



Full length article



P2Y₂ receptor activation promotes esophageal cancer cells proliferation via ERK1/2 pathway

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ARTICLE INFO

Keywords:

Esophageal cancer
Purinergic system
Nucleotide
P2Y₂R
Proliferation
Signaling pathways

ABSTRACT

Esophageal cancer is a prominent worldwide illness that is divided into two main subtypes: esophageal squamous cell carcinoma and esophageal adenocarcinoma. Mortality rates are alarming, and the understanding of the mechanisms involved in esophageal cancer development, becomes essential. Purinergic signaling is related to many diseases and among these various types of tumors. Here we studied the effects of the P2Y₂ receptor activation in different types of esophageal cancer. Esophageal tissue samples of healthy controls were used for P2Y₂R expression quantification. Two human esophageal cancer cell lines Kyse-450 (squamous cell carcinoma) and OE-33 (adenocarcinoma) were used to perform in vitro analysis of cell proliferation, migration, adhesion, and the signaling pathways involved in P2Y₂R activation. Data showed that P2Y₂R was expressed in biopsies of patients with ESCC and adenocarcinoma, as well as in the two human esophageal cancer cell lines studied. The RT-qPCR analysis demonstrated that OE-33 cells have higher *P2RY2* expression than Kyse-450 squamous cell line. Results showed that P2Y₂R activation, induced by ATP or UTP, promoted esophageal cancer cells proliferation and colony formation. P2Y₂R blockage with the selective antagonist, AR-C 118925XX, led to decreased proliferation, colony formation and adhesion. Treatments with ATP or UTP activated ERK 1/2 pathway in ESCC and ECA cells. The P2Y₂R antagonism did not alter the migration of esophageal cancer cells. Interestingly, the esophageal cancer cell lines presented a distinct profile of nucleotide hydrolysis activity. The modulation of P2Y₂ receptors may be a promising target for esophageal cancer treatment.

1. Introduction

Esophageal cancer (EC) is a very aggressive illness that affects patients worldwide and ranks the 8th most common incident cancer in the world (Bray et al., 2018). The high mortality rates are mainly due to poor diagnosis, and 15%–25% of patients reach five years of survival when they are diagnosed in early stages (Pennathur et al., 2013; Zhang, 2013). There are two main subtypes of EC: esophageal squamous cell

carcinoma (ESCC) and esophageal adenocarcinoma (EAC) (Murphy et al., 2017). ESCC is the most incident type, however, cases of EAC have increased significantly over the years (Napier et al., 2014). Beyond the cell type, epidemiological features, anatomic localization, risk factors, and molecular characteristics differ between the two subtypes (Lin et al., 2016; The Cancer Genome Atlas Research, 2017).

Purinergic signaling has been related to several kinds of cancer, and there are mounting interests in the role of nucleosides and nucleotides

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<https://doi.org/10.1016/j.ejphar.2020.173687>

Received 22 September 2020; Received in revised form 23 October 2020; Accepted 26 October 2020

Available online 31 October 2020

0014-2999/© 2020 Published by Elsevier B.V.

and their therapeutic potential for cancer treatment (Burnstock, 2004; Burnstock and Di Virgilio, 2013). Purinergic receptors are classified into two major classes. P1 receptors are G-protein coupled and are divided into four subtypes (A_1 , A_{2A} , A_{2B} , and A_3) (Fredholm et al., 2011). P2-receptors are distinguished in P2XR ionotropic receptors, containing a central ion channel with seven members described (P2X1-7); and, P2Y metabotropic receptors (P2YR) with eight members described in humans (P2Y_{1,2,4,6,11,12,13,14}) (Abbraccio and Burnstock, 1994).

P2YR can be activated by different nucleotides according to its affinity for each receptor subtypes (Franke et al., 2012). Different P2YR subtypes are widely distributed in the body, playing an essential role in physiological and pathological processes (Abbraccio et al., 2006). P2Y₂R acts by altering intracellular levels of cAMP, modulating adenylate cyclase or increasing intracellular levels of calcium via PLC, and its main agonists are ATP and UTP (Burnstock, 2004). Maaser et al. (2002), demonstrated that the P2Y₂R activation by high ATP concentrations leads to apoptosis and cell cycle arrest in an esophageal squamous carcinoma cell line (Kyse-140). However, P2Y₂R has also been related with tumor growth, invasion and metastasis in other types of tumors, for example, breast (Jin et al., 2014), prostate (Li et al., 2013) and hepatocellular cancer (Xie et al., 2014). Recently, the P2Y₂R became visible as a potential pharmacological target, promising results in the cancer research field both through its blockage and its activation (Nylund et al., 2007; Buzzi et al., 2010).

There is scarce literature focusing on the P2Y receptors in different types of esophageal carcinomas. Most of the studies are focused on ESCC, however, it is important pay attention to esophageal adenocarcinoma, which had a considerable increase in the number of cases in the last years (Lagergren et al., 2017). Thus, our hypothesis is that P2Y₂R activation is related to proliferation via ERK1/2 or Akt signaling pathways in esophageal cancer. We analyzed P2Y₂R expression on human biopsies of esophageal cancer. Likewise, we explored the pharmacological blockage of P2Y₂R in the proliferation, adhesion and migration of ESCC and EAC cells.

2. Materials and methods

2.1. Chemical compounds

ATP (PubChem CID: 5957), and UTP (PubChem CID: 6133) were purchased from Santa Cruz Biotechnology (CA, USA). The P2Y₂R antagonist, AR-C 118925XX (PubChem CID: 54210200) was obtained from Tocris Bioscience (MO, USA). Penicillin (PubChem CID: 2349), streptomycin (PubChem CID: 19649) and fungizone (PubChem CID: 5280965), thiazolyl blue tetrazolium bromide (MTT) (PubChem CID: 64965), Hoechst 33258 (PubChem CID: 2392) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Subjects

The tissue samples used in this study came from the pathology department of São Lucas Hospital of the Pontifical Catholic University of Rio Grande do Sul (PUCRS). The clinical data of patients were obtained through the patients' medical records. Inclusion criteria: patients older than 18 years of both sexes, who underwent esophageal biopsy between the years of 2014 and 2016. Exclusion criteria: tumor in advanced stage (T4), inadequate or non-existent sample. The final number of biopsies samples collected to immunohistochemistry was, HC = 11 cases (7 men and 4 women) ESCC = 12 (9 men and 3 women) cases and EAC = 7 cases (5 men and 2 women). This study was carried out following the Declaration of Helsinki, and all subjects signed an Informed Consent Form previously approved by the Local Ethics Committee (number: 1.645.764).

2.3. Immunohistochemistry and tissue cytometry

Human histological samples of normal esophagus, ESCC and EAC were collected to verify P2Y₂R expression. The final number of biopsy samples collected to immunohistochemistry was, HC = 10 cases, ESCC = 16 cases and EAC = 7 cases. Formalin-fixed, paraffin-embedded tumor blocks were used to obtain 2 μ m thick sections. Thereafter, the sectioned slides were deparaffinized in xylene and dehydrated in serial ethanol dilutions. The next steps were performed according to Braganhol et al. (2009), sections were incubated overnight at 4 °C with the rabbit polyclonal P2Y₂R antibody (dilution 1:100), then sections were washed in PBS and incubated with the secondary anti-rabbit IgG antibody at room temperature for 2 h. Lastly, samples were incubated with 3,3'-diaminobenzidine (DAB) for 5 min, followed by hematoxylin counterstaining. Imaging cytometry scanning and analysis of the cell cultures using TissueFAXS™ Cytometry platform (TissueGnostics GmbH, Vienna, Austria) was done. P2Y₂R expression was distinguished in brown and nuclei in blue, and quantification was performed using StrataQuest software (TissueGnostics GmbH). The software detects different color shades and builds a mask for nuclei (blueish), and other mask(s) contains the cytoplasm and membrane of cells, which accounts for the P2Y₂R by detecting brownish shades. Cellular data were provided in dot-plots to show multiple measurement parameters of single cell morphological features. The area and the number of cells were determined by using detection cellular masks (Pillat et al., 2016).

2.4. Culture of esophageal cancer cells

Esophageal cancer cell line, OE-33, was donated by Dr. Luis Felipe Ribeiro Pinto (INCA) and cell lines Kyse-450 and Kyse-30 were obtained commercially from Deutsche Sammlung von Mikroorganismen und Zellkulturen. For all the experiments, OE-33 and KYSE-450 cells were used until passage 10. Cells were maintained in RPMI medium supplemented with 10% fetal bovine serum (FBS) (both purchased from Gibco, NY, USA), 50 units of penicillin/ml, 50 mg streptomycin/ml, and fungizone (250 μ g/ml) were purchased from Sigma Aldrich (MO, EUA) were maintained at controlled cell incubator (37 °C, 5% CO₂, and 95% humidity).

2.5. RNA isolation and real-time qPCR

The gene expression pattern of purinergic receptors P2Y was determined by RT-qPCR. The total RNA was isolated from esophageal cancer cell lines Kyse-450 and OE-33 (1×10^6 cells, in triplicate) with TRIzol® Reagent (Life Technologies) in accordance with the manufacturer's instructions. RNA purity (Abs 260/280 nm–2.0) and concentration were determined by Nanodrop® Lite, and after, treated with Deoxyribonuclease I (Sigma-Aldrich) to eliminate genomic DNA contamination in accordance with the manufacturer's instructions. The cDNA was synthesized with ImProm-II™ Reverse Transcription System (Promega, WI, USA) from 1 μ g of the total RNA, following the manufacturer's instruction. Quantitative PCR was performed using SYBR® Green I (Invitrogen) to detect double-strand cDNA synthesis on the 7500 Real-time PCR System (Applied Biosystems, CA, EUA). The PCR cycling conditions were an initial polymerase activation step for 5 min at 95 °C, 40 cycles of 15 s at 95 °C for denaturation, 35 s at 60 °C for annealing and 15 s at 72 °C for elongation. At the end of cycling protocol, a melting-curve analysis was included, and fluorescence measured from 60 to 99 °C to confirm the specificity of primers and absence of primer-dimers, and showed in all cases, one single peak. All real time assays were carried out in quadruplicate and, in all cases, substituting the templates for DNase/RNase-free distilled water in each PCR reaction included a reverse transcriptase negative control. We analyzed the optimal number of internal control genes for normalization with geNorm. The results indicate the combination of the three most stable genes for normalization purposes. GAPDH, B2M and 18S were used as reference genes for

normalization. The sequences of reverse and forward primers are in Table 1. The efficiency per sample was calculated using LinRegPCR 2016.1 Software (<http://LinRegPCR.nl>) and the stability of the reference genes, and the optimal number of reference genes according to the pairwise variation (V) was analyzed by GeNorm 3.5 Software (<http://medgen.ugent.be/genorm/>). Relative mRNA expression levels were determined using the $2^{-\Delta\Delta C_q}$ method (Bustin et al., 2013).

2.6. Immunocytochemistry

Esophageal cancer cell lines Kyse-450 and OE-33 were seeded on glass coverslips and cultured to 48 h. After this time, cells were washed twice with PBS pH 7.4, fixed with 3.7% paraformaldehyde during 20 min, washed twice again and permeabilized with 0.1% Triton X-100 in PBS for 20 min. Then, cells were newly washed twice with PBS and blocked with 1% BSA for 45 min. In sequence, cells were incubated overnight with primary antibody rabbit anti-P2Y₂ receptor (APR-010, Alomone Labs, dilution 1:100). Next, cells were incubated for 1 h with secondary anti-rabbit Alexa-Fluor 488 (A-21206, Invitrogen, Thermo Fisher Scientific) at dilution 1:500 and finally incubated with Hoechst 33258 (B-1155, Sigma Aldrich) diluted 1:1000 for 5 min, as a nuclear stain. Cells were washed three times with PBS and dried at room temperature. The slices were mounted and analyzed under a confocal laser scanning microscope (Leica TCS SP8, Leica Microsystems, Germany) (Pillat et al., 2016). Fifty single confocal sections with a z-step size of 0.1 μ m were acquired with a 63 \times (numeric aperture 1.40) oil-immersion objective (HC PLAPO CS2 63X/1.40 OIL, Leica Germany). The final images obtained were analyzed with ImageJ software and the figure was generated using Adobe® Photoshop® CS5.

2.7. Cell counting

Cells were seeded at density of 2×10^4 per well in 24-well plates. The nucleotides (ATP and UTP) and the P2Y₂R antagonist (AR-C 118925XX) were used. To perform the experiments, the three cell lines were induced to G1-phase through FBS starvation by reducing the concentration of 10%–5% and finally to 0.5% for 24 h. After, cells were divided in six treatment groups as follows: (1) Control group: received RPMI medium (0.5% FBS), (2) ATP (100 μ M), (3) UTP (100 μ M), (4) AR-C 118925XX (20 μ M), (5) AR-C 118925XX (20 μ M) plus ATP (100 μ M), (6) AR-C 118925XX (20 μ M) plus UTP (100 μ M). After 24 h of treatment, the medium was removed, the cells were washed with calcium and magnesium-free medium (CMF) and 100 μ l of 0.23% trypsin/EDTA solution were used to detach the cells. Absolute cell number was acquired by Countess FL cell counter (Life Technologies, CA, USA). This experiment was performed three times in triplicate.

2.8. Clonogenic assay

This experiment was performed according to Rafehi et al. (2011). Briefly, Kyse-450 (100 cells) and OE-33 (300 cells) were seeded in 24-well plates. Since it is a long-term experiment, we chose to use RPMI medium with 5% of FBS supplementation. After 24 h, cells are treated with ATP (100 μ M), UTP (50 and 100 μ M) alone or in combination with AR-C 118925XX (20 μ M), cells of control group received RPMI 5% FBS only. Every two days, the medium was replaced, and the wells that received nucleotides initially, got a nucleotide pulse together with new

medium. On the 7th day, cells were washed twice with PBS and fixed with formalin 4% for 10 min; colonies were stained with 200 μ l of crystal violet for 10 min, and then washed with PBS. Colonies with 50 cells or more were considered, and counting was performed using the ImageJ software®. This assay was performed in quadruplicate in two independent experiments.

2.9. Determination of signaling pathways Phospho-Akt and Phospho-ERK1/2

In order to analyze intracellular signaling pathways, the expression of p-ERK1/2 and p-Akt was measured by cytometry using the Phosflow Kit from BD Biosciences (CA, USA). Cells were plated (30×10^4) in 12-well plates and FBS was reduced to 0.5% for 24 h. The cells were treated for 5, 15, 30 or 60 min with ATP and UTP at 100 μ M concentration, the positive control received RPMI 10% FBS, and RPMI control 0.5% FBS, and maintained in incubation at 37 °C. After treatment, the cells will be trypsinized, and fixed with Phosflow Buffer for 10 min at 37 °C. After, the cells were washed with PBS and permeabilized with Phosflow Perm Buffer for 30 min, conditioned on ice. After permeabilization, cells were washed twice and labeled with FITC anti-p-ERK 1/2 and anti-p-Akt antibodies for 30 min conditioned on ice. Data were obtained using cytometer FACSCantoII (Beckton Dickinson) and BD FACSDiva software, and subsequently analyzed using Flowjo v10. Flow cytometry was conducted in triplicate in a single experiment.

2.10. Adhesion assay

To evaluate cell adhesion, Kyse-450 and OE-33 were seeded (5×10^4 cells) in 96-well plates. For this experiment, the cells were treated together with plating and incubated for 2 h at 37 °C. Treatment: (1) Control group: received RPMI medium (10% FBS), (2) ATP (100 μ M), (3) UTP (100 μ M), (4) AR-C 118925XX (20 μ M). Passed the incubation time, cells were washed three times with PBS to remove non-adherent cells. Adherent cells were fixed with formalin for 10 min and stained with crystal violet (0.5%) diluted in methanol (20%). Then, cells were cleaned three times with Milli-Q™ water, and 100 μ l of 10% acetic acid (v/v) was added in each well for elution. Lastly, the cell adhesion was evaluated by measuring optical density (OD) at 570 nm in a plate reader (Spectra Max M2e, Molecular Devices) (Wang et al., 2008). This experiment was conducted in triplicate in two independent experiments.

2.11. Wound healing assay

To determine the cell capacity of migration, wound healing assay was performed according to previous studies (Valster et al., 2005; Garay et al., 2013). Kyse-450 (15×10^4) and OE-33 (15×10^4) cells were seeded on 24-well plates in RPMI 10% FBS for 24 h at 37 °C under 5% de CO₂. After, Kyse-450 cells reduced the concentration of 10% FBS to 5%, and subsequently to 0.5%, for 24 h. OE-33 cells did not resist FBS starvation to 0.5% for long periods, so the experiment was carried out with 5% FBS. Using a p200 pipette tip, a line was scraped into the well to simulate an injury. Cells were washed three times with PBS to remove the debris/serum and treated with ATP (100 μ M), UTP (100 μ M), AR-C 118925XX (20 μ M), and controls received only media supplemented with FBS 0.5% or 5% (OE-33). Kyse-450 and OE-33 were photographed at time point zero, 24 h and 48 h. All images were obtained via an

Table 1

Primer sequences for RT-qPCR experiments included in the study.

Gene	Forward primer	Reverse primer	Reference
18S	5'-GTAACCCGTTGAACCCATT-3'	5'-CCATCCAATCGGTAGTAGCG-3'	Rho et al., 2010
B2M	5'-ACTGAATCACCCCACTGA-3'	5'-CCTCCATGATGCTGCTTACA-3'	Rho et al., 2010
Gapdh	5'-TGCACCACCAACTGCTTA-3'	5'-GGATGCAGGGATGATGTTTC-3'	Rho et al., 2010
P2Y₂	5'-CACCCGCACCCCTACTACT-3'	5'-CCTTGTAGGCCATGTTGATG-3'	Designed by authors

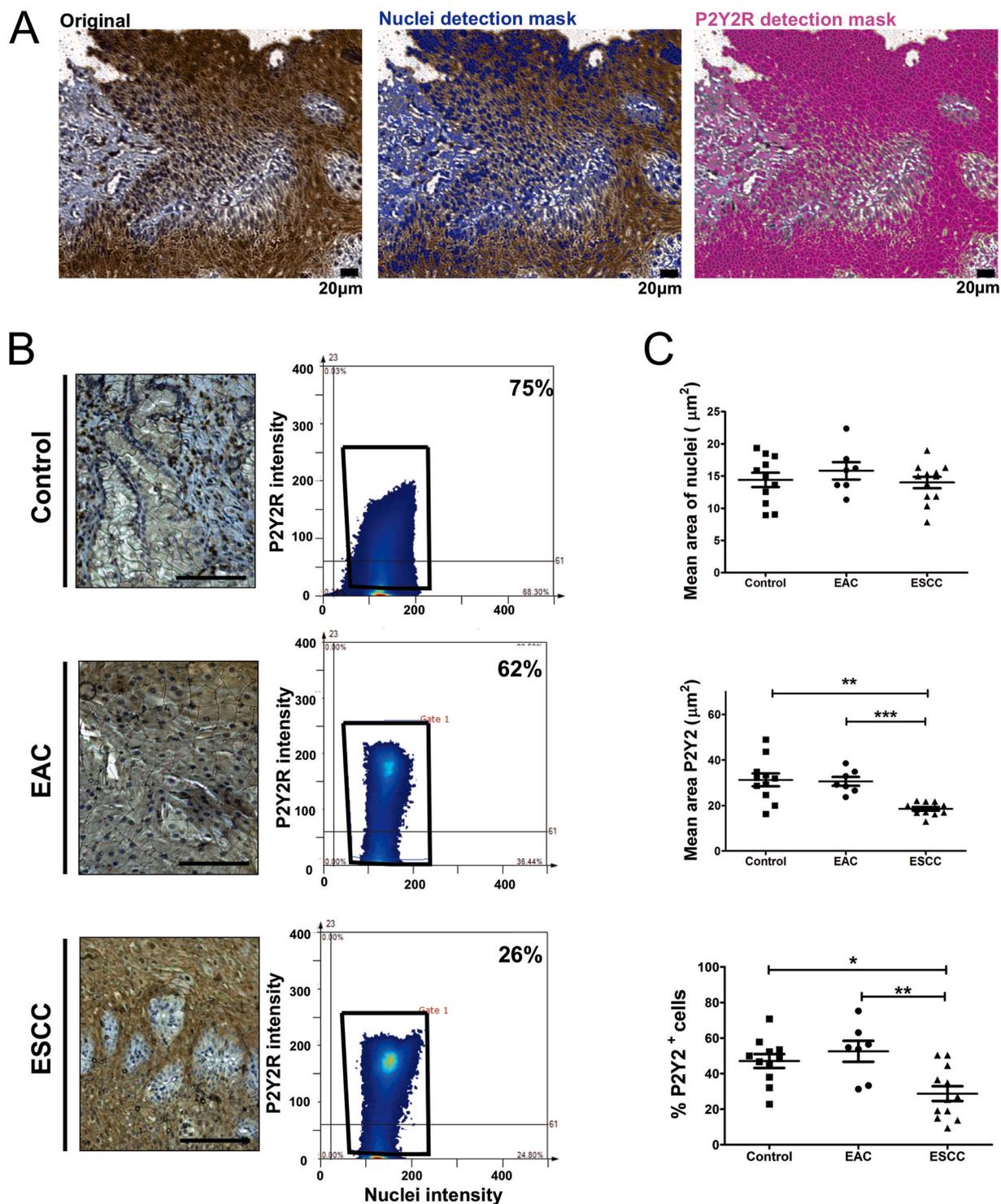


Fig. 1. Human biopsies stained for P2Y2R tissue cytometry analysis. Brown staining indicates P2Y2R presence. Scale bars = 100 μm (A). Expression of P2Y2R in healthy control, ESCC and EAC. Brightfield microscopy images are representative of cell nuclei staining with HE and P2Y2 receptor with DAB. Designed masks for measurements with StrataQuest software are highlighted by color blue (HE) and pink (P2Y2R). Scale bars = 100 μm (B). Quantitative analyses were performed using Strata Quest software, a representative sample image of each sample and their density plot quantification by tissue cytometry at the right (C). Data represent mean values \pm S.D., of independent biopsies samples. One-Way ANOVA followed by Tukey post-test was used to calculate statistical difference, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

inverted optical microscope (10 \times objective) with a capturing digital image system (Olympus IX71), always in the same field. The images obtained were analyzed by using Adobe[®] Photoshop[®] CS5. This assay was conducted in duplicate in three independent experiments.

2.12. Enzymatic activity

In order to determine ectonucleotidases activities, we used the protocol described by (Wink et al., 2003). Kyse-450 and OE-33 cells (4 \times 10⁴) were trypsinized and included to solution containing 50 mM

Tris-HCl (pH 8.0) and 5 mM CaCl₂ (ectonucleotidase activities) or 50 mM Tris-HCl (pH 7.2) and 5 mM MgCl₂ (CD73 activity) in a final volume of 200 µl. Samples were preincubated for 10 min at 37 °C before starting the reaction with the addition of substrates (ATP, ADP, AMP or UTP) to a final concentration of 100 µM. The reaction was stopped after 30 min with the addition of 200 µl of trichloroacetic acid at a final concentration of 5%. Samples were incubated on ice for 10 min and was added 1 ml of a colorimetric solution containing 2.3% polyvinyl alcohol, 5.7% ammonium molybdate, and 0.08% malachite green was added in order to determine the inorganic phosphate released (Pi). The quantification of Pi released was reached using a spectrophotometer at 630 nm (Spectra Max M2e, Molecular Devices). This assay was conducted in triplicate in three independent experiments and results were expressed as nmol Pi released/min/mg of protein.

2.13. Statistical analysis

Data were analyzed by Student's t-test, one-way analysis of variance (one-way ANOVA), followed by Tukey or Dunnett's post hoc test or two-way analysis of variance (two-way ANOVA) followed by Bonferroni post-test for multiple comparisons, using GraphPad Software 6.0 (San Diego, CA, U.S.A.). Data of in vitro analyzes are presented as mean ± S.E.M. Data of tissue samples images presented as mean ± SD. P values < 0.05 indicate statistical significance.

3. Results

3.1. P2Y₂R immunostaining and clinical data of patients

Human samples of esophageal biopsies of normal esophagus, squamous cell carcinoma, and adenocarcinoma were analyzed regarding P2Y₂R expression by immunohistochemistry, following scanning and quantification through imaging tissue cytometry using TissueFAXS™ Cytometry platform (TissueGnostics GmbH, Vienna, Austria). Analysis of nuclei and P2Y₂R immunostaining in human biopsies using the detection masks is demonstrated in Fig. 1A. Our data demonstrated that ESCC and normal appearing esophageal mucosa showed positive immunostaining for P2Y₂R (Fig. 1B), whereas immunoreacted receptor expression levels were markedly increased in EAC (Fig. 1C). Regarding the EAC cells P2Y₂R intensity and density of expression, we demonstrated in all plots that EAC has higher levels when compared to ESCC, but not when compared to control (Fig. 1C). Socio-demographic and

Table 2
Patients clinical data.

Clinical data of patients	Control (n = 11)	Squamous cell carcinoma (n = 12)	Adenocarcinoma (n = 7)
Age	51 (±14,28)	62,50 (±9,58)	76 (±10,55)
Sex			
Men	7 (63,64%)	9 (75%)	5 (71,43%)
Women	4 (36,36%)	3 (25%)	2 (28,57%)
Dysphagia/odynophagia	-	10 (83,33%)	3 (42,85%)
Weight loss	-	10 (83,33%)	2 (28,57%)
Surgery	-		
Lymphadenectomy	-	2 (16,66%)	-
Esophagectomy	-	7 (58,33%)	1 (14,28%)
Radiotherapy	-	9 (75%)	3 (42,85%)
Chemotherapy	-	6 (50%)	4 (57,14%)
Tobacco use	2 (18,18%)	9 (75%)	2 (28,57%)
Alcohol use	Absent data	4 (33,33%)	Absent data
Death	-	10 (83,33%)	1 (14,28%)

Data shown as mean (SD) or absolute number and relative number (%).

clinical data of patients, age, sex prevalence, treatments and mortality rates, are expressed in Table 2.

3.2. Esophageal cancer cell lines express P2Y₂ receptors

Since patients with esophageal cancer display the expression of the P2Y₂ receptor in EAC and ESCC biopsies, we decided to investigate the gene expression of this receptor in these two different subtypes of esophageal cancer human cell lines. To evaluate the P2RY gene expression in Kyse-450, Kyse-30 and OE-33, we performed the RT-qPCR technique for determining expression levels of eight receptor subtypes already described in humans (Supplementary Table 1 and Table 2). Since the profile of P2Y receptors expression was similar between the two ESCC cells (Kyse-450 and Kyse-30), we have performed the next experiments comparing the two different types of esophageal cancer cells (OE-33 and Kyse-450). The results obtained demonstrate that the EAC OE-33 cell line had a higher gene expression of P2RY2 compared with Kyse-450 (Fig. 2A). In addition, we evaluated the expression of P2Y₂R in cell lines by immunocytochemistry (Fig. 2B), and the qualitative analysis showed that the esophageal cell lines presented P2Y₂R protein expression following the same pattern evidenced into P2RY2 mRNA analysis.

3.3. Extracellular nucleotides induce proliferation and colony formation in esophageal cancer cells

Esophageal cancer cells were treated with extracellular nucleotides (ATP and UTP) and with the specific P2Y₂R antagonist AR-C 118925XX. Kyse-450 (Fig. 3A) and OE-33 cells (Fig. 3B), showed a significant increase in cell number when treated with ATP 100 µM or UTP 100 µM, when compared to control. Besides, the two cell lines presented similar responses when received the P2Y₂R antagonist, showing a significant decrease in the number of cells. This effect remained even with the addition of the P2Y₂ agonists ATP or UTP in both cell lines (Fig. 3C and D).

Conversely, in the clonogenic assay the ESCC lineage Kyse-450 (Fig. 3E), UTP (100 µM) treatment increased the number of colonies after 7 days. Treatment with the specific P2Y₂ antagonist decreased the number of colonies, and the subsequent addition of UTP was able to reverse this effect. Furthermore, UTP or ATP treatments increased the number of colonies on adenocarcinoma OE-33 cells when compared to control, and the antagonist AR-C 118925XX diminished this effect. Interestingly, the addition of UTP was able to reverse the colony number when compared to the group treated with AR-C 118925XX alone (Fig. 3F).

3.4. ATP and UTP promote activation of ERK1/2 and Akt signaling pathways

In order to investigate if the ERK1/2 and Akt signaling pathways were involved in the regulation of P2Y₂ receptor activation and proliferation, we treated ESCC and EAC cells with ATP or UTP (100 µM) at different time-points (3, 15, 30 and 60 min). Kyse-450 cells treated with ATP exhibited higher expression in p-Akt after 30 min treatment. Also, p-ERK1/2 expression was increased in 30 and 60 min after ATP treatment in comparison to control (Fig. 4A). When Kyse-450 was treated with UTP, no difference was observed in p-Akt pathway. Conversely, p-ERK1/2 expression presented an elevated expression after 3, 15 and 30 min when compared to control (Fig. 4B).

Once we stimulated the EAC OE-33 cell line with ATP, there was no difference in p-Akt expression in any time-point tested. On the contrary, p-ERK1/2 expression was elevated at 3, 15, 30, 60 min after ATP stimulus when compared with control. When we stimulated the cells with UTP, p-Akt expression was elevated in 30 min when compared to control, and p-ERK1/2 expression was higher than control at 3, 15 and 30 min (Fig. 4C).

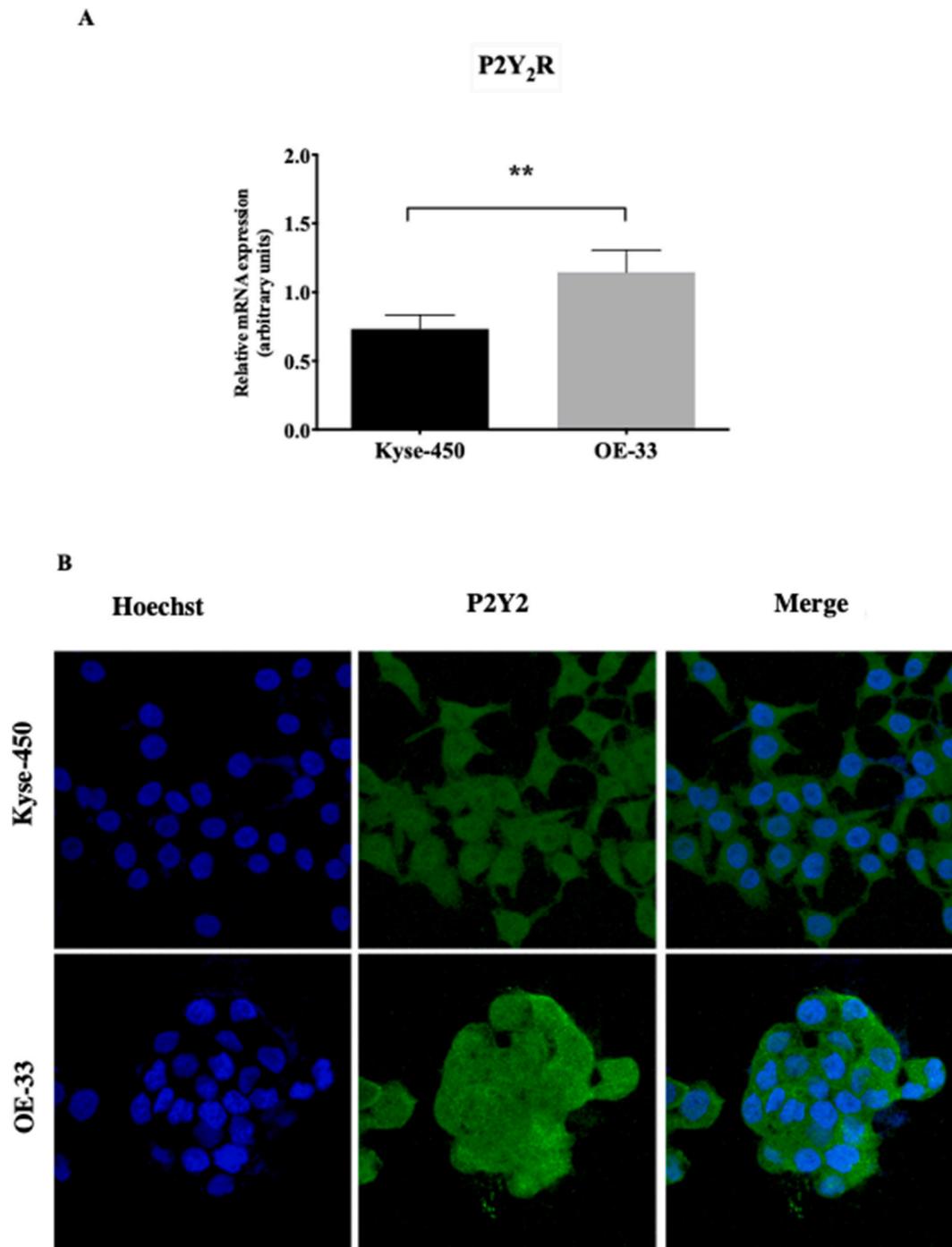


Fig. 2. Expression of *P2Y₂R* in human ESCC and EAC cell lines. (A) Analysis of *P2Y₂R* mRNA expression performed by Real time PCR. *GAPDH*, *B2M* and *18S* were used as reference genes for normalization. Results were expressed as median of each cell line were $n = 3$. Student T test was used to calculate statistical difference between cell lines. Data showed as \pm S.E.M., $**P < 0.01$. (B) Qualitative evaluation of *P2Y₂R* protein expression (green) performed by immunocytochemistry. Nucleus were stained with DAPI (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.5. *P2Y₂R* antagonism decreases adhesion in adenocarcinoma esophageal cells

Additionally, we evaluated the role of *P2Y₂R* in cell adhesion capacity. Treatment with the selective antagonist in adenocarcinoma OE-33 lineage showed a significant decrease in cell adhesion when treated with the antagonist AR-C 118925XX when compared to control group. The tested antagonist treatment did not alter ESCC Kyse-450 cell adhesion (Fig. 5A and B). We further investigated if the inhibition of *P2Y₂R* with the selective *P2Y₂R* antagonist was able to favor cell

migration through the wound-healing method. No differences were observed in both cell lines migration with any treatments used (Figs. 5C and D). Fig. 5 E-F show representative images of the two cell lines studied at 0 and 24 h after treatment.

3.6. Esophageal cancer cell lines present distinct profile of nucleotide hydrolysis

In order to understand how nucleotide hydrolysis, occur in the cell lines used in this study, we evaluated the ATPase, ADPase, AMPase and

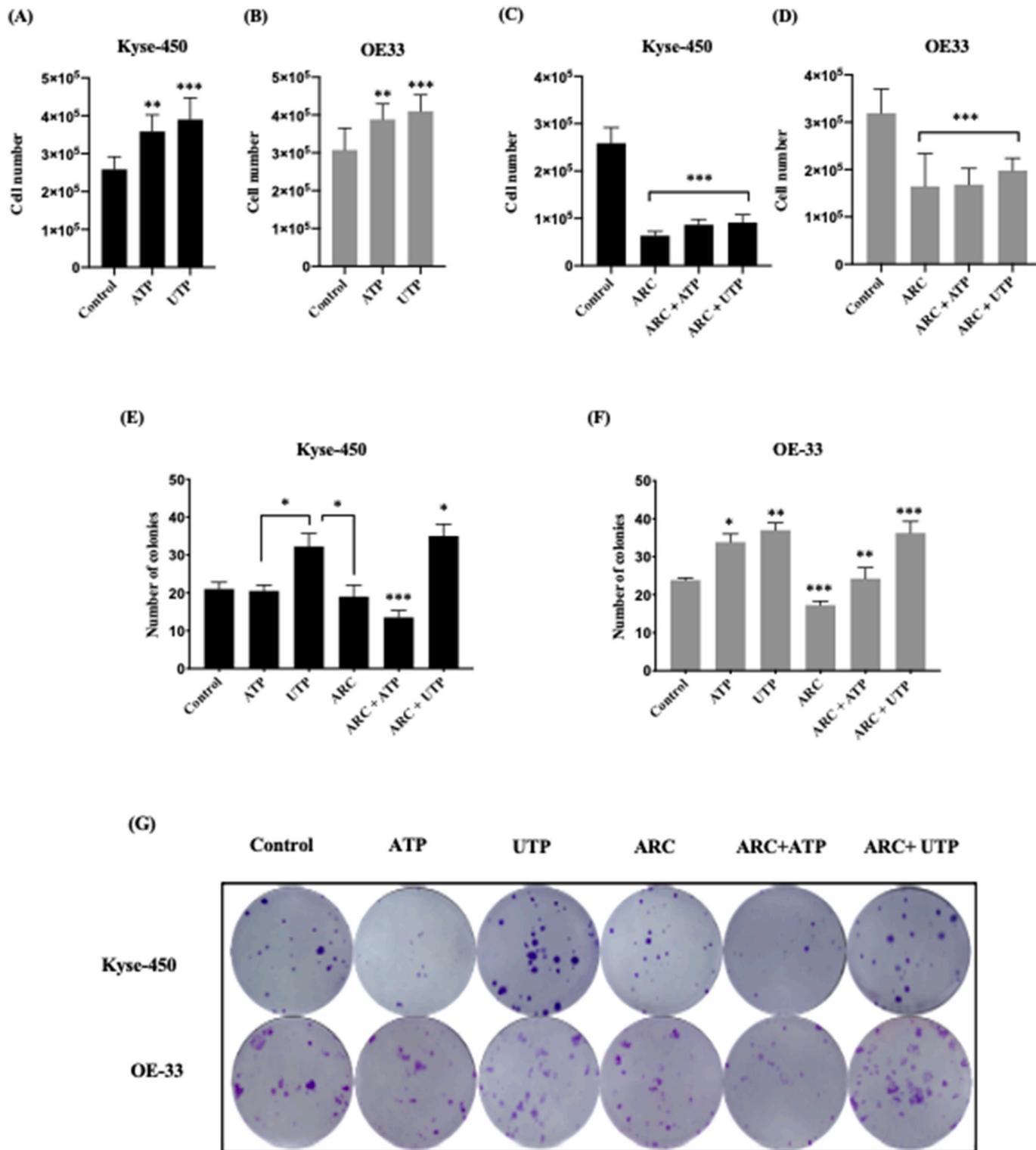


Fig. 3. Effects of nucleotides and P2Y₂R antagonist on human esophageal cancer cells proliferation. Cell number of Kyse-450 (A) and OE-33 (B) were determined 24 h after treatment with ATP or UTP (100 μM). Kyse-450 (C) and OE-33 (D) treated with AR-C 118925XX (20 μM) alone and in combination with ATP or UTP (100 μM) were counted by Countess FL cell counter (Life Technologies, CA, USA). These experiments were performed three times in triplicate for each cell line and the results were presented as median of life cells ± S.E.M. Quantification of colony number was performed after 7 days of treatment with ATP or UTP (100 μM), AR-C 118925XX (20 μM) (ARC in the graph) alone and in combination with ATP or UTP (100 μM), in Kyse-450 (E) OE-33 (F) and representative images of colony formation (G). This experiment was performed three times in duplicate for each cell line and data were presented as median ± S.E.M. The statistical differences were determined by One-Way ANOVA analysis followed Tukey post-test, where *P < 0.05, **P < 0.01, ***P < 0.001.

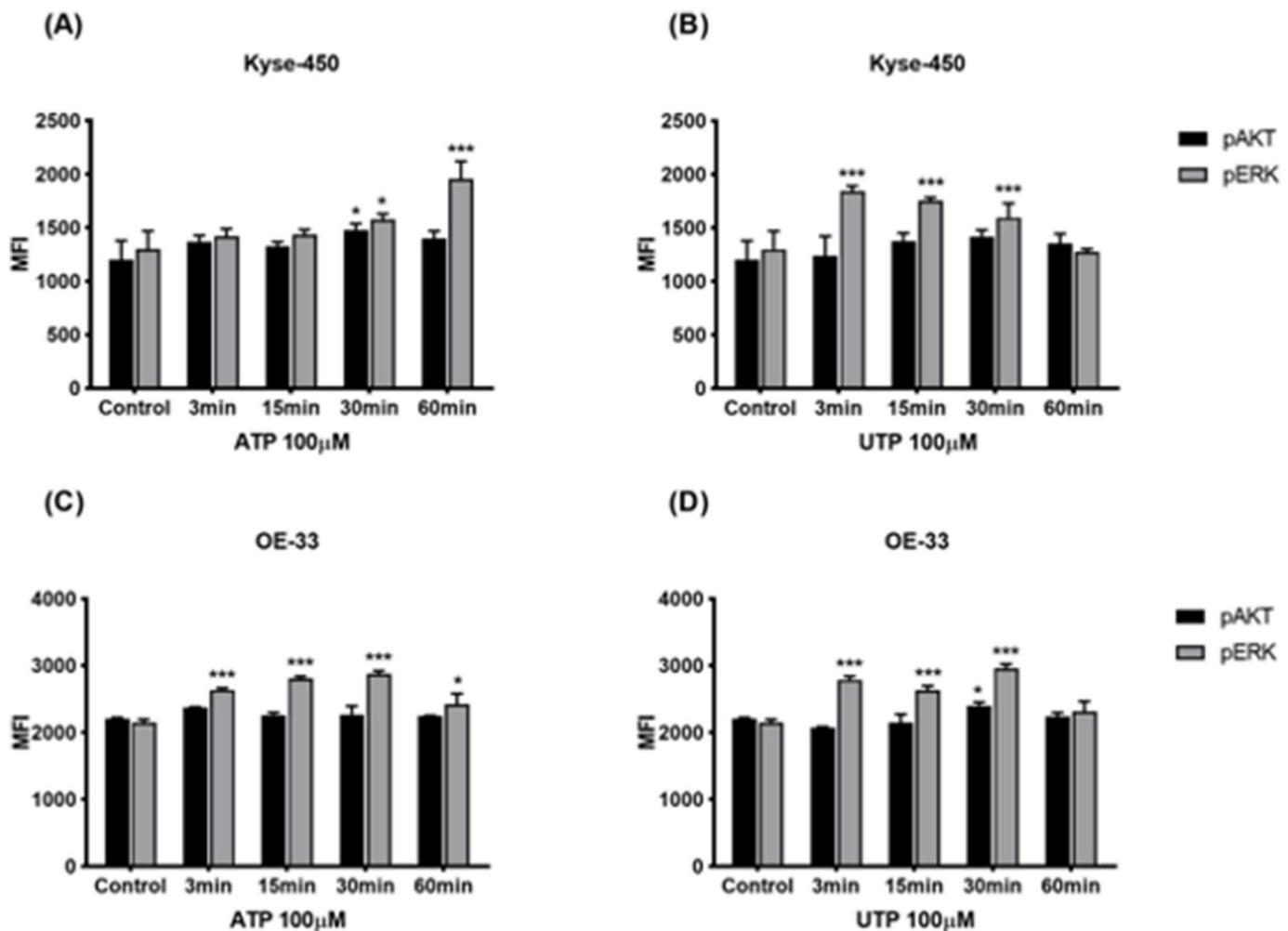


Fig. 4. Involvement of $P2Y_2$ receptor in $ERK1/2$ and Akt cell signaling pathways. Activation on signaling pathways expression after stimulus with agonists of $P2Y_2R$ ATP 100 μM (left graphics) and UTP (right graphics). Flow cytometry was performed at 3, 15, 30 and 60 min after nucleotide stimulation in Kyse-450 (A-B), and OE-33 (C-D). Two-Way ANOVA followed by Bonferroni post-test calculates statistical differences between time-points. Each bar was compared to control and data are present as \pm S.E.M., where * $P < 0.05$ and *** $P < 0.001$.

UTPase activity. Kyse-450 cells presented a similar ATP, ADP, AMP and UTP hydrolysis profile (Fig. 6A). OE-33 lineage presented a higher ATP hydrolysis when compared to ADP, AMP and UTP. Moreover, the hydrolysis of ADP and AMP was higher than UTP in the adenocarcinoma cells (Fig. 6B). The hydrolysis profile for adenocarcinoma cells OE-33 was ATPase > ADPase > AMPase > UTPase. Finally, adenocarcinoma cell line OE-33 showed higher ATPase, ADPase and UTPase activity when compared to squamous cell carcinoma cell line Kyse-450 (Supplementary Fig. 1).

4. Discussion

The association of purinergic signaling and several types of cancer is broadly studied. Since the high signaling potential of ATP and UTP as intra and extracellular messengers, and the vast amounts of ATP existing in the tumor microenvironment (Burnstock, 2006), the understanding of signaling triggered by these molecules in esophageal cancer can explain different mechanisms involved in both cell proliferation and death. We hypothesized that esophagus tissues with different histological origins would express $P2Y_2$ purinergic receptor. Through the analysis of images obtained from healthy esophageal tissues, ESCC and EAC, we showed that adenocarcinoma tissue expressed $P2Y_2R$ compacted in a smaller area with higher expression intensity, while in ESCC tissue this expression is diffuse and less intense. Regarding age, sex prevalence and mortality rates, the clinical data of patients showed by our study was in

accordance with the global epidemiological characteristics of esophageal cancer (Murphy et al., 2017; Smyth et al., 2017).

Further, we performed in vitro experiments to compare cells from two different histological types, human ESCC (Kyse-450) and EAC (OE-33), considering the role of $P2Y_2R$ in various cellular processes that occur in cancer. Taking into account the gene expression of $P2Y_2R$, EAC cell line OE-33 displays higher gene expression than ESCC cell lines Kyse-450 and Kyse-30. Previous studies have described the association of $P2Y$ receptors in non-pathological functions related to muscle control (contraction and relaxation) and neurotransmission (Wan et al., 2016). Also, $P2Y_2R$ expressed in primary human esophageal epithelial cells, are involved in pro-inflammatory responses when activated (Wu et al., 2017). Moreover, another cell line, Kyse-140, representative of human ESCC, expressed several purine receptors, including the $P2Y_2R$, and the activation of this receptor was related to activation of cell death mechanisms (Maaser et al., 2002). In human hepatocellular carcinoma cells, the expression of $P2Y_2R$ was higher than normal hepatocytes (Li et al., 2014), evidence that this receptor develops essential function in the tumor microenvironment.

Concerning nucleotide stimulus succeeding by cell counting, the two cell lines utilized in this study responded in similar ways. The Kyse-450 and OE-33 showed an increase in cell number after ATP and UTP stimulus. When ESCC and EAC cells were treated with $P2Y_2R$ antagonist, similar effect was observed, with a decrease in cell number that was not reversed by the next addition of nucleotides. Recent studied showed that

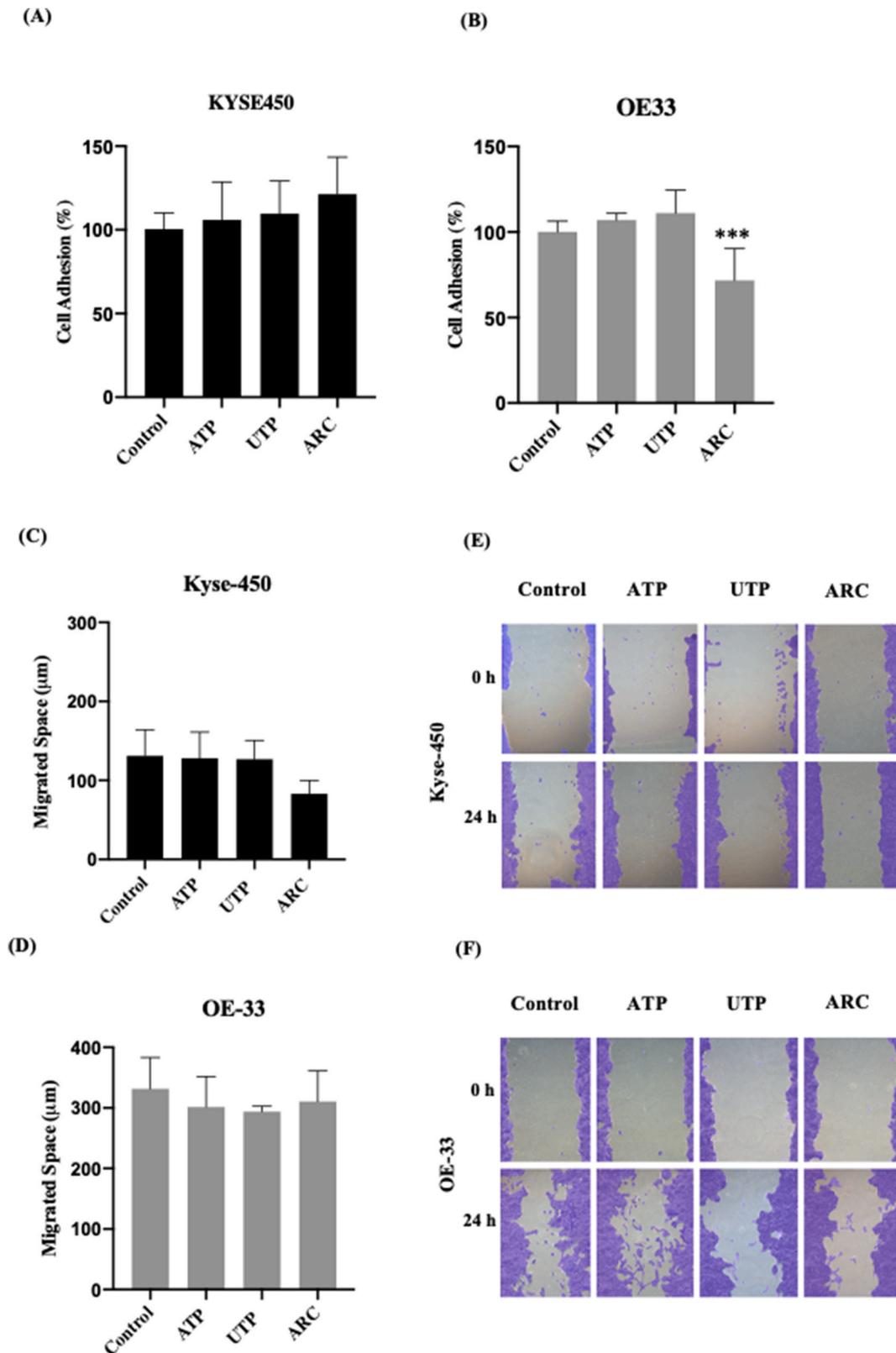


Fig. 5. Evaluation of adhesion and wound healing in esophageal cancer cell lines. Evaluation of cell adhesion was performed in Kyse-450 (A) and OE-33 (B) after 2 h of treatment with ATP or UTP (50 and 100 μ M), P2Y₂ antagonist AR-C 118925XX (ARC in the graph) (20 μ M) alone or in combination with ATP or UTP (50 and 100 μ M). The experiments were performed three time in triplicate and results were expressed as percentage of adherent cells in relation to control. Cell migration of Kyse-450 (C) and OE-33 (D) were performed as described in Material and Methods section. Fig. 5 E-F show representative images. After confluence, the scratch was performed, treatments were added, and images were obtained at 0 and 24 h of treatment. Experiments were performed three times and results were expressed as median \pm S.E.M. The statistical analysis was performed by One-way ANOVA followed by Tukey post-test, where *P < 0.05; ***P < 0.001.

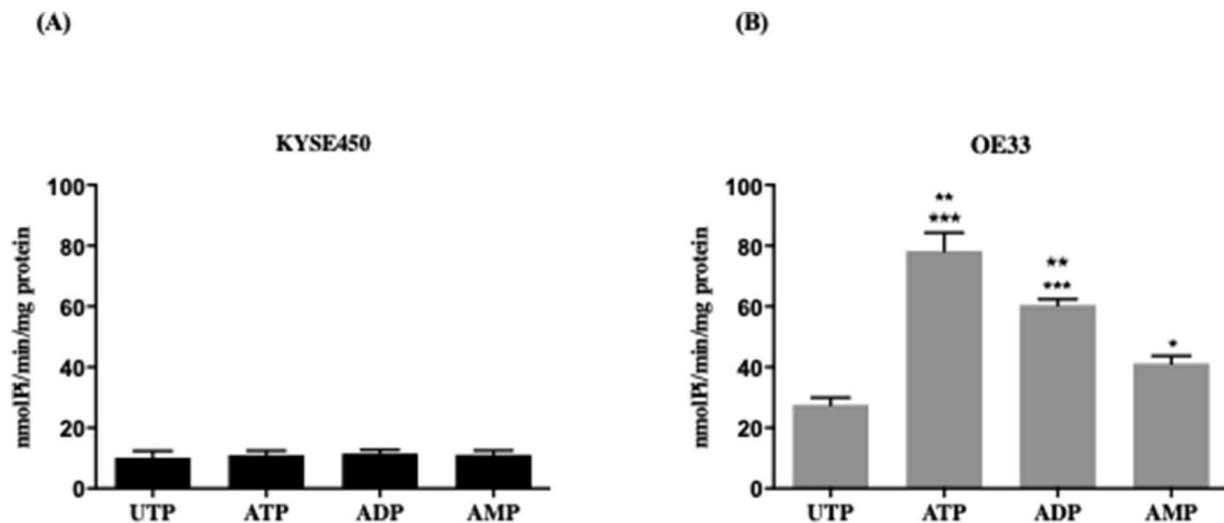


Fig. 6. Evaluation of hydrolysis of ATP, ADP, AMP and UTP in adenocarcinoma and squamous cell carcinoma cell lines. ATPase, ADPase, AMPase and UTPase activity was performed by malachite green method as described in the Material and Methods. Comparison among nucleotide hydrolysis in Kyse-450 (A) and OE33 (B) cells. One-Way ANOVA analysis followed Tukey post-test were used to calculate statistical differences. Data showed as \pm S.E.M., * $p < 0.05$, ** $p < 0.01$ or *** $p < 0.001$.

UTP increased cell proliferation in gastric adenocarcinoma lineages, and this effect was prevented by specific purinergic antagonists. The authors also found high variability of P2Y₂R expression in different tumor-derived biopsies (Hevia et al., 2019). Likewise, it has already seen that ATP and UTP treatment after 24 and 48 h were able to induce proliferation in different glioma cell lines (Morrone et al., 20013). The P2Y₂ receptor is mostly related to tumor growth, extravasation and invasion maintenance and metastasis promotion, in several types of cancer (Di Virgilio and Adinolfi, 2017). Xie et al. (2014), reported the involvement of P2Y₂R and its activation through ATP in hepatocellular carcinoma cells. They also concluded that when P2Y₂R is antagonized, ATP is unable to promote cell proliferation. Recently, our group demonstrated the involvement of the P2X7 receptor in colony formation of ESCC treated with different concentrations of ATP (1–5 mM). The results obtained indicate that high levels of ATP, for long periods, can cause cell death (Santos et al., 2017).

Likewise, in this study we aimed to investigate the role of P2Y₂R in cell ability to form colonies. Intending to evaluate how long the effect of P2Y₂R pharmacological blockade lasts, the cells received the P2Y₂R antagonist only on the first day of treatment; the cells received nucleotides every two days, and the experiment was finished on the 7th day. Kyse-450 cells increased cell colonies number when stimulated UTP the nucleotides. On the other hand, OE-33 cells had an increase in the number of colonies formed after treated with ATP and UTP. Both, ESCC (Kyse-450) and adenocarcinoma cells (OE-33) that received the P2Y₂R antagonist showed an inhibition effect in relation to control; supporting the idea that pharmacological blockade of P2Y₂R continued over seven days in this type of cells. Interestingly, the colonies formed into wells that received UTP were more consistent, which means that they were formed by a higher number of cells, than those that received ATP. Previous reports demonstrate that P2Y₂R activation by ATP and UTP causes cell migration and proliferation in MDA-MB-231 and MCF-7 breast cancer cells, and also, it is linked to pro-inflammatory cascade activation (Wagstaff et al., 2000; Xie et al., 2014), and in A-549 human lung cancer cells, ATP and UTP also support cancer cells growth (Schafer et al., 2003). Conversely, the data shown here allows us to postulate that P2Y₂R has an essential role in the maintenance of living cells and is related to proliferation in cell lines here analyzed.

The activation of signaling pathways such as ERK1/2 provide cell proliferation, differentiation, and survival (Samatar et al., 2014), and PI3K (Akt is the main effector located downstream of PI3K) are involved with cell survival, migration capacity, inflammation and cancer

progression (Foukas et al., 2010). Our data showed that ATP and UTP exert activation in both ERK1/2 and Akt signaling pathways, in different times and cell types. Both nucleotides induced the activation of ERK1/2, and UTP promoted more prolonged activation in both ESCC and EAC cells. This finding is in accordance with the study carried out by Hoffmann et al. (2008), which elucidated that ATP and UTP nucleotides have different profiles of ERK activation. Several studies have demonstrated the nucleotide capacity in activating signaling pathways in diverse tumors. In MCF-7 cells UTP activates the phosphorylation of ERK1/2, but this did not occur when PD98059 inhibited ERK1/2. In HeLa cells, ATP and UTP evoked the activation of P2Y₂R and lead to ERK1/2 and PI3K phosphorylation (Muscella et al., 2003). Glioma cells that received ATP showed increased cell proliferation that occurred via ERK and Akt after the activation of the purinergic receptors (Jacques-Silva et al., 2004).

Here, we also demonstrated that P2Y₂ antagonist decreased cell adhesion in adenocarcinoma OE-33 cells, and this impairment might occur due to adhesion molecules impairment caused by P2Y₂R blockage. In MB-MDA-231 breast cancer cells, the activation of P2Y₂R by ATP or UTP was capable of upregulating the expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), and this expression was inhibited in cells transfected with P2Y₂RshRNA (Jin et al., 2014). Also, in MB-MDA-231 the overexpression of CD73 was related to support cell adhesion, migration and invasion by producing large amounts of adenosine (Wang et al., 2008). In different human prostate cell lines, the P2Y₂R activation by ATP/UTP promotes invasion and migration, and these nucleotides activate epidermal growth factor receptor (EGFR) and ERK1/2 (Li et al., 2015). Whereas in human breast cancer cells, ATP and UTP can stimulate cell migration mainly via ERK1/2, and when the P2Y₂R is silenced this effect is lost (Chadet et al., 2014). Cell migration is an important mechanism utilized by cancer cells to spread and generates metastasis, and studies have focused on the participation of P2Y₂R in cell invasion and migration. In our study, nor ATP, UTP or P2Y₂R antagonist were able to induce cell migration of the cell lines studied.

Lastly, we demonstrated that the adenocarcinoma representative cell line OE-33 exhibited a higher capacity to hydrolyze the extracellular nucleotides tested when compared to ESCC cells. The purine metabolism focused here occurs through the enzymatic activity developed by the CD39 ectonucleotidase, that converts ATP to ADP, ADP to AMP and UTP to UDP (Zimmermann et al., 2012). Previous study from our group showed that ESCC cells presented higher expression ecto-5'-NT, E-NTPDases 1 and 2 when compared to normal esophageal cells (Santos

et al., 2017). Here, the expression and activity of the ectonucleotidases have been related to different stages of the tumor establishment such as proliferation, adhesion, and invasion. Furthermore, ATPase and ADPase presented higher hydrolysis profile when compared to UTPase in adenocarcinoma cells. These data suggest that P2Y₂R agonist UTP can be available for more extended periods in extracellular space in OE-33 cells, modulating cell proliferation observed in this study. The results also demonstrated that both cell lines studied are able to hydrolyze AMP. It is known that CD73 is very important into the final step of extracellular ATP catabolism, producing extracellular adenosine, which favors cancer cell progression and adhesion. Moreover, Santos et al. (2017) showed that ESCC cell lines presented an elevated expression of CD73 when compared to normal EPC2 cells, and this enzyme is active to hydrolyze AMP (Santos et al., 2017).

5. Conclusion

Considering the high rates of mortality in esophageal cancer, the understanding of the mechanisms that induce cell proliferation become fundamental in the search for new therapeutic targets. Here, we showed that P2Y₂R is expressed in biopsies of patients with esophageal cancer, and in two different types of human esophageal cancer cell lines. Both cell lines demonstrated an increase in cell number and colony formation when treated with P2Y₂ agonists ATP or UTP, and these nucleotides activated the primary signaling ERK1/2 pathway. Our data indicate that nucleotides induced the P2Y₂R activation, and the selective antagonist reversed this effect. These results allow us to postulate that P2Y₂R blockage could interfere in human esophageal cancer in cell proliferation and as a consequence, control tumor growth.

CRedit authorship contribution statement

Aline Zaparte: Writing - review & editing, Formal analysis, participated in the research design, conducted the experiments. **Angélica R. Cappellari:** Writing - review & editing, Formal analysis, participated in the research design, conducted the experiments. **Caroline A. Brandão:** conducted the experiments. **Júlia B. de Souza:** conducted the experiments. **Thiago J. Borges:** conducted the experiments. **Luíza W. Kist:** conducted the experiments. **Maurício R. Bogo:** contributed with analytic tools. **Luiz F. Zerbini:** contributed with analytic tools. **Luis Felipe Ribeiro Pinto:** contributed with analytic tools. **Talita Glaser:** conducted the experiments. **Maria Carolina B. Gonçalves:** conducted the experiments. **Yahaira Naaldijk:** conducted the experiments. **Henning Ulrich:** Writing - review & editing, Formal analysis, contributed with analytic tools. **Fernanda B. Morrone:** Writing - review & editing, Formal analysis, participated in the research design.

Declaration of competing interest

The authors declare that they have no competing interests.

Acknowledgements

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, CAPES, Brasil (Finance Code 001); Fundação de Amparo à Pesquisa do Rio Grande do Sul, FAPERGS, PqG 02/2017 (grant number 17/2551-0001016-7); Conselho Nacional de Desenvolvimento Científico e Tecnológico, CNPq (grant number 310317/2018-5), São Paulo Research Foundation, FAPESP (grant number 2012/50880-4); and FINEP “Implantação, Modernização e Qualificação de Estrutura de Pesquisa da PUCRS”, PUCRSINFRA (grant number #01.11.0014-00). Zerbini L.F. was supported by the International Centre for Genetic Engineering and Biotechnology (ICGEB). This study was also supported by Latin American Cooperative Oncology Group (LACOG).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ejphar.2020.173687>.

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