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Research Article

BERGAMOTTIN POTENTIALIZES THE CYTOTOXIC ACTIVITY OF GEMCITABINE THROUGH INHIBITION OF MDR1 ON PANCREATIC CANCER CELL LINE ASPC-1

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A B S T R A C T

Pancreatic cancer is one of the most aggressive cancers, with only about 5% of patients surviving 5 years past the initial diagnosis. Gemcitabine monotherapy is the standard of treatment for patients with metastatic pancreatic cancer. Here, we examined the efficacy of combined treatments of bergamottin and gemcitabine in human pancreatic cancer AsPC-1 cells. For that purpose, the pro- apoptotic effects of gemcitabine were studied in presence or absence of bergamottin, in order to evaluate if this latter is able to potentialize gemcitabine cytotoxicity. Our study aims to investigate the implication of MDR1 in resistance to gemcitabine and if bergamottin could target these drug efflux pumps in AsPC-1 cells by flow cytometric. We observed that 5 µg/ml gemcitabine in combination with 15 µg/ml bergamottin was more effective than gemcitabine alone as shown by increasing in the percentage of dead cells up to 60%. In addition, the combination of gemcitabine and bergamottin increased the expression levels of cleaved caspase-3 and p53. Moreover our results demonstrated that bergamottin inhibits the efflux activity of MDR1. In conclusion, our study in vitro suggests therefore that chemotherapy with gemcitabine might be significantly increased upon combination with bergamottin and then may be promising agents for novel combination therapy.

INTRODUCTION

Pancreatic cancer is the eighth major form of cancer-related death worldwide, causing 227 000 deaths annually, (Torre *et al*., 2015). In 2016 it was estimated that 53,070 patients will be diagnosed with pancreatic cancer and 41,780 patients with pancreatic cancer will die in the United States, most of them dying within the first year of diagnosis, (Jemal *et al*., 2010).

For most patients with pancreatic cancer, surgery is a pivotal curative therapeutic approach. However, the success rate of resection surgery remains very low because about 80% to 85% patients diagnosed with a pancreatic cancer were already in an advanced stage. So, only 10% to 20% of patients with pancreatic cancer are eligible for surgical resection after being primarily diagnosed, (Paulson *et al*., 2013). Therefore, as standard treatment approaches for patients with pancreatic cancer, chemotherapy and radiotherapy are considered, especially for locally advanced or metastatic patients, (Perkhofer *et al*., 2015).

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Gemcitabine is the most commonly used chemotherapeutic agent over the past decade. Current treatment modalities for advanced pancreatic cancer include gemcitabine as a single agent or in combination with multiple chemotherapeutic agents, (Cunningham *et al*., 2009) (Louvet *et al*., 2005).

Numerous phase III trials testing gemcitabine combined with other cytotoxic drugs have failed to reveal any additional benefit compared with gemcitabine alone. Recently ithas been reported that a combination of an antiepidermal growth factor receptor agent (Erlotinib) with gemcitabine may be more effective than gemcitabine alone, (Donadelli *et al*., 2007).

The major cause of chemotherapy failure is the drug resistance to multiple chemotherapeutic agents, (Kornmann *et al*., 2003). Overexpression of MDR1 has been shown to induce resistance to various anticancer drugs, (Cantwell *et al*., 1988). MDR1 acts as an energy-dependent drug efflux pump, thereby decreasing the intracellular drug concentration and causing drug resistance.For example, colorectal cancer express high levels of MDR1, and this expression may contribute to the general resistance of colorectal cancer to anticancer drugs, (Linn & Giaccone, 1995). The very limited use of chemotherapy for pancreatic cancer patients is associated with the inherent chemoresistant nature of this aggressive disease.

However, MDR1 the ATP-dependentmembrane-bound drug efflux pumps, is mediators of clinically relevant chemoresistance, (Bradley & Ling, 1994). Studies of the prevalence of MDR1 in pancreatic tumours have been limited. To determine the potential involvement of these drug effluxpumps, we study MDR1 activity in pancreatic tumours in AsPC-1 cells.

To enhance the anticancer therapeutic efficacy and reduce the side effects, natural productswere combined with standard chemotherapy and radiotherapy, (Mohammed *et al*., 2015). In this context, the use of natural products, as a supplementary approach, to treat pancreatic cancer holds a great promise with minimal side effects, (Zhu, *et al*., 2016). Many studies with polyphenols,such as flavonoids from fruits and vegetables, many studies have shown that they are efficient a chemopreventative agentssince theyare able to promote apoptosis in a variety ofcancer cells, (Nakagawa *et al*., 2009). To enhance intracellular anticancer drug accumulation by impairing the MDR1 efflux function, the process of chemosensetization involves usually a co-administration of a MDR1 inhibitor with an anticancer,(Gollapudi *et al*.,1995). Numerous compounds have been shown to inhibit the drug efflux function of MDR1 and therefore, increase the intracellular concentration of cytotoxic anti cancer agents and consequently decrease in the cellular resistance, (Louisa *et al*., 2014). Grape fruit juice contained dihydroxybergamotin and other furanocoumarins, such as bergamottin; bergaptol and bergapten that are know toinhibit the drug efflux transporters, such as MDR1and increased the uptake of vinblastine by Caco-2 cells. Bergamottin is also found in the oil of bergamot orange and in the essential oils of other citrus fruits from which it was first isolated and from which its name is derived, (Ohnishi *et al*., 2000).

The aim of our study is to establish whether bergamottin could have the ability to induce apoptosis and enhance the chemotherapeutic effect of gemcitabine in vitro in an established human pancreatic cancer cell line (AsPC-1) without causing damage on the normal cells.

MATERIALS AND METHODS

Chemicals and Drugs

Rhodamine 123 (RH 123) was purchased from Invitrogen. Verapamil, bergamottin and gemcitabine were purchased from Sigma-Aldrich. Final concentration of DMSO applied to cells during incubation with tested drugs was 0.5%. In the tested setup these concentrations had no adverse effects on cell viability or cell morphologyor on rhodamine-123 efflux.

Cell culture and maintenance

The human pancreatic adenocarcinoma cell line AsPC-1 cells (CRL-1682) and the fibroblastic cell line BJ (CRL-2522) all purchased from ATCC (LGC Standards, Molsheim, France)were cultivated in the physiological nutrient-rich DMEM-based media (Sigma-Aldrich, Saint-Quentin Fallavier, France) supplemented with 10% (v/v) foetal bovine serum (Lonza, Verviers, Belgium), 2 mM glutamine, P/S (100 unit/ml and 100 lg/ml) (Sigma-Aldrich). Cells were grown in petri dishes to 70-80% confluency prior to treatment. All plates were incubated in a humidified incubator at 37°C and 5% CO_2.Then cells were incubated with gemcitabine and bergamottin for 24 hours.

Detection of apoptosis by annexin-FITC

Pancreatic cancer cells AsPC-1 and BJ cells death was assessed using AnnexinV-FITC Kit (Miltenyi Biotec) according to manufacturer's protocol. Briefly, Aspc1 and BJ cells were incubated with the gemcitabine, bergamottin and their combination for 24 hours. Cells were then washed with phosphate buffered saline (PBS) and stained with AnnexinV-FITC and PI followind the manufacturer's protocol. The fluorescence intensity of AnnexinV-FITC stained cells at 530/540 nm and PI stained cells at 675/630 were analyzed by Guava EasyCyte Plus capillary flow cytometer (Merck Millipore, Life Science division, Merck KgaA, Darm- stadt, Germany) and computed using the Guava ExpressPro software (Merck/Millipore/Guava Tech). The apoptotic potential of the tested drugs was compared to the apoptotic potential of celastrol, which is known to be a positif control of apoptosis.

MDR-1 function assay

MDR1-mediated efflux of rhodamine 123 was monitored on a Guava EasyCyte Plus capillary flow cytometer equipped with a 488 nm excitation laser. The accumulated intracellular fluorescence intensity of rhodamine 123 at 530/540 nm was computed on the Guava ExpressPro software (Merck/Millipore/Guava Tech) in terms of x-geometric mean arbitrary units. Dead cells were excluded based on propidium iodide staining. The inhibitory potential of tested compounds on rhodamine-123 efflux was expressed relative to maximum inhibition obtained with 100 µM verapamil in the same experiment. The experiments were repeated three times.

Western Blot analysis

After incubating AsPC-1 cells for 24 hours with the gemcitabine in presence and absence of bergamottin cells were collected after trypsinisation transferred to 15 ml tubes and centrifuged at 500 *g* for 5 minutes. Cell pellets were washed three times with cold 10 mM Tris- HCl, pH 7.4, 150 mM NaCl, and solubilized in lysis buffer (RIPA) (150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 0.5 M Tris pH 8.0). After spinning for 20 minutes at 10,000 *g* at 4°C, protein concentrations were determined in supernatants using Bradford assay. All samples, were denatured by heating for 5 minutes at 95° C, before being loaded onto a 10% gel for p53 detection and 15% gel for cleaved caspase 3. After electrophoresis, Western gels were blottedand the transferred nitrocellulose membrane was blocked with 5% milk powder in 50mM Tris-HCl, 150 mM NaCl, 0.1 % Tween 20 at room temperature for 1 hour. The membrane was then immunoblotted with a rabbit monoclonal anti-cleaved caspase 3 (Cell Signaling Technology) oranti-p53 antibody (Santa Cruz Biotechnology, Heidelberg, Germany) (diluted 1/1000 in TBS tween 0.1%) overnight at 4°C. Subsequently the membrane was washed three times with 50 mM Tris- buffered saline and 0.1% Tween 20. Following incubation with horseradish peroxidase conjugated anti-mouse polyclonal anti-beta tubulin secondary antibody (Abcam) (1:10000) for 1 hour at room temperature, the blots were developed using enhanced chemiluminescence (ECL) (GE Healthcare). The experiments were repeated three times.

Statistical Analysis

Data are expressed as mean +/- standard error of mean (S.E.M.) and analysed using GraphPad Prism5" (La Jolla, CA,

USA). Statistical analysis was performed with either the one-way ANOVA test followed by Student's t-test. A P value <0.05 was considered significant. Experiments were performed at least in three separate experiments.

RESULTS

Apoptotic potential of bergamottin and gemcitabine

Non-cancerous fibroblast cells (BJ) were treated with bergamottin or gemcitabine toevaluate their toxicity. Our results demonstrates BJ to be unaffected when treated with bergamottin in the range up to 3µg /ml as shown in figure 1A and gemcitabine up to 2.5 µg /ml as shown in figure 1B. In addition, the combination of 0, 05 µg /ml gemcitabine with bergamottin did not enhanced apoptosis in fibroblast cells (figure 1C).

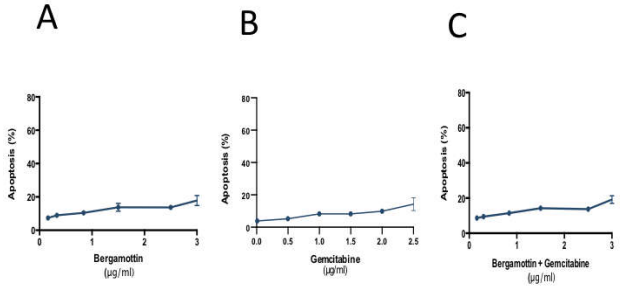

Figure 1 Effect of bergamottin (A), gemcitabine (B) and their combination (C) on BJ. Fibroblast cells were treated with bergamottin or gemcitabine. Cell death was assessed following the simultaneous staining of cells with AnnexinV-FITC and PI by capillary flow cytometry. Data are represented as Mean ± S.E.M (n=3).

AsPC-1 cells treated for 24 hours with gemcitabine do not exhibit a significant apoptotic potential in the range tested (0.05-25 µg /ml) (figure 2) when compared to the negative control. Moreover, administration of bergamottin in the range tested (1-30 µg /ml) alone did not enhanced apoptosis in AsPC-1.

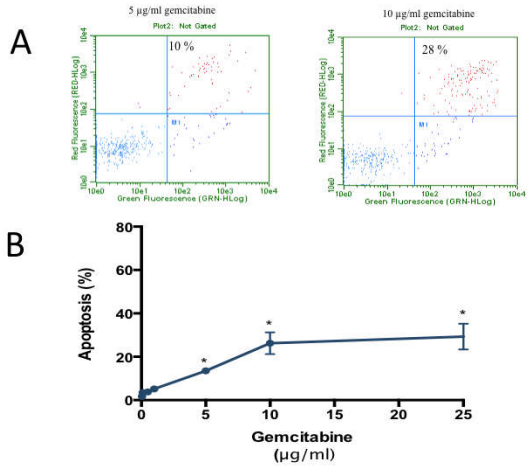

Figure 2 Apoptotic effect of gemcitabine on Aspc1 cells. After incubating Aspc1 cells 24h with gemcitabine, cells death was assessed following the simultaneous staining of cells with AnnexinV-FITC and PI by capillary flow cytometry. (A) Shows the results of representative scatter plots obtained after the different treatments, cells of the lower left quadrant are viable, cells on the upper and lower right quadrants are in late and early apoptosis, respectively. The number of cells in the late apoptosis, expressed as percent relative to the total cell number, is indicated. (B) Recapitulates in a dose response curves from the percentage of cells undergoing apoptosis after different treatments. Data are represented as Mean ± S.E.M (n=3). * represents P < 0.5 and refers to the variation in apoptotic potential between non-treated and treated AsPC-1 cells with gemcitabine.

Based on the above results and literature, we choose 5 µg /ml of gemcitabine for subsequent experiments to test the effects of gemcitabine in combination with bergamottin. In the range tested, the combined drugs exhibited more potent apoptotic effects than when they are administered alone. The results demonstrated therefor that, gemcitabine synergizes with bergamottin to promote cellular apoptosis (figure 3). The apoptotic potential of the investigated drugs is elucidated by an increase in the fluorescence of AnnexinV-FITC/PI stained cells.

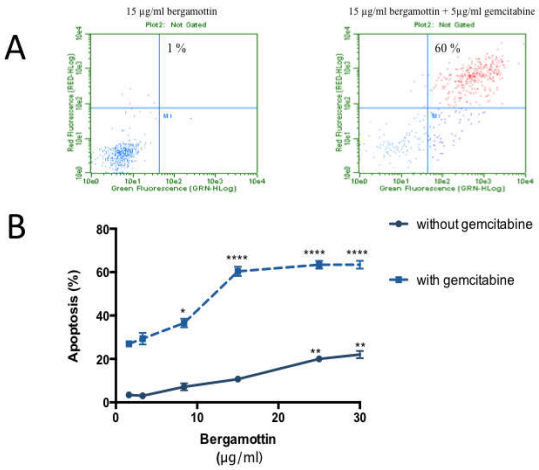

Figure 3 Effect of bergamottin and its combination with gemcitabine on AsPC-1 cells.

AsPC-1 cell cytotoxicity was induced by treatment with gemcitabine. Gemcitabine at 5 µg/mL in combination or not with 15 µg/mL of bergamottin reduces the cell viability up to 60%. (A) Shows the results of representative scatter plots obtained after the different treatments, cells of the lower left quadrant are viable, cells on the upper and lower right quadrants are in late and early apoptosis, respectively. The number of cells in the late apoptosis, expressed as percent relative total cell number, is indicated. (B) Recapitulates in a dose response curves from the percentage of cells undergoing apoptosis after different treatments. Data are represented as Mean ± S.E.M (n=3). **** represents P < 0.001 and refers to the variation in apoptotic potential between non-treated and treated AsPC-1 cells with the combination of bergamottin and gemcitabine.

Bergamottin effect on MDR1 Activity

Based on above results, we choose 15 µg /ml of bergamottin and 5 µg /ml of gemcitabine to evaluate their impacton MDR1. The study of the effect of bergamottin, gemcitabineor their combination on the efflux activity of MDR1 shown that in the

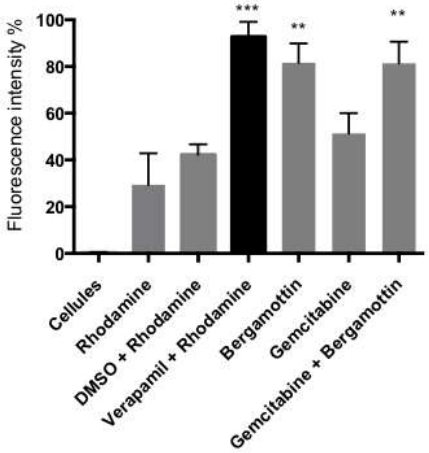

Figure 4 Effect of bergamottin on the MDR1-mediated efflux of rhodamine 123. AsPC-1 cells were incubated for 24 h, in the presence of bergamottin, gemcitabine or in combination. Bars represent the geometric mean values of % fluorescence intensity SD. The experiments were repeated three times. ** represents P < 0.001.

presence of bergamottin as an inhibitory potential on the efflux activity of MDR1 when compared to the specific MDR1 inhibitor verapamil. For instant the inhibition is concludedthrough an increase in the intracellular fluorescence of rhodamine 123-loaded cells. Their inhibitory potential on the efflux activity of MDR1 as demonstrated by the increase in fluorescence of rhodamine 123-loaded cells (figure 4 and 5). It should be noted that even verapamil, which is considered as the standard MDR1 inhibitor.

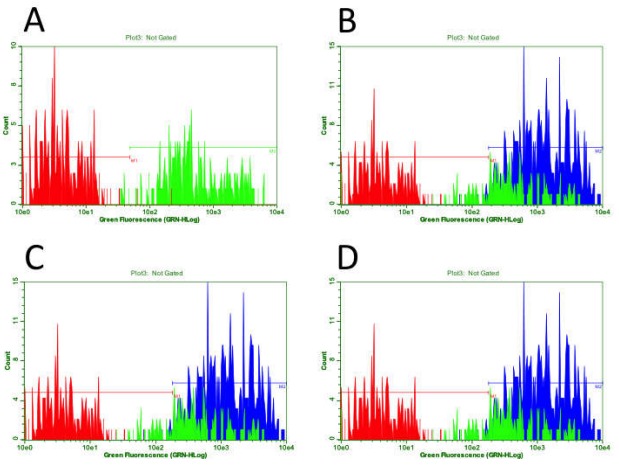

Figure 5 Intracellular retention of rhodamine 123 in AsPC-1cells.
In the presence of 0.5 µM rhodamine 123 (A), 100 µM Verapamil (B), 15 µg/mL bergamottin (C), and 15 µg/mL bergamottin in combination with 5 µg/mL gemcitabine (D). Prior to cytometry analysis cells were treated as described under Materials and Method. Histograms represent the cell counter numbers versus geo-mean fluorescent intensity (AU). In each figure, three overlaid histograms represent, from left to right, the peak from auto-fluorescence of control cells without rhodamine 123, the peak from rhodamine loaded-cells and the peak from rhodamine loaded-cells in the presence of verapamil (B), bergamottin (C), and bergamottin in combination with gemcitabine (D).

Additionof Bergamottin to Gemcitabine Increases Apoptosis Rate in Cancer Pancreatic Cells Via A Caspase-Dependent Mechanism

Based on above results, we chose 15 µg /ml of bergamottin and 5 µg /ml of gemcitabine for subsequent experiments to test the effects of this drug combination on the expression of

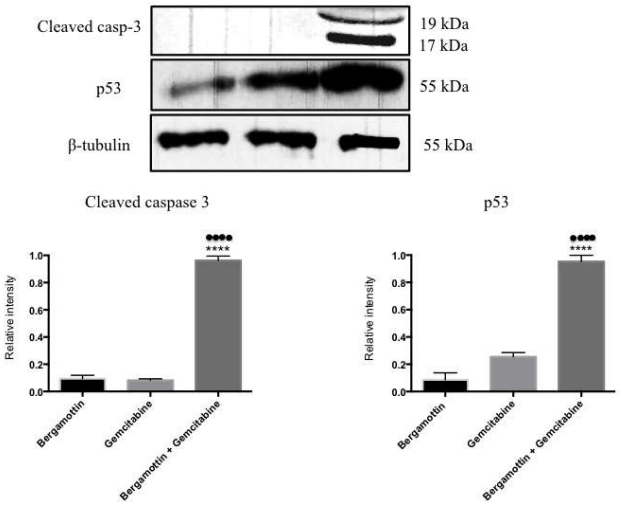

Figure 6 Effect of gemcitabine, bergamottin or their combination on the expression of p53 and cleaved caspase 3 in AsPC-1 cells.
After incubating, AsPC-1 cells treated with gemcitabine in absence or presence of bergamottin. The quantification of p53 and cleaved caspase 3expressions was analysed using Image J and represented as bar graphs. Data are represented as Mean ± S.E.M (n=3). **** represents $P < 0.0001$ and refers to the variation in p53 or cleaved caspase 3 expression between cells treated with gemcitabine and the combination of bergamottin and gemcitabine. •••• represents $P < 0.0001$ and refers to the variation in p53 or cleaved caspase 3 expression between the cells treated with bergamottin and the combination of bergamottin and gemcitabine

apoptos is regulator p53 and the apoptosis effector caspase 3 (in its cleaved form) in AsPC-1 cell line. Our results show that cleaved caspase 3 and p53 expression level are increased when the two drugs are combined ifcompared tothe cells treated with bergamottin or gemcitabine alone. These results indicate that the combination of gemcitabine and bergamottin induced apoptosis and increased the expression of cleaved caspase 3 and p53 in pancreatic cancer cells (figure 6).

DISCUSSION

Due to its aggressive nature, frequent late detection with advanced disease, and chemoresistant properties, pancreatic cancer is one of the most challenging solid organs, (Torre *et al*., 2015). In a number of cancer types, the drug efflux pumps have been associated with chemoresistance (multiple drug resistance/MDR), (O'Driscoll *et al*., 2007). However, their presence with resistance in pancreatic cancer remains to be elucidated.

Until 1997, 5-FU was used as standard treatment for pancreatic cancer. After that gemcitabine has been the drug of choice for its safety quality, (Snady *et al*, 2000) (Burris *et al*, 1997).

Gemcitabine, which has been the frontline chemotherapeutic agent against pancreatic cancer, has offered some relief over the past two decades, (Burris *et al*, 1997). But frequently, gemcitabine failed in the overall survival benefit, (Heinemann, 2001). Trials in mixed regimens including gemcitabine had variable success. National Comprehensive Cancer Network (NCCN) guidelines indicated the success improved in the gemcitabine based combination regimen, (Zhao, 2011). In addition, recent studies indicated that natural products could provide additional strategies for monotherapy or combination treatments in pancreatic cancer due to their efficacy and low toxicity, (Mohammed *et al*., 2015). Here, we examined the efficacy of combined treatments of gemcitabine and bergamottin in human pancreatic cancer cells. Bergamottin a natural furanocoumarin found in oil of bergamot, grapefruit juice and other citrus fruits. People who eat a large amount of grapefruit have show a greater bioavailability of certain drugs, these observations have been explained by the inhibition of the activity of certain cytochrome P450 enzymes, especially CYP3A4 by bergamottin and its related metabolites present in grapefruit, (Dresser *et al*, 2002). Many studies demonstrated the inhibitory action of flavonoids, but also furanocoumarins of drug rejection, including MDR1. The importance of this protein inhibition is to increase the sensibilisation of cancer cells to anti-cancer drugs and the enhancing of chemosensetization process, (Abdallah *et al*., 2015).To establish whether comparable toxicity occurs in pancreatic cancer, AsPC-1 was used and results compared with those for gemcitabine and combinationwith bergamottin, to evaluate any supra-additive effect. BJ cell line was also tested, as a no transformed cell type which are similar to host components in tumours that are important for progression. BJ cells were unaffected when treated with bergamottin up to the highest concentration used 3µg /ml. These cells are not transformed, yet similar phenotypes would be represented in in vivo cancers, and tumour vasculature has proved a popular therapeutic target. No normal pancreatic cells wereavailable; such cells are not indexed in most catalogues. MDR1 inhibiting agents are pharmacologically active in vitro in concentration range from 1 to 15 µg /ml, (Abdallah *et al*., 2015).A range of 1-30 µg /ml of bergamottin was selected for

AsPC-1 cells in this study, at minimally cytotoxic doses of the micronutrient, the combination with gemcitabine showed a strong supra-additive effects at lower doses of the anti-cancer drug.

Many studies indicate that the expression of drug efflux pumps MDR1 is common in pancreatic tumours and so potentially could contribute, at least in part, to the chemoresistant properties of this cancer,(O'Driscoll *et al*., 2007). MDR1 blockade might have a very important role in the intracellular accumulation and the cellular pharmacokinetics of many anticancer drugs. Ourresults showed that bergamottin possesses a potent inhibitory potential on MDR1 mediated efflux of rhodamine 123 when compared to verapamil and then increasing in the intracellular accumulation of gemcitabine, in the pancreatic cancer cells used, loading to more apoptosis when gemcitabine were combined with bergamottin.

An effective strategy to halt tumour growth is the induction of cell apoptosis by chemotherapeutic agents in cancer cells. The tumour suppressor p53 is a cell cycle checkpoint protein that contributes to the preservation of genetic stability by mediating apoptosis in response to DNA damage. To repair the DNA damage, p53 induce apoptosis to eliminate the damaged cells, (Yamaguchi *et al*., 2008) (Roos & Kaina, 2013).In our study, treatment with gemcitabine and bergamottin in combination increased expression of p53 and induced apoptosis in AsPC-1 cells compared to pancreatic cancer cells treated with gemcitabine only. Also, the combination of gemcitabine and bergamottin up-regulated cleaved caspase 3 expression at the protein level AsPC-1 cells compared to AsPC-1 cells treated with gemcitabine only, which contributed to the induction of apoptosis.

In conclusion, our present data indicate that the combined treatment of bergamottin and gemcitabine induces apoptosis in the established pancreatic cell line used indicating that the effects seen are potentialize and it is strongly recommended to study and find a natural and non-toxic MDR1 blockers to potentialize the efficacyof anticancer drugs.

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