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Cancer Spheroid Proliferation Is Suppressed by a Novel Low-toxicity Compound, Pyra-Metho-Carnil, in a Context-independent Manner

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Abstract. *Background/Aim: In a screen of compounds to selectively suppress the growth of cancer spheroids, which contained mutant (mt) KRAS, NPD10621 was discovered and associated derivatives were investigated. Materials and Methods: Spheroid areas from HCT116-derived HKe3 spheroids expressing wild type (wt) KRAS (HKe3-wtKRAS) and mtKRAS (HKe3-mtKRAS) were treated with 12 NPD10621 derivatives and measured in three-dimensional floating (3DF) cultures. Several cancers were treated with NPD1018 (pyra-metho-carnil: PMC) in 3DF cultures. In a nude mouse assay, 50% cell growth inhibition (GI_{50}) values were determined. Results: From these 12 derivatives, PMC was the most effective inhibitor of HKe3-mtKRAS spheroid growth with the least toxicity. Furthermore, PMC-mediated growth suppression was observed in all tested cancer cell lines, independent of tissue context, driver gene mutations, and drug resistance, suggesting that the PMC target(s) was crucial for cancer growth in a context-independent manner. The GI_{50} value of PMC in nude mice assay was 7.7 mg/kg and nude mice that were administered 40 mg/kg PMC for 7 days did not show any abnormal blood cell count values. Conclusion: PMC is a low-toxicity compound that inhibits the growth of different tumor cell types.*

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Key Words: Cancer spheroid, KRAS, 3D floating culture, low-toxic, context-independent.

The human gene encoding *KRAS* GTPase is among the most frequently mutated cancer drivers (1). Mutant KRAS (mtKRAS), with activating missense mutations, constitutively activates numerous signaling pathways implicated in cell proliferation and survival, and therefore promotes cancer development, metastasis, and therapy resistance (2, 3). Oncogenic *KRAS* mutations are frequently identified in hard-to-treat cancers such as pancreatic cancers (86%-96% with oncogenic *KRAS* mutations) (4), colorectal cancers (CRCs) (40%-54%), and non-small cell lung cancers (15%-20%) (5, 6). Hence, considerable efforts have been focused on developing drugs targeting the activated KRAS or KRAS-related tumor-promoting pathways.

Recently, AMG510 (sotorasib), which directly targets the KRAS G12C mutation, was developed (7) and approved by the United States Food and Drug Administration. However, the G12C mutation accounts for approximately 10% of mutations in oncogenic mtKRAS (5, 7-12); therefore, oncogenic mtKRAS remains an "undruggable" target. In addition, while molecular-targeted drugs against KRAS-related signaling factors (such as BRAF and EGFR) are clinically approved and effective, intrinsic or acquired drug resistance is an important unsolved issue in the present cancer treatment. Therefore, novel agents, which overcome KRAS-targeted therapy limitations, are highly anticipated.

Different natural compounds have served as important discovery sources for effective anticancer agents, while canonical anticancer agents from natural products, such as

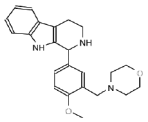
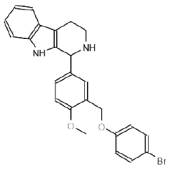
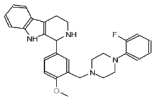
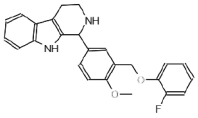
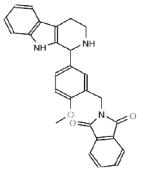
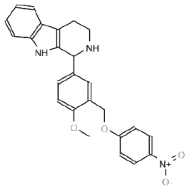
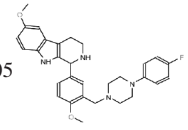
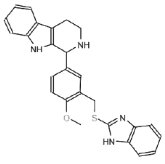
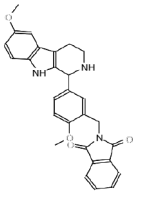
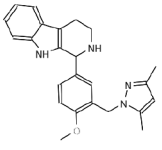
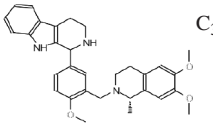
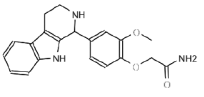
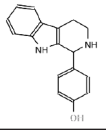
Table I. *Efficacy of growth suppression by PMC on several types of cancer spheroids.*

Tissue	Cell line	Type of RAS mutation	Other mutations of hub genes	Effect of PMC	KRAS gene effect
Lung (meta)	Calu-6	KRAS(Q61K)	TP53	Positive	–1,924979
Colorectal	HCT 116	KRAS(G13D)	CDKN2A PIK3CA CTNNB1	Positive	–1,577539
Pancreas	MiaPaka2	KRAS(G12C)	TP53 CDKN2A	Positive (high conc.)	–1,553559
Colorectal	SW 837	KRAS(G12C)	APC TP53	Positive (high conc.)	–1,550585
Colorectal (meta)	SW 620	KRAS(G12V)	APC TP53	Positive	–1,452649
Lung	A-427	KRAS (G12V, G12D)	CDKN2A CTNNB1	Positive	–1,299873
Pancreas (meta)	Hs 766T	KRAS(Q61H)	TP53 CDKN2A CTNNB1	Positive (high conc.)	–1,21037
Colorectal	DLD-1	KRAS(G13D)	APC TP53 PIK3CA	Positive (high conc.)	–1,12538
Colorectal	LS 180	KRAS(G12D)	APC CTNNB1 PTEN PIK3CA BRAF	Positive	–1,070264
Endometrium	HEC-1-B	KRAS(G12D)	APC TP53 PIK3CA BRAF	Positive (high conc.)	–0,9731834
Lung	A549	KRAS(G12S)	CDKN2A	Positive (high conc.)	–0,958262
Breast (meta)	MDA-MB-231	KRAS(G13D)	TP53 BRAF (G464V) CDKN2A	Positive (high conc.)	–0,9540346
Colorectal (meta)	LoVo	KRAS(G13D)	APC CDKN2A CTNNB1	Positive	–0,8052327
Gastric (meta)	Hs 746T	WT	APC TP53	Positive	–0,6759476
Colorectal	HCT-15	KRAS(G13D)	APC DCC TP53 PIK3CA	Positive (high conc.)	–0,5670731
Breast (meta)	MCF-7	WT	PIK3CA CDKN2A	Positive (high conc.)	–0,5231921
Colorectal (meta)	COLO 201	WT	APC CTNNB1 BRAF (V600E)	Positive	–0,4896021
Cervix	SiHa	WT		Positive	–0,4693851
Colorectal (meta)	CCK-81	WT	APC TP53 CTNNB1 PIK3CA BRAF	Positive	–0,4509773
Liver	Hep G2	NRAS(Q61L)	CTNNB1	Positive	–0,4485559
Prostate (meta)	DU145	WT	TP53 CDKN2A CTNNB1RB1	Positive (high conc.)	–0,4242528
Colorectal	COLO 205	WT	APC TP53 BRAF (V600E)	Positive	–0,4100692
Breast	HCC1937	WT	TP53 BRCA1 PTEN	Positive	–0,3932961
Breast	Hs 578T	HRAS(G12D)	TP53	Positive (high conc.)	–0,3919313
Colorectal	SW 48	WT	APC DCC CTNNB1 TP53 PIK3CA	Positive	–0,3740498
Cervix	C-33A	WT	DCC TP53 PTEN PIK3CA CTNNB1BRAF RB1	Positive	–0,347293
Breast (meta)	MDA-MB-468	WT	RB1 TP53 SMAD4 PTEN	Positive	–0,3325101
Prostate (meta)	LNCaP	WT	APC CTNBB1 PTEN	Positive	–0,3270123
Breast	BT549	WT	RB1 TP53 PTEN	Positive (high conc.)	–0,2206648
Ovarian	Caov-3	WT	TP53 PTEN	Positive (high conc.)	–0,050929
Colorectal	HKe3-mtKRAS	KRAS(G13D)	CDKN2A PIK3CA CTNNB1	Positive	unknown
Pancreas (meta)	Hs 700T	KRAS(G12C)	TP53	Positive	unknown
Endometrium	AN3 CA	KRAS(G12D)	TP53 PTEN	Positive	unknown
Colorectal	WiDr	WT	BRAF(V600E)	Positive	unknown
Skin	SK-MEL-28	WT	TP53 BRAF(V600E)	Positive	unknown
Kidney	A-498	WT	APC CDKN2A	Positive	unknown
Bladder	J82	WT	APC TP53 PIK3CA PTEN RB1	Positive (high conc.)	unknown
Breast	BT-20	WT	TP53 PI3CA RB1	Positive	unknown
Cervix	HeLa	WT	DCC	Positive	unknown

camptothecin (topoisomerase I inhibitor) and paclitaxel (mitotic inhibitor), are highly toxic and cause severe side effects (13). We previously developed a novel drug-screening system using three-dimensional (3D) floating (3DF) cultures to identify low-toxicity inhibitors of the mtKRAS-mediated oncogenic pathway. In this system, two HKe3-derived cell lines were used to examine if the compounds could selectively suppress cancer cell spheroid growth (14): one expressing wt KRAS (HKe3-wtKRAS; normal cell model) and another expressing KRAS G13D mutant (HKe3-mtKRAS; cancer cell model) with the same genetic backgrounds other than KRAS mutation. Using this system and natural product libraries, we previously identified several new compounds displaying selective growth-suppressive effects in cancer cells harboring mtKRAS but not in normal cells (15, 16). We also used this system to validate the anti-tumor effects of MK615 (Japanese apricot extract), apremilast (PDE4 inhibitor), and UHA6052 (resveratrol-derivative) against human CRC cells harboring mtKRAS (17-19).

In this study, we report the identification of NPD1018 (pyra-metho-carnil: PMC), from natural product libraries, as an effective low-toxicity inhibitor of HKe3-mtKRAS tumor growth in 3DF cultures and a nude mouse xenograft model. PMC has the potential to become a useful anticancer agent, as it exhibits strong efficacy toward many cancer cell types independent of tissue context, driver gene mutations, and drug resistance.

Table II. *List of NPD10621 derivatives*.

Name	Number	Structure	Formula	M.W.	Distance	Name	Number	Structure	Formula	M.W.	Distance
NPD10261	#102		$C_{23}H_{27}N_3O_2$	377,49	0	NPD1022	#109		$H_{23}BrN_2O_2$	463,37	0,43
NPD10259	#103		$C_{29}H_{31}FN_4O$	470,59	0,258	NPD10254	#110		$H_{23}FN_2O_2$	402,47	0,441
NPD9443	#104		$C_{27}H_{23}N_3O_3$	437,5	0,264	NPD981	#111		$_3H_{23}N_3O_4$	429,47	0,447
NPD71	#105		$C_{30}H_{33}FN_4O_2$	500,62	0,338	NPD591	#112		$_3H_{24}N_4OS$	440,57	0,455
NPD9444	#106		$C_{28}H_{25}N_3O_4$	467,52	0,349	NPD1018	#113		$_4H_{26}N_4O$	386,5	0,456
NP980	#107		$C_{31}H_{35}N_3O_3$	497,64	0,359	NPD10256	#114		$_3H_{21}N_3O_3$	351,4	0,464
NPD8264	#108		$C_{17}H_{16}N_2O$	264,32	0,395						

Materials and Methods

Compounds. A natural product compound library was provided by RIKEN NPDepo (Saitama, Japan). Chemical distances were determined using the Jaccard similarity index (16, 20). PMC (IUPAC Name: 1-{3-[(3,5-dimethylpyrazol-1-yl)methyl]-4-methoxyphenyl]-2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole) was synthesized by Kyushu-University, Graduate School of Pharmaceutical Sciences and Namiki Shoji Co., Ltd. (Tokyo, Japan).

Cell culture. HKe3, HKe3-wtKRAS, and HKe3-mtKRAS cultures were established and maintained as previously described (14, 16, 21). Other cells (Table I), including HCT116 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Human bladder cancer KK47 cells, cisplatin-resistant KK47 cells (KK47/DDP20), patient-derived melanoma cells, and a vemurafenib-resistant subline were obtained and maintained as previously described (22, 23).

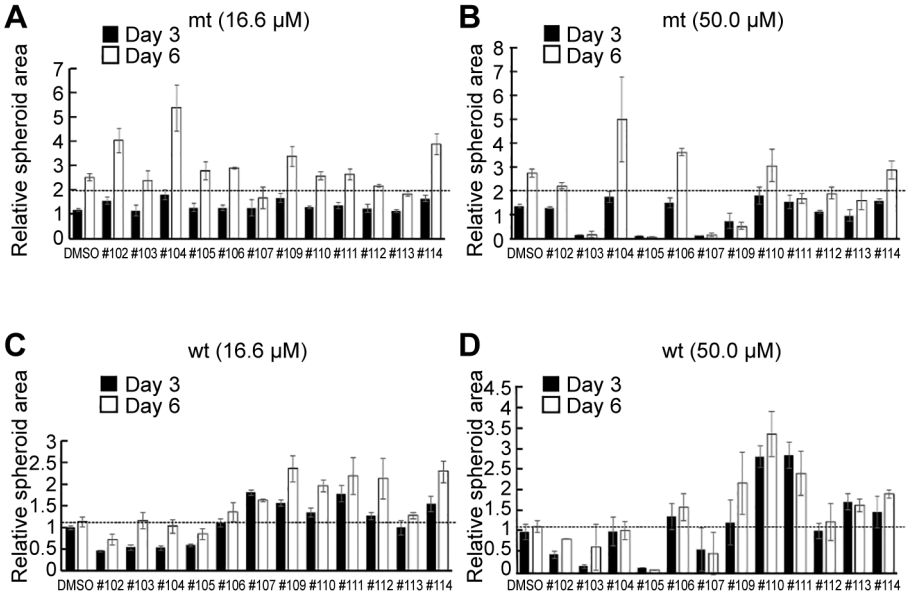


Figure 1. *NPD10621 derivatives inhibit HKe3-mutant (mt) KRAS spheroid growth. The relative spheroid area of HKe3-mt KRAS (A and B) and HKe3-wild type (wt) KRAS (C and D) cells treated with NPD10621 derivatives on day 3 and 6 compared with HKe3-wt KRAS treated with dimethyl sulfoxide (DMSO control) on day 3. The NPD10621 derivative concentration was 16.6 μM (A and C) and 50 μM (B and D).*

3DF cell culture. Cells were seeded in 96-well plates with round-bottoms and ultralow attachment surfaces (product number 7007; Corning Inc., Corning, NY, USA) and treated with derivatives at day 0 as described previously (14-16).

Spheroid area measurements. Photomicrographs of the cells were taken and analyzed using an IN Cell Analyzer 1000 (GE Healthcare, Little Chalfont, UK) and an IN Cell Developer Toolbox (GE Healthcare). Relative growth rates were calculated by comparing control spheroid areas on day 3.

Tumorigenicity assays. Four-week-old female SCID Hairless Outbred (SHO) mice (Crlj:SHO-*Prkdc*^{scid}*Hr*^{hr}) were purchased from Charles River Laboratories (Yokohama, Japan). For implantation, HCT116 cells were trypsinized and re-suspended in a 1:1 mixture of phosphate-buffered saline and Matrigel (BD Bioscience, Bedford, MA, USA). Further, a 100 μl aliquot containing 1.5×10^6 HCT116 cells was subcutaneously injected into the flank of mice as previously described (17).

Gene effect scores. The DepMap portal was used to determine *KRAS* gene effect scores, which was calculated using Chronos algorisms (24).

Data presentation. All the experiments were performed in triplicate and data are presented as mean±standard deviation.

Results

NPD10621 derivatives inhibit HKe3-mtKRAS growth in 3DF cultures. During the first screening of compounds from natural products, NPD10621 was identified as a candidate drug that specifically inhibited HKe3-mtKRAS spheroid growth but not that of HKe3-wtKRAS spheroids (data not shown). Using the chemical distances, 12 NPD10621 derivatives were selected from RIKEN natural product libraries (20) (Table II). Further, cells grown in 3DF cultures were treated with 16.6 μM and 50.0 μM derivatives (#103–#114) to examine their effects on cell proliferation. While the HKe3-mtKRAS spheroid areas treated with dimethyl sulfoxide (DMSO) were 2.53-fold larger than control HKe3-wtKRAS spheroids treated with DMSO on day 3, HKe3-mtKRAS spheroid areas treated with 16.6 μM #107 and #113 were 1.69- and 1.85-fold larger on day 6, respectively, when compared with control spheroids on day 3 (Figure 1A). This suggested that these derivatives suppressed cancer cell growth.

The areas of HKe3-mtKRAS spheroid treated with 50.0 μM #103, #105, #107, #109, #111, #112, and #113 were 0.19, 0.09-, 0.17-, 0.54-, 1.71-, 1.91-, and 1.63-fold larger on day 6, respectively, when compared with those of HKe3-wtKRAS spheroids treated with DMSO on day 3 (Figure 1B).

The areas of HKe3-wtKRAS spheroids treated with 16.6 μM #107, #109, #110, #111, #112, and #114 were on day 6 1.63-, 2.36-, 1.96-, 2.19-, 2.13-, and 2.30-fold larger, respectively, when compared with those of HKe3-wtKRAS spheroids treated with DMSO on day 3 (Figure 1C). The areas of HKe3-wtKRAS spheroid treated with 50.0 μM #106, #109, #110, #111, #113, and #114 were on day 3 1.59, 2.17-, 3.33-, 2.40-, 1.64-, and 1.91-fold larger, respectively, when compared with those of HKe3-wtKRAS spheroids

Table III. *Scoring method.*

Chemical No. (NPD No.)	A	B	C	D	E=A+B+C+D
	The area of HKe3-mtKRAS spheroids with 16.6 μM drugs at day6 is 0~2-fold increased:3p, 2~3-fold increased:2p, 3~4-fold increased:1p, over 4-fold increased:0p, compared to that of HKe3-wtKRAS spheroids with DMSO control at day3.	The area of HKe3-mtKRAS spheroids with 50.0 μM drugs at day6 is 0~2-fold increased:3p, 2~3-fold increased:2p, 3~4-fold increased:1p, over 4-fold increased:0p, compared to that of HKe3-wtKRAS spheroids with DMSO control at day3.	The area of HKe3-wtKRAS spheroids with 16.6 μM drugs at day6 is over 1.5-fold increased:2p, 1.1~1.5-fold increased:1p, 0~1.1-fold increased:0p, compared to that of HKe3-wtKRAS spheroids with DMSO control at day3.	The area of HKe3-wtKRAS spheroids with 50.0 μM drugs at day6 is over 1.5-fold increased:2p, 1.1~1.5-fold increased:1p, 0~1.1-fold increased:0p, compared to that of HKe3-wtKRAS spheroids with DMSO control at day3.	Total:10p
#102 (NPD10261)	0	2	0	0	2
#103 (NPD10259)	2	3	1	0	6
#104 (NPD9443)	0	0	0	0	0
#105 (NPD71)	2	3	0	0	5
#106 (NPD9444)	2	1	1	2	6
#107 (NP980)	3	3	2	0	8
#109 (NPD1022)	1	3	2	2	8
#110 (NPD10254)	2	1	2	2	7
#111 (NPD981)	2	3	2	2	9
#112 (NPD51)	2	3	2	1	8
#113 (NPD1018)	3	3	1	2	9
#114 (NPD10256)	1	2	2	2	7

treated with DMSO alone on day 3 (Figure 1D). These observations suggested that the toxicity of these derivatives was low in normal cells. To select the best compounds from these derivatives, we scored toxicity in the normal model (HKe3-wtKRAS) and growth suppression efficacy in the cancer model (HKe3-mtKRAS) at low or high doses (Table III). When combined, these results indicated that #113 (NPD1018) had the highest score suggesting that it is a good candidate for further analyses. We renamed NPD1018 as PMC from its three functional groups: pyrazole, methoxyphenyl, and β-carboline.

PMC suppress growth of several cancer spheroids with or without KRAS mutations. The parental HKe3 cells were derived from HCT116 cells by disrupting the *KRAS* G13D mutation by homologous recombination. PMC selectively suppressed HCT116 spheroid growth in a dose-dependent manner (Figure 2A). To examine PMC efficacy against other cell lines, 3DF cultures were treated with 5, 15, 45, and 90 μM (high concentration) PMC (Figure 2B and Table I). Notably, PMC suppressed not only cells with KRAS mutations, such as Calu-6 (colon) and SW620 (colon) cells, but also cells without KRAS mutations (Figure 2B). These cells had other driver mutations, such as BRAF [WiDr (colon) and SK-MEL28 (skin) cells] or PTEN [MDA-MB-468 (breast) and LNCaP (prostate) cells], suggesting that PMC could effectively inhibit the growth of cancer cells with different driver mutations. Indeed, PMC suppressed growth of all examined cell lines, independent of tissue or gene mutations (Table I). Furthermore, PMC suppressed the growth of cisplatin-resistant bladder cancer KK47 spheroids and patient-derived melanoma spheroids with vemurafenib resistance (Figure 2C) (22, 23). The effect of PMC on growth suppression was also independent of KRAS dependency (high KRAS dependency: KRAS gene effect <–0.5) (Table I). These results suggested that the PMC target(s) was independent of tissue context and driver gene mutations and was not closely associated with KRAS dependency.

The effects of PMC on in vivo human colorectal cancer cell (HCT116) tumorigenicity. HCT116 cells were subcutaneously injected into flanks of nude mice to examine the effects of PMC on HCT116 cell tumorigenicity. PMC was administered from day 0. In control mice, tumor volume was 2,362 mm^3 on day 7. In contrast, in mice treated with 10 mg/kg, 40 mg/kg, and 80 mg/kg PMC, tumor volumes were 829 mm^3, 525 mm^3, and 201 mm^3, respectively, on day 7 (Figure 3A). The 50% cell growth inhibition (GI_{50}) value was 7.7 mg/kg. Furthermore, mice that administered 40 mg/kg PMC for 7 days showed no abnormal blood cell count values (Figure 3B), suggesting that PMC inhibited *in vivo* tumor growth with low toxicity.

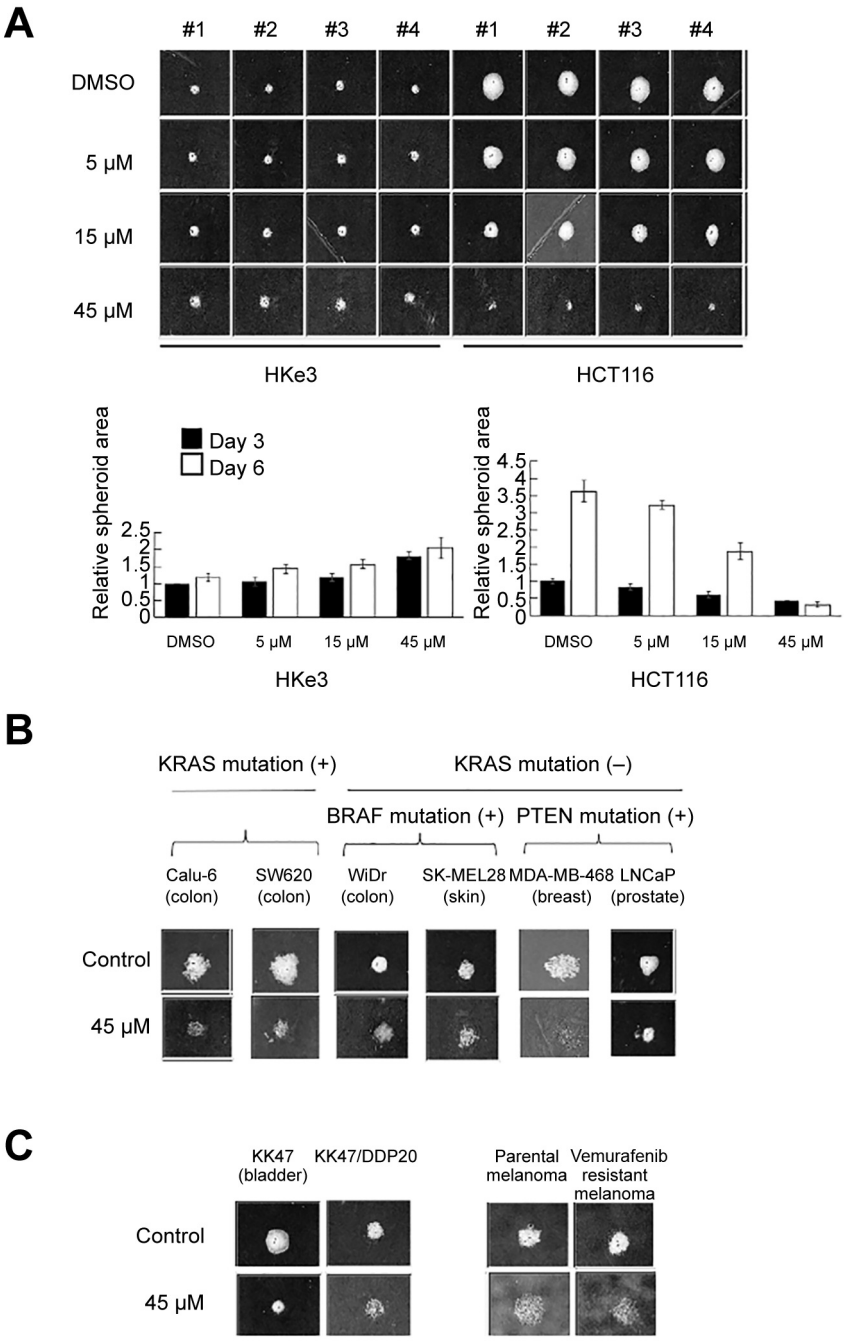


Figure 2. *Pyra-metho-carnil (PMC) inhibits cancer cell growth in three-dimensional floating (3DF) cultures. A: HCT116 or HKe3 cells were grown in 3DF cultures and treated with dimethyl sulfoxide (DMSO) or PMC (5, 15, and 45 μM) on day 0. Spheroid images of four replicates on day 6 are shown (upper panel). Relative spheroid areas on day 3 and 6 compared with HKe3 spheroid areas treated with DMSO on day 3 are shown (lower panel). B and C: Spheroid images of the indicated cancer cells treated with DMSO (control) or PMC (45 μM) on day 6 in 3DF cultures.*

Discussion

In this study, we identified PMC as a potent drug for the treatment of several cancers. Furthermore, PMC showed low toxicity and suppressed mtKRAS CRC growth *in vivo*. The PMC structure consists of β-carboline with methoxyphenyl and pyrazole at the C1 position. β-carbolines such as harmine are often identified in medicinal plants and exert antiproliferative effects toward several cancers (25, 26). In addition, β-carboline hybrids with pyrazole at the C3 position

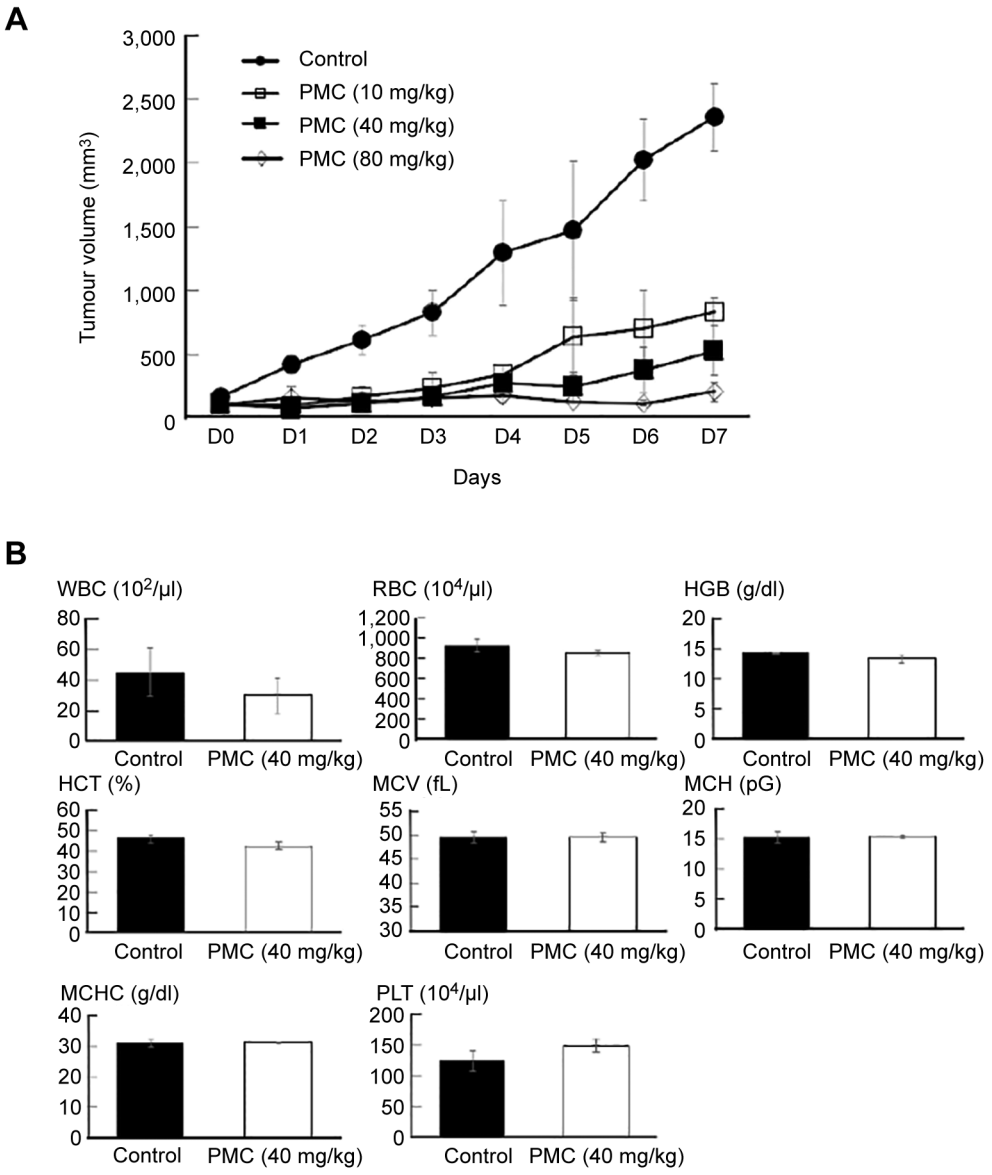


Figure 3. *The effects of pyra-metho-carnil (PMC) on human colorectal cancer (HCT116) cell tumorigenicity in vivo. A: HCT116 cells were subcutaneously injected into the flanks of nude mice. Relative tumor volumes are shown for mice treated with and without PMC. Derivatives were administered each day from day 0. B: Mice were administered DMSO (control) or 40 mg/kg PMC each day for 7 days. Blood biochemistry parameters were unchanged. WBC: White blood cell; RBC: red blood cell; HGB: hemoglobin; HCT: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; PLT: platelets.*

exhibit similar anticancer properties toward A549 (lung), DU145 (prostate), MCF-7 (breast), and Hela (cervix) cells through targeting topoisomerase I (27). PMC was effective in several *in vitro* cancer cell lines regardless of driver gene mutations (Figure 2 and Table I), suggesting that the direct PMC target(s) is not associated with hub genes that are canonically associated with mtKRAS-related signals. Moreover, PMC was effective in cell lines with wt KRAS and low KRAS dependency (KRAS gene effect >–0.5) (Table I). Furthermore, PMC was similarly effective toward parental and drug-resistant cells, suggesting the PMC target(s) was not involved in drug-resistance mechanisms (Figure 2C). PMC was also effective *in vivo* with low toxicity (Figure 3), suggesting that PMC was different for canonical anticancer drugs targeting cell proliferation with cytotoxicity. The identification of PMC targets will be crucial for the identification of the Achilles' heel of cancers. We are currently performing pull-down assays to determine PMC targets.

Conflicts of Interest

The Authors declare no conflicts of interest in relation to this study.

Authors' Contributions

K.Y., Kensuke N, and T.T. performed experiments, analyzed the data, and wrote the first manuscript draft. S.I., Kazuhiko N., R.Y., and M.S. participated in study design, data collection, and analysis. T.O. and S.S. conceived the idea, designed the study, interpreted the data, provided important intellectual content, and obtained final approval for manuscript submission.

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