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What is This?
Antitumor Activities of Rauwolfia vomitoria Extract and Potentiation of Gemcitabine Effects Against Pancreatic Cancer

Jun Yu, PhD1 and Qi Chen, PhD1

Abstract
Pancreatic cancer is one of the most lethal malignancies with very limited treatment option. In the effort of enhancing the effect of the conventional chemotherapeutic drug gemcitabine against pancreatic cancer, we investigated in vitro and in vivo the anticancer effect of a β-carboline-enriched extract from the plant Rauwolfia vomitoria (Rau), either alone or in combination with gemcitabine, in preclinical pancreatic cancer models. Rau induced apoptosis in pancreatic cancer cells in a concentration-dependent manner, and completely inhibited colony formation of PANC-1 cells in soft agar. The combination of Rau and gemcitabine had synergistic effect in inhibiting cell growth with dose reduction effect for gemcitabine. In an orthotopic pancreatic cancer mouse model, PANC-1 tumor growth was significantly suppressed by Rau treatment. Metastasis was inhibited by Rau. Adding Rau to gemcitabine treatment reduced tumor burden and metastatic potential in the gemcitabine non-responsive tumor. These data suggest that Rau possesses anti–pancreatic cancer activity and could improve effect of gemcitabine.

Keywords
pancreatic cancer, Rauwolfia vomitoria, plant extract, gemcitabine, synergy, combination therapy

Introduction
With estimates of 43,920 new cases and 37,390 deaths in the United States in 2012 (>100 deaths per day), pancreatic cancer has the highest fatality rate among cancers.1,2,3 This is likely because of disappointing treatment efficacy in addition to late detection and fulminant disease course.4,5 The 1- and 5-year overall survival rates are 26% and 6%, respectively, which remain unchanged for the recent 30 years.2 More than half of pancreatic cancer patients are diagnosed at a distant stage, for whom 5-year survival is only 2%.1 Gemcitabine as a single agent remains to be the first-line therapy of metastatic pancreatic cancer. Gemcitabine provides benefit at early stages of the disease; however, it has little impact on median overall survival for patients with locally advanced or metastatic disease, who comprise the majority of cases.6,7 Recent clinical trials adding agents to gemcitabine had statistical significance, but are not really meaningful for patients.8-14 A new gemcitabine-free regimen FOLFIRINOX combining 5-fluorouracel, leucovorin, irino-tecan, and oxaliplatin provided a 5-month survival benefit over gemcitabine.16 However, this regimen added adverse effects. Lack of effective therapeutic options, lack of adjuvant therapy, significant side effects with existing chemotherapies, and radiation therapies or their combinations remain major problems in the treatment of pancreatic cancer.

Natural products have long been proven a bountiful resource for bioactive anticancer agents. One of the advantages of natural products is their low toxicity compared with conventional chemo-drugs. Combination of natural compounds to standard chemotherapeutic drugs may exert additive or synergistic effects in killing cancer cells, which would in turn allow lower and safer doses to be used. Herbal preparation of Rauwolfia vomitoria, a tropical shrub in the family Apocynaceae, is a traditional folk medicine in Africa used to treat a variety of ailments, including hypertension,17-20 fever,21,22 general weakness,23-25(q75-76,24pp174-176)

Abbreviations
Cl, Combination Index; DRI, Dose-reduction index; Gem, Gemcitabine; H&E, Haematoxylin-eosin; i.p., intraperitoneal; MTT, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; PARP, poly-ADP ribose polymerase; PI, Propedium Iodine; Rau, Rauwolfia vomitoria extract.

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Materials and Methods

Experimental Materials, Cell Lines, and Viability Assay

Rauwolfia vomitoria extract (Rau) was provided by Natural Source International (New York, NY). Rau and gemcitabine (Sigma, St Louis, MO) were prepared in sterile water and stored at −20°C.

Human pancreatic cancer cell lines PANC-1, MIA PaCa-2, AsPC-1, HPAF-II, and BxPC-3 were obtained from the American Type Culture Collection (Manassas, VA). Immortalized human lung epithelial cells MRC-5 were provided by Dr Sittampalam at the University of Kansas Medical Center, and were used as a comparison to cancer cells. All the cells were cultured at 37°C in 5% CO2/95% air in recommended growth media containing 10% fetal bovine serum, with or without 400 µg/mL Rau.

Anchorage-independent colony formation assay in soft agar containing 0.5% agar, Dulbecco’s modified Eagle medium, and 10% fetal bovine serum, with or without 400 µg/mL Rau. The solid agar base (lower layer) contained 0.75% agar and the complete growth medium, with or without 400 µg/mL Rau. Cells were incubated for 20 days. Colonies were visualized by crystal violet staining and counted.

Apoptosis Detection by Flow Cytometry

Cells were exposed to various concentrations of Rau for 48 hours. Cells were washed in phosphate-buffered saline, resuspended in binding buffer, and subjected to fluorescein isothiocyanate conjugated (FITC)-conjugated annexin V and propidium iodide (PI) staining according to the manufacturer’s protocol (BD Biosciences, San Jose, CA). Cells were analyzed by flow cytometry. Annexin V positive and annexin V–PI double positive cells were identified as apoptotic cells, whereas PI single positive cells were identified as necrotic cells.

Western Blot

Forty micrograms of protein were loaded for sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Primary and secondary antibodies were from Cell Signaling Technology Inc (Danvers, MA): rabbit anti-poly-(ADP-ribose)-polymerase (PARP) (1:2000), rabbit anti-caspase-3 (1:1000), rabbit anti-caspase-8 (1:1000), mouse anti-β-actin (1:1000), and goat anti-rabbit or anti-mouse IgG (1:5000). Blots were developed using immobilon chemiluminescent substrate (Thermo Scientific, Waltham, MA).

Intraperitoneal Pancreatic Cancer Mouse Model

Through an intrapancreas surgical procedure, PANC-1 cells were orthotopically implanted into the pancreas of nude mice at 3 × 10^5/mouse. To allow in vivo imaging, PANC-1 cells were transfected with luciferase gene prior to inoculation. Luciferin was given intraperitoneally each time prior to imaging to luminescence the tumor cells. Ten days after tumor cells were implanted, mice were imaged and grouped to 8 mice per group based on tumor burden. Then treatment began with intraperitoneal injection of gemcitabine (Gem; 20 mg/kg, every 4 days), Rau (20 or 50 mg/kg daily), the respective combination of Gem and Rau, and saline as control. After 48 days of treatment, mice were euthanized. All tumor lesions in the peritoneal cavity were collected and weighted. Metastasis lesions were assessed and counted. Major organs such as liver, kidney, and spleen were fixed in 4% formaldehyde and subjected to histological analysis for any damage due to potential drug toxicity.

Data Analysis

MTT data were normalized to their corresponding untreated controls for each condition (drug, cell type) and were expressed as percentage viability. Dose reduction index...
Yu and Chen

(DRI) values for gemcitabine were calculated by the equation \( \text{DRI}_{\text{Gem}} = \frac{D_{\text{Gem}}}{D_{\text{Gem} + \text{Rau}}} \), where \( D_{\text{Gem}} \) is the dose of gemcitabine alone required to produce an ICx level of cytotoxicity, and the divisor \( D_{\text{Gem} + \text{Rau}} \) is the dose of gemcitabine needed to produce the same ICx level of cytotoxicity when it is combined with Rau (at a given molar ratio). DRIGem is defined with respect to gemcitabine. SPSS15.0 was used for additional statistical analysis.

Results

Effect of Rauwolfia vomitoria Extract (Rau) Against Pancreatic Cancer Cells

Human pancreatic cancer cell lines PANC-1, MIA PaCa-2, AsPC-1, HPAF-II, and BxPC-3 were compared to an immortalized non-cancerous epithelial cell line MRC-5 for sensitivity to Rau. The dose–response curves showed that cancer cells were more sensitive to Rau treatment than the normal cells MRC-5 (Figure 1A). The cancer cells had IC50 values ranging from 83 to 283 µg/mL. The noncancerous cell MRC-5 had IC50 of 553 µg/mL, more than 2-fold higher than those of the cancer cells.

Colony formation in soft agar was used to assess the survival of tumorigenic cancer cells in a longer time, which has been positively correlated to in vivo tumorigenicity of the cancer cells in animal models.42,43 The PANC-1 cancer cells formed colonies at a rate of 12% when untreated. Rau at 400 µg/mL completely inhibited formation of colonies of PANC-1 cells in soft agar (Figure 1B), indicating no survival of tumorigenic cancer cells with this treatment.

To assess Rau-induced death pathway, annexin V/PI staining was performed to detect apoptosis versus necrosis in PANC-1 cells treated with Rau. Data from flow cytometry demonstrated a predominant apoptotic cell death was induced by Rau treatment. With 0, 50, 100, 200, and 400 µg/mL Rau treatment, the percentage of apoptotic cells were 9.5%, 25.9%, 73%, 80.5%, and 94%, respectively (Figure 2A). The induction of apoptosis was clearly dependent on the concentration of Rau and was the major form of cell death induced by Rau. Necrosis (cells that were PI positive only) contributed only 4% to 19% of total cell death across all the different Rau concentrations. Consistent with this finding, Western blot analysis detected extensive cleavage of caspase-8, caspase-3, and PARP in Rau-treated PANC-1 cells in a dose- and time-dependent manner (Figure 2B).

Synergistic Effect of Rau in Combination With Gemcitabine Against Pancreatic Cancer Cells

After determining the dose–response relationships for Rau (Figure 1A), the dose–response relationships for Gem cytotoxicity were established in PANC-1, MIA PaCa-2, AsPC-1, HPAF-II, and BxPC-3 cells (Figure 3A, dotted lines). These cells demonstrated a spectrum of resistance to Gem. Chou–Talalay’s constant ratio design44 was used to systematically examine combination dose–response relationships between Gem and Rau. The molar ratio of Gem:Rau was chosen as \( \frac{IC_{50\text{Gem}}}{IC_{50\text{Rau}}} \). Combination data were presented in terms of Gem concentration. Despite the different inherent sensitivity of these cell lines to Gem, the results unambiguously showed an enhanced cytotoxicity with the curves.
in Gem + Rau combinations for all cell lines compared with the corresponding curves with Gem alone (Figure 3A).

To quantitatively assess whether gemcitabine effect was enhanced by pairing with Rau, the DRI for Gem was calculated. The DRI indicates decrease in Gem concentration when combined with Rau than that of gemcitabine alone to achieve the same cytotoxic effect. As shown in Figure 3B, DRI was >1 for all cell lines, and across the aimed cytotoxic effect of 60% to 90% (fraction affected, or fa). Depending on cell lines and the aimed level of effect, DRI can reach 100 to 10 000 (Figure 3B). These data unequivocally support the conclusion that the concentration of Gem can be decreased to produce an equitoxic effect on pancreatic cancer cells when Rau is combined.

**In Vivo Tumor Inhibitory Effect of Rau Either Alone or in Combination With Gemcitabine**

An orthotopic pancreatic cancer mouse model was used to evaluate the effect of Rau and gemcitabine plus Rau (Gem + Rau) treatment. Compared with the subcutaneous model, this model better mimics clinical condition of human pancreatic cancer, because the local environment for pancreatic cancer development was represented.

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**Figure 2.** Apoptosis in PANC-1 cells induced by *Rauwolfia vomitoria* (Rau). (A) Flow cytometry detection of apoptotic cells. PANC-1 cells were treated with Rau at indicated concentrations. At 48 hours of treatment cells were subjected to fluorescein isothiocyanate conjugate (FITC)-affiliated annexin V and propidium iodine (PI) double staining and flow cytometry. Cells in Q2 and Q4 were identified as apoptotic cells, and cells in Q1 (PI positive only) were identified as necrotic cells. Percentage of apoptotic cells in each treatment was quantified and shown in the bar graph. (B) Cleavage of caspase-8, caspase-3, and poly-ADP ribose polymerase (PARP) in PANC-1 cells treated with Rau. Cells were treated with Rau at different concentrations (left panel) and for different time (right panel) as indicated. The cleavage of caspase-8, caspase-3, and PARP were detected by Western blots.
Ten days after PANC-1 cells were inoculated; treatment was carried out as described in the Materials and Methods section. Longitudinal tumor progress was monitored through live animal images. Representative images are shown in Figure 4A. As the tumors did not respond to gemcitabine, Rau at either 20 mg/kg or 50 mg/kg provided observable inhibition in tumor growth. The combination of Gem + Rau at both doses also inhibited tumor progression. When tumor burden was quantified by photon flux, the results were consistent with the observation (Figure 4B). Rau treatment groups at both doses had less tumor burden than the control group and gemcitabine-treated group at the end of treatment. The combination groups showed less tumor burden than the control and gemcitabine-treated groups; however, these were not different from the Rau treatment alone groups. Notably, there were 2 mice in the Gem + Rau50 group that had complete tumor regression, an effect that had not been seen in any other treatment groups.

Necropsy confirmed the imaging results at the end of the treatment (Figure 5). Gemcitabine at the used dose did not provide any inhibition in tumor weight. Rau alone decreased tumor weight by 53% at the daily dose of 20 mg/kg, and 46% at the daily dose of 50 mg/kg, compared with saline-treated control. By combining Rau with gemcitabine, the decreases in tumor weight were 56% at both Rau doses. The improvement in tumor inhibition provided by the combination was significant compared with gemcitabine alone; however, it was not significant compared with Rau treatment alone (Figure 5A).

On assessing tumor metastasis, 12% mice in the control group (saline-treated) and in the Rau 20 mg/kg group did not form metastasis, whereas all mice in gemcitabine group formed metastasis. The percentage of metastasis free mice increased to 40% with both Rau 50 mg/kg treatment and Gem + Rau, suggesting that Rau provided benefit in reducing metastatic potential while gemcitabine did not (Figure 5B). Number of metastasis lesions was also decreased with Rau 50 mg/kg treatment, or the combination treatments of gemcitabine and Rau (Figure 5C). As gemcitabine did not provide any benefit in inhibiting metastasis, Rau or Gem + Rau combinations significantly improved the effect versus gemcitabine alone.

Proteins were extracted from tumor samples of the treated and control mice. Western blot analysis showed cleavage of caspase-3 and PARP in Rau and Gem + Rau treatment groups at all the doses used (Figure 5D). These results confirmed the *in vitro* data that Rau induced apoptosis in tumor cells.

None of the mice demonstrated observable toxicity associated with the treatments. At the end of the experiments, major organs (kidney, liver, and spleen) were subjected to hematoxylin and eosin staining and pathological analysis. No tissue damage was detected in the treatment groups, and there were no significant differences between the control group and treated groups (Figure 5E). These data demonstrated that Rau at the doses used, either alone or combined with gemcitabine, was of low toxicity.
Discussion

Gemcitabine as the first line of pancreatic cancer care failed to provide impact in the median survival for patients with locally advanced or metastatic pancreatic cancer.\textsuperscript{14,45,46} Our study had consistent observation that gemcitabine at a commonly used dose in mice did not produce inhibition in tumor burden or metastasis in our animal model. Quite in contrast, the extract of \textit{R vomitoria} (Rau) exhibited significant inhibition in PANC-1 tumors, and ended with >50\% tumor inhibition and reduced chance of metastasis and numbers of metastasis lesion formed. Consistent with the \textit{in vitro} dose reduction effect for gemcitabine, the combination of Rau and gemcitabine had better effect than gemcitabine \textit{in vivo}. However, the combination did not make a real difference than Rau-alone treatment \textit{in vivo}, although Gem + Rau20 showed a tendency of less metastasis than Rau20 alone. This is likely because the tumors did not respond to gemcitabine treatment in the \textit{in vivo} experiment. The gemcitabine nonresponsive tumor still responded to Rau treatment. These results greatly raise the potential for using \textit{R vomitoria} in
Yu and Chen

Pancreatic cancer treatment, alone or in addition to gemcitabine. Moreover, our data showed that Rau had relatively low toxicity toward normal cells. The low toxicity was evident in mice treated with Rau where major organ toxicities were absent. Although pathological changes were also absent in gemcitabine treated mice at our used dose, gemcitabine did not show any efficacy. By the dose reduction effect Rau exhibited, Rau could allow lower doses of gemcitabine while achieving an equivalent efficacy than seen with higher gemcitabine doses alone. This may allow decrease of toxicity associated with chemotherapy.

While potential benefit was suggested by our study, mechanism(s) of Rau-induced anticancer effect warrant further study. It has been reported that Rau induced DNA damage and cell cycle inhibition of prostate cancer cells.31

**Figure 5.** Inhibition of tumor weight and metastasis by *Rauwolfia vomitoria* (Rau) and the combination of Rau and gemcitabine in vivo. Mice bearing orthotopic PANC-1 pancreatic tumors were treated for 48 days. Tumor weight and metastasis were assessed at the end of treatment. (A) Tumor weight shown as mean ± standard error of the mean (mean ± SEM). (B) Percentages of mice that did not form metastasis. (C) Number of metastatic lesions in each mouse that had metastasis. The black bars indicate the median number of metastasis in each group. (D) Cleavage of capase-3 and poly-ADP ribose polymerase (PARP) in tumor samples from different treatment groups. (E) Hematoxylin and eosin staining of major organs from different treatment group (400× magnitudes). Kidney, liver, and spleen were collected from each treatment group and fixed in 4% formaldehyde, and later subjected to histological analysis. *P < .05 relative to control group; †P < .05 relative to gemcitabine (Gem)-treated group.
Activation of these pathways could lead to apoptosis, which is a powerful tumor-suppressive pathway preventing the uncontrolled proliferation of cancerous cells and potentially depleting stem-like and progenitor cancer cell pools.\textsuperscript{47,48} Our study showed that Rau mainly induced apoptosis both \textit{in vitro} and \textit{in vivo} as shown by flow cytometry and the cleavage of caspase-8, caspase-3, and PARP. Caspase-8 is generally regarded as the initiator caspase for the extrinsic apoptotic pathway. Once it was activated by Rau treatment, the downstream cascade of execution caspses were activated, such as caspases-3. Caspase-3 in turn cleaved cytoskeletal and nuclear proteins such as PARP. PARP plays a critical role in the maintenance of DNA integrity and its expression has been associated with overall prognosis in cancer.\textsuperscript{49} Our data suggest that by activating this apoptotic pathway, Rau completely inhibited the \textit{in vitro} tumorigenic capacity of pancreatic cancer cells in soft agar. However, as this plant preparation contains a complex mixture of natural compounds, there is potential to affect multiple molecular targets and pathways that lead to cell death.

The β-carboline-enriched \textit{R} \textit{vomitoria} extract could contain compounds that possess potent anticancer activity. The data presented herein are part of the initial step in identifying the anticancer activity of Rau. Active components could be isolated and developed for optimizing efficacy, toxicity, and other profiles that could lead to anticancer drug development.

**Conclusion**

Taken together, our data demonstrate anti–pancreatic cancer activity of \textit{R} \textit{vomitoria} extract \textit{in vitro} and \textit{in vivo}. Continued investigation is needed for the use of this plant extract in the treatment of pancreatic cancer.

**Declaration of Conflicting Interests**

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**References**

17. La Barre J. Hypotensive effects of the completely derserpinised extract of \textit{Rauwolfia vomitoria}. \textit{Arzneimittelforschung}. 1973;23:600-605.