Schedule-Dependent Effects of Kappa-Selenocarrageenan in Combination with Epirubicin on Hepatocellular Carcinoma

Yu-Bin Ji¹, Na Ling¹*, Xiao-Jun Zhou², Yun-Xiang Mao³, Wen-Lan Li¹, Ning Chen¹

Abstract

Hepatocellular carcinoma (HCC) has a relatively higher incidence in many countries of Asia. Globally, HCC has a high fatality rate and short survival. Epirubicin, a doxorubicin analogue, may be administered alone or in combination with other agents to treat primary liver cancer and metastatic diseases. However, the toxic effects of epirubicin to normal tissues and cells have been one of the major obstacles to successful cancer chemotherapy. Here, we investigated the effects of epirubicin in combination with kappa-selenocarrageenan on mice with H22 implanted tumors and HepG-2 cell proliferation, immune organ index, morphology, cell cycle and related protein expressions in vivo and in vitro with sequential drug exposure. The inhibitory rate of tumor growth in vivo was calculated. Drug sensitivity was measured by MTT assay, and the King’s principle was used to evaluate the interaction of drug combination. Morphological changes were observed by fluorescent microscopy. Cell cycle changes were analyzed by flow cytometry. Expression of cyclin A, Cdc25A and Cdk2 were detected by Western blotting. In vivo results demonstrated that the inhibitory rate of EPI combined with KSC was higher than that of KSC or EPI alone, and the Q value indicated an additive effect. In addition, KSC could significantly raise the thymus and spleen indices of mice with H22 implanted tumors. In the drug sensitivity assay in vitro, exposure to KSC and EPI simultaneously was more effective than exposure sequentially in HepG-2 cells, while exposure to KSC prior to EPI was more effective than exposure to EPI prior to KSC. Q values showed an additive effect in the simultaneous group and antagonistic effects in the sequential groups. Morphological analysis showed similar results to the drug sensitivity assay. Cell cycle analysis revealed that exposure to KSC or EPI alone arrested the cells in S phase in HepG-2 cells, exposure to KSC and EPI simultaneously caused accumulation in the S phase, an effect caused by either KSC or EPI. Expression of cyclin A, Cdc25A and Cdk2 protein was down-regulated following exposure to KSC and EPI alone or in combination, exposure to KSC and EPI simultaneously resulting in the lowest values. Taken together, our findings suggest that KSC in combination with EPI might have potential as a new therapeutic regimen against HCC.

Keywords: Kappa-selenocarrageenan - epirubicin - combination therapy - HCC - cell cycle

Introduction

Human hepatocellular carcinoma (HCC) is one of the most common malignancies all around the world, especially in Asia (Norsa’adah et al., 2013; Wiangnon et al., 2012). It occurs with great frequency and is becoming more common as a complication of chronic Hepatitis B or Hepatitis C (Zekri et al., 2012; Liang et al., 2013; Yeo et al., 2013). Until now, many therapeutic approaches have been applied clinically such as surgery, interventional or micro-traumatic techniques, physical or chemical methods (Mao et al., 2012). But the high incidence of tumor recurrences, possibly from micrometastasis of tumor cells prior to curative surgery, further reduces patients’ 5-year survival (Li et al., 2013; Zhu et al., 2013). And this cancer seems to be stubborn as it is resistant to any chemical agents developed until now and chemical therapies used to treat cancer are highly toxic and often nonspecific (Kong et al., 2013).

Epirubicin (EPI), an anthracycline antitumour antibiotic which is structurally related to doxorubicin, is among the most active single agents used in the management of patients with breast cancer and other human tumour cells. Epirubicin is most active in S and G2 phases of the cell cycle, although the drug exhibits activity in all phases of the cell cycle (Coulkell et al., 1997). Epirubicin may be administered alone or in combination with other agents such as vinorelbine, fluorouracil, cyclophosphamide, docetaxel to patients with early breast cancer and to those with metastatic disease and shows more effective with less toxicity (Findlay et al., 1998; Yan et al., 2010; Roche et al., 2012; Cao et al., 2013; Chen et al., 2013). Comparative and noncomparative clinical trials have demonstrated that regimens containing conventional...
doses of epirubicin achieved equivalent even higher objective response rates and overall median survival as similar doxorubicin-containing regimens in the treatment of advanced and early breast cancer, non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC), non-Hodgkin’s lymphoma, ovarian cancer, gastric cancer and nonresectable primary hepatocellular carcinoma (Nair et al., 1998; Khasraw et al., 2012). Recently, dose-intensive regimens of epirubicin have achieved high response rates in a number of malignancies including early and advanced breast cancer and lung cancer (Unek et al., 2012; Vici et al., 2012). However, improved overall survival has not yet been demonstrated. The major adverse effects of epirubicin are acute dose-limiting hematological toxicity and cumulative dose-related cardiac toxicity. In vitro and clinical studies have shown that epirubicin is less myelotoxic and cardiotoxic than equimolar doses of doxorubicin. Other major adverse effects of epirubicin administration include mucositis, nausea and vomiting, reversible alopecia and local cutaneous and vesicant reactions (Launchbury et al., 1993). Reducing drug side-effects would be a most important approach to improve the success of anticancer chemotherapy.

Selenium is an essential trace element, the deficiency of which is associated with an increased incidence of some human diseases (Alatise et al., 2013; Kryczyk et al., 2013; Lener et al., 2013). Dietary supplementation with selenium has been reported to produce a decrease in the incidence of some human cancers (Wrobel et al., 2013; He et al., 2013). Kappa-selenocarrageenan (KSC) is a new-type Se-containing polysaccharide, which consists of selenium and kappa-carrageenan (Lin et al., 1993). KSC has been demonstrated effective in inhibiting proliferation of hepatocarcinoma, osteosarcoma, breast cancer and multidrug-resistant K562/ADM cells (Wei et al., 2006; Ling et al., 2009). The possible molecular mechanisms of anti-tumor were through antioxidation, induction of tumor cell apoptosis, blockade of cell cycle, and enhancement of immunity (Zhang et al., 2005). In addition, the combined treatment of KSC with other chemotherapeutic drug can better improve anti-tumor efficacy, enhance immune functions and decrease its toxic effects (Hu et al., 1997; Li et al., 2000; Ling, 2012).

In this study, we investigated the anti-tumor effects of KSC in combination with epirubicin on hepatoma both in vivo and in vitro and the possible mechanisms, and used different sequences to determine the role of cell cycle on cytotoxicity of the combinations, which may help design appropriate treatment schedules for clinical application.

Materials and Methods

Drugs and reagents

Kappa-selenocarrageenan (organic selenium containing 1.68% selenium and polysaccharide, solid powder, made by TianCiFu Biological Engineering Co., Ltd); Epirubicin was obtained from Sigma Chemical Co., Ltd. RPMI1640 medium was bought from GIBCO BRL, Life Technologies Inc. (New York, USA). Fetal bovine serum (FBS) was obtained from Hangzhou SiJiQing Life Technologies. 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT), Hoechst 33258, propidium iodide (PI), Triton X-100, and ribonuclease-A were purchased from Sigma (St. Louis, MO, USA). Rabbit anti-human Cyclin A, Cdk2 and Cdc25A polyclonal antibody were purchased from Santa Cruz Biotechnology Inc.

Animals and cell line

Mice with hepatoma H22 were obtained from Chinese Academy of Medical Sciences and Kunming mice (female and male weighing, 18 g-22 g) from the Experimental Research Center of Changchun high and new Medical Animal. Human hepatoma HepG-2 cells were obtained from the Institute for Cancer Research of Heilongjiang Cancer Hospital and maintained in our laboratory. HepG-2 cells were cultured in RPMI1640 medium with 10% fetal bovine serum and 100U/mL penicillin-streptomycin at 37°C in an atmosphere containing 5% CO₂.

Measurement of anticancer activity in vivo

The mice with hepatocarcinoma H22 were killed and their ascites extracted, adjusted to 1×10⁶/mL and implanted by subcutaneous injection 200 μL to each mouse. Eighty mice with implanted H22 tumor were randomly divided into control group (saline), and groups of EPI alone (6 mg·kg⁻¹·d⁻¹), KSC alone (20 mg·kg⁻¹·d⁻¹, 40 mg·kg⁻¹·d⁻¹, 80 mg·kg⁻¹·d⁻¹), EPI combined with KSC (EPI 6 mg·kg⁻¹·d⁻¹+KSC 20, 40, 80 mg·kg⁻¹·d⁻¹). EPI and combined groups were injected intravenously and KSC groups intragastric administration 24 h after transplatation once a day for 10 days continuously. The mice were killed on the 11th day after the treatment and the tumor, thymus and spleen isolated and weighed. The inhibitory rate of tumor was calculated as follows: Inhibitory rate of tumor (%)=(1-mean tumor weight in experiments/mean tumor weight in controls)×100%, thymus index=thymus weight/mice weight, spleen index=spleen weight/mice weight.

In vitro drug sensitivity assay

HepG-2 cells in exponential growth were washed with phosphate buffered saline (PBS), trypsinized with 0.25% trypsin for 5 min at 37°C, counted and seeded in 96-well plates at a density of 4×10⁴ cells/well in 100 μL RPMI1640 containing 10% FBS. After 24h, various concentrations of KSC and EPI were added to the medium for 24h. After drug exposure, the drug-containing medium was aspirated from the plate, fresh medium was added and the cultures incubated for 24 h. Control wells without anticancer drugs were treated identically. After discarding the media, 100 μL tetrazolium (MTT, 0.5 mg/mL) was pipetted into each well and incubated for 4 h at 37°C. Sucking out the media, added 150 μL dimethyl sulfoxide to dissolve the violet-crystal and measured the absorption at 570 nm. Five wells were used for each drug concentration. Experiments were triplicated. The inhibitory rate was calculated as follows: IR (%)=(1-mean absorption in experiments/mean absorption in controls)×100%. The drug concentrations inhibiting cell growth by 50% (IC₅₀) were obtained by graphical analysis.

Five different protocols were used to investigate the interaction of KSC and EPI: Protocol 1: exposure to KSC for 48 h; Protocol 2: exposure to EPI for 48 h; Protocol 3:
exposure to KSC and EPI simultaneously for 48 h (SIM); Protocol 4: exposure to KSC for 24 h prior to incubation in drug-free medium for 24 h and finally exposure to EPI for 24 h (K+K); Protocol 5: exposure to EPI for 24 h prior to incubation in drug-free medium for 24 h and finally exposure to KSC for 24 h (E+K).

At the end of treatment, cells were washed with PBS and incubated at 37°C in an atmosphere containing 5% CO₂ for 48 h. Cell inhibition ratios were determined as described above.

**Evaluation of drug interaction**

The interaction between KSC and EPI was evaluated by Q value, which was calculated as follows: Q=E (AB)/ [EA+ (1-EA)×EB]. E (AB) is the inhibiting tumor rate of the combination and EA or EB is that of a drug alone. When Q value was equal to 0.85-1.15, or less than 0.85 or more than 1.15, additive or antagonistic or synergistic interaction was thought to occur (Wang et al., 2001).

**Hoechst 33258 staining for morphological analysis**

HepG-2 cells at 2×10⁴ cells/well were cultured in 6-well plates containing cover slips overnight. After incubation with KSC and EPI alone or in combination for 48 h, the cover slips were washed twice with PBS, fixed in 0.5 mL solution (the ratio of methanol to ice-cold acetate acid was 3 to 1) at 4°C for 30 min, rinsed twice with PBS, and stained with Hoechst 33258 (5 μg/mL) at room temperature for 30 min, rinsed twice with PBS, and wet mounted using 20 μL of the cell suspension. The cover slips were observed under a Leica DMI3000B fluorescence microscope.

**Cell cycle analysis by flow cytometry**

HepG-2 cells were cultured at 2×10⁵ cells per 25 mm dish. The same protocols as described for in vitro cytotoxicity assay were used. After incubation, the cells were harvested by trypsinization, washed twice with PBS, and fixed in 70% ethanol at 4°C for 1h. The cells were stained with propidium iodide (PI) solution (50 μg/mL of PI, 100 mg/L RNase, and 0.1% Triton X-100 in PBS) at 4°C for 30 minutes and analyzed for cell cycle distribution by flow cytometry (EPICS XL, BECKMAN COULTER). Data from 10, 000 cells per sample were collected and analyzed with CellQuestTM program.

**Western blotting analysis**

HepG-2 cells were cultured at 1×10⁶ cells per 90 mm dish. The cells were treated using the protocols described above. Cells were collected, washed twice with PBS, lysed in lysis buffer (10 mmol/L Tris, pH 7.4, 5 mmol/L EDTA, 150 mmol/L NaCl, 1% Triton X-100 and 0.1 mmol/L PMSF) and then centrifuged at 12, 000 g for 20 min at 4°C and the amount of protein was determined using a DC protein assay (Bio-Rad). The lysates were boiled for 5 min, separated by 15% SDS-PAGE, and transferred to polyvinylidene difluoride (PVDF) membranes (Pall Corporation) in transblotting buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3). The membranes were incubated for 2 h at room temperature with a blocking buffer (5% non-fat milk, 0.1% Tween 20 in TBS), and then incubated with KSC and EPI alone or in combination

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg·d)</th>
<th>Tumor weight (g)</th>
<th>Inhibition</th>
<th>Q value (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>1.48±0.0775</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KSC1</td>
<td>20</td>
<td>1.207±0.1745</td>
<td>18.45%</td>
<td></td>
</tr>
<tr>
<td>KSC2</td>
<td>40</td>
<td>0.810±0.0576</td>
<td>45.46%</td>
<td></td>
</tr>
<tr>
<td>KSC3</td>
<td>80</td>
<td>0.700±0.0051</td>
<td>52.68%</td>
<td></td>
</tr>
<tr>
<td>EPI</td>
<td>6</td>
<td>0.443±0.0675</td>
<td>70.06%</td>
<td></td>
</tr>
<tr>
<td>EPI+KSC1</td>
<td>6 20</td>
<td>0.305±0.0307</td>
<td>79.34%</td>
<td></td>
</tr>
<tr>
<td>EPI+KSC2</td>
<td>6 40</td>
<td>0.158±0.0265</td>
<td>89.51%</td>
<td></td>
</tr>
<tr>
<td>EPI+KSC3</td>
<td>6 80</td>
<td>0.122±0.0149</td>
<td>91.71%</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1. Effect of KSC and/or EPI on H22 Implanted Tumor in Mice (n=10)**

**Figure 1. Effect of EPI Combined with KSC on Thymus Index and Spleen Index of Mice with H22 Implanted Tumor. **

![Figure 1](https://example.com/figure1.png)

The effect of KSC and EPI on H22 implanted tumor

The inhibiting tumor rate of EPI in combination with KSC was higher than that of KSC or EPI alone and Q value was less than 1.15 but more than 0.85, which indicated an additive effect (Table 1). In addition, KSC can raise significantly thymus index and spleen index of mice with H22 implanted tumor as compared with EPI alone (P<0.01) (Figure 1).

**Cytotoxicity of KSC and EPI on HepG-2 cells**

MTT assays were performed to evaluate the potential cytotoxic effects of combining KSC and EPI. As shown in Figure 2, either KSC or EPI alone inhibited HepG-2 activity in a dose-dependent manner, but the effect was greater when the two agents were combined
Yu-Bin Ji et al


Table 2. Q value of KSC Combined with EPI on HepG-2 Cells in Different Administration Ways

<table>
<thead>
<tr>
<th>Groups</th>
<th>EPI (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>SIM</td>
<td>0.802</td>
</tr>
<tr>
<td>K+E</td>
<td>0.5602</td>
</tr>
<tr>
<td>E+K</td>
<td>0.53</td>
</tr>
</tbody>
</table>

Figure 2. Dose Response Curves for (a) KSC and (b) KSC in Combination with EPI in HepG-2 Cells for 48 h. Drug exposure protocols 1-5 as described in Methods. The points represent the means±SD of three determinations. Isobolograms at IC_{50} were generated based upon these dose-response curves for the combinations

Figure 3. The Morphological Changes of HepG-2 cells. A) Control; B-F) Drug exposure protocols 1-5 as described in Materials and methods

Figure 4. Effects of KSC and EPI on Cell Cycle and Apoptosis Rates of HepG-2 Cells. a) Distribution charts of cell cycle by flow cytometry. b) The statistical analysis of FCM results. Data represented as means±SD of three independent experiments. *p<0.05, **p<0.01, significantly different from the control group

Morphological changes
Morphological evidence of apoptosis was demonstrated by Hoechst-33258 fluorescence staining. Cells treated with KSC and EPI showed typically apoptotic changes, such as chromatin condensation, membrane blebbling, deformed and fragmented nuclei and apoptosis bodies (Figure 3). In agreement with the MTT results, EPI alone appear to cause a significant increase in the apoptotic process and decrease in cell number. Addition of KSC significantly increased the number of apoptotic cells.

Effect of KSC and EPI on cell cycle
DNA flow cytometry studies were performed to determine the effect of different KSC-EPI combinations on the cell cycle distribution. In general, HepG-2 cells treated with KSC or EPI alone or in combination resulted in a significant decrease in G1 and G2/M and an increase in S phase as compared to control, which indicated S phase arrest. Protocol 3 with exposure to KSC and EPI simultaneously arrested cells in S phase more significantly, while cells in G2/M phase decreased sharply. For the study of sequential applications, exposure to KSC for 24 h prior to EPI (protocol 4) resulted in an increase in S and G2/M and a decrease in G1 phase as compared to control. For the reversed sequences, exposure to EPI prior to KSC...
toxicity, raise the possibility that it could be effective in tumor and immunoregulatory properties, along with low immunoregulatory activities (Zhang et al., 2005). Its anti-demonstrated that KSC had anti-tumor and cisplatin-induced MMP-2 upregulation (Chen et al., 2013). The use of less toxic doses in combination with other drugs or other treatment modalities (Zhu, 2006; Berk et al., 2013). However, high doses of these drugs lead to severe toxicities, which have a negative effect on patients’ survival (Berk et al., 2013).

Discussion

Currently, a variety of cytotoxic and antiproliferative agents have been tested in Hepatocellular carcinoma (HCC) treatment, which are used alone, or in combination with other drugs or other treatment modalities (Zhu, 2006; Boulin et al., 2011; Berk et al., 2013). However, high doses of these drugs lead to severe toxicities, which have a negative effect on patients’ survival (Berk et al., 2013). The use of less toxic doses in combination with other anti-proliferative agents would be desirable (Lee et al., 2004; Abou-Alfa et al., 2010). Genistein could reinforce anti-proliferative agents would be desirable (Lee et al., 2004). Our flow cytometry results indicated that exposure to KSC or EPI only increased the proportion of cells in the S phase. In HepG-2 cells exposed to KSC and EPI simultaneously were remarkably arrested at the S phase. Exposure to KSC prior to EPI or exposure to EPI prior to KSC caused similar cell cycle perturbations, which was greater at lower concentrations. The possible reason is that the anti-tumor activity of an individual drug is saturated at high concentrations and difficult to increase after combination or there was antagonistic action to some extent between two drugs in combination and counteracted part of anti-tumor activity of a drug. Q values showed that in vitro the nature of interaction is additive between KSC and EPI in the simultaneous group and antagonistic in the sequential groups. On the basis of the experiments in vivo, low dose EPI combined with KSC was applied to treat H22 tumor implanted in mice, and inhibitory rate of tumor evidently increased as compared with that of a drug alone. Q value showed that in vivo markedly additive interaction between KSC and EPI were thought to occur, which agreed with the results in vitro. Meanwhile, thymus index and spleen index of mice with H22 implanted tumor were raised significantly as compared with EPI alone. These results suggested that low-dose EPI and KSC in combination could increase evidently anti-hepatocarcinoma effect. Epirubicin is the main anti-HCC agent, but its toxicities in kidney, liver or heart restrict its clinical application, as a result patients cannot tolerate the high dose agents whereas low dose is difficult to achieve satisfactory results (Li et al., 2012). Considering selectivity inhibitory effect of KSC on HCC in vitro and unobvious toxicity in vivo, the effect may be improved evidently without increased toxicities or keep satisfactory in poorly-tolerated patients with low dose of EPI when KSC and EPI in combination are applied to treat hepatocarcinoma.

KSC induces cell cycle arrest at the S phase (Wu et al., 2004). Our flow cytometry results indicated that exposure to KSC or EPI only increased the proportion of cells in the S phase. In HepG-2 cells exposed to KSC and EPI simultaneously were remarkably arrested at the S phase. Exposure to KSC prior to EPI or exposure to EPI prior to KSC caused similar cell cycle perturbations, blocking the cells in the S phase. These findings indicate that accumulation at the S phase might be an effect of KSC or EPI. We conjecture that different cytotoxicities in the same cell line following different drug exposure sequences were caused by different mechanisms. Besides,
Yu-Bin Ji et al

KSC and/or EPI could cause significant decrease in the expression of Cyclin A, Cdc25A and Cdk2 in HepG-2 cells. The possible mechanism of S phase arrest may be that the treatments activate the S phase checkpoint, and Chk2 activity is activated with a catalytic activity of Chk2 by phosphorylation of Cdc25A to promote its ubiquitination degradation and thus Cdk2 activation is suppressed, which will block the formation of Cdk2 and CyclinA complex that lead to S phase arrest and hinder the process of cell-cycle (Weinert, 1998).

In summary, in vitro studies further extend observations on selenium’s antitumor actions per se and as a potential synergistic agent with anticancer drugs. The present results can provide new hope for chemotherapy of hepatoma cancer. Furthermore, an improved understanding of the interactions between phytochemicals with the genes that are critical to the regulation of cancer cell growths will provide strong armaments to cancer therapy.

Acknowledgements

This research is supported by the Science and Technology Research Project of Heilongjiang Education Department (No. 12531151).

References


Norsa’adah B, Nurhzalalini-Zayani CG (2013). Epidemiology


