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Papadopoulou, M. V., Bloomer, W. D., Taylor, A. P., Hernandez, M., Blumenthal, R. D. and Hollingshead, M. G. Advantage of Combining NLCQ-1 (NSC 709257) with Radiation in Treatment of Human Head and Neck Xenografts. *Radiat. Res.* **168**, 65–71 (2007).

NLCQ-1 (NSC 709257), a hypoxia-selective cytotoxin that targets DNA through weak intercalation, was investigated for efficacy in combination with single or fractionated radiotherapy of human head and neck xenografts. A staged tumor experiment was performed in tumor-bearing female athymic nude mice that were locally irradiated with or without NLCQ-1. Tumor hypoxia was assessed by immunohistochemistry for pimonidazole adducts in tumors of varying weight. Fractionated radiation, depending on the dose, was administered either once daily for 4 days or once daily for 4 days followed by a 7-day rest and repeat. NLCQ-1 was administered i.p. at 15 mg/kg alone or 45 min before each radiation dose. Hypoxia (1–52%) was detected in all tumors and was positively correlated with tumor size. NLCQ-1 alone resulted in about 10 days of tumor growth delay, measured at sixfold the tumor's original size, without causing toxicity. All combination treatments with NLCQ-1 were more effective than treatments with radiation alone. Radiation at 1 Gy given once daily for 4 days on days 20 and 30 caused 3.5 days of tumor growth delay, whereas in combination with NLCQ-1 it caused 14.5 days of growth delay. Radiation at 5 Gy given in two doses 10 days apart resulted in 3.5 days of tumor growth delay, whereas more than 20 additional days of delay were observed in combination with NLCQ-1. Radiation given as a single dose of 10 Gy resulted in about 7 days of tumor growth delay, whereas in combination with NLCQ-1 about 30 additional days of delay were seen. These results suggest a significant advantage in combining radiation with NLCQ-1 in treatment of human head and neck tumors, which are known to have hypoxic areas. © 2007 by Radiation Research Society

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INTRODUCTION

Head and neck malignancies have a poor prognosis, and a majority of patients die from uncontrolled local disease. The main treatments are surgery and radiotherapy with or without chemotherapy. The ineffectiveness of treatment is related to tumor hypoxia, one of the microenvironmental features of solid tumors that contributes to tumor progression and limits tumor response to radiotherapy and chemotherapy (*1, 2*). This has been substantiated in head and neck cancers, in which a correlation was found between tumor hypoxia and local control, disease-free survival and overall survival (*3, 4*).

Hypoxia-targeted chemotherapy not only supplements modalities such as radiation or chemotherapy (which primarily attack aerobic proliferating cells) but often interacts synergistically with them and therefore constitutes a promising tool against tumor hypoxia (*5*). For example, the hypoxia-targeted bioreductive compound mitomycin C has been employed successfully in the past as an adjuvant to radiotherapy for the treatment of head and neck cancer (*6, 7*). Recent clinical trials of the hypoxic cytotoxin tirapazamine (3-amino-1,2,4-benzotriazine-1,4-dioxide, SR-4233, TPZ) in various combination protocols demonstrate clinical proof-of-principle that drugs that exploit hypoxia can be of clinical value (*8–11*).

4-[3-(2-Nitro-1-imidazolyl)-propylamino]-7-chloroquinoline hydrochloride (NLCQ-1; NSC 709257) is a 2-nitroimidazole-based hypoxia-selective cytotoxin that does not fall into the category of classical nitroimidazole-based bioreductive drugs because it possesses two unique features: (a) binding to DNA through weak intercalation and (b) increasing hypoxic potency and selectivity with time (*12*). These features allow for fast dissociation from DNA, therefore facilitating extravascular diffusion and penetration to hypoxic tumor regions, without compromising efficacy and ultimately enabling effectiveness *in vivo*. NLCQ-1 synergistically enhances the effect of radiotherapy, radioimmunotherapy or chemotherapy against murine tumors and human xenografts without a concomitant enhancement in bone

marrow, hypoxia-dependent retinal or systemic toxicity (*13, 14*). Moreover, NLCQ-1 exhibits good stability in human plasma (*15*), and reversible toxicity and favorable pharmacokinetics in mice and dogs.[2,3]

In a recent study, we showed that NLCQ-1 interacts synergistically with single or fractionated radiation doses against advanced-stage human glioma U251 xenografts, resulting in complete regressions and increased survival (*16*). In the present study, we investigated the effect of NLCQ-1 and radiation in treatment of advanced-stage human head and neck xenografts and its correlation with the hypoxic status of the tumors.

MATERIALS AND METHODS

Drugs

NLCQ-1 (provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute) was dissolved in saline at 1.5 mg/ml and was injected intraperitoneally (i.p.) at 0.1 ml/10 g body weight for a dose of 15 mg/kg. Fresh solutions were prepared just prior to the injection.

Mice and Tumors

Xenografts were established from cells of the WSU-HN-31 human head and neck tumor cell line (kind gift of Pervin Anklesaria, Targeted Genetics Corporation, Seattle, WA) that were cultured in RPMI-1640 medium (Quality Biologicals, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) and 2 m*M* glutamine (Quality Biologicals).

Cells were implanted subcutaneously (1.0×10^7 cells/0.1 ml per mouse) at the base of the tail of female athymic nude mice (nu/nuNCr) (NCI Animal Production Program, Frederick, MD). All mice developed tumors, and the median tumor weight was 196 mg (ranging from 172–196 mg) at the time treatment was initiated. Therefore, the tumors were characterized as staged tumors (*17*). The initial median tumor doubling time was 11.6 days. Mice were housed in sterile, filter-capped, polycarbonate cages (Allentown Caging, Allentown, NJ), maintained in a barrier facility on 12-h light/dark cycles, and provided with sterilized food and water *ad libitum* (*17*). Animals were randomized into groups of 8 (treated groups) and 16 (control group). The mice were housed in an AAALACi-approved animal facility and all studies were conducted on an Animal Study Protocol (ASP) approved by the NCI-Frederick Animal Care and Use Committee.

Immunohistochemistry for Detection of Hypoxia

Mice (*n* = 20) bearing WSU-HN-31 human head and neck tumors varying in size were treated 3 h before tumor harvest with 100 mg/kg of hypoxic, viable cell targeting pimonidazole HCl, given i.p. (Chemicon International, Temecula, CA) (*18*). Tumors were fixed in 10% formalin/PBS and embedded in paraffin. Pimonidazole adducts were detected by

immunohistochemistry using the Hydroxyprobe-1 Plus Kit (Chemicon) according to the manufacturer's protocol. Briefly, paraffin-embedded sections were deparaffinized and rehydrated, followed by quenching of tissue peroxidase in 3% aqueous H_2O_2 and antigen retrieval (Pronase, Biomeda, Foster City, CA). Slides were blocked with serum-free protein block (Dako, Carpinteria, CA) and treated with the primary antibody (FITC-conjugated anti-pimo mAb) diluted 1:50 or an irrelevant control antibody (Ag8). A second antibody (HRP-conjugated anti-FITC mAb) was added, followed by the peroxidase substrate, 3,3′-diaminobenzidine (DAB) (Sigma, St. Louis, MO). Slides were counterstained with hematoxylin (Sigma). Each slide contained two or more sections, and each sample was stained at least twice. Live-appearing tumor tissue was evaluated microscopically for positive signal at 100×. Necrotic areas were not counted. Serial micrographs (100×) of the tumors were evaluated by using the pixel quantification method (*19*). This technique is based on selection of similar color features on a digital micrograph using Photoshop software. The Magic Wand tool is used to select either the specific stain (brown) or the counterstain (blue). After initial selection with the tool, the Select Similar command is used to select similar areas of the micrograph, which are then highlighted. The Image Histogram is then used to show the "luminosity" (color) of all pixels that are similar to the selected color. The number of pixels of the selected color is also given. To obtain the percentage positive hypoxia staining for each sample, the sum of the number of pixels for the specific (brown) stain from the micrographs was divided by the total number of pixels (brown + blue counterstain) to obtain the percentage of the area that was positive for the hypoxia stain. We analyzed two or three micrographs/sample with three to five samples per tumor size. The values for all samples from each of the size groups were averaged, and the standard deviation was calculated.

Irradiation

Unanesthetized mice were irradiated (Pantak 300 kV X-ray Irradiator, Pantak, Solon, OH) with or without NLCQ-1 in lead jigs (Engineering Specialties, Silver Spring, MD) with either single or fractionated radiation doses. Fractionated radiation, depending on the dose, was administered either once daily for 4 days or once daily for 4 days followed by a 7-day rest and repeat. The radiation doses were chosen based on an initial experiment performed in this tumor line in which a response to radiation was observed only at high single doses (15 and 20 Gy) and at relatively high multiple fractionated doses (3, 4 or 5 Gy once daily for 4 days). NLCQ-1 was administered i.p. at 15 mg/kg alone or 45 min before each radiation dose. Growth of the solid tumors was monitored using caliper measurements to determine tumor size. Weights (mg) were calculated from two perpendicular dimensions (length and width) using the formula for a prolate ellipsoid and assuming a specific gravity of 1.0 g/cm³ (*20*). Tumor size and body weight were measured approximately two times per week.

Antitumor activity was assessed by calculating optimal %T/C values from the formulas

$$\%\text{T/C} = (\Delta\text{T}/\Delta\text{C}) \times 100, \quad \text{where } \Delta\text{T} > 0, \quad \text{or} \tag{1}$$

$$\%\text{T/C} = (\Delta\text{T}/\text{T}_I) \times 100, \quad \text{where } \Delta\text{T} < 0, \tag{2}$$

where ΔT and ΔC are changes in tumor weight in the treated and control groups, respectively, and are obtained by subtracting the median tumor weight on the day of first treatment (staging day) from the median tumor weight on the observation day, and T_I is the median tumor weight at the start of treatment (*17*). Tumor growth delays were determined from individual tumor growth curves at sixfold (and in two cases at 2.5-fold) the initial median tumor weight. Both drug-related deaths and maximum percentage relative mean net body weight losses were determined (*17*). Multiple comparisons between groups were performed using the Student's *t* test.

TABLE 1
Evaluation of Tumor Hypoxia in WSU-HN-31
Human Head and Neck Xenografts by
Pimonidazole Staining

Tumor size	Average hypoxic area (% ± SD)	Range (% hypoxic area)
Small (0–299 mg)	3 ± 3	1–7
Medium (300–599 mg)	23 ± 16	14–40
Large (>600 mg)	25 ± 15	18–52

RESULTS

Tumors processed for pimonidazole staining were classified by size in three groups (Table 1). Three out of twenty tumors (two large and one medium) were not evaluated because they could not be cut. All of the remaining tumors stained positively for hypoxic live cells (Fig. 1). Minimal to no background staining was detected for any of the samples stained with an irrelevant antibody. The irrelevant antibody stained and anti-pimonidazole-stained slides were compared to each other. Larger tumors tended to have a thin layer of hypoxic live cells surrounding a necrotic core, whereas smaller tumors contained relatively less necrotic area than large tumors. The mean hypoxic content was 3 ± 3%, 23 ± 16% and 25 ± 15% for small, medium and large tumors, respectively (Table 1). Therefore, medium and large-size tumors have almost identical content of hypoxia.

The response of staged human head and neck xenografts to NLCQ-1 with and without radiation treatments is summarized in Table 2. NLCQ-1 alone at 15 mg/kg given i.p. on a schedule of a single dose per day for 4 days, 7 days rest and repeat had a significant effect on tumor growth ($P = 0.0009$ compared to control) (Table 2). This was consistent with the presence of hypoxia in tumors, especially during the second cycle of treatment, when tumors were above 200 mg (Figs. 1 and 2C).

Fractionated radiation alone at small doses such as 1 or 2 Gy, given on a schedule of a single dose per day for 4 days, 7 days rest and repeat had no effect (1 Gy; $P = 0.11$ compared to control) or a minimal effect (2 Gy) on tumor growth ($P = 0.02$ compared to control). A tumor growth delay of 3.5 days, measured at sixfold its original size, was obtained with 16 Gy given in eight fractions of 2 Gy (Table 2). A longer tumor growth delay of 6.1 days was obtained with 16 Gy given as four fractions of 4 Gy compared to the vehicle-treated control ($P = 0.0007$). Two doses of 5 Gy administered with a 10-day interval resulted in a 3.3-day tumor growth delay (Table 2), which was marginally significant compared to control ($P = 0.054$). Finally, a sin-
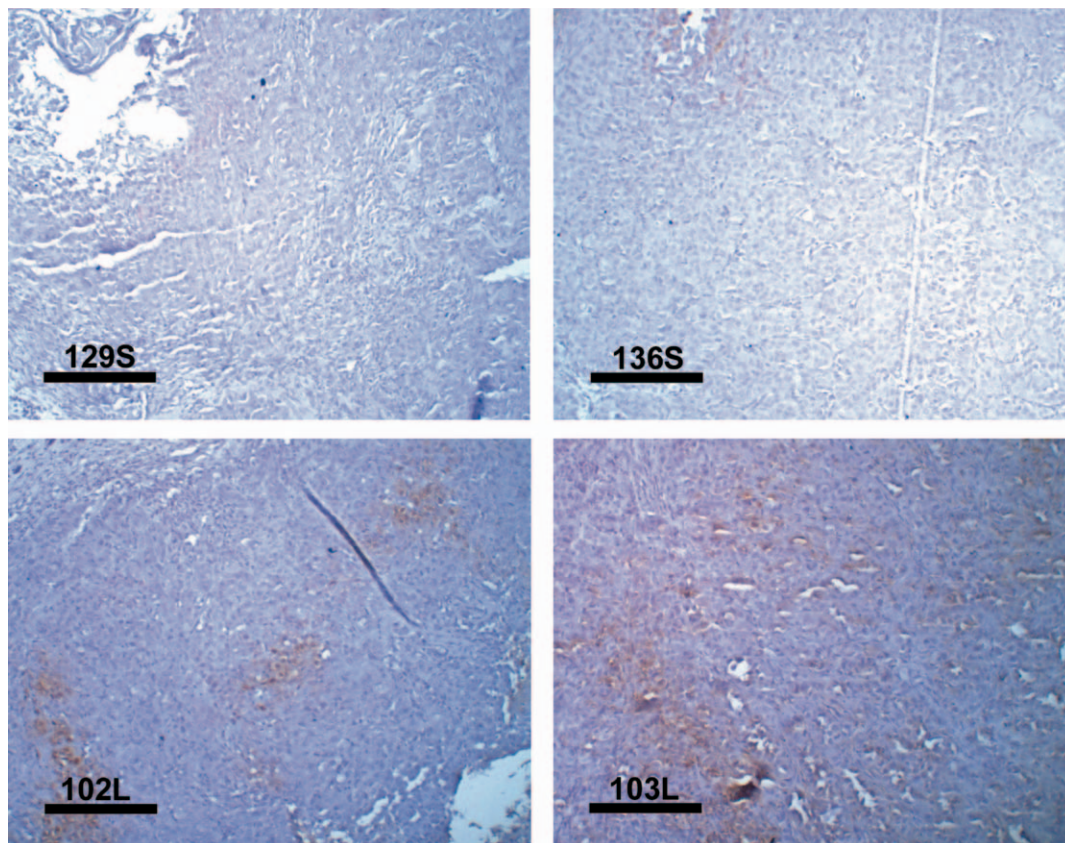

FIG. 1. Immunohistochemistry micrographs: paraffin-embedded tumors sections were deparaffinized and stained by IHC for hypoxia using designated antibodies. Positive staining is brown. Two small (S, 0–299 mg) and two large (L, >600 mg) samples are depicted. Bar is 0.2 mm.

TABLE 2
Response of Advanced-Stage Subcutaneous WSU-HN-31 (Head and Neck) Tumor
Xenografts to NLCQ-1 with or without Radiation

Treatment (first day of each cycle)	No. of mice	Optimal %T/C (day)	Median days to 6× or (2.5×)[a] weight	Tumor growth delay[b] (days)
Saline	16	—	49.5 (35.0)	—
5 Gy Q10D × 2[c] (20, 30)	8	62 (27)	52.8	3.3
10 Gy QD × 1 (20)	8	45 (48)	67.1 (41.9)	6.9
1 Gy QD × 4 (20, 30)	8	71 (27)	46.0	−3.5
2 Gy QD × 4 (20, 30)	8	59 (27)	53.0	3.5
4 Gy QD × 4 (20)	8	44 (42)	55.6	6.1
NLCQ-1[d] 15 mg/kg QD × 4 (20, 30)	8	47 (27)	59.5[e]	10.0
NLCQ-1 + 5 Gy Q10D × 2 (20, 30)	8	14 (52)	~73.0[f]	~23.5
NLCQ-1 + 10 Gy QD × 1 (20)	8	2 (52)	ND (71.5)[g]	36.5
NLCQ-1 + 1 Gy QD × 4 (20, 30)	8	46 (58)	64.0	14.5
NLCQ-1 + 2 Gy QD × 4 (20, 30)	8	53 (52)	62.0	12.5
NLCQ-1 + 4 Gy QD × 4 (20)	8	45 (48)	65.0	15.5

Notes. The initial median tumor weight was 196 mg. ND: Not determined

[a] The numbers in parentheses indicate the median time (days) for a 2.5-fold increase in the tumor size.

[b] Normally calculated at sixfold increase in the tumor size.

[c] Q10D × 2: two doses given 10 days apart; QD × 1: single dose; QD × 4: one dose per day for 4 consecutive days.

[d] NLCQ-1 was given i.p. in saline 45 min before each radiation dose.

[e] Mean ± SD: 59.0 ± 1.6 compared to 49.0 ± 4.3 ($P = 0.0009$ compared to control).

[f] Mean ± SD: 72.6 ± 2.3 compared to 52.3 ± 1.8 ($P = 0.0001$ compared to radiation alone).

[g] Mean ± SD: 70.0 ± 7.0 compared to 41.5 ± 5.1 ($P < 0.0001$ compared to radiation alone).

gle high dose of 10 Gy resulted in a 6.9-day of tumor growth delay ($P = 0.001$ compared to control), measured at 2.5-fold the original size.

All combination treatments with NLCQ-1 given 45 min before irradiation were more effective than radiation alone (Table 2). Tumor growth delays of 14.5, 12.5 and 15.5 days were obtained compared to control, with the 1-, 2- or 4-Gy fractionated protocols, respectively, measured at sixfold the tumor's original size. The combination treatments were significantly different from the corresponding radiation-alone treatments, with P values of <0.0001 (1 Gy), 0.049 (2 Gy) and 0.0004 (4 Gy). The most impressive results were ob-
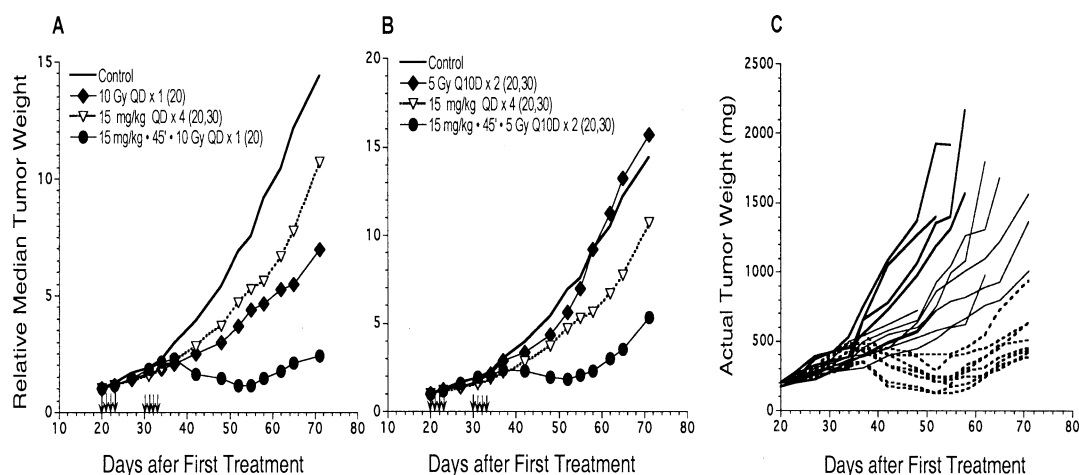

FIG. 2. Response of staged WSU-HN-31 human head and neck xenografts to NLCQ-1 with or without radiation treatment. Panels A and B: Relative median tumor weight was plotted as a function of time after treatment. NLCQ-1 (15 mg/kg) was given 45 min before each radiation dose. Sixteen and eight mice per group were used for control and treated groups, respectively. Treatment is indicated on the plots, and the first day of each cycle is shown in parentheses. Arrows indicate days of treatment with NLCQ-1 alone. Panel C: Actual tumor weight of untreated and treated individual mice was plotted as a function of time after treatment. Solid thick lines represent the untreated control (four mice only); solid thin lines represent the group treated with radiation alone (10 Gy on day 20, eight mice); dotted lines represent the group treated with NLCQ-1 + 10 Gy (eight mice). The software program used allows for only 20 plots at a time; thus not all of the data for the 16 control mice could be presented. See Table 2 for an explanation of abbreviations.

tained when NLCQ-1 was combined with either one single large radiation dose of 10 Gy or two doses of 5 Gy given with a 10-day interval. In the first case, 36.5 days of tumor growth delay was obtained, measured at 2.5-fold the tumor's original size (Fig. 2A), which is almost 30 days more than the delay obtained with radiation alone ($P < 0.0001$). A distinct difference in the growth of treated and untreated tumors is apparent in Fig. 2C, in which the weight of individual tumors is plotted as a function of time. Similarly, in combination with 5 Gy given in two doses 10 days apart, a tumor growth delay of about 23.5 days was observed at sixfold tumor's original size (Fig. 2B), which is 20 days more than treatment with radiation alone ($P = 0.0001$).

In terms of optimal %T/C values, all treatments with radiation alone were inactive (%T/C > 40) or marginally active (%T/C ~ 40) (17) (Table 2). Minimal tumor inhibition in terms of %T/C values was also obtained with NLCQ-1 plus fractionated radiation at small doses (Table 2). However, when NLCQ-1 was combined with 10 Gy given as a single dose or 5 Gy given in two doses 10 days apart, %T/C values of 2 and 14 were obtained, respectively (17).

Extended survival was observed in three of the combination treated groups. The survival was 100, 75 and 63% on day 71 (when the experiment ended) for NLCQ-1 plus a single dose of 10 Gy, NLCQ-1 plus 5 Gy given as two doses 10 days apart, and NLCQ-1 plus 1 Gy given once daily for 4 days (days 20 and 30), respectively, compared to 38, 25, 13 and 13% for the corresponding radiation-alone groups and untreated control, respectively.

No drug-related deaths or weight loss were observed in any of the combination treated groups. Minimal ($<5\%$) weight loss was observed in the groups receiving a single dose of 10 Gy or 5 Gy given as two doses 10 days apart.

DISCUSSION

Head and neck cancers have generally been treated with radiation, with local-regional recurrence and tumor hypoxia as the major causes for treatment failure. Therefore, head and neck cancer has been used as a model to prove the hypothesis that bioreductively activated hypoxia-selective cytotoxins and radiosensitizers can improve local-regional control by radiotherapy, which translates to better survival. However, the results of clinical trials are still inconclusive. The classic 2-nitroimidazole-based radiosensitizers misonidazole and etanidazole, which are also hypoxia-selective cytotoxins at higher, clinically unachievable doses, underwent several clinical trials with radiation against head and neck cancers. No statistically significant difference was found between groups treated with radiation alone or radiation plus sensitizer (21–23). However, in a meta-analysis of data on 7,000 patients treated in 50 randomized trials, a small benefit in loco-regional control and overall survival was seen in sensitizer-treated patients (24). A significantly better loco-regional control rate than in the control group

was also seen with the 5-nitroimidazole-based radiosensitizer nimorazole (25, 26). In addition, several clinical trials have demonstrated the benefit of the bioreductive alkylating agent, mitomycin C, as an adjunct to radiotherapy (7), continuous hyperfractionated accelerated radiotherapy (27), or radiochemotherapy (28) in the management of head and neck cancer. Finally, clinical studies confirm the usefulness of the bioreductive agent tirapazamine as an adjunct to radiotherapy (8) or radiochemotherapy in patients with advanced head and neck cancers (29).

In the present study, we demonstrated that a therapeutic benefit can be achieved by combining the weakly DNA-intercalating hypoxia selective cytotoxin NLCQ-1 with radiotherapy in a head and neck cancer model in which tumor-hypoxia is present. Indeed, all ≥ 200 mg WSU-HN-31 human head and neck tumors xenografts were stained positively for hypoxia (Fig. 1). Even though hypoxia was measured in untreated tumors from a separate experiment, the same cell line was used, and the xenografts were implanted in the same strain of mouse; it is likely that tumor hypoxia would not vary substantially between experiments. Radiation and other treatments tend to cause increased tumor hypoxia, and this is partially due to treatment-related damage to blood vessels and tumor blood supply. Tumors from the treated animals, if assayed for hypoxia, would most likely have been more hypoxic than the tumors from untreated animals. The following limitations are of concern when performing assays of hypoxia with a single injection of pimonidazole as the tracer. The information obtained from this assay depends on penetration of the tissue by pimonidazole during the time between administration and harvest of the tumor. The pimonidazole may not penetrate into areas of tumor farthest from the capillaries, where cells are likely to be hypoxic, so the extent of hypoxia may be greater than shown. When pimonidazole is given as a single injection, a "snapshot" of the hypoxia is obtained and may not reflect the dynamic nature of tumor hypoxia because diffusion of pimonidazole is dependent on capillary perfusion, which is subject to local fluctuations. Cells near capillary beds that are currently "shut down" would more likely be hypoxic, yet the pimonidazole may not be delivered as well as if the tissue capillaries were fully open. Pimonidazole does not affect tissue hypoxia (30).

NLCQ-1 plus radiation treatment significantly increased the tumor growth delay and survival of mice bearing head and neck xenografts without causing additional toxicity (Table 2). Consistent with current clinical practice for head and neck cancers, WSU-HN-31 tumors responded best to NLCQ-1 administered in conjunction with a single or two relatively large radiation doses. In addition, efficacy was seen with fractionated radiation regimens (Table 2), suggesting that tumor reoxygenation can be overcome to some degree.

The benefit of adding NLCQ-1 to radiation treatment has been demonstrated previously in studies of human glioma xenografts (16) and murine tumors (31). In the first study,

nine out of 10 complete tumor regressions with long duration were obtained, in addition to a very significant tumor growth delay, when a fractionated regimen of radiation and NLCQ-1 was used, implying restoration of hypoxia (*32*) of human glioma xenografts between treatments. In the case of the murine tumors, where the excision assay was used as an end point, NLCQ-1 interacted synergistically with single or fractionated radiation doses and caused significant tumor cell killing (*31*). In the same study, NLCQ-1 demonstrated a greater *in vitro* therapeutic index than TPZ (*31*).

In the current study, the existence of tumor hypoxia was confirmed and was consistent with resistance to radiation and sensitivity to NLCQ-1 alone. Furthermore, WSU-HN-31 was only moderately responsive even to relatively high doses of radiation alone, as is commonly seen clinically in patients suffering from head and neck tumors. The response of these tumors to NLCQ-1 in combination with radiation is suggestive of potential clinical value since the results for treatment with radiation alone mirrored the clinical experience.

In previous studies of human colon tumor xenografts, NLCQ-1 demonstrated significant antitumor activity in combination with radioimmunotherapy only when it was administered 14 days after radioimmunotherapy, when the radioimmunotherapy-induced tumor hypoxia (measured with oxygen microelectrodes, anti-PIMO staining or the radiotracer ^{3}H-MISO) was maximum (*33*). NLCQ-1 also caused significant growth delay on its own only in EMT6 mouse mammary tumors positive to the hypoxia tracer ^{18}F-FAZA (unpublished results). Apparently, the presence of hypoxia and a proper reductive enzyme are prerequisites for NLCQ-1 activation and enhancement of the effect by radiation treatment. NLCQ-1 is activated by cytochrome P450 and cytochrome b5 reductases (*34*). Additional studies in human MDA-MB-231 and HT1080 cancer cells transfected with a virus encoding for P450 reductase showed increased hypoxic selectivity of NLCQ-1 compared to non-transfected controls, confirming the involvement of cytochrome P450 reductase in the bioreductive activation of NLCQ-1.[4]

In conclusion, NLCQ-1 could be beneficial in the management of head and neck cancer by radiotherapy or perhaps radiochemotherapy, since synergistic interactions between NLCQ-1 and cisplatin or 5-fluorouracil have been observed in preclinical studies as well (*13*). In addition, the toxicology and pharmacokinetic studies[2,3] suggest a milder toxicity of NLCQ-1 in humans compared to TPZ (*32*).

ACKNOWLEDGMENTS

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