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Short communication

Effect of PPADS on achondroplasic chondrocytes: Inhibition of FGF receptor type 3 over-activity

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Abstract

Achondroplasia, results from a mutation in the FGF receptor type 3, leading to receptor hyperactivation and subsequent amplification of FGF receptor type 3 signals. We have tested the ability of pyridoxal-5'-phosphate-6-azophenyl-2', 4'-disulfonate (PPADS) to decrease the overactivation and signalling of FGF receptor type 3 in achondroplasic chondrocytes. PPADS reduced the tyrosine phosphorylation of FGF receptor type 3 triggered by fibroblast growth factor 9 (FGF9) (50% reduction), as well as the activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) pathway. As a consequence of this inhibitory effect on ERK1/2 activity the loss of extracellular matrix was also reversed by PPADS. The action of PPADS seems to be due to a mechanism independent of P2 receptor antagonism.

Keywords: Achondroplasia; Chondrocytes; FGF receptor type 3; ERK1/2; PPADS

1. Introduction

Achondroplasia is one of the most representative types of congenital skeletal dysplasias. In this pathology the mutant receptor (FGF receptor type 3^{G380R}), shows a gain of function, yielding an alteration of the normal equilibrium between proliferation and maturation inhibiting the normal growth of the bone (Naski et al., 1996).

In the presence of heparan sulfate proteoglycans, specific fibroblast growth factors (FGFs) bind to FGF receptor type 3, which induces receptor dimerization and subsequent autophosphorylation at the intracellular kinase domain. Autophosphorylated tyrosine residues provide binding sites for different signalling proteins which initiate downstream cascades (signal transducer and activators of transcription pathway, extracellular signal-regulated kinases 1 and 2 cascade, phosphoinositide-3 kinase pathway), leading to a cellular response (Eswarakumar et al., 2005). In the case of ERK1/2, this pathway seems to inhibit proliferation (Raucci et al., 2004) involved as well in chondrocyte differentiation process (Murakami et al., 2004). In addition, ERK1/2 pathway reduces the synthesis of the extracellular matrix components (Yasoda et al., 2004).

Achondroplasia is an orphan pathology with no pharmacological treatment so far. Strategies for the treatment of this disease are focused on limiting the FGF receptor type 3 activity. A possible approach to achieve the down-regulation of mutated FGF receptor type 3 activity is the inhibition of extracellular receptor activation disrupting the direct interaction of fibroblast growth factor with the receptor or alternatively, interfering with the heparan sulphate-FGF-FGF receptor interaction. The compounds, depicting the second way of action, are mainly polyanionic molecules, such as suramin and suradistas (Botta et al., 2000), pentosan polysulfate (Zugmaier et al., 1999) and phosphorothioate oligodeoxynucleotides (Guvakova et al., 1995). We developed the hypothesis that pyridoxal-5'-phosphate-6-

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azophenyl-2',4'-disulfonate (PPADS), being a polyanionic compound with a sulfonated-derivatized aromatic ring, might also prevent the activation of achondroplasic FGF receptor type 3. The role of PPADS as a non-selective P2 receptor antagonist is well-known (Lambrecht et al., 2002). P2 receptors are stimulated by nucleotides and two major families have been described: a P2X family of ligand-gated ion channel receptors and a P2Y family of G protein-coupled receptors. To characterize the different P2X and P2Y subtypes according to pharmacological response, a great number of compounds, among them, PPADS, have been used to block P2 receptor activity. Apart from this role, a mechanism of action independent of P2 receptors have also been suggested for PPADS (Shehnaz et al., 2000; Vigne et al., 1998).

The aim of the present experimental work is to investigate whether or not PPADS can modify the prolonged signalling triggered by over-activated mutant FGF receptor type 3, therefore becoming a potential therapeutic compound which possibly could be used for the treatment of achondroplasia.

2. Materials and methods

2.1. Reagents and antibodies

Tetracycline, α-MEM, heat-inactivated foetal bovine serum and antibiotics were supplied by Invitrogen (Carlsbad, CA, USA).

The P2Y receptor antagonists: reactive blue 2 (Rb2), 2′-Deoxy-N^6-methyladenosine-3′,5′-bisphosphate (MRS2179), P1, P5-di[inosine-5'] pentaphosphate (Ip_5I) and FGF9 were purchased from Sigma (St. Louis, MO, USA). Tris, sodium chloride (NaCl), Triton X-100, phenylmethylsulfonyl fluoride (PMSF), sodium fluoride (NaF), sodium orthovanadate (Na_3VO_4), sodium docecyl sulphate (SDS), Tween 20, bovine serum albumin (BSA), p-formaldehyde, aprotinin, pepstatin, leupeptin were also obtained from Sigma.

Pyridoxal, pyridoxal phosphate, PPADS, pyridoxal-5'-phosphate-6-(2'-naphthylazo-6'-nitro-4',8'-disulfonate) (PPNDS) were supplied by Tocris (Bristol, United Kingdom).

Antibodies against phospho-ERK1/2, ERK2, FGF receptor type 3, and horseradish peroxidase-conjugated goat anti-mouse were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 4G10 antibody was obtained from Upstate (Lake Placid, NY, USA).

2.2. Cell lines and cell culture

RCJ3.1C5.18 cells, a chondrogenic precursor cell line derived from multipotential mesenchymal rat stem cells were transfected with full-length human mutant FGF receptor type 3, ACH FGF receptor type 3 (FGF receptor type 3^{G380R}), as described elsewhere (Monsonego-Ornan et al., 2000). Expression of FGF receptor type 3 was regulated by a tetracycline suppression system, the receptor is expressed in the absence of tetracycline in the culture medium.

Standard culture medium was α-MEM supplemented with 15% heat-inactivated foetal bovine serum and antibiotics. Cells were incubated at 37 °C with 5% CO_2.

Cell viability was determined by trypan blue staining. Viable (unstained) cells were counted using a hemocytometer. Experiments were performed in triplicate.

2.3. Signal transduction studies

In order to analyze tyrosine-phosphorylation of FGF receptor type 3 or phosphorylation status of ERK1/2, 2×10^4 cell/cm^2 cells were plated onto tissue culture dishes. Two days later the culture medium was replaced by fresh one without tetracycline and cells were incubated during for 16 h. Cells were serum starved for 4 h before treatment with FGF9 (25 ng/ml) alone or with other compounds for 10 min. When pyridoxal, pyridoxal phosphate, PPADS, PPNDS, or P2Y receptor antagonists (RB2, MRS2179, Ip_5I) were used cells were pre-treated for 30 min prior to FGF9 treatment. After treatment cells were lysed in buffer (50 mM Tris–HCl pH 7,4, 150 mM NaCl, nonidet P40 (NP-40) 1%, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM PMSF, 1 mM NaF, 1 mM Na_3VO_4, 1 μg/ml aprotinin, pepstatin and leupeptin). Lysates were clarified at 13,000 ×g for 20 min at 4 °C. Protein concentration was determined by the Bio-Rad protein assay (Bio-Rad, CA, USA).

2.4. Immunoprecipitation and western blot analysis

For immunoprecipitation, 1 mg of total protein was incubated for 16 h at 4 °C with 2 μg of anti-FGF receptor type 3 C-terminus antibody. Immunocomplexes were purified using A/G agarose (Santa Cruz Biotechnology, CA, USA) and analyzed by western blot with anti-phosphotyrosine 4G10 (1:1,000) (Upstate, Lake Placid, NY, USA).

To analyze phosphorylation of ERK1/2, lysates from each sample (45 μg) were subjected to 10% SDS-polyacrilamide gels and were transferred to nitrocellulose membranes. Subsequently membranes were blocked and incubated overnight with p-ERK1/2 (1:1,000). After washing, blots were incubated with peroxidase-conjugated secondary antibody. Development was performed using ECL system (Amersham, Buckinghamshire, UK).

To verify equal loading, membranes were stripped in 62.5 mM Tris–HCl pH 6.8, 2% SDS and 100 mM 2-mercaptoethanol and re-blotted with proper antibodies. Films were scanned and densitometric analysis was performed using Kodak GL 200 Imaging system and Kodak Molecular Imaging software (Kodak, Rochester, NY, USA). All data shown are results representative of three independent experiments.

2.5. Extracellular matrix studies

Cartilage matrix deposition in chondrocytes was quantified by Alcian blue staining. Cells were seeded at a density of 2×10^4 cells/well in six-well dishes. After reaching the confluence (day 4), the differentiation was induced by adding 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid to the medium. Differentiating cultures were fed with supplemented media every 3 days. FGF9 (25 ng/ml) alone and FGF9 together with PPADS (100 μM) were also added to the fresh growth medium. Cultures were monitored over a total period of 14 days. At

different time points, extracellular matrix was quantified by Alcian Blue staining of cell layers, as previously described (Lunstrum et al., 1999).

2.6. Statistical analysis

The differences between the mean values were analyzed with SigmaPlot v8.02 (SPSS, Chicago, IL, USA) and by using Student's *t*-test; statistical significance was considered to be achieved at a $P<0.05$ level.

3. Results

3.1. Effect of PPADS on tyrosine-phosphorylation of achondroplasic FGF receptor type 3 and ERK1/2 phosphorylation

The achondroplasic FGF receptor type 3 showed a slight constitutive phosphorylation even in absence of FGF9, the preferred ligand for FGF receptor type 3 (Fig. 1A, left panel). This basal activation may occur due to high expression levels of ACH FGF receptor type 3, which led to spontaneous

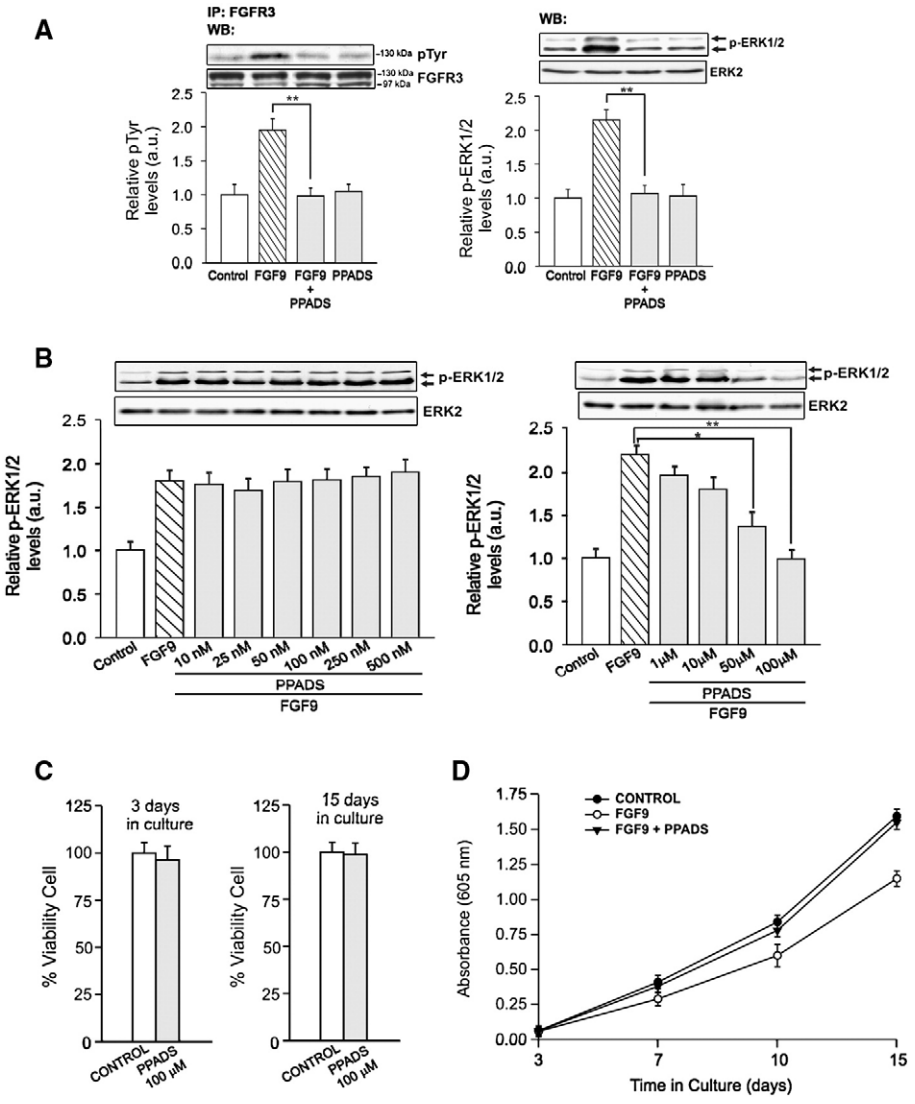

Fig. 1. Effect of PPADS on ACH FGF receptor type 3 activity and signalling. A. Chondrocytes precursors transfected with ACH FGF receptor type 3 were stimulated with FGF9 (25 ng/ml), FGF9 together with PPADS (100 μM) or PPADS alone for 10 min. FGF receptor type 3 was immunoprecipitated by anti-FGF receptor type 3 and analyzed for tyrosine phosphorylation by western blot with the 4G10 antibody (left panel). The levels of total FGF receptor type 3 detected by western blot serves as loading control for immunoprecipitation. Two FGF receptor type 3 isoforms were detected, the upper band is the mature, membrane-associated glycoprotein (130 kDa) and the lower band is the immature form (97 kDa), only the fully glycosylated isoform was phosphorylated. On the other hand, cell lysates were successively immunoblotted with antiphospho-ERK 1/2 and anti-ERK2 antibody to verify equal loading (right panel). B. Chondrocytes precursors expressing ACH FGF receptor type 3 were treated with FGF9 (25 ng/ml) or FGF9 together with different concentrations of PPADS (ranging from 10 nM to 100 μM) for 10 min. ERK1/2 phosphorylation levels were analyzed by western blot as in A. The histograms represent the levels of tyrosine phosphorylated FGF receptor type 3 (A) or phosphorylated ERK1/2 (A, right panel and B). Data (mean ± SD) are represented in arbitrary units (a.u.) and normalized to the intensity of the band corresponding control. *$P<0.05$, **$P<0.01$. C. Viable chondrocytes precursors were determined by trypan blue exclusion assay. The number of viable cells in the control (without PPADS) was normalized to 100% and was used as the reference for cells treated with PPADS. D. Chondrocytes precursors transfected with ACH FGF receptor type 3 were treated as described in Materials and methods. At the indicated times, extracellular matrix was quantified by Alcian Blue staining of cell layers, following solubilization, dye uptake was quantified at 605 nm for triplicate samples.

dimerization and tyrosine phosphorylation. However, significant levels of tyrosine phosphorylation were discovered only after FGF9 exposure. Since the tyrosine phosphorylation of the receptor enhanced substantially after FGF9 addition, the activation of ACH FGF receptor type 3 cannot be considered as ligand-independent. When PPADS (100 μM) together with FGF9 was added, a significant decrease on tyrosine phosphorylation was detected (50% compared to FGF9 alone). The application of PPADS alone did not modify the degree of tyrosine phosphorylation, when it was compared to the control.

The effect of receptor activation on downstream signalling pathways was also examined. We focused on ERK1/2 pathway, since its important involvement in patho-physiological events occurring in achondroplasia has already been demonstrated (Yasoda et al., 2004). A two-fold increase in ERK1/2 phosphorylation was observed, after the application of FGF9, when it was compared to the control (Fig. 1A, right panel). Consistent with the decrease observed on FGF9-mediated tyrosine phosphorylation of ACH FGF receptor type 3, PPADS treatment also reduced the level of ERK1/2 phosphorylation induced by FGF9 (50% compared to FGF9 alone). In relation to the treatment with PPADS alone, as in the case of ACH FGF receptor type 3 tyrosine phosphorylation, the compound did not produce a significant variation on ERK1/2 phosphorylation.

3.2. Dose–response studies with PPADS

After determining the PPADS ability, in counteracting ERK1/2 phosphorylation triggered by FGF9 in achondroplasic chondrocytes precursors, we calculated the minimum concentration of PPADS required to elicit its effect on ERK1/2 activity. As shown in Fig. 1B PPADS was assayed over a wide range of concentrations. PPADS used in nanomolar concentrations (10–500 nM) was completely ineffective (Fig. 1B, left panel). When low micromolar concentrations (1–10 μM) were assayed, it was detected a slight decrease on ERK1/2 phosphorylation, compared to sole application of FGF9 (Fig. 1B, right panel). Only at high micromolar concentration (50–100 μM) a marked reduction of ERK1/2 phosphorylation was found. In particular, 39% of decrease in the degree of ERK1/2 phosphorylation was assessed after the application of 50 μM PPADS together with FGF9. ERK1/2 phosphorylation returned to control level when the dose of PPADS was 100 μM. The analysis of the concentration–response curve provided a pD_2 value of −4.27±0.02, which was equivalent to an IC_{50} value of 53.70 μM.

3.3. Effect of PPADS treatment on cell viability

To evaluate the hypothetical PPADS toxicity, the viability of cells treated with the compound was assessed (Fig. 1C). No significant changes in cell viability were observed after short (left panel) or long term (right panel) treatments with PPADS (100 μM).

3.4. Effect of PPADS on FGF9-induced loss of extracellular matrix

It has been reported that sustained activation of ERK1/2 pathway that was elicited by activated FGF receptor type 3, promotes the loss of extracellular matrix in chondrocytes (Krejci et al., 2005; Yasoda et al., 2004). Taking into account the reduction of FGF9-mediated ERK1/2 phosphorylation caused by PPADS, we examined whether PPADS could also reverse the decrease of extracellular matrix. The amount of extracellular matrix was evaluated by Alcian blue staining in achondroplasic chondrocytes precursors, treated with plain FGF9 or FGF9 with PPADS (Fig. 1D). As expected, FGF9-treated chondrocytes precursors, showed a clear reduction of Alcian blue stainable extracellular matrix. On the contrary, after exposing PPADS (100 μM) together with FGF9, achondroplasic chondrocytes precursors showed an Alcian blue staining, comparable with control cells, indicating that the extracellular matrix was restored by PPADS.

3.5. Effect of P2Y receptor antagonists and PPADS analogues on ERK1/2 phosphorylation

To obtain more information regarding how PPADS works on ERK1/2 phosphorylation stimulated by FGF9, we investigated the behaviour of other P2 receptor antagonists. As shown in Fig. 2A different receptor antagonists were tested: the non-selective P2 receptor antagonist, RB2; the selective P2Y1 receptor

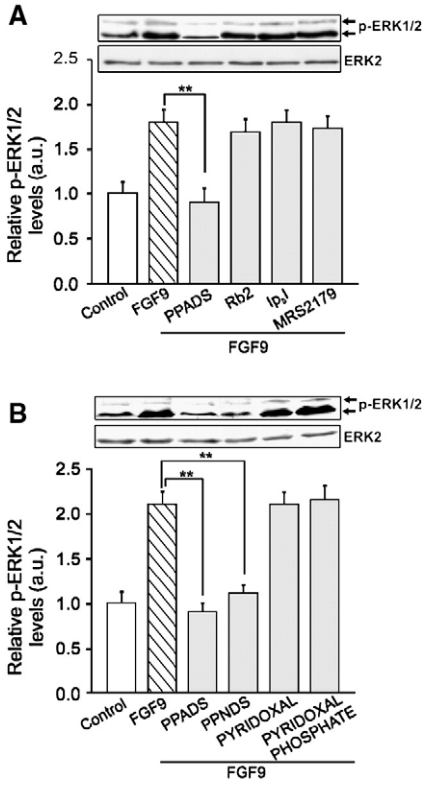

Fig. 2. Analysis of P2 receptor antagonists and chemical PPADS analogues action on ERK1/2 phosphorylation. Chondrocytes precursors expressing achondroplasic FGF receptor type 3 were incubated in the presence of FGF9 alone, or together with P2 receptors antagonists (all 100 μM) (A) or PPADS analogues (100 μM) (B) for 10 min. Cell lysates were successively immunoblotted with antiphospho-ERK 1/2 and anti-ERK2 antibody to verify equal loading. The histograms represent the levels of phosphorylated ERK 1/2. Data (mean±SD) are represented in arbitrary units (a.u.) and normalized to the intensity of the band corresponding control. $^{**}P<0.01$.

antagonist, MRS2179, and Ip_5I, which has been described as antagonist for the diadenosine polyphosphate receptor (Pintor et al., 1997) and for some P2X receptors (Ford et al., 2005). None of these compounds was able to reproduce the action of PPADS on FGF9-mediated ERK1/2 phosphorylation, indicating that PPADS' effect does not involve the antagonism of a P2 receptor.

Additionally, other compounds chemically related to PPADS were checked (Fig. 2B). The PPADS' synthesis precursors of, pyridoxal and pyridoxal-5'-phosphate failed to reduce ERK1/2 phosphorylation. In contrary PPNDS, a naphthylazo derivative bearing two sulfonic group as substituents, mimicked the inhibitory action of PPADS on ERK1/2 phosphorylation with 48% reduction compared to FGF9 treatment.

4. Discussion

In this work, we investigated the effect of PPADS, a typical non-selective P2 receptor antagonist, on achondroplasic FGF receptor type 3 activity. As it was discovered PPADS dramatically reduced the FGF9-mediated tyrosine phosphorylation of mutant FGF receptor type 3, showing that PPADS acts as a potent inhibitor of FGF receptor type 3 activity.

Furthermore, we examined the effect of PPADS on signal transduction pathways initiated by the activated FGF receptor type 3 receptor. In particular, we analyzed the ERK1/2 pathway. PPADS also counteracted FGF9-induced ERK1/2 phosphorylation, being in accordance to the previously mentioned decrease in FGF receptor type 3 activity.

These results have a special value for achondroplasic chondrocytes, where the over activation of the mutant receptor and subsequent sustained signalling, alters profoundly the biochemical behaviour of chondrocytes (Aviezer et al., 2003). Therefore, the attenuation of FGF receptor type 3 signalling would permit the pathological phenotypes showed by achondroplasic chondrocytes to be reversed. In fact, we have experimentally confirmed this statement by evaluating the extracellular matrix.

It has previously reported that ERK1/2 pathway activated in a constitutive manner, accomplishes the FGF-mediated matrix loss, which appears to originate from both FGF-induced inhibition of synthesis and stimulation of proteoglycan matrix degradation (Krejci et al., 2005). We identified that PPADS, probably acting through the inhibition of ERK1/2 pathway, was able to prevent the FGF9-mediated loss of extracellular matrix. This finding reinforces the key role of PPADS to inhibit the over-stimulated FGF receptor type 3 mutant as well the cascade of biochemical events induced by the activated receptor.

Regarding the mechanism used by PPADS to attenuate ACH FGF receptor type 3 activity, though not fully determined, some ideas could be inferred. The current study was carried out with the initial expectation that PPADS might have a different role than being a P2 receptor acting as antagonist. Supporting this idea, other P2 receptor antagonists were unable to abolish over-activated FGF receptor type 3 signalling.

In our experiments, the FGF9 presence was necessary to observe the decrease of FGF receptor type 3 activity and signalling elicited by PPADS. This fact indicates that PPADS could disrupt the interaction between FGF9 and FGF receptor type 3, diminishing FGF receptor type 3 activity. Thus, PPADS would disturb the direct binding of FGF9 to FGF receptor type 3, and/or alternatively, the compound could interfere with the heparansulfate-FGF9-FGF receptor type 3 interaction. FGF ligands require the presence of cell surface heparan sulfate proteoglycans for binding and activation of their receptor (Yayon et al., 1991). In particular, FGF9 contains three potential heparin-binding sites, which would allow to establish the required interaction with extracellular matrix heparan sulfates (Hecht et al., 2001).

Interestingly, PPADS possesses in its structure two sulfonated moieties. The presence of sulfonated groups is usually a common chemical feature between heparin mimetic compounds such as suramin or suradistas. These compounds compete with heparan sulfates and block the heparin-binding sites of FGFs, being their sulfonated functional groups involved in this process (Lozano et al., 1998). Therefore, it is tempting to suggest that PPADS could act in a similar way by interfering in the interaction between FGF9 and heparan sulfates.

In this action, the sulfonated groups of PPADS would have also a key role, as in the case of suramin and suradistas. Consistent with this notion, PPNDS, whose structure resembles PPADS with two sulfonic groups as substituents, it was the only compound able to induce a significant decrease of ERK1/2 phosphorylation.

Nevertheless, the presence of sulfonated groups is not the only requirement and other structural features of the compounds must be considered like a space between sulfonic groups and their position as substituents, as well as the rigidity and planarity of the molecule (Botta et al., 2000). For instance, the non-selective P2 receptor antagonist tested, RB2, shows a polysulfonated skeleton, but despite that, it did not produce any significant change on FGF9-induced ERK1/2 phosphorylation.

In summary, we show evidence supporting that PPADS, acting through a mechanism which needs to be fully investigated, exerts a potent inhibition of achondroplasic FGF receptor type 3 activity and to the signalling induced by FGF9. As a consequence, the biological responses elicited by sustained FGF receptor type 3 signalling such as the loss of extracellular matrix were also antagonized. Our findings, render PPADS as a highly attractive candidate agent for the treatment of Achondroplasia.

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