The Association of Propranolol with 2DG Reduces the Metabolism and the Proliferation of Cutaneous Squamous Cell Carcinoma A431 Cell Line

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Research Article

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Abstract

The non-selective β-blocking (±)-Propranolol Hydrochloride was demonstrated to improve the progression-free survival of patients and to reduce the incidence of different cancer types. Since the expression of β-adrenoceptors (β-AR) in the A431 squamous cell carcinoma (cSCC) human cells was described, we had suggested that cSCC proliferation may be controlled by using β-AR-blockers. Thus, we hypothesized that the topical application of a β-AR-blocker over the tumor lesion may decrease/restrain its extension before the surgical excision becoming an adjuvant\therapy against cSCC. However, it is known that β-AR-blocker anti-cancer activity as a single agent is limited. Hence, we suggested that the combination of Propranolol with the glucose analog 2-Deoxy-D-glucose (2-DG) could improve its antiproliferative effect through the induction of metabolic stress. Our results have demonstrated that the addition of 2DG to (±)-Propranolol Hydrochloride therapy can improve its effect on A431 cells metabolism and proliferation, suggesting that the combination of (±)-Propranolol Hydrochloride with low dose of 2DG could be a promising treatment to be topically applied as an adjuvant pre-surgical therapy against cSCC, aiming to decrease the size of the injury before the surgical procedure, avoiding systemic adverse effects to the patient.

Introduction

The cutaneous squamous cell carcinoma (cSCC) is the second most frequent skin cancer in white ethnic populations worldwide[1], and even if most of the cases are easily cured by surgical removal, this cancer remains the cause of the majority of non melanoma skin cancer (NMSC) deaths. This is due to “high-risk SCCs”, which are associated with significant metastasis, morbidity, and death[2]. Among the main cause of cSCC, the DNA damage by ultraviolet (UV) radiation exposure is the most common, since it causes the deregulation of important signaling pathways that are involved on cell cycle, apoptosis, DNA repair and cell differentiation[3]. Other risk factors to cSCC promotion are immunosuppression, human papilloma virus (HPV) infection[4], genetic disorders and smoking. Sporadically, cSCC can be also associated with non-healing wounds/scarring, or chronic lesions preceded by chronic inflammatory processes[5].

Usually, *in situ* cSCC may be controlled by different interventions, including electrodessication and curettage, topical therapy, cryotherapy, and photodynamic therapy; however, since these treatments are not appropriate for invasive cSCC, surgical excision is usually indicated. The surgical procedure creates wounds that could be small, superficial, and amenable to primary closure, but often they can be large, deep, and extensive needing more complex closure and covering. Specifically scalp injuries, due to its low elasticity, can be devastating and can require significantly more extensive surgeries, concerning both the number and complexity. Consequently, we believe that an effective therapeutic strategy could be to control the extension of the lesion before its excision aspiring for a less invasive surgery with less devastating wounds.

Multiple intracellular signal transduction pathways that are involved events such as cellular replication, inflammation, angiogenesis, apoptosis, cell motility and trafficking, activation of tumor- associated

viruses, DNA damage repair, cellular immune response and epithelial–mesenchymal transition (reviewed by Coelho M et al) are regulated through interactions of α- and β-adrenoceptors (AR) and Catecholamine (CA) neurotransmitters. Tumor cells may express β-AR, and the involvement of β-adrenergic signaling in the progression of malignant diseases has been increasingly recognized[-]. The use of beta-blocker therapy can reduce the incidence of prostate cancer and improve the prognosis of patients with breast and hepatocellular cancer. The expression of β-AR in the A431 cSCC human cells was described in 1987 by Kashles and Levitzki, which leads us to believe that cSCC proliferation may be controlled by using β-AR-blockers. Thus, we hypothesized that the topical application of a β-AR-blocker over the tumor lesion may decrease/restrain its extension before the surgical excision becoming an adjuvant\therapy against cSCC. The topical application of β-AR-blocker (*timolol*[-] and *propranolol*) was already described on infantile hemangioma, with no collateral effects.

Recent evidence has shown that non-selective β-blocking (±)-Propranolol Hydrochloride, improved the progression-free survival of breast cancer patients, and reduced the risk of developing head and neck, prostate, esophagus, stomach, and colon cancers. It is known that the (±)-Propranolol Hydrochloride anti-cancer activity is due to its ability to inhibit the mitochondrial metabolism, which can increase the cell glycolytic activity resulting in elevated metabolism and switch towards aerobic glycolysis, which could stimulate the tumor progression and drug resistance. However, its anti-cancer activity as a single agent was demonstrated to be limited.

Hence, based on the well-known Warbur effect, cancer cells boost glucose uptake and conversion into lactate in the presence of high oxygen tension, exploiting the aerobic glycolysis, we suggested the combination of propranolol with the glucose analog 2-Deoxy-D-glucose (2-DG) aiming to improve its antiproliferative effect. 2DG is a well-known antidiabetic drug, which by competition can inhibits glucose uptake, blocking the first critical step of glucose metabolism and the mitochondrial respiration, inducing a metabolic stress. 2DG increases autophagy, a ubiquitous cellular catabolic process that under conditions of protracted stresses suppresses tumorigenesis. 2DG treatment alone does not significantly induce cancer cells death, but it may use with specific agents or to exert a synergistic therapeutic action.

To confirm these hypotheses, we performed in vitro assays using the human A431 cSCC cell line. We demonstrated that the addition of 2DG to (±)-Propranolol Hydrochloride therapy can improve its effect on A431 cells metabolism and proliferation.

Results

Combination of (±)-Propranolol Hydrochloride and low dose of 2DG decreases the proliferation of human cSCC cells

To determinate sensitivity of A431 cells to different concentrations of (±)-Propranolol Hydrochloride and 2DG, we performed the MTT test that establishes cell metabolism by measuring the functionality of

mitochondrial dehydrogenases. Results revealed that 48h treatment of Propranolol Hydrochloride reduced A431 cell metabolism in a dose dependent manner (Fig. 1A), starting from 200uM.

2DG treatment reduced the cells' metabolism, starting from 0.5mM; however, we did not detect statistical differences between concentrations from 0.5mM to 5mM (Fig. 1B).

Usually, the aim of combined two or more medicines is increasing the effects minimizing the collateral effects of each medicine. Therefore, all subsequently experiments were performed by using the lower effective doses of propranolol (200uM) and 2DG (0.5mM).

Because the MTT assays measures the mitochondrial metabolic rate, and it indirectly reflect the viable cell numbers, this assay is commonly performed as a cell proliferation assay. However, to confirm the obtained results of MTT, we performed a cell proliferation assay by counting the alive cells after 48 hours of treatment. Our results demonstrated that the combination of 200μM of (±)-Propranolol Hydrochloride and 0.5mM of 2DG determined a significant reduction of A431 cells' metabolism (< 20%) (Fig. 2A) and proliferation compared to 2DG or propranolol alone (Fig. 2B).

Ki-67 is an antigen associated with mitosis in mammalian cells, vastly used as a cell proliferation marker. Our results demonstrated that after 24h, a time point in which the differences start to being seen on cell proliferation (Fig. 2B), the Ki-67 expression was reduced A431 cells treated with propanolol, 2DG or combination (Fig. 3). The decreased expression of Ki-67 in 2DG treatments is consistent with the decrease of cell numbers at 24 hours (Fig. 2B). We believe it is because in tumor cells, glycolysis contributes less than 50% for energy production, then the early effect of 2DG by itself could be circumvented by oxidative phosphorylation's process.

The combination of Propranolol 2DG slightly increases the apoptosis and does not affect cell cycle in A431 cells

Aiming to ascertain the cytotoxic and/or cytostatic effects of propranolol and propranolol + 2DG, we performed apoptosis and cell cycle assays. Our data demonstrated no significant apoptotic role of 200μM propanolol, which corroborates with preview studies. The same was observed in the treatment with 0.5mM 2DG, which did not induce apoptosis. However, the combination of propranolol and 2DG had a slightly superior apoptotic effect than the drugs singularly, but still very limited (± 5%) (Fig. 4).

Cell cycle analysis revealed that treatment with (±)-Propranolol Hydrochloride determined a modest increase of A431 G1 phase cells, but without statistical significance. 2DG alone reduced the percentage of cells in S and G2 + M, apparently by arresting cells in G1, which corroborates to other studies[7]. On the other hand, the combination of Prop + 2DG had slightly increased the percentage of cells on G2 phases (Fig. 5).

Discussion

The adrenoceptors belong to the G protein-coupled receptor family (GPCRs), and are divided into α and β. The β-ARs in turn, are subdivided in three subtypes: β1, β2, and β3. Their activation triggers a range of transcriptional regulators pathways that modulate the expression of numerous genes including interleukin-6 (IL-6), vascular endothelial growth factor (VEGF), interleukin-8 (IL-8), and matrix metalloproteinases, which promote angiogenesis, cellular invasion, and inflammation. Consequently, an increasing number of studies have been establishing on different tumor types the β-ARs subtypes, their expression, and their role on cancer processes (Reviewed by Tang J, *et al.*).

In the present study, we have demonstrated that doses up to 200uM of the non-selective β-AR (±)-Propranolol Hydrochloride can decrease the proliferation of A431 cell line. Even if no substantial statistical differences on cell cycle and apoptosis assay were observed, cells that were treated with 200μM of Propranolol had demonstrated an important decrease of Ki67 expression, which suggest that cell proliferation and growth were affected by the treatment.

However, we had verified that the association of Propranolol with the glucose analog 2DG improves the cSCC cells sensitivity to the treatment, by decreasing their metabolism and consequently the proliferation of these cells. It was confirmed by the decrease of the cell growth (about 58%) and the expression of the Ki-67 (about 78%) after 48h of the treatment. We also observed that differently of single treatments, the association of propranolol + 2DG has slightly increased the apoptotic events.

Since it is known that the oral assumption of propranolol can cause some reversible, but inconvenient adverse effects, such as lower heart rate, diarrhea, dry eyes, hair loss, nausea, hypoglycemia, weakness or tiredness[7], we believe that topical application of propranolol could be a promising option for cSCC therapy being a promising strategy to decrease the area of the tumor before a surgical removal, avoiding large dimensions wounds caused by the biopsies procedures that tend to be difficult to heal, mainly in the scalp region. The topical application of propranolol is already used for infant hemangioma therapy[7], with the vantages of the absence of the systemic side effects of oral administration. Even the topical use of 2DG can be an effective pharmacological agent if used in appropriate vehicle and at the proper dosage.

Propranolol as a dermatologic therapeutic tool was first described in 2008, and its relatively low adverse risk profile makes it a versatile tool to use both systemically and topically. It was reporter that Propranolol blocks the late phase of autophagy then, when in condition of enhancing autophagy flux, cancer cells have demonstrated to be especially sensitive to propranolol. A modulation of autophagy may provide a promising avenue to cancer therapy but considering complex relationship between autophagy and cancer investigating the effect of propranolol on autophagy in cSCC cell line would be desirable. In this way, even being aware that the present results are preliminary and *in vivo* experiments should be performed, we hardly believe that these are the first steps to the development of a promising medicine that could be topically applied as an adjuvant pre-surgical therapy against cSCC, aiming to decrease the size of the injury before the surgical procedure, avoiding large wounds or scars, without systemic adverse effects to the patient.

Material And Methods

Propranolol solution

(±)-Propranolol Hydrochloride (Sigma St. Louis, MO, USA) dilutions were made using 1:1 (v: v) Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12 Ham (SF-DMEM: F12) (Sigma St. Louis, MO, USA) in a stock solution of 400uM/L, filtered with a 0.22µm (Millipore, Burlington, MA, USA). The solution was diluted freshly prior to each experiment to different concentrations as indicated in each assay. Corroborating with the results of Bustamante *et al*. 2019 whom have demonstrated that on melanocytes 200µM/L of Propranolol Hydrochloride has cytotoxic effect, but not with 50µM, we started to see effects only with concentrations over 100µM (data not shown), thus, we performed the experiments using concentrations from 100µM to 300µM (100, 150, 200 and 300µM).

2DG solution

2-Deoxy-D-glucose (Calbiochem, San Diego, CA, USA) dilutions were made using 1:1 (v:v) Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12 Ham (SF-DMEM:F12) (Sigma St. Louis, MO, USA) in a stock solution of 5mM/L, filtered with a 0,22µm (Millipore, Burlington, MA, USA). The solution was diluted freshly prior to each experiment to different concentrations as indicated in each assay. Based on the literature, we have investigated the cytotoxic effect of 2DG in five different concentrations: 1mM, 2mM, 3mM, 4mM and 5mM.

Cell culture

The epidermal squamous cell carcinoma A431 (ATCC® CRL-1555™) (Cell Applications, San Diego, CA, USA) were cultivated on DMEM/Ham's F12 medium (Sigma St. Louis, MO, USA), added with 1x nonessential amminoacids (Lonza™ BioWhittaker™. Basel, CH), L-glutamine (2.5 mM,) gentamicin (1ul/ml) and FBS 10%. Medium was routinely changed every 3 days and at confluence cells were subcultured (split ratio 1:5) by trypsinization (0.5% trypsin/0.02% EDTA).

Metabolic viability – MTT assay

To evaluate the effect of different doses of propranolol on the cellular metabolic activity, we used the colorimetric assay with MTT [3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]. Briefly, cells ($5x10^3$cells/well) were seeded into 96-well plates. After 24 hours of incubation, the medium was added with the specific doses of propanolol diluted in complete cell culture medium (150uM/L – 350uM/L), or complete medium which was used as a reference. After 48h, MTT phosphate buffered solution (final concentration of 0.1 mg/ml) was added to each well and cultures were incubated at 37°C for 3h. The supernatant was removed from the wells by slow aspiration and replaced with DMSO (100µl per plate) to solubilize the MTT tetrazolium dye. At the end of incubation time, the optical density (OD) was measured at 550nm wavelength using a microplate reader (Spectrafluor Plus; TECAN Austria GmbH, Grödig, Austria). Three replications' wells were used for each analysis. The percentage of cell viability was calculated vs. the complete medium (assumed as 100%).

Cell proliferation assay

Cells were seeded ($5x10^3$ cells/well) in a 96-wells plate and incubated for 24 hours. Them we treated the cells with 200uM of propranolol, 0,5mM 2DG or propranolol/2DG. Control was made by adding fresh complete medium. Three replications' wells were used for each analysis. Cells were them detached and alive cells were manually counted in three times: before treatments, 24h and 48h after the treatments.

Cell proliferation assay by Ki67 antigen

Cells were grown over night on glass coverslips and then treated with 200uM of propranolol during 24h. Cells were washed twice with 1 mL of cold phosphate buffered saline (PBS), fixed for 20 min in 3.7% paraformaldehyde in PBS and permeabilized with 0.3% Triton X-100 in PBS for 5 min. Cells were incubated in blocking buffer (5% FBS and 0.3% Triton X-100 in PBS) for 1 h at room temperature. Then, the cells were incubated overnight at 4°C with primary antibody Ki-67 (sc-23900) (Santa Cruz Biotechnology, Dallas, TX, USA) and successively for 1 h with the anti-mouse Alexa Fluor 488 (Cat #A21121) (ThermoFisher, Waltham, MA, USA) at room temperature. After staining of the nuclei with Hoechst 33242 dye (4',6-diamidino-2-phenylindole; (Sigma St. Louis, MO, USA), the cells were dried, mounted onto glass slides with DPX Mountant for histology (Sigma St. Louis, MO, USA), and examined with a confocal microscopy using a Nikon Eclipse TE2000-U (Nikon, Tokyo, Japan). A single composite image was obtained by superimposition of 6 optical sections for each sample observed. The collected images were analyzed by ImageJ software. All the experiments were repeated three times.

Apoptosis assay

Apoptosis was detected by flow cytometry by using (BV421)-Annexin-V and the nonvital dye 7-amino-actinomycin D (7AAD) double staining (BD Biosciences). A431 cells were inoculated into six-well plates with $5×10^5$ cells/well and cultured for 24 h. The growth of cells converged to approximately 70%. Cells were then treated with 200uM of (±)-Propranolol Hydrochloride, 0.5mM of 2DG or Prop + 2DG. After 24 hours of treatment, floating and adherent cells were collected and resuspended in binding buffer (BD Biosciences). (BV421)-Annexin V and 7AAD were added, the samples were incubated for 20 min in the dark at 4 C° and analyzed by FACSCanto II and FlowJo software (BD Biosystem). Experiments were performed three times.

Cell Cycle analysis

Flow cytometry analysis of DNA content was performed to assess the cell cycle phase distribution in control conditions (no treated) or after the treatments were added to logarithmically growing A431 cells. After 48 h exposure, A431 cells were harvested by trypsinization, and a solution containing containing 50 µg/mL propidium iodide (Sigma Aldrich), 0.1% w/v trisodium citrate and 0.1% NP40 was added. Samples were then incubated for 30 min at 4°C in the dark and nuclei analysed with a FACSCanto II flow cytometer and FlowJo software (BD Biosciences) Experiments were performed three times.

Statistical analysis

Differences in the experimental groups were assessed using analysis of variance (ANOVA). To avoid bias due to the variability between the experiments, the factor defining the different experimental groups was crossed with a second factor defining the different experiments (two-way ANOVA). p-values lower than 0.05 were considered statistically significant. Figures are representative from all experiments that were realized during the study, using the Graphpad Prism software (GraphPad Software, Inc., version 6.0).

Declarations

Author contributions

C.V.A carried out the experiment with support from M.B and M.L. and wrote the manuscript with support from M.L. G.S and P.L helped supervise the project.

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Figures

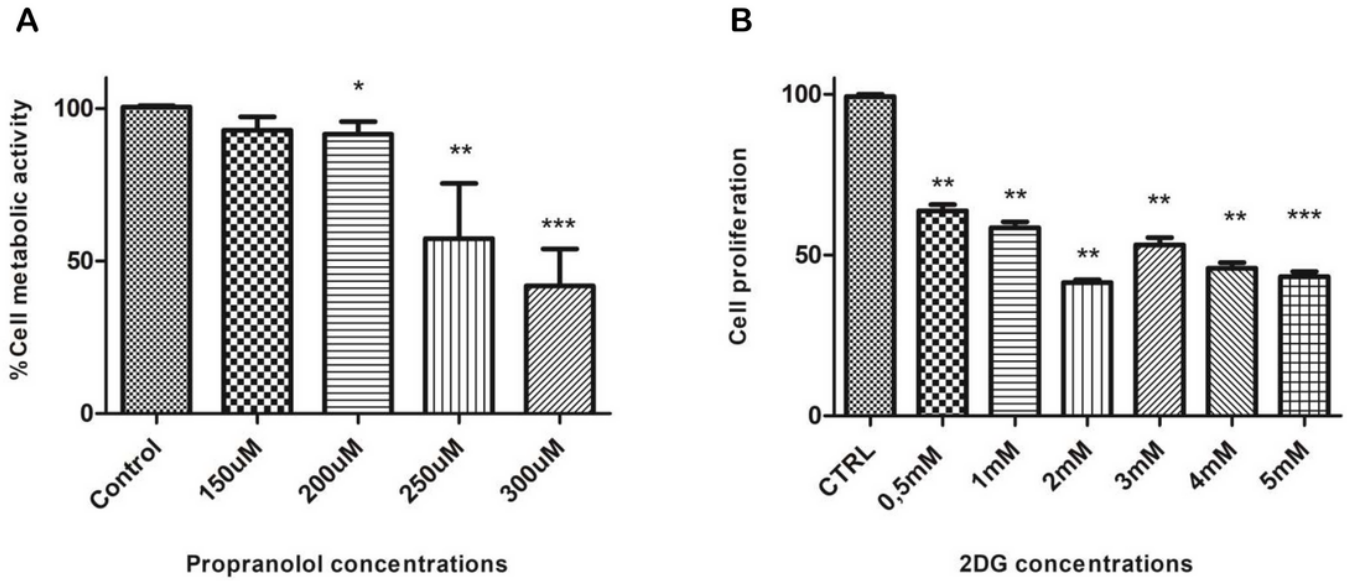

Figure 1

Effect of different concentrations of (±)-Propranolol Hydrochloride and 2DG on squamous carcinoma cell metabolic activity. MTT assay from A431 cell line (A) treated with different concentrations of (±)-Propranolol Hydrochloride. (B) A431 cells were treated with different concentrations of 2DG. Cells were treated during 48h with different concen-trations of (±)-Propranolol Hydrochloride (150µM-350µM) and 2DG (0.5mM-5mM), and a MTT assay was performed. The histograms represent the medium of 3 experiments (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

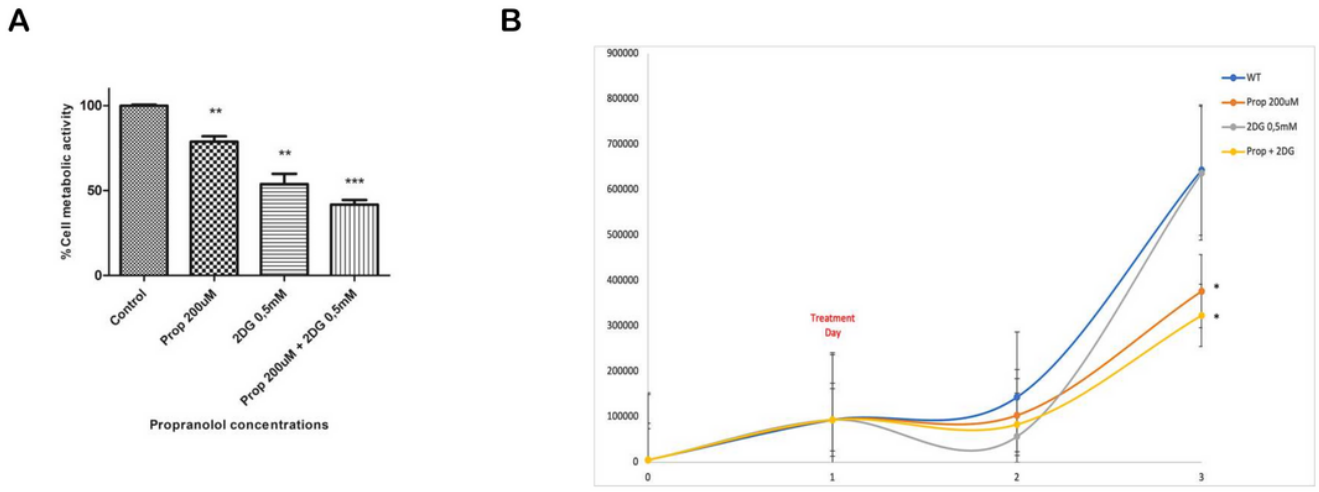

Figure 2

Effect of combined (±)-Propranolol Hydrochloride and 2DG on squamous carcinoma cell metabolic activity and cell proliferation. (A) MTT assay from A431 cells lines treated with combined (±)-Propranolol Hydrochloride and 2DG. A431 cells were treated during 48h with combined (±)-Propranolol Hydrochloride (200μM) and 2DG (0.5mM) and a MTT assay was performed. (B) Cell growth curve of 72 hours of culture and 48h of treatment. A431 cells were seeded and incubated for 24h, when the specifics treatments were add-ed. The alive cells were counted each 24hours. The combination of 2DG on propranolol treatment had significantly increased the antiproliferative effect. There was no difference in cell proliferation in the samples treated only with 2DG. The graphics represent the me-dium of 3 experiments (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

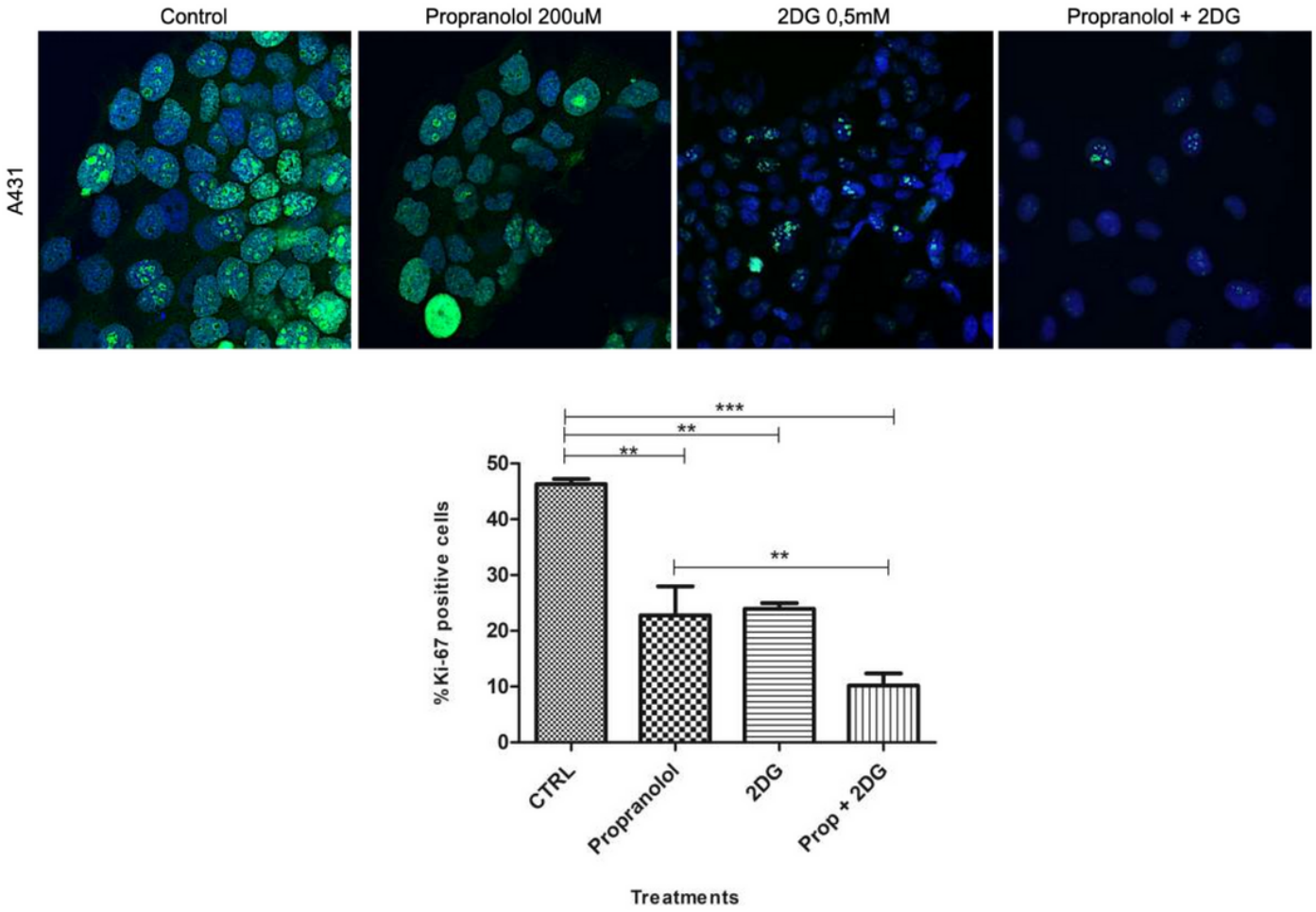

Figure 3

The effect of (±)-Propranolol Hydrochloride, 2DG and Propranolol + 2DG on A431 cell pro-liferation by Ki-67 immunofluorescence assay. A431 cells were treated during 24 hours with the three treatments. Cells

were fixed and labelled with anti-Ki67 (green) and Hoechst 33242 dye (blue). The histogram represents the medium of 3 experiments (** p ≤ 0.01; *** p≤ 0.001).

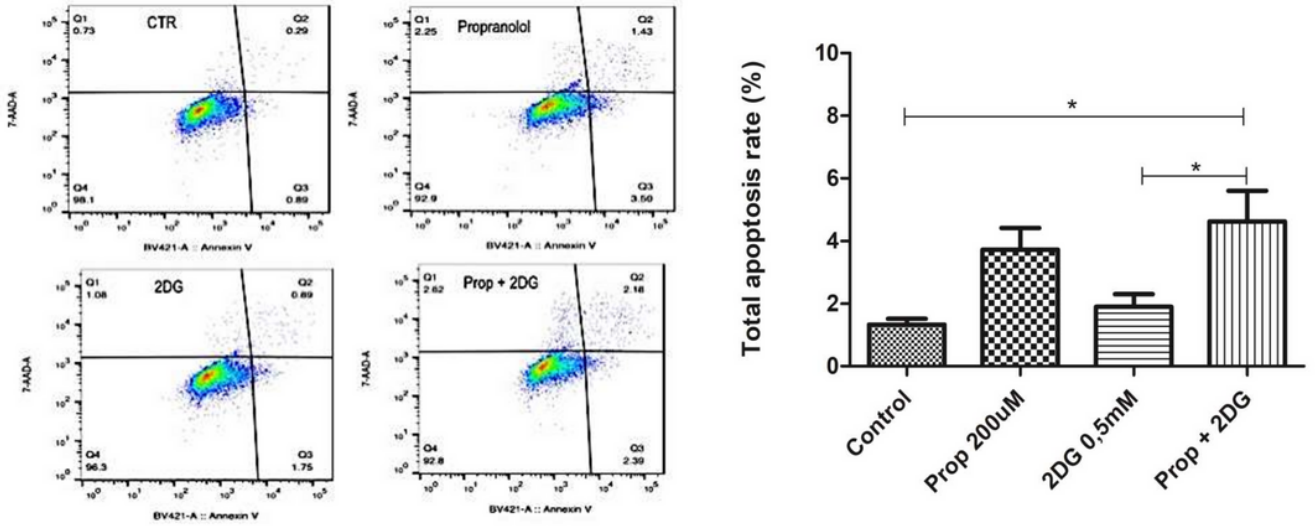

Figure 4

Cell apoptosis assay. A431 cells were treated with 200uM of (±)-Propranolol Hydrochloride, 0.5mM of 2DG or 200uM of (±)-Propranolol Hydrochloride and 0.5mM of 2DG (Prop + 2DG) for 48 hours. The histogram represents the medium of 3 experiments (* p≤0,05).

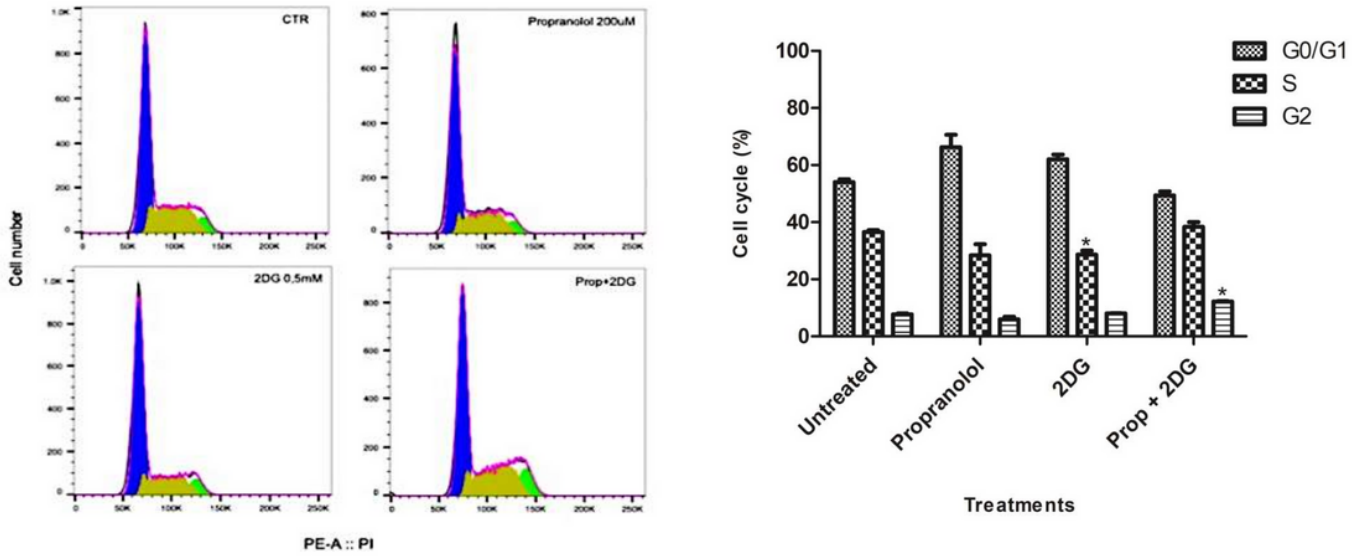

Figure 5

Cell cycle assay. A431 cells were seeded and incubated overnight. In the next day, cells were treated with 200uM of propranolol, 0,5mM of 2DG or propranolol + 2DG during 48h. The graphics represent the medium of 3 experiments (* $p \leq 0,05$).