NLCQ-1 (NSC 709257) in Combination with Radiation Against Human Glioma U251 Xenografts

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Abstract. Background: The efficacy of the weak DNA-intercalative hypoxia-selective cytotoxin NLCQ-1 (NSC 709257) was investigated in combination with single or fractionated doses of radiation against human glioma U251 xenografts. Two "advanced stage" experiments were performed in female athymic nude mice. Materials and Methods: Tumor-bearing mice were allocated in groups of 8-10 (treated) or 10-20 (control) and irradiated in the presence or absence of NLCQ-1. Fractionated radiation was administered either qd x 4 or qd x 2, followed by a 9-day rest and repeated dosing. NLCQ-1 was administered i.p. 45 min before each radiation dose. Results: NLCQ-1 alone did not show antitumor activity or toxicity. Radiation at the highest single dose used (5.0 Gy) showed antitumor activity without weight loss (optimal T/C = –45). Lower single radiation doses (2.0 or 3.0 Gy) were marginally effective (optimal T/C of 34 and 40, respectively). The addition of NLCQ-1 to the treatment with each single radiation dose provided better optimal T/C values (e.g., –64 with 5.0 Gy). Fractionated radiation at 1.0 Gy qd x 4 showed minimal effectiveness (T/C = 27) but, in combination with NLCQ-1, the T/C value was improved to 19. Radiation alone, given on a 3.0 Gy qd x 2, 9-day rest and repeat schedule was very effective (T/C = –57) without toxicity and resulted in 5 out of 10 complete regressions up to 42 days. When NLCQ-1 was added to the above protocol an optimal T/C value of –100 and 9 out of 10 complete regressions were obtained with a follow-up of 52 days. Conclusion: The above results suggest a significant advantage in combining radiation with NLCQ-1 against glioma tumors.

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Key Words: NLCQ-1, radiation, human gliomas, hypoxia.

Malignant gliomas remain refractory to intensive radiotherapy, mainly due to their hypoxic content (1-4). It has been reported that the median pO2 for high-grade gliomas, studied under anesthesia, was approximately 5-7 mm Hg, whereas significant proportions of pO2 values were less than 2.5 mm Hg (3, 4). Hypoxia has been recognized as one of the microenvironmental features of solid tumors that contributes to tumor progression and limits tumor response to radiotherapy and chemotherapy (5-7). However, tumor hypoxia also presents opportunities for the use of compounds that are selectively activated under hypoxic conditions, otherwise known as bioreductive drugs (8). Recent clinical trials of the hypoxic cytotoxin tirapazamine (3-amino-1,2,4-benzotriazine-1,4-dioxide, SR-4233, TPZ) in various combination protocols demonstrated clinical proof-of-principle that drugs properly designed to exploit hypoxia can be of clinical value (9-11). 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 kinetics from DNA, thus facilitating extravascular diffusion and penetration to hypoxic tumor regions and, ultimately, enabling effectiveness in vivo. Thus, in agreement with the above, NLCQ-1 synergistically enhances the effect of radiotherapy, radioimmunotherapy or chemotherapy against murine tumors and human colon and prostate xenografts without a concomitant enhancement in bone marrow or hypoxia-dependent retinal toxicity (13, 14). Moreover, studies at the NCI and recent IND-directed studies showed that NLCQ-1 exhibits good stability in human plasma and favorable pharmacokinetics in mice and dogs (15 and unpublished results).

The present study was initiated to investigate interactions between NLCQ-1 and single or fractionated radiation doses.
against advanced stage human glioma U251 xenografts, which, as has been mentioned above, contain significant regions of hypoxia.

**Materials and Methods**

**Drugs.** NLCO-1 (provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute, USA) was dissolved in saline at 1.5 mg/ml and was injected intraperitoneally (i.p.) at 0.1 ml/10 g body weight.

**Mice and tumors.** Xenografts were established from U251 human tumor cell lines (DCTD Tumor Repository, NCI-Frederick, MD, USA) which were cultured in RPMI 1640 medium (Quality Biologicals, Gaithersburg, MD, USA) supplemented with 10% FBS (Hyclone, Logan, UT, USA) and 2 mM glutamine (Quality Biologicals). Cells were implanted subcutaneously (1.0 x 10^7 cells / 0.1 ml/mouse) at the base of the tail of female athymic nude mice (NCI Animal Production Program, NCI-Frederick, MD, USA). At the time of treatment, the median tumor weight ranged from 158-196 mg (advanced stage). Mice were housed in sterile, polycarbonate, filter-capped Microisolator™ cages (Lab. Products, Inc.), maintained in a barrier facility on 12-h light/dark cycles, and provided with sterilized food and water ad libitum (16). Eight to 10 animals per treated group and 16-20 animals in the control groups were used.

**Irradiation.** Unanesthetized mice were irradiated (Pantak 300 kV X-ray Irradiator, Pantak, Solon, OH, USA) with or without NLCO-1 in lead jigs (Engineering Specialties, Silver Spring, MD, USA) with either single or fractionated radiation doses. Fractionated radiation was administered either qd x 4, or qd x 2 followed by a 9-day rest and repeated dosing. In combination treatments, NLCO-1 was administered i.p. at 15 mg/kg, 45 min before each radiation dose. The initial median tumor doubling time of untreated controls was 5.7 and 6.5 days for the two experiments. Growth of the solid tumors was monitored using in situ caliper measurements to determine tumor size. Weights (mg) were calculated from measurements (mm) of two perpendicular dimensions (length and width) using the formula for a prolate ellipsoid and assuming a specific gravity of 1.0 g/cm^3 (17). Individual tumor weights were expressed as a fraction of their weight on the day of treatment. Tumor size and body weights were obtained approximately 2 times/wk.

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

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

\[
%T/C = (\Delta T/T_1) \times 100 \quad \text{where} \quad \Delta T < 0 \quad (2)
\]

where AT and AC are changes in tumor weight in treated and control groups, respectively, and 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 T1 is the median tumor weight at the start of treatment (16). Tumor growth delay was determined at 2-fold the initial tumor weight. Both drug-related deaths (DRDs) and maximum percent relative mean net body weight losses were determined (16). Multiple comparisons between groups were performed using the Student's t-test.

**Results**

The response of human glioma U251 xenografts to NLCO-1 ± radiation treatments, from the two experiments performed, is summarized in Table I. All mice developed tumors by the day of treatment. The median time for control tumors to reach 2-fold the initial weight was 16.9 and 12.6 days for the two experiments, respectively. NLCO-1 alone at 15 mg/kg, given i.p. for 4 consecutive days did not have any effect on tumor response in both individual experiments (Table I). Radiation alone at low doses had a minimal effect on tumor response, which however was significant. Thus, 3.0 Gy qd x 1 or 1.0 Gy qd x 4 caused only 1.6 days of additional tumor growth delay compared to vehicle-treated control (p<0.01 and 0.05, respectively). The same small radiation doses, in combination with NLCO-1 given 45 min before irradiation, caused 4.4 and 6.1 days of additional tumor growth delay, respectively, compared to the vehicle-treated control (Table I). Higher radiation doses, single or fractionated, resulted in a much more significant tumor growth delay. Therefore, a single dose of 5 Gy resulted in 19.7 days of additional tumor growth delay (p<0.001 vs control), whereas with 3.0 Gy qd x 2, given on days 6 and 17 (total of 12.0 Gy), 44.8 days of additional tumor growth delay was achieved (p<0.0001 vs control) (Table I). Adding NLCO-1 to the above protocols further increased the tumor response. Thus, NLCO-1 combined with a single radiation dose of 5.0 Gy resulted in 25.1 days of additional tumor growth delay compared to the vehicle-treated control (p<0.001) or 5.4 additional days compared to the corresponding radiation alone treated group (p=NS).

Moreover, NLCO-1 combined with the fractionated radiation protocol of 3.0 Gy qd x 2, given on days 6 and 17 caused 70.0 days of additional tumor growth delay compared to the vehicle-treated control (p<0.0001) or 25.2 additional days compared to the corresponding radiation alone-treated group (p<0.02) (Table I).

In terms of optimal %T/C values, marginal tumor inhibition (16) was observed with the lower doses of radiation alone (Table I). Thus, 3.0 Gy qd x 1 and 1.0 Gy qd x 4, yielded %T/C values of 40 and 27, respectively. Better tumor inhibition was obtained when NLCO-1 was combined with the previous low radiation dose protocols (%T/C values of 9 and 19, respectively). Tumor stasis (%T/C range 0 to –49) was obtained with 5.0 Gy x 1 alone, whereas the fractionated radiation of a 3.0 Gy qd x 2, 9-day rest and repeat schedule resulted in 5 complete regressions out of 10 mice, for up to 42 days (Table II). The optimal %T/C value in this case was –57 (on day 38) (Table I). When NLCO-1 was added to the above protocol, the treatment proved very effective, resulting in 9 out of 10 complete regressions with a follow-up of 52 days (Table II and Figure 1). The optimal %T/C value was –100 from day...
38 to day 52 (Table I). For “advanced stage” tumor tests, complete regression is defined as a decrease in tumor burden below 63 mg, but regrowth occurs before the end of the experiment.

No drug-related deaths or relative mean net weight loss were observed in any of the treated groups (Table I).

Table I. Response of advanced stage SC U251 CNS tumor xenografts to NLCQ-1 ± radiation.

<table>
<thead>
<tr>
<th>Treatment (day)</th>
<th>No. of mice</th>
<th>Drug deaths</th>
<th>Max % rel. mean net wt. loss (day)</th>
<th>Opt % T/C (day)</th>
<th>Median days to 2-fold wt.</th>
<th>TGD$^1$ (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (10 or 6)</td>
<td>16 or 20</td>
<td>0</td>
<td>0; 2.4 (14)</td>
<td>16.9; 12.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.0 Gy, QD x 1 (10)</td>
<td>8</td>
<td>0</td>
<td>12.4 (28)</td>
<td>40 (14)</td>
<td>18.5</td>
<td>1.6</td>
</tr>
<tr>
<td>5.0 Gy, QD x 1 (10)</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>–45 (17)</td>
<td>36.6</td>
<td>19.7</td>
</tr>
<tr>
<td>1.0 Gy, QD x 4 (10)</td>
<td>8</td>
<td>0</td>
<td>1.1 (14)</td>
<td>27 (14)</td>
<td>18.5</td>
<td>1.6</td>
</tr>
<tr>
<td>3.0 Gy, QD x 2 (6, 17)</td>
<td>10</td>
<td>0</td>
<td>1.6 (14)</td>
<td>–57 (38)</td>
<td>57.4</td>
<td>44.8</td>
</tr>
<tr>
<td>NLCQ-1$^2$, 15 mg/kg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>QD x 4 (10 or 6)</td>
<td>8 or 10</td>
<td>0</td>
<td>0; 0</td>
<td>93 (24)</td>
<td>15.3; 11.9</td>
<td>–1.6; –0.7</td>
</tr>
<tr>
<td>NLCQ-1 + 3.0 Gy QD x 1 (10)</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>9 (17)</td>
<td>21.3</td>
<td>4.4</td>
</tr>
<tr>
<td>NLCQ-1 + 5.0 Gy QD x 1 (10)</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>–64 (25)</td>
<td>42</td>
<td>25.1</td>
</tr>
<tr>
<td>NLCQ-1 + 1.0 Gy QD x 4 (10)</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>19 (21)</td>
<td>23</td>
<td>6.1</td>
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<tr>
<td>NLCQ-1 + 3.0 Gy QD x 2 (6, 17)</td>
<td>10</td>
<td>0</td>
<td>1.0 (14)</td>
<td>–100 (38-52)</td>
<td>82.6</td>
<td>70</td>
</tr>
</tbody>
</table>

$^1$Tumor growth delay.

$^2$NLCQ-1 was given i.p. in saline 45 min before each radiation dose.

Discussion

In a previous study against murine tumor-models, where the excision assay was used, the hypoxia-selective cytotoxin NLCQ-1, in combination with single or fractionated radiation doses, caused significant tumor cell killing due to a synergistic interaction with radiation (18). In the same study, NLCQ-1 demonstrated a greater in vitro therapeutic index than the leading bioreductive drug tirapazamine (18). This is the first study against human glioma xenografts where NLCQ-1 was combined with radiation. Hypoxia has been demonstrated in this type of tumor by polarographic oxygen electrode measurements (3) and by positron emission tomography with $[^{18}\text{F}]$fluoromisonidazole (2). Moreover, the presence of bioreductive enzymes in human gliomas (3), in addition to hypoxia, further justifies the use of this experimental model for investigation of NLCQ-1 activity. In the present study, NLCQ-1 failed to demonstrate activity on its own, as has been seen before at such low NLCQ-1 doses. However, in a previous study against human colonic xenografts, where hypoxia was measured with oxygen microelectrodes and was found to be present, NLCQ-1 did demonstrate a significant antitumor effect on its own (19). In addition, studies at the NCI with U251 xenografts transfected to express luciferase operating off the hypoxia-responsive element promoter showed increased expression of luciferase in the xenografts when the tumors reached 300 mg in size (20). This means that perhaps the initial hypoxic status of tumors was not significant enough for NLCQ-1 activation. However, in all cases NLCQ-1 improved the antitumor effect of radiation.

The experimental protocol was designed according to results obtained in pilot studies where the sensitivity of U251 xenografts to radiation was assessed. It is clear from the results that low doses of radiation alone caused only a minimal tumor response, which was statistically significant and which was further improved with the addition of NLCQ-1. The higher single radiation dose of 5.0 Gy qd x 1
resulted in a substantial tumor growth delay of 19.7 days ($p<0.001$), which was further increased to 25.1 days with the addition of 15 mg/kg NLCQ-1 (Table I). Similar tumor growth delay (24.4 days) of glioma xenografts was obtained in our pilot studies with 8.0 Gy of total radiation, given on a 2.0 Gy qd x 4 schedule (data not shown). Therefore, it seems that in the present study, a single i.p. dose of 15 mg/kg NLCQ-1 given 45 min before irradiation accounted for the same antitumor effect caused by a single radiation dose of 3 Gy. Furthermore, since NLCQ-1 treatment alone did not result in any tumor growth delay, NLCQ-1 interacted with radiation in a synergistic way. In a relatively recent study against intracranial U251 gliomas, 53.4 mg/kg (0.3 mmol/kg) of tirapazamine combined with a single radiation dose of 5.0 Gy did not cause any statistically significant difference in survival compared to control or radiation alone treatment (21). This single tirapazamine dose represented 68% of its LD$_{50}$ (22) and it was 7.36-fold greater than the single NLCQ-1 molar dose of 0.0407 mmol/kg (15 mg/kg, 42.8% LD$_{50}$) used in the present study.

However, the most impressive result was obtained when the fractionated schedule of NLCQ-1 plus 3.0 Gy qd x 2, 9 days rest and repeat was applied. In this case, 9 out of 10 complete tumor regressions with long duration were obtained (Table II), besides the very significant tumor growth delays, which were statistically significant compared to the untreated control or radiation alone-treated groups ($p<0.001$ and $<0.02$, respectively). This is interesting because it implies that, since NLCQ-1 is activated only under hypoxic conditions, rehypoxiation (23) perhaps occurred between the fractionated treatments of human gliomas with radiation. Thus, NLCQ-1 could be beneficial against gliomas in the clinic where a fractionated radiation protocol is usually followed.

In a similar study against U251 human glioma xenografts, fractionated radiation was combined with tirapazamine (24). When tirapazamine was given i.p. at 0.08 mmol/kg (14.24 mg/kg) daily for 6 consecutive days prior to 2-Gy irradiation, the tumor volume doubling time was increased to 35±2.5 days compared to 22±2.5 days for radiation alone and 10±0 days for untreated control. The total tirapazamine dose used was 0.48 mmol/kg (85.44 mg/kg), whereas the total radiation administered was 12 Gy. In our fractionated protocol, 12 Gy of total radiation was also administered, whereas the total NLCQ-1 dose used was 0.163 mmol/kg (60 mg/kg), namely about 3-fold less molar dose than tirapazamine. However, a much more substantial increase in tumor doubling time was obtained (25.2 extra days compared to radiation alone treatment) (Table I). Moreover, the above protocol resulted in extended survival (Figure 2). Thus, 3.0 Gy qd x 2 on days 6 and then 17 extended survival for 48 days, whereas the addition of NLCQ-1 to the protocol further prolonged survival for 17 additional days.
The presented results suggest a significant advantage in combining radiation with NLCQ-1 against glioma tumors, especially when following a fractionated protocol.

Acknowledgements
This work was supported by internal NCI grants and by NCI Contract NO1-CM-12400 to SAIC-Frederick, Inc. It was presented in part at the 94th Annual Meeting of the American Association for Cancer Research, March 27-31, 2004, Orlando, FL as well as at the 7th International Conference of Anticancer Research, October 25-30, 2004, Corfu, Greece.

References

Received February 2, 2005
Accepted March 1, 2005