

Failure of 2-Deoxy-D-glucose and 5-Thio-D-glucose to Kill Hypoxic Cells of Two Murine Tumors¹

Ian F. Tannock,² Patricia Guttman, and A. Michael Rauth

Department of Medicine [I. F. T.] and Division of Physics [I. F. T., P. G., A. M. R.], Ontario Cancer Institute, and Department of Medical Biophysics [I. F. T., A. M. R.], University of Toronto, Toronto, Ontario, Canada M4X 1K9

ABSTRACT

We have administered the glycolysis inhibitors 2-deoxy-D-glucose and 5-thio-D-glucose to C3H/HeJ mice bearing KHT or 16/C transplantable tumors to seek evidence for hypoxic cell toxicity *in vivo*. The drugs were given (a) with or without insulin, (b) as large single doses or as multiple hourly injections, and (c) alone or immediately after the tumors had received radiation to kill most of the aerobic cell population. Tumor response was assessed by growth delay or by lung colony assay.

Limiting toxicity of 2-deoxy-D-glucose and 5-thio-D-glucose was neurological, leading to seizures and/or death, and this toxicity was increased by insulin. The drugs had at most minimal effects on the growth of either untreated or irradiated tumors at maximal tolerated doses. Despite the known selective toxicity of these glucose analogues for hypoxic cells in tissue culture, we have found them to be ineffective in killing hypoxic cells of two murine tumors.

INTRODUCTION

Solid tumors are known to contain hypoxic cells that are resistant to ionizing radiation. Hypoxic cells may also be resistant to some anticancer drugs because of poor drug distribution from blood vessels (12), because they are predominantly noncycling (7, 23), or because their metabolic state might convey resistance to some drugs. Our previous results have demonstrated *in vivo* resistance of hypoxic cells in the 16/C mouse mammary tumor to Adriamycin (26), while reports of limited penetration of Adriamycin and other drugs into multicellular spheroids *in vitro* (11, 22, 29) suggest that hypoxic cells in solid tissue may escape drug toxicity because of a limited drug concentration in their environment. These results raise the possibility of improving therapeutic index by combining radiation or some conventional anticancer drugs with agents that are selectively toxic for hypoxic cells.

The glucose analogues 2-DG³ and 5-TG are small water-soluble molecules that should penetrate rapidly into tumor tissue and which are known to have selective toxicity for hypoxic cells *in vitro* (8, 14, 18, 20, 21). The drugs inhibit glycolysis at several steps including uptake of glucose into the cell, competition for hexokinase, and competition by 2-deoxy-D-glucose 1-phosphate and 5-thio-D-glucose 1-phosphate for the isomerase which catalyzes the conversion of glucose 1-phosphate to glucose 6-phosphate (2, 10, 30). The drugs have

been found to exert minor effects on the growth of some experimental tumors when used alone (1, 9, 17, 18) and at concentrations above 5 mM led to selective toxicity for hypoxic cells in multicellular tumor spheroids (20, 21). When used with radiation and hyperthermia, 5-TG was reported to prolong survival of mice bearing a transplanted tumor (19).

Drugs which have specific toxicity for hypoxic cells would be expected to have only minor effects when used alone to treat solid tumors but might demonstrate considerable activity when used after a dose of radiation sufficient to kill most of the aerobic cells. Hypoxic cell toxicity *in vivo* by 2-DG and 5-TG may be expected to depend on duration of drug availability and on their uptake into cells; insulin might be expected to influence drug toxicity by stimulating uptake into cells or by lowering glucose concentration (15). We therefore report a study of 2-DG and 5-TG used to treat 2 murine tumors; we have administered the drugs as single or multiple doses with and without insulin and have given the drugs either alone or in addition to tumor irradiation.

MATERIALS AND METHODS

Two syngeneic transplantable tumors, the KHT fibrosarcoma and the 16/C mammary adenocarcinoma (3, 6, 25), have been maintained by serial transplantation of tumor pieces in the flanks of male C3H/HeJ mice with reestablishment from a stock of frozen tumor cells at approximately 6-month intervals. For generation of tumors to be used in experiments which assessed the effect of treatment on tumor growth, a single-cell suspension was prepared by mechanical means (28), and approximately 2×10^5 cells were injected in 0.05 ml 0.9% NaCl solution into the muscle of the left hind leg of recipient mice. For both tumors, palpable masses appeared 10 to 12 days later, and tumor diameter was estimated to the nearest 0.5 mm by passing the tumor-bearing leg through a series of graded holes drilled in lucite. Mice were coded with ear tags and, when tumors attained a mean diameter of ~9 mm (tumor weight, ~0.3 g), the mice were randomized into groups of 7 to 8 animals for treatment.

The drugs 2-DG and 5-TG (Sigma Chemical Co., St. Louis, Mo.) were dissolved in 0.9% NaCl solution, and appropriate dilutions were injected i.p. in volumes of 0.01 to 0.04 ml/g body weight. Some animals received i.p. injections of regular insulin (Connaught Laboratories, Toronto, Ontario, Canada) given at the same time as the glucose analogues. Groups of mice received either single injections of drug(s) or a course of 6 injections given at hourly intervals. To seek evidence for specific toxicity to hypoxic cells, a dose of 15 grays irradiation was given immediately before a single injection of 2-DG or 5-TG (\pm insulin) or between the third and fourth dose in experiments using 6 hourly injections of drug; most cells surviving this dose of irradiation are expected to be hypoxic (5, 26, 27). Local tumor irradiation was undertaken by using a specially designed double-headed 100-kV X-ray machine at a dose rate of 11.4 grays/min (16). For irradiation, awake mice were restrained in a Lucite container, and the tumor-bearing leg was secured with tape in the radiation field (16).

For assessment of treatment on tumor growth, mice were reassigned

¹ Supported by a Research Grant from the National Cancer Institute of Canada and funds from the Ontario Cancer Treatment and Research Foundation.

² To whom requests for reprints should be addressed.

³ The abbreviations used are: 2-DG, 2-deoxy-D-glucose; 5-TG, 5-thio-D-glucose.

Received August 9, 1982; accepted November 29, 1982.

to their original cages, and tumor diameter was estimated 3 times weekly by an observer who was unaware of their treatment history. Mice were killed humanely when their tumors attained a mean diameter of ~15 mm. Tumor growth curves were constructed from a previously defined calibration curve relating tumor weight to mean tumor diameter. The end point used to determine response to treatment was mean delay in time to grow to 1 g (mean diameter, ~12.5 mm) as compared to control animals.

In some experiments with the KHT tumor, we used an *in vivo* clonogenic assay to assess cell survival. To seek evidence for either toxicity or radiosensitization of hypoxic cells by 5-TG, groups of 3 to 5 mice bearing tumors in both flanks received various i.p. doses of the drug given 45 min before 25 grays whole body irradiation. Irradiation was delivered to nonanesthetized mice using ^{137}Cs γ -rays at a dose rate of 0.9 grays/min (4). Mice were killed either immediately or 24 hr after radiation, tumors were removed and pooled from each group, single-cell suspensions were prepared (28), and cell survival was assayed by using an *in vivo* lung colony assay (6).

RESULTS

Toxicity of Glucose Analogues with or without Insulin. The 50% lethal dose values in mice for single doses of 2-DG and 5-TG were reported to be about 4.5 and 5.5 g/kg body weight, respectively (9, 19). We observed that doses of either drug (2 g/kg) caused a period of somnolence for about 0.5 hr after injection, but all of the mice recovered subsequently. A higher dose of 5-TG (4 g/kg) caused disorientation and seizures in all of the mice during the first hr after injection, but most of them survived. We also gave 6 injections of the drugs at hourly intervals; 6 doses of 500 mg/kg body weight of either drug led to effects of somnolence and inactivity, but all animals survived without seizures. In experiments designed to assess drug effects on tumor growth, we used a single dose of 2 g/kg, and 6 multiple hourly doses of 500 mg/kg for both drugs.

We did not measure blood glucose, but mice tolerated single i.p. doses of 5 units insulin alone, or 6 doses of 1 unit insulin given at hourly intervals, without apparent side effects.

In several experiments, insulin was injected at the same time as the glucose analogues and was found to increase their toxicity. For 2-DG, the effect was relatively small, with 7 of 34 mice (20%) dying at short intervals after a single dose of 2-DG (1 g/kg) plus 5 units insulin. Insulin had a much larger effect on the toxicity of 5-TG and necessitated a reduction in dosage of 5-TG by more than 100-fold. Seizures and death were observed at single doses as low as 5-TG (20 mg/kg) plus 5 units insulin, and we selected a single dose of 5-TG (10 mg/kg) plus 5 units insulin for treatment-related experiments. Multiple dose experiments were performed using 6 injections at hourly intervals of 1 unit insulin plus 2-DG (75 mg/kg) or 5-TG (5 mg/kg). These doses were tolerated, but higher doses led to seizures and/or death.

Response of Tumors to Treatment. The effects of treatment on growth of the KHT and 16/C tumors are summarized in Tables 1 and 2, and representative tumor growth curves are shown in Chart 1. Untreated tumors grew from a mean diameter of 9 mm (~0.3 g) to 12.5 mm (~1.0 g) in 4 to 5 days, and this was not prolonged significantly by single or multiple doses of 2-DG or 5-TG used with or without insulin.

Local tumor irradiation with a dose of 15 grays delayed tumor growth by about 5 and 7 days for the KHT and 16/C tumors respectively, and most of the cells surviving this dose

Table 1
Influence of treatment with 2-DG and 5-TG with or without insulin on growth of the untreated or irradiated KHT sarcoma

Drug	Dose and schedule	Mean delay in growth to 1 g (days) ^a	
		Without radiation	With 15 grays radiation
2-DG	2 g/kg	0.9 ± 0.6 ^b	-0.1 ± 0.8
	1 g/kg + 5 units insulin	1.1 ± 0.7	0.2 ± 0.9
	500 mg/kg q 1 hr × 6 ^c	0.9 ± 0.7	-0.1 ± 1.0
	75 mg/kg + 1 unit insulin q 1 hr × 6	0.5 ± 0.7	1.6 ± 0.9
5-TG	2 g/kg	0.0 ± 0.3	0.2 ± 1.0
	10 mg/kg + 5 units insulin	0.0 ± 0.3	1.8 ± 1.3
	500 mg/kg q 1 hr × 6	0.3 ± 0.4	1.7 ± 1.7
	5 mg/kg + 1 unit insulin q 1 hr × 6	0.0 ± 0.4	-0.4 ± 0.6

^a Growth delay is expressed as mean of the differences for groups of 5 to 8 animals receiving the designated treatments and controls receiving 0.9% NaCl solution or radiation alone. Radiation (15 grays) delayed growth by 5.4 ± 0.6 and 4.7 ± 1.1 days in 2 separate experiments.

^b Mean ± S.E.

^c q 1 hr × 6, hourly for 6 hr.

Table 2
Influence of treatment with 2-DG and 5-TG with or without insulin on growth of the untreated or irradiated 16/C carcinoma

Drug	Dose and schedule	Mean delay in growth to 1 g (days) ^a	
		Without radiation	With 15 grays radiation
2-DG	2 g/kg	-0.7 ± 0.8 ^b	0.7 ± 2.5
	1 g/kg + 5 units insulin	0.2 ± 0.4	-1.2 ± 2.4
	500 mg/kg q 1 hr × 6 ^c	0.7 ± 0.6	-0.4 ± 1.9
	75 mg/kg + 1 unit insulin q 1 hr × 6	-0.6 ± 0.6	1.5 ± 2.1
5-TG	2 g/kg	1.5 ± 0.5	0.9 ± 0.9
	10 mg/kg + 5 units insulin	0.6 ± 0.4	1.5 ± 1.2
	500 mg/kg q 1 hr × 6	0.1 ± 0.4	0.3 ± 1.1
	5 mg/kg + 1 unit insulin q 1 hr × 6	0.4 ± 0.5	1.0 ± 1.0

^a Growth delay is expressed as mean of the difference for groups of 5 to 9 animals receiving the designated treatments and controls receiving 0.9% NaCl solution or radiation alone. Radiation (15 grays) delayed growth by 7.2 ± 1.8 and 8.4 ± 0.7 days in 2 separate experiments.

^b Mean ± S.E.

^c q 1 hr × 6, hourly for 6 hr.

are presumed to be hypoxic immediately after radiation. This delay in irradiated tumor growth was not prolonged by 2-DG or 5-TG (± insulin) in any of the doses and schedules used. Thus, we were unable to detect evidence for killing of hypoxic cells in these 2 murine tumors using a regrowth assay.

A limited number of experiments were performed to assess the effects of single doses of 5-TG on the radiation response of the KHT tumor using a clonal assay. Drug doses up to 2.5 g/kg given 45 min before 25 grays irradiation had no effect on cell survival assessed immediately after irradiation (Chart 2a) or 24 hr later (Chart 2b). These results are in agreement with those obtained from the regrowth assay and fail to show an effect of 5-TG on hypoxic cells of the KHT tumor.

DISCUSSION

Our 2 experimental tumors have been shown to contain cells that are radiobiologically hypoxic (5, 26). Hypoxic cells are spared by radiation and may also survive treatment with some

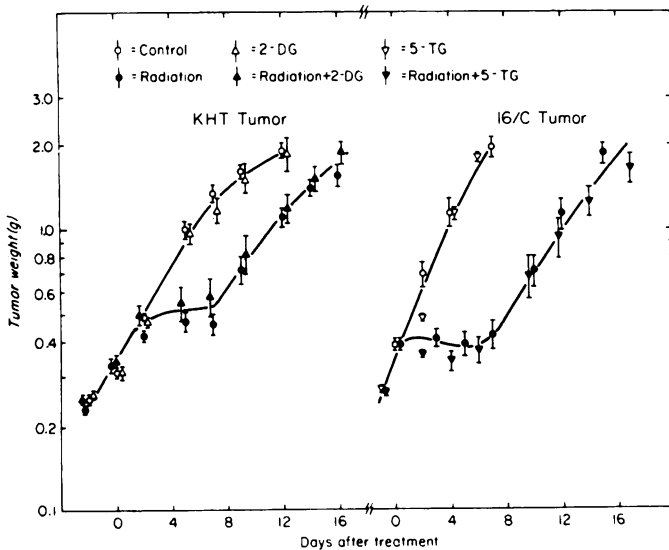


Chart 1. Growth curves for KHT sarcoma treated with 0.9% NaCl solution, radiation (15 grays), and/or multiple doses of 2-DG (500 mg/kg \times 6) and 16/C carcinoma treated with 0.9% NaCl solution, radiation (15 grays), and/or multiple doses of 5-TG (500 mg/kg hourly for 6 hr). Points, mean groups of 7 to 8 mice; bars, S.E.

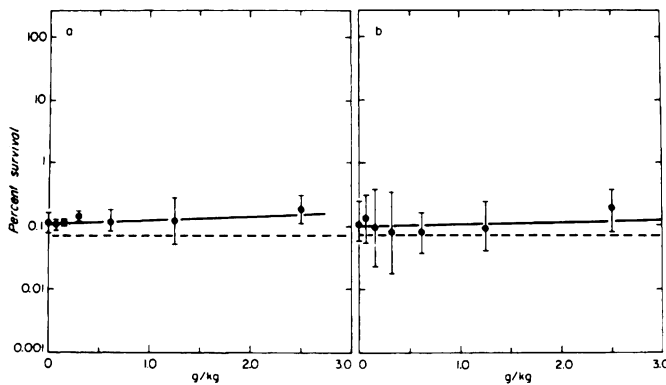


Chart 2. Survival of KHT tumor cells determined by lung colony assay after injection into tumor-bearing C3H mice of graded doses of 5-TG followed 45 min later by 25 grays irradiation. Assay was performed immediately (a) or 24 hr after radiation (b). Percentage of survival is expressed relative to unirradiated control cells. ---, the expected survival of KHT tumor cells following 25 grays radiation based on previous work; Points, mean from 4 experiments; bars, S.E.

anticancer drugs. Thus, there is potential for improving therapeutic index by using agents that have selective toxicity for hypoxic and poorly nourished cells in combination with radiotherapy or chemotherapy. Such drugs might have minimal activity when used alone because eradication of all severely hypoxic cells in tumors [typically $\leq 30\%$ (24)] would be expected to have only small effects on tumor growth and animal survival. Drugs that kill only hypoxic cells would fail the currently used screens for active anticancer drugs but should demonstrate considerable activity when used with radiation.

The glucose analogues 2-DG and 5-TG are known to be selectively toxic to hypoxic cells *in vitro* and in spheroids (8, 18, 20, 21), but we were unable to demonstrate significant toxicity for hypoxic cells in 2 murine tumors with the dosage and schedules used. The addition of insulin had a profound effect on the toxicity of 5-TG, presumably by stimulating drug uptake into cells and/or by reduction of serum glucose (15);

however, insulin led to no therapeutic benefit. Our failure to observe hypoxic cell toxicity at tolerated doses *in vivo* could have been due to an inadequate concentration \times time in hypoxic regions of the tumors. *In vitro* killing of hypoxic cells at 37° has generally required drug concentration in excess of 5 mM maintained for several hours (14, 20, 21), although shorter incubation times are sufficient at higher temperature (8). If the glucose analogues were freely diffusible in body fluids, large single doses of 2 g/kg body weight would be expected to achieve an initial concentration of the order of 10 mM in serum, but rapid recovery of mice from the neurological side effects implies rapid elimination of drug. Chronic administration of 2-DG or 5-TG for several days (e.g., in the drinking water) might have greater potential for killing hypoxic cells, but effects reported by others are modest (1, 9, 17, 18).

Another possible explanation for lack of *in vivo* effectiveness of these glucose analogues is that the time spent by cells in the hypoxic cell compartment is short compared to the drug exposure time needed for cell killing. This could arise from a short transit time through this compartment or because of cycling of cells from a hypoxic to an aerobic state (13, 23). Reoxygenation of hypoxic cells which survive tumor irradiation may also occur within hr (27). *In vitro* experiments have shown that very low oxygen concentration is required for hypoxic cell toxicity of 5-TG (14) and that high levels of D-glucose greatly reduce this hypoxic cell toxicity (15). Thus, failure to observe killing of hypoxic cells by 5-TG may reflect the time-dependent oxygenation state of tumor cells as well as the level of sugars within these tumors.

Toxic doses of 2-DG and 5-TG give symptoms that are similar to those of insulin-induced hypoglycemia. Unfortunately, the central nervous system is sensitive to inhibition of glycolysis, and it seems unlikely that modification of dosage schedule of 2-DG and 5-TG will lead to large variations in relative toxicity to hypoxic tumor cells and to central nervous system for these small freely diffusible molecules. A more successful approach to *in vitro* toxicity of hypoxic cells will probably require inhibitors of glycolysis that have adequate diffusion to hypoxic tumor cells but which have limited penetration of the blood-brain barrier.

REFERENCES

- Ball, H. A., Wick, A. N., and Sanders, C. Influence of glucose antimetabolites on the Walker tumor. *Cancer Res.*, 17: 235-239, 1957.
- Chen, M., and Whistler, R. L. Action of 5-thio-D-glucose and its 1-phosphate with hexokinase and phosphoglucosmutase. *Arch. Biochem. Biophys.*, 169: 392-396, 1957.
- Corbett, T. H., Griswold, D. P., Jr., Roberts, B. J., Peckham, J. C., and Schabel, F. M., Jr. Biology and therapeutic response of a mouse mammary adenocarcinoma (16/C) and its potential as a model for surgical adjuvant chemotherapy. *Cancer Treat. Rep.*, 62: 1471-1488, 1978.
- Cunningham, J. R., Bruce, W. R., and Webb, H. P. A convenient ¹³⁷Cs unit for irradiating cell suspensions and small laboratory animals. *Phys. Med. Biol.*, 10: 381-384, 1965.
- Hill, R. P. Radiation-induced changes in the *in vivo* growth rate of KHT sarcoma cells: implications for the comparison of growth delay and cell survival. *Radiat. Res.*, 83: 99-108, 1980.
- Hill, R. P., and Bush, R. S. A lung colony assay to determine the radiosensitivity of the cells of a solid tumor. *Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med.*, 15: 435-444, 1969.
- Hirst, D. G., and Denekamp, J. Tumour cell proliferation in relation to the vasculature. *Cell Tissue Kinet.*, 12: 31-42, 1979.
- Kim, S. H., Kim, J. H., and Hahn, E. W. Selective potentiation of hyperthermic killing of hypoxic cells by 5-thio-D-glucose. *Cancer Res.*, 38: 2935-2938, 1978.

9. Laszlo, J., Humphreys, S. R., and Goldin, A. Effects of glucose analogs (2-deoxy-D-glucose, 2-deoxy-D-galactose) on experimental tumors. *J. Natl. Cancer Inst.*, 24: 267-281, 1960.
10. Markoe, A. M., Risch, V. R., Emrich, J., Fala, S., and Brady, L. W. Tissue distribution and retention of 5-thio-D-glucose in animal tumor models. *In: L. W. Brady (ed.), Radiation Sensitizers*, pp. 287-295. New York: Masson Publishing USA Inc., 1980.
11. Nederman, T., Carlsson, J., and Malmquist, M. Penetration of substances into tumor tissue—a methodological study on cellular spheroids. *In Vitro (Rockville)*, 17: 290-298, 1981.
12. Ozols, R. F., Locker, G. Y., Doroshow, J. H., Grotzinger, K. R., Myers, C. E., and Young, R. C. Pharmacokinetics of Adriamycin and tissue penetration in murine ovarian cancer. *Cancer Res.*, 39: 3209-3214, 1979.
13. Rauth, A. M., Paciga, J. E., and Mohindra, J. K. *In vivo* studies of the cytotoxicity of hypoxic cell radiosensitizers. *In: L. W. Brady (ed.), Radiation Sensitizers*, pp. 207-214. New York: Masson Publishing USA Inc., 1980.
14. Schulz, R. J., and Bongiorno, P. J. The effect of oxygen tension and temperature on the toxicity of 5-thio-D-glucose to mammalian cells. *Radiat. Res.*, 83: 483, 1980.
15. Schulz, R. J., and Bongiorno, P. J. Antagonistic action of D-glucose to the selective toxicity of 5-thio-D-glucose in hypoxic cells. *Radiat. Res.*, 91: 373, 1982.
16. Siemann, D. W., Bronskill, M. J., Hill, R. P., and Bush, R. S. The relationship between mouse arterial partial pressure of oxygen (PaO₂) and the effectiveness of localized tumour irradiation. *Br. J. Radiol.*, 48: 662-667, 1975.
17. Sokoloff, H. B., Eddy, W. H., Saelhoff, C. C., and Beach, J. Glucose antagonists in experimental cancer. *Arch. Pathol. Lab. Med.*, 59: 729-732, 1955.
18. Song, C. W., Clement, J. J., and Levitt, S. H. Preferential cytotoxicity of 5-thio-D-glucose against hypoxic tumor cells. *J. Natl. Cancer Inst.*, 57: 603-605, 1976.
19. Song, C. W., Kang, M. S., Stettner, S. L., and Levitt, S. H. *In vivo* effects of 5-thio-D-glucose on tumor. *In: L. W. Brady (ed.), Radiation Sensitizers*, pp. 296-301. New York: Masson Publishing USA Inc., 1980.
20. Song, C. W., Sung, J. H., Clement, J. J., and Levitt, S. H. Cytotoxic effect of 5-thio-D-glucose on chronically hypoxic cells in multicell spheroids. *Br. J. Cancer*, 37(Suppl. 3): 136-140, 1978.
21. Sridhar, R., Koch, C. J., Stroude, E. C., and Inch, W. R. Cell survival in V79 multicell spheroids treated with dehydroascorbate, 5-thio-D-glucose and 2-deoxy-D-glucose. *Br. J. Cancer*, 37(Suppl. 3): 141-144, 1978.
22. Sutherland, R. M., Eddy, H. A., Bareham B., Reich, K., and Vanantwerp, D. Resistance to Adriamycin in multicellular spheroids. *Int. J. Radiat. Oncol. Biol. Phys.*, 5: 1225-1230, 1979.
23. Tannock, I. F. The relation between cell proliferation and the vascular system in a transplanted mouse mammary tumour. *Br. J. Cancer*, 22: 258-273, 1968.
24. Tannock, I. F. Oxygen diffusion and the distribution of cellular radiosensitivity in tumours. *Br. J. Radiol.*, 45: 515-524, 1972.
25. Tannock, I. F. *In vivo* interaction of anti-cancer drugs with misonidazole or metronidazole: methotrexate, 5-fluorouracil and Adriamycin. *Br. J. Cancer*, 42: 861-870, 1980.
26. Tannock, I. F. Response of aerobic and hypoxic cells in a solid tumor to Adriamycin and cyclophosphamide and interaction of the drugs with radiation. *Cancer Res.*, 42: 4921-4926, 1982.
27. Thomlinson, R. H. Changes of oxygenation in tumours in relation to irradiation. *Front. Radiat. Ther. Oncol.*, 3: 109-121, 1968.
28. Thomson, J. E., and Rauth, A. M. An *in vitro* assay to measure the viability of KHT tumor cells not previously exposed to culture conditions. *Radiat. Res.*, 58: 262-276, 1974.
29. West, G. W., Weichselbaum, R., and Little, J. B. Limited penetration of methotrexate into human osteosarcoma spheroids as a proposed model for solid tumor resistance to adjuvant chemotherapy. *Cancer Res.*, 40: 3665-3668, 1980.
30. Wick, N. R., Drury, R., Naxada, H. L., and Wolf, J. B. Localization of the primary metabolic block produced by 2-DG. *J. Biol. Chem.*, 244: 963-969, 1957.