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A novel p38 MAPK docking groove-targeted compound is a potent inhibitor of inflammatory hyperalgesia

Hanneke L.D.M. Willemen^{*,†}, Pedro M. Campos^{‡,†,§}, Elisa Lucas^{‡,||}, Antonio Morreale^{||,1}, Rubén Gil-Redondo^{||,2}, Juan Agut³, Florenci V. González³, Paula Ramos^{‡,||}, Cobi Heijnen^{*,4}, Federico Mayor Jr.^{‡,||}, Annemieke Kavelaars^{*,4,5}, and Cristina Murga^{‡,||,5}

^{*}Laboratory of Neuroimmunology and Developmental Origins of Disease, University Medical Center Utrecht, Utrecht, The Netherlands [†]Departamento de Biología Molecular and Centro de Biología Molecular “Severo Ochoa” UAM-CSIC, Madrid, Spain ^{||}Instituto de Investigación Sanitaria La Princesa, Madrid, Spain ¹Centro de Biología Molecular “Severo Ochoa”, UAM-CSIC, Madrid, Spain ³Departament de Química Inorgànica i Orgànica, Universitat Jaume I, Castelló, Spain ⁴Department of Symptom Research, University of Texas MD Anderson Cancer Center, Houston, TX, USA

Synopsis

The mitogen activated protein kinase (MAPK) p38 is an important mediator of inflammation and of inflammatory and neuropathic pain. We recently described that docking-groove dependent interactions are important for p38 MAPK-mediated signal transduction. Thus, virtual screening was performed to identify putative docking groove-targeted p38 MAPK inhibitors. Several compounds of the benzooxadiazol family were identified with low micromolar inhibitory activity both in a p38 MAPK activity assay, and in THP-1 human monocytes acting as inhibitors of LPS-induced TNF α secretion. Positions 2 and 5 in the phenyl ring are essential for the described inhibitory activity with a chloride in position 5 and a methyl-group in position 2 yielding the best results with an IC₅₀ of 1.8 μ M (FGA-19 compound). Notably, FGA-19 exerted a potent and long-lasting analgesic effect *in vivo* when tested in a mouse model of inflammatory hyperalgesia. A single intrathecal injection of FGA-19 completely resolved hyperalgesia, being ten times as potent and displaying longer lasting effects than the established p38 MAPK inhibitor SB239063. FGA-19 also reversed persistent pain in a model of post-inflammatory hyperalgesia (in LysM-GRK2^{+/-} mice). These potent *in vivo* effects put forward p38 MAPK docking-site targeted inhibitors as a potential novel strategy for the treatment of inflammatory pain.

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Corresponding authors: Dr. Cristina Murga, Departamento de Biología Molecular and Centro de Biología Molecular “Severo Ochoa”, Universidad Autónoma de Madrid (UAM-CSIC), Madrid, Spain, Phone: + 34 91 196 4641/ + 34 91 497 4413, cmurga@cbm.uam.es. Dr. Annemieke Kavelaars, Department of Symptom Research, University of Texas MD Anderson Cancer Center, 1400 Pressler Street, Unit 1450 Houston, TX 77030. Phone: +713-794-4453, akavelaars@mdanderson.org.

[†]equal contribution to this work

[§]current address: Allinix Biopharma, Parque Científico de Madrid, Madrid, Spain.

¹current address: Repsol, Technology Center. Ctra. de Extremadura, A-5, km 18, 28935 Móstoles, Madrid, Spain

²current address: SmartLigs Bioinformática S.L., Fundación Parque Científico de Madrid, Campus de Cantoblanco UAM, Madrid, Spain

⁵co-corresponding authors

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Keywords

p38 MAPK; inflammatory hyperalgesia; pain; docking; mitogen-activated protein kinases; persistent pain

Introduction

The mitogen activated protein kinase (MAPK) p38 is a key signaling protein involved in regulating the production of multiple inflammatory mediators. p38 MAPK has thus been the target of intensive research in academic groups and in many different pharmaceutical companies [1]. p38 belongs to the MAPK family of serine/threonine kinases that share a common overall structure and a similar mode of activation via phosphorylation at the activation loop by upstream activators, including MKK3 and MKK6 for p38 MAPK (reviewed in [2]). Of the four p38 MAPK isoforms, p38 α and β are ubiquitously expressed and in inflammatory cells p38 α is the most prominent. Many different extracellular stimuli activate p38 MAPK, among them inflammatory cytokines, lipopolysaccharide (LPS) or other bacterial products, and different stressors such as thermal, mechanical or hypoxic insults [3]. In turn, p38 MAPK directly phosphorylates important transcription factors including myocyte enhancer factor 2 (MEF-2) and activating transcription factor 2 (ATF-2). p38 MAPK also controls the activation state of downstream kinases such as MAPK-activated protein kinases 2 and 3 (MK2 and 3) that in turn phosphorylate heat shock protein-27 (Hsp-27) and other cellular proteins. A p38-dependent route regulates the stability and expression of mRNAs coding for cytokines such as TNF α , IL-1 β , IL-6, and IL-8 [4]. In the context of pain, p38 MAPK is activated in the spinal cord in several models of chronic pain, and pain hypersensitivity can be transiently blocked by administration of p38 MAPK inhibitors [5–8]. These findings indicate that p38 MAPK may be an interesting target for the development of therapeutic drugs to treat chronic inflammatory pain. However, so far human clinical trials using p38 MAPK inhibitors have not yielded the expected results [9].

p38 MAPK is able to specifically interact with upstream regulators and also with downstream substrates by virtue of a specific site called docking domain that lays opposite to the activation loop [2]. The specific features of these interactions have been characterized in detail and the structure of p38 α co-crystallized with peptides derived from the interacting motifs in MKK3b and MEF-2A has been resolved [10]. Interestingly, the interactions mediated by this docking domain can be regulated by means of phosphorylation of either the substrate or p38 kinase itself [11]. This characteristic provides a feasible mechanism for intervention into the p38 MAPK route by providing a target for therapeutic drugs potentially more specific protein than the currently most widely used domain: the ATP binding site. The latter shares a high overall homology with that of other protein kinases [12], what likely underlies the failure of the first and second generation ATP-site targeted compounds during human clinical trials [1]. Consequently, the need for novel strategies utilizing unexplored interactive domains in p38 is an avenue that certainly deserves further research. Recently, we described that p38 MAPK can be phosphorylated at the docking groove resulting in reduced binding of upstream kinases and downstream targets and impairing activity of p38

MAPK [11]. With this basis, we performed a virtual screening of a chemical library of compounds to search for potential novel p38 MAPK inhibitors targeting the docking groove.

Materials and Methods

Virtual Screening (VS)

All VS calculations were performed within the automated platform VSDMIP [13, 14]. For the sake of clarity, we briefly describe here the main steps comprising the protocol.

Receptor preparation—The three-dimensional structure of p38 MAPK as found in complex with MEF2A peptide (PDB: 1LEW) [10] was used as the receptor. All atoms other than those from the receptor were removed. AMBER ff99 force field [15] was then used to assign atom types and charges for each atom in the receptor. Addition of missing hydrogen atoms and computation of the protonation state of ionizable groups at pH 6.5 were carried out using the H++ Web server [16], which relies on AMBER parameters and finite difference solutions to the Poisson–Boltzmann equation. A salt concentration of 0.15 M and an internal and external dielectric constant of 4 and 80, respectively, were used.

Binding site definition and characterization—To delimit the binding site we selected MEF2A as the core around which to build the docking box by adding a 5.0 Å cushion to the maximum dimensions of the peptide. A equally spaced grid of 0.5 Å was then built, and CGRID [17] calculated protein interaction fields (a 12–6 Lennard-Jones term and an electrostatic term modeled with a sigmoidal dielectric screening function) using common atom probes (C, N, O, S, P, H, F, Cl, Br, and I) at each grid point. Next, benzene, water and methanol probes were docked with CDOCK [17] to generate intermolecular interaction energy maps aimed at capturing the most likely areas of interest for hydrophobic, hydrophilic and hydrogen bond interactions, respectively. These areas were further compressed into gaussian functions using GAGA algorithm [18] producing a negative image of the interaction site. The putative active ligands in the library must conform to this approximate shape.

Chemical library preparation—Ligands for VS consisted on a selection of ~1 million non-redundant molecules obtained from the publicly available ZINC database [19], mainly from ChemBridge and ASINEX companies, in SMILES format [20]. Multiple protonation states and tautomeric forms were considered as implemented by default in ZINC. The molecules were then processed within VSDMIP as usually: (a) conversion from SMILES to 3D MOL2 using CORINA [21], (b) atomic charge calculations with MOPAC [22] (MNDO ESP method) on every single structure provided by CORINA; and (c) atom type assignment according to the AMBER ff99 force field and conformational analysis using ALFA [23].

Filter 1—An initial filter was performed with the docking program DOCK to quickly discard those molecules that do not geometrically fit within the binding site. The spheres needed by DOCK were those previously generated with GAGA. We used DOCK contact as the scoring function, normalizing the score values ($score_i$) by converting them into ZScore using mean (average score) and standard deviation (σ) values ($ZScore_i = (score_i - \text{average}) / \sigma$).

score)/ σ). Only those molecules with a ZScore beyond a cut off value of 2.5 were selected (45,488) and passed onto the next step.

Filter 2—Selected molecules from the previous step were docked with the more accurate docking algorithm CDOCK. CDOCK exhaustively docks each molecule within the binding site of the receptor using the interaction energy grids previously calculated with CGRID. This was achieved by an exhaustive exploration of the location and orientation of each molecule by positioning its center of mass on grid points and performing discrete rotations of 27° on each axis (poses). Finally, the energy of the poses was evaluated by the molecular mechanics force-field scoring function implemented in CDOCK, which beside including a 12–6 Lennard-Jones term and an electrostatic term modeled with a sigmoidal dielectric screening function, also accounts for ligand and receptor desolvations as well as for hydrogen bonding interactions [24].

Molecular dynamics simulations and MM-GBSA calculations—The top ranked 200 molecules according to CDOCK's scoring function were subjected to better binding free energy estimation by a combination of molecular dynamics (MD) trajectories and MM-GBSA [25] calculation on these trajectories. The 200 complexes were hydrated by using boxes containing explicit water molecules with added counter ions to maintain electro neutrality, energy minimized, heated (20 ps), and equilibrated (100 ps). After equilibration, MD trajectories were continued for 200 ps. From this last part, structures were homogeneously sampled each 10 ps and stored for postprocessing. All the simulations were performed at constant pressure and temperature (1 atm and 300 K) with an integration time step of 2 fs. SHAKE [26] was used to constrain all the bonds involving H atoms at their equilibrium distances. Periodic boundary conditions and the Particle Mesh Ewald methods were used to treat long-range electrostatic effects [27]. AMBER ff99 and TIP3P [28] force-fields were used in all cases. Finally, the effective binding free energies were qualitatively estimated using the MM-GBSA approach, which calculates the free energy of binding as a sum of a Molecular Mechanics (MM) interaction term, a solvation contribution through a Generalized Born (GB) model and a Surface Area (SA) contribution to account for the non-polar part of desolvation. A 12–6 Lennard-Jones term was used to model the MM contribution. For GB, the solute dielectric constant was set to 4 while that of the solvent was set to 80, and the dielectric boundary was calculated using a solvent probe radius of 1.4 Å. The SA contribution was approximated as a linear relationship to the change in SASA (Solvent Accessible Surface Area):

$$\Delta G_{np} = a + b \cdot \Delta SASA$$

where a is 0.092 kcal · mol⁻¹, b is 0.00542 kcal · mol⁻¹ · Å⁻², G_{np} is the SA contribution, and the change in SASA refers to the complex SASA minus the sum of that of the protein and the ligand alone. In addition, interaction energy analysis between the ligands and the more relevant residues in the binding site were computed (with MM-GBSA). All the trajectories and analysis were performed using the AMBER 8 computer program and associated modules [29].

Selection of candidates—Averaged structures along the trajectories were obtained and energy minimized in vacuum with the ff99 force field, without periodic boundary conditions and during 1000 steps (the first 500 with the steepest descent method and the rest with the conjugated gradient) solely to alleviate the possible clashes that may be originated by averaging the coordinates. These structures were used for graphical representation and comparison of the binding modes. From the highest scoring compounds, and upon visual examination, 18 candidates were finally selected, purchased (see below), and tested experimentally. All visualizations were done within the molecular graphics program PyMOL [30]. Unfortunately, not all the compounds were available from the vendors. In these cases similarity search was performed employing two complementary strategies based on: (a) the 2D topological representation of the molecules and the search/compare engine implemented in ZINC; and (b) the 3D structure of the molecules, the shape-matching algorithm implemented in the program ROCS [31], and the ZINC database as implemented in VSDMIP. The similarity between structures was assessed by the Tanimoto coefficient (T_c), selecting only those candidates with a $T_c \geq 0.9$ to be docked into the receptor and further analysis. The final goal was to choose one or two purchasable analogues for each not-found compound presenting as similar interactions as possible when compared to the originally selected molecules.

Hit to lead optimization (H2L)

The scheme for the H2L optimization cycle is shown in figure 1. A typical optimization step involves a finer docking study of the best compounds from the experimental assays over an energy minimized MD-averaged structure followed by MD simulation and MM-GBSA analysis. In brief, for every docked molecule we: (a) selected the 100 best docking solutions; (b) clustered them according to their structural resemblance; (c) selected a representative structure from each of the cluster based on its CDOCK energy; (d) run a MD simulation for a period of up to 10 ns; (e) estimated their free energy of binding *via* MM-GBSA method; (f) selected the best pose based on MM-GBSA energy; and (g) visually inspected and analyzed the structures from (f). In this last analysis, the essential ligand-receptor interactions were evaluated to define possible chemical modifications to enhance binding towards the receptor. These suggestions represented new candidates for synthesis. Following synthesis, the compounds are experimentally assayed and the theoretical models revised. This process (a)–(f) was performed twice resulting in two final lead compounds.

Chemical Synthesis

N-(5-chloro-2-methylphenyl)-7-nitrobenzo[*c*][1,2,5]oxadiazol-4-amine (FGA-19) was synthesized as follows: a solution of 4-chloro-7-nitrobenzofurazan (NBD-Cl) (798.2 mg, 4 mmol) and 5-chloro-2-methylaniline (1.26 g, 8 mmol) in *N,N*-dimethylformamide (50 mL) was refluxed for 24 h. Then the reaction mixture was allowed to warm up to room temperature and was quenched with a saturated aqueous sodium bicarbonate solution, then extracted with ethyl ether (3 × 30 mL), the combined organic layers were washed with a saturated aqueous sodium chloride solution and dried (sodium sulfate), filtered and concentrated to afford a crude oil which was purified using chromatography over silica-gel and hexanes:ethyl acetate (7:3) as eluent. The desired compound was obtained as a red oil

(608 mg, yield = 50%) which was crystallized (MeOH:H₂O (1:1)) to afford pure compound FGA-19 as red crystals.

Compounds FGA-17, FGA-20, FGA-23, FGA-29, FGA-32 and FGA-34 were synthesized as follows: a solution of 4-chloro-7-nitrobenzofurazan (NBD-Cl) (798.2 mg, 4 mmol) and the corresponding aniline (8 mmol) in ethyl acetate (50 mL) was refluxed for 48 h. Then the reaction mixture was allowed to warm up to room temperature and worked up and purified as described above. The yields and physical state of each one of these final products are specified below:

Compound	Physical State	Yield (%)
FGA-17	Red crystals	67
FGA-19	Red crystals	50
FGA-20	Yellow solid	55
FGA-23	Orange solid	64
FGA-29	Deep red solid	58
FGA-32	Orange solid	54
FGA-34	Orange solid	52

Animals

Female C57BL/6 mice (aged 12 to 14 weeks) and female mice with cell-specific reduction of GRK2 in LysM-positive macrophages (LysM-GRK2^{+/-} mice) were used [32]. LysM-Cre mice and GRK2-flox mice were obtained from Jackson Laboratories (Jackson Laboratories, Bar Harbor, ME, USA) to generate LysM-GRK2^{+/-} and control LysM-GRK2^{+/+} mice (WT littermates). Peritoneal macrophages were collected from global GRK2 hemizygous male mice [33] as described [11]. All mice were bred and maintained in the animal facility of the University of Utrecht, The Netherlands or of the Universidad Autonoma de Madrid, Spain. Experiments were performed in accordance with international guidelines and approved by the experimental animal committee of each Center.

Mice received an intraplantar injection in the hind paw of 20 µl λ-carrageenan (2% w/v; high dose; Sigma-Aldrich, St. Louis, MO, USA) or 5 µl λ-carrageenan (1% w/v; low dose; Sigma-Aldrich) diluted in saline. Heat withdrawal latency times were determined using the Hargreaves test (IITC Life Science, Woodland Hills, CA) as described [34]. Paw Thickness was measured using a Digimatic Micrometer (Mitutoyo, Veenendaal, the Netherlands). Different concentrations of FGA-19 were applied intrathecally (5 µl/mouse) while the animals were under light isoflurane anesthesia. All behavioral experiments were performed by an experimenter blinded to treatment and mice were assigned to the various treatment groups in a randomized fashion.

Fluoro-Jade B staining

Two days after intrathecal FGA-19 or vehicle mice were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and perfused intracardially with 4% paraformaldehyde in PBS.

Spinal cords were removed and paraffin-embedded. Spinal cord sections were deparaffinized, pretreated with 0.06% potassium permanganate and stained with 0.001% Fluoro-Jade B solution (Millipore Bioscience Research Reagents, Hampshire, UK) in 0.1% acetic acid to visualize damaged neurons.

TNF α determination in tissue lysates

Spinal cords were homogenized in PBS and centrifuged at 13,000g for 15 min at 4°C. TNF α content in the supernatant was quantified by ELISA (R&D systems, San Diego, CA, USA).

Electrophoresis and Western blotting

Proteins were resolved by SDS-PAGE, and transferred to nitrocellulose membranes for Western blotting analysis. The antibodies used were as follows: anti-p-p38 (T180/Y182 #9215), anti-p38 (#9212), anti-p-MK2 (T334, #3041), anti-MK2 (#3042), anti-p-HSP27 (#2405), anti-MEF2A (#9736), anti-p-ERK1/2 (#9101), anti-SAPK/JNK (#9252) and anti-p-SAPK/JNK (T183/Y185) (#9251) antibodies were purchased from Cell Signaling. Anti-p-MEF2A (T312, #ab30644) was obtained from Abcam. Anti-ERK1 (C-16, sc-93) and anti ERK2 (C-14, sc-154) were mixed for blotting and purchased from Santa Cruz Biotechnology,

***In vitro* inhibition of p38 MAPK activation and activity by FGA-19**

Purified p38 α (75 nM) was pre-incubated for 15 minutes at 30°C with FGA-19 at concentrations between 0.5 and 50 μ M in buffer (25 mM Tris pH 7.5, 50 μ M EGTA, 50 μ M NaVO₄ and 0.05% β -ME). Constitutively active MKK6 (from Upstate, 10 nM), magnesium acetate (2.5 mM) and ATP (25 μ M) were added and a kinase reaction was performed for 15 minutes at 30°C. The reaction was terminated by adding Laemmli buffer and proteins were resolved by SDS-PAGE to detect phospho-p38 (pTGY) by Western blot analysis. *In vitro* activity assays were performed using recombinant activated p38 α (from ProQinase, 1 nM), which was pre-incubated for 15 minutes at 30°C with the small molecule in the same buffer. Full length GST-MEF2A (purified in our laboratory, 10 nM), magnesium acetate and ATP were added and the kinase reaction was allowed to proceed. Phospho-MEF2A was detected by Western blot analysis.

Pull down assay

To examine the interaction between p38 and its substrate MK2 in the presence or absence of FGA-19, full length mouse p38 α -GST and full length MK2-His were produced in bacteria and purified using the ProfiniaTM Purification Protein System (Biorad) according to manufacturer protocols. 150 nM p38-GST was pre-incubated with 25 μ M FGA-19 or vehicle (DMSO 0.05%) in binding buffer (25 mM Tris-HCl pH 7.5, 0.25 M NaCl, 10 mM MgCl₂ and 5 mM NaF) at 30°C for 30 minutes. Then 150nM MK2-His was added for 15 minutes and the reaction was incubated with glutathione-Sepharose 4B (GE Healthcare) at 30 °C for 15 min. Beads were washed twice with washing buffer (25 mM Tris-HCl pH 7.5, 0.25 M NaCl, 10 mM MgCl₂, 5 mM NaF, 0.5% Triton X-100). GST-p38-bound proteins were analyzed by SDS-PAGE and Western blot analysis.

Inhibition of TNF α secretion by FGA-19 and the structurally similar compounds FGA-23 and FGA-29

THP-1 cells, growing in log phase, were re-suspended in RPMI-1640 to a final concentration of 2×10^6 cells/ml and distributed in 24-well plates. FGA molecules were added to the wells to a final concentration ranging from 10 nM to 100 μ M and plates were incubated at 37°C and 5% CO₂ in humidified atmosphere. Cells were stimulated 1h later with LPS to a final concentration of 0.5–2 μ g/ml and incubated for 3h followed by centrifugation to pellet the cells. Supernatants were collected and stored at –20°C for further analysis. TNF α secretion was measured by ELISA (GE-Healthcare) following manufacturer's indications.

Cell culture and viability analysis

THP1 human monocytic cells were maintained in suspension in RPMI media containing 10% fetal calf serum, pyruvate 1 mM, glutamine 2mM and antibiotics. The potential in vitro toxicity of FGA-19 was quantified using Propidium Iodide (PI), which specifically labels dying cells. Cells were resuspended in buffer (PBS, 1% BSA, 1% FBS, 0.01% NaN₃ and 1 μ g/ml PI) and kept on ice for 30–60 minutes and then analysed by flow cytometry using a FACSCalibur (Becton Dickinson). Data were analyzed with Cell Quest Pro Software (Becton Dickinson).

Statistical analysis

All data are presented as mean $\pm$ SEM. Measurements were compared using Student's T-tests or one-way ANOVA followed up by post-hoc Tukey or two-way ANOVA with Bonferroni post-hoc tests using Prism 5 software.

Results

Virtual screening and identification of a lead compound

The virtual screening (VS) protocol employed here is summarized in figures 1A and 1B and briefly described in the methods section. An essential part of the procedure comprises characterization of the shape of the active site (the docking groove of p38 MAP kinase). For this purpose we used the space occupied by the MEF-2A peptide co-crystallized with p38 MAPK and the GAGA algorithm to obtain a negative image of the binding site. Although different X-ray structures were available to be used as the receptor for the VS, we selected the one contained in the PDB ID 1LEW because of its high resolution, in particular in the area where the docking was to be performed. Moreover, superimposition of several structures (PDB IDs 1LEW, 3P4K, 1P38, 2FSL, 2FSM, 2FSO and 2FSZ) showed very low RMSD values considering equivalent Ca carbon atoms, supporting the use of a single, rigid receptor structure in the docking process.

Upon characterizing the binding site, a library of around 1 million compounds was first screened using DOCK and the negative image of the binding site. After applying a Z-Score cut off value of 2.5 on the DOCK ranked list, 45,488 molecules passed onto the next step. These molecules were then re-docked with CDOCK and scored with its molecular mechanics energy function, which explicitly included solvent and hydrogen bonding terms.

The 200 highest scoring compounds were submitted to MD simulation in explicit solvent and their binding energies estimated and pair-wise decomposed by MM-GBSA calculation over a large collection of snapshots homogeneously sampled along the trajectories.

Finally, from the top scoring compounds, and upon visual examination, 18 candidates were selected, purchased, and tested experimentally. The most promising candidate, the highly symmetrical compound 978604, was not available from the vendor. In addition, and according to Lipinski's rule of five (RoF) [35], this compound presented a very high logP value [36] (~8.00). Therefore, a similarity search was performed resulting in a new compound, 5380, that was found to be structurally equivalent to one half of the original one due to its symmetry. On the one hand, 5380 due to its reduced size is better suited for chemical modifications; on the other hand its logP value was drastically reduced being within the RoF (5.00) criteria. This compound was purchased, experimentally tested and submitted to two optimization rounds. From the first optimization round a lead candidate was obtained, FGA-19, with enhanced pharmacological profile and logP value (4.35). Upon studying the binding mode of FGA-19, a second optimization cycle led to the discovery of a second lead compound FGA-29 in which the methyl group of FGA-19 was replaced by a hydroxyl moiety, thus further reducing its logP value (3.68).

Due to their structural resemblance FGA-19 and FGA-29 display a common interaction pattern with residues I116, L122 and L130, or H126, P129, V158, and C161. In addition, the interaction between residue Q120 and FGA-29 (not present in FGA-19) is of the same magnitude as the interaction between residue Q133 and FGA-19 (not present in FGA-29) counterbalancing each other. Finally, there are some subtle differences in their binding modes (Fig. 1D). In particular, the interactions with residues N159 and E160, which are geometrically optimized in FGA-29 as compared to FGA-19, are mainly hydrophobic in nature and account for a total energy difference of about $2 \text{ kcal} \cdot \text{mol}^{-1}$. However, this magnitude is within the error of these calculations, and therefore, no firm conclusions can be drawn regarding possible differences in their potential activities, which is in agreement with the experimental observations.

***In vitro* effects of FGA-19 on p38 MAPK activation and inhibition of the p38 MAPK route in human monocytic cells**

We next set out to investigate the possible effects of the FGA-19 compound on p38 MAPK in an *in vitro* kinase activity assay using purified p38 MAPK-dependent phosphorylation of a well-characterized docking-dependent p38 MAPK substrate: the MEF-2A protein. When adding increasing doses of the FGA-19 molecule to the reaction mix containing the active kinase and a saturating amount of substrate in the presence of ATP, we observed a dose-dependent inhibition of p38 MAPK-mediated phosphorylation of MEF-2A at the specific site targeted by this kinase (T312) (Figure 2A and 2B). Densitometric analysis of the Western blot data of MEF-2A phosphorylation in the presence of increasing amounts of FGA-19 in several experiments revealed an estimated mean inhibitory concentration (IC₅₀) of $6.31 \pm 2.32 \text{ } \mu\text{M}$. An almost complete inhibition of p38 kinase activity towards MEF-2A was observed at the 50 μM dose (Fig. 2A and 2B).

Next we analyzed the effects of the FGA-19 compound on the p38 MAPK signaling route in the human monocytic cell line THP-1. As can be seen in Figure 2C–D, in the presence of FGA-19 we detected clear inhibition of p38 MAPK phosphorylation at the activation loop by upstream kinases. FGA-19 also inhibited the activity of p38 MAPK in THP-1 cells as measured by a decrease in the phosphorylation of the downstream substrates MK-2 and Hsp-27. Quantification of these inhibitory effects of FGA-19 revealed a α 80% inhibition for phosphorylation of p38 MAPK and α 100% inhibition for MK2 phosphorylation at the 5 μ M dose of FGA-19 (Fig. 2C–D). Interestingly, no feedback activation effects were detected at the 1 μ M dose for the JNK or ERK1/2 pathways (Fig 2D, lower panel), and, in fact, both kinases appear to be inhibited to a certain extent by FGA-19 at the highest dose tested (5 μ M), although with a much lower efficacy compared to the effect observed on the p38 MAPK pathway

Interestingly, as shown in figure 2E, FGA-19 was capable of impairing the *in vitro* interaction between p38 and the docking-dependent substrate MK2, which was almost absent from p38 sediments in the presence of FGA-19 while it was clearly detected in the vehicle (DMSO) control samples. These results suggest that competitive inhibition towards docking-dependent partners is a likely mechanism for the actions of FGA-19 in agreement with the kinetic constants of the phosphorylation of MEF2 by constitutively active p38 in the presence or absence of FGA-19 (Supplementary Table 1)

A broad specificity analysis was performed using a commercially available kinase screen (MRC-PPU Express Screen, International Centre for Kinase profiling, University of Dundee). As can be seen in Supplementary Table 2, FGA-19 at a dose of 10 μ M has little or no effect on a variety of kinases representative of the different groups comprised in the human kinome. In agreement with its docking-groove targeting design, FGA-19 had no inhibitory activity on p38 α in this screening, since this assay is performed using myelin basic protein as a substrate, a protein that apparently lacks a functional docking site [37].

The effects of FGA-19 were then analyzed in the well established cellular system of cytokine secretion by THP-1 cells stimulated with LPS [4]. Secretion of TNF α by these cells is p38 MAPK-dependent, and thus this system has been broadly utilized to study the effects of different p38 MAPK inhibitors. As shown in figure 2C, FGA-19 has a dose-dependent inhibitory effect on TNF α secretion in this cell system with an IC₅₀ of 1.8 ± 0.006 μ M reaching an almost maximal inhibition at the 10 μ M concentration. For comparison, the IC₅₀ for SB203580 (commercially available p38 MAPK inhibitor targeting the ATP binding site) in the same system was 1 μ M (data not shown), and the effect of SB203580 at the 10 μ M dose was similar to that obtained for FGA-19. Of note, we did not detect significant cell death as determined by FACS analysis of propidium iodide staining at concentrations up to 100 μ M FGA-19, indicating that there was no cellular toxicity (Fig. 3A).

Identification of the structural determinants of the inhibitory effect

Comparative analysis of FGA-19 and several structurally-related compounds, all belonging to the benzooxadiazol family, allowed us to conclude that the substituents in positions 2 and 5 of the phenyl ring are essential for the inhibitory activity towards p38 MAPK. When the

substituents in positions 2 and 5 present in the FGA-19 molecule (a chloride and a methyl group respectively) are located in other positions of the phenyl ring (as so happens in the FGA-17, FGA-20 and FGA-23 compounds), the IC₅₀ for inhibition of monocyte TNF α secretion decreased by at least one logarithmic unit (Figure 3B and C and Table 1). This was the case for Cl and CH₃ located in positions 4 and 5 (FGA-17), or 4 and 6 (FGA-20), with the IC₅₀ shifting from 1.8 μ M to 17 μ M and 28 μ M respectively, or for OH and CH₃ at positions 2 and 4 (FGA-23, IC₅₀ higher than 100 μ M). The presence of a single substituent at different positions of the ring also resulted in IC₅₀ higher than 100 μ M in at least three other compounds (data not shown). On the other hand, when other substituents are placed in the same positions of the ring where the chloride and the methyl group are located in the FGA-19 molecule, an IC₅₀ similar to that obtained for FGA-19 is detected. This is the case for the substitution of positions 2 and 5 by OH and Cl (in FGA-29), by Cl and OCH₃ (in FGA-34) and by F (FGA-32). These three compounds yielded IC₅₀s in the low micromolar range (3.5 μ M, 3.2 μ M and 2.8 μ M, respectively), which is comparable to the IC₅₀ of FGA-19 (1.8 μ M) (Table 1). Altogether, these results establish that a substituent at positions 2 and 5 in the phenyl ring is essential for the described inhibitory activity and, furthermore, that a chloride at position 5 and a methyl group at position 2 are the most effective chemical groups among those tested so far.

***In vivo* effect of FGA-19 on inflammatory hyperalgesia**

To determine the *in vivo* efficacy of FGA-19, we examined its effect in the high dose carrageenan model of persistent inflammatory hyperalgesia in C57BL/6 mice. In response to intraplantar injection of 20 μ l 2% carrageenan, mice develop thermal hyperalgesia that lasts at least 11 days (Fig. 4A). We injected increasing doses of FGA-19 intrathecally at day 6 after intraplantar carrageenan. The data show that hyperalgesia completely resolved in response to a single injection of 1 μ g FGA-19. A lower dose of 0.5 μ g FGA-19 transiently inhibited carrageenan-induced hyperalgesia (Figure 4A). For comparison, we used the established p38 MAPK inhibitor SB239063. At the maximal dose that could be injected using 20 % DMSO as a solvent, *i.e.* 5 μ g SB239063, carrageenan-induced hyperalgesia was only transiently attenuated (Fig. 4A). Thermal sensitivity was not affected by FGA-19 in control saline-treated mice (Fig. 4B). The structurally similar FGA-23 compound, that *in vitro* has an IC₅₀ of > 100 μ M, did not have any effect on the course of carrageenan-induced hyperalgesia (Figure 4B). To determine whether the inhibition of hyperalgesia by FGA-19 was associated with cellular damage in the spinal cord, we analyzed the effect of FGA-19 on staining with Fluor-Jade, a widely used marker of neurodegeneration. We did not observe an increase in Fluoro-Jade positive cells in the spinal cord at 48 hrs after injection of 1 μ g FGA-19, indicating that there was no effect of this dose of FGA-19 on neuronal cell death (Fig. 4C). Our cell culture experiments showed that FGA-19 inhibits LPS-induced TNF α production (Fig. 2C) and spinal cord production of pro-inflammatory cytokines such as TNF α is known to contribute to the persistent hyperalgesia in the carrageenan model of inflammatory pain [38–39]. To investigate whether *in vivo* FGA-19 treatment affects TNF α levels, we injected carrageenan or saline intraplantarly, followed by intrathecal FGA-19 or vehicle on day 6, and isolated lumbar spinal cord 2 days later for analysis of TNF α levels by ELISA. As anticipated, intraplantar carrageenan causes a significant increase in spinal cord TNF α (Fig. 4D). Intrathecal FGA-19 treatment significantly inhibited this carrageenan-

induced rise in spinal cord TNF α levels. FGA-19 alone does not have any effect on TNF α production in the spinal cord (Fig. 4D). Six days after intrathecal FGA-19 treatment mice still show increased paw thickness and redness, indicating that intrathecal FGA-19 treatment did not directly affect peripheral inflammatory activity (Fig. 4E).

Since p38 MAPK can be phosphorylated by G protein-coupled kinase 2 (GRK2) at the docking groove [11], it was tempting to hypothesize that cells with decreased levels of GRK2 and lower amounts of docking site-phosphorylated p38 protein would display differential susceptibility to inhibition via docking-targeted compounds. For instance, inflammatory cells from patients of autoimmune diseases or murine models of these diseases contain decreased levels of GRK2 [40], and this is recapitulated in hemizygous GRK2 macrophages [11]. We thus set out to investigate whether TNF α secretion by macrophages isolated from GRK2 $^{+/-}$ mice was differentially inhibited by FGA-19 when compared to macrophages from WT mice. Interestingly macrophages collected from GRK2 $^{+/-}$ mice are more sensitive to FGA-19-dependent blockade of TNF α secretion than WT macrophages (Figure 5A).

The next question we addressed is whether FGA-19 treatment also reverts the persistent hyperalgesia that develops in mice with a targeted deletion of GRK2 in lysozyme M-positive macrophages/monocytes. We have recently described that LysM-GRK2 $^{+/-}$ mice develop persistent hyperalgesia lasting at least 20 days in response to an intraplantar injection of a low dose of carrageenan (5 μ l of a 1% solution). In this model of transient hyperalgesia, the pain response in WT mice resolves within 2 days [38]. The LysM-GRK2 $^{+/-}$ mice can be considered as a model of persistent post-inflammatory hyperalgesia as we do not observe ongoing peripheral inflammation (no redness or thick paws, no detectable increase in inflammatory cytokines in comparison to control paws [32]). As depicted in Figure 5B, intrathecal administration of FGA-19 also completely attenuates hyperalgesia in this model of post-inflammatory hyperalgesia. The lowest effective dose providing a long lasting analgesic effect was 0.5 μ g of FGA-19, which is lower than that the 1 μ g dose found in the high dose carrageenan model in WT mice (Figure 4A).

Discussion

The search for clinically applicable inhibitors of p38 MAPK has rendered, so far, no fruitful therapeutic results. The reasons underlying this lack of success include low efficacy in some cases and the identification of certain toxicities during clinical trials that were not uncovered in surrogate animal models of the different diseases tested [41]. These toxicities can have several etiologies. On the one hand, first and second generation p38 MAPK inhibitors were designed to target the ATP binding pocket of p38 MAPK, thus exploiting a common domain found in many different kinases, likely triggering off-target effects [1]. On the other hand, even when new allosteric site inhibitors were developed to overcome these problems, it became apparent that a strong downregulation of the p38 MAPK pathway can trigger the upregulation of other signaling pathways by virtue of the concomitant stimulation of interconnecting feedback mechanisms (see below). These final undesired consequences were apparently underestimated [3]. Lastly, the systemic administration of p38 MAPK inhibitors has been proven to induce toxicities in organs other than those important for the

disease they were meant to combat, including liver and brain [41]. For all these reasons, in this study we set out to characterize a novel family of inhibitors that take advantage of the existence of docking domains in the p38 MAPK protein providing the possibility of modulating the activity and activation of this kinase independently of its intrinsic catalytic activity in a more subtle but possibly more efficacious manner. Our strategy relied on our previous results showing that p38 MAPK can be phosphorylated at the docking domain, specifically at T123 of murine p38 α . This phosphorylation of T123 causes the loss of interaction with different p38 binding partners and reduced activation and activity of p38 MAPK [11]. On this basis, we performed a virtual screening of a chemical library of compounds over a model based on pre-existing structures of p38 MAPK bound to peptides derived from upstream regulators or downstream substrates [10]. This search yielded the identification of compounds of different chemical nature that were analyzed for their *in vitro* inhibitory capacity towards p38 activity or towards cytokine secretion induced by LPS in a human monocytic cell line. Only some molecules were inhibiting these events with IC₅₀s in the micromolar range. Out of these, the family of benzoxadiazol-based compounds described in this study, with FGA-19 as a lead candidate, was found to have a biological effect both in cells and in two animal models of hyperalgesia. The fact that FGA-19 contains several putative sites susceptible for chemical modifications highlights this molecule as a good lead compound for further drug development strategies.

In vitro experiments support the notion that the biochemical mechanism of action of FGA-19 involves its ability to interfere with binding of docking-dependent substrates to p38, as indicated by kinetic competition experiments and the inhibition of pull-down of purified MK2 by p38MAPK. Accordingly, FGA-19 does not efficiently inhibit the activity of p38 towards myelin basic protein (MBP), a substrate that does not rely on a functional docking site for its phosphorylation by members of the p38 kinase family [37]. Moreover, 10 μ M FGA-19 had little or no effect on the activity of a variety of kinases representative of the human kinome, supporting the notion that by targeting the docking groove of p38, FGA-19 displays less cross-inhibition with other kinases and thus increased specificity. However, the possibility exists that FGA-19 might interfere with the phosphorylation of specific docking-dependent substrates of other members of the p38 MAPK family, or of the related MAPK family members JNK1 or ERK1/2, and this will be subject of future research.

The strategy utilized to identify FGA-19 aimed at interfering with docking interactions between p38 MAPK and substrates or activators of p38 MAPK [11]. This particular characteristic was utilized as it could in principle yield a different mode of action leading to a less abrupt blockade of the pathway flow and thus, at least theoretically, less toxic effects. For instance, one of the off-target effects described for classical p38 MAPK inhibitors is the hyperactivation of the JNK cascade. This is thought to happen because strong inhibition of p38 MAPK can abrogate some negative feedback loops that p38 kinase maintains towards upstream kinases such as MLK2/MLK3 or TAK1 which also function upstream of JNK [42]. Thus, abolition of the negative feedback control exerted by the p38 MAPK branch redirects the pathway flow towards a higher activation of the JNK pathway. However, we did not detect increased activation of JNK in cells in the presence of FGA-19. If anything, the JNK pathway was inhibited, but to a lesser extent than the p38 MAPK route by FGA-19.

These findings suggest that this type of unwanted effects may not occur for docking-based inhibitors or for inhibitors that do not fully ablate p38 MAPK catalytic activity. The possibility that the more refined, less potent inhibition of p38 MAPK such as that obtained by FGA-19 can prevent other undesired side effects such as activation of cell death routes certainly deserves further investigation.

The *in vivo* effects of FGA-19 surpass those detected for other well established p38 MAPK inhibitors in our mouse models of inflammatory pain both in potency and in persistence of the pharmacological effect. Of note, the FGA-19 molecule is almost ten times as potent as the SB239063 inhibitor, since a 0.5 µg dose of FGA-19 exerts the same transient analgesic effect as 5 µg of SB239063 while both molecules have similar molecular weights (368.40 g/mol for SB239063 and 304.45 g/mol for FGA-19). Moreover, longer-lasting effects are detected in mice treated intrathecally with FGA-19 at the 1 µg dose, which abrogates inflammatory hyperalgesia for at least 5 days post-injection. For comparison, the pharmacological effect of SB239063 at the highest injectable dose did not last longer than 6 hours. These results suggest that a very promising family of molecules could be derived from the benzoxadiazol structure to treat chronic inflammatory pain. Interestingly, recent studies have shown that mice with a reduced level of GRK2 in macrophages develop chronic hyperalgesia in response to inflammatory mediators that induce only transient hyperalgesia in WT mice (reviewed in [43]). We therefore used these mice to test whether FGA-19 also inhibits the hyperalgesia in this model of post-inflammatory pain. We show that the persistent hyperalgesia that develops in LysM-GRK2^{+/-} mice is completely reversed by a single intrathecal injection of FGA-19. Moreover, macrophages obtained from GRK2^{+/-} – mice are more sensitive to FGA-19-dependent blockade of TNFα secretion. These findings are particularly interesting because decreased levels of GRK2 are found in cells from patients with various inflammatory and autoimmune diseases [34] and it is tempting to suggest that our docking-groove targeting inhibitors would be especially suited for treatment of inflammation and pain in these conditions. Collectively, our findings indicate that FGA-19 is a potential candidate for the treatment of inflammatory hyperalgesia.

Clinical trials performed for p38 MAPK in the context of chronic inflammation have been unsuccessful so far due to both lack of efficacy and toxic effects [41]. However, although the importance of p38 MAPK in regulating nociception is well established in mice and in humans [43], few studies have been conducted so far to assess the efficacy of p38 inhibition in pain therapeutics. In particular, different laboratories have shown in rodent models that phosphorylated p38 MAPK is increased in spinal cord microglia after nerve ligation or spinal cord injury which are two widely used models of chronic neuropathic pain. More importantly, intrathecal infusion of p38 MAPK inhibitors attenuates neuropathic pain in animal models (see references in [44]). A small study has described a potent analgesic effect of p38 MAPK inhibition for post-surgical dental pain in humans [45] and several recent studies have described the contribution of p38 MAPK in animal models of neuropathic post-surgical pain and cancer-pain [46]. It is also well established that increased levels of phosphorylated p38 MAPK are detected in spinal cord microglia in animal models of chronic inflammatory pain (see references in [43]). In accordance with these published studies, our own results indicate that inhibition of spinal cord p38 MAPK by intrathecal

administration of specific inhibitors efficiently reverses ongoing hyperalgesia ([47] and this work). However, the effect observed using these classic p38 MAPK inhibitors was much more transient than that observed using the FGA-19 inhibitor, which at a 1 µg dose shows an effect that lasted all the way along the 5 days of duration of our experimental setup.

In conclusion, we present evidence that a novel family of small molecules targeting the docking groove of p38 MAPK, the benzooxadiazol-based compounds, are potent inhibitors of cytokine production in human monocytic cells. In addition, we show that a member of this family, the FGA-19 molecule, inhibits inflammatory and post-inflammatory pain in murine models. We suggest that this novel compound can represent the basis for further research on similar compounds for their specificity and potency in these models and, ultimately, for translation into a clinical setting.

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Figure 1. Virtual screening and identification of a lead compound

(A) Schematic representation of the computational protocol employed in this study (left and right panels) and the drug design cycle established with the experimental counterpart (central panel); (B) Structural pathway from the identified virtual hit 978604 to the hit candidate 5380 and to the lead compounds FGA-19 and FGA-29 after two rounds of optimization; (C) Chemical reaction for the preparation of FGA-19 starting from 4-chloro-7-nitrobenzofurazan and 5-chloro-2-methylaniline; (D) Proposed binding modes for FGA-19 and FGA-29. The receptor is represented in cartoons (white in FGA-19 complex and black in FGA-29 complex), whereas the ligands and main interacting residues (see text) are shown in sticks. Hydrogen atoms have been omitted for clarity. Residue T123 that is targeted by GRK2 is also indicated.

Figure 2. Effects of FGA-19 on the activity of p38 MAPK *in vitro* and on the p38-MK2-HSP27 signaling route

(A) Recombinant active p38 (1 nM) and MEF2A (10 nM) were incubated with different concentrations of FGA-19 in kinase buffer for 5 minutes. Proteins were resolved by SDS-PAGE and p38 MAPK activity was measured as phosphorylated MEF2A using p-MEF2A (T312) specific antibody. Average results are plotted as percent phosphorylation relative to controls (DMSO) of two independent experiments performed in duplicate. (B) Autoradiogram of a representative experiment. (C–D) THP-1 cells were preincubated with indicated doses of FGA-19 for 1 hour and stimulated with LPS for 30 or 60 minutes. Cell lysates were resolved by SDS-PAGE and the activation status of the p38MAPK signaling route and the JNK and ERK1/2 pathways was

detected with specific antibodies and films quantified by densitometric analysis. Average results of two independent experiments with duplicates are plotted in C as percent phosphorylation relative to cells with vehicle at the 60min time point.

Autoradiograms of a representative experiment are shown. (E) Effect of FGA-19 on p38 interaction with its substrate MK2. p38-GST (150 nM) was preincubated with 25 μM FGA-19 or DMSO. Then 150 nM His-MK2 and Glutathione-sepharose beads were added. Bound fusion proteins were detected by Western Blot using specific antibodies. Data represent mean ± SEM. N=3.

*P<0.05

Figure 3. Inhibition of TNF α secretion by FGA-19, FGA-23 and FGA-29

(A) THP-1 cells were preincubated with the indicated doses of FGA-19 for 1 hour and stimulated with LPS for 3 hours. Cells were stained with Propidium Iodide and surviving cells were counted with a FACS Calibur cytometer. TNF α secretion in cell supernatants was quantified using a TNF α ELISA kit and referred to control (DMSO). Results are average of 3 independent experiments each in duplicate. (B) THP-1 cells growing in log phase were pre-incubated for 1 hour with indicated doses of the different compounds or with 10 μ M SB203580 (SB) as a control, and then stimulated with LPS for 3 additional hours to analyze supernatants for TNF α secretion by ELISA. Results are the mean of 2 independent experiments performed in duplicate and are referred to controls with DMSO (vehicle). (C) FGA-19 and structurally-related compounds.

Figure 4. Effect of FGA-19 treatment on high-dose carrageenan-induced persistent hyperalgesia in C57BL/6 mice

Mice received an intraplantar injection of 20 µl 2% λ-carrageenan or saline. At day 6, carrageenan-treated mice were still hyperalgesic. At this time point, mice received an intrathecal injection of (A) different doses of FGA-19 or (B) 1 µg of the inactive compound FGA-23, and the percentage change in heat withdrawal latency was determined (n = 4 to 8 per group). Two days after intrathecal injection, lumbar spinal cord was collected and (C) sections were stained for Fluoro-Jade B to investigate the potential neurotoxicity of FGA-19 and (D) TNFα levels were determined by ELISA (n = 4). (E) As a measure for paw-inflammation, the effect of FGA-19 treatment on the paw thickness was determined (n = 8). Data are expressed as means ± SEM. * or # P<0.05, *** or ### P<0.001 (Figure A: * for 0.5 µg FGA-19 versus vehicle; # for 5 µg SB239063 versus vehicle).

Figure 5. Effect of FGA-19 treatment in GRK2^{+/-} macrophages and on low dose carrageenan-induced persistent hyperalgesia in LysM-GRK2^{+/-} mice

A) GRK2^{+/-} peritoneal macrophages were stimulated with LPS in the presence of increasing FGA-19 doses and TNFα secretion was measured as described above. The inhibition observed in GRK2^{+/-} macrophages was larger than that obtained for WT cells along all FGA-19 doses. Results from two independent experiments performed in triplicate are shown. *, p<0.01; ***, p<0.001 (T-test); p<0.0001 (ANOVA). B) LysM-GRK2^{+/-} mice received a low dose of carrageenan (5 μl of 1% α-carrageenan) and at day 6 mice were still hyperalgesic. At this time point, the mice received an intrathecal injection with different doses of FGA-19 and the percentage change in heat withdrawal latency was determined (n = 4). Data are expressed as means ± SEM

Table 1
Comparative analysis of the effects on TNF α secretion of FGA-19 and several structurally-related compounds belonging to the benzooxadiazol family

THP-1 cells were preincubated with indicated doses of several benzooxadiazol-derived compounds for 1 hour and stimulated with LPS for 3 hours. TNF- α secretion in cell supernatants was quantified using a TNF α ELISA kit and referred to control (DMSO). Values were used to approximate graphically an IC₅₀ value in μ M units. When the substituents in positions 2 and 5 present in the FGA-19 molecule (namely a chloride and a methyl group) are located in other positions of the phenyl ring, the IC₅₀ for the inhibition of TNF α secretion by monocyctic cells decreases.

	FGA-17	FGA-32	FGA-20	FGA-23
2D molecular structure
^a IC ₅₀	17	2.8	28	>100
	FGA-19	FGA-29	FGA-34	
2D molecular structure
^a IC ₅₀	1.8	3.5	3.2	

^a Units in μ M; TNF α secretion in LPS-stimulated THP1 cells.