RESEARCH ARTICLE

Suppression of PRKAR1A Expression Enhances Anti-proliferative and Apoptotic Effects of Protein Kinase Inhibitors and Chemotherapeutic Drugs on Cholangiocarcinoma Cells

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Abstract

Suppression of protein kinase A regulatory subunit 1 alpha (PRKAR1A) has been proven to inhibit cholangiocarcinoma (CCA) cell growth and enhance apoptosis. In the present study, we aimed to determine synergistic and/or additive effects of chemotherapeutic agents, including protein kinase inhibitors (i.e. sorafenib, sunitinib, gefitinib, Met inhibitor) and conventional chemotherapeutic drugs (i.e. 5-fluorouracil, doxorubicin, paclitaxel, gemcitabine), in PRKARIA knockdown CCA cell lines. The results revealed that PRKAR1A suppressed CCA cell lines demonstrated enhanced sensistivity to some chemotherapeutic drugs when compared to control cells. Moreover, PRKAR1A knockdown in combination with either sorafenib or 5-fluorouracil increased apoptotic effects on CCA cell lines. Therefore, selective inhibition of PRKAR1A appears to enhance the growth inhibitory effects of chemotherapeutic drugs as well as induce apoptotic cell death. Our findings suggest that additional suppression of PRKAR1A expression may increase the efficacy of conventional CCA chemotherapeutic treatment. Clinical studies in CCA patients now need to be conducted.

Keywords: Cholangiocarcinoma - PRKAR1A knockdown - kinase inhibitors - chemotherapeutic drugs

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Introduction

Cholangiocarcinoma (CCA) is a highly invasive malignancy that is difficult to diagnose 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 (Uttaravichien et al., 1999; Ohtsuka et al., 2003). However, a complete resection is often impossible, typically resulting in subsequent metastasis and local recurrence (Olnes and Erlich, 2004). The survival of CCA patients can most likely be improved with more advanced surgical techniques, as well as with more potent drug regimens. Thus, chemotherapy is considered a preferred treatment option of preventing postsurgical cancer recurrence (Todoroki, 2000). In patients with advanced CCA, however, the treatment options are unsatisfactory and new therapeutic approaches are required.

Medical treatment of cancer is mainly based on the use of chemotherapeutic drugs acting on intracellular targets. Nevertheless, the therapeutic benefits derived from such regimens seem to have limitations imposed by the resistance of cancer cells to chemotherapeutic drugs (Tannock, 2001; Krishan and Arya, 2002; Lonning, 2003). Therefore, inhibiting molecular targets relevant to the processes of neoplastic transformation and progression represents a new challenging opportunity in cancer treatment. As the efficacy of anticancer drugs can be improved by a combination of two or more drugs, future cancer therapy strategies may be based on the integration of conventional therapies with novel inhibitors of signals involved in cancer development.

Recently, our group has reported that protein kinase A regulatory subunit 1 alpha (PRKAR1A/PKAI) is overexpressed in CCA cells and plays an important role in regulating CCA cell growth. The abrogation of PRKAR1A expression using RNA interference technology led to growth inhibition and induction of apoptosis in CCA cells (Loilome et al., 2011). Moreover, other studies suggested that overexpression of the PRKAR1A/PKAI pathway is involved in neoplastic transformation and tumor growth of different types of cancers, including CCA (Handschin and Eppenberger, 1979; Simpson et al., 1996; Miller et al., 1997; McDaid et al., 1999; Loilome et al., 2006; Loilome et al., 2011). Therefore, targeting this pathway in combination with anticancer drug treatment would likely be a more effective approach for this bile duct cancer. There is growing evidence supporting that combination of chemotherapeutic drugs with 8-Cl-cAMP, a cyclic adenosine monophosphate analogue which selectively

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inhibits PKAI or with a series of modified antisense oligonucleotides targeting PRKAR1A (AS-PKAI) is found to be a promising therapeutic innovation toward the treatment of cancer. Results from *in vitro* and *in vivo* studies have demonstrated that both 8-Cl-cAMP and antisense PRKAR1A could inhibit both PKAI expression and function which in turn promotes PKAII formation, leading to cancer cell growth arrest in several cancer cell types (Tortora et al., 1991; Rohlff et al., 1993; Cho-Chung et al., 1995; Nesterova and Cho-Chung, 1995).

In this combination approach, a number of experimental studies have demonstrated that selective inhibition of PRKAR1A/PKAI has a synergistic growth inhibitory effect with chemotherapeutic drugs. The 8-Cl-cAMP and a selective inhibitor of PKAI (AS-PKAI), are proven to be able to cooperate with a variety of anticancer drugs, such as taxanes, topoisomerase II inhibitors, and platinum derivatives. These drugs can cause 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., 1997; Tortora and Ciardiello, 2000). Furthermore, antisense PRKAR1A Gem231 in combination with the cytotoxic drug, hydroxycamptothecin (HCPT) has shown synergistic effects on tumor cell growth inhibition and apoptosis induction in colon and prostate cancer cell lines (Cho and Cho-Chung, 2003). In this particular study, the combined agents have resulted in up-regulation of pro-apoptotic proteins (Bax and Bad) and down-regulation of anti-apoptotic proteins (Bcl-2). In addition, it was found either in changes of cell morphology by exhibiting a flattened shape and an increase cytoplasm: nucleus ratio or changes indicative of apoptosis, such as chromatin condensation, nuclear fragmentation and increased apoptotic nuclei counts. Furthermore, treatment with a combination of 8-Cl-cAMP and sulfinosine resulted in synergistic effects on growth inhibition, cell cycle arrest, and induction of apoptosis in a 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 interaction may provide the basis for the development of a therapeutic strategy based on the combination of their selective inhibitors (Tortora et al., 1997). The blocking of EGFR and PKAI signaling pathways by specific inhibitors, PD153035 and Rp-cAMP leads to a synergistic cell growth inhibition of prostatic cancer cells concomitant with an arrest in G1 phase of the cell cycle. This 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, the combination synergistically inhibited the growth of different human cancer cell lines *in vitro* and *in vivo* (Ciardiello et al., 1995; Ciardiello et al., 1996).

The present study aimed to investigate whether selective down-regulation of PRKAR1A/PKAI by shPRKAR1A in combination with various chemotherapeutic drugs or protein kinase inhibitors had any cooperative effect on cell growth inhibition in CCA cell lines. We used M156 and OCA17 cell lines as representative of the non-papillary and papillary types of CCA that exhibit different prognoses and survival times (Yeh et al., 2004) as a model.

Materials and Methods

Cell lines and cell culture

Two stable PRKAR1A knockdown human CCA cell lines; KKU-OCA17, KKU-M156 were constructed and maintained as previously described (Loilome et al.).

Small molecule kinase inhibitors and chemotherapeutic drugs

Protein kinase inhibitors; sorafenib, sunitinib were purchased from LC Laboratories (Woburn, MA). Gefitinib was from AstraZeneca plc (London) 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), paclitaxel and gemcitabine were dissolved in NH4OH, 50% methanol and 0.9% NaCl, respectively. Doxorubicin was dissolved in water. Stock solutions of the tested drugs were prepared at a concentration of 10 mM and stored at -20 °C until used.

Combination of PRKAR1A silencing CCA cell lines and drugs treatment

To test the chemosensitivity of PRKAR1A silencing combined with protein kinase inhibitors and chemotherapeutic drugs, PRKAR1A stable knockdown CCA cell lines (M156 and OCA17) and their empty viral transfection controls were treated with various concentrations of each drug for 72 h. Cell proliferation and apoptosis were further evaluated by the sulforhodamine B (SRB) assay and subsequently by flow cytometry using Annexin-V-FLUOS staining kit (Roche, Penzberg). The antiproliferative and apoptosis induction effects of the combinations were compared to their empty viral transfection controls.

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 $P < 0.05$.

Results

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% growth inhibition on CCA cells as demonstrated in Figures 1 and 2, we therefore sought further possible cooperative effects on cell proliferation inhibition of PRKAR1A suppression when coupled with a series of protein kinase inhibitors or chemotherapeutic drugs. PRKAR1A stable knockdown CCA cell lines (M156 and OCA17) were treated with different concentrations of designated drugs

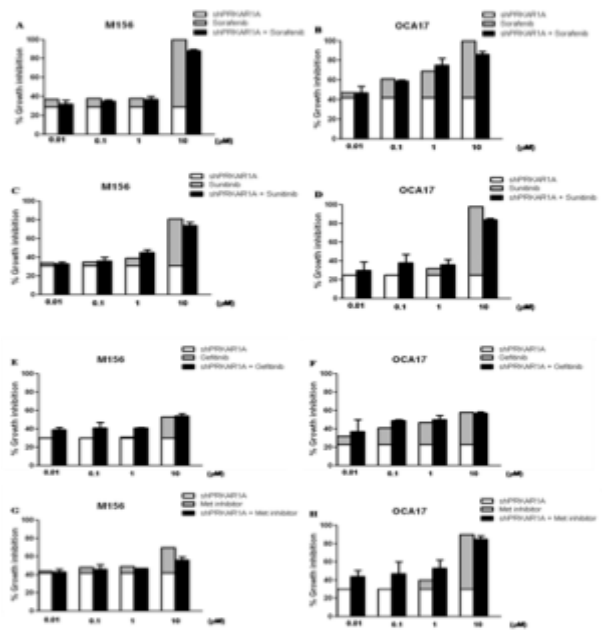

Figure 1. The Antiproliferative Effect of shPRKAR1A and Protein Kinase Inhibitors in CCA Cell Lines (M156 and OCA17). The shPRKAR1A stable knockdown CCA cell lines and their empty viral transfection controls were treated with different concentrations of Sorafenib (A and B), Sunitinib (C and D), Gefitinib (E and F) and Met inhibitor (G and H) for 72 h. Data are expressed as a percentage of growth inhibition in reference to the growth of untreated control cells 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 the 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 were 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, differences between heights of the paired bars reflect the magnitude of the additive effect on growth inhibition. These data represent means and SD of triplicate determinations of at least two independent experiments

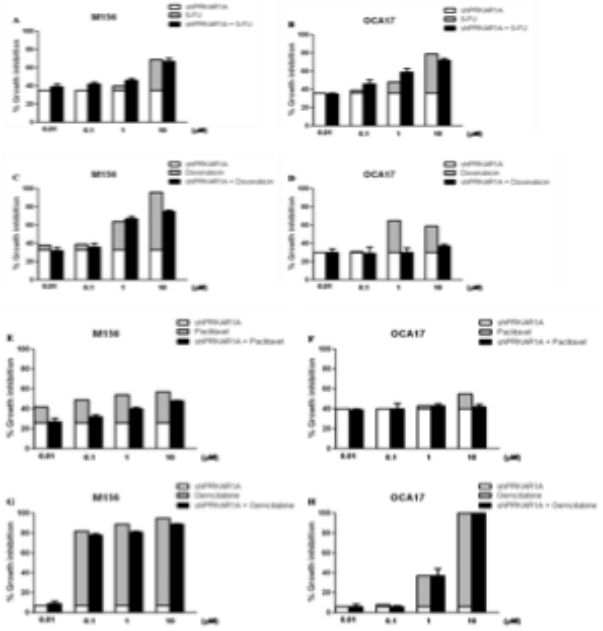

Figure 2. The Antiproliferative Effect of shPRKAR1A and Chemotherapeutic Drugs in CCA Cell Lines (M156 and OCA17). The shPRKAR1A stable knockdown CCA cell lines and their empty viral transfection controls were treated with different concentration of 5-FU (A and B), Doxorubicin (C and D), Paclitaxel (E and F) and Gemcitabine (G and H) for 72 h. Data are expressed as a percentage of growth inhibition in reference to the growth of untreated control cells 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 values for the chemotherapeutic drugs as indicated in respective legends. The height of the stacked bars on the left represents sums of the individual agent effects and expected percentage growth inhibition if drugs were additive when used in the combination. The total height of the solid bars 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 the additive effect of growth inhibition. These data represent means and SD of triplicate determinations of at least two independent experiments

(0-10 μM) for 72 h. Cell proliferation was subsequently determined by the SRB assay.

The treatment of empty viral transfection controls in both CCA cell lines with protein kinase inhibitors including sorafenib, sunitinib, gefitinib and Met inhibitor (0.01-10 μM) as single agents, showed growth inhibition between 15% and 80% (Figure 1), whereas cells treated with chemotherapeutic drugs including 5-FU, doxorubicin, paclitaxel and gemcitabine (0.01-10 μM) alone caused between 15% and 60% growth inhibition (Figure 2). An additive effect was observed when PRKAR1A silencing cell lines were treated with almost any drug used in the experiments (Figures 1 and 2). For example, in M156 (Figure 1), the treatment of shPRKAR1A in combination with either 1 μM of sorafenib, sunitinib, gefitinib or 5-FU, which were used alone showed an increase in the percentage of growth inhibition of 9%, 8%, 4% and 5%, respectively, and caused a total growth inhibition of 37%, 45%, 41% and 46%, respectively. However, the PRKAR1A silencing of the OCA17 CCA cell line had no such remarkable effect when combined with doxorubicin or paclitaxel (Figure 2). In addition, the IC_{50} value of each drug was found to be decreased after combination as shown in Table 1, except for two cases that are when the M156 was combined with paclitaxel and the OCA17 was treated with doxorubicin.

We next sought to determine if an additive effect could be found when parental M157 and OCA17 cells were treated with the combination of the cAMP analogues, 8-Cl-cAMP and 8-Br-cAMP (100 μM) or with the same series of protein kinase inhibitors and cytotoxic drugs; unfortunately no such effects were observed (data not shown).

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

The effect of PRKAR1A silencing CCA cell lines in combination with protein kinase inhibitors or cytotoxic drugs on cellular apoptosis was studied by treating the PRKAR1A silencing CCA cell lines with various concentrations of either sorafenib, a protein kinase

Table 1. IC_{50} Values of Combination Treatment of shPRKAR1A and Protein Kinase Inhibitors or Chemotherapeutic Drugs on Inhibiting CCA Cells Proliferation.

Drugs	IC_{50} (μM) value			
	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	11.87 (± 6.3)	11.60 (± 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)*
Gemcitabine	0.006 (± 0.0)	0.004 (± 0.0)	2.50 (± 0.8)	2.10 (± 0.5)

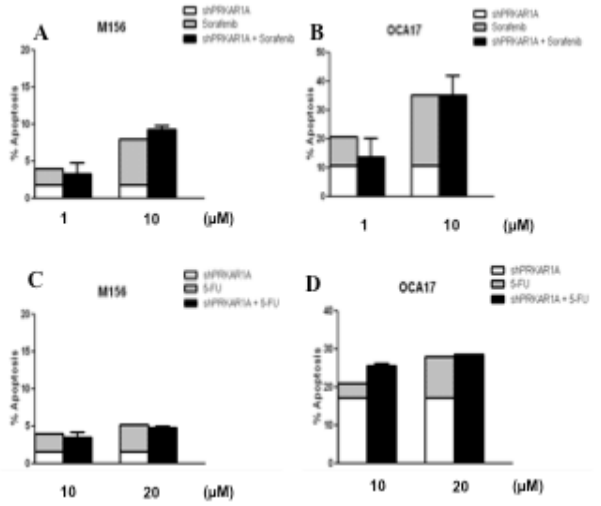

Figure 3. The Apoptotic Effect of shPRKAR1A in Combination with 10 μM Sorafenib (A and B) or 20 μM 5-FU (C and D) in M156 and OCA17. Apoptosis was determined after 24h treatment by flow cytometric analysis. The open portion of the bars represents the percentage of apoptotic cells for shPRKAR1A. The striped portion of the bars represents the percentage of apoptotic cells for the drugs as indicated in the 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 were 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

inhibitor, or 5-FU, a chemotherapeutic drug for 24 h. The numbers of apoptotic cells were determined by Annexin V for early apoptotic cells and propidium iodide staining for late apoptotic cells using flow cytometry.

As shown in Figure 3, the numbers of cells with positive Annexin V in PRKAR1A silencing cells were mainly observed in both early and late apoptotic cells. These results showed a higher percentage of apoptotic cells when compared to those of the controls in both M156 and OCA17 cell lines. Moreover, an increase in the percentage of cells entering apoptosis was observed when PRKAR1A silencing was combined with sorafenib and 5-FU in both cell lines, but most notably in the OCA17 case.

Discussion

Selective down-regulation of specific targets involved in the processes of neoplastic transformation and progression is an interesting strategy for the treatment of several cancers. This study revealed that PRKAR1A is over expressed in CCA and involved in CCA cell growth. An increase in growth inhibition occurred when treating PRKAR1A silencing cell lines with the tested drugs, particularly in the cases of sorafenib, sunitinib, gefitinib, and 5-FU. This indicates an additive effect of the PRKAR1A expression in the drug treatment. Interestingly, 5-FU, a widely used chemotherapeutic agent to treat CCA patients, showed a good additive effect on growth inhibition when combined with the treatment of PKA inhibitors and cAMP analogs. Therefore, it may be possible to enhance antineoplastic activity of drugs, notably 5-FU by targeting PRKAR1A/PKAI in addition, however, it should be noticed that the types of CCA cell lines may affect the treatment response. In the combination treatment of doxorubicin with OCA17, there was no such significant effect. Moreover, most of the studied drugs exhibited lower IC_{50} values than those of the controls, suggesting that the suppression of PRKAR1A may allow drugs to act more potently with the same amount of drug used. Besides, the abrogation of this protein induced apoptosis of CCA cells, which may further present promising results for the treatment of CCA both in the animal model and in humans. Overall, these findings indicate the impact of suppressing PRKAR1A in CCA treatment *in vitro* and showed additive effects toward tumor cell growth inhibition and the induction of apoptosis by combining PRKAR1A silencing with protein kinase inhibitors and chemotherapeutic drugs in CCA cell lines. These results were consistent with previous research on the effect of PRKAR1A/PKAI conduced in other types of cancers.

We suggest that PRKAR1A may be a potential target-molecule for improving the efficacy of anticancer drugs in CCA treatment. Down-regulation of PRKAR1A in combination with anticancer drugs could be considered as the therapy of choice in treating CCA.

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