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Radiosensitization and Hypoxic Cell Toxicity of NLA-1 and NLA-2, Two New Bioreductive Compounds

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Two new bioreductive compounds, 9-[3-(2-nitro-1-imidazolyl)propylamino]acridine hydrochloride (NLA-1) and 9-[2-(2-nitro-1-imidazolyl)ethylamino]acridine hydrochloride (NLA-2), have been prepared. They feature an acridine ring to intercalate with DNA, a 2-nitroimidazole ring as the radiosensitizing moiety and an amino functionality for increased DNA-binding and hydrophilicity. Time and concentration dependent cytotoxicity as well as radiosensitization efficacy of the two compounds under hypoxic or aerobic conditions were determined *in vitro* using V-79 cells and an MTT colorimetric or clonogenic assay. The isosensitization point (ISP), defined as that drug concentration which results in the same survival decrement upon exposure of hypoxic or oxygenated cells to a given radiation dose, has been determined for both compounds at 7.5 Gy and the values are significantly lower than the ISPs of 5-[3-(2-nitro-1-imidazolyl)propyl]phenanthridinium bromide, 2-(2-nitro-1-imidazolyl)ethylamine or misonidazole (MISO). NLA-1 and NLA-2 are potent hypoxic cytotoxins and on a concentration basis, more potent than MISO as radiosensitizers *in vitro*. The sensitization enhancement ratios were significantly increased when 1 h drug preincubation under hypoxia at 37°C was applied, before irradiation at room temperature.

Key words: Bioreductive agent — Acridine — Intercalation — Radiosensitizer

There is increasing interest in targeting radiosensitizers to DNA as a way of improving their efficiency.[1,2] In one recent study,[3] sensitizer concentration within DNA, rather than the average intracellular concentration, was suggested to be the critical determinant of radiosensitization. New research efforts involve combinations of alkylating and radiosensitizing moieties within the same drug. Because of its DNA-alkylating aziridine attached to the 2-nitroimidazole radiosensitizing moiety, the bifunctional agent RSU-1069 is a potent hypoxic cell radiosensitizer.[4] The use of transition metal coordination complexes such as platinum and ruthenium to target nitroaromatic radiosensitizers to DNA has also been attempted.[5–9] Unfortunately, these complexes are often less or no more efficient than the free sensitizer itself, even though the one electron reduction potential, an index of radiosensitization efficacy, can be increased relative to the free sensitizer ligand.[10] Another interesting strategy of targeting radiosensitizers to DNA is through an intercalating system such as phenanthridine or acridine. 5-[3-(2-Nitro-1-imidazolyl)propyl]phenanthridinium bromide (NLP-1), a 2-nitroimidazole-linked phenanthridine, has been synthesized and its hypoxic cell toxicity and radiosensitization estimated.[11] Nitracrine (a 1-nitro-acridine derivative) is a potent hypoxia-selective cytotoxin[12] and radiation sensitizer in tissue culture,[13,14] but rapid metabolism limits its radiosensitization efficacy *in vivo*.[15] Non-covalent binding of a sensitizer to DNA, such as through intercalation, might permit significant mobility to different sites of the DNA where radiation-induced target radicals can exist. This probably explains why 1-nitracrine, which exhibits faster dissociation kinetics of the DNA-drug complex, is a 20 times more potent sensitizer than other isomers.[14]

The present report describes our initial *in vitro* cytotoxicity and radiosensitization studies of two new bioreductive compounds, 9-[3-(2-nitro-1-imidazolyl)propylamino]acridine hydrochloride (NLA-1), and 9-[2-(2-nitro-1-imidazolyl)ethylamino]acridine hydrochloride (NLA-2). In these compounds, the 2-nitroimidazole radiosensitizing moiety is linked to an acridine ring at the 9-position, through either a propyl-amino or ethyl-amino chain (Fig. 1). The amino functionality increases hydrophilicity and therefore it might decrease chronic toxicity. On the other hand, the amino group could improve the binding[16] with DNA by stabilizing the positive charge on the protonated acridine ring. Since the 2-nitroimidazole ring possesses mobility due to the rotational freedom of the alkyl chain, the possibility of capturing radiation-induced radicals on DNA may be enhanced.

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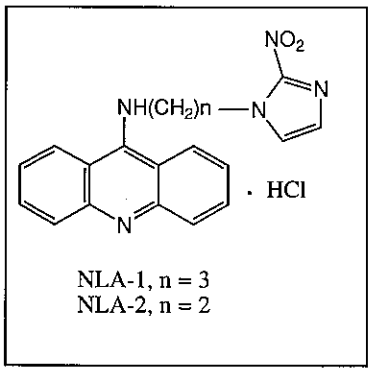

Fig. 1. The chemical structure of NLA-1 and NLA-2.

MATERIALS AND METHODS

Chemicals NLP-1,[11] NEA (2-[2-nitro-1-imidazolyl]-ethylamine)[17] and MISO (misonidazole; 1-[2-nitro-1-imidazolyl]-2-hydroxy-3-methoxypropane)[18] were prepared in our lab according to the literature. The synthesis of NLA-1 and NLA-2 will be described in detail elsewhere. Briefly, the sodium salt of azomycin (2-nitroimidazole) was treated with an equivalent amount of the proper N-(ω-bromoalkyl)phthalimide in dimethyl sulfoxide at room temperature. The produced N-[ω-(2-nitro-1-imidazolo)alkylphthalimides were hydrazinolized to the corresponding 2-nitro-1-imidazolo-alkylamines, which were subsequently coupled with 9-chloroacridine to give NLA-1 or NLA-2. Both are stable in aqueous solution for weeks at room temperature. All drugs were prepared as aqueous solutions and then diluted to appropriate concentrations with tissue culture medium.

Cells Exponentially growing V79 cells as monolayer cultures in RPMI 1640 medium supplemented with 10% fetal calf serum were trypsinized, centrifuged (750g) for 5 min, harvested and suspended in 25 ml Erlenmeyer flasks fitted with rubber caps at 5×10^5 cells per ml (5 ml). The flasks were shaken (100 rpm) at 37°C under aerobic conditions or made hypoxic[19] by gassing with 97% N_2 plus 3% CO_2 humidified gas mixture for 1 h, at which time the drug was added.

Acute toxicity To determine the acute toxicity of NLA-1 and NLA-2, samples of cells were exposed either for 1 h under hypoxia or air to drug concentrations ranging from 0 to 0.1 mM, or for various periods of time, to fixed drug concentrations (15 and 25 μM for NLA-1; 5 and 10 μM for NLA-2). To determine survival, samples were removed periodically, diluted and plated to give 10^2 to 10^4 cells on 60 mm Linbro multi-well plates (Flow Laboratories, McLean, Virginia). The plates were incubated at 37°C for 5 days, stained with crystal violet and examined for colony formation. Colonies of 50 cells or greater were counted. The MTT colorimetric assay[20] was also used in these experiments, for cell survival determination and gave comparable results with the clonogenic assay.

Radiosensitization Radiosensitization studies were performed in a manner similar to the acute toxicity experiments, except that samples were removed after receiving various radiation doses. Drugs were added to aerated or hypoxic cells at 37°C, 15 min or 1 h before irradiation at room temperature (^{137}Cs, 4 Gy/min). Hypoxia was maintained until the end of irradiation. Survival curves were normalized for the hypoxic cytotoxicity of NLA-1 or NLA-2, observed during the 1 h interval between drug addition and irradiation. Sensitization enhancement ratios (SER) were determined from the ratio of the radiation doses required to reduce cell survival to 1% of the control value obtained from hypoxic survival curves in the presence and absence of test compounds. $C_{1.6}$ values (concentration yielding an SER of 1.6) were determined by plotting SER values against concentration of compound tested and only for the 15 min drug exposure experiments, as described above.

Isosensitization point determination The isosensitization point (ISP), defined as that drug concentration which results in the same survival decrement upon exposure of hypoxic or oxygenated cells to a given radiation dose, has been determined for NLA-1, NLA-2, NLP-1, NEA, and MISO at 7.5 Gy. Each drug was added at concentrations ranging from 0 to 1 mM to aerated or hypoxic cells at 37°C for 1 h before 7.5 Gy irradiation at room temperature. Hypoxic cells were maintained hypoxic during irradiation. Cell survival was plotted against drug concentration and the ISP determined from the intersection of the hypoxic and aerobic survival curves.

RESULTS

Acute cytotoxicity NLA-1 and NLA-2 both demonstrate time- (Fig. 2 A, B) and dose- (Fig. 2C) dependent selective hypoxic cytotoxicity. No aerobic cytotoxicity was observed at concentrations <50 μM and a 1 h incubation time at 37°C (points >60 μM under air are not shown, Fig. 2C). Longer incubation times increased the aerobic cytotoxicity of NLA-1 (Fig. 2A). The $EC_{50/A}$ or $EC_{50/H}$, the effective concentration of drug for 50% killing under air (A) or hypoxia (H), after 1 h incubation at 37°C was calculated using the "median effect plot"[21] and the concentration-dependent cytotoxicity data. The $EC_{50/A}$ and $EC_{50/H}$ for NLA-1 are 84.2 ± 0.7 μM and 15.3 ± 1.1 μM, respectively, while for NLA-2 they are 90.2 ± 1.0 μM and 4.0 ± 0.3 μM, respectively.

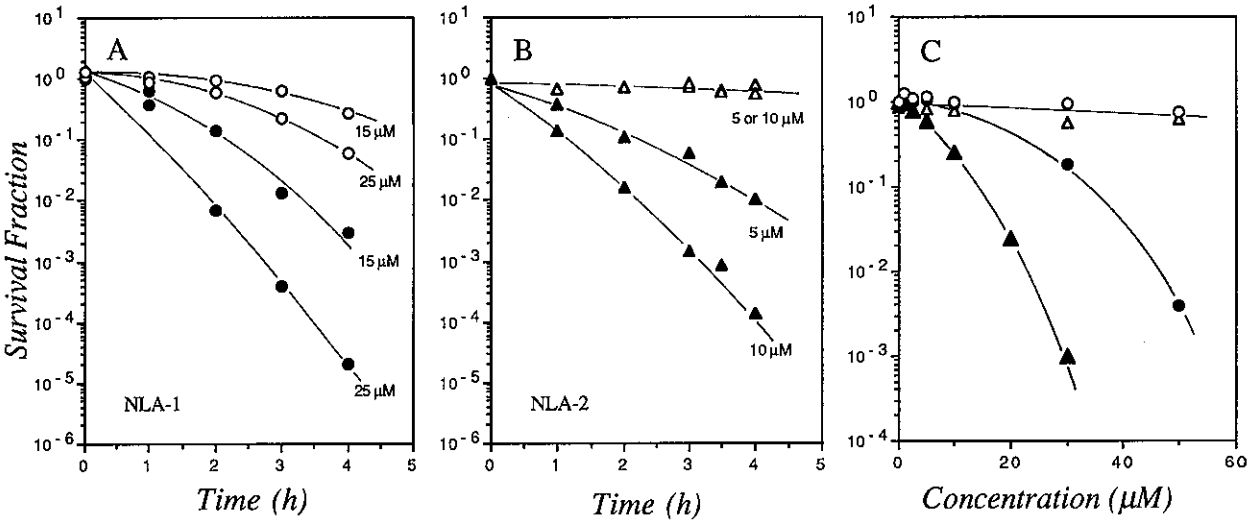

Fig. 2. The time-dependent (A, B panels) and concentration-dependent (C panel) cytotoxicity of NLA-1 (●, ○) and NLA-2 (▲, △) towards hypoxic (solid symbols) or aerobic (open symbols) V-79 cells in suspension culture. For the concentration-dependent cytotoxicity graphs, cells were incubated under hypoxia or air for 1 h. Each point represents the mean value of 3 experiments. The SE bars are not shown for clarity.

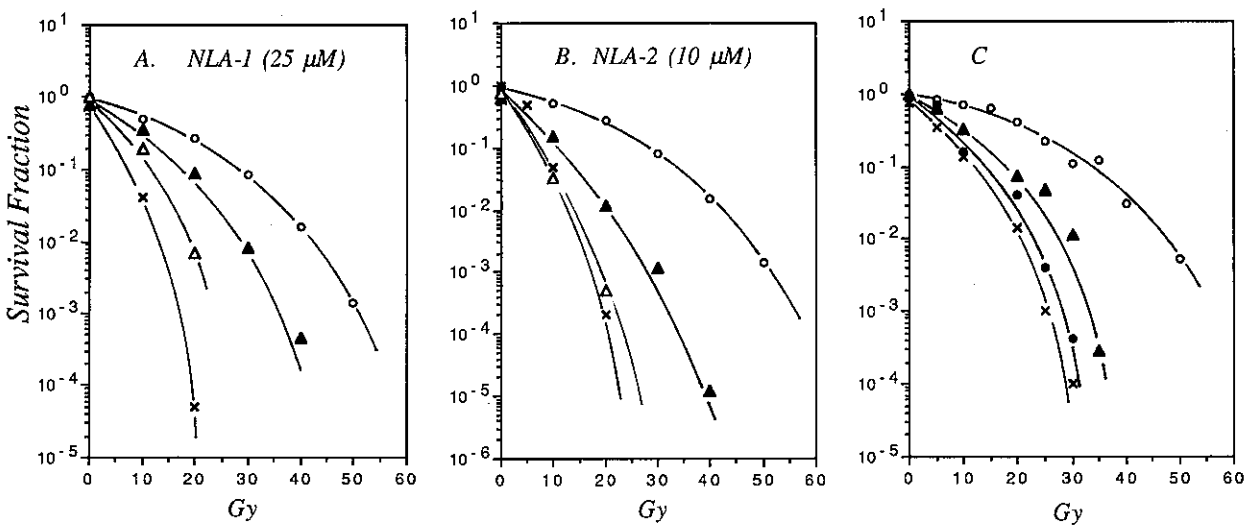

Fig. 3. Radiosensitization curves of NLA-1 (25 μM, ▲, △, A panel) and NLA-2 (10 μM, ▲, △) under hypoxia (solid symbols) or air (open symbols). Hypoxia alone (○); air alone (×). Drugs were given to the hypoxic or aerated cells 15 min before irradiation. No hypoxic cytotoxicity due to the drugs was observed in this case. In panel C, hypoxic cells were exposed to NLA-1 (25 μM, ●) or NLA-2 (10 μM, ▲) for 1 h at 37°C before irradiation at room temperature. Curves in this case were normalized for the hypoxic toxicity of the drugs. Each point represents the mean value of 3 experiments. The SE bars are not shown for clarity.

Radiosensitization The SERs (at 1% survival) are 1.6 for 25 μM of NLA-1 and 1.9 for 10 μM of NLA-2 when irradiation at room temperature follows 15 min hypoxic exposure to the drugs at 37°C; the oxygen enhancement ratio (OER) was 3.2. No hypoxic toxicity was observed due to the compounds in this case (Fig. 3A, B). However, when cells were exposed to the drugs under the same conditions except for 1 h instead of 15 min, and

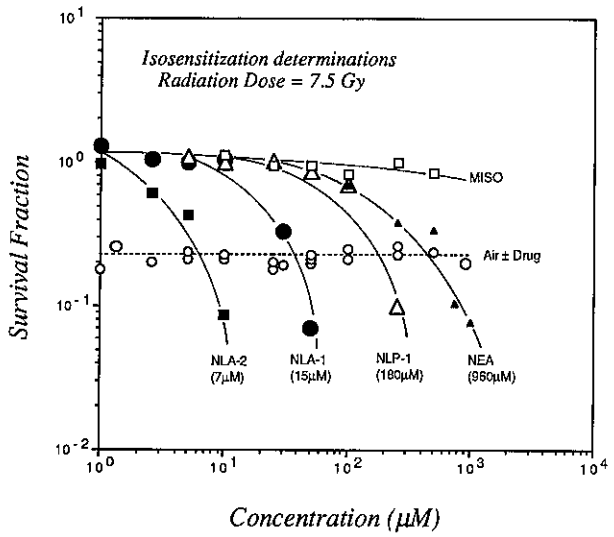

Fig. 4. Survival curves for the determination of the ISPs of NLA-1, NLA-2, NLP-1, NEA, and MISO. V-79 cells were irradiated at 7.5 Gy, after 1 h exposure under air (○) or hypoxia (other symbols), at different concentrations of the above compounds. ISPs were calculated from the graph, as ordinates of the intersection points between curves under hypoxia and air. All curves under aerobic conditions are identical straight lines independent of drug concentration in the observed range. Dotted line represents the air-survival level without any drug. Each point represents the mean value of 3 experiments.

then irradiated, NLA-1 (25 μM) was almost as effective as oxygen. Radiosensitization curves were normalized, in this case, for the 1 h hypoxic toxicity of each compound (Fig. 3C). Slight but reproducible radioprotection, as with the phenanthridine-linked 2-nitroimidazoles,[22] was observed for oxygenated cells in the presence of either compound (Fig. 3A, B).

Isosensitization points The ISP is an easily determined indicator for comparing the potency of different compounds as combined radiosensitizers and hypoxic cytotoxins at a given radiation dose and in relation to oxygen. As a prerequisite, the tested concentrations of each compound should not be toxic under aerobic conditions. Thus, survival level under aerobic conditions represents only the oxygen effect at the given radiation dose and remains constant through the whole concentration range, while ISP represents the isoeffective dose of each compound, to the oxygen, at that particular radiation dose. The ISPs at 7.5 Gy for NLA-1 and NLA-2 are 15 μM and 7 μM, respectively. The corresponding ISPs for NLP-1 and NEA are 180 and 960 μM, respectively, while for misonidazole the ISP is not reached at concentrations up to 2000 μM (Fig. 4).

DISCUSSION

NLA-1 and NLA-2 are potent selective hypoxic cytotoxins with $\approx$5.5 and $\approx$22.5 fold hypoxic selectivity, respectively. On a concentration basis, both compounds are much more potent than MISO[20] ($EC_{50/H}$ $\approx$4 mM). Only NLA-2 has greater hypoxic selectivity than MISO, whose hypoxic selectivity in V79 cells is 11.[20] The shorter alkyl chain between the two rings in NLA-2 may explain its greater hypoxic selectivity in terms of reduced lipophilicity. Indeed, recent cytotoxicity studies using two other NLA analogs with longer chains show less selective hypoxic cytotoxicity due to increased lipophilicity (unpublished data). This shorter alkyl chain in NLA-2 may also explain in part its increased cytotoxicity due to better approach of the 2-nitroimidazole to DNA via acridine intercalation.

The SERs of NLA-1 and NLA-2 are concentration-dependent, as with other 2-nitroimidazole derivatives (data not shown). On the basis of $C_{1.6}$ values (concentration yielding an SER of 1.6), NLA-2 is a more potent radiosensitizer than NLA-1 (4 versus 25 μM). Furthermore, both compounds are more potent radiosensitizers than MISO ($C_{1.6}$=0.3 mM) and pimonidazole ($C_{1.6}$=0.1 mM) *in vitro* and under similar conditions.[23] When hypoxic cells were irradiated after 1 h exposure to the drugs, 25 μM NLA-1 was almost as effective as oxygen, while 10 μM NLA-2 gave almost the same SER obtained within 15 min drug-exposure (Fig. 3C). In the case of NLA-1, it is apparent that a synergistic effect between radiation and the sensitizer's hypoxic toxicity occurs. The lack of this synergism in the case of NLA-2 could be mainly attributed to its low actual concentration during irradiation, probably due to rapid hydrolysis (HPLC observations, unpublished results). Therefore, even though the 1 h hypoxic toxicity of 10 μM NLA-2 is very significant, not much drug is available during the following irradiation for radiosensitizing effect.

For ISP determinations, we chose 7.5 Gy as a relatively low radiation dose. It is clear from Fig. 4 that, under non-toxic aerobic conditions, NLA-1 and NLA-2 are more potent compounds than NLP-1, NEA and MISO. Based upon ISP determinations, potency increases in the following order: neutral system without intercalator (MISO) < basic system without intercalator (NEA) < basic system, phenanthridine intercalation (NLP-1) < basic system, acridine intercalation (NLA-1, NLA-2). Assuming that the ultimate goal of therapy is total killing of tumor cells, hypoxic cytotoxicity in combination[24] with radiosensitizing action may be a fruitful area of research.

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