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Review Article

Exploiting hypoxia in solid tumors with DNA-targeted bioreductive drugs

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CONTENTS

Abstract

Targeting bioreductive drugs to DNA via alkylation or strong intercalation can improve their potency as hypoxia-selective cytotoxins and radio/chemosensitizers *in vitro* but not *in vivo*. Covalent DNA binding or slow DNA dissociation kinetics constitute obstacles for an efficient extravascular diffusion and penetration to the hypoxic tumor tissues, and can also cause non-bioreduction-mediated toxicity. Our strategy focuses on the development of bioreductive compounds that bind weakly to DNA through intercalation so that toxicity due to DNA affinity can not be compromised by inefficient extravascular diffusion and penetration to hypoxic tumor tissue. In this review, several examples of bioreductive compounds will be discussed, with emphasis on DNA-targeted and weak DNA-intercalating hypoxia-selective cytotoxins.

Introduction

One of the recognized microenvironmental features of solid tumors is the existence of hypoxic regions which are resistant to both ionizing radiation and chemotherapy and can negatively affect cure rates (1-4). In addition, an increasing number of reports suggest that hypoxia-induced proteome and genome changes in tumors can lead to a more aggressive phenotype and malignant progression (5-7). However, because hypoxia constitutes a major difference between tumor and normal tissues, it also presents opportunities for exploitation, such as the use of compounds known as bioreductive drugs or hypoxia-selective cytotoxins. Such compounds are inactive prodrugs that can be irreversibly activated by reductive enzymes in a hypoxic environment, whereas this process is reversible in the presence of oxygen (8, 9). Therefore, bioreductive agents, as tumor-activated prodrugs, demonstrate minimal toxicity to aerobic normal tissues, whereas upon hypoxic activation in tumors, they release toxic metabolites that cause cell damage and death.

Other strategies for tumor-specific activation of bioreductive prodrugs besides hypoxia include tumor-specific enzymes, low tumor pH, exogenous enzymes delivered to tumors through gene-directed enzyme-prodrug therapy (GDEPT), tumor-specific antigens for antibody-directed enzyme-prodrug therapy (ADEPT) and the radiolytic action of ionizing radiation delivered in tumors (9). However, the coverage of all these strategies for activation is beyond the scope of this review.

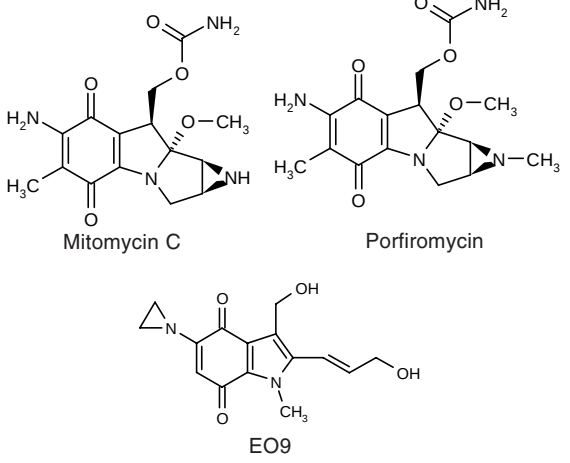
Hypoxia-selective cytotoxins target the hypoxic subpopulation in tumors and therefore are not expected to have substantial antitumor activity on their own. However, they do not only supplement other modalities, *e.g.*, radiation or chemotherapy (which primarily attack aerobic, proliferating cells), but often interact in a synergistic way with them. Thus, a therapeutic benefit has been achieved in preclinical studies with a number of bioreductive compounds (10-18). Based on chemical structure, the main categories of hypoxia-activated bioreductive agents include quinones, aromatic and aliphatic *N*-oxides, transition metals and nitroaromatics.

Classes of hypoxia-activated bioreductive compounds

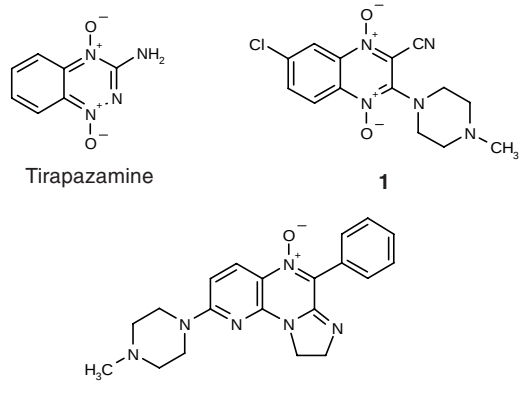
Quinones

Quinone analogues were among the first bioreductive compounds studied as hypoxia-selective cytotoxins. The most well-known quinone-based bioreductive compounds which have undergone clinical evaluation include

Scheme 1: Quinones



Scheme 2: Aromatic *N*-oxides



mitomycin C, porfiromycin and the aziridoquinone EO9 (19-21) (Scheme 1). In all these cases, cytotoxicity under hypoxia was the result of drug metabolite-DNA covalent binding, after one-electron reduction of the parent compound by cytochrome P-450 reductase (22). However, quinone-based bioreductive compounds demonstrate low hypoxic selectivity because they are also good substrates for two-electron reductases, such as DT-diaphorase (23), and have shown limited success in the clinic (19-21).

Aromatic N-oxides

Tirapazamine is the best-known aromatic *N*-oxide bioreductive agent and has shown significant promise in preclinical studies (10, 11, 16). Tirapazamine is currently undergoing phase II/III clinical trials in combination with radio/chemotherapy, with promising results (24-28). However, because of its dose-limiting toxicity (27) and evidence that activation in the nucleus rather than the cytoplasm is predominantly responsible for its hypoxic toxicity via the formation of DNA double-strand breaks (29, 30), efforts have been made recently to synthesize more potent analogues that target DNA (31, 32). Other compounds in this class include quinoxaline 1,4-di-*N*-oxides such as **1** (33) and the imidazo[1,2-*a*]pyrido[2,3-*e*]-pyrazine *N*-oxide RB-90740 (34) (Scheme 2).

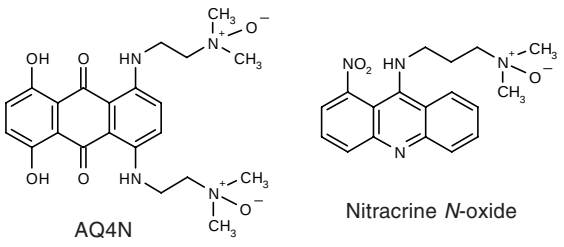
Aliphatic N-oxides

Compounds in this class bear a DNA-intercalating polycyclic unit and tertiary amine side-chains in which the amines have been converted to their *N*-oxides. Upon reduction of the *N*-oxides under hypoxia, tight binding to DNA is obtained, which leads to inhibition of topoisomerases and thus toxicity. Representative compounds of this class are the di-*N*-oxide of 1,4-bis[2-(dimethylamino)ethylamino]-5,8-dihydroxyanthracene-9,10-dione (AQ4N) and nitracrine *N*-oxide (Scheme 3). AQ4N has been evaluated in preclinical studies, where it demonstrated efficacy in combination with radiation and chemotherapy (17, 18), and it recently entered phase I clinical trials (35). Nitracrine *N*-oxide is a bis-bioreductive agent, since it contains two different reducible groups (nitro and *N*-oxide). Because of this feature, it demonstrates exceptional hypoxic selectivity *in vitro* (36). However, because of its rapid metabolism and low extravascular diffusion rate, nitracrine *N*-oxide and improved derivatives have little activity *in vivo* (37, 38).

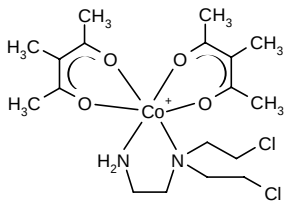
Transition metals

Complexes of transition metals have also been developed as hypoxia-activated bioreductive agents (39). Upon reduction of the metal, a highly toxic ligand (*e.g.*, mustard) is usually released, as in the cobalt complex **2** (Scheme 4). However, none of these compounds to date has shown activity against hypoxic cells in tumors (39). Complexes of reducible radioactive transition metals have also been developed as hypoxia-imaging agents (40). The latter should not be confused with complexes of radioactive transition metals such as **3** (Scheme 4), in which a nitroaromatic ligand is usually reduced and

Scheme 3: Aliphatic *N*-oxides



Scheme 4: Transition metals



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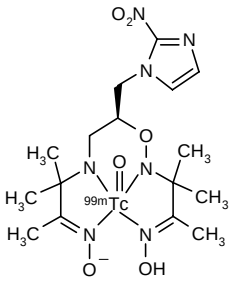


3

bound to intracellular elements, thereby trapping the marker to the hypoxic tissues (41).

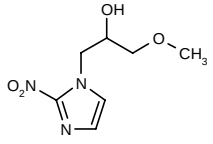
Nitroaromatics

Nitroaromatics such as misonidazole and etanidazole (Scheme 5) have been extensively studied, initially as oxygen-mimetic radiosensitizers. These compounds are 2-nitroimidazole derivatives which fulfill the appropriate reduction potential requirements, known to be –330 to –450 mV (42). Therefore, further development of nitroimidazole-based bioreductive compounds was pursued in an attempt to identify more potent, less lipophilic DNA-targeted hypoxia-selective cytotoxins. RSU-1069 (43), CI-1010 (44, 45), nitroimidazole-linked phenanthridines (NLPs) (46), nitroimidazole-linked acridines (NLAs) (47), bisnitroimidazoles **4** and **5** (48, 49), and compound **6** (50) are examples (Scheme 5). Similarly, nitracrine and derivatives have been developed (51) as DNA-targeted hypoxia-selective cytotoxins (Scheme 6).

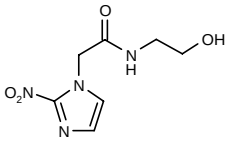
However, compounds that bind covalently to DNA cause systemic toxicity. Thus, RSU-1069 and CI-1010 cause gastrointestinal (43) and retinal (52) toxicity, respectively. Bisnitroimidazoles, which were developed as cross-linking agents, produce fewer DNA single-strand breaks than mononitroimidazoles at equivalent toxicity (53). On the other hand, nitroimidazoles tethered to strongly DNA-intercalating chromophores (such as NLPs and NLAs) or nitracrines are ineffective *in vivo* due to slow extravascular diffusion (54, 55) and non-bioreduction-mediated toxicity associated with topoisomerase I/II inhibition (56). Furthermore, in the case of nitracrines, rapid metabolism due to a high one-electron reduction potential intensifies their lack of *in vivo* efficacy (57). Efforts to decrease the strong DNA affinity of nitracrines resulted in the development of 5-nitroquinolines (Scheme 6), which showed superior hypoxic selectivity in cell cultures but not *in vivo*, due to their rapid metabolism (58).

Aromatic nitrogen mustards **7** have also been developed as hypoxia-activated alkylating bioreductive agents and as prodrugs of the dinitroaziridine CB-1954 (Scheme 6). CB-1954 constitutes a substrate for the aerobic

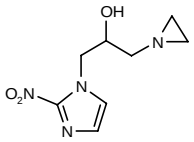
Scheme 5: Nitroaromatics



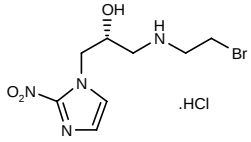
Misonidazole



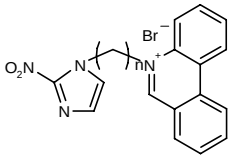
Etanidazole



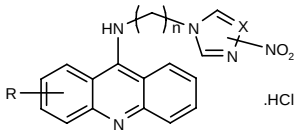
RSU-1069



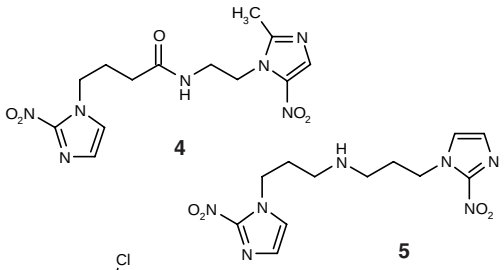
CI-1010



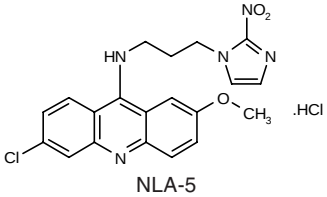
NLPs



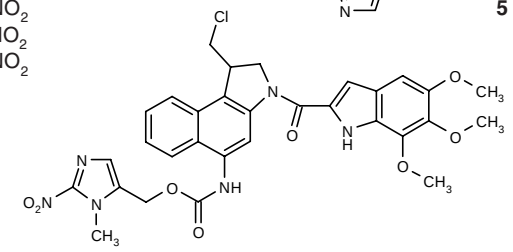
NLAs (X = C or N, n = 2, 3, 4)
NLA-1: X = C, R = H, n = 3, 2-NO_2
NLA-2: X = C, R = H, n = 2, 2-NO_2
NLA-3: X = C, R = H, n = 3, 4-NO_2
NLA-4: X = C, R = H, n = 4, 2-NO_2



4 **5**



NLA-5



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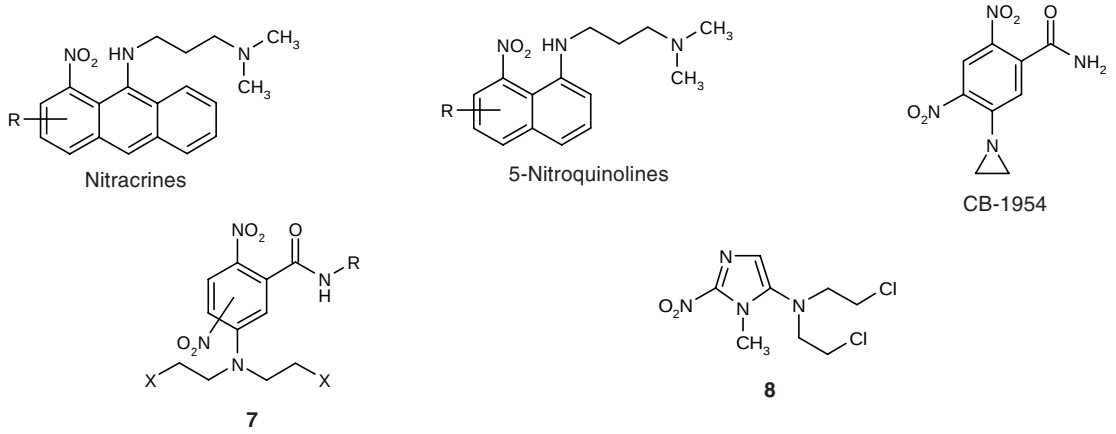
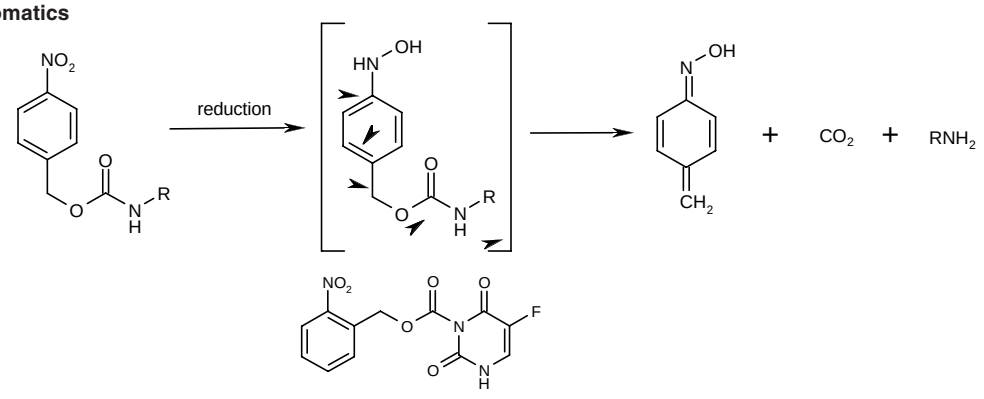
Scheme 6: Nitroaromatics



Scheme 7: Nitroaromatics



nitroreductase DT-diaphorase and demonstrates modest hypoxic selectivity (59). Upon reduction of the nitro group in the *para*-position of **7**, the electron density on the mustard nitrogen increases, resulting in an increased alkylating reactivity (60). However, nitrobenzenes in general have a much lower reduction potential (< –500 mV) than the optimal one and they are therefore not easily reduced (61). On the other hand, substitution with electron-withdrawing groups increases their reduction potential but also reduces their alkylating reactivity. Nitroheterocyclic mustards, such as **8** (Scheme 6), with higher reduction potential than compounds **7** did not show, however, improved hypoxic selectivity (62).

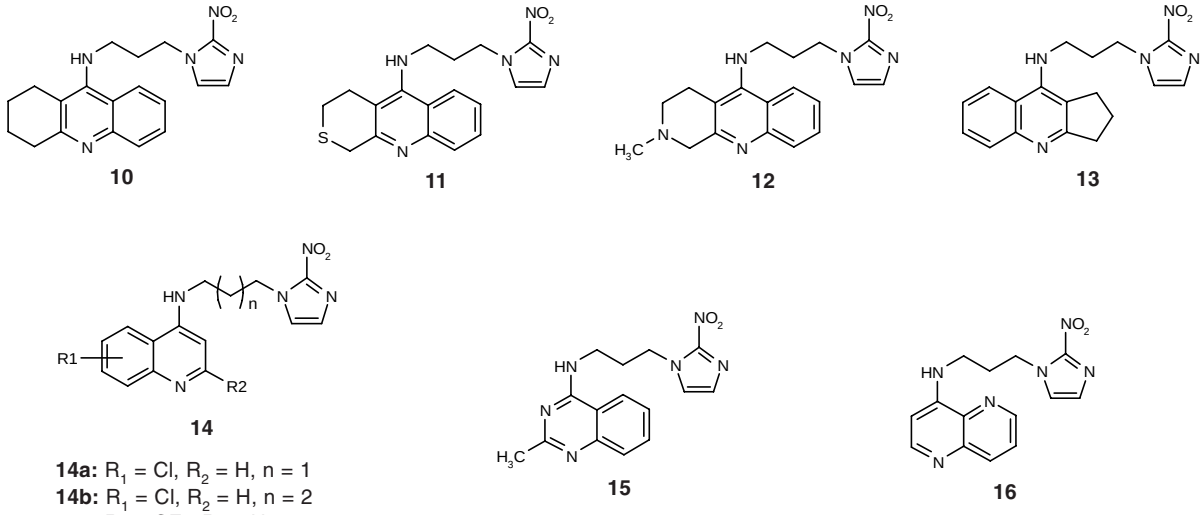
Another subclass of nitroaromatic bioreductive compounds are the nitrobenzylcarbamates such as **9** (Scheme 7), which undergo fragmentation and release toxins ("effectors") upon multielectron reduction (63). This strategy was characterized as the "trigger-linker-effector" concept (64). However, such compounds demonstrate a low reduction potential and require different methods of reductive activation other than hypoxia, such as GDEPT or radiolytic reduction (65).

Weak DNA-intercalating bioreductive compounds

Definition

From the above retrospection of the classes of compounds and techniques developed for improving the potency and efficacy of hypoxia-activated bioreductive compounds *in vivo*, it is apparent that three factors are crucial: 1) a favorable reduction potential; 2) relative stability under aerobic conditions; and 3) noncovalent binding to DNA that allows for fast dissociation kinetics. Therefore, as the next logical step, our strategy involved the development of weak DNA-intercalating bioreductive compounds with a favorable reduction potential and DNA affinity high enough to produce toxicity yet low enough to permit efficient extravascular diffusion and penetration to hypoxic tumor tissue. We defined as weak DNA intercalators compounds whose range of concentrations (C_{50}) for 50% ethidium bromide (EB) displacement (from an EB-DNA complex) lies between 40 and 100 μM. We have synthesized nitroimidazole-based derivatives which bear a quinolinic, quinazolinic, pyridopyridinic or a fused

Scheme 8: Weak DNA-affinity compounds



14a: R_1 = Cl, R_2 = H, n = 1
14b: R_1 = Cl, R_2 = H, n = 2
14c: R_1 = CF_3, R_2 = H, n = 1
14d: R_1 = H, R_2 = Me, n = 1

tricyclic quinolinic scaffold (of disturbed planarity) for weak DNA intercalation (Scheme 8).

All of these derivatives fulfill the favorable reduction potential criteria (*ca.* –390 mV), since they are 2-nitroimidazole-based compounds. DNA-binding studies using the EB displacement assay showed that the quinolinic derivatives **14a and 14c** and the fused tricyclic quinolines **10-13** bind weakly to DNA through intercalation, since they demonstrate C_{50} values in the desirable range of 40-100 μM (Fig. 1). However, compounds **14b** and **14d,** as well as quinazoline **15** and pyridopyridine **16,** demonstrate C_{50} values > 100 μM, and they were therefore not considered weak DNA intercalators. In the case of compounds **15** and **16,** DNA binding is probably prohibited due to electronegative repelling forces of two nitrogens in the aromatic chromophore.

Toxicity in cell culture

In vitro toxicity measurements in various cell lines showed that all compounds in Scheme 8, except **12,** demonstrate superior hypoxic selectivities compared to the strong DNA-intercalating NLAs (14, 66-71). However, only compound **14a** demonstrated up to 30-fold hypoxic selectivity in several rodent and human tumor cell lines. In addition, the hypoxic selectivity of **14a** increased significantly over time, up to 386-fold with 4.5 h of exposure, due to a concomitant increase and decrease in its hypoxic and aerobic potencies, respectively (67). We also showed that the fluorinated compound **14c** is accumulated in hypoxic cells and can be used as a marker for hypoxia with ^{19}F-MRS techniques *in vitro* (69). With some exceptions, a nonlinear correlation existed between DNA binding affinity and CT_{50A} values (concentration for 50% reduction in clonogenicity under aerobic conditions) of compounds **10-16**. Aerobic toxicity was increased with increasing DNA binding affinity, and it was maximum in the case of the strong DNA intercalator NLA-1. However, no correlation existed between DNA binding affinity and CT_{50H} values (concentration for 50% reduction in

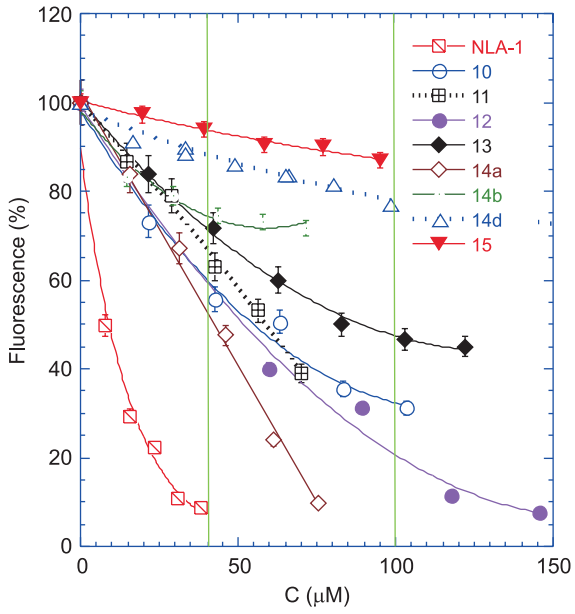


Fig. 1. DNA binding data of compounds in Scheme 8 using the ethidium bromide (EB) displacement assay. The fluorescence due to the EB-DNA complex was recorded and plotted *versus* compound concentration. The C_{50} values were determined from the plots and constitute a direct measure of DNA binding. Bars represent SD of quadruplicate measurements.

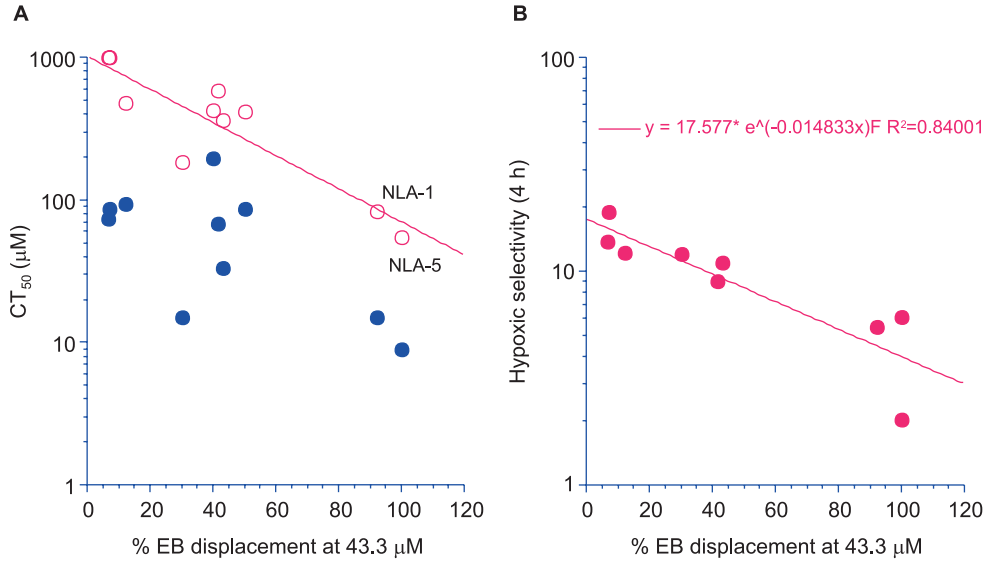


Fig. 2. Correlation plots between CT_{50} values under aerobic (O) or hypoxic (●) conditions and DNA binding affinity (**A**), and between hypoxic selectivity and DNA binding affinity (**B**), for compounds in Scheme 8.

clonogenicity under hypoxic conditions) (Fig. 2A). A weak negative correlation also existed between hypoxic selectivity (CT_{50A}/CT_{50H}) and DNA binding affinity (Fig. 2B).

Interaction with topoisomerases

Decreased aerobic toxicity of compounds **10-12** was associated with lack of topoisomerase I/II inhibition. Thus, topoisomerase I-mediated relaxation of supercoiled DNA was inhibited by **10** at concentrations of ≥ 1000 μM (66). Furthermore, topoisomerase II-mediated decatenation of kinetoplast DNA remained unaffected *in vitro,* even at aerobically toxic concentrations of **10** (66). Similar results were obtained with compounds **11** and **12**, whereas the strong DNA-intercalating analogue NLA-1 completely inhibited the topoisomerase I-mediated relaxation of supercoiled DNA at 30 μM and the topoisomerase II-mediated decatenation of kinetoplast DNA at 250 μM (manuscript in preparation).

Radiosensitization in vitro

The ability of compounds **10-16** to radiosensitize hypoxic cells *in vitro* was also examined in relation to their DNA binding ability. As can be seen in Figure 3A, in general, radiosensitizing potency ($C_{1.6}$ value: concentration for a sensitization enhancement ratio [SER] of 1.6) increases with DNA binding affinity, but the correlation is not linear. Thus, the weak DNA intercalators **10**, **13** and **14a** have an equivalent $C_{1.6}$ value to the strong DNA-intercalating analogue NLA-1, but, because of their lower aerobic toxicity, higher *in vitro* therapeutic indices ($CT_{50A}/C_{1.6}$) were obtained with these compounds (14, 66, 72). In addition, greater SER values were obtained *in vitro* compared to NLA analogues with all weak DNA-intercalating compounds in Scheme 8 at nontoxic concentrations (Fig. 3B).

Chemosensitization in vitro

Compounds **10**, **13, 14a, 15** and **16** have been investigated *in vitro* in combination with various chemotherapeutic agents under hypoxic pretreatment or post-treatment conditions (depending on the chemotherapeutic agent). All tested compounds interacted with chemotherapy in a synergistic way, yielding dose modification factors (DMFs) of 2-13 at low, inactive concentrations (13, 15, 70, 71, 73). Potentiation was observed even in chemotherapy-resistant cell lines such as OVCAR-3 under hypoxic pretreatment conditions with **10** (13). In general, the synergistic effect was dependent on the concentration of both hypoxia-selective cytotoxin and chemotherapeutic agent, and on the duration of the hypoxic pre-exposure to the bioreductive compound (13, 15, 71). No synergistic interaction was observed between each tested bioreductive compound and chemotherapeutic agent under aerobic coexposure conditions (13, 15).

Mechanistic studies in V79 cells revealed that, in the case of alkylating agents such as cisplatin and melphalan, hypoxic pre-exposure of cells to **10** or **13** increased the number of alkylating agent-induced DNA cross-links and apoptosis, caused significant delay in passage through the S phase, potentiated the chemotherapy-induced DNA, RNA and protein synthesis inhibition, and caused suppression of potential lethal damage repair (74, 75). EM9 cells, which lack the functional *XRCC1* gene involved in base excision repair and thus are unable to

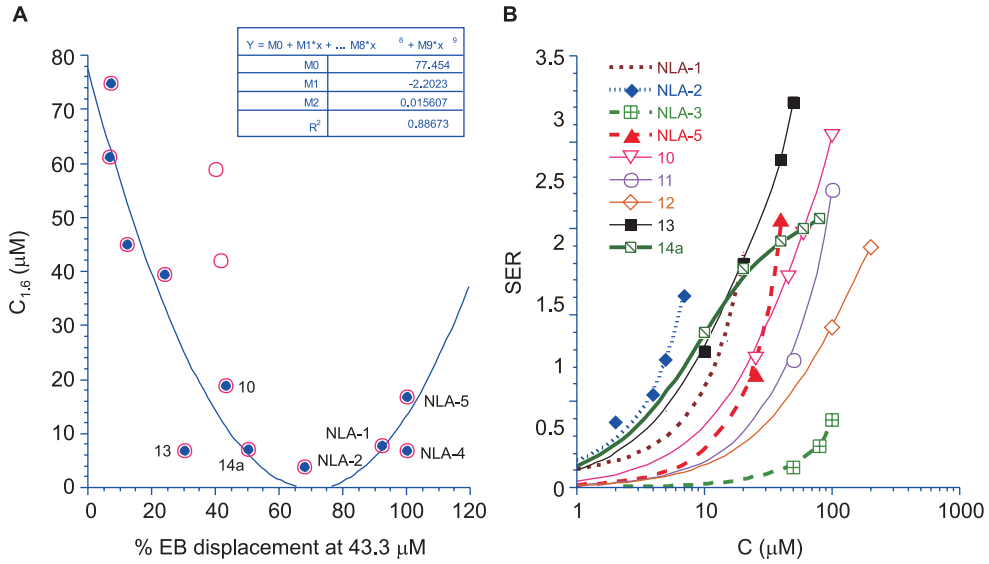


Fig. 3. Correlation plot between $C_{1.6}$ values (sensitizing ability) and DNA binding affinity of compounds in Scheme 8 (**A**); sensitization enhancement ratios (SERs) *versus* concentration plots for several strong and weak DNA-intercalating compounds (**B**).

efficiently repair DNA single-strand breaks, were 3.7 times more sensitive to **14a** than the parental AA8 cells. In addition, UV41 cells, which are defective in the essential gene for repair of DNA interstrand cross-links (*ERCC4/XPF*), and thus hypersensitive to DNA cross-linking agents, were 4.1 times more sensitive to **14a** than AA8 cells (76). These results suggest the involvement of **14a** in the formation of DNA single-strand breaks, and perhaps interstrand cross-links under hypoxic conditions. In potentiation studies of **14a** with melphalan or cisplatin, synergy was observed in AA8 cells but not in EM9 or UV41 cells, again suggesting an enhancement in melphalan/cisplatin-induced DNA interstrand cross-links by **14a**, possibly as a result of an inhibited repair mechanism of these lesions (76).

The mechanisms of paclitaxel and 5-FU potentiation by **14a** were investigated in V79 cells (77). When **14a** was administered under hypoxic conditions 2 or 3 h after the antimitotic agent paclitaxel or the thymidylate synthase inhibitor 5-FU, chemotherapy-induced apoptotic mechanisms were enhanced and occurred earlier. Thus, for instance, nucleosome formation was enhanced by **14a** by a factor of 2.5 and 1.3, respectively, 24 and 36 h post-treatment compared to paclitaxel alone (77). On the other hand, compound **14a** alone did not increase nucleosome formation compared to control at all time intervals examined. Apoptosis was also confirmed by detection of caspase 3 activity, particularly 30 h after combination treatment with either paclitaxel or 5-FU plus **14a**. Unrepairable DNA damage and persistent inhibition of DNA, RNA and protein synthesis were also some of the mechanisms involved in the potentiation of paclitaxel or 5-FU by **14a**, only under conditions of hypoxic exposure to this compound (77).

Evaluation in vivo

As mentioned earlier, our hypothesis in developing weak DNA-intercalating hypoxia-activated bioreductive drugs was that weak DNA binding through intercalation can overcome the problems of decreased extravascular diffusion and inability to reach hypoxic tumor tissues *in vivo*, problems associated with strong DNA intercalation. Therefore, the most promising derivatives **10**, **13** and **14a** were evaluated *in vivo* in combination with radiation (14, 72, 78, 79) or chemotherapeutic drugs (13, 15, 78, 80-86). The lead compound **14a** was also tested *in vivo* in combination with radioimmunotherapy (87).

All three compounds demonstrated substantial activity against murine tumors *in vivo* in combination with radiation or chemotherapy, without a concomitant enhancement of radio/chemotherapy-induced systemic toxicity, including bone marrow toxicity. Thus, compound **10** at 40-45 mg/kg (57-64% of its maximum tolerated dose [MTD] in mice) enhanced the killing effect of 20 Gy against EMT6 tumors approximately 1-2 logs beyond additivity when given 1 h before radiation. This corresponded to an *in vivo* SER value of 1.6-2.2 (78). Furthermore, compound **10,** administered i.p. at 30 mg/kg 3 h before cisplatin (5 mg/kg) to BALB/c mice bearing EMT6 tumors, increased the killing effect of cisplatin about 7-fold beyond additivity. Similarly, a dose of 45 mg/kg of **10** caused a 22-fold enhancement beyond additivity when administered 3 h before 8 mg/kg cisplatin in the same tumor model (13, 78).

Compound **13** at 15 mg/kg (< 30% of its MTD) increased the killing effect of 20 Gy about 5-fold beyond additivity when given i.p. 1 h before radiation in BALB/c mice bearing EMT6 tumors (14). In the same tumor model, compound **13**, 15 mg/kg i.p. 2-2.5 h before each

chemotherapeutic agent, potentiated the killing effect of melphalan (5 mg/kg), cisplatin (5 mg/kg) or cyclophosphamide (100 mg/kg) between 0.5 and 1.5 logs beyond additivity (15, 80). A synergistic interaction was also observed in the above tumor model with paclitaxel (20 mg/kg) when compound **13** was given i.p. at 15 mg/kg 3-3.5 h after the chemotherapeutic agent (80). It should be emphasized that compound **13** was ineffective on its own at a single dose of 15 mg/kg.

However, accumulated *in vivo* results were obtained only with the lead compound **14a** (NLCQ-1, NSC-709257), which was investigated against murine tumors (72, 78, 81-86) and human tumor xenografts (79, 82, 83, 87). In addition, pre-IND toxicological and pharmacokinetic studies have been completed or are in progress in mice, rats and dogs (88-91). Highlights of NLCQ-1 are presented in the following section.

The lead compound NLCQ-1 (NSC-709257)

Diffusion through cell multilayers

NLCQ-1 binds weakly to DNA through intercalation, with a C_{50} of 44 μM (Fig. 1), and possesses structural similarities with the antimalarial drug chloroquine, which binds to the minor groove of DNA (92). A recent study (93) showed that NLCQ-1 diffuses very well through oxic HT-29 multilayers, with a diffusion coefficient of 6.06 x 10^{-7} cm^2sec^{-1}, which makes it 1.5-fold faster than tirapazamine (diffusion coefficient = 3.97 x 10^{-7} cm^2sec^{-1}). As a result, NLCQ-1 is a more efficient radio/chemosensitizer than tirapazamine *in vivo*, as has been demonstrated in comparative studies using murine tumors (72, 82-86). Following our weak DNA intercalation approach, efforts have recently been made by Dr. Denny's group to synthesize improved tirapazamine derivatives (31, 32).

Metabolic activation

The hypoxic cytotoxicity of NLCQ-1 (expressed as the product of exposure time and concentration for 50% survival) ranges between 11.5 and 136 μM·h for five human and rodent tumor cell lines (A549, OVCAR-3, EMT6, SCCVII and V79) at an input concentration of 30 μM. Since uptake was similar in all these cell lines, the differences in hypoxic potency presumably reflect differences in the enzymatic profile and rate of reductive metabolism among the cell lines (67).

Nitroaromatics are reduced in cells by a number of flavoprotein enzymes, which effect stepwise addition of up to six electrons. Enzymatic reduction of NLCQ-1 with isolated rat liver microsomes and NAD(P)H showed that, under hypoxic conditions, *ca*. 60% of the parent compound is reduced within 1 h of incubation (37 °C) and that P-450 reductase and cytochrome b_5 reductase are responsible for the reduction. Similarly, reduction kinetics with human P-450 reductase-expressing microsomes showed *ca*. 75% and 50% reduction of NLCQ-1 under hypoxic and aerobic conditions, respectively, after 2-h incubation. Reduction of ≤ 5% was observed with recombinant human DT-diaphorase and NADPH or NADH under hypoxic or aerobic conditions, indicative of the absence of the involvement of DT-diaphorase, at least in the initial steps of NLCQ-1 reduction (94). In recent studies, it was shown that human breast cancer cells transfected with the P-450 reductase gene were up to 358 times more sensitive to NLCQ-1 under hypoxic conditions and up to 49 times more sensitive to NLCQ-1 under aerobic conditions than wild-type cells, which implies that NLCQ-1 could be used in the GDEPT approach (Dr. Kaye Williams and Natasha Wind, University of Manchester, personal communication).

Combined with radiation

In general, NLCQ-1 had weak *in vivo* antitumor activity on its own at the doses used. However, in combination with a single radiation dose, a synergistic interaction was observed (> 1 log kill) when 10 mg/kg (27 μmol/kg) NLCQ-1 was given 45-60 min before irradiation. An *in vivo* SER value of 1.58 was obtained with 10 mg/kg NLCQ-1, similar to that obtained with an equitoxic dose of tirapazamine, in SCCVII tumors using a fractionated radiation regimen (72).

When NLCQ-1 (15 mg/kg) was combined with clinically relevant single or fractionated doses of radiation against human glioma U-251 xenografts, efficacy was improved (79). Radiation alone (3.0 Gy/day x 2, 9-day rest and repeat schedule) resulted in 5 complete regressions in 10 mice, lasting for up to 42 days, and provided an optimal T/C value of –57 on day 38. When NLCQ-1 was added to the above protocol, 9 complete regressions were obtained with a follow-up of 52 days and the optimal T/C value was –100 from day 38 to day 52.

In a recent study at the National Cancer Institute (NCI), NLCQ-1 (15 mg/kg) was combined with radiation against head and neck cancer xenografts (unpublished results). Substantial tumor growth delays were obtained when NLCQ-1 was administered i.p. 45 min before each of 2 radiation doses of 5 Gy (days 20 and 30 after tumor implantation) or with 1 dose of 10 Gy (day 20 after tumor implantation), with additional tumor growth delays of 20 and 30 days, respectively, compared to radiation alone (Fig. 4).

Combined with radioimmunotherapy

In a series of studies using electrophysiology, immunohistochemistry and radiotracers, it was demonstrated that radioimmunotherapy (RAIT) induces a prolonged state of hypoxia in most tumors, without affecting the pO_2 levels in normal tissues, and that the maximum effect is observed about 14 days post-treatment and is independent of initial tumor size (87). When NLCQ-1 was

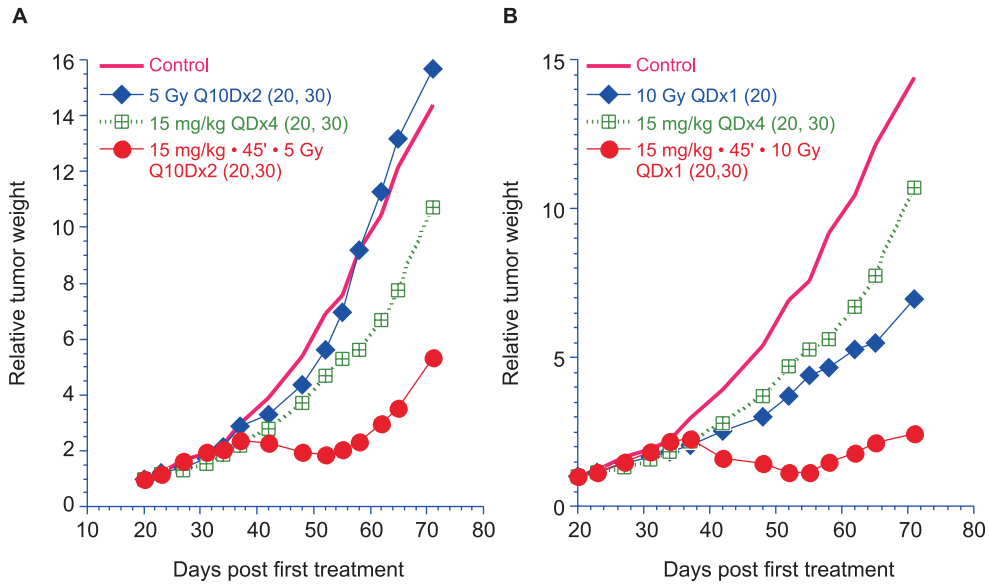


Fig. 4. Response of WSU-HN-31 xenografts to NLCQ-1 with or without radiation treatment. NLCQ-1 (15 mg/kg) alone was given i.p. daily for 4 consecutive days, starting on days 20 and 30 postinoculation. In combination with radiation, NLCQ-1 was given 45 min before each radiation dose at a 5 Gy per 10-day schedule on days 20 and 30 (**A**), or at a 10 Gy/day schedule on day 20 (**B**).

administered i.p. 14 days after a single dose of ^{131}I-MN-14 anti-CEA IgG to nude mice bearing various human colon tumor xenografts, tumor size significantly declined compared to treatment with RAIT alone. A similar decline in tumor size was observed with RAIT and tirapazamine, but at a 4.8-fold higher molar dose than NLCQ-1 (87).

Combined with chemotherapy

Various chemotherapeutic agents with different mechanisms of action have been tested in combination with NLCQ-1 *in vivo* using murine tumors or human tumor xenografts. A schedule-dependent synergistic interaction was observed in most cases (86). Synergy was optimal when NLCQ-1 was administered before an alkylating agent such as cisplatin, cyclophosphamide or melphalan (81, 83, 86), and before or after the antimitotic drug paclitaxel (82, 85, 86) and the thymidylate synthase inhibitor 5-FU (84, 86). In studies using EMT6 and SCCVII murine tumors, dose modification factors of 1.7-5.7 were obtained with a single NLCQ-1 dose of 10 mg/kg, whereas the corresponding factor for bone marrow toxicity was always about 1. NLCQ-1 was also superior to tirapazamine as a chemosensitizer in terms of achievable therapeutic indices (84-86). Significant tumor growth delays were observed in FSaIIC tumors when a single i.p. dose of NLCQ-1 was administered at an optimal interval with melphalan, cisplatin or cyclophosphamide (81). For instance, a delay of 18.1 ± 3.2 days was obtained when NLCQ-1 was combined with 10 mg/kg of melphalan *versus* 5.8 ± 3.6 days with melphalan alone.

Significant tumor growth delays were also achieved when multiple NLCQ-1 doses were combined with multiple small doses of a chemotherapeutic agent against murine tumors or human tumor xenografts (82-84). Thus, 10 mg/kg NLCQ-1 given i.p. 90 min after an inactive paclitaxel dose (8 mg/kg twice a day, 4 h apart, on days 0 and 9) provided 10 days of additional tumor growth delay compared to paclitaxel alone in the SCCVII/C3H model. An equitoxic tirapazamine dose gave 4.5 days of additional delay in the same model (82).

Toxicology in vivo

The LD_{50} and MTD of NLCQ-1 in non-tumor-bearing BALB/c mice were 35 and 30 mg/kg, respectively, when given as a single i.p. injection (81). However, NLCQ-1 can be administered in mice as multiple i.p. injections far exceeding the single LD_{50} dose without causing any significant systemic toxicity in terms of weight loss or other signs of toxicity (72). In combination with chemotherapeutic agents, NLCQ-1 given i.p. at 10 mg/kg/day for 4 or 5 consecutive days did not cause any additional toxicity in mice bearing human tumor xenografts (82, 83). No additional bone marrow toxicity was observed in murine tumor models in combination with various chemotherapeutic agents known to cause myelosuppression (81, 83-85). No hypoxia-dependent retinal toxicity was observed with 10 or 22 mg/kg of NLCQ-1 alone, or 10 mg/kg NLCQ-1 followed by 100 mg/kg cyclophosphamide an hour later, in BALB/c mice. On the other hand, statistically significant retinal toxicity was observed with 52 mg/kg of tirapazamine alone, which is equitoxic to 22 mg/kg of NLCQ-1

(90). Similar toxicity has been reported for tirapazamine in female C57B16 mice (95).

IND-directed toxicological studies in rats and dogs showed that NLCQ-1 can be safely administered i.v. at 12 mg/kg/day x 5 (male rats), 16 mg/kg/day x 5 (female rats) or 10.95 mg/kg/day x 5 (dogs) (88, 89 and unpublished results). In addition, daily administration of 9 (male rats) or 12 (female rats) mg/kg of NLCQ-1 1 h after i.v. administration of paclitaxel (3.5 mg/kg) had no effect on paclitaxel toxicity (89).

Pharmacokinetics in mice and dogs

NLCQ-1 was rapidly distributed and eliminated following i.v., i.p. or p.o. administration in CD2F1 mice (91). The plasma elimination was described by a two-compartment open model. A peak plasma concentration of 1481 ng/ml was achieved immediately after i.v. injection of 2.5 mg/kg NLCQ-1 and the concentration fell below 10 ng/ml 90 min postinjection. The plasma clearance (Cl) and volume of distribution (V_{ss}) after i.v. administration were 69.9 ml/min/kg and 2.04 l/kg, respectively, and the half-life ($t_{1/2\beta}$) was 41.3 min. A peak plasma concentration of 8900 ng/ml was achieved 5 min after an i.p. injection of 10 mg/kg. The $t_{1/2\beta}$ was 18.5 min and the bioavailability was 85%. No NLCQ-1 was detected in plasma after 2 h. NLCQ-1 was rapidly absorbed following oral administration at 10 mg/kg, but eliminated more slowly. The p.o. $t_{1/2\beta}$ was 83.9 min and the bioavailability was 28%. The 24-h urinary recovery of NLCQ-1 was 6.4% of the administered dose. NLCQ-1 plasma concentrations after i.p. administration of an active dose (10 mg/kg) remained above a concentration with demonstrated *in vivo* activity (3 μM) for approximately 30 min. The mouse data suggest that oral administration may provide plasma concentrations and systemic exposure similar to those observed after i.v. administration (91).

When NLCQ-1 was administered i.v. at 10.95 mg/kg/day x 5 in beagle dogs, its plasma levels showed apparent biphasic elimination, with an estimated half-life of 20 ± 5 and 30 ± 5 min for male and female dogs, respectively (unpublished results).

Summary

In this article we have briefly reviewed representatives of all classes of hypoxia-activated bioreductive drugs, with emphasis on those compounds that have entered preclinical or clinical testing. In addition, we have focused on compounds that target DNA and to problems associated with covalent or noncovalent but strong DNA binding. Finally, we have discussed our weak DNA intercalation approach for the development of more effective and less toxic *in vivo* bioreductive drugs and the results we have obtained with our lead compound NLCQ-1, which is about to enter clinical trials.

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