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Growth inhibition and mechanism of action of *p*-dodecylaminophenol against refractory human pancreatic cancer and cholangiocarcinoma

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ABSTRACT

Pancreatic cancer and cholangiocarcinoma are aggressive and drug-resistant refractory cancers. Based on *N*-(4-hydroxyphenyl)retinamide (**3**), a synthetic amide of all-*trans*-retinoic acid (RA), *p*-dodecylaminophenol (**1**) was developed to be an effective anticancer agent without key side-effects of these agents. Compound **1** suppresses cell growth of pancreatic cancer (MIA Paca2) and cholangiocarcinoma (HuCCT1), potentially by inhibiting ras expression and signaling through ERK pathways in MIA Paca2 cells and both ERK and Akt pathways in HuCCT1 cells. Compound **1** inhibits proliferation of these cells to a greater extent than either RA or **3**. Compound **1** may represent a potent and useful anti-cancer drug for use against pancreatic cancer and cholangiocarcinoma that lacks their key side-effects.

1. Introduction

Pancreatic cancer is a highly aggressive and drug-resistant malignancy.[1–6] The poor prognosis associated with pancreatic cancer is attributable to its tendency for late presentation, aggressive local invasion, early metastases, and poor response to chemotherapy.[7] In terms of death certificate only to incident cases (DCO/I), pancreatic cancer is the first and second leading cause of death based on proportion of cases for both females and males, respectively. Moreover, cholangiocarcinoma is proportionally the fourth leading cause of death based on DCO/I data.[8] Presently, pancreatic cancer and cholangiocarcinoma are refractory cancers that are difficult to cure.

One of the earliest genetic events in pancreatic cancers is mutation of the K-ras oncogene,[9] which is also reported in cholangiocarcinoma.[10] Since mutational activation of ras proteins is seen with high frequency (90%) in pancreatic ductal adenocarcinoma, it is reasonable to assign a major role for K-ras in pancreatic carcinogenesis. Targeting this signaling pathway could be fundamental to improving clinical response.[11]

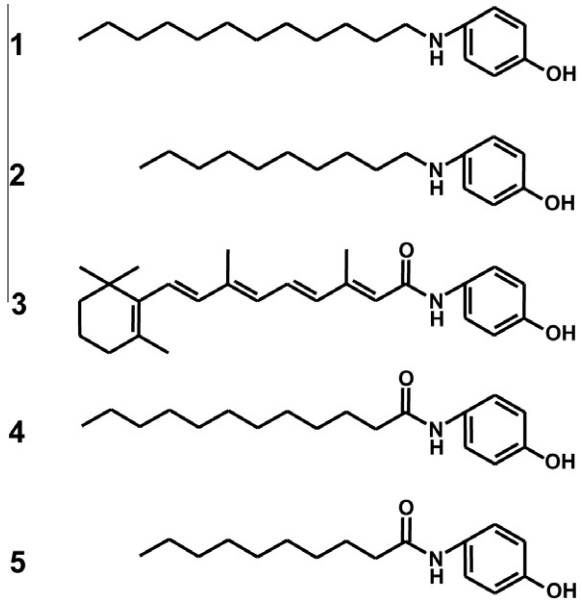

Figure 1. Chemical structures of **1, 2, 3, 4** and **5**.

N-(4-Hydroxyphenyl)retinamide (**3**, fenretinide) (Fig. 1), a synthetic amide of all-*trans*-retinoic acid (RA), is an effective anticancer

Abbreviations: **1**, *p*-dodecylaminophenol, 4-(dodecylamino)phenol; **2**, *p*-decylaminophenol, 4-(decylamino)phenol; **3**, *N*-(4-hydroxyphenyl)retinamide, fenretinide; **4**, *N*-(4-hydroxyphenyl)dodecananamide, *p*-dodecanoylaminophenol; **5**, *N*-(4-hydroxyphenyl)decananamide, *p*-decanoylaminophenol; RA, all-*trans*-retinoic acid; DMSO, dimethylsulfoxide; BSA, bovine serum albumin; PBS, phosphate-buffered saline (1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 136.9 mM NaCl, pH 7.2); MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; EDTA, ethylenediaminetetraacetic acid; RT-PCR, reverse transcription-polymerase chain reaction; PI3K, phosphatidylinositol 3-kinase; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase kinase; SD, standard deviation.

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drug, which is used against a wide variety of tumor types.[12–16] Compound **3** currently is in clinical trials for the treatment of breast, bladder, renal, and neuroblastoma malignancies.[17–22] However, studies have shown that treatment with **3** is accompanied by night blindness because of a decrease in serum retinol levels.[23] It appears that this side-effect occurs by the displacement of retinol from serum retinol binding protein (RBP), which results in reduced delivery of retinol to the eyes (nyctalopia).[24–26]

To maintain potent anticancer activity with reduced side-effects, we have developed agents whose design is based on the assumption that the cyclohexene portion of both **3** and RA, is responsible for their association with RBP, which is a regulator of plasma retinol levels.[27,28] We have shown that the *p*-methylaminophenol moiety in **3** contributes significantly to its anticancer activity as compared to the 4-aminophenol and *p*-aminoacetophen moieties.[29] Elongation (C_1–C_8) of the poly-methylene chain in *p*-methylaminophenol increases anticancer activity.[27] Recently, we synthesized four new compounds, which had lengths of carbon side chains similar to that of **3**; *p*-dodecylaminophenol (**1**), *p*-decylaminophenol (**2**), *N*-(4-hydroxyphenyl)dodecananamide (**4**), and *N*-(4-hydroxyphenyl)decananamide (**5**) (Fig. 1).[30] Among *p*-aminophenols having differing lengths of alkyl chains, **1** was the most potent inhibitor of cell growth when examined against a set of cancer cell lines, including human cancer cell lines that are resistant to RA.[28,30–32] Herein, we evaluate these derivatives of **3** (Fig. 1), with particular emphasis on **1**. In the current studies, we examine whether aminophenols, including **1**, **2**, **4** and **5**, are effective against refractory pancreatic cancer and cholangiocarcinoma cell lines[33–35] and whether ras pathways are involved in their actions against these refractory cancers.

2. Results

2.1. Effects of RA, 3, and 1 on the growth of human pancreatic cancer and cholangiocarcinoma

There are few reports regarding antiproliferative activities of RA and **3** against human pancreatic cancer and cholangiocarcinoma. We examined the effects of RA, **3** and **1** (Fig. 1) on cell growth of MIA Paca2 (pancreatic cancer) and HuCCT1 (cholangiocarcinoma) cells. Growth of MIA Paca2 and HuCCT1 cells was inhibited by approximately 15% and 17% at 4 μM RA and approximately 18% and 28% at 10 μM RA, respectively as compared with controls (Fig. 2A and B). Significant growth inhibition was also observed in MIA Paca2 (approximately 8%) and HuCCT1 (approximately 17%) cells treated with 4 μM of **3**. Compound **3** reduced cell growth drastically at 10 μM and showed cytotoxicity. In contrast, growth of MIA Paca2 cells were significantly suppressed to the extent of approximately 20%, 45% and 50% by **1** at concentrations of 1, 4 and 10 μM, respectively (Fig. 2A). Against HuCCT1 cells at concentrations of 1, 4 and 10 μM **1**, significantly inhibited growth to the extent of approximately 35%, 85% and 84%, respectively (Fig. 2B). Thus, **1** gradually suppressed cell growth beginning at low concentrations without cytotoxicity as compared with **3**. IC_{50} values of **1** and **3** estimated from the data in Fig. 2A and B were approximately 10 and 7 μM for MIA Paca2 cells and approximately 2 and 6 μM for HuCCT1 cells, respectively.

Compounds **1** and **3** are also known to induce apoptosis of prostate cancer and neuroblastoma.[31,32] We found that MIA Paca2 and HuCCT1 cells are not induced to undergo apoptosis by **1** and **3** under the same conditions that inhibited cell growth (data not shown). These results suggest that **1** exhibits potent effects that are superior against refractory cancers as compared with RA and **3**. In addition, **3**, which showed cytotoxicity at high concentrations, was a potent agent against refractory cancers within a narrow concentration range.

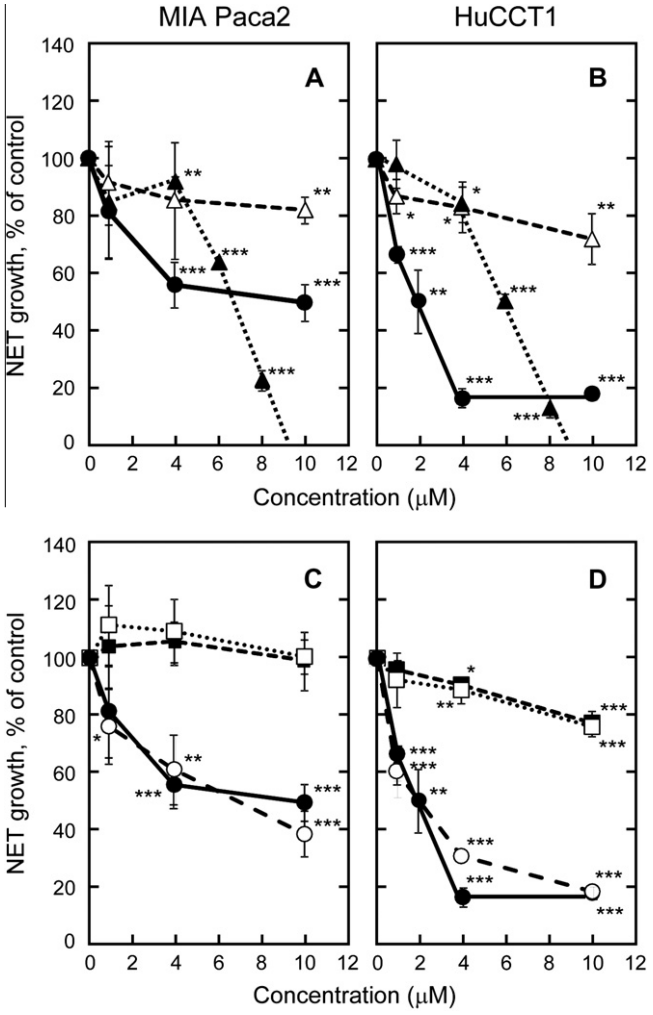

Figure 2. Growth inhibition of refractory cancer cells by retinoids, *p*-alkylaminophenols and *p*-acylaminophenols. MIA Paca2 (A, C) and HuCCT1 (B, D) cells were treated with various concentrations of RA (△), **3** (▲), **1** (●), **2** (○), **4** (■), or **5** (□) for 72 h. Relative cell growth was determined as described in the Section 4. Data shown represent the mean ± SD of three separate studies. $^{*}p$ <0.05, $^{**}p$ <0.01, and $^{***}p$ <0.001 versus control as compared by the Student's *t*-test.

2.2. Growth inhibition of refractory cancer cells treated with *p*-aminophenols (*p*-alkylaminophenols and *p*-acylaminophenols)

Next we examined growth inhibition using other *p*-aminophenols. As shown in Fig. 2C and D, against MIA Paca2 and HuCCT1 cells, growth inhibition with *p*-alkylaminophenols (**1** and **2**) was much greater than with *p*-acylaminophenols (**4** and **5**). Moreover, the extent of cell growth inhibition was approximately equal for **1** and **2** (approximately 55% for MIA Paca2 cells and 82% for HuCCT1 cells at 10 μM concentration), and for **4** and **5** (approximately 0% for MIA Paca2 cells and 23% for HuCCT1 cells at 10 μM concentration). Growth of HuCCT1 cells was suppressed by *p*-aminophenols to a much greater extent than MIA Paca2 cells in dose-dependent manner. These results suggest that *p*-alkylaminophenols are potent agents against pancreatic cancer and cholangiocarcinoma, with broad effective ranges. Against these refractory cancers, elongation of the alkyl moiety in the *p*-alkylaminophenols did not enhance cell growth inhibition. It also appeared that the

carbonyl moieties in the *p*-acylaminophenols might play a role in suppressing growth inhibition.

2.3. Incorporation of 1 into refractory cancer cells

Cell growth inhibition may occur through the regulation of existing pathways and/or gene expression, which are associated with signaling of extracellular or intracellular events. Our findings raise the question as to whether the level of incorporation of **1** into cells affects growth inhibition. Therefore, we examined incorporation of **1** into MIA Paca2 and HuCCT1 cell lines.

After incubation at 37 and 4 °C in the presence of 10 μM of **1**, drug was extracted from cells and quantified by HPLC. Cellular uptake at 4 °C (data not shown) was extremely low as compared to uptake at 37 °C. At 37 °C, the incorporation of **1** into HuCCT1 cells was less than into MIA Paca2 cells (Fig. 3). The incorporation of **1** into MIA Paca2 cells increased with prolongation of incubation time, and achieved near saturation after 30 min. In contrast, the uptake of **1** into HuCCT1 cells increased until saturation at 30 min and then decreased from 30 min to 60 min (Fig. 3). At 30 min, the amount of **1** in MIA Paca2 cells was approximately 1.8-fold higher than in HuCCT1 cells. These results indicate that cellular incorporation of **1** does not correlate with antiproliferative activities of **1** against each cancer cell line, because **1** incorporated poorly into HuCCT1 cells, which were more sensitive to **1**. This suggested that the mechanism of growth inhibition by **1** is highly dependent on the signaling of intracellular events rather than on extracellular events.

2.4. Comparison of oncogenic ras gene expression in refractory cancer cells

It is well known that the ras family of proteins regulate cell survival and cell differentiation, and that the K-ras gene is highly mutated in pancreatic cancer and cholangiocarcinoma. Mutations of the K-ras gene in two cell lines were analyzed using PCR-amplified cDNA. Both cell lines contained K-ras mutations at codons 12 and 61, but not at codon 13 (Table 1). Mutation at codon 12 was a GGT-TGT substitution (Gly-Cys) in MIA Paca2 cells and a GGT-GAT substitution (Gly-Asp) in HuCCT1 cells. Mutation at codon 61 was a CAT-CAA substitution (His-Gln) in both cell lines. Therefore, K-ras genes in the two cell lines exhibit different mutations at codon 12.

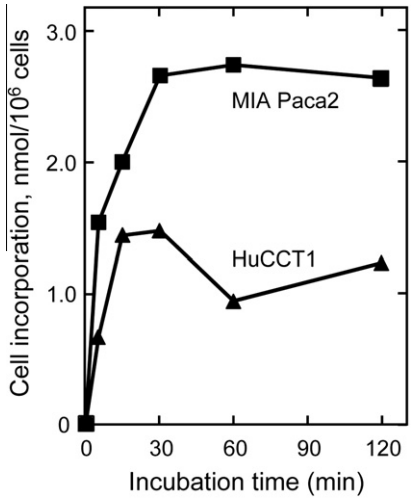

Figure 3. Incorporation of **1** into human refractory cancer cells. MIA Paca2 (■) and HuCCT1 (▲) cells (1×10^6 cells/ml) were incubated in the presence of **1** at 10 μM concentration in medium containing 10% FBS for the indicated time. Cells were harvested and measurements of cell incorporation were performed using HPLC with a LUNA® 5 μ C_{18} (2) column (150 × 4.6 mm, Phenomenex) as described in the Experimental section. Compound **1** was eluted with 80% MeOH, 20% H_2O, and 10 mM ammonium acetate at a flow rate of 1.0 ml/min. Each point is the mean of at least three measurements. The SD of each point is ⩽8% of the mean.

Table 1
Mutations in K-ras genes in human pancreatic cancer and cholangiocarcinoma

Cell line	Codon 12	Codon 13	Codon 61
MIA Paca2	GGT > TGT (Gly > Cys)	GCC (Wild)	CAT > CAA (His > Gln)
HuCCT1	GGT > GAT (Gly > Asp)	GCC (Wild)	CAT > CAA (His > Gln)

We examined whether expression of ras genes were distinct among the refractory cancers. In order to examine the levels of oncogenic ras gene expression, we measured the expression levels of the ras family genes (K-ras, H-ras, and N-ras) in the two cancer cells. The levels of N-ras gene expression in HuCCT1 cells were significantly higher (approximately 1.75-fold) than in MIA Paca2 cells (Fig. 4A). These results indicate that ras oncogene expression is distinct between the two cell lines and they suggest that signaling through the ras family may contribute to the growth of pancreatic cancer and cholangiocarcinoma.

2.5. Alteration of oncogenic ras gene expression in refractory cancer cells treated with 1 and 3

Since **1** is a potent antiproliferative agent against both MIA Paca2 and HuCCT1 cells, but to different extents (Fig. 2), we examined the effects of **1** and **3** on ras expression in refractory cancer cells at IC_{50} concentrations. The levels of K-ras, H-ras and N-ras gene expression were analyzed and quantified by agarose gel electrophoresis (Fig. 4B–D). The levels of K-ras gene expression were inhibited effectively by treatment with both **1** and **3** in MIA Paca2 and HuCCT1 cells (Fig. 4C). In addition, in HuCCT1 cells, **1** inhibited H-ras and N-ras gene expression, while **3** suppressed H-ras gene expression (Fig. 4B and D). These results suggest that inhibition of ras family gene expression by **1** (and **3**) might be one mechanism affecting their cell growth inhibition, and that the inhibition of K-ras gene expression among the three ras family members might be especially relevant to growth inhibition by **1**.

2.6. Effects of 1 and 3 on phosphorylation of ERK and Akt proteins in refractory cancer cells

The reduction of K-ras gene expression in refractory cancer cells by treatment with **1** led us to examine whether signal transduction pathways involving activated ERK or Akt are affected by **1**. The protein levels of phosphorylated ERK, ERK, phosphorylated Akt and Akt in cells treated with **1** or **3** at IC_{50} concentrations, were analyzed by immunoblotting. Compound **1** reduced by approximately 50% the phosphoryled ERK/ERK levels in MIA Paca2 cells (Fig. 5A), and by approximately 21% the phosphoryled ERK/ERK levels and by approximately 20% the phosphorylated Akt/Akt levels in HuCCT1 cells (Fig. 5B). In contrast, **1** did not affect the levels of phosphorylated Akt/Akt in MIA Paca2 cells. Compound **3** increased by approximately 2.4-fold the phosphoryled ERK/ERK levels and suppressed by approximately 30% the phosphoryled Akt/Akt levels in MIA Paca2 cells (Fig. 5A). However, **3** did not affect phosphorylated ERK/ERK or phosphorylated Akt/Akt levels in HuCCT1 cells. These results indicate that the inhibitory effects on cell growth by **1**, but not by **3**, correlate with inhibition of ERK and Akt pathways. This might be due to suppression of ERK phosphorylation for MIA Paca2 cells and both ERK and Akt phosphorylation for HuCCT1 cells.

3. Discussion

Herein, we have shown inhibitory effects of **1** and **3** (Fig. 1) against the growth of human pancreatic cancer (MIA Paca2) and

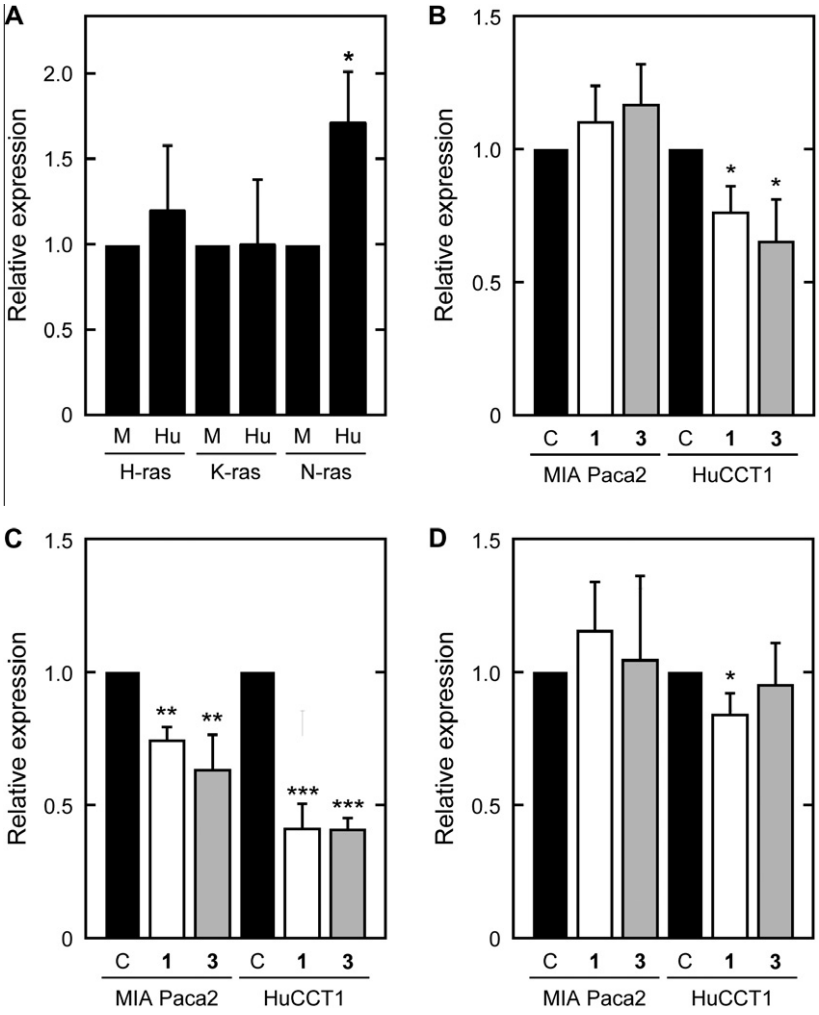

Figure 4. Effects of **1** and **3** on the levels of ras family mRNAs in human refractory cancer cells. (A, B) Expression levels of ras family mRNAs in MIA Paca2 (M) and HuCCT1 (Hu) cells were analyzed by RT-PCR, followed by agarose gel (2%) electrophoresis. The intensities of controls in MIA Paca2 cells were defined as 1.0. Data shown represent the mean ± SD of three separate studies. $^{*}p$ <0.05 versus MIA Paca2 cells as compared by Student's *t*-test. (C, D, E, F) Cells were treated with DMSO (C: black column), **1** (white column), or **3** (gray column) at IC_{50} concentrations for 72 h. Expression levels of ras family mRNAs were analyzed by RT-PCR, followed by an agarose gel (2%) electrophoresis. The intensities of controls were defined as 1.0. Data shown represent the mean ± SD of three separate studies. $^{*}p$ <0.05, $^{**}p$ <0.01, and $^{***}p$ <0.001 versus control as compared by the Student's *t*-test.

cholangiocarcinoma (HuCCT1) cell lines (Fig. 2). Analogue **1** was more effective than **3**, exhibiting milder effects and a broader effective range. Compound **3** displayed more drastic and greater cytotoxic effects (Fig. 2A and B). We also demonstrated that **2** inhibits cell growth of MIA Paca2 and HuCCT1 cells to the same extent as **1**, while *p*-acylaminophenols (**4** and **5**) did not affect these cells (Fig. 2C and D). The cellular incorporation of **1** was higher in MIA Paca2 cells than in HuCCT1 cells (Fig. 3). The cell growth inhibition by **1** was the opposite (Figs. 2 and 3). In MIA Paca2 cells, **1** suppressed K-ras gene expression and ERK phosphorylation as compared to controls (Figs. 4C and 5A). In contrast, expression of H-ras, K-ras and N-ras genes and ERK/Akt phosphorylation in HuCCT1 cells were inhibited by **1** (Figs. 4B–D and 5B). Cell sensitivities to **1** correlated with the extent of ras gene expression. These results suggest that **1** suppresses cell growth by inhibiting growth/differentiation signaling through ras-MEK-ERK pathways for MIA Paca2 cells and through both ras-MEK-ERK and ras-PI3K-Akt pathways for HuCCT1 cells.

The design of the aminophenol analogues was based on the structure of **3**, which is a potent anticancer agent. Compound **1** exhibits the most potent anticancer activity among the *p*-alkylaminophenols examined, suppressing cell growth in a variety of cancer cell lines.[28,32] In the current study, **1** and **2** showed inhibitory effects on cell proliferation of refractory pancreatic cancer and cholangiocarcinoma, while **4** and **5** failed to inhibit growth in these cell lines (Fig. 2C and D). These results are similar to those in other cancers, including leukemia, neuroblastoma and breast and prostate cancer cell lines.[31,32] The insertion of a carbonyl moiety into the *p*-alkylaminophenol structure diminished anticancer activity. The data suggest that **1** and **2** may be promising anticancer agents against drug-resistant and refractory cancers.

Previous studies have shown that **3**, which is a synthetic derivative of RA, is an effective chemopreventive and anti-proliferative agent,[12–16] and that it is effective against a wide variety of tumor types. There are no reports that **3** as a single agent exhibits anti-proliferative activity against human pancreatic cancer or cholangiocarcinomas, although studies have shown that **3** inhibits the growth of hamster pancreatic duct carcinoma.[36] The current study is the first to show that **3** in the range of 4–8 μM inhibits cell growth of refractory human cancers. The effects of **3** were weaker than **1** at concentrations between 4 and 6 μM and it was cytotoxic at a concentration of 10 μM (Fig. 2). The inhibitory effects on cell growth by **3** did not correlate with inhibition of ras-ERK and ras-Akt pathways (Fig. 5), which is in contrast to **1**. These results

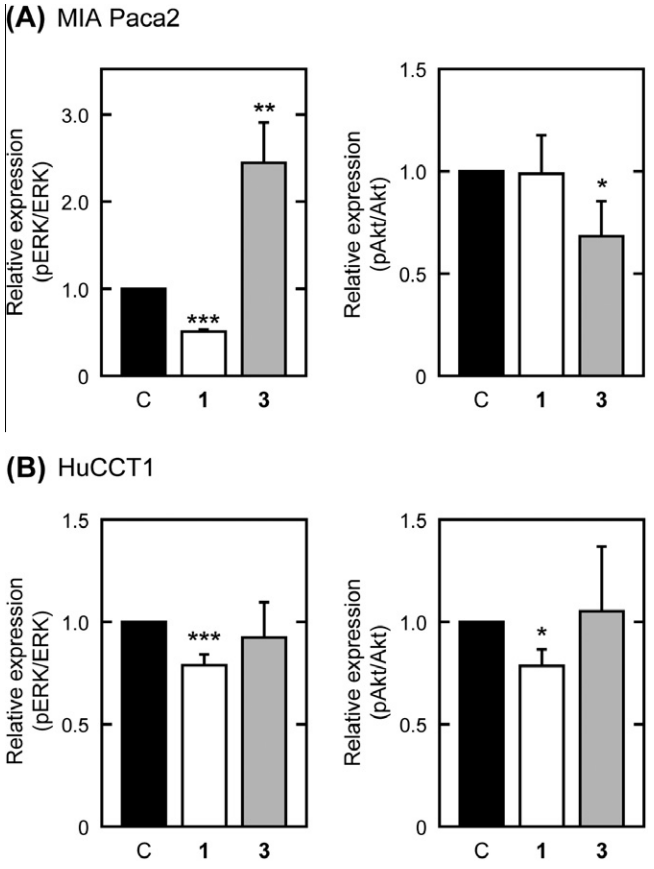

Figure 5. Protein levels of phosphorylated-ERK, ERK, phosphorylated-Akt and Akt in human refractory cancer cells. MIA Paca2 cells (A) and HuCCT1 cells (B) were treated with DMSO (C: control), **1**, or **3** at IC_{50} concentrations for 48 h. The protein levels of phosphorylated-ERK, ERK, phosphorylated-Akt and Akt were analyzed by Western blot analysis with specific antibodies against phosphorylated-ERK, ERK, phosphorylated-Akt, Akt, and β-actin as described in the Section 4. Intensities of bands were analyzed by scanning densitometry. Each bar represents the mean ± SD of each group. $^{*}p$ <0.05, $^{**}p$ <0.01, and $^{***}p$ <0.001 versus control as compared by the Student's *t*-test.

indicate that the mechanisms of action of **3** and **1** against refractory cancers may be distinct. Previous studies have shown that **3** and **1** may inhibit cell growth via induction of cell apoptosis mediated through caspase-3 and bcl-2.[31,32] Another mechanism of action of **3** has been shown to involve the generation of reactive oxygen species.[37,38] It is possible that growth inhibition by **3**, including cytotoxicities that were not seen following treatment with **1**, might be due to the latter mechanism, since **3** and **1** do not induce apoptosis against refractory cancer cells (data not shown). It would be interesting to compare apoptosis systems in refractory cancers (e.g., pancreatic cancer and cholangiocarcinoma) and sensitive cancers (e.g., neuroblastoma and prostate cancers).

Activated oncogenes most often associated with human neoplasia are members of the ras gene family (K-ras, H-ras and N-ras).[39] These highly conserved genes encode similar membrane-bound proteins that possess guanine nucleotide-binding and hydrolytic activities. It is known that ras proteins are involved in signal transduction processes across cell membranes. Single point mutations at codon 12 in the K-ras gene are seen in a majority of human pancreatic carcinomas.[40–43] The ras oncogene is aberrantly activated in up to 90% of pancreatic cancers and it plays a role in the development of this malignancy. On the other hand, K-ras codon 12 mutations have been seen often in biliary tract tumors.[10] In the current study, MIA Paca2 and HuCCT1 cells exhibited K-ras mutation at codon 12 and codon 61 (Table 1). Compound **1** significantly suppressed K-ras mRNA expressions in MIA Paca2 and HuCCT1 cells, and it inhibited expression of H-ras and N-ras genes in HuCCT1 cells. Alterations in ras expression correlated with cell growth inhibition by **1** in these two cell lines.

Oncogenic ras promotes tumorigenesis through activation of multiple downstream pathways, including MEK/ERK and PI3K/Akt.[44,45] In the current study, we showed that growth of MIA Paca2 and HuCCT1 cells is activated through ras/MEK/ERK or ras/PI3K/Akt pathways. Compound **1** inhibited the phosphorylation of ERK in MIA Paca2 cells and the phosphorylation of ERK and Akt in HuCCT1 cells (Fig. 5). These results suggest that the mechanism of cell growth inhibition by **1** may involve ras-related pathways through the inhibition of MEK/ERK pathways in pancreatic cancer and MEK/ERK and PI3K/Akt pathways in cholangiocarcinoma. Compound **1** is an extremely effective anticancer drug against pancreatic cancer and cholangiocarcinoma. The results reported herein support potential clinical applications of **1** in the treatment of refractory cancers.

4. Experimental

4.1. Chemicals

RA, ethyrenediaminetetraacetic acid (EDTA), and dimethylsulfoxide (DMSO) were obtained from Sigma Chemical (St. Louis, MO, USA). Compound **3** was provided by Dr. R. C. Moon, University of Illinois, Chicago, IL, USA. Compounds **1**, **2**, **4**, and **5** were synthesized as described previously (Fig. 1).[30] All other chemicals were of reagent grade.

4.2. Cell lines and culture conditions

Human pancreatic cancer cell line MIA Paca2[46] and human cholangiocarcinoma cell line HuCCT1[47] were obtained from the RIKEN Cell Bank (Tsukuba, Ibaraki, Japan). MIA Paca2 cells were maintained in DMEM (low-glucose) (Wako Pure Chemical Ind., Osaka, Japan) supplemented with 10% fetal bovine serum (FBS, GIBCO, Grand Island, NY, USA). HuCCT1 cells were maintained in RPMI 1640 medium (GIBCO) supplemented with 10% FBS. Attached cells were removed from tissue-culture flask surface with Trypsin-EDTA (GIBCO).

All cells described above were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. Cell number was estimated using an electric particle counter (Coulter Electronics, Hialeah, FL, USA).

4.3. Cell culture

Cells (1×10^4 cells/ml) were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. After 24 h, various concentrations of compounds were added to the cultures, and then cells were incubated for 48 h or 72 h. Controls were prepared with cells treated with culture medium containing 0.1% DMSO.

1) Cell growth: Viable cell number was estimated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described previously.[48,49] Values for percent net cell growth were calculated with the following formula: [(absorbance of experimental cell concentration) − (absorbance of initial cell concentration)/(absorbance of control cell concentration) − (absorbance of initial cell concentration)] × 100.
2) Preparation of cell extract: Cells were harvested and lysed using Buffer A (25 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.5% NP-40, 4 mM NaF, 0.1 mM Na_3VO_4 and protease inhibitor cocktail). After centrifugation (13,000*g*, 20 min), the supernatants were separated by one-dimensional polyacrylamide gel electrophoresis (1D-PAGE) and then were immunostained.

4.4. Incorporation of 1 into cells

Incorporation of **1** into cells was examined as described previously.[28] Briefly, cells (1×10^6 cells/ml) treated with 10 μM of **1** were incubated for the indicated time at 37 °C or at 4 °C. Compound **1** was incorporated into cells and extracted with ethyl acetoacetate. Compound **1** contained in organic layer was quantified by high performance liquid chromatography (HPLC) with mobile phase solvent of 80% methanol, 20% H_2O, and 10 mM ammonium acetate, using a Shimadzu LC-6A high pressure pump, Shimadzu CTO-10AS column oven including injector, and an SPD-6A UV spectrophotometric detector (Shimadzu Co. Ltd, Kyoto, Japan). A LUNA® 5 μ C_{18} (2) column (150 × 4.6 mm, Phenomenex, Rancho Palos Verdes, CA, USA) was used to separate compounds. The column was eluted with mobile phase solvent at a flow rate of 1.0 ml/min. Compound **1** was detected with UV monitoring at 240 nm, which is the maximum absorbance of **1**. Retention time of **1** was 18 min. Measurements were made using the ratio of peak areas to internal standards. Values for cell incorporation were calculated based on the extraction efficiencies of **1**.

4.5. RNA isolation and semi-quantitative RT-PCR

Total RNA was extracted from cells treated with DMSO, **1** or **3** using an ISOGEN RNA extraction kit (Nippon Gene, Toyama, Japan). cDNA was synthesized from 2.5 μg of RNA using SuperScript® VILO® cDNA Synthesis Kit (Invitrogen Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions. The RT-PCR reactions were then amplified with specific primers for ras oncogenes (*K-ras*: 299 bp [*UniSTS: 155355*]: sense 5′-AGG CAT ACT AGT ACA AGT GG-3′, antisense 5′-ACA CCA ACA TTC ACA CTT GG-3′; *H-ras*: 209 bp [*UniSTS: 64957*]: sense 5′-TCA GCA GCC TCC CTT GTG-3′, antisense 5′-GGA TGT TCA AGA CAG TCT GTG C-3′; *N-ras*; 331 bp [*UniSTS: 24005*]: sense 5′-GAA TGC ACC TCT TGT TAC TCC C-3′, antisense 5′-CAG ATG CCA GTT TAG AGA ATA GAG C-3′) and β-*actin* (202 bp: sense 5′-CCT TCC TGG GCA TGG AGT CCT G-3′, antisense: 5′-GGA GCA ATG ATC TTG ATC TTC-3′).[50] The PCR products were separated by electrophoresis in 2% agarose gel and visualized.

4.6. Amplification and sequencing of *K-ras*

Template DNAs (100 ng) were amplified by PCR in a reaction mixture (50 μl) containing sense and antisense *K-ras* primers (sense: 5′-CAT TTC GGA CTG GGA GCG A-3′ antisense: 5′-TAC TAG GAC CAT AGG TAC ATC-3′) and TaKaRa Ex Taq® (TAKARA BIO INC., Shiga, Japan). DNA amplification was performed in an ABI 2027 thermal cycler (Applied Biosystems, Inc., Foster City, CA, USA). PCR products were purified with Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) according to the manufacturer's instructions. The purified PCR products were sequenced using an ABI 3730 automated sequencer (Applied Biosystems) at Operon Biotechnologies (Tokyo, Japan). The *K-ras* reference sequence was based on BC013572 from the National Center for Biotechnology Information database.

4.6.1. 1D-PAGE and immunostaining

1D-PAGE was performed according to Laemmli's method.[51] Briefly, sample solutions were heated, and then electrophoresis on 10% polyacrylamide gels (0.75 mm thick) was performed with prestained protein molecular weight markers obtained from New England BioLabs Ltd. (Ipswich, MA, USA). Proteins in gels were transferred to a polyvinylidene difluoride membrane (PVDF, Millipore Co., Bedford, MA, USA) using an electroblotter (Bio Craft, Tokyo, Japan). Antigen reactivities using antibodies against K-ras (Santa Cruz Biotechnology Inc., USA), phosphorylated-ERK, ERK, phosphorylated-Akt, Akt (Cell Signaling Technology Inc., Danvers, MA, USA) or β-actin (Santa Cruz) were visualized by chemiluminescence staining using a Western blotting starter Kit (ECL plus, GE Healthcare UK Ltd. Buckinghamshire, UK) according to the manufacturer's instructions. Gels were fixed and stained with Coomassie Brilliant Blue R-250 (Sigma).

4.7. Statistical analysis and presentation of results

The statistical significance of the data was evaluated by the Student's *t*-test. $p < 0.05$, $p < 0.01$, and $p < 0.001$ were considered significant. All experiments were repeated at least three times with consistent results, and results are shown as means ± SD of the three or four assays.

Conflicts of interest

None declared.

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