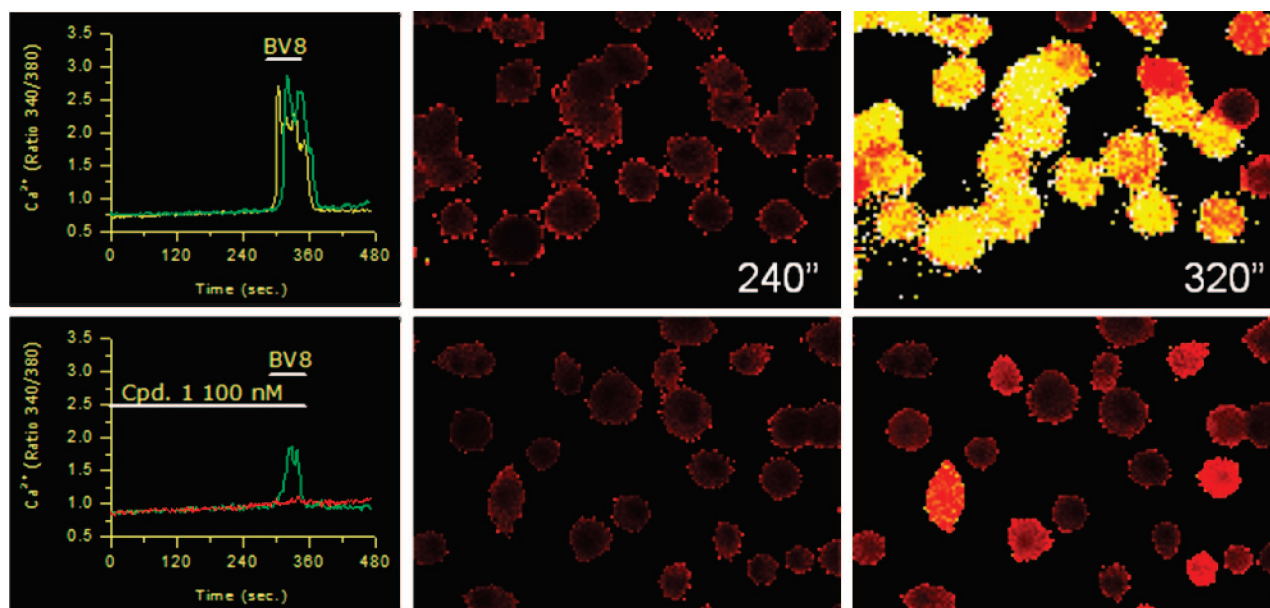
are required for biological activity: deletions and substitutions in these conserved residues produces antagonist molecules.<sup>13,14</sup> The N-terminal deletion of alanine and valine, in Bv8 molecule (dAV-Bv8), yields an analogue lacking any biological activity but still able to bind the receptors acting as PKR-antagonist in vitro and in vivo.<sup>14</sup> In rats and mice, dAV-Bv8 dose-dependently reduced and abolished Bv8-induced hyperalgesia.<sup>14</sup> Owing to the involvement of the prokineticin system in various biological and pathological functions, the availability of effective antagonists of the PKRs may be useful in the treatment and prevention of various mammalian disease states, for example, visceral pain that is associated with irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD). Furthermore, PK receptor antagonists could be useful in treating cancer-specific angiogenesis, hence in

preventing tumoral development/progression. Recently, some patents regarding the synthesis and pharmacological characterization of non-peptide prokineticin antagonists were deposited by Janssen Pharmaceutica and Merck.<sup>15–18</sup> General structures of patented compounds, summarized in Figure 1, are as follows: 1,3,4-trisubstituted 1,2,3,6-tetrahydro-2,6-dioxopyrimidines, 1,2,5-trisubstituted 1,4,5,6-tetrahydro-4,6-dioxo-1,3,5-triazines, and *N*,4-disubstituted morpholine-2-carboxamides, respectively. Considering the completely different structures reported in Figure 1, we selected to study the structure/activity relationship related to the triazine/pirimidine scaffold on the basis of better pharmacological data reported for its analogues.

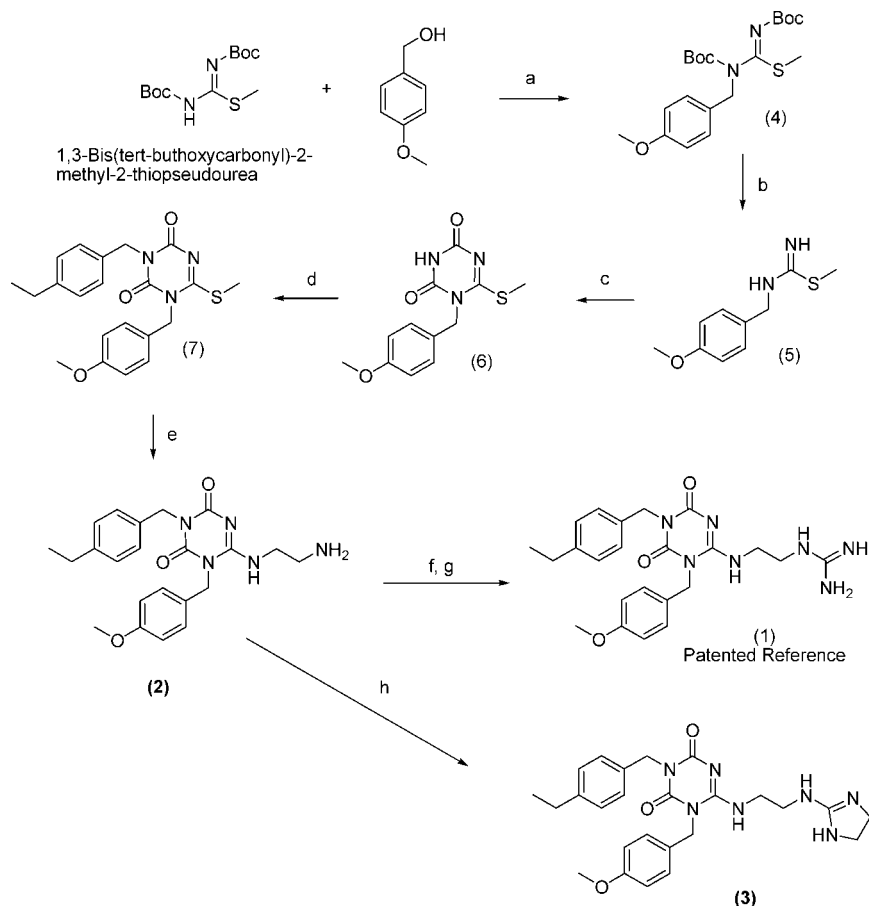
With the aim to ameliorate the synthetic way to triazine compounds, among all patented 1,3,5-triazine prokineticin antagonists, on the basis of its pharmacological data and its easy synthetic feasibility, we focalized our attention on compound 1 [(2-(5-(4-ethylbenzyl)-1-(4-methoxybenzyl)-1,4,5,6-tetrahydro-4,6-dioxo-1,3,5-triazin-2-ylamino)ethyl)guanidine] reported in the patent WO2006104713 as compound 46 (Figure 2).

## Chemistry

A useful amelioration in the new synthetic way for the synthesis of compound 1 (Scheme 1) regards the preparation of the intermediate 1-(4-methoxybenzyl)-2-methylisothiourea. In the patented scheme (Supporting Information, Scheme 2), it was prepared by alkylation of 1-(4-methoxybenzyl)thiourea with methyl iodide. This reaction per se is not difficult, but the *N*-alkylation of thiourea often is a source of side reactions and it is quite easy to obtain considerable amounts of the disubstituted thiourea with an important yield decrease. To avoid this drawback, it is convenient to start the synthesis from the commercially available (Sigma-Aldrich) 1,3-bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopsedourea that, under Mitsunobu conditions, is treated with 4-methoxybenzyl alcohol to yield the *N,N'*-Boc diprotected 1-(4-methoxybenzyl)-2-methylisothiourea (4), which upon TFA treatment gives the Boc deprotected (5) in good yield. The reaction of (5) with the commercially available *N*-(chlorocarbonyl) isocyanate provides the ring closure to the triazine intermediate (6) in 31% yield, in accord with the corresponding patented reaction. A second Mitsunobu reaction



**Figure 3.** Effect of 1 on 1 nM Bv8-triggered cytoplasmic  $\text{Ca}^{2+}$  signals.

Scheme 1. Synthesis of the Triazine Compounds (1–3)<sup>a</sup>

<sup>a</sup> Reagents: (a) THF, DEAD, PPh<sub>3</sub>, room temp. *Y* = 78%; (b) TFA, room temp. *Y* = 91%; (c) DCM, Cl(C=O)-N=C=O, DIPEA, *Y* = 31%; (d) THF, DEAD, PPh<sub>3</sub>, 4-ethylbenzyl alcohol, room temp. *Y* = 80%; (e) toluene, NH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NH<sub>2</sub>, reflux *Y* = 95%; (f) 1,3-bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea, THF/H<sub>2</sub>O, 50 °C, *Y* = 77%; (g) TFA, room temp. *Y* = 95%; (h) 4,5-dihydro-2-(methylthio)-1*H*-imidazole, THF/H<sub>2</sub>O, 50 °C, *Y* = 65%.

inserts the second benzyl group providing the intermediate (7), which upon treatment with ethylenediamine yields the compound 2. Finally, (2) can be converted to the reference (1) by the reaction with 1,3-bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea and subsequent TFA deprotection or to compound 3 by the reaction with 4,5-dihydro-2-(methylthio)-1*H*-imidazole.

## Results and Discussion

### Binding of Triazine Compounds to Prokineticin Receptors.

The affinity of the new compounds for the prokineticin receptors was expressed as inhibition constant (*K<sub>i</sub>*) of the binding of <sup>125</sup>I-MIT on PKR1 and PKR2 as detailed in the Supporting Information. The results from the binding assay indicated that the reference (1) is a PKR1 preferring ligand with an apparent affinity about 70-fold higher for PKR1 (*K<sub>i</sub>* = 22 nM) than for PKR2 (*K<sub>i</sub>* = 1610 nM). PKR1 affinity of 2 (*K<sub>i</sub>* = 440 nM) and 3 (*K<sub>i</sub>* = 4719 nM) was 20- and 200-fold lower than that of (1). PKR2 affinity of (2) and (3) is negligible, on the order of μM (Figures 4–5, Supporting Information).

The competitive displacement of <sup>125</sup>I-MIT from PKR1 and PKR2 by graded concentration of compounds 2 and 3 indicated that the lack of the guanidine function reduced the affinity for both receptors.

**Effect of Triazine Compounds on Bv8 Triggered [Ca<sup>2+</sup>]<sub>i</sub>.** Fura-2 loaded CHO cells stably transfected with the PKR1 or PKR2<sup>14</sup> were utilized to evaluate the antagonistic effect of compounds 1–3 on Bv8-triggered cytoplasmic Ca<sup>2+</sup> signals.

Bv8 induced transient increases in [Ca<sup>2+</sup>]<sub>i</sub> in a concentration-dependent number of cells.<sup>14</sup> As shown in Figure 3, PKR1 expressing cells were challenged with 1 nM Bv8 in the absence (upper panels) or presence (lower panels) of 100 nM (1).

In the left panels is reported the time-course of the intracellular Ca<sup>2+</sup> concentration (expressed as ratio between the emissions at 340 and 380 nm). In the right panels are reported the pseudocolor images of the emission intensity ratios recorded just before (240 s) and during (320 s) Bv8 challenge. Compound 1 dose dependently antagonized the 1 nM Bv8-induced intracellular Ca<sup>2+</sup> mobilization ([Ca<sup>2+</sup>]<sub>i</sub>) in PKR1- and PKR2-transfected CHO cells (IC<sub>50</sub> = 100 nM; IC<sub>100</sub> = 300 nM). 2 and 3 slightly reduced Bv8-induced [Ca<sup>2+</sup>]<sub>i</sub> at 10 and 100 μM. Compound 2 completely blocked Bv8-induced intracellular Ca<sup>2+</sup> mobilization at 1 mM concentration (Table 1).

## Conclusion

Here we improved the synthesis and confirmed the activity of a non-peptidic prokineticin antagonist characterized by a structure much more simple than the corresponding cysteine-rich small proteins endowed with the same activity. Making a comparison between the two synthetic ways to the reference 1, our method, reported in Scheme 1, provides an overall yield of the final compound of about 13% vs an overall yield of about 3% calculated in accord with the synthesis reported in the patent (Scheme 2, Supporting Information). The same synthetic method we applied to (1), can be extended to a

wide range of triazine analogues deriving for example by the insertion of different benzyl groups. Interestingly, (1) showed comparable affinity but higher selectivity for PKR1 than the nonselective prokineticin receptor antagonist dAV-Bv8 obtained from Bv8 by N-terminal deletion of alanine and valine residues.<sup>14</sup> Preliminary in vivo studies (data not shown) indicate that (1) and (2) both behave as antihyperalgesic and anti-inflammatory drugs.

## Experimental Section

**Chemistry. 1,3-Bis-(*tert*-butoxycarbonyl)-1-(4-methoxybenzyl)-2-methyl-3-thiopseudourea (4).** To a solution of 1,3-bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea (2.0 g, 6.90 mmol), 4-methoxybenzyl alcohol (0.94 mL, 7.59 mmol) and triphenylphosphine (1.99 g, 7.59 mmol), at 0 °C in anhydrous THF, was added a solution of diethyl azodicarboxylate (1.4 mL, 7.59 mmol) dissolved in anhydrous THF. After 10 min, the reaction was warmed at room temperature and stirred overnight. The solvent was removed under vacuum, and the crude intermediate was purified by flash chromatography (EtOAc/Pe, 1:9, v/v): yield 2.21 g (78%); *R*<sub>f</sub>(A) 0.60; HPLC *K'* 9.54; mp oil; *m/z* 412 (M + H)<sup>+</sup>. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 7.27–7.25 (d, 2H, *J* = 8.4 Hz), 6.84–6.82 (d, 2H, *J* = 8.4 Hz), 4.69 (s, 2H), 3.77 (s, 3H), 2.24 (s, 3H), 1.50 (s, 9H), 1.40 (s, 9H). <sup>13</sup>C NMR (CDCl<sub>3</sub>): 163.28, 158.99, 158.04, 152.00, 129.53, 129.35 (2 carbon atoms), 113.69 (2 carbon atoms), 82.59, 81.74, 55.21, 51.82, 28.01 (6 carbon atoms), 15.57.

**1-(4-Methoxybenzyl)-2-methyl-3-thiopseudourea (5).** Intermediate (4) (1.51 g, 3.68 mmol) was treated with TFA (10 mL) for 2 h. at room temperature. TFA was removed under vacuum, and the deprotected intermediate was precipitated from Et<sub>2</sub>O: yield 1.08 g (91%); *R*<sub>f</sub>(B) 0.32; HPLC *K'* 6.51; mp >250 °C; *m/z* 211 (M + H)<sup>+</sup>.

**1-(4-Methoxybenzyl)-6-(methylthio)-1,3,5-triazine-2,4(1H,3H)-dione (6).** To a solution of (5) (0.5 g, 1.54 mmol) in dichloromethane (10 mL) at 0 °C, diisopropylethylamine (0.79 mL, 4.63 mmol) was added. At the same temperature, *N*-chlorocarbonyl isocyanate (0.12 mL, 1.54 mmol), dissolved in dichloromethane (3 mL), was added dropwise. The reaction mixture was allowed to stir while slowly warming to room temperature (1 h) and was then stirred for an additional 24 h. The solvent was evaporated, and the residue was partitioned between EtOAc and H<sub>2</sub>O. The EtOAc layer was washed with brine and dried over Na<sub>2</sub>SO<sub>4</sub>. The solution was filtered, the solvent evaporated, and the residual oil was precipitated from methanol: yield 0.13 g (31%); *R*<sub>f</sub>(C) 0.25; HPLC *K'* 5.33; mp 210–212 °C; *m/z* 280 (M + H)<sup>+</sup>. <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>): δ 11.60 (bs, 1H), 7.24–7.22 (d, 2H, *J* = 7.6 Hz), 6.91–6.89 (d, 2H, *J* = 7.6 Hz), 4.97 (s, 2H), 3.73 (s, 3H), 2.45 (s, 3H). <sup>13</sup>C NMR (CDCl<sub>3</sub>): 171.08, 158.62, 152.15, 149.98, 128.34 (2 carbon atoms), 126.86, 113.87 (2 carbon atoms), 55.00, 46.13, 14.68.

**3-(4-Ethylbenzyl)-1-(4-methoxybenzyl)-6-(methylthio)-1,3,5-triazine-2,4(1H,3H)-dione (7).** To a solution of intermediate (6) (0.55 g, 1.97 mmol), triphenylphosphine (0.57 g, 2.17 mmol) and 4-ethylbenzyl alcohol (0.29 mL, 2.17 mmol), at 0 °C in anhydrous THF, a solution of diethyl azodicarboxylate (0.4 mL, 2.17 mmol) in anhydrous THF (3 mL) was added dropwise. The reaction mixture was stirred overnight at room temperature, the solvent was evaporated in vacuo, dissolved in EtOAc, and washed twice with water (20 mL each). After solvent evaporation, the residue was purified by flash chromatography (EtOAc/Pe, 1:2, v/v) to give a colorless oil: yield 0.62 g (80%); *R*<sub>f</sub>(D) 0.35 HPLC *K'* 6.23; mp oil; *m/z* 398 (M + H)<sup>+</sup>. <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>): δ 7.27–7.16 (m, 6H), 6.92–6.88 (d, 2H, *J* = 8 Hz), 5.02 (s, 2H), 4.91 (s, 2H), 4.05–4.01 (q, 2H, *J* = 7 Hz) 3.73 (s, 3H), 3.43 (s, 3H), 1.10–1.04 (t, 3H, *J* = 7 Hz). <sup>13</sup>C NMR (CDCl<sub>3</sub>): 170.06, 158.77, 151.58, 150.35, 142.88, 133.78 128.52 (2 carbon atoms), 127.73 (2 carbon atoms), 126.72 (2 carbon atoms), 113.87 (2 carbon atoms), 63.29, 55.10, 47.56, 44.72, 27.85, 15.69, 14.75.

**6-(2-Aminoethylamino)-3-(4-ethylbenzyl)-1-(4-methoxybenzyl)-1,3,5-triazine-2,4(1H,3H)-dione (2).** To a solution of intermediate (7) (0.38 g, 0.95 mmol) in toluene (10 mL) at room temperature,

ethylenediamine (0.38 mL, 5.7 mmol) was added. The reaction mixture was refluxed for 18 h. After solvent evaporation, the residue was dissolved in EtOAc and washed twice with water (2 × 10 mL). The organic layer was dried (Na<sub>2</sub>SO<sub>4</sub>) and evaporated to afford the final compound 2 as a pale-yellow oil: yield 0.37 g (95%); *R*<sub>f</sub>(B) 0.92; HPLC *K'* 5.22; mp oil; *m/z* 410 (M + H)<sup>+</sup>. <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>): δ 7.21–7.10 (m, 6H), 6.92–6.88 (d, 2H, *J* = 8 Hz), 5.02–4.98 (d, 2H, *J* = 8.8 Hz), 4.86 (s, 2H), 4.08–3.98 (q, 2H, *J* = 7 Hz) 3.73 (s, 3H), 3.28–3.25 (t, 2H, *J* = 6.4), 2.53–2.50 (t, 2H, *J* = 6.4 Hz), 1.14–1.10 (t, 3H, *J* = 7 Hz). Anal. C<sub>26</sub>H<sub>29</sub>F<sub>6</sub>N<sub>5</sub>O<sub>7</sub>: C; H; N.

**(2-(5-(4-Ethylbenzyl)-1-(4-methoxybenzyl)-1,4,5,6-tetrahydro-4,6-dioxo-1,3,5-triazin-2-ylamino)ethyl)-*N,N'*-di-*tert*-butyloxycarbonyl-guanidine (*N,N'*-di-Boc-1).** To a solution of 1,3-bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea (0.15 g, 0.51 mmol) in distilled THF (5 mL) and H<sub>2</sub>O (50 μL) (2) (0.2 g, 0.49 mmol) was added at room temperature. The reaction was heated at 50 °C for 3 h. The solvent was evaporated under vacuum and the crude intermediate was precipitated from Et<sub>2</sub>O/Pe (1:9, v/v) to give a white solid: yield 0.25 g (77%); *R*<sub>f</sub>(D) 0.62; HPLC *K'* 6.45; mp 132–134 °C; *m/z* 653 (M + H)<sup>+</sup>. <sup>1</sup>H NMR (CDCl<sub>3</sub>): 7.41–7.14 (m, 6H), 6.95 (bs, 1H), 6.87–6.85 (d, 1H, *J* = 8.8), 6.75–6.73 (d, 1H, *J* = 8.8), 6.43 (bs, 1H), 5.07 (s, 2H), 5.00 (s, 2H), 3.79 (s, 3H), 3.46–3.27 (m, 4H), 2.62–2.58 (q, 2H, *J* = 7.6), 1.52 (s, 9H), 1.42 (s, 9H), 1.22–1.18 (t, 3H, *J* = 7.6).

**(2-(5-(4-Ethylbenzyl)-1-(4-methoxybenzyl)-1,4,5,6-tetrahydro-4,6-dioxo-1,3,5-triazin-2-ylamino)ethyl)-guanidine (reference 1).** *N,N'*-di-Boc-1 (0.21 g, 0.32 mmol) was treated with TFA (2 mL) for 0.5 h at room temperature. Et<sub>2</sub>O/Pe (1:1, v/v) were added to the solution until the product precipitated: yield 0.21 g (95%); *R*<sub>f</sub>(D) 0.46; HPLC *K'* 5.34; mp >250 °C; *m/z* 453 (M + H)<sup>+</sup>. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 8.24 (bs, 1H), 7.78 (bs, 1H), 7.26–7.10 (m, 6H), 6.92–6.87 (d, 2H, *J* = 8.8 Hz) 5.06 (s, 2H), 4.86 (s, 2H), 3.72 (s, 3H), 3.51 (q, 2H, *J* = 7 Hz), 2.57–2.50 (m, 4H), 1.78–1.10 (t, 3H, *J* = 7 Hz). <sup>13</sup>C NMR (CDCl<sub>3</sub>): 163.03, 158.55, 157.04, 153.89, 153.35, 150.99, 142.58, 134.51, 128.21 (2 carbon atoms), 127.58 (2 carbon atoms), 127.20 (2 carbon atoms), 113.83 (2 carbon atoms), 55.02, 44.16, 40.65, 38.57, 27.74, 24.37, 15.63. Anal. C<sub>27</sub>H<sub>31</sub>F<sub>6</sub>N<sub>7</sub>O<sub>7</sub>: C; H; N.

**6-(2-(4,5-Dihydro-1H-imidazol-2-ylamino)ethylamino)-3-(4-ethylbenzyl)-1-(4-methoxybenzyl)-1,3,5-triazine-2,4(1H,3H)-dione (3).** To a solution of 4,5-dihydro-2-(methylthio)-1H-imidazole, (0.03 g, 0.25 mmol) in distilled THF (5 mL) and H<sub>2</sub>O (50 μL) (2) (0.1 g, 0.24 mmol) was added at room temperature. The reaction was heated at 50 °C for 3 h. The solvent was evaporated under vacuum, and the crude intermediate was precipitated from Et<sub>2</sub>O/Pe (1:9, v/v) to give a pale-yellow solid: yield 0.11 g (65%); *R*<sub>f</sub>(D) 0.53; HPLC *K'* 6.08; mp 220–222 °C; *m/z* 479 (M + H)<sup>+</sup>. <sup>1</sup>H NMR (CDCl<sub>3</sub>): 7.41–7.14 (m, 6H), 6.95 (bs, 1H), 6.87–6.85 (d, 1H, *J* = 8.8), 6.75–6.73 (d, 1H, *J* = 8.8), 6.43 (bs, 1H), 5.07 (s, 2H), 5.00 (s, 2H), 3.83 (s, 4H) 3.79 (s, 3H), 3.46–3.27 (m, 4H), 2.62–2.58 (q, 2H, *J* = 7.6), 1.22–1.18 (t, 3H, *J* = 7.6). <sup>13</sup>C NMR (CDCl<sub>3</sub>): 163.03, 158.55, 157.04, 153.89, 153.35, 150.99, 142.58, 134.51, 128.21 (2 carbon atoms), 127.58 (2 carbon atoms), 127.20 (2 carbon atoms), 113.83 (2 carbon atoms), 55.02, 51.3, 44.16, 40.65, 38.57, 33.6, 27.74, 24.37, 15.63. Anal. C<sub>29</sub>H<sub>33</sub>F<sub>6</sub>N<sub>7</sub>O<sub>7</sub>: C; H; N.

**Pharmacology. Receptor Binding Assay.** Affinity of compounds 1–3 for prokineticin receptors was assayed on membrane preparation from PKR1- or PKR2-transfected CHO cells.<sup>14</sup> The prokineticin binding sites were labeled with <sup>125</sup>I-MIT (*K*<sub>d</sub> = 4 pM for PKR1; *K*<sub>d</sub> = 1.24 pM for PKR2, PerkinElmer, Membrane Target Systems). The inhibition constant (*K*<sub>i</sub>) of the three compounds was calculated from competitive binding curves with the PRISM software (GraphPad Software, San Diego, CA)

**Intracellular Ca<sup>2+</sup> Imaging.** PKR1- or PKR2-transfected CHO cells, were loaded for 50 min at room temperature with 2 μM-Fura-2-AM in a balanced saline solution. Bv8 1 nM induced increases in [Ca<sup>2+</sup>]<sub>i</sub> in 10–40 s in about 95% of cells. Compounds were added and incubated for 4 min, and then 1 nM Bv8 was added and the fluorescent signal was evaluated for 2 min. The IC<sub>50</sub> is

defined as the amount of a given compound required to inhibit 50% of the maximum signal that is generated by 1 nM Bv8.

**Acknowledgment.** This study was supported in part by the University of Cagliari (G.B.), and the University of Ferrara (S.S.). Pharmacological tests were supported by grants of the University of Rome "La Sapienza" (L.N.).

**Supporting Information Available:** Chemistry general methods, patented synthetic scheme of Reference 1, receptor binding assay, figures 1 and 2 related to prokineticin receptors binding, elemental analysis and MS data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

## References

- (1) Mollay, C.; Wechselberger, C.; Mignogna, G.; Negri, L.; Melchiorri, P.; Barra, D.; Kreil, G. Bv8, a small protein from frog skin and its homologue from snake venom induce hyperalgesia in rats. *Eur. J. Pharmacol.* **1999**, *374*, 189–196.
- (2) Kaser, A.; Winklmayr, M.; Lepperdinger, G.; Kreil, G. The AVIT protein family. Secreted cysteine-rich vertebrate proteins with diverse functions. *EMBO Rep.* **2003**, *4*, 469–473.
- (3) Li, M.; Bullock, C. M.; Knauer, D. J.; Ehlert, F. J.; Zhou, Q. Y. Identification of two prokineticin cDNAs: recombinant proteins potently contract gastrointestinal smooth muscle. *Mol. Pharmacol.* **2001**, *59*, 692–698.
- (4) Lin, D. C.; Bullock, C. M.; Ehlert, F. J.; Chen, J. L.; Tian, H.; Zhou, Q. Y. Identification and molecular characterization of two closely related G protein-coupled receptors activated by prokineticins/endocrine gland vascular endothelial growth factor. *J. Biol. Chem.* **2002**, *277*, 19276–19280.
- (5) Masuda, Y.; Takatsu, Y.; Terao, Y.; Kumano, S.; Ishibashi, Y.; Suenaga, M.; Abe, M.; Fukusumi, S.; Watanabe, T.; Shintani, Y.; Yamada, T.; Hinuma, S.; Inatomi, N.; Ohtaki, T.; Onda, H.; Fujino, M. Isolation and identification of EG-VEGF/prokineticins as cognate ligands for two orphan G-protein-coupled receptors. *Biochem. Biophys. Res. Commun.* **2002**, *293*, 396–402.
- (6) Soga, T.; Matsumoto, S.; Oda, T.; Saito, T.; Hiyama, H.; Takasaki, J.; Kamohara, M.; Ohishi, T.; Matsushime, H.; Furuichi, K. Molecular cloning and characterization of prokineticin receptors. *Biochim. Biophys. Acta* **2002**, *1579*, 173–179.
- (7) LeCouter, J.; Kowalski, J.; Foster, J.; Hass, P.; Zhang, Z.; Dillard-Telm, L.; Frantz, G.; Rangell, L.; Deguzman, L.; Keller, G.; Peale, F.; Gurney, A.; Hillan, K.; Ferrara, N. Identification of an angiogenic mitogen selective for endocrine gland endothelium. *Nature* **2001**, *412*, 876–884.
- (8) Shojaei, F.; Wu, X.; Zhong, C.; Yu, L.; Liang, X. H.; Yao, J.; Blanchard, D.; Bais, C.; Peale, F. V.; Van Bruggen, N.; Ho, C.; Ross, J.; Tan, M.; Carano, R. A.; Meng, Y. G.; Ferrara, N. Bv8 regulates myeloid-cell-dependent tumour angiogenesis. *Nature* **2007**, *450*, 825–831.
- (9) Zhou, Q. Y. The prokineticins: a novel pair of regulatory peptides. *Mol. Interv.* **2006**, *6*, 3330–3338.
- (10) Negri, L.; Lattanzi, R.; Giannini, E.; Melchiorri, P. Bv8/Prokineticin proteins and their receptors. *Life Sci.* **2007**, *81*, 1103–1116.
- (11) Negri, L.; Lattanzi, R.; Giannini, E.; Melchiorri, P. Modulators of pain: Bv8 and prokineticins. *Curr. Neuropharmacol.* **2006**, *4*, 207–215.
- (12) Negri, L.; Lattanzi, R.; Giannini, E.; Colucci, M.; Margheriti, F.; Melchiorri, P.; Vellani, V.; Tian, H.; De Felice, M.; Porreca, F. Impaired nociception and inflammatory pain sensation in mice lacking the prokineticin receptor PKR1: focus on interaction between PKR1 and the capsaicin receptor TRPV1 in pain behaviour. *J. Neurosci.* **2006**, *26*, 6716–6727.
- (13) Bullock, C. M.; Li, J. D.; Zhou, Q. Y. Structural determinants required for the bioactivities of prokineticins and identification of prokineticin receptor antagonists. *Mol. Pharmacol.* **2004**, *65*, 582–588.
- (14) Negri, L.; Lattanzi, R.; Giannini, E.; Colucci, M. A.; Mignogna, G.; Barra, D.; Grohovaz, F.; Codazzi, F.; Kaiser, A.; Kreil, G.; Melchiorri, P. Biological activities of Bv8 analogues. *Br. J. Pharmacol.* **2005**, *146*, 625–632.
- (15) Coats, S. J.; Dyatkin, A. B.; He, W.; Lisko, J.; Ralbovsky, J. L.; Schultz, M. J. Prokineticin 1 receptor antagonists. Patent WO2006104715, 2006.
- (16) Coats, S. J.; Dyatkin, A. B.; He, W.; Lisko, J.; Ralbovsky, J. L.; Schultz, M. J. Pyrimidindione derivatives as prokineticin 2 receptor antagonists. Patent WO2006104713, 2006.
- (17) Coats, S. J.; Dyatkin, A. B.; He, W.; Lisko, J.; Miskowski, T. A.; Ralbovsky, J. L.; Schultz, M. J. Prokineticin 2 receptor antagonists. Patent WO2007079214, 2007.
- (18) Thompson, W. J.; Melamed, J. Y. Preparation of morpholinecarboxamides as prokineticin 2 receptor antagonists. Patent WO2007067511, 2007.

JM800854E