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Discovery of 2-pyridineformamide thiosemicarbazones as potent antiausterity agents

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ABSTRACT

Series of 2-pyridineformamide thiosemicarbazones were synthesized. Their preferential cytotoxicity in nutrient deprived medium (NDM) was evaluated using PANC-1 human pancreatic cancer cells by employing an antiausterity strategy. 2-Pyridineformamide thiosemicarbazones induced apoptosis and exhibited preferential cytotoxic activity toward PANC-1 cells in NDM, with potencies in the submicromolar range. These compounds are potential candidates for the development of therapeutics against pancreatic cancer.

Pancreatic cancer is one of the most deadly forms of cancer. It is associated with the lowest 5-year survival rate known for human cancers (<5%).[1] Almost all patients with pancreatic cancer rapidly develop metastases and die within a short period after diagnosis.[2] It is resistant to conventional chemotherapeutic agents, including paclitaxel, doxorubicin, cisplatin, and camptothecin, and there are currently no reliable chemotherapeutic agents available to treat this disease. Therefore, there is an urgent need for the discovery of novel agents to treat this disease.[3] Pancreatic cancers are hypovascular in nature, which causes an inadequate supply of nutrition and oxygen to aggressively proliferating tumor cells.[4] However, these tumor cells show an extraordinary tolerance to nutrient starvation for a prolonged period of time, enabling them to survive in the hypovascular (austere) tumor microenvironment.[5] Development of drugs that specifically target the resistance of tumor cells to nutrient starvation has been termed the antiausterity therapeutic strategy.[6–11]

Thiosemicarbazones are an important class of compounds that have long attracted interest among medicinal chemists owing to their incredible biological activities, which include antibacterial, antiviral, antimalarial, and anti-tumor activities.[12–14] Marboran® (methisazone), which was marketed for the treatment of smallpox, is a notable example of a successful commercial thiosemicarbazone drug.[15] A more recent development was the discovery of Triapine® (3-aminopyridine-2-carboxaldehyde thiosemicarbazone, 3-AP, Fig. 1), which has undergone both phase II clinical trial in patients with metastatic squamous cell carcinoma of the head and neck[16] and phase II clinical trial, in combination with gemcitabine, in patients with advanced non-small cell lung cancer.[17] Triapine®, a potent antiproliferative that is effective against many cancer types, presents a marked selectivity for tumor cells.[18] It obstructs tumor growth by inhibiting ribonucleotide reductase (RR), a key enzyme involved in the conversion of ribonucleotides into deoxyribonucleotides, the building blocks of DNA synthesis.[19] Overexpression of RR, which has been reported in human pancreatic adenocarcinoma, is associated with resistance to gemcitabine,[19,20] a drug that has been prescribed most frequently for the management of advanced pancreatic cancer.

In our antiausterity strategy-based anticancer drug discovery program, we found that almost all of the conventional chemotherapeutic agents, including gemcitabine, are virtually ineffective against pancreatic cancer cells in the tumor mimicking austere environment of nutrient starvation.[21] In contrast, thiosemicarbazones have been reported to show improved activity against gemcitabine resistant human cancers.[22] Therefore, we speculated that thiosemicarbazones could be the new antiausterity agents and may possess the ability to diminish cancer cells' tolerance to nutrient starvation. To test this hypothesis, we synthesized series of 2-pyridineformamide thiosemicarbazone derivatives with variations in their ring and N-4 substitution. The synthetic route is illustrated in Scheme 1. The common intermediate 4-methyl-4-phenyl-3-thiosemicarbazide (**I**) was first prepared according to the procedure described by Scovill.[21] Transamination of **I** with an amine gave

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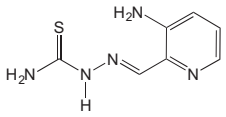
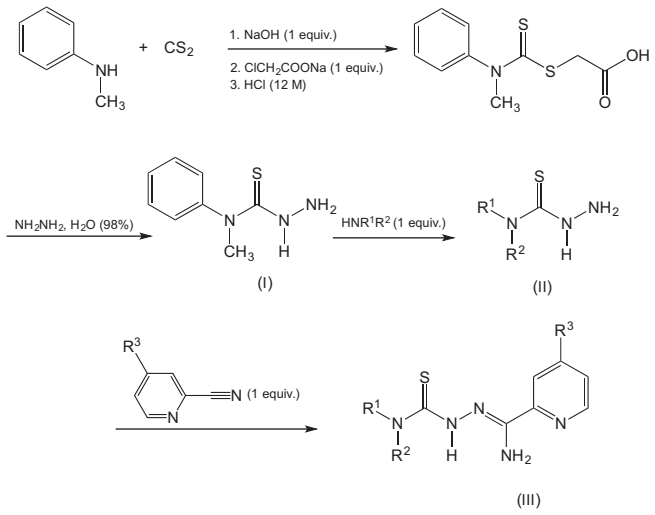


Figure 1. Structure of Triapine®.

the corresponding N-disubstituted thiosemicarbazides (**II**) which was converted into thiosemicarbazone (**III**) (see Supplementary data S2–S4).[23] For this study, we prepared twelve 2-pyridineformamide thiosemicarbazones with varied substituents.[24,25] Their structures and yields are presented in Table 1. The structures of all synthesized compounds were established using NMR spectroscopic data and HRFABMS data (See Supplementary data S4–S7).

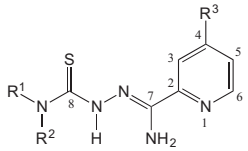
All of these synthesized compounds were tested for their preferential cytotoxicity against PANC-1 human pancreatic cancer cell line under both nutrient-rich and nutrient-deprived conditions, utilizing an antiausterity strategy[26] (Fig. 2). The concentration at which 50% of the cells were preferentially killed in the nutrient deprived medium (PC_{50}) are also presented in Fig. 2. Arctigenin, an antiausterity strategy-based anticancer agent, was used as a positive control. It showed preferential cytotoxicity with a PC_{50} value of 0.85 μM. Of interest, all thiosemicarbazones were highly active against PANC-1 human pancreatic cancer cell lines. Moreover, presence or absence of methyl substituent at C-4 position did not found to alter the activity of thiosemicarbazones. **7** showed the most potent preferential cytotoxicity among the tested compounds with a PC_{50} of 0.37 μM, approximately two fold as potent as the positive control arctigenin (PC_{50}, 0.86 μM). Among others, **1**, **6**, **7**, and **12** also displayed more potent activity than the positive control. Paclitaxel, a well known anti cancer agent, was virtually inactive. Similarly, gemcitabine, a clinically used anticancer drug for the treatment of pancreatic cancer, was virtually inactive after 24 h in both NDM and Dulbecco's modified Eagle medium (DMEM) at the maximum tested dose, with PC_{50} >200 μM (Fig. 2). This evidence suggests that thiosemicarbazones are powerful lead candidates for antipancreatic cancer drug development and demand urgent further investigation.

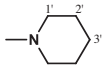
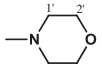
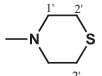
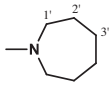
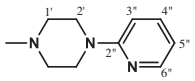
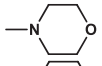
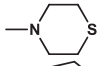
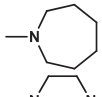
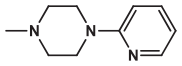
Among the synthesized compounds, **6** was selected for further study because it was synthesized in larger quantities. PANC-1 cells were treated with 1 μM **6** for 24 h in NDM, stained with ethidium bromide/acridine orange (EB/AO) reagent, and then visualized under fluorescent and phase contrast modes of the microscope.[27]



Scheme 1. Synthesis of 2-pyridineformamide thiosemicarbazones.

Table 1
2-Pyridineformamide thiosemicarbazones



Compd	$-NR^1R^2$	R^3	Yield of III (%)
1		H	46
2		H	21
3		H	60
4		H	59
5		H	24
6		H	45
7		4-CH_3	24
8		4-CH_3	29
9		4-CH_3	41
10		4-CH_3	32
11		4-CH_3	45
12		4-CH_3	63

AO is a cell permeable dye that emits bright green fluorescence in viable cells. EB is impermeable and does not stain viable cells. In late apoptotic and necrotic cells, the integrity of the plasma and nuclear membranes decreases, allowing EB to pass through the membranes, intercalate into DNA and other nucleic acids, and emits red fluorescence. As shown in Figure 3a, control cells showed intact cell morphology with bright green AO fluorescence, suggesting the cells are viable. However, cells treated with a 1 μM **6** showed exclusively red fluorescence due to EB, indicative of nonviable cells (Fig. 3b). Phase contrast microscopic observation of the treated cells showed a dramatic alteration in the PANC-1 cell morphology (Fig. 3c), including swelling, rupture of cell membranes, and disruption of cellular organelles.

We further performed western blot analysis[28] to check whether **6** modulated the key proteins involved in cell survival mechanisms (Fig. 4). A number of antiausterity agents have been found to inhibit Akt activation, leading to preferential cell death under nutrient-deprived conditions.[6] However, in present study, **6** was not found to inhibit p-Akt (S437) or p-Akt (T308), suggesting that Akt signaling is unlikely to be a target of **6**. However, **6** was found to activate apoptosis, resulting in the cleavage of caspase-3 even at a very short exposure time of 4 h in a concentration dependent manner (Fig. 4). Therefore, much stronger effect at lower concentration could be expected with increase in the exposure time period. The evidence for apoptosis was further con-firmed using an annexin

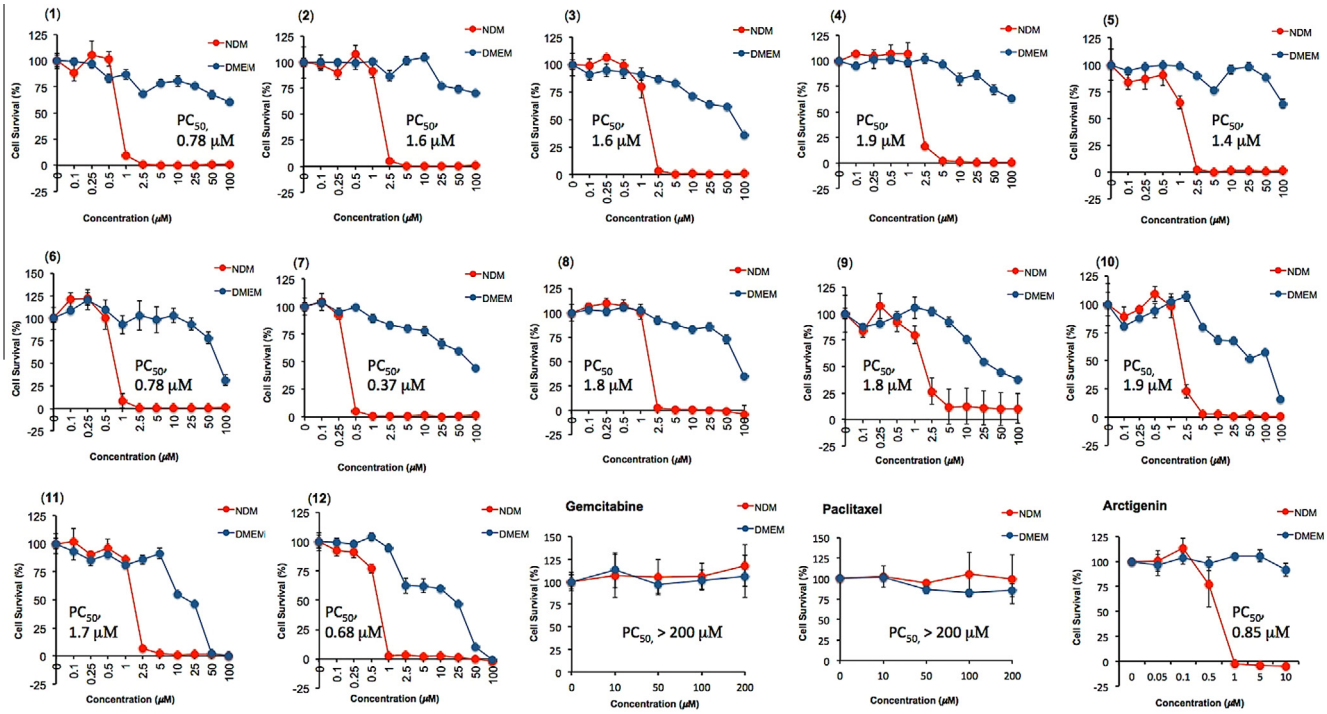


Figure 2. Preferential cytotoxic activity of synthesized 2-pyridineformamide thiosemicarbazones against the PANC-1 human pancreatic cancer cell line in nutrient deprived medium (NDM) and Dulbecco's modified Eagle medium (DMEM).

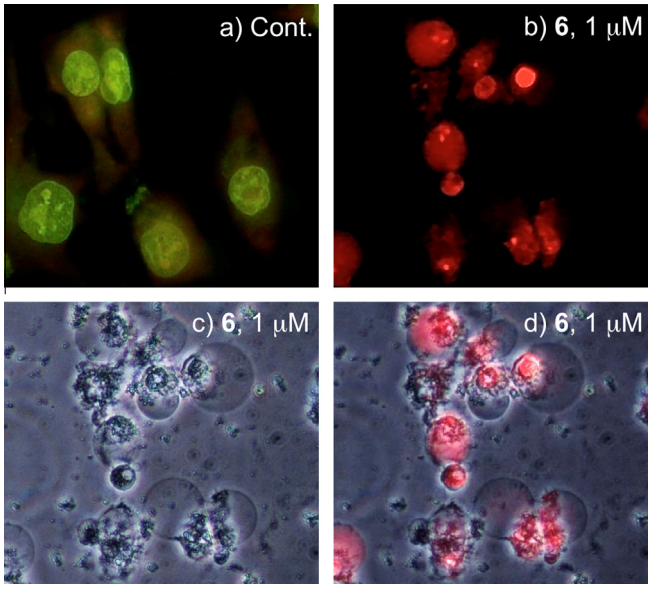


Figure 3. Fluorscent [ethidim bromide (EB)/acridine orange (AO)] and phase contrast images of PANC-1 cells at 24 h. (a) Control, live cells stained only with AO emitted bright green fluorescence, (b) treatment with **6** (1 μM) led to total death of PANC-1 cells within 24 h; cells stained with EB emitted red fluorescence, (c) phase contrast image of PANC-1 cells treated with **6** (1 μM) showing morphological alteration. (d) Merged image of phase contrast and red fluorescence image of PANC-1 cells treated with **6** (1 μM).

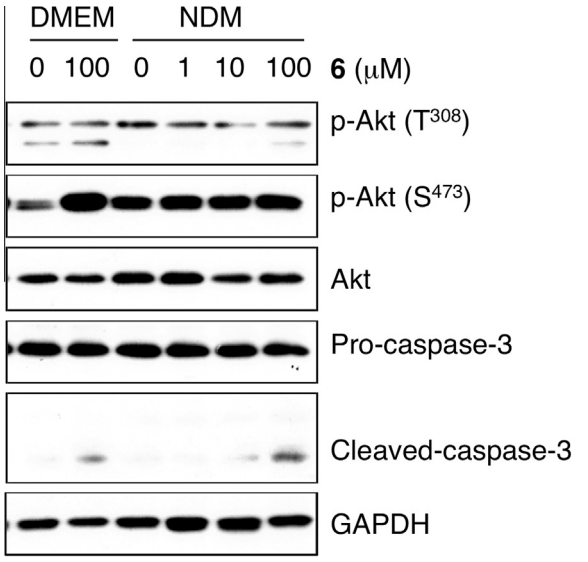


Figure 4. Western blot of the effect of **6** for 4 h against Akt, pAkt, pro-caspase 3, and cleaved-caspase 3.

v (AV) and propidium iodide (PI) staining assay. During apoptosis, alterations in the plasma membrane lead to the translocation of phosphatidylserine (PS), which is exposed at the external surface of the cell. AV specifically binds to the exposed PS, which is detected by green fluorscence (Supplementary data S8). Pancreatic cancer cells are known to be resistant to apoptosis, which is one of the key reasons for the failure of chemotherapy, and lead to aggressive growth and metastasis. In the present study, apoptosis induced by **6** indicated that 2-pyridineformamide thiosemicarbazones are potential candidates for antipancreatic cancer drug development.

In conclusion, 2-pyridineformamide thiosemicarbazones are novel powerful antiausterity agents for the development of therapeutics against deadly pancreatic cancers. Detailed studies of both their mechanism of action and their in vivo activity are under consideration.

Acknowledgments

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Supplementary data

Supplementary data (general experimental procedures, annexin-V/PI staining assay, synthetic protocol, and NMR spectral data for compounds **1**–**12**) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.12.044.

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25. Compound **5** was previously reported by Santos, I. G.; Hagenbach, A.; Abram, U. *Dalton Trans.* **2004**, *21*, 677.
26. *PANC-1 cell line, culture and in vitro preferential cytotoxicity:* The PANC-1 human pancreatic cancer cell lines were purchased from Riken BRC cell bank. These cell lines were maintained in standard D-MEM 10% FBS supplement, 0.1% $NaHCO_3$, and 1% antibiotic antimycotic solution. For preferential cytotoxicity experiment, PANC-1 cells were seeded in 96-well plates (1.5×10^4/well) and incubated in fresh DMEM at 37 °C under humidified 5% CO_2 and 95% air for 24 h. After the cells were washed with D-PBS, the medium was changed to serially diluted test samples in DMEM or NDM, with the control and blank in each plate. After 24 h incubation, cells were washed twice with D-PBS and 100 μL of DMEM containing 10% WST-8 cell counting kit solution was added in each well. After 3 h incubation, the absorbance at 450 nm was measured (PerkinElmer EnSpire multilabel reader). Cell viability was calculated from the mean values from three wells using the following equation:
Cell viability (%) = [(Abs(test sample) − Abs(blank))/(Abs(control) − Abs(blank))] × 100
27. *Morphological assessment of cancer cells:* Cells for the morphological change study were seeded in 60 mm dishes (1×10^6) and incubated in humidified CO_2 incubator for 24 h for the cell attachment. The cells were then washed twice with PSB, and treated with 1 μM of synthesized compound **6** in DMEM and in NDM. After 24 h incubation, 10 μL of ethidium bromide/acridine orange (EB/AO) reagent (dye mixture 100 μg/ml AO and 100 μg/ml EB in PBS) was added, and then visualized under fluorescent and phase contrast modes on inverted Nikon Eclipse TS 100 microscope (40 × objective). Images were taken with a Nikon DS-L-2 camera directly attached to the microscope.
28. *Western blot analysis:* Rabbit polyclonal antibodies to Akt, phosphoryl Akt (S473), caspase 3 was purchased from Cell Signaling Technology (Danvers, MA). A rabbit polyclonal antibody to β-GAPDH was purchased from GeneTex (Irvine, CA, USA). Horseradish peroxidase (HRP)-labeled goat polyclonal anti-rabbit immunoglobulins, HRP-labeled rabbit polyclonal anti-goat immunoglobulins and HRP-labeled goat polyclonal anti-mouse immunoglobulins were purchased from DakoCytomation (Glostrup, Denmark). The proteins were separated by gel electrophoresis on a polyacrylamide gel containing 0.1% SDS (SDS–PAGE) and then transferred to PVDF membranes. The membranes were blocked with Block Ace (DS Pharma Medical, Suita, Japan), washed with PBS containing 0.1% polyoxyethylene(20) sorbitan monolaurate (Wako Pure Chemical), then incubated overnight at room temperature with primary antibodies diluted Can Get Signal (Toyobo, Osaka, Japan). After washing, the membranes were incubated for 45 min at room temperature with HRP–conjugated anti-rabbit, mouse or goat immunogloblins as the secondary antibody. The bands were detected with an enhanced chemiluminescence solution (PerkinElmer., Waltham, MA, USA).