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Pancreatic anticancer activity of a novel geranylgeranylated coumarin derivative

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ABSTRACT

A series of hydroxycoumarin derivatives has been synthesized and evaluated against human pancreatic PANC-1 cancer cells under nutrient-deprived conditions. Several compounds exhibited 100% preferential cytotoxicity at low micromolar concentrations under nutrition starvation, and showed no cytotoxicity under nutrient-rich conditions. In this study, a novel geranylgeranylated ether coumarin derivative **9** was found to exhibit the highest cytotoxic activity of 6.25 μM within 24 h. The preferential anti-tumor activity exhibited by compound **9** against PANC-1 under low oxygen and nutrient environment illustrates its great potential as a promising lead structure for the development of novel agents to combat pancreatic cancer.

Pancreatic cancer is one of the most devastating human cancers and is the fourth leading cause of overall cancer-related mortality in the United States.[1,2] Currently, there is no clinically effective therapy for pancreatic cancer. The only chance for complete recovery is surgical resection; however, only 15–20% of patients have a resectable tumor, and of those only 20% survive up to five years after surgery.[1] Pancreatic cancer metastasizes early and extensively, and invades surrounding tissues aggressively.[1] In addition to its rapid progression, nearly all conventional chemotherapies and radiation treatments are ineffective against pancreatic cancer.[2] Even when diagnosed early, patients with pancreatic cancer have <1% chance of a complete recovery.[2] The high resistance of pancreatic cancer to available conventional chemotherapies along with its aggressive nature highlights the urgent need to develop novel effective adjunct therapies to combat this devastating cancer.

Pancreatic cancer cells are known to be remarkably tolerant to nutrient and oxygen deprivation under hypovascular conditions.[3] Hypoxic and nutrient-deprived pancreatic cells which have adapted to survive under these conditions in tumors, both elude conventional anticancer therapies and also drive forward disease progression. Therefore, agents that eliminate the ability of cancer cells to survive under nutrient starvation conditions were considered a novel modality in anticancer drug discovery.[4–8] In 2006, a novel coumarin-based natural product, angelmarin, was isolated from the root of the Japanese medicinal plant, *Angelica pubescens*.[9] Angelmarin was found to exhibit cytotoxicity of 0.01 μg/mL against the pancreatic cancer cell line PANC-1 under nutrient starvation conditions within 24 h. Given the potential of angelmarin in pancreatic cancer chemotherapy, our laboratory initiated efforts towards the total synthesis of this natural product and structure–activity relationship (SAR) studies to discover novel compounds that could possess superior pancreatic anticancer activities.

Angelmarin is a coumarin-based natural product with a molecular formula of $C_{23}H_{20}O_6$ and a chemical name of 11-*O*-(*p*-hydroxycinnamoyl) columbianetin. Coumarins are ubiquitous structures present in a large number of natural compounds comprising a broad range of powerful physiological activities including anticancer, anti-viral, antifungal, anti-inflammatory, antioxidant, anticoagulant, antibacterial, antitubercular, and analgesic activities.[10] Because of their diverse pharmacological properties, coumarins have attracted intense interest in recent years. Among these properties, the cytotoxic activities of coumarins have been the most extensively studied and coumarin compounds have served as valuable leads for further design and synthesis of more active anticancer analogues. Among the wide variety of naturally occurring active coumarin-based compounds, simple hydroxycoumarin derivatives have been shown to possess desirable pharmacological activities. For example, several prenyl and prenyloxycoumarins isolated from plants have been shown to exhibit promising antitumor, anti-inflammatory and anti-viral effects.[11]

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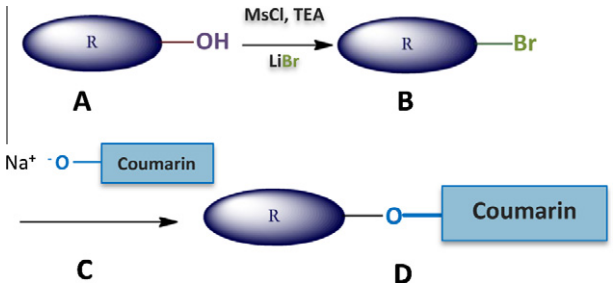


Scheme 1. General synthetic scheme for reaction of commercially available alcohols **A** or bromides **B** and respective hydroxycoumarins **C** to generate corresponding coumarin ethers **D**.

Even though the application of coumarin derivatives in anticancer therapy constitutes an exploitable source of new anticancer agents, the details of the relationship between the structure and biological activity of these coumarins remains to be fully understood. Based on our ongoing work towards the total asymmetric synthesis of angelmarin and analogues, we began to conduct structure–activity relationship studies of hydroxycoumarin based-compounds and evaluate their in vitro cytotoxic activity against PANC-1 cells under nutrient-deprived conditions. The studies presented herein represent the results of the first preliminary SAR study of simple hydroxycoumarin compounds as potential anti-austerity agents.

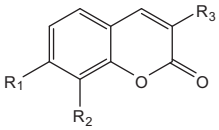
In the present study, several substituted hydroxycoumarin derivatives were synthesized, where various alkyl and prenyl groups were linked to the benzopyrone ring of three hydroxycoumarin scaffolds through the phenoxy group via an ether bond in order to investigate their cytotoxic activity as novel anti-austerity agents. To our delight, several compounds exhibited promising in vitro preferential cytotoxicity against human pancreatic cancer cell line, PANC-1 at low micromolar concentration within 24 h.

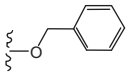
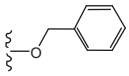
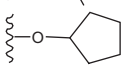
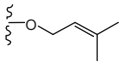
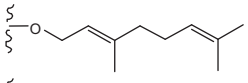
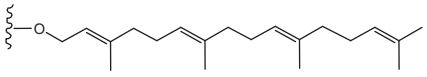
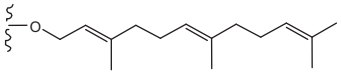
In order to generate the desired coumarin ethers **D** via Williamson ether synthesis, treatment of the respective hydroxycoumarin with sodium hydride followed by reaction with suitably activated prenyl or alkyl electrophiles **B** was envisioned (Scheme 1).

Since not all desired bromides were commercially available, preparation of the bromides **B** from commercially available alcohols **A** was accomplished by following a procedure previously published by Corey and coworkers.[12] To this end, geranylgeraniol was converted to the corresponding mesylate and was subsequently treated with lithium bromide to afford the desired geranylgeranyl bromide. Alkylation of the hydroxycoumarin **1** with geranylgeranyl bromide successfully afforded compound **9** (Table 1).[13] Employing this procedure, several bromides were then reacted with the appropriate coumarin scaffold, which allowed for the synthesis of a total of eight compounds (**3**–**10**), which are architecturally diverse in terms of type of aliphatic or aromatic ether chain, isoprenyl chain length, type of hydroxycoumarin scaffold, and ether chain saturation. Compound **2** was successfully prepared by treating **1** in a basic medium with aqueous KI_3.[14] All compounds were purified by flash column chromatography or preparative thin layer chromatography and were thoroughly characterized by ^{1}H- and ^{13}C NMR spectroscopy and High Resolution Mass Spectrometry.

The compounds shown in Table 1 were tested for their in vitro preferential cytotoxicity against human pancreatic cancer cell line, PANC-1.[15] The simple coumarin scaffolds **1** and **2** showed no appreciable cytotoxicity even at 200 μM, and compounds **3** and **4** also did not exhibit any cytotoxicity, demonstrating that the presence of a benzyl group or an iodo substituent appear not to be

Table 1
Hydroxycoumarin derivatives **3**–**10** synthesized via Scheme 1



Compound	R_1	R_2	R_3
1	OH	H	H
2	OH	I	H
3		H	H
4		I	H
5		H	H
6		H	H
7		H	H
8		H	H
9		H	H
10	H	H	

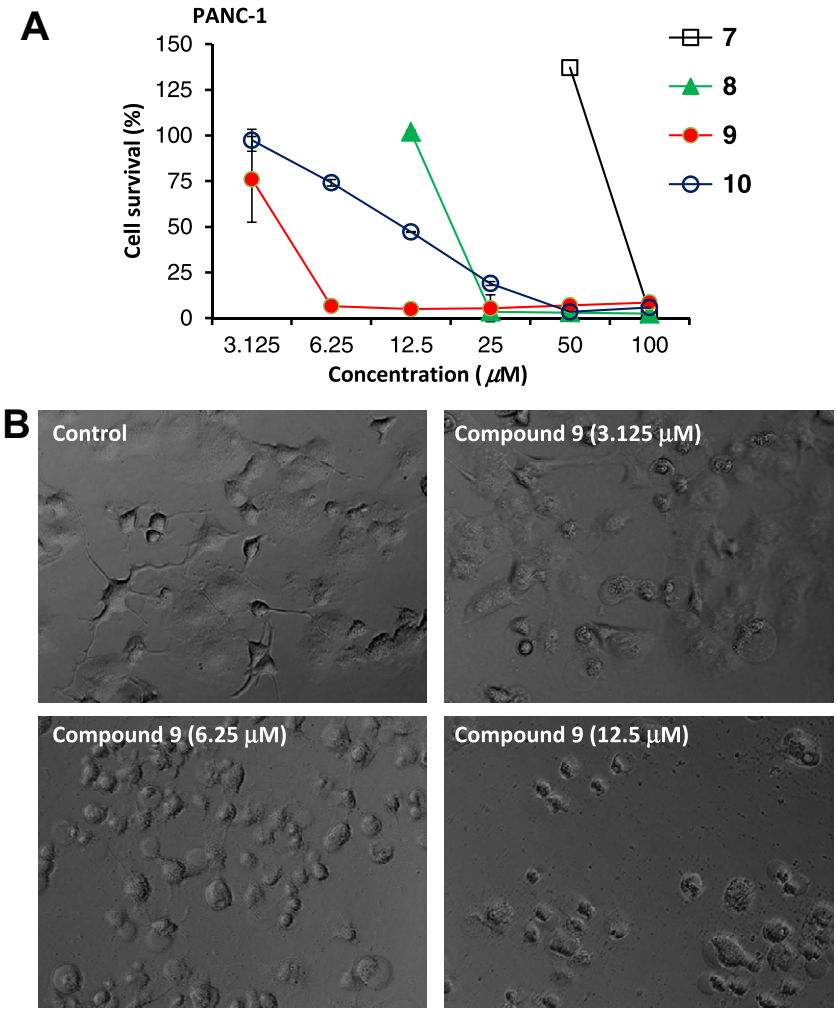


Figure 1. (A) Survival of human pancreatic cancer PANC-1 cells under nutrient-deprived conditions within 24 h by **7**–**10**. Data are means of ±SEM, $n = 3$. (B) Morphological change of human pancreatic cancer PANC-1 cells under nutrient-deprived medium after 24 h exposure (control) and with compound **9** at 3.125, 6.25 and 12.5 μM.

important for the biological activity of these hydroxycoumarin derivatives. Compound **5**, possessing a short 4-carbon acyclic alkyl ether chain, did not show any appreciable activity while compound **6** with a 5-carbon cyclic alkyl chain exhibited 100% preferential cytotoxicity (PC100) against PANC-1 cells under nutrient-deprived medium (NDM) at a high concentration of 200 μM. The 5-carbon isoprenyl derivative compound **7** also showed 100% preferential cytotoxicity against PANC-1 at a high concentration of 100 μM (Fig. 1A). Since compounds **6** and **7** both possess a 5-carbon ether chain and exhibited activity at high concentrations, it is possible that the observed cytotoxic activity is linked to both increased hydrophobicity conferred by the additional carbon on the ether chain, as well as the specific isoprenyl molecular structure. Evaluation of the 10-carbon, 15-carbon and 20-carbon isoprenyloxy derivatives, compounds **8**, **10** and **9** respectively, suggests that the presence of longer ether chains correlates to higher cytotoxic activity in this series of compounds as seen in Figure 1A. Also, these results suggest that the precise location of the isoprenyloxy chain is not as important for cytotoxic activity as the length of the ether chain as demonstrated by the activity of compound **10**. Additional studies are ongoing to further map the SAR of these compounds. Of all the compounds evaluated, the geranylgeranyl derivative, compound **9**, exhibited the highest preferential cytotoxicity at 6.25 μM showing total PANC-1 cell death within 24 h. In addition, compound **9** also induced apoptosis-like morphological changes to PANC-1 cells within the 24 h treatment period (Fig. 1B). It is important to note that the tested compounds showed selective preferential cytotoxicity only in nutrient deprived conditions. No cytotoxicity in nutrient rich medium was observed.

In conclusion, we have synthesized several alkylated and prenylated hydroxycoumarins in order to evaluate their efficacy as preferential cytotoxic agents against PANC-1 cells under nutrient deprived conditions. This study led to the discovery of a novel geranylgeranylated hydroxycoumarin-based compound (**9**) which exhibited 100% preferential cytotoxicity against PANC-1 cells under nutrient-deprived medium at 6.25 μM making this compound a powerful new lead structure for the development of novel anti-austerity agents. Further studies on the synthesis of compounds with improved therapeutic activity, as well as their mechanism of action and in vivo anti-tumor activity, are underway and will be reported in due course.

Acknowledgments

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13. *Synthesis of geranylgeranyl bromide:* To an oven-dried 25-mL round-bottom flask equipped with a magnetic stirring bar, rubber septum and a nitrogen inlet, 326.8 mg (1.125 mmol) of geranylgeraniol and 7.5 mL of anhydrous tetrahydrofuran were added. The solution was stirred and cooled to −45 °C. To the cold solution, methanesulfonyl chloride (167.5 mg, 113 μL, 1.46 mmol) was slowly added to the reaction flask via syringe. To this solution, triethylamine (227.7 mg, 313 μL, 2.25 mmol) was then added via syringe over 5 min and the resulting suspension was stirred at −45 °C for 45 minutes. A solution of lithium bromide (390.8 mg, 4.5 mmol) in 2.5 mL of tetrahydrofuran was added dropwise via syringe. The suspension was allowed to warm to 0 °C and was subsequently allowed to stir at 0 °C for 1 h. The reaction mixture was poured into 8 mL of iced water and the aqueous layer was extracted with *ice-cold* diethyl ether (3 × 4 mL). The combined organic extracts were washed with *ice-cold* saturated $NaHCO_3$ (10 mL) and brine (10 mL), and dried over anhydrous $MgSO_4$. The organic layer was filtered and concentrated *in vacuo* to give the crude geranylgeranyl bromide which was then was used for the next reaction immediately without further purifications. *Synthesis of 7-((2E,6E,10E)-3,7,11,15-tetramethylhexadeca-2,6,10,14-tetraenyl-oxy)-2H-chromen-2-one (**9**):* To an oven dried 50-mL round-bottom flask equipped with a magnetic stirring bar, rubber septa and a nitrogen inlet, umbelliferone (**1**) (19.3 mg, 0.12 mmol) and 10 mL of anhydrous DMF were added. The solution was cooled to 0 °C and sodium hydride (4.2 mg of 60% mineral oil suspension) was added. The reaction mixture was allowed to stir for 25 min at 0 °C, and the crude geranylgeranyl bromide from the previous step was dissolved anhydrous DMF (2 mL) and added dropwise to the reaction flask via syringe. The reaction was allowed to slowly warm to room temperature and was stirred overnight. The desired compound was isolated via preparative thin layer chromatography using 3:7 ethyl acetate:hexanes solvent system. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.51–1.87 (m, 15H), 1.92–2.18 (m, 12H), 4.60 (d, J = 6.6 Hz, 2H), 5.10 (m, 3H), 5.47 (t, J = 6.3 Hz, 1H), 6.25 (d, J = 9.5 Hz, 1H), 6.80–6.88 (m, 2H), 7.36 (d, J = 8.4 Hz, 1H), 7.64 (d, J = 9.5 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 16.1, 16.8, 17.7, 20.9, 26.6, 29.4, 29.7, 30.0, 39.5, 39.7, 39.8, 65.5, 101.6, 112.4, 113.0, 113.2, 118.4, 123.5, 124.4, 129.7, 131.3, 135.6, 136.2, 142.4, 143.5, 155.9, 161.3, 162.2. HRMS (EI) Calcd for $C_{29}H_{38}O_3$: 434.28210; found: 434.28330.
14. *Synthesis of 7-hydroxy-8-iodo-2H-chromen-2-one* (**2**): Umbelliferone (**1**) (200 mg, 1.23 mmol) was dissolved in a 20% NH_4OH solution (5 mL) to which a solution of I_2 (313 mg, 1.23 mmol) dissolved in aqueous KI (5%, 10 mL) was added dropwise. After 1.5 h, the reaction was quenched with 2.5 N·H_2SO_4 until acidic and precipitation occurred. The solid was filtered and purified with silica gel column chromatography using 5:95 ethyl acetate:dichloromethane to afford the desired product as a yellow solid (318 mg, 90%). ^{1}H NMR (300 MHz, CD_3OD): δ 8.01 (d, J = 9 Hz, 1H), 7.66 (d, J = 6 Hz, 1H), 7.05 (d, J = 9 Hz, 1H), 6.41 (d, J = 9 Hz, 1H). ^{13}C NMR (75 MHz, CD_3OD): δ 74.7, 112.4, 113.2, 130.0, 145.3, 156.3, 162.4, 162.6. HRMS (EI) Calcd for $C_9H_5O_3I$: 287.92838; found: 287.92906.
15. *In vitro preferential cytotoxicity and morphological change observation:* PANC-1 cells were seeded in 96-well plates (2×10^4 cells per well) and incubated in fresh Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceuticals; Tokyo, Japan) at 37 °C under 5% CO_2 and 95% air for 24 h. The cells were then washed with PBS (Nissui Pharmaceuticals), followed by addition of serially diluted test samples in DMEM or nutrient deprived medium (absence of glucose, amino acid and serum), and incubated for 24 h incubation. Then, the cell morphology was observed under the inverted microscope (TS100, Nikon) and was photographed by digital camera (Degital Sight DS-L2, Nikon). The cells were then washed again with PBS, and 100 ml of DMEM with 10% WST-8 cell counting kit solution was added to each well. After 3 h incubation, absorbance of the wells at 450 nm was measured. Cell viability was calculated from the mean values of data from three wells by using the following equation: (%) Cell viability = $[\{Abs_{(test\ sample)} - Abs_{(blank)}\}/Abs_{(control)} - Abs_{(blank)}] \times 100$.