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ORIGINAL ARTICLE

Radiation enhancing effects with the combination of sanazole and irinotecan in hypoxic HeLa human cervical cancer cell line

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Summary

Purpose: *This study was conducted to determine the synergistic radiation sensitizing effects of the combination of sanazole and irinotecan in hypoxic cervical cancer HeLa human tumor cell line.*

Methods: *The 3-(4,5 dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay was used to evaluate the number of surviving cells. Cell cycle was determined by flow cytometry. Surviving cell fractions were determined by the standard in vitro colony formation assay.*

Results: *The MTT assay showed that the presence of irinotecan with or without sanazole reduced significantly the cells' viability. Flow cytometry demonstrated that the combination of sanazole and irinotecan led to more HeLa cells blocked in G_2 phase. Cell colony formation assay indicated that the radiosensitivity of hypoxic HeLa cells was enhanced by sanazole and/or irinotecan.*

Conclusion: *This study showed that the radiation enhancing effects produced by the combination sanazole and irinotecan was significant in hypoxic HeLa cells, which were arrested in the G_2 phase of the cell cycle. This study may provide a new combination modality of radiosensitizers in the radiotherapy of cervical cancer.*

Key words: *HeLa cell line, irinotecan, radiation enhancing effect, sanazole*

Introduction

Tumor hypoxia is an important factor that negatively affects the prognosis of cancer patients [1].The inability of radiotherapy to eradicate completely certain human tumors may be due to the presence of hypoxic cells [2].To overcome the problem of tumor hypoxia, a number of strategies have been attempted. Radiosensitizers such as sanazole and irinotecan can sensitize hypoxic cells, thus improve the efficacy of radiotherapy in controlling human tumors [3-5].

Topoisomerase inhibitors including irinotecan are being studied as potential radiosensitizers and are clinically available [6,7]. Topoisomerase I-targeting drugs exert their cytotoxic effects by producing enzyme-mediated DNA damage, rather than by directly inhibiting enzyme catalytic activity [8]. DNA topoisomerase I recently has been established as a biochemical mediator of radiosensitization in cultured mammalian cells by camptothecin derivatives.

Sanazole and irinotecan can be used in combination since they have different dose-limiting toxicities [9].This approach minimizes the overall toxicity and enhances the radiosensitizing effects. This kind of strategy was used to explore the radiation-ehancing effects of the combination of sanazole and irinotecan.

As sanazole and irinotecan have different dose-limiting toxicities, it may be possible to combine the two agents so as to improve the therapeutic advantage without increasing toxicity. This study was conducted to determine the radiation-enhancing effects of sanazole and irinotecan when administered together at appropriate concentrations in hypoxic HeLa human cervical cancer cell line.

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Methods

Compounds

Sanazole was kindly offered by the Central Lab of China Medical University and irinotecan was purchased from Aventis Pharma (CTP-11, Campto®, Antony, France). Sanazole was dissolved in phosphate-buffered saline (PBS) at 1 mM concentration and irinotecan was dissolved in PBS at 3μM concentration. For the experiments, cells were exposed to sanazole at 1 μM for 30 min and irinotecan at 3μM for 4 h, respectively.

Cells culture and hypoxic condition

The human HeLa cervical cancer cells were kindly provided by Central Laboratory of Shengjing Hospital of China Medical University. The cells were cultured as monolayers in Dublecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37˚C. In the experiments, the cells were grown in 96-well tissue culture plates and used at the stage of the exponential growth phase. They were made hypoxic by flushing 95% N_2 and 5% CO_2 gas for 24 h.

Irradiation

Irradiation was delivered by linear accelerator (6 MV Photon Beam, SIEMENS Primus) at room temperature. The dose rate was 300cGy/min.The irradiation doses were 0, 2, 4, 6, 8, and 10 Gy, respectively. After irradiation, the medium with drugs was absorbed and replaced with fresh DMEM medium containing 10% FBS.

MTT assay

The MTT assay was used to evaluate the number of surviving cells. The HeLa cells were plated at 500 cells per well in 96-well tissue culture plates and allowed to attach for 24 h. After irradiation (6Gy), 20μl of MTT (5mg/ml) were added to each well and the plates returned to the incubator for 4 h. Extra care was taken when removing untransfered MTT by aspiration in order not to disturb the blue formazan crystals. 200μl dimethyl sulfoxide (DMSO) were then added to each well to dissolve the formazan crystals while slightly agitating the cells on an automated shaker. Then the absorbance was measured at 497 nm.

Flow cytometry analysis

HeLa cells were plated at 1×10^5 cells per well in 6-well culture plates and allowed to attach for 24 h. Hypoxic condition, administration of drugs and irradiation at 6 Gy were carried out as described above (Cells culture and hypoxic condition; Irradiation). The cells were fixed overnight with cold 70% ethanol and centrifuged at 800 rpm for 5 min. Then the supernatant was aspirated and the pellet was resuspended in 1ml PBS, following staining with propidium iodide (PI) solution containing 50 μg/ml PI and 10 μg/ml RNase. After incubation at room temperature for 30 min, cells were analyzed by flow cytometry (FACSCalibur; Becton Dickinson, USA).

Table 1. The radiosensitizing effects of sanazole and/or irinotecan in hypoxic HeLa cells irradiated at 6 Gy

Time after irradiation (Days)	*Sanazole (mM)*	*Irinotecan concentration (μM)* 0 mean±SD	3 mean±SD
1	0	89.50±2.86[a]	47.33±3.35[c]
	1	72.20±2.54[b]	30.37±1.69[d]
2	0	85.03±4.38[a]	40.75±7.38[c]
	1	68.10±1.85[b]	23.37±2.79[d]
3	0	86.25±5.24[a]	44.44±4.41[c]
	1	72.79±3.95[b]	28.47±2.07[d]

a vs b, a vs c, a vs d, b vs d and c vs d : $p<0.05$ for all comparisons. SD: standard deviation

Cell survival assay

The HeLa cells were plated at 500 cells per well in 6-well tissue culture plates and allowed to attach for 24 h. The cells were incubated in hypoxic condition for 24 h. Immediately after exposure of the cells to 0,2,4,6,8, and 10 Gy of radiation respectively in the presence or absence of sanazole and/or irinotecan, the medium was replaced with fresh DMEM supplemented with 10% FBS. Cells were incubated under standard growth conditions for 14 days, and the resultant colonies were stained with Giemsa. Colonies containing 50 or more cells were scored manually.

Statistics

Five plates were used per experimental point, and all the experiments were performed in triplicate. All the data were expressed as means± standard deviation (SD). Analysis of Variance (ANOVA) was used to determine the differences between groups. The level of significance set at $p<0.05$. SPSS, v. 13.0 software was used for all analyses.

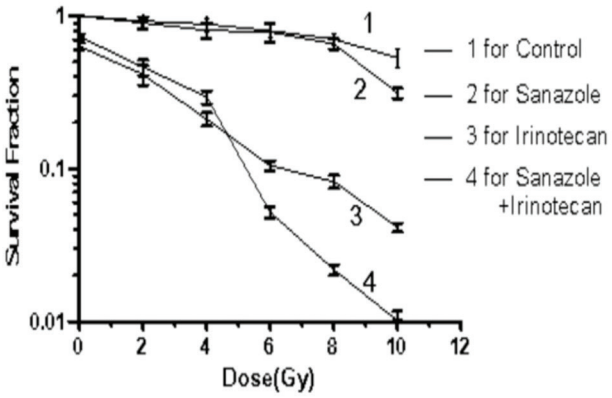

Figure 1. Cell survival fraction according to the irradiation dose for hypoxic HeLa cells with sanazole and/or irinotecan. 1 vs 2, 1 vs 3, 1 vs 4, 2 vs 3, 2 vs 4 and 3 vs 4: $p<0.05$ for all comparisons.

Table 2. Cell cycle distribution of hypoxic HeLa cells incubated with 1mM sanazole and/or 3 μM irinotecan 24 h after irradiation at 6 Gy

Treatment	G_1 (%) *mean±SD*	*S (%) mean±SD*	G_2/*M (%) mean±SD*
Control (no drugs)	55.8±9.9	15.1±5.1	29.0±5.2[a]
Sanazole	40.9±2.3	15.8±6.3	43.4±4.0[b]
Irinotecan	28.2±7.1	14.1±6.6	57.6±2.7[c]
Sanazole+irinotecan	15.6±1.4	13.2±4.4	71.1±5.4[d]

a vs b, a vs c, a vs d, b vs d and c vs d : $p<0.05$ for all comparisons. SD: standard deviation

Results

MTT assay

The survival rates of the cells at different time points after treatment with the two drugs and then irradiation are shown in Table 1. Irinotecan reduced the survival rate significantly alone or in combination with sanazole. Irinotecan 3μM led to a remarkable radiosensitizing effect in HeLa cells. Meanwhile,1mM sanazole indicated radiation sensitizing enhancing effect on HeLa cells. Significant synergistic radiosensitizing effects of the two drugs were seen on HeLa cells.

Cell cycle distribution

After irradiation, there was significant statistical difference of G_2 arrest between groups. The combination of the two drugs led to more HeLa cells arrested in G_2 phase (Table 2).

Cell survival analysis

Figure 1 shows that the radiosensitivity of hypoxic HeLa cells was enhanced by sanazole or irinotecan. The combination of the two drugs also displayed a radiation enhancing effect at the irradiation doses of 6, 8 and 10 Gy. The radiosensitizing effect of 3 μM irinotecan was more significant than that of 1mM sanazole. The survival rate of non-irradiated cells also decreased, mainly due to the cytotoxicity of irinotecan.

Discussion

During the past decades, much progress has been made in exploring hypoxic cell radiation sensitizing agents [10-12]. A number of radiosensitizers have been invented and applied *in vitro* and *in vivo* [13-15]. Nevertheless, owing to a number of adverse effects, their clinical application is somehow limited. So different administration modalities are sought to offer appropriate radiosensitizers for clinical use [16].Currently, using combinations of different radiation sensitizing agents is a hot topic with obvious clinical interest [17]. This study was conducted to investigate the possibility of combining sanazole and irinotecan to achieve a radiation enhancing effect in human HeLa hypoxic cervical cancer cells.

MTT assay indicated that both sanazole and irinotecan had a significant radiation enhancing effect in hypoxic HeLa cells. The combination of the two drugs resulted in a more significant radiosensitizing effect in hypoxic HeLa cells during the 3 days after irradiation and this effect was much more pronounced on the 2nd day.

Flow cytometry analysis demonstrated that the radiation-enhancing effect of sanazole or irinotecan was expressed by the ability of drugs to arrest tumor cells in the G_2 phase of the cell cycle. With the combination of sanazole and irinotecan to hypoxic HeLa cells, the proportion of cells in the G_2 phase increased compared to cells exposed to either drug alone.

In order to disclose the synergistic radiosensitizing effects of the two drugs, cells cultured with sanazole and/or irinotecan were treated with different doses of irradiation. The cell survival curve (Figure 1) showed that the administration of sanazole and/or irinotecan led to an enhancement in the fraction of cells becoming clonogenically incompetent; the radiosensitizing effect of irinotecan was more significant than that of sanazole at the administered doses and significant radiation-enhancing effects were observed with the combination of both drugs. Therefore, the coadminstration of the two drugs in conjunction with radiotherapy may result in a more pronounced therapeutic activity on hypoxic cervical cancer cells.

In conclusion, when administered at proper concentrations, the combination of sanazole and irinotecan resulted in radiation-enhancing effects in hypoxic HeLa cervical cancer cell line. This study indicated that the combination of sanazole and irinotecan *in vitro* is possible. However, there is not sufficient experimental evidence that the radiosensitizing effects of the combination of sanazole and irinotecan are synergistic *in vivo*. So, further *in vivo* studies are needed to provide data validating the synergistic radiosensitizing effects of the combination of the two drugs.

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