CHAPTER III

PART II: ANTIPROLIFERATIVE EFFECT INDUCED BY TARGETING OF PRKAR1A/PKAI IN COMBINATION WITH CHEMOTHERAPEUTIC DRUGS OR PROTEIN KINASE INHIBITORS ON HUMAN CHOLANGIOCARCINOMA CELL LINES

3.1 Introduction

Cholangiocarcinoma (CCA) is a highly invasive/metastasis malignancy that is difficult to be diagnosed until the advanced or disseminated stage, resulting in poor prognosis. At present, only surgical resection of all detectable tumors is correlated with the improvement in 5-year survival (Ohtsuka *et al.*, 2003; Uttaravichien *et al.*, 1999). However, a complete resection is often impossible, typically resulting in subsequent metastasis and local recurrence (Olnes and Erlich, 2004). Improvements in the survival of CCA patients are probably not only rely on more aggressive or advanced surgical techniques but also from the improvement in treatment with chemotherapy. Chemotherapy is thus considered a preferred treatment option in order to prevent postsurgical cancer recurrence (Todoroki, 2000). However, treatment options of advanced CCA are unsatisfactory and new therapeutic approaches are challenging.

Medical treatment of cancer is mainly based on the use of chemotherapeutic drugs acting on intracellular targets that are generally common to both cancer and normal cells. However, the therapeutic benefits derived from such regimens seem to have limitation because cancerous cells are commonly resisted to chemotherapeutic drugs (Krishan and Arya, 2002; Lonning, 2003; Tannock, 2001). Therefore, the inhibition of molecular targets relevant for the process of neoplastic transformation and progression represents a new challenging opportunity in cancer treatment (Sathornsumetee *et al.*, 2007; Sawyers, 2004). Recently, the combination therapy is highly desirable in cancer treatment including CCA. Combinations of two or more drugs can improve the efficacy of cancer treatment (Doval *et al.*, 2004; Huether *et al.*,

2007; Malik *et al.*, 2003) and future cancer therapy strategies may base on the integration of conventional therapies with novel inhibitors of signals involved in cancer development.

Recently, our previous study in chapter II was found that PRKAR1A/PKAI is overexpressed and plays an important role(s) in regulating CCA cell growth. This work also indicates that abrogation of PRKAR1A expression by RNA interference technology leads to induce CCA cells growth inhibition and apoptosis. The others have shown that overexpression of PRKAR1A/PKAI pathway is involved in neoplastic transformation and tumor growth of many types of cancer including CCA that is proposed to act as a potential drug target for CCA treatment. There is growing evidence supporting that selective inhibition of PKAI by the use of site selective cAMP analogue 8-Cl-cAMP and a series of modified antisense oligonucleotides targeting PRKAR1A (AS-PKAI) alone or in combination with other drugs can be a promising therapeutic innovation toward treatment of cancer. Both 8-Cl-cAMP and antisense PRKAR1A are able to inhibit PKAI expression and function and to promote PKAII formation, causing cancer cell growth arrest, *in vitro* and *in vivo*, in a wide variety of cancer cell types (Cho-Chung *et al.*, 1995; Nesterova and Cho-Chung, 1995; Rohlff *et al.*, 1993; Tortora *et al.*, 1991).

In combination approach, a number of experimental studies have demonstrated that selective inhibition of PRKAR1A/PKAI has a synergistic growth inhibitory effect with chemotherapeutic drugs. 8-Cl-cAMP as well as the AS-PKAI, are able to cooperate with a variety of anticancer drugs, such as taxanes, topoisomerase II inhibitors, and platinum derivatives, causing a synergistic antitumor activity associated with increased apoptosis in a wide variety of human cancer types *in vitro* and in nude mice bearing human cancer xenografts (Tortora *et al.*, 1997a; Tortora and Ciardiello, 2002). Furthermore, antisense PRKAR1A Gem231 in combination with the cytotoxic drug, hydroxycamptothecin (HCPT) have shown synergistic effects of tumor cell growth inhibition and apoptosis induction in colon and prostate cancer cell lines (Cho and Cho-Chung, 2003). In particular study, the combined agents resulted in up-regulation of pro-apoptotic proteins, Bax and Bad and down-regulation of anti-apoptotic protein, Bcl-2. In addition, it was found either change in cell morphology by exhibiting a flattened shape and an increase cytoplasm: nucleus ratio or changes

indicatives of apoptosis, such as chromatin condensation, nuclear fragmentation and increased apoptotic nuclei count. Besides, treatment with a combination of 8-Cl-cAMP and sulfinosine resulted in synergistic effects on growth inhibition, cell cycle arrest, and induction of apoptosis of human neuroblastoma cell line (Jankovic *et al.*, 2006).

Several studies have revealed a functional interaction between neoplastic transformation involving the epidermal growth factor receptor (EGFR) and PKAI. This may provide the basis for the development of a therapeutic strategy based on the combination of their selective inhibitors (Tortora and Ciardiello, 2000). The blockade of EGFR and PKAI signaling pathways by specific inhibitors, PD153035 and Rp-cAMP, respectively lead to a synergistic cell growth inhibition of prostatic cancer cells concomitant with an arrest in G1 phase of cell cycle and also caused an increase apoptotic/necrotic death of these prostatic cancer cells when compared with drug alone (Mimeault *et al.*, 2003). Moreover, 8-Cl-cAMP, in combination with a monoclonal antibody blocking the EGFR, synergistically inhibits the growth of different human cancer cell lines *in vitro* and *in vivo* (Ciardiello *et al.*, 1995; Ciardiello *et al.*, 1996).

Taken together of the above information, we sought whether selective down-regulation of PRKAR1A/PKAI by shPRKAR1A or site selective cAMP analogues (8-Cl-cAMP, 8-Br-cAMP) in combination with various chemotherapeutic drugs or protein kinase inhibitors has any cooperative effect on cell growth inhibition in CCA cell lines.

3.2 Materials and methods

3.2.1 Cell lines and cell culture

Two stable PRKAR1A knockdown human CCA cell lines; KKU-OCA17, KKU-M156 and their empty viral transfection control which were constructed by lentivirus stable knockdown technique as described in chapter II and two parental CCA cell lines; KKU-OCA17, KKU-M156 were cultured in HAM-F12 (Gibco/BRL, Grand Island, NY) supplemented with 10% inactivated fetal bovine serum, 2 mg/ml sodium bicarbonate and 1% antibiotic-antimycotic solution (Life Technologies, Inc., Gaithersburg, MD). The cultured cell lines were incubated at 37°C in a humidified incubator maintained with an atmosphere of 5% CO_2. Subculture was done when

the cell reached the confluent stage and the media were changed once every two to three days.

3.2.2 cAMP analogues, small molecule kinase inhibitors and chemotherapeutic drugs

The site-selective cAMP analogues, 8-Cl-cAMP and 8-Br-cAMP were purchased from Calbiochem (La Jolla, CA) and dissolved in water. Protein kinase inhibitors; sorafenib, sunitinib were purchased from LC Laboratories (Woburn, MA). Gefitinib was from AstraZeneca plc (London, UK) and Met inhibitor was from Calbiochem (La Jolla, CA). All protein kinase inhibitors were dissolved in DMSO. Chemotherapeutic drugs were purchased from Sigma (St. Louis, MO). 5-Fluorouracil (5-FU) and paclitaxel were dissolved in NH_4OH and 50% methanol, respectively. Doxorubicin was dissolved in water. All drugs were prepared at stock concentration of 10 mM and stored at -20°C until used.

3.2.3 Combination treatment studies

3.2.3.1 Combination of PRKAR1A silencing CCA cell lines and drugs treatment

To test the chemosensitivity of PRKAR1A silencing combine with protein kinase inhibitors (sorafenib, sunitinib, gefitinib and met inhibitor) or chemotherapeutic drugs (5-fluorouracil (5-FU), doxorubicin and paclitaxel), PRKAR1A stable knockdown CCA cell lines (M156 and OCA17) and their empty viral transfection control were treated with various concentrations of each drug for 72 h. Cell proliferation and apoptosis were further evaluated by sulforhodamine B (SRB) assay and flow cytometry. The antiproliferative and apoptotic induction effects of the combinations were compared to their empty viral transfection control.

3.2.3.2 Combination of cAMP analogue and drugs treatment

Combination of the site-selective cAMP analogue (8-Cl-cAMP and 8-Br-cAMP) and protein kinase inhibitors or chemotherapeutic drugs was studied. Parental CCA cell lines (M156 and OCA17) were treated with 100 μM of cAMP analogue alone and in combination with increasing concentrations of the designed drugs for 72 h. Then, cell proliferation was evaluated using sulforhodamine B (SRB) assay. The antiproliferative effects of the combinations were compared to those of each drug alone.

3.2.3.3 Sulforhodamine B (SRB) assay

The SRB assay was performed to measure the efficiency of combination treatment on CCA cells proliferation as previously described (Papazisis *et al.*, 1997; Skehan *et al.*, 1990) with minor modifications. After 72 h of the cells with designed drugs, the culture medium was aspirated prior to fixation of the cells by adding 200 µl of 10% cold trichloroacetic acid (Merck, Germany). After 1 h incubation at 4^0C, cells were washed five times with deionized water, then cells were stained with 100 µl of 0.4% (w/v) SRB (Sigma-Aldrich St. Louis, MO) dissolved in 1% (v/v) acetic acid (BDH Laboratory, UK) for 30 min and subsequently washed five times with 1% acetic acid to remove unbound stain. The plate was left to dry at room temperature and bound protein stain was solubilized with 200 µl of 10 mM unbuffered Tris base (pH 10.5) then gyratory shaken for 1 h. The amount of SRB was measured by reading the absorbance at 540 nm using ELISA plate reader (Perkin Elmer, Finland).

3.2.3.4 Detection of apoptosis by flow cytometry

To determine the effect of PRKAR1A silencing combined with Protein kinase inhibitors and chemotherapeutic drugs on apoptosis of CCA cells, Annexin-V-FLUOS staining kit (Roche, Germany) containing Annexin-V-FLUOS and propiodium iodide was used to analyze the apoptotic rate by flow cytometry. Briefly, stable PRKAR1A knockdown CCA cell lines (M156 and OCA17) and their empty viral transfection control were seeded in 6 well plates at a cell density of 2×10^5 cells per well overnight. Then, cells were treated with designed concentrations of each drug for 24 h. After 24 h of incubation, the attached and floating cells were gently trypsinized and collected by centrifugation at 1,000×g, 4°C for 5 min. After that, washed with ice-cold PBS then cell pellets were resuspended in 100 µl of Annexin-V-FLUOS labeling solution and incubated at room temperature for 15 min. Cell were then analyzed over the flow cytometer (Beckman Coulter, Fullerton, CA) at an excitation of wavelength of 488 nm and two emission wavelength; 518 nm for green fluorescence (Annexin-V-Fluorescein) and 617 nm for red fluorescence (propiodium iodide). At least 10,000 cells were count per sample and cells were analyzed for apoptosis rate using CXP analysis software. The experiment was performed in duplication.

3.2.4 Statistical analysis

All results were reported as mean ± SD and the differences between control and test groups were tested using Student's *t* test. Results were considered statistically significant between control and test treatment at the level of $P < 0.05$.

3.3 Results

3.3.1 Antiproliferative potency of PRKAR1A silencing in combination with protein kinase inhibitors or chemotherapeutic drugs

Based on the fact that silencing of PRKAR1A expression in CCA cell lines exhibited approximately 20% on CCA cell growth inhibition as demonstrated in 2.3.4., therefore we sought further possible cooperative effect on cell proliferation inhibition of PRKAR1A suppression plus a series protein kinase inhibitors or chemotherapeutic drugs. PRKAR1A stable knockdown CCA cell lines (M156 and OCA17) were treated with different concentration of designed drugs (0-10 μM) for 72 h and cell proliferation was determined by SRB assay.

Treatment of empty viral transfection control in both CCA cell lines with protein kinase inhibitors including sorafenib, sunitinib, gefitinib and Met inhibitor (0.01-10 μM) as a single agent, showed growth inhibition 23% to 86% whereas cells treated with chemotherapeutic drugs including 5-FU, doxorubicin and paclitaxel (0.01-10 μM) alone showed 14% to 63% growth inhibition (Figure 3.1 and 3.2). An additive effect was observed when PRKAR1A silencing cell lines were treated with almost drug used in the experiment. For example in M156 (Figure 3.1A, C, E and 3.2A), treatment of shPRKAR1A in combination with 1 μM of sorafenib, sunitinib, gefitinib or 5-FU, which used alone showed 9%, 8%, 4% and 5% growth inhibition, respectively, caused a growth inhibition of 37%, 45%, 41% and 46%, respectively. However, in PRKAR1A silencing OCA17 CCA cell line had no remarkably such effect when combined with doxorubicin (Figure 3.2D) as similar as PRKAR1A knockdown M156 cells treated with paclitaxel (Figure 3.2E). In addition, IC_{50} value of each drug was found to be decreased after the combination as shown in Table 3.1 except M156 combined with paclitaxel and OCA17 treated with doxorubicin which the IC_{50} value seemed to be increased.

We next sought to determine if an additive effect can be found when parental M157 and OCA17 cells were treated with the combination of cAMP analogue 8-Cl-cAMP and 8-Br-cAMP (100 μM) with the same series of protein kinase inhibitors and cytotoxic drugs. Results reveal that no additive effect was observed (Appendix B, Figure B.3 and B.4).

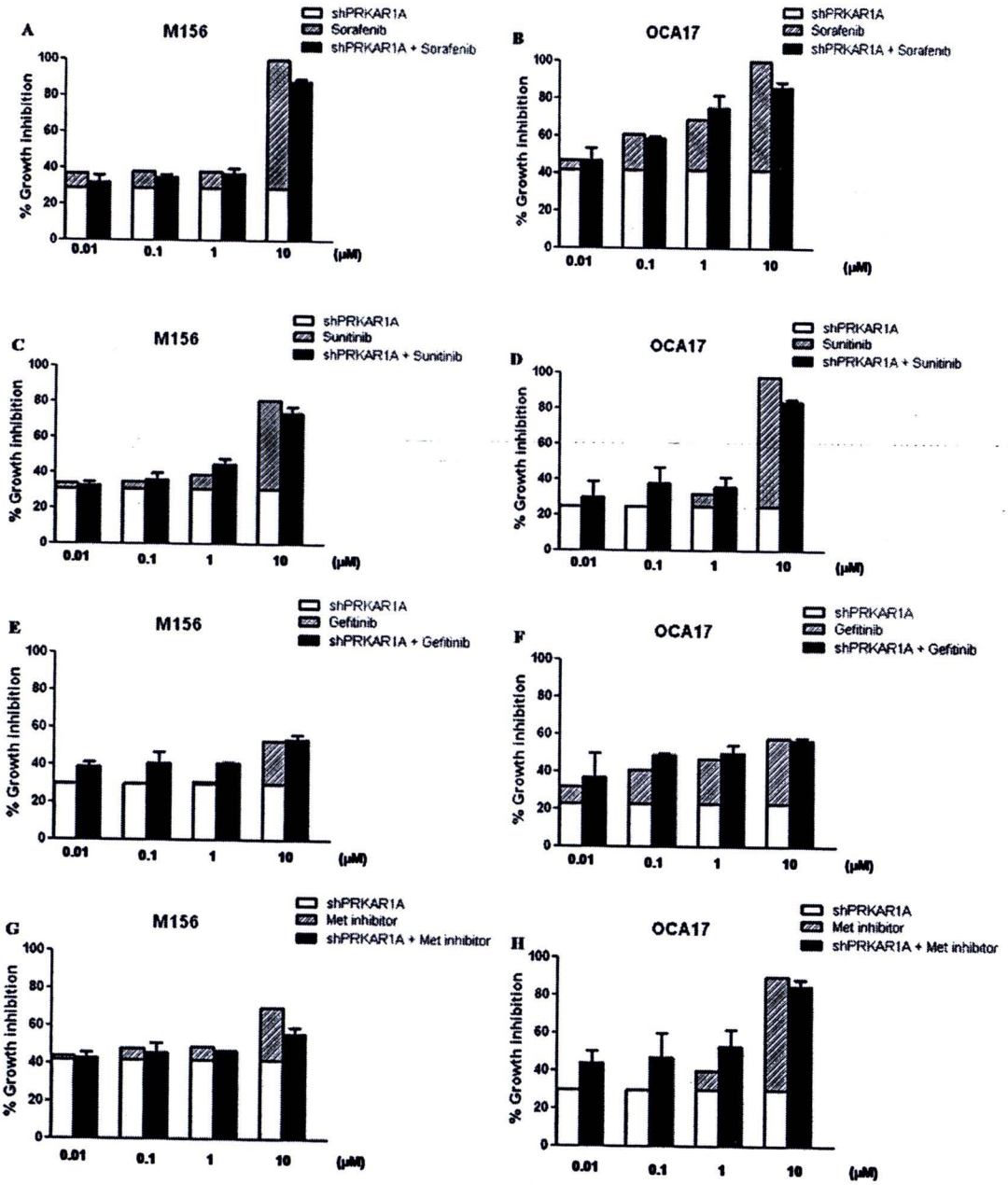

Figure 3.1 Antiproliferative effect of shPRKAR1A and protein kinase inhibitors in CCA cell lines. shPRKAR1A stable knockdown CCA cell lines and their empty viral transfection control were treated with different

concentration of sorafenib (A and B), sunitinib (C and D), gefitinib (E and F) and Met inhibitor (G and H), respectively for 72 h. Data are expressed as a percentage of growth inhibition in reference to the growth of untreated control cells and as indicated in the respective legends. The open portion of the bars represents the percentage of growth inhibition value for shPRKAR1A. The striped portion of the bars represents the percentage growth inhibition value for the kinase inhibitors as indicated in respective legend. The height of the stacked bars on the left represents sum of the individual agent effects and expected percentage growth inhibition if drugs are additive when used in the combination. The total height of the solid bar indicates the actual observed growth inhibition when drugs were used in combination. Therefore, the differences between the heights of the paired bars reflect the magnitude of additive effect of growth inhibition. The data represent means and SD of triplicate determinations of at least two independent experiments.

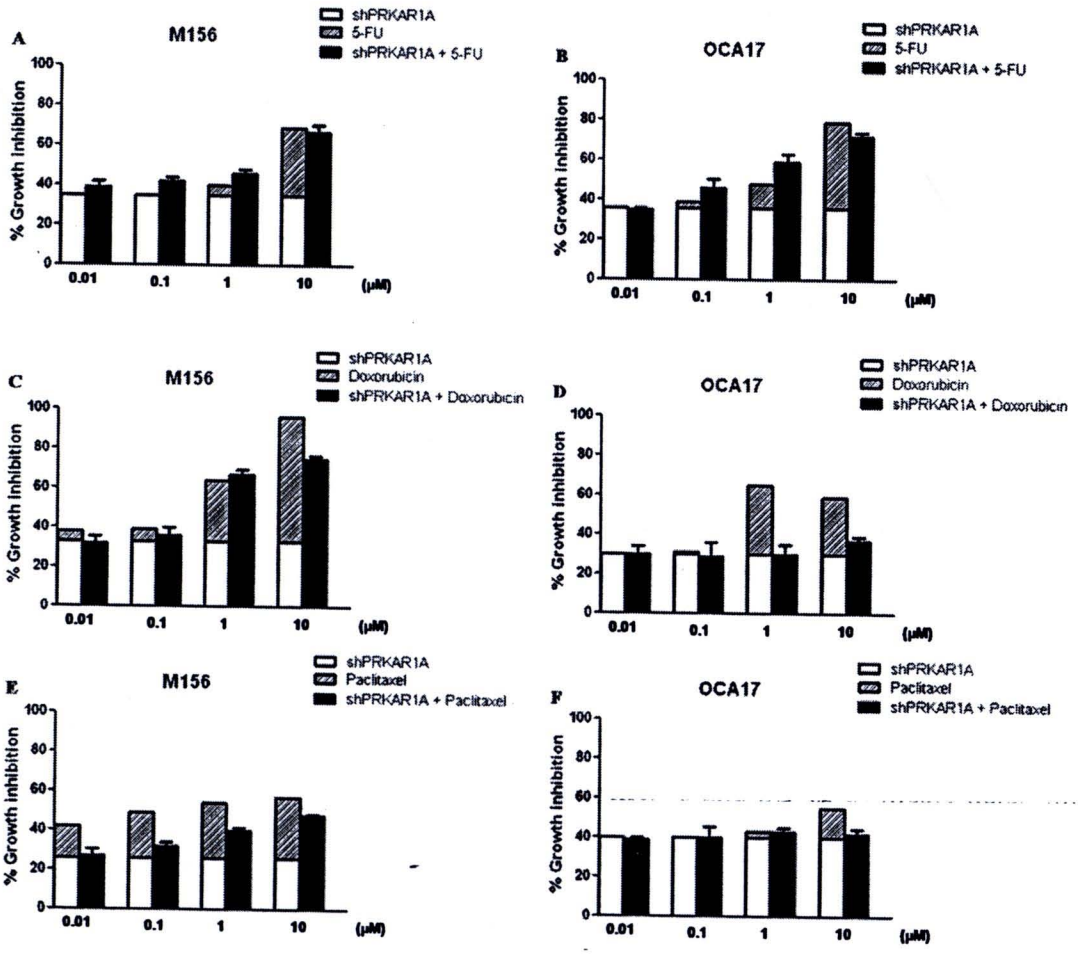

Figure 3.2 Antiproliferative effect of shPRKAR1A and chemotherapeutic drugs in CCA cell lines. shPRKAR1A stable knockdown CCA cell lines and their empty viral transfection control were treated with different concentration of 5-FU (A and B), doxorubicin (C and D) and paclitaxel (E and F) for 72 h. Data are expressed as a percentage of growth inhibition in reference to the growth of untreated control cells and as indicated in the respective legends. The open portion of the bars represents the percentage of growth inhibition value for shPRKAR1A. The striped portion of the bars represents the percentage growth inhibition value for the chemotherapeutic drugs as indicated in respective legend. The height of the stacked bars on the left represents sum of the individual agent effects and expected percentage growth inhibition if drugs are additive when used in the combination. The total height of the solid bar indicates the actual observed growth inhibition when

drugs were used in combination. Therefore, the differences between the heights of the paired bars reflect the magnitude of additive effect of growth inhibition. The data represent means and SD of triplicate determinations of at least two independent experiments.

Table 3.1 IC_{50} values of combination treatment of shPRKAR1A and protein kinase inhibitors or chemotherapeutic drugs.

	IC_{50} (μM) value			
Drugs	**M156 empty virus**	**M156 shPRKAR1A**	**OCA17 empty virus**	**OCA17 shPRKAR1A**
Protein kinase inhibitors				
Sorafenib	4.50 (± 1.1)	3.78 (± 0.2)	2.37 (± 0.7)	1.60 (± 1.4)
Sunitinib	14.60 (± 0.3)	8.70 (± 0.08)*	7.50 (± 2.0)	6.70 (± 0.9)
Gefitinib	53.62 (± 0.8)	32.58 (± 2.1)*	47.80 (± 1.6)	46.74 (± 1.4)
Met inhibitor	6.30 (± 0.2)	6.40 (± 0.7)	4.35 (± 0.04)	3.37 (± 0.03)*
Chemotherapeutic drugs				
Paclitaxel	0.16 (± 0.03)	0.64 (± 0.0)*	0.20 (± 0.01)	0.32 (± 0.04)
Doxorubicin	1.44 (± 0.05)	0.37 (± 0.04)*	0.23 (± 0.0)	0.79 (± 1.1)
5-FU	9.33 (± 0.3)	6.92 (± 2.2)	4.50 (± 0.6)	1.24 (± 0.6)*

The data represent mean±SD from two independent experiments. * $P < 0.05$ compared to control

3.3.2 Effect of shPRKAR1A in combination with protein kinase inhibitors or chemotherapeutic drugs on apoptosis induction

We then analyzed the effect of PRKAR1A silencing CCA cell lines in combination with protein kinase inhibitors and chemotherapeutic drugs on cellular apoptosis. PRKAR1A silencing CCA cell lines were treated with varied concentrations of sorafenib and 5-FU, respectively for 24 h. Then, the numbers of apoptotic cells were determined by Annexin-V (for early apoptotic cells) and propidium iodide (for late apoptotic cells) staining using flow cytometry. As shown in Figure 3.3 and 3.4, the numbers of cells with positive Annexin-V in PRKAR1A silencing cells were counted in areas 2 and 3 which represent early and late apoptotic cells (Figure 3.3A). The higher percentage of apoptotic cells than those of the control in both M156 and OCA17 were shown. Moreover, the increased in the percentage of cells entering

apoptosis was observed when PRKAR1A silencing combined with sorafenib and 5-FU in both cell lines especially in OCA17

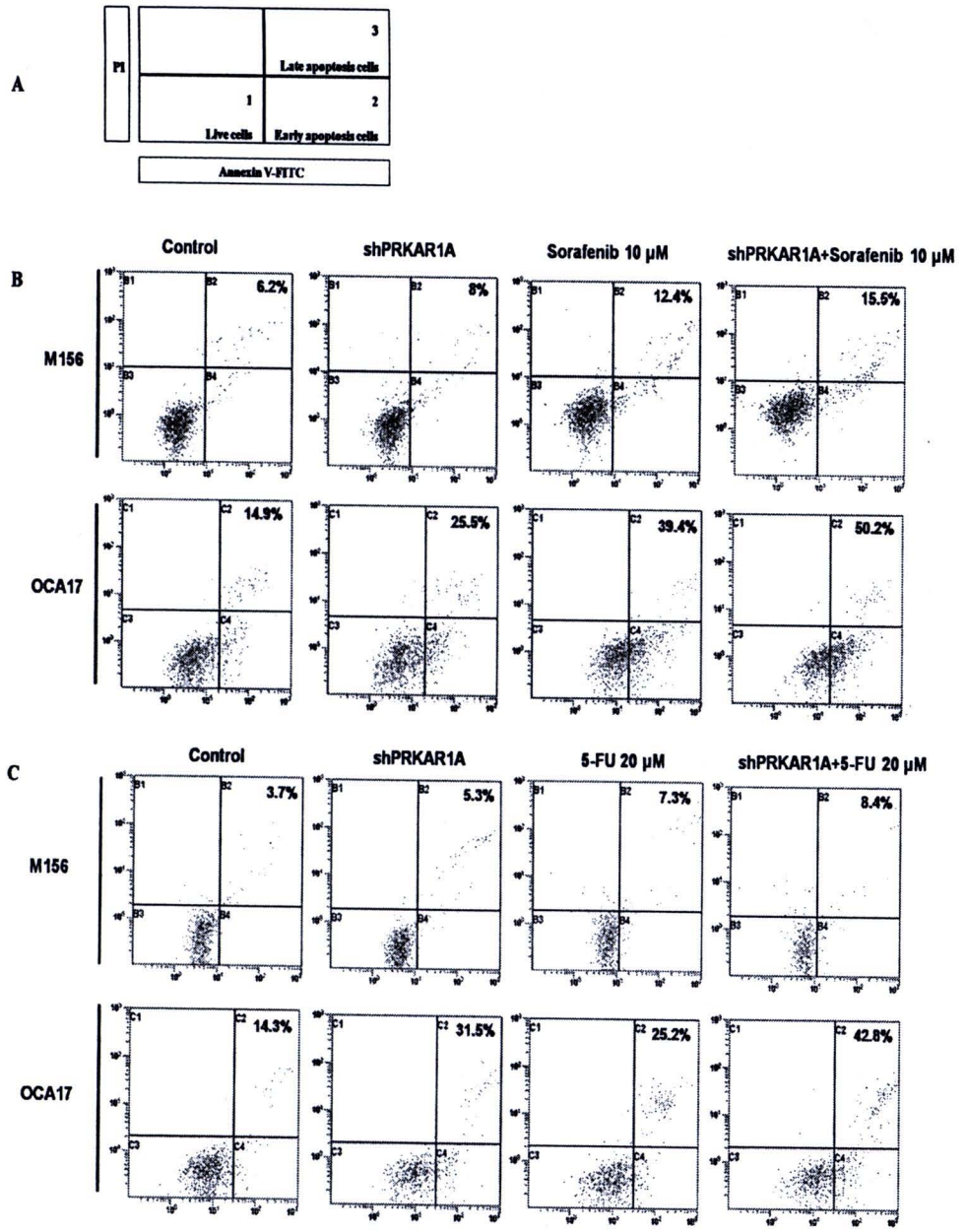

Figure 3.3 Flow cytometric analysis of combination treatment on apoptosis. shPRKAR1A in combination with 10 μM sorafenib (B) or 20 μM 5-FU (C) in M156 and OCA17. Annexin-V and PI (propidium iodide) staining was performed, followed by flow cytometry. The percentage of apoptotic cells was counted (Figure A, areas 2 and 3) after treatment for 24 h.

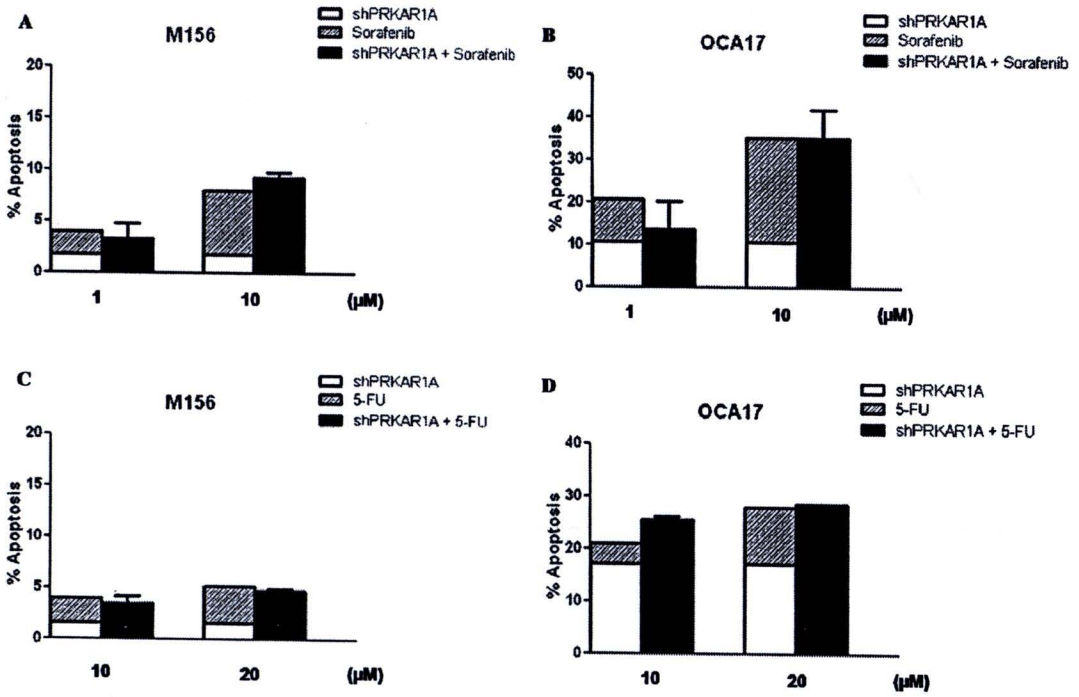

Figure 3.4 The effect of combination treatment of shPRKAR1A and sorafenib or 5-FU on apoptosis. shPRKAR1A in combination with 1, 10 μM sorafenib (A and B) or 10, 20 μM 5-FU (C and D) in M156 and OCA17. Apoptosis was determined after 24h treatment by flow cytometric analysis. The open portion of the bars represents the percentage of apoptotic cells for shPRKAR1A. The striped portion of the bars represents the percentage of apoptotic cells for the drugs as indicated in respective legend. The height of the stacked bars on the left represents sum of the individual agent effects and expected percentage of apoptotic cells if drugs are additive when used in the combination. The total height of the solid bar indicates the actual observed of apoptotic cells when drugs were used in combination. Therefore, the differences between the heights of the paired bars reflect the magnitude of additive effect of apoptosis induction.The results represent mean±SD of two independent experiments.

3.4 Discussion

Treatment options of CCA are unsatisfactory, and the prognosis of patients suffering from CCA is poor. Moreover, the high recurrence rate is observed in particular cancer. Therefore, effective therapeutic approaches are much needed. Emerging novel strategies of cancer treatment are based on the selective down-regulation of specific targets involved in the process of neoplastic transformation and progression. PRKAR1A/PKAI seems to be a relevant target for such therapeutic intervention. Accumulated evidence has indicated that selective inhibition of PRKAR1A/PKAI have shown promising results in inhibiting cancer cell growth both *in vitro* and *in vivo* (Cho-Chung *et al.*, 1999; Mantovani *et al.*, 2008; Nesterova and Cho-Chung, 1995; Nesterova and Cho-Chung, 2004). Since we have demonstrated in the present study that overexpression of PRKAR1A is a common occurrence in CCA and provides the pathway signaling by which PRKAR1A/PKAI is involved in CCA cell growth. This work also indicates that abrogation of PRKAR1A expression leads to induce CCA cells growth inhibition and apoptosis. In addition, we have demonstrated a relevant additive effect toward tumor cell growth inhibition as well as apoptosis induction can be achieved *in vitro* by combining PRKAR1A silencing with protein kinase inhibitors and chemotherapeutic drugs. However, particular cell types as well as drugs used in combination need to be considered.

These results support the others studies which have been reported that selective inhibition of PRKAR1A/PKAI show a synergistic growth inhibitory effect with chemotherapeutic drugs. 8-Cl-cAMP as well as the AS-PKAI, are able to cooperate with a variety of anticancer drugs, such as taxanes, topoisomerase II inhibitors, and platinum derivatives, causing a synergistic antitumor activity associated with increased apoptosis in a wide variety of human cancer types *in vitro* and in nude mice bearing human cancer xenografts (Tortora *et al.*, 1997a; Tortora and Ciardiello, 2000; Tortora *et al.*, 1997b). Furthermore, antisense PRKAR1A Gem231 in combination with the cytotoxic drug, hydroxycamptothecin (HCPT) have shown synergistic effects of tumor cell growth inhibition and apoptosis induction in colon and prostate cancer cell lines (Cho et al., 2003). Moreover, the experimental study demonstrated that combination of selective inhibition of PKAI by 8-Cl-cAMP with gamma ionizing

radiation exhibited enhancement of apoptosis induction in human cancer cells (Vucic et al., 2008).

Interestingly, chemotherapeutic agents, 5-FU, the widely used anticancer agents and frequently applied for CCA patients, also showed additive effect on growth inhibition. Therefore, instead of increasing drug concentration to supratoxic levels, it may be possible to enhance antineoplastic activity of 5-FU by addition of targeting PRKAR1A/PKAI. Unfortunately, cooperative effect was not observed when cAMP analogues were used in combination with those drugs. This finding was consistent with result as shown in 2.3.6 that cAMP analogues tested seem to be less effective in CCA cell lines and high concentration of drugs was need. It implies that at present available selective inhibitors for PKAI are ineffective in CCA cell growth inhibition. Therefore, new PKA inhibitors need to be developed.

In conclusion, our data demonstrated that PRKAR1A can be a new target-molecule for improving the efficacy of anticancer drugs in CCA. PRKAR1A knockdown by RNAi induce cell growth inhibition and apoptotic activity, hence enhance the cytotoxicity of these anticancer drugs. Therefore, combination of down-regulation of PRKAR1A and these anticancer drugs could be the therapy of choice in treating CCA. Our study provides the rationale for testing the effectiveness of particular combination for *in vivo* CCA treatment, then translating this strategy into a clinical setting.