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Original Article

Chinese herb derived-Rocaglamide A is a potent inhibitor of pancreatic cancer cells

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Abstract: Pancreatic cancer ranks No.1 in mortality rate worldwide. This study aims to identify the novel anti-pancreatic cancer drugs. Human pancreatic carcinoma cell lines were purchased from ATCC. CPE-based screening assay was used to examine the cell viability. Patient derived tumor xenografts in SCID mice was established. The Caspase-3 and 7 activities were measured using the Caspase Glo 3/7 Assay kit. Soft agar colony formation assay was used to evaluate the colony formation. Wound healing assay was employed to determine the cell migration. We screened a Chinese herbal product library and found three "hits" that kill cancer cells at nanomolar to micromolar concentrations. One of these compounds, rocaglamide, was found to be potent inhibitors of a wide spectrum of pancreatic cancer cell lines. Furthermore, Rocaglamide reduced the tumor size in a patient-derived pancreatic cancer xenograft mouse model without noticeable toxicity *in vivo*. Rocaglamide also inhibits pancreatic cancer cell migration and invasion. In conclusion, these data support that Rocaglamide may be a promising anti-pancreatic cancer drug.

Keywords: Pancreatic cancer, high throughput screening, Rocaglamide, cell death

Introduction

Pancreatic cancer ranks 1st in death rate worldwide with the 5-year survival rate remains less than 5% [1]. Patients with pancreatic cancer often have an extremely poor prognosis, because most diagnoses are made at a very late stage. Additionally, currently approved drugs fail to significantly expand the lifespan of pancreatic cancer patients. As such, there is a major unmet medical need to identify and develop novel anti-Pancreatic cancer drugs.

Traditional Chinese herbs are rich sources for natural compounds that may potentially be used in chemotherapy. For example, herbal extracts from traditional Chinese medicine (TCM) reportedly reduced chemotherapy-induced side effects *in vivo* [2-5]. The notion that combination of Chinese and Western medicine may offer new treatments for pancreatic cancers arises from those studies where introduction of Chinese medicine into chemotherapy successfully improved life expectancy of patients with liver, lung, colorectal cancers, and osteosarcoma [6]. It is foreseeable that TCM-derived compounds may greatly expand the repertoire of anti-pancreatic cancer drugs.

In this study, we attempted to establish an assay that can be adapted for high-throughput screen of natural compounds that kill pancreatic cancer cells. Toward this goal, we assembled a library of compounds derived from Chinese herbs. We further characterized one of the identified "hits", Rocaglamide, which inhibits pancreatic cancer cells both *in vitro* and *in vivo*. Our results demonstrated that Rocaglamide holds great promise for further investigation as a novel anti-pancreatic cancer compound.

Materials and methods

Cells and reagents

Human pancreatic carcinoma cell line PANC-1 (CRL-1469), AsPC-1 (CRL-1682), BxPC-3 (CRL-1687), HPAF-II (CRL-1997), Capan-2 (HTB-80),

Hs-766T (HTB-134), and MiaPACA-2 (CRL-1420) were purchased from ATCC (American type culture collection) and maintained as instructed by the manufacturer. The natural product library containing 238 compounds were assembled at Central South University. Isolation of pancreatic islets cells were performed under a protocol that is detailed elsewhere [7], using resected pancreas from patients seen at the Department of General Surgery, Xiangya Hospital, Central South University. All patients gave written informed consent, and the tissue donation protocol was approved by Central South University Institutional Review Board.

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CPE-based screening assay

PANC-1 cells were seeded at 2×10^4 cells/well in a 96 well format 24 hours before the experiment. After cells had reached 80% confluency they were treated with compounds at the concentration of 5 μM for 48 hours. The cell viability was measure by Cell Titer GLO kit according to manufacturer's instruction with a GloMax 96 microplate luminometer (Promega). % Cell Death = $100\text{-}RLU_{inhibitor}/RLU_{DMSO}*100$ where $RLU_{inhibitor}$ represents the luciferase counts obtained from inhibitor treated wells and RLU_{DMSO} represents luciferase counts obtained from treated with DMSO. The Z-factor, which reflects the assay robustness and reproducibility, was calculated according to the published method [8]. The Z values are calculated from triplicates from within the same experiment and our results showed a Z-factor of 0.8±0.1 between experiments. An assay with a Z-factor between 0.5 and 1.0 is considered highly consistent and reproducible [8].

Patient derived tumor xenografts in SCID mice

SCID mice were purchased from HFK Bioscience Ltd (Beijing, China). Animal experiments were performed in accordance with national regulations, and research protocols were approved by Central South University IACUC committee. Patient-derived pancreatic tumor cells (5×10^6), suspended in 100 μl mix (equal volumes of DMEM and Matrigel), were implanted subcutaneously into the right flank of 10 female SCID mice (5-week-old) and then randomly divided into two equal groups, one of which received an intraperitoneal injection of rocaglamide (1.5 mg/kg; n=5) and the other, used as a vehicle control, received olive oil alone (n=5). These treatments were performed once daily for 52 days and the tumor volumes of the animals were measured once every four days. The tumor volumes (mm^3) were calculated using the following formula: Tumor volume = $LS^2/2$, where L is the longest diameter and S is the shortest. Mice used in the study were monitored every other day for signs of discomfort such as loss of appetite and slow in movement. No unexpected death was observed throughout the study. No anesthesia was given. Mice were euthanized by CO_2 and cervical dislocation when their tumors reached the maximum of 2500 mm^3. The survival time of these mice in each group (RocA-treated group, n = 5; Vehicle control group, n = 5) was recorded. To observe the potential effect of Roc-A treatment on growth, SCID mice were treated with Roc-A (1.5 mg/kg; n=3) or olive oil daily for 24 days, body weight was measured once every four days.

Animal experiments were performed in accordance with national regulations, and research protocols were approved by Central South University IACUC committee.

Caspase 3/7 assays

PANC-1 cells or primary human pancreatic islet cells (2×10^5/well) were seeded in a 48-well plate. Cells were treated with Roc-A at indicated concentrations or DMSO for 24 hours. The Caspase-3 and 7 activities were measured using the Caspase Glo 3/7 Assay kit (Promega) following the manufacturer's instruction.

Soft agar colony formation assay

PANC-1 cells (4000 cells/well) were suspended in RPMI 1640 containing 0.2% agar and then placed into a 6-well culture plate containing a 0.5% hard agar base. The cultures were incubated at 37°C and replenished with 500 of complete growth medium every other day. The plates were incubated for 10-14 days. Colonies (≥50 cells/colony) were then fixed with 70% ethanol, stained with crystal violet solution, and counted.

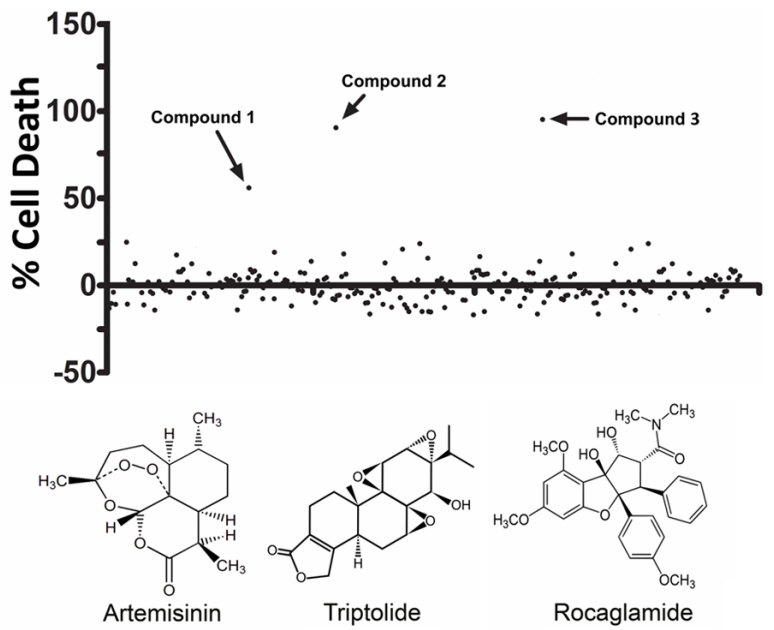

Figure 1. Identification of anti-pancreatic cancer Chinese herbal compounds. The initial screen of the 238 natural compounds yielded 3 positive hits (over 50% inhibition of PANC-1 cell viability). The concentration of each compound was 5 mM. The three compounds that were found to inhibit are indicated with arrows.

Wound healing assay

1×10^6 PANC-1 cells were cultured to confluency in a 6-well plate. The wound was created by scratching three separate wounds through the cells by moving a 200 μl pipet tip. Phase-contrast pictures were taken at 0 or 48 hours after scratching with a Nikon TS100 microscope equipped with a digital camera.

Statistics

Data presented in this study were described as means ± SD of at least three independent experiments. The mean values were compared using Student's *t*-test for significant variation between treatment and control groups. *P*-values less than 0.05 were considered statistically significant.

Results and dcussion

In order to identify novel anti-pancreatic cancer compounds, we assembled a library containing 238 traditional Chinese medicinal compounds. We treated human pancreatic cancer cell line PANC-1 with each compound for 48 hours at the concentration of 5 μM. The cell viability was measured by a commercial luciferase assay that monitors cellular ATP levels, which positively correlate with cellular viability. The Z factor for the assay was 0.8±0.1. Initial screen of the 238 traditional Chinese medicinal compounds identified Artemisinin (Compound 1), Triptolide (Compound 2), and Rocaglamide (Roc-A) (Compound 3), as potent inhibitors by more than 50% (**Figure 1**). To validate the screening results, we treated PANC-1 cells with increasing concentrations of the three compounds and determined the 50% tissue culture cytotoxicity (CC_{50}) of each inhibitor. Rocaglamide displayed a CC_{50} of 80 nM, being the most potent one (**Figure 2**).

Roc-A was first discovered in 1982 by King *et al*. from *Aglaia elliptifolia*. Since then more than 100 naturally occurring derivatives of rocaglamide have been isolated and characterized from over 30 *Aglaia* species [9]. Roc-A contains a flavonoid unit and a cinnamic acid amide moiety [10]. Interestingly, Roc-A was first shown to be an immunosuppressant by inhibiting NF-kB activity [11]. Others then reported activities including insecticidal [12], anti-fungal [13], anti-tumor [14-19], cardioprotective [20] and neuroprotective effects [21].

To determine whether Rocaglamide effectively kills other pancreatic cancer cells, we determined its CC_{50} on primary pancreatic island cells and the pancreatic cancer cell lines (**Table 1**). Impressively, the CC_{50} on isolated primary pancreatic island cells was around 20 μM, whereas the CC_{50} on other pancreatic cancer cell lines range from 50 nM to 200 nM, which yields a therapeutic index window over at least 100. This result indicates that Roc-A selectively kills pancreatic tumor cells than healthy pancreatic cells.

To further evaluate the *in vivo* efficacy of Roc-A, we established a patient derived tumor xenografts (PDTX) model. SCID mice were subcuta-

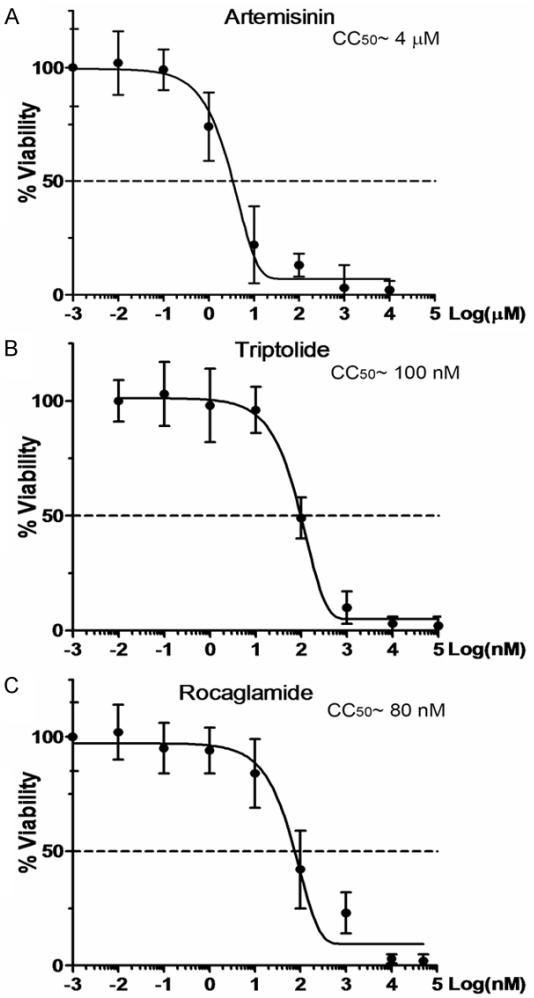

Figure 2. Cytopathic profiles of three "hits" on PANC-1 cells. (A) Artemisinin (Compound 1), (B) Triptolide (Compound 2), and (C) Rocaglamide (Roc-A) (Compound 3).

Table 1. Cytotoxicity of Roc-A on a Panel of Pancreatic Cell Lines

Cell Line	CC_{50} (nM)	CC_{90} (nM)
AsPC-1	60	75
BxPC-3	100	140
HPAF-II	70	80
PANC-1	80	100
Capan-2	120	160
Hs-766T	90	110
MiaPACA-2	130	160
Primary pancreatic islet cells	20000	30000

neously injected pancreatic tumor cells from a patient and then treated with Roc-A or vehicle, which was administered intraperitoneally once per day. Without the xenograft, SCID mice tolerated Roc-A treatment at 1.5 mg/kg well, will all treated animals survived during the course of study without showing any noticeable signs of discomfort or loss of body weight (**Figure 3A**). Notably, patient-derived pancreatic tumor cells grew significantly slower in Roc-A treated animals as shown by reduced tumor volumes and the extended death curve (**Figure 3B**, **3C**). All together, these data unambiguously demonstrated that Roc-A potently inhibited the growth of pancreatic tumor *in vivo* and is likely to display a favorable pharmacokinetics profile. Future study will be designed to investigate along this line.

In order to understand how Roc-A induced cell death of pancreatic tumor cells, we measured the Caspase activation by Roc-A and found that Roc-A at 80 nM significantly induced the activation of Caspase 3 and 7 in PANC-1 cells (**Figure 4A**, **4B**). By contrast, such dose did not induce Caspase 3 and 7 activation in primary human pancreatic islets cells (**Figure 4C**, **4D**). These results suggest that Roc-A induced apoptosis by activating the Caspase 3 and 7 pathways.

Lastly, we sought to evaluate the effect of Roc-A on tumor migration and invasion. To this end, we performed soft agar colony formation assay and found Roc-A drastically reduced the ability of colony formation by more than 100-fold (**Figure 5A**, **5B**). Subsequent results from the wound healing assay indicate that Roc-A treatment also decreased the ability for pancreatic cancer cells to migrate (**Figure 5C**).

Several studies have investigated the potential anti-tumor activity by Roc-A and its derivatives through identifying its cellular targets. To date, the known molecular targets of Roc-A include Prohibitins (PHBs) [18], a highly conserved protein family found on the inner mitochondrial membrane; and the RNA helicase eIF4A [22], a component of the eukaryotic translation initiation complex; and most recently transcription factor HSF1 [23]. Four mechanisms potentially account for Roc-A mediated anti-cancer activities: (1) inhibition of translation initiation by inhibiting phosphorylation of the mRNA cap-binding translation initiation factor eIF4E and by destabilizing the RNA-binding of the translation initiation factor eIF4A in the eIF4F complex; (2) block of cell cycle progression by activating the ATM/ATR-Chk1/Chk2 checkpoint pathway;

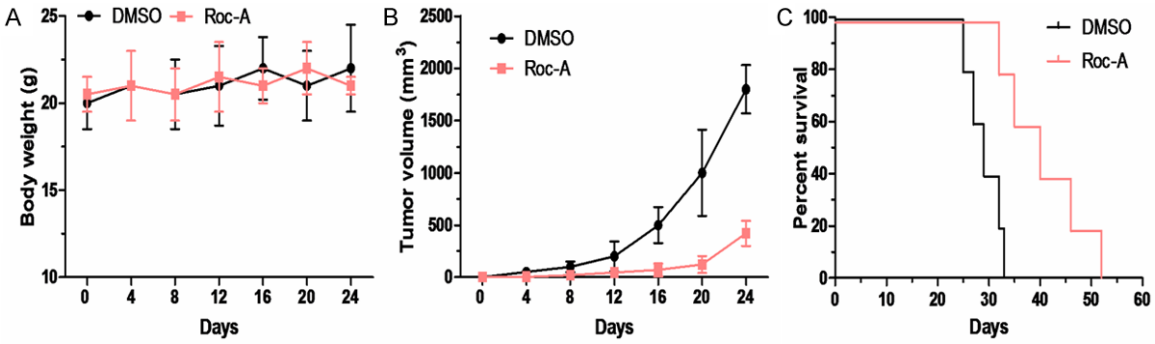

Figure 3. Rocaglamide inhibits pancreatic tumor growth in mouse xenograft models. (A) SCID mice (n=3) were treated with Roc-A (1.5 mg/kg) or olive oil daily for 24 days, body weight was measured once every four days. (B) Patient-derived tumor cells were implanted subcutaneously into SCID mice and treated with either Roc-A or vehicle for the indicated time period. Tumor volume was measured as detailed in "Materials and Methods". (C) Same as in (B), except survival rate was recorded.

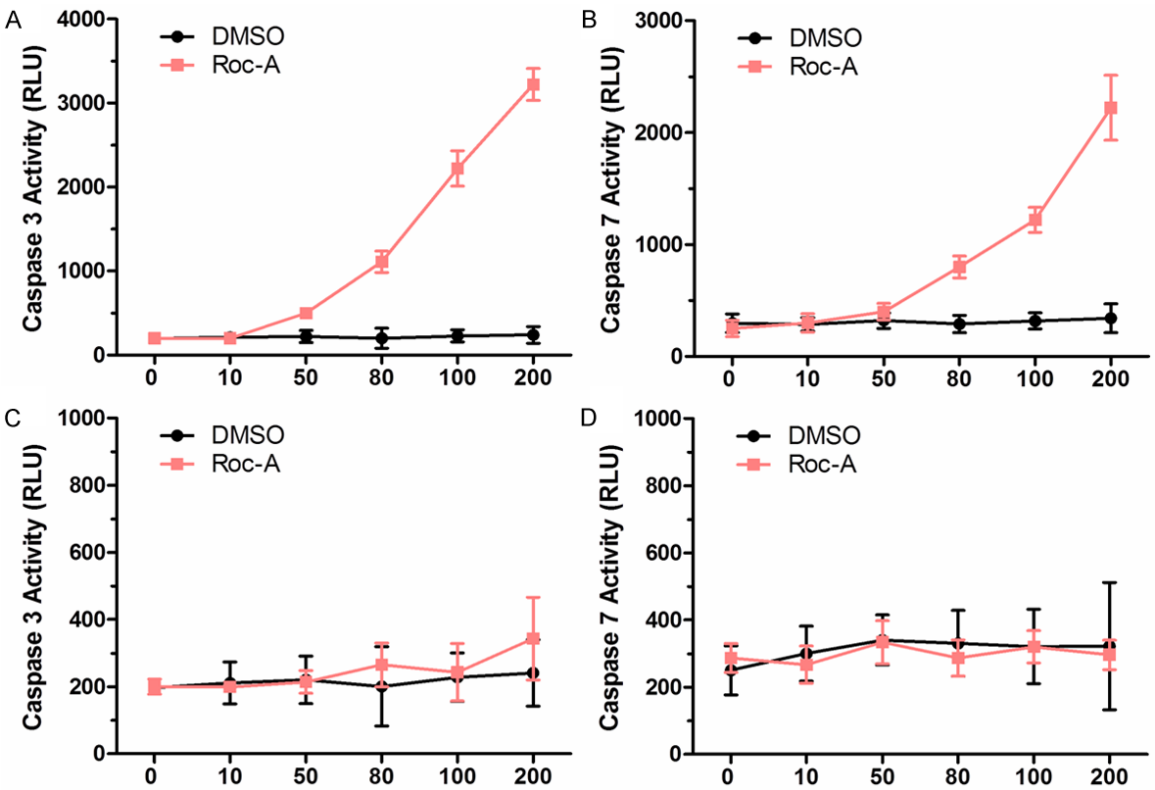

Figure 4. Rocaglamide treated pancreatic cancer cell lines showed enhanced Caspase 3 and 7 activity. (A, B) Panc-1 cells were treated with DMSO or Roc-A (80 nM) for 24 hours following by Caspase Glo 3/7 assay. (C, D) Same as in (A, B) except that primary human pancreatic islet cells were used.

(3) inactivation of HSF1, resulting in up-regulation of thioredoxin-interacting protein and reduction of glucose uptake [24]; (4) induction of apoptosis through activating the MAPK p38 and JNK pathways while inhibiting the Ras-CRaf-MEK-ERK signaling pathway [16]. Besides the anti-cancer activities, Roc-A has also been shown to protect primary cells from chemotherapy-induced cell death [17]. Interestingly, Lee et al. reported that PHB is expressed in pancreatic beta-cells and protects against oxidative and proapoptotic effects of ethanol [25]. Luan

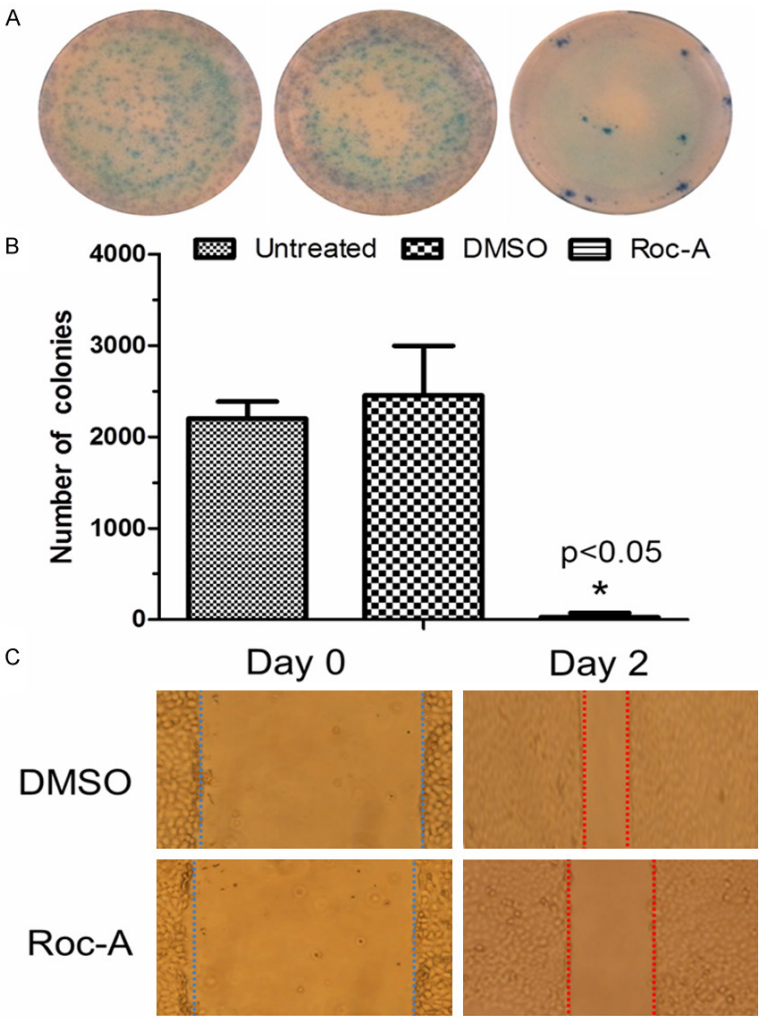

Figure 5. Rocaglamide inhibits pancreatic cancer cell migration and invasion. A. Panc-1 cells were treated with DMSO or Roc-A (80 nM) and subjected to soft agar colony formation assay as described in "Materials and Methods". Pictures of stained colonies were shown. B. Quantitative results from A were plotted in bar graph. Data are presented as means ± SD, n=3. C. Represented images of wound healing assay on Panc-1 cells treated with DMSO or Roc-A (40 nM).

et al., recently showed that PHB expression levels positively correlate with the maintenance of ERK-driven pancreatic tumorigenesis [26]. The study also reported that Roc-A treatment resulted in a significant increase of the lifespan of tumor-bearing mice without any detectable toxicity [26]. While we cannot confirm that Roc-A is inducing death to pancreatic tumor cells in this study, it is clear that Roc-A treatment induced massive Caspase activation. Furthermore, we did *in vivo* study using a PDTX model, which shows that Roc-A treatment was able to reduce tumor volume and increase survival rate of the animals. This observation is significant because it clearly shows the promise of Roc-A in chemotherapy. Although we have not done pharmacokinetics and toxicology studies on Roc-A, its *in vivo* anti-pancreatic tumor efficacy suggests that it is likely to be tolerated well and stable enough to deliver the anti-tumor effect. Ongoing experiments are characterizing all its drug-like properties.

In summary, we have identified three natural compounds that that induce death of pancreatic tumor cells through a robust screen. Characterization of Roc-A reveals potent anti-tumor activity both *in vitro* and *in vivo*. While perhaps there is still a long way from turning Roc-A into a drug, our findings strongly suggest that natural compounds like Roc-A may add more weapons to the future arsenal of chemotherapy.

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Disclosure of conflict of interest

None.

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