Nitroimidazole-based bioreductive compounds bearing a quinazoline or a naphthyridine chromophore

Maria V. Papadopoulou and William D. Bloomer

In our search for novel bioreductive agents with weak DNA-binding characteristics, we have synthesized two new 2-nitroimidazolyl derivatives tethered to a fused aromatic-ring chromophore with two nitrogen atoms: 4-[3-(2-nitro-1-imidazolyl)-propylamino]-2-methyl-quinazoline hydrochloride (NLQZ-1) and 4-[3-(2-nitro-1-imidazolyl)-propylamino]-1,5-naphthyridine hydrochloride (NLPP-1). DNA binding was evaluated by using the ethidium bromide displacement assay. Cytotoxicity, radiosensitization, and interaction with chemotherapeutic agents were evaluated in V79 and A549 cells by using the clonogenic assay. Both compounds are not DNA intercalators and showed relatively low uptake characteristics in V79 cells. A slightly increasing hypoxic selectivity [HS = $IC_{50(A)}/IC_{50(H)}$ (IC_{50} is the product of a compound's concentration and the time necessary for 50% reduction in clonogenicity under aerobic (A) or hypoxic (H) conditions)] was observed with incubation time in the case of NLPP-1 (12–19 and 15–26 in V79 and A549 cells, respectively, with 1–3 h of incubation). The HS of NLQZ-1 was approximately 14, independently of incubation time. Good radiosensitization of hypoxic V79 cells was obtained with both compounds at nontoxic concentrations [the concentration for a sensitization enhancement ratio of 1.6 ($C_{1.6}$) was 61.4 and 75.0 μmol/l for NLQZ-1 and NLPP-1, respectively]. For NLPP-1, a $C_{1.6}$ of 44.1 μmol/l was obtained in A549 cells. Both compounds interacted synergistically with cisplatin or melphalan in V79 cells, under hypoxic preexposure conditions and dose modification factors values of approximately 2.5 were obtained at 10% survival. It is concluded that although compounds that do not bind to DNA are in general less potent hypoxic cytotoxins, they can still show good HS values and interact synergistically with radiation/chemotherapeutic agents. Therefore, a further in-vivo evaluation of NLQZ-1 and NLPP-1 is worthwhile. *Anti-Cancer Drugs* **20:493–502** © **2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

Tumor-associated hypoxia is 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 only been recognized in the last 20 years when it became clear that hypoxia is a major determinant of therapeutic response and it portends poor prognosis, regardless of treatment, even when the tumors are surgically excised [2]. This resulted in an increased interest in the development of potent bioreductive agents, or otherwise, hypoxia-activated prodrugs. Such compounds not only selectively kill hypoxic tumor cells but also interact synergistically with other treatment modalities [3].

For potency and efficacy of hypoxia-activated bioreductive compounds *in vivo*, three factors are crucial: favorable reduction potential [4], relative stability under aerobic conditions [5], and a noncovalent binding to DNA that allows for fast dissociation kinetics [6]. To this end, we have previously developed 2-nitroimidazole derivatives that bind to DNA through weak intercalation through a quinolinic chromophore or a fused tricyclic quinolinic scaffold with disturbed planarity [7–10]. The lead compound of this series, 4-[3-(2-nitro-1-imidazolyl)-propylamino]-7-chloroquinoline hydrochloride (NLCQ-1), has shown excellent in-vivo activity with various treatment modalities such as radiation, radioimmunotherapy, or chemotherapy, because of its efficient extravascular diffusion and penetration to the hypoxic tumor tissues [3,9,11–13].

To further explore the field of bioreductive agents with weak DNA-binding characteristics, we have tethered 2-nitroimidazole to a fused aromatic-ring chromophore containing two nitrogen atoma, to form 4-[3-(2-nitro-1-imidazolyl)-propylamino]-2-methyl-quinazoline hydrochloride (NLQZ-1) and 4-[3-(2-nitro-1-imidazolyl)-propylamino]-1,5-naphthyridine hydrochloride (NLPP-1). The compounds were evaluated *in vitro* as hypoxia-selective cytotoxins and radio/chemosensitizers.

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Materials and methods

Chemistry

Solvents and chemicals were purchased from Aldrich (Milwaukee, Wisconsin, USA). NLQZ-1 and NLPP-1 were synthesized as outlined in Fig. 1.

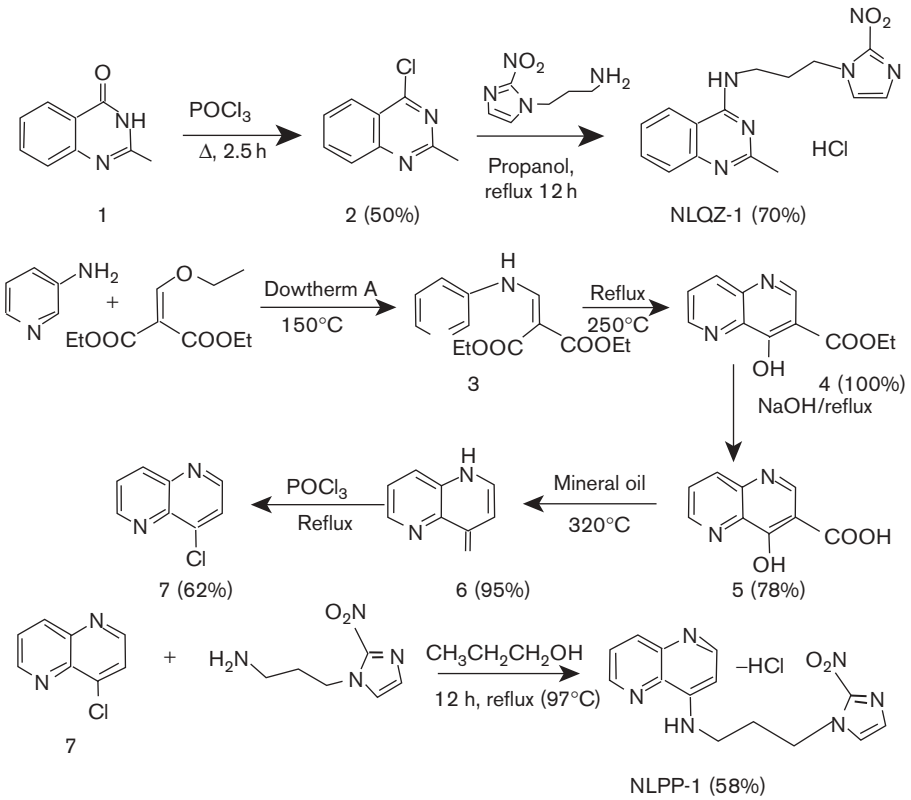
4-[3-(2-Nitro-1-imidazolyl)-propylamino]-2-methyl-quinazoline hydrochloride

2-Methylquinazolin-4(3H)-one was first converted to the 4-chloro-2-methylquinazoline by refluxing (2.5 h) in $POCl_3$ [14]. Under an atmosphere of nitrogen and exclusion of moisture, 148 mg (1 equivalent) of 4-chloro-2-methylquinazoline and 189 mg (1.34 equivalent) of 3-(2-nitro-1-imidazolyl)-propylamine were dissolved in 3 ml absolute propanol and refluxed for 12 h. The desired product was precipitated as its HCl salt and collected by filtration (201 mg, 70% yield). Ultraviolet (UV) (Hewlett Packard UV spectrophotometer, Agilent, Palo Alto, California, USA) (water): 314 nm (NO_2). ^{1}H NMR (GEN-500, 500 MHz spectrometer, GE NMR Instruments, Fremont, California, USA) (D_2O) δ: 7.80 [doublet (d), $J = 8.8$ Hz, 1 H] 7.74 [triplet (t), $J = 7.4$ Hz, 1 H], 7.46 (t, $J = 7.4$ Hz, 1 H), 7.42 (d, $J = 8.8$ Hz, 1 H), 7.28 [singlet (s), 1 H], 6.83 (s, 1 H), 4.41 (t, $J = 7.3$ Hz, 2 H), 3.65 (t, $J = 6.1$ Hz, 2 H), 2.40 (s, 3 H), 2.17 [multiplet (m), 2 H). LRMS (VG 70–250SE mass spectrometer, VG Analytical, Manchester, UK): calculated for $C_{15}H_{17}N_6O_2$ ($M + H^+$) mass/charge (m/z) 313. Found: 313.

4-[3-(2-Nitro-1-imidazolyl)-propylamino]-1,5-naphthyridine hydrochloride

This was synthesized by coupling the 4-chloro-1, 5-naphthyridine (193.5 mg, 1 equivalent) with 3-(2-nitro-1-imidazolyl)-propylamine (200 mg, 1 equivalent) by refluxing in absolute propanol (3.5 ml) for 12 h under an atmosphere of nitrogen (Fig. 1). The reaction mixture was cooled down and filtered to remove some unreacted 3-(2-nitro-1-imidazolyl)-propylamine as its hydrochloride salt. The filtrate was evaporated, redissolved in a small volume of MeOH, and chromatographed on alumina preparative thin layer chromatography (ethyl acetate). The free amine of NLPP-1 was isolated ($R_f = 0.25$) as a viscous liquid, dissolved in anhydrous acetone, and converted to its HCl salt (white precipitant) by addition of 1mol/l HCl gas in dry ether. Yield 58%; melting point= 230°C (decomposition). UV (water):

Fig. 1



Schematic synthesis of 4-[3-(2-nitro-1-imidazolyl)-propylamino]-2-methyl-quinazoline hydrochloride (NLQZ-1) and 4-[3-(2-nitro-1-imidazolyl)-propylamino]-1,5-naphthyridine hydrochloride (NLPP-1). 1: 2-methylquinazolin-4(3H)-one; 2: 4-chloro-2-methylquinazoline; 3: diethyl (3-pyridinamino)-methylenemalonate; 4: 3-carboethoxy-4-hydroxy-1,5-naphthyridine; 5: 3-carboxy-4-hydroxy-1,5-naphthyridine; 6: 1,4-dihydro-1,5-naphthyridin-4-one; 7: 4-chloro-1,5-naphthyridine.

333 nm (NO_2), 243 nm. ^{1}H NMR (D_2O) δ: 8.90 [doublet doublet (dd), J = 5.5, 1.0 Hz, 1 H), 8.35 (d, J = 7.0 Hz, 1 H), 8.26 (dd, J = 10.0, 1.5 Hz, 1 H), 7.93 (dd, J = 8.8, 4.5 Hz, 1 H), 7.48 (s, 1H), 7.05 (s, 1 H), 6.90 (d, J = 7.0 Hz, 1 H), 4.67 (t, J = 7.0 Hz, 2 H), 3.77 (t, J = 6.5 Hz, 2 H), 2.44 (quintet, J = 6.5 Hz, 2 H). LRMS (VG 70-250SE mass spectrometer): calculated for $C_{14}H_{15}N_6O_2$ ($M + H^+$) m/z 299. Found: 299.

The precursors 4-chloro-1,5-naphthyridine, 4-hydroxy-1, 5-naphthyridine, 3-carboxy-4-hydroxy-1,5-naphthyridine, and 3-carboethoxy-4-hydroxy-1,5-naphthyridine were prepared as depicted in Fig. 1, according to the literature [15] and identified with IR (Perkin-Elmer Corp., Fair Oaks, California), ^{1}H NMR, and mass spectroscopy. The reaction between 3-aminopyridine and diethyl methylenemalonate did not give directly the compound 3-carboethoxy-4-hydroxy-1,5-naphthyridine (as expected from the literature) but diethyl (3-pyridinamino)-methylenemalonate, which was converted to 3-carboethoxy-4-hydroxy-1,5-naphthyridine by additional refluxing at 250°C for 2.5 h.

4-Chloro-1,5-naphthyridine: ^{1}H NMR ($CDCl_3$) δ: 9.11 (dd, J = 5.1, 1.0 Hz, 1 H), 8.87 (d, J = 5.1 Hz, 1 H), 8.46 (dd, J = 8.2, 1.0 Hz, 1 H), 7.78 (d, J = 5.1 Hz, 1 H), 7.74 (dd, J = 8.2, 4.1 Hz, 1H). HRMS: calculated for $C_8H_5ClN_2$ (M^+): m/z 164.01413. Found: 164.01417.

1,4-Dihydro-1,5-naphthyridin-4-one: ^{1}H NMR (D_2O + DMSO-6d) δ: 9.03 [s, broad (br), 1 H], 8.37 (d, J= 10.0 Hz, 1 H), 8.32 (d, J = 7.1 Hz, 1 H), 8.03 (m, 1 H), 6.73 (d, J = 7.1 Hz, 1H). LRMS: calculated for $C_8H_6N_2O$ (M^+): m/z 146. Found: 146.

3-Carboxy-4-hydroxy-1,5-naphthyridine: ^{1}H NMR (D_2O) (as the Na salt) δ: 8.58–8.50 (m, 2 H), 8.02 (d, J = 8.33 Hz, 1 H), 7.54–7.49 (m, 1 H). LRMS: calculated for $C_9H_6N_2O_3$ (M^+): m/z 190. Found: 190, 172 $(M\text{-}H_2O)^+$, 146, $(M\text{-}CO_2)^+$.

3-Carbethoxy-4-hydroxy-1,5-naphthyridine: this compound was in equilibrium with the keto form in the $CDCl_3$ solution and gave a complicated spectrum in the aromatic area. ^{1}H NMR ($CDCl_3$) δ: 8.75–6.64 (d, s-br, d, d, dd, d, dd, d, 4 H), 4.38 (m, 2 H), 1.38 (m, 3 H). LRMS: calculated for $C_{11}H_{11}N_2O_3$ ($M + H^+$): m/z 219. Found: 219.

Diethyl (3-pyridinamino)-methylenemalonate: melting point= 64°C. ^{1}H NMR ($CDCl_3$) δ: 11.02 (d-br, J = 13.5 Hz, 1 H), 8.54–8.34 (m, 3 H), 7.49 (d-br, 1 H), 7.32 (m, 1H). 4.30 (quartet, J = 7.2 Hz, 2H), 4.25 (quartet, J = 7.2 Hz, 2H), 1.38 (t, J = 7.2 Hz, 3H), 1.32 (t, J = 7.2 Hz, 3H). LRMS: calculated for $C_{13}H_{16}N_2O_4$ (M^+): m/z 264. Found: 264, 218 $(M\text{-}EtOH)^+$.

DNA binding was evaluated using the ethidium bromide (EB) fluorescence displacement assay at pH 8 and low ionic strength [16]. Excitation was set to 525 nm and emission was measured at 600 nm using a Cambridge Technology 7625 Microplate Fluorometer (Watertown, Massachusetts, USA).

Cells

A549 (human lung adenocarcinoma) and V79 (Chinese hamster lung) cells were purchased from American Type Culture Collection (Rockville, Maryland, USA). Exponentially growing cells as monolayer cultures in RPMI 1640 medium supplemented with 10% fetal bovine serum were trypsinized, centrifuged (750 g for 5 min), harvested, and resuspended in 25-ml Erlenmeyer flasks fitted with rubber caps, normally at 5×10^5 cells/ml (5 ml). The cells were allowed to recover from the trypsin treatment for 1 h at 37°C, in a humidified incubator. Then the flasks were shaken (100 rpm) at 37°C and gassed with humidified gas mixtures containing either 95% air plus CO_2 (aerobic conditions) or 95% N_2 plus 5% CO_2 (hypoxic conditions) for 1 h before drug was added. The plating efficiency of untreated V79 and A549 cells was 71.2% (59.5–80.5%) and 54.3% (45.0–63.0%), respectively.

Acute toxicity

Acute toxicity was determined by exposing suspended cells to a fixed drug concentration under hypoxic or aerobic conditions for various periods of time at 37°C, as has been described before [8]. Survival was determined by plating 2×10^2 to 2×10^4 cells/well in quadruplicates, on 60-mm Linbro multi-well plates (Flow Laboratories, McLean, Virginia, USA). The plates were incubated for 14 days, stained with crystal violet, and examined for colony formation. Colonies of 50 cells or greater were counted and survival was expressed as a fraction of the control (cells similarly treated but not drug exposed).

Drug uptake measurements

Intracellular and extracellular drug concentrations were determined in V79 cells as has been described by us before [8]. Briefly, 10^7 cells/flask were exposed to various drug concentrations or no drug for 30 min, under aerobic conditions, at 37°C. Afterwards, the cells were pelleted, lysed with 90 µl distilled water, and deproteinized with 0.9 ml acetonitrile. A small volume of the supernatant (200 µl) was combined with nine equal volumes (1.8 ml) of acetonitrile and the remaining supernatant was discarded. After centrifugation and filtration, all samples were stored at −70°C until UV spectroscopic analysis at 333 or 314 nm (absorption of the nitro group for NLPP-1 and NLQZ-1, respectively) for intracellular and extracellular drug concentrations determination. The efficiency of drug recovery from cell lysates or supernatant was determined in parallel studies in which lysates and supernatant of untreated cells were spiked with

known drug concentrations. Mean intracellular concentration of drug was calculated using a value of 810 fl as the intracellular water content of log-phase cells [17] and taking in account the efficiency of recovery.

Interaction with radiation *in vitro*

These studies were performed as has been described before [7]. Briefly, each compound 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. Colonies were stained with crystal violet and counted. Survival was expressed as the fraction of the untreated controls and plotted versus radiation dose.

Interaction with cisplatin/melphalan *in vitro*

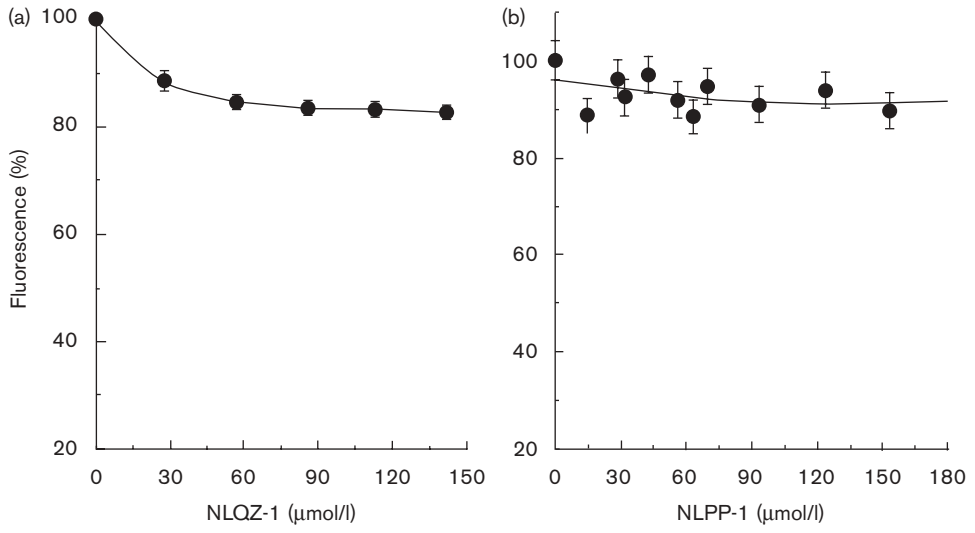
V79 cells, while in suspension, were exposed to a fixed concentration of NLPP-1/NLQZ-1 under hypoxic conditions (2 h, 37°C) followed by an aerobic coexposure (1 h, 37°C) to cisplatin (cisDDP)/melphalan (L-PAM) at varying concentrations, and then processed for clonogenicity to determine dose modification factors (DMF). In addition, V79 cells were exposed to each bioreductive agent from 1 to 5 h under hypoxic conditions followed by 1 h aerobic coexposure to a fixed concentration of cisDDP/L-PAM to determine the time effect. Untreated controls or controls treated with each agent alone were always included. Synergistic interactions were evaluated by using the fractional product analysis [18], which can apply in instances of drugs with independent action. In our experimental model, each bioreductive compound was administered to the cells under hypoxic pretreatment conditions, whereas the chemotherapeutic agent was administered sequentially under aerobic conditions. The dose of the bioreductive compound was minimally toxic under hypoxic conditions and not toxic under aerobic exposures.

Results

The synthesis of NLQZ-1 and NLPP-1 was straightforward and with relatively good yields (Fig. 1). Both compounds did not bind to DNA, as EB was not displaced to a significant degree from its DNA complex at increasing compound concentrations (Fig. 2). Thus, no C_{50} values (concentration for 50% reduction in fluorescence) were determined by the EB-displacement assay. The EB-displacement assay is not specific for intercalators. Groove binding compounds can also displace ethidium. However, minor-groove binding is dominated by electrostatic interactions between the negatively charged backbone of DNA and the cationic form of a compound. As the assay was run at pH 8, we assume that the binding motif would be dominated by intercalation rather than electrostatic interactions.

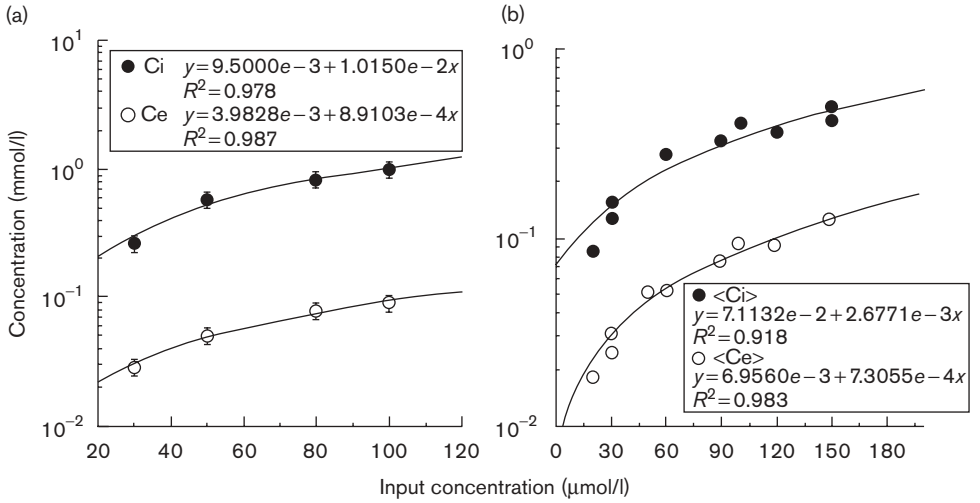
Both compounds showed relatively low uptake characteristics in V79 cells, with maximum uptake factors (intracellular to extracellular concentration ratio) of about 5.5. An uptake factor of 11 was obtained for NLQZ-1 in serum free medium, indicative of a possible binding of the compound with proteins in complete medium (Fig. 3).

Fig. 2



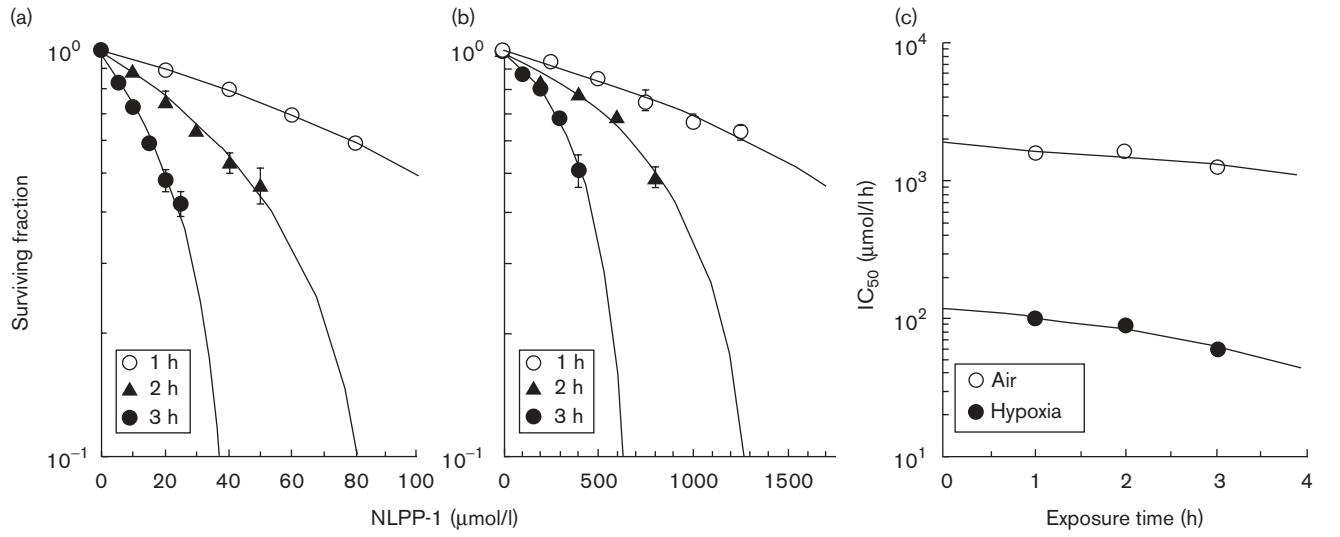
DNA-binding results for 4-[3-(2-nitro-1-imidazolyl)-propylamino]-2-methyl-quinazoline hydrochloride (NLQZ-1) (a) and 4-[3-(2-nitro-1-imidazolyl)-propylamino]-1,5-naphthyridine hydrochloride (NLPP-1) (b). The fluorescence because of the DNA-ethidium bromide complex was recorded and plotted versus increasing concentration of each compound. C_{50} values (concentration for 50% reduction in fluorescence) were not determined because of the asymptotic nature of the curves.

Fig. 3



Uptake of 4-[3-(2-nitro-1-imidazolyl)-propylamino]-2-methyl-quinazoline hydrochloride (NLQZ-1) (a) and 4-[3-(2-nitro-1-imidazolyl)-propylamino]-1, 5-naphthyridine hydrochloride (NLPP-1) (b) in V79 cells. Cells were incubated for 30 min (37°C) under aerobic conditions with varying concentrations of each compound. In the case of NLQZ-1, the uptake in serum-free cells is shown. Mean intracellular (<Ci>) and extracellular (<Ce>) concentrations were determined by ultraviolet spectroscopy.

Fig. 4



Cytotoxicity of 4-[3-(2-nitro-1-imidazolyl)-propylamino]-1,5-naphthyridine hydrochloride (NLPP-1) in A549 cells under hypoxic (a) and aerobic (b) conditions. IC_{50} values (concentration × time to reduce survival to 50% of control) were plotted versus exposure time (c) to generate the hypoxic selectivity graphs in Fig. 5.

Examples of cytotoxicity studies under hypoxic/normoxic conditions are shown in Fig. 4. $IC_{50(A)}$ [the product of a compound's concentration and the time necessary for 50% reduction in clonogenicity under aerobic (A) conditions] values in V79 cells varied from 998.6 (1 h) to 1486.7 μmol/l (3 h) for NLQZ-1, and from 1000.0 (1 h) to 962.2 μmol/l (3 h) for NLPP-1 (Table. 1). The corresponding values of NLPP-1 in A549 cells were 1668.8 (1 h) to 1311.6 μmol/l (3 h). A slightly increasing hypoxic selectivity [HS = $IC_{50(A)}/IC_{50(H)}$] with exposure time was observed in the case of NLPP-1. Thus, HS was ranging from 12–19 and 17–22 in V79 and A549 cells, respectively, with 1–3 h of incubation. The HS of NLQZ-1 was approximately 14, independently of exposure time (Fig. 5).

Good radiosensitization of hypoxic V79 cells was obtained with each compound at nontoxic concentrations (Fig. 6). Consequently, $C_{1.6}$ values [concentration for a sensitization

Table 1 Various parameters of the hypoxia-selective cytotoxins NLQZ-1 and NLPP-1 and comparison with NLCQ-1 in V79 cells

Compound	$IC_{50(A)}$[a] (μmol/l h)	$IC_{50(H)}$[a] (μmol/l h)	HS[b]	$C_{1.6}$[c] (μmol/l)	$C_{i1.6}$[d] (μmol/l)	ThI[e]
NLQZ-1	998.6–1486.7	73.6–108.4	14	61.4	347.9 ± 6.0	16–24
NLPP-1 (V79)	1000–962.2	86.1–51.9	12–19	75.0	271.9 ± 3.5	13
NLPP-1 (A549)	1668.8–1311.6	97.6–59.7	17–22	44.1	ND	30–38
NLCQ-1	414.2–958.9[f]	86–14.7[f]	5–65[f]	7.2	62.5 ± 1.5	57–133[f]

NLCQ-1, 4-[3-(2-nitro-1-imidazolyl)-propylamino]-7-chloroquinoline hydrochloride; NLPP-1, 4-[3-(2-nitro-1-imidazolyl)-propylamino]-1,5-naphthyridine hydrochloride; NLQZ-1, 4-[3-(2-nitro-1-imidazolyl)-propylamino]-2-methyl-quinazoline hydrochloride.

[a]The product of a compound's concentration and the time necessary for 50% reduction in clonogenicity under aerobic (A) or hypoxic (H) conditions. Since cytotoxicity was exposure time dependent, range of values for 1–3 h exposure to each compound is shown.

[b]Hypoxic selectivity: $IC_{50(A)}/IC_{50(H)}$.

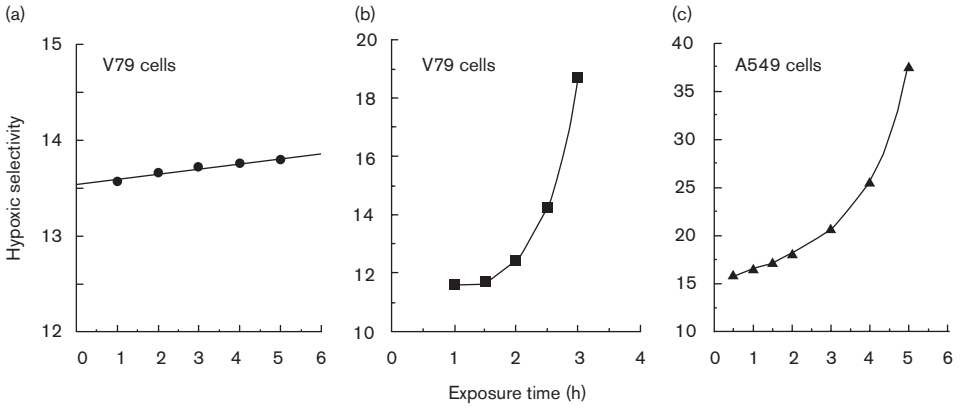
[c]Concentration for an SER of 1.6. SER, sensitization enhancement ratio.

[d]Intracellular concentration at $C_{1.6}$, determined as described in Materials and methods.

[e]In-vitro therapeutic index: $IC_{50(A)}/C_{1.6}$.

[f]Range of values for 1–4 h exposure is shown for NLCQ-1.

Fig. 5



Hypoxic selectivity versus exposure time for 4-[3-(2-nitro-1-imidazolyl)-propylamino]-2-methyl-quinazoline hydrochloride (NLQZ-1) (a) and 4-[3-(2-nitro-1-imidazolyl)-propylamino]-1,5-naphthyridine hydrochloride (NLPP-1) (b, c).

enhancement ratio (SER) of 1.6] of 61.4 and 75.0 μmol/l were obtained in V79 cells for NLQZ-1 and NLPP-1, respectively. For NLPP-1, a $C_{1.6}$ value of 44.1 μmol/l was obtained in A549 cells (Table 1). Although both compounds were similarly potent as radiosensitizers (similar $C_{1.6}$ values), greater SERs were reached with NLPP-1 (Fig. 6).

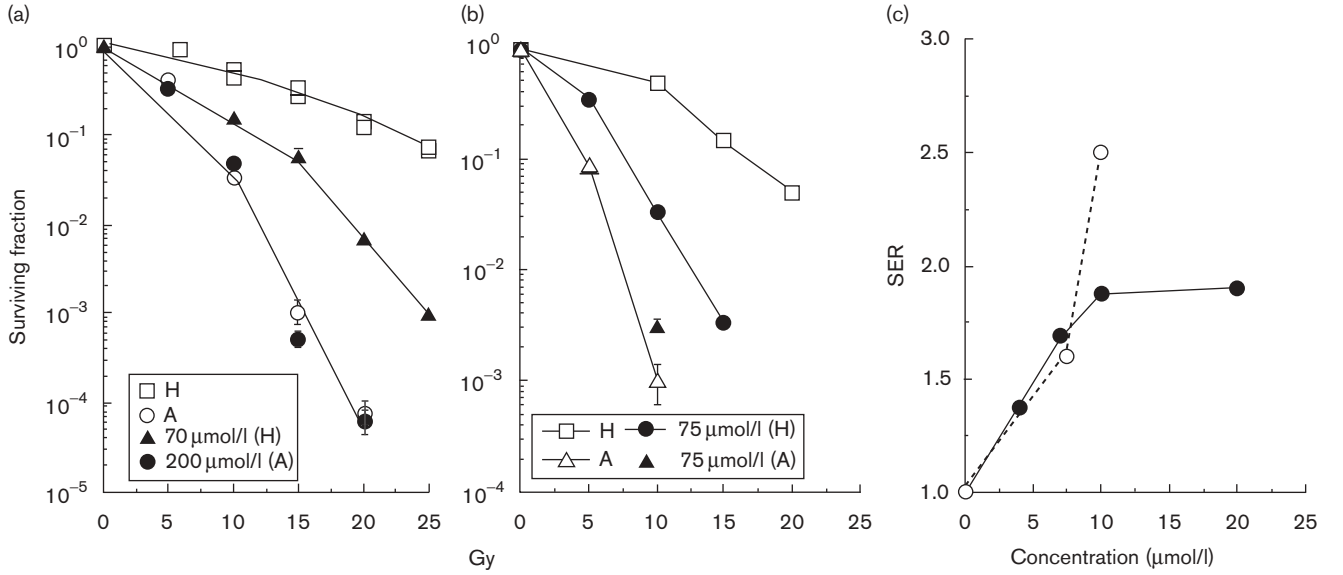
When NLQZ-1, at the nontoxic concentration of 80 μmol/l, was combined with L-PAM or cisDDP under hypoxic preexposure conditions in V79 cells, a synergistic interaction was observed (Fig. 7). Thus, DMF of approximately 2.5 were obtained for both chemotherapeutic agents, at 10% survival. The synergism was preexposure time-dependent, initially increasing with it and eventually declining at longer hypoxic exposures (Fig. 7b and c). Similar results were obtained with NLPP-1 in V79 cells (Fig. 8).

Discussion

Both NLQZ-1 and NLPP-1 were slightly more hydrophilic than the quinolinic analog NLCQ-1, presumably because of the second nitrogen atom in the aromatic chromophore, which allows for additional hydrogen bonding. This was also confirmed from the predicted log*P* values, by using the Marvin calculator (*www.chemaxon.com*). We found log*P* values of 1.36, 2.72, and 2.8 for NLPP-1, NLQZ-1, and NLCQ-1, respectively. The uptake factors for both compounds were relatively low (presumably because of their hydrophilicity), even in serum-free medium, but not significantly different from those of NLCQ-1 [19], consistent with the log*P* values.

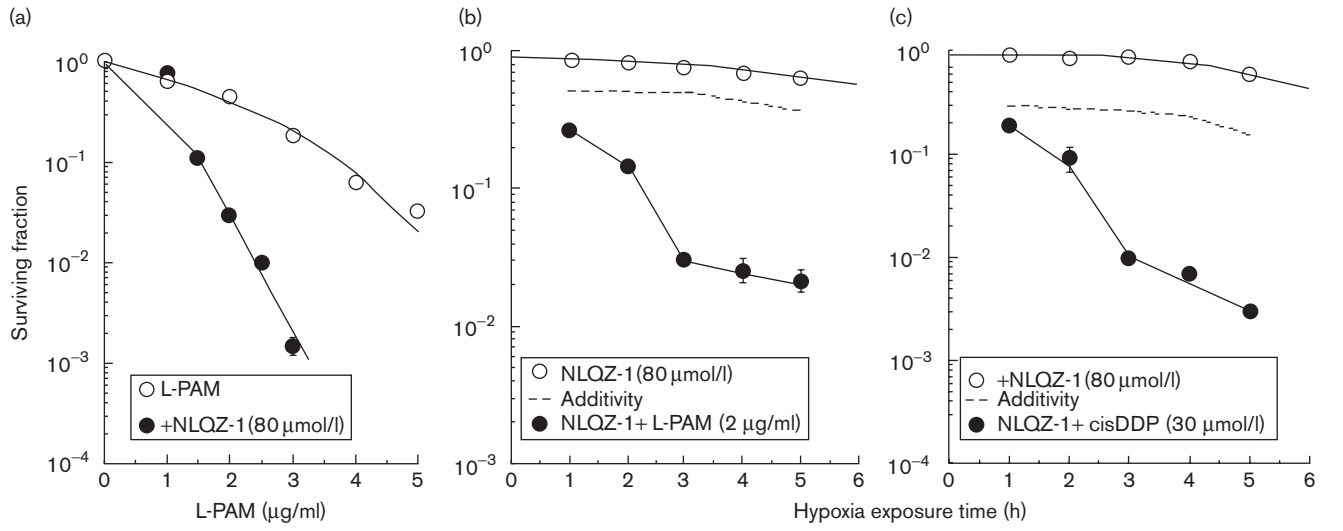
Although several quinazolines are known to intercalate DNA [20,21], NLQZ-1 did not seem to bind DNA through intercalation. However, this is not surprising if we consider that the two very similar ErbB-1 quinazolininic inhibitors PD153035 and AG1478 interact completely differently with DNA; namely, the bromo derivative PD153035 intercalates DNA, whereas the chloro analog AG1478 does not [21]. In contrast, affinity to DNA at physiological pH could be driven mainly by the pKa value, for example, the amount of cationic species available, rather than complementarity to DNA. Using the Marvin pKa calculator (*www.chemaxon.com*), we have calculated pKa

Fig. 6



Radiosensitization of V79 cells by 4-[3-(2-nitro-1-imidazolyl)-propylamino]-2-methyl-quinazoline hydrochloride (NLQZ-1) (a) or A549 cells by 4-[3-(2-nitro-1-imidazolyl)-propylamino]-1,5-naphthyridine hydrochloride (NLPP-1) (b). Sensitization enhancement ratio (SER) values calculated for NLQZ-1 (closed symbols) and NLPP-1 (open symbols) in V79 cells (c). A, aerobic conditions; H, hypoxic conditions.

Fig. 7

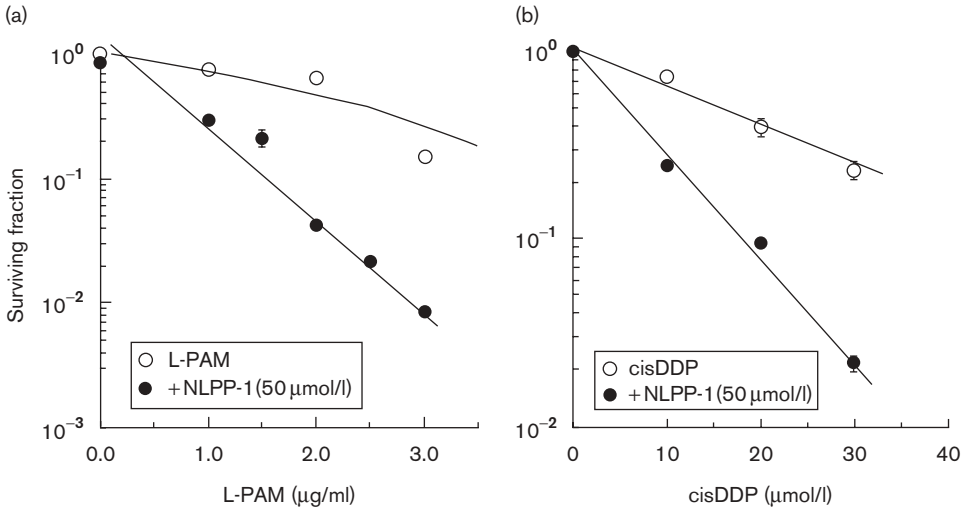


Potentiation of melphalan (L-PAM) or cisplatin (cisDDP) by 4-[3-(2-nitro-1-imidazolyl)-propylamino]-2-methyl-quinazoline hydrochloride (NLQZ-1) in V79 cells. Cells were exposed under hypoxic condition to NLQZ-1 for 2 h (a) or up to 5 h (b, c), followed by 1 h aerobic coexposure to L-PAM (a, b) or cisDDP (c). Dotted lines represent the additive effect of NLQZ-1 plus each chemotherapeutic agent (see Materials and methods).

values for NLQZ-1, NLPP-1, and NLCQ-1 to be 5.87, 6.81, and 7.76, respectively. These pKa values mean that 85, 40, and 7% of NLCQ-1, NLPP-1, and NLQZ-1, respectively is protonated at physiological pH 7. Thus, both NLPP-1 and especially NLQZ-1, being predominately in their neutral form at pH 7 because of their decreased basicity, they also show decreased affinity for DNA through electrostatic interactions.

NLPP-1 showed a slightly increased hypoxic selectivity with incubation time in V79 and A549 cells (Fig. 5b and c). Increased hypoxic selectivity with incubation

Fig. 8



Potentiation of melphalan (L-PAM) and cisplatin (cisDDP) by 4-[3-(2-nitro-1-imidazolyl)-propylamino]-1,5-naphthyridine hydrochloride (NLPP-1) in V79 cells. Cells were exposed under hypoxic conditions to NLPP-1 for 2 h followed by 1 h aerobic coexposure to L-PAM (a) or cisDDP (b).

time is usually associated with compounds possessing two redox systems with different one electron reduction potential [22]. In the case of NLPP-1, however, this should not be the case. A plausible explanation could be the induction of inducible NO synthase (iNOS), mediated by HIF-1α activation in response to hypoxia [23]. In the literature, A549 cells could strongly upregulate iNOS, as a result of HIF-1α activation by cytokines, as soon as 30 min posttreatment [24]. Furthermore, ras/myc-SFME tumorigenic cells, stimulated by 1% oxygen treatment (hypoxic conditions), strongly upregulated iNOS expression [25]. iNOS has a strong homology in its C-terminal reductase domain with P450 reductase, a known activator of bioreductive agents. Thus, we speculate that upregulation of iNOS under hypoxia could lead to further reductive activation of NLPP-1 with time. We have seen, for instance, that the HS of NLCQ-1 was doubled in HT1080 (human fibrosarcoma cancer) cells, stably transfected to overexpress iNOS, compared with the wild type HT1080 cells, because of the decrease in the hypoxic IC_{50} values, whereas the aerobic IC_{50} values remained unchanged [26]. Similarly, the HS of NLCQ-1 was increased from 7.22 to 95.07 in MDA231 (human breast cancer) cells stably transfected to overexpress iNOS, when the oxygen decreased from 1 to 0.001% (anoxia). The corresponding HS value in the wild type MDA231 cells was increased from 2.71 to 18.89 [26].

The decreased DNA-binding affinity of NLQZ-1 and NLPP-1 could also explain their decreased potency as hypoxic cytotoxins and radiosensitizers, compared with NLCQ-1 (Table 1). Both compounds showed $C_{1.6}$ values significantly larger than NLCQ-1 (Table 1). However, as both compounds were significantly less toxic under aerobic conditions than NLCQ-1, therapeutic indexes greater than 10 could be obtained. With regard to SER values, NLQZ-1 gave a dose-dependent SER, which however reached a plateau value of 1.8 at 100 μmol/l. In contrast, NLPP-1 gave exponentially increasing SERs up to 2.5 at 100 μmol/l, suggesting a better radiosensitizing ability than NLQZ-1. At this point, it is difficult to explain this improved radiosensitizing behavior, especially as one electron reduction potentials ($E_{1/2}$) have not been determined yet for the two compounds. Nitroimidazoles can show a broad range of $E_{1/2}$ values, depending on the substitution on the imidazolic ring. Thus, for example, 1-methyl-2-nitro-5-vinyl-imidazole, 1-methyl-2-nitro-5-methyl-imidazole, and 1-methyl-2-nitro-5-carboethoxy-imidazole have an $E_{1/2}$ value of −355, −443, and −195 mV, respectively [27].

NLQZ-1 and NLPP-1 interacted in a synergistic way with alkylating agents such as cisDDP and L-PAM, under hypoxic pretreatment conditions (Figs 7 and 8). For instance, NLQZ-1 at 80 μmol/l provided a surviving fraction of 0.86 in V79 cells after 2 h hypoxic incubation (37°C). L-PAM at 3 μg/ml, given for 1 h under aerobic conditions to hypoxia-pretreated (2 h) V79 cells provided a surviving fraction of 0.1 (Fig. 7a). However, the two compounds in combination, given as described above, provided a surviving fraction of 0.002 after correction for the effect of the NLQZ-1 alone (Fig. 7a). Thus, NLQZ-1 at a minimally toxic concentration under hypoxic pretreatment conditions could decrease the survival of aerobically treated cells with L-PAM about two extra logs. Similarly, NLPP-1 at 50 μmol/l (minimally toxic in V79 cells after 2 h hypoxic treatment) was able to

increase the aerobic toxicity of L-PAM or cisDDP about 15-fold (Fig. 8a and b), providing DMF values of 2–2.5. No synergistic interaction was observed when NLQZ-1 and L-PAM were coadministered under aerobic conditions (data not shown), consistent with our previous results with the weak DNA-intercalating bioreductives 9-[3-(2-nitro-1-imidazolyl)propylamino]-1,2,3,4-tetrahydroacridine hydrochloride and 9-[3-(2-nitro-1-imidazolyl)propylamino]-cyclopenteno[b]quinoline hydrochloride, indicating that conditions for bioreductive activation are necessary for synergy [28,29]. Potentiation of both L-PAM and cisDDP was also increased with the hypoxia pretreatment time with each bioreductive agent (example is given with NLQZ-1 in Fig. 7b and c), although a plateau in surviving fraction was reached at later time-points, presumably because of the bioreductive compound's consumption [28,29].

Furthermore, timing was also important for synergistic interaction, as we have seen before with NLCQ-1 and alkylating agents *in vitro* as well as *in vivo* [30,31]. Thus, once again, potentiation was associated with delivery of the bioreductive agent about 2 h before the alkylating drug. In the case of NLCQ-1, hypoxic pretreatment with NLCQ-1 resulted in the formation of DNA single strand breaks which predisposed V79 cells to alkylating agent-induced formation of DNA interstrand cross-links [30]. This mechanism of potentiation of alkylating drugs is independent of DNA interaction and has been observed also with bioreductives that do not bind to DNA [32,33]. In addition, we have found in previous studies with NLCQ-1 (or tirapazamine) that no potentiation of alkylating agents occurs in cells with deficiency in DNA damage repair mechanisms, suggesting inhibition of such mechanisms by NLCQ-1 [34]. Therefore, we can speculate that similar mechanisms may take place in the case of NLQZ-1-mediated and NLPP-1-mediated potentiation of L-PAM and cisDDP, although additional experiments are needed to verify such speculations.

From the above, it is concluded that although nitroimidazole-based bioreductive compounds bearing a quinazoline or a pyrido-pyridine chromophore do not bind to DNA, mainly because of their decreased basicity, they can still be of value as hypoxia-selective cytotoxins and radio/chemosensitizers. The representative compounds NLQZ-1 and NLPP-1 are in general less potent hypoxic cytotoxins than weak DNA-intercalative bioreductives, but their potency is still showed at micromolar concentrations. Both compounds have relatively good HS and interact synergistically with radiation or alkylating chemotherapeutic agents at nontoxic doses. Thus, a further in-vivo evaluation of NLQZ-1 and NLPP-1 is worthwhile.

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