Final Report

Project Title

Anticancer effects of the selective glucocorticoid receptor modulator, compound A, in cholangiocarcinoma cells

By

Mutita Junking

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Mutita Junking Mahidol University

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Abstract

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Cholangiocarcinoma (CCA) is an aggressive cancer arising from biliary epithelium. This cancer is the most common cancer in northeast Thailand. Surgery is the only curative treatment for patients and the use of chemotherapy had poor results. It is crucial need to search for a new compound for improves treatment of CCA. Carcinogenesis of CCA is a multistep cellular process. The inflammatory cytokines such as IL-6 have an important role in the pathogenesis and growth of CCA by activate several survival signaling. Glucocorticoids (GC) are one of the potent anti-inflammatory agents that have been used in many cancers. As a result, decreasing in pro-inflammatory cytokines and survival genes, leading to the tumor cell death. Recently, a small plant-derived GR modulator, compound A (CpdA), showed anti-inflammatory activity and has fewer side effects compared with glucocorticoids. CpdA shows anti-inflammatory activity, strongly inhibits growth, and decreases survival of many cancers. Since CpdA exerts the inhibition of cancer cell progression, the effect of this compound in chemotherapy of CCA seems very promising. In this project, we evaluated the anticancer effect of CpdA in CCA cells. The results showed that CCA cell lines express glucocorticoid receptor (GR), a receptor for CpdA, and CpdA affects CCA cell survival. Cytotoxicity of CpdA on CCA cell lines was determined. CpdA reduced *IL-6* expression in CCA cell lines. Moreover, effect of CpdA on cell proliferation was determined by propridium iodide staining. The results showed that CpdA inhibited CCA cell lines proliferation and G1 cell cycle arrest was investigated. Antitumor activity of CpdA either using CpdA alone or combination with the traditional anticancer agents, cisplatin were observed. This study will provide a novel strategy for chemotherapy of CCA patients.

Keywords : cholangiocarcinoma, bile duct cancer, glucocorticoid, compound A, interleukin-6

บทคัดย่อ

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ชื่อโครงการ : บทบาทของ compound A ในการยับยั้งการเจริญของเซลล์มะเร็งท่อน้ำดี

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โรคมะเร็งท่อน้ำดี เป็นโรคมะเร็งที่เกิดจากเซลล์เยื่อบุผิวของท่อน้ำดี เป็นมะเร็งที่มีความรุนแรงโรคสูง มะเร็งท่อน้ำดีพบได้น้อยในประเทศที่พัฒนาแล้วและในทั่วโลก แต่เป็นโรคที่พบได้สูงในแถบเอเชียตะวันออกเฉียงใต้ โดยเฉพาะประเทศไทยที่พบโรคนี้สูงที่สุด การรักษาหลักของมะเร็งท่อน้ำดีคือการผ่าตัด และอาจร่วมกับรังสีรักษา หรือยาเคมีบำบัด แต่เนื่องจากมะเร็งชนิดนี้มีการตอบสนองต่อยาเคมีบำบัดต่ำ การวิจัยเพื่อค้นหายาหรือวิธีการรักษาโรคมะเร็งท่อน้ำดีจึงมีวามสำคัญ กระบวนการเกิดมะเร็งท่อน้ำดีมีกระบวนการหลายขั้นตอน และมีระยะเวลานาวนานหลายปี ในกระบวนการนี้มีไซโตไคน์ที่ทำหน้าที่ในการเกิดการอักเสบร่วมด้วย เช่น IL-6 ซึ่งได้มีการศึกษามากมายรายงานว่า IL-6 มีบทบาทสำคัญในกระบวนการเจริญเติบโตของเซลล์มะเร็งท่อน้ำดี และได้มีการศึกษาการใช้ Glucocorticoids (GC) ซึ่งเป็น compound ที่สามารถลดการแสดงออกของไซโตไคน์ และสามารถลดการเจริญเติบโตของเซลล์มะเร็งได้หลายชนิด แต่ GC ที่ใช้ในปัจจุบันยังมีผลข้างเคียงอยู่มาก จึงได้มีการศึกษาสารสกัดจากธรรมชาติที่ให้ผลลดไซโตไคน์เช่นเดียวกับ GC แต่ไม่มีผลข้างเคียง สารสกัดดังกล่าวคือ compound A (CpdA) ซึ่งได้มีการรายงานแล้วว่า CpdA สามารถต้านการอักเสบได้โดยการลดการแสดงออกของไซโตไคน์ และยับยั้งการเจริญเติบโตของเซลล์มะเร็งได้หลายชนิด โครงการวิจัยนี้จึงได้ทำาารศึกษาผลของ CpdA ต่อการเจริญเติบโตของเซลล์มะเร็งท่อน้ำดี โดยใช้ความเข้มข้นของ CpdA ที่ไม่เป็นพิษต่อเซลล์ปกติ ทดสอบในเซลล์มะเร็งท่อน้ำดี พบว่า CpdA ลดการแสดงออกของไซโตไคน์ IL-6 ในเซลล์มะเร็งท่อน้ำดี นอกจากนี้ยังยับยั้งการเจริญเติบโตของเซลล์มะเร็งท่อน้ำดี โดยหยุดกระบวนการแบ่งเซลล์ไว้ที่ระยะ G1 อย่างไรก็ตามเมื่อมีการทดสอบการใช้ CpdA ร่วมกับยาเคมีบำบัด Cisplatin ไม่ได้ทำให้เซลล์มะเร็งท่อน้ำดีตายได้มากขึ้น ซึ่งอาจจะเป็นผลจากการที่ Cisplatin ไปเพิ่มไซโตไคน์ที่ช่วยให้เซลล์มะเร็งแบ่งตัวได้ดีขึ้น เช่น ไซโตไคน์ TGF-β1 แต่อย่างไรก็ตามผลการวิจัยดังกล่าวสามารถนำไปประยุกต์ใช้ในการพัฒนาวิธีการรักษาโรคมะเร็งท่อน้ำดีสำหรับคนไทยได้ต่อไป

คำหลัก : cholangiocarcinoma, bile duct cancer, glucocorticoid, compound A, interleukin-6

Executive summary

Cholangiocarcinoma (CCA) is an aggressive and lethal cancer arising from biliary epithelia within biliary tracts. This cancer is the most common liver cancer in northeast Thailand. The malignancy of CCA is normally difficult to diagnose until the disease become advanced, at which the prognosis is poor. Surgery is the only curative treatment for patients with CCA; however, less than one-third of patients are resectable at diagnosis. Moreover, the use of chemotherapy for CCA had poor results and studies were small and conflicting. It is crucial need to search for a new drug or compound that can utilize its effect either alone or in combination with the present chemotherapy for improve treatment of CCA.

Carcinogenesis of CCA is a multistep cellular process. CCA cells express altered molecular mechanisms, which enhance cell proliferation, decrease apoptosis, and increase the capacity of tissue invasion, stromal proliferation, and angiogenesis. The inflammatory cytokines such as IL-6 have an important role in the pathogenesis and growth of CCA by activate several pathways involved in survival signaling and contributes to chemoresistance. Glucocorticoids (GC) are one of the potent anti-inflammatory agents that have been used in chronic inflammatory disease and many cancers. As a result, decreasing in pro-inflammatory cytokines, anti-apoptotic genes, and survival genes, leading to the tumor cell death. Recently, a small plant-derived GR modulator, compound A (CpdA) showed anti-inflammatory activity and has fewer side effects compared with glucocorticoids. CpdA shows anti-inflammatory activity, strongly inhibits growth, and decreases survival of many cancers. Since CpdA strongly interacts with GR and exerts the inhibition of cancer cell progression, the effect of this compound in chemotherapy of CCA seems very promising.

In this project, we evaluated the anticancer effect of CpdA in CCA cells. The results showed that CCA cell lines express glucocorticoid receptor (GR), a receptor for CpdA, and CpdA affects CCA cell survival. Cytotoxicity of CpdA on CCA cell lines was determined. CpdA decreased *IL-6* expression in CCA cell lines as determined by using realtime PCR. Moreover, effect of CpdA on cell proliferation was determined by propridium iodide staining. The results showed that CpdA inhibited CCA cell lines proliferation and G1 cell cycle arrest was investigated. The antitumor activity of CpdA either using CpdA alone or combination with the traditional anticancer agents, cisplatin were observed and found that combination treatment did not increased effect of CpdA. However, this study will provide a novel strategy for chemotherapy of CCA patients.

Objectives

1. To observe the effects of CpdA on cell proliferation and apoptosis in CCA cell lines
2. To determine the molecular mechanism(s) of CpdA on cell proliferation and apoptosis in CCA cell lines
3. To determine the combination effects of CpdA and anticancer drugs in CCA cells

Research methodology

1. Cells and reagents

1.1 CCA cell lines

Human CCA cell lines, KKU-100 (JCRB1568) and KKU-M213 (JCRB1557), and an immortalized cholangiocyte cell line, MMNK-1 (JCRB1554), were available at the Japanese Collection of Research Bioresources Cell Bank (JCRB), National Institute of Biomedical Innovation, Japan. All cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco), containing 10% fetal bovine serum (Gibco), 100 Units/ml penicillin and 100 μg/ml streptomycin at 37^{O}C with 5% CO_2 and sub-cultured twice per week following a standard trypsinization protocol.

1.2 Primary normal cells

Skin fibroblast cells (SF-A4) were a gift from Assoc. Prof. Chanitra Thuwajit, Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University. Peripheral blood mononuclear cells (PBMCs) from 3 healthy donors were isolated by Ficoll–Hypaque (GE Healthcare) gradient centrifugation. PBMCs were subsequently resuspended in RPMI (Gibco) with various concentration of CpdA. Human umbilical vein endothelial cells (HUVECs) were isolated from 3 healthy term pregnancies. HUVECs were isolated from a 6-inch umbilical cord segment via trypsin digestion. HUVECs were cultured using M199 (Gibco). The protocol for PBMCs and HUVECs collection was approved by the Siriraj Institutional Review Board (COA No. *Si*405/2013).

1.3 Cpd A, Dexamethasone (Glucocorticoid), and cisplatin

Purified CpdA is a gift from Prof. Guy Haegeman The Laboratory of Eukaryotic Gene Expression and Signal Transduction (LEGEST), Department of Physiology, Faculty of Sciences, Ghent University, Belgium and Prof. Irina Budunova, Department of Dermatology, Northwestern University, Chicago, Illinois, USA. Dexamethasone, a classical glucocorticoid, were purchased from Sigma. Anticancer agents Cisplatin was purchased from Sigma.

2. Determination of glucocorticoid receptor expression in CCA cell lines

2.1 Polymerase chain reaction and real-time PCR

To examine mRNA expression of the glucocorticoid receptor genes in CCA cells, total RNA was prepared by Trizol reagent (Invitrogen) and chloroform extraction. RNA samples were

reverse transcribed then complementary DNA (cDNA) templates were assayed for level of transcription using a pair of specific primers. The PCR reaction was performed by using GoTaq® DNA polymerase (Promega) in a thermocycler (Biometra). For level of transcription of glucocorticoid receptor in each cell, LightCycler® 480 SYBR Green I Master (Roche) was used and the reactions was recorded and analyzed using LightCycler® 480 Instrument equipped with a 96-well thermal cycler (Roche).

2.2 Western blotting

The expression of glucocorticoid receptor protein was determined by western blotting. After wash cells with PBS and lysed with lysis buffer, then protein was determined for protein content and separated in a micro-slab SDS-PAGE. Immunobloting was done using antibody against glucocorticoid receptor and secondary antibody conjugated with HRP for subsequence detection by SuperSignal West Pico Chemiluminescent Substrate (Pierce).

3. Observation the anticancer effect of CpdA in CCA cells

3.1 Cell proliferation assay

Effect of CpdA on the cell proliferation was determined by using PrestoBlue® Reagent (Invitrogen). CCA cell suspension was seed into 96-well plates (Costar) then after 24 h incubation, cells were treated with various concentration of CpdA and Dexamethasone (which is classical glucocorticoid; Sigma) for 24 and 48 h. PrestoBlue® dye was added to the plate and incubate for further 2 h then the absorbance of the color complex was read at 570 nm with a reference wavelength set at 600 nm using a BIO-TEK Power-Wave XS multiwell plate reader.

3.2 Cell cycle distribution assay

After treated CCA cells with CpdA, cells were detached and washed with PBS, then fixed with ice-cold 70% ethanol. Cells were further resuspended in Propidium iodide (PI) for 30 min. The cell cycle were determined using FACSort™ flow cytometer (Becton Dickinson) and data was analyzed using Cell Quest software (Becton Dickinson).

4. Determination of *IL-6* expression

The expression of *IL-6* was done after CpdA treatment in CCA cells using specific primers for real-time PCR according to the method in **2.1**.

5. **Observation the anticancer effect of CpdA and chemotherapeutic drugs**

Combination treatment between CpdA and cisplatin were performed in CCA cells, which has been reported to resistance to this chemotherapeutic drug. The anticancer effect was observed according to the methods in **3.1**.

6. **Statistical analysis**

The statistical significance of the differences observed between experimental groups were determined using Student's t-test. $P<0.05$ was considered significant.

Results

1. CCA cell lines expressed GR as determined by PCR and western blotting

The expression of glucocorticoid receptor (GR) at mRNA level was observed by conventional PCR in 3 CCA cell lines namely KKU-100, KKU-M213, and KKU-M055 and one cholangiocyte cell line, MMNK-1. Total RNA was prepared and reverse transcribed. Then complementary DNA (cDNA) templates were observed by conventional PCR. All CCA cell lines and cholangiocyte expressed GR (Figure 1).

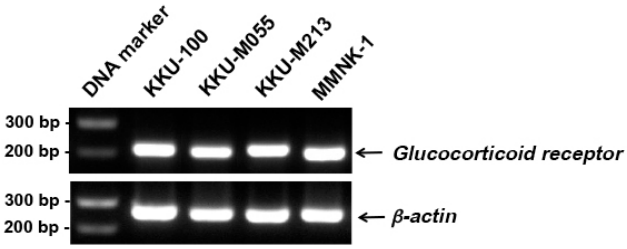


Figure 1 Detection of GR mRNAs in CCA cell lines, KKU-100, KKU-M055, KKU-M213, and cholangiocyte, MMNK-1, by conventional PCR. β-actin mRNA was detected as an internal control.

The expression of GR at protein level was also observed by western bloting. Protein lysate from each cell line was prepared by using cell lysis buffer. The lysate was subjected to SDS-PAGE, and the protein expression of GR detected by anti-GR antibody (Abcam). Anti-β-actin antibody (SantaCruz) was used as an internal control. The membrane was probed with horseradish peroxidase-conjugated antibody. The immunoreactive proteins were visualized by SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) which were then detected by using a G:BOX chemiluminescence imaging system (Syngene). As shown in Figure 2, KKU-M055 and MMNK-1 clearly expressed GR protein, KKU-M213 slightly expressed GR protein, while GR protein could not be detected by immunobloting (Figure 2).

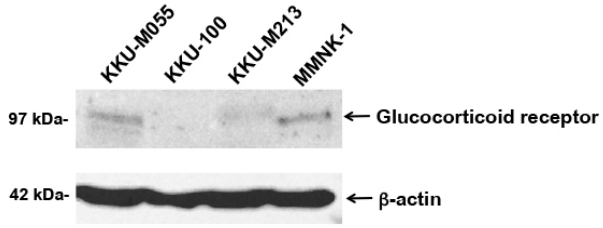


Figure 2 Detection of GR protein in CCA cell lines, KKU-M055, KKU-100, KKU-M213, and cholangiocyte, MMNK-1, by immunobloting. β-actin was detected as an internal control.

2. Observation the effect of compound A (CpdA) on cell survival in primary cells and CCA cell lines

To observed the cytotoxicity of CpdA on normal cells, three primary cells were used in this study. Skin fibroblast cells (SF-A4) were a gift from Assoc. Prof. Chanitra Thuwajit, Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University. Peripheral blood mononuclear cells (PBMCs) were repared from heathy donor. Human umbilical vein endothelial cells (HUVECs) were obtained from umbilical cord. The effect of CpdA on cell survival was perfomed to observed the cytotoxic concentration 50 (CC50) of compound. Three primary normal cells and CCA cell lines were treated with CpdA at concentration 1.56 – 50 μM for 24 h and 48 h (Figure 3 and 4). CC50 of CpdA primary normal cells and CCA cell lines were shown in Table 1.

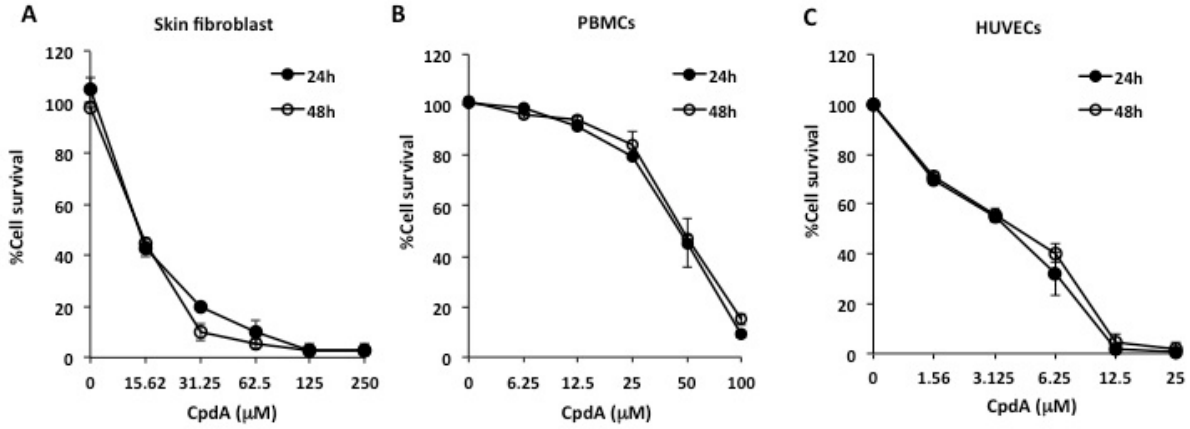


Figure 3 Cell viability of primary cells after treated with various concentration of CpdA at 24 and 48 hours. **(A)** Skin fibroblast, **(B)** PBMCs, and **(C)** HUVECs.

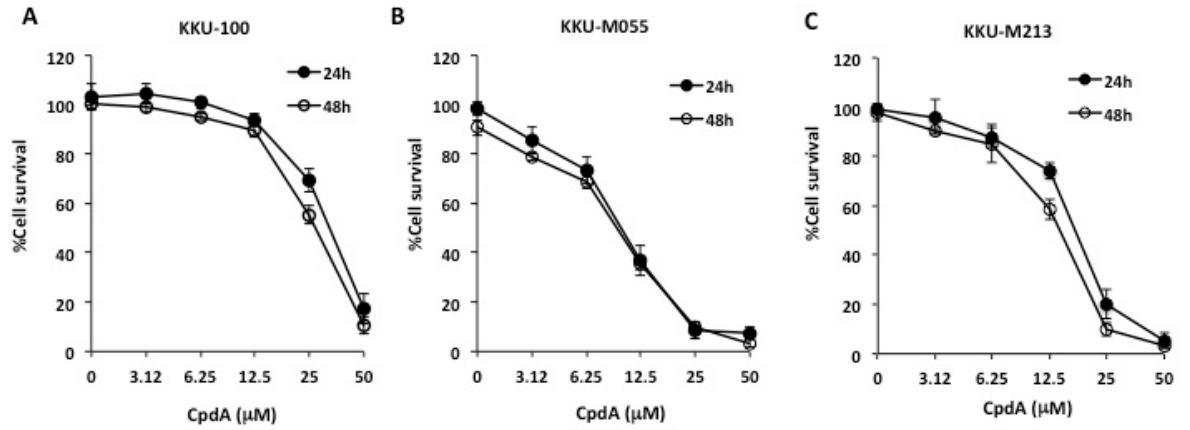


Figure 4 Cell viability of CCA cell lines after treated with various concentration of CpdA at 24 and 48 hours. **(A)** KKU-100, **(B)** KKU-M055, and **(C)** KKU-M213.

Table 1 IC50 of primary cells and CCA cell lines

Time after CpdA treatment	IC50 of CpdA (mM)					
	SF-A4	PBMCs	HUVECs	KKU-100	KKU-M055	KKU-M213
24 hours	15.44 + 1.50	49.86 + 3.33	3.22 + 0.61	25.22 + 0.14	12.28 + 0.34	12.99 + 0.31
48 hours	13.23 + 3.38	49.82 + 2.16	3.20 + 0.71	24.84 + 1.12	12.32 + 0.32	12.71 + 0.17

3. Observation the effect of compound A (CpdA) on *IL-6* expression in CCA cell lines

To observed the effect of CpdA on *IL-6* expression in KKU-100, KKU-M055, and KKU-M213, sub-lethal doses of CpdA were used to treated the cells for 24 h. Total RNA were then prepared by Trizol reagent (Invitrogen) and chloroform extraction. RNA samples were reverse transcribed then complementary DNA (cDNA) templates were assayed for level of transcription using a pair of *IL-6* primers. The PCR reaction were performed by using LightCycler® 480 SYBR Green I Master (Roche) and the reactions were recorded and analyzed using LightCycler® 480 Instrument equipped with a 96-well thermal cycler (Roche). *IL-6* expression was decreased in CCA cells that treated with CpdA in dose dependent manner (Figure 5).

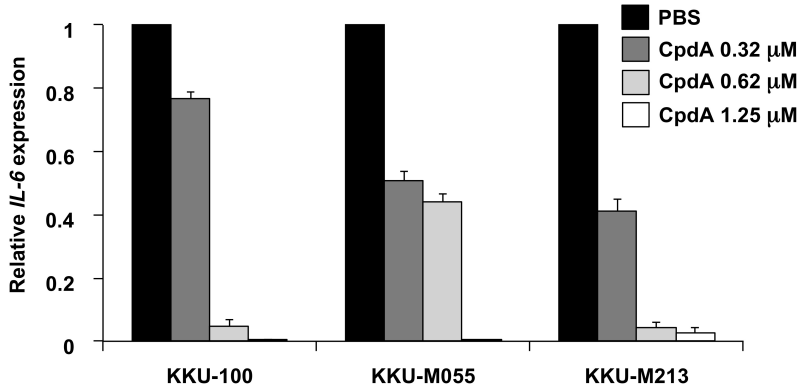


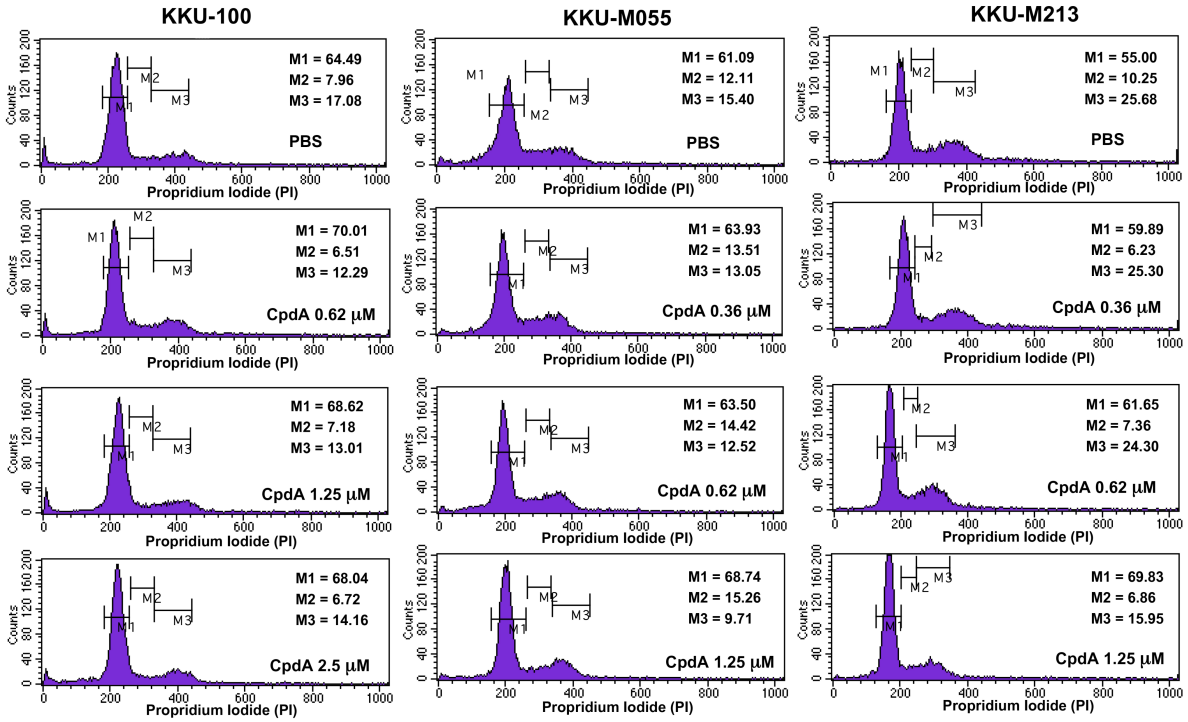
Figure 5 The mRNA expression of *IL-6* was measured in in KKU-100, KKU-M055, and KKU-M213 cells in the presence of CpdA at indicated concentration for 24 hours. Relative gene expression (fold change) of *IL-6* was determined by real-time PCR.

4. Observation the effect of compound A (CpdA) on CCA cell cycle

The effect of CpdA on cell proliferation was investigated by using propidium iodide (PI) staining. Cells were treated with CpdA at appropriate concentration for 24 h. Treated cells were fixed with 70%ethanol at -20^{o}C for 1 h. Then fixed cells were treated with RNase A at 37^{o}C for 30 min before stained with PI. Cell cycle at G1, S, and G2-M phase were analysed by using flow

cytometry. As shown in Figure 5, CpdA slightly increased number of cells at G1 phase in KKU-100 and KKU-M055 cells at 24 h after CpdA treatment. However, increasing number of cell at G1 phase was significantly observed in KKU-M213. The effect of CpdA on cell cycle arrest will further be observed at 48 h. after CpdA treatment. However, the results at 24 h suggested that CpdA inhibited cell proliferation by inhibited cell cycle at G1 phase.

A



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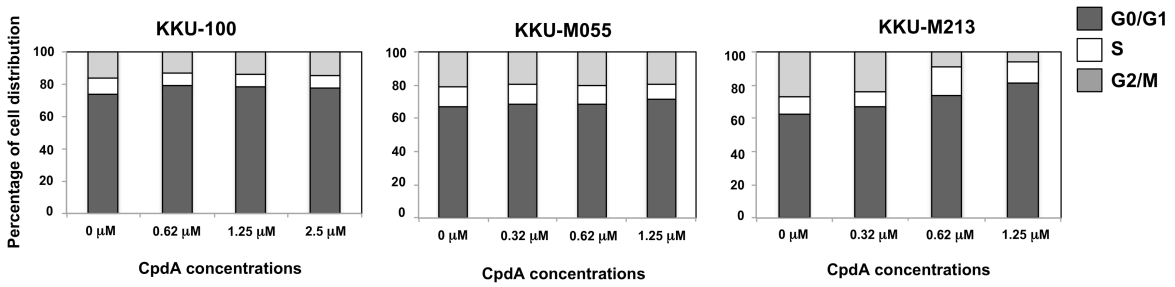

Figure 6 Effect of CpdA on cell cycle distribution of CCA cell lines. KKU-100, KKU-M055, and KKU-M213 were treated with PBS or CpdA at indicated concentration for 24 h. Cell cycle distribution was determined by propidium iodide staining then analysed by flow cytometry. **(A)** Histogram plot of flow cytometry analysis. M1, M2, and M3 represent G0/G1, S, and G2/M phase, respectively. **(B)** The percentage of cells in each phase of cell cycle. The data represent as mean from two independent experiments.

5. Combined effect of CpdA and Cisplatin on CCA cell survival

IL-6 has an important role in the pathogenesis and growth of CCA by activate several pathways involved in survival signaling. From previous report, we found that CpdA reduced *IL-6* expression and arrested cell cycle at G1 phase. Since the anti-cancer drug resistance has been reported in CCA patients, which result in using drugs at high dose, we planed to combined sub-lethal dose of CpdA with cisplatin in CCA cell lines. We expected to observe that dose of cisplatin treatment should reduced when combined with CpdA in activating cancer cell death. CpdA and cisplatin were combined and test with CCA cell lines; KKU-100, KKU-M055, and KKU-M213 cells. For KKU-100, CpdA was used at concentration 0.625, 1.25, and 2.5 μM while CpdA at 0.321, 0.625, and 1.25 μM were used to treated KKU-M055 and KKU-M213. The combined CpdA and cisplatin (at 1, 10, and 100 μM) treatments showed higher number of KKU-100 cell survival than cisplatin treatment alone (Figure 6). The same results were observed with KKU-M055 (Figure 7) and KKU-M213 (Figure 8) cells.

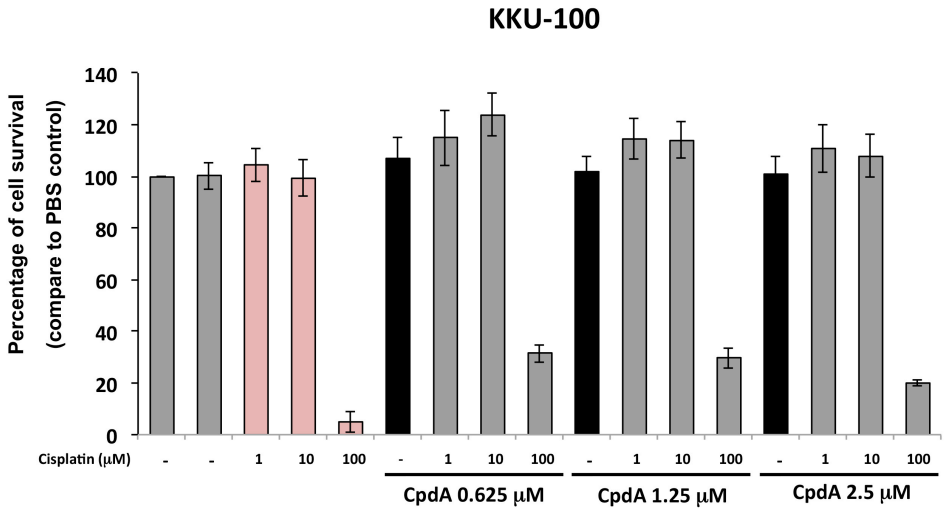

Figure 6 Cell viability (presented as percentage) determined by using Presto Blue dye assay of KKU-100 cells that were treated with CpdA alone or in combination with cisplatin at the indicated concentrations.

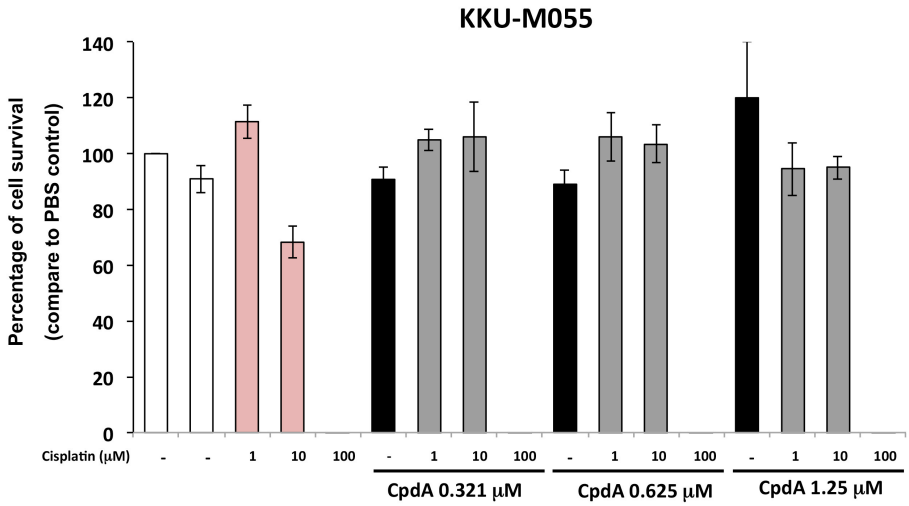

Figure 7 Cell viability (presented as percentage) determined by using Presto Blue dye assay of KKU-M055 cells that were treated with CpdA alone or in combination with cisplatin at the indicated concentrations.

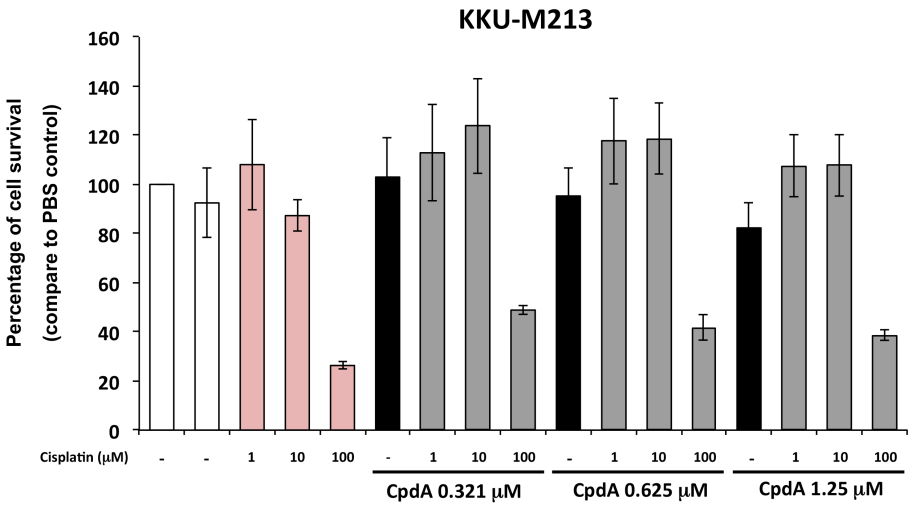

Figure 8 Cell viability (presented as percentage) determined by using Presto Blue dye assay of KKU-M213 cells that were treated with CpdA alone or in combination with cisplatin at the indicated concentrations.

Conclusion and discussion

Cholangiocarcinoma (CCA) is an aggressive and lethal cancer arising from biliary epithelia within biliary tracts (Kambakamba and DeOliveira, 2014). This cancer is rare worldwide but it is the most common liver cancer in northeast Thailand (Sripa and Pairojkul, 2008). Several conditions associated with chronic inflammation have been identified as risk factors for CCA. Infection with the liver fluke *Opisthorchis viverrini* is the most common risk factor for CCA in Thailand and in Southeast Asia (Sithithaworn et al., 2014). The malignancy of CCA is normally difficult to diagnose until the disease become advanced or disseminated, at which the prognosis is poor. Surgery is the only curative treatment for patients with CCA; however, less than one-third of patients are resectable at diagnosis. CCA is well known to recur after surgery, and there is a high mortality rate even if patients receive a curative operation (Rosen et al., 2008). Moreover, the use of chemotherapy for CCA had poor results and studies were small and conflicting (Nakajima et al., 2003, Yoon et al., 2011, Lin et al., 2012). It is crucial need to search for a new drug or compound that can utilize its effect either alone or in combination with the present chemotherapy for improve treatment of CCA.

Carcinogenesis of CCA is a multistep cellular process evolving from a normal condition of the epithelial biliary cells through a chronic inflammation status ending with malignant transformation (Fava et al., 2007, Fava and Lorenzini. 2012). CCA cells express altered molecular mechanisms, which enhance cell proliferation, decrease apoptosis, and increase the capacity of tissue invasion, stromal proliferation, and angiogenesis. The inflammatory cytokines such as IL-6 have an important role in the pathogenesis and growth of CCA by activate several pathways involved in survival signaling and contributes to chemoresistance (Okada et al., 1994, Park et al., 1999, Park et al., 1999). Glucocorticoids (GC) are one of the potent anti-inflammatory agents that have been used in chronic inflammatory disease and many cancers (Pazdur et al., 1999). In CCA, GC namely Dexamethasone, has been use in clinical trial phase II but various metabolic side effects were found (Pazdur et al., 1999). Since CpdA strongly interacts with GR and exerts the inhibition of cancer cell progression, the effect of this compound in chemotherapy of CCA seems very promising.

In this project, we aim to evaluate the anticancer effect of CpdA in CCA cells. The expression of glucocorticoid receptor (GR) at mRNA level was observed by conventional PCR in CCA cell lines namely KKU-100, KKU-M213, and KKU-M055 and cholangiocyte cell line, MMNK-1. The result showed that CCA cell lines and cholangiocyte expressed GR, which

could be response to GR or CpdA. The expression of GR at protein level was also observed by western bloting. KKU-M055 and MMNK-1 clearly expressed GR protein, KKU-M213 slightly expressed GR protein, while GR protein could not be detected by immunobloting (Figure 2). Then the effect of CpdA on cell survival was perfomed to observe the cytotoxic concentration 50 (CC50) of compound (Figure 3).

CpdA has been reported to inhibit cytokine expression and secretion in many inflammatory diseases (Reber et al., 2012). IL-6 is one of cytokines that increases in CCA patients and promoted CCA cell proliferation (Johnson et al., 2012). We then determined the effect of CpdA on *IL-6* expression in KKU-100, KKU-M055, and KKU-M213 by real-time PCR. *IL-6* expression was decreased in CCA cells that treated with CpdA in dose dependent manner (Figure 4). These studies demonstrated that CpdA acts as anti-inflammatory agent by reduce inflammatory cytokine production such as IL-4, IL-5, and IL-13 (Reber et al., 2012). Moreover, according to the mechanism in Figure 3B, CpdA binds to GR and induces its nuclear translocation. Subsequently it represses the expression of inflammatory genes; IL-6 and matrix metalloproteinase-1 (Dewint et al., 2008). The anti-inflammatory effect of CpdA has been reported that involves with reduction of DNA-binding activity as well as interferes with transactivation potential of NF-kB (De Bosscher et al., 2005). There is a recent study showed CpdA can reduce TNF-stimulated-IkBa degradation and NF-kB p65 nuclear translocation in heat shock factor 1-dependent mannerin A549 lung epithelial cells. This diminished the expression of NF-kB-driven genes such as IL-6 and IL-8 (Beck et al., 2013).

IL-6 has an important role in the pathogenesis and growth of CCA by activate several pathways involved in survival signaling (Johnson et al., 2012). Since CpdA reduced *IL-6* expression in CCA cell lines, we then investigated the effect of CpdA on cell proliferation by using propidium iodide (PI) staining. CpdA slightly increased number of cells at G1 phase in KKU-100 and KKU-M055 cells at 24 h after CpdA treatment. However, increasing number of cell at G1 phase was significantly observed in KKU-M213. These results suggested that CpdA inhibited cell proliferation by inhibited cell cycle at G1 phase. The antitumor effect of CpdA has been reported. In leukemia cell lines CEM and K562, CpdA inhibited cell growth and induced pro-apoptosis in these cells by activated transrepression of NF-kB and AP-1 factor (Lesovaya et al., 2011). CpdA has an anticancer by inhibited both growth and cell survival of highly malignant prostate cancer cells in glucocorticoid receptor-dependent fashion (Robertson et al., 2010). In human T-B-lymphoma, and multiple myeloma cells, CpdA strongly inhibited growth and viability (Lesovaya et al., 2013). In combination with proteasome inhibitor, Bortezomib, CpdA potently suppressed growth and survival that cause by accumulation of glucocorticoid receptor

(Lesovaya et al., 2013). Since the anti-cancer drug resistance has been reported in CCA patients, which result in using drugs at high dose, we then combined sub-lethal dose of CpdA with cisplatin in CCA cell lines., CpdA that were combined with did not show any additive or synergistic effect (Figure 6, 7, and 8). These results are contrast with our expectation that combined treatment of CpdA and cisplatin did not enhance the CCA cell death. However, our finding has been supported by the study of Chen YX, et al. in ovarian cancer cell lines. Combined treatment of dexamethasone and cisplatin increased cell survival of HO-8910 and SKOV3 cells by increasing of TGF-β1 (Chen et al., 2010). Since dexamethasone is the glucocorticoid as CpdA, increasing of CCA cells survival in this study might be the action of TGF-β1, which should be confirmed.

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Output

1. International Journal Publication

   1.1 Project-related output

   - Manuscript will be submitted to publish in “Journal of Molecular Medicine” with title “Anticancer effects of compound A in CCA cells”.
   - Rattanaburee T, **Junking M**, Panya A, Sawasdee N, Songprakhon P, Suttitheptumrong A, Limjindaporn T, Haegeman G, Yenchitsomanus PT. Inhibition of dengue virus production and cytokine/chemokine expression by ribavirin and compound A. Antiviral Res. 2015 Nov 2;124:83-92.

   1.2 Non project-related output

   - **Junking M**, Sawasdee N, Duangtum N, Cheunsuchon B, Limjindaporn T, Yenchitsomanus PT*. Role of adaptor proteins and clathrin in the trafficking of human kidney anion exchanger 1 (kAE1) to the cell surface. Traffic. 2014;15(7):788-802.
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2. Application

   -

3. Others e.g. national journal publication, proceeding, international conference, book chapter, patent

Poster presentation

Junking M, Rattanaburee T, Panya A, Wongkham S, Haegeman G, Yenchitsomanus P. *Anticancer effect of the selective glucocorticoid receptor modulator, compound A, in cholangiocarcinoma* [abstract]. In: Proceedings of the TRF-OHEC Annual Congress 2016 (TOAC2016); 2016 Jan 6-8; Phetchaburi, Thailand.

Anticancer Effect of the Selective Glucocorticoid Receptor Modulator, Compound A, in Cholangiocarcinoma

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INTRODUCTION and OBJECTIVES

Cholangiocarcinoma (CCA) is an aggressive and lethal cancer arising from biliary epithelium. This cancer is the most common cancer in northeast Thailand. Surgery is the only curative treatment for patients and the use of chemotherapy had poor results. It is crucial need to search for a new compound for improves treatment of CCA. Glucocorticoids (GC) are one of the potent anti-inflammatory agents that have been used in many cancers. As a result, decreasing in pro-inflammatory cytokines and survival genes, leading to the tumor cell death. Recently, a small plant-derived GR modulator, compound A (CpdA), showed anti-inflammatory activity and has fewer side effects compared with glucocorticoids. In this project, we aim to evaluated the anticancer effect of CpdA in CCA cells. This study will provide a novel strategy for chemotherapy for cholangiocarcinoma.

METHODS

Determination of cytotoxicity of CpdA on primary cells and CCA cell lines by Presto Blue dye assay

Determination the effect of CpdA on *IL-6* expression in CCA cell lines by real-time PCR

Observation the effect of CpdA on cell cycle arrest in CCA cell lines by flow cytometry

RESULTS

The cytotoxicity of CpdA was observed in normal cells (Figure 1A) and CCA cell lines (Figure 1B). Cells were treated with CpdA at various concentrations for 24 and 48 h. Cytotoxic concentration (CC50) of each condition is shown in Table 1.

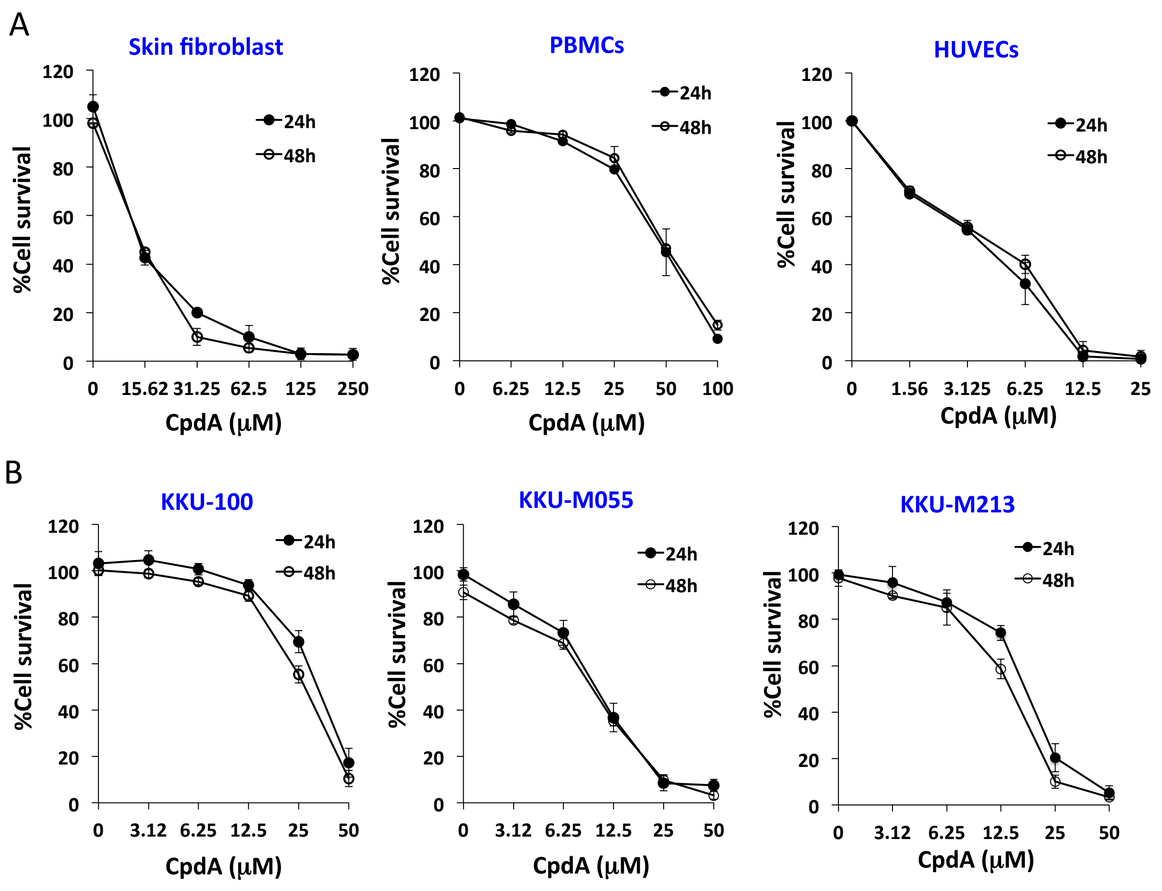

Figure 1 Cell viability of cells after treated with CpdA at indicated concentration for 24 and 48 h. A. Normal cells. B. CCA cell lines. The data represent as mean from three independent experiments.

Table 1 Cytotoxic concentration (CC50) of CpdA in normal cells and CCA cell lines.

Time after Cpd A treatment	CC50 of Cpd A (μM)					
	SF-A4	PBMCs	HUVECs	KKU-100	KKU-M055	KKU-M213
24 h	15.44 ± 1.5	49.86 ± 3.33	3.22 ± 0.61	25.22 ± 0.14	12.28 ± 0.34	12.99 ± 0.31
48 h	13.23 ± 3.38	49.82 ± 2.16	3.20 ± 0.71	24.84 ± 1.12	12.32 ± 0.32	12.71 ± 0.17

RESULTS

CpdA has been reported to inhibit cytokine expression and secretion in many inflammatory diseases. IL-6 is one of cytokines that increases in CCA patients and promoted CCA cell proliferation. We then determined the effect of CpdA on *IL-6* expression in CCA cell lines by real time PCR using a pair of *IL-6* primers. *IL-6* expression was decreased in CCA cells that treated with CpdA in dose dependent manner (Figure 2).

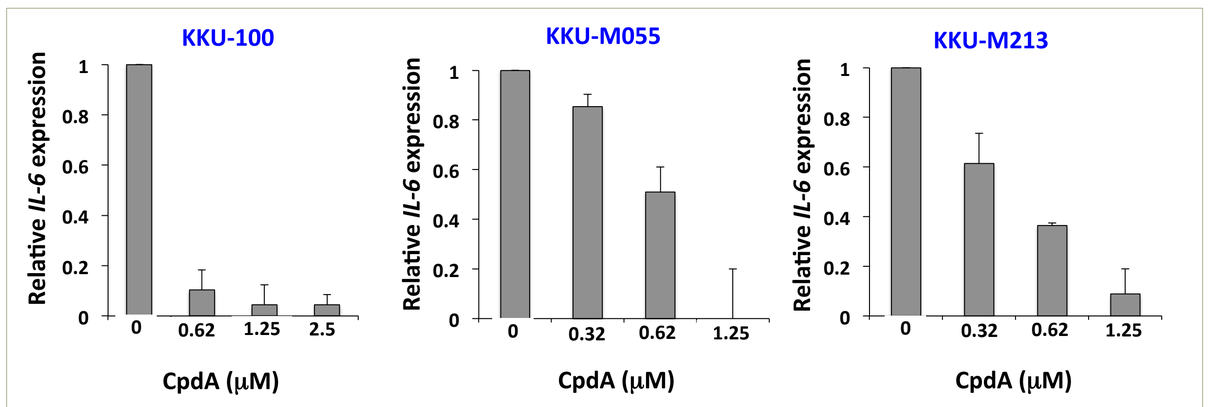

Figure 2 The mRNA expression of *IL-6* was measured in in KKU-100, KKU-M055, and KKU-M213 cells in the presence of CpdA at indicated concentration for 24 h. Relative gene expression (fold change) of *IL-6* was determined by real-time PCR.

The effect of CpdA on cell proliferation were investigated by using propidium iodide (PI) staining. Cell cycle were analysed by using flow cytometry. CpdA increased number of cells at G1 phase in CCA cells at 24 h after CpdA treatment, suggesting that CpdA inhibited cell proliferation by inhibited cell cycle at G1 phase.

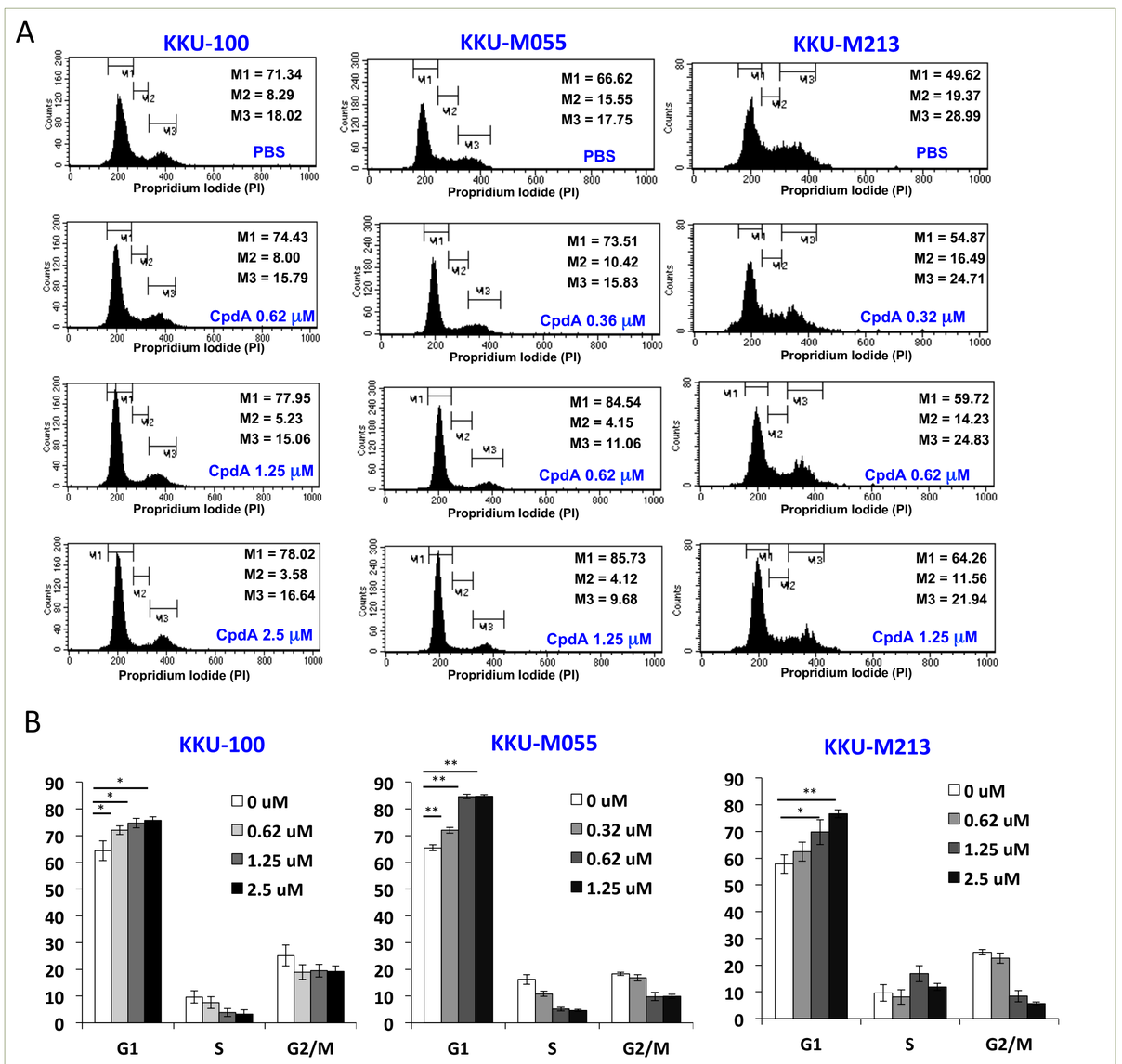

Figure 3 Effect of CpdA on cell cycle distribution of CCA cell lines. A. Histogram plot of flow cytometry analysis. M1, M2, and M3 represent G0/G1, S, and G2/M phase, respectively. B. The percentage of cells in each phase of cell cycle. The data represent as mean from three independent experiments.

CONCLUSION

The anticancer effects of CpdA in CCA cell lines were observed in this study. Cytotoxicity of CpdA on normal cells and CCA cell lines was determined. CpdA reduced *IL-6* expression in CCA cell lines. Moreover, CpdA inhibited CCA cell lines proliferation and G1 cell cycle arrest was investigated. Further study will be observed antitumor activity of CpdA either using CpdA alone or combination with the traditional anticancer agents, cisplatin. This study will provide a novel strategy for chemotherapy of CCA patients.

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Inhibition of dengue virus production and cytokine/chemokine expression by ribavirin and compound A

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ABSTRACT

Dengue virus (DENV) infection is a worldwide public health problem with an increasing magnitude. The severity of disease in the patients with DENV infection correlates with high viral load and massive cytokine production – the condition referred to as "cytokine storm". Thus, concurrent inhibition of DENV and cytokine production should be more effective for treatment of DENV infection. In this study, we investigated the effects of the antiviral agent – ribavirin (RV), and the anti-inflammatory compound – compound A (CpdA), individually or in combination, on DENV production and cytokine/chemokine transcription in human lung epithelial carcinoma (A549) cells infected with DENV. Initially, the cells infected with DENV serotype 2 (DENV2) was studied. The results showed that treatment of DENV-infected cells with RV could significantly reduce both DENV production and cytokine (IL-6 and TNF-α) and chemokine (IP-10 and RANTES) transcription while treatment of DENV-infected cells with CpdA could significantly reduce cytokine (IL-6 and TNF-α) and chemokine (RANTES) transcription. Combined RV and CpdA treatment of the infected cells showed greater reduction of DENV production and cytokine/chemokine transcription. Similar results of this combined treatment were observed for infection with any one of the four DENV (DENV1, 2, 3, and 4) serotypes. These results indicate that combination of the antiviral agent and the anti-inflammatory compound offers a greater efficiency in reduction of DENV and cytokine/chemokine production, providing a new therapeutic approach for DENV infection.

1. Introduction

Dengue virus (DENV) infection, a mosquito-borne viral disease, is a major public health problem worldwide (Gubler, 2011; Halstead, 2007). DENV is transmitted by *Aedes aegypti* and *Aedes albopictus* mosquitoes, which are widespread in the tropical and subtropical regions (Bhatt et al., 2013; Kyle and Harris, 2008). Approximately 390 million people worldwide are at risk for DENV infection (Bhatt et al., 2013) with 500,000 dengue hemorrhagic fever (DHF) cases and more than 22,000 deaths each year (Gubler, 2002; Murray et al., 2013; Shepard et al., 2011). DENV is a member of *Flaviviridae* family and *Flavivirus* genus; its genome is a single positive-strand RNA with approximately 10.6 kilobases (Qi et al., 2008). It consists of four antigenically related serotypes, DENV 1, DENV 2, DENV 3, and DENV 4 (Blok, 1985).

The clinical manifestations of DENV infection range from asymptomatic or undifferentiated febrile illness, dengue fever (DF), dengue haemorrhagic fever (DHF), to dengue shock syndrome (DSS) (Simmons et al., 2012). Currently, there is neither licensed vaccine for prevention nor specific antiviral drug for treatment of DENV infection. Several studies have shown that severity of disease in the patients with DENV infection correlates with high viral load and host immune response, especially elevation of cytokines (Green and Rothman, 2006; Guilarde et al., 2008; Tricou et al., 2011; Vaughn et al., 2000, 1997). In the severe forms of DENV infection,

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0166-3542/

DHF/DSS, there are marked increases of pro-inflammatory cytokines, immunosuppressive cytokines, and chemokines such as IL-6, IP-10, RANTES, TNF-α, and IFN-γ (Castro et al., 2011; Malavige et al., 2012; Nguyen et al., 2004; Rathakrishnan et al., 2012; Restrepo et al., 2008; Suharti et al., 2003). The correlation between severity of DENV infection and high viral titer as well as extreme cytokine production – the condition referred to as "cytokine storm" prompts us to hypothesize that concurrent inhibition of DENV and cytokine production should be more effective for treatment of DENV infection. Thus, the use of combined antiviral and anti-inflammation drugs or a single drug with both effects is a promising therapeutic strategy for DENV infection.

An antiviral drug, ribavirin (RV), has been used for treatment of hepatitis C virus (HCV) infection (Abdel-Hady et al., 2014; Bansal et al., 2015) and in experimental studies with flaviviruses including DENV (Diamond et al., 2002; Lee et al., 2012; Takhampunya et al., 2006), suggesting potential for use in combinations against other flaviviruses such as DENV. Combination of RV with α-glucosidase inhibitor efficiently inhibited DENV infection of cultured human cells (Chang et al., 2011). As anti-inflammatory drug, dexamethasone (DEX) – a synthetic glucocorticoid, was tested for its modulation in DENV infection, showing optimistic results in both decreasing cell infection rates and inhibiting TNF-α, IFN-α and IL-10 production (Reis et al., 2007). However, the use of the glucocorticoid for treatment of DENV infection is controversial because of its efficacy and side effects (Halvorsen et al., 2003; Heimdal et al., 1992; Vardy et al., 2006). Our group has recently reported the results of *in vitro* studies using compound A (CpdA) as anti-inflammatory compound in DENV infection (Khunchai et al., 2015; Suttitheptumrong et al., 2013). CpdA is a plant-derived phenyl aziridine precursor extracted from *Salsola tuberculatiformis* Botschantzev, a Namibian shrub (De Bosscher et al., 2005; Louw and Swart, 1999; Louw et al., 1997), that contains an anti-inflammatory effect and acts as a dissociated non-steroidal glucocorticoid receptor modulator. CpdA has been studied in inflammatory diseases such as rheumatoid arthritis (Dewint et al., 2008; Gossye et al., 2009; Rauch et al., 2011) and multiple sclerosis (Wust et al., 2009). Our recent study showed that CpdA reduced DENV-induced cytokine secretion and DENV production in the infected human hepatocellular carcinoma (HepG2) cell line (Suttitheptumrong et al., 2013). CpdA reduced inflammatory cytokines via inhibition of NF-κB transcriptional factors (Gossye et al., 2009; Rauch et al., 2011; Reber et al., 2012). We also demonstrated that CpdA, which acts as an NF-κB inhibitor, could suppress RANTES in DENV-infected human embryonic kidney cells (Khunchai et al., 2015).

In this study, we investigated the effects of the antiviral drug (RV) and the anti-inflammatory compound (CpdA), individually and in combination, on DENV production and cytokine transcription in human lung epithelial carcinoma (A549) cells, which were primarily infected with DENV2. The effects of combined treatment were then observed for the infection with either DENV1, 2, 3 or 4. The results of our study indicate that combination of RV and CpdA offers greater effects on reduction of DENV and cytokine production, paving a new way for treatment of DENV infection.

2. Materials and methods

2.1. DENV propagation

DENV serotype 1 (DENV1) strain Hawaii, DENV serotype 2 (DENV2) strains 16681, DENV serotype 3 (DENV3) strains H87, and DENV serotype 4 (DENV4) strains H241 were propagated in C6/36 cells. The C6/36 cells were separately infected with each DENV serotype at a multiplicity of infection (MOI) of 0.1 in maintenance medium and incubated for 3 h at room temperature on a shaker. Subsequently, unbound viruses were removed and fresh maintenance medium was added. Then, the infected cells were incubated at 28 °C for 5–7 days or until cells showed cytopathic effect (CPE). The culture supernatant containing DENV was collected and stored at −70 °C until used.

2.2. Foci-forming unit assay (FFU)

The Vero cells were cultured with ten-fold dilutions of DENV and incubated at 37 °C, 5% CO_2 for 2 h. After virus absorption, overlay medium was added and incubated for 3 days. The overlay media was removed and the infected cells were saved. Cells were fixed with formaldehyde/PBS and permeabilized with 0.2% Triton-X100/PBS for 15 min. Infected cells containing DENV antigen were identified by incubation with mouse monoclonal anti-DENV-E protein (4G2) antibody followed by HRP-conjugated rabbit anti-mouse IgG for 60 min (Dako, Denmark) and then stained with DAB (3,3′-diaminobenzidine) substrate solution. The foci-forming units were counted under light microscope.

2.3. A549 cell culture

The A549 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (GIBCO BRL, USA), supplemented with 10% fetal bovine serum (FBS, GIBCO, USA), 36 μg/ml penicillin G (Sigma, USA), 60 μg/ml streptomycin (Sigma, USA), 2 mM L-glutamine (Sigma, USA), 1 mM non-essential amino acids (GIBCO BRL, Invitrogen, USA), and 1 mM sodium pyruvate (Sigma, USA), at 37 °C on a humidified atmosphere with 5% CO_2.

2.4. DENV infection and compound treatment

DENV1-4 serotypes were prepared in 2% DMEM and then taken to infect in A549 cells at MOI 5. The A549 cells without DENV infection were used as mock control. After absorption onto the cells, unbound DENV was removed. After 24 h post-infection, the infected cells were treated individually or in combination with RV (Sigma, USA) dissolved in PBS, CpdA [Laboratory of Eukaryotic Gene Expression & Signal Transduction (LEGEST), Department of Physiology, Ghent University, Belgium] dissolved in PBS, or DEX (Sigma, USA) dissolved in DMSO at concentrations as indicated in the experiments, which was then added into 2% DMEM for 24 h. The supernatant were then collected to determine DENV production by the FFU assay. The treated A549 cells were collected to measure cytokine transcription by real-time PCR method.

2.5. Real time reverse transcription-polymerase chain reaction (RT-PCR)

To examine cytokine/chemokine transcription, total RNA was prepared from mock and DENV-infected cells, with or without treatment as indicated, by using Trizol™ reagent (Invitrogen, New Zealand), following the manufacturer's procedure. Cytokine/chemokine mRNA was quantified by real-time RT-PCR technique using specific primers (Table 1). cDNA was synthesized using Superscript®III reverse transcriptase (Invitrogen, New Zealand). The cDNA synthesis was performed by following the manufacturer's guidelines. Amplification of cDNA by real-time PCR was carried out in a reaction mixture (Roche, Germany). The real-time PCR profile

Table 1
Sequences of primers for cytokine transcription study.

Primer	Orientation	Sequence (5′-3′)
IP-10_F	Forward	GAATCGAAGGCCATCAAGAA
IP-10_R	Reverse	AAGCAGGGTCAGAACATCCA
RANTES_F	Forward	TCCTGCAGAGGATCAAGACA
RANTES_R	Reverse	TCCTGCAGAGGATCAAGACA
IL-6_F	Forward	GTACATCCTCGACGGCATC
IL-6_R	Reverse	AGCCACTGGTTCTGTGCCT
TNF-α_F	Forward	TGCTTGTTCCTCAGCCTCTT
TNF-α_R	Reverse	ATGGGCTACAGGCTTGTCACT
GAPDH_F	Forward	CGACCACTTTGTCAAGCTCA
GAPDH_R	Reverse	AGGGGTCTACATGGCAACTG

was set for: (i) pre-incubation at 95 °C for 5 min; (ii) PCR for 35 cycles (consisting of 95 °C for 10 s, 60 °C for 10, and 72 °C for 20 s, per cycle); (iii) melting curve analysis. The relative mRNA expression was normalized against GAPDH mRNA level by using a comparative Ct (delta delta Ct) method.

2.6. Calculation of additive and synergistic effects

The combinatory effects of RV and CpdA on DENV production and cytokine transcription were calculated by comparing theoretically additive effect with combinatory effect of two agents according to the fractional product method (Webb, 1961). The formula is as follows:

$$\text{Theoretically additive (or predicted) effect} = (\text{fu})_{\text{RV}} \times (\text{fu})_{\text{CpdA}}$$

$(\text{fu})_{\text{RV}}$ = fractional unaffected or the result from experiment with RV treatment alone.
$(\text{fu})_{\text{CpdA}}$ = fractional unaffected or the result from experiment with CpdA treatment alone.

Combinatory (or actual) effect = fractional unaffected or the result from experiment with combined treatment.

The result of theoretically additive effect and combinatory effect were plotted to compare and evaluate the combined effect. Additive effect was assigned when the theoretically additive effect was found to be equal to the combinatory effect. Synergistic effect was assigned when the combinatory reduction was found to be greater than the theoretically additive reduction.

2.7. Enzyme-linked immunosorbent assay (ELISA)

The level of IP-10 and RANTES production were measured in the supernatant collected from RV and CpdA treated DENV2 infected A549 cells by ELISA (R&D Systems) by following the manufacturer's instruction.

2.8. Statistical analysis

Statistical analyses were conducted by using Graph-Pad Prism 6 Software (GraphPad Software, Inc.). Mean and standard error of mean (SEM) from three independent experiments were calculated. The difference in experimental results was analyzed by one-way ANOVA, followed by Tukey's *pos hoc* test. A p-value less than 0.05 were considered statistically significant.

3. Results

3.1. RV and CpdA reduced DENV production and cytokine/chemokine transcription in DENV2-infected A549 cells

Initially, the experiments were conducted by using DENV2. Cell viability in each condition was more than 90% (Fig. 1A) and the infection efficiency was examined by flow cytometry (Fig. 1S). The supernatant was collected to determine DENV production by the FFU assay. The mRNA levels of IL-6, TNF-α, IP-10, and RANTES were determined by real-time RT-PCR method.

The anti-viral drug (RV) reduced DENV2 production in dose dependent manner (Fig. 1B) when compared to untreated, PBS-treated or DMSO-treated controls, while DENV2 production was not significantly reduced by treatment with either CpdA at 5, 10, or 20 μM ($p > 0.99$, $p = 0.98$, and $p = 0.52$, respectively) or DEX at 50 μM ($p = 0.90$). Treatment of DENV2-infected cells with RV decreased the transcription of IL-6 (Fig. 1C), TNF-α (Fig. 1D), IP-10 (Fig. 1E) and RANTES (Fig. 1F), when compared to that of PBS-treated. CpdA at 20 μM reduced cytokine/chemokine transcription in DENV2-infected cells (Fig. 1C, D, and F), although IP-10 (Fig. 1E) mRNA level reduction was not statistically significant ($p = 0.06$). DEX at 50 μM also reduced cytokine/chemokine transcription in DENV2-infected cells (Fig. 1C, D, and E), although RANTES (Fig. 1F) mRNA level reduction was not statistically significant ($p = 0.19$). Thus, individual treatment of DENV2-infected cells with RV reduced DENV production, and IL-6, TNF-α, IP-10, and RANTES transcription while CpdA reduced IL-6, TNF-α, and RANTES transcription but did not reduce DENV production and IP-10 transcription.

3.2. Combined treatments of RV and CpdA on DENV production and cytokine/chemokine transcription in DENV2-infected A549 cells

To investigate combined effects of RV and CpdA on DENV2 production and cytokine/chemokine transcription, RV and CpdA were tested with DENV2-infected A549 cells. The combined RV (at 50 and 100 μM) and CpdA (at 5, 10, and 20 μM) treatments to DENV2-infected A549 cells showed significant reduction of DENV2 production (Fig. 2B) when compared with that of individual treatments, with cell viability greater than 90% (Fig. 2A). The infection efficiency of DENV2-infected cells was examined by flow cytometry (Fig. 2S). Similarly, the combined RV and CpdA treatments also showed significant reduction of cytokine/chemokine transcription, including IL-6 (Fig. 2C), TNF-α (Fig. 2D), IP-10 (Fig. 2E), and RANTES (Fig. 2F), in the DENV2-infected A549 cells. The effect of combined RV and CpdA treatments on the reduction of DENV2 production and cytokine/chemokine transcription was greater than that of the individual treatments.

3.3. Individual and combined treatments of RV and CpdA reduced DENV production and cytokine/chemokine transcription in A549 cells infected with DENV1, DENV2, DENV3, or DENV4

To examine whether or not individual or combined RV and CpdA treatment could reduce virus production and cytokine/chemokine transcription in A549 cells infected with other DENV serotypes, A549 cells were infected with DENV1, DENV2, DENV3 or DENV4 at MOI 5. Then, the infected cells were incubated, individually or in combination, with RV at 50 μM and CpdA at 20 μM. The cell viabilities were more than 90% (Fig. 3A). The infection efficiency of DENV-infected were observed by flow cytometry (Fig. 3S).

The results showed that treatments of A549 cells infected

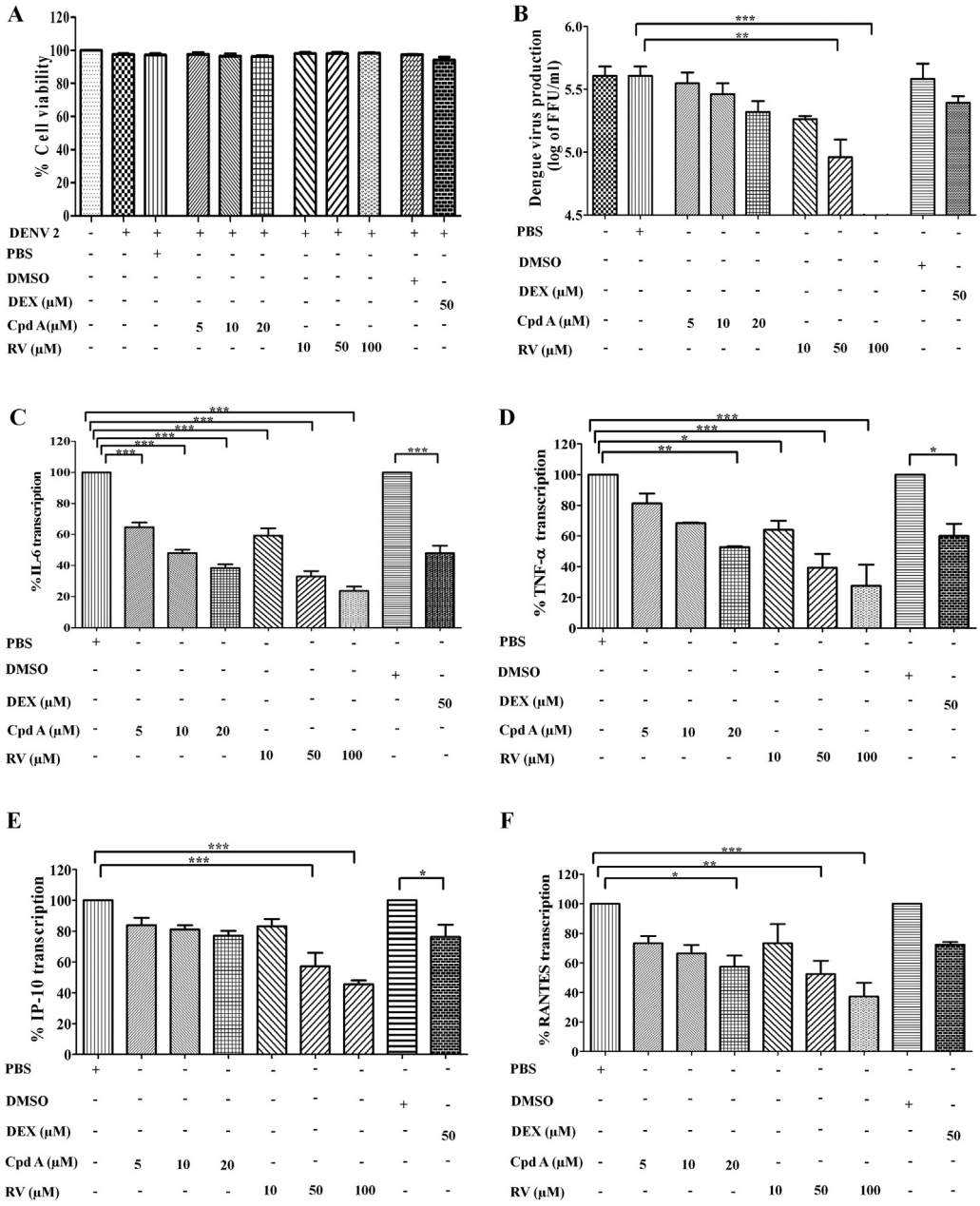

Fig. 1. Effects of ribavirin (RV) and compound A (CpdA) on DENV production and cytokine transcription. A549 cells were infected with DENV2 at MOI 5 and then individually treated with RV or CpdA. **(A)** Cell viability (presented as percentage) was determined by trypan blue exclusion assay of DENV2-infected A549 cells that were individually incubated with RV or CpdA at the indicated concentrations. **(B)** DENV production in cell culture supernatant analyzed by the foci-forming unit (FFU) assay. **(C–F)** Transcription of IL-6, TNF-α, IP-10, and RANTES in DENV2-infected A549 cells determined by real-time RT-PCR technique. The results were conducted in five independent experiments and mean ± SEM were calculated and plotted (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

with either DENV1, DENV2, DENV3 or DENV4 with combined RV at 50 μM and CpdA at 20 μM could significantly reduce DENV production for all DENV serotypes (Fig. 3B). Treatment of A549 cells infected with either DENV1, DENV2, DENV3 or DENV4 with combined RV at 50 μM and CpdA at 20 μM could also significantly reduce IL-6 (Fig. 3C), TNF-α (Fig. 3D), IP-10 (Fig. 3E), and RANTES (Fig. 3F) transcription.

The effects of combined RV and CpdA treatments on the reduction of DENV1, DENV2, DENV3, and DENV4 productions and cytokine/chemokine transcription were generally greater than that of individual treatments with RV at 50 μM.

3.4. Calculation of additive and synergistic effects of RV and CpdA

The combinatory effects of RV and CpdA on DENV production and cytokine/chemokine transcription were calculated by comparing the theoretically additive (or predicted) effects of RV and CpdA with the combinatory (or actual) effects of the two agents from the experiments by using fractional product method. The results of theoretically additive effect and combinatory effect were plotted (Fig. 4) to evaluate their combined effects. The effects of combined RV and CpdA treatments on DENV2 production and cytokine/chemokine transcription obtained from the

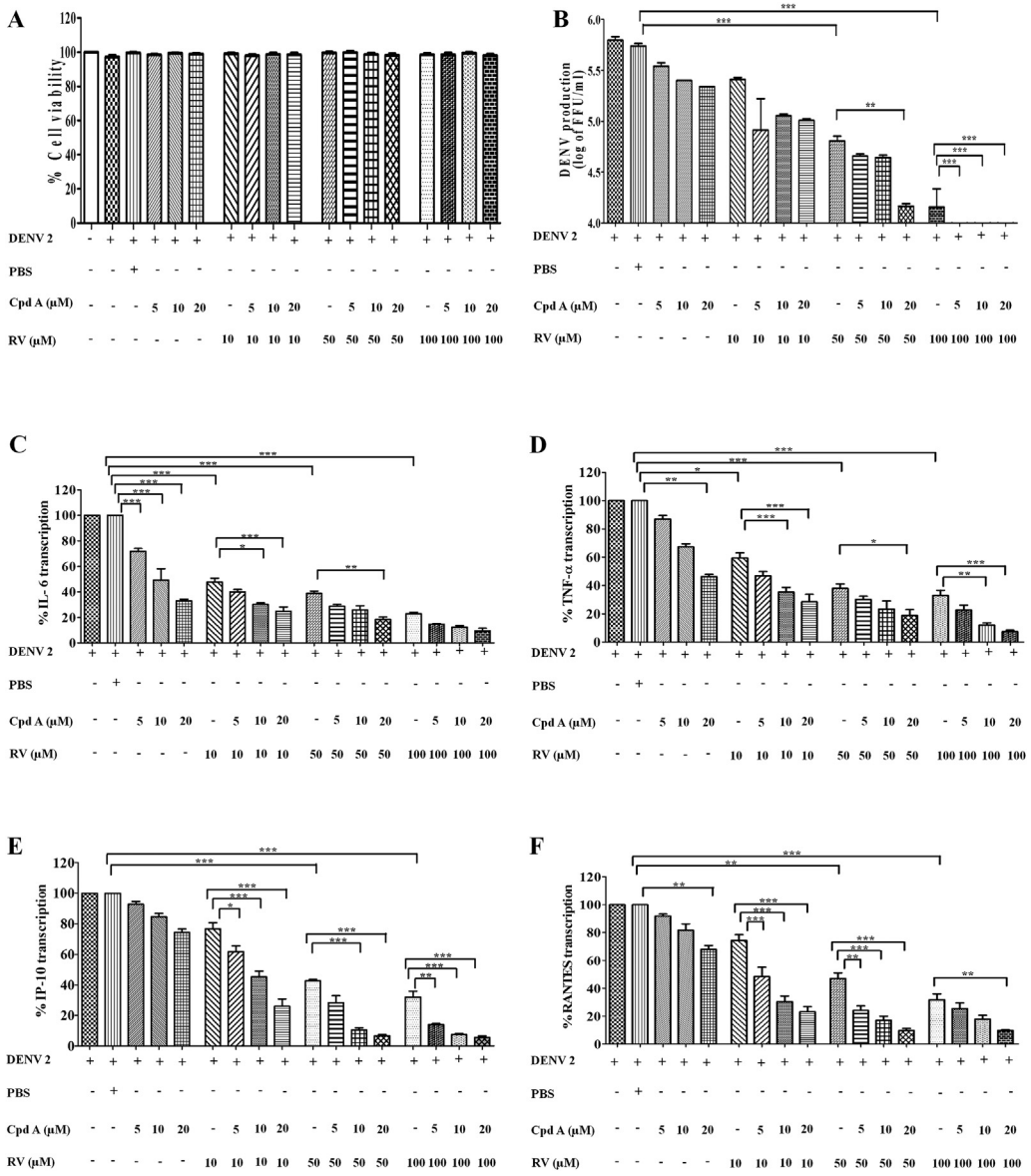

Fig. 2. Combined effects of ribavirin (RV) and compound A (CpdA) on DENV2 production and cytokine transcription. A549 cells were infected with DENV2 at a MOI 5 and subjected to individual or combined treatment with RV and CpdA. **(A)** Cell viability (presented as percentage) determined by the trypan blue exclusion assay of DENV2-infected A549 cells that were incubated with RV alone or in combination with CpdA at the indicated concentrations. **(B)** DENV production in cell culture supernatants analyzed by the foci forming unit (FFU) assay. **(C–F)** Transcription of IL-6, TNF-α, IP-10, and RANTES in DENV2-infected A549 cells determined by real-time RT-PCR technique. The results were conducted in five independent experiments and mean ± SEM were calculated and plotted (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

calculation for theoretically additive effect and combinatory effect were compared (Fig. 4A–E). It was found that combined RV and CpdA treatments on the DENV2-infected A549 cells resulted in the reduction of DENV2 production and cytokine (IL-6, and TNF-α) transcription in additive effect model, since the theoretical additive effects were equal to the combinatory effects (Fig. 4A–C). An effect in which two substances or actions used in combination produced a total effect on the DENV2-infected A549 cells led to the reduction of chemokine (IP-10 and RANTES) transcription in synergistic effect model, since synergistic effect was assigned when the combinatory effect was significantly more than expected for a theoretically additive effect (Fig. 4D and E).

The additive or synergistic effect model of combined treatments was then evaluated by using fractional product method for four DENV serotypes. The results supported the previous finding that the combined RV and CpdA treatments of the A549 cells infected with either DENV1, DENV2, DENV3 or DENV4 resulted in the reduction of virus production (Fig. 5A) in additive model because the combinatory effects were not greater than the theoretically additive effect ($p = 0.34$, $p = 0.46$, $p = 0.85$, and $p = 0.17$, respectively). The additive effect was also observed with cytokine (IL-6 and TNF-α) transcription (Fig. 5B and C). In contrast, the combined RV and CpdA treatment of the A549 cells-infected with either DENV1, DENV2, DENV3 or DENV4 led to the reduction of chemokine (IP-10 and RANTES) transcription in the synergistic effect model (Fig. 5D and E). Thus, combined RV and CpdA treatment reduced virus production and cytokine/chemokine transcription in the A549 cells infected with any one of the 4 DENV serotypes. The combined treatment showed an additive effect on virus production

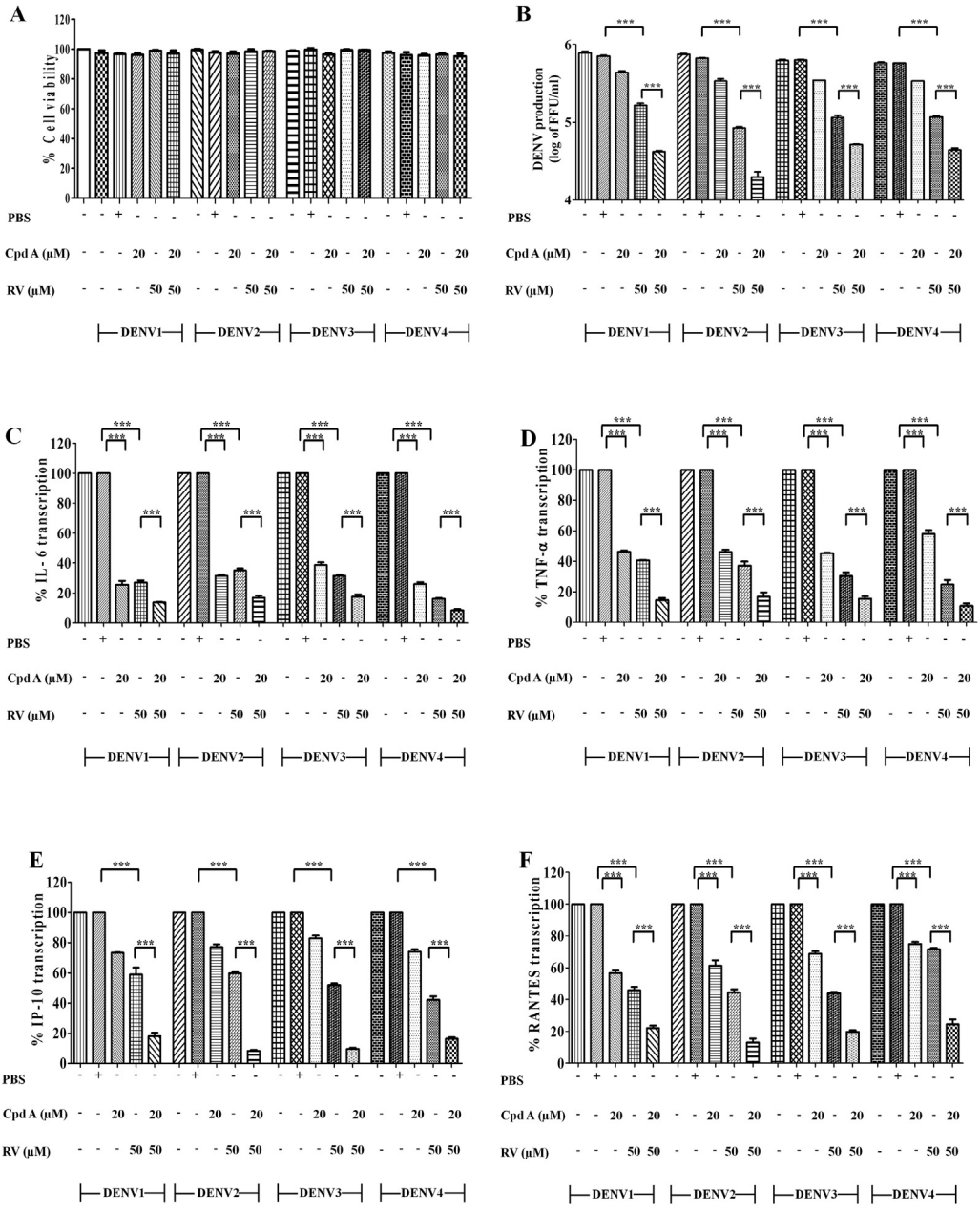

Fig. 3. Combined effects of ribavirin (RV) and compound A (CpdA) on DENV production and cytokine transcription in A549 cells infected with DENV serotypes 1, 2, 3, and 4. A549 cells were infected with each serotype of DENV at MOI 5 and then incubated with RV at 50 μM or CpdA at 20 μM or their combination. **(A)** Cell viability (presented as percentage) determined by the trypan blue exclusion assay of DENV-infected A549 cells that were incubated with RV or CpdA alone or their combination. **(B)** DENV production in cell culture supernatants analyzed by the foci-forming unit (FFU) assay. **(C–F)** Transcription of IL-6, TNF-α, IP-10, and RANTES in DENV-infected A549 cells determined by the real-time RT-PCR technique. The results were conducted in five independent experiments and mean ± SEM were calculated and plotted (* = $p < 0.05$, *** = $p < 0.001$).

and cytokine transcription but a synergistic effect on chemokine transcription.

Since the synergistic effect of the combined RV (50 μM) and CpdA (20 μM) on the DENV-infected A549 cells in the reduction of IP-10 and RANTES transcription was observed, the protein levels of IP-10 and RANTES in the supernatants of DENV2-infected A549 cells were determined by ELISA. As shown in Fig. 6, the protein levels of IP-10 (Fig. 6A) was reduced when treated with RV alone. The protein levels of RANTES (Fig. 6B) were not significantly reduced by RV alone ($p = 0.07$). Individual treatment of CpdA reduced IP-10 and RANTES proteins but did not reach significant levels ($p = 0.74$ and $p = 0.47$, respectively). The combined treatment of RV and CpdA significantly reduced IP-10 and RANTES protein levels, when compared with the result of either RV or CpdA treatment alone.

4. Discussion

We conducted the *in vitro* study by infecting A549 cells with DENV and treating the infected cells with the antiviral drug – ribavirin (RV) and anti-inflammatory compound – compound A (CpdA) for inhibition of DENV production and the cytokine/chemokine expression, respectively. The A549 cells were used in this study because they are susceptible to DENV infection and showed

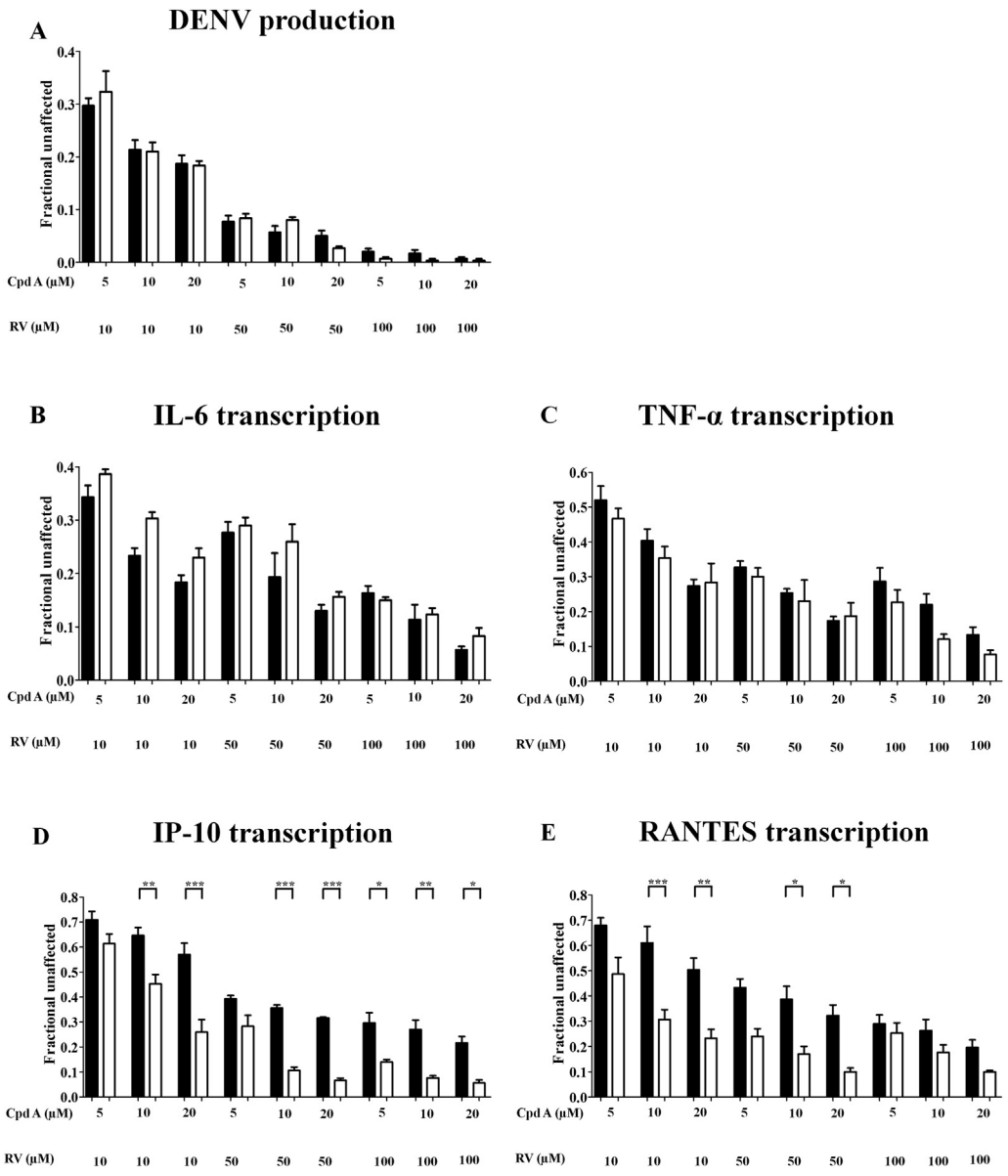

Fig. 4. Calculation of combinatorial effects of ribavirin (RV) and compound A (CpdA) together on DENV production and cytokine transcription. A549 cells were infected with DENV2 at MOI 5 and incubated with RV and CpdA as shown in Fig. 2. **(A)** DENV production, and **(B–E)** transcription of IL-6, TNF-α, IP-10, and RANTES, calculated by fractional product method. Black and white bars represented theoretical additive (or predicted) effects and combinatory (or actual) effects, respectively. The results were conducted in five independent experiments and plotted (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

the highest virus replication rate when compared to other cells (Yohan et al., 2014). Moreover, lung is a human organ affected by DENV infection (Povoa et al., 2014; Rodrigues et al., 2014). The antiviral drug, RV, is a broad antiviral agent that is currently used for treatment of HCV infection and has previously been reported to reduce DENV production in hepatoma cell lines (Diamond et al., 2002). Although it failed to inhibit DENV infection in AG129 mouse model (Schul et al., 2007), the combination between RV and glucosidase inhibitor enhanced the antiviral activity in this animal model in synergistic manner (Chang et al., 2011). The anti-inflammatory compound, CpdA, has recently been tested with DENV-infected HepG2 cells by our group (Suttitheptumrong et al., 2013), in which it was found to reduce cytokine (CXCL10 and TNFα) secretion and also DENV production. CpdA also suppressed RANTES production in DENV-infected HEK293 cells (Khunchai et al., 2015).

By using DENV2 at the beginning, we have shown in the present study that at the concentrations that did not affect A549 cell viability, RV could significantly reduce both DENV2 production and cytokine/chemokine (IL-6, TNF-α, IP-10 and RANTES) transcription (Fig. 1) while CpdA could significantly reduce cytokine/chemokine (IL-6, TNF-α, and RANTES) transcription but did not reduce DENV production and IP-10 transcription. Since RV could significantly reduce DENV production, which may subsequently lead to the reduction of cytokine/chemokine transcription in DENV infected cells. In addition to anti-DENV, RV may also have immune modification mechanisms. However, when it was combined with CpdA, DENV production could be greatly reduced. This indicates that, possibly in the initial phase of its infection, DENV may require cytokine/chemokine for activation of the cells to promote DENV replication.

The combination of RV and CpdA could even more

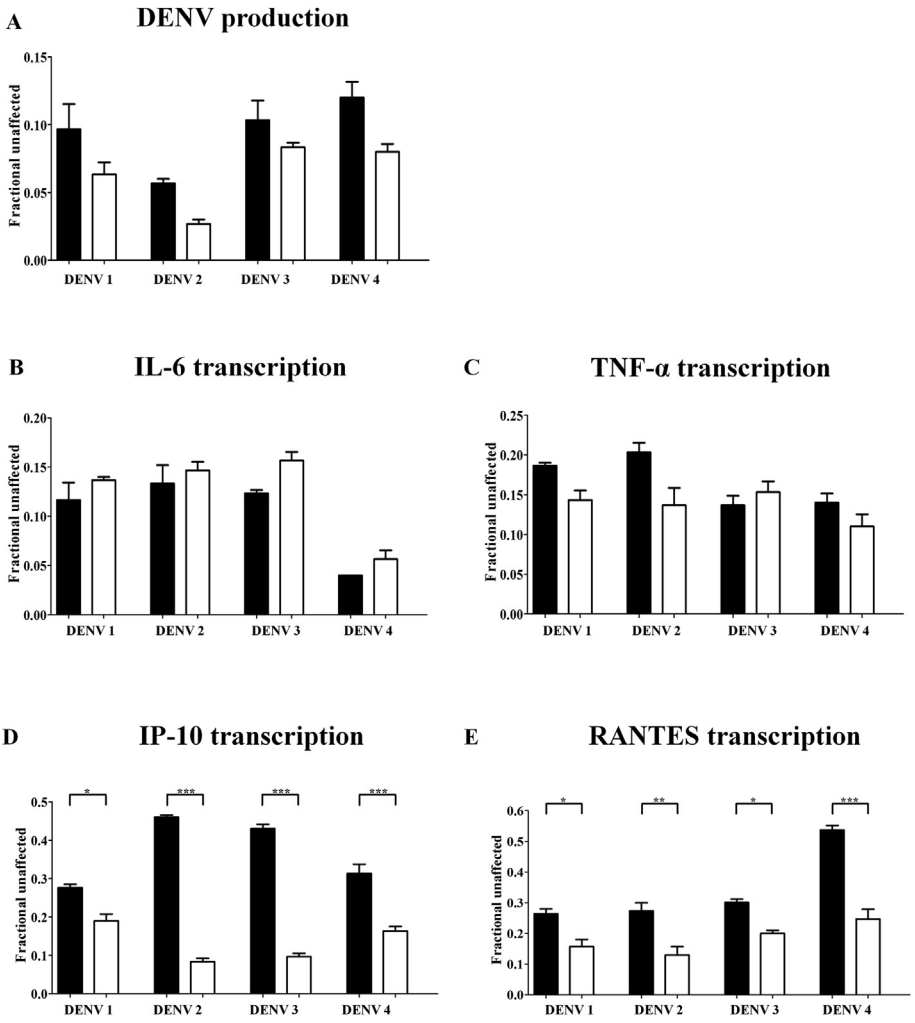

Fig. 5. Calculation of combinatorial effects of ribavirin (RV) and compound A (CpdA) together on DENV production and cytokine transcription in A549 cells infected by DENV serotype 1, 2, 3, and 4. A549 cells were infected with DENV serotypes 1, 2, 3, and 4 at MOI of 5 and incubated with combined of RV and CpdA as shown in Fig. 3. **(A)** DENV production, and **(B–E)** transcription of IL-6, TNF-α, IP-10, and RANTES, calculated by the fractional product method. Black and white bar represented theoretical additive (or predicted) effects and combinatory (or actual) effects respectively. The results were conducted in five independent experiments and plotted (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

significantly reduce DENV2 production and cytokine/chemokine transcription in dose-dependent manner, when it was compared with that of individual treatments to the DENV2-infected A549 cells (Fig. 2). This combined effect of RV and CpdA on the reduction of both virus production and cytokine/chemokine transcription in the infected A549 cells was not only observed for the infection with DENV2 but also for that with all four DENV serotypes (Fig. 3). Interestingly, the combined RV and CpdA treatment on the DENV2-infected A549 cells resulted in the reduction of viral production and cytokine (IL-6, and TNF-α) transcription in an additive effect model (Fig. 4A–C), whereas the same treatments caused the reduction of chemokine (IP-10 and RANTES) transcription in a synergistic effect model (Fig. 4D–4E). Additionally, similar findings were observed for the infection with all four DENV serotypes (Fig. 5). It has been known that TNF-α and IL-6 are pro-inflammatory cytokines, that promote systemic inflammatory response (Hernandez-Rodriguez et al., 2004), while IP-10 and RANTEs are chemokines that have the action to recruit immune cells to the site of infection, resulting in tissue damage (Agostini et al., 1998; Rathakrishnan et al., 2012; Sundstrom et al., 2001). These cytokines/chemokines were frequently investigated in the patients with severe DENV infection (Castro et al., 2011; Nguyen et al., 2004; Rathakrishnan et al., 2012; Restrepo et al., 2008; Suharti et al., 2003; Vennemann et al., 2012). The additive effect model of the combined RV and CpdA treatment on the reduction of DENV production and cytokine (IL-6, and TNF-α) transcription might result from the collective direct-inhibitory effects of RV and CpdA on the DENV-infected cells. However, the synergistic effect model of the combined RV and CpdA treatment on the reduction of chemokine (IP-10 and RANTES) transcription, and also resulting in the reduction of their protein levels, may be explained by the inhibition in two fold, from both direct-inhibitory effects of RV and CpdA, and also from the indirect inhibitory effect on the reduction of cytokine (IL-6, and TNF-α) expression that might in turn affect the reduction of chemokine. The transcription of chemokines (IP-10 and RANTES) is regulated by TNF-α (Ammit et al., 2002; Yeruva et al., 2008), which was also reduced by combined treatment of RV and CpdA. Taken together, the result of this study indicates that the combined treatment by anti-viral

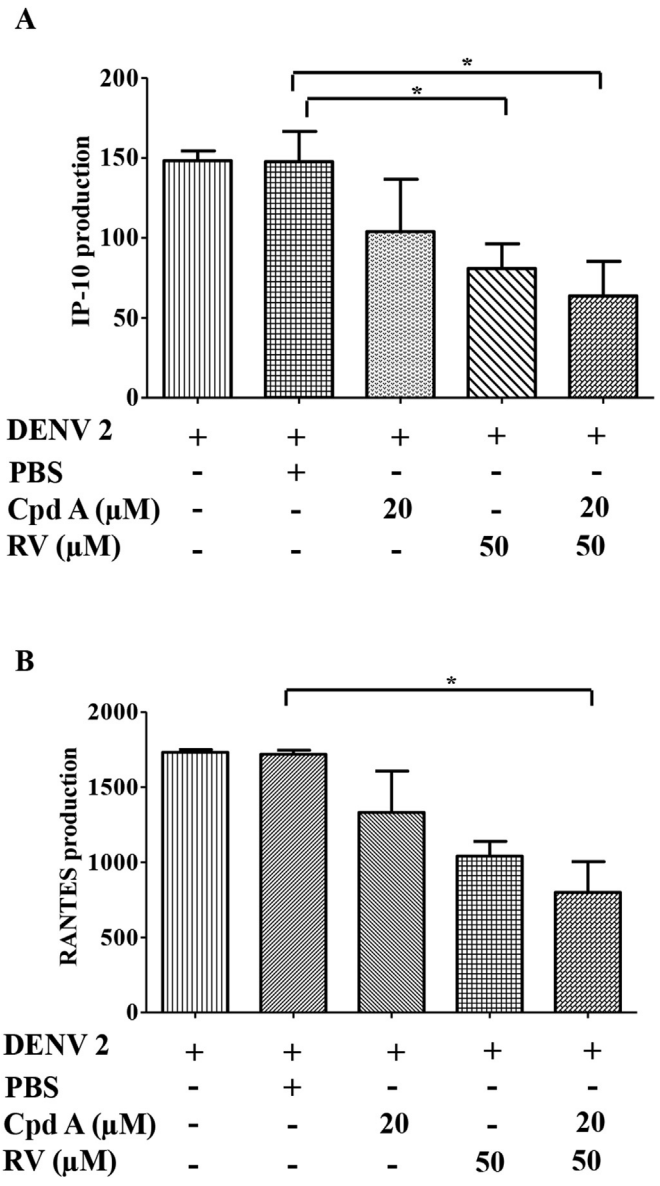

Fig. 6. Combined effects of RV and CpdA on IP-10 and RANTES protein production on DENV2 infected cells. A549 cells were infected with DENV2 at a MOI of 5 and incubated with RV at 50 μM, CpdA at 20 μM, and combinations of RV and CpdA for 24 h. Supernatants were collected and analyzed for **(A)** IP-10 and **(B)** RANTES protein production by ELISA (* = $p < 0.05$, ** = $p < 0.01$).

(RV) and anti-inflammatory (CpdA) agents offers a greater inhibitory effect on DENV production and host cytokine/chemokine transcription than the treatment by individual compound. Thus, the therapeutic approach using combined anti-viral and anti-inflammatory drugs for treatment of DENV infection is proposed, which merits further studies.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.antiviral.2015.10.005.

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