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Cetuximab and epirubicin HCl-combined application as a possibility to treat both parental and epirubicin HCl-resistant liver cancer cells

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Background and aims: Targeted chemotherapeutics such as cetuximab can cause many side effects such as skin toxicity when used in high concentrations. In addition, cancer cells can develop resistance to some of the anticancer agents during treatment. The lack of the desired success in chemotherapy and the development of resistance to chemotherapeutics, such as epirubicin HCl, suggest that there is a need for combined therapies. The combination of targeted chemotherapeutics and conventional chemotherapy drugs may lead to the emergence of new strategies in the treatment of cancer. In this study, cytotoxic, antiproliferative, cell cycle inhibitive, oxidative stress generation, and apoptotic effects and effect mechanisms of cetuximab alone and together with epirubicin HCl on parental liver cancer cells (P-Hep G2) and epirubicin HCl-resistant liver cancer cells (R-Hep G2) were investigated. *Materials:* Cytotoxic effects of cetuximab alone and with epirubicin-HCl on cells were determined by Cell Titer-Blue® Cell Viability and Lactate Dehydrogenase Activity tests. Cell cycle distributions and apoptosis were detected by reverse transcription polymerase chain reaction (RT-PCR). *Results:* Cetuximab with epirubicin HCl treatment increased the cytotoxic effect on both cells. Caspase-3/7 activity increased 3 and 1.5 times in comparison with control group in P-Hep G2 and R-Hep G2 cells, respectively, after treating with cetuximab alone, whereas the increase was found to be approximately 4.7 and 2.5 times when cetuximab was treated with epirubicin HCl in P-Hep G2 and R-Hep G2 cells, respectively. Both cetuximab alone and together with epirubicin HCl treatments caused increases in Bax/Bcl-2 ratio in both cells. *Discussion:* Treatment of cetuximab with epirubicin HCl to P-Hep G2 and R-Hep G2 cells was found to be more effective in cytotoxic effect and inducing apoptosis comparison to cetuximab alone treatment. In addition, combination treatment showed different effects on pro-apoptotic/anti-apoptotic genes expression according to cells drug resistance properties.

Original Article

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of cancer death (Forner et al., 2012). Several kinds of treatments might be beneficial for HCC, such as surgical resection, liver transplantation, percutaneous ethanol injection, transarterial chemoembolization, and transarterial radioembolization. Moreover, patients with HCC usually exhibit poor tolerance to systemic chemotherapy because of their abnormal liver function (Yang & Roberts, 2010).

Molecular targeted-therapy has drawn broad attentions (Ashworth & Wu, 2014; Chuma et al., 2015). Yet, only sorafenib has been utilized for the clinical treatment of HCC. This multikinase inhibitor improves overall survival for some HCC patients (Escudier et al., 2007; Llovet & Bruix, 2008; Spinzi & Paggi, 2008). Thus, there is an urgent need to explore other molecular-targeted agents for HCC (Ashworth & Wu, 2014; Chuma et al., 2015; Stotz et al., 2015).

The epidermal growth factor receptor (EGFR) is dysregulated in HCC, which is associated with less differentiated tumors, tumor recurrence, and poor survival. Therefore, EGFR represents a valuable treatment target for HCC. EGFR signal pathways are involved in the control of cell survival, cell cycle, angiogenesis, migration, invasion, and metastatic potential of cells. Cetuximab is a chimeric human–murine monoclonal antibody of EGFR, directly against EGFR, which binds

to EGFR with an affinity that is approximately 5–10 times higher than that of endogenous ligands. Cetuximab blocks binding of endogenous EGFR ligands resulting in inhibition of receptor function and induces EGFR internalization (Harding & Burtness, 2005).

Epirubicin HCl, an anthracycline derivative, is used in colorectal cancers, in liver, breast, lung, stomach, and ovary tumors, and in Hodgkin's lymphomas and non-Hodgkin's lymphomas (Parfitt et al., 1999; Radford et al., 1991; Rothenberg & Blanke, 1999; Trissel et al., 1991). There is a cardiotoxicity risk in the use of epirubicin HCl (Ellence, 1999). Life-threatening congestive heart failure-like symptoms are mostly associated with high-dose administrations.

The use of drugs in combination is widespread in cancer chemotherapy. Combination therapy is usually more toxic than single-agent therapy, but occasionally, combinations of drugs with different mechanisms of actions and side effect profile can be as or less toxic than single-agent therapy. Therefore, it is sometimes possible to decrease serious dose-limiting side effects through combined drug therapy. In addition, combination chemotherapy can decrease the resistance of tumor cells (Williams & Lokich, 1992).

Tumors are heterogeneous in several ways. Chemotherapeutic susceptibility is one of them (Rihova et al., 2002). Resistance to chemotherapeutics in liver cancer is one of the biggest problems preventing treatment. A group of drug-resistant cells can develop in tumors during the chemotherapy.

It is well known that liver is the main target organ of toxicity (Nishiwaki-Matsushima et al., 1992). Therefore, hepatocytes or hepatoma cell lines have been increasingly used as *in vitro* models to evaluate the toxicity of drugs. Human HCC (Hep G2) cells can display the morphology and biochemical activities of healthy hepatocytes (Javitt, 1990) and hence have been widely used to evaluate the toxic effects of various toxicants on hepatocytes for many years (Carvalho et al., 2014; Van et al., 1997).

The lack of success in chemotherapy and the development of resistance to chemotherapeutics, such as epirubicin HCl, suggest that there is a need for combined therapies in HCC. Therefore, when determining the strategies to be applied in cancer treatment, it is necessary to consider the drug-resistant cells in the tumor. The fact that the chemotherapeutic doses used in the treatment of cancer are capable of killing the drug-resistant cells among the cancer cells is a desirable treatment. There is no study comparing the effects of cetuximab alone and with epirubicin HCl treatments, which are used as chemotherapeutic agents in liver cancer treatment, on parental and epirubicin HCl-resistant (drug-resistant) liver cancer cells (Hep G2). Therefore, in this study, we used cetuximab alone and also with epirubicin HCl.

In this study, cytotoxic, antiproliferative, cell cycle inhibitive, oxidative stress generation and apoptotic effects and effect mechanisms of cetuximab, which is a monoclonal antibody targeted to EGFR, alone and together with epirubicin HCl on parental liver cancer cells (P-Hep G2), and epirubicin HCl-resistant liver cancer cells (R-Hep G2) were investigated. The mechanisms of action of cetuximab alone and in combination with epirubicin HCl in P-Hep G2 and R-Hep G2 cells have been evaluated from different perspectives and tried to shed light on the identification of mechanism of action. The results obtained from this study will help to identify new strategies and contribute to the literature knowledge by drawing attention to the differences of the responses of cetuximab alone and together with epirubicin HCl in the treatment of P-Hep G2 and R-Hep G2.

MATERIALS AND METHODS

Chemicals and drugs

Cetuximab (also called as C225-03, IMC-C225, C225, and ch225) was purchased from Merck Serono (Kenilworth, NJ, USA) and epirubicin HCl from Calbiochem (San Diego, CA, USA). Epirubicin HCl was dissolved in water under sterile condition. Drugs were diluted immediately before use in growth medium. The CellTiter-Blue® Cell Viability Assay and ApoTox-Glo™ Triplex Assay used for caspase-3/7 activity were purchased from Promega (Madison, WI, USA). Lactate Dehydrogenase Activity Assay Kit was purchased from Sigma-Aldrich (St. Louis, MO, USA). RNeasy Mini Kit used for purification of high-quality RNA from cells was purchased from Qiagen (Chatsworth, CA, USA). Titan One Tube RT-PCR System Kit was purchased from Roche Applied Science (Mannheim, Germany).

Cell cultures and treatments

Human liver cancer cell line Hep G2 was obtained from American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 units/ml), respectively, in a humidified atmosphere with 5% CO_2 at 37 °C. When the cells grew to approximately 80% confluency, they were subcultured or treated with drugs.

The epirubicin HCl-resistant R-Hep G2 cells were derived from the parental line by stepwise selection in increasing concentrations of epirubicin HCl until the cells were capable of propagating in 50 ng/ml drug, as described previously (Jansson et al., 1999; Ozkan, 2007). Typically, the resistant cells were grown in medium lacking epirubicin HCl as minimum four passages before they had been used in experiment.

Cytotoxicity assay

The CellTiter-Blue® Cell Viability Assay provides a homogeneous, fluorometric method for estimating the number of viable cells present in multiwell plates. It uses the indicator dye resazurin to measure the metabolic capacity of cells, an indicator of cell viability. Viable cells retain the ability to reduce resazurin into resorufin, which is highly fluorescent. Non-viable cells rapidly lose metabolic capacity, do not reduce the indicator dye, and thus do not generate a fluorescent signal.

For this purpose, 96-well plates were set up, containing medium, cells to the desired density, and the substances to be analyzed, appropriately diluted in medium.

More precisely, 10^4 P-Hep G2 and R-Hep G2 cells per well were seeded in a volume of medium, so that the final volume was 200 µl/well. The cells were treated with cetuximab alone and together with epirubicin HCl for 72 h. At the expiry of treatment, the reagent (20 µl/well) was appropriately added, and the plate was incubated in the dark for 1 h at 37 °C. Finally, the fluorescence (560 Ex/590 Em) was detected using the spectrofluorometer (PerkinElmer LS 55, Waltham, MA, USA). The fluorescence produced is directly proportional to the number of viable cells. The data were expressed as average values obtained from eight wells for each concentration. Percentage of viable cells was calculated based on the number of live cells divided by the control cells (contained only the culture medium without the test sample). The IC_{50} concentration (resulting in 50% growth inhibition) for cetuximab treatment and IC_5, IC_{10}, IC_{20}, IC_{30}, and IC_{40} ($<IC_{50}$) concentrations for epirubicin HCl were calculated for 72 h.

Moreover, each cell was treated with cetuximab (IC_{50}) and epirubicin HCl (IC_5, IC_{10}, IC_{20}, IC_{30}, and IC_{40}) together. As a result of these treatments, the concentration of cetuximab and epirubicin HCl combination, which has the highest cytotoxic effect for each cell, was calculated.

Measurement of lactate dehydrogenase (LDH) release

Cytotoxicity was further quantified using LDH activity assay in cultured media. Briefly, after 72-h exposure with cetuximab alone (IC_{50}) and combined with epirubicin HCl (the most effective combination concentrations in cytotoxicity) or control group (contained only the culture medium without the test sample), cell supernatant was collected and assayed for LDH activity using a Lactate Dehydrogenase Activity Assay Kit (MAK066, Sigma-Aldrich) according to the manufacturers' instructions. In this kit, LDH reduces nicotinamide adenine dinucleotide (NAD) to nicotinamide adenine dinucleotide-hydrojen (reduced; NADH). The product was measured using a microtiter plate reader at 450 nm. LDH activity measurements were made as three replicates. LDH activity was calculated using the following formula provided by the instructions. The results are given in milliunits/ml. One unit of LDH activity is defined as the amount of enzyme that catalyzes the conversion of lactate into pyruvate to generate 1.0 µmol of NADH per minute at 37 °C.

LDH Activity = The amount of NADH that occurs between the first and last measurements (nmol) × sample dilution factor/reaction time × sample volume (ml).

Glutathione peroxidase (GSH-Px) activity

After incubation and treatment with cetuximab alone (IC_{50}) and combined with epirubicin HCl (the most effective combination concentrations in cytotoxicity), the medium and cells were separated and collected, respectively. After being washed twice by phosphate-buffered saline (PBS), the P-Hep G2 and R-Hep G2 cells was lysed and followed by centrifugation. Then, the cell precipitation was collected, scraped into ice-cold PBS, and homogenized with sonication in 50 mM potassium phosphate, pH 7.2, containing 1 mM fluoride, and 1 µg/ml of leupeptin. Afterward, the homogenate was centrifuged (150,000 g, 45 min, 4 °C) and the supernatant was collected for the following determination. Activity of GSH-Px was determined according to Flohe and Gunzler (1984) with tert-butyl hydroperoxidase as substrate. One unit of enzyme activity results in the oxidation of 1 µmol GSH/min. GPx activity measurements were made as three replicates. Protein was determined by the Bradford method with bovine serum as a standard (Bradford, 1976).

Caspase-3/7 activity

ApoTox-Glo Triplex Assay (Promega) was used for assessing caspase-3/7 activity in P-Hep G2 and R-Hep G2 cells. Caspase-3/7 activity was determined after P-Hep G2 and R-Hep G2 cells had been exposed to cetuximab alone (IC_{50}) and combined with epirubicin HCl (the most effective combination concentrations in cytotoxicity) for 72 h. The cells (1×10^4 cells) were cultured in 96-well assay plates. After the incubation, the caspase-Glo 3/7 reagent was added into each well, and the plates were briefly mixed by an orbital shaker and incubated for 1 h at room temperature. In this assay, activated caspases cleave a luminogenic peptide substrate, which releases a luminescent signal by a luciferin/luciferase reaction. Caspase activation was determined by measuring luminescence with a microplate reader to assess apoptosis. Caspase-3/7 activity measurements were made as three replicates. Caspase-3/7 activity was measured in relative light unit.

RNA extraction and study of gene expression

Reverse transcription polymerase chain reaction (RT-PCR) was used for determining the rate of proliferating cell nuclear antigen (PCNA), cyclin D1, Bax, and Bcl-2 gene expressions at the transcriptional level in P-Hep G2 and R-Hep G2 cells.

P-Hep G2 and R-Hep G2 cells were incubated in presence of either cetuximab alone (IC50) and combined with epirubicin HCl (the most effective combination concentrations in cytotoxicity). Gene expression studies were made as three trials. Total RNA were isolated from the cells using RNeasy mini kit (Qiagen) following the manufacturer's instructions after 72 h of incubation. MEM was used as control. cDNA was synthesized from 1 mg of total RNA using Titan One Tube RT-PCR System kit (Roche Applied Science) following the manufacturer's instructions.

The experimental protocol for PCR reaction is as follows: 30 cycles were used; denaturation: 1 min, 94 °C; annealing: 1 min, at 55 °C (glyceraldehyde-3-phosphate dehydrogenase and Bax), 58 °C (Cyclin D1) and 59 °C (PCNA), respectively; elongation: 2 min, 72 °C, final elongation: 72 °C, 90 s. Touchdown PCR was used for the Bcl-2 gene. It was first maintained at 73 °C and decreased to 63 °C by reducing 1 °C in each cycle (10 cycles). After 63 °C, the PCR was completed by making 25 cycles. The RT-PCR was performed in triplicate and the negative controls included water. GAPDH gene was used as control. Genes and primers, which were used, are shown in Table 1.

Table 1. Sequence of utilized primers

Gene	F-primer sequence (5′–3′)	R-primer sequence (5′–3′)
PCNA	CGCGCAGAGGGTTGGTAGTT	AAGCCTTCGGAGCGCAGAGT
Bcl-2	TGCACCTGACGCCCT TCAC	AGACAGCCAGGAGAAATCAAACAG
Bax	ACCAAGAAGCTGAGCGAGTGTC	ACAAAGATGGTCACGGTCTGCC
GAPDH	TTCATTGACCTCAACTACAT	GAGGGGCCATCCACAGTCTT
Cyclin D1	CCG TCC ATG CGG AAG ATC	ATG GCC AGC GGG AAG AC

PCR products were analyzed by electrophoresis in agarose 2.5% with ethidium bromide (1 µg/ml) in TBE 1× buffer (Tris 40 mM, EDTA 1 mM, and boric acid 44 mM) for 30 min at 120 V (constant voltage) with 100 bp ladder as molecular weight markers. Adobe Photoshop CS4 program was used for analysis of band density. The density of the PCR bands was divided by that of the housekeeping gene and expressed as percent of the control band density (Bijwaard et al., 2001; Huang et al., 2003).

Data analysis

The results of the replicates were pooled and expressed as mean ± standard deviation (SD). Analysis of variance (ANOVA) was carried out. ANOVA was used to determine whether there were any significant differences between the means of three or more independent (unrelated) groups on some variable. Tukey's multiple comparisons tests were used. Statistical differences were considered significant at $p < .05$ (Kirkman, 1996). Statistical analyses were performed using the Minitab program (http://www.minitab.com/products), release 13.0.

RESULTS

Effect of cetuximab and epirubicin HCl on the viability of P-Hep G2 and R-Hep G2 cells

The cytotoxic effects of cetuximab alone and combined with epirubicin HCl on P-Hep G2 and R-Hep G2 cells were measured using the CellTiter-Blue® Cell Viability Assay Kit. P-Hep G2 and R-Hep G2 cells were exposed to increasing concentrations of cetuximab and epirubicin HCl for 72 h (Figs 1 and 2). A decrease in cell viability was observed in parallel with the increase in concentration of cetuximab and epirubicin HCl in P-Hep G2 cells. After incubations of cetuximab and epirubicin HCl for 72 h, IC_{50} values were calculated, respectively, as 1,000 and 0.80 µg/ml for P-Hep G2. P-Hep G2 cells were incubated with cetuximab (IC_{50}) and IC_{50}, IC_{10}, IC_{20}, IC_{30}, and IC_{40} epirubicin HCl concentrations for 72 h. The most effective cytotoxic concentrations were found as IC_{50} cetuximab + IC_{40} epirubicin HCl ($40 > 30 > 20 > 5 > 10$) (Fig. 3).

Cell viability was reduced due to increased concentration in R-Hep G2 cells exposed to cetuximab and epirubicin HCl (Figs 1 and 2). The fastest decline was observed between 1,000 and 4,000 µg/ml cetuximab concentrations. After 72 h incubation, the IC_{50} values of cetuximab and epirubicin HCl were calculated to be 2,279 and 0.95 µg/ml, respectively. An important feature of drug resistance is that development of resistance to one drug can lead to resistance to other drugs. R-Hep G2 cells developed resistance to epirubicin HCl also developed cross-resistance to cetuximab. When parental cells are compared to drug-resistant cells, drug-resistant cells showed approximately 2.3-fold more resistance to cetuximab. Drug-resistant cells showed approximately 1.2-fold more resistance to epirubicin HCl than parental cells at the IC_{50} concentration. The most effective cytotoxic concentrations were found as IC_{50} cetuximab + IC_5 epirubicin HCl ($5 > 20 > 10 > 40 > 30$) for R-Hep G2 cells (Fig. 3). Those most effective cytotoxic concentrations for each cell were used following experiments.

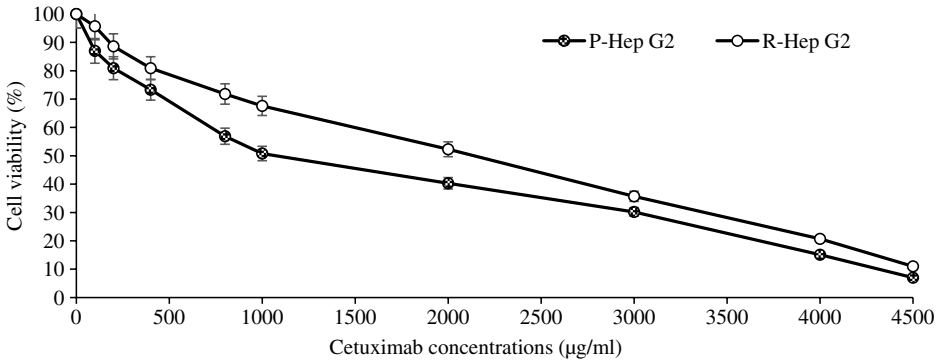

Fig. 1. The cytotoxic effects of cetuximab (100–4,500 µg/ml) for 72 h on parental Hep G2 (P-Hep G2) and drug-resistant Hep G2 (R-Hep G2) measured by CellTiter-Blue Cell Viability Assay. Results are presented as viability ratio compared with the control group (contained only the culture medium without the test-sample-untreated cells). Values are expressed as the mean of three separate experiments. Error bars represent standard deviation (SD) of the mean from eight replications (ANOVA with Tukey's test, $p < .05$). Critical F value = 3.18

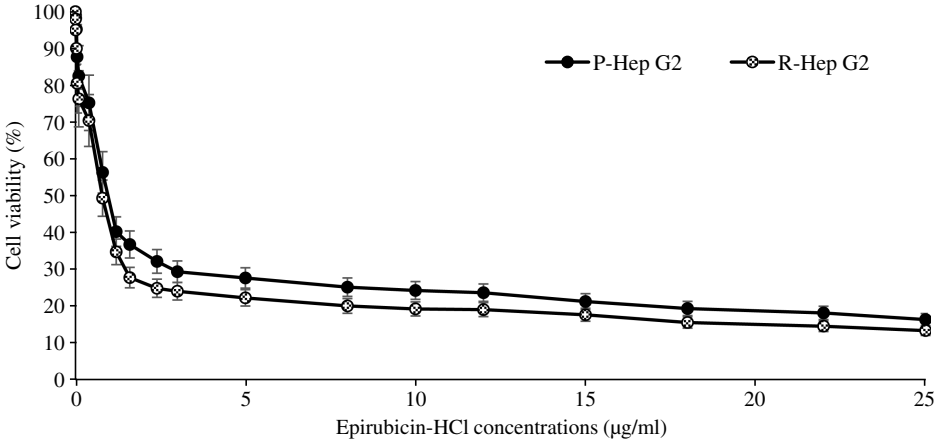

Fig. 2. The cytotoxic effects of epirubicin HCl (0.01–25 µg/ml) for 72 h on parental Hep G2 (P-Hep G2) and drug-resistant Hep G2 cells (R-Hep G2) as measured by CellTiter-Blue Cell Viability Assay. Results are presented as viability ratio compared with the control group (contained only the culture medium without the test-sample-untreated cells). Values are expressed as the mean of three separate experiments. Error bars represent standard deviation (SD) of the mean from eight replications (ANOVA with Tukey's test, $p < .05$). Critical F value = 2.33

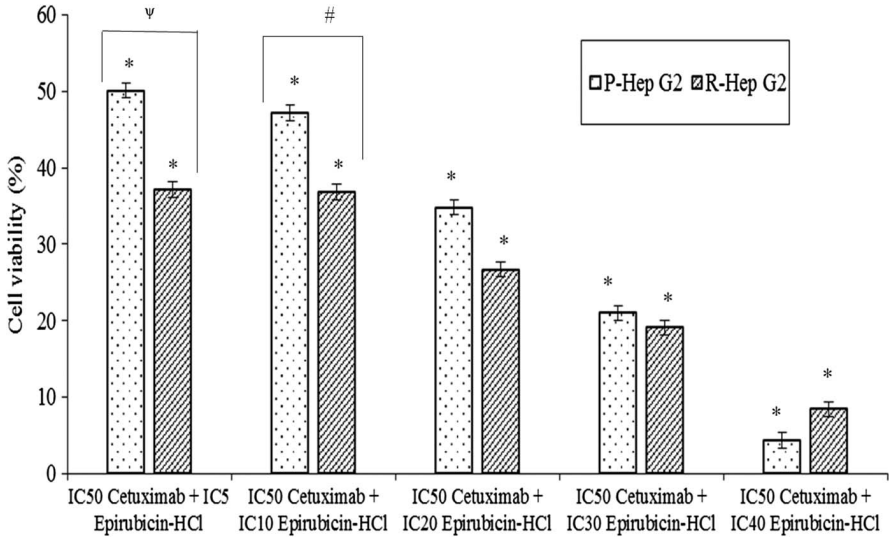

Fig. 3. Combined cytotoxic effects of cetuximab and epirubicin HCl (IC_5, IC_{10}, IC_{20}, IC_{30}, and IC_{40}) for 72 h on parental Hep G2 (P-Hep G2), and drug-resistant Hep G2 cells (R-Hep G2). Results are presented as viability ratio compared with the control group (contained only the culture medium without the test-sample-untreated cells). Values are expressed as the mean of three separate experiments. Error bars represent standard deviation (SD) of the mean from eight replications. *Significantly different from control (untreated cells). #Significantly different that IC_{50} cetuximab and IC_{10} epirubicin HCl combination treatment in P-Hep G2 and R-Hep G2 cells from each other. ΨSignificantly different that IC_{50} cetuximab and IC_5 epirubicin HCl combination treatment in P-Hep G2 and R-Hep G2 cells from each other (ANOVA with Tukey's test, $p < .05$). Critical F value = 2.98

In both parental and drug-resistant cells, treatment of IC_{50} cetuximab together with IC_5, IC_{10}, IC_{20}, IC_{30}, and IC_{40} epirubicin HCl was statistically different from control (contained only the culture medium without the test sample) ($p < .05$). In parental cells, treatment of IC_{50} cetuximab together with IC_{40} epirubicin HCl was statistically different from IC_{50} cetuximab together with IC_{10}, IC_{20}, and IC_{30} treatments ($p < .05$), whereas in drug-resistant cells, treatment of IC_{50} cetuximab together with IC_5 epirubicin HCl was statistically different from IC_{50} cetuximab together with IC_{20}, IC_{30}, and IC_{40} epirubicin HCl treatments ($p < .05$).

Effect of cetuximab and epirubicin HCl on LDH activity in P-Hep G2 and R-Hep G2 cells

Besides the cell viability, the release of LDH is also a vital index of cytotoxicity. LDH, a stable cytoplasmic enzyme inside the cells, is released into the culture medium after the damage of cell membrane (Ramalingam & Kim, 2014).

Changes in LDH enzyme activity were measured after P-Hep G2 cells had been exposed to IC_{50} cetuximab alone and together with IC_{40} epirubicin HCl (combination concentrations showing the most potent cytotoxic effect) for 72 h (Table 2). It was found that the LDH enzyme activity

Table 2. Lactate dehydrogenase, caspase-3/7, and glutathione peroxidase activities and gene expression levels in P-Hep G2 and R-Hep G2 cells after treated with cetuximab alone and with epirubicin HCl

Concentrations	LDH activity (miliunits/ml) Mean ± *SD*	Caspase-3/7 activity (RLU) Mean ± *SD*	GSH-Px activity (U/mg protein) Mean ± *SD*	PCNA mRNA expression (% control)	Cyclin D1 mRNA expression (% control)	Bax mRNA expression (% control)	Bcl-2 mRNA expression (% control)
Cet (IC_{50}), P-Hep G2	20.0 ± 0.8*	9,000 ± 1.8*	6.8 ± 1.4*	72.7	50.3	124.8	60.8
Cet + Epi, P-Hep G2	33.3 ± 0.3$^{\#*}$	14,000 ± 1.2$^{*\#}$	9.8 ± 0.4$^{*\#}$	95.7	77.2	166.7	52.9
Control, P-Hep G2	11.7 ± 0.4	3,000 ± 0.3*	5.6 ± 0.3	100	100	100	100
Cet (IC_{50}), R-Hep G2	17.3 ± 0.7*	3,000 ± 0.3*	9.2 ± 1.6*	79.5	61.0	115.0	79.4
Cet + Epi, R-Hep G2	27.3 ± 1.2*	5,000 ± 0.8$^{*\#}$	13.5 ± 1.4$^{*\#}$	72.0	33.3	139.7	52.9
Control, R-Hep G2	12.0 ± 0.4	2,000 ± 0.3	5.9 ± 1.7	100	100	100	100

Note. Data are expressed as mean ± standard deviation (SD). Cet: cetuximab; Epi: epirubicin HCl; RLU: relative light unit.
*Significantly different from control (ANOVA with Tukey's test, $p < .05$).
$^{\#}$Significantly different from cetuximab alone treatments ($p < .05$), Critical F value = 1.67. All activity measurements were made as three replicates. One unit of LDH activity is defined as the amount of enzyme that catalyzes the conversion of lactate into pyruvate to generate 1.0 µmol of NADH per min at 37 °C.

increased 2.8-fold in the cells subjected to combination concentrations and 1.7-fold in the cells treated with cetuximab alone compared to the control group (contained only the culture medium without the test sample). The LDH enzyme activity in the cells treated with cetuximab alone and combined with epirubicin HCl was found to be statistically different ($p < .05$) from the control group.

LDH activity in R-Hep G2 cells treated with cetuximab alone and combined with IC_5 epirubicin HCl was approximately 1.4-fold and 2-fold higher than control group, respectively (Table 2). The LDH enzyme activity in cells treated with cetuximab alone and combined with epirubicin HCl was found to be statistically different ($p < .05$) from LDH enzyme activity in the control groups. LDH activities were found higher in cetuximab + epirubicin HCl treatment than cetuximab alone treatment for both cells compared to control groups.

Cetuximab and epirubicin HCl increases GSH-Px activity in P-Hep G2 and R-Hep G2cells indicative of enhanced oxidative stress

GSH-Px is one of the antioxidant enzymes in the cells. Cells synthesize this enzyme to protect themselves against oxidative stress when exposed to a prooxidant/oxidant agent. Changes in GSH-Px activity, indicative of oxidative stress in cells, were determined after administration of cetuximab alone (IC_{50} concentration) and also combination with epirubicin HCl (combination concentrations showing the most effective cytotoxic effect) in P-Hep G2 and R-Hep G2 cells for 72 h.

It was observed that after parental cells exposed to cetuximab for 72 h, GSH-Px activity increased by 21% according to control group (contained only the culture medium without the test sample) and this increase was found to be statistically significant ($p < .05$). When cetuximab and epirubicin HCl were treated together with P-Hep G2 cells for 72 h, the GSH-Px activity was found to increase by 75% relative to the control group, and this increase was statistically significant ($p < .05$). After P-Hep G2 cells treated with cetuximab and epirubicin HCl together, the activity of GSH-Px was found to be 1.4 times greater than in cetuximab-alone-treated cells (Table 2). GSH-Px activity in cetuximab-alone-treated R-Hep G2 cells was observed to be 56% higher than control cells, and this increase was statistically significant ($p < .05$). The activity of GSH-Px in R-Hep G2 cells treated with cetuximab and epirubicin HCl together was about 1.5 times higher than in only cetuximab treated cells (Table 2).

Cetuximab and epirubicin HCl induces caspase-3/7 mediated apoptosis in P-Hep G2 and R-Hep G2 cells

Apoptosis induction potentials were determined by measuring the activity of caspase-3/7, one of the apoptotic pathway enzymes, after cetuximab alone and combined with epirubicin HCl treatments for 72 h in P-Hep G2 and R-Hep G2 cells. The caspase-3/7 activity in cetuximab-alone-treated P-Hep G2 cells showed threefold increase compared to the control (contained only the culture medium without the test sample), whereas it was increased about 4.7-fold when cetuximab applied with epirubicin HCl (Table 2). When cetuximab applied with epirubicin HCl, the caspase-3/7 activity in P-Hep G2 cells was found to be 1.6 times greater than in cetuximab-alone-treated cells. It was determined that the highest increase in caspase-3/7 activity according to the control was observed after both cetuximab alone and with epirubicin HCl together treatments in P-Hep G2 cells. The caspase-3/7 activity in R-Hep G2 cells treated with cetuximab showed 1.5-fold increase compared to the control, whereas caspase-3/7 activity increased approximately 2.5-fold when cetuximab coadministered with epirubicin HCl (Table 2). When cetuximab was applied with epirubicin HCl, it was found that caspase-3/7 activity in R-Hep G2 cells was 1.7 times higher than in cetuximab-alone-treated cells.

Regulation of proliferation and apoptosis

Expression levels of the PCNA, cyclin D1, Bax, and Bcl-2 genes were assessed using RT-PCR to determine the

antiproliferative and apoptotic effect of cetuximab alone (IC_{50} concentration) and combined with epirubicin HCl.

When cetuximab was applied to P-Hep G2 cells, mRNA expression of PCNA decreased by 27.3% compared to control cells (contained only the culture medium without the test sample), while PCNA mRNA expression was decreased by 4.3% when cetuximab and epirubicin HCl were coadministered. When R-Hep G2 cells were treated with cetuximab, PCNA mRNA expression was found to decrease by 20.5%, whereas it was reduced by 28% compared to control group when cetuximab and epirubicin HCl administered together. Cetuximab alone treatment resulted in more effective reduction of PCNA mRNA expression in P-Hep G2 cells, whereas coadministration of cetuximab and epirubicin HCl resulted in a more effective reduction of PCNA mRNA expression in R-Hep G2 cells. Combined treatment showed more antiproliferative effect in R-Hep G2 cells, whereas cetuximab alone treatment showed more antiproliferative effect in P-Hep G2 cells (Table 2).

When cetuximab was treated with P-Hep G2 cells, mRNA expression of cyclin D1 was reduced by 49.7% compared to control cells, whereas it was reduced by 22.8% when cetuximab and epirubicin HCl treated together. When cetuximab was treated with R-Hep G2 cells, mRNA expression of cyclin D1 was found to decrease by 39% compared with control cells, whereas it was reduced by 66.7% when cetuximab and epirubicin HCl treated together. Cetuximab alone treatment in P-Hep G2 cells resulted in a more effective reduction of cyclin D1 mRNA expression, while coadministration of cetuximab and epirubicin HCl resulted in more effective reduction of cyclin D1 mRNA expression in R-Hep G2 cells (Table 2).

It is known that antiapoptotic Bcl-2 balance with Bax, a proapoptotic protein that releases cytochrome c from mitochondria, is important in determining the survival or death of cells. The ratio of Bax/Bcl-2 is influential in regulating the susceptibility of the apoptosis in a cell. In both P-Hep G2 and R-Hep G2 cells, cetuximab alone and also coadministration of cetuximab and epirubicin HCl enhanced the expression of Bax relative to control cells. When cetuximab was treated alone with P-Hep G2 cells, the expression of Bax was found to be 24.8% higher than the control group, and it was calculated to be 66.7% when cetuximab and epirubicin HCl were treated together. Bax expression was found to increase by 15% in R-Hep G2 cells compared to the control group when only cetuximab was treated, whereas it was calculated to be 39.7% when cetuximab and epirubicin HCl were coadministered. Both applications caused an increase in Bax expression in P-Hep G2 and R-Hep G2 cells. Combined treatment was more effective in increasing Bax mRNA expression than cetuximab alone treatment in both P-Hep G2 and R-Hep G2 cells (Table 2).

When cetuximab was treated with P-Hep G2 cells, the expression of Bcl-2 was decreased by 39.2% compared to the control cells, whereas the reduction of Bcl-2 expression was calculated to be 47.1% when cetuximab and epirubicin HCl were treated together. When cetuximab was treated with R-Hep G2 cells, it was found that Bcl-2 expression decreased by 20.6% compared to the control group, whereas the reduction of Bcl-2 expression was calculated to be 34.6% when cetuximab and epirubicin HCl were coadministered. Both treatments caused more reduction in Bcl-2 expression in P-Hep G2 cells than R-Hep G2 cells. Combined treatment was more effective in reducing Bcl-2 mRNA expression than cetuximab alone treatment in both P-Hep G2 and R-Hep G2 cells. Thus, cetuximab alone and combined with epirubicin HCl treatment resulted in an increase in Bax/Bcl-2 in both P-Hep G2 and R-Hep G2 cells (Table 2).

DISCUSSION

HCC is the fifth most common type of cancer in the world and is estimated to cause half a million deaths per year (Bosetti et al., 2014). It cannot be cured due to the poor prognosis of HCC and the wide variety of clinical problems. Thus, it is necessary to develop new treatment options to increase the survival rate of patients with HCC and to increase the success rates of current treatment modalities used (Chen et al., 2012).

HCC cell line Hep G2 cells are highly differentiated and display many of the genotypic features of normal liver cells. The liver cells play a key role in most metabolic processes, especially detoxification, so antioxidant enzymes are expected to have higher activity in liver cells relative to other cells. Thus, elimination of free radicals caused by epirubicin HCl (involved in oxidation/reduction reactions by generating cytotoxic free radicals) can be done more effectively in liver cancer cells. Therefore, the parental cells may not have developed much resistance (approximately 1.2-fold) to epirubicin HCl due to their cellular properties.

Today, chemotherapy is one of the most important methods used in the treatment of cancer. The most important problem that confronts the use of doxorubicin and epirubicin HCl as anthracycline analogues is the cumulative dose-limiting cardiotoxicity (Nasr et al., 2014). It has been demonstrated in animal systems that epirubicin HCl has nearly equivalent antitumor activity with doxorubicine and almost exclusively cause less cardiotoxicity in half of it (Berthiaume & Wallace, 2007; Han et al., 2015). The most important reason why we prefer epirubicin HCl in this study is that it causes less cardiotoxicity than doxorubicine and other anthracyclines. However, chemotherapy is not achieving the desired success, and the development of epirubicin HCl resistance of cancer cells shows that combined therapies are needed.

Drug resistance developed in cancer patients leads to the prolongation of treatment and the use of high-dose drugs, leading to increased side effects in patients and making treatment more difficult (Gao et al., 2013). Therefore, we consider the need to develop new treatment strategies that will kill parental cancer cells as well as drug-resistant cancer cells, when dealing with increasing therapeutic efficacy of existing drugs. One of the new treatment strategies developed recently is the coadministration of targeted chemotherapeutics with conventional chemotherapeutics.

One of the most important targets of targeted chemotherapeutics (antibodies and small molecule inhibitors) in cancer treatment is the EGFR, which is abnormally and continuously exaggerated in many cancer cells. Several studies have shown that EGFR contributed to the aggressive growth

properties of tumors is frequently expressed in HCC (Buckley et al., 2008; Lupberger et al., 2011).

In studies on cetuximab, it has been shown that cetuximab inhibited the proliferation of various human cancer cells, such as breast, colon, lung, kidney, and prostate, by inhibiting phosphorylation of EGFR, AKT, and mitogen-activated protein kinases by inducing apoptosis (Chen et al., 2014; D'angelo et al., 2014; Mao et al., 2010). However, treatment with cetuximab only showed the desired effect in a small number of cancers, such as rectal and non-small cell lung cancer (Harari, 2004), and failed to show the expected results in clinical trials with HCC patients (Gruenwald et al., 2007; Llovet & Bruix, 2008; Zhu et al., 2007). Furthermore, there are serious side effects found in the patients of cetuximab alone application. The most serious ones are infusion reactions, cardiopulmonary arrest, dermatological toxicity and radiation dermatitis sepsis, renal failure, interstitial lung disease, and pulmonary embolism.

It was shown that there was a positive correlation between early recurrence of tumor with excessive expression of EGFR in HCC (Daveau et al., 2003) and extrahepatic metastasis formation (Zhao et al., 2004). For this reason, EGFR is a promising goal to develop new treatment strategies in HCC. In addition, the desired success cannot be achieved from cetuximab and epirubicin HCl in liver cancer treatments. The aim of this study is to shed light on treatment strategies that will overcome the drug resistance and simultaneously reduce cetuximab and epirubicin HCl side effects in the treatment of liver cancer.

Cetuximab inhibited the proliferation of wild-type p53 expressing HCC cells (Hep G2) by up to 57% (1,000 µg/ml in 72 h administration) depending on time and dose by the crystal-violet staining method. Furthermore, Huh-7 cells expressing mutant p53 and Hep G2 cells showed different sensitivity to cetuximab, which was suggested to be caused by different level of p53 between two cell lines. When cetuximab applied together with tyrosine kinase inhibitors (erlotinib or AG1024), HMG-CoA reductase inhibitor (fluvastatin), doxorubicin, and cisplatin to Hep G2 and Huh-7 cells, only synergistic antiproliferative effect was observed in Hep G2 cells (Huether et al., 2005). However, no studies were conducted on these synergistic cytotoxic mechanisms and apoptotic mechanisms of action. In this study, the antiproliferative effects and mechanisms of action of cetuximab and epirubicin HCl were demonstrated.

The fact that cells feature differing levels of sensitivity toward treatment methods may be attributable to the differences in their natural antioxidant protection systems. Antioxidant systems protect cells against oxidative damage. It was shown that H1299 (human non-small cell lung cancer cell line) cells that have developed epirubicin HCl resistance causing oxidative stresses have higher GSH-Px and glutathione-S-transferase enzyme activities than parental cells (Ozkan, 2007). GSH-Px activity increased after cetuximab alone and with epirubicin HCl together compared to controls in both parental and drug-resistant cells. However, the increase in drug-resistant cells was higher than the increase in parental cells compared to controls. This may be due to the difference in drug resistance between the two cells.

Caspase-3 is responsible for the breakdown of key cellular proteins, such as skeletal proteins, which cause typical morphological changes observed in apoptotic cells. For this reason, apoptosis is a critical player. Many chemotherapeutic agents exhibit cytotoxic effects by inducing apoptosis. Cetuximab alone treatment in both P-Hep G2 and R-Hep G2 cells may have increased caspase-3/7 activation by inducing cell lysis. Epirubicin HCl used in combination may have contributed to caspase-3/7 activity increase by increasing production of reactive oxygen species and nitric oxide production, Fas, FasL, and Fas-related death domain expression.

mRNA expressions of the PCNA, which exhibits antiproliferative effect and is one of the DNA polymerase components, involved in replication and mRNA expressions of cyclin D1 genes that are active in the transition from the G1 phase to the S phase of the cell cycle and exhibit cell cycle arresting effect, were examined by RT-PCR. Coadministration of cetuximab with epirubicin HCl was found to be more effective in increasing Bax mRNA expression, a pro-apoptotic gene, than cetuximab alone administration in both P-Hep G2 and R-Hep G2 cells. When cetuximab coadministered with epirubicin HCl, Bcl-2, an anti-apoptotic gene, was reduced more in both P-Hep G2 and R-Hep G2 cells than cetuximab alone administration. Thus, both cetuximab alone and coadministration of cetuximab with epirubicin HCl resulted in an increase in Bax/Bcl-2 in both cells. Increasing this ratio both in P-Hep G2 and R-Hep G2 cells showed that both administrations induced apoptosis in these cells, with cetuximab alone and also concomitant administration with epirubicin HCl. Cetuximab has been shown to have decreased expression of PCNA and cyclin D1 in various cells, but different studies have shown that cetuximab do not have any effect on PCNA and cyclin D1 expressions in some cells (Rampias et al., 2014; Yang et al., 2015). This suggests that the effect of cetuximab on PCNA and cyclin D1 expressions may vary depending on the cell type. In this study, cetuximab alone was found to be more effective in reducing PCNA and cyclin D1 expressions than combined treatment in P-Hep G2 cells, whereas combined treatment was more effective in R-Hep G2 cells. This result may be due to the difference in drug resistance between two cell types.

Coadministration of cetuximab with epirubicin HCl increased the cytotoxic effect more than cetuximab alone administration both in P-Hep G2 and R-Hep G2 cells. Therefore, this combined application could be shed light for the development of new treatment strategies for cancer cells resistant to conventional drugs, such as epirubicin HCl or cetuximab. Cetuximab alone treatment was found to have more antiproliferative and cell cycle-arresting effect in P-Hep G2 cells compared with the combined treatment, whereas combined treatment was more effective in R-Hep G2 cells. These results suggest that different strategies should be followed in the treatment of parental and drug-resistant cells. Cetuximab showed different effects according to cell type, this will vary depending on EGFR, insulin-like growth factor receptor, and p53 expression level and mutation status. At present, biomarkers to be used in the identification of patient populations for which molecularly targeted drugs such as cetuximab are used are extremely low and inadequate. The work that will enable these biomarkers to be identified is extremely important and interesting.

The use of these drugs in the right patient populations in line with the results of these studies to be performed in future periods can lead to higher treatment response rates and also a significant gain in the economic direction.

CONCLUSION FOR FUTURE BIOLOGY

The results obtained from this study, about that cetuximab alone and in combination with epirubicin HCl applied to P-Hep G2 and R-Hep G2 cells for the first time, may provide a rationale for future clinical studies in which cetuximab combination therapy is used. As a result of clinical trials, side effects such as skin redness can be reduced or eliminated by providing the use of lower doses of cetuximab in combination than single administration. In addition, it is possible to achieve more response from the treatment and to reduce the burden of patients economically. Different responses of parental and drug-resistant cells to drug applications may provide some guide in determining new strategies for cancer treatment.

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