IJPSR (2014), Vol. 5, Issue 11 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 07 July, 2014; received in revised form, 26 September, 2014; accepted, 20 October, 2013; published 01 November, 2014

HALOGENATED TRIAZINEDIONES BEHAVE AS ANTAGONISTS OF PKR_1: *IN-VITRO* AND *IN-VIVO* PHARMACOLOGICAL CHARACTERIZATION

R Lattanzi [1], C Congiu [2], V Onnis [2], A Deplano [2], S Salvadori [3], V Marconi [1], D Maftei [1], A Francioso [4], C Ambrosio [5], I Casella [5], T Costa [5], G Caltabiano [6], M T Matsoukas [6], G Balboni [2], L Negri [1*]

Department of Physiology and Pharmacology [1] "Vittorio Erspamer", Sapienza University of Rome, I-00185 Rome, Italy.
Department of Life and Environmental Sciences [2], Unit of Pharmaceutical, Pharmacological and Nutraceutical Sciences, University of Cagliari, I-09124, Cagliari, Italy.
Department of Chemical and Pharmaceutical Sciences [3], University of Ferrara, I-44-100 Ferrara, Italy.
Department of Biochemical Sciences [4] "Rossi Fanelli" Sapienza University of Rome, Italy.
Dipartimento del Farmaco [5], Istituto Superiore di Sanita, I-00161 Rome, Italy.
Laboratory of Computational Medicine [6], Biostatistics Unit, Faculty of Medicine, Autonomous University of Barcelona, Bellaterra, Spain.

Keywords:

PKR_1 (Prokineticin receptor 1), PKR_2 (Prokineticin receptor 2), Prokineticin receptor antagonist Triazinedione derivatives

Correspondence to Author:
Lucia Negri
Professor of Pharmacology
Dept. of Physiology and Pharmacology, Sapienza University of Rome, P.le Aldo Moro 5, 00185 Rome, Italy

E-mail: lucia.negri@uniroma1.it

ABSTRACT: Different prokineticin receptor antagonists, based on the triazinedione scaffold, were synthesized by a new efficient method. Here we demonstrated that 5-benzyl triazinediones substituted in position para of the benzyl group with halogens provide compounds endowed with interesting selectivity for the Prokineticin receptor 1 (PKR_1). BRET technology indicates that such substitution results in increased affinity for the PKR_1.The affinity for PKR_2, always in μM range, was never significantly affected by the para-halogen-benzyl pharmacophores. The analog bearing a para-bromobenzyl pharmacophore (PC-25) displayed the highest affinity for PKR_1 (~18 times higher than the reference PC-1 that bears a para-ethyl benzyl group) and the highest selectivity (~300 times). The other halogen substituted analogs (PC-7, PC-18 and PC-35), showed selectivity for PKR_1 more than 100 times higher than for PKR_2. Using transgenic mice lacking one of the two PKRs we demonstrated that all these compounds were able to abolish the Bv8-induced hyperalgesia in mice still expressing the PKR_1 at doses lower than those necessary to abolish hyperalgesia in mice expressing only the PKR_2. The dose ratio reflected the *in-vitro* evaluated receptor selectivity.

INTRODUCTION: The prokineticins (among them the mammalian molecules named Prokineticin 1, PROK1, prokineticin 2, PROK2, and their Amphibian homologous, Bv8) make up a new family of chemokines [1, 2] which, in mammals, activate two G-protein linked receptors (prokineticin receptor 1 and 2, PKR_1 and PKR_2).

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.5(11).5066-74

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.5(11).5066-74

Intensive research of the prokineticin system over the past decade has revealed a dazzling array of physiological functions [3]. In addition, the disruption of prokineticin system has been implicated in several pathological conditions, including cancer [4], immunological response [5, 6] and persistent pain [3].

In animal models of inflammatory [7] and neuropathic [8] pain, in which the prokineticin system is highly activated, we already demonstrated that the prokineticin receptor antagonist PC-1 [9] not only abolishes pain, hindering the nociceptor sensitization, but also reduces the over expression of the endogenous

mediator, the PROK2 [10]. By using mice lacking the *prokr1* or the *prokr*2 gene we demonstrated that the up-regulation of PROK2 as well as its proinflammatory and immunomodulatory effects are both mediated by the receptor PKR_1 [6, 7, 11].

Our purpose is to find out molecules endowed with higher affinity and/or selectivity for the PKR_1. We foresee that these molecules might be novel drugs effective in controlling development of immuno-inflammatory processes which underlay several pathological conditions.

In previous papers we described the synthesis of a series of prokineticin-receptor antagonists endowed with PKR_1 selectivity [12]. In *in- vivo* screening we demonstrated that molecules containing a fluorine atom in para position of the benzyl pharmacophore, were effective in abolishing the Bv8-induced hyperalgesia at very low doses (fmol ranges).

Considering this behaviour it could be interesting to prepare and evaluate the affinity and selectivity of the other four halogen substituted PC1-analogs *in -vitro*, using the BRET technology, and *in- vivo*, testing the compounds in transgenic mice expressing only one of the PKRs i.e. mice lacking the *prokr1* or the *prokr2* gene.

In both experimental setting we evaluated the ability of the new compounds to quench the effects of Bv8. Bv8 is the Amphibian homologue of PROK2 which, like PROK2, displays no selectivity for either receptors, but about 10 times higher affinity, and we demonstrated to be a very good pharmacological tool to mimic the activity of the endogenous agonist PROK2 [13].

MATERIALS AND METHODS:

In- vitro experiments:

cDNA Constructs and preparation of cell lines: To prepare cDNA constructs expressing prokineticin receptors fused to the N-terminus of *Renilla* Luciferase (Rluc), PCR fragments coding for the *PROKR1* and *PROKR2* sequences were inserted into pRluc-C vectors (Packard), upstream of the Rluc-coding sequence. Each receptor-Rluc chimeric sequence was then transferred into a pQCIXN retroviral vector (Clontech) expressing the neomycin resistance gene. The preparation of bovine $G\beta_1$ tagged at the N-terminal with RGFP *(Renilla* green fluorescent protein, Prolume) was described in Molinari *et al.*[14]

SH-SY5Y human neuroblastoma cells were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium and F-12 supplemented with 10% (v/v) foetal bovine serum in a humidified atmosphere of 5% CO2 at 37 °C. Cell lines stably co-expressing each luminescent receptor (PKR_1-Rluc, PKR_2-Rluc) in association with RGFP-Gβ1 were obtained by infecting cells sequentially with retroviruses encoding the fusion proteins, followed by selection with G418 (500 µg/ml) in combination with Hygromicin B (100 µg/ml). The expression level of chimaeric proteins was determined by measuring the intrinsic luminescence and fluorescence of cell-membrane preparations [14].

BRET (Bioluminescence Resonance Energy Transfer) assays: The use of *Renilla* photoproteins as reporters of protein–protein interactions has been described previously [14]. G-protein-coupling assays were performed in membranes (prepared by differential centrifugation as described [15]) from neuroblastoma SHSY5Y cells expressing luminescent PKR_1 or PKR_2 and fluorescent $G\beta_1$. Membranes (5 mg of proteins) were incubated in sterile 96-well white plastic plates (Packard View Plate) containing 2–5 µM coelenterazine (Prolume) and different concentrations of Bv8 (10^{-12} - 10^{-6} M) in PBS for 10 min. Luminescence was recorded sequentially using two band pass filters (blue, 450/20 nm, and green, 510/20 nm, 3rd Millenium, Omega Optical, VT) in a plate luminometer (VICTOR light, PerkinElmer). To measure the effect of PKR antagonists on Bv8-induced receptor-G protein coupling, concentration-response curves of antagonists were generated in the presence of 5 nM Bv8.

Data analysis: RET ratios were calculated as the ratios of high energy (donor) and low energy (acceptor) emissions sequentially recorded through the 450 nm and 510 nm filters corrected for spectral overlap, i.e.,

RET ratio = $(CPS_{510} \times T_{450} / CPS_{450} \times T_{510}) - 1$, where CPS indicates photon counts per second and *T* is the relative transmittance of the filters, as reported by the manufacturer.

***In- vivo* experiments:**

Animals:

Male C57Bl6 PKR_1(-/-) or PKR_2(-/-) mice (Lexicon Genetics, The Woodlands, TX) weighing 25-30 g were used for behavioral 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.

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 (NT_{TS}). The effect of the tested drug was calculated as the percentage change in nociceptive threshold from baseline threshold (%ΔNT) according to the following equation: $\%\Delta NT = 100 \times (NT_{TS} - NT_B)/ NT_B$

Drug injections:

Bv8 was extracted from the skin secretion of the frog *Bombina variegata* and purified to 98% (HPLC), as previously described [16]. Bv8 was dissolved in saline and injected in a volume of 20 µl into one hind paw (intraplantar, i.pl.) of mice at the dose of 630 fmol .The antagonists were dissolved in a saline and injected in a volume of 20µl, i.pl., in the same paw, 5 min before Bv8. After drugs administration the animals were observed for three hours at the established time intervals. For each drug dose, a different group of 5 male mice from each genotype was used.

Statistical analysis:

The data are presented as mean ± S.E.M. Statistical analyses were performed using two-way ANOVA followed by Bonferroni post test. ** $P<0.01$ *** $P < 0.001$, Bv8+PC1 *vs.* Bv8+saline.

Molecular Modelling:

Homology modelling:

Modeller v9.13 was used to build homology models of human Prokinetic in receptors PKR_1 and PKR_2 (Uniprot codes Q8TCW9 and Q8NFJ6 respectively) using the crystal structures of human kappa opioid (PDB code 4DJH) and neurotensin 1 (PDB code 4GRV) receptors as templates. The highly conserved $N^{1.50}$ in TM 1, $D^{2.50}$ in TM 2, $R^{3.50}$ in TM 3, $W^{4.50}$ in TM 4, $P^{5.50}$ in TM 5, $P^{6.50}$ in TM6, and $P^{7.50}$ in TM 7, which define the Ballesteros and Weinstein numbering scheme, were used as reference points in TM sequence alignments.

Docking of Prokineticin receptor antagonists:

All docking calculations were performed using Discovery Studio. The Flexible docking protocol was used, assigning side chains to selected residues of the orthosteric binding site using ChiFlex. The ligand docking algorithm was LibDock, and the refinement of the selected protein side-chains in the presence of the rigid ligand was made with ChiRotor. The docked poses were evaluated with the CDOCKER scoring function.

RESULTS AND DISCUSSION:

***In vitro* assay:**

In BRET assay the Bv8-induced interaction between PKR_1 or PKR_2 and the β subunit of heterotrimeric G-proteins resulted in a dose-dependent enhancement of RET signal (**Fig. 1A, B**). Bv8-association curves with the PKR_1 (EC_{50} = 4.9 nM) and the PKR_2 (EC_{50} = 2.9 nM) were similar, confirming that Bv8, like the mammalian

ligand PROK2, binds PKR_1 and PKR_2 with the same affinity, hence is a good ligand to generate displacement curves from PKR_1- and PKR_2-preparations directly comparable. PC-1 dose-dependently reduced the Bv8-induced RET signal and displayed 20 times higher affinity for PKR_1 (IC_{50} = 144 nM) than for PKR_2 (IC_{50} = 2964 nM) (**Fig. 1C, D**) confirming the binding results measured as competition for ^{125}I MIT binding [9].

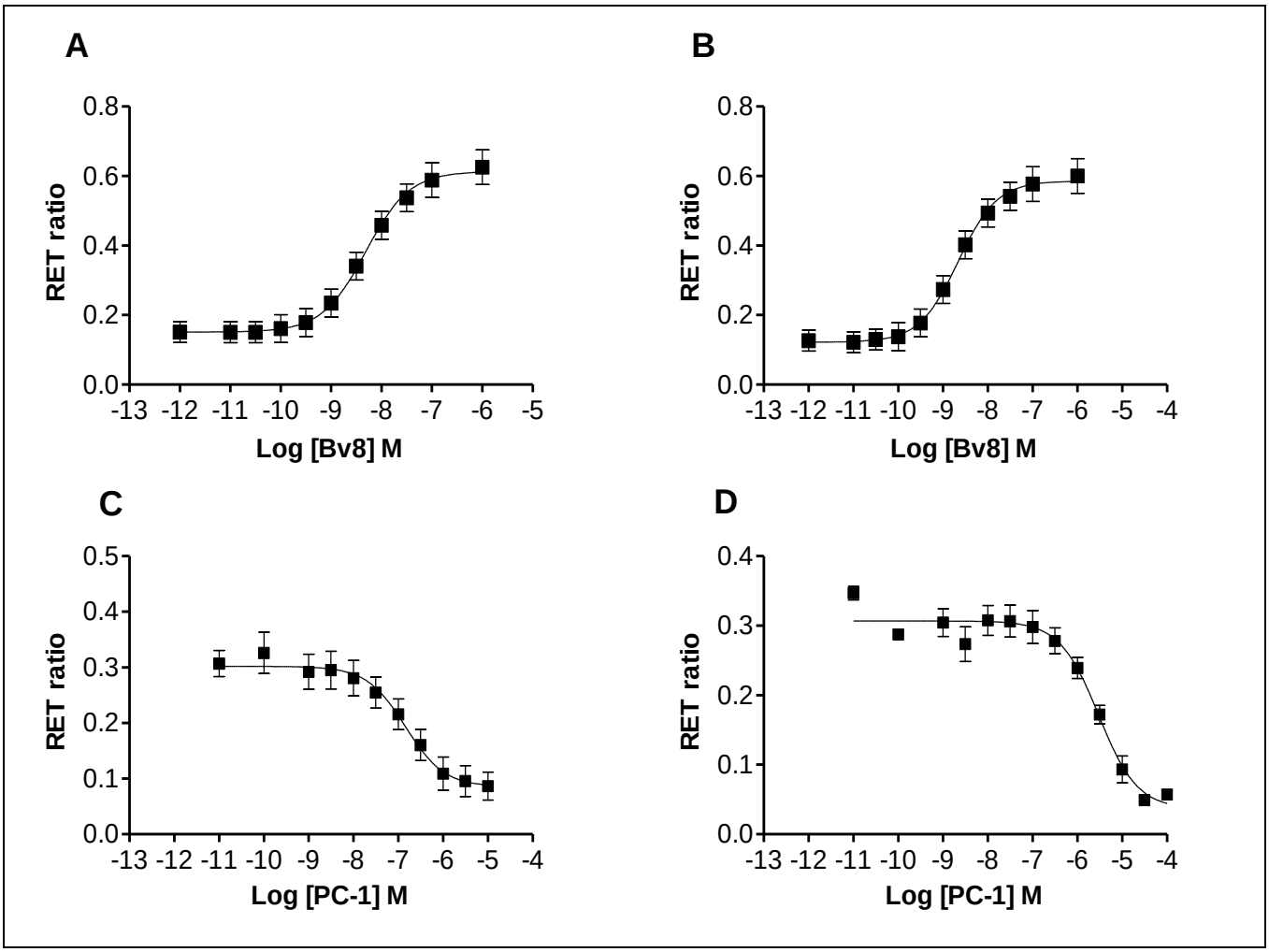


FIGURE 1: CONCENTRATION-RESPONSE CURVES FOR Bv8-INDUCED ENHANCEMENT OF RET RATIO IN MEMBRANES PREPARED FROM SHSY5Y CELLS CO-EXPRESSING FLUORESCENT Gβ AND LUMINESCENT PKR_1 (A) OR PKR_2 (B). EC_{50} VALUES ARE 4.9 ± 0.5 nM AND 2.9 ± 0.3 nM FOR PKR_1 AND PKR_2, RESPECTIVELY. CONCENTRATION-DEPENDENT INHIBITION OF 5 nM Bv8-INDUCED-RET BY PC-1 IN PKR_1 (C) OR PKR_2 (D) CELL MEMBRANES. IC_{50} VALUES ARE 144 ± 15 nM AND 2964 ± 215 nM FOR PKR_1 AND PKR_2, RESPECTIVELY. DATA POINTS ARE MEANS ± S.E.M. OF THREE EXPERIMENTS.

PC-7 (**Fig. 2A**) resulted about 100 times more selective for PKR_1 (IC_{50} = 36 nM) than for PKR_2 (IC_{50} = 4400 nM) and displayed 4 times higher affinity for PKR_1 than the lead compound PC-1. Again ^{125}I MIT-binding assay (not shown) gave affinity values for PKR_1 (IC_{50} = 50 nM) and PKR_2 (IC_{50} = 5700 nM) comparable to those obtained with BRET assay. PC-18 and PC-35 **(Fig 2 C, D)**, bearing a para-iodine and para-chlorine, behaved very similar to PC-7: they maintain the same low affinity for PKR_2 and a comparable affinity for PKR_1.

PC-25 displayed the highest affinity for PKR_1 (~18 times higher than PC-1) and the highest selectivity for PKR_1 (~300 times) (**Fig. 2B** and **Table1**).

These data clearly indicate that para-halogen substitution of the benzyl group in the 5-position of triazinedione scaffold significantly increased the affinity for the PKR_1, while it did not affected the affinity for the PKR_2. The compounds containing the other halogens, chlorine, iodine and fluorine (compd. PC-18, PC-35 and PC-7, respectively) showed about 5 times lower affinity than the best compound PC-25, containing bromine.

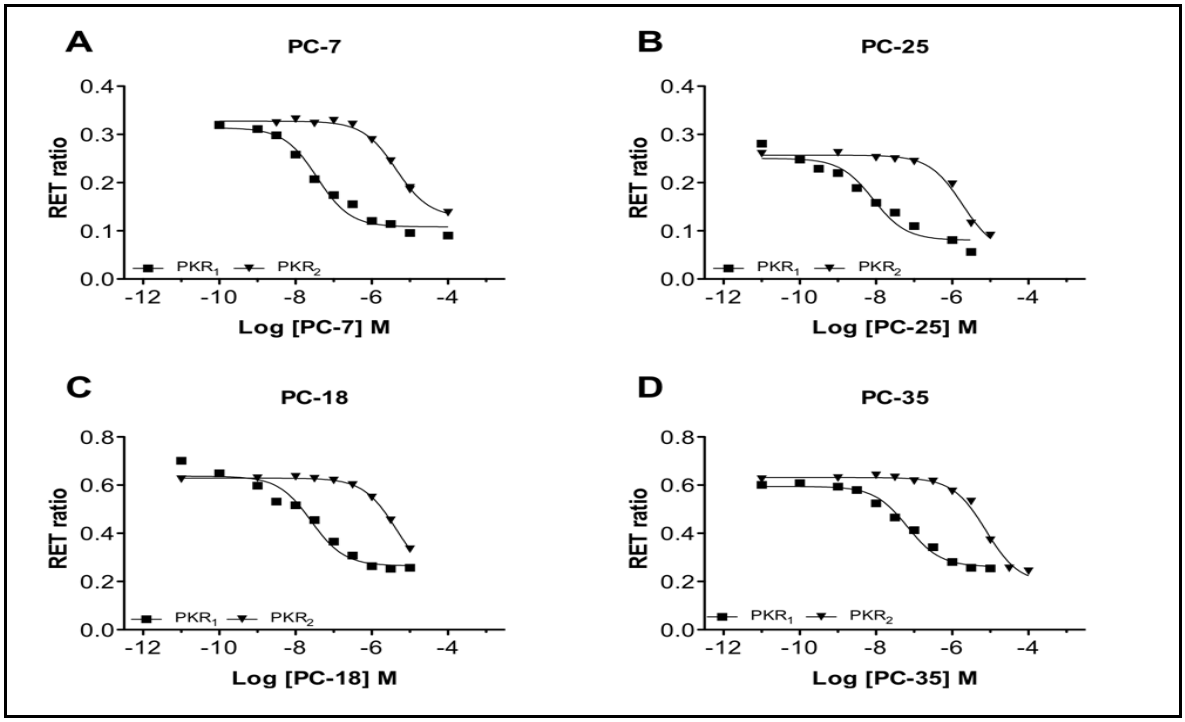
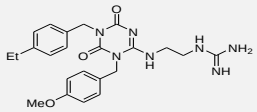
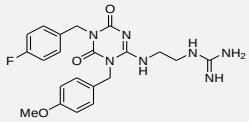
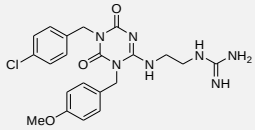
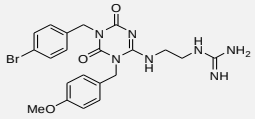
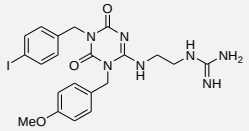


FIGURE 2: CONCENTRATION-DEPENDENT INHIBITION OF Bv8 (5 nM)-INDUCED RET SIGNAL IN PKR_1 (■) OR PKR_2 (▼) CONTAINING CELL MEMBRANES BY HALOGENATED PC-1-ANALOGS. REPRESENTATIVE CURVES OF THREE SEPARATE EXPERIMENTS. IC_{50} VALUES FOR PKR_1 AND PKR_2 ARE REPORTED IN TABLE 1.

TABLE 1. AFFINITY AND SELECTIVITY OF THE HALOGENATED PC_S EXPRESSED AS IC_{50} VALUES. DATA ARE MEANS ± S.E.M. OF THREE EXPERIMENTS

Triazinediones	PKR_1 IC_{50} (nM)	PKR_2 IC_{50} (nM)	Selectivity (IC_{50} PKR_2 / IC_{50} PKR_1)
 **PC-1** ***(reference)***	*144 ± 15*	*2964 ± 215*	*20.6*
 **PC-7**	36 ± 6.1	4399 ± 340	122
 **PC-18**	28 ± 3.2	4400 ± 310	157
 **PC-25**	8 ± 0.5	2162 ± 180	270
 **PC-35**	39 ± 4.9	4440 ± 385	114

In- vivo assay:

In PKR_1(-/-) and PKR_2(-/-) mice i.pl. injection of Bv8 (5 ng = 630 fmol) induced comparable hyperalgesia evaluated as decrease of the nociceptive threshold to thermal stimuli. Hyperalgesia was already evident in 15 min, peaked in 60 min and lasted for about 3 h. Pre-injection (-5 min) of the compounds, into the paw, antagonized the Bv8-induced hyperalgesia in dose-dependent manner. In PKR_1(-/-) mice a dose of 150 pmol of PC-1 abolished thermal hyperalgesia induced by Bv8, whereas in PKR_2(-/-) mice a 10 folds lower dose (15 pmol) of PC-1 was enough to obtain the same effect (**Fig. 3**).

Evaluation of the dose-effect relationship was obtained considering the area under the curves (AUC) for each tested dose. This analysis confirmed that the antihyperalgesic effect of PC-1 is more than 10 times higher in PKR_2(-/-) mice respect to PKR_1(-/-) mice accordingly with its preferential affinity for PKR_1, the receptor still present in PKR_2(-/-) mice (**Fig. 3**).

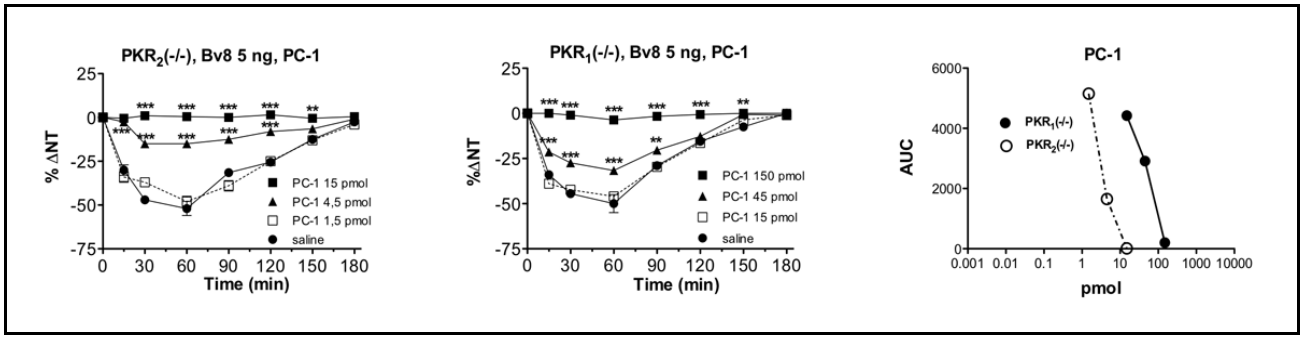


FIGURE 3: EFFECTS OF INTRAPLANTAR (i.pl.) PRE-INJECTION (-5 min) OF VARIOUS DOSES OF PC-1 ON THERMAL HYPERALGESIA INDUCED BY Bv8 (5 ng, i.pl.) IN PKR_2(-/-) AND PKR_1(-/-) MICE. THE ANTIHYPERALGESIC EFFECT WAS EVALUATED AS AUC OF THE TIME-RESPONSE CURVE FOR EACH DOSE.

As demonstrated in **Fig. 4**, PC-7 and PC-25 antagonized the Bv8-induced thermal hyperalgesia in PKR_1-KO mice at doses of 15 pmol and 14 pmol, but in PKR_2-KO mice at doses of 0.15 pmol and 0.04 pmol, respectively, confirming a selectivity of 100 and 300 times for PKR_1.

PC-18 and PC-35 appeared less effective than PC-7, being necessary significantly higher doses to counteract the Bv8 induced hyperalgesia, however the ratio of the effective doses in PKR_1 (-/-) and PKR_2 (-/-) mice was consistent with their selectivity: about 100 folds.

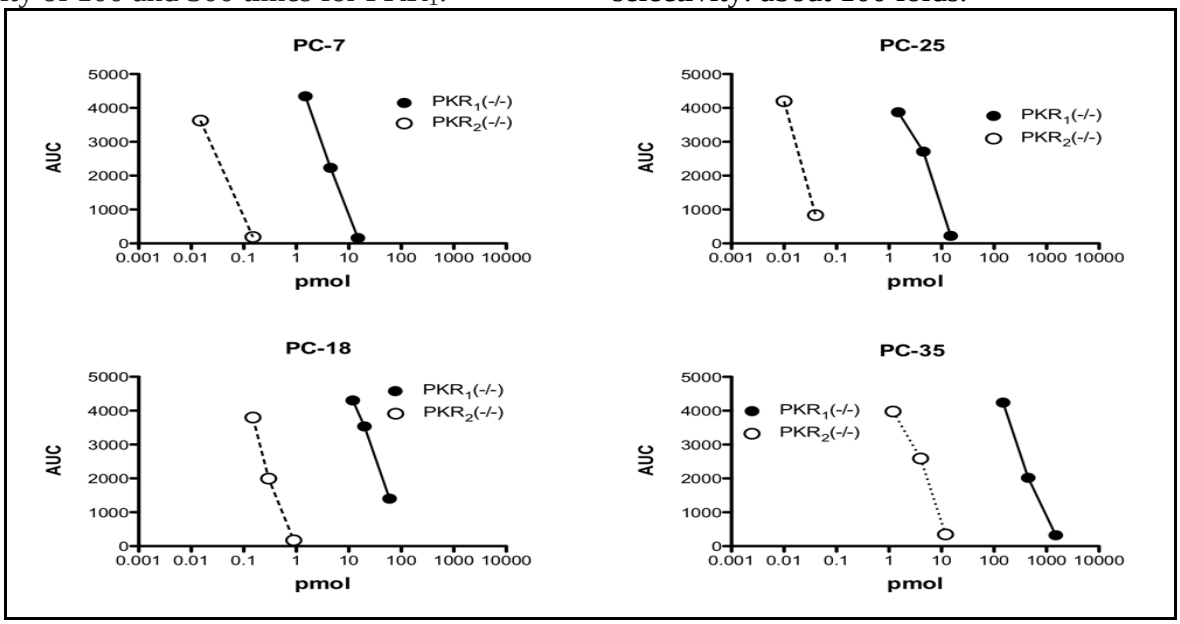


FIGURE 4: DOSE-DEPENDENT INHIBITION OF Bv8-INDUCED HYPERALGESIA BY PRETREATMENT WITH VARIOUS DOSES OF PC-7, PC-18, PC-25 AND PC-35 IN PKR_2(-/-) AND PKR_1(-/-) MICE. THE ANTIHYPERALGESIC EFFECT OF VARIOUS DOSES OF THE HALOGENATED COMPOUNDS WAS EVALUATED AS AREA UNDER THE TIME-RESPONSE CURVE (AUC).

Molecular Modelling

Using a flexible docking method (see Materials and Methods), the known antagonist PC-1 was docked to the binding site of the structural model of the PKR_1 receptor. Residue side chains that were considered flexible were E2.61, R3.32, T4.64, Qi+2 (at position i+2, relative to the conserved C180i engaged in a disulfide bond with C3.25 in TM 3), R6.58 and E7.39. The highest scored poses were ranked and inspected visually, to determine whether they satisfy the experimental results. Based on the docking results, as seen in **Fig. 5A**, the guanidine group interacts with E2.61 and F7.35, while one of the carbonyl oxygens interacts with R6.58. The anisole oxygen forms a hydrogen bond with N3.29 and the ethyl benzene groups is located in hydrophobic vicinity formed by T4.64 and F4.63. The interactions with PKR_2 are similar, with the difference of the A4.64 instead of the threonine (**Fig. 5B**). In the case of PC-25, the bromine of the benzene group is interacting with the T6.46 hydroxyl group of PKR_1 through a halogen bond (**Fig. 5C** see Conclusion), whereas the absence of this interaction can be seen on **Fig. 5D**.

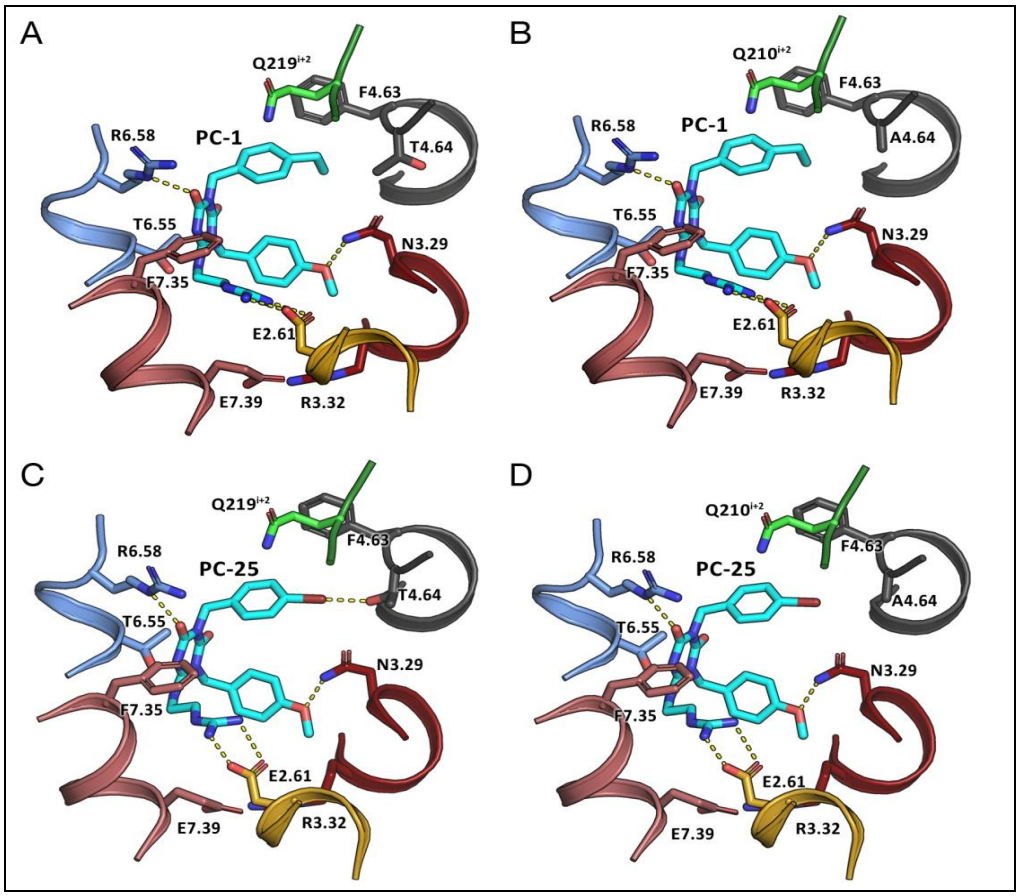


FIGURE 5. DOCKING MODELS OF PC-1 AND PC-25. DIFFERENCES IN THE BINDING OF PC-1 (CYAN STICKS) INTERACTING WITH THE BINDING SITE RESIDUES OF PKR_1 (PANEL A) AND PKR_2 (PANEL B). DIFFERENCES IN THE BINDING OF PC-25 (CYAN STICKS) INTERACTING WITH THE BINDING SITE RESIDUES OF PKR_1 (PANEL C) AND PKR_2 (PANEL D). THE COLOUR CODE OF THE HELICES IS TM 2 IN YELLOW, 3 IN RED, 4 IN GRAY, 6 IN BLUE, AND 7 IN LIGHT BROWN, WHERE EL2 IS IN GREEN

CONCLUSIONS: The primary structures of PKR_1 and PKR_2 are highly homologous, having a sequence identity of 91% and BLOSUM62 similarity of 96%. All residues that are located in the different binding pockets (mayor and minor binding pocket as well as the extracellular entrance, the latter often involved in allosteric modulation of class A GPCR) are fully conserved with the exception of position 4.64 in which PKR_2 has an alanine (A201 in absolute numeration) instead of a threonine (T192), featured in PKR_1. Most sequence variation between the PKR subtypes is concentrated in the extracellular N terminal region, which contains a nine-residue insert in PKR_1 compared with PKR_2, as well as in the second intracellular loop (ICL2) and in the C terminal tail. A conserved disulfide bridge connects the second extracellular loop (ECL2) with the extracellular end

of TM3 a characteristic shared with more than 80% of class A GPCR. Levit *et al.*[17], analyzing the human PKRs, identified a putative extracellular surface binding site, which most likely binds the endogenous PKR ligands, but also confirmed PKRs are able to use a pocket located in the upper part of the TM bundle among TMs 3,4,5,6, and 7, the extracellular entrance, were synthetic small-molecule supposedly bind.

In- vitro experiments using BRET technology confirmed results we previously obtained for PC-1 and PC-7, from competitive binding experiments labelling the PKRs with ^{125}I MIT [9].

Our docking models show residues at position 4.64 (T192 in PKR_1 and A201 in PKR_2, the only non-conserved residue of the binding sites between PKR_1 and PKR_2), are responsible of the selectivity of PC-1 towards PKR_1. T4.64 (T192) in PKR_1 potentially stabilizes the ethyl benzene group by offering an extra methyl group, enhancing the hydrophobic of this region. All the compounds containing a halogen atom instead of the ethyl group in *para*-position of the benzyl pharmacophore of the 5-position of the triazinedione scaffold resulted more selective and displayed higher affinity for PKR_1.

Interestingly, PC-25, which contains a bromine atom displayed the highest affinity for PKR_1 (~18 times higher than PC-1) and the highest selectivity (~300 times). Despite the fact PC-35, containing iodine, has not the highest affinity, the observed trend in binding is likely ascribable to the formation of a halogen bond. Halogen bond, is a specific semi-directional molecular interaction between a halogen atom, acting as a Lewis acid, and an electron-rich partner (O, N, or S acting as Lewis basis) and which strength follow the size of the positively charged σ-hole on the tips of the atoms, thus I>Br>Cl>F.

They are abundant in biological systems[18] and database surveys reveal that halogen bonding is the prevalent interaction between halogenated ligand and target protein [19, 20]. Moreover around 25% of the "top 200 brand name drugs by retail dollar in 2009" possess halogen atoms in their molecular structures [21]. Therefore, halogens have a key role in drug development. The trend in affinity of halogenated compounds to PKR_1 follow the halogen bond trend, I>Br>Cl>F with the exception of iodine, while no clear trend is observed in the affinity to PKR_2, as expected since it misses a Lewis base in position 4.64. Following and confirming the docking of PC-1, compounds PC-7, PC-18, PC-25 and P-35, are likely forming halogen bonds with the hydroxyl group of T4.64 (**Fig. 5C**) explaining both the higher affinity of these compounds to PKR_1 as well as their selectivity.

Despite their affinity trend is clearly following the halogen bond strength trend, no statistically difference is observed in the binding of PC-7, PC-18 and PC-35. Wilcken *et al.*[21], in an analysis of the halogen bonds found in PDB and involving threonines, observed that most of the -I···O- distances are of in the range of 5.50-6Å, while most of the Cl···O was of around 3.5Å.

The calculated distances between the halogens of PC-7, PC-18, PC-25 and P-35 and the oxygen of T4.64 are of 3.0Å±0.2, which perfectly fits with many of the observed [21] halogen bonds between Br-containing compounds and threonines, thus explaining why PC-25 is statistically binding stronger PKR_1 than PC-7, PC-18 and PC-35. Moreover the iodine of PC-35 is likely too close to the oxygen of T4.64 thus limiting the quality of its halogen bond.

In-vivo all these compounds were able to antagonize dose-dependently the hyperalgesia induced by the i.pl. injection of 5 ng Bv8. In PKR_1(-/-) mice, in which only the receptor-2 is still present, higher doses were necessary than in PKR_2(-/-) mice which still express the receptor-1. The dose ratio well mirrored the selectivity for the PKR_1. Hence this *in-vivo* test performed on mice expressing only one of two PKRs is a quick and reliable method to evaluate antagonists of the prokineticin system.

ACKNOWLEDGMENTS: This study was supported by grants of the University of Rome "La Sapienza" (L.N.), by the Italian Ministero dell'Istruzione, Universita e della Ricerca (PRIN 2010-2011, Prot. no. 20105YY2HL_002), by Research Funds of the University of Cagliari to (G.B., C.C., V.O.) and of the University of Ferrara (S.S.).

REFERENCES:

1. Lattanzi R, Giannini E, Melchiorri P and Negri L: Pharmacology of Bv8: a new peptide from amphibian skin. British Journal of Pharmacology 2001; 133: 45P.
2. Kaser A, Winklmayr M, Lepperdinger G andKreil G: The AVIT protein family. Secreted cysteine-rich vertebrate proteins with diverse functions. EMBO Reports 2003; 4: 469–473.
3. Negri L, Lattanzi R, Giannini E, Canestrelli M, Nicotra A and Melchiorri P: Bv8/prokineticins and their receptors: a new pronociceptive system. International Review of Neurobiology 2009; 85: 145-157.
4. Shojaei F, Wu X, Zhong C, Yu L, Liang XH, Yao J, Blanchard D, Bais C and Peale FV, van Bruggen N, Ho C, Ross J, Tan M, Carano RAD, Meng YG and Ferrara N: Bv8 regulates myeloid-cell-dependent tumour angiogenesis. Nature 2007; 450: 825-831.
5. Dorsh M, Qui Y, Soler D, Frank N, Duong T, Goodearl A, O'neil S, Lora J and Fraser C: PK1/EG-VEGF induces monocyte differentiation and activation. Journal of Leukocyte Biology 2005; 78: 426-434.
6. Franchi S, Giannini E, Lattuada D, Lattanzi R, Tian H, Melchiorri P, Negri L, Panerai AE and Sacerdote P: The prokineticin receptor agonist Bv8 decreases IL-10 and IL-4 production in mice splenocytes by activating prokineticin receptor-1.BMC Immunology 2008; 9: 60-73.
7. Giannini E, Lattanzi R, Nicotra A, Campese A F, Grazioli P, Screpanti I, Balboni G, Salvadori S, Sacerdote P and Negri L: The chemokine Bv8/prokineticin 2 is up-regulated in inflammatory granulocytes and modulates inflammatory pain. Proc. Natl. Acad. Sci. USA. 2009; 106: 14646-14651.
8. Maftei D, Marconi V, Florenzano F, Giancotti LA, Castelli M, Moretti S, Borsani E, Rodella LF, Balboni G, Luongo L, Maione S, Sacerdote P, Negri L and Lattanzi R: Controlling the activation of the Bv8/Prokineticin system reduces neuroinflammation and abolishes thermal and tactile hyperalgesia in neuropathic animals. British Journal of Pharmacology 2014; 171**:** 4850–4865.
9. Balboni G, Lazzari I, Trapella C, Negri L, Lattanzi R, Giannini E, Nicotra A, Melchiorri P, Visentin S, De Nuccio C and Salvadori S: Triazine compounds as antagonists at Bv8-prokineticin receptors. Journal of Medicinal Chemistry 2008; 51: 7635-7639.
10. Negri L and Lattanzi R: Bv8/PK2 and prokineticin recepors: a druggable pronociceptive system Current Opinion in Pharmacology 2012; 12: 62-66.
11. Martucci C, Franchi S, Giannini E, Tian H, Melchiorri P, Negri Land Sacerdote P:Bv8, the amphibian homologue of the mammalian prokineticins, induces a proinflammatory phenotype of mouse macrophages. British Journal of Pharmacology 2006; 147 (2): 225-234.
12. Congiu C, Onnis V, Deplano A, Salvadori S, Marconi V, Maftei D, Negri L, Lattanzi R and Balboni G: A new convenient synthetic method and preliminary pharmacological characterization of triazinediones as prokineticin receptor antagonists. European Journal of Medicinal Chemistry 2014; 81: 334-340.
13. Negri L and Lattanzi R: Bv8-prokineticins and their receptors: modulators of pain. Current Pharmaceutical Biotechnology 2011; 12(10):1720-1727.
14. Molinari P, Casella I and Costa T: Functional complementation of high efficiency resonance energy transfer: a new tool for the study of protein binding interactions in living cells. Biochemistry Journal 2008; 409: 251–261.
15. Molinari P, Ambrosio C, Riitano D, Sbraccia M, Gro MC and Costa T: Promiscuous coupling at receptor-Gα fusion proteins. The receptor of one covalent complex interacts with the α-subunit of another. Journal of Biological Chemistry 2003; 278: 15778-15788.
16. Mollay C, Wechsel berger C, Mignogna G, Negri L, Melchiorri P, Barra D and Kreil G: Bv8, a small protein from frog skin and its homologue from snake venom induce hyperalgesia in rats. European Journal of Pharmacology 1999; 374: 189-196.
17. Levit A, Yarnitzky T, Wiener A, Meidan R and Niv MY. Modeling of human prokineticin receptors: interactions with novel small-molecule binders and potential off-target drugs. PLoS One 2011; 6 (11): e27990.
18. Auffinger P, Hays FA, Westh of E and Ho PS: Halogen Bonds in Biological Molecules. Proc Natl Acad Sci USA 2004; 101: 16789–16794.
19. LuY, Shi T, Wang Y, Yang H, Yan X, Luo X, Jiang H and Zhu W: Halogen Bonding: A Novel Interaction for Rational Drug Design? Journal of Medicinal Chemistry 2009; 52: 2854–2862.
20. Xu Z, Liu Z, Chen T, Chen T, Wang Z, Tian G, Shi J, Wang X, Lu Y, Yan X, Wang G, Jiang H, Chen K, Wang S, Xu Y, Shen J and Zhu W: Utilization of Halogen Bond in Lead Optimization: a Case Study of Rational Design of Potent Phosphodiesterase Type 5 (PDE5) Inhibitors. Journal of Medicinal Chemistry 2011; 54: 5607–5611.
21. Wilcken R, Zimmermann MO, Lange A, Joerger AC and Boeckler FM: Principles and Applications of Halogen Bonding in Medicinal Chemistry and Chemical Biology. Journal of Medicinal Chemistry 2013; 56: 1363–1388

How to cite this article:
Lattanzi R, Congiu C, Onnis V, Deplano A, Salvadori S, Marconi V, Maftei D, Francioso A, Ambrosio C, Casella I, Costa T, Caltabiano G, Matsoukas MT, Balboni G, Negri L: Halogenated Triazinediones Behave As Antagonists of Pkr_1: *In -Vitro* and *In -Vivo* Pharmacological Characterization. Int J Pharm Sci Res 2014; 5(11): 5066-74.doi: 10.13040/IJPSR.0975-8232.5 (11).5066-74.