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ORIGINAL ARTICLE

The influence of cisplatin, doxorubicin, pegylated doxorubicin, oxaliplatin and gemcitabine on mahlavu cell line

Sevil Uygun Ilikhan[1], Muammer Bilici[1], Hatice Sahin[1], Ayse Semra Demir Akca[2], Huseyin Engin[3], Cemil Bilir[4], Nergis Sevinc[5], Nil Banu Pelit[6], Ishak Ozel Tekin[5]

Bulent Ecevit University, Faculty of Medicine, [1]Department of Internal Medicine, [2]Department of Family Medicine, Zonguldak; [3]Department of Medical Oncology, and [5]Department of Immunology, Zonguldak; [4]Recep Tayyip Erdogan University, Faculty of Medicine, Department of Medical Oncology, Rize; [6]Acibadem University, Vocational School of Health Services, Department of Cardiovascular Perfusion, Istanbul, Turkey

Summary

Purpose: *Hepatocellar carcinoma (HCC) remains a major health problem being the third leading cause of deaths due to cancer worldwide. Because HCC is known to be highly resistant to conventional systemic therapies, single-agent or combination of systemic therapies have been investigated. Today, sorafenib, a multikinase inhibitor, is the only approved systemic agent for the first line treatment of advanced HCC. In this study, we aimed to investigate the influence of different concentrations of cisplatin, doxorubicin, pegylated doxorubicin (PLD), oxaliplatin and gemcitabine by applying these agents either single or in combinations on mahlavu cell line.*

Methods: *HCC mahlavu cell line was used for the experiments. Cell death was measured by flow cytometry at 48 hrs after incubation with various concentrations (0.1 μg/ml, 1.0 μg/ml and 10 μg/ml) of the drugs.*

Results: *Cell death due to gemcitabine was found to be significantly higher than cell deaths caused by the other single agents including cisplatin, oxaliplatin, doxorubicin and PLD ($p<0.001$, $p<0.001$, $p<0.001$ and $p=0.0049$, respectively). There was no significant difference between gemcitabine and both the gemcitabine combination with doxorubicin and PLD ($p=0.992$ and $p=0.441$, respectively).*

Conclusion: *This is a preliminary analysis evaluating the effect of the conventional chemotherapeutic agents on mahlavu cell line in vitro. The findings of this study suggest that gemcitabine-based therapies keep on being the prefered therapeutic approach for the treatment of HCC.*

Key words: *cell death, chemotherapy, hepatocellular carcinoma, mahlavu cell line*

Introduction

HCC is mainly diagnosed at advanced stage and has low response rate to known chemotherapeutic agents. In addition, patients have some systemic disorders related to the severity of underlying background liver disease that may reduce tolerance to chemotherapy. Thus HCC remains a major cause of death due to cancer worldwide [1-3]. At an early stage, the potentially curative options such as resection, ablation, and transplantation should be applied so that early diagnosis may offer a long-term disease control [4]. Although the improvements of survival rates for HCC have been achieved by applying local and systemic therapies [5], the median survival of HCC remains less than 12 months from diagnosis [6]. The treatment options at an advanced stage include hormonal therapy, immunotherapy, cryotherapy, selective internal radiotherapy and systemic or local chemotherapy [7].

HCC is known to be highly resistant to con-

Correspondence to: Ayse Semra Demir Akca , MD. Bulent Ecevit University Faculty of Medicine, Department of Family Medicine, 67100 Esenkoy, Kozlu, Zonguldak, Turkey. Tel: +90 542 687 0849, Fax: +903722610155, E-mail: aysesemra@hotmail.com
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ventional systemic therapies, as a result of having a high incidence of expression of the multidrug resistance gene-1 (MDR1) and high levels of P-glycoprotein (P-gp) that are related to poor response to chemotherapy [8,9]. Hence, single-agent or combinations of various systemic chemotherapeutic agents have been investigated for the treatment of this disease in recent years. However, many authors have not been able to yield the expected effectiveness of systemic agents in HCC [10-12]. Today, sorafenib, a multikinase inhibitor, is the only approved systemic agent for first-line treatment of advanced HCC [13].

Prior to the approval of sorafenib, the cytotoxic agents such as cisplatin, doxorubicin, oxaliplatin and gemcitabine have been investigated in advanced HCC [7].

Cisplatin has been widely used in the treatment of various malignancies including testicular, ovarian, bladder, lung, head and neck cancers. The effect of cisplatin appears via interacting with DNA, RNA, nuclear and cytoplasmic proteins that may result in cytotoxicity and apoptosis. One of the major disadvantages of cisplatin is the existence or development of drug resistance [14,15]. Oxaliplatin may be used instead of cisplatin in the treatment of resistant cancers. Oxaliplatin has a very similar activity profile against cancers and is better tolerated than cisplatin [16].

Another chemotherapeutic agent that has been most commonly used for the treatment of unresectable advanced or metastatic HCC is doxorubicin. Doxorubicin is a topoisomerase II inhibitor that causes eventual DNA breaks with subsequent inhibition of DNA synthesis. In contrast to doxorubicin, PLD has lower toxicity, and it has shown higher therapeutic efficacy owing to longer circulation time and preferential accumulation in tumor tissue [17-19].

Gemcitabine is a pyrimidine antimetabolite which inactivates the ribonucleotide reductase (RNR) enzyme irreversibly, and leads to cell apoptosis. Gemcitabine has shown antitumor activity against HCC and is used as part of combination regimens in the treatment of HCC [7,17].

In this study, we aimed to investigate the influence of different doses of cisplatin, doxorubicin, PLD, oxaliplatin and gemcitabine in mahlavu cell line by applying either single-agent or combinations of these agents.

Methods

Cell line

Human HCC (Mahlavu) cells were used in cell culture experiments. This cell line was provided by Acibadem University, Istanbul, Turkey.

Chemicals

The drugs were purchased from the different companies (Cisplatin- Kocak Farma, Istanbul, Turkey; Doxorubicin-Saba, Istanbul, Turkey; PGL- Ben Venue Laboratories, Ohio, US; Oxaliplatin and Gemcitabine-Actavis Italy SpA, Milan, Italy).

Cell culture and cytotoxicity analysis

Mahlavu cells were grown in RPMI 1640 medium containing 10% fetal bovine serum (FBS) in a humidified atmosphere containing 5% CO_2 at 37°C. Cell culture media were supplemented with 100 U/ml penicillin, 100 µg /ml streptomycin and 2 nmol/l L-glutamine. Cells were plated and grown overnight until they reached 80% confluence, and then treated with cisplatin, oxaliplatin, gemcitabine doxorubicin, PLD alone and gemcitabine plus doxorubicin and gemcitabine plus PLD combinations at different concentrations (0.1 µg /ml, 1 µg/ml, 10 µg /ml) in 24-well cell culture plates for 48 hrs. RPMI 1640 medium containing 10% FBS was used as control sample. Following incubation, detached cells in the medium were collected, and the remaining adherent cells were harvested by trypsinization. After double washing, propidium iodide was added in each sample. Cell death was measured by using flow cytometry (FC500-Beckman Coulter, Brea CA, USA).

Statistics

Statistical analyses were performed with SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). Results were expressed as mean±standard deviation. Differences among groups were analyzed by the Kruskal–Wallis test. A p value less than 0.05 was considered statistically significant for all tests.

Results

Each of the therapies was significantly cytotoxic compared to controls ($p<0.001$ for all groups). The percentages of cell death at 48 hrs after incubation with various concentrations (0.1 µg /ml, 1.0 µg/ml and 10 µg /ml) of the conventional chemotherapeutic agents which were used in this study and controls are shown in Table 1. Gemcitabine was found to be most effective agent among the chemotherapeutic drugs on Mahlavu cell culture *in vitro*. Cell death due to gemcitabine was significantly different from cisplatin, oxaliplatin, doxorubicin and PLD ($p<0.001$, $p<0.001$, $p<0.001$ and $p=0.049$, respectively). There was no significant difference between gemcitabine and both the gemcitabine combination with doxorubicin and PLD ($p=0.992$ and $p=0.441$, respectively). Also, the

Table 1. Cell death due to the single agents at different concentrations on mahlavu cell line

Concentration (μg/ml)	*Cisplatin (mean ± SD)*	*Oxaliplatin (mean ± SD)*	*Doxorubicin (mean ± SD)*	*PLD (mean ± SD)*	*Gemcitabine (mean ± SD)*	*Control (mean ± SD)*
0.1	29.08±1.84	27.11±1.96	36.40±2.54	25.78±1.25	47.61±5.11	
1	34.22±2.84	31.71±3.26	43.50±3.11	38.82±3.23	52.36±4.48	
10	43.68±4.57	46.70±7.24	*	68.56±3.47	58.80±5.78	
Total	35.66±6.92	35.17±9.66	39.95±4.58	44.39±18.48	52.92±6.79	24.73±1.57

PLD: pegylated liposomal doxorubicin, *: doxorubicin 10 μg/ml dose was excluded from the analysis, SD: standard deviation

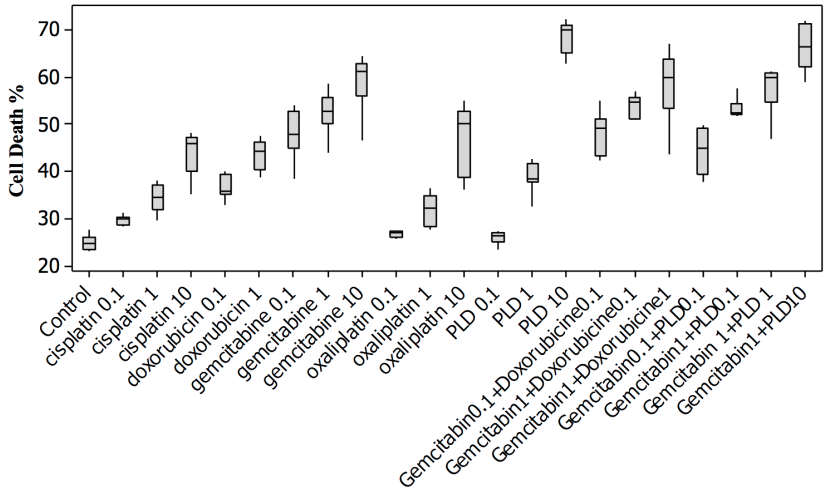

Figure 1. The cytotoxic effects of chemotherapeutics on Mahlavu cells. Cell death was measured at 48 hrs after incubation with various concentrations (0.1 μg /ml, 1.0 μg/ml and 10 μg /ml) of the chemotherapeutics on Mahlavu cell line. All agents exerted significant cytotoxicity on Mahlavu cells in a concentration-dependent manner. The maximal cell death occurred at the 10μg/ml concentration of PLD among all of the drugs. PLD: pegylated liposomal doxorubicin

Table 2. Comparison of the effects of gemcitabine-based combinations

Concentrations (μg/ml)	*Gemcitabine-Doxorubicin (mean±SD)*	*Gemcitabine-PLD (mean±SD)*	*p value*
0.1+0.1	47.92±4.46	44.26±4.81	0.279
1.0+0.1	52.82±4.59	52.45±3.32	0.721
1.0+1.0	58.25±7.50	57.40±4.93	0.505
1.0+10	*	66.31±5.06	
Total	53.00±6.94	55.10±9.21	0.345

PLD: pegylated liposomal doxorubicin, *: Doxorubicin 10 μg/ml dose was excluded from the analysis, SD:standard deviation

effect of combinations of gemcitabine with doxorubucin and PLD were not significantly different compared to each other at similar concentrations (Table 2). Cell death due to oxaliplatin was not significantly different from cisplatin (p=0.477). Doxorubicin was found to be significantly more effective than cisplatin (p=0.025), and no significant difference was noticed between doxorubicin and PLD (p=0.795). The maximal percentage of cell death occurred at the 10 μg/ml concentration of PLD among all of the drugs (Table 1). Analysis of doxorubicin at 10 μg/ml concentration could not be completed because of its high autofluorescence and therefore doxorubicin at this concentration was excluded from analysis. We did not observe autofluorescence in PLD at the dose of 10 μg/ml. The effect of cisplatin, oxaliplatin, doxorubicin, PLD, gemcitabine, gemcitabine with doxorubicin and gemcitabine with PLD administration on Mahlavu cell lines were dependent on the concentration of these agents (Figure 1) and the relation of dose-response differed significantly in each group (p<0.001, p<0.001, p=0.002, p<0.001,

p=0.004, p=0.006 and p<0.001, respectively). The percentage of maximum cell death for each drug was measured at 48 hrs after incubation with the highest concentration of cisplatin, oxaliplatin, doxorubicin, PLD, gemcitabine and the combinations of gemcitabine with doxorubucin and PLD (Tables 1,2).

Discussion

HCC is one of the most common reasons of death due to cancer and it remains a major health problem worldwide. The majority of HCC cases occur in patients with chronic liver disease such as hepatitis B-virus (HBV), hepatitis C-virus (HCV) infection, alcoholic liver diseases, and non-alcoholic fatty liver diseases [2,20]. Because advanced HCC patients have poor prognosis, various combinations of chemotherapeutic drugs have been investigated to detect their antitumor activity against HCC. Currently sorafenib is recommended for the first line treatment of advanced HCC [13]. However, survival rate remains low and no desired improvements for the prognosis of patients with advanced HCC have been achieved yet and therefore the need to further working on the chemotherapeutic drugs used in the treatment of advanced HCC is real. In the present study, we aimed to investigate the antitumor activity of cisplatin, oxaliplatin, doxorubicin, PLD and gemcitabine via measuring cell death in HCC mahlavu cells.

Tozawa et al. have constructed a model that illustrated the efficiency of oxaliplatin against cisplatin-resistant gastric cancer cell line [21]. In their study, however, the intracellular concentration of cisplatin was not detectable up to 48 hrs after exposure to cisplatin (1.0 μg/ml). The concentration of oxaliplatin in the cells was not significantly different up to 72 hrs in this study. This might show lack of cisplatin accumulation in resistant cells. The authors also demonstrated that oxaliplatin is more effective than cisplatin against cisplatin-resistant cells at similar doses. Rixe et al. have investigated the activities of platinum compounds including cisplatin, oxaliplatin, carboplatin, tetraplatin in cisplatin-resistant cell lines [22]. Reduced cellular accumulation of these drugs in resistant cell lines was found in that study. The activity of oxaliplatin was more clear compared to cisplatin in resistant cells. Thus, different mechanisms were thought to play roles in oxaliplatin activity against cisplatin-resistant cells. Hence, the authors suggested that cisplatin and oxaliplatin might show possible synergistic effects in cisplatin-refractory patients. In mahlavu cell culture, we observed that both cisplatin and oxaliplatin displayed an effective antitumor activity against cancer cells. However, there was no significant difference between cisplatin and oxaliplatin at similar doses (0.1 μg/ml, 1.0 μg/ml and 10 μg/ml) regarding the cell death that was measured at 48 hrs after incubation.

Inoue et al. have compared the effect of doxorubicin and cisplatin in a murine neuro-blastoma cell line [23]. Although they observed minimal cell death at 24 hrs after incubation with doxorubicin (<0.1 μM), the major cell death rate (>98%) occurred at the over 1.6 μM concentrations of doxorubicin. In addition, they have seen the same effect with cisplatin which caused the major cell death rate (>98%) at 72 hrs after incubation with cisplatin (>0.01 mg/ml). They suggested that doxorubicin has an immunological advantage over cisplatin in the treatment of neuroblastoma.

We applied cisplatin, doxorubicin and PLD to mahlavu cell line at similar doses to compare one another's cytotoxic effects by measuring the percentages of cell death 48 hrs later. A consistent increase in response with increased dose has been shown for each of these drugs *in vitro*. Doxorubicin and PLD in increasing doses seemed to be more effective than cisplatin on mahlavu cell line. And also, the highest percentage of cell death was observed in PLD at dose of 10 μg /ml. However, there was no significant difference between the effect of doxorubicin and PLD. In addition, the application of doxorubicin at the concentration of 10 μg/ml has been excluded from analysis because a high autofluorescence that might cause a failure of analysis has been detected. But this special feature of doxorubicin at 10 μg/ml has not been observed following PLD administration at similar dose. This feature may depend on molecular differences between PLD and doxorubicin.

Taïeb et al. administered a combination of gemcitabine and oxaliplatin to patients with HCC which resulted to a median overall survival of 12 months with an overall response rate of 19%. They suggested that this combination of gemcitabine-oxaliplatin might have potential for improving the prognosis of patients with advanced HCC [24]. Louafi et al. also have observed the efficacy of gemcitabine with oxaliplatin combination in patients with advanced HCC [25]. The median overall survival of patients was 11.5 months with overall response rate of 18%. Gemcitabine-oxaliplatin combination was found to be more effective in patients who had an underlying nonalcoholic liver disease than the others in their study [25].

The median progression-free survival in both previous studies using the gemcitabine-oxaliplatin combination were 5 and 6.3 months, respectively [24,25]. This might be considered as an achievement of this combination in advanced HCC patients with the median overall response rate of less than 20%. Poh et al. investigated the effectiveness of the PLD with capecitabine and PLD plus gemcitabine as salvage therapy in advanced HCC but they have not observed sufficient efficacy of these combinations in HCC patients [18]. Conversely, Lombardi et al. have reported that PLD plus gemcitabine combination could be used safely as an effective treatment choice, especially in patients with impaired liver function [17]. Furthermore, the demographic characteristics of patients who were enrolled in the previous two studies were not identical and PLD-based combination therapies were well tolerated in patients with advanced HCC in both of these two studies [17,18].

In our study, gemcitabine appeared to have a more potent antitumor effect than the single usage of cisplatin, oxaliplatin, doxorubicin and PLD on mahlavu cell line. Furthermore, there was no significant difference between gemcitabine and gemcitabine-based combinations (gemcitabine with doxorubicin and gemcitabine with PLD) in terms of percentage of cell death at 48 hrs after incubation with the drugs. Probably, PLD and doxorubicin are not able to contribute to the antitumor effect in their combinations with gemcitabine sufficiently because gemcitabine has a potent cytotoxicity against cancer cells.

Conclusions

To our knowledge, this is the first study in the literature evaluating the antitumor activity of conventional chemotherapeutic agents including cisplatin, oxaliplatin, doxorubicin, PLD and gemcitabine via measuring cell death in HCC mahlavu cells. Mahlavu cell culture system seems to be an eligible model to work on HCC *in vitro*. The findings of this study suggest that the gemcitabine-based therapies are worth working with. More in vitro studies are needed before recommending the new drugs and their combination strategies.

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