

Cytogenetic Damages from Iododeoxyuridine -induced Radiosensitivity with and without Methoxyamine in Human Glioblastoma Spheroids

Ali Neshasteh-Riz, Ph.D.^{1‡}, Mohammad Saki, M.Sc.², Samideh Khoei, Ph.D.²

1. Radiology Department, Iran University of Medical Sciences
2. Medical Physics Department, Iran University of Medical Sciences

‡Corresponding Address: P.O. Box: 14155-6183, Medical Physics Department,
Iran University of Medical Sciences, Tehran, Iran
Email: neshastehriz@yahoo.com

Abstract

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Objective: Iododeoxyuridine-induced Radiosensitivity (IUdR) is a halogenated thymidine analogue recognized to be effective *in vitro* and *in vivo* radiosensitizer in human cancers. It is reported that Methoxyamine (MX) potentiates DNA damages in cancer cells with blocking the repair pathway of IUdR damages. But studies, entirely, are restricted on monolayer culture cells from human colon cancer cells. Spheroids are 3D form of cells that aggregate and grow together which resemble *in vivo* tumor models in several aspects and the results of such studies can be extended to tumor *in vivo*. The aim of the current study was to evaluate DNA damages from IUdR and gamma rays with and without Methoxyamine in human Glioblastoma spheroids.

Materials and Methods: The DNA induced damages in U87MG cell line were compared using alkaline comet assay method. Experiments were performed with two different sizes of spheroids (100µm and 300µm).

Results: Evaluation of the effects of IUdR with and without MX pretreatment on spheroids following ionizing radiation showed that MX increased the cell damages of IUdR with and without irradiation in both diameters spheroids. The damages were further increased in 100µm compared with 300µm diameter.

Conclusion: Comparisons of tail moments in spheroids with 100 and 300µm diameter showed that cell damages in larger spheroids, 300µm, are lesser than smaller one, 100µm. This could be due to existence of G₀ cells and cells with longer cycle which IUdR was less incorporated into them. Thus, decrease in IUdR radiosensitization and base excision repair (BER), results in reduction of MX activities. Using agents for Inhibiting the activities of proteins which are responsible for carrying the cells to G₀ may be beneficial in solving such problems.

Keywords: Radiosensitization, Spheroid, IUdR, Methoxyamine, BER

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Introduction

The incidence of malignant glioma has been reported to be 18,300 new cases and its mortality rate has been estimated to be approximately 13,100 patients just in 2003 in the United States. The primary management is surgery, followed by radiotherapy (1-4). Unfortunately, the irradiation effective enough to control the tumors far exceeds the tolerance of normal brain tissues (5). Thus, to avoid such unfavorable outcomes, methods which sensitize the tumor cells to ionizing radiation (IR) are used.

Iododeoxyuridine is a halogenated pyrimidine that can be incorporated into DNA instead of thymidine during DNA replication. This halogenated pyrimidine is considered as clinical radiosensitizer, where the extent of radiosensitization correlates directly with the level of halogenated pyrimidine-DNA incorporation (6-8). Although, the cellular and molecular mechanisms of IUdR radiosensitization are not clearly understood, it is presumed that IUdR sensitizes cells through enhancing

the formation of IR-induced double-strand breaks (9-12).

Recent studies have shown that IUdR incorporated into DNA can be recognized by base excision repair (BER) proteins (13, 14). Therefore, the cellular status of tumor cells repaired by BER may alter the degree of IUdR radiosensitization. Methoxyamine (MX) is a specific chemical inhibitor of BER mediated by tight binding to AP sites generated by cleavage of BER glycosylases and rendering the phosphodiester bonds adjacent to the AP site refractory to the catalytic activity of AP endonuclease (14). Studies have indicated that MX potentiated IUdR-induced radiosensitization in human cancer cells (14, 15). Since most of the previous studies conducted on evaluating the effectiveness of the IUdR/MX radiosensitization have been confined to laboratory trials on monolayer cell culture in which the corresponding cells were extracted from human colon cancer, in the present study, the genetic damages of IUdR/MX radiosensitization were investigated in spheroid cell culture of glioblastoma cell lines.

The multicellular spheroid represents an *in vitro-in vivo* transition model which is important *in vivo* solid tumors (16). These include intimate cell-cell contact (17), individual hypoxic cell populations (18), and cycle times that range from as those of exponential monolayer rates through an essentially nondividing state (19). In brief, they combine the relevance of organized tissues with the accuracy of *in vitro* methodology (20-25). It has been suggested that some physiological differences may exist between cell growth in two dimensional contact (monolayer) and cells grown in three-dimensional contact (multicellular tumor spheroids) (26, 27). A research conducted on the growth of human glioma cells in these two systems showed different degrees of sensitivity to radioionated IUdR (28). Several authors have reported higher radioresistance of cells in spheroids compared with monolayer cultures (29-32). In this paper, we have studied the radiosensitivity of glioblastoma cell line U87MG multicellular spheroids using IUdR in combination with MX. Due to the existence of hypoxic and

G₀ in larger spheroids compared with lesser one, experiments were performed with two different sizes of spheroids (100µm and 300µm in diameter).

Materials and Methods

Cell Line

Human glioblastoma cell line, U87MG, were provided from Pastor Institute of Iran. This cell line was maintained in MEM (GIBCO) supplemented with 10% fetal bovine serum (FBS) (GIBCO), 500U/ml of penicillin (SIGMA) and 200 mg/lit of streptomycin (JABEREbn-HAYAN).

Monolayer culture

Cells were cultured as monolayer at a density of 10⁴ cells/cm² in T-25 tissue culture flasks (NUNC). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂. Cultures were propagated or cells were harvested by trypsinizing cultures with 1mM EDTA/ 0.25% Trypsin (w/v) in Phosphate Buffer Saline (PBS).

Spheroid Culture

Spheroids were initiated using the Liquid Overlay technique (26). A medium including 10⁵ U87MG cells were seeded into 100 mm dishes coated with a thin layer of 1% agar (Bacto Agar, Difco, Detroit, MI) with 10ml of MEM supplemented with 10% FBS. The plates were incubated at 37 °C in a humidified atmosphere of 5% CO₂. Half of the culture medium was replaced with fresh medium twice per week.

Spheroid Growth curve

After 3 days of culture, spheroids were moved into multiwell plates (24 wells/plate) (NUNC) coated with a thin layer of 1% agar with 10ml of MEM supplemented with 10% FBS, whereas one spheroid was located into one well. The multiwells were incubated at 37°C in a humidified atmosphere of 5% CO₂. for one month, two perpendicular diameters of spheroids were measured and their volume calculated according to the following equation:

V (Volume) = $a \times b^2 \times \pi / 6$ (1), in that, (a) and (b) are lesser and greater in diameters, respectively. Then the volume growth curve

were calculated according to the duration. In the logarithmic phase of curve, spheroids follow the equation:

$$V = V_0 \times e^{kt} \quad (2)$$

Where (V_0) is primary spheroids volume, (V) equals the spheroids volume after the duration (t) and k shows the gradient of the logarithmic phase of the curve. The volume doubling time of spheroids is achieved according to gradient of logarithmic phase of the curve.

Drug-IR Treatment

IuDR ($1\mu\text{M}$) and MX (6mM) in U87MG spheroids were treated for 67h in MEM containing 10% FBS. After 67 h, drug-containing medium was removed, the cultures were washed with PBS and the spheroids were immediately irradiated (2Gy). IR was irradiated using ^{60}Co at a dose rate of 1.14 cGy/s .

Comet assay

The induction of DNA damages in U87MG cell populations by IuDR alone ($1\mu\text{M}$ for 67 h) or in combination with MX 6 mM for 67h) were determined by SCGE (comet assay). This assay was performed according to the Singh et al protocol (33). Comet tail moment was determined by measuring the fluorescence intensity using the Comet scor software.

Statistical analysis

Data were given as mean values \pm SEM, with n denoting the number of experiments. Student's t test was considered appropriate and applied. A value of $p \leq 0.05$ was considered to be significant.

Results

The U87MG cells were able to form spheroids in liquid overlay cultures. Fig 1 shows the phase contrast micrographs of these spheroids in two different diameters. The growth curve of U87MG cells in spheroid culture is shown in Fig 2.

The volume doubling time calculated from this curve was approximately 67 ± 0.91 hours which was applied as drug treatment time consequently.

Comet assay were used for evaluation of DNA

damages after drug treatment and ionization radiation. Experiments were performed with two different sizes of spheroids ($100\mu\text{m}$ and $300\mu\text{m}$ in diameter) and for evaluation of MX effects on IuDR radiosensitization, spheroids were treated with IuDR \pm MX for one volume doubling time. Figs 3 (A-H) and 4 (A-H) shows the microphotographies of the cells from spheroids during comet assay between the treatment and the control groups. The photos were analyzed by comet score software and the tail moments were calculated as a parameter related to DNA damages.

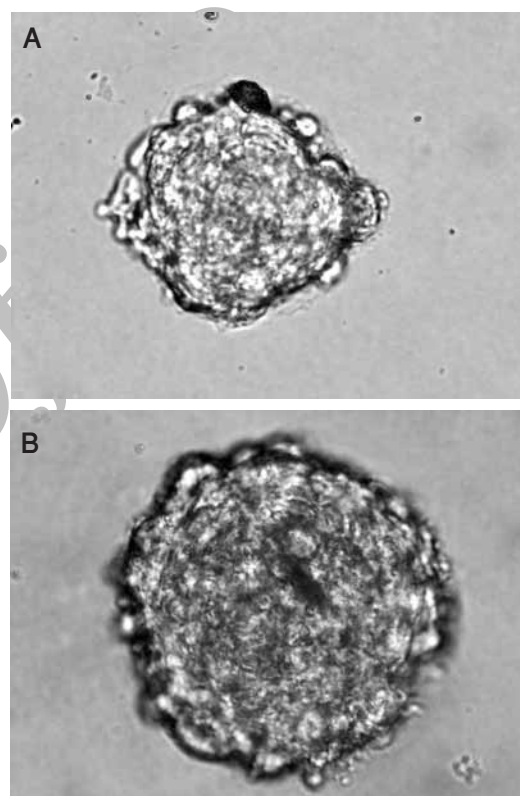


Fig 1: Phase contrast micrographs of U87MG spheroids. (A) $100\mu\text{m}$ spheroid at day 11 and (B) $300\mu\text{m}$ spheroid at day 23 after initiation of measuring the diameters.

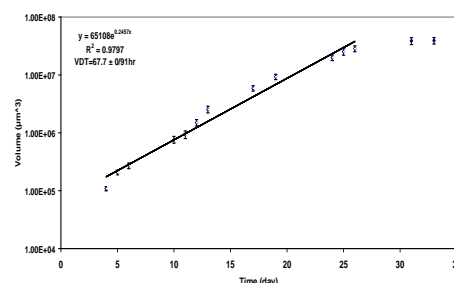


Fig 2: The growth curve of U87MG cell line in the spheroid cultures. The days 4 to 26 show the log phase of curve and used to measuring volume doubling time (67 h). Mean \pm SEM of 3 experiments

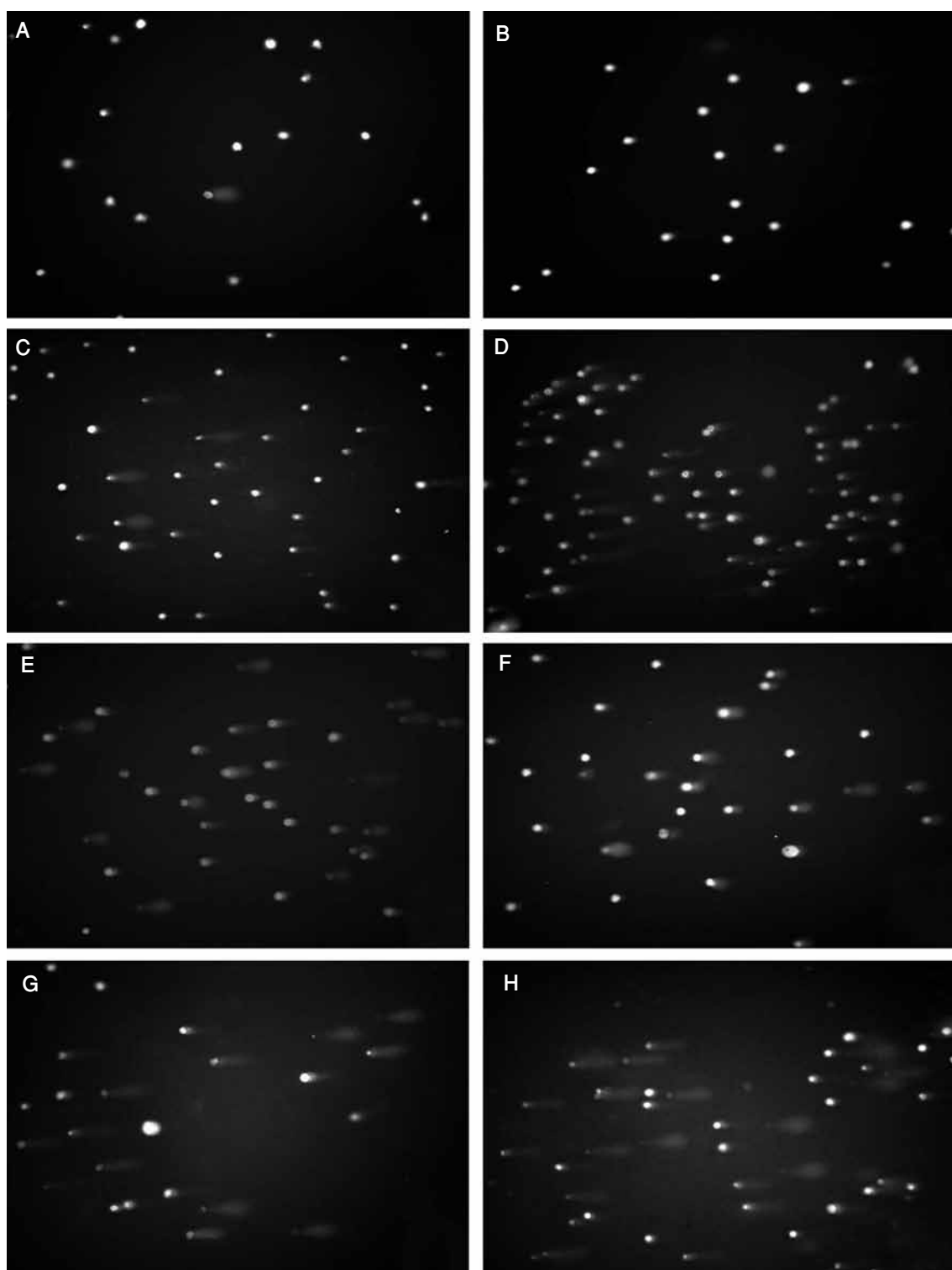


Fig 3: SCGE (Comet Assay) of U87MG cells of 100µm spheroids after treatment for 67 h with 1µM IUdR alone or simultaneous treatment with 6mM MX. microphotographies are representative samples of following slides: A) control, B) MX, C) IUdR, D) IUdR+ MX, E) Gamma, F) MX+Gamma, G) IUdR+Gamma, H) IUdR MX+Gamma

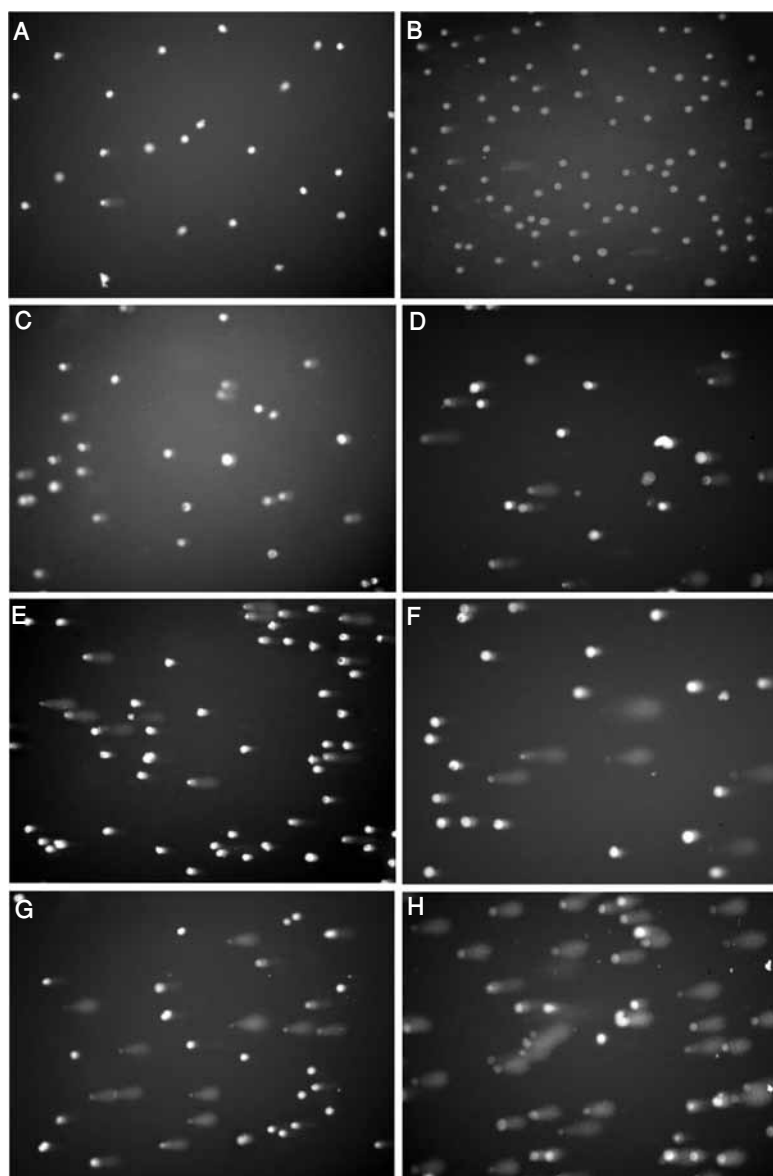


Fig 4: SCGE (Comet Assay) of U87MG cells of 300µm spheroids after treatment for 67 h with 1µM IudR alone or simultaneous treatment with 6mM MX. microphotographies are representative samples of following slides: A) control, B) MX, C) IudR, D) IudR+ MX, E) Gamma, F) MX+Gamma, G) IudR+Gamma, H) IudR+MX+Gamma

Fig 5 shows the effect of IudR/MX pretreatment on DNA damages following IR in 100 and 300 micrometer spheroids. According to the figure, MX alone is not effective in changing IR-related cytotoxicity in both diameters. In contrast IudR/MX treatment, significantly ($p < 0.001$) increased the tail moments before and after IR compared with the control group in both spheroid diameters. On the other hand, except control and MX treated groups, the DNA damages in 100 micrometer spheroids were more significantly increased than 300 micrometer spheroids.

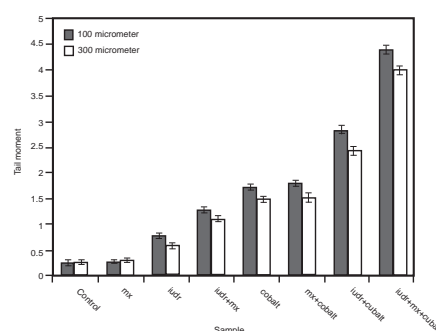


Fig 5: Comparative chart of comet tail moment in U87MG cell line of 100 and 300µm spheroids after treatment with 1µM IudR alone or simultaneous treatment with 1µM IudR and 6mM MX. Spheroids were treated and processed as described in "Material and Methods." Comet tail moment is calculated as the product of tail length and fraction of DNA in the tail. Data are expressed as fold increase compared with tail moment in untreated control cells; Mean±SEM of 3 experiments.

Tab 1 shows the Comparison of DNA damages (tail moments) in 100 and 300µm spheroids treated with IUdR/MX/IR in comparison with IUdR/IR. The combined treatment with IUdR/MX for 67 hours before IR induced tail moments to a greater extent (42% increase for 100µm and 34% increase for 300µm spheroids) than IUdR pretreatment alone.

Table 1: Percentages of increasing damages in U87MG spheroids from combination IUdR and Methoxyamine with and without IR in 100µm and 300µm diameters.

Spheroid diameter	Percentage of increasing damages after pretreatment with IUdR+MX	Percentage of increasing damages after pretreatment with IUdR+MX+IR
100µm	100µm	42.34%
300µm	19.47%	33.8%

Discussion

IUdR is known as radiosensitizer that increases IR-induced DNA DSBs (6-12). It is reported that MX potentiated IUdR-induced radiosensitization in human tumor cells (14, 15). IUdR with MX radiosensitization studies, entirely, are restricted to laboratory researches on monolayer culture cells from human colon cancer and it remain unclear whether these results could be extended into tumor in vivo. on the other hand, in glioma cells which are one of the most important choices for IUdR indications, there is a debate whether MX induces IUdR radiosensitization.

Spheroid cultures are interesting and valuable in vitro model systems, which allow many properties of in vivo tumor systems to be studied quantitatively (34-35). In the present study, we have studied the IUdR radiosensitization combined with MX in spheroid cultures of human glioblastoma cell line U87MG. To better clarify the role of hypoxic and G₀ cells in large spheroids (36), we performed this experiment with two different sizes of spheroids (100µm and 300µm in diameter). Using comet assay (Fig 5), IUdR pretreatment significantly sensitized cells to IR, and the radiosensitization was further increased when cells were pretreated with IUdR/MX compared with control in both spheroid diameters. These results showed for the first time that, in human glioma

cells, the use of MX significantly increases the DNA damages induced from IUdR radiosensitization.

It is not completely understood that why MX alone is not effective in changing IR-related cytotoxicity in these cell lines. A study proposed that MX may inhibit short-patch BER by blocking APE activity, therefore enhancing long-patch BER to remove MX-AP (14). Comparisons of tail moments in spheroids with 100 and 300µm diameters showed that, except for MX treated and control groups, cell damages in larger spheroids, 300µm, are lesser than smaller one, 100µm. This may be due to the existence of G₀ cells and also hypoxic cells in 300µm spheroids which IUdR was not incorporated into them and therefore MX couldn't perform its inhibition activity. To solve this problem and increase the efficacy of this method. Agents can be used to inhibit the proteins which make cells hypoxic and carry them to G₀ phase, this can also increase the efficacy of modality treatment.

Conclusion

Pretreatment of cells with IUdR and MX before irradiation enhances tumor radiosensitization and may improve therapeutic index for IR.

References

1. Jemal A, Murray T, Samuels A, Chafour A, Ward E, Thun MJ. Cancer statistics. CA Cancer JCLIN. 2003; 53(1): 5-26
2. Green SB, Byar DP, Walker MD. Comparison of carmustine, procarbazine, and high-dose methylprednisolone as additions to surgery and radiotherapy for the treatment of malignant glioma. Cancer Treat Rep. 1983; 67: 121-132
3. Walker M, Alexander E, Hunt WE. Evaluation of BNCU and/or radiotherapy in the treatment of anaplastic gliomas: A cooperative clinical trial. J Neurosurg. 1978; 49: 333-342
4. Walker M, Green S, Byar D. Randomized comparisons of radiotherapy and nitrosoureas for the treatment of malignant glioma after surgery. N Engl Med. 1980; 303: 1323-1329
5. Scheline GE, Wara W, Smith V. Therapeutic irradiation and brain injury. Int J Radiat Oncol Biol Phys. 1980; 6: 1215-1228
6. Miller EM, Fowler JF, Kinsella TJ. Linear-quadratic analysis of radiosensitization by halogenated pyrimidines. I. Radiosensitization of human colon cancer cells by iododeoxyuridine. Radiat Res 1992; 131: 81-89

7. Lawrence TS, Davis MA, Maybaum J, Stetson PL, Ensminger WD. The dependence of halogenated pyrimidine incorporation and radiosensitization on the duration of drug exposure. *Int J Radiat Oncol Biol Phys* 1990; 18: 1393-1398
8. Kinsella TJ, Dobson PP, Mitchell JB, Fornace AJ, Jr. Enhancement of X-ray induced DNA damage by pre-treatment with halogenated pyrimidine analogs. *Int J Radiat Oncol Biol Phys* 1987; 13: 733-739
9. Jones GD, Ward JF, Limoli CL, Moyer DJ, Aguilera JA. Mechanisms of radiosensitization in iododeoxyuridine-substituted cells. *Int J Radiat Biol* 1995; 67: 647-653
10. Lawrence TS, Davis MA, Maybaum J, Stetson PL, Ensminger WD. The effect of single versus double-strand substitution on halogenated pyrimidine-induced radiosensitization and DNA strand breakage in human tumor cells. *Radiat Res* 1990; 123: 192-198
11. Watanabe R, Nikjoo H. Modelling the effect of incorporated halogenated pyrimidine on radiation-induced DNA strand breaks. *Int J Radiat Biol* 2002; 78: 953-966
12. Fornace AJ, Jr., Dobson PP, Kinsella TJ. Enhancement of radiation damage in cellular DNA following unifilar substitution with iododeoxyuridine. *Int J Radiat Oncol Biol Phys* 1990; 18: 873-878
13. Turner DP, Loh T, Kinsella TJ, Bellacosa A. Kinetic and mutational assessment of the DNA N-glycosylase activity of the base excision repair enzyme MED1: preference for halogenated pyrimidines. *Cancer Res* In press
14. Taverna P, Hwang HS, Schupp JE, et al. Inhibition of base excision repair potentiates iododeoxyuridine-induced cytotoxicity and radiosensitization. *Cancer Res* 2003; 63: 838-846
15. Yan T, Seo Y, Schupp JE, Zeng X, Desai AB, and Kinsella TJ. Methoxyamine potentiates iododeoxyuridine-induced radiosensitization by altering cell cycle kinetics and enhancing senescence. *Mol Cancer Ther.* 2006; 5(4): 893-902
16. Kunz-Schughart LA, Kreutz M, Knuechel R. Multicellular spheroids: a three-dimensional in vitro culture system to study tumour biology. *Int. J. Exp. Pathol.* 1998; 79: 1-23
17. Santini MT, Rainaldi G, Indovina PL. Apoptosis, cell adhesion and the extracellular matrix in the three-dimensional growth of multicellular tumor spheroids. *Crit Rev. Oncol. Hematol.* 2000; 36: 75-87
18. Woods ML, Koch CJ, Lord EM. Detection of individual hypoxic cells in multicellular spheroids by flow cytometry using the 2-nitroimidazole, EF5, and monoclonal antibodies. *Int. J. Radiat. Oncol. Biol. Phys.* 1996; 34: 93-101
19. Durand RE, Olive PL. Tumour cell kinetics and heterogeneity: insights from multicell spheroids. *BJR Suppl.* 1992; 24: 79-83
20. Gunther W, Pawlak E, Damasceno R, Arnold H, Terzis AJ. Temozolomide induces apoptosis and senescence in glioma cells cultured as multicellular spheroids. *Br. J. Cancer.* 2003; 88: 463-469
21. Kosaka K, Fujiwara H, Tatsumi K, Yoshioka S, Higuchi T, Sato Y, Nakayama T, Fujii S. Human peripheral blood mononuclear cells enhance cell-cell interaction between human endometrial epithelial cells and BeWo-cell spheroids. *Hum. Reprod.* 2003; 18:19-25
22. Wartenberg M, Schallenberg M, Hescheler J, Sauer H. Reactive oxygen species-mediated regulation of eNOS and iNOS expression in multicellular prostate tumor spheroids. *Int. J. Cancer.* 2003; 104: 274-282
23. Yamauchi N, Yamada O, Takahashi T, Imai K, Sato T, Ito A, Hashizume K. A Threedimensional Cell Culture Model for Bovine Endometrium: Regeneration of a Multicellular Spheroid Using Ascorbate, Placenta. 2003; 24: 258-269
24. Olsnes C, Heimdal JH, Kross K, Olofsson J, Aarstad HJ. Mechanisms for monocyte activation in co-culture with autologous tumor spheroids. *Cell Immunol.* 2002; 219: 11-21
25. Simiantonaki N, Jayasinghe C, Kirkpatrick CJ. Differential endothelial CAM-expression after stimulation with supernatants of LPS- and cytokine-stimulated HT-29 and ST-ML-12 tumor cells growing as monolayer cultures and multicellular spheroids. *Anticancer Res.* 2002; 22(5): 2641-2649
26. Wigle JC, Sutherland RM. Increased thermoresistance developed during growth of small multicellular spheroids. *J. Cell Physiol.* 1985; 122: 281-289
27. Dobrucki J, Bleehen NM. Cell-cell contact affects cellular sensitivity to hyperthermia. *Br. J. Cancer.* 1985; 52: 849-855
28. Neshasteh-Riz A, Mairs RJ, Angerson WJ, Stanton PD, Reeves JR, Rampling R, Owens J, Wheldon TE. Differential cytotoxicity of [123I] IUDR, [125I] IUDR and [131I] IUDR to human glioma cells in monolayer or spheroid culture: effect of proliferative heterogeneity and radiation cross-fire. *Br. J. Cancer.* 1998; 77: 385-390
29. Kerbel RS, Rak J, Kobayashi H, Man MS, St Croix B, Graham CH. Multicellular resistance: a new paradigm to explain aspects of acquired drug resistance of solid tumors. *Cold Spring Harb. Symp. Quant. Biol.* 1994; 59: 661-672
30. Olive PL, Durand RE. Drug and radiation resistance in spheroids: cell contact and kinetics. *Cancer Metastasis Rev.* 1994; 13: 121-138
31. Desoize B, Gimonet D, Jardillier JC. Cell culture as spheroids: an approach to multicellular resistance. *Anticancer Res.* 1998; 18: 4147-4158
32. Desoize B, Jardillier J. Multicellular resistance: a paradigm for clinical resistance. *Crit Rev. Oncol. Hematol.* 2003; 6: 193-207
33. Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels

of DNA damage in individual cells. *Exp Cell Res* 1988; 175(1): 184-191

34. Boyd M, Mairs SC, Stevenson K, Livingstone A, Clark AM, Ross SC, et al. Transfectant mosaic spheroids: a new model for evaluation of tumour cell killing in targeted radiotherapy and experimental gene therapy. *J Gene Med* 2002; 4: 567-576

35. Grill J, Lamfers ML, Van Beusechem VW, Dirven CM, Pherai DS, Kater M, et al. The organotypic

multicellular spheroid is a relevant three-dimensional model to study adenovirus replication and penetration in human tumors in vitro, *Mol. Ther*, 2002; 6: 609-614

36. Neshasteh-Riz A, Angerson WJ, Reeves JR, Smith G, Rampling R, Mairs RJ. Incorporation of iododeoxyuridine in multicellular glioma spheroids: electron emitters. *Br J Cancer*, 1997; 75(4): 493-499

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