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Enhancement of radiotherapeutic effectiveness by temperature-sensitive liposomal 1-methylxanthine

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ABSTRACT

Most of methylxanthine derivatives including caffeine have been known to radiosensitize cancer cells, but the obstacles such as toxicity, request of high dose and poor solubility hinder their preclinical evaluations and clinical applications. In this study, we evaluated the efficacy of 1-methylxanthine (1-MTX), a caffeine metabolite as a radiosensitizer and the *in vivo* effectiveness of the temperature-sensitive liposomal 1-methylxanthine (tsl-MTX) in combination with ionizing radiation and regional hyperthermia. In human colorectal and lung cancer cells, treatment of 1-MTX sensitized cells to ionizing radiation. To evaluate the *in vivo* capability of 1-MTX to radiosensitize tumors, we developed temperature-sensitive liposomal 1-MTX using DPPC:DMPC:DSPC (4:1:1 molar ratio) with intention of overcoming lethal toxicity of 1-MTX and controlling drug-release. The particle size of the liposomes was approximately 200 nm in diameter. The release of 1-MTX from the liposomes was responding to increase of temperature. In xenograft tumor-bearing mice, the tsl-MTX administered using the *i.p.* route showed delay of tumor growth. Importantly, tsl-MTX in combination with radiation and regional hyperthermia exhibited marked delay of tumor growth, suggesting that 1-MTX effectively enhanced radiation-induced suppression of tumor growth. In conclusion, tsl-MTX has highly efficacious anticancer competence *in vivo*, enhancing radiotherapeutic effectiveness, and feasibility for further clinical applications.

1. Introduction

The advance in nanomedicine that consists of effective anticancer drugs and a tumor-targeting carrier may bring new hope for cancer therapy. Although many anticancer drugs show pharmacological effectiveness in cancer treatment, they are limited in their preclinical evaluations and clinical translations because of their serious toxicity and poor aqueous solubility. Not only to overcome these difficulties but also to accomplish the successful clinical translation, researchers have emphasized the development of nano-sized anticancer drug carriers which improve therapeutic efficacy and also reduce unwanted side effects (Duncan, 2003; Kopecek, 2003). Among these carriers, temperature-sensitive liposomes have been developed in cancer chemotherapy to increase the release efficiency of the anticancer drug at tumor sites in combination with hyperthermia and to reduce the severities of the side effects associated with the drug (Kono et al., 1999; Needham and Dewhirst, 2001).

In response to various genotoxic stresses, cells activates DNA damage checkpoint to protect their genomic integrity (Sancar et al., 2004). Following exposure of ionizing radiation, DNA double strand breaks (DSBs) occur and cells undergo G2/M cell cycle arrest to block replication and segregation of damaged chromosomes, allowing time for DNA repair (Iliakis et al., 2003). DSBs are recognized by protein complex that contains both signaling kinases and repair activity. The most important kinases are ataxia telangiectasia mutated (ATM) and ataxia telangiectasia mutated and Rad3 related (ATR) protein, although G2/M checkpoint induced by DSBs

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is more dependent on ATM (Ismail et al., 2005). The ATM kinase activity is rapidly triggered by irradiation phosphorylates various downstream substrates including NBS1, p53, the E3 ubiquitin ligase MDM2, checkpoint kinase 1 (Chk1) and Chk2, histone 2AX (H2AX), and BRCA1 (Ahn et al., 2000; Cortez et al., 1999; Fernandez-Capetillo et al., 2002; Lavin et al., 2005; Matsuoka et al., 1998). The activated Chk1 and Chk2 through phosphorylation by ATM inhibit the entry into mitosis by downregulation of Cdc25 phosphatase (Matsuoka et al., 1998) and subsequently Cdc2 kinase, resulting in G2 phase arrest (Blasina et al., 1997).

Methylxanthine derivatives including caffeine, pentoxifylline, and staurosporine derivative, 7-hydroxystaurosporine (UCN-01) have been demonstrated to increase radiosensitivity of cells by inhibiting G2 checkpoint activation (Jha et al., 2002; Sarkaria et al., 1999; Teicher et al., 1991; Wang et al., 1996). Caffeine had been initially thought to inhibit DNA repair, but it was found that caffeine abrogated G2 arrest, and drove cells through a lethal mitosis (Lau and Pardee, 1982). The ATM kinase was finally identified as a molecular target for caffeine, as well as the AT-related homolog ATR for abrogating G2 arrest and radiosensitization (Choi et al., 2006). The inhibition of ATM kinase activity by caffeine leads to inhibition of Chk1/Chk2 kinase activity and activation of Cdc2 kinase, consequently abrogating G2 arrest. Increased cytotoxicity by caffeine-induced abrogation of G2 arrest is resulted from an inadequate time to repair the damaged DNA prior to mitosis. Additionally, caffeine-induced radiosensitization is mediated by inhibition of DNA double strand break repair requiring homologous recombination repair (HRR) (Asaad et al., 2000; Wang et al., 2004). These observations imply that the radiosensitization induced by caffeine is the combined result of G2 checkpoint abrogation and direct inhibition of DNA repair. However, caffeine was unfortunately not a possible clinical drug because patients cannot bear taking the millimolar concentrations required for the effect. Other checkpoint inhibitor, staurosporine was identified (Tam and Schlegel, 1992), but it was too toxic for administration to humans. UCN-01, a protein kinase C (PKC) inhibitor, was developed as a more potent inhibitor than caffeine at abrogating cell cycle arrest in human cells (Bunch and Eastman, 1996). The molecular target for UCN-01 was finally identified as Chk1, while the target for caffeine was ATM and the AT-related homolog ATR (Busby et al., 2000; Graves et al., 2000). The necessary concentration of UCN-01 was well tolerated in murine tumor models where UCN-01 enhanced the activity of DNA-damaging agents (Akinaga et al., 1993). UCN-01 was, however, found to bind avidly to α1 acid glycoprotein in human plasma, leading to plasma concentrations in excess of 30 μM (Fuse et al., 1998; Sausville et al., 2001), while only 10 nM of UCN-01 is required to abrogate cell cycle arrest (Kohn et al., 2002). Although the clinical trial of UCN-01 in combination with DNA-damaging agents has been continued, it has been thought to be difficult to obtain an applicable amount of free drug without threatening patient safety. Therefore, it is necessary to continue the search for better inhibitors abrogating cell cycle checkpoint.

In the present study, the efficacy of 1-MTX as a radiosensitizer and the effectiveness of the temperature-sensitive liposomal 1-methylxanthine (tsl-MTX) in combination with ionizing radiation and regional hyperthermia were evaluated. 1-MTX sensitized cancer cells to ionizing radiation. The severe toxicity of 1-MTX *in vivo* caused by NaOH that is a sole solvent for 1-MTX was overcome by encapsulation of 1-MTX in the liposome. Furthermore, the tsl-MTX was designed for not only passive targeting to tumor by EPR effect, but also the accurate release of the drug by hyperthermia. By administration of tsl-MTX, it became evident that 1-MTX encapsulated in temperature-sensitive liposome has a potent capability to radiosensitize xenograft tumors and attenuate the tumor growth rate *in vivo*.

2. Materials and methods

2.1. Cells and treatment

RKO human colorectal cancer cells and A549 human lung cancer cells obtained from the American Type Culture Collection (ATCC) were maintained in tissue culture flasks at 37 °C with Dulbecco's modified Eagle's medium (DMEM, Hyclone) supplemented with 10% fetal bovine serum (Hyclone) and 1% penicillin/streptomycin (Invitrogen) in a humidified 5% CO_2 incubator. Cells were used for the experiments when they were in exponential growth phase. 1-MTX purchased from Sigma Chemical Co. was dissolved at a concentration of 300 mM in 0.1N NaOH. The culture media was supplemented with 25 mM HEPES to minimize the change of pH when drugs were added. 1-MTX was added to the cell culture 30 min prior to radiation. Cells were irradiated with ^{137}Cs irradiator at 5 Gy/min (CIS Bio International), and then incubated for the designated time.

2.2. Clonogenic survival assay

Cells were plated in 25T tissue culture flasks, incubated overnight, and then irradiated in the presence or absence of 1-MTX. Cells were further maintained in the drug solution for 72 h, and then cultured in fresh complete medium for 14 days. After staining with 0.4% crystal violet (Sigma Chemical Co.), the colonies containing more than 50 cells were counted and the surviving fractions were calculated.

2.3. Western blot analysis

Cells were harvested by trypsinization and lysed in solubilizing buffer as we previously described (Choi et al., 2004; Park et al., 2003). Aliquots of 30 μg proteins were separated by 10% SDS-PAGE and blotted onto PVDF membrane (GE Healthcare) in transfer buffer. Blots were blocked with 5% non-fat dry milk in 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20 (TBST), incubated with primary antibodies, and then treated with horseradish peroxidase-conjugated secondary antibody. Immunoreactive bands were visualized using ECL Western Blotting Detection Reagents (GE Healthcare). Equal loading of sample was confirmed with anti-actin. Primary antibodies used in this study were anti-caspase-3 (Cell signaling Technology), anti-PARP (BD Biosciences) and anti-actin (Calbiochem).

2.4. Preparation of temperature-sensitive liposomal 1-MTX

Liposomes were prepared by a reverse-phase evaporation (REV) method (Szoka and Papahadjopoulos, 1978) as recently reported (Lim et al., 2007). Briefly, DPPC:DMPC:DSPC at a molar ratio of 4:1:1 (10 mM total) was dissolved in 1 ml chloroform. After evaporation of solvent, dry lipid film was suspended in 1 ml of freshly hydrated diethyl ether and 1 ml solution of 1-methylxanthine. After sonicating the mixtures for 3 min, the organic solvent was removed by rotary evaporation. Free (unencapsulated) drug was separated out by gel chromatography on a 1 cm × 12 cm Sephadex® G-75 column eluted with PBS. Liposomes were downsized by extrusion through 0.2 μm polycarbonate membranes 10–20 times using a Lipex™ extrusion device (Avestin Inc.). PEGylation for long-circulation was done as reported (Lim et al., 2007). 1,2-Dipalmitoyl-rac-glycero-3-phosphocholine (DPPC), 1,2-dimylistoyl-rac-glycero-3-phosphocholine (DMPC), and D,1-α-phosphatidylcholine, distearoyl (DSPC) were perchased from Sigma Chemical Co.

2.5. Scanning electron microscope (SEM) and size distribution analysis

After lyophilization, liposomes were coated with palladium and the surface was examined under JSM-5410 Scanning Microscope (JEOL, Japan). The size distribution of liposomes was determined using dynamic laser-light scattering using a laser particle analyzing system (Particle Sizing Systems, Inc., Santa Barbara, CA) at 23 °C with a He–Ne laser light source (at 632.8 nm).

2.6. Release of 1-MTX from tsl-MTX

1-MTX was loaded into the temperature-sensitive liposomes (DPPC:DMPC:DSPC at the molar ratio of 4:1:1) or non-temperature-sensitive liposomes (DPPC:DSPC at the molar ratio of 1:2) prepared by REV method. 0.1 ml of tsl-MTX or liposome-MTX were mixed with 0.9 ml of phosphate-buffered saline in each tube, and incubated at 25, 35, 37, 39, 41, 43, and 45 °C for 10 min using a heating block. The absorbance was determined using UV–vis spectrophotometer. The absorbance was determined again after heating at 60 °C for 10 min. The release (%) was calculated by the followed formula.

$$\text{Release}\,(\%) = ((F_T - F_{25})/(F_{60} - F_{25})) \times 100$$

where F_T is the absorbance after incubation at specific temperature, F_{25} is the absorbance after incubation at 25 °C and F_{60} is the absorbance after incubation at 60 °C.

2.7. Tumor xenograft studies in nude mice

RKO or A549 cells in exponential growth phase in culture were harvested, and then 4×10^5 cells suspended in serum free medium were injected subcutaneously into the right hind leg of BALB/c-nuscle nude mice weighing about 20 g. When the tumors grew to 120–130 mm^3, the host mice were randomized and divided into the experimental groups ($n = 10$ per group). The mice were injected intraperitoneally or not injected with 100 mg/kg of 1-MTX encapsulated in temperature-sensitive liposome (tsl-MTX). 1 h later the tumors of the mice injected with tsl-MTX were heated in 42 °C water-bath for 1 h. The tumors were irradiated or not irradiated with 5 Gy using 6MV photon beam linear accelerator (CL/1800, Varian, CA, USA). A customized photon beam shielding block was used to irradiate the right hind leg bearing the tumor only. The 3 perpendicular tumor diameters were measured with a caliper every 2 or 3 days and the tumor volume was calculated from the measured length (R_1), width (R_2), and height (R_3) on the assumption that the tumors were hemi-ellipsoids using following formula; $V = (4\pi/3) \times (R_1/2) \times (R_2/2) \times (R_3/2)$. At the end of experimental period, the tumors were isolated and the photograph was taken. For the test of tolerance against soluble 1-MTX dissolved in 0.1N NaOH, mice were *i.p.* injected with 100 mg/kg soluble 1-MTX. All experiment was done following the protocol approved by The University of Ulsan Animal Use Committee.

2.8. Immunohistochemical analysis

Tumor tissues were obtained from A549 xenograft mice after 2 days of treatment. Formalin-fixed, paraffin-embedded tumor blocks were sectioned to 5 μm, mounted onto lysine-coated glass slides, deparaffinized with xylene, and rehydrated through graded alcohols, and then incubated with protease-K for 10 min in a 40 °C humidity chamber. Endogenous peroxidase was blocked by 3% H_2O_2 for 15 min. Tissues were incubated overnight at 4 °C in humidity chamber with antibodies to phospho-histone H3 (Upstate Biotechnology), cleaved caspase-3 (Cell Signaling Technology) and DcR2 (Stressgen Bioreagents). For TUNEL staining, tissues were incubated with terminal transferase and Biotin-16-dUTP (Roche Applied Science). The detection was performed with ChemMate DAKO Envision (DAKO Corporation) and 3,3′-diaminobenzidine (DAKO Corporation). The slides were counterstained with Harris hematoxylin, dehydrated in graded alcohols, and covered with a cover slip.

3. Results

3.1. 1-MTX sensitizes cells to ionizing radiation

To examine the effect of 1-MTX on radiation-induced cytotoxicity, RKO and A549 cells were treated with different doses of radiation in the presence or absence of 1-MTX, and then the clonogenic survival rates of cells were measured. As shown in Fig. 1A, the survival rate of RKO was decreased by radiation alone in a dose-dependent manner. Compared to the survival rate decreased by radiation, additional treatment with 1-MTX displayed more decreases in the survival rate which is gradually aggravated by increasing dose of radiation and 1-MTX. In A549 cells, similar pattern of decrease in the survival rate was observed by treatment with 1-MTX (Fig. 1B). The effect of 1-MTX without radiation on the survival rate was also determined (Fig. 1C). The survival rate of RKO cells was very slightly affected by 1-MTX alone, suggesting that 1-MTX is quite less toxic in this condition. Although 1-MTX displayed more toxicity in A549 cells than in RKO cells, it was quite less than the toxicity of combined treatment of 1-MTX and radiation. To explore the effect of 1-MTX in a short period, RKO cells were treated with 4 Gy radiation alone or in a combination of 3 mM 1-MTX and the cleavage of caspase-3 and PARP, the markers of apoptosis induction, was determined at 24 h and 48 h. Caspase-3 and PARP in the cells treated with radiation alone were remained as a form of uncleaved till 48 h (Fig. 1D), suggesting that the induction of apoptosis is not sufficient. In contrast, both proteins were completely cleaved at 48 h in the cells irradiated in the presence of 1-MTX. These results indicate that 1-MTX is a potent radiosensitizer enhancing the killing effect of radiation in cancer cells.

3.2. Preparation and characterization of temperature-sensitive liposomes

Since we ascertained that 1-MTX has a property to radiosensitize cancer cells, we addressed to test whether 1-MTX exerts an anti-tumor activity *in vivo*. On a trial test for the tolerance, mouse immediately died after *i.p.* injection with a minimal dose of 1-MTX dissolved in 0.1N NaOH. The lethality happened in the mouse was accompanied with having swollen stomach and their nails and claws turned to red. The same lethality was observed in the mouse injected with the equal volume of 0.1N NaOH without 1-MTX, indicating that the death was due to the 0.1N NaOH, only in which 1-MTX is dissolved. Hence, we designed to envelop the 1-MTX in a drug delivery carrier to overcome the lethality of it. 1-MTX was encapsulated in temperature-sensitive liposome (tsl-MTX) that is responding to increasing temperature, and the problem of lethality was completely overcome with this tsl-1-MTX. Another advantage that we aimed to get with the tsl-MTX was to control the release of drug only in a specific site. Although it was expected that the liposomal drug would passively targeted to tumor site by EPR effect, we designed a strategy of hyperthermia to increase the accuracy of drug-release only in the tumor site, but not in other unwanted sites. After preparation of tsl-MTX, we characterized the tsl-MTX for micrographic surface, size and *in vitro* drug release. Fig. 2A shows the typical surface morphology of the tsl-MTX that was observed under scanning electron microscope. The distribution of particle size determined by dynamic

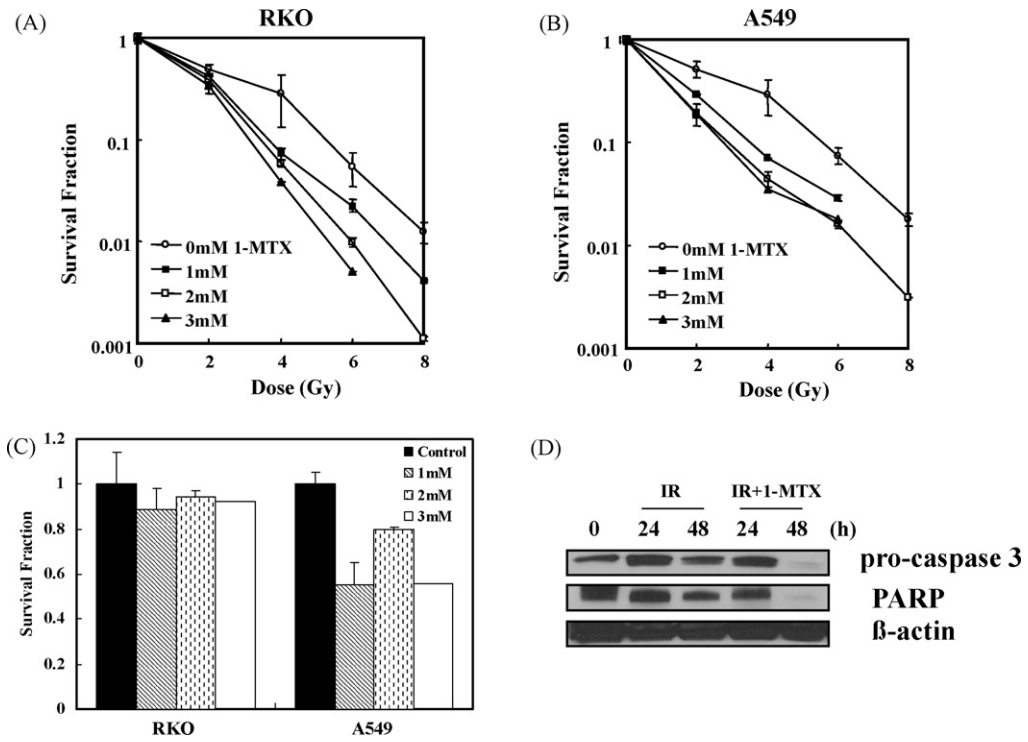

Fig. 1. 1-MTX sensitizes cells to ionizing radiation. Clonogenic surviving fraction of RKO (A) or A549 cells (B). Cells were irradiated in a gradually increased dose in the presence or absence of 1, 2, 3 mM of 1-MTX, and cultured in a 5% CO_2 incubator for 14 days. The numbers of colonies formed with more than 50 cells were counted, and the average survival (%) was calculated. The values of all concentrations at 0 Gy were normalized. The results are expressed as means ± S.D. for six independent experiments. (C) Effect of 1-MTX on clonogenic survival of RKO or A549 cells. Cells were treated with 1, 2, 3 mM of 1-MTX without radiation. (D) Western blot analysis against caspase-3 and PARP. Total cell lysates were prepared at 0, 24, 48 h after the treatment, and aliquots of 30 μg protein were subjected to western blot analysis against caspase-3 and PARP. Equal sample loading was confirmed with anti-actin.

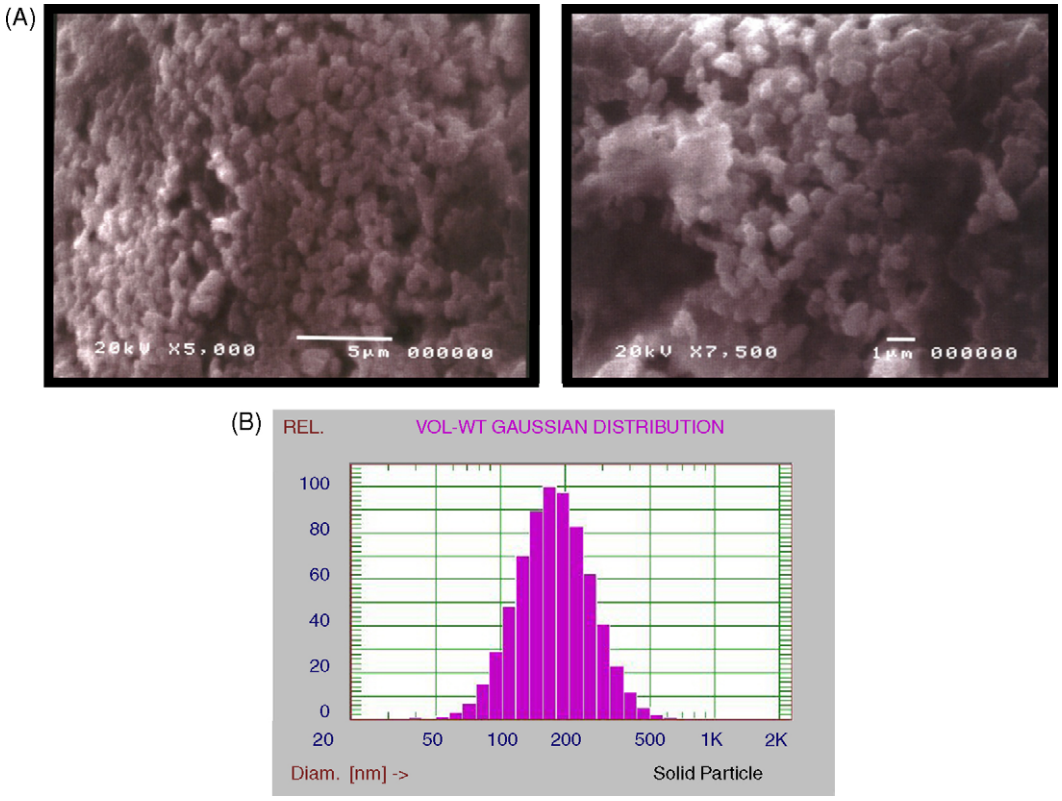

Fig. 2. Scanning electron microscope (SEM) and size distribution analysis. Typical micrograph of liposomes containing 1-MTX (A). After lyophilization, liposomes were coated with palladium and the surface was examined under JSM-5410 Scanning Microscope (JEOL, Korea). (B) The size distribution of liposomes was determined using dynamic laser-light scattering (Particle Sizing Systems, Inc., Santa Barbara, CA, USA).

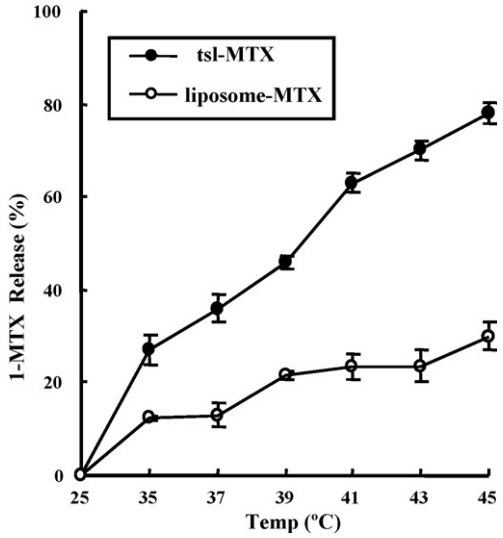

Fig. 3. Temperature dependent release of 1-MTX from tsl-MTX. 1-MTX loaded into the temperature-sensitive liposomes (tsl-MTX, DPPC:DMPC:DSPC in the molar ratio of 4:1:1) or non-temperature-sensitive liposomes (liposome-MTX, DPPC:DSPC in the molar ratio of 1:2) were mixed with 0.9 ml of phosphate-buffered saline in each tube, and incubated for 10 min at various degrees. The absorbance was determined using UV–vis spectrophotometer and the release (%) was calculated. Results are given as mean ± S.D. for three independent experiments.

laser-light scattering indicated that the liposomes after extrusion were approximately 200 nm in diameter (Fig. 2B). The loading efficiency of 1-MTX in the temperature-sensitive liposome was about 35% (0.322 mg 1-MTX/mg lipid on the final liposomes). *In vitro* drug release studies in phosphate-buffered saline showed that the release of 1-MTX from the temperature-sensitive liposome was increased in a temperature-dependent manner, while that from non-temperature-sensitive liposome was quite less released (Fig. 3). The absorbance of liposome-MTX and tsl-MTX was significantly lower than the absorbance of released 1-MTX after heating. Since the tsl-MTX was designed for *in vivo* experiment combined with hyperthermia, we focused on within the boundary of temperature available for *in vivo* hyperthermia. This result indicates that the tsl-MTX would be *in vivo* effective when it is combined with hyperthermia.

3.3. 1-MTX encapsulated in temperature-sensitive liposome inhibits tumor growth in vivo

The *in vivo* effect of tsl-MTX was examined using nude mice bearing xenograft tumors established by implantation with RKO or A549 cells in the hind leg. The xenograft animals were administered with tsl-MTX suspension at a dose of 100 mg/kg of 1-MTX by *i.p.* injection to give sufficient dose and treated as described in Section 2. The injection of tsl-MTX resulted in marked inhibition of tumor growth in combination with radiation and regional hyperthermia, as well as by itself, for both RKO (Fig. 4A) and A549 xenograft (Fig. 5A), although the tumor growth of RKO was more potently inhibited than that of A549. The volume of untreated tumor in RKO xenograft increased 5 times in 13.3 ± 0.5 days while the volume of tumors in the host injected with tsl-MTX increased 5 times in 21.5 ± 1.9 days, indicating that there was a growth delay of about 8 days by tsl-MTX similarly to the growth delay caused by 5 Gy of radiation in a single dose (Fig. 4A). The predominant inhibition observed in the host mice irradiated after injection with tsl-MTX was that the volume of tumors increased about 2 times during 26 days. In addition, tsl-MTX without hyperthermia showed meaningful suppressive effect on the tumor growth, which was more augmented by combination with radiation. The killing effect of tsl-MTX without radiation and external heat could be due to leakage of liposome contents *in vivo*, similarly to the previous report showing that the content release of liposome even without external heat (Ferraretto et al., 1996). It is also interesting that tsl-MTX without radiation displayed suppressive effect on the tumor growth *in vivo*, while free 1-MTX was observed not fatal to the survival rate of cultivated RKO cells (Fig. 1C). To examine the influence of hyperthermic effect on the retardation of tumor growth caused by radiation, RKO xenograft tumors were heated or not heated before radiation. As shown in Fig. 4B, notable difference in tumor growth between heated or not heated was not observed, indicating that the effect of tumor growth by tsl-MTX was not reflected by hyperthermic effect in this study of xenograft model.

For A549 tumors (Fig. 5A), the volume of untreated tumor increased 5 times in 16.3 ± 2.5 days while the volume of tumors in the host injected with tsl-MTX alone, irradiated alone, and the combination of tsl-MTX and radiation increased 5 times in 18.2 ± 3.1 days, 25.6 ± 5.7 days, and 29.6 ± 4.2 days, respectively. In consequence, compared to the growth of untreated tumors for the 5 times increase, the growth delay caused by tsl-MTX, radiation and the combination were about 2 days, 9 days and 13 days, respectively. At the end of experimental period, a photograph was taken for comparison of A549 tumors isolated from the host mice after excluding the biggest and smallest tumors in each group (Fig. 5B). Therefore, the tsl-MTX exerts a potent *in vivo* anti-tumor activity in xenograft animals, especially when it is combined with radiation. During the *in vivo* experiment using A549 xenograft mice, tumor tissues were isolated from the animal in each group at 2 days after treatment. The tumor tissues were subjected to TUNEL staining or immunohistochemistry against marker proteins for cell proliferation, apoptosis, and senescence. As shown in Fig. 6, many of cells presenting phosphorylated his-

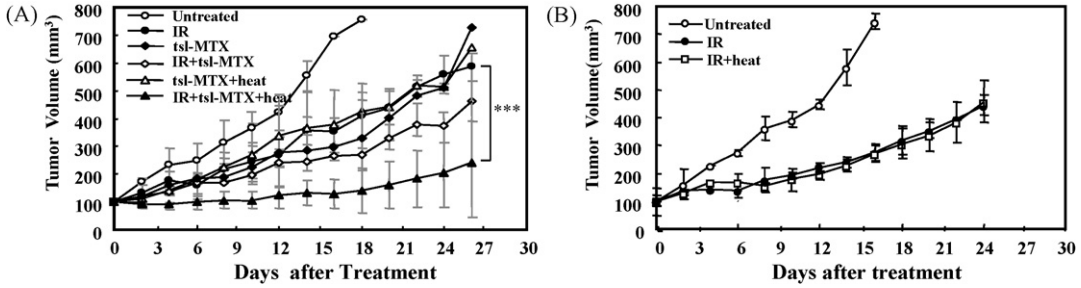

Fig. 4. The effect of tsl-MTX on the growth of RKO xenograft tumors. (A) The nude mice bearing RKO xenograft tumors on the right hind leg were injected intraperitoneally or not injected with 100 mg/kg of tsl-MTX, and 1 h later the tumors were heated or not in 42 °C water-bath for 1 h for the mice injected with tsl-MTX. The tumors were irradiated or not irradiated with 5 Gy in a single dose, the 3 perpendicular tumor diameters were measured every 2 days and the growth rates were plotted. ***$P < 0.001$ as compared to irradiation. (B) RKO xenograft tumors without injection of tsl-MTX were heated in 42 °C water-bath for 1 h or not. The tumors were irradiated with 10 Gy in a single dose, and the 3 perpendicular tumor diameters were measured every 2 days and the growth rates were plotted.

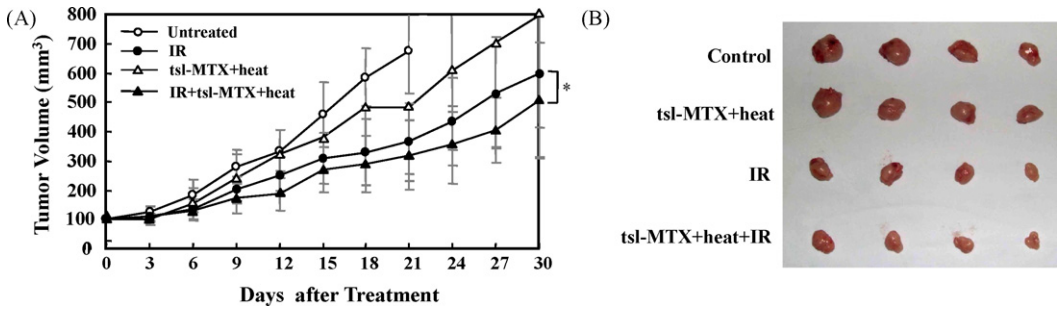

Fig. 5. The effect of tsl-MTX on the growth of A549 xenograft tumors. (A) The nude mice bearing A549 xenograft tumors on the right hind leg were injected intraperitoneally or not injected with 100 mg/kg of tsl-MTX, and 1 h later the tumors were heated in 42 °C water-bath for 1 h for the mice injected with tsl-MTX. The tumors were irradiated or not irradiated with 5 Gy in a single dose, the 3 perpendicular tumor diameters were measured every 3 days and the growth rates were plotted. $^{*}P < 0.05$ as compared to irradiation. (B) The photograph of the tumors isolated from the A549 xenograft animals at the end of the experimental period.

tone H3, a marker for proliferation, were appeared in tumor tissues untreated or treated with tsl-MTX in combination of hyperthermia. Distinctively, phosphorylated histone H3 was not shown in tissues irradiated alone or irradiated with administration of tsl-MTX, suggesting that the cellular proliferation was inhibited in these tumor tissues. Apoptotic cell detected by TUNEL staining was not shown in untreated tissue, but several apoptotic cells were detected in both tissues treated with tsl-MTX and irradiated alone. More potent induction of apoptosis was observed in the tissue treated in combination of tsl-MTX and radiation. For cleaved caspase-3, an apoptotic marker, many cells in tumor tissue irradiated with administration of tsl-MTX were immunodetected by cleaved caspase-3 antibody, but not in tissues untreated or treated with tsl-MTX without radiation. Few cells in tumor irradiated without tsl-MTX were immunoreacted with the antibody. These results indicate that tsl-MTX radiosensitizes tumor tissue and induces apoptosis *in vivo*, which contribute to lead the tumor growth delay. DcR2, a senescence marker protein, was detected in the tumor tissue that was isolated from the mouse administrated with tsl-MTX and irradiated, while it was not appeared in the tumor tissue from untreated mouse, implying that tsl-MTX induces senescence which might be associated also with the tumor growth delay occurred by tsl-MTX.

4. Discussion

In the present study, we showed that 1-MTX, a caffeine derivative, has a potency of radiosensitizer both *in vitro* and *in vivo*. In RKO human colorectal and A549 human lung cancer cells, the results obtained through clonogenic cell survival assay and western blot analysis against caspase-3 and PARP showed that treatment with 1-MTX sensitized cells to ionizing radiation. Although 1-MTX showed its potent pharmacological influence to enhance the killing effect against cancer cells by radiation, the lethality of 1-MTX to animal model as a form of aqueous drug in 0.1N NaOH hampered the further preclinical evaluation of this drug. To overcome this obstacle, we applied the temperature-sensitive liposomes prepared by reverse-phase evaporation method using DPPC:DMPC:DSPC at 4:1:1 molar ratio, in which 1-MTX was adequately loaded. The tsl-MTX was characterized to have approx-

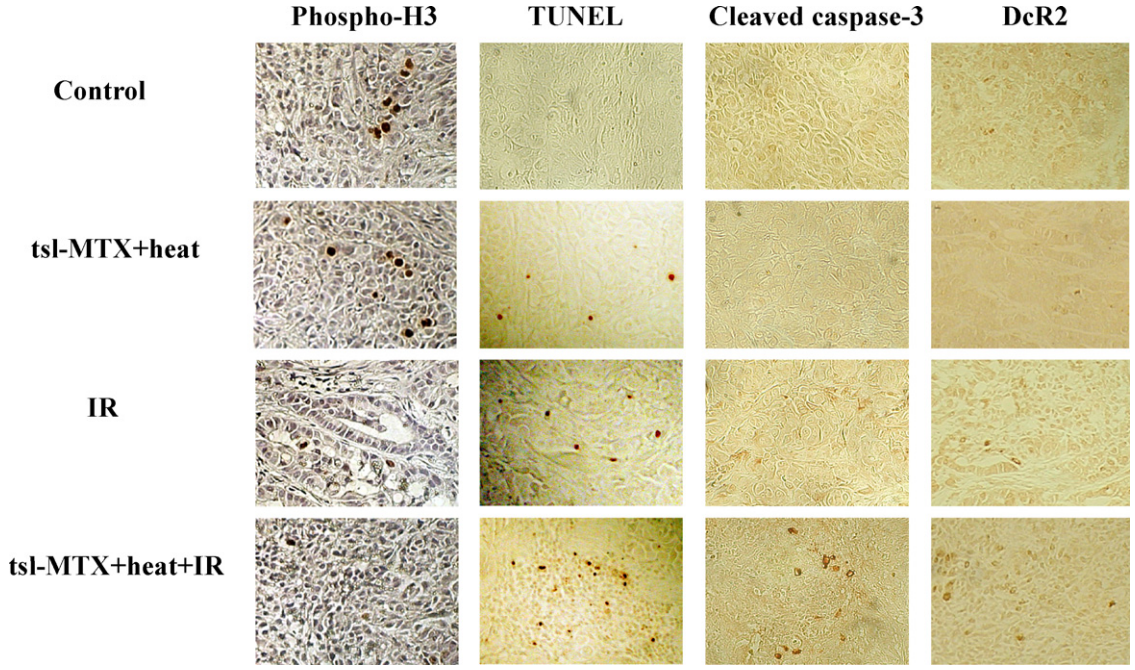

Fig. 6. Immunohistochemical analysis in tumor tissues. Tumor tissues were isolated from A549 xenograft mice at 2 days after treatment. Tissues were subjected to TUNEL staining or immunodetection with antibodies against phospho-histone H3, cleaved caspase-3 and DcR2. The detection was performed with ChemMate DAKO Envision and 3,3′-diaminobenzidine. The tissues for phospho-histone H3 were counterstained with Harris hematoxylin.

imately 200 nm size in diameter and temperature-based release kinetics. In xenograft mice, the temperature-sensitive liposomes containing 1-MTX displayed notable anticancer effect, which was culminated in its effectiveness when combined with ionizing radiation. This study demonstrates that the delivery of 1-MTX to solid tumors using the temperature-sensitive liposomes can potentially reduce the toxicity and increase the efficacy of the targeted compound.

It has been known that methylxanthine derivatives including caffeine, pentoxifylline, and staurosporine derivative 7-hydroxystaurosporine (UCN-01) increase radiosensitivity of cancer cells by inhibiting G2 checkpoint activation (Jha et al., 2002; Sarkaria et al., 1999; Teicher et al., 1991; Wang et al., 1996). Although we have not shown the accurate molecular mechanism of 1-MTX that enhances radiosensitivity, it was evident that 1-MTX increases the radiation-induced apoptosis in cancer cells and xenograft tumor tissues, which implies the possibility that 1-MTX enhances the apoptosis induction by abrogating radiation-induced G2 checkpoint. Many checkpoint inhibitors have been discovered with an intention to sensitize tumors to certain genotoxic and chemotherapeutic drugs. Caffeine, a methylxanthine derivative was the first inhibitor of G2 checkpoint (Walters et al., 1974). Other compounds such as pentoxifylline, lisofylline, staurosporine, UCN-01 and SB-218078 have been discovered as a G2 checkpoint inhibitor (Jackson et al., 2000; Russell et al., 1996; Tam and Schlegel, 1992; Wang et al., 1996). Despite the many pharmacological activities, their toxicity at the high concentrations required for clinical use and problem of solubility unfortunately preclude them from clinical application (Anderson et al., 2003). 1-MTX basically has a problem of lethality in experimental animal, which is due to the solubility of 1-MTX limited in only NaOH solution. To overcome the problem, we have developed tsl-MTX and this approach shown in this study should be principally applicable to any other chemotherapeutic drug including methylxanthine derivatives that has poor property in solubility.

In conclusion, 1-MTX radiosensitized cancer cells *in vitro*, and tsl-MTX has a potent capability to radiosensitize xenograft tumors, resulting in augmentation of apoptosis induction and attenuation of the tumor growth rate *in vivo*. Further study is warranted to examine the feasibility of using tsl-MTX to enhance the response of human tumors to radiotherapy.

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References

Ahn, J.Y., Schwarz, J.K., Piwnica-Worms, H., Canman, C.E., 2000. Threonine 68 phosphorylation by ataxia telangiectasia mutated is required for efficient activation of Chk2 in response to ionizing radiation. Cancer Res. 60, 5934–5936.

Akinaga, S., Nomura, K., Gomi, K., Okabe, M., 1993. Enhancement of antitumor activity of mitomycin C in vitro and in vivo by UCN-01, a selective inhibitor of protein kinase C. Cancer Chemother. Pharmacol. 32, 183–189.

Anderson, H.J., Andersen, R.J., Roberge, M., 2003. Inhibitors of the G2 DNA damage checkpoint and their potential for cancer therapy. Prog. Cell Cycle Res. 5, 423–430.

Asaad, N.A., Zeng, Z.C., Guan, J., Thacker, J., Iliakis, G., 2000. Homologous recombination as a potential target for caffeine radiosensitization in mammalian cells: reduced caffeine radiosensitization in XRCC2 and XRCC3 mutants. Oncogene 19, 5788–5800.

Blasina, A., Paegle, E.S., McGowan, C.H., 1997. The role of inhibitory phosphorylation of CDC2 following DNA replication block and radiation-induced damage in human cells. Mol. Biol. Cell. 8, 1013–1023.

Bunch, R.T., Eastman, A., 1996. Enhancement of cisplatin-induced cytotoxicity by 7-hydroxystaurosporine (UCN-01), a new G2-checkpoint inhibitor. Clin. Cancer Res. 2, 791–797.

Busby, E.C., Leistritz, D.F., Abraham, R.T., Karnitz, L.M., Sarkaria, J.N., 2000. The radiosensitizing agent 7-hydroxystaurosporine (UCN-01) inhibits the DNA damage checkpoint kinase hChk1. Cancer Res. 60, 2108–2112.

Choi, E.K., Roberts, K.P., Griffin, R.J., Han, T., Park, H.J., Song, C.W., 2004. Effect of pH on radiation-induced p53 expression. Int. J. Radiat. Oncol. Biol. Phys. 60, 1264–1271.

Choi, E.K., Ji, I.M., Lee, S.R., Kook, Y.H., Griffin, R.J., Lim, B.U., Kim, J.S., Lee, D.S., Song, C.W., Park, H.J., 2006. Radiosensitization of tumor cells by modulation of ATM kinase. Int. J. Radiat. Biol. 82, 277–283.

Cortez, D., Wang, Y., Qin, J., Elledge, S.J., 1999. Requirement of ATM-dependent phosphorylation of brca1 in the DNA damage response to double-strand breaks. Science 286, 1162–1166.

Duncan, R., 2003. The dawning era of polymer therapeutics. Nat. Rev. Drug Discov. 2, 347–360.

Fernandez-Capetillo, O., Chen, H.T., Celeste, A., Ward, I., Romanienko, P.J., Morales, J.C., Naka, K., Xia, Z., Camerini-Otero, R.D., Motoyama, N., Carpenter, P.B., Bonner, W.M., Chen, J., Nussenzweig, A., 2002. DNA damage-induced G2-M checkpoint activation by histone H2AX and 53BP1. Nat. Cell. Biol. 4, 993–997.

Ferraretto, A., Sonnino, S., Soria, M.R., Masserini, M., 1996. Characterization of biotinylated liposomes sensitive to temperature and pH: new tools for anticancer drug delivery. Chem. Phys. Lipids 82, 133–139.

Fuse, E., Tanii, H., Kurata, N., Kobayashi, H., Shimada, Y., Tamura, T., Sasaki, Y., Tanigawara, Y., Lush, R.D., Headlee, D., Figg, W.D., Arbuck, S.G., Senderowicz, A.M., Sausville, E.A., Akinaga, S., Kuwabara, T., Kobayashi, S., 1998. Unpredicted clinical pharmacology of UCN-01 caused by specific binding to human alpha1-acid glycoprotein. Cancer Res. 58, 3248–3253.

Graves, P.R., Yu, L., Schwarz, J.K., Gales, J., Sausville, E.A., O'Connor, P.M., Piwnica-Worms, H., 2000. The Chk1 protein kinase and the Cdc25C regulatory pathways are targets of the anticancer agent UCN-01. J. Biol. Chem. 275, 5600–5605.

Iliakis, G., Wang, Y., Guan, J., Wang, H., 2003. DNA damage checkpoint control in cells exposed to ionizing radiation. Oncogene 22, 5834–5847.

Ismail, I.H., Nystrom, S., Nygren, J., Hammarsten, O., 2005. Activation of ataxia telangiectasia mutated by DNA strand break-inducing agents correlates closely with the number of DNA double strand breaks. J. Biol. Chem. 280, 4649–4655.

Jackson, J.R., Gilmartin, A., Imburgia, C., Winkler, J.D., Marshall, L.A., Roshak, A., 2000. An indolocarbazole inhibitor of human checkpoint kinase (Chk1) abrogates cell cycle arrest caused by DNA damage. Cancer Res. 60, 566–572.

Jha, M.N., Bamburg, J.R., Bernstein, B.W., Bedford, J.S., 2002. Caffeine eliminates gamma-ray-induced G2-phase delay in human tumor cells but not in normal cells. Radiat. Res. 157, 26–31.

Kohn, E.A., Ruth, N.D., Brown, M.K., Livingstone, M., Eastman, A., 2002. Abrogation of the S phase DNA damage checkpoint results in S phase progression or premature mitosis depending on the concentration of 7-hydroxystaurosporine and the kinetics of Cdc25C activation. J. Biol. Chem. 277, 26553–26564.

Kono, K., Nakai, R., Morimoto, K., Takagishi, T., 1999. Thermosensitive polymer-modified liposomes that release contents around physiological temperature. Biochim. Biophys. Acta 1416, 239–250.

Kopecek, J., 2003. Smart and genetically engineered biomaterials and drug delivery systems. Eur. J. Pharm. Sci. 20, 1–16.

Lau, C.C., Pardee, A.B., 1982. Mechanism by which caffeine potentiates lethality of nitrogen mustard. Proc. Natl. Acad. Sci. U.S.A. 79, 2942–2946.

Lavin, M.F., Birrell, G., Chen, P., Kozlov, S., Scott, S., Gueven, N., 2005. ATM signaling and genomic stability in response to DNA damage. Mutat. Res. 569, 123–132.

Lim, S.K., Park, H.J., Choi, E.K., Kim, J.S., 2007. Long-circulating, temperature sensitive and EGFR-targeted liposomes for drugs delivery. Key Eng. Mater. 342–343, 537–540.

Matsuoka, S., Huang, M., Elledge, S.J., 1998. Linkage of ATM to cell cycle regulation by the Chk2 protein kinase. Science 282, 1893–1897.

Needham, D., Dewhirst, M.W., 2001. The development and testing of a new temperature-sensitive drug delivery system for the treatment of solid tumors. Adv. Drug Deliv. Rev. 53, 285–305.

Park, H.J., Lee, S.H., Chung, H., Rhee, Y.H., Lim, B.U., Ha, S.W., Griffin, R.J., Lee, H.S., Song, C.W., Choi, E.K., 2003. Influence of environmental pH on G2-phase arrest caused by ionizing radiation. Radiat. Res. 159, 86–93.

Russell, K.J., Wiens, L.W., Demers, G.W., Galloway, D.A., Le, T., Rice, G.C., Bianco, J.A., Singer, J.W., Groudine, M., 1996. Preferential radiosensitization of G1 checkpoint-deficient cells by methylxanthines. Int. J. Radiat. Oncol. Biol. Phys. 36, 1099–1106.

Sancar, A., Lindsey-Boltz, L.A., Unsal-Kacmaz, K., Linn, S., 2004. Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. Annu. Rev. Biochem. 73, 39–85.

Sarkaria, J.N., Busby, E.C., Tibbetts, R.S., Roos, P., Taya, Y., Karnitz, L.M., Abraham, R.T., 1999. Inhibition of ATM and ATR kinase activities by the radiosensitizing agent, caffeine. Cancer Res. 59, 4375–4382.

Sausville, E.A., Arbuck, S.G., Messmann, R., Headlee, D., Bauer, K.S., Lush, R.M., Murgo, A., Figg, W.D., Lahusen, T., Jaken, S., Jing, X., Roberge, M., Fuse, E., Kuwabara, T., Senderowicz, A.M., 2001. Phase I trial of 72-hour continuous infusion UCN-01 in patients with refractory neoplasms. J. Clin. Oncol. 19, 2319–2333.

Szoka Jr., F., Papahadjopoulos, D., 1978. Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. Proc. Natl. Acad. Sci. U.S.A. 75, 4194–4198.

Tam, S.W., Schlegel, R., 1992. Staurosporine overrides checkpoints for mitotic onset in BHK cells. Cell. Growth Differ. 3, 811–817.
Teicher, B.A., Holden, S.A., Herman, T.S., Epelbaum, R., Pardee, A.B., Dezube, B., 1991. Efficacy of pentoxifylline as a modulator of alkylating agent activity in vitro and in vivo. Anticancer Res. 11, 1555–1560.
Walters, R.A., Gurley, L.R., Tobey, R.A., 1974. Effects of caffeine on radiation-induced phenomena associated with cell-cycle traverse of mammalian cells. Biophys. J. 14, 99–118.
Wang, Q., Fan, S., Eastman, A., Worland, P.J., Sausville, E.A., O'Connor, P.M., 1996. UCN-01: a potent abrogator of G2 checkpoint function in cancer cells with disrupted p53. J. Natl. Cancer Inst. 88, 956–965.
Wang, H., Boecker, W., Wang, X., Guan, J., Thompson, L.H., Nickoloff, J.A., Iliakis, G., 2004. Caffeine inhibits homology-directed repair of I-SceI-induced DNA double-strand breaks. Oncogene 23, 824–834.