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Potentiation of 1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea's Toxicity *in vitro* by Two New Bioreductive Agents

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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), which behave as hypoxic cytotoxins and radiosensitizers, have been investigated for potentiation of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea's (CCNU) cytotoxic activity *in vitro* using V-79 cells. The preincubation effect as well as conditions of coadministration of CCNU with each sensitizer have been examined. In this latter case, the median-effect analysis was applied to evaluate whether the phenomenon was additive or synergistic. A clonogenic assay was used to score survival. Both bioreductive compounds, even at very low concentrations, significantly enhance the cytotoxic activity of CCNU under conditions of hypoxic preincubation. The enhancement of CCNU cytotoxicity is dependent upon preincubation time and the concentrations of both CCNU and the specific bioreductive agent. Coincubation of cells under hypoxia with CCNU and each bioreductive agent led to some potentiation, but only at lower survival levels. No chemosensitization was observed under aerobic conditions with either sensitizer.

Key words: Bioreductive agent — Acridine — Intercalation — Chemosensitizer

The effectiveness of many clinically useful anticancer drugs can be limited by the existence of hypoxic cells, which are known to be present in certain solid tumors. The drug uptake by such cells may be low because of their relatively slow proliferation, compared to the aerobic cells within the tumor.[1,2] It is well established that bioreductive compounds, such as nitroimidazole-based radiosensitizers, potentiate the cytotoxic effects of several chemotherapeutic agents towards hypoxic tumor cells, both *in vitro* and *in vivo*.[3–7] Thus, the combination of such compounds with conventional anti-cancer drugs might overcome the problem of hypoxic cells in cancer therapy. Chemosensitization *in vitro* is usually demonstrated by pretreating cells with a sensitizer under hypoxic conditions before exposure to the chemotherapeutic drug, usually an alkylating agent, under aerobic conditions. This "preincubation effect"[8–10] is largely attributable to the nitroreduction of sensitizer which occurs under hypoxic conditions,[11–13] and its existence has been observed *in vivo* as well.[6]

We recently reported the radiosensitizing efficacy and selective hypoxic cytotoxicity 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 V-79 cells.[14] These compounds contain a 2-nitroimidazole moiety which is biologically activated under hypoxic conditions, which are believed to exist in certain solid tumors, and an acridine ring which intercalates[15] with DNA, and therefore, undergo selective subcellular localization and bioactivation. Besides their role as hypoxia-bioactivated compounds, which are known to cause DNA sub-lethal damage and glutathione depletion,[6] as intercalating agents they could have other targets of action, such as DNA topoisomerases,[16,17] which affect a number of vital biological functions, including the replication and repair of DNA.[18] Nitrosoureas are commonly used to test for chemosensitization by nitroimidazoles.[19–21]

In this report we investigate (a) if any chemosensitization of V-79 cells to 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) is caused by either NLA-1 or NLA-2; (b) the preincubation effect with either sensitizer as well as the effects of preincubation time, sensitizer concentration and CCNU concentration upon chemosensitization, and (c) the coincubation effect under hypoxia or air. In this latter case the median effect analysis described by Chou and Talalay[22] was used to quantify the type and degree of interaction between CCNU and either NLA-1 or NLA-2. CCNU has been used before, in combination with misonidazole or nitrofurans, for potentiation studies in V-79 spheroids and the median effect analysis has been successfully applied to interpret the results.[23,24]

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MATERIALS AND METHODS

Cell culture techniques Exponentially growing V-79 cells as monolayer cultures in RPMI 1640 medium supplemented with 10% fetal calf serum were trypsinized, centrifuged (750*g*) for 5 min, harvested and suspended in 25 ml Erlenmeyer flasks fitted with rubber caps at 5×10^5 cells per ml (5 ml total volume). The flasks were shaken (100 rpm) at 37°C under aerobic conditions or made hypoxic by gassing with 97% N_2 plus 3% CO_2 humidified gas mixture for 1 h. Cells were rinsed in fresh medium after appropriate drug exposure, counted, plated [4×10^2 to 4×10^5 cells per well, on 60 mm Linbro multiwell plates (Flow Laboratories, McLean, Virginia)], incubated at 37°C for 5 days and assayed for colony formation. Cell survival was determined using a crystal violet clonogenic assay; only colonies containing ≥ 50 cells were scored. All data-points presented represent the mean of 3 to 4 experiments.

Drugs and treatment NLA-1 and NLA-2[14, 15] (Fig. 1) were prepared as aqueous solutions and then diluted to appropriate concentrations with tissue culture medium. CCNU (98% pure) (Aldrich Chemical Company, Inc., Milwaukee, Wisconsin) was dissolved in adsolute ethanol (5 mg/ml), immediately before addition to the growth medium of the cells. In all cases, the final concentration of ethanol was $\leq 1\%$; ethanol at this level did not influence the plating efficiencies.

When the preincubation effect of the sensitizers was to be examined, cells were exposed to either NLA-1 (5 μM) or NLA-2 (1 μM) for 2 h under hypoxic conditions, subsequently exposed to varying concentrations of CCNU for 1 h under aerobic conditions and then assayed for colony formation. In evaluating the effect of preincubation time on chemosensitization, cells were exposed to 4 μM NLA-1 or NLA-2 under conditions of hypoxia for 0 to 4 h, subsequently exposed to 2 μg/ml of CCNU under aerobic conditions and then assayed for colony formation. The effects of NLA-1 or NLA-2 concentration on chemosensitization was also investigated. Cells were exposed to varying concentrations of either NLA-1 or NLA-2 for 2 h under conditions of hypoxia, subsequently exposed to fixed concentrations of CCNU under aerobic conditions for 1 h and assayed for colony formation. In all experimental circumstances, appropriate NLA-1,NLA-2 and CCNU controls were used. Survival curves were normalized for the hypoxic cytotoxicity of NLA-1 or NLA-2, in order to determine the dose modification factors (DMF) [i.e., the ratio of CCNU concentrations required to reduce cell survival to the same defined level (0.25) alone or in combination with sensitizer]. Envelopes of additivity were determined as well, according to the fractional product concept,[25] which can be applied in cases of independent action of drugs.

When the effect of coincubation of sensitizer with CCNU was examined (where it was not clear whether or not the actions of the two drugs were independent), cells were exposed to varying concentrations of CCNU, NLA-1, or NLA-2 alone, and to varying concentrations of the combined drugs at fixed ratios (CCNU:NLA-1, 1:1; CCNU:NLA-2, 5:1) under hypoxic or aerobic conditions for 1 h, and then plated for colony formation. Dose-response survival curves were generated for each drug alone and for each combination of drugs. The median-effect principle of the mass action law,[22] based on survival (S) being related to dose (D), was adopted to analyze the resultant data. This method is used to determine if two or more drugs have additive, synergistic or antagonistic effects. The median effect equation states that:

$$(1-S)/S=(D/D_m)^m \quad (1)$$

or, restated,

$$D=D_m[(1-S)/S]^{1/m} \quad (2)$$

where D is the dose giving survival S, D_m is the dose required to produce the median effect (50% killing) and m is a coefficient related to the sigmoidicity of the dose-effect curve.

The median effect equation (1) can be linearized by taking the logarithms of both sides (eq. (3)):

$$\log[(1-S)/S]=m\log D-m\log D_m \quad (3)$$

The linearity of such a log-log plot determines the applicability of the method. This so-called median effect plot provides m and D_m values from which the value of any D can be calculated at any level of S, using equation (2). Parallel curves for log $[(1-S)/S]$ versus log D for two test drugs indicate that the agents can be added by dose, so if a constant dose ratio is used, the fractional part of the effect (f) due to each drug varies with its concentration (D):

$$f_1=D_1/(D_1+D_2);\ f_2=D_2/(D_1+D_2)$$

Thus, the so-called combination index (CI) can be determined at any desired survival level (x), in terms of the calculated single (D_1, D_2) or combined ($D_{1,2}$) dosages needed to reach that end point:

$$\mathrm{CI}=\frac{(D_x)_{1,2}(f_1)}{(D_x)_1}+\frac{(D_x)_{1,2}(f_2)}{(D_x)_2}+\frac{[(D_x)_{1,2}(f_1)][(D_x)_{1,2}(f_2)]}{(D_x)_1\,(D_x)_2} \quad (4)$$

where the last term in equation (4) is required only if the agents are nonexclusive (i.e., m, the slope of the combination curve, is greater than that for either single agent alone). In essence, the combination index is the sum of the ratios of each fractional part of the combination dose to the (isoeffective) single agent dose; and thus CI values >1 indicate antagonistic effect, values$=1$ indicate summation and values <1 indicate synergism.

RESULTS

The preincubation effect studies Figure 1 shows that both NLA-1 and NLA-2 potentiate the aerobic cytotoxicity of CCNU in V-79 cells when such cells have previously been exposed to either 5 μM NLA-1 or 1 μM NLA-2 under hypoxic conditions for 2 h. The shaded areas indicate envelopes of expected additive cytotoxicity, calculated according to the fractional product concept,[25] which assumes totally independent actions of CCNU and sensitizer (mutually nonexclusive). This assumption is reasonable, since NLA-1 and NLA-2 are not aerobically toxic at the concentrations tested and therefore, their effect is expected to occur during the hypoxic preincubation period.

According to the fractional product method, the combined action of two independently acting drugs equals the product of the survivals obtained of each drug acting alone. If this survival calculated from the product expression is larger than the observed one, then the two drugs act synergistically. For example, the survival fraction of hypoxic (for 2 h) cells exposed under air (for 1 h) to 4 μg/ml CCNU is 0.262, while the survival of cells exposed to 5 μM NLA-1 alone (for 2 h under hypoxia followed by 1 h exposure under air) is 0.683. On the other hand, exposure of cells first to NLA-1 followed by exposure to CCNU under these same conditions yields a survival fraction of 0.026, a 7-fold smaller value than the product ($0.683 \times 0.262 = 0.179$), which represents additivity.

The ratio between expected and observed survival fractions of combined treatment, the so-called potentiation index (PI), is dependent upon CCNU concentration, when the concentrations of NLA-1 or NLA-2 are constant (Table I).

The dose modification factors (DMF) at 25% survival level are 1.97 and 2.03 for NLA-1 (5 μM) and NLA-2 (1 μM), respectively, and were calculated as described in "Materials and Methods" (data not shown).

Because chemosensitization is usually dependent upon sensitizer concentration[26] and bacause 5 μM NLA-1 and 1 μM NLA-2 are well below[14] the doses giving aerobic toxicity in V-79 cells, we investigated if chemosensitization of CCNU can be maximized with higher doses of NLA-1 or NLA-2. The results of these experiments are shown in Table II. The magnitude of potentiation increases as a function of sensitizer concentration. Furthermore, the PI of CCNU by NLA-2 exceeds that of NLA-1.

Table I. Potentiation Indices with Varying CCNU Concentrations

CCNU	NLA-1 (5 μM)			NLA-2 (1 μM)		
(μg/ml)	S_e[a]	S_o[b]	PI[c]	S_e	S_o	PI
1.0	0.489	0.414	1.18	0.627	0.349	1.80
2.0	0.382	0.156	2.45			
3.0	0.268	0.102	2.63	0.344	0.113	3.00
4.0	0.179	0.026	6.88	0.229	0.064	5.58

V-79 cells were incubated with NLA-1 (5 μM) or NLA-2 (1 μM) under hypoxic conditions for 2 h and subsequently with varying concentrations of CCNU under aerobic conditions for 1 h, then assayed for colony formation.
a) S_e: Expected survival fraction from simple additivity of CCNU and NLA-1 or NLA-2 effects.
b) S_o: Observed survival fraction.
c) PI: Potentiation index $= S_e/S_o$.

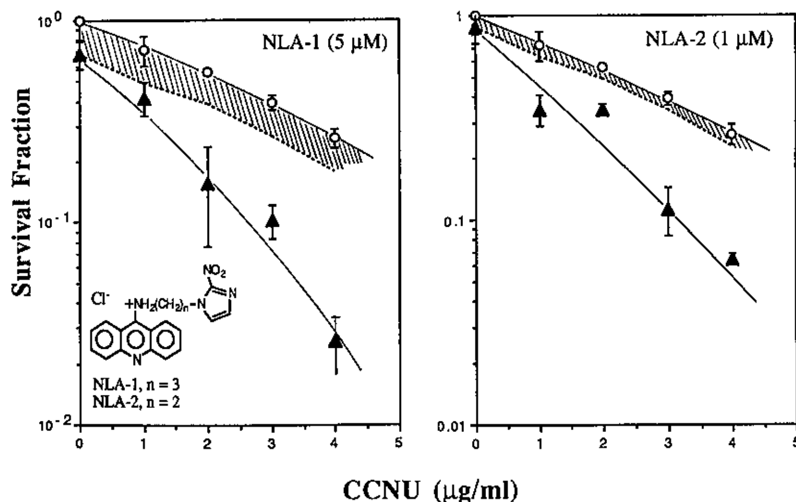

Fig. 1. Effects of sensitizer preincubation. V-79 cells were preincubated with NLA-1 (5 μM, solid triangles, left panel) or NLA-2 (1 μM, solid triangles, right panel) for 2 h under hypoxia and subsequently exposed to varying concentrations of CCNU for 1 h under aerobic conditions, then assayed for colony formation. Open circles represent cells exposed to hypoxia alone for 2 h, followed by 1 h aerobic exposure to CCNU. Shaded areas indicate envelopes of additivity.

Table II. Potentiation Indices with Varying Sensitizer Concentrations

CCNU (0.77 μg/ml)				CCNU (2.77 μg/ml)			
NLA-1 (μM)	S_e[a]	S_o[b]	PI[c]	NLA-2 (μM)	S_e	S_o	PI
3.0	0.779	0.610	1.30	1.0	0.348	0.304[d]	1.14
4.0	0.668	0.436	1.53	3.0	0.175	0.049	3.57
6.0	0.559	0.295	1.89	5.0	0.052	0.0055	9.45

V-79 cells were incubated with varying concentrations of either NLA-1 or NLA-2 under hypoxic conditions for 2 h and subsequently with fixed concentrations of CCNU under aerobic conditions for 1 h, then assayed for colony formation.

a) S_e: Expected survival fraction from simple additivity of CCNU and NLA-1 or NLA-2 effects.
b) S_o: Observed survival fraction.
c) PI: Potentiation index $= S_e/S_o$.
d) Expected to be smaller according to the data in Table I.

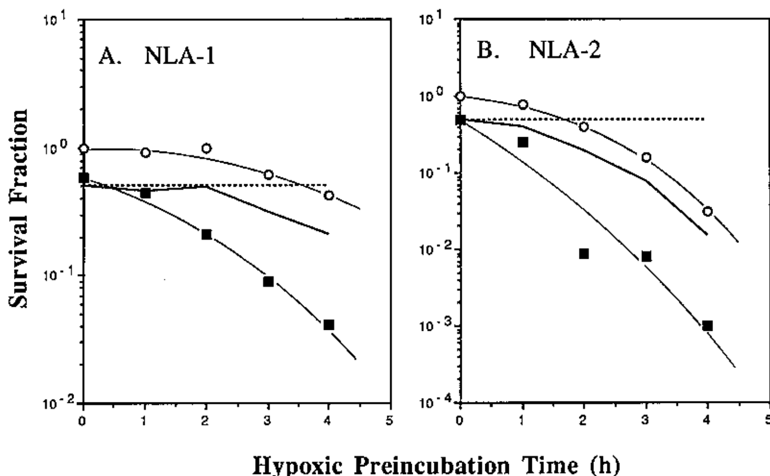

Fig. 2. Preincubation time-dependent chemosensitization by NLA-1 (4 μM, panel A) or NLA-2 (4 μM, panel B). Dotted line represents the mean aerobic toxicity of 2 μg/ml CCNU alone after 0 to 4 h pretreatment under hypoxic conditions. Open circles represent the hypoxic toxicity of NLA-1 of NLA-2 alone, at the tested periods of tine. Solid lines represent the calculated additive cytotoxicity of CCNU and either NLA-1 or NLA-2. Solid squares represent the observed combined effect.

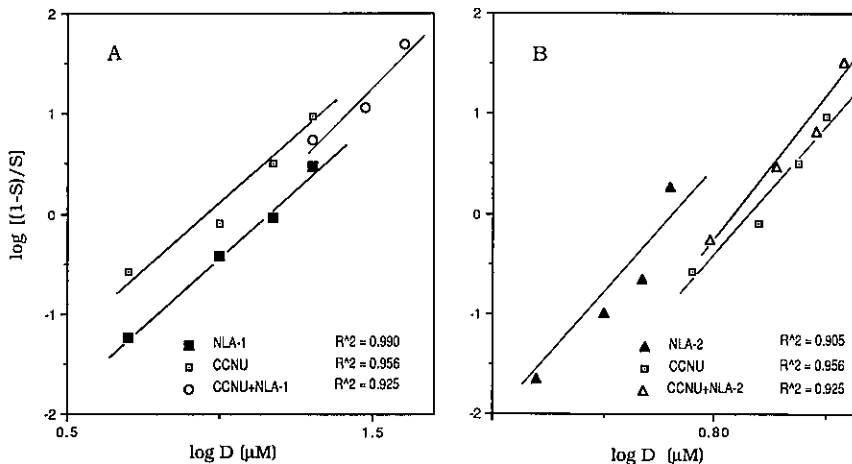

Fig. 3. Median effect plots for CCNU and NLA-1 alone or in their 1:1 combination (panel A) or CCNU and NLA-2 alone or in their 5:1 combination (panel B). V-79 cells were incubated for 1 h under hypoxic conditions with each drug either alone or in combination.

Figure 2 shows the effect of hypoxic preincubation time on survival fraction and PI. Longer pretreatment of cells with NLA-1 or NLA-2 (4 μM) under hypoxia increases the potentiation of CCNU cytotoxicity. NLA-2 is the more potent sensitizer for CCNU. For example, NLA-2 decreases 15-fold the expected survival fraction, after 4 h hypoxic pretreatment versus a 5.12-fold decrease observed with the same concentration of NLA-1 under identical conditions. Potentiation of CCNU occurs with NLA-1 or NLA-2 even with short preincubation time periods.

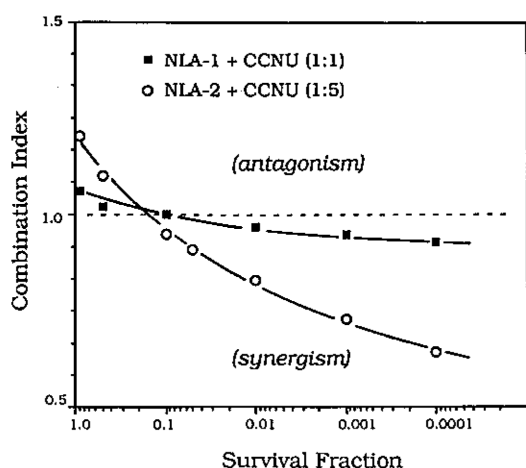

Fig. 4. Combination indices for CCNU:NLA-1 (1:1) and CCNU:NLA-2 (5:1) were plotted as functions of survival fraction. CI < 1, CI = 1 and CI > 1 suggest synergy, additivity or antagonism, respectively.

The coincubation studies Figure 3A depicts median effect plots for CCNU alone, NLA-1 alone, and their 1:1 combination while Figure 3B depicts the corresponding plots for CCNU, NLA-2 and their 5:1 combination, both sets of data having been obtained under hypoxic conditions. In both situations the plots have correlation coefficients for regression lines >0.9; therefore, the median effect analysis can be applied. The parallelism observed between the median effect plots of CCNU alone and either NLA-1 or NLA-2 alone permits the addition of individual agent doses. The upward displacement of the combined modality data indicates a synergistic response. Similar conclusions have been previously reported with CCNU and nitro sensitizers.[23, 24]

Using the combination index equation, we calculated and plotted CI values versus survival fraction (Fig. 4). Both NLA-1 and NLA-2 yield synergistic effects with CCNU at survival fractions <0.2. On the other hand, at high survival levels, slight antagonism seems to occur.

Figure 5 depicts the cytotoxic effects of CCNU, CCNU:NLA-1 (1:1) and CCNU:NLA-2 (5:1) on V-79 cells under aerobic conditions. The cytotoxicity of varying doses of CCNU for 1 h exposure under either aerobic or hypoxic conditions is identical (Fig. 5A). Because the cytotoxicity of CCNU alone or in combination with either NLA-1 or NLA-2 (at a fixed concentration ratio) for 1 h under aerobic conditions is identical, there is no aerobic chemosensitization of CCNU by either NLA-1 or NLA-2 (Fig. 5B).

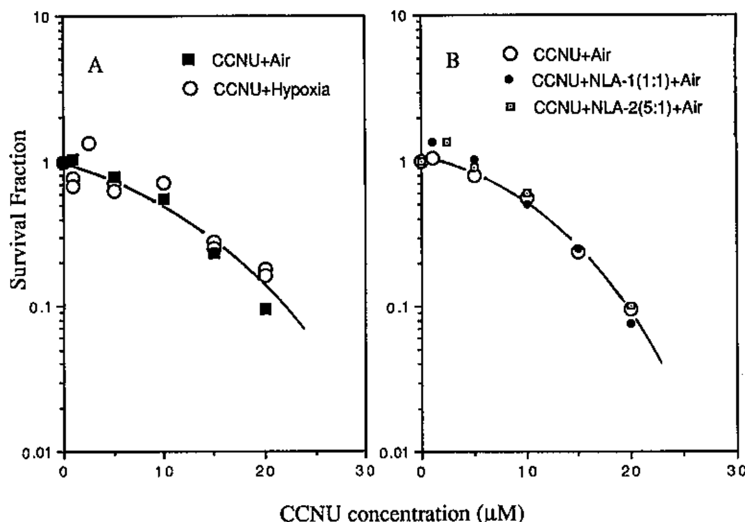

Fig. 5. Aerobic and hypoxic toxicity of CCNU after 1 h incubation in V-79 cells (panel A). Aerobic toxicity of CCNU, CCNU:NLA-1 (1:1) and CCNU:NLA-2 (5:1) after 1 h incubation in V-79 cells (panel B).

DISCUSSION

NLA-1 and NLA-2 behave like other 2-nitroimidazole-based bioreductive agents, exhibiting potentiation of chemotherapeutic activity only under hypoxic conditions. It is hypothesized that the anaerobic metabolism of these bioreductive agents causes intracellular glutathione (GSH) depletion by converting GSH to its oxidized form GSSG.[6, 27] GSH protects target macromolecules in cells from damage by noxious agents. GSH depletion appears to be dependent upon sensitizer concentration, a fact which may explain our results showing dependence of CCNU potentiation upon sensitizer concentration. Because of its great carbamoylating activity,[28] CCNU prevents the recycling of GSH by inhibiting GSSG reductase, an enzyme which catalyzes the reduction of GSSG to GSH. Therefore, carbamoylation is related to DNA repair inhibition also. The substantial potentiation of CCNU cytotoxicity observed when cells were pretreated with sensitizer for longer periods of hypoxia suggests that complete GSH depletion occurred due to the sensitizer, while GSH regeneration was then blocked by the subsequent exposure to CCNU. Relatively long hypoxic pretreatments of V-79 cells with other nitroaromatic sensitizers did not potentiate the cytotoxicity of melphalan,[29] a chemotherapeutic agent with only alkylating rather than carbamoylating activity, a fact which may correspond to the lack of GSH recycling inhibition in this case. Assuming that the rate of GSH depletion is related to the rate of sensitizer nitroreduction, both compounds should potentiate CCNU to the same degree, since they demonstrate similar reduction rates; i.e., the nitroreduction half-lives[15] are 13 and 17 min for NLA-1 and NLA-2, respectively. Surprisingly, NLA-2 is a more potent chemosensitizer, on a concentration basis, than NLA-1. This probably is related to the greater potency of NLA-2 as a hypoxic cytotoxin.[14] DNA-binding alone can not explain the greater potency of NLA-2 either, since both compounds have similar binding constants (3.04×10^5 mol^{-1} for NLA-1; 2.23×10^5 mol^{-1} for NLA-2)

The potentiation observed with NLA-1 and NLA-2 might also be related to their abilities to intercalate with DNA.[15] Both compounds exhibit similar binding constants, determined with the ethidium bromide displacement fluorescence assay.[30] It is well known that nitroacridines (a) interact with DNA by intermolecular forces, forming complexes that resist DNA strand separation, (b) inhibit the DNA polymerase reaction by altering the template and/or enzyme activity, (c) impair the basic macromolecular biosyntheses of DNA, RNA and protein and (d) inhibit cell growth.[31] NLA-1 and NLA-2, as acridine derivatives, intercalate with DNA and may therefore exhibit some or all of the above activities, subsequently enhancing CCNU cytotoxicity.

Independently of the precise mechanism of action by which CCNU cytotoxicity in V-79 cells is potentiated by either NLA-1 or NLA-2, we conclude that: micromolar amounts of either NLA-1 or NLA-2 (concentrations at which neither agent exhibits any aerobic or even substantial hypoxic cytotoxicity) provide significant potentiation; whereas 3–5 m*M* misonidazole is needed for chemosensitization of nitrosoureas.[27] Even with short preincubation times, NLA-1 and NLA-2 exhibit substantial potentiation, probably due to their availability for causing DNA sublethal damage through intercalation. Pretreatment of cells with NLA-1 or NLA-2 under hypoxic conditions, followed by CCNU exposure under aerobic conditions, is superior to hypoxic coincubation of CCNU with either sensitizer. This agrees with the *in vivo* results obtained by Brown,[6] where greater DMF values were achieved for cyclophosphamide (CYC) in RIF-1 tumor-bearing mice when misonidazole was given 5.5 h prior to CYC injection versus 0.5 h. Lack of CCNU chemosensitization by NLA-1 or NLA-2 under aerobic conditions strongly suggests that nitroreduction is necessary for sensitization to occur and speaks well for the potential specificity of chemosensitization in the clinic, where hypoxia in tumor tissue still constitutes a problem.

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