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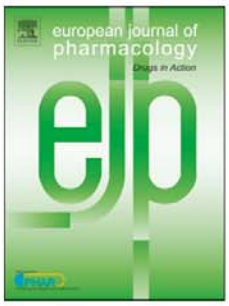

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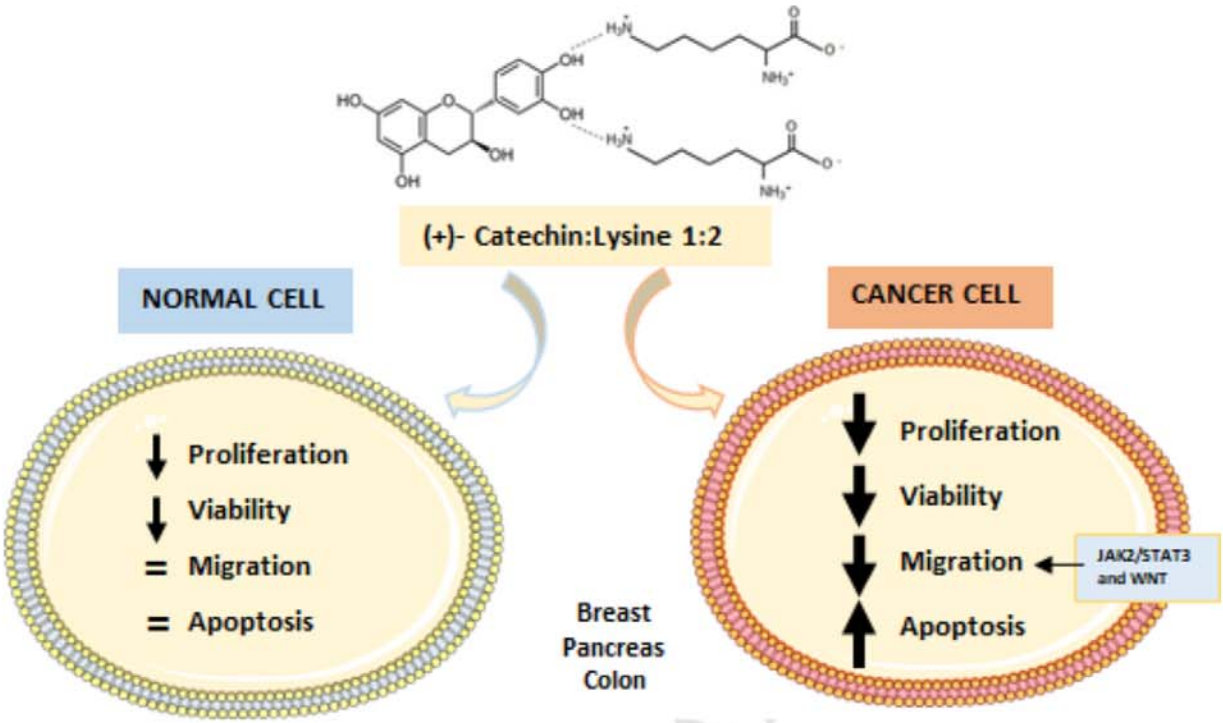

Selective pro-apoptotic and antimigratory effects of polyphenol complex catechin:lysine 1:2 in breast, pancreatic and colorectal cancer cell lines

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Abstract

Cancer is a major cause of death in both developed and developing countries. Polyphenols, abundantly found in plants, possess many anticarcinogenic properties, including inhibition of cancer cell proliferation, tumor growth, angiogenesis, metastasis and inflammation, as well as pro-apoptotic effects. Our study aimed to investigate the effects of a complex of (+)-catechin with 2 lysines (Cat:Lys) on cancer and non-cancer cells. For this, the *in vitro* effects of Cat:Lys on the viability, growth, proliferation, apoptosis, nutrient uptake and migration of breast, pancreatic and colorectal cancer and non-cancer cell lines was evaluated.

We found that Cat:Lys exerted antiproliferative and cytotoxic effects in all breast, pancreatic and colorectal cell lines tested, but with a much less marked amplitude in non-cancer cell lines. It nevertheless interfered with nutrient (^{3}H-deoxy-*D*-glucose and ^{3}H-lactate) uptake and with lactate production in both cancer and non-cancer cell lines. Cat:Lys was found to possess selective antimigratory effects in breast, pancreatic and colorectal cancer cell lines compared to non-cancer cell lines. Cat:Lys also exerted pro-apoptotic effects in all the cancer cell lines that we tested, but not in non-cancer breast and pancreatic cell lines. The antimigratory, but not the pro-apoptotic, effects of Cat:Lys were found to be mediated by JAK2/STAT3 and Wnt pathway inhibition.

In conclusion, Cat:Lys is a strong candidate for the development of a new, effective anticancer agents against cancer.

1 Introduction

Cancer is a group of diseases involving abnormal cell proliferation with the potential to spread to other parts of the body. It is presently a major cause of death in both developed and developing countries (Jemal et al., 2011). According to the World Health Organization (WHO), the average number of new diagnosed cases of cancer in the World overcomes 14 million per year and, among these, more than 60% result in patient dead (8.8 million in 2015) (WHO, 2017). Importantly, cancer incidence continues to escalate (Siegel et al., 2017).

Despite efforts aimed at increasing awareness, early diagnosis and novel therapeutic interventions, the occurrence of drug resistance, elevated costs of treatment as well as secondary toxicity and adverse drug reactions associated with current chemotherapies have hindered progress made (Singh et al., 2016; Singh and Singh, 2018). Due to these concerns, individuals with cancer are increasingly turning to complementary medicines. In this context, the pharmaceutical industry is in search of natural products to treat cancer.

Polyphenols constitute one of the most numerous and widely distributed groups of natural products in the plant kingdom. These secondary metabolites are essential for plant physiology, being involved in functions such as pigmentation, pollination, inhibition of pathogen development and protection against UV radiation, photosynthetic stress, reactive oxygen species, wounds and herbivores (Romero et al., 2004). The growing interest for dietary polyphenols arose from epidemiological studies

reduced risk of several chronic diseases in humans, including cancer (Scalbert et al., 2005; Vauzour et al., 2010).

A number of epidemiological, clinical and experimental researches has indicated that consumption of green tea may have anticancer activities (Li et al., 2018), although not in all cancer types (Najaf Najafi M1 et al., 2018). The health benefits associated with green tea have been predominantly attributed to polyphenols, and the major phenolic constituents of green tea are catechins, including (-)-epicatechin, (-)-epicatechin gallate, (-)-epigallocatechin, (-) epigallocatechin-3-gallate (EGCG), (+)-catechin and (+)-gallocatechin (Massounga Bora et al., 2018).

(+)-Catechin (also known as cyanidanol-3) is more stable than EGCG, the main catechin found in green tea and currently tested in 37 clinical trials for cancer patients [clinicaltrials.gov]), as EGCG undergoes auto-oxidation and epimerization in water (Das and Griffiths, 1967; Sang et al., 2005). Of further interest, (+)-catechin has been reported to form complexes with one or two lysines, which increases its water solubility and brings a net positive charge to the catechin at physiological pH (Niebes et al., 1981). We recently verified that, whereas EGCG, (+)-catechin and (+)-catechin:lysine complexes had similar general antioxidant properties, (+)-catechin:lysine 1:2 (Cat:Lys), but not the other tested catechins, was able to prevent metastatic take of melanoma cells to the lungs of mice (Payen et al., 2017). Indeed, compared to general antioxidants that act unpredictably on cancer, because of its positive charge Cat:Lys is believed to preferentially inactivate mitochondrial reactive oxygen species that drive the metastatic process of several cancer types (Porporato et

vitro anticancer properties of Cat:Lys in breast, pancreatic and colorectal cancer cell lines.

2 Materials and Methods

2.1 Cells and cell culture

We used breast, pancreatic and colorectal cancer and non-cancer cell lines. Breast cell lines were: MCF-7 (an estrogen receptor (ER)-positive human breast epithelial adenocarcinoma cell line; ATCC HTB-22; passage numbers 78-107), MDA-MB-231 (a triple negative human breast adenocarcinoma cell line; ATCC HTB-26; passage numbers 25-49) and MCF12A (a non-tumorigenic epithelial cell line; ATCC CRL-10782; passage numbers 8-21). Pancreatic cell lines were: PANC-1 (a human pancreatic cell line with low metastatic rate; ATCC CRL-1469; passage numbers 13-28), AsPC-1 (a human pancreatic cell line with high metastatic rate; ATCC CRL-1682; passage numbers 7-19) and H6C7 (a normal pancreatic duct cell line; Kerafast catalog nº ECA001; passages numbers 3-10). Colorectal cell lines were: HT-29 (a human colorectal adenocarcinoma cell line; ATCC HTB-38; passage numbers 20-31), Caco-2 (a human colorectal adenocarcinoma cell line; ATCC HTB-37; passage numbers 10-14) and NCM460 (a non-tumorigenic human colorectal epithelial cell line, passage numbers 8-12). MCF-7, MDA-MB-231, MCF12A, PANC-1, AsPC-1, Caco-2 and HT-29 cells were from ATCC (Manassas, VA, USA), H6C7 from Kerafast (Boston, MA, USA) and NCM460 cells were kindly provided by Prof. Fernando Magro (Department of Biomedicine, Unity of Pharmacology and Therapeutics, Faculty of Medicine of Porto, Portugal).

Cells were maintained in a humidified atmosphere of 5% CO_2-95% air and were grown in MEM (catalogue #31095-052, Gibco, Life Technologies Corporation, CA, USA)

MI, USA) supplemented with 2 mM *L*-glutamine, 10 mM sodium bicarbonate, 15% heat-inactivated FBS and 1% antibiotic/antimycotic (MDA-MB-231 cells); DMEM:Ham's F12 medium (1:1) (catalogue #FG4815, Biochrom, Berlin, Germany) supplemented with 20 ng/ml human epidermal growth factor (Gibco), 100 ng/ml cholera toxin, 0.01 mg/ml bovine insulin, 500 ng/ml hydrocortisone (Sigma), 5% heat-inactivated horse serum and 1% antibiotic/antimytotic (MCF12A); DMEM with 4.5 g/L glucose (catalogue #D5796, Sigma), 2 mM *L*-glutamine, 10 mM sodium bicarbonate, 10% heat-inactivated FBS and 1% antibiotic/antimycotic (PANC-1 cells); RPMI 1640 (catalogue #R6504, Sigma) supplemented with 2 mM *L*-glutamine, 1 mM sodium pyruvate, 10% heat-inactivated FBS and 1% antibiotic/antimycotic (AsPC-1 cells); keratinocyte serum-free medium (catalogue #17005-075, Gibco) supplemented with 2.5 µg/µl epidermal growth factor, 25 µg/ml bovine pituitary extract (Gibco) and 1% antibiotic/antimycotic (H6C7 cells); DMEM with 4.5 g/L glucose (catalogue #D5796, Sigma), 2 mM *L*-glutamine, 10 mM sodium bicarbonate, 10% heat-inactivated FBS and 1% antibiotic/antimycotic (HT-29 cells); MEM (catalogue #M-0643, Sigma) supplemented with 25 mM HEPES, 26.2 mM sodium bicarbonate, 15% heat-inactivated FBS and 1% antibiotic/antimycotic (Caco-2 cells); or RPMI 1640 (catalogue #R6504, Sigma) supplemented with 2 mM *L*-glutamine, 10% heat-inactivated FBS and 1% antibiotic/antimycotic (NCM460 cells). Culture medium was renewed every 2-3 days, and the culture was split every 7 days.

For the determination of viability, proliferation, growth, migration and lactate production, cells were seeded on 24-well culture dishes and used at 90% confluence.

used at 50-60% confluence. For RNA extraction, cells were seeded on 21 cm^2 plates and used at 100% confluence.

2.2 Cell treatments

To test the acute effect of Cat:Lys (BePharBel Manufacturing, Courcelles, Belgium) on viability, proliferation and culture growth, cells were exposed to the compound for 26 min in GF-HBS buffer (composition in mM: 20 HEPES–NaOH, 5 KCl, 140 NaCl, 2.5 $MgCl_2$, 1 $CaCl_2$, pH 7.4). For transport experiments, cells were exposed to Cat:Lys during a 20 min pre-incubation and an additional 6-min incubation time with ^{3}H-2-deoxy-*D*-glucose (^{3}H-DG, specific activity 60 Ci/mmol, American Radiolabeled Chemicals, St. Louis, MO, USA) or ^{3}H-lactic acid (^{3}H-L, specific activity 15 Ci/mmol, American Radiolabeled Chemicals) in GF-HBS buffer. To test the chronic effects of Cat:Lys on cell proliferation, viability, apoptosis, migration, culture growth and glucose metabolism, cells were exposed to Cat:Lys for 24 h in serum-free culture medium. For transport experiments, cells were exposed to Cat:Lys and/or transport inhibitors 2-DG (2-deoxy-*D*-glucose) and NPPB (5-nitro-2-[3-phenypropylamino]benzoic acid) (both from Sigma) for 24 h in serum-free culture medium, followed by a 20-min pre-incubation and a 6-min incubation with ^{3}H-DG or ^{3}H-L in GF-HBS buffer.

Tested drugs were dissolved in water (Cat:Lys complex and 2-DG) or DMSO (tyrphostin AG 490, XAV939 and NPPB; all from Sigma). The final concentration of solvents in buffer and culture media was 1% (v/v). Controls for the drugs were run in the presence of solvent.

Cells were exposed to Cat:Lys for 26 min (0.001-5 mM) or 24 h (0.001-1 mM). After this period of time, cellular leakage of lactate dehydrogenase (LDH) to the extracellular culture medium was determined, as described (Azevedo et al., 2015). LDH activity was expressed as the percentage of extracellular activity in relation to total cellular LDH activity.

2.4 Cell growth

Cells were exposed to Cat:Lys for 26 min (0.001-5 mM) or 24 h (0.001-1 mM). At the end of treatment, culture growth was determined by the sulforhodamine B (SRB) assay, which reports on intracellular protein content, as described (Azevedo et al., 2015).

2.5 Cell proliferation

Cells were exposed to Cat:Lys for 24 h (0.001-1 mM), and cell proliferation rates were determined by a ^{3}H-thymidine incorporation assay, as described (Azevedo et al., 2015). DNA synthesis rate was expressed relatively to the incorporation of ^{3}H-thymidine (mCi/mg total protein).

2.6 Cell migration

Cell migration rates were determined by a scratch injury assay. Briefly, cell monolayers were scratched with a 10 µl pipette tip and were afterwards treated for 24 h with Cat:Lys (0.01-1 mM) or vehicle. Images were obtained at 0 and 24 h after the scratch, and quantification was performed using the ImageJ software (NIH, Bethesda, MD,

2.7 Apoptotic index

Cells were seeded on glass coverslips and were exposed to Cat:Lys (0.01-1 mM), tyrphostin AG 490 and/or XAV939 for 24 h. Then, the apoptotic index was determined by the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, by using the In Situ Cell Death Detection kit (Roche Diagnostics, Basel, Switzerland), as described (Correia-Branco et al., 2015). The apoptotic index was calculated as the percentage of apoptotic cells respective to total cell number.

2.8 Glucose and lactate uptake

Culture medium was discarded and the cells were washed with 300 µl GF-HBS buffer at 37°C. Then, cell monolayers were pre-incubated for 20 min in buffer at 37°C. Uptake was initiated by the addition of 200 µl GF-HBS buffer at 37°C containing 10 nM ^{3}H-DG or ^{3}H-L. Incubation was stopped after 6 min by removing the incubation medium, placing the cells on ice, and rinsing them with 500 µl ice-cold GF-HBS buffer. Cells were then solubilized with 300 µl 0.1% (v/v) Triton X-100 (in 5 mM Tris-HCl, pH 7.4) and placed at 4 °C overnight. Intracellular radioactivity was measured by liquid scintillation counting (LKB Wallac 1209 Rackbeta, Turku, Finland). Results were normalized for total protein content (Bradford method).

2.9 Lactate release

After exposure to Cat:Lys for 24 h (0.01-1 mM), extracellular lactate was measured with the lactate oxidase/peroxidase colorimetric assay, as described (Azevedo et al.,

2.10 RT-qPCR

Total RNA was extracted from MCF-7, MDA-MB-231 and MCF12A cells treated for 24 h with Cat:Lys (1 mM), and RT-qPCR was carried out as described (Azevedo et al., 2015). The primer pair for human glucose transporter 1 (*SLC2A1*; *GLUT1*) was: 5'-GAT GAT GCG GGA GAA GAA GGT-3' (forward) and 5'-ACA GCG TTG ATG CCA GAC AG-3' (reverse). The primer pair for human monocarboxylate transporter 1 (*SLC16A1*, *MCT1*) was: 5'-CAC CGT ACA GCA ACT ATA CG-3' (forward) and 5'-CAA TGG TCG CCT CTT GTA GA-3' (reverse). The amount of *GLUT1* and *MCT1* mRNA was normalized to the amount of mRNA of housekeeping gene *human β-actin*. The primer pair for *β-actin* was: 5'-AGA GCC TCG CCT TTG CCG AT-3' (forward) and 5'-CCA TCA CGC CCT GGT GCC T-3' (reverse). Data were collected using the LightCyclerw 96 SW 1.1 analysis software (Roche, Mannheim, Germany), and results were analyzed by the comparative Ct (ΔΔCT) method (Schmittgen and Livak, 2008). *β-actin* mRNA expression levels were not affected by the Cat:Lys treatment (*data not shown*).

2.11 Total protein determination

The protein content of cell monolayers was determined as described by Bradford (1976) (Bradford, 1976), using human serum albumin as a standard.

2.12 Western blotting

MCF-7 cells treated for 24 h with Cat:Lys (1 mM) were lysed with RIPA Lysis Buffer (150 mM NaCl, 0,1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl

using the BCA assay (Pierce). Proteins (20 µg) from each sample were subjected to SDS/PAGE on TGX Stain-Free Any-kD gels (BioRad). Gels were subsequently stain-free activated for 1 min and imaged on the BioRad ChemiDoc™ system. Activated gels were then transferred onto nitrocellulose membranes (GE Healthcare) that were also subjected to stain-free imaging.

Membranes were blocked with 4% BSA in TBS-Tween 0.1% and incubated overnight with primary antibodies diluted in the same solution as follows: Stat3 phospho-Y705 (Abcam 76315) 1:500; Stat3 (Abcam 109085) 1:1,000; Caspase-3 (Abcam 184787) 1:1,000; β-Catenin (Abcam 223075) 1:1000. Incubation with secondary antibody (GE Healthcare NA934V, 1:10,000 dilution) was performed for 1 h at room temperature and chemiluminescent signal was detected using Clarity Western ECL Substrate (BioRad).

2.13 Statistics

Data are expressed as means ± S.E.M. *n* indicates the number of replicates of at least 2 independent experiments. Student's *t* test and two-way ANOVA with Dunnett or Tukey posthoc tests were used where convenient. $P < 0.05$ was considered to be statistically significant.

3 Results

3.1 Cat:Lys decreases cancer and non-cancer cell proliferation, viability and growth

Based on the hypothesis that Cat:Lys would exert anticancer activities in breast, pancreatic and colorectal cancer cell lines, we first measured the effects of a 24 h treatment with Cat:Lys on cell proliferation, viability and growth (**Fig. 1**). With respect to cell proliferation, Cat:Lys induced a concentration-dependent decrease in the proliferation rates of all breast, pancreatic and colorectal cancer and non-cancer cell lines that we tested. However, the effect was less pronounced in non-cancerous breast cell lines than in the two breast cancer cell lines, especially for the lower concentrations of the compound. No such difference was observed in pancreatic and colorectal cells. With respect to viability, Cat:Lys induced a concentration-dependent decrease in the viability of breast, pancreatic and colorectal cancer cell lines. A less marked effect was observed in non-cancer cell lines; it was without effect in pancreatic non-cancer cells. With respect to culture growth, Cat:Lys caused a marked and concentration-dependent decrease in the growth of breast and colorectal cancer cell lines, and, interestingly, a much less marked effect in the non-cancer cell lines. In contrast, the effects of Cat:Lys on the growth of cancer and non-cancer pancreatic cell lines were similar (**Fig. 1**). Compared with Cat:Lys, Cat and *L*-Lys alone present much less potent effects in relation to cell proliferation, viability and culture growth (**Fig.S1**).

The effects of Cat:Lys on cell viability and growth were also investigated after a shorter exposure time of 26 min in breast cells. In the three cell lines, decreased

antiproliferative effects. Such a rapid effect can be associated with necrosis, but this point was not further investigated.

3.2. Cat:Lys has variable effects on cellular glucose uptake

We used ^{3}H-DG to measure cellular glucose uptake. In cancer cell lines, Cat:Lys (24 h) either decreased (MCF-7 and Caco-2), had no effect (Panc-1) or increased (MDA-MB-231, AspC-1 and HT-29) ^{3}H-DG uptake **(Fig. 2A)**. A similar variability was found in the non-cancer cell lines, with increased (MCF12A) or decreased (H6C7 and NCM460) ^{3}H-DG uptake (**Fig. 2A**). Interestingly, a shorter exposure time (26 min) to Cat:Lys caused similar changes in^{3}H-DG uptake in all the breast cell lines that we tested (**Fig. S3A**).

In the three breast cell lines that we tested, ^{3}H-DG uptake involves a GLUT-mediated mechanism, as it was significantly reduced in the presence of 2-DG, a GLUT and glycolysis substrate/inhibitor (**Fig. S3B**). Results obtained with Cat:Lys in the presence of 2-DG suggest that Cat:Lys stimulates GLUT-mediated ^{3}H-DG uptake in MDA-MB-231 and MCF12A cell lines, and that a non-GLUT-mediated mechanism is inhibited by Cat:Lys in MCF-7 cells (**Fig. S3B**).

In MDA-MB-231 and MCF12A cells, Cat:Lys increased ^{3}H-DG uptake, whereas it decreased ^{3}H-DG uptake in MCF-7 cells. This could be linked to changes in GLUT expression or activity. GLUT1 overexpression has been consistently observed in many cancers, including breast, lung, renal, colorectal and pancreas (Medina and Owen, 2002), and GLUT1 is also the main mediator of glucose uptake in breast cancer cells (Grover-McKay et al., 1998; Krzeslak et al., 2012). Here, we observed that Cat:Lys (24

increased *GLUT1* mRNA expression in MDA-MB-231 cells, which could explain how it increased ^{3}H-DG uptake (**Fig. 2B**).

3.3 Cat:Lys has variable effects on cellular lactate transport

We used ^{3}H-L to measure cellular lactate uptake. Interestingly, Cat:Lys had a distinct effect on cancer and non-cancer cell lines, namely decreasing ^{3}H-L uptake in cancer cell lines, while having no effect or increasing it in non-cancer cell lines (**Fig. 2C**). A similar effect of Cat:Lys on ^{3}H-L uptake by breast cancer and non-cancer cell lines was found after a shorter time (26 min) exposure (**Fig. S4A**).

By using NPPB, a MCT inhibitor, we observed that ^{3}H-L uptake by MCF-7 and MCF12A cells involves an MCT-mediated mechanism, whereas ^{3}H-L uptake by MDA-MB-231 is not MCT-mediated (**Fig. S4B).** Analysis of the effects of a combination of Cat:Lys with NPPB on ^{3}H-L uptake revealed that, in MCF-7 cells, Cat:Lys inhibited both MCT- and non-MCT mediated ^{3}H-L uptake (**Fig. S4B**). In agreement with these results, Cat:Lys decreased *MCT1* mRNA levels in MCF-7 cells (**Fig. 2B**). Comparatively, combining Cat:Lys with NPPB in MDA-MB-231 cells revealed that Cat:Lys inhibited non MCT-mediated ^{3}H-L uptake in this cell line (**Fig. S4B**), even if it increased *MCT1* mRNA expression (**Fig. 2B**). Finally, we observed no effect of Cat:Lys on MCT and non-MCT mediated ^{3}H-L uptake in non-cancer cell lines (**Fig. S4B**). Cat:Lys did not modify *MCT1* mRNA expression in MCF12A cells (**Fig. 2B**).

We also tested the effects of Cat:Lys on lactate release by the cells. In breast cell lines, the effects of Cat:Lys on lactate production (**Fig. 2D**) were similar to its effects on

production. In contrast, in the pancreatic and colorectal cell lines, the effects of Cat:Lys on lactate production were variable, and, except for Panc-1, Caco-2 and NCM460 cell lines, distinct from its effects on ^{3}H-DG uptake (**Fig. 2D**).

3.4 Cat:Lys has antimigratory effects

We next examined the effect of Cat:Lys on cell migration. In scratch injury assays, Cat:Lys had antimigratory effects in all the cancer cell lines that we tested (**Fig. 3A; Fig. S5**). The response was less marked in non-cancer cell lines, and in non-cancer pancreatic and colorectal cell lines, Cat:Lys was devoid of any effect on cell migration.

JAK2/STAT3 and Wnt signaling pathways are well-known to promote migration of human cancer cells (Anastas and Moon, 2013; Teng et al., 2014), and several polyphenolic compounds are known to interfere with these signaling pathways (Iftikhar and Rashid, 2014; Yang et al., 2016; Zhang et al., 2014). We therefore investigated the involvement of these pathways in the response to Cat:Lys by using specific inhibitors of JAK2/STAT3 (AG490 [5 μM]) and Wnt (XAV939 [10 μM]) signaling in breast (MCF-7) and pancreatic (Panc-1 and AsPc-1) cell lines. Interestingly, cell migration was decreased when using JAK2/STAT3 and Wnt inhibitors (although not significantly so in MCF-7 cells), indicating that these pathways are involved in the migratory ability of these cell lines (**Fig. 3B**). Scratch injury assays in the presence of JAK2/STAT3 and Wnt inhibitors suggested that the antimigratory effects of Cat:Lys in pancreas and breast cancer cells involves JAK/STAT3 and Wnt inhibition, as the antimigratory effect of Cat:Lys and JAK2/STAT3 and Wnt inhibitors was not additive

analysis (**Fig. 4**), indicating that Cat:Lys negatively regulates the Wnt and JAK2/STAT3 signaling pathways, respectively.

3.5 Cat:Lys has pro-apoptotic effects

Finally, we examined the effects of Cat:Lys on the apoptosis index of breast, pancreatic and colon cell lines by TUNEL. Cat:Lys induced apoptosis in all the cancer cell lines, except HT-29 (**Fig. 5A**). Importantly, it had no effect in non-cancer cell lines (**Fig. 5A**). In agreement, the protein levels of caspase-3 (a major regulator of apoptotic cell death) were increased over two-fold by Cat:Lys treatment of MCF-7 cells, when compared to untreated controls (**Fig. 4**).

In addition to their capability to induce cell migration, JAK2/STAT3 and Wnt signaling pathways are well known to protect cancer cells against apoptosis (Bowman et al., 2000) and several polyphenolic compounds are known to interfere with these signaling pathways (Iftikhar and Rashid, 2014; Yang et al., 2016; Zhang et al., 2014). Because the pro-apoptotic effects of Cat:Lys were selective for breast and pancreatic cancer cell lines, we investigated the involvement of these pathways in the response to Cat:Lys. In contrast to what was verified for cell migration, apoptosis index was not changed by JAK2/STAT3 and Wnt inhibitors, indicating that apoptotic index in these cell lines does not depend on these pathways (**Fig. 5B, 5C**). Moreover, although western blot analysis had revealed that Cat:Lys is an inhibitor of JAK2/STAT3 and Wnt pathways (**Fig. 4**), the effects of Cat:Lys (increase) and of JAK2/STAT3 and Wnt inhibitors (no effect) on the apoptosis index were very distinct (**Fig. 5B, C**). So, we

of Cat:Lys in breast and pancreatic cancer cell lines was negatively affected by inhibitors of both JAK2/STAT3 and Wnt pathways (**Fig. 5B, C**). This effect may be attributable to the fact that these inhibitors affect not only JAK2/STAT3 and Wnt pathways, but other pathways as well. In this context, AG-490 was described to be an inhibitor of tyrosine kinases including not only JAK2, but also EGFR, HER2 and JAK3 (Gazit et al., 1991; Wang et al., 1999); and XAV939 was also found to possess effects not related to Wnt inhibition (Dittfeld et al., 2018). This hypothesis needs, however, confirmation.

4. Discussion

Polyphenols, abundantly found in plants, possess many anticarcinogenic properties, including inhibition of cancer cell proliferation, tumor growth, angiogenesis, metastasis and inflammation, as well as pro-apoptotic effects. Most of the above effects are linked to the antioxidant and anti-inflammatory properties of polyphenols (Hussain et al., 2016). Interestingly, by acting on multiple molecular and cellular targets, polyphenols are able to influence the three phases of chemoprovention, namely primary prevention (inhibition of cancer initiation), secondary prevention (inhibition of cancer promotion) and therapy (inhibition of cancer progression) (Oyenihi and Smith, 2019). Indeed, the effects of polyphenols are often multifaceted, with the most consistently reported mechanisms related to stimulation of apoptosis (Curti et al., 2017) and autophagy (Gali-Muhtasib et al., 2015), regulation of cellular signaling cascades (Lewandowska et al., 2016), epigenetic modulation (Li et al., 2016) and inhibition of cell proliferation, angiogenesis and metastasis (Morbidelli, 2016; Weng and Yen, 2012). One of major problems with using polyphenols as anticancer agents individually is their poor bioavailability in the human body and their interactions with other natural compounds in a diet, which may hinder or complicate consistency of their efficacy (Williamson and Manach, 2005). In this context, several studies previously indicated that the anticarcinogenic efficacy of polyphenols can be enhanced by combining them with compounds such as amino acids and vitamins (Niedzwiecki et al., 2016). The aim of this study was to investigate the effect of a complex of (+)-

As expected, we found that Cat:Lys exerted cytotoxic and antiproliferative effects in all breast, pancreatic and colorectal cancer cell lines and, interestingly, a much less marked effect in non-cancer cell lines. One of the molecular hallmarks of cancer is a deviant energetic metabolism, whereby the rate of cellular glucose uptake is significantly increased, associated with a high rate of glycolysis and lactate production. Primarily, the switch from an oxidative to a glycolytic metabolism is an adaptive response to cancer-associated hypoxia, but it can also occur independently of the presence of oxygen (Hanahan and Weinberg, 2011; Strickaert et al., 2017; Warburg, 1956). These processes, known as "anaerobic glycolysis" and "aerobic glycolysis" (or the Warburg effect, or "aerobic lactatogenesis") (Martel et al., 2016), respectively, are believed to be cancer cell fingerprints that can be used as a specific molecular therapeutic targets. *In vitro* studies have shown that several polyphenols act as specific inhibitors of glucose transport in breast cancer cells, and an association between their anticarcinogenic effects and inhibition of cellular glucose uptake was also described (Azevedo et al., 2015; Martel et al., 2016; Moreira et al., 2013; Uifalean et al., 2016). Moreover, some polyphenols are able to inhibit lactate transport in breast cancer cells and, importantly, some are able to inhibit both glucose and lactate transport, thus depleting breast cancer cells of their two most important energetic substrates (Martel et al., 2016). For this reason, we investigated the effect of Cat:Lys on the cellular uptake of ^{3}H-DG and ^{3}H-L.

We report that the effects of Cat:Lys on ^{3}H-DG uptake are variable. In cancer cell lines, the complex either decreased (MCF-7 and Caco-2 cells), had no effect (Panc-1) or

found. Interestingly enough, a shorter exposure time of MCF-7 cells to Cat:Lys caused an increase in ^{3}H-DG uptake, an effect also found with (+)-catechin (Azevedo et al., 2015). Mechanistically, the use of a GLUT inhibitor in breast cell lines led us to conclude that (a) there is a contribution of a GLUT-mediated mechanism in ^{3}H-DG uptake in the three breast cell lines, (b) Cat:Lys stimulates GLUT-mediated ^{3}H-DG uptake in MDA-MB-231 and MCF12A cell lines, and (c) Cat:Lys inhibits non-GLUT-mediated ^{3}H-DG uptake in MCF-7 cells, a conclusion that was supported by the results of *GLUT1* mRNA expression. Non-GLUT-mediated ^{3}H-DG uptake might correspond to a sodium-dependent glucose co-transporter (*SLC5; SGLT*)-mediated mechanism, as *SGLT1* and *SGLT2* overexpression has been found in a number of cancers (Koepsell, 2017).

In relation to ^{3}H-L uptake, the effects of Cat:Lys were quite interesting, as the complex had a distinct effect on cancer (decrease) and non-cancer (no effect or increase) breast, pancreatic and colorectal cell lines. Moreover, by using an MCT inhibitor in breast cell lines, we verified that (a) the contribution of MCT- and non MCT-mediated mechanisms supporting ^{3}H-L uptake in the three cell lines is distinct (higher contribution in MCF-7, lower in MCF12A and no contribution in MDA-MB-231 cells), and (b) that Cat:Lys has an inhibitory effect on both MCT- and non-MCT-mediated ^{3}H-L uptake in MCF-7 cells, an inhibitory effect on non-MCT1-mediated ^{3}H-L uptake in MDA-MB-231 cells and no effect on both MCT- and non-MCT ^{3}H-L uptake in the non-cancer cell line. These conclusions were supported by the results of *MCT1* mRNA expression.

was unraveled, with the complex reducing lactate uptake by cancer cells, while having no effects on the non-cancer cell lines that we tested. Of importance, Cat:Lys was able to simultaneously reduce ^{3}H-DG and ^{3}H-L uptake in MCF-7 cells, thus potentially depleting them of glucose and lactate, *i.e.*, two important energy sources. This possibility is supported by the fact that another polyphenol, kaempferol, showed a similar double inhibitory effect in this cell line, and this effect was involved in its anticarcinogenic effects (Azevedo et al., 2015).

Analysis of the effects of Cat:Lys on lactate production showed that its effect on ^{3}H-DG uptake were sometimes associated to downstream effects on lactate production (all breast cell lines plus Caco-2 and NCM460), while distinct effects of Cat:Lys on both parameters were observed in the other cell lines (all pancreatic cell lines and HT-29 cells). These observations suggest that different metabolic adaptations could arise in response to Cat:Lys, involving the relative contributions of glucose and glutamine to lactate production.

We also investigated the effect of Cat:Lys on cell migration and apoptosis. Cat:Lys was found to exert antimigratory effects in all breast, pancreatic and colorectal cancer cell lines, which showed up to be much less potent in non-cancer cell lines. In pancreatic and colorectal non-cancer cell lines, Cat:Lys was in fact devoid of antimigratory effects. It is of note that our migration assay does not allow to normalize for cell death and proliferation. In relation to apoptosis rates, Cat:Lys exerted pro-apoptotic effect in all breast, pancreatic and colorectal cancer cell lines, and, importantly, it was without effect in non-cancer breast and pancreatic cell lines. We

and pancreatic cancer cells and selective pro-apoptotic effects in breast and pancreatic cancer cell lines.

For this reason, we decided to further investigate the mechanisms involved in this effect of Cat:Lys, by investigating the involvement of the JAK2/STAT3 and Wnt signaling pathways. These intracellular signaling pathways promote migration and reduce apoptosis of cancer cells (Anastas and Moon, 2013; Bowman et al., 2000; Teng et al., 2014) and several polyphenolic compounds are known to interfere with them (Iftikhar and Rashid, 2014; Yang et al., 2016; Zhang et al., 2014). More specifically, STAT3 is a transcription factor that relays signals from cell surface receptors to the nucleus (Brivanlou and Darnell, 2002). It regulates the transcription of several genes that play a significant role in cell survival, proliferation and angiogenesis (Teng et al., 2014; Yu and Jove, 2004). Enhanced and persistent activation of STAT3 is frequently found in human cancers and is associated with cell proliferation, cell survival, invasion, angiogenesis and metastasis (Bowman et al., 2000; Yu and Jove, 2004). Conversely, targeting STAT3 activation inhibits tumor growth and metastasis both *in vitro* and *in vivo* without affecting normal cells, thus suggesting that STAT3 is a valid molecular target for cancer therapy (Jing and Tweardy, 2005). As to the Wnt signaling pathway, it is a complex network that regulates gene transcription. Wnts and their downstream effectors regulate various processes that are important for cancer progression, including tumor initiation, tumor growth, cell senescence, cell death, differentiation and metastasis. Accordingly, overactivation of Wnt signaling is thought to be closely associated to cancer development (Anastas and Moon, 2013; Clevers and Nusse, 2012;

Analysis of the results using specific inhibitors of JAK2/STAT3 and Wnt pathways suggests that migration, but not apoptosis, of breast cancer cell line MCF-7 and pancreatic cancer cell lines Panc-1 and AspC-1 is dependent on both JAK2/STAT3 and Wnt activation under control conditions. The results also suggest that the antimigratory effects of Cat:Lys in both breast and pancreatic cell lines involve inhibition of the JAK2/STAT3 and Wnt signaling pathways. In agreement with this conclusion, levels of β-catenin and phosphorylated STAT-3 were downregulated by Cat:Lys. In contrast, we concluded that the pro-apoptotic effect of Cat:Lys do not involves inhibition of JAK2/STAT3 or Wnt signaling pathways. So, the antimigratory, but not the pro-apoptotic, effects of Cat:Lys is dependent on JAK2/STAT3 and Wnt inhibition.

A last point concerns the concentrations of Cat:Lys tested. The highest concentrations of Cat:Lys tested (1 and 5 mM) are not attainable *in vivo*, and different mechanisms can be acting at these high concentrations, when compared with the lower ones. However, it is important to note that the results obtained with lower and higher concentrations of Cat:lys were very consistent.

In conclusion, we report that Cat:Lys concentration-dependently inhibits proliferation, viability and migration and stimulates apoptosis in breast, pancreatic and colon cancer cell lines. This compound was also found to interfere with cellular glucose and lactate uptake and with lactate production in these cell lines. Importantly, the effects of Cat:Lys on non-cancer cell lines, especially its antimigratory and pro-apoptotic effects, were less evident. The antimigratory, but not the pro-apoptotic

Conflict of interest

JS has interests in patent BPH-001-EP: oral composition comprising (+)- catechin. JS is a consultant for and an investor in BePharBel Manufacturing. Authors have no other conflicts of interest to declare.

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Figure legends

Figure 1. Cat:Lys decreases cancer and non-cancer cell proliferation, viability and growth. A-C, Effects of Cat:Lys (24 h) on cell proliferation rates (*left graphs*), viability (*medium graphs*) and culture growth (*right graphs*) **A,** Effects of increasing concentrations of Cat:Lys on cancer (MCF-7 and MDA-MB-231) and non-tumorigenic (MCF12A) breast cell lines (n = 6-8). **B,** Effects of increasing concentrations of Cat:Lys on cancer (Panc-1 and Aspc-1) and normal (H6C7) pancreatic cell lines (n = 6-16). **C**, Effects of increasing concentrations of Cat:Lys on cancer (HT-29 and Caco-2) and normal (NCM460) colorectal cell lines (n=6-8). Data show means ± S.E.M. * $P < 0.05$ *versus* control, by two way ANOVA with Dunnett post-hoc test.

Figure 2. Cat:Lys exerts variable effects on glucose uptake and lactate transport. A, Effects of Cat:Lys (24 h) on ^{3}H-2-deoxy-glucose (^{3}H-DG) uptake by cancer (MCF-7 and MDA-MB-231) and non-tumorigenic (MCF12A) breast cell lines (n = 8-16) (*left graph*); cancer (Panc-1 and Aspc-1) and normal (H6C7) pancreatic cells (n = 8-12) (*middle graph*); and cancer (HT-29 and Caco-2) and normal (NCM460) colorectal cells (n = 8). **B,** Effects of Cat:Lys(24h) on *GLUT1* and *MCT1* mRNA levels in breast cancer and non-tumorigenic cells (n = 5-6). **C,** Effects of Cat:Lys (24h) on ^{3}H-Lactate (^{3}H-L) uptake in cells as in **A**, but with n = 8 for all treatment conditions. **D,** Effect of Cat:Lys on lactate production in cells as in **A**, but with n = 8 for breast cells, n = 4 for pancreatic cells and n = 4 for colorectal cells. Data show means ± S.E.M. * $P < 0.05$ *versus* control by two-way ANOVA (A, C, D *middle* and *right*) with Dunnett post-hoc test or Student's *t* test (B and D *left*).

Figure 3. Cat:Lys selectively decreases cancer cell migration. A, Effects of Cat:Lys (24 h) on the migration of cancer (MCF-7 and MDA-MB-231) and non-tumorigenic (MCF12A) breast cells (n = 8-12) (*left graph*); cancer (Panc-1 and Aspc-1) and normal (H6C7) pancreatic cells (n = 8) (*middle graph*); and cancer (HT-29 and Caco-2) and normal (NCM460) colorectal cells (n = 8) (*right graph*). **B,** Effects of

(24h; *n* = 8 all). Data show means ± S.E.M. * *P* < 0.05 *versus* control; *ns*, not significant, by two-way ANOVA with Dunnett (A) or Tukey post-hoc (B and C) test.

Figure 4. Cat:Lys treatment results in decreased STAT-3 phosphorylation and β-Catenin expression, and in overexpression of apoptosis main effector protease, Caspase-3. Effects of Cat:Lys (24 h, 1 mM) on protein levels of β-Catenin (n=6), phosphorylated STAT3 (p-STAT3; n=12), STAT3 (n=12) and caspase-3 (n=6) in breast cancer (MCF-7) cells as assessed by Western blotting. Data were normalized to total protein (stain free gel technology) and are shown relative to untreated controls (=100%). * P < 0.05 by Student's t-test.

Figure 5. Cat:Lys selectively causes apoptosis in cancer cells. A, Effects of Cat:Lys (24h) on apoptotic rates of cancer (MCF-7 and MDA-MB-231) and non-tumorigenic (MCF12A) breast cells (*n* = 8) (*left*); cancer (Panc-1 and Aspc-1) and normal (H6C7) pancreatic cells (*n* = 8) (*middle*); and cancer (HT-29 and Caco-2) and normal (NCM460) colorectal cells (*n* = 6) (*right*). **B,** Effects of Cat:Lys ± 5 μM of JAK2/STAT3 inhibitor AG490 (24h) on the apoptosis of breast cancer cells (*left*) and pancreatic cancer cells (*right*) (*n* = 8 all). **C,** As in **B** but using 10 μM of Wnt inhibitor XAV939 10 μM (24h; *n* = 8 all). Data show means ± S.E.M. * *P* < 0.05 *versus* control; *ns*, not significant, by two-way ANOVA with Dunnett (A) or Tukey post-hoc (B and C) test.

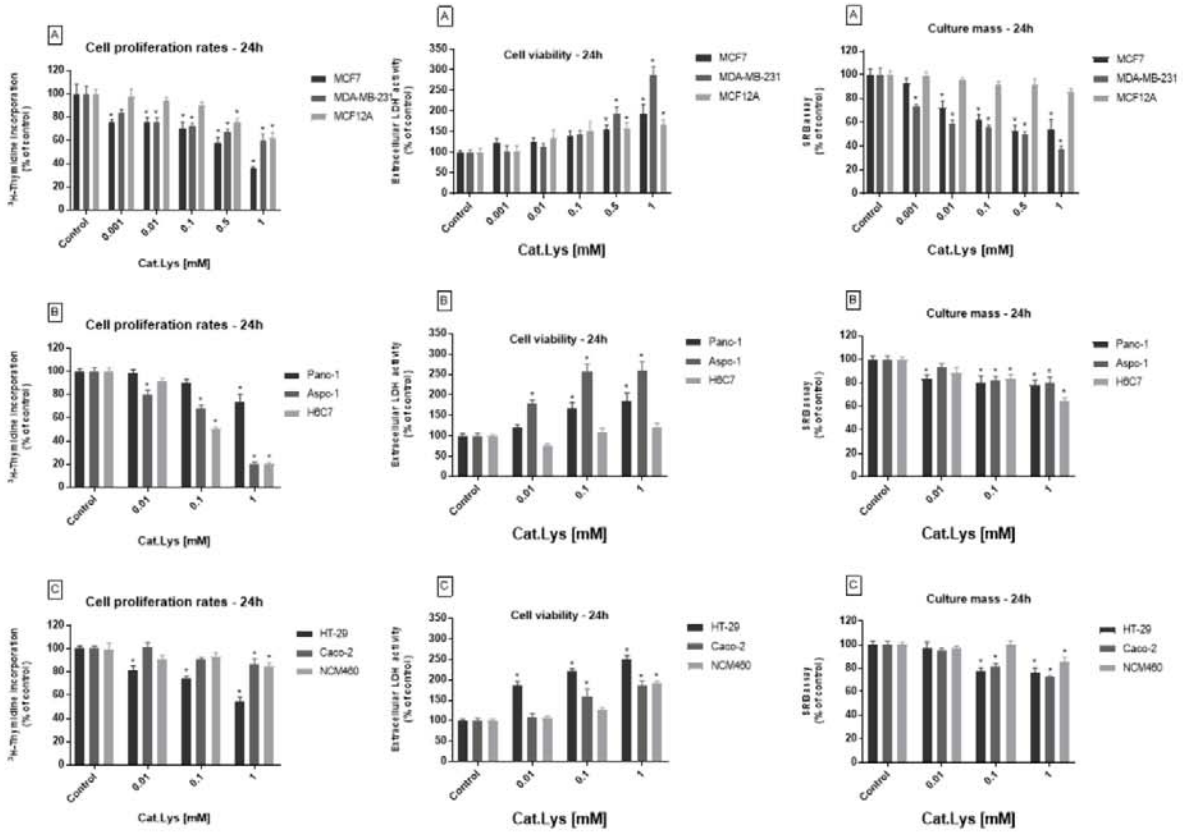
Figure S1. Separately, Cat and L-Lys have more discrete effects on breast cancer cell proliferation, viability and growth than Cat:Lys. A, Effects of increasing concentrations of Cat and L-Lys (24 h) on cell proliferation rates. **B,** Effects of increasing concentrations of Cat and L-Lys (24 h) on cell viability. **C,** Effects of increasing concentrations of Cat and L-Lys (24 h) on culture mass (*n* = 6-8). Data show means ± S.E.M. * P < 0.05 *versus* control, by two way ANOVA with Dunnett post-hoc test.

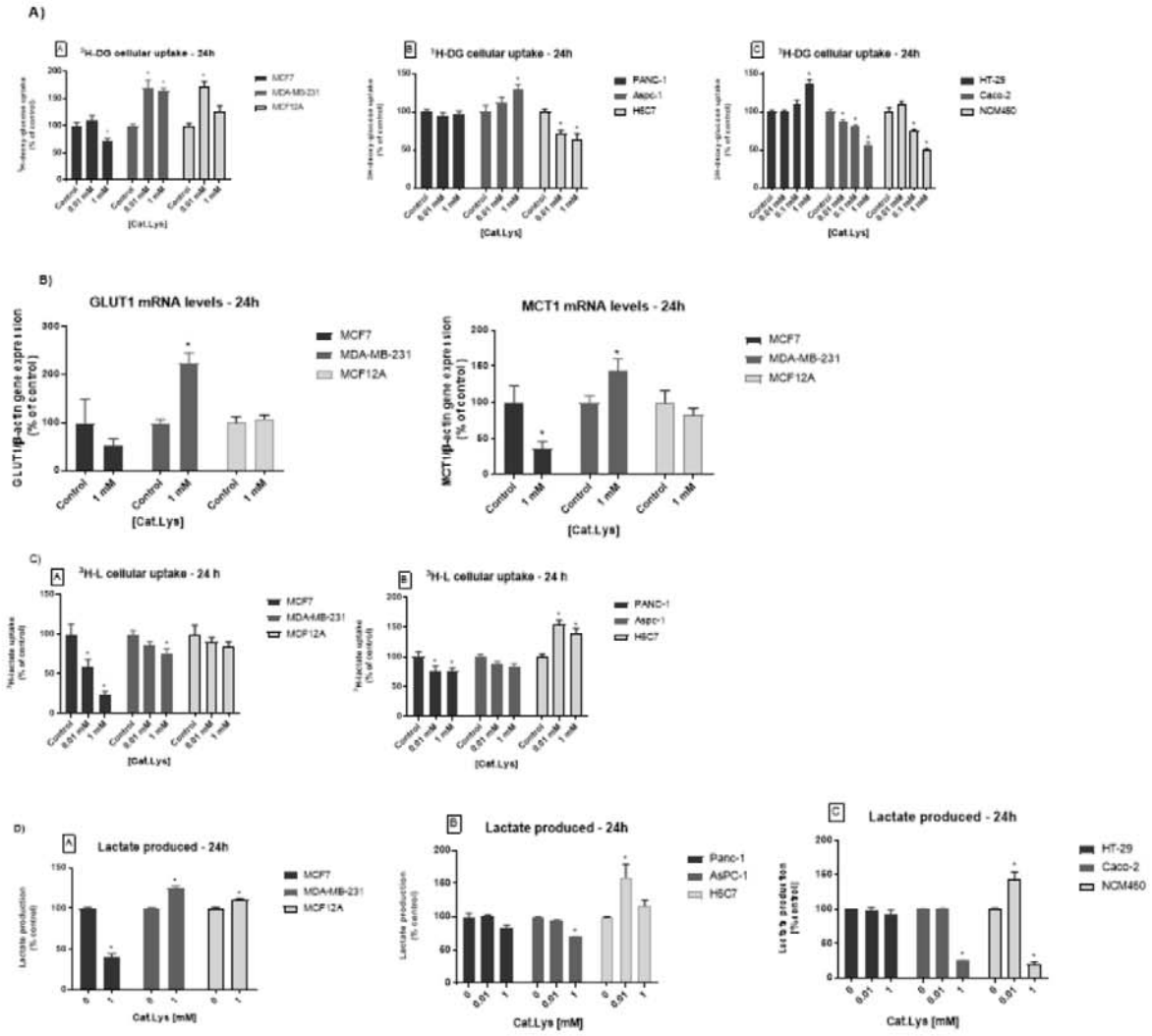
Figure S2. Cat:Lys acutely decreases breast cell growth and viability. A, Effects of increasing doses of Cat:Lys (26 min) on breast cancer (MCF-7 and MDA-MB-231) and non-tumorigenic (MCF12A) cell growth (n = 8). **B,** as in **A** but measuring cell viability (n = 8). Data show means ± S.E.M. * $P < 0.05$ *versus* respective control, by two-way ANOVA with Dunnett post-hoc test.

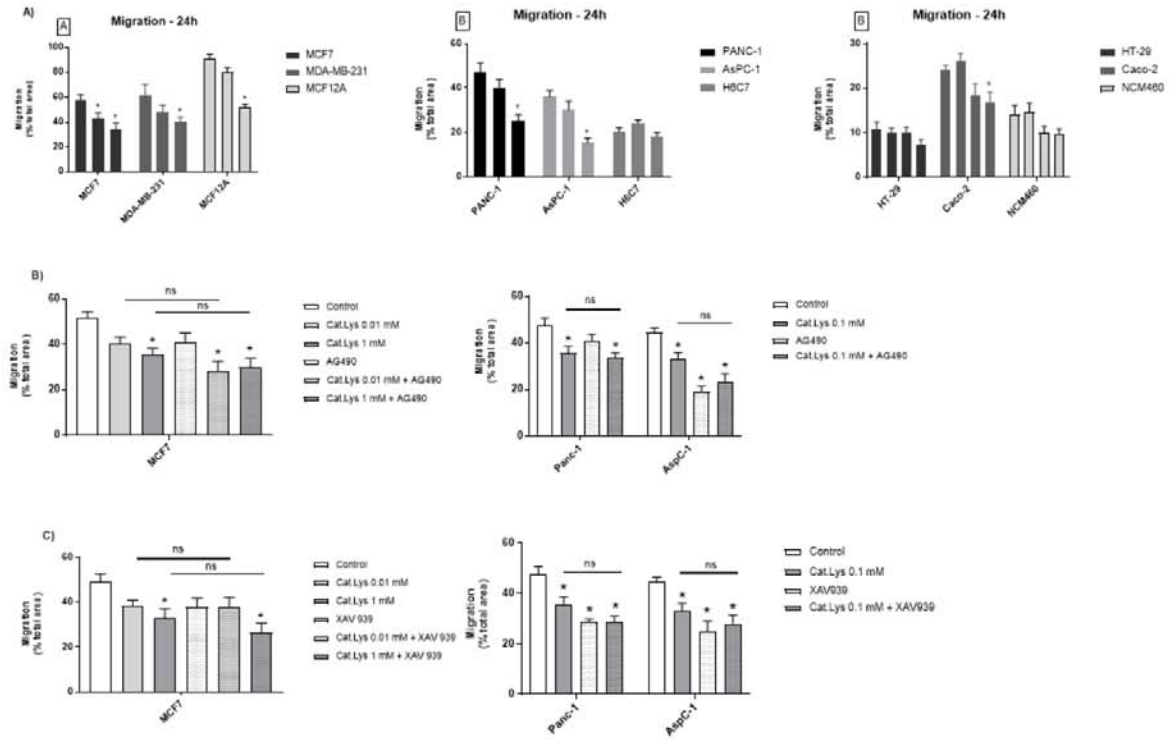
Figure S3. Cat:Lys modulates glucose uptake in breast cells. A, Effect of Cat:Lys (26 min) on ^{3}H-DG uptake by breast cancer (MCF-7 and MDA-MB-231) and non-tumorigenic (MCF12A) cells (n = 8-16). **B,** as in **A** but treating the cells for 24 h (n = 6). Data show means ± S.E.M. * $P < 0.05$ *versus* control; $^{\#}$ $P < 0.05$ *versus* the condition indicated; *ns*, not significant; by two-way ANOVA with Dunnett (A) or Tukey (B) post-hoc test.

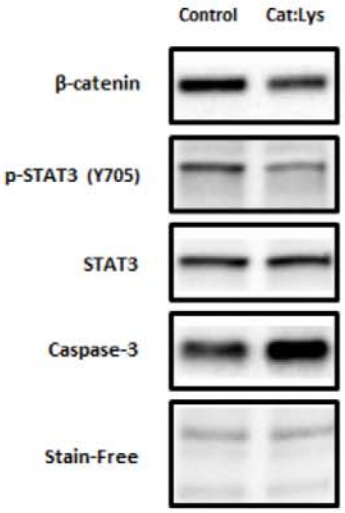
Figure S4. Cat:Lys modulates glucose uptake in breast cells. A, Effect of Cat:Lys (26 min) on ^{3}H-L uptake by breast cancer (MCF-7 and MDA-MB-231) and non-tumorigenic (MCF12A) cells (n = 7-8). **B,** as in **A** but treating the cells for 24 h (n = 6). Data show means ± S.E.M. * $P < 0.05$ *versus* control; $^{\#}$ $P < 0.05$ *versus* the condition indicated; *ns*, not significant; by two-way ANOVA with Dunnett (A) or Tukey (B) post-hoc test.

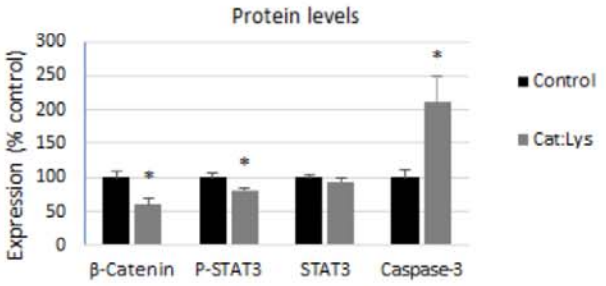
Figure S5. Cat:Lys selectively decreases cancer cell migration. Representative images of cell migration determined by the injury assay. Cell monolayers were scratched with a pipette tip and then treated for 24 h with Cat:Lys 1 mM. **A**, cancer (MCF-7 and MDA-MB-231) and non-tumorigenic (MCF12A) breast cells (n=8-12). **B**, cancer (Panc-1 and Aspc-1) and normal (H6C7) pancreatic cells (n=8). **C**, cancer (HT-29 and Caco-2) and normal (NCM460) colorectal cells (n=8). Imagens were obtained at 0 h and 24 h after scratching. Scale bar: 200 µm.

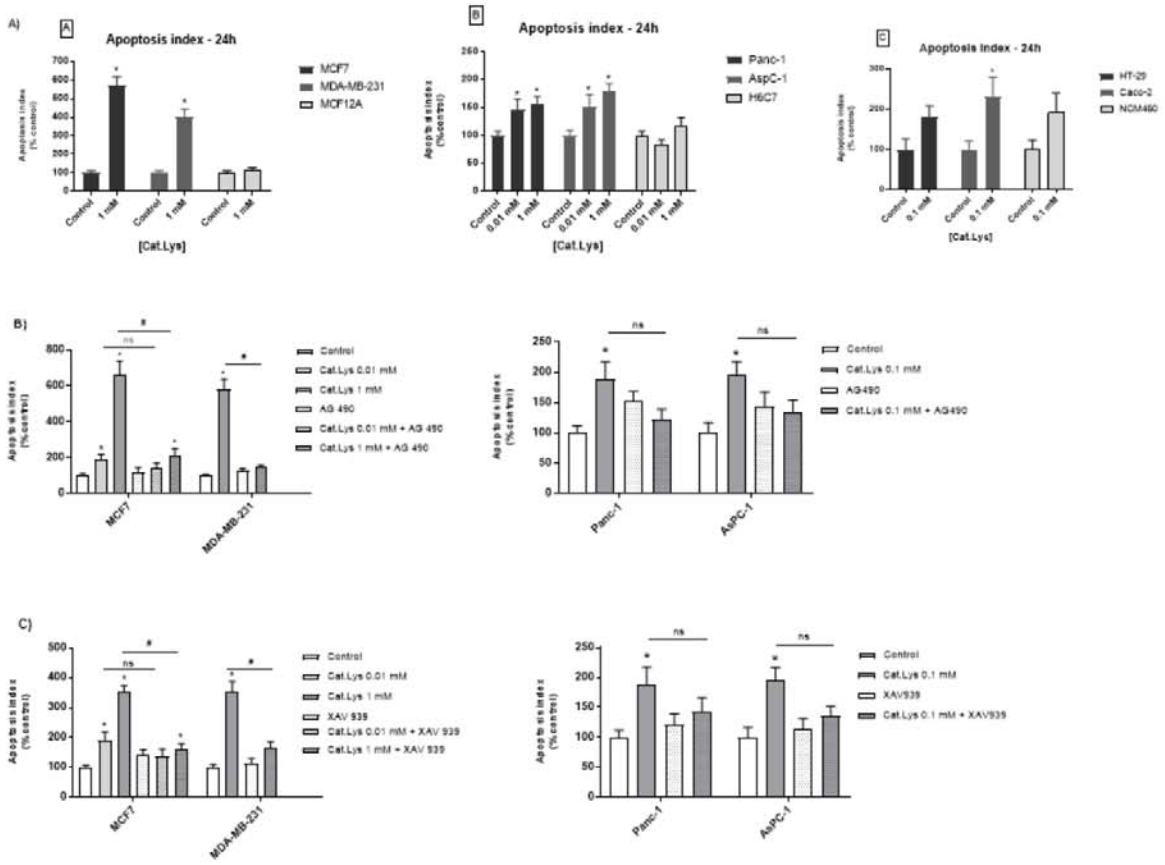












CRediT author statement

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