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NLNQ-1, a 2-[3-(2-Nitro-1-Imidazolyl) Propylamino]-3-Chloro-1,4-Naphthoquinone, as a Hypoxia-selective Cytotoxin and Radiosensitizer

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Abstract. *Background: Compounds bearing two independent redox centers are considered bis-bioreductive agents and usually demonstrate increased hypoxic selectivity with exposure time due to different requirements for reduction of each center. We have synthesized a novel 2-[3-(2-nitro-1-imidazolyl)propylamino]-3-chloro-1,4-naphthoquinone (NLNQ-1), through Michael addition. NLNQ-1, which combines a naphthoquinone (with a relatively high one electron reduction potential) with a 2-nitroimidazole (with a relatively low one electron reduction potential), could perform as a more potent hypoxia-selective cytotoxin and radiosensitizer. Materials and Methods: NLNQ-1 was evaluated in V79 cells under hypoxic/normoxic conditions, alone or with radiation, by using the clonogenic assay. Results: Clearly NLNQ-1 was a more potent cytotoxin than the 2-alkylsulfonyloxy-naphthoquinones (VH-compounds), developed previously in our lab, demonstrating hypoxic and aerobic IC_{50} values at μM rather than mM concentrations. As a radiosensitizer of hypoxic cells, NLNQ-1 was superior to the best bis-nitroimidazolic compound, NNB (which combines a 2-nitroimidazole with a 5-nitroimidazole), demonstrating a $C_{1.6}$ value of 25.4 μM (ca. 25 fold lower than that of NNB), whereas its in vitro therapeutic index ($IC_{50A}/C_{1.6}$) ranged from 5.3-13.2. Conclusion: NLNQ-1 could be used as a novel scaffold for bis-bioreductive agents that can be properly modified for further optimization of their hypoxia-selective toxicity and radiosensitization properties.*

Tumor-associated hypoxia has long been recognized as a major problem in radiotherapy because it renders solid tumors more resistant to ionizing radiation (1). However, the importance of tumor hypoxia to the wider cancer research community has become more apparent in the last 20 years, as it has become clear that hypoxia is a major determinant of the response to therapy and it signals poor prognosis, regardless of treatment, even when tumors are surgically excised (2). Therefore, there is increasing interest in the development of potent hypoxia-activated prodrugs. Such compounds not only selectively kill hypoxic tumor cells, but they also interact in a synergistic way with other modality treatments, including radiation (3).

One major class of bioreductive prodrugs are the nitroimidazoles, which undergo enzymatic reduction of the nitro group via the one electron reduction product. This first step in nitroreduction is totally reversed in the presence of oxygen, resulting in preferential metabolism in hypoxic cells (4). However, the prototype bioreductive agent was the quinone alkylating agent mitomycin (MMC) (5). Mitomycin (and other quinones) is reduced under hypoxic conditions by the one-electron NADPH/cytochrome P450 reductase, a process that facilitates opening of the aziridine moiety and alkylation of DNA (6). In other words, MMC is a bi-functional bioreductive agent.

Denny and his group thought to make a "bis-bioreductive" agent by combining two nitroimidazoles with different redox potentials (7, 8). In this case an increased hypoxic selectivity can be achieved with exposure time due to different requirements for reduction of each redox center (8). Increased hypoxic selectivity was previously seen with another bis-bioreductive compound, the nitracrine N-oxide (9). However, bis-nitroimidazoles demonstrated solubility problems and low potency as radiosensitizers and were not developed further (7).

Another approach to bis-bioreductive agents is to combine a nitroimidazole with a quinone moiety via an amine linker for better solubility. Therefore, we have synthesized 2-[3-(2-nitro-1-imidazolyl)propylamino]-3-chloro-1,4-naphthoquinone (NLNQ-1), through Michael addition of 3-(2-nitro-1-imidazolyl)propylamine to 2,3-dichloro-1,4-naphthoquinone. In this way we combined a naphthoquinone moiety (with a relatively high one electron reduction potential) with a 2-nitroimidazolic moiety (with a relatively low one electron

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Key Words: NLNQ-1, hypoxic cytotoxins, radiosenzitizers, bis-bioreductive agents.

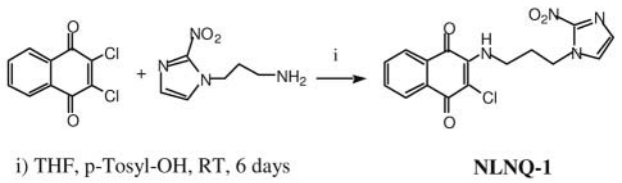

Figure 1. *Schematic synthesis of NLNQ-1.*

reduction potential) in an effort to end up with a more potent hypoxia-selective cytotoxin and radiosensitizer.

Materials and Methods

Chemicals. The synthesis of NLNQ-1 is outlined in Figure 1. Briefly, in a 2-necked flask and under nitrogen atmosphere, 154 mg (0.665 mmoles) of 2,3-dichloro-1,4-naphthoquinone and a catalytic amount of p-TsOH were dissolved in anhydrous THF (8 ml). Then, a solution of 170 mg (1 mmole) of 3-(2-nitro-1-imidazolyl)propylamine in dichloromethane (7 ml) was added dropwise over 30 min, at room temperature, under stirring conditions. The reaction mixture was stirred at room temperature for 6 days, then evaporated and chromatographed on a silica gel preparative TLC plate with 70% ethyl acetate and 30% petroleum ether as eluents. The product of interest was isolated (R_f=0.58) as an orange/red solid (40-50% yield). Mp. 153-156˚C (dec.). IR (nujol): 3290 cm^{-1} (NH); 1680 cm^{-1} (CO); 1570 cm^{-1} (NO_2). ^1H NMR ($CDCl_3$) δ: 8.16 (d, J=8.5 Hz, 1H), 8.04, (d. J=8.5 Hz, 1H), 7.75 (t, J=7.1 Hz, 1H), 7.65 (t, J=7.1 Hz, 1H), 7.20 (s, 1H), 7.15 (s, 1H), 6.13 (br, 1H), 4.56, (t, J=5.6 Hz, 2H), 3.95 (m, 2H), 2.31 (m, 2H). LRMS: Calculated for $C_{16}H_{14}ClN_4O_4$ (M+H): *m/z* 361. Found: 361. For *in vitro* experiments, NLNQ-1 was dissolved in DMSO at 5.55 mM and then diluted appropriately with cell culture medium so that the final DMSO concentration was ≤0.5%.

NQ-1. This compound (Figure 2) was synthesized and evaluated for comparison purposes. 2-Chloro-3-hydroxy-1,4-naphthoquinone (2 mmoles) was dissolved in 25 ml anhydrous THF and the solution was cooled down to 0˚C under a N_2 atmosphere. Then, a THF solution of 2-hydroxyethylamine was added slowly (30 min), and the reaction mixture was stirred for 4 h at room temperature. The product was filtered off as a red precipitant. Yield 65-70%. Mp. 164-167˚C. ^1H NMR (CD_3OD) δ: 8.00 (d, J=8.3 Hz, 1H), 7.94 (d, J=8.3 Hz, 1H), 7.68 (t, J=6.0 Hz, 1H), 7.56 (t, J=6.0, 1H), 3.75 (t, J=2.4 Hz, 2H), 3.04 (t, J=3.5 Hz, 2H). LRMS: Calculated for $C_{12}H_{12}NO_4$ (M+H): *m/z* 234. Found: 234.

Cells. V79 cells (Chinese hamster lung; ATCC, Rockville, MD, USA), exponentially growing as monolayer cultures in RPMI 1640 medium supplemented with 10% FBS were used in all experiments.

Toxicity studies. Cells were trypsinized, centrifuged (750 g) for 5 min, harvested and suspended in 25 ml glass Erlenmeyer flasks fitted with rubber caps, at $5x10^5$ cells/ml (5 ml) for exposure to NLNQ-1 under hypoxic/aerobic conditions in a humidified incubator at 37˚C. Hypoxia was induced by gassing the shaken glass flasks (100 rpm) through inlets and outlets with a humidified mixture of 95% N_2 plus 5 % CO_2 for 1 h prior to addition of NLNQ-1. Cells were exposed under hypoxia to NLNQ-1 for various time intervals. For aerobic exposures, flasks were gassed with a humidified mixture of 95 % air plus 5 % CO_2. After treatment, cells were processed for clonogenicity (11).

Table I. *Cytotoxicity and radiosensitization parameters of NLNQ-1, NNB, VH-3 and NQ-1.*

Compound	IC_{50H} (μM x h)	HS	$C_{1.6}$ (μM)	*In vitro* TI
NLNQ-1	34.5-48.2	4-7	25.4	5.3-13.2
NNB (7, 8)	370-1200*	8-200*	630	11
VH-3 (11)	1800	1.8	400	2.75
NQ-1	>4500	ND	ND	ND

IC_{50}: Concentration x time for 50% reduction in clonogenicity under aerobic (A) or hypoxic (H) exposure to the compound; HS: hypoxic selectivity equals $IC_{50(A)}/IC_{50(H)}$; $C_{1.6}$: concentration of a compound to obtain a sensitization enhancement ratio (SER) of 1.6; TI: therapeutic index equals $IC_{50A}/C_{1.6}$. *This value represents the IC_{10H}, otherwise concentration x time to reduce clonogenicity to 10% of the control, under hypoxic conditions. ND: Not determined.

Radiosenzitization studies. These studies were performed as has been described before (10). Briefly, NLNQ-1 was added to aerated or hypoxic cells at 37˚C, 1 h before irradiation at room temperature (^{60}Co, 1.534 Gy/min). Hypoxia was maintained until the end of irradiation. Then, cells were washed free of drug and processed for clonogenicity. Survival was expressed as the fraction of the untreated controls. The plating efficiency of untreated V79 cells was 71.2 % (59.5-80.5%).

Results and Discussion

NLNQ-1 was clearly a more potent cytotoxin, on a concentration basis, than the 2-alkylsulfonyloxy-naphthoquinones (VH-compounds, Figure 2), developed previously in our lab (11), demonstrating hypoxic and aerobic IC_{50} values at μM rather than mM concentrations (Table I). Thus, the IC_{50H} and IC_{50A} value (concentration x exposure time for 50% reduction in clonogenicity under hypoxic and aerobic conditions, respectively) was ranging from 34.5 to 48.2 μM x h and 135 to 335 μM x h, respectively, both increasing slightly over a period of 8 h exposure. Therefore, the hypoxic selectivity (IC_{50A}/IC_{50H}) of NLNQ-1 was increased from ca. 4 to 7 over 8 h of exposure not because of an expected increase in the hypoxic toxicity but rather the slight decrease observed in the aerobic toxicity over time (Figure 3). NLNQ-1 was also a much more potent cytotoxin compared to the structurally more related compound NQ-1 (Figure 2) which, however, lacks the 2-nitroimidazole redox center (Table I). As expected, NLNQ-1 was a more potent hypoxic cytotoxin than misonidazole, a mono-nitroimidazolic bioreductive agent (7). In addition, NLNQ-1 was more potent on a

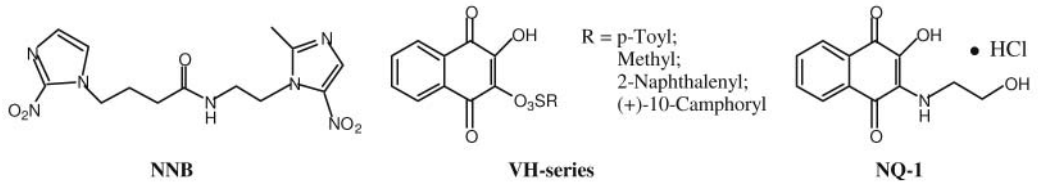

Figure 2. *Chemical structures of NNB, VH (s) and NQ-1.*

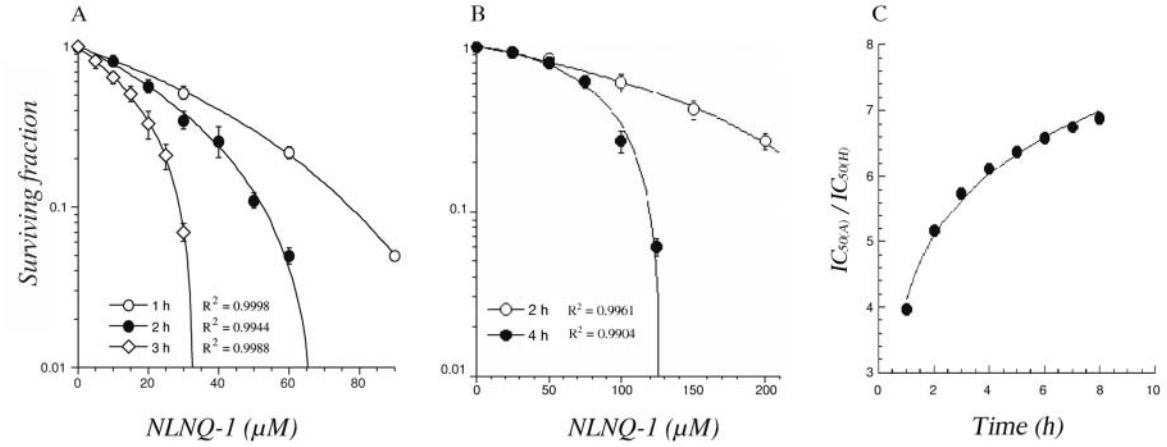

Figure 3. *Examples of concentration-dependent cytotoxicity of NLNQ-1 in V79 cells under hypoxic (A) or aerobic (B) conditions. Bars represent SD of quadruplicate measurements. Changes in hypoxic selectivity (aerobic IC_{50}/hypoxic IC_{50}) of NLNQ-1 with time in V79 cells (C). IC_{50} is the concentration x exposure time to reduce survival to 50% of controls.*

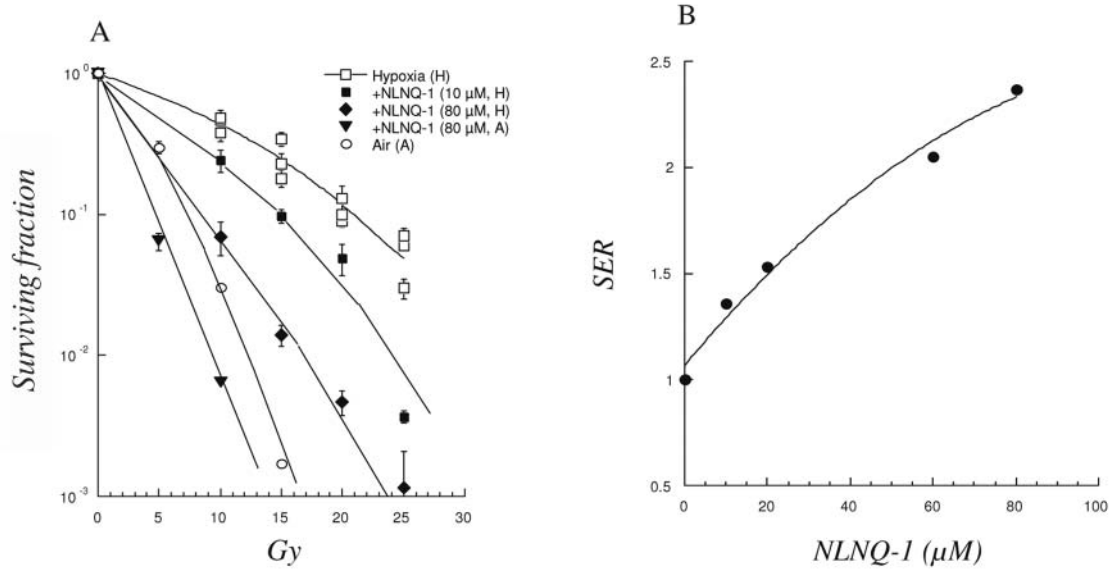

Figure 4. *Radiosensitizing effect of NLNQ-1 on V79 cells. Cells were exposed to various concentrations of NLNQ-1 under hypoxic or aerobic conditions for 1 h at 37°C, before their subsequent irradiation under similar conditions, at room temperature (A). Bars represent SD of quadruplicate measurements. SER values obtained at 10% survival from plots in panel A for various NLNQ-1 concentrations (B).*

concentration basis than all tested bis-nitroimidazoles (7), confirming our hypothesis that combination of a nitroimidazole moiety with an easily reduced naphthoquinone (12) will increase the potency of the bioreductive agent. However, the fact that the hypoxic toxicity of NLNQ-1 is not increased over time deserves further investigation. For bis-nitroimidazoles, it was suggested that a linker chain length of more than 5 atoms may be required between the two redox systems for increasing hypoxic selectivity (7). This may be true in the case of NLNQ-1 as well, since the two redox systems are relatively close and may interfere in each other's activation.

As a radiosensitizer of hypoxic cells, NLNQ-1 was superior to the best bis-nitroimidazole compound NNB (which combines a 2-nitroimidazolic moiety with a 5-nitroimidazolic moiety), demonstrating a $C_{1.6}$ value (concentration for an SER of 1.6) of 25.4 μM (ca. 25 fold lower than that of NNB (7)). Thus, an *in vitro* therapeutic index ($TI=IC_{50A}/C_{1.6}$) ranging from 5.3-13.2 was achieved (Table I). The corresponding TI value for NNB was 11 (7). Sensitization enhancement ratios (SERs) were calculated from radiosensitization curves obtained under hypoxic conditions (Figure 4A) and plotted *versus* concentration (Figure 4B). A maximum SER value of 2.5 was achieved at 80 μM of NLNQ-1, whereas the SER_{max} for NNB was only 2 at ca. 5 mM (7). Interestingly, aerobic radiosensitization was observed also at the aerobically non-toxic concentration of 80 μM (Figure 4A), something unusual for nitro-bioreductive compounds.

In conclusion, NLNQ-1 is a novel bis-bioreductive compound, combining a 2-nitroimidazole with a 1,4-naphthoquinone via an alkylamino-chain linker. NLNQ-1 demonstrated increased potency as a hypoxic cytotoxin and radiosensitizer *in vitro*, compared to the best bis-nitroimidazole bioreductive agent NNB. However, only a modest hypoxic selectivity was achieved. Therefore, NLNQ-1 could be used as a novel scaffold for further modification and optimization of bis-bioreductive agents combining two different redox moieties.

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