Rationale for the use of P-MAPA immunotherapy alone and in association with Gemcitabine in the treatment of pancreatic cancer

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Highlights

- Pancreatic cancer was induced in five animal groups. Control group had no cancer; all DMBA-induced and Gemcitabine-treated animals had invasive pancreatic cancer.
- P-MAPA-treated animals presented 40% of histopathological recovery, expanded to 80% in P-MAPA + Gemcitabine-treated animals.
- P-MAPA Immunotherapy associated with Gemcitabine chemotherapy showed a synergistic effect to block tumor progression and increase apoptosis, without additional toxicity.
- The drug association induced the interferon-signaling pathway, specifically IFN-γ, a powerful inducer of tumor cell death.
- In other experiments, P-MAPA downregulated the cytokine IL-10 levels; the immunosuppressive effect of IL-10 can aggravate pancreatic cancer.

Overview

Pancreatic cancer (PC) is a highly aggressive and lethal cancer characterized by invasiveness, local and extensive dissemination at time of diagnosis and resistance to treatment.

Pancreatic intraepithelial neoplasia (PanIN) is the presumed precursor lesion to infiltrating ductal adenocarcinoma of the pancreas (Andrea A et al., 2003, Hruban RH et al, 2008).

The management modalities of pancreatic cancer may include surgical resection, chemotherapy, radiotherapy, and chemoradiation. Due to rapid cancer cell proliferation, aggressive local invasion, metastasis, high rate of local recurrence and resistance to most forms of treatment, pancreatic cancer remains highly lethal even when surgically resected.

The median survival for locally advanced pancreatic cancer is only between 9 and 15 months, according to The American Cancer Society. Only 18% of the patients with advanced pancreatic ductal adenocarcinoma remain alive at 1 year, and 4% at 5 years (Hidalgo M *et al.*, 2015).

The prognostic is dismal for metastatic disease. Studies report median survival ranges for patients presenting metastatic disease between 2.8 and 5.7 months (Carrato A *et al.*, 2015).

Current Treatment Options

Gemcitabine therapy has been the standard first-line treatment for patients with unresectable locally advanced or metastatic pancreatic cancer, but the treatment has only marginally improved the median survival rate.

Various gemcitabine-based schemes have been attempted to improve the clinical results. One of them, Gemcitabine plus Cisplatin (GemCis), has showed synergistic effects; high toxicity rates, however, were observed (Ouyang G et al, 2016).

FOLFIRINOX (FFX) – a combination of fluorouracil [5-FU], leucovorin, irinotecan and oxaliplatin – was introduced into clinical practice in 2010 in the treatment of metastatic PC and is associated with better survival time (W Marsh R De W *et al.*, 2015, Conroy T *et al.*, 2011).

Meantime, FFX regime and/or FFX-type combinations can be highly toxic and their side effects can be more intense than the standard therapy (gemcitabine) alone.

Despite advances, the median survival time of advanced and /or metastatic PC patients remains between 4 and 11 months (Von Hoff D *et al.*, 2013). Therefore, new treatment options are urgently needed to improve the survival rates of patients with PC.

New Approaches for Treatment of Pancreatic Cancer

As dysregulation of the immune system facilitates PC development, immunotherapy would represents a valuable therapeutic strategy for the treatment of PC (Paniccia A *et al.*, 2015).

However, pancreatic cancer has proven difficult to treat with standard conventional drugs, and it has proven resistant to several immunotherapy approaches. Taking into account this scenario, association of chemotherapy with immunotherapy may provides advances in treatments and prolongs survival (Paniccia A *et al.*, 2015, Kunk PR et al, 2016).

Acting through direct killing of cancerous cells, chemotherapeutic agents indirectly lead to the release of pro-inflammatory molecules and by this way may facilitate the development of a more efficacious effector immune response against cancer cells. Chemotherapy can also suppress the inhibitory mechanism in the tumor microenvironment.

Indeed, reduction of the number of T_{regs} cells and myeloid derived suppressor cells (MDSC) and their related cytokines (IL-17 and IL-15) are one of the recognized positive effects of chemotherapy on tumor microenvironment. However, chemotherapy has also potential immunosuppressive effects, which should be mitigated to maximize the efficacy of combined treatments (Paniccia A *et al*., 2015).

To explore this strategy, new approaches are being proposed for treatment of PC such as the combinations described by Ghansah and Chang – Gemcitabine chemotherapy associated respectively with a dendritic cell vaccine and a TLR2 agonist – leading to a significant decrease of pancreatic tumor in animal model (Ghansah T *et al*., 2013, Chang LS *et al*., 2016).

In addition, several clinical trials have showed positive results of Gemcitabine associated with immunomodulator ones (e.g., Hirooka Y *et al*., 2009, Kimura Y *et al*., 2012).

After evaluating 18 clinical studies, from which 14 on immunotherapy associated with chemotherapy and 4 on immunotherapy alone, Zhang concluded that the drug association is more effective than immunotherapy alone (Zhang B *et al*., 2016).

In this scenario, Toll-like receptors (TLRs) agonists may play an important role in the treatment of PC in association with chemotherapy. For instance, TLR-2 and TLR-4 signaling pathways promotes cytokines production, such as interferons, which are powerful inducers of tumor cell death. Specifically, IFN-γ stimulates immune cells, blocks tumor growth and enhances tumor immunogenicity (Beatty GL et al., 2001, Sun D et al. 2006).

In addition, IFN-γ is involved in Th1-mediated immune responses by regulating the differentiation, activation and homeostasis of T cells and inhibits Th2 cell development. (Agnello D.et al, 2003).

Scientific rationale for treatment of pancreatic cancer using P-MAPA immunotherapy

Immunotherapy in association with chemotherapy may be advantageous in the treatment of PC (Hirooka Y *et al*., 2009, Kimura Y *et al*., 2012, Paniccia A *et al*., 2015, Zhang B *et al*., 2016, Kunk PR et al, 2016). Considering this strategy, immunotherapy with P-MAPA would represent an option for treatment of PC.

P-MAPA is an immunomodulator in late stage of development led by the research network Farmabrasilis. Several results from *in vivo* and *ex vivo* studies strongly suggest that P-MAPA modulates the innate and adaptive immune systems. In addition, P-MAPA at therapeutic dosages did not show signs of systemic or topical toxicity in animal models, as well as in clinical trials phase I (Nunes IS et al, 2009, Lenaerts AJ et al, 2012, Farmabrasilis 2010).

P-MAPA stimulates the *in vivo* production of granulocytes, lymphocytes, NK and other cells of the immune system, which have cytotoxic effect against tumors. P-MAPA acts on TLR-2 and TLR-4, induces or restores the Type-1 (Th1) response and possibly may downregulate the Type-2 (Th2) one (Justo GZ *et al*., 2003, Lenaerts AJ et al, 2012, Garcia, PV et al, 2016).

P-MAPA downregulated IL-10 levels in animal model, reestablishing NK cells activity and downregulating IL-10 levels in other diseases related to an impaired immune status (Justo GZ *et al*., 2003, Santiago ME *et al*., 2013). Concerning treatment of pancreatic cancer, the findings are relevant; once PC has been associated with a Th2 biased immune response (Wörmann SM *et al*., 2015 , Paniccia A *et al*., 2015)

In summary, studies carried out in several animal models have indicated that P-MAPA modulates the innate and adaptive immune systems, increases IL-2 and IFN-γ levels and decreases IL-10 levels, reversing the tumor-induced immunosuppression and blocking the tumor progression. The result is the host protection and high survival rates of the animals.

Importantly, as previous studies evidenced, P-MAPA also shows potential to be used in association with other drugs without additional toxicity: P-MAPA immunotherapy showed synergistic effects against bladder cancer when used in association with chemotherapy and androgen-deprivation therapy (Dias QC *et al*., 2016, Garcia PV et al, 2015, Godoy G. et al, 2016).

Taking together, the data pave the way and provides the scientific rationale for the experimental use of P-MAPA immunotherapy alone and in association with chemotherapy in the treatment of PC. Therefore, in order to explore the possibilities of P-MAPA immunotherapy for treatment of pancreatic cancer an experiment using an animal model with PC induced with the use of 7, 12-Dimethylbenz (a) anthracene (DMBA) was carried out.

In this experiment, P-MAPA acts on TLR4 and IFN-γ, and P-MAPA immunotherapy alone and in association with Gemcitabine showed impressive results in the treatment of PC (Santos MM., 2016), as detailed below.

Aims

1) to explore and compare the effects of P-MAPA monotherapy and associated with Gemcitabine in the treatment of chemically induced PC;

2) to verify if P-MAPA monotherapy or associated with Gemcitabine would be able to induce effects on cytokines associated with a Th-1 type response;

3) to verify if P-MAPA immunotherapy associated with Gemcitabine would present signs of additional toxicity.

Experimental Design

PC was induced in 50 rats (Fischer 344) using 200 µg of 7, 12-Dimethylbenz (a) anthracene (DMBA) in the pancreas head. Other 10 rats (not induced) composed the Control Group. After 120 days of induction, all animals were subdivided into five groups (10 animals per group): **Group 1**: Control Group; **Group 2**: DMBA Group; **Group 3**: P-MAPA; **Group 4**: Gemcitabine Group; **Group 5**: P-MAPA + Gemcitabine Group.

Control group (1): animals received intraperitoneal applications of 5 mL/kg 0.9% physiological solution, three times per week for six weeks; **DMBA (cancer) group (2):** animals received the same treatment of the control group; **P-MAPA group (3):** animals received intraperitoneal applications of 5 mg/kg P-MAPA (Farmabrasilis, São Paulo, Brazil), three times per week for six weeks; **DMA + Gemcitabine group (4):** animals received intraperitoneal applications of 10 mg/kg Gemcitabine once a week for six weeks; **P-MAPA + Gemcitabine group (5):** animals received intraperitoneal applications of 5 mg/kg P-MAPA three times by week for three weeks followed by intraperitoneal application of 10 mg/kg Gemcitabine once a week for the following three weeks.

Statistical Analyzes: Histopathological analyzes, Western Blotting and Proliferative and Apoptotic Indexes were performed for all groups. Proportion test was used for histopathological analyzes, and 1% type-I error was considered statistically significant. Analysis of variance (ANOVA), followed by Tukey post hoc test for multiple comparison of means, was used for statistical analysis of Western Blotting, Proliferative and Apoptotic Indexes. The significance level of 5% was adopted and the results were expressed as mean ± standard deviation.

P-MAPA immunotherapy alone and associated with Gemcitabine chemotherapy blocked DMBA-induced neoplastic changes and reduced the aggressiveness of DMBA- induced premalignant lesions

DMBA induced tumors in **100% of the animals**, from which **60%** were invasive carcinoma and **40%** were **PanIN-3** (Table, Fig.1.c, d, e, f).

Gemcitabine chemotherapy **reduced the aggressiveness** of DMBA-induced malignant lesions, but was not effective for tissue recovery. PanIN-1, PanIN-2 (Figure 1g), PanIN-3 (Figure 1h) and **invasive carcinoma** (Figure 1i, 1j) responded for 20%, 20%, 40% and 20% of the lesions, respectively, in **DMBA + Gemcitabine** group (Table, Figure 1, g, h).

P-MAPA immunotherapy blocked the tumor progression, compared to DMBA + Gemcitabine group (Table); 40% of the animals presented hyperplasia ductal (Figure 2a, Table), 40% PanIN-1 (Table, Figure 2b) and 20% PanIN-2 (Table, Figure 2c) in **DMBA + P-MAPA group**.

P-MAPA immunotherapy associated with Gemcitabine chemotherapy was highly effective for tissue recovery, **resulting in 80% of tumor non-progression** (Table); 60% of the animals presented normal ductal morphology (Table, Figure 5d) and 20%, ductal hyperplasia (Table, Figure 2e). **Premalignant lesions** (**PanIN-1)** were observed in only **20% of the animals** (Table, Figure 2f).

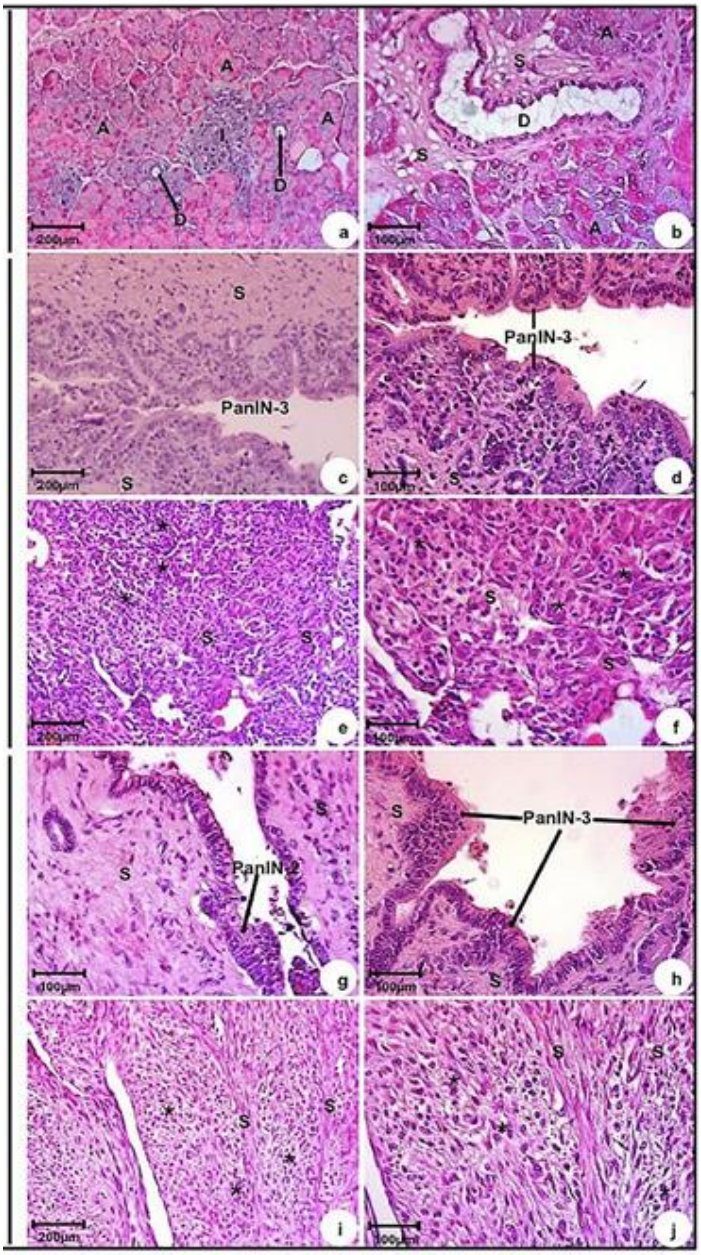
Table: Histopathological changes in rat pancreas (%)

Histopathology	Groups				
	Control (n = 5)	DMBA (cancer) (n = 5)	DMBA + P-MAPA (n = 5)	DMBA + Gemcitabine (n = 5)	DMBA + P-MAPA + Gemcitabine (n = 5)
Normal	5 (100%)*	0	0	0	3 **(60%)***
Hyperplasia ductal	0	0	2 (40%)*	0	1 **(20%)***
PanIN-1	0	0	2 (40%)*	1 (20%)	1 (20%)*
PanIN-2	0	0	1 (20%)*	1 (20%)*	0
PanIN-3	0	2 (40%)*	0	2 (40%)*	0
Invasive carcinoma	0	3 (60%)*	0	1 (20%)	0

* Statistically significant (proportion test, $p < 0.0001$)
PanIN: Pancreatic intraepithelial neoplasia (PanIN)

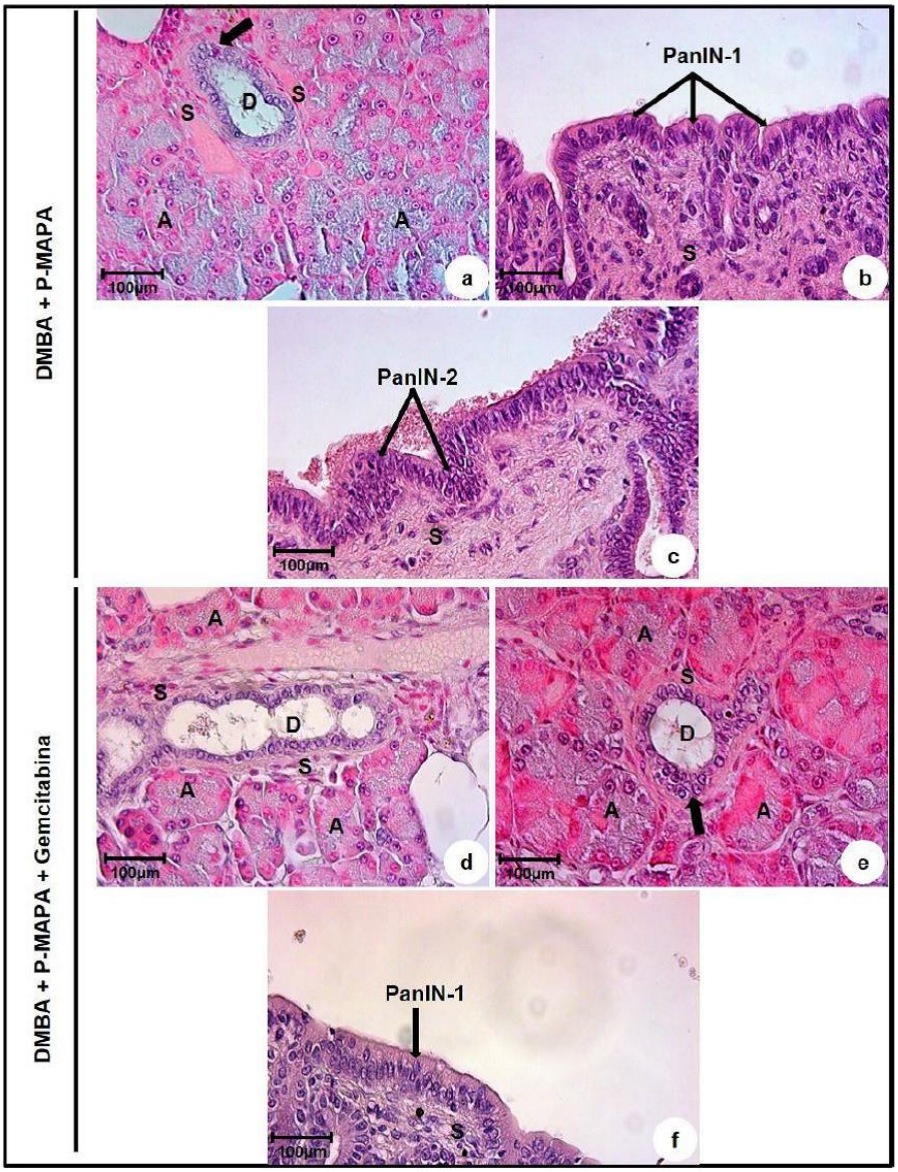
Histopathological Analysis

Figure 1 – Photomicrographs of rat pancreas from control group (a, b), DMBA (c, d, e, f), and DMBA + Gemcitabine (g, h, i, j) groups. (A), (b).



Normal pancreas, characterized by rounded serous acini (A) with basophilic cells and Islet of Langerhans (I), forming the endocrine region; interlobular ducts (D) composed of simple cubic epithelium surrounded by stromal cells (S) with abundant collagen fibers and blood vessels. (C), (d), (h) PanIN-3, characterized by papillary or micropapillary lesions with a high degree of dysplasia; Massive nuclei, pleomorphic, with evident nucleoli and loss of cellular polarity. (E), (f), (i), (j) Invasive carcinoma *, characterized by neoplastic cells with intense cellular atypia, arranged in nests or cords (* and **) infiltrating the desmoplastic (S) stroma; Nuclei of bulky neoplastic cells, pleomorphic, with evident nucleoli. (G) PanIN-2, characterized by moderate degree of dysplasia, nuclei enlarged in size and number, with loss of cell polarity, hyperchromasia and pseudostratification. * Classification of Duct Lesions in the Pancreas; ** PaIN-1 to PaIN-3: Pancreatic Intraepithelial Neoplasia.

Figure 2 – Photomicrographs of rat pancreas from DMBA + P-MAPA (a, b, c), and DMBA + P-MAPA + gemcitabine (d, e, f) groups.



(a), (e). Interlobular ducts (D) with proliferation of cubic ductal cells (arrows), characterizing ductal hyperplasia; Rounded serous acini (A) and stromal cells (S). (B), (f) PanIN-1, characterized by flat lesions with low degree of dysplasia, composed of columnar epithelial cells with uniform and round nuclei perpendicular to the basement membrane of the ducts. (C) PanIN-2, characterized by moderate degree of dysplasia, nuclei enlarged in size and number, with loss of cell polarity, hyperchromasia and pseudostratification. (D) Normal pancreas, characterized by rounded serous acini (A) with basophilic cells; Interlobular ducts (D) composed of simple cubic epithelium, surrounded by stromal cells (S) with abundant collagen fibers and blood vessels. * Classification of Duct Lesions in the Pancreas; ** PaIN- 1 to PaIN-3: Pancreatic Intraepithelial Neoplasia.

P-MAPA immunotherapy alone and associated with Gemcitabine chemotherapy increased interferon signaling pathway, blocking tumor proliferation and increasing apoptosis.

TLR4 protein levels

TLR4 protein levels were significantly higher in Control, DMBA + P-MAPA and DMBA + P-MAPA + Gemcitabine groups than in DMBA and DMBA + Gemcitabine groups (Figure 3a).

IRF3 protein levels

IRF3 protein levels were significantly higher in DMBA + PMAPA + Gemcitabine group than in other groups (Figure 3b). Additionally, the levels were significantly higher in Control and DMBA + P-MAPA groups than in DMBA and DMBA + Gemcitabine groups (Figure 3b).

IFN-γ protein levels

Similarly, IFN-γ protein levels were significantly higher in Control, DMBA + P-MAPA and DMBA + P-MAPA + Gemcitabine groups than in DMBA and DMBA + Gemcitabine groups (Figure 3c).

MyD88 protein levels

MyD88 protein levels were significantly higher in DMBA + P-MAPA and DMBA + P-MAPA + Gemcitabine groups than in other groups (Figure 3d).

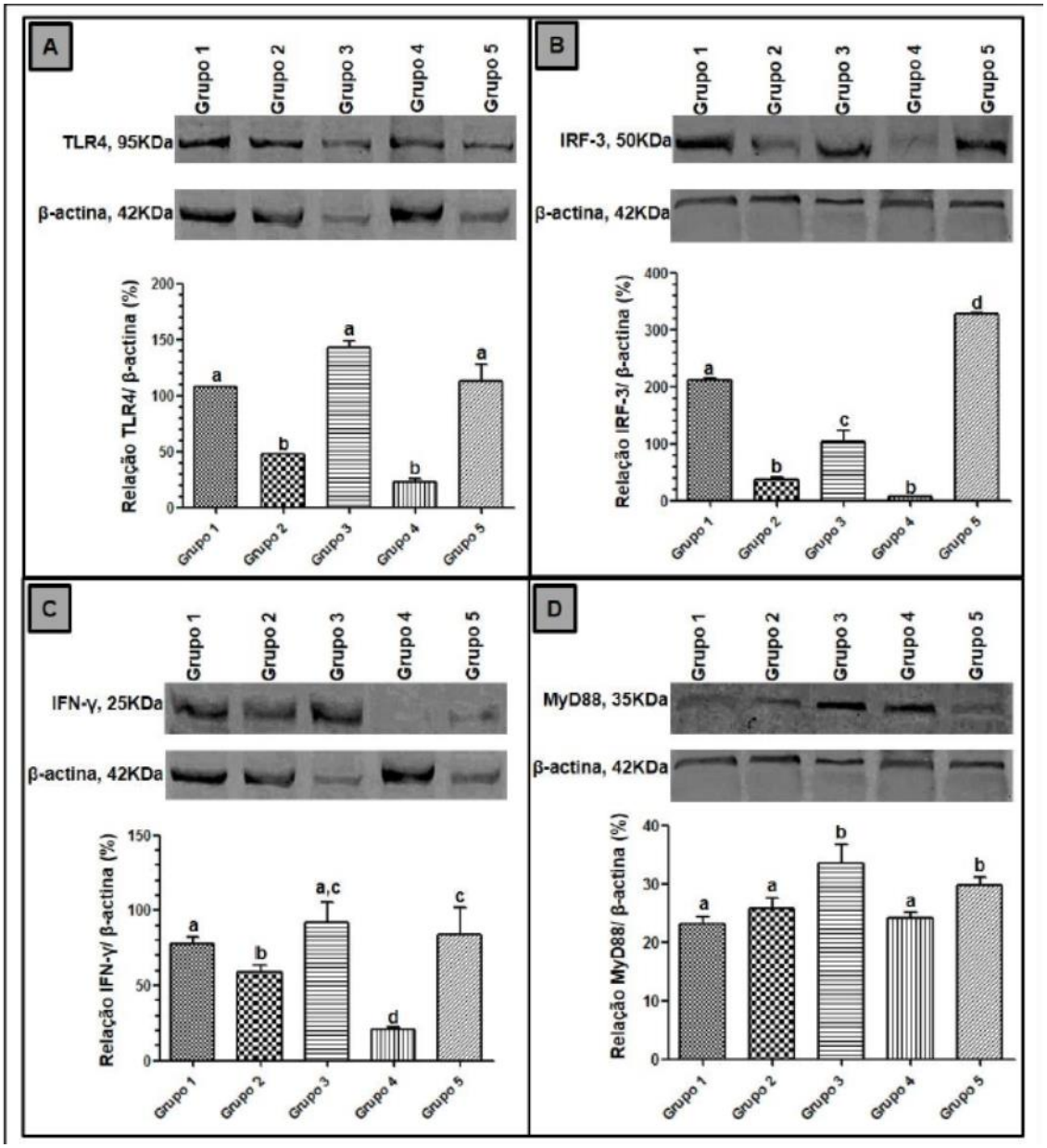


Figure 3: Groups: Control (Group 1); DMBA (Group 2); DMBA + P-MAPA (Group 3); DMBA + Gemcitabine (Group 4) and DMBA + P-MAPA + Gemcitabine (Group 5). All data were expressed as mean ± standard deviation. Different lowercase letters (a, b, c, d) indicate significant differences ($p < 0.05$) between groups after Tukey test.

Proliferative index, apoptotic index and P/A ratio.

There was no significant difference between DMBA + Gemcitabine and DMBA + P-MAPA groups (Figure 4a). There was a significant decrease in **proliferative index** in Control and DMBA + P-MAPA + Gemcitabine groups compared to other treated groups (Figure 4a).

Apoptotic index

There was a significant decrease in **apoptotic index** in DMBA and DMBA + Gemcitabine compared to other groups (Figure 4b). There was a significant increase in apoptotic index in P-MAPA-treated animals and no significant difference between Control and DMBA + P-MAPA + Gemcitabine groups (Figure 4b).

Proliferation/apoptosis ratio (P/A)

There was a significant **increase** in **proliferation/apoptosis ratio (P/A)** ratio in **DMBA** and (DMBA + Gemcitabine groups) compared to other groups (Figure 4c).

There was a significant **decrease** in **proliferation/apoptosis ratio (P/A)** ratio between Control, (DMBA + P-MAPA) and (DMBA + P-MAPA + Gemcitabine groups) (Figure 4c).

Importantly, there was no significant difference in **P/A ratio** between **Control**, (DMBA + **P-MAPA)** and (**DMBA** + **P-MAPA + Gemcitabine)** groups (Figure 4c).

Figure 4 – (A) Proliferative index, (B) apoptotic index, and (C) proliferation/apoptosis (P/A) ratio of pancreas rats

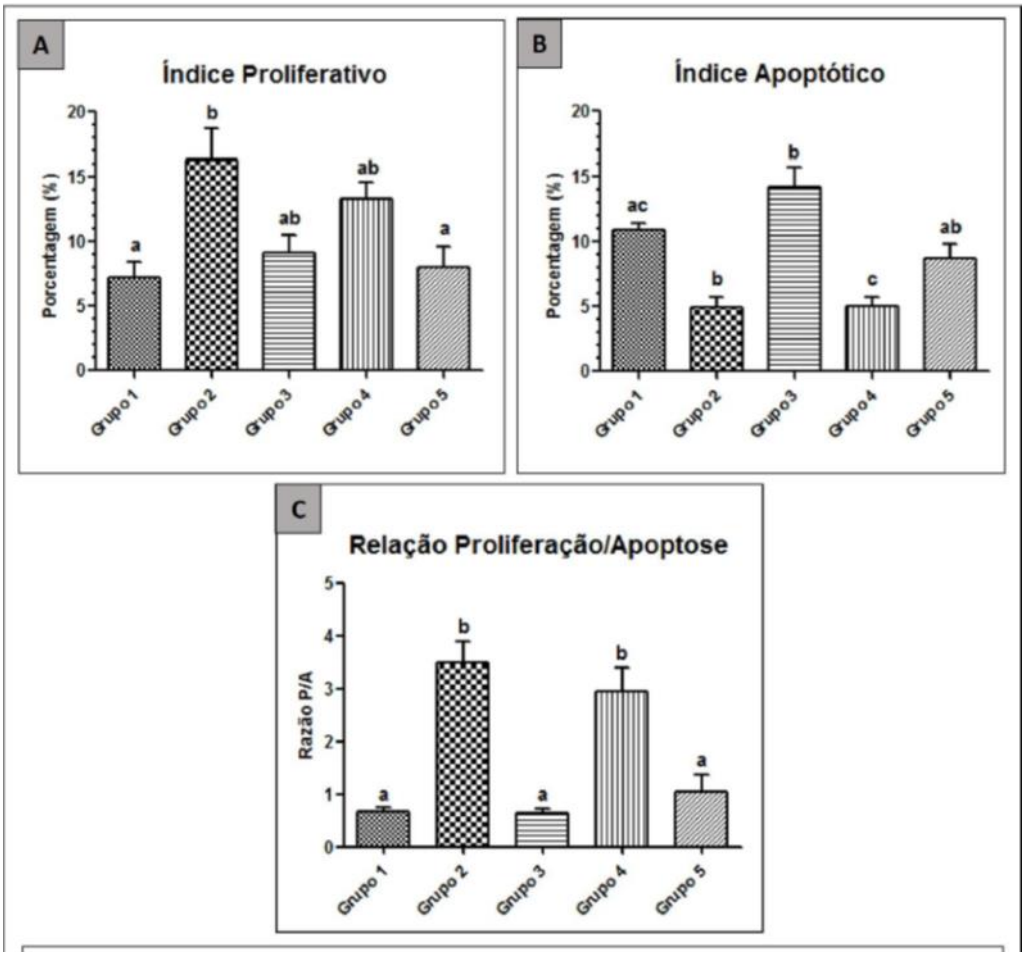


Figure 4: Groups: Control (Group 1); DMBA (Group 2); DMBA + P-MAPA (Group 3); DMBA + Gemcitabine (Group 4) and DMBA + P-MAPA + Gemcitabine (Group 5). Data were expressed as mean ± standard deviation. The lower case letters indicate a significant difference (P < 0.05) between the groups by the Tukey test.

Discussion

Chemotherapeutic agents kill cancer cells, stimulate the production of pro-inflammatory molecules and may facilitate the development of a more efficacious effector immune response against cancer cells. However, chemotherapy has also potential immunosuppressive effects, which should be mitigated to maximize the efficacy of treatments (Paniccia A *et al*., 2015).

Pancreatic intraepithelial neoplasia is (PanIN) is the presumed precursor lesion to invasive ductal adenocarcinoma of the pancreas. Thereby, new therapies aiming to prevent or treat these lesions would have potential to cause a significant impact on pancreatic cancer mortality (Andrea A et al., 2003, Hruban RH et al, 2008).

Invasive carcinoma (60%) and PanIN-3 (40%) were observed in **DMBA-treated** animals (Table, Figure1).

PanIN-1, PanIN -2, and PanIN -3, and invasive carcinoma (20%, 20%, 40% and 20% respectively) were observed in **Gemcitabine-treated** animals (Figure 1, Table).

In sharp contrast, there was a 40% of PanIN-1 and 20% of PanIN-2 in **DMBA + P-MAPA** Group (Figure 2, Table) and **only 20%** of **PanIN-1** in **DMBA+ P-MAPA+ Gemcitabine-treated** animals.

The **40% of non-tumor progression** for P-MAPA monotherapy and **80%** for the association with Gemcitabine, are consistent with the increase in the IFN-γ levels, suggesting that P-MAPA enhances or restores a **Type-1 (Th1) response** in treated animals (Table, Figure 3).

Importantly, **histological recovery** and **80% of non-tumor progression** (60% normal and 20% reactive hyperplasia) and only **20% of PanIN-1** respectively, were observed in P-MAPA plus Gemcitabine-treated animals.

Tough the major dose -limiting toxicity of Gemcitabine is myelosuppression (Ely Lilly); this drug did not induce immunosuppressive effects when associated with P-MAPA as well as the association not presents any signs of additional toxicity for the treated animals. On contrary, TLR4 protein and IFN-γ levels were significantly higher in Control and P-MAPA + Gemcitabine groups (Figure 3).

The results strongly suggest that the **drug association** (P-MAPA and Gemcitabine) **have a synergistic effect** against **premalignant lesions and tumor progression** in animal model.

Conclusions

A) The model of PC induced by DMBA effectively induced tumors in **100% of the animals**, from which **60%** were invasive carcinoma and **40%** were **PanIN-3**;

B) Gemcitabine **alone** showed PanIN-1, PanIN-2, and PanIN-3, and invasive carcinoma in 20%, 20%, 40% and 20% of treated animals, respectively. In contrast, **P-MAPA monotherapy** was more effective against PC, **showing 40% of non-tumor progression,** 40% of **PanIN-1** and 20% of **PanIN-2**, respectively.

C) P-MAPA immunotherapy **associated** with Gemcitabine chemotherapy was the **best treatment strategy** to treat PC, resulting in **80% of non-tumor progression** and **20% of PanIN-1** respectively;

D) P-MAPA immunotherapy **associated with Gemcitabine** chemotherapy stimulated the innate immune system through TLR4/IRF3/IFN-γ signaling pathway;

E) TLR4 signaling pathway was dependent on Myd88, which was important to induce IFN-γ production;

F) P-MAPA immunotherapy **alone** and **associated with Gemcitabine** chemotherapy increased apoptotic levels; the P/A ratio favored cell death and tissue repair;

G) P-MAPA immunotherapy associated with Gemcitabine did not present signs **of immunosuppression** or **additional toxicity**;

H) All data have strengthened the validity of use **of P-MAPA immunotherapy associated with Gemcitabine** in the treatment of PC.

Invitation

Based on this scientific rationale, Farmabrasilis teams will be happy to discuss the possibilities of use of P-MAPA immunotherapy with patient advocates, cancer advocacy networks, research groups, institutions and pharmaceutical companies interested in establishing collaborations to move forward this novel therapy for treatment of pancreatic cancer.

Our action can make the difference in the life of patients with pancreatic cancer

Send your questions, comments and/or proposal to alliances@farmabrasilis.org please write P-MAPA in the subject line. You will receive a reply as soon as possible.

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Farmabrasilis is a non-profit research network based in Brazil who runs the development of P-MAPA in collaboration with research centers and universities in Brazil, the U.S. and Europe.

For more information about Farmabrasilis and P-MAPA, visit the website www.farmabrasilis.org and the PubMed database, https://www.ncbi.nlm.nih.gov/pubmed/?term=p-mapa.