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Identification of PARP-1 in cancer stem cells of gastrointestinal cancers: A preliminary study

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Advanced-stage gastrointestinal tumors have high mortality due to chemotherapy limitations. One of the causes of treatment failure is the presence of cancer stem cells (CSCs), which show resistance mechanisms against DNA damage, such as poly (adenosine diphosphate-ribose) polymerase 1 (PARP-1). However, little is known about the relevance of PARP-1 in these tumor cells. Our purpose is to analyze the expression of PARP-1 in cancer cells and CSCs from gastrointestinal tumors, its relationship with the DNA damage repair process and its modulation by cytotoxic and PARP-1 inhibitors. We used pancreatic, liver and colon cancer cell lines and isolated CSCs using Aldefluor technology to analyze PARP-1 expression. In addition, we examined the effect of classic cytotoxic drugs (Doxorubicin, Gemcitabine, Irinotecan and 5-Fluorouracil) and a PARP-1 inhibitor (Olaparib) in cultured cells and 3D tumorspheres. We demonstrated that PARP-1 is highly expressed in pancreatic, liver and colon tumor cells and that this expression was significantly higher in cell populations with CSC characteristics. In addition, Doxorubicin and Gemcitabine increased their cytotoxic effect when administered simultaneously with Olaparib, decreasing the formation of 3D tumorspheres. Our findings suggest that PARP-1 is a common and relevant resistance mechanism in CSCs from gastrointestinal tumors and that the use of PARP-1 inhibitors may be an adjuvant therapy to increase apoptosis in this type of cells which are responsible to cancer recurrence and metastasis.

Keywords. Cancer stem cells; colon cancer; liver cancer; Olaparib; pancreatic cancer; PARP-1

1. Introduction

Gastrointestinal (GI) cancer is composed of different types of tumors that develop in the GI tract and the accessory organs of digestion (Martin-Guerrero *et al.* 2017). Worldwide, colorectal and liver cancer rank third and sixth in frequency, followed by pancreatic cancer in twelfth place. Colorectal cancer (CRC) causes the highest number of deaths annually (over 880,000), followed closely by liver cancer (Bray *et al.* 2018). Hepatocellular carcinoma (HCC) and pancreatic cancer have high mortality due to late diagnosis, with a

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5-year survival rate of 18% and 9%, respectively (Fateen and Ryder 2017; Siegel *et al.* 2020). In addition, both tumors are highly resistant to chemotherapy and radiotherapy (Zhang *et al.* 2017; Sukowati 2019).

Multiple GI cancer therapies based on the use of different molecules, including Doxorubicin (DOXO), 5-Fluorouracil (5-FU), Irinotecan (IRI) or Gemcitabine (GEM), are currently being used. In fact, GEM is the drug of choice in advanced pancreatic tumors while 5-FU continues to be used as a first-line treatment for colon cancer (Adamska *et al.* 2018; Abdel-Rahman 2019). In addition, 5-FU is often used in combination with other chemotherapy drugs (Engstrom *et al.* 2009). GEM is an antimetabolite whose active form competes with deoxycytidine triphosphate to bind DNA polymerase and inhibit DNA elongation and ribonucleotide reductase causing replication stress (Artin *et al.* 2009; Ramón-López *et al.* 2012). 5-FU causes damage to DNA and RNA by disrupting the activity of the enzyme thymidylate synthase (Longley *et al.* 2003). Finally, DOXO is a DNA intercalating agent (Nguyen *et al.* 2015); while IRI is a pro-drug that, in its active form (SN-38), acts by binding to the topoisomerase I-DNA complex during replication and transcription, maintaining breaks in the DNA strand and leading to cell death (Kümler *et al.* 2015). Despite the progress in the use of these drugs, the development of resistance hinders the treatment of patients with these types of tumors (Biancur and Kimmelman 2018; Van Der Jeught *et al.* 2018; Yu *et al.* 2019).

The presence of cancer stem cells (CSCs) in a tumor is one of the main causes of the development of drug and radiation resistance, as well as metastasis and recurrence (Taniguchi *et al.* 2016). This resistance could be explained by the over-expression of multidrug resistance (MDR) proteins in these cells, the overactivation of DNA damage repair mechanisms, or their ability to remain in a quiescent state (Raha *et al.* 2014; Morata-Tarifa *et al.* 2016). In GI tumors, CSCs have been detected in oesophageal, stomach, liver, pancreatic and colorectal cancers (Mikhail and Zeidan 2014). Gastrointestinal CSCs express surface markers such as CD24, CD26, CD44, CD90, CD133, and CD166, show high ALDH1 activity (Ilmer *et al.* 2016), and form spheres when cultured under non-adherent conditions (Visvader and Lindeman 2008; Taniguchi *et al.* 2016). Deep knowledge of the characteristics of CSCs in GI cancer could allow the development of a specific and targeted therapy that aims to achieve a more effective and lasting response.

On the other side, PARP family includes a large group of enzymes that catalyze the polymerization of ADP-ribose monomers (poly(ADP-ribosyl)ation or PARylation) on target proteins (Jiang *et al.* 2015; Zai *et al.* 2019) and has been associated with multiple functions such as cellular stress response, hormone signaling and epithelial-mesenchymal transition (Rajawat *et al.* 2017). In this context, the multifunctional nuclear protein Poly-(ADP-Ribose) Polymerase 1 (PARP-1) (Jiang *et al.* 2015), the most abundant member of the PARP family, has been linked to the detection and repair of DNA damage (Schiewer *et al.* 2018) and, therefore, to cellular resistance to drugs (Martin-Guerrero *et al.* 2017; Rajawat *et al.* 2017). In fact, PARP-1 overexpression has been described in several human tumors, including colorectal, breast, skin, lung, and hepatocellular cancers (Ossovskaya *et al.* 2010; Dziaman *et al.* 2014). In HCC, PARP-1 has been associated with tumor occurrence, progression and growth (Martin-Guerrero *et al.* 2017). In addition, it has recently been shown that PARP inhibition together with suppression of autophagy may have a synergistic effect on HCC cell lethality (Zai *et al.* 2019). In CRC, PARP-1 has been associated in the early stages with increased expression of β-catenin, c-Myc, MMP-7 and cyclin D1 (Nosho *et al.* 2006). Recently, cytoplasmic PARP-1 has been correlated with the regulation of death receptor-5-activated apoptosis and tumorigenesis in pancreatic cancer (Xu *et al.* 2019). In CSCs of GI tumors, the presence and function of PARP-1 remain unknown. However, it has been described that, in other types of tumors, PARP1 is overexpressed in CSC populations (Gilabert *et al.* 2014).

Furthermore, PARPi has shown numerous benefits in the treatment of homologous recombination repair (HRR)-deficient tumors due to the concept of "synthetic lethality" (Poggio *et al.* 2018; Golan *et al.* 2019, Bryant *et al.* 2005). Numerous PARPis, including Olaparib, have been designed to be combined with traditional chemotherapy drugs such as Temozolomide, DOXO and IRI (LoRusso *et al.* 2016; Pishvaian *et al.* 2018; Farago *et al.* 2019); and, with PI3K inhibitors (BKM120) (Matulonis *et al.* 2017). In addition, another study showed that Olaparib can suppress PARylation and associated NF-κβ signaling, and induce cell death in Olaparib-sensitive head and neck cancer cells; whereas in cells with low basal levels of NF-κβ, Olaparib led to apoptosis by activating p53 (Kwon *et al.* 2016).

The aim of this study is to analyse the levels of PARP-1 expression in tumor lines of several GI tumors and to establish possible differences in the expression of CSCs derived from these lines. Moreover, the

relationship of PARP-1 with the growth of cell populations and the possible modulation by PARPi will be determined.

2. Materials and methods

2.1 *Cell culture and reagents*

Pancreatic cancer cell lines MIA-PaCa2, Bx-PC3 and PANC-1, liver cancer cell lines HEPG2, HEP3B and PLCPRF5, colon cancer cell lines DLD-1, HT29, HCT-116 and T84 and liver WRL68 and colon CCD18 non-tumor cell lines were obtained from the Center of Scientific Instrumentation (Granada University). MIA-PaCa2, PANC-1 and PLCPRF5 were cultured using DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin (P/S); T-84 was cultured in Ham´s DMEM-F12 medium supplemented with 2 mM glutamine, 10% FBS and 1% P/S; HEP3B, HEPG2, WRL68, Bx-PC3 and DLD1 were cultured in RPMI medium supplemented with 2 mM glutamine, 10% FBS and 1% P/S; HCT-116 and HT-29 were cultured in McCoy's 5A supplemented with 10% FBS and 1% P/S. All cell lines were maintained at 37°C and 5% CO_2.

2.2 *RT- PCR analysis*

Cells were trypsinized and centrifuged at 250 g for 5 min. The pellet was resuspended in Trizol Reagent (Sigma-Aldrich, San Luis, Missouri, EEUU) Total RNA was extracted according to RNeasy Mini Kit (Qiagen, Hilden, Germany) protocol and quantified using Nanodrop 1000 (Thermo Fisher, Waltham, Massachusetts, EEUU). The reverse transcription of RNA was performed using iScript cDNA Synthesis (BioRad, Hercules, California, EEUU) according to the fabricant protocol. The quantitative PCR was completed using SYBR Green (Takara, Kyoto, Japan) following the fabricant protocol. The primers used were PARP-1 forward 5′-AGG GCA AGC ACA GTG TCA AA-3′ and reverse 5′-TAC CCA TCA GCA ACT TAG CG-3′ and two genes as housekeeping: *homo sapiens ubiquitin C (UBC)* forward 5′-TGG GAT GCA AAT CTT CGT GAA GAC CCT GAC-3′ and reverse 5′-ACC AAG TGC AGA GTG GAC TCT TTC TGG ATG-3′; *peptidylprolyl isomerase A (PPIA)* forward 5′-CCA TGG CAA ATG CTG GAC CCA ACA CAA ATG-3′ and reverse 5′-TCC TGA GCT ACA GAA GGA ATG ATC TGG TGG-3′.

2.3 *Cancer stem cells isolation*

Isolation of CSCs was performed using ALDEFLUOR™ kit (StemCell Technologies, Vancouver, Canada). Cells were trypsinized and centrifuged at 250g and 4°C for 5 min. Then, cells were counted and resuspended in ALDEFLUOR Assay Buffer at (8×10^5 cell/ml) and divided into two tubes (negative control and test). The negative control tube was treated with aldehyde dehydrogenase inhibitor DEAB at 10 µl/ml and then, both tubes were incubated with ALDEFLUOR reagent 5 µl/ml at 37°C for 40 min. Finally, cells were centrifuged at 250g and 4°C for 5 min and were resuspended in 200 µl ALDEFLUOR buffer assay. Results were analyzed and cells were sorted using FACS Aria II (BD BioSciences, New Jersey, EEUU) obtaining both ALDEFLUOR (+) (CSCs) and (−) cells. The gates were established using the DEAB-treated sample as a negative control (supplementary figure 1).

2.4 *Apoptosis analysis*

MIA-PaCa2 cell line was seeded 24 h before treatments in a 6-well plate at 2×10^5 cells/well. After 3 days of exposure to DOX (1 µg/ml), GEM (10 µg/ml), 5-FU (30 µM), IRI (5 µg/ml) and PARP-1 inhibitor (Olaparib) (10 µM), cells were washed with PBS and trypsinized. Then, cells were centrifuged at 250 g 4°C for 5 min and fixed with Fixation Buffer (BD BioSciences, New Jersey, USA) for 15 min at 4°C. Thereafter, cells were stained using PI/RNase Staining Buffer (BD BioSciences) for 15 min in the darkness. Finally, tubes were analyzed in FACS Aria II (BD BioSciences) cell cytometer.

2.5 *Immunofluorescence analysis*

Cells were trypsinized and centrifuged at 250 g. Pellet was resuspended in the cell medium and cells were seeded in Chamber Slides (Thermo Fisher, Waltham, Massachusetts, EEUU) wells (4×10^5 cells/ml) 24 h after seed, cells were treated with different drugs for 2h (for PARylation analysis) or overnight (to study DNA damage induced by drugs and later adding a second treatment for 5 h) and incubated at 37°C. Later, cells were fixed and permeabilized for 10 min using 1:1 methanol-acetone solution. Then, antigens of cells were blocked using non-fat milk 5% in PBS-tween for 30 min and then the chambers were washed three times with PBS. Later, primary antibody was added to the chamber: Poly(ADP-ribose) monoclonal antibody

(10H) (Enzo Life Sciences, Farmingdale, NY, EEUU) at 1:400 and PE Anti-H2AX (pS139) (BD BioSciences Pharmigen, New Jersey, EEUU) at 1:1000 both diluted in non-fat mil 5% in PBS-tween and were incubated for 1 h. After 3 washes with PBS, the secondary antibody Dylight 488 FITC anti-mouse (Biolegend, San Diego, California, EEUU) at 1:800 in non-fat mil 5% in PBS-tween was incubated for 1 h, subsequently washing 3 times using PBS. The nuclei were stained using propidium iodide or DAPI for 5–10 min respectively in darkness. Finally, cells were visualized in a Spectral Confocal Laser microscopy (Leica, Wetzlar, Germany).

2.6 *Tumorsphere formation assays and treatment*

Tumorsphere formation assays were carried out with ALDH- and ALDH+ cell MIA-PaCa2 cells cell obtained by FACS sorting (see above). Both types of cells were seeded in a 96-well plate (500 cells/well) and cultured in a tumor stem cell induction medium composed of DMEM-F12 medium, 1% penicillin-streptomycin solution, hydrocortisone, heparin, human epithelial growth factor and human fibroblast growth factor (all from Sigma-Aldrich, San Luis, Missouri, EEUU) and ITS-G and B27 vitamin (Thermo Fisher, Waltham, Massachusetts, EEUU). The spheres were cultured in suspension for 14 days in a 37°C and 5% CO_2 atmosphere, making a medium change every 4–5 days. In addition, tumorspheres were treated after seed (4 and 7 days) with DOXO (0.7 μg/ml), Olaparib (10 μM), DOXO+Olaparib. Finally, tumorspheres photos were taken using DM-IRB (Leica, Wetzlar, Germany) microscope with DP74 (Olympus, Shinjuku, Tokyo, Japan) camera attached. Tumorospheres were measured using ImageJ software (NIH).

2.7 *Statistical analysis*

Statistical analyses were performed using Student's *t*-test. Data were expressed as the mean ± SEM (Standard Error of the Mean) and a p-value of less than 0.05 was considered statistically significant.

3. Results

3.1 *PARP-1 expression in non-tumor and tumor cell lines*

PARP-1 expression levels in cell lines were determined using RT-qPCR. As shown in figure 1, a significant difference was observed between non-tumor and tumor cell lines. MIA-PaCa-2 cells showed the highest PARP-1 expression among pancreatic cancer cells (figure 1A). In addition, all colon cancer cell lines showed high PARP-1 expression. DLD-1 and HT29 showed the highest levels of PARP-1 expression in relation to CCD18 normal colon cells (figure 1B). Finally, hepatic cancer cell lines (PLCPRF5, HEPG2 y HEP3B) also showed increased PARP-1 expression (2 to 3-fold) compared to the WRL68 non-tumor cell line (figure 1C).

3.2 *PARP-1 expression in CSCs from gastrointestinal tumors*

To determine the relevance of PARP-1 expression in CSCs from different GI tumors, a sorter of ALDH(+) and ALDH(−) cells was performed using ALDEFLUOR™. In colon cancer cell lines, T84 showed the greatest ALDH activity (60% ALDH(+) cells), followed by HCT-115, DLD-1 and HT-29 (50.6%, 12.9%, and 1%, respectively) (figure 2). On the other hand, in pancreatic cell lines, MIA-PaCa-2 showed the highest proportion of ALDH(+) cells (37%) while PANC-1 and BxPC3 showed 17% and 7%, respectively (figure 2). In hepatic cancer cell lines, ALDH(+) cells accounted for 84.4%, 55% and 33.2% in HEP3B, HEPG2 and PLCPRF5, respectively (figure 2). Interestingly, non-tumor colon and liver cells showed no ALDH activity (0.1% in CCD18 and 0% in WRL68).

To demonstrate the correlation between CSCs phenotype and PARP-1 expression, ALDH(+) and (−) cells were analyzed by RT-PCR. As shown in figure 3, a higher expression of PARP-1 was detected in ALDH(+) pancreatic tumor cell lines (MIA-PaCa-2 and Bx-PC3) compared to ALDH(−) cells (figure 3A). Likewise, a higher PARP-1 expression was demonstrated in two ALDH(+) hepatic tumor cell lines (HEP3B and HEPG2) in comparison with ALDH(−) cells. Despite a higher PARP-1 expression in PLC ALDH(+) cells was detected in comparison to PLC ALDH (−) cells, no statically significant differences could be demonstrated (figure 3C). Finally, no statically significant differences were detected between ALDH(+) and ALDH(−) colon cancer cell lines (figure 3B).

3.3 *Drug-induced PARP-1 activation in MIA-PaCa-2 cells*

Prior to conducting the tumorsphere assays, PARP-1 activation induced in MIA-PaCa-2 cells by drugs such

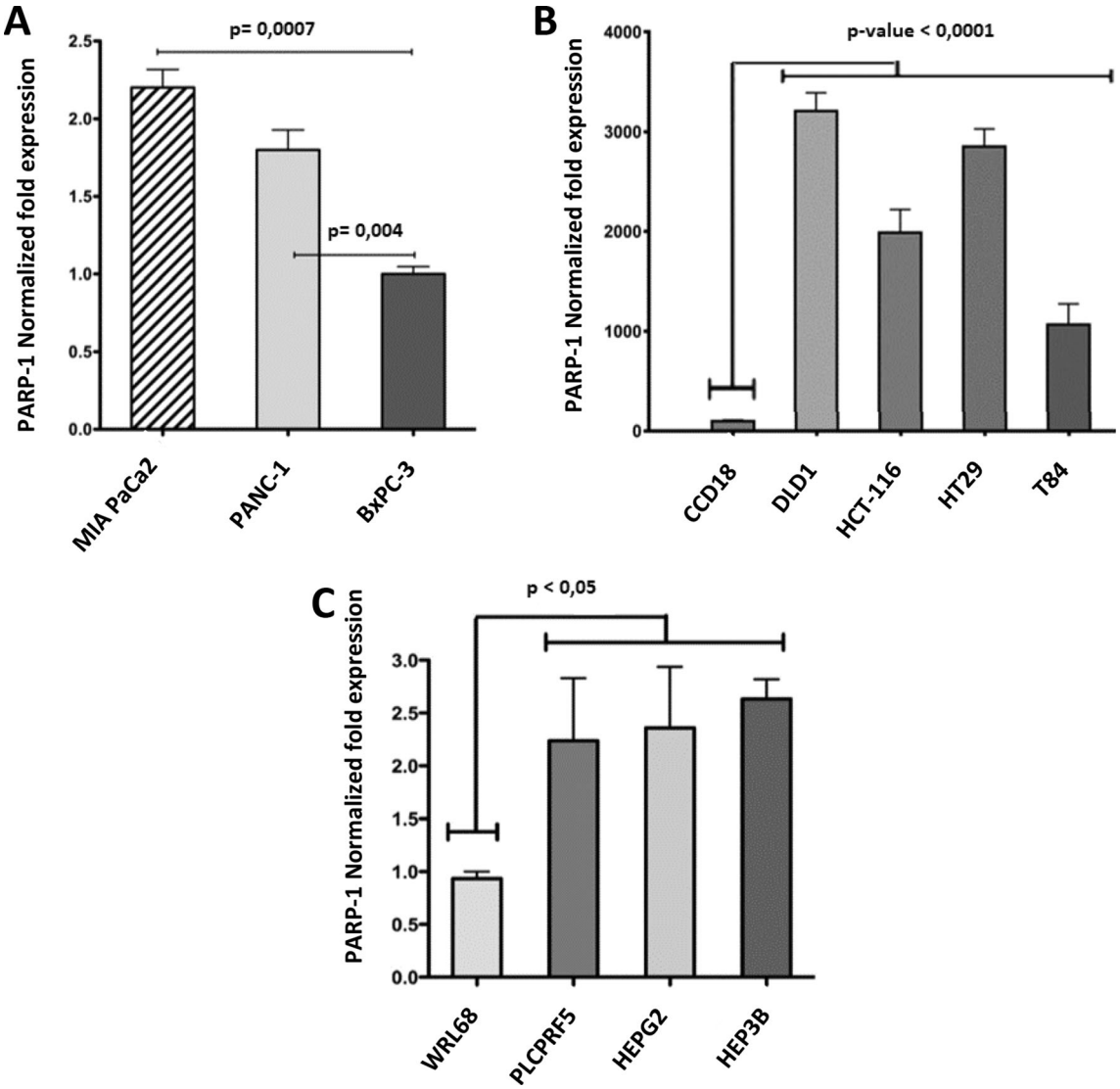

Figure 1. RT-qPCR of PARP-1 expression in non-tumor and tumor cells. (**A**) Pancreatic tumor cell lines (MIA-PaCa-2, PANC-1 and Bx-PC3). (**B**) Colon tumor (DLD1, HCT-116, HT-29 y T84) and non-tumor (CCD18) cell lines. (**C**) Hepatic tumor (PLCPRF5, HEPG2 y HEP3B) and non-tumor (WRL68) cells. Tumor cell showed a higher PARP1 expression than non-tumor lines. PARP1 expression was normalized using two genes (PPIA and Ubiquitin C) and statistical differences were assessed using a non-tumoral line, except for pancreatic lines (**A**) where MIA PaCa2 tumor cell line was used as reference. Data were represented as the mean ± SEM (n = 3). Significance of the results ($p<0.05$) were indicated.

as DOXO, 5-FU, IRI and GEM was determined. As shown in figure 4A, DOX and GEM caused the greatest PARP-1 activation. In addition, the percentage of apoptotic cells after drug treatment with and without Olaparib – a PARP-1 specific inhibitor – was determined. As shown in figure 4B, DOX and GEM treatment showed statistical differences when used with and without Olaparib. Conversely, the addition of Olaparib was not associated with significant differences in the case of IRI and 5-FU. In addition, we used the pH2AX to corroborate the accumulation of DNA damage (without repair) by Olaparib. As shown in figure 4, the two treatments that induced more apoptosis in MIA-PaCa-2 cells (DOX/Olaparib and GEM/Olaparib) showed higher pH2AX expression than the drugs alone (figure 4C and 4D, respectively), indicating that the inhibition of PARP-1 by Olaparib increased DNA damage caused by cytotoxic agents.

3.4 *Influence of Olaparib and DOX treatment on MIA-PaCa-2 tumorspheres formation*

We selected the MIA PaCa-2 cell line to carry out the analysis of tumorspheres formation

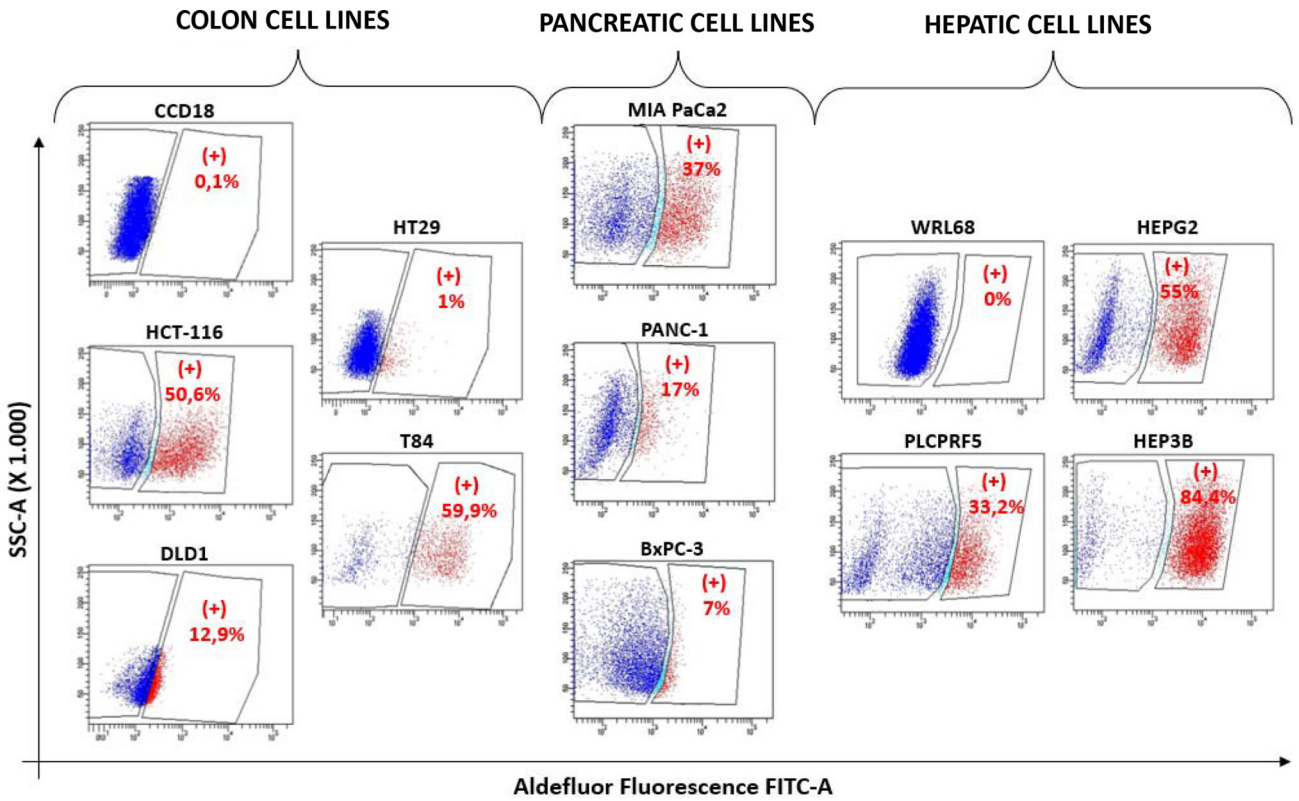

Figure 2. ALDH activity in colon, pancreatic and hepatic (non-tumor and tumor) cell lines. Representative image of FACS analysis showing the percentage of ALDH (+) cells. Tumor cell lines present variable percentages of ALDH (+) cells. Non-tumor cell lines do not express the ALDH marker. The gates were established in each line individually using the ALDH inhibitor DEAB, allowing the comparison between cells expressing low and high expressions of ALDH. Percentages were represented as the mean of three replicates (n = 3).

(ALDEFLUOR assay) and the effect of a cytotoxic drug (DOX) and PARP-1 inhibitor (Olaparib). Once the efficacy of the combined drug treatment in MIA-PaCa-2 cell was determined, we selected DOX and Olaparib combined treatment to conduct tumorspheres experiments. ALDH+ MIA-PaCa-2 cells formed tumorspheres 17 days after growth in CSC medium (figure 5A). Culture of MIA-PaCa-2 cells in CSCs medium with DOX significantly decreased tumorspheres formation and cell proliferation compared to the control group from the tenth day (figure 5B). Administration of Olaparib at day 7 led to decreased cell proliferation inhibition regarding DOX, and sustained growth during the post-treatment days was observed. While its administration post-formation of the tumorspheres prevents CSC tumorspheres from growing (figure 5B). In addition, the combination of DOX and Olaparib boosted the cytotoxic effect of DOX in ALDH+ MIA-PaCa-2 cells although no significant differences were found between the individual treatment using DOX and the combined treatment (figure 5B).

4. Discussion

Gastrointestinal tumors have a high incidence worldwide, representing one-sixth of all diagnosed tumors and one-third of all cancer-related deaths. Specifically, pancreatic, liver and colorectal tumors show high prevalence and/or mortality (Siegel *et al.* 2020) and current therapies still have low efficacy, thus the development of new therapeutic strategies is required. The PARP protein family has been linked to drug resistance in several tumors, including GI tumors such as pancreatic cancer (Xu *et al.* 2019). Thus, the study of PARP could improve the current therapies used in GI tumors.

We determined that colon and liver tumor lines showed higher PARP-1 expression than non-tumor lines, supporting previous studies (Dörsam *et al.* 2018). This

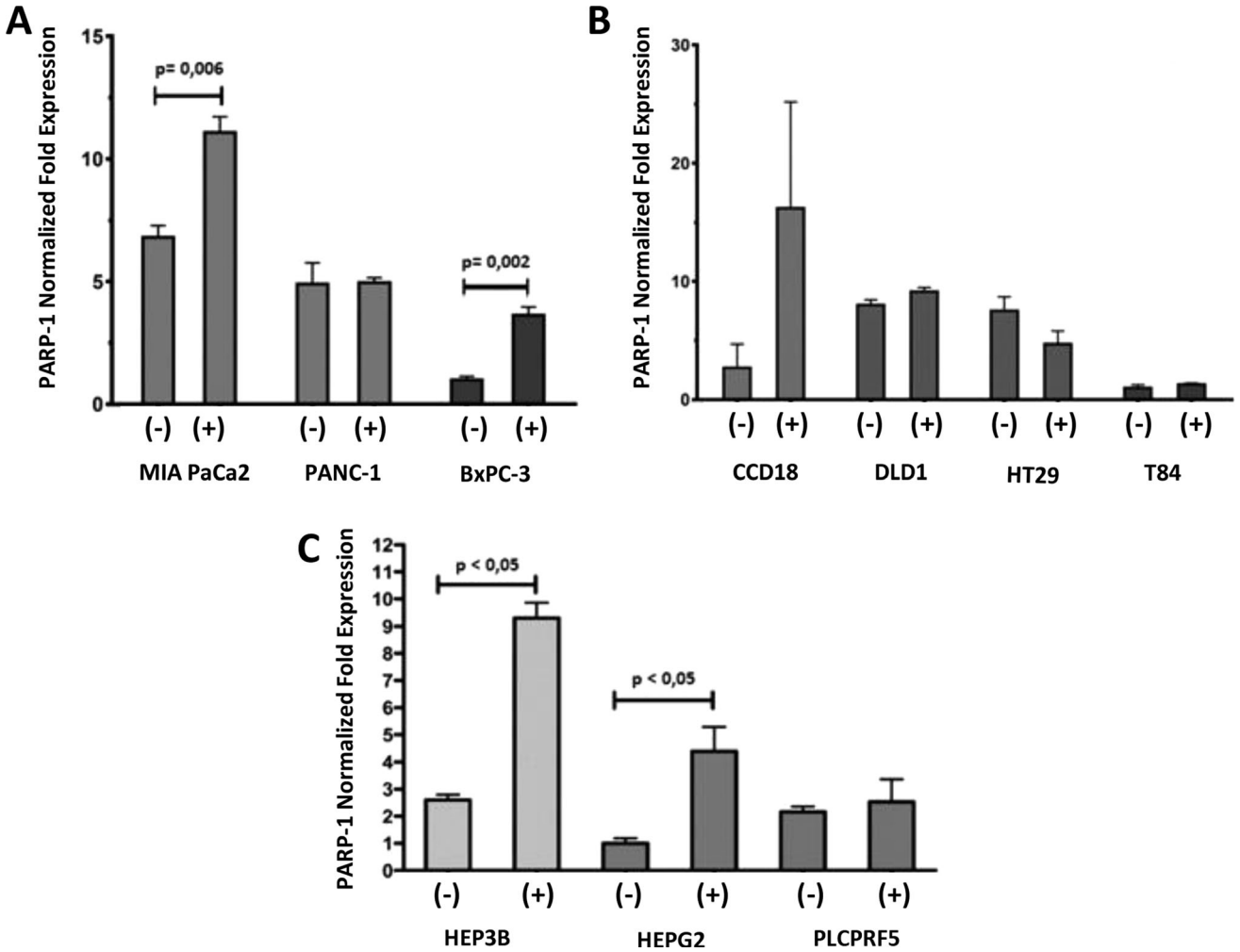

Figure 3. PARP-1 expression in ALDH (−) and ALDH (+) cells. Graphic represent the relative PARP-1 expression in pancreatic tumor cells (**A**), colon tumor cells (**B**) and liver tumor cells (C). Most of the isolated ALDH (+) cells showed a higher PARP1 expression than ALDH (−). PARP1 expression was normalized using two genes (PPIA and Ubiquitin C). Data are represented as the mean ± SEM (n = 3). p-value of significant results was indicated.

difference could not be observed in pancreatic tumors due to the absence of non-tumor lines. Because high ALDH activity has been associated with CSCs (Tomita *et al.* 2016), we used this method to select and analyze these cells regarding PARP-1 expression. In the Mia-PaCa-2 pancreatic cancer cell line, a greater PARP-1 expression was detected in ALDH(+) cells, supporting previous studies in which PARP-1 loss was associated with decreased expression of CSC markers and loss of stem cell phenotype (Venere *et al.* 2014). Furthermore, studies in the BxPC3 pancreatic cancer cell line and HEP3B and HEPG2 liver cancer cell lines confirmed that the expression of ALDH correlated with a high PARP-1 expression. This trend was also seen in three other tumor lines, e.g. PLC, HCT116 and DLD1, although no significant differences were observed. Non-tumor colon and liver cells that were used as control did not express ALDH.

Moreover, studies in the Mia-PaCa-2 cell line showed high PARP-1 activity after exposure to genotoxic drugs like DOX (Shin *et al.* 2015) whereas other drugs such as IRI and 5-FU did not cause PARP-1 activation. Further, our combined studies using cytotoxic drugs and PARP-1 inhibitors showed that DOX and GEM display synergistic effects with PARP-1 inhibitors (Olaparib), causing a high percentage of apoptosis and DNA damage accumulation (determined by the pH2AX marker) in Mia-PaCa-2 cells. These synergistic results coincide with studies carried out with Doxorubicin, Gemcitabine, 5-FU and their combination with PARP inhibitors in osteosarcoma, non-small-cell lung cancer and colon cancer respectively, where it is observed that PARP is essential for the survival of the cells. against Doxorubicin and Gemcitabine but it is not essential in the

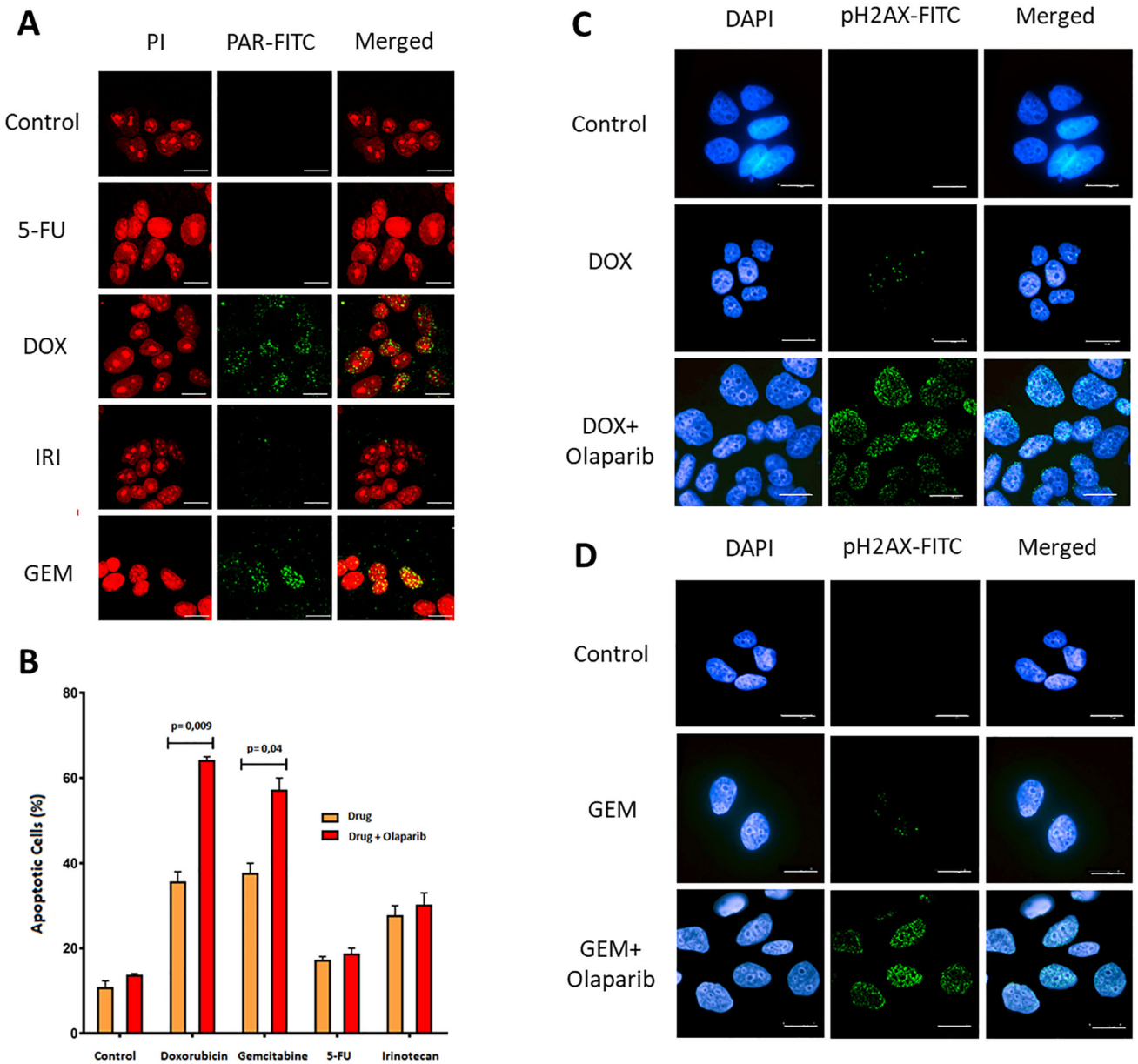

Figure 4. PARP-1 activation analysis in MIA-PaCa-2 cell line. (**A**) Representative immunofluorescence images of PARP-1-induced activity (PARylation; green). Nuclei were dyed using propidium iodide (red). Drugs used were IRI, DOX, GEM and 5-Fluorouracil (5-FU). Overlap of both markers represented PAR formation in nuclei (yellow). (**B**) Apoptosis analysis using different drugs, alone or combined with the PARP-1 inhibitor Olaparib. Data were represented as the mean ± SEM (n=3). Representative immunofluorescence images revealed DNA damage (pH2AX, green) caused by DOX (**C**) and GEM (**D**) after 6 h of treatment alone and in combination with Olaparib. Nuclei were dyed with DAPI (blue). Scale bar, 20 μm.

response to 5-FU (Geng *et al.* 2011; Park *et al.* 2018; Jiang *et al.* 2019). However, we cannot replicate the results obtained with Irinotecan in colon cancer, where PARP inhibitors do establish a synergistic effect with the drug (Augustine *et al.* 2019). The proposed mechanism for this synergy is that the accumulation of DNA damage cannot be repaired due to the absence of the enzyme.

Once the synergistic effect was confirmed, DOX and Olaparib were used for toxicity studies in MIA-PaCa-2 CSC tumorspheres. Our results showed that DOX was more effective than Olaparib to inhibit the growth of tumorspheres derived from ALDH+ isolated cells and demonstrated the effectiveness of the DOX-Olaparib combination on tumorspheres growth. In addition, as observed in cell line

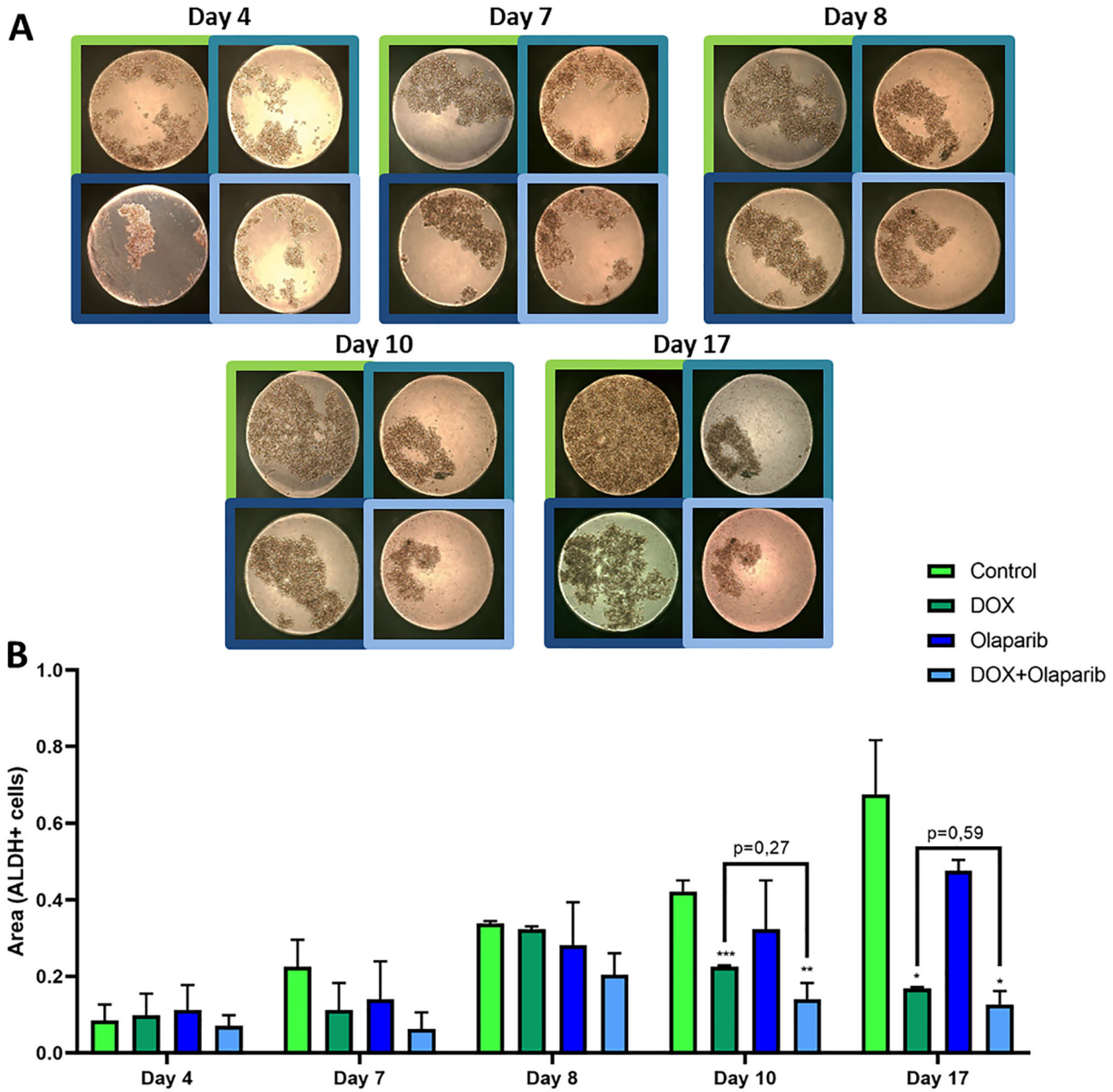

Figure 5. Treatment effect in MIA PaCa-2 tumorospheres formation. (**A**) Representative images of tumorospheres at different times (4 to 17 days). (**B**) Graphic representing modulation of MIA PaCa-2 ALDH+ tumorosphere area in CSC medium after being treated with DOX drugs (1μg/ml), Olaparib (10 μM) and DOX+Olaparib. Tumorospheres without treatment were used as a control. Data were represented as the mean ± SEM (n = 6). Asterisks indicated significant differences between controls and treatments on the same day * = $p \leq 0,05$, ** = $p \leq 0,01$, *** = $p \leq 0,001$. The significance between DOX and DOX+Olaparib treatments was also indicated.

treatments, the simultaneous treatment based on DOX and Olaparib allows a greater inhibition of CSC proliferation, decreasing CSC growth since the start of the treatment. This may be due to the same effect previously observed, in which CSCs would be unable to repair the damage caused by DOX.

In conclusion, our study reveals that a high proportion of GI tumor cell lines analyzed showed high PARP-1 expression and that such expression was very significant in comparison with non-tumor colon and liver cells –although not all the GI tumor cell lines analyzed could be compared-. On the other hand, studies in pancreatic cancer –and, specifically, in MIA-PaCa-2 cell- lines, showed that drugs such as DOX and GEM induced PARP-1 activation. Interestingly, the use of the PARP-1 inhibitor Olaparib increased the DNA

damage caused by both cytotoxic drugs with a synergistic effect. These results support the use of PARP inhibitors as a promising treatment to kill CSCs, which are responsible for tumor treatment failure and cancer relapse. However, more studies are needed to completely elucidate the role played by PARP-1 in GI tumors and its influence on drug resistance.

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