In vitro studies with an AKT inhibitor, ARQ 092, provide evidence for a new and more effective therapeutic option in PIK3CA Related Overgrowth Spectrum (PROS) patients

D. C. Loconte[1], V. Grossi[1], S. Di Tommaso[1], C. Ranieri[1], R. Bagnulo[1], A. Peserico[1], G. Forte[2], P. Sanese[1], A. Giglio[3], Y. Yu[4], G. Abbadessa[5], C. Simone[1,2], N. Resta[1]

[1] Division of Medical Genetics, DIMO, University of Bari "Aldo Moro", Italy; [2]Cancer Genetics Laboratory, IRCCS "S. de Bellis", Castellana Grotte (BA), Italy; [3]Department of Emergency and Organ Transplantation-Urology, Andrology and Kidney Transplantation Unit, University of Bari "Aldo Moro", Italy; [4]Biology, ArQule, Inc., Burlington, Massachusetts, USA; [5]Clinical Development, ArQule, Inc., Burlington, Massachusetts, USA.

Summary and Background

In PROS (PIK3CA-related overgrowth spectrum), PIK3CA germline and postzygotic mutations constitutively activate the PI3K/AKT/mTOR pathway, causing congenital segmental overgrowth, while are absent in surrounding healthy tissues (1). mTOR inhibitors are empirically tested in these patients, with sporadic limited benefit (2). We aim to assess the effects of pathway blockage upstream of mTOR on PROS patient-derived cells by the potent and selective allosteric AKT inhibitor ARQ 092, an experimental drug with activity and long-term tolerability in cancer patients, that is also tested in patients with Proteus syndrome.

We performed targeted deep sequencing of pathway genes in six PROS patient-derived cells to identify causative mutations and immunoblots to assess the phosphorylation status of AKT and its downstream targets (pS6, pPRAS40, pFOXO3a, pBAD, pGSK3a-b). Anti-proliferative effect of ARQ 092 and PI3K/AKT/mTOR inhibitors (wortmannin, LY294002, rapamycin) was evaluated, with or without serum, in PROS cells from six patients (3). ARQ 092 potently inhibited AKT signaling and exerted a strong anti-proliferative effect by inducing cell death more efficiently than comparators, with 50% of cells surviving after 60 hours of treatment at a 5 uM dose. Our data show that PROS cells are 'addicted' to AKT and PROS treatment benefits more from AKT than mTOR inhibition. Clinical development of ARQ 092 in PROS patients is warranted.

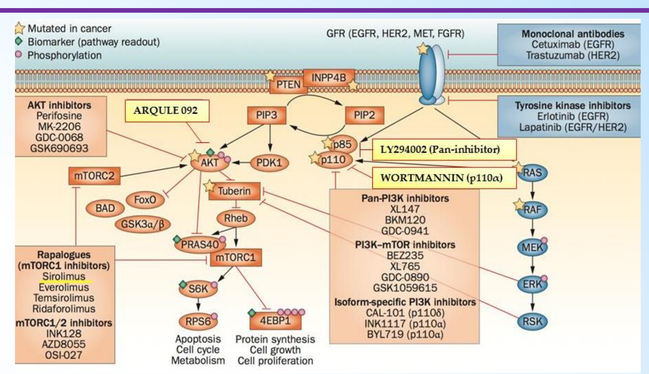
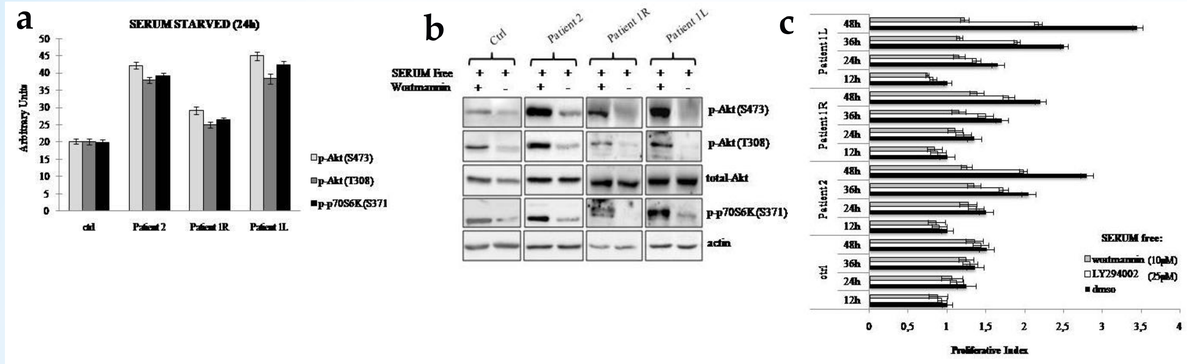


Figure 1. Key elements of the studied pathway (AKT, PI3K p110, PRAS40, pS6) and their pharmacological inhibitors (4).

Overactivation of the PI3K/AKT pathway is abrogated by pharmacological inhibition of PI3K



PROS Patients	Clinical Phenotype	PIK3CA mutation	Mutation Frequency (%)
1	MCAP*	p.Glu81Lys	21,5
2	Macrodactyly	p.His1047Arg	9
3	CLOVES**	p.His1047Arg	57,1
4	CLOVES	p.His1047Arg	46,5
5	MCAP	p.Glu726Gly	37
6	MCAP	p.His1047Tyr	25

* Megalencephaly-Capillary Malformation;
** Congenital Lipomatous Overgrowth, Vascular Malformations, Epidermal Nevi, Scoliosis/Skeletal and Spinal syndrome

Figure 2.

a. The indicated values are the result of the densitometric analysis of the phosphorylated forms of AKT and p70S6K normalized against total AKT and the loading control, respectively.

b. Immunoblot analysis of phospho-AKT (Ser473), phospho-AKT (Thr308), total AKT and phospho-p70S6K (Ser371) in mutant cells (fibroblasts from biopsies of patient 2, and from left [L] and right [R] leg biopsies of patient 1) compared to IMR90 primary human normal fibroblasts (Ctrl). Cells were treated with the PI3K inhibitor wortmannin (10μM) for 24 hours in the absence of growth factor stimulation.

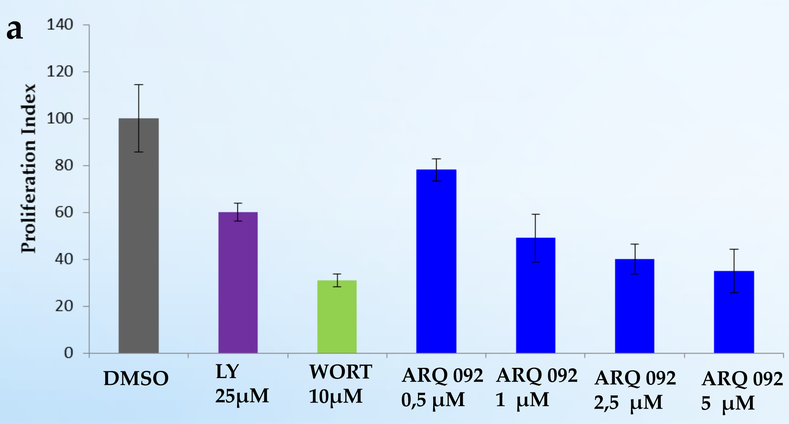
c. Patients' affected cells are dependent on PI3K activity for proliferation. Primary fibroblasts obtained from biopsies were cultured in the presence or absence of wortmannin (10μM) and LY294002 (25μM). At the indicated time points, the proliferation index was determined using the WST-1 assay (5).

Methods

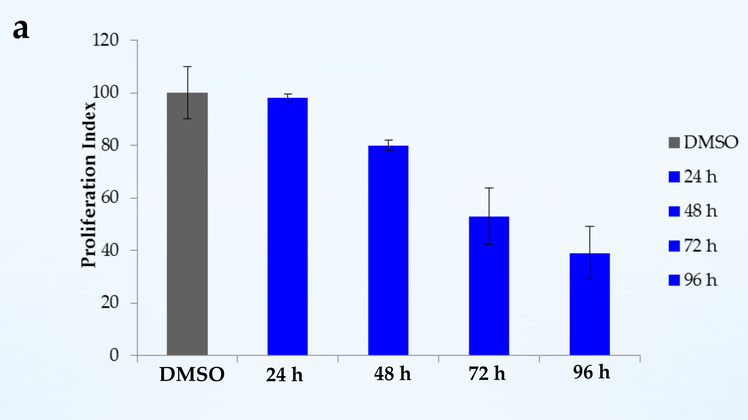
Entire coding region of PIK3CA was sequenced and analyzed according to our previous report (5). Patients-derived primary fibroblasts were grown in RPMI supplemented with 10% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin and 1% L-glutamine. For phosphorylation studies, cells were grown in RPMI 10% fetal bovine serum or transferred to serum free medium for 6 hours prior to test the drugs. Wortmannin, and Rapamycin were purchased from Sigma-Aldrich (Poole, UK) and LY294002 from Selleckchem (Houston, TX). Anti-proliferative effect of ARQ 092 and PI3K/AKT/mTOR inhibitors (wortmannin, LY294002, rapamycin) was evaluated, with or without serum, in PROS cells from six patients. Cell proliferation was determined using the Cell Proliferation Reagent WST-1 (Roche, Mannheim, Germany) according to manufacturer's instructions. Briefly, cells were seeded into 96-well plates one day before treatment. After 24, 48, 72, 96 hours after treatment, The absorbance was measured on a microplate reader (BioTek, Seattle, USA) at 450/655 nm. Each assay was performed in 3 replicates. Immunoblotting analyses were performed according to Cell Signaling Technology instructions (Beverly, USA). All antibodies used to perform immnublot analyses were from Cell Signaling Technologies except to antibody against β-actin (Sigma- Aldrich). Signal intensities were measured by Image Lab software (version 5.1- Biorad) using raw data from scans.

Results

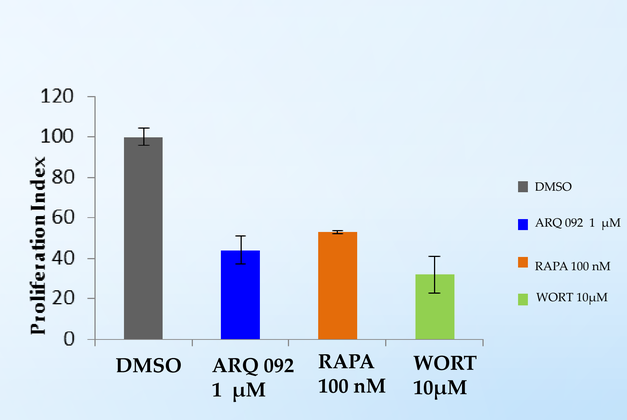
ARQ 092 decreases phosphorylation of AKT and PRAS40 in patients-derived primary fibroblasts in a dose-dependent manner



ARQ 092 decreases phosphorylation of AKT and PRAS40 in patients-derived primary fibroblasts in a time-dependent manner



The pharmacodynamic of ARQ 092 is different from wortmannin and rapamycin in primary fibroblasts



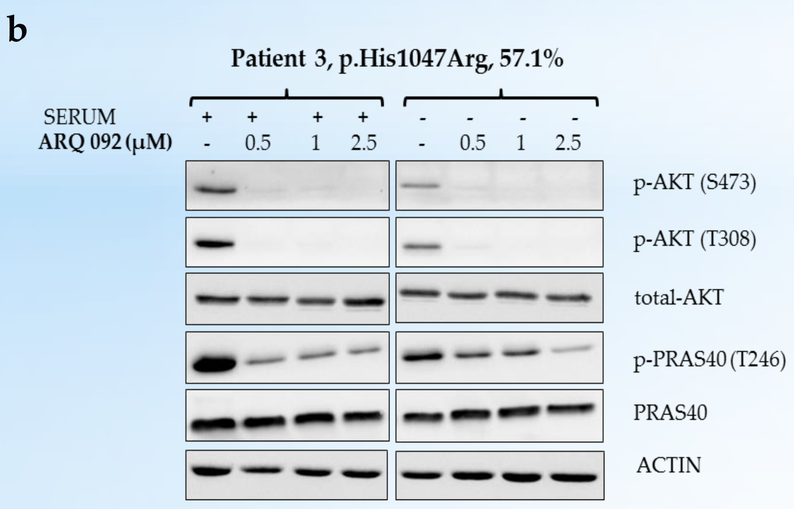


Figure 3. a. Primary fibroblasts obtained from biopsies of patient 3 were incubated in the absence of serum, LY294002 (25μM), wortmannin (10μM) and ARQ 092 (0.5 μM, 1μM, 2.5 μM, 5 μM). At the indicated time points, the proliferation index was determined using the WST-1 assay. **b.** Immunoblot analysis of phospho-AKT (Ser473), phospho-AKT (Thr308), total AKT and phospho-PRAS40 (T 246) and total PRAS40 in primary fibroblasts (from biopsies of patient 3), which were incubated in the presence or absence of serum and ARQ 092 (0.5 μM, 1μM, 2.5 μM) and collected at 72h. pAKT and pPRAS40 levels are reduced with increasing doses of ARQ 092 when grown +/- serum.

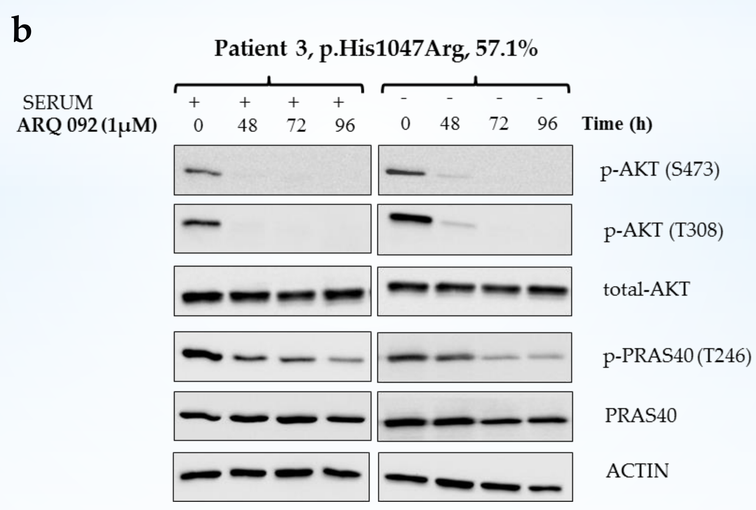


Figure 4 a. Primary fibroblasts (from biopsies of patient 3) were incubated in the absence of serum and ARQ 092 (1μM). At the indicated time points (24h, 48h, 72h, 96 h), the proliferation index was determined using the WST-1 assay. **b.** Immunoblot analysis of phospho-AKT(Ser473), phospho-AKT (Thr308), total AKT and phospho-PRAS40 (T 246) in primary fibroblasts (from biopsies of patient 3), which were incubated in the presence or absence of serum and ARQ 092 (1μM) and collected at different time points.

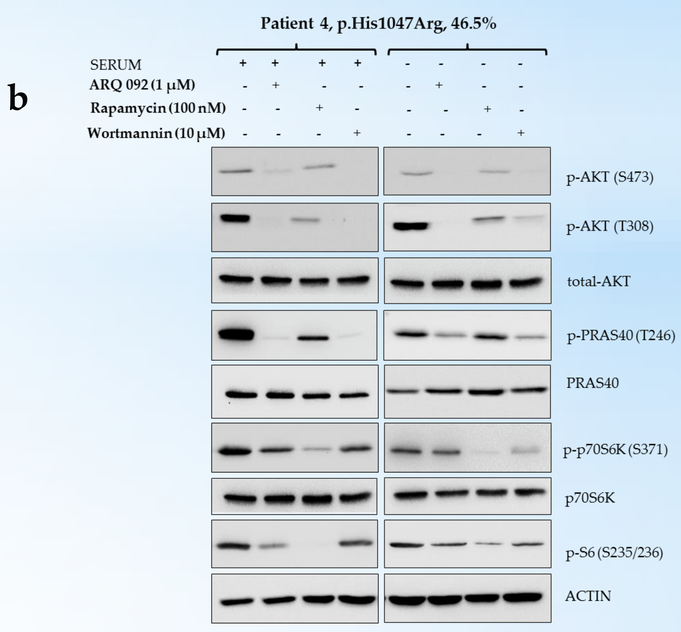


Figure 5. Primary fibroblasts (from biopsies of patient 4) were incubated in the presence or absence of serum, ARQ 092 (1μM), rapamycin (100 nM), wortmannin (10 μM) for 96 h. **a.** The proliferation index was determined using the WST-1 assay. **b.** Immunoblot analysis of phospho-AKT (Ser473), phospho-AKT (Thr308) and total AKT, phospho-PRAS40 (T 246) and total PRAS40, phosho-p70S6K (S371) and total p70S6K, p-S6 (S235/236). ARQ 092 inhibits phosphorylation of AKT and PRAS40 more efficiently than rapamycin, while phosphorylation of p70S6K and S6 are drastically reduced by rapamycin. Whereas, very high concentrations of wortmannin showed comparable inhibition of AKT/mTOR pathaway as ARQ 092.

ARQ 092 potently inhibits phosphorylation of AKT and PRAS40 in PROS patient-derived cells

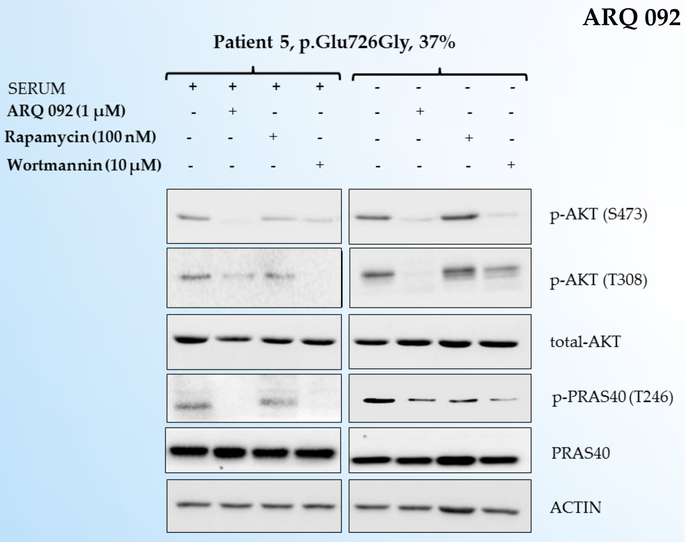


Figure 6.
Primary fibroblasts (from biopsies of patient 5) were incubated in the presence or absence of serum, ARQ 092 (1μM), rapamycin (100 nM), wortmannin (10 μM) for 96 h. Immunoblot analysis of phospho-AKT (Ser473), phospho-AKT(Thr308) and total AKT, phospho-PRAS40 (T246) and total PRAS40. ARQ 092 inhibits phosphorylation of AKT and PRAS40 more efficiently than rapamycin, in both serum and no serum.

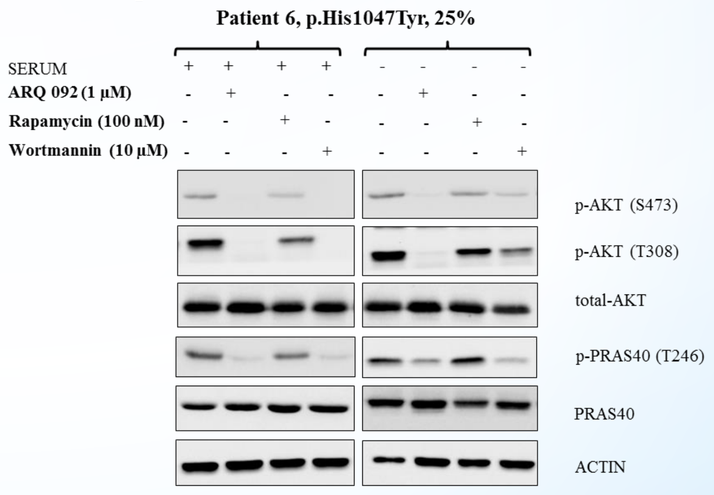


Figure 7.
Primary fibroblasts from biopsies of patient 6 were incubated in the presence or absence of serum, ARQ 092 (1μM), rapamycin (100 nM), wortmannin (10 μM) for 96 h. Immunoblot analysis of phospho-AKT (Ser473), phospho-AKT (Thr308) and total AKT, phospho-PRAS40 (T 246) and tot PRAS40. ARQ 092 inhibits phosphorylation of AKT and PRAS40 more efficiently than rapamycin, in presence of serum and not.

References

1. Keppler-Noreuil KM, Sapp JC, Lindhurst MJ, et al (2015). Clinical delineation and natural history of the PIK3CA-related overgrowth spectrum. Am J Med Genet A. 2015 167A:907-9.
2. www.clinicaltrials.org, clinicaltrials.gov Identifier NCT02443818: Sirolimus effect on Hypertrophic Syndrome s Related gene PIK3CA.
3. Yu Y, Savage RE, Eathiraj S, Meade J, Wick MJ, Hall T, Abbadessa G, Schwartz B. (2015). Targeting AKT1-E17K and the PI3K/AKT Pathway with an Allosteric AKT Inhibitor, ARQ 092. PLoS One. 10:e0140479.
4. J. Rodon R. Dienstmann et al. (2013). Development of PI3K inhibitors: Lessons Learned from early clinical trials. Nat. Rev. Clin. Oncol. 10, 143-153
5. Loconte DC, Grossi V, Bozzao C, Forte G, Bagnulo R, Stella A, Lastella P, Cutrone M, Benedicenti F, Susca FC, Patruno M, Varvara D, Germani A, Chessa L, Laforgia N, Tenconi R, Simone C, Resta N. (2015). Molecular and Functional Characterization of Three Different Postzygotic Mutations in PIK3CA-Related Overgrowth Spectrum (PROS) Patients: Effects on PI3K/AKT/mTOR Signaling and Sensitivity to PIK3 Inhibitors. PLoS One. 10:e0123092.

Conclusions

- **ARQ 092 targets AKT, and its constitutive activation caused by different PIK3CA activating mutations identified in PROS patients with different phenotypes (from macrodactyly to MCAP)**
- **Targeting AKT permits the inhibition of the PI3K pathway immediately downstream of AKT but upstream of mTOR, achieving even better results than treatment with rapalogues (sirolimus)**
- **The use of an AKT inhibitor offers the possibility to circumvent additional pathways dependent on multiple classes and isoforms of PI3K kinases (class I, II , and III)**
- **A clinical trial with ARQ 092 in PROS patients is being planned**

Poster presented at the 2016 conference of the European Society of Human Genetics in Barcelona, Spain