Combined antitumor activity of the nitroreductase/CB1954 suicide gene system and γ-rays in HeLa cells in vitro

GELING TENG1,2*, YUANRONG JU1*, YEPENG YANG3, HU HUA2, JINGYU CHI2 and XIUAN MU4

1Department of Respiratory Medicine, Shandong Provincial Hospital Affiliated with Shandong University, Jinan, Shandong 250021; 2Department of Respiratory Medicine, Chest Hospital, Jinan, Shandong 250013; 3Department of Radiation Medicine, Peking University Health Science Centre, Beijing 100191; 4Foreign Language Department, Shandong Medical College, Jinan, Shandong 250002, P.R. China

Received September 24, 2015; Accepted October 11, 2016

DOI: 10.3892/mmr.2016.5917

Abstract. Escherichia coli nitroreductase (NTR) may convert the prodrug CB1954 (5-(aziridin-1-yl)-2,4-dinitrobenzamide) into a bifunctional alkylating agent, which may lead to DNA crosslinks and the apoptosis of cancer cells. NTR/CB1954 has been demonstrated to be an effective gene therapy in cancer cells. The present study examined whether the NTR/CB1954 suicide gene system had cytotoxic effects on HeLa cells and may improve the radiosensitivity of HeLa cells to γ-rays. It was observed that the NTR/CB1954 suicide gene system exerted marked cytotoxic effects on HeLa cells. The combined therapeutic effects of NTR/CB1954 and γ-rays on HeLa cells demonstrated a synergistic effect. CB1954 at concentrations of 12.5 and 25 µmol/l increased the sensitization enhancement ratio of HeLa cells to 1.54 and 1.66, respectively. Therefore, when compared with monotherapy, the combined therapy of NTR/CB1954 and γ-rays may increase the apoptotic rate and enhance the radiosensitivity of HeLa cells. The combined therapy of γ-ray radiation and the NTR/CB1954 suicide gene system may be a novel and potent therapeutic method for the treatment of cervical carcinoma.

Introduction

Gene-directed enzyme-prodrug therapy (GDEPT), or suicide gene therapy, is a promising treatment strategy, which acts by tumor-targeted delivery of an exogenous genes that may express an enzyme capable of converting a non-toxic prodrug into an activated cytotoxic agent, which may then result in apoptosis of tumor cells (1-3). Various suicide gene therapy systems have been previously investigated, including the herpes simplex thymidine kinase/prodrug GCV (HSV1-tk-GCV) and cytosine deaminase and 5-fluorocytosine (CD/5-FC) (4-6).

Nitroreductase/(5-(aziridin-1-yl)-2,4-dinitrobenzamide) (NTR/CB1954) is another GDEPT strategy that has been previously investigated in clinical trials (7). NTR is responsible for the conversion of CB1954, a weak monofunctional alkylating agent, into 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide, a DNA inter-strand cross-linking agent that may trigger apoptosis of cancer cells that express the enzyme NTR. Tumor cells are radiation resistant when they are in the S phase of the cell cycle. Previous studies have confirmed that compared with the HSV1-tk-GCV system, the advantages of the NTR/CB1954 suicide gene system are as follows: i) Independence from the cell cycle; ii) ability to target both dividing and growth-arrested cancer cells; and iii) induction of a potent bystander effect on the cell cycle (8-11).

Radiotherapy has been used due to its efficacy against various tumor types, including head and neck, lung and gastrointestinal tumors. Radiotherapy is usually used alone or in combination with surgery and chemotherapy, and is important for the successful clinical treatment of patients with cancer. However, the modality of this treatment is associated with serious side-effects, including damage to normal tissues, thus requiring restriction of the doses used. A radiosensitizing agent or system may provide the opportunity to circumvent these issues (12,13).

Previous studies have confirmed that gene-directed enzyme prodrug therapy may sensitize tumor cells to the effects of ionizing radiation, and have demonstrated excellent results, including enhanced radiosensitivity of numerous tumor cells in vitro and in vivo (14-17). These observations have promoted the use of cancer gene therapy to enhance the effect of ionizing radiation.

The present study investigated whether the NTR/CB1954 suicide gene system and γ-rays have a combined effect and whether NTR/CB1954 may enhance the cytotoxic effect of γ-rays on cervical carcinoma cells in vitro.

Materials and methods

Materials. Dulbecco's modified Eagle's medium (DMEM) with high glucose and 10% fetal calf serum (FCS) was
purchased from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Reverse transcription-polymerase chain reaction (RT-PCR) assay kits were obtained from Beijing Modern Gold Biotechnology Co., Ltd. (Beijing, China). The endonucleases and ligase enzymes used for molecular cloning technology were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). Lipofectamine 2000, G418, CB1954 and MTT were purchased from Sigma-Aldrich; Merck Millipore (Darmstadt, Germany).

**Plasmid vector construction.** The nfsB sequence was amplified by PCR from the *Escherichia coli* k12 genome using the following primers: 5'-CGGGATCCATGGATATCATTT CTGTCTC-3' and 5'-CGGAATTCCTACCCTCCTGTTAAG GTG-3'. This sequence contained BamHI and EcoRI sites for insertion into the pcDNA3 plasmid. Following endonuclease digestion and DNA sequencing, the successful insertion of the nfsB gene was confirmed and was re-cloned into the pcDNA3 plasmid. The following the following thermocycling conditions: 94°C for 3 min, 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min and 72°C for 5 min, 35 cycles. The resulting plasmid was termed pcDNA3-nfsB.

**Cell culture and transfection.** HeLa cells were maintained in DMEM, supplemented with 10% FCS, 100 U/ml penicillin and 100 mg/ml streptomycin and were incubated at 37°C in a 5% CO₂ atmosphere. All of the cultures were demonstrated to be free of mycoplasma. The HeLa cells were transfected with the pcDNA3 plasmid and pcDNA3-nfsB with G418 selection at a concentration of 400 µg/ml. Following 1 month of selection, several independent clones were selected and the NTR mRNA and protein expression levels were determined. Subsequently, the concentration of G418 was reduced to 200 µg/ml.

RT-PCR and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Total RNA was extracted from the pcDAN3-nfsB-HeLa, pcDNA3-HeLa and non-transfected control HeLa cells using TRIzol reagent. RT was performed according to the manufacturer's protocol (Transgen Biotech Co., Ltd. (Beijing, China). The reaction mixture was placed in a 42°C water bath for 50 min, the temperature was increased to 70°C for 15 min to inactivate Transcript RT and synthesise cDNA. For nfsB, the following primers were used: Sense, 5'-ATGGACGATGTCTGCGTGA-3' and antisense 5'-AAC GCTGATGACCTACCG-3'. Endogenously expressed human β-actin mRNA was used as an internal control with the following primers: Sense 5'-GGCATCTCACCCTGAA TA-3' and antisense 5'-GGGTTGTTGAGGTCTCAA-3'. A DNA product of 208 base pairs (bp) was then amplified. A total of 500 ng cDNA were used. The following the following thermocycling conditions: 94°C for 3 min, 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min and 72°C for 5 min, 35 cycles. The PCR products of nfsB and β-actin were separated by electrophoresis on 1.5% agarose gel. The DNA bands were visualized and analyzed by staining with ethidium bromide. The proteins were collected from the three cell lines and SDS-PAGE was performed with a 10% acrylamide separating gel and 4% acrylamide, 10 µg protein were loaded per lane. The samples were prepared in a Tris-glycine buffer with 1% SDS at pH 8.8. Electrophoresis was conducted at a current of 10 mA for 5 h in electrophoretic Tris-glycine buffer with 0.1% SDS. Following electrophoresis, the gel sheets were stained for proteins with 0.25% coomassie brilliant blue-R250 and then were destained with 10% acetic acid and 20% methanol. Next, the protein bands were visualized and analyzed.

**Cell growth curve and the determination of the cell survival fraction and survival curves following transfection of HeLa cells.** The pcDNA3-nfsB-HeLa, pcDNA3-HeLa and HeLa cells were incubated for 96 h and counted every 12 h. The number of cells was determined using trypan blue staining. Subsequently, a cell growth curve was generated and the differences between the three cell lines were compared. pcDNA3-nfsB-HeLa, pcDNA3-HeLa and HeLa cells of different concentrations were irradiated with different doses of γ-rays. The cells were subsequently incubated for 12 days and the survival fraction of the cells was detected by a colony-formation assay. The cell survival curve was generated using SPSS software, version 15.0 (SPSS, Inc., Chicago, IL, USA) and Microsoft Excel (Microsoft Corporation, Redmond, WA, USA).

Analysis of apoptosis by Hoechst 33258/propidium iodide (PI) fluorescent vital staining. Hoechst 33258/PI fluorescent vital stain was used to quantitatively determine the percentage of apoptotic cells. Briefly, the cells were washed with PBS, and Hoechst 33258 (15 µg/ml) was added. Following a 50 min incubation at ambient temperature, PI (10 µg/ml) was added, followed by another incubation for 10 min. The samples were analyzed using fluorescence microscopy. The nuclei of apoptotic cells appeared bright fluorescent blue, whereas the nuclei of necrotic cells were bright fluorescent red. The nuclei of normal cells appeared only weakly fluorescent blue (Fig. 1). This method was used to analyze the effects of the NTR/CI954 suicide gene system in combination with γ-ray treatment on HeLa cells. Bradford assay (Leagene Biotech Co., Ltd., Beijing, China) was used for quantification.

**Hydroploid HeLa cell formation detected by flow cytometry following NTR/CB1954 treatment.** The three cell lines were plated at a density of 1.0x10⁴ cells/well in a 6-well plate. Following incubation with CB1954 at various concentrations (0, 12.5, 25, 37.5 and 50 µmol/l) for 36 h, the cells were collected, washed twice with ice-cold PBS (pH 7.4), fixed with 70% alcohol for a minimum of 18 h and then stained with PI (50 µg/ml) in the presence of 20 µg/ml RNase A for a minimum of 30 min prior to flow cytometric analysis. The data were analyzed with ModFit version 3.2 (Verity Software House, Inc., Topsham, ME, USA) and CellQuest version 7.5.3 (BD Biosciences, Franklin Lakes, NJ, USA).

**Detection of the combined effect of γ-rays and the NTR/CB1954 suicide gene system via Hoechst 33258/PI fluorescent vital staining.** pcDNA3-nfsB-HeLa cells in the exponential growth phase were incubated for 24 h at 37°C and CB1954 was added at concentrations of 0, 12.5 and 25 µmol/l. After 24 h the cells were irradiated at doses of 0, 2, 4 and 6 Gy with Co⁶⁰ γ-rays. Subsequently, the cells were incubated at 37°C with an atmosphere of 5% CO₂ for 48 h. Detection of apoptosis was performed using Hoechst/PI fluorescent vital staining.
Determination of cell survival via colony-formation assay following treatment with NTR/CB1954 combined with γ-rays. Cells were cultured in 25 cm² flasks. Single suspension was obtained, the cells were diluted to 1x10⁴/ml and 1x10³/ml. Next, 0.2, 0.4, 0.6, 0.8 and 1 ml from the concentration of 1x10³/ml, and a cell suspension of 0.3, 0.8 and 1 ml from the concentration of 1x10⁴/ml, all of which were cultured in 50 cm² flasks, and the number of cells were 200, 400, 600, 800, 1,000, 5,000, 8,000 and 10,000 from low to high. Following the addition of CB1954 at concentrations of 0, 12.5 or 25 µmol/l, the cells were irradiated at doses of 0, 0.5, 1, 1.5, 2, 4, 6 and 8 Gy with Co⁶⁰ γ-rays, according to the number of cells from low to high. Next, the cells were incubated at 37°C with 5% CO₂ atmosphere for 2-15 days. Following this, the medium was removed, the cell culture plate was washed with PBS, and the cells were fixed in methanol for 15 min. The colonies were then stained with a solution of crystal violet for 20 min. Colonies that were visible to the naked eye were counted and the cell survival fraction (SF) was calculated using the following formulas: Plating efficiency (PE) = cell colonies formed / cells inoculated and SF = cell colonies formed / cells inoculated x PE.

Statistical analysis. Differences between treatment groups were determined using Student’s t-test and one-way analysis of variance. Statistical analysis was performed using Statistics Analysis System version 8.0 (SAS Institute, Inc., Cary, NC, USA). Data are presented as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

Results

NTR mRNA expression in HeLa cells. NTR mRNA expression level was determined using RT-PCR. In the pcDNA3-nfsB-HeLa cells, a 381 bp DNA fragment of nfsB was amplified, whereas no mRNA was detected in either the control pcDNA3-HeLa cells or in the non-transfected HeLa cells. A 208 bp human β-actin mRNA fragment, which was used as an internal control, was amplified in the three different cell lines (Fig. 2A). NTR protein expression level was determined using SDS-PAGE. In the pcDNA3-nfsB-HeLa cells, a 24 kDa protein was identified, which coincided with the
TENG et al.: SYNERGISTIC ANTITUMOR ACTIVITY OF NTR/CB1954 AND γ-RAYS IN HeLa CELLS

size of NTR. However, in the control pcDNA3-HeLa cells and in the non-transfected HeLa cells, no corresponding protein band was detected (Fig. 2B). These observations indicated that NTR was stably and correctly expressed in the transfected HeLa cells.

Transfection of the nfsB gene had no effect on cell growth or on the reaction of HeLa cells to γ-ray irradiation. The cell counting method was used following the transfection with the nfsB gene to observe the growth and proliferation of the three groups of cells. No significant differences were observed between the pcDNA3-nfsB-HeLa cells and the control cells (Fig. 3), which indicated that the nfsB gene did not affect the growth and proliferation of HeLa cells. Additionally, the apoptotic activity of the pcDNA3-nfsB-HeLa, pcDNA3-HeLa and non-transfected HeLa cells was examined following γ-ray irradiation (6 Gy) using Hoechst 33258/PI fluorescent vital staining. No significant differences were observed among the three groups of cells in terms of apoptotic rate over time (0-72 h) (Fig. 4A). The cell survival fraction in the three groups of cells was also quantified. Following treatment with various doses of γ-ray irradiation, the cells were incubated for 12 days. No significant differences were identified among the three groups of cells in terms of the cell survival fraction (Fig. 4B). These observations demonstrated that transfection with the nfsB gene alone did not have an influence on the reaction of the cells to γ-rays.

CB1954 increased the cytotoxicity in NTR-expressing cells and led to an extended S phase. The rate of apoptosis of pcDNA3-nfsB-HeLa, pcDNA3-HeLa and HeLa cells treated with CB1954 was determined using Hoechst 33258/PI fluorescent staining. Apoptosis was observed in the pcDNA3-nfsB-HeLa cells 12 h following treatment with CB1954 (Fig. 5A). No apoptosis was observed in the pcDNA3-HeLa cells or in the wild-type HeLa cells. The rate of apoptosis of the pcDNA3-nfsB-HeLa cells peaked at 36 h of treatment (Fig. 5A). Additionally, an MTT assay and flow cytometry were used to determine cell viability and apoptotic rates, with the observations consistent with the previous experiments (data not shown). The pcDNA3-nfsB-HeLa, pcDNA3-HeLa and non-transfected HeLa cells were treated with various concentrations of CB1954 (0, 12.5, 25 and 50 µmol/l) for 36 h. Flow cytometry was then used to analyze the number of cells in the different cell cycle phases, and the results demonstrated that the NTR/CB1954 suicide...
gene system primarily affected HeLa cells in S phase. When the concentration of CB1954 was 50 µmol/l, the percentage of cells in S phase was 70.51%, whereas no clear alterations were observed in the control cell groups, the pcDNA3-HeLa and HeLa cells (Fig. 5B).

CB1954 combined with γ-ray irradiation increased cytotoxicity and reduced the cell survival fraction in pcDNA-nfsB-HeLa cells. pcDNA-nfsB-HeLa cells were treated with CB1954 at various concentrations (0, 12.5 and 25 µmol/l) for 16 h prior to γ-ray irradiation (0, 2, 4 and 6 Gy), and the rate of apoptosis was determined using Hoechst 33258/PI fluorescent vital staining. The cytotoxicity was proportional to the radiation dose in pcDNA-nfsB-HeLa cells and the CB1954 concentration used. Cytotoxicity increased with the combination of radiation and CB1954 at 12.5 and 25 µmol/l. For example, when the concentration of CB1954 was 12.5 µmol/l, the apoptotic rate of pcDNA3-nfsB-HeLa cells was 12.92% and at a dose of γ-ray irradiation of 6 Gy, the rate of apoptosis was 12.84%. However, with the combination of 12.5 µmol/l of CB1954 and 6 Gy of γ-ray irradiation, the rate of apoptosis rate increased to 39.9%, which was increased compared with irradiation or CB1954 alone for pcDNA3-nfsB-HeLa cells (Fig. 6A). These observations demonstrated that the cytotoxic effect on HeLa cells was due to the interaction between the suicide gene system NTR/CB1954 and radiation, and was not a simple additive effect. In addition, the present study determined the cell survival fraction with a colony-formation assay. The cells were treated with CB1954 at various concentrations (0, 12.5 and 25 µmol/l) for 16 h prior to the delivery of various doses of γ-rays (0, 0.5, 1, 1.5, 2, 4, 6 and 8 Gy). Next, the cell survival fraction was then determined using a colony-formation assay. Following treatment with 12.5 and 25 µmol/l of CB1954, reduced cell survival fraction was evident with the combination of radiation and CB1954. SER was obtained from the cell survival curve. At concentrations of 12.5 and 25 µmol/l CB1954 radiosensitivity ratios were 1.54 and 1.66, respectively (Fig. 6B). These results indicated that the NTR/CB1954 suicide gene system may significantly enhance the sensitivity of HeLa cells to radiation.

Discussion

The GDEPT treatment approach may sensitize tumor cells to ionizing radiation. Previous studies determined that the HSV1-tk-GCV and CD5-FC suicide gene therapy systems may improve the sensitivity of tumor cells to radiotherapy, and these systems have demonstrated considerable advantages both in vitro and in vivo (5,18). The NTR/CB1954 gene therapy system is another form of GDEPT and demonstrated an effective tumor reducing treatment on various cells in vitro (19,20). The NTR/CB1954 system has been previously investigated in clinical trials. The present study aimed to investigate this system and determine whether it may be used as a potential radiosensitizing gene therapy.

The present study cloned the nfsB gene from the E. coli K12 genome using PCR. This gene was then cloned into the eukaryotic expression vector pcDNA3 to obtain the pcDNA3-nfsB vector. Following the transfection of HeLa cells, RT-PCR and SDS-PAGE were performed to determined that the DNA fragment and protein size were consistent with those of the nfsB gene and NTR. The transfection was deemed successful as the fragment was missing in the control groups (HeLa and pcDNA3-HeLa cells). The present study demonstrated that the cloned nfsB gene was correct and that the eukaryotic expression vector pcDNA3-nfsB was stable and functional, which laid a foundation for the observation of the effects of NTR/CB1954 suicide gene therapy in the subsequent experiments.

The growth and proliferation of HeLa cells following the transfection were determined. No significant differences were identified between the pcDNA3-nfsB-HeLa cells and the control groups (pcDNA3-HeLa and HeLa cells). Therefore, the nfsB gene itself did not affect the cytotoxicity of NTR/CB1954 and γ-rays. Following the transfection, the rate of apoptosis of the three groups of HeLa cells following γ-ray irradiation was quantified and it was confirmed that transfection of the nfsB gene did not affect the fraction of surviving cells. Therefore, these observations indicated that the HeLa cell line and the nfsB gene were suitable for the present experiments, ensuring the potential for further cytotoxicity research.
The cytotoxic effects of NTR/CB1954 were also evaluated and it was confirmed that the NTR/CB1954 suicide gene system exerted a specific cytotoxic effect on HeLa cells. CB1954 exerted selective cytotoxicity against pcDNA3-nsfB-HeLa cells compared with the controls (pcDNA3-HeLa cells and HeLa cells). The present study determined that the mechanism of cytotoxicity was primarily via apoptosis; however, the peak effect of cytotoxicity was observed after 36 h, which was sooner compared with other suicide gene systems, such as the HSV/TK suicide gene therapy system (21). Cell cycle analysis of pcDNA3-HeLa cells was performed following treatment with CB1954. The results indicated that the NTR/CB1954 suicide gene system led to S-phase arrest. This was not in agreement with a previous study performed by White et al (18). White et al (18) concluded that the NTR/CB1954 gene therapy system acted independently of the cell cycle, which was the greatest advantage of this system compared with other types of GDEPT (18). However, following multiple replications of the experiments in the present study, the results were still in contradiction with that of White et al (18). This may be due to the cell line that was used; therefore, it may be useful for future studies to use different cell lines to verify this.

The present study examined the combined effect of the NTR-CB1954 gene system and γ-ray irradiation in pcDNA3-nsfB-HeLa cells. Increased cell apoptosis was observed when CB1954 treatment was combined with γ-ray radiation in pcDNA3-nsfB-HeLa cells. Subsequent experiments were performed to demonstrate the reduced level of surviving cells in the pcDNA3-nsfB-HeLa cell group that received CB1954 and γ-ray radiation compared with cells that only received CB1954 or γ-ray radiation. Based on the survival curve, the SER was 1.54 and 1.56 when the concentrations of CB1954 were 12.5 and 25 µmol/l. Therefore, this indicated that NTR/CB1954 may enhance the radiosensitivity of HeLa cells and that this was due to a synergistic effect as opposed to an additive effect.

The current study demonstrated that the NTR/CB1954 suicide gene system led to S phase arrest in HeLa cells. As cells in S phase are resistant to radiation, the S phase arrest may be the result of DNA synthesis inhibition, which was not reduced when a combination of NTR/CB1954 and γ-ray radiation was used. However, the result of this combination was cooperative. This may be due to the fact that the cells were treated when they were in the S phase, and they became damaged by NTR/CB1954, which resulted in S phase arrest. It is possible that the cells remained in the S phase in order to undergo repairs of either lethal or sublethal damage as the purpose was to enter the next phase of the cell cycle to enable the cells to proliferate and grow. However, at this time point, as the cells were exposed to γ-ray radiation they became lethally injured whilst in the S phase. Therefore, S-phase arrest may be due to the combined effect of the NTR/CB1954 and the γ-ray radiation. However, additional studies are required to test this hypothesis.

The use of suicide genes in combination with radiation therapy is currently underway and whilst progress has been achieved, it may take a considerable amount of time for this to become a widely used treatment. Efforts should be focused on the identification of a potential means for the development of strategies that aim to use GDEPT to enhance radiotherapy in clinical treatments.

Acknowledgements

The present study was supported by the Shandong Province Science and Technology Development Projects (grant nos. 2011GGH21820 and 2011GGH21837).

References

1. Carruthers KH, Metzger D, During MJ, Muravlev A, Wang C and Kochak E: Gene-directed enzyme prodrug therapy for localized chemotherapeutics in allograft and xenograft tumor models. Cancer Gene Ther 21: 434–440, 2014.
2. Zawilska JB, Wojcieszak J and Olejniczak AB: Prodrugs: A challenge for the drug development. Pharmacol Rep 65: 1-14, 2013.
3. Both GW: Gene-directed enzyme prodrug therapy for cancer. A glimpse into the future? Discov Med 8: 97-103, 2009.
4. Huang Q, Liu XZ, Kang CS, Wang GX, Zhang Y and Pu PY: The anti-glioma effect of suicide gene therapy using BMSC expressing HSV/TK combined with overexpression of Cx43 in glioma cells. Cancer Gene Ther 17: 192-202, 2010.
5. Takahashi M, Valdes G, Hiraoka I, Inagaki A, Kamijima S, Micewicz E, Gruber HE, Robbins JM, Jolly DJ, McBride WH et al: Radio sensitization of gliomas by intra cellular generation of S-fluorouracil potentiates prodrug activator gene therapy with a retroviral replicating vector. Cancer Gene Ther 21: 405-410, 2014.
6. Wester HJ: Nuclear imaging probes: From bench to bedside. Clin Cancer Res 13: 6339-481, 2007.
7. Patel P, Young JG, Mautner V, Ashdown D, Bonney S, Pineda RG, Collins SI, Searle PF, Hull D, Peers E, et al: A phase I/II clinical trial in localized prostate cancer of an adenovirus expressing nitroreductase with CB1954 [correction of CB1984]. Mol Ther 17: 1292-1299, 2009.
8. Bhaumik S, Sekar TV, Depuy I, Khimj J and Paulmurugan R: Noninvasive optical imaging of nitroreductase gene-directed enzyme prodrug therapy system in living animals. Gene Ther 19: 295-302, 2012.
9. Dachs GU, Hunt MA, Syddall S, Singleton DC and Patterson AV: Bystander or no bystander for gene directed enzyme prodrug therapy. Molecules 14: 4517-4545, 2009.
10. Palmer DH, Milner AE, Kerr DJ and Young LS: Mechanism of cell death induced by the novel enzyme-prodrug combination, nitroreductase/CB1954, and identification of synergism with S-fluorouracil. Br J Cancer 89: 944-950, 2003.
11. Anlezark GM, Vaughan T, Fashola-Stone E, Michael NP, Murdoch H, Sims MA, Stubbs S, Wigley S and Minton NP: Bacillus amyloliquefaciens orthologue of Bacillus subtilis nfsB may take a considerable amount of time for this challenge for the drug development of pcDNA3-HeLa cells and that this was due to a synergistic effect as opposed to an additive effect.

The current study demonstrated that the NTR/CB1954 suicide gene system led to S phase arrest in HeLa cells. As cells in S phase are resistant to radiation, the S phase arrest may be the result of DNA synthesis inhibition, which was not reduced when a combination of NTR/CB1954 and γ-ray radiation was used. However, the result of this combination was cooperative. This may be due to the fact that the cells were treated when they were in the S phase, and they became damaged by NTR/CB1954, which resulted in S phase arrest. It is possible that the cells remained in the S phase in order to undergo repairs of either lethal or sublethal damage as the purpose was to enter the next phase of the cell cycle to enable the cells to proliferate and grow. However, at this time point, as the cells were exposed to γ-ray radiation they became lethally injured whilst in the S phase. Therefore, S-phase arrest may be due to the combined effect of the NTR/CB1954 and the γ-ray radiation. However, additional studies are required to test this hypothesis.

The use of suicide genes in combination with radiation therapy is currently underway and whilst progress has been achieved, it may take a considerable amount of time for this to become a widely used treatment. Efforts should be focused on the identification of a potential means for the development of strategies that aim to use GDEPT to enhance radiotherapy in clinical treatments.

Acknowledgements

The present study was supported by the Shandong Province Science and Technology Development Projects (grant nos. 2011GGH21820 and 2011GGH21837).

References

1. Carruthers KH, Metzger D, During MJ, Muravlev A, Wang C and Kochak E: Gene-directed enzyme prodrug therapy for localized chemotherapeutics in allograft and xenograft tumor models. Cancer Gene Ther 21: 434–440, 2014.
2. Zawilska JB, Wojcieszak J and Olejniczak AB: Prodrugs: A challenge for the drug development. Pharmacol Rep 65: 1-14, 2013.
3. Both GW: Gene-directed enzyme prodrug therapy for cancer. A glimpse into the future? Discov Med 8: 97-103, 2009.
4. Huang Q, Liu XZ, Kang CS, Wang GX, Zhang Y and Pu PY: The anti-glioma effect of suicide gene therapy using BMSC expressing HSV/TK combined with overexpression of Cx43 in glioma cells. Cancer Gene Ther 17: 192-202, 2010.
5. Takahashi M, Valdes G, Hiraoka I, Inagaki A, Kamijima S, Micewicz E, Gruber HE, Robbins JM, Jolly DJ, McBride WH et al: Radiosensitization of gliomas by intra cellular generation of S-fluorouracil potentiates prodrug activator gene therapy with a retroviral replicating vector. Cancer Gene Ther 21: 405-410, 2014.
6. Wester HJ: Nuclear imaging probes: From bench to bedside. Clin Cancer Res 13: 6339-481, 2007.
7. Patel P, Young JG, Mautner V, Ashdown D, Bonney S, Pineda RG, Collins SI, Searle PF, Hull D, Peers E, et al: A phase I/II clinical trial in localized prostate cancer of an adenovirus expressing nitroreductase with CB1954 [correction of CB1984]. Mol Ther 17: 1292-1299, 2009.
8. Bhaumik S, Sekar TV, Depuy I, Khimj J and Paulmurugan R: Noninvasive optical imaging of nitroreductase gene-directed enzyme prodrug therapy system in living animals. Gene Ther 19: 295-302, 2012.
9. Dachs GU, Hunt MA, Syddall S, Singleton DC and Patterson AV: Bystander or no bystander for gene directed enzyme prodrug therapy. Molecules 14: 4517-4545, 2009.
10. Palmer DH, Milner AE, Kerr DJ and Young LS: Mechanism of cell death induced by the novel enzyme-prodrug combination, nitroreductase/CB1954, and identification of synergism with S-fluorouracil. Br J Cancer 89: 944-950, 2003.
11. Anlezark GM, Vaughan T, Fashola-Stone E, Michael NP, Murdoch H, Sims MA, Stubbs S, Wigley S and Minton NP: Bacillus amyloliquefaciens orthologue of Bacillus subtilis nfsB may take a considerable amount of time for this
18. White CL, Menghistu T, Tigger KR, Searle PF, Bhide SA, Vile RG, Melcher AA, Pandha HS and Harrington KJ: *Escherichia coli* nitroreductase plus CB1954 enhances the effect of radiotherapy in vitro and in vivo. Gene Ther 15: 424-433, 2008.

19. Cobb LM, Connors TA, Elson LA, Khan AH, Mitchley BC, Ross WC and Whisson ME: 2,4-Dinitro-5-ethyleneiminobenzamide (CB1954): A potent and selective inhibitor of growth of the Walker carcinoma 256. Biochem Pharmacol 18: 1519-1527, 1969.

20. Chung-Faye G, Palmer D, Anderson D, Clark J, Downes M, Baddeley J, Hussain S, Murray P, Searle P, Seymour L, et al: Virus-directed, enzyme prodrug therapy with nitroimidazole reductase: A phase I and pharmacokinetic study of its prodrug. CB1954. Clin Cancer Res 7: 2662-2668, 2001.

21. Sekar TV, Foygel K, Ilovich O and Paulmurugan R: Noninvasive theranostic imaging of HSV1-sr39TK-NTR/GCV-CB1954 dual-prodrug therapy in metastatic lung lesions of MDA-MB-231 triple negative breast cancer in mice. Theranostics 4: 460-474, 2014.