Multidrug resistance protein 4 (MRP4/ABCC4) is overexpressed in clear cell renal cell carcinoma (ccRCC) and is essential to regulate cell proliferation

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Abstract

Kidney cancer accounts for 2.5% of all cancers, with an annual global incidence of almost 300,000 cases leading to 111,000 deaths. Approximately 85% of kidney tumors are renal cell carcinoma (RCC) and their major histologic subtype is clear cell renal cell carcinoma (ccRCC). Although new therapeutic treatments are being designed and applied based on the combination of tyrosine kinase inhibitors and immunotherapy, no major impact on the mortality has been reported so far.

MRP4 is a pump efflux that transporters multiple endogenous and exogenous substances. Recently it has been associated with tumoral persistence and cell proliferation in several types of cancer including pancreas, lung, ovary, colon, ostesarcoma, etc.

Herein, we demonstrate for the first time, that MRP4 is overexpressed in ccRCC tumors, compared to control renal tissues. In addition, using cell culture models, we observed that MRP4 pharmacological inhibition produces an imbalance in cAMP metabolism, induces cell arrest, changes in lipid composition, increase in cytoplasmic lipid droplets and finally apoptosis.

These data provide solid evidence for the future evaluation of MRP4 as a possible new therapeutic target in ccRCC.

KEY WORDS: cAMP; MRP4; lipidomics, renal carcinoma.

1. Introduction

Renal cell carcinoma (RCC) is the most common type of adult kidney malignant tumor. Clear cell Renal Cell Carcinoma (ccRCC) is the predominant histological subtype of RCC (75–80 %) and is usually associated with severe prognosis and high mortality [1]. About 30% of ccRCC are diagnosed at the metastatic stage and it is treated by nephrectomy, however a third of the patients after resection of the tumor will have a recurrence [2].

Conventional systemic therapeutic treatments on advanced ccRCC, like cytotoxic drugs and radiotherapy resulted largely ineffective and did not improve patient survival [3]. Currently, recommended first-line targeted therapy options are single-agent tyrosine kinase inhibitors (TKIs) including pazopanib, sunitinib, axitinib and cabozantinib, or temsirolimus, which targets mTOR. In addition, immune checkpoint inhibitors were introduced, with encouraging results. Nevertheless, RCC incidence has been stable and death rates have been falling only 0.9% each year from 2007 through 2016 [4].

Recently it has been described the existence of drug/metabolites efflux proteins pumps, on the basolateral or apical side of cells, that might be involved in the persistence of tumors modulating levels of physiological mediators. [5–7].

Among them, multidrug resistance protein 4 (MRP4) is member of ATP-binding cassette (ABC) transporters (C4 subtype), responsible for ATP-driven transmembranous transport of substrates [8]. MRP4 (MOAT-B; multi- specific organic anion transporter) is a lipophilic anion efflux pump that was categorized as a short MRP, and has similar membrane topologies as MRP5, 8, and 9 [9]. To date, nine MRPs have been identified and are able to transport a wide variety of endogenous and xenobiotic organic anions out of the cell [10,11]. Each MRP has its own membrane location, tissue distribution,

and substrate specificity. Especially, MRP4 has a particular broad substrate specificity, encompassing cyclic nucleotides (especially cAMP), ADP, eicosanoids, urate, steroid hormones, folate, and bile acids among others endogenous substrates and several antiviral, antibiotic, cardiovascular, cytotoxic (methotrexate, 6-thioguanine, 6-mercaptopurine, topotecan) exogenous drugs [12,13]. Additionally, there are other carriers related to MRPs, such as OATP4C1 that has a reverse transport flow of metabolites, compared to MRP4 [14].

It has been reported in different cancer types, that MRP4 dysregulation is associated with cell proliferation and malignancy [15]. Moreover, it was observed in neuroblastoma that high levels of MRP4 expression were significantly associated with poor clinical prognosis, and it was proposed as a prognostic biomarker and therapeutic target [8,16]. Although historically the role of MRPs proteins in cancer was associated with chemotherapy resistance, nowadays, there are many additional experimental evidences that relate poor prognosis with endobiotics transport, cAMP and prostaglandins, which are vital for tumor progression and malignancy [17–19].

On the other hand, the cAMP balance is critical for the lipids metabolism and formation of lipid droplets in cancer [20], a particular phenotypic characteristic of ccRCC [21].

There are no previous reports describing the MRP4 expression and its role in ccRCC related to cAMP metabolism. So it is likely that MRP4 is particularly involved in tumor characteristics and be responsible, at least in part, for the clinically observed chemoresistance [3].

The objective of this work was to evaluate the expression and functionality of MRP4 in ccRCC and its association with malignancy, in order to postulate it as a potential

biomarker of prognosis and / or pharmacological target for the development of more effective therapeutic strategies.

2. Materials and methods

2.1. In Silico Analysis of MRP4/ABCC4 expression in ccRCC patients

In order to determine the prognostic value of MRP4/ABCC4 expression in patients diagnosed with ccRCC, we first evaluated it expression levels, using related studies hosted in Array Express. Through a script, the sequencing platform's ID used in each study was retrieved from NCBI's site, then the platform's data table containing the probe's ID for ABCC4 were downloaded. The expression matrix file for each study was downloaded through NCBI's ftp site. Then ABCC4's expression levels for each sample was extracted along with the sample's description. This was used to evaluate if there was significant differential expression among sample types (ccRCC vs adjacent non-tumoral renal cells)[22,23].

2.2. Patients and sampling procedures

Surgical specimens were obtained from 32 patients with ccRCC. Patients were treated by radical nephrectomy at the Urology Unit of the J.R. Vidal Hospital (Corrientes, Argentina) between 2013 and 2018. Surgically removed ccRCC and counterpart normal tissues (tissue sections away from the tumor in the same kidney) were collected from specimens, transported aseptically to the laboratory, and quickly frozen at -80°C. Specimens were fixed for histopathology and immunohistochemistry procedures.

The design and methods of this research have been approved by the Bioethics Committee of the School of Medicine of the Northeastern National University and

by the Department of Medical Research of the J.R. Vidal Hospital from Corrientes, Argentina.

2.3. *RT-qPCR*

MRP4 messenger RNA (mRNA) expression was extracted using the TRIzol reagent method (Invitrogen) and purified using Ambion® TURBO DNA-free™according to the manufacturer's protocol. First-strand cDNA was obtained by using the MMLV-RT (Promega) from 2 μg of RNA. qPCRs were then performed using specific primers for MRP4 as follows: 5'-GGACAAAGACAACTGGTGTGCC-3' (forward), 5'-AATGGTTAGCACGGTGCAGTGG-3' (reverse) with a product of 156 bp. GAPDH: 5'-ATGGGGAAGGTGAAGGTCG-3' (forward); 5'- GGGGTCATTGATGGCAACAATA-3' (reverse) with a product of 108 bp or β-Actin: 5'-CATGTACGTTGCTATCCAGGC-3' (forward); 5´- CTCCTTAATGTCACGCACGAT-3´ (reverse) with a product of 250 bp were used as housekeeping genes and no differences were overserved between them. All primers were tested for specificity using the Blast program available at the National Center for Biotechnology Information web site. Cycling conditions were as follows: 1 cycle at 95 °C for 10min, 35 cycles at 95°C for 30 s, 58°C for 45 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min.

2.4. *Western blot analysis*

Expression of MRP4 was determined by immunoblotting using total cell lysates. Samples from ccRCC patients and a distal section of renal normal tissue of each patient were homogenized and lysed in an ice-cold buffer [10 mM HEPES pH 7.4, 10 mM KCl, 1.5 mM MgCl2, 0.5 mM dithiothreitol, 0.1% IGEPAL (Sigma Co, MO, USA)], supplemented with a protease inhibitor cocktail.

Total proteins (50 µg) were then separated by 8% SDS-PAGE and blotted on nitrocellulose membranes (Bio-Rad, CA, USA). Membranes were then treated with 1:500 dilutions of primary antibodies purchased at Santa Cruz® biotechnologies and after that probed with the corresponding antibodies followed by HRP-conjugated secondary antibodies in TBS solution. β-Actin was used as a load control. The immunoblots were visualized using enhanced luminescence (ECL, BIO-RAD). Densitometry was performed on scanned images using ImageJ software (NIH; Ver 1.52a), and values were normalized for the corresponding controls of each experiment. All experiments were performed at least 3 times and representative results of one experiment are shown.

2.5. Immunohistochemistry (IHC)

Paraffin-embedded sections were deparaffinized and rehydrated in graded alcohols using routine protocols. Briefly, sections (4 µm) were stained with anti-MRP4 antibody (Santa Cruz® biotechnologies, 1:100) with overnight incubation at 4°C. Slides of adult colon were used as positive controls to test anti-MRP4 antibody. Immunostaining was performed using the avidin-biotin-peroxidase complex technique (Vectastain Elite ABC kit, Vector Laboratories, CA). The reactions were developed with 3-3'diaminobenzidine (DAB) as described previously [24]. After immunohistochemistry, the specimens were lightly counterstained with 10% hematoxylin, dehydrated, and mounted. All negative controls were obtained by excluding the primary antibody from the reaction. Slides were analyzed using light microscopy by two independent investigators who were blinded to the patient data. The immunohistochemical expression was evaluated and categorized in four groups: negative (-), weak (+), moderate (++) and strong (+++) IHC expression.

2.6. *Cell lines and proliferation tests*

Two established human renal carcinoma cell lines Caki-1 and Caki-2 were used. The former was derived from a human tumor skin metastasis, in opposition, Caki-2 was established from a primary ccRCC. Both cell lines were grown in 25 cm^2 flasks at 37°C, in a humidified 5% CO_2 atmosphere, in DMEM medium supplemented with 10% fetal bovine serum, and 50 µg/mL gentamicin. HCT-116, cell line was use as positive controls in RT-qPCR or immunocytochemistry (ICC) experiments. Cell proliferation was measured by trypan blue staining and Neubauer chamber counting or using the XTT® kit according to the manufacturer's instructions.

2.7. *Immunocytochemistry (ICC)*

Caki-1 or Caki-2 cells were grown on glass coverslips. The cells were washed with 1% BSA (Sigma-Aldrich) in PBS and permeabilizated with Triton X100 (1%, PBS) for 5 min. Then, cells were incubated overnight at 4°C with primary anti-MRP4 antibodies (Santa Cruz® biotechnology; dilution 1:100). Incubations with a secondary antibody, staining and scoring were done as previously described for IHC assays.

2.8. *cAMP Radiobinding Protein Assay*

In all cases, cells were seeded in 24-well plates at a density of 1.10^4 cells/well, and, before starting each experiment, culture media was replaced with phenol red-free media (Sigma-Aldrich) without FBS. Cells were then exposed to PBS (controls) or 25 µM Forskolin (FSK), and 1 mM 3-isobutyl-1-methylxanthine (IBMX) at different time points as indicated in the corresponding figure legend. After treatment, at indicated times, cell monolayer and supernatants were extracted with 95% v/v ethanol in order to obtain intracellular cAMP (i-cAMP) and extracellular (e-cAMP) contents

respectively. Extracts were then evaporated, and residues were resuspended in radiobinding protein (RBP) buffer (50 mM Tris-HCl, 4 mM EDTA, pH 7.4, 0.1% bovine serum albumin). cAMP content was determined by a competitive RBP assay for PKA using $^{[3H]}$cAMP, as previously described [25]. Briefly, titrated PKA was incubated in equilibrium conditions (2 hours, 4°C) with different samples or cAMP standards (0.1–90 pmol) in the presence of 2 nM $^{[3H]}$cAMP (20.7 Ci/mmol, NET1161250UC; PerkinElmer) in RBP buffer. The bound fraction was separated by carbon–dextran precipitation, followed by centrifugation (2000g, 15 minutes, 4°C), and Optiphase HiSafe3 scintillation cocktail (PerkinElmer) was added to each supernatant for counting in a Pharmacia Wallac 1410 counter. cAMP Sample concentrations were determined by interpolating from the displacement curves obtained from cAMP standards using Prism 8.0 (GraphPad Software). Duplicate samples of at least three independent experiments were analyzed. For concentration–response assays, we fit the pooled data from all experiments into a single equation.

2.9. Lipidomics approaches

Lipidomic determinations of the content of fatty acids (FA) in the triacylglycerides (TAG) and cholesterol esters (CE) fractions were determined using gas chromatography coupled to mass spectrometry (GC-MS) according to the procedures previously described [26]. Analysis of eicosanoids by LC/MS was carried out exactly as described elsewhere [27], using an Agilent 1260 Infinity high-performance liquid chromatograph equipped with an Agilent G1311C quaternary pump and an Agilent G1329B Autosampler, coupled to an API2000 triple quadrupole mass spectrometer (Applied Biosystems, Carlsbad, CA, USA). Quantification was carried integrating the chromatographic peaks of each species

and by comparing with an external calibration out by integrating the chromatographic peaks of each species and by comparing with an external calibration curve made with analytical standards [28].

2.10. Cell cycle assessment

Caki-2 cells were seeded in a 6-well plate at 2.5 x 10^5 cells/well. After treatments, cells were washed with PBS and harvested using Tripsin-EDTA (GIBCO) and then fixed with cold Ethanol 70% and a final concentration was adjusted to 10^6 cells/mL. The cells were treated with RNAse A (Sigma, Darmstadt, Germany) for 15 minutes at 37°C and stained using propidium iodine (PI) 1 mg/mL (Sigma, Darmstadt, Germany) for 15 minutes. The DNA content of the cells were analyzed using FACSCalibur (BD Biosciences, San Jose, CA, USA) and the data was analyzed with FlowJo (BD Biosciences, San Jose, CA, USA).

2.11. Confocal microscopy

The cells were plated on coverslips and then fixed with 4% paraformaldehyde in PBS containing 3% sucrose for 20 min. Afterward, paraformaldehyde was removed by washing the cells thrice with PBS, and BODIPY493/503 (2 μg/ml) and DAPI (1 μg/ml) staining were carried out. Coverslips were mounted on microscopy slides with 25 μl of a polyvinyl alcohol solution until analysis by fluorescence microscopy. Lipid drops were analyzed using oleic acid (30 μM) as a positive control. In contrast, untreated cells were used as negative controls. Fluorescence was monitored by microscopy using a confocal BioRad laser scannig system Radiance 2100. Images were analyzed with the ImageJ software (NIH; Ver 1.52a).

2.12. Statistics

Statistics were performed using GraphPad Prism 8.0 via an unpaired *t* test or one-way analysis of variance (ANOVA) followed by Bonferroni's or Tukey's comparison test. Differences were considered statistically significant at $p<0.05$.

3. Results

3.1. MRP4 is overexpressed in ccRCC

MRP4 is an apical organic anion transporter and the known efflux pump for cAMP in human kidney proximal tubules [29]. While MRP4 extrudes drugs to the proximal tubular lumen and metabolites from the cellular cytoplasm, OATP4C1 has a reverse flow of substance transport [14,30]. In order to establish the implication that the rate of both transporters would have as cellular modulators of cAMP, we first performed an *in-silico* analysis to determine the level of expression of MRP4 and OATP4C1 mRNAs in ccRCC and normal patients, using specific probes as described in materials and methods.

MRP4 showed a clear overexpression in tumors (n=69), in comparison with normal patients (n=32; difference of median 169.3; *P*<0.0001). In opposition, OATP4C1 showed lower expression levels than normal samples (**Figure 1A**). This opposite expression of both proteins could explain the peculiar phenotype exhibited by these tumors. In parallel, the overexpression of MRP4 could mean a hyper-functionality, susceptible of being treated therapeutically. Thus, we focused on MRP4. We next evaluated the expression and localization of this protein in human tumor samples of ccRCC. First, we determined the expression level of MRP4 mRNA transcripts in renal control samples and tumors by RT-qPCR. ccRCC tumors are solid and compact

tissue masses with distinguishable macroscopic features between the center and the periphery of the tumor. While the center shows a high lipid content, the periphery of the tumor exhibits abundant fibrous tissue with less friable consistency. In order to determinate the MRP4 expression profile, we analyzed the protein expression in two different areas of the tumor mass: *Core* (center) and *Periphery* (other zones in the border) (n=18). **Figure 1B** shows that the expression level of this messenger in the aforementioned tumoral sections, is at least 20-fold higher than in normal tissues, considering the core (media= 47.46) and periphery (media= 21.34). Similarly, when we analyzed the expression levels of the protein in tumors, in all analyzed cases we found at least 5-10-fold increase, when compared to the control samples (**Figure 1B**).

Immunohistochemical staining was tested to confirm the enhanced MRP4 expression and to determine its sub-cellular localization (**Figure 1C-J**). First, we investigated the MRP4 expression in non-neoplastic tissues (Normal; n=10). This protein showed a homogeneous and intense membranous pattern in all samples. MRP4 was circumscribed only to the renal proximal tubular cells, since a complete absence was observed in the glomerular components (**Figure 1C** and **F**). On the other hand, tumoral MRP4, displayed a very strong and rather heterogeneous expression with zone-dependent differences: high levels (score 2+ or 3+) of membranous MRP4 expression in the core (80 %) and in contrast, mild and moderate protein expressions (score +1 or 2+) in peripheral tissues (100%; **Figure 1J**). Additionally, **Figure 1C-E and F-H** illustrate two patients with different expression levels of MRP4. The first one shows a more balanced expression of the

protein between sectors, while the second denotes clear differences between the core and the periphery (Panels **D-E** and **G-H** respectively).

Opposite to the observations in peripheral tissues, tumor core shows a high heterogeneous expression of this protein with intense foci (arrows)(**Figure 1J**).

→ **Figure 1**

3.2. MRP4 expression in renal cell lines: Caki-1 and Caki-2

Given the differential expression of MRP4 in tumors and normal tissue, we set out to design a model that allows us to validate this protein as a therapeutic target or a prognosis marker. We measured the expression level of this protein in the two cell lines representing ccRCC with dissimilar degrees of differentiation. Caki-1 is a cell line derived from a metastatic skin clear cell renal carcinoma tumor (well-differentiated) and Caki-2 is another human clear cell renal carcinoma line that also displays epithelial morphology and grows in adherent culture (less-differentiated)[31,32]. Following the same rationale used in IHQ (**Figure 1I**), we used HCT-116, a colonic tumoral cell line, as positive control to measure the basal expression of MRP4. **Figure 2A** shows that Caki-2 is the cell line that displays the maximum expression of MRP4 mRNA (~3-fold higher than Caki-1 cells). Monolayers from these cell lines were scraped and proteins were extracted for western blot analysis. **Figure 2B** shows that both cell lines exhibit MRP4 but clearly, Caki-2 cells express 1.5-fold increased. This difference was analyzed and quantified as shown in materials and methods using ImageJ software (**Figure 2C**).

Similarly to previous assays, MRP4 expression was also confirmed by ICC. No quantitative differences in protein expression were observed nor specific localizations between Caki cell lines. Cytoplasmic aggregates of proteins were found

in Caki cells (light blue and red arrows), in opposition to the remarkable membranous pattern observed for HCT-116 cells (Black arrows; **Figure 2D**).

→ **Figure 2**

3.3. cAMP efflux is mediated by MRP4 in Caki-1 and Caki-2 cells and it is engaged in cell proliferation

MRP4 has been described as the main transporter involved in cAMP extrusion in other cancer cell lines [17,18]. Thus, we next evaluated cAMP dynamics in Caki-1 and Caki-2 cells by kinetic experiments. To study the efflux and intracellular accumulation of cAMP, Caki cell lines were treated with IBMX (1 mM), a phosphodiesterase (PDE) inhibitor and FSK (25 μM), an adenylyl cyclase direct activator. Then the supernatant or scraped cells were collected for the radiometric measurement of extracellular cAMP (e-cAMP) and intracellular cAMP (i-cAMP) respectively as it was previously described in M&M. Thus, in order to show differences in the extrusion capacity of this nucleotide for both lines, we calculated the area under the curve (AUC) values for both e-cAMP and i-cAMP levels and plotted their ratio (**Figure 3A**). Caki-2 cells presented the highest AUC e-cAMP/AUC i-cAMP, in contrast with Caki-1 (difference between means 0.073; P= 0.007). Strikingly, the ratio between both extrusion capacities is approximately 1.36 ± 0.07, which is in agreement with the protein difference found by western blotting or even more by RT-qPCR, for both cell lines.

To further characterize cAMP efflux, Caki cells were incubated in the aforementioned experimental conditions (IBMX + FSK), and also two different MRP4 inhibitors: MK-571 (25 μM; 5 min) or Probenecid (0.5 mM; 5 min) were added. The synergistic effect of increased cAMP synthesis, inhibition of its degradation and

simultaneous inhibition of its extrusion, produced a significant rise in the accumulation of i-cAMP compared to controls in Caki-2. As seen in **Figure 3B**, the pharmacological inhibition of MRP4 leads to significant decreases in the e-cAMP levels in both cell lines, denoting an effective inhibition of the extrusion of this metabolite. In summary, these results indicate that that MRP4 is the carrier responsible for modulating cAMP levels in both, intra and extracellular space, in Caki-2 cell line. In light of the above observations, we next investigated the role of MRP4 in ccRCC proliferation. If the extrusion of cAMP was directly related to the amount of MRP4 expressed, the Caki-2 cells would proliferate with greater speed [15,18]. Effectively, in all the developed experiments we have observed that Caki-2 innately proliferates faster (**Figure 3C**). Additionally, in order to demonstrate that e-cAMP is engaged in proliferation, cells were depleted from FBS-supplemented medium and different concentrations of this cyclic nucleotide were added to the culture medium and then cell proliferation was evaluated (**Figure 3D**). Low cAMP concentrations (such as 10 μM) induced up to 100% increase in proliferation in Caki-2 cells. Simultaneously, this effect seems to have a saturation limit, since no dose-dependent effect was observed. Strikingly, Caki-2 cells, which extrude more cAMP, better show this responding effect to e-cAMP. Similar results were obtained when cell proliferation was evaluated using both Neubauer chamber or XTT cell proliferation Kit®. Additionally, Caki-2 cells were treated with cAMP (10 μM) and cell cycle stages were analyzed using flow cytometry. Treated cells showed a significant increase in Phase S (35.43%) compared to control cells (**Figure 3E**).

→ **Figure 3**

3.4. The pharmacological inhibition of MRP4 activity induce phenotypical changes, inhibits Caki cell proliferation and finally triggers apoptosis cell death

Previous reports showed that the e-cAMP/i-cAMP balance is crucial for cell proliferation[18]. The increase in i-cAMP induces proliferation inhibition associated with cell differentiation in AML [17], and decrease in i-cAMP is associated with cell growth inhibition and cell cycle arrest in PDAC models [18]. Considering that it is pharmacologically possible to modulate the e-cAMP/ i-cAMP balance (**Figure 3**), we next investigated its biochemical cellular effects. Since the Caki-2 line has a greater degree of undifferentiation (increased proliferative capacity and malignancy), greater expression of MRP4 and, consequently, greater cAMP extrusion capacity or accumulation of i-cAMP (if MRP4 is inhibited), we focused on this renal cell line.

When Caki-2 cells were exposed to MK-571 (25 μM; selective pharmacological inhibitor of MRP4) and simultaneously stimulated the production of i-cAMP with FSK (25 μM) for 18-24 h, they showed very clear changes in cell morphology (cytoplasmic stretching and cell thinning) compatible with cell stress (**Supplementary Figure 1**). We and others [33] have shown that a remarkable measure of cellular stress assessment is the observation of lipid droplets (LD). Using different approaches, such as flow cytometry analysis and confocal microscopy, we determined that the number of LD increases under the experimental conditions mentioned above. **Figure 4A-C** shows an increase in the media fluorescence intensity (MFI) of treated cells (MK-571 + FSK, both 25 μM) compared to control cells (difference between means: 81,33 ± 5,81; *P*= 0.0002), after staining them with Bodipy®, a specific LD dye. Simultaneously, Caki-2 cells were observed with confocal microscopy using oleic acid (OA; 30 μM) as positive control for the LD biogenesis

[34,35]. While the OA-induced LDs, show an apical order denoting a cellular polarization, the microscopic image of the cells treated with the MRP4 inhibitor and FSK showed rather a cytoplasmic profile distribution of the fluorescence, compatible with a greater number of smaller LD (**Figure 4D-F**).

→ **Supplementary Figure 1**

In order to determine if the biochemical composition of these LDs had quantitative differences, we analyzed the lipidomic profile of fatty acids (FA) in phospholipids (PLs), triacylglycerides (TAG) and cholesterol esters (CE) fractions using GC-MS. **Figure 4G** panels show greater accumulation of saturated fatty acids, mainly 16:0 and 18:0 (palmitic acid and stearic acid respectively) in both TAG and CE fractions, on cells simultaneously treated with FSK and MK-571. In contrast, the PLs fraction showed no major changes in FA composition between treated cells and controls (**Supplementary Figure 2**).

→ **Supplementary Figure 2**

Longer treatment times, beyond 24 h, and even only exposed to MRP4 inhibitor (MK-571 or Probenecid) drastically affected the cells and progressively, an impaired proliferative capacity was observed (**Figure 4H**). The compromised cell proliferation observed at 48 h is compatible with the increase in the Sub G_0 stage of cells under the same experimental conditions (19.1%) analyzed by flow cytometry (**Figure 4I**). Evidently, the MRP4 pharmacological inhibition, resulted in apoptosis, as revealed by morphological changes (membrane bubbles) observed by optical and electron microscopy (data not shown) and by activation of caspases (Caspase-3; apoptosis effector pathway) measured by ICC (**Figure 4J**).

Different chemotherapeutic drugs and endogenous physiological mediators, such as eicosanoids, have been previously reported as important substrates of MRP4

[12]. In order to determine if any others MRP4 substrate metabolites, in addition to cAMP, could accumulate in the cytoplasmic compartment and subsequently interfere with cell proliferation, we measured the profile of eicosanoids in Caki-2 cells under the experimental conditions outlined above. Using solid phase extraction and HPLC coupled to tandem mass spectrometry (TQP), we determined a set of 20 eicosanoids in the intracellular fractions and in the cell culture supernatant. Then, we evaluated their production in a wide range of temporal exposure (1-24 h). After an exhaustive analysis, we did not found differences in the profile of eicosanoids in the intracellular space. By contrast, the extracellular space does show the accumulation of some eicosanoids such as 12-HETE, tetranor-12-HETE and 14-HDoHE. This is more obvious at 18 h of exposure, at which time we have already shown that cellular stress begins and subsequently the consequent cell death by apoptosis (24 h) (**Supplementary Figure 3**). These results are compatible with the hypothesis that cAMP is the metabolite hitherto mainly involved in the biological effects previously described.

Taking together, all these data suggest that, an inhibition of MRP4 function leads to an imbalance of MRP4 substrates and this provokes an inhibition of cell proliferation and subsequently induction of apoptosis. Simultaneously this pharmacological inhibition caused phenotypic changes associated to the cellular lipid profile.

→ **Figure 4**

→ **Supplementary Figure 3**

3.5. MRP4 is overexpressed under hypoxic conditions

We have already shown that MRP4 has a differential expression pattern in tumors versus normal tissues, and we also observed that there is a heterogeneous expression of MRP4 in intratumoral sectors: MRP4 has greater expression in the

core than in the periphery. In addition, unpublished data from our laboratory suggests that the core cells of this tumor show greater mitotic capacity than those from the periphery (measured by Ki-67 expression). In line with this idea, our Caki cell model showed that there is a direct association between the level of MRP4 expression and the proliferation capacity of these cell lines. On the other hand, numerous reports indicate that cells in the tumor core are often in hypoxia due to their impaired ability to access blood capillaries [36,37]. Considering all of the above, we set out to investigate whether there is a direct association between both phenomena: Hypoxia and MRP4 expression. In order to develop this, we previously designed a chemical hypoxia assay by treatment of Caki-2 cells with $CoCl_2$ [38,39]. First, we determined the range of non-cytotoxic concentrations of this salt for Caki-2 cells (**Figure 5A**). Subsequently, we evaluated whether these experimental conditions represent a hypoxic micro-environment, confirming the increased expression of HIF-2α by both qPCR **(Figure 5B)** and ICC **(Figure 5C-D)**. We observed that the control cells showed a weak and homogeneous cytoplasmic staining pattern, in contrast to treated cells (eg. 300 μM) that show HIF-2α nuclear translocation and exhibit a marked perinuclear or nuclear expression **(Figure 5D,** arrows**)**.

In this context, in the experimental conditions mentioned above, we found a dose-dependent increase in MRP4 mRNA. Particularly with 300 μM $CoCl_2$, we detected a 10-fold increase (**Figure 5E)**. Higher concentrations involve cell cytotoxicity and this probably explain the lower MRP4 mRNA expression at 400 μM.

→ **Figure 5**

4. Discussion

Despite the therapeutic advances achieved using combinations of TK receptor inhibitors and / or immunotherapy, kidney cancer still causes more than 100,000 deaths a year worldwide. Due to the aforementioned mortality, it is urgent to develop reliable, safe and effective therapeutic strategies based on the mechanisms that govern this pathology [4,40].

It has long been known that the mechanisms of resistance acquired by tumors are based on the existence of efflux proteins dependent on the ATP hydrolysis (ABCs)[9,41]. Among them, MRP4 has been described as responsible for chemoresistance or essential for cell proliferation in many tumors, such as Pancreas [42], Neuroblastoma [8], Acute myeloid leukemia [17], Lung [7], Ovarium [43], Esophagus [44], Colon [45,46] as well as a prognostic marker due to its overexpression compared to normal tissues [43,47].

While other MRPs have been reported overexpressed in different types of tumors, in this work to the best of our knowledge, we report for the first time, that MRP4 is overexpressed in ccRCC.

Thus, using search algorithms in virtual platforms, we developed an *in-silico* analysis of the mRNA expression of two transporters with opposite direction of pumping: MRP4 and OATP4C1 [48]. As shown in the obtained results, the combination of the overexpression of MRP4 and the downregulation of OATP4C1 shows the ideal scenario to propose MRP4 as a possible target of pharmacological action. In this sense, all ccRCC tumors examined in this study were found to express MRP4, and its overexpression was significantly high. This is very distinguishable, since normal renal tissues exhibit low MRP4 levels. A proteomic study that evaluated all functional transporters in the healthy renal cortex, showed that MRP4 is expressed at a very low level (less than 1 pg/mg.

Protein), which is in line with the western blotting made in this work [49]. Thus, the MRP4 overexpression observed is comparable to those detected in pancreas [18,42], colon [46], osteosarcoma [50] tumors and it seems to be far above compared to leukemia [51] or lymphoma [52].

ccRCC tumors characteristically show a bright yellow color in the center as a result of its abundant lipid content, and a variegated appearance in the border with hemorrhage, necrosis and/or fibrosis with a well-circumscribed capsule or pseudo-capsule that separates the tumor from adjacent tissues [53]. Since that, we arbitrarily separated the tumor into two zones: *Core* and *Periphery*, and we focused on the analysis of the expression of MRP4 in both zones. We observed a marked heterogeneity of expression of this transporter along tumor tissues, something already described for MRP4 in tumors like prostate [47].

Given the possible therapeutic implications that have been described for MRP4 in other cancer cells, and in order to develop some functional test, we studied its expression in cell lines to validate a model for this transporter. As mentioned before, we first used two cell lines with different degrees of differentiation, Caki-1 obtained from a skin metastasis of renal carcinoma (well-differentiated)[31], and Caki-2 cell line model of clear cell renal carcinoma (less-differentiated)[32,54]. Thus, in line with what was observed by Carozzo and collaborators, who analyzed the correlation between the degree of differentiation and the expression of MRP4 in three pancreatic cancer cell lines [18], we observed that MRP4 levels in Caki-2 are slightly higher than those of Caki-1 in both mRNA and protein. Similarly the same trend was observed in leukemias [17,51] and lymphomas [52], where the malignancy and the degree of dedifferentiation are remarkably linked.

Since both Caki lines express MRP4, we next evaluated their functional faculty to extrude cAMP in *in vitro* cultures. As expected, Caki-2 showed a higher extrusion capacity (~30%) of cAMP confirming that this cell line actually shows a greater amount of MRP4. Additionally, we confirmed the responsibility for extrusion of this cyclic nucleotide by MRP4, using selective pharmacological inhibitors of this carrier. Thus, we abolished the role that MPR4 possesses to modulate cAMP levels both in the intra and extracellular compartments. Based on these findings, as shown in results, Caki-2 proliferates faster, as demonstrated using XTT reagent. These data confirmed that, also in renal carcinomas, a higher expression of MRP4 is associated with higher cell duplication rate and in consequence, greater malignancy.

Different studies show that MRP4, MRP5, and MRP8 induce the extrusion of cyclic nucleotides in various cell types; however, MRP4 has emerged as the main transporter for cAMP [12]. In this sense, Copsel and collaborators, further characterized cAMP efflux by MRP4, in human myeloid leukemia cells and the effect of its pharmacological blockade on cell proliferation and differentiation to a non-neoplastic phenotype [17]. This inspired us to use the same model of pharmacological inhibition to modulate the cAMP pathway in renal carcinoma. In fact, both inhibitors, probenecid and MK-571, were effective to increase the concentration of i-cAMP or depleting the extracellular medium of it, and therefore, decline cell proliferation in Caki-2 cells (see below). This pharmacological model was verified for other types of cancer, and also using shRNA technology, this transporter was silenced or overexpressed and thus cAMP levels could be modulated, leading to equivalent results [18]. These data allowed us to assume, that similar to what was observed in leukemia and pancreas carcinoma, cAMP has a dual role in ccRCC depending on its compartmentalization (extra o intracellular). Thus, our data

and those obtained by others, would allow us to infer that e-cAMP induce cell proliferation and on the other hand, uncontrolled increments of i-cAMP would induce cell stress and apoptosis.

Ultrastructural features reported for Caki-2 cells include microvilli and microfilaments with few mitochondria, and abundant LD [55]. As mentioned before, a remarkable characteristic of the phenotype of ccRCC is their high lipid content [53] with specific lipidomic signatures [21], so the cell lines that represent it, also exhibit these lipid-filled organelles. LDs are formed under very different conditions but undoubtedly cellular stress or an imbalance in lipid metabolism are basic pathognomonic signs that trigger the biogenesis of these organelles. In these cases, it is hypothesized that fatty acids in LDs may serve as protectors against the stressors [33,56]. The combination of drugs used in our model (FSK and/or inhibitors) to rise i-cAMP, induced remarkable cellular morphological changes, already visible only with optical microscopy in the first hours of exposure. A further analysis of the cells under these conditions, using flow cytometry and confocal microscopy, confirmed the LDs biogenesis. Additionally, we also performed a lipidomic analysis and determined the FA content of LD. Cells treated with FSK alone underwent a marked thinning and showed much lower number of LD compared to control cells. Although there was an apparent decrease of 16:0, 18:0 and 18:1, no significant statistical variations were found. Something similar occurs with the decrease in size in LD stimulated with FSK in 3T3-L1 adipocytes [57].

When the cells were treated with the combination of FSK + MK-571, similar morphologic changes (stretching and cell thinning) were observed to those only treated with FSK. However, significant increases were detected in TAG and CE fractions in these cells, demonstrating, that it is the inhibition of the function of MRP4 that generates the

change of cellular lipid composition. First, we thought that in consonance to what was found for other cell lines (*e.g.* myeloid leukemia), these experimental conditions tended to induce a cellular differentiation towards a non-neoplastic phenotype similar to adipocytes. However, we observed that prolonged exposure times to FSK + MK-571, induces apoptosis [58,59].

Although the idea of cell transdifferentiation to an adipocyte-like phenotype is an attractive research option, there are controversies. Several studies have shown that tumor cells reactivate *de novo* lipid synthesis [60] and particularly, Saito and collaborators using an untargeted lipidomic approach clearly showed that CE and TAG are accumulated in ccRCC tumor tissues [21]. However, our data shows that when this increase in lipid biosynthesis is achieved in combination with the blockade of MRP4, the cells stop their proliferation and trigger cell apoptosis. So, it remains to be determined whether the increase in lipid synthesis in these tumor cells is positive or not.

Another key point that intertwines and governs many important essential metabolic processes in tumor cells is hypoxia. We previously described the relationship among proliferation, survival, and apoptosis with the expression of key molecules related to tumoral hypoxia in ccRCC tumors (hypoxia-inducible factor (HIF)-1α, erythropoietin (EPO), vascular endothelial growth factor (VEGF), and their receptors (EPO-R, VEGFR-2) [24]. In addition, in this work we also showed that MRP4 is upregulated in these tumors. Thus, we investigated whether there is a direct relationship between both phenomena, in order to know if MRP4 function is also engaged with the phenotype of this tumor. We demonstrated that, in the chemical hypoxia model set up, MRP4 exhibited a significant increase in expression. Although this is a novel data for ccRCC, it is perfectly consistent with the previous data obtained since that: **a)** we observed a differential expression of

MRP4 in core and peripheral in ccRCC tumors; **b)** there is an increased expression of hypoxia markers in the core; **c)** tumor cells exhibit an increased proliferative capacity under hypoxia conditions; and **d)** we observed a higher expression of MRP4 in Caki-2 cells, which are the ones cells with the greatest proliferative capacity.

Although these results are preliminary and additional experiments are needed to determine the mechanism of activation of the synthesis of MRP4, these findings would indicate that the target points that regulate hypoxia could modulate the expression of MRP4 and in consequence cAMP levels.

All in all, data presented herein show direct evidence that MRP4 is a differential marker in solid ccRCC tumors, which is involved in the cAMP signaling pathway and therefore, could be a new pharmacological target to be employed in therapeutic strategies for the treatment of ccRCC so far not addressed.

5. Acknowledgements

The authors thank Montse Duque for the excellent technical assistance.

6. Author contributions

Juan Pablo Melana Colavita: Conceptualization, Methodology, Writing. **Maximiliano de Sousa:** Software. **Juan Santiago Todaro, Leandro Ferrini, María May, Carlos Guijas, Natalia Gomez, Agustin Yaneff, Nicolas Di Siervi**: Methodology, Supervision, Data Curation. **María Victoria Aguirre, Carlos Davio** and **Juan Pablo Rodríguez**: Conceptualization, Writing- Reviewing, Editing and Project administration.

7. Funding

This research was funded by Universidad Nacional del Nordeste, SGCyT-UNNE PI18-I009; UBACYT-20020170100674BA-2018 and MINCYT-FONCYT- PICT-2015-2996.

8. Conflict of Interest Statement

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results

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10. Legends

Figure 1. ***Differential expression of MRP4 in tumors and normal samples.*** **A**. *in-silico* analysis showing MRP4 over differentially expressed in Clear Cell Renal Carcinoma (ccRCC) samples when compared to their proper non-tumoral samples (n=69). In opposition OATP4C1 is downregulated. The probe's ID for MRP4 and OATP4C1 are shown in both cases. *Student's t-test* was used to determine differences. **** indicate statistical significance at the p<0.0001 level. **B**. MRP4 mRNA was quantified by RT-qPCR, normalized by β-actin mRNA and expressed relative to normal in tumor (Core or Periphery; n= 18). MRP4 is overexpressed in most of the protein homogenates obtained from ccRCC (Core or Periphery) compared to distal normal renal samples from the same patient. One patient was selected for illustration. Differences among groups were analyzed by one-way analysis of variance (ANOVA; **** p<0.0001; *** p<0.001; * p<0.05). Densitometric analysis of immunoreactive MRP4 band intensities (in arbitrary units) were normalized with those of β-actin. **C – J.** Two representative patients of the analyzed samples were selected for immunohistochemical staining for membranous MRP4 expression in ccRCC samples. **C** & **F**. In normal renal tissue, a full absence of glomerular protein expression is observed in opposition to the highly ordered and polarized proximal tubular expression. **D** & **G**. The expression of MRP4 in the tumor Core appears disordered with foci of intense expression. **E** & **H.** In contrast peripheral tumor tissues show a weak reduction of MRP4 with infrequent intense pockets of protein expression. **I.** Normal positive control of MRP4 expression in Colon. **J.** Semi-quantification of MRP4 expression and localization in ccRCC. Magnification 400x.

Figure 2. ***MRP4 expression in renal cell lines***. **A**. MRP4 mRNA was quantified by RT-qPCR in Caki-1 and Caki-2 cell lines. The MRP4 expression was normalized by β-actin mRNA. HCT-116 cell line was used as positive control. Differences among groups were analyzed by one-way analysis of variance (ANOVA; * p<0.05). **B.** Western blot analysis showing the protein expression in cell lines. **C.** Densitometric analysis of immunoreactive MRP4 band intensities in renal cell lines. *Student's t-test* was used to determine differences. * indicate statistical significance at the p<0.05 level. **D-F.** ICC detection and localization of MRP4 in HCT-116 (**D**), Caki-1 (**E**), Caki-2 (**F**). Note the only membranous expression profile in colon cells (black arrows) compared to the intense cytoplasmic expression of both Caki cells (light blue and red arrows). Magnification 400x.

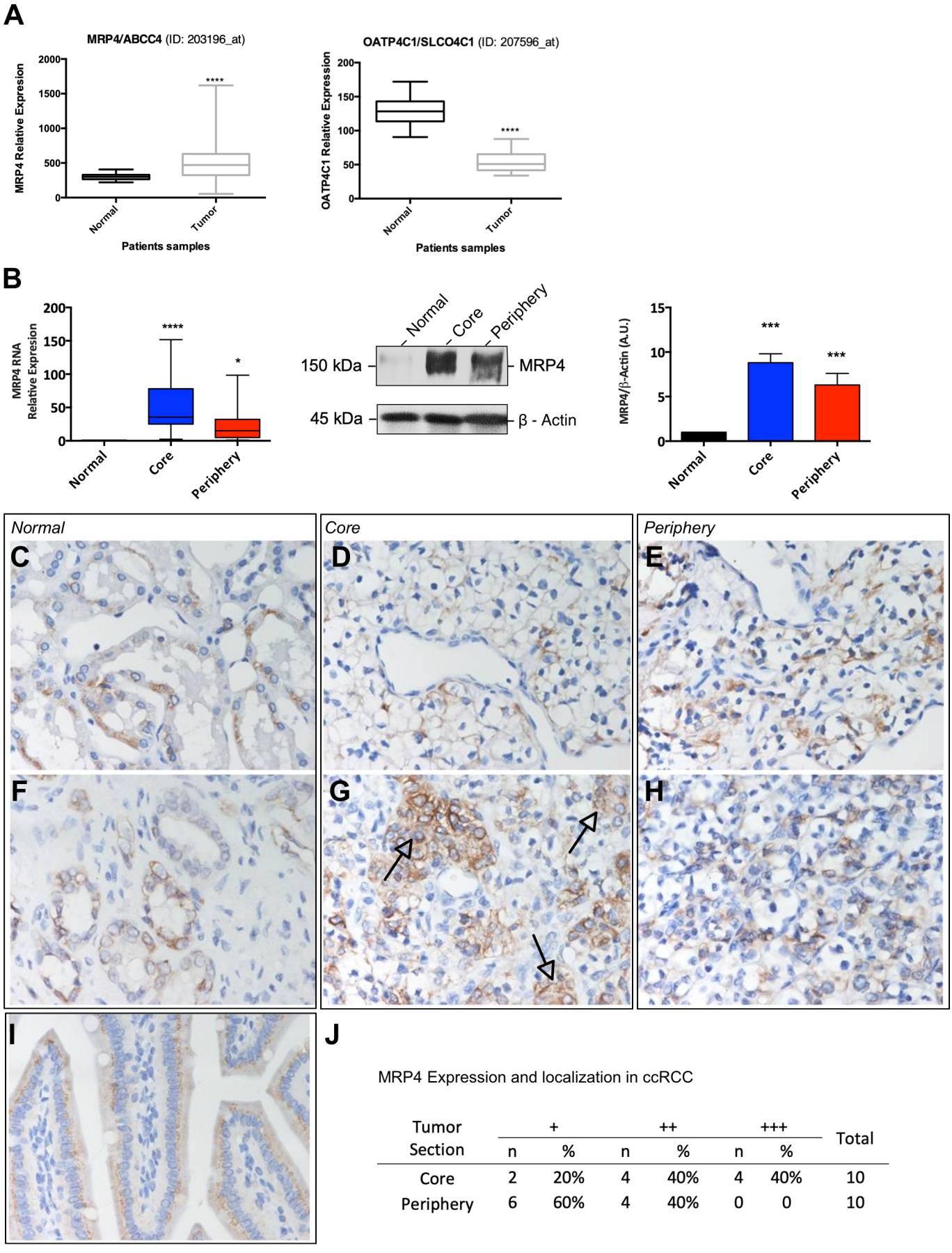
Figure 3. ***cAMP efflux is mediated by MRP4 in Caki-1 and Caki-2 cells and it is engaged in cell proliferation*****. A.** Cells were incubated with IBMX (1 mM) and subsequently stimulated with FSK (25 μM) for

5 minutes. e-cAMP was measured in supernatants, whereas i-cAMP was obtained from cellular scrapings. **B.** Pharmacological inhibition of MRP4 with MK-571 (25 μM) or Probenecid (0.5 mM) induced an accumulation of the cyclic nucleotide in the intracellular space in Caki-2 (light-blue). **C.** The role of MRP4 in cell proliferation was assayed in both Caki cell lines. Note that Caki-2 line has a shorter time of cellular duplication as demonstrated by XTT® proliferation assay. **D.** Cells monolayers were grown up to confluence and then were depleted of FBS-supplemented culture medium. Two hours later, different cAMP concentrations were added and cell proliferation was evaluated 48 h later. **E.** Cell cycle stages were evaluated using propidium iodide (PI) staining in flow cytometer. cAMP-treated cells showed a significant increase in Phase S.

Figure 4. ***Pharmacological inhibition of MRP4 induced phenotypical changes, produced inhibition of cell proliferation and triggered apoptotic cell death***. **A.** Gating strategy in control and treated cells. **B.** Caki-2 control cells (red) and treated with FSK + MK-571 (blue) for 18 h, an then they were stained with Bodipy® and acquired (2.10^4 events) in linear mode. A shift to the right of the treated cells was observed, indicating an increase in cytoplasmic LD (cell stress signal). **C.** Media fluorescence intensity (MFI %) of the control and treated populations. **D.** Confocal microscopy of LD in Caki-2 controls. **E.** Positive control of LD biogenesis induced by OA (30 μM). **F.** Caki-2 treated cells (MK-571 + FSK; both 25 μM). Original magnification 400x. **G.** Total lipid extract of control and treated cells were obtained and the content of the TAG and CE fractions (LD) was analyzed by CG-MS. Fatty Acids (FA) esterified in cellular glycerol (TAG) and into cholesterol is shown. An elevation in FA content (particularly 16:0 and 18:0) is observed in cells treated with FSK + MK-571. **H.** Caki cells treated with FSK + MK-571 or FSK + Probenecid for 48 – 72 h exhibited an impaired cell proliferation. **I**. Cells treated with FSK + MK-571 (25 μM) for 48 h showed a cellular accumulation in the Sub G_0 stage measured by staining with PI and evaluated by flow cytometry. **J**. Apoptosis cells death were studied analyzing caspase 3 activation by ICC. Cells exposed to FSK + MK-571 (both 25 μM) by 48 h showed extensive deposition of caspase 3, similarly to those cells treated with staurosporin (STP; 1 μM). Magnification 400x. Data are representative of three different experiments and are shown as mean ± SD. Differences among groups were analyzed by ANOVA or *t*-student test correspondingly (* $p<0.05$ or *** $p<0.001$).

Figure 5. ***MRP4 is overexpressed under hypoxic conditions***. **A.** Caki-2 cell viability in the range of 0-400 μM $CoCl_2$ concentrations. Cell cytoxicity was observed when cells where exposed to 400 μM $CoCl_2$. **B.** Overexpression of HIF-2α evaluated by qPCR under hypoxic conditions *in vitro*. **C-D.** ICC of HIF-2α. Control cells show a weak and homogeneous cytoplasmic staining pattern of HIF-2α; in opposition, cell treated with 300 μM $CoCl_2$ showed perinuclear or nuclear recruitment of the nuclear factor HIF-2α (black arrows). Both panels **C** and **D** 100x magnification (lower right quadrants 400x). **E.** MRP4 mRNA expression measured by qPCR in normoxic and hypoxic conditions.

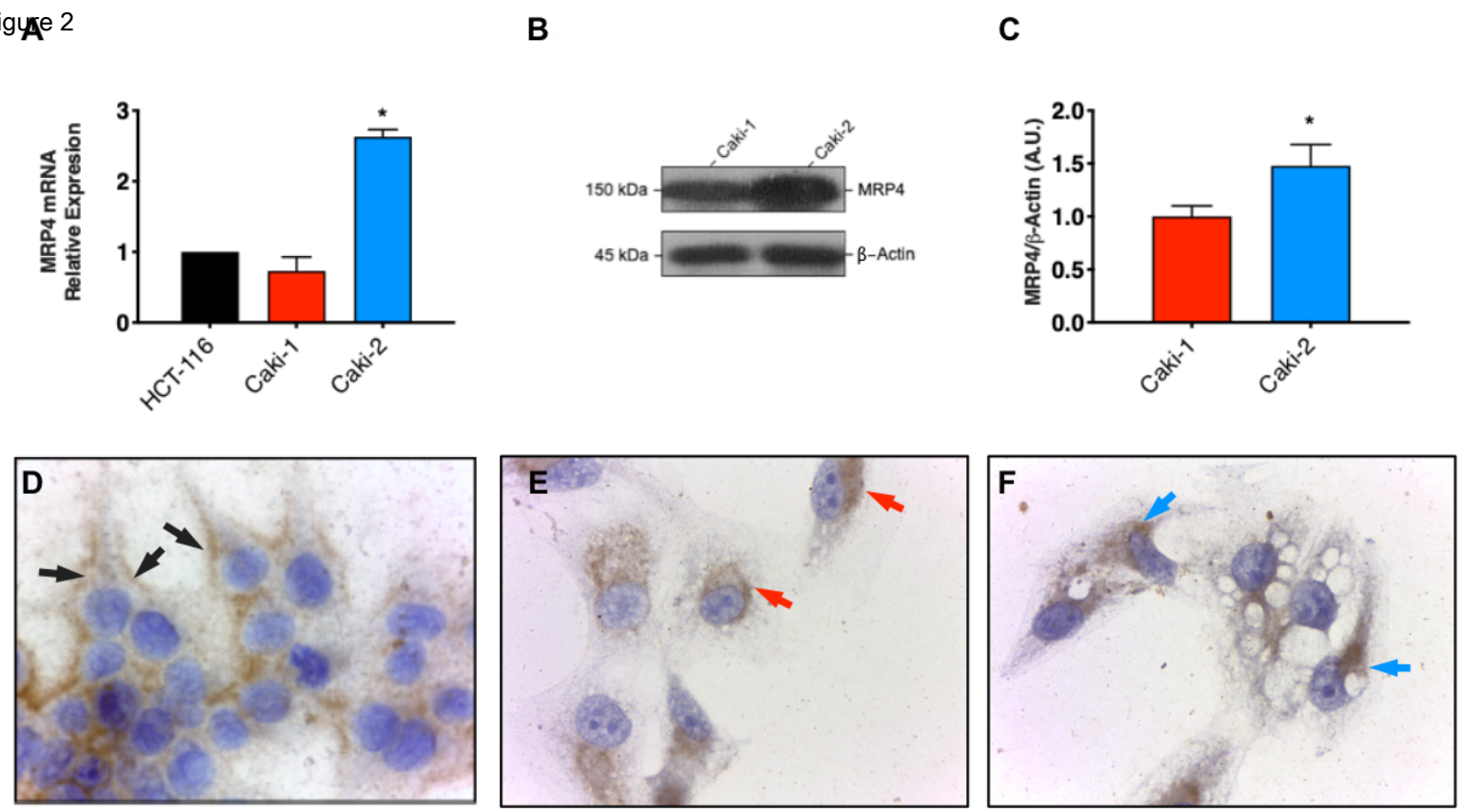
Figure 1



MRP4 Expression and localization in ccRCC

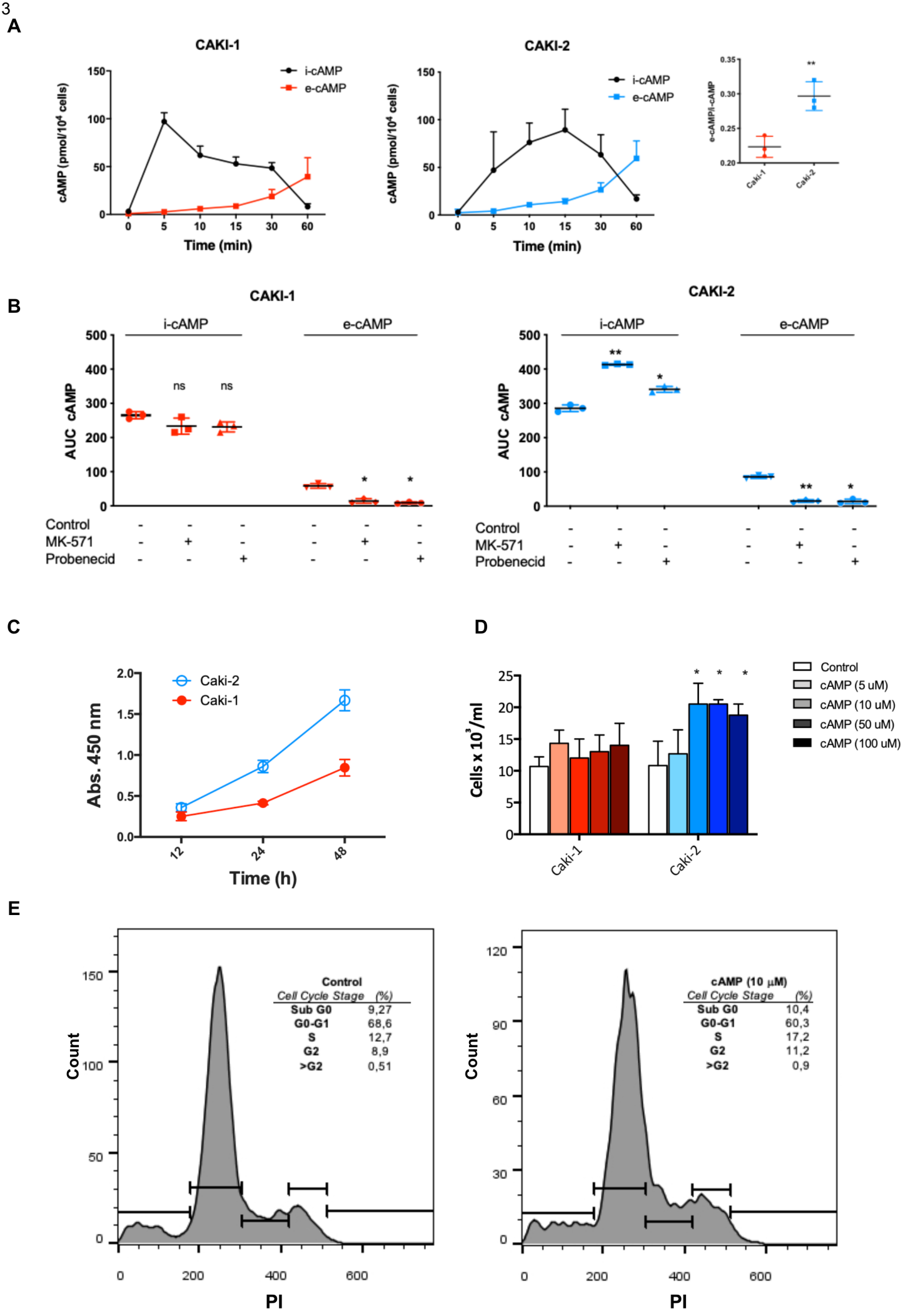
Tumor Section	+		++		+++		Total
	n	%	n	%	n	%	
Core	2	20%	4	40%	4	40%	10
Periphery	6	60%	4	40%	0	0	10

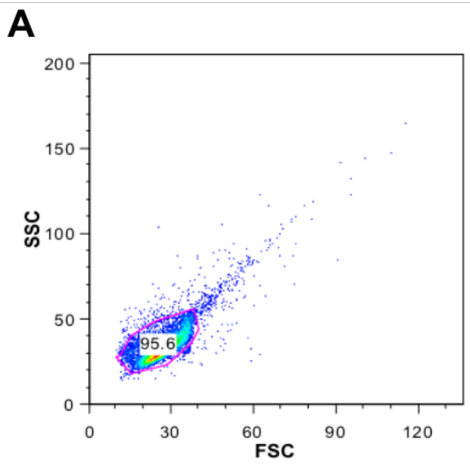
Figure 2

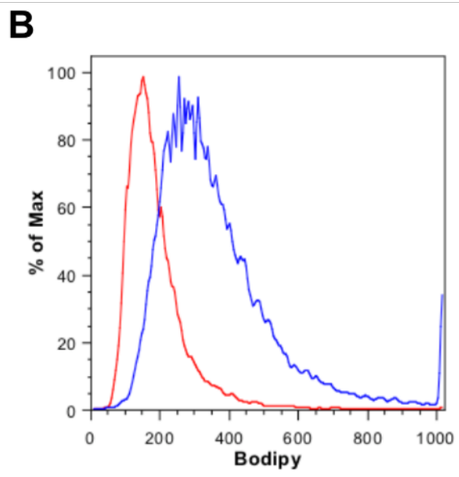

A
MRP4 mRNA Relative Expresion
3
2
1
0
*
HCT-116
Caki-1
Caki-2
B
Caki-1
Caki-2
150 kDa
MRP4
45 kDa
β-Actin
C
MRP4/β-Actin (A.U.)
2.0
1.5
1.0
0.5
0.0
*
Caki-1
Caki-2

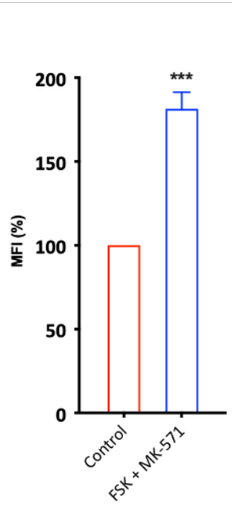
D
E
F

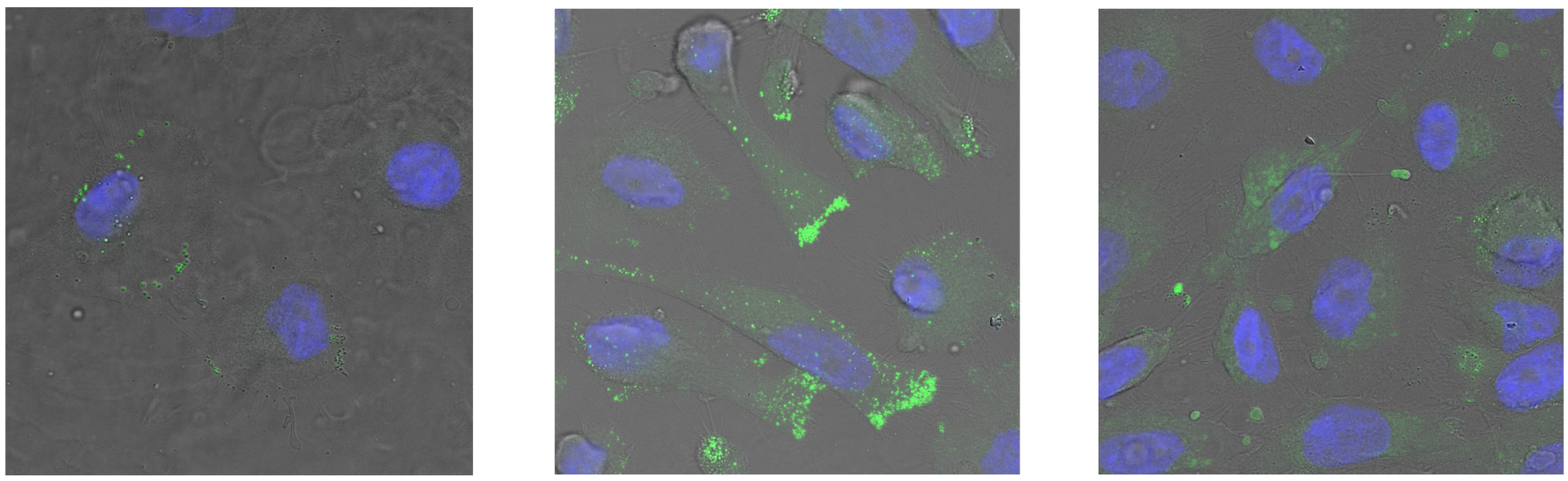
Figure 3

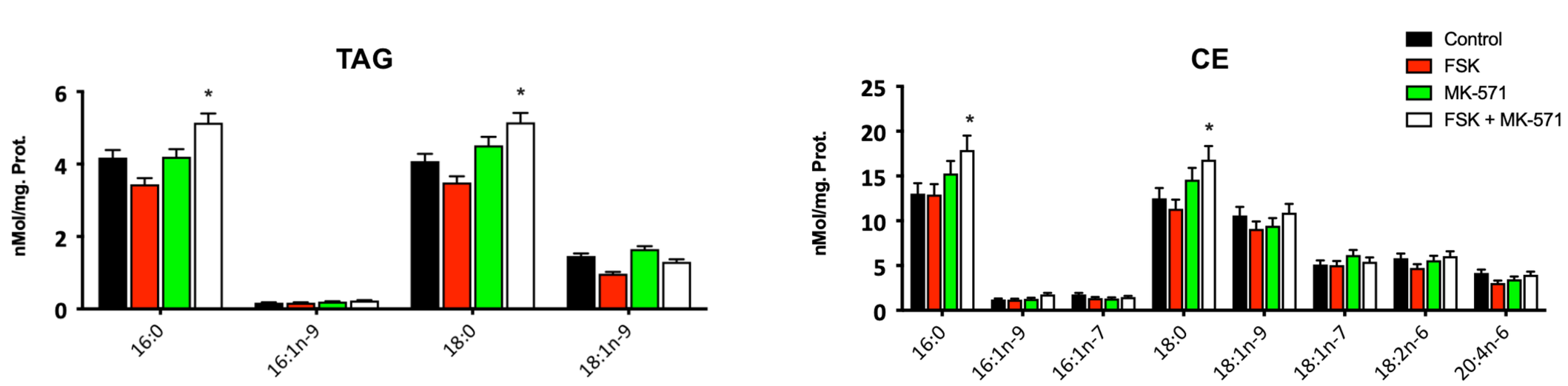

A
CAKI-1
i-cAMP
e-cAMP
cAMP (pmol/10⁴ cells)
Time (min)
CAKI-2
e-cAMP/i-cAMP
Caki-1
Caki-2
B
CAKI-1
CAKI-2
i-cAMP
e-cAMP
AUC cAMP
ns
Control
MK-571
Probenecid
C
Caki-2
Caki-1
Abs. 450 nm
Time (h)
D
Control
cAMP (5 uM)
cAMP (10 uM)
cAMP (50 uM)
cAMP (100 uM)
Cells x 10³/ml
E
Control
Cell Cycle Stage (%)
Sub G0 9,27
G0-G1 68,6
S 12,7
G2 8,9
>G2 0,51
cAMP (10 µM)
Sub G0 10,4
G0-G1 60,3
S 17,2
G2 11,2
>G2 0,9
Count
PI

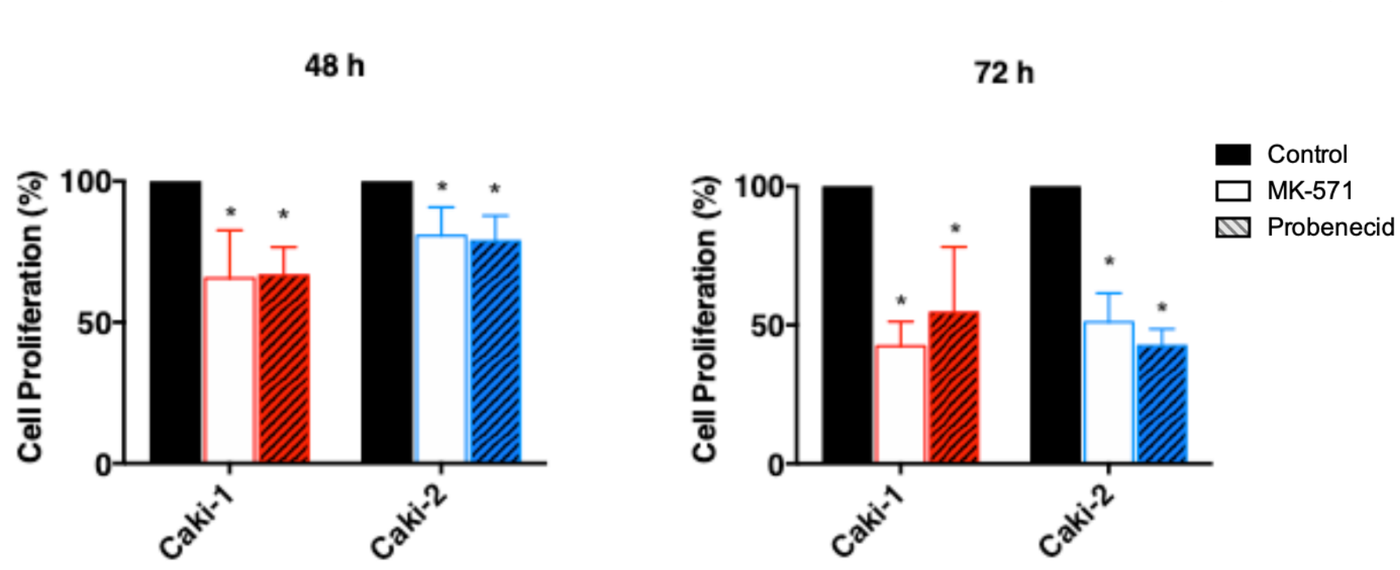

A
SSC
FSC
95.6

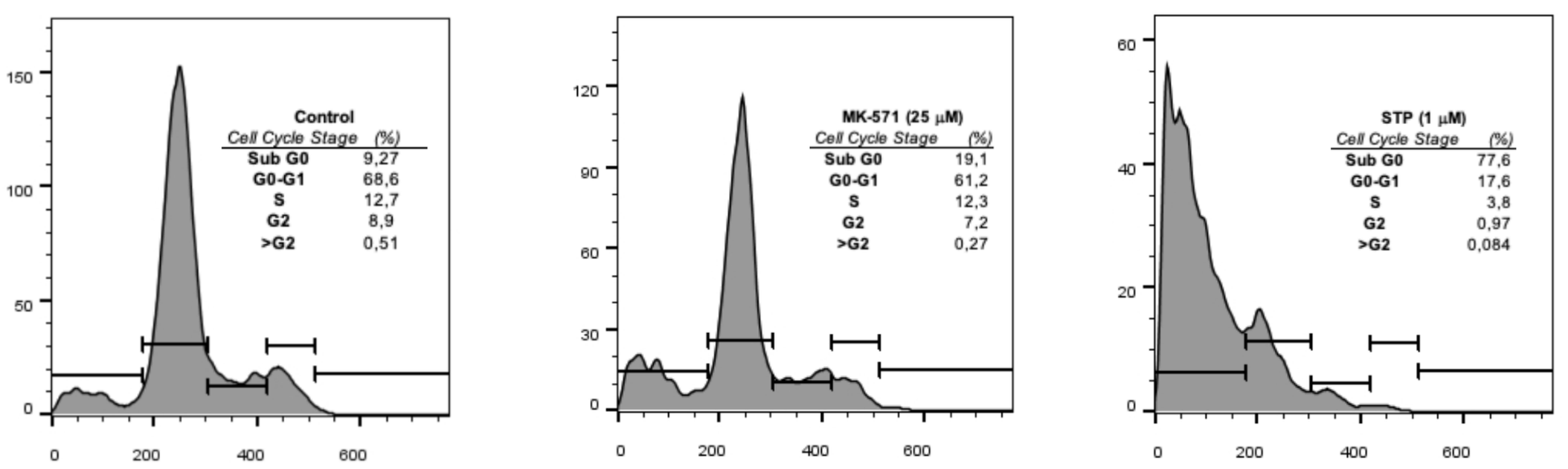

B
% of Max
Bodipy


C
MFI (%)
***
Control
FSK + MK-571


D
E
F


G
TAG
CE
nMol/mg. Prot.
Control
FSK
MK-571
FSK + MK-571
16:0
16:1n-9
16:1n-7
18:0
18:1n-9
18:1n-7
18:2n-6
20:4n-6


H
48 h
72 h
Cell Proliferation (%)
Control
MK-571
Probenecid
Caki-1
Caki-2


I
Control
Cell Cycle Stage (%)
Sub G0 9,27
G0-G1 68,6
S 12,7
G2 8,9
>G2 0,51
MK-571 (25 µM)
Cell Cycle Stage (%)
Sub G0 19,1
G0-G1 61,2
S 12,3
G2 7,2
>G2 0,27
STP (1 µM)
Cell Cycle Stage (%)
Sub G0 77,6
G0-G1 17,6
S 3,8
G2 0,97
>G2 0,084

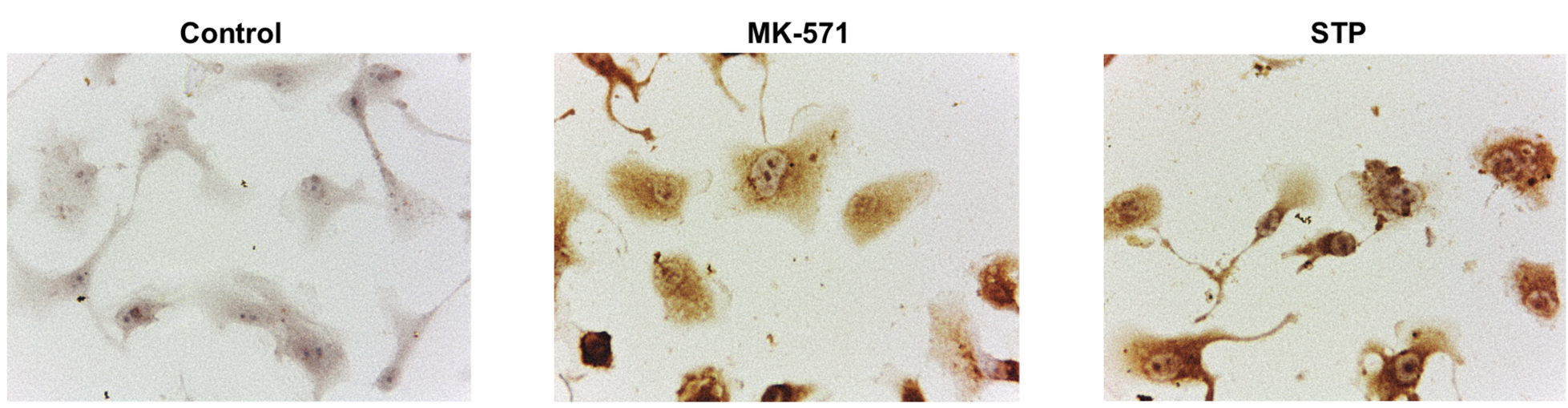
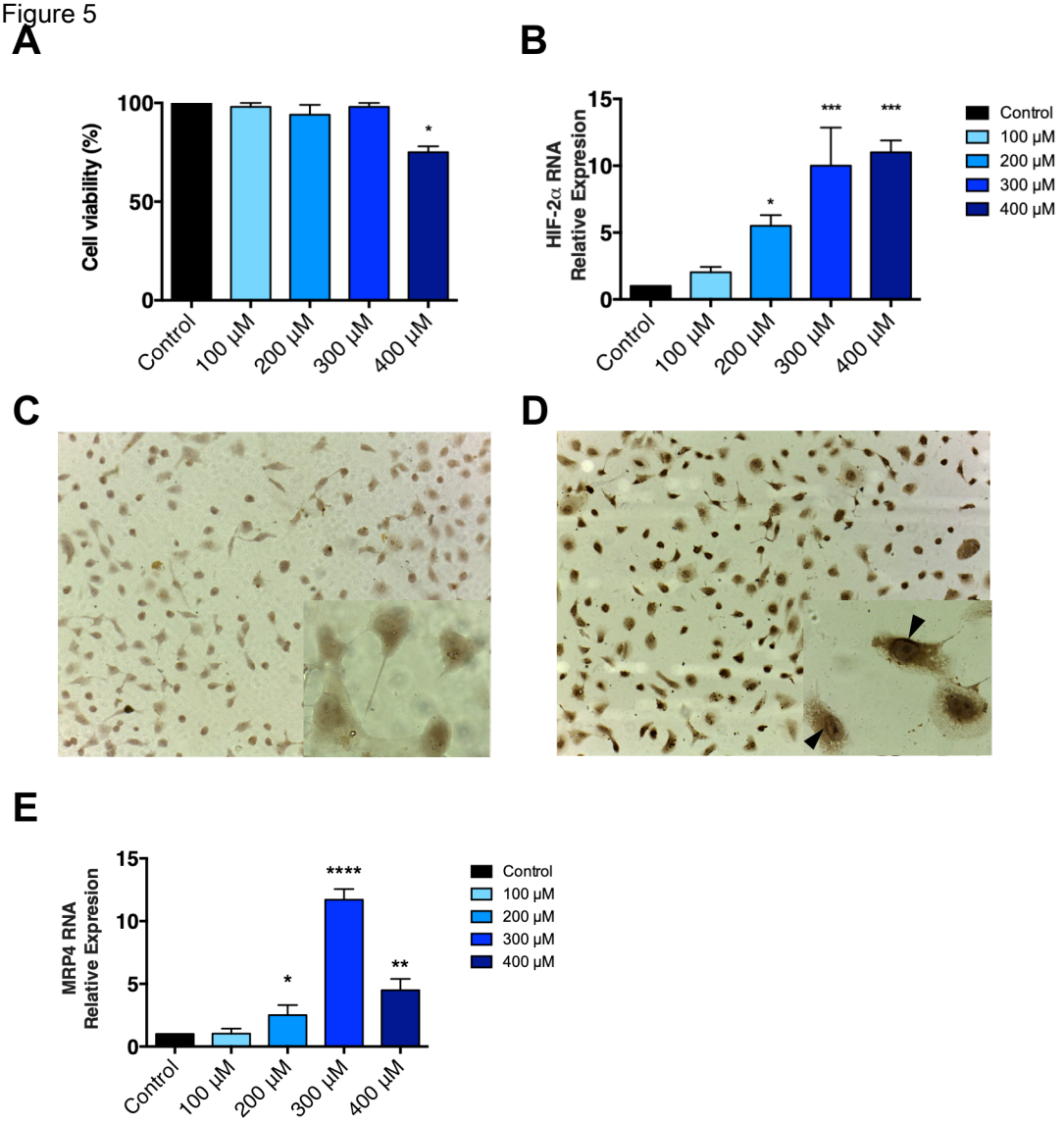

J
Control
MK-571
STP

Figure 5


A
Cell viability (%)
100
50
0
*
Control
100 μM
200 μM
300 μM
400 μM
B
HIF-2α RNA Relative Expresion
15
10
5
0
*
***
***
Control
100 μM
200 μM
300 μM
400 μM
C
D
E
MRP4 RNA Relative Expresion
15
10
5
0
*
****
**
Control
100 μM
200 μM
300 μM
400 μM