

BIOLOGY CONTRIBUTION

ENHANCED RESPONSE OF HUMAN HEAD AND NECK CANCER XENOGRFT TUMORS TO CISPLATIN COMBINED WITH 2-DEOXY-D-GLUCOSE CORRELATES WITH INCREASED ¹⁸F-FDG UPTAKE AS DETERMINED BY PET IMAGING

ANDREAN L. SIMONS, PH.D.,* MELISSA A. FATH, R.Ph., PH.D.,* DAVID M. MATTSON, M.D.,*
BRIAN J. SMITH, PH.D.,† SUSAN A. WALSH, M.A.,* MICHAEL M. GRAHAM, M.D., PH.D.,‡
RICHARD D. HICHA, PH.D.,‡ JOHN M. BUATTI, M.D.,* KEN DORNFELD, M.D., PH.D.,*
AND DOUGLAS R. SPITZ, PH.D.*

Departments of *Radiation Oncology, †Biostatistics, and ‡Radiology, The Holden Comprehensive Cancer Center, University of Iowa, Iowa City, IA

Purpose: To determine whether the response of human head and neck cancer xenografts to cisplatin (CIS) could be enhanced with 2-deoxy-D-glucose (2DG); whether 2-[¹⁸F]-fluoro-2-deoxy-D-glucose (FDG) uptake correlated with responses to this drug combination; and whether 2DG would enhance CIS-induced radiosensitization.

Methods and Materials: Clonogenic survival responses to CIS + 2DG were determined in FaDu and Cal-27 cells and reduced/oxidized glutathione levels were monitored as parameters indicative of oxidative stress. The efficacy of CIS + 2DG was determined in FaDu and Cal-27 xenografts, and FDG uptake was determined by using positron emission tomography.

Results: Use of CIS + 2DG enhanced cell killing of FaDu and Cal-27 cells compared with either drug alone while increasing the percentage of oxidized glutathione *in vitro*. Use of CIS + 2DG inhibited FaDu and Cal-27 tumor growth and increased disease-free survival compared with either drug alone. The Cal-27 tumors showed greater pretreatment FDG uptake and increased disease-free survival when treated with 2DG + CIS relative to FaDu tumors. Treatment with 2DG enhanced CIS-induced radiosensitization in FaDu tumor cells grown *in vitro* and *in vivo* and resulted in apparent cures in 50% of tumors.

Conclusions: These results show the enhanced therapeutic efficacy of CIS + 2DG in human head and neck cancer cells *in vitro* and *in vivo* compared with either drug alone, as well as the potential for FDG uptake to predict tumor sensitivity to 2DG + CIS. These findings provide a strong rationale for evaluating 2DG + CIS in combined-modality head and neck cancer therapy with radiation in a clinical setting. © 2007 Elsevier Inc.

Cisplatin, 2-Deoxy-D-glucose, Squamous cell carcinoma of the head and neck (HNSCC), 2-[¹⁸F]-fluoro-2-deoxy-D-glucose (FDG)—positron emission tomography (PET), Oxidative stress, Radiation.

INTRODUCTION

Squamous cell carcinoma of the head and neck (HNSCC) comprises 3–5% of all cancers in the United States and is diagnosed annually in 40,000 patients, with a 5-year survival rate of 56% (1). Management of locally advanced or recurrent HNSCC usually involves treatment with cisplatin (CIS) alone or in combination with other chemotherapeutic agents, radiotherapy, and/or surgery (2). Concurrent use of CIS and radiotherapy results in improved survival relative to radio-

therapy alone both in unresectable cases (3) and when used as adjuvant therapy after resection (4, 5). However, concurrent use of CIS-based chemotherapy leads to significant toxicity, including myelosuppression, nephrotoxicity, and enhanced toxicities of radiotherapy, including dysphagia and voice dysfunction (6, 7), decreased quality of life (8), and increased treatment-related deaths (4, 5). Therefore, combining CIS with agents selectively toxic to cancer cells has potential advantages, including use of lower CIS doses,

Reprint requests to: Douglas R. Spitz, Ph.D., Free Radical and Radiation Biology Program, Department of Radiation Oncology, B180 Medical Laboratories, University of Iowa, Iowa City, IA 52242. Tel: (319) 335-8019; Fax: (319) 335-8039; E-mail: douglas-spitz@uiowa.edu

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fewer side effects, loss of chemoresistance, and enhanced therapeutic response.

One common abnormal biochemical characteristic associated with cancer cells, including head and neck malignancies, is increased intracellular use of glucose (9). We previously reported that glucose deprivation induced selective cytotoxicity and oxidative stress in transformed human cells vs. normal cells (10). Additionally, increased prooxidant production and disruptions in thiol metabolism consistent with metabolic oxidative stress were noted in cancer cells during glucose deprivation or when treated with the glycolytic inhibitor 2-deoxy-D-glucose (2DG) (11). The 2DG-induced cytotoxicity and increases in parameters indicative of oxidative stress were inhibited by the thiol antioxidant *N*-acetylcysteine (12, 13), as well as overexpression of enzymes that scavenge such reactive oxygen species as superoxide and hydrogen peroxide (13). These results led to the hypothesis that glucose deprivation in cancer cells causes metabolic oxidative stress by limiting hydroperoxide metabolism (10, 11). Although the mechanisms responsible for increased glucose metabolism in cancer cells are not fully understood, this phenomenon proved useful in locating metabolically active cancer cells based on preferential uptake of 2-[¹⁸F]-fluoro-2-deoxy-D-glucose (FDG) coupled with positron emission tomography (PET) imaging (14, 15). The FDG-PET is now a standard test in patients with HNSCC for staging pretreatment (16) and surveillance or restaging posttreatment (17). Uptake of FDG-PET also has prognostic value because several groups reported poor survival for patients with tumors showing high pretreatment FDG uptake (18, 19).

We propose that inhibition of glucose metabolism with 2DG would cause increased oxidative stress and cytotoxicity, thereby sensitizing cancer cells (relative to normal cells) to conventional cancer therapies that increase oxidative stress (*i.e.*, radiation and some chemotherapies). Following the same logic, we propose that the relative increase in pretreatment FDG uptake will be proportional to the degree of 2DG-induced chemosensitization.

The current study identifies 2DG + CIS with or without radiation as an effective antitumor combination *in vivo* in FaDu and Cal-27 human xenograft tumors in mice. Pretreatment FDG uptake in FaDu and Cal-27 xenograft tumors also was determined, and increased uptake correlated with improved tumor responses to 2DG + CIS.

METHODS AND MATERIALS

Cells and culture conditions

FaDu and Cal-27 human HNSCC cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium containing 4.5 g/L of glucose, 4 mM of L-glutamine, and 1 mM of sodium pyruvate (FBS; Hyclone, Logan, UT). Cultures were maintained in 5% carbon dioxide and humidified in a 37°C incubator. Experiments were performed with cells from Passages 3–20.

In vitro drug treatment and clonogenic cell survival experiments

The 2DG was obtained from Sigma Chemical Co. (St. Louis, MO). *Cis*-diamminedichloroplatinum(II) (CIS) was obtained from Bedford Laboratories (Bedford, OH). Drugs were added to cells at 20 mM of 2DG and 0.5 μM of CIS. Cells were placed in a 37°C incubator and harvested at the times indicated. Clonogenic cell survival experiments were performed as described previously (13).

In vitro radiation treatment

Radiation was delivered using a J.L. Shepherd cesium irradiator (J.L. Shepherd, San Fernando, CA) with a dose rate of 0.805 Gy/min. Cells were irradiated at 2 Gy at room temperature at the end of 2DG and/or CIS drug treatment. Cells were plated for clonogenic survival immediately after radiation.

Glutathione assay

Total glutathione content was determined by using the method of Anderson (20). Reduced (GSH) and oxidized glutathione (GSSG) were distinguished as described previously (21). All glutathione determinations were normalized to the protein content of whole homogenates by using the method of Lowry *et al.* (22).

Tumor cell implantation

Eighty female 4–5-week-old athymic-nu/nu nude mice were purchased from Harlan Laboratories (Indianapolis, IN). All mice were housed in a pathogen-free barrier room in the Animal Care Facility at the University of Iowa and handled using aseptic procedures. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Iowa and conformed to the guidelines established by the National Institutes of Health. Mice were allowed 3 days to acclimate before beginning experimentation, and food and water were made freely available. Tumor cells were inoculated into mice by subcutaneous injection of 0.1-ml aliquots of sterile saline containing 4×10^6 FaDu cells or 8×10^6 Cal-27 cells into the right flank using 26-G needles.

Tumor measurements

The first measurable tumor appearance was considered when individual tumor volumes measured 0.01 cm³. Mice were evaluated daily, and tumor measurements were obtained three times weekly using Vernier calipers. Tumor volumes were calculated by using the formula: tumor volume = (length × width²)/2, where length was the longest dimension and width was the dimension perpendicular to length. Mice were killed by means of carbon dioxide gas asphyxiation or lethal overdose of sodium pentobarbital (100 mg/kg) when tumor length exceeded 1.5 cm in any dimension.

In vivo drug administration

The mentioned nude mice were divided into five groups ($n = 6$ –12 mice/group). In the 2DG group, 2DG was dissolved in saline and administered intraperitoneally (*i.p.*), 0.5 g/kg every other day, for six total doses during 2 weeks (Days 1, 3, 5, 8, 10, and 12). In the CIS group, CIS was dissolved in saline and administered *i.p.*, 2 mg/kg every other day, for six total doses during 2 weeks. In the 2DG + CIS group, mice were administered *i.p.* 0.5 g/kg of 2DG plus 2 mg/kg of CIS every other day for six total doses during 2 weeks. In the 2DG + CIS* group, mice were administered *i.p.* 0.5 g/kg of 2DG every day (weekends off) plus 6 mg/kg of CIS on Days 2 and 9 of treatment for a total of ten 2DG doses and two CIS doses during 2 weeks. In the control group, mice were administered *i.p.*

saline every other day. Treatment began approximately 2 weeks after tumor inoculation.

Radiation treatment

FaDu tumor cells (4×10^6) were inoculated into 32 mice by subcutaneous injection into the right flank. Mice were divided into five groups ($n = 5-6$ mice/group) and treated with 2DG and/or CIS as mentioned. Mice were anaesthetized with ketamine and xylazine mix i.p. containing ketamine, 87.5 mg, and xylazine, 12.5 mg/kg of body weight. Tumor tissue was exposed to fractionized ionizing radiation (FIR) in 2-Gy fractions (250-kVp x-rays filtered with 0.25 mm of Cu and 0.25 mm of aluminum from a Pantak Therapax DXT 300 x-ray machine [Elimpex, Modling, Austria]) after drug treatment twice weekly for 2 weeks (Days 3, 5, 10, and 12) for a total dose of 8 Gy (2-Gy fractions four times). Dosimetry was confirmed by using a Victoreen R meter (Victoreen Instrument Company, Cleveland, OH). The remainder of the body was shielded in a lead box to reduce exposure of normal tissues.

FDG-PET imaging of FaDu xenografts

On the day of imaging, food was removed 4 hours before injection of FDG. Blood glucose levels were checked before injection of FDG by using a Therasense Freestyle Glucometer (Abbott, Alameda, CA) and were determined to be within normal limits. Conscious mice (mildly sedated with midazolam, 5 mg/kg i.p.) were injected through the tail vein with 23 ± 6.7 MBq of FDG in 0.2 ml and returned to their cage for a 30-minute uptake period with access to drinking water. After the labeled-FDG uptake period, the mouse was placed in a temperature-controlled imaging holder. The mouse underwent imaging in a darkened and quiet environment. Mouse body temperature was maintained, and warmed oxygen was administered during acquisition. The imaging holder was affixed and remotely translated into the center of the axial field of view of the Philips MOSAIC animal PET scanner (12 cm) (Philips Medical Systems, Milpitas, CA). The entire mouse was positioned within the sensitive region of the scanner. Images (three frames at 5 minutes each) from one bed position were acquired because the MOSAIC scanner has an axial field of view larger than the typical nude mouse longitudinal body dimension. A total of 240 transaxial slices of 0.5 mm were reconstructed, spanning the total body of the mouse.

Quantitative image analysis

Transaxial slices were reconstructed by means of a three-dimensional algorithm using projection data corrected for random coincidences, scatter, and dead time. Three orthogonal views (transverse, sagittal, and coronal), as well as rotating projection images, were displayed postreconstruction. A commercial software package, MIMVISTA (Cleveland, OH), was used to obtain standardized uptake values (SUVs) from regions drawn on the coronal slices through the tumor and normal muscle on the contralateral side of the animal. The SUV of the tumor was determined from the maximum voxel within the tumor volume. This was compared with the SUV from the normal muscle region by using the average activity concentration in the muscle region.

Data analysis

To determine differences among three or more means, one-way analysis of variance with Bonferroni post tests were performed. Two-way analysis of variance was used to determine differences and interaction effects between cell lines and treatment groups in the *in vitro* experiments. Individual tumor measurements were seri-

ally recorded, and corresponding volumes were calculated for each mouse. Mean tumor volumes for all treatment groups were calculated by averaging all individual tumor volumes per measurement date. Survival curves were estimated by using the Kaplan-Meier method. Disease-free survival was defined as no evidence of tumor at the time of death and an apparent cure of the disease. Cox proportional hazards regression was used to estimate and compare survival across treatment regimens and cell lines. To determine statistical differences in tumor volumes over time, a generalized linear model was fit to the data. Model estimates were obtained by using the method of generalized estimating equations to account for the repeated tumor measurements for each subject (23). An autoregressive structure was specified for within-subject correlation in the generalized estimating equation analysis. All statistical tests were two sided and carried out at the 5% level of significance using the SAS statistical software package (SAS Institute, Cary, NC).

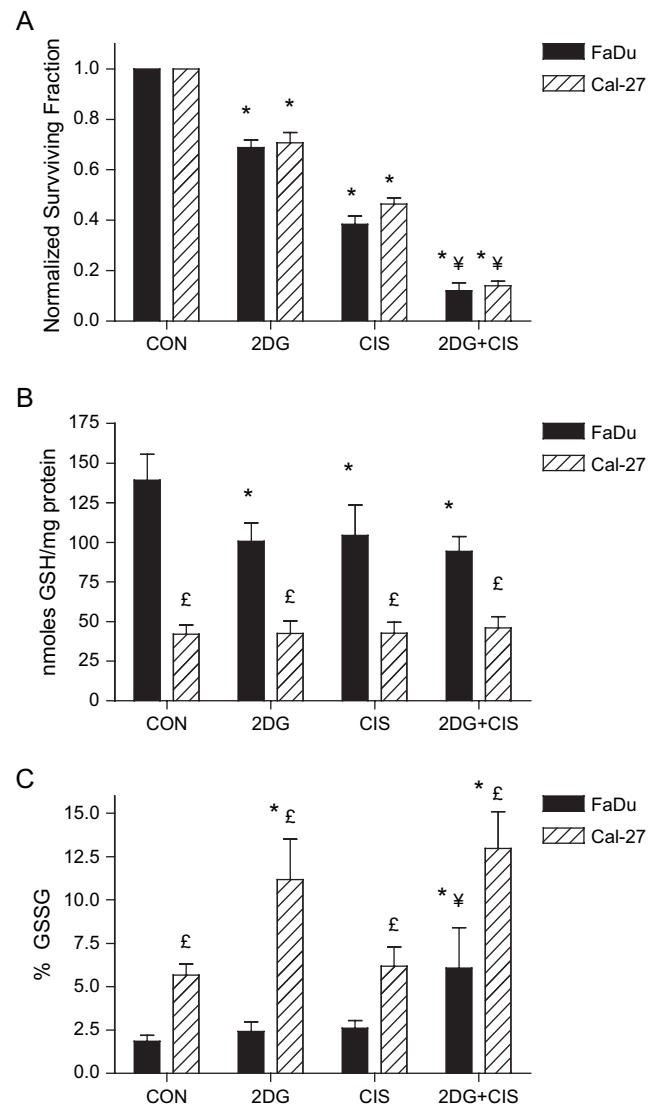


Fig. 1. Effect of 2-deoxy-D-glucose (2DG) and cisplatin (CIS) on (A) cytotoxicity, (B) total glutathione, and (C) percentage of oxidized glutathione (% GSSG) levels in FaDu and Cal-27 cells. Clonogenic cell-survival data were normalized to control (CON). Error bars represent the SEM of $N = 3$. * $p < 0.001$ vs. control; ‡ $p < 0.001$ vs. 2DG and CIS alone; £ $p < 0.001$ FaDu vs. Cal-27.

RESULTS

2DG-enhanced CIS-induced cytotoxicity in vitro

Treatment with 2DG caused 32% and 30% cell killing in FaDu and Cal-27 cells relative to untreated control cells, respectively ($p < 0.001$; Fig. 1A), whereas CIS caused 62% and 54% cell killing, respectively ($p < 0.01$; Fig. 1A). The combination of 2DG and CIS caused a significant increase in cell killing in both cell lines (88% and 86%, respectively; $p < 0.001$), showing an additive effect of 2DG and CIS (Fig. 1A).

Use of 2DG in combination with CIS induced disruptions in glutathione metabolism

FaDu cells showed a decrease in total GSH when treated with 2DG and CIS alone and in combination ($p < 0.01$; Fig. 1B) and a significant increase in percentage of GSSG when treated with the combination of 2DG and CIS (Fig. 1C) compared with untreated cells ($p < 0.01$). Cal-27 cells showed no changes in total GSH with drug treatment

(Fig. 1B), but significant increases in the percentage of GSSG with 2DG alone ($p < 0.001$) and the combination of 2DG and CIS ($p < 0.001$; Fig. 1C). These results suggest that the toxicity of 2DG in combination with CIS was mediated by disruptions in thiol metabolism consistent with oxidative stress.

Use of 2DG in combination with CIS inhibited growth of HNSCC xenografts

We examined *in vivo* activity of 2DG and CIS in FaDu and Cal-27 tumor-bearing athymic nude mice. The control and 2DG groups showed no differences in tumor growth for both FaDu and Cal-27 tumors ($p > 0.05$; Fig. 2A and 2B). Additionally, Cal-27 tumors did not show growth delay when treated with CIS ($p > 0.05$; Fig. 2B), whereas FaDu tumors showed a slight, but significant, growth delay ($p = 0.01$; Fig. 2A) when treated with CIS. The combination of 2DG and CIS showed pronounced inhibition of growth in both FaDu and Cal-27 tumors for both dosing schedules compared with control and 2DG alone ($p < 0.01$; Fig. 2A

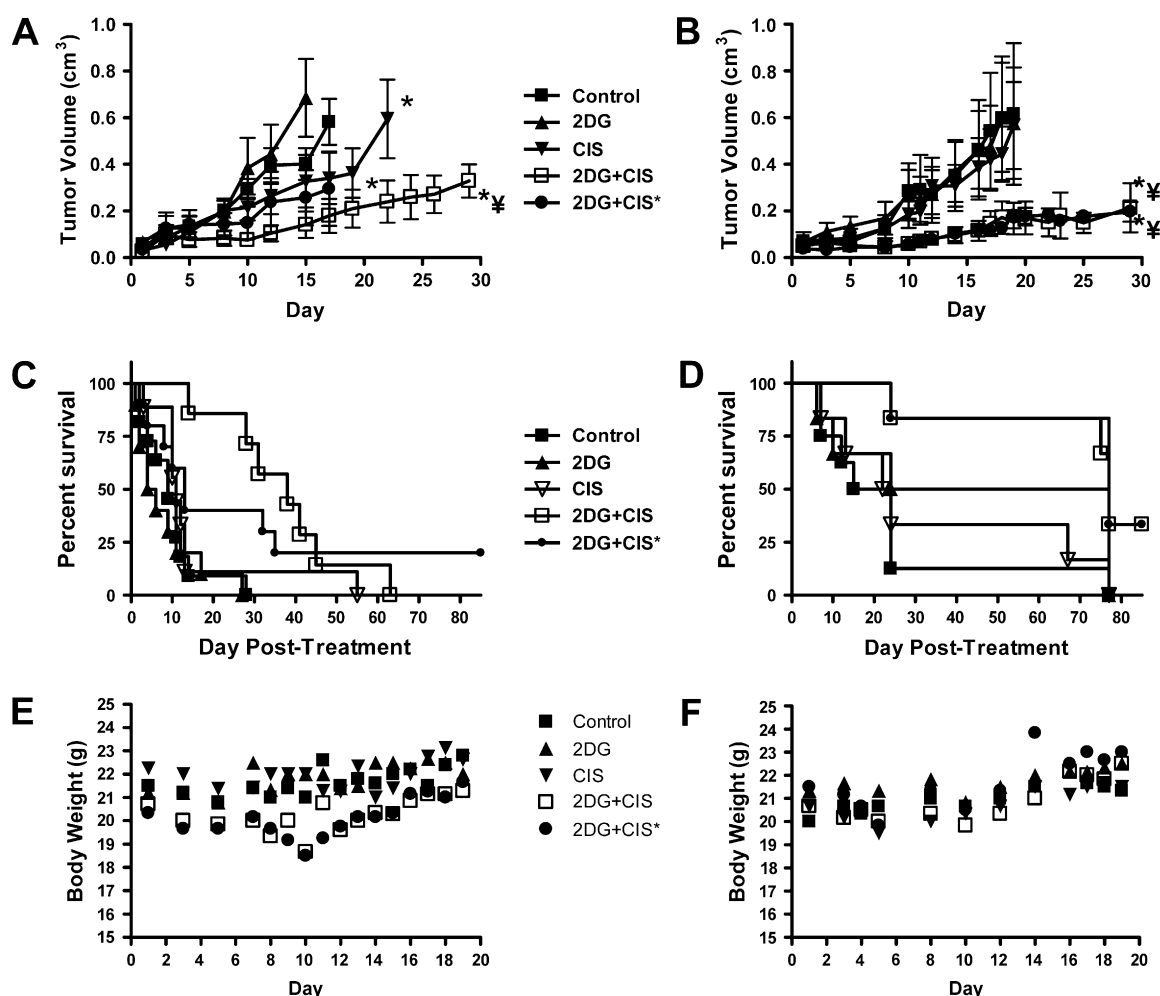


Fig. 2. Tumor growth curves for athymic (nu/nu) mice with (A) FaDu and (B) Cal-27 tumors treated with 2-deoxy-D-glucose (2DG) and cisplatin (CIS). * $p < 0.001$ vs. control; $\yenumber{p} < 0.001$ vs. 2DG and CIS alone. Data points represent average values for seven to 12 mice. Survival analyses of athymic nu/nu mice bearing (C) FaDu and (D) Cal-27. Body weight measurements of mice bearing (E) FaDu and (F) Cal-27 tumors.

and 2B). However, the combination of 2DG + CIS*, where CIS was administered as two bolus doses of 6 mg/kg once weekly, showed significant growth delay in only Cal-27 tumors compared with CIS alone ($p = 0.02$; Fig. 2B). When CIS was administered in six doses of 2 mg/kg over the course of 2 weeks in combination with 2DG, both FaDu and Cal-27 showed significant inhibition of growth compared with CIS alone ($p < 0.01$; Fig. 2A and 2B). These results show that 2DG in combination with CIS caused significant inhibition of growth in HNSCC tumors *in vivo* compared with either agent alone, consistent with the *in vitro* data shown in Fig. 1A.

Use of 2DG in combination with CIS increased disease-free survival of mice bearing HNSCC tumors

There was no significant increase in overall disease-free survival of mice treated with 2DG or CIS alone compared with control mice regardless of tumor type ($p > 0.05$; Fig. 2C and 2D; Table 1). Mice bearing FaDu tumors treated with 2DG in combination with CIS had significantly longer median survival times than control ($p < 0.001$), 2DG-alone ($p < 0.001$) and CIS-alone groups ($p = 0.012$) when administered in the low daily dosing schedule (2DG + CIS; Fig. 2B; Table 1). Mice administered 2DG and CIS in the high weekly dosing schedule (2DG + CIS*) had significantly longer median survival times than control ($p = 0.002$) and 2DG-alone mice ($p < 0.00001$; Fig. 2C; Table 1). Interestingly, 2DG and CIS administered to FaDu tumor-bearing mice in the high weekly dosing schedule appeared to produce more disease-free survivors, yet led to shorter median survival ($p = 0.058$; Fig. 2C; Table 1). Conversely, mice bearing Cal-27 tumors treated with 2DG and the combination of 2DG and CIS had significantly longer median survival times compared with control mice regardless of the dosing schedule ($p < 0.05$; Fig. 2D; Table 1). The overall disease-free surviving fractions of the FaDu and Cal-27 tumor-bearing mice at 150 days posttreatment are listed in Table 1. There were no surviving mice at Day 150 posttreatment in the control, 2DG, or CIS-alone groups for both tumor types

Table 1. Survival analysis of athymic nu/nu mice bearing FaDu and Cal-27 tumors treated with 2DG and CIS at 150 days posttreatment

	Disease-free surviving fraction		Median survival (d)	
	FaDu	Cal-27	FaDu	Cal-27
Control	0 (0/12)	0 (0/6)	9	19.5
2DG	0 (0/11)	0 (0/6)	5	50.5* [†]
CIS	0 (0/11)	0 (0/6)	11	23
2DG + CIS	0 (0/7)	0.33 (2/6)	38 [‡]	77 [‡]
2DG + CIS*	0.22 (2/9)	0.33 (2/6)	13*	77 ^{†‡}

Abbreviations: 2DG = 2-deoxy-D-glucose; CIS = cisplatin.

Numbers in parenthesis indicate disease-free animals relative to total animals/group.

* $p < 0.01$ vs. control.

[†] $p < 0.05$ vs. FaDu.

[‡] $p < 0.05$ vs. 2DG and CIS alone.

(Table 1). However, 33% of Cal-27 tumor-bearing mice treated with 2DG in combination with CIS in both dosing schedules were disease free and alive at Day 150 posttreatment, whereas 22% of FaDu tumor-bearing mice were disease free and alive at 150 days when administered 2DG and CIS in the high weekly CIS dosing schedule despite the poor median survival observed for this dosing schedule (Table 1). There were no FaDu animals surviving at 150 days in the daily CIS dosing schedule (Table 1). When surviving fraction and median survival times were compared between the two tumor types (Table 1), mice with Cal-27 tumors showed greater disease-free surviving fractions when treated with the combination of 2DG + CIS at both dosing schedules at the termination of the experiment and longer median survival times when treated with 2DG and the combination of 2DG and CIS (Table 1). There were no significant changes in body weight of mice receiving 2DG and CIS alone or in combination during or after treatment (Fig. 2E and 2F). Collectively, all these data (Fig. 2; Table 1) indicate that 2DG and CIS increased overall survival for both tumor types, and mice bearing Cal-27 tumors appeared to respond better after 2DG-based chemotherapy than mice bearing FaDu tumors.

Comparison of pretreatment FDG-PET scans in FaDu and Cal-27 xenografts in vivo

Figure 3 shows representative PET images from mice bearing FaDu (Fig. 3A) and Cal-27 (Fig. 3B) tumors after injection of FDG. The images show that the tumors were clearly identified from such normal tissues as the bladder and other organs (Fig. 3A and 3B) to identify adequate regions of interest for quantitative analysis. The FDG distribution across normal tissues was consistent with expected rates of glucose uptake and label excretion (high in brain, brown fat, and bladder) and similar in mice bearing FaDu and Cal-27 tumors. The SUVs for FaDu and Cal-27 tumor types were determined for each tumor. When all FaDu and Cal-27 tumor SUVs were compared and matched according to smaller (0.01–0.1 cm³; Fig. 3C) and larger (0.2–0.4 cm³; Fig. 3D) tumors, it was clear that Cal-27 tumors showed greater average SUVs relative to FaDu tumors regardless of tumor size ($p = 0.015$). These data show that pretreatment uptake of FDG was greater in Cal-27 tumors relative to FaDu tumors, and differences in FDG uptake can be quantified by FDG-PET imaging in these model systems.

2DG and CIS-induced radiosensitization in vitro

To determine the effect of 2DG on CIS-induced radiosensitization in FaDu cells, 2 Gy caused 55% killing and 2DG + 2 Gy caused 67% cell killing (Fig. 4A). Use of CIS + 2 Gy caused 78% cell killing and 2DG + CIS + 2 Gy significantly increased cell killing to 93% in FaDu cells compared with all other treatments (Fig. 4A). These results show that CIS-induced radiosensitization could be enhanced further by 2DG in FaDu cells in at least an additive manner *in vitro*.

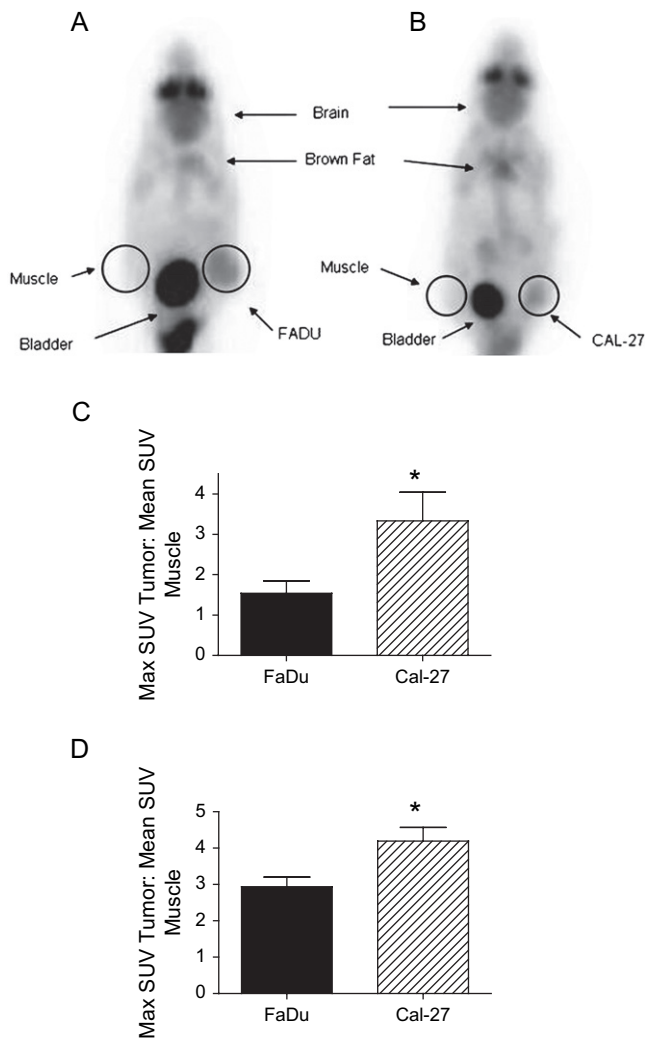


Fig. 3. Comparison of glucose uptake determined by using 2-[¹⁸F]-fluoro-2-deoxy-D-glucose (FDG)-positron emission tomography (PET) imaging of FaDu and Cal-27 xenografts. (A) FaDu and (B) Cal-27 tumor-bearing mice were imaged from each tumor type. Three tumor-bearing animals of each tumor type with average volumes of (C) 0.04 ± 0.03 and (D) 0.27 ± 0.05 cm³ underwent imaging using FDG-PET, and standardized uptake volumes (SUVs) were determined from regions overlaid on tumor (right flank) and contralateral muscle (left flank). Errors are ± 1 SD. * $p < 0.01$ vs. FaDu.

2DG and CIS-induced radiosensitization in vivo

Animals were treated with 2DG and CIS in protocols that were the same as in Fig. 2. Tumors treated with 2 Gy fractions four times (FIR) showed very little inhibition of growth compared with control tumors (Fig. 4B) in the first 30 days, and only one animal showed disease-free survival at 125 days when treated with this radiation dose (Fig. 4C; Table 2). Using this modest radiation dosing schedule, 2DG + FIR had no pronounced antitumor activity and was not significantly different from the control or FIR-alone groups (Fig. 4B and 4C; Table 2). Use of CIS + FIR significantly inhibited tumor growth and median survival (but not disease-free survival) compared with controls ($p < 0.01$; Fig. 4B and 4C; Table 2). The combination of 2DG + CIS + FIR administered in the lower daily dose CIS treatment

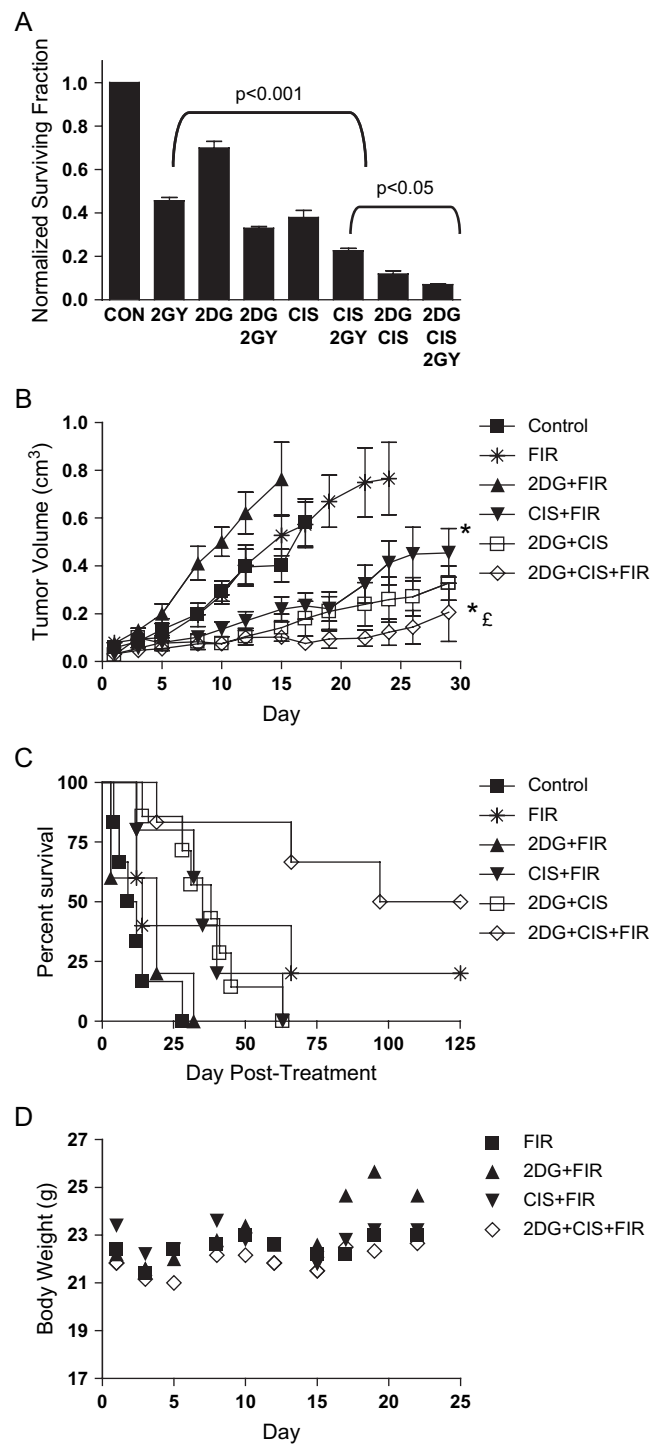


Fig. 4. 2-Deoxy-D-glucose (2DG) and cisplatin (CIS)-induced radiosensitization in FaDu cells (A) *in vitro* and (B) *in vivo*. Clonogenic cell survival data were normalized to control (CON). Error bars represent ± 1 SD of $N = 3$ experiments. Data points on tumor growth graphs represent average values for five to six mice. * $p < 0.001$ vs. respective treatment without 2GY; † $p < 0.01$ vs. CIS + 2 GY. (C) Survival analysis of athymic nu/nu mice bearing FaDu tumors and (D) body weight measurements of mice bearing FaDu tumors treated with fractionized ionizing radiation (FIR).

schedule significantly enhanced tumor growth inhibition, median survival, and overall disease-free survival (50%) compared with any other treatment group ($p < 0.05$;

Table 2. Survival analysis of athymic nu/nu mice bearing FaDu tumors treated with 2DG, CIS, and x-ray radiation (2 Gy) at 125 days posttreatment

	Disease-free surviving fraction	Median survival (d)
Control	0 (0/5)	10.5
FIR	0.2 (1/5)	14
2DG + FIR	0 (0/5)	19
CIS + FIR	0 (0/5)	35*
2DG + CIS	0 (0/6)	38*
2DG + CIS + FIR	0.50 [†] (3/6)	123.5 [†]

Abbreviations: 2DG = 2-deoxy-D-glucose; CIS = cisplatin; FIR = fractionized ionizing radiation.

Numbers in parenthesis indicate disease-free animals relative to total animals per group.

* $p < 0.001$ vs. control.

[†] $p < 0.001$ vs. CIS + 2 Gy.

Fig. 4B and 4C; Table 2) without showing significant changes in body weight (Fig. 4D). Interestingly, 2DG + CIS* + FIR administered in the higher weekly CIS dosing schedule was not as effective at inhibiting tumor growth, median survival, or disease-free survival (data not shown) as the lower dose CIS schedule combined with FIR, which is consistent with findings for FaDu tumors treated with 2DG + CIS without radiotherapy in Fig. 2C. These data show that 2DG is able to significantly enhance CIS-induced radiosensitivity and overall disease-free survival when administered in the low daily CIS dosing schedule without showing overt signs of morbidity and mortality.

DISCUSSION

Clinical trials showed that concomitant CIS/radiation therapy is effective in many HNSCC disease sites (3, 4). Concomitant chemoradiotherapy with CIS has emerged as the standard of care for locally advanced unresectable tumors for laryngeal preservation in appropriately selected patients (24). Currently, the most widely used standard regimen is 100 mg/m² of CIS every 3 weeks combined with radiation (5, 24). A common alternative is lower daily CIS dosing during radiotherapy for those unable to tolerate high-dose therapy (25, 26). Although this is an accepted standard, consistently high locoregional failure rates of 30–40% were reported, and this regimen caused severe toxic side effects, which include nephrotoxicity, ototoxicity, nausea, weight loss, and vomiting (5, 27). Consequently, multiagent chemoradiotherapy has been difficult to use because of the incidence of adverse effects. Cisplatin was combined with 5-fluorouracil (28), paclitaxel (29), and cetuximab (30) in various chemoradiotherapy regimens, which showed favorable results in overall patient survival, but frequently caused increased morbidity.

Several studies showed that 2DG enhanced the cytotoxic effects of therapeutic agents *in vitro*, such as tumor necrosis factor α in lymphoma cells (31); topoisomerase inhibitors such as etoposide, camptothecin, and Hoechst 3342 in

cerebral glioma cells (32); and L-buthionine-sulfoximine (BSO) in breast cancer cells (11). Consistent with these results, we show that 2DG significantly enhanced the cytotoxicity of CIS in HNSCC cell lines *in vitro* (Fig. 1A). In addition to *in vitro* data, we found similarly enhanced anti-tumor responses with HNSCC xenografts in nude mice in which the combination of 2DG and CIS significantly inhibited FaDu and Cal-27 tumor growth and increased overall disease-free survival compared with either agent alone (Fig. 2A–2D).

Efforts to decrease the toxicity of CIS-based regimens typically used more frequent dosing with lower doses to avoid high peak serum levels (29, 33). In an attempt to mimic these different types of CIS dosing strategies, we used two CIS dosing regimens; one with frequent small doses and another with less frequent, but higher, doses. Use of 2DG in combination with CIS was administered in low daily doses and high weekly dosing schedules. The lower daily dosing schedule was chosen because preliminary studies showed these doses to be well tolerated, whereas the high weekly dosing schedule was chosen for comparison to a common clinically relevant schedule. Although 2DG in combination with CIS in both dosing schedules resulted in significant tumor growth inhibition in both tumor types compared with control or tumors treated with 2DG only (Fig. 2A and 2B), 2DG + CIS in the high weekly dosing schedule (2DG + CIS*) did not inhibit growth as effectively in FaDu tumors as in Cal-27 tumors (Fig. 2A and 2B). The reason for this is not known at this time, but we speculate that CIS may be more bioavailable in the lower daily dosing schedule. Overall, these data strongly suggest that 2DG may be able to enhance the cytotoxic effects of chemotherapeutic agents currently used in the clinic that were suggested to exert their toxicity through oxidative stress.

Because CIS-based chemoradiotherapy is the standard of care for many patients with head and neck cancer, we determined whether the combination of 2DG with CIS plus radiation would improve anticancer responses *in vitro* and *in vivo*. Figure 4A shows in FaDu human head and neck cancer cells that 2DG + CIS + FIR significantly enhanced tumor growth inhibition *in vitro*, as well as increased overall disease-free survival *in vivo* (Fig. 4B and 4C; Table 2) compared with CIS + FIR. These results provide strong justification for pursuing the potential of 2DG to serve as a relatively non-toxic adjuvant to the standard of care (CIS + FIR) for patients with head and neck cancer.

Integration of 2DG into the therapy for patients with head and neck cancer exploits a basic difference between normal and cancer cells and has the advantage of being linked to imaging assessment in which treatment and imaging can be seamlessly integrated. Fundamental differences in glucose metabolism between transformed and normal cells are used clinically to image cancerous tissues by using tracer amounts of FDG with PET (15). The FDG uptake measurements using PET imaging suggested that glucose metabolism may correlate directly with degree of malignancy and resistance to treatment (16). Although the precise mechanisms responsible

for these relationships were not determined, there seemed to be strong correlations between glucose uptake, glycolysis, and treatment resistance in tumors (34). More specifically, tumors with lower FDG uptake tended to respond better to standard treatments than those with greater FDG uptake (18, 35). These results suggest that new adjuvants to chemoradiotherapy are needed to treat patients with head and neck tumors with high FDG uptake.

Tumors with high FDG uptake may represent tumors with high metabolic production of hydroperoxides and thus increased susceptibility to 2DG-induced radio-/chemosensitization. Using this logic, we predicted that 2DG should sensitize tumors with greater FDG uptake to a greater extent to agents that further increase hydroperoxide production and metabolic oxidative stress relative to low-FDG-uptake tumors. At all tumor sizes, Cal-27 tumors had significantly greater SUVs than FaDu tumors (Fig. 3A and 3B). Additionally, Cal-27 responded very well to 2DG + CIS in both dosing schedules, whereas FaDu only responded well to 2DG + CIS at the low dosing schedule with respect to tumor growth (Fig. 2A and 2B). In addition, more apparent differences between tumor types were noted in overall median survival times (Table 1). Differences were pronounced in the 2DG treatment group in which the Cal-27 tumor-bearing mice

had more than 10 times the median survival time compared with FaDu tumor-bearing mice (Table 1). Additionally, Cal-27 tumor-bearing animals had significantly longer median survival for 2DG + CIS in both dosing schedules (Table 1) relative to mice with FaDu tumors. Furthermore, overall disease-free survival in the Cal-27 tumor-bearing animals with 2DG + CIS was greater than that in animals with FaDu tumors treated in a similar fashion. This suggests that the increased FDG uptake in Cal-27 tumors correlated positively with greater sensitivity to 2DG and 2DG + CIS compared with FaDu tumors, which showed lower FDG uptake. These data support our hypothesis that HNSCC tumors with greater FDG uptake (determined *in vivo* by means of PET imaging) are more susceptible to 2DG and 2DG + CIS.

The present studies support the hypothesis that 2DG in combination with CIS could be useful in enhancing the efficacy of the standard of care (CIS + radiotherapy) for HNSCC without obvious signs of enhanced normal tissue toxicity. Additionally, FDG uptake using PET imaging may be useful as a predictor of responses to 2DG + CIS in human head-and-neck cancers. Overall, these data provide a rationale for initiating clinical trials to study the efficacy of 2DG in combination with CIS chemo/radiotherapy.

REFERENCES

- Carvalho AL, Nishimoto IN, Caifano JA, *et al.* Trends in incidence and prognosis for head and neck cancer in the United States: A site-specific analysis of the SEER database. *Int J Cancer* 2005;114:806–816.
- Forastiere AA. Overview of platinum chemotherapy in head and neck cancer. *Semin Oncol* 1994;21:20–27.
- Bourhis J, Le Maitre A, Baujat B, *et al.* Individual patients' data meta-analyses in head and neck cancer. *Curr Opin Oncol* 2007;19:188–194.
- Marcu L, van Doorn T, Olver I. Cisplatin and radiotherapy in the treatment of locally advanced head and neck cancer. *Acta Oncol* 2003;42:315–325.
- Cooper JS, Pajak TF, Forastiere AA, *et al.* Postoperative concurrent radiotherapy and chemotherapy for high-risk squamous-cell carcinoma of the head and neck. *N Engl J Med* 2004;350:1937–1944.
- Zackrisson B, Mercke C, Strander H, *et al.* A systematic overview of radiation therapy effects in head and neck cancer. *Acta Oncol* 2003;42:443–461.
- Dornfeld K, Simmons JR, Karnell L, *et al.* Radiation doses to structures within and adjacent to the larynx are correlated with long-term diet- and speech-related quality of life. *Int J Radiat Oncol Biol Phys* 2007;68:750–757.
- El-Deiry M, Funk GF, Nalwa S, *et al.* Long-term quality of life for surgical and nonsurgical treatment of head and neck cancer. *Arch Otolaryngol Head Neck Surg* 2005;131:879–885.
- Warburg O. On the origin of cancer cells. *Science* 1956;132:309–314.
- Spitz DR, Sim JE, Ridnour LA, *et al.* Glucose deprivation-induced oxidative stress in human tumor cells: A fundamental defect in metabolism? *Ann NY Acad Sci* 2000;899:349–362.
- Andringa KK, Coleman MC, Aykin-Burns N, *et al.* Inhibition of glutamate cysteine ligase (GCL) activity sensitizes human breast cancer cells to the toxicity of 2-deoxy-D-glucose. *Cancer Res* 2006;66:1605–1610.
- Lin X, Zhang F, Bradbury CM, *et al.* 2-Deoxy-D-glucose-induced cytotoxicity and radiosensitization in tumor cells is mediated via disruptions in thiol metabolism. *Cancer Res* 2003;63:3413–3417.
- Simons AL, Ahmad IM, Mattson DM, *et al.* 2-Deoxy-D-glucose (2DG) combined with cisplatin enhances cytotoxicity via metabolic oxidative stress in human head and neck cancer cells. *Cancer Res* 2007;67:3364–3370.
- Wahl RL, Cody RL, Hutchins GD, *et al.* Primary and metastatic breast carcinoma: Initial clinical evaluation with PET with the radiolabeled glucose analogue 2-[F-18]-fluoro-2-deoxy-D-glucose. *Radiology* 1991;179:765–770.
- Hannah A, Scott AM, Tochon-Danguy H, *et al.* Evaluation of ¹⁸F-fluorodeoxyglucose positron emission tomography and computed tomography with histopathologic correlation in the initial staging of head and neck cancer. *Ann Surg* 2002;236:208–217.
- Schwartz DL, Rajendran J, Yueh B, *et al.* Staging of head and neck squamous cell cancer with extended-field FDG-PET. *Arch Otolaryngol Head Neck Surg* 2003;129:1173–1178.
- Yao M, Graham M, Smith R, *et al.* Value of FDG PET in assessment of treatment response and surveillance in head-and-neck cancer patients after intensity modulated radiation treatment: A preliminary report. *Int J Radiat Oncol Biol Phys* 2004;60:1410–1418.
- Dobernt N, Kovacs AF, Menzel C, *et al.* The prognostic value of FDG PET in head and neck cancer. Correlation with histopathology. *J Nucl Med Mol Imaging* 2005;49:253–257.
- Schwartz DL, Rajendran J, Yueh B, *et al.* FDG-PET prediction of head and neck squamous cell cancer outcomes. *Arch Otolaryngol Head Neck Surg* 2004;130:1361–1367.
- Anderson ME. Handbook of methods for oxygen radical research. Florida: CRC; 1985. p. 317–323.
- Griffith OW. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Anal Biochem* 1980;106:207–212.

22. Lowry OH, Rosebrough NJ, Farr AL, *et al.* Protein measurement with the folin phenol reagent. *J Biol Chem* 1951;193:265–275.
23. Liang KY, Zeger SL. Longitudinal data analysis using generalized linear models. *Biometrika* 1986;73:13–22.
24. Forastiere AA, Goepfert H, Maor M, *et al.* Concurrent chemotherapy and radiotherapy for organ preservation in advanced laryngeal cancer. *N Engl J Med* 2003;349:2091–2098.
25. Jeremic B, Shibamoto Y, Stanisavljevic B, *et al.* Radiation therapy alone or with concurrent low-dose daily either cisplatin or carboplatin in locally advanced unresectable squamous cell carcinoma of the head and neck: A prospective randomized trial. *Radiother Oncol* 1997;43:29–37.
26. Jeremic B, Shibamoto Y, Milicic B, *et al.* Hyperfractionated radiation therapy with or without concurrent low-dose cisplatin in locally advanced squamous cell carcinoma of the head and neck: A prospective randomized trial. *J Clin Oncol* 2000;18:1458–1464.
27. Bachaud JM, Cohen-Jonathan E, Alzieu C, *et al.* Combined postoperative radiotherapy and weekly cisplatin infusion for locally advanced head and neck carcinoma: Final report of a randomized trial. *Int J Radiat Oncol Biol Phys* 1996;36:999–1004.
28. Taylor SG, Murthy AK, Griem KL, *et al.* Concomitant cisplatin/5-FU infusion and radiotherapy in advanced head and neck cancer: 8-Year analysis of results. *Head Neck* 1997;19:684–691.
29. Garden AS, Harris J, Vokes EE, *et al.* Preliminary results of Radiation Therapy Oncology Group 97-03: A randomized phase II trial of concurrent radiation and chemotherapy for advanced squamous cell carcinomas of the head and neck. *J Clin Oncol* 2004;22:2856–2864.
30. Pfister DG, Su YB, Kraus DH, *et al.* Concurrent cetuximab, cisplatin, and concomitant boost radiotherapy for locoregionally advanced, squamous cell head and neck cancer: A pilot phase II study of a new combined-modality paradigm. *J Clin Oncol* 2006;24:1072–1078.
31. Halicka HD, Ardelt B, Li X, *et al.* 2-Deoxy-D-glucose enhances sensitivity of human histiocytic lymphoma U937 cells to apoptosis induced by tumor necrosis factor. *Cancer Res* 1995;55:444–449.
32. Dwarakanath BS, Khaitan D, Ravindranath T. 2-Deoxy-D-glucose enhances the cytotoxicity of topoisomerase inhibitors in human tumor cell lines. *Cancer Biol Ther* 2004;3:864–870.
33. Jeremic B, Milicic B, Dagovic A, *et al.* Radiation therapy with or without concurrent low-dose daily chemotherapy in locally advanced, nonmetastatic squamous cell carcinoma of the head and neck. *J Clin Oncol* 2004;22:3540–3548.
34. Stokkel MPM, ten Broek FW, van Rijk PP. The role of FDG PET in the clinical management of head and neck cancer. *Oral Oncol* 1998;34:466–471.
35. Kitagawa Y, Nishizawa S, Sano K, *et al.* FDG-PET for the prediction of tumor aggressiveness and response to intra-arterial chemotherapy and radiotherapy in head and neck cancer. *Eur J Nucl Med* 2003;30:63–71.