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Antitumoral effects of glycoconjugates on primary cultures of human pancreatic cancer cells obtained by echoendoscopy

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Summary

Pancreatic ductal adenocarcinoma (PDAC), which constitutes 90% of pancreatic cancers, is the fourth leading cause of cancer-related deaths in the world. PDAC belongs to one of the most chemo resistant cancers, in part due to its molecular and genetics features. Currently, available therapeutic options are surgery, radiation, chemotherapy, and immunotherapy. Most of the available treatments are palliative, with the objective of relieving disease-related symptoms. Our aim was to evaluate the antitumoral effect as a second line therapy, of enzymatically glycosylated compounds, hydroquinone-rutinoside, on primary pancreatic tumoral cells. We found that glycoconjugates combined with antitumor agents, such as gemcitabine, improved single-treatment effects. These findings suggest that emerging trends towards glycoconjugates could be a promising approach for cancer therapies.

Key words. *Pancreatic adenocarcinoma, hydroquinone, vacuole-membrane protein 1, fine needle aspirate, biopsy.*

Abbreviations
PDAC: Pancreatic ductal adenocarcinoma.
HQ: Hydroquinone.
VMP1: Vacuole-membrane protein 1.
FNA: Fine-needle aspiration biopsy.

Pancreatic ductal adenocarcinoma (PDAC) involves the 90% of pancreatic tumors. PDAC is one of the deadliest cancers worldwide, with an estimation of 367.000 new cases worldwide during 2015, and with 359.000 deaths in the same year.[1] Nowadays pancreatic cancer constitutes the fourth leading cause of cancer in developing countries, however, if there are no improvement in the therapies, the disease will be the second cause of death in the next decade.[2] The reason of this prognostic is due, in part, to the characteristic stromal composition of these cells, which acts as a mechanical barrier, and the subsequent reduced vascularization of the cellular environment, both of which, interfere with the ability of drugs to reach the target cells.[3] Furthermore, biological, molecular

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and genetic features of pancreatic cancer cells, which impair drug entry into the cells, or affect cellular metabolism, may increase the chemo-resistance of PDAC. Thus, although surgical resection has shown survival rates of up to 40%, 85% of patients with PDAC are not eligible for surgery. Of the 15% who undergo surgery, 85% present with tumor relapse.[4-8]

Autophagy is an evolutionarily preserved degradation process of cytoplasmic cellular constituents, which serves as a survival mechanism in starving cells. Autophagy is characterized by the sequestration of bulk cytoplasm and organelles in double - membrane vesicles called autophagosomes, which eventually acquire lysosomal-like features completing the autophagy flow with the degradation of the sequester material.[9, 10] Autophagy plays important physiological roles in human health and disease. This catabolic process is involved in the turnover of long-lived proteins and the elimination of damage/old organelles, thus maintains quality control of essential cellular components. In addition to its role in cellular homeostasis, a cytoprotective role is playing when cells encounter environmental stresses such as nutrient starvation, hypoxia, oxidative stress, pathogen infection, radiation or anticancer drug treatment, the level of autophagy can be dramatically augmented resulting in adaptation and survival.[11, 12] The role of the autophagic process in human health and disease has been described as a double-edge sword.[13] This decision depends on the carcinogenesis status, cell-tissue context, molecular characteristics of the target and nature of the stress.[14-16] Yang and coworkers have demonstrated that autophagy is constitutively activated in tumors bearing the KRAS mutation. KRAS is involved in almost all pancreatic cancers, including the three preneoplasic lesions: PanINs, *pancreatic intraepithelial neoplasias*, papillary mucinoses neoplasias and quistic mucinoses neoplasis.[17]

The genetic or pharmacological inactivation of autophagy prevents KRAS-mediated tumorigenesis. We have previously shown that KRAS induces the expression of VMP1 (vacuole membrane protein 1) in pancreatic cancer cells. VMP1 is expressed early during numerous pathologies, including diabetes *mellitus*, inflammatory processes of the pancreas (pancreatitis) and pancreatic cancer.[18, 19]

Flavonoids are phenolic compounds with a benzopyronic functional group.[20] Hesperidin (hesperetin 7-O-rutinoside), which is found in plants of the citrus genus, is composed of the aglycone hesperetin and the disaccharide rutinose (6-*O*-α-L- rhamnopyranosyl-β-D-glucopyranose).[21-23] It has been shown to have pharmacological activities, including antioxidant, vasoprotective, and antineoplasic effects.[24-31]

The enzymatic synthesis of glycosidic bonds has been studied for more than 60 years and different strategies have been developed to avoid or complement the chemical synthesis, which generally requires multiple stages of protection and desprotection to obtain the desired oligosaccharides.[32] In nature, glycoside transferases (GTs) are the main catalysts for the synthesis of glycosidic bonds. However, due to difficulties in its expression and purification, combined with the high economic cost to obtain it, its applications are restricted. An alternative is to use glycoside hydrolases (GHs) enzymes as catalysts for glycosidic bonds, since they are more abundant, easier to obtain, and have a wide range of specificity, as well as being more economical.[33]

In our laboratory, a GH was isolated and characterized, called 6-*O*-α-rhamnosyl-glucoisdase from the fungus *Acremonium* sp. DSM 24697. This enzyme is capable of transferring the rutin disaccharide, from hesperidin, to various hydroxylated receptors, such as hydroquinone.[34-35] In this work we aimed to evaluate the effect of the hydroquinone-rutinoside on primary pancreatic tumor cells.

Methods

Tumoral Samples

The protocols to be used to obtain tumor samples, the inclusion and exclusion criteria of patients in the study, and the statistical models were approved by the Ethics and Independent Patagonic Research Committee (Ceip). The tumor samples were obtained from FNA (Fine-needle aspiration biopsy) guided by echoendoscopy (EE) from patients diagnosed with pancreatic cancer without having received treatment prior to the puncture. This project was approved by the Independent Patagonian Ethics Committee.

Criteria for choosing patients

Inclusion criteria: patients older than 18 years with suspicion of tumor or diagnosis that lead to a surgical procedure or endoscopic ultrasound.

Exclusion criteria: any person in an emergency situation, or an adult subject to a legal protection measure or any person incapable of giving their consent, or a final diagnosis other than PDAC.

The study of the pancreas was performed with a linear echoendoscope (UCT-140 AL5, Olympus, Tokyo, Japan). Tumor lesions were punctured with 22 Gauge needles (EchoTip, Wilson Cook, Winston Salem, NC, USA) (Expect, Boston Scientific, Burlington, MA, USA). Applying the fanning technique after removing the stylet and applying suction by attaching a syringe (20 cc with negative pressure), the needle under vacuum made at least

20 passages inside the lesion. The needle was removed and the stylet was reintroduced, the first protruded material was placed on glass holders and fixed in 96% alcohol (cytology). Then with a syringe with physiological solution, the aspirated material was recovered in a tube with preservation fluid (BD CytoRich Red Preservative, destined for cell culture). Another puncture was performed repeating the same steps; some samples were recovered, fixed in 10% Formol and sent for pathological anatomy study.

Cell culture

Within a safety culture room, the tumor samples were treated as follows: Centrifugation of the piece at 1200 rpm/7 minutes at room temperature (R/T). The pellet obtained was then resuspended in 3 mL of collagenase (1 mg/ml) (Sigma) and incubated at 37°C in a water bath. After 10' of incubation, the samples were centrifuged at 700 rpm 4' at R/T. The cell pellet was resuspended in 5ml of lysis plug for red blood cells (1 x RBC Lysis Buffe, eBiosciences). They were gently shaken 5' and centrifuged at 700 rpm 4' R/T. Finally, the pellet was resuspended in DMEM F10 medium (Invitrogen) in a plate previously covered with an extracellular matrix. Incubate the cells at 37°C in an environment with 5% CO_2.

Each primary culture was maintained in complete culture medium until the appearance of confluence, which varied from 15 days to 30 days, depending on the tumor. When the cells reached 70% confluence, they were treated with alphazime (PAA LAboratories) for 20'.

Histology

The tumor samples were embedded in 4% paraffin and stained with hematoxylin and eosin (H&E) and analyzed with a Nikon E200 trinocular Microscope attached to a digital camera.

RNA extraction and purification

RNA extraction from cell cultures was carried out with Trizol reagent (Invitrogen). The cells were grown in a 6-multiwell and then the desired treatment was performed. For purification of the RNA the cells were washed with PBS, 1 ml of Trizol was added per well and pipetted to lift the cells. The content of each well was then passed to a 1.5 ml tube, where 0.2 ml of chloroform was added. Next, it was centrifuged at 12,000 xg for 15 minutes at 4°C. The aqueous phase was transferred to a new tube where 0.5 ml of isopropanol was added. It was left incubating 10 min and then centrifuged at 12000 xg for 10 min at 4°C. The RNA pellet obtained after the centrifugation was washed with 75% ethanol, centrifuged again and suspended in water. The RNA concentrations were determined by measuring the absorbance at 260 nm. To check its quality, an agarose gel run was performed and visualized by Etidio Bromide staining.

RT-PCR and Real-Time RT-PCR

2 µg of the purified RNA was used and treated with 1 µl of RNase-free DNase I (Invitrogen) in a final volume of 10 µl containing 1X RNAse buffer. It was incubated for 15 minutes at R/T and then incubated at 65°C for 10 minutes. Next, 10 µl of DNAse treatment was incubated with 100 nM random primers (N6) at 70°C for 5 minutes. Following, the mixture of RNA plus random primers was used and 1 mM dNTPs and 1 µl of reverse transcripase enzyme MMLV (Promega) were added in a final volume of 20 µl containing 1X MMLV buffer. The mixture was incubated at 25°C for 5 minutes and then at 37°C for 1 hour. For the PCR reaction, 0.5 µl of RT reaction was used in a mix with 200 µM dNTPs, 4 mM $MgCl_2$, 200 nM of first Forward and Reverse and 1 µl of Go Taq enzyme (Promega) in a volume of 25 µl containing Go Taq 1X buffer. The conditions of the PCR and the primers used were developed in the Physiopathology subsector of the IBIMOL.

Synthesis of Compounds

Hydroquinone (HQ) (benzene-1,4-diol) was obtained from Sigma-Aldrich (St. Louis, USA). HQ-rutinoside was synthesized enzymatically at 30°C using 50 mM sodium phosphate buffer pH 6. The reaction mixture contained: 1.8 mM of hesperidin, 1.8 mM of HQ, 0.02 U ml-1 of 6-*O*-α-rhamnosyl-β-glucosidase, and 2% v/v of DMSO. The reaction was finished in a boiling water bath at 100°C for 10 minutes. The products of the reaction were purified using the Sephadex LH20 column (1.5 × 150 cm; flow 0.1 ml min^{-1}). The transglycosylation product was identified by Nuclear Magnetic Resonance (NMR) as 4-hydroxyphenyl β-rutinoside (HQ-rutinoside).

Chemograms

Chemosensitivity was evaluated by performing cell proliferation curves. 5 x 10^3 cells per well were plated in a 96-well multiplate in SFDM. 24 hours later the medium was supplemented with 200 M Gemcitabine (Lilly), and incubated for 4 hours. Then, increasing doses (0 to 1000 µM) of HQ-rutinoside were added and after 24 hours the cytotoxicity was evaluated. Each experiment was performed in duplicate. Cell proliferation was estimated after incubation with the Presto Blue agent (Life Technologies) for 3 hours, according to the manufacture protocol.

Statistical analysis

The data were expressed as the mean ± ES (standard error). A factorial analysis of variance (ANOVA) was performed to compare the samples. The differences were considered significant when *p < 0.05; **$p < 0.01$ vs. control or § $p < 0.05$; §§ $p < 0.001$ vs. GEM.

Results

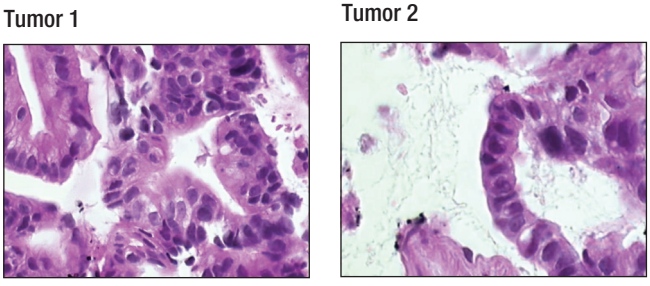
Clinic-pathological data of patients

Table 1 shows the characteristics of the patients and the evolution of the tumor. Figure 1 shows the histology of the tumors. Tumor 1 presented a glandular differentiation and some tubular forms. In the picture of tumor 2 we observed a desmoplasmic stroma and glandular formations.

Establishment of pancreatic tumor lines

In order to corroborate the tumorigenicity of the primary cell lines, we performed the characterization of the KRAS mutational state by PCR. PCR results were followed by DNA sequencing. It has been previously published, that the KRAS mutation is present in 75-95% of pancreatic adenocarcinomas. The cell lines studied carried the Kras mutation, on chromosome 12, exon 1 at the level of codons 12 (most frequent mutation). The mutated forms contain GAT (aspartic acid) and GTT (valine) (Table 2). The presence of these mutations in the established cell lines indicates the epithelial origin of a pancreatic tumor process.

Figure 1. *Hematoxylin-Eosin staining of the tumors obtained by Echoendoscopy. (A) Tumor 1; (B) Tumor 2; (HE, 1000X).*



Expression of VMP1 in the tumor lines

The presence of VMP1, a protein involved in the early stages of the autophagy process in established cell lines, has been described. VMP1 is a stress protein, which is over expressed after subjecting the cells to stress such as pancreatitis or pancreatic cancer.

In order to evaluate the expression of VMP1 in the established cell lines from human samples, the expression of the mRNA of VMP1 in the tumor lines was determined and compared with the expression in HeLa cells by Real Time RT-PCR. The mRNA levels of VMP1 were relativized to the β-actin mRNA. The results showed that the basal expression of VMP1 mRNA of pancreatic tumor cells was significantly higher than that of HeLa cells (Figure 2). These results suggested that the primary pancreatic cancer cells could present higher levels of autophagy, indicated by a higher expression of the VMP1 protein.

Evaluation of chemo sensitivity of tumor lines

The sensitivity of primary pancreatic cancer cells to gemcitabine therapy was analyzed. The primary cell lines were treated with 200 µM of gemcitabine. After 4 hours, 0 to 1000 µM of HQ-rutinoside were added and at 24 hours the cytotoxicity was evaluated and the percentage of viable cells was determined. Figure 3A shows the chemical structure of enzymatically synthesized HQ-rutinoside. After treatment with HQ-rutinoside for 24 hours, we observed that the administration of 15 µM HQ-ruti-

Table 1. *Clinical history and clinical-pathology characteristics of the patients.*

Tumor	Age (years) /Sex	Risk factors	Early symptoms	Pathology	Tumor Stage (TNM)	Survival without recurrence/ progression	Global Survival	Global Evolution
1	77/M	Tabaq.	Jaundice and pain 4 months of evolution	Pancreatic cancer, not resectable; Located in the cephalic portion, with invasion of the hepatic artery and extension towards retroperitoneum.	pT3N1M0	1 month	2 months	Death
2	62/F	Tabaq.	Jaundice and pain of 1 month of evolution	Pancreatic cancer, not resectable; Located in the cephalic portion, with invasion of the trunk Mesenteric-porta.	pT3N1M0	3 months	8 months	Death

Table 2. *Sequencing of the Kras mutational state of the two primary cell lines (LT1 and LT2). It is observed that in both tumors Kras is mutated.*

Name	Cellular Morphology	Kras	Codon	State
LT1	Rounded-Spherical	G(G>A)TGGC	12	mutated
LT2	Prismatic	G(G>A)TGGC	12	mutated

noside combined with 200 µM gemcitabine significantly affected cell survival compared to treatment with gemcitabine alone. We can conclude that the cytotoxic effect of a glycocompound as the second line of therapy to gemcitabine, significantly improves the effectiveness of the treatment against PDAC.

Figure 2. *VMP1 mRNA was measured by RT-PCR Real Time. It was observed that expression of VMP1 is higher in the primary cell lines (LT1 and LT2) from pancreatic tumors. ** p < 0.001 versus HeLa control cells.*

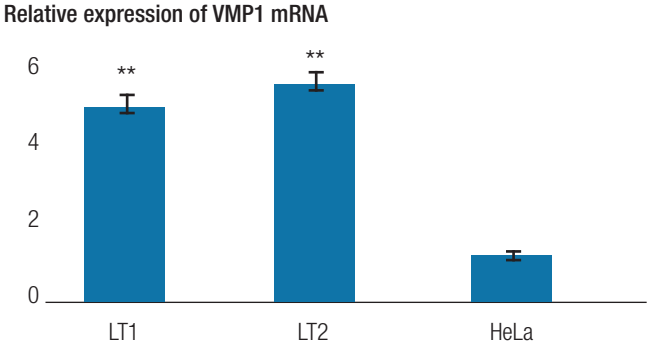
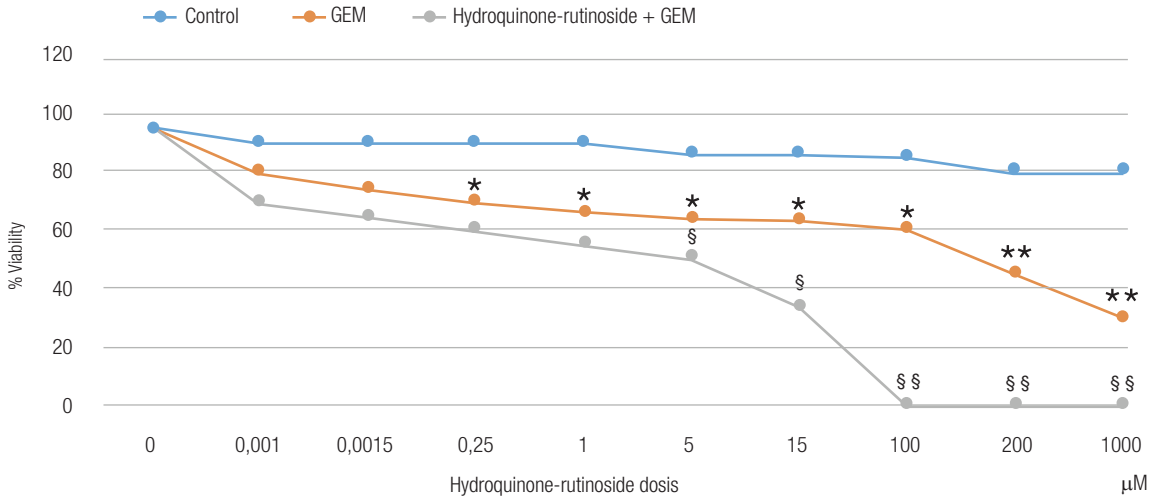


Figure 3. *Primary tumor cell lines were treated with 200 µM Gemcitabine (GEM) alone or combined with increased doses (0 to 1000 µM) of Hydroquinone-rutinoside. It was compared with untreated cells (control). The viability percentage was obtained by absorbance at 490 nm. Each point represents duplicates (± SD). *p < 0.05; **p < 0.001 versus control cells; § p < 0.0, §§ p < 0.001 vs. treatment with GEM.*



Discussion

Pancreatic cancer is one of the most lethal diseases in the world, and due to its poor prognosis, more effective treatments are needed. The causes of PDAC are still unknown, and many patients with PDAC are at an advanced stage of the disease at the time of diagnosis. Even if the tumor is resected, the postoperative recovery is unsatisfactory, and the prognosis continues to be poor. Gemcitabine, a base analogue nucleoside, is the currently treatment for PDAC. However, due to the high resistance to gemcitabine presented by pancreatic tumor cells, the discovery of agents that increase sensitivity to the drug is required, seeking to improve the PDAC prognosis.[36]

We developed a strategy in which biopsies of pancreatic tumors from 2 patients were collected and their cells were maintained culture. This culture allowed us to analyze the sensitivity to gemcitabine of the two tumors and to test new drugs as a second treatment. Methodologically, it should be noted that we were able to obtain samples from FNAP-EE. This strategy is important since it allows us to think about clinical applications, if we can grow cells from FNAP-EE we have access to the possibility of performing a tumor biopsy,

studying the sensitivity of tumor cells to drugs and in this way apply a personalized treatment.

We also studied the expression of the RNA of the VMP1 gene by RT-PCR. We observed an increase in the expression of this gene in pancreatic tumor cells. Previous experimental studies showing that the expression of VMP1 in tumor cells is related to tumor resistance.[37, 38] These data of increased expression of VMP1 in tumor cells bearing oncogenic Kras may suggest a role for this protein in resistance from the cells to the treatment.

On the other hand, we studied the sensitivity of tumor cells from human biopsies to treatment. We obtained a resistance profile associated with the treatment. We observed that the IC_{50} of gemcitabine was when 200 μM of drug was used.[39] Notably, the IC_{50} of gemcitabine decreased to 5 μM after treatment with a second line of therapy, such as HQ-rutinoside. This result suggests that treatment with HQ-rutinoside significantly improves the effectiveness of gemcitabine for the treatment of PDAC. Despite the numerous investigations and efforts made in recent years, conventional strategies for the treatment of various types of cancer, including surgery, radiotherapy, chemotherapy, have had a moderate impact on the prognosis of tumors and especially on gastrointestinal cancers. Most commercial drugs have the great disadvantage of not having systems that direct them to the target organ or tissue, so much of the drug that remains circulating in the bloodstream increases side effects causing toxicity in patients. An interesting targeting strategy is the glycosylation of molecules due to the high specificity of the interaction with carbohydrates and the wide range of cellular receptors to which they can be specifically directed, allowing the selective administration of drugs to the desired active sites.[40] On the other hand, the glycosylation of bioactive molecules can modify their pharmacokinetics and modulate their toxicity. It was shown that not only the number of residues is important, but also the type of sugar that is added to produce effects on the toxicity, biological activity or on the solubility of the original molecule.[41] This is of interest, since the profile of sensitivity can detect the percentage of drug resistant cells, and therefore serve as a tool in the selection of the second line of therapy.

In conclusion, in this work we show that it is possible to perform primary cultures of samples obtained by FNAB-EE. Although, promising these results demonstrate the possibility of obtaining a sample in which the sensitivity of the tumor cells to the treatment could be predicted. Also, the study model, using samples of human tumor tissue, gives us the possibility of detecting proteins that would serve as resistance markers. Finally, we describe a second line of promising therapy for the treatment of PDAC to be used in combination with gemcitabine. These results suggest the possible use of glycoconjugate compounds as complementary therapies against pancreatic cancer.

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Conflict of Interest. *The authors have declared that no conflict of interest exists.*

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