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APPENDICES

APPENDIX A

Reagents for laboratory experiments

Reagents for RNA extraction

1. 70% Ethanol	**Final conc.**	**Volume (ml)**
Absolute ethanol	70%	35
DW		15

Mix the solution and store at room temperature.

2. Diethylpyrocarbonate (DEPC) treated water	**Final conc.**	**Volume (ml)**
DEPC (MW 162.141)	0.1%	1
DW		999

Mix well for at least 1 hr, let the solution stand at room temperature for an overnight then subject to autoclave, and stored at room temperature or aliquots store at -20 °C for longer time.

Reagents for reverse transcription (RT) reaction

1. RT solution I (freshly prepared)	**Final amount**	**Volume (μl)**
Total RNA	2.5 μg	11.5
Random hexamer (0.5 μg/μl)	0.5 μg	1

Mix the solution then spin down and incubated at 70 °C for 10 min.

After incubation, keep it on ice. Quickly spin down the solution and keep on ice.

2. RT solution II (freshly prepared)	**Final conc.**	**Volume (μl)**
5X RT buffer	20%	4
RNase inhibitor (RNasin®; 40U/μl)	1U/μl	0.5
10 mM dNTP mix	0.5 mM	1
MMuLV (reverse transcriptase)	10U/μl	1
RT solution I		12.5
	Total volume	**20**

Reagents for TaqMan real time RT-PCR

1. Reaction mixture	**Final conc.**	**Volume (µl)**
2X TaqMan® Universal PCR Master Mix (ABI)	50%	10
20X Gene Expression Assay Mix (each target genes; ABI)	5%	1
cDNA	25%	5
DEPC treated water		4
	Total volume	**20**

Reagents for SDS-PAGE and Western Blotting

1. 30% Acrylamide solution (30% Acrylamide, 0.8% Bis)	**Final conc.**	**Amount**
Acrylamide (MW 71.08)	30%	300 g
Bis (N, N,-methylenebisacrylamind, MW 154.17)	0.8%	8 g
Distilled water	up to	1000 ml

Dissolve the component in distilled water and adjust volume (using volumetric flask) upto 1000 ml or 500 ml. Filter through filter paper (Number 1 or 2_ low grade) and store in dark bottle (for light protection). Store at 4°C.

2. 1.5 M Tris-HCL pH 8.8	**Final conc.**	**Amount**
Tris (MW 121.14)	1.5 M	181.7 g

Dissolve the component in 800 ml distilled water and adjust pH to 8.8* by using Conc.HCl (~ 20 ml). Then adjust volume upto 1000 ml. Store at 4°C.

3. 0.5 M Tris-HCL pH 6.8	**Final conc.**	**Amount**
Tris (MW 121.14)	0.5 M	60.57 g

Dissolve the component in 800 ml distilled water and adjust pH to 6.8* by using Conc.HCl (~ 20 ml). Then adjust volume upto 1000 ml. Store at 4°C

4. 10% (w/v) SDS solution	**Final conc.**	**Amount**
SDS (MW 288.38)	10%	5 g
Distilled water	up to	50 ml

Filter with 0.22 □m filter and keep at room temperature.

5. 10% Ammonium persulfate (APS) (Freshly prepare)	**Final conc.**	**Amount**
Ammonium persulfate (MW 71.08)	10%	0.5 g
Distilled water	up to	5 ml

6. Water-saturated buthanol	**Final conc.**	**Volume**
N, I or t- buthanol	-	50 ml
Distilled water	-	10 ml

Shake well and store at room temperature.

7. 10 x SDS buffer for electrophoresis	**Final conc.**	**Volume**
Trisma base (MW 121.14)	250 mM	30.25 g
Glycine (MW 75.07)	1.92 M	144.10 g
SDS (MW 288.38)	1.0 % (w/v)	10 g
Distilled water	up to	1000 ml

Shake well and store at room temperature

8. 4x SDS loading buffer (4x sample buffer)	**Final conc.**	**Volume**
1M Tris- HCL pH 6.8	0.25 M	2.5 ml
SDS	8%	0.8 g
Glycerol	40%	4 ml
2-mercaptoethanol	20%	2 ml
Distilled water	up to 10 ml	< 1.5 ml

Dissolve all of components and add bromophenol blue 0.0001 g (estimate)

Dilute the solution with PBS to 3x, 2x and 1x SDS loading buffer. Aliquot and keep at - 20 °C.

9. TBS	**Final conc.**	**Volume**
1 M Tris – HCL pH 7.4	30 ml	10 ml
5 M NaCl	60 ml	20 ml
Distilled water	60 ml	1000 ml

Shake well and store at room temperature

10. TBS-T (0.1 % Tween 20 in TBS)	**Final conc.**	**Volume**
TBS		500 ml
Tween-20	0.1%	500 µl

Shake well and store at room temperature

11. Tris-Glycine buffer	**Final conc.**	**Volume**
Tris (MW 121.14)	25 mM	3 g
Glycine	192 mM	14.4 g
DW	-	600 ml
Methanol	10%	100 ml
Distilled water	up to	1000 ml

Shake well and store at room temperature

12. 5 % skim milk in TBS (Freshly prepare)	**Final conc.**	**Volume**
Skim milk	5%	1.5 g
TBS		30 ml

Shake well and store at room temperature

13. 0.1% CBB (Coomasie Brilliant Blue)	**Final conc.**	**Volume**
0.2% CBB stock solution	0.1%	200 ml
20% Acetic acid	10%	200 ml

Shake well and store at room temperature

14. Destain solution	**Final conc.**	**Volume**
Ethanol	25%	750 ml
Acetic acid	8%	240 ml
Distilled water	up to	3000 ml

Shake well and store at room temperature

Reagent for cell lysis buffer

1. RIPA buffer	**Final conc.**	**Volume**
1 M Tris-HCl (pH 8.0)	20 mM	0.2 ml
0.5 M EDTA	5 mM	0.1 ml
5 M NaCl	150 mM	0.3 ml
10% NP-40	1%	1 ml
10% SDS	0.025%	0.025 ml
1 M NaF	10 mM	0.1 ml
0.2 M $NaVO_4$	2 mM	0.1 ml
Proteinase inhibitor cocktail (100X)	1X	0.1 ml
Distilled water	up to	10 ml

Shake well and store at -20 °C

Reagent for Protein kinase A assay

1. PKA extraction buffer	**Final conc.**	**Volume**
1M Tris-HCl (pH 7.4)	25 mM	2.5 ml
EDTA (MW 372.24)	0.5 mM	19 mg
EGTA (MW 380.35)	0.5 mM	19 mg
β-mercaptoethanol	10 mM	0.07 ml
Leupeptin (2.5 mg/ml)	1 µg/ml	0.04 ml
Aprotinin (1 mg/ml)	1 µg/ml	0.1 ml
Distilled water	up to	100 ml

Store at -20 °C. Just before use, add 0.5 ml of PMSF stock solution (100 mM PMSF in

2. PKA dilution buffer	**Final conc.**	**Volume**
K_2PO_4 (pH 6.8) (MW 212.27)	350 mM	3.7 g
DTT (MW 154.24)	0.1 mM	0.75 mg
Distilled water	up to	50 ml

Shake well and store at room temperature
100% ethanol) per 100 ml of PKA extraction buffer.

3. 50 mM Tris-HCl (pH 8.0)	**Final conc.**	**Volume**
Tris (MW 121.14)	50 mM	6.057 g

Dissolve the component in 800 ml distilled water and adjust pH to 8.0* by using Conc.HCl. Then adjust volume up to 1000 ml. Store at 4°C

Reagent for Sulforhodamin B assay

1. 10% w/v TCA	**Final conc.**	**Amount**
Trichloroacetic acid	10%	5 g
DW	up to	50 ml

Store at 4 °C

2. 0.4% Sulforhodamine B in 1% acetic acid	**Final conc.**	**Amount**
Sulforhodamine B	0.4%	0.2 g
1% acetic acid		50 ml

3. 1% acetic acid	**Final conc.**	**Volume**
100% acetic acid	1%	10 ml
DW	up to	1000 ml

4. 10 mM unbuffed Tris base pH 10.5	**Final conc.**	**Amount**
Trisma-base (MW 121.1)	10 mM	121.14 mg

Dissolve the component in 60 ml distilled water and adjust pH to 6.8* by using Conc.HCl. Then adjust volume up to 100 ml. Store at room temperature.

Reagent for cell culture

1. Ham's F12 media	**Final conc.**	**Amount**
Ham's F12 Nutrient mixture (Gibco)		10.6 g
Solid $NaHCO_3$	14 mM	1.176 g
Sterile distilled water		900 ml
Adjust pH to 7.3-7.4 by using 1N HCl		
Penicillin/streptomycin		10 ml
Fetal bovine serum		100 ml
Stored at 4 °C		

2. 1X Phosphate buffer saline (PBS)	**Final conc.**	**Amount**
$NaHPO_4$	12 mM	1.48 g
KH_2PO_4	3 mM	0.43 g
NaCl	0.12 M	7.2 g
Adjust pH to 7.4		
DW	up to	1000 ml
Autoclave at 121°C, 15 psi for 20 min and keep at 4 °C		

APPENDIX B

Supplementary data

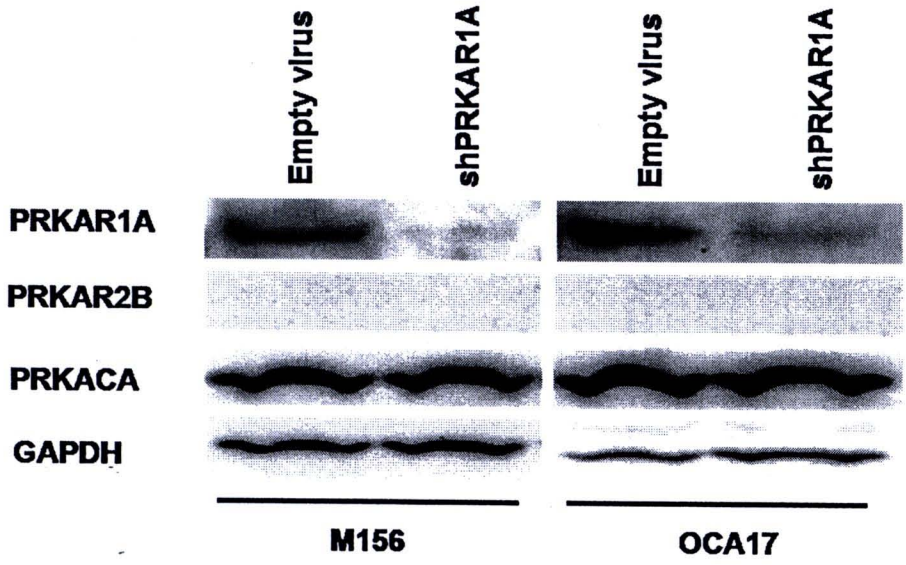


Figure B.1 Effect of shPRKAR1A on PRKACA expression.

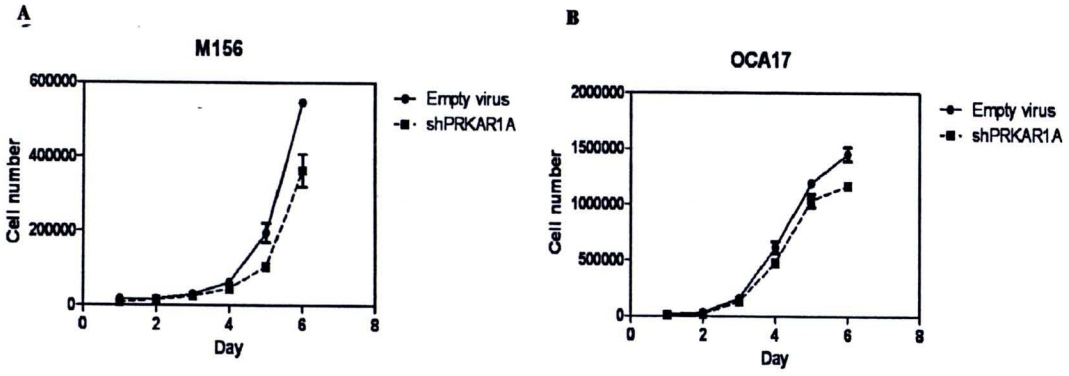


Figure B.2 Growth curve of PRKAR1A silencing cells in M156 (A) and OCA17 (B). The number of cells was determined by cell counting at indicated time. Each point represents average values from triplicates.

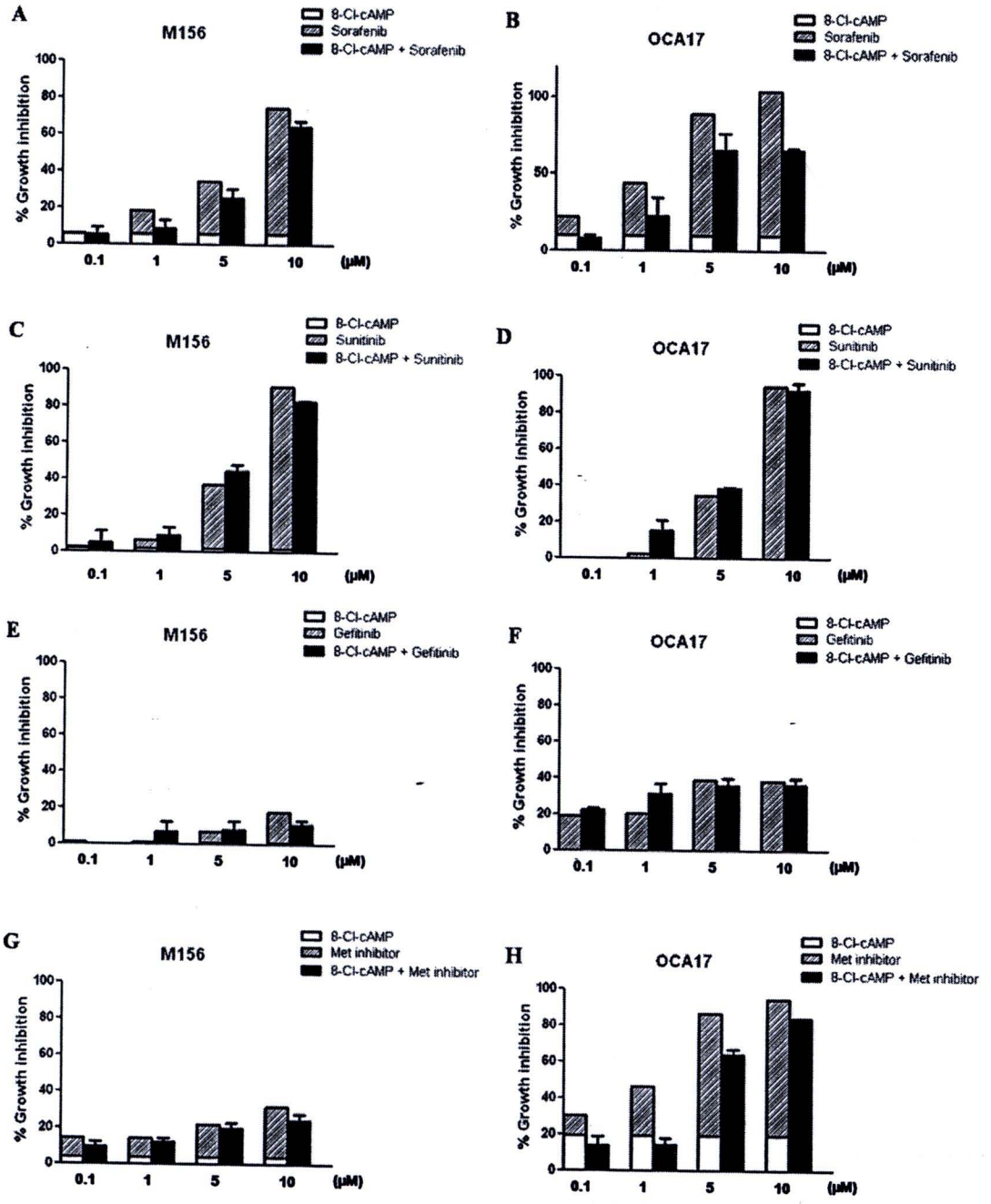


Figure B.3 Antiproliferative effect of 8-Cl-cAMP and protein kinase inhibitors in CCA cell lines. Cells were treated with 100 μM 8-Cl-cAMP plus different concentration of sorafenib (A and B), sunitinib (C and D), gefitinib (E and F) and Met inhibitor (G and H) for 72 h.

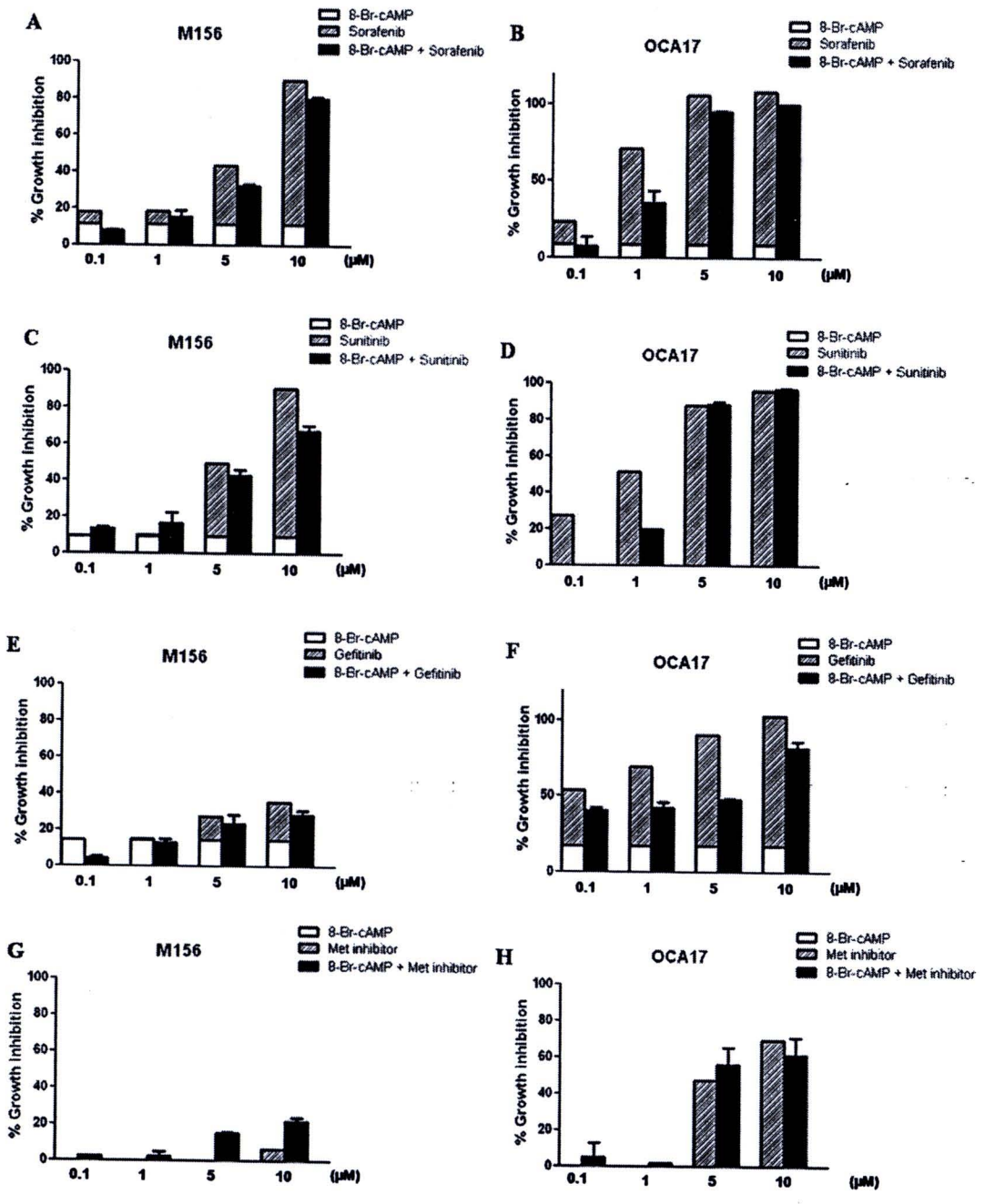


Figure B.4 Antiproliferative effect of 8-Br-cAMP and protein kinase inhibitors in CCA cell lines. Cells were treated with 100 μM 8-Br-cAMP plus different concentration of sorafenib (A and B), sunitinib (C and D), gefitinib (E and F) and Met inhibitor (G and H) for 72 h.

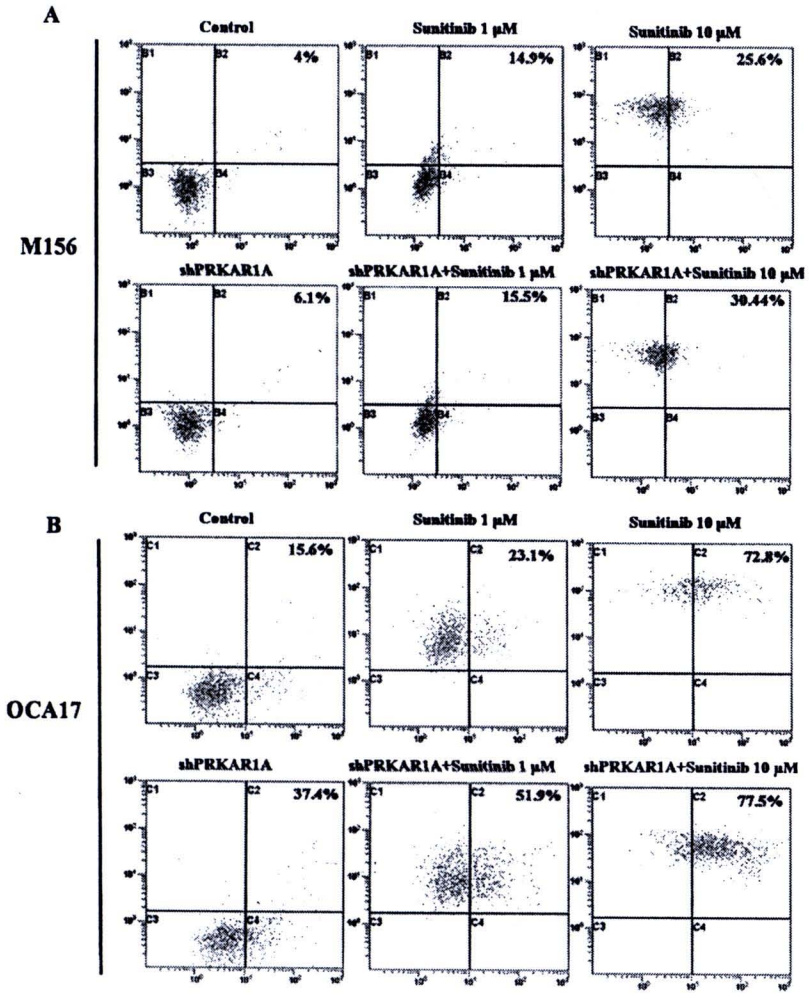


Figure B.5 Effect of shPRKAR1A in combination with 1μM and 10μM sunitinib on induction of apoptosis in M156(A) and OCA17(B). Annexin-V and PI (propidium iodide) staining was performed, followed by flow cytometry.

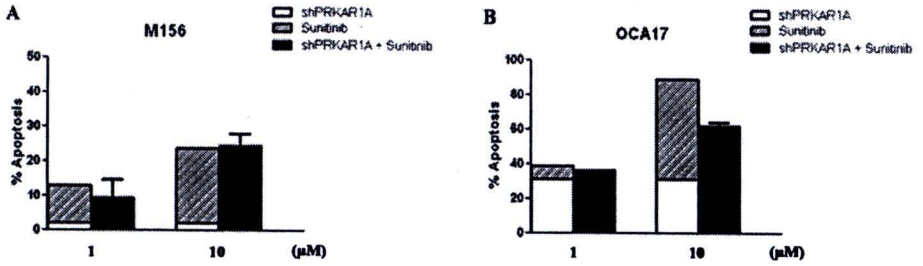


Figure B.6 Percentage of apoptosis of shPRKAR1A in combination with sunitinib in M156(A) and OCA17(B). Annexin-V and PI (propidium iodide) staining was performed, followed by flow cytometry.

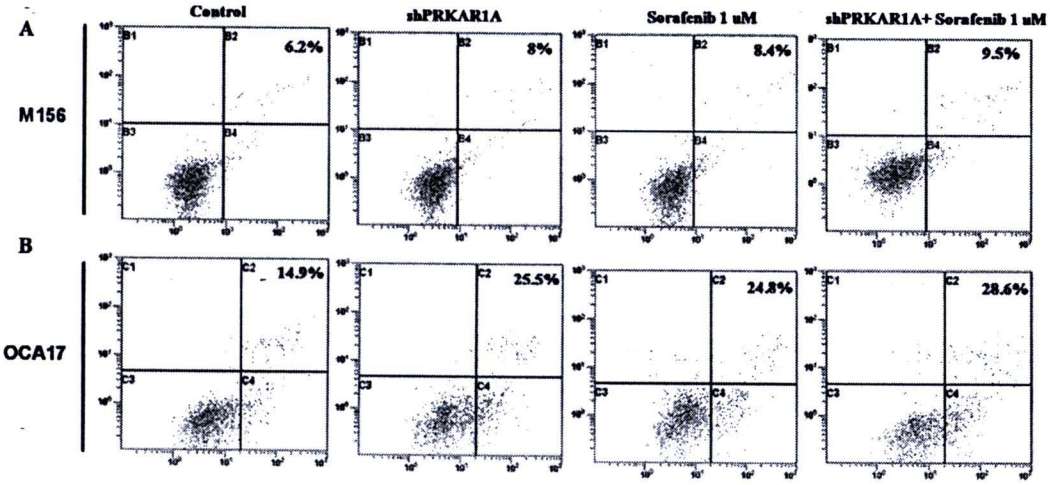


Figure B.7 Effect of shPRKAR1A in combination with 1μM sorafenib on induction of apoptosis in M156(A) and OCA17(B). Annexin-V and PI (propidium iodide) staining was performed, followed by flow cytometry.

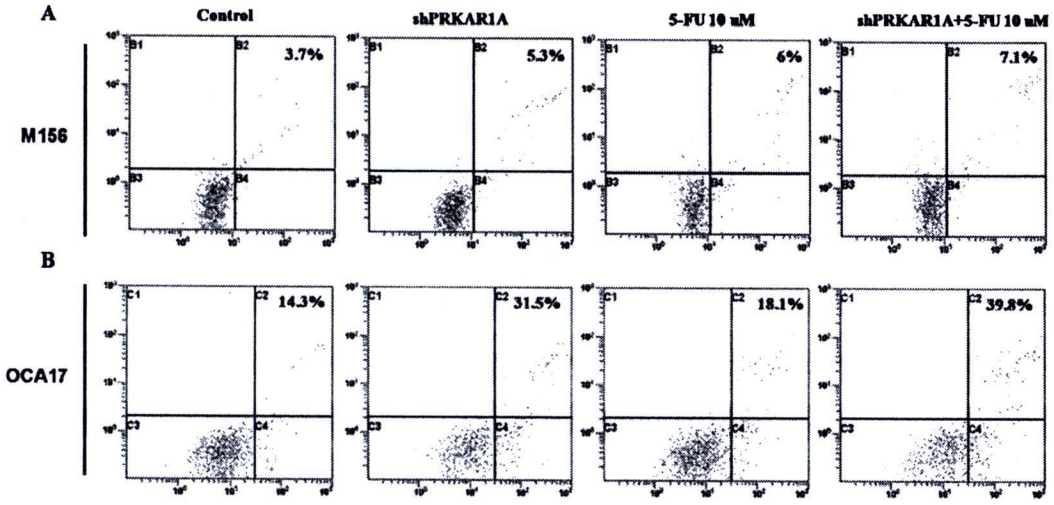


Figure B.8 Effect of shPRKAR1A in combination with 10μM 5-FU on induction of apoptosis in M156 (A) and OCA17(B). Annexin-V and PI (propidium iodide) staining was performed, followed by flow cytometry.

Table B.1 Effect of PRKAR1A silencing cells in combination with protein kinase inhibitors or chemotherapeutic drugs on CCA cells growth inhibition. This table represents growth inhibitory effect of either each agent alone or in combination and the expected percentage growth inhibition if drugs are additive when used in the combination.

		% Growth inhibition									
		M156					OCA17				
Drugs	Conc. (μM)	Control	shPRKAR1A	Drug	shPRKAR1A + drug	Expected additive effect	Control	shPRKAR1A	Drug	shPRKAR1A + drug	Expected additive effect
Sorafenib	0	0	29				0	42			
	0.01			8	32	37			5	47	47
	0.1			9	35	38			19	59	61
	1			11	37	40			27	75	69
	10			72	12	101			86	86	128
Sunitinib	0	0	31				0	25			
	0.01			3	33	34			0	30	25
	0.1			4	36	35			0	38	25
	1			8	45	39			7	36	32
	10			50	74	84			73	84	98

Table B.1 Effect of PRKAR1A silencing cells in combination with protein kinase inhibitors or chemotherapeutic drugs on CCA cells growth inhibition. This table represents growth inhibitory effect of either each agent alone or in combination and the expected percentage growth inhibition if drugs are additive when used in the combination (Cont.).

		% Growth inhibition									
		M156					OCA17				
Drugs	Conc. (μM)	Control	shPRKAR1A	Drug	shPRKAR1A + drug	Expected additive effect	Control	shPRKAR1A	Drug	shPRKAR1A + drug	Expected additive effect
Gefitinib	0	0	30				0	23	9	37	32
	0.01			0	39	30			18	49	41
	0.1			0	41	30			24	50	47
	1			1	41	31			35	57	58
	10			23	54	53					
Met inhibitor	0	0	42				0	30			
	0.01			2	43	44			0	44	30
	0.1			6	46	48			0	47	30
	1			7	47	49			10	53	40
	10			28	56	70			60	85	90

Table B.1 Effect of PRKAR1A silencing cells in combination with protein kinase inhibitors or chemotherapeutic drugs on CCA cells growth inhibition. This table represents growth inhibitory effect of either each agent alone or in combination and the expected percentage growth inhibition if drugs are additive when used in the combination (Cont.).

		% Growth inhibition									
		M156					OCA17				
Drugs	Conc. (μM)	Control	shPRKAR1A	Drug	shPRKAR1A + drug	Expected additive effect	Control	shPRKAR1A	Drug	shPRKAR1A + drug	Expected additive effect
Paclitaxel	0	0	26				0	40			
	0.01			16	27	42			1	40	41
	0.1			23	32	49			1	46	41
	1			28	40	54			2	48	42
	10			31	48	57			14	53	54
Doxorubicin	0	0	33				0	30			
	0.01			5	32	38			0	30	30
	0.1			6	36	39			1	29	31
	1			31	67	64			35	30	65
	10			63	75	96			29	37	59

Table B.1 Effect of PRKAR1A silencing cells in combination with protein kinase inhibitors or chemotherapeutic drugs on CCA cells growth inhibition. This table represents growth inhibitory effect of either each agent alone or in combination and the expected percentage growth inhibition if drugs are additive when used in the combination (Cont.).

% Growth inhibition											
		M156					OCA17				
Drugs	Conc. (μM)	Control	shPRKAR1A	Drug	shPRKAR1A + drug	Expected additive effect	Control	shPRKAR1A	Drug	shPRKAR1A + drug	Expected additive effect
5-FU	0	0	35				0	36			
	0.01			0	39	65			0	35	36
	0.1			0	42	65			3	46	39
	1			5	46	40			12	59	48
	10			34	67	69			43	72	79
Gemcitabine	0	0	7				0	6			
	0.01			0	9	7			0	6	6
	0.1			75	78	153			2	6	8
	1			82	81	88			31	37	37
	10			88	89	95			97	100	103

Table B.2 Effect of PRKAR1A silencing cells in combination with protein kinase inhibitors or chemotherapeutic drugs on induction of apoptosis. This table represents percentage apoptosis of either each agent alone or in combination and the expected percentage apoptosis if drugs are additive when used in the combination.

		% Apoptosis									
		M156					OCA17				
Drugs	Conc. (μM)	Control	shPRKAR1A	Drug	shPRKAR1A + Drug	Expected additive effect	Control	shPRKAR1A	Drug	shPRKAR1A + drug	Expected additive effect
Sorafenib	1	0	1.81	2.19	3.25	4	0	10.62	9.93	13.66	20.55
	10		1.81	6.14	9.28	7.95		10.62	24.53	35.26	35.15
Sunitinib	1	0	2.06	10.91	11.44	12.97	0	31.305	7.48	36.29	38.79
	10		2.06	21.55	26.38	23.6		31.305	57.38	63.41	88.69
5-FU	10	0	1.6	2.36	3.43	3.96	0	17.14	3.8	25.49	20.94
	20		1.6	3.59	4.72	5.19		17.14	10.87	28.46	28.01

1. KKU-M156

Cell Name	**KKU-M156**
Species	*Homo Sapiens*
Sex	Male
Age	68-year-old
Diagnosis	Cholangiocarcinoma
Histological type	Moderately differentiated
Establisher	Sripa, B. et al.
Medium	Ham's F-12 medium with 10% fetal bovine serum
Passage Method	Cells treated with 0.25% Trypsin-EDTA for 5 min at 37°C

2. KKU-OCA17

Cell Name	**KKU-OCA17**
Species	*Homo Sapiens*
Sex	Male
Age	38-year-old
Diagnosis	Cholangiocarcinoma
Histological type	Well differentiated
Establisher	Sripa, B. et al.
Medium	Ham's F-12 medium with 10% fetal bovine serum
Passage Method	Cells treated with 0.25% Trypsin-EDTA for 5 min at 37°C

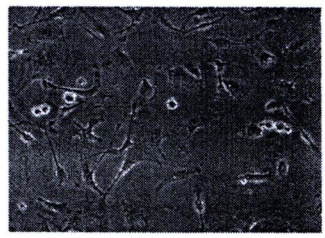
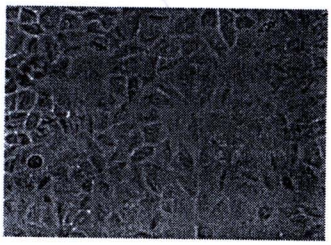


Figure B.9 Human CCA cell lines which were used in this study.

3. KKU100

Cell Name	**KKU-100**
Species	*Homo Sapiens*
Sex	Female
Age	65-year-old
Diagnosis	Cholangiocarcinoma
Histological type	Poorly differentiated
Doubling time	36 h
Establisher	Sripa, B. et al.
Medium	Ham's F-12 medium with 10% fetal bovine serum
Passage Method	Cells treated with 0.25% Trypsin-EDTA for 5 min at 37°C



4. KKU-M214

Cell Name	**KKU-M214**
Species	*Homo Sapiens*
Sex	Male
Age	52-year-old
Diagnosis	Cholangiocarcinoma
Histological type	Well differentiated
Doubling time	23 h
Establisher	Sripa, B. et al.
Medium	Ham's F-12 medium with 10% fetal bovine serum
Passage Method	Cells treated with 0.25% Trypsin-EDTA for 5 min at 37°C

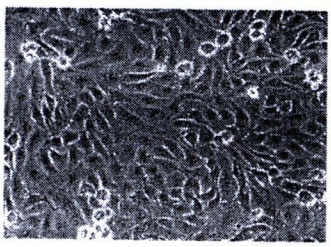


Figure B.9 Human CCA cell lines which were used in this study. (Cont.).

APPENDIX C

Researchpublications & Presentations

1. RESEARCH PUBLICATIONS

1. Sirinun Juntana, Watcharin Loilome, Nisana Namwat, Raynoo Thanan, Apinya Jusakul, Janpen Puetkasichonpasutha, Sasithorn Yooyeun, Gregory J. Riggins, Puangrat Yongvanit. Antiproliferative effect induced by targeting of PRKAR1A/PKAI and chemotherapeutic drug on human cholangiocarcinoma cell lines. Proceeding. The 2nd Biochemistry and Molecular Biology (BMB) Conference, 140-143.
2. Watcharin Loilome, Nisana Namwat, Raynoo Thanan, Anchalee Techasen, Sirinun Juntana, Puangrat Yongvanit. Antiproliferative effect of PRKAR1A gene suppression by RNA interference in cholangiocarcinoma cell line., KKU Res J, 903-910.
3. Watcharin Loilome, Sirinun Juntana, Nisana Namwat, Anucha Puapairoj, Paiboon Sithithaworn, Masanao Miwa, Hideyuki Saya, Gregory J. Riggins and Puangrat Yongvanit. PRKAR1A is over- expressed and represents a possible therapeutic target in-human cholangiocarcinoma. Submitted to *Int J Cancer* (Under revision).

2. RESEARCH PRESENTATIONS

1. Sirinun Juntana *et al.* Antiproliferative effect induced by targeting of PRKAR1A/PKAI and chemotherapeutic drug on human cholangiocarcinoma cell line. The 2nd Biochemistry and Molecular Biology (BMB) Conference : Biochemistry and Molecular Biology for Regional Sustainable Development. Khon Kaen University, Khon Kaen Thailand 2009. ***(Poster presentation)*** May 7-8.
2. Sirinun Juntana *et al.* PRKAR1A acts as a possible therapeutic target in human cholangiocarcinoma. Annual Meeting of Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand 2009. ***(Oral presentation)*** October 15.

VITAE

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2003-2006	Bachelor Degree of Science (Medical technology, Second Class Honor) Faculty of Associated Medical Science, Khon Kaen University, Khon Kaen, Thailand
2007-2010	Graduate student of Master degree in Medical Biochemistry Program, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand
Sep 2009-Jan 2010	Exchange research student, Department of Neurological surgery, School of Medicine, Johns Hopkins University, Baltimore, MD, USA

Scholarship:

2007-2008	Liver fluke and Cholangiocarcinoma Research Center Scholarship
Sep 2009- Jan 2010	University Mobility in Asia and the Pacific (UMAP) Scholarship