Radiation enhancing effects of sanazole and gemcitabine in hypoxic breast and cervical cancer cells *in vitro*

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Aim of the study: Sanazole and gemcitabine have been proven clinically as hypoxic cell radiosensitisers. This study was conducted to determine the radiation enhancing effects of sanazole and gemcitabine when administered together at relevant concentrations into hypoxic human MCF-7 and HeLa cells.

Material and methods: A 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. Cell surviving fractions were determined by the standard in vitro colony formation assay.

Results: The cell colony formation assay indicated that the radiosensitivity of hypoxic MCF-7 and HeLa cells was enhanced by sanazole or gemcitabine. The combination of the two drugs displayed significant radiation enhancing effects at the irradiation doses of 6, 8, and 10 Gy in both cell lines, which were arrested in the S phase.

Conclusions: This study indicated that the co-administration of the two drugs may result in a beneficial gain in radiotherapy for hypoxic breast cancer and cervical cancer.

Key words: Sanazole, gemcitabine, radiosensitiser, HeLa cells, MCF-7 cells.

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Introduction

Tumour hypoxia is an important factor that negatively affects the prognosis of cancer patients [1]. The inability of radiotherapy to completely eradicate certain human tumours may be due to the presence of hypoxic tumour cells [2]. A number of strategies have been attempted to overcome the problem of tumour hypoxia. Radiosensitisers such as sanazole and gemcitabine can sensitise hypoxic cells and thereby improve the efficacy of radiotherapy in controlling human tumours [3–5].

Sanazole, a 3-nitrotriazole derivative also known as substance AK-2123 (N-2'-methoxy ethyl)-2-(3''-nitro-1''-triazolyl), has the potential to be used as a hypoxic cell radiosensitiser due to its low neurotoxicity and high radiosensitising effect. Early studies testing sanazole as a radiosensitiser with irradiation in various tumours showed encouraging results [6, 7].

Gemcitabine (dFdC) (2′, 2′-difluoro-2′-deoxycytidine) is a deoxycytidine analogue well known for its antitumor activity in different tumour types. It is also one of the most effective drugs to sensitise cells to radiation; its radiation enhancing properties have been demonstrated both *in vivo* and *in vitro* [8–10]. Gemcitabine itself is not active. It is transported into the cell by facilitated diffusion, where it is phosphorylated to its active metabolites, dFdCMP, dFdCDP, and dFdCTP, by deoxycytidine kinase. All three of the metabolites interfere with different steps in the processing of DNA. dFdCTP is incorporated into DNA and as such can obstruct DNA replication and repair [11]. Ionising radiation induces an array of lesions in DNA, including base damage, single-strand breaks, and double-strand breaks (DSBs), and damage to the phosphodiester backbone. Double-strand breaks are generally thought to be the most relevant lesion in radiation-induced killing of cells. Sanazole is considered to sensitise hypoxic cells selectively by capturing macromolecular free radicals, thereby acting to fix damage as well as to inhibit DNA repair [12].

Sanazole and gemcitabine can be used in combination since they have different dose-limiting toxicities [13, 14]. This approach minimises the overall toxicity and enhances the radiosensitising effects.

This study was conducted to determine the radiation enhancing effects of sanazole and gemcitabine when administered together at relevant concentrations into two hypoxic human tumour cell lines: MCF-7, a breast cancer cell line, and HeLa cells, a cervical cancer cell line.

Material and methods

Compounds

Sanazole was kindly offered by the Central Lab of China Medical University. Gemcitabine was purchased from Eli Lilly and Company. Sanazole

was dissolved in phosphate-buffered saline (PBS) at a concentration of 1 mM. Gemcitabine was dissolved in PBS at a concentration of 5 μM. Cells were exposed to 1 mM sanazole for 30 minutes and to 5 μM gemcitabine for 4 hours. After irradiation, the dishes were washed with PBS and the DMEM medium containing 10% FBS was replaced.

Cell culture and hypoxic conditions

The human breast cancer cells (MCF-7) and human cervical cancer cells (HeLa) were kindly provided by the 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% foetal bovine serum (FBS), penicillin, and streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. The cells were subcultured twice weekly. In the experiments, the cells were grown in 96-well tissue culture plates and used when they reached the exponential growth phase. They were made hypoxic by flushing with 95% N_2 and 5% CO_2 gas for 24 hours.

Irradiation

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

MTT assay

The 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to evaluate the number of surviving cells. The MCF-7 and HeLa cells were plated at 500 cells per well in 96-well tissue culture plates and allowed to attach for 24 hours. After irradiation (8 Gy), 20 μl of MTT (5 mg/ml) was added to each well and the plates were returned to the incubator for 4 hours. Extra care was taken when removing untransferred MTT by aspiration in order not to disturb the blue formazan crystals. 200 μl dimethyl sulfoxide (DMSO) was 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

MCF-7 and HeLa cells were plated at 1×10^5 cells per well in six-well culture plates and allowed to attach for 24 hours. Hypoxic conditions, administration of drugs, and irradiation at 8 Gy were carried out as described above (cell culture and hypoxic condition; irradiation). The cells were fixed overnight with cold 70% ethanol and centrifuged at 800 rpm for 5 minutes. Then the supernatant was aspirated and the pellet was resuspended in 1 ml PBS, followed by staining with propidium iodide (PI) solution containing 50 μg/ml PI and 10 μg/ml RNase. After incubation at room temperature for 30 minutes, cells were analysed by flow cytometry (FACSCalibur; Becton Dickinson, USA).

Cell survival assay

The MCF-7 and HeLa cells were plated at 500 cells per well in six-well tissue culture plates and allowed to attach for 24 hours. The cells were incubated under hypoxic conditions for 24 hours. Immediately after exposure of the cells to 0, 2, 4, 6, 8, and 10 Gy of radiation in the presence or absence of sanazole and/or gemcitabine, 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.

Statistical analysis

Five dishes were used per experimental point, and all the experiments were repeated three times. All the data were expressed as mean ± standard deviation (SD). Analysis of variance (ANOVA) was used to determine the differences between groups (SPSS13.0 statistical software) with the level of significance set at $p < 0.05$.

Results

MTT assay

The survival rates of the two hypoxic tumour cell lines at different time points after treatment with the two drugs and then irradiation are shown in Table 1. Gemcitabine reduced the survival rate significantly alone or in combination with sanazole. 5 μM gemcitabine led to a remarkable radiosensitising effect in both MCF-7 and HeLa cells.

Table 1. The radiosensitising efficacy of sanazole and/or gemcitabine on MCF-7 and HeLa cells irradiated at 8 Gy

Day after irradiation(d)	Cell line	Drug free controls	Sanazole (1 mM)	Gemcitabine (5 μM)	Sanazole (1 mM) + gemcitabine (5 μM)
1	MCF-7	88.68 ±0.61[a]	74.36 ±0.62[b]	64.28 ±1.46[c]	43.82 ±0.50[d]
	HeLa	88.53 ±1.53[a]	69.47 ±1.07[b]	50.00 ±0.67[c]	27.10 ±0.63[d]
2	MCF-7	88.64 ±0.33[a]	71.35 ±0.23[b]	55.80 ±0.88[c]	38.41 ±1.32[d]
	Hela	86.84 ±0.57[a]	59.34 ±1.34[b]	48.92 ±2.14[c]	22.52 ±3.15[d]
3	MCF-7	88.86 ±0.90[a]	76.17 ±1.23[b]	61.24 ±0.84[c]	41.40 ±1.87[d]
	HeLa	88.28 ±0.29[a]	70.37±0.16[b]	47.03 ±1.44[c]	26.82 ±1.03[d]

Compare: a vs. b, a vs. c, c vs. d and b vs. d, $p < 0.05$, for all groups.

Table 2. Cell cycle distributing of MCF-7 and HeLa cells incubated with 1 mM sanazole and/or 5 μM gemcitabine 24 hours after irradiation at 8 Gy

Cells	Treatment	G1 (%)	S (%)	G2/M (%)
CMF-7	drug-free controls	28.21 ±2.04	26.10 ±2.95	45.62 ±4.94
	sanazole	53.74 ±2.83[a,b]	26.62 ±1.48	19.60 ±0.77
	gemcitabine	28.87 ±3.08	54.47 ±2.06[a,b]	16.89 ±1.71
	gemcitabine + sanazole	30.40 ±1.55	62.33 ±0.94	7.20 ±1.23
HeLa	drug-free controls	29.96 ±2.34	28.56 ±0.59	41.65 ±2.57
	sanazole	53.06 ±1.54[a,b]	27.33 ±1.54	19.78 ±2.73
	gemcitabine	25.41 ±1.22	58.49 ±0.63[a,b]	16.25 ±1.21
	gemcitabine + sanazole	20.53±1.17	70.05±1.63	9.54±0.86

[a]p<0.05 vs. control without drug; [b]p<0.05 vs. combination of drugs

1 mM sanazole also exerted a radiosensitising effect in HeLa cells. The MTT assay showed that the presence of sanazole with or without gemcitabine reduced the cell viability significantly.

Cell cycle distribution

After irradiation, sanazole alone led to more cells arrested in the G1 phase. Gemcitabine alone led to more cells arrested in the S phase, and the combination of gemcitabine and sanazole led to more cells arrested in the S phase (Table 2).

Cell survival analysis

Figures 1 and 2 show the cell survival curves of MCF-7 and Hela cells. The radiosensitivity of hypoxic MCF-7 and Hela cells was enhanced by sanazole or gemcitabine. The combination of the two drugs also displayed a radiation enhancing effect at the irradiation doses of 6, 8, and 10 Gy in both cell lines. The radiosensitising effect of 5 μM gemcitabine was higher than that of 1 mM sanazole in both MCF-7 and HeLa cells. The radiosensitivity of HeLa cells was affected more than that of MCF-7 cells by the two drugs used either alone or in combination. The survival rate of non-irradiated cells also decreased, mainly due to the cytotoxicity of gemcitabine.

Discussion

During the past decades, much progress has been made in exploring hypoxic cell radiation sensitising agents [15–18]. A number of radiosensitisers have been invented and applied *in vitro* and *in vivo* [19–21]. Nevertheless, owing to some adverse effects, they are limited in clinical application. Different administration modalities are therefore sought to offer appropriate radiosensitisers for clinical use [22]. Currently, using combinations of different radiation sensitising agents may be a popular approach [23].

This study was conducted to investigate the possibility of combining sanazole and gemcitabine to achieve a radiation enhancing effect in human hypoxic MCF-7 and HeLa cells. MTT assays indicated that both sanazole and gemcitabine had a significant radiosensitising effect in both cell lines. The combination of the two drugs resulted in a more significant radiosensitising effect during the three days after irradiation, with the effect being greatest on the second day.

Flow cytometry analysis demonstrated that the radiation enhancing effect of sanazole or gemcitabine resulted in arrest of MCF-7 cells in the S phase of the cell cycle and HeLa cells in G1. Upon treatment with a combination of sanazole and gemcitabine the proportion of cells in the S phase increased in both cell lines compared to cells

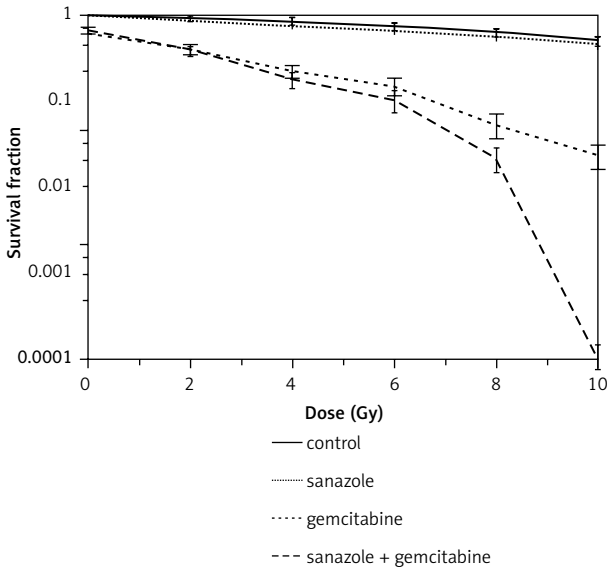

Fig. 1. Colony formation assay: cell survival fraction versus irradiation dose for hypoxic MCF-7 cells with sanazole and/or gemcitabine

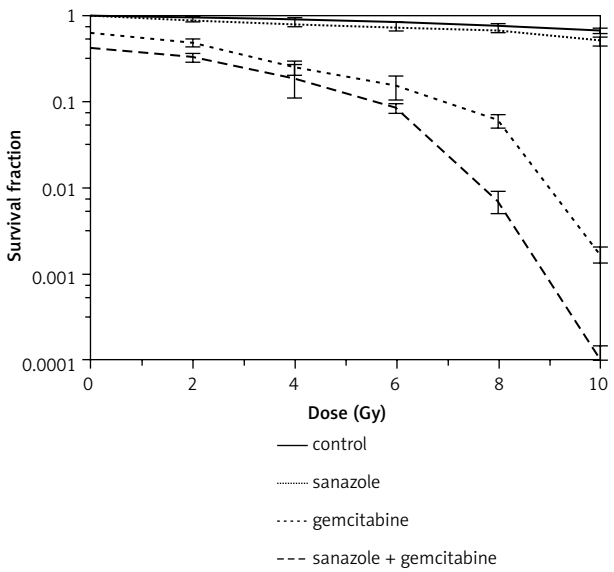

Fig. 2. Colony formation assay: cell survival fraction versus irradiation dose for hypoxic HeLa cells with sanazole and/or gemcitabine

treated with either drug alone. Therefore, it could be hypothesised that before progressing in the S phase, tumour cells were blocked and partially synchronised at the more radiosensitive G1/S boundary. Furthermore, cells progressing past the block might accumulate proapoptotic signals caused by radiation and/or two agents, which would also result in cell death [8, 12, 24].

In order to better understand the radiosensitising effect of the two drugs, cells cultured with sanazole and/or gemcitabine were exposed to different doses of irradiation. The cell survival curves (Figs. 1 and 2) show that: the administration of sanazole and/or gemcitabine led to an enhancement in the fraction of cells becoming clonogenically incompetent; the radiosensitising effect of gemcitabine was more significant than that of sanazole at the doses we administered; and there is a more significant radiation enhancing effect with the combination of the two drugs in human hypoxic MCF-7 and HeLa cells.

The radiation enhancing effects of sanazole plus radiation are low; this is probably due to the use of relatively low radiation doses. It seemed that gemcitabine was more effective than sanazole. The reason for this might be that gemcitabine destroyed tumour cells by its own cytotoxic action and additionally enhanced the effects of radiotherapy.

In conclusion, the co-administration of the two drugs may result in a beneficial gain in radiotherapy for hypoxic breast cancer and cervical cancer. However, there is not yet enough experimental evidence to claim that these drugs are synergistic *in vivo*. The synergistic effects of the two agents, the molecular characteristics of the cells, and further studies *in vivo* require further study. What genetic modification makes the cells more susceptible to radiation are required to exploit the clinical ramifications of the radiation enhancing effects described here. What is more, there are some other questions that need to be explored in future studies, for example: why was the effect greatest on the second day; why were HeLa cells more sensitive to radiation or to the combination; and why did the agents in combination arrest cells in S phase, whereas alone in G1, in HeLa cells.

The authors declare no conflict of interest.

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