**N-[2-(2-Methyl-5-nitroimidazolyl)ethyl]-4-(2-nitroimidazolyl)butanamide (NSC 639862), a Bisnitroimidazole with Enhanced Selectivity as a Bioreductive Drug**

John W. Moselen, Michael P. Hay, William A. Denny, and William R. Wilson

Section of Oncology, Department of Pathology [J. W. M., W. R. W.] and Cancer Research Laboratory [M. P. H., W. A. D.], University of Auckland School of Medicine, Private Bag 92019, Auckland, New Zealand

**ABSTRACT**

Compounds containing two redox centers, both of which must be reduced for full expression of cytotoxicity by oxygen-inhibitable pathways (bis-bioreductive drugs), have potential as cytotoxic agents for hypoxic tumor cells. The bisnitroimidazole N-[2-(2-methyl-5-nitroimidazolyl)ethyl]-4-(2-nitroimidazolyl)butanamide (NNB, NSC 639862), in which a 2-nitroimidazole and 5-nitroimidazole moiety are joined via a carboxamide linker, is highly selective for hypoxic AA8 Chinese hamster cells (200-fold by 8 h) relative to mononitroimidazoles (5-25-fold). A bis-bioreductive mechanism is consistent with the marked increase in hypoxic potency and selectivity of NNB with time and the apparent requirement that the two nitro groups be present in the same molecule. NNB differed from mononitroimidazoles in inducing fewer DNA single strand breaks at equivalent toxicity, suggesting that a duplex DNA lesion (locally doubly damaged site) may be responsible for cell killing. Alkaline elution studies and the lack of hypersensitivity of the repair-defective UV4 cell line indicate that the cytotoxic lesion is not a DNA interstrand cross-link. NNB shows greater hypoxic selectivity than the alkylating 2-nitroimidazolylethyl-4-(2-nitroimidazolyl)butanamide (RB 6145) against AA8 cells and is active in combination with radiation when administered in multiple doses against the MDAH-MCa-4 mouse mammary carcinoma.

**INTRODUCTION**

There is evidence that many human tumors contain hypoxic cells (1) and that the radioreistance of these cells can be a limiting factor in radiotherapy (2–6). Studies with murine tumors have shown that nitroimidazoles can partially substitute for oxygen to radiosensitize hypoxic cells (7, 8); some trials in humans have also shown radiosensitization by nitroimidazoles (5) but results have generally been disappointing at clinically achievable doses.

In addition to their radiosensitizing activity, nitroimidazoles are selectively toxic to hypoxic cells (9–11) as a consequence of their reductive activation via oxygen-inhibitable metabolic pathways. Such “bioreductive drugs” have the potential to improve tumor response to radiotherapy by killing hypoxic cells (12–14) and might be preferable to hypoxic cell radiosensitizers inasmuch as they offer potential for turning hypoxia to advantage (15–17). The selective metabolic activation of 2-nitroimidazoles (and resulting covalent binding) in hypoxic regions of tumors is well documented (18–21), but the low cytotoxic potency of the reduction products of simple 2-nitroimidazoles precludes their use as bioreductive drugs (22).

The alkylating 2-nitroimidazolylethyl-4-(2-nitroimidazolyl)butanamide (RB 6145) linked to a 5-nitroimidazole were identified as having a hypoxic selectivity greater than related mononitroimidazoles (33). In the present study the more soluble of these two compounds, NNB (NSC 639862; Fig. 1), is examined to test the hypothesis that it acts as a bis-bioreductive agent under hypoxic conditions and to evaluate the therapeutic potential of this approach. The hypoxic toxicity and mechanism of action of NNB are compared with those of mononitroimidazoles, including the close analogue INB which lacks the 5-nitro group (Fig. 1).

**MATERIALS AND METHODS**

**Chemicals.** MISO was a gift from the National Cancer Institute (Bethesda, MD), METRO was from May & Baker NZ, Ltd. (Lower Hutt, Wellington, New Zealand), and RB 6145 was from Parke-Davis Pharmaceutical Research Division, Warner Lambert Co. (Ann Arbor, MI). NNB and INB were synthesized as described (33). The purities of these compounds were >99% by HPLC based on peak areas at 320 nm. All were dissolved in CM and filter sterilized before use. Concentrations were determined by spectrophotometry, using extinction coefficients in 0.01 N HCl of 7,750 M⁻¹ cm⁻¹ at 326 nm for MISO, 5,320 M⁻¹ cm⁻¹ at 280 nm for METRO, 12,480 M⁻¹ cm⁻¹ at 322 nm for NNB, and 4,760 M⁻¹ cm⁻¹ at 326 nm for INB. Initial concentrations of RB 6145 were estimated similarly with reference to a standard solution prepared in CM and

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: dsb, double strand break; CM, culture medium (oMEM containing 5% FBS plus 100 IU/ml penicillin and 100 μg/ml streptomycin); C₁₀₀ or C₁₀₀₀ concentration or concentration × time to reduce survival to 10% of controls; INB, N-[2-(2-methyl-5-nitroimidazolyl)ethyl]-4-(2-nitroimidazolyl)butanamide; LD₅₀, locally doubly damaged site; METRO, metronidazole; MISO, misonidazole; NNB, N-[2-(2-methyl-5-nitroimidazolyl)ethyl]-4-(2-nitroimidazolyl)butanamide; ssb, single strand break; T₉₀, time to reduce survival to 10% of control; [¹⁴C]Thrd, [⁵-methyl-¹⁴C]thymidine; [¹⁴H]Thrd, [⁵-methyl-¹⁴H]thymidine.
allowed to react to completion (34) (final extinction coefficient, 7,620 M⁻¹ cm⁻¹ at 326 nm). [¹⁴C]dThd and [³H]dThd were purchased from NEN Research Products (Boston, MA) and proteinase K was from Sigma Chemical Co. (St. Louis, MO).

**Cell Culture.** AA8 (35) and UV4 (36) Chinese hamster cell lines were passaged as monolayer cultures using CM without antibiotics and grown for experiments in 250-ml glass spinner flasks in CM with the fetal bovine serum raised to 10%. Log-phase cultures were adjusted to 5 X 10⁵ (AA8) or 3 X 10⁵ (UV4) cells/ml to give, 20 h later, early plateau phase cultures at about 1 X 10⁶ (AA8) and 5 X 10⁶ (UV4) cells/ml.

**Assessment of Hypoxia-selective Cytotoxicity.** Aerobic (20% O₂) and hypoxic cytotoxicity was assessed by clonogenic assay of stirred, continuously gassed cell suspensions (10⁶ cells/ml) as detailed elsewhere (37), with drug solutions in CM gassed with 5% CO₂ in N₂ or air for 60 min prior to addition of a concentrated suspension of hypoxic cells. O₂ concentrations in solution were determined using a Clark-type O₂ electrode with a high-stability voltage supply and amplifier (38). Rates of killing were assessed by fitting the survival data with second order regressions to interpolate Tₜ₀ values. The reproducibility of the cytotoxicity assay was evaluated by treating hypoxic AA8 cells with NNB at 3.2 mM, providing an intraexperiment mean Tₜ₀ of 1.6 h (coefficient of variation 7%) for 4 replicate cultures and an interexperiment mean of 1.7 h (coefficient of variation 6%) in 3 independent experiments.

**DNA Elution.** Cells for alkaline elution were grown to 5 x 10⁵ (AA8) or 3 x 10⁵ (UV4) cells/ml and labeled with either [¹⁴C]dThd (0.37 kBq/ml, 2.1 GBq/mmol) or [³H]dThd (3.7 kBq/ml, 710 MBq/mmol) for 20 h before centrifugation to remove unincorporated label. [¹⁴C]dThd-labeled cells were exposed to drugs for 5 h under hypoxic conditions as above. A sample was then removed for determination of cell survival and the remainder were centrifuged and resuspended in ice-cold PBS at 5 x 10⁵ cells/ml. In some experiments cells were then γ-irradiated (3 Gy) as stirred aerobic suspensions on ice, using an Eldorado G ⁶⁰Co teletherapy unit (Nordion, Ltd., Ottawa, Ontario, Canada) at a dose rate of 2.2 Gy/min, for determination of DNA cross-links. Non-drug-treated [³H]dThd-labeled cells were irradiated (3 Gy) to provide an internal standard. Samples containing 5 x 10⁵ each of [¹⁴C]- and [³H]-labeled cells were lysed with proteinase K and sodium dodecyl sulfate at pH 10 for 1 h on polycarbonate filters and eluted at pH 12.1 as described by Kohn et al. (39). ssb frequencies, expressed relative to radiation, were quantitated by interpolating the relative retention, R (fraction of ¹⁴C retained on the filter when 50% of the internal ³H standard had eluted). A standard curve for radiation was determined from two experiments with ¹⁴C-labeled AA8 cells irradiated as oxygenated suspensions on ice. The relationship between log₁₀ R and the radiation dose D (in Gy) was linear (log₁₀ R = –0.11 D; r = 0.99) over the range investigated (0–7.5 Gy). Nondenaturing elution (pH 9.6) was performed as described by Bradley and Kohn (40); [¹⁴C]dThd-labeled cells were exposed to drug and loaded onto polycarbonate filters as above, but using [³H]dThd-labeled cells irradiated to a dose of 75 Gy as the internal standard.

**Activity against MDAH-MCa-4 Tumors.** The MDAH-MCa-4 mouse mammary carcinoma (41) was obtained from Dr. Helen B. Stone, Radiation Oncology Research Laboratory, UCSF, CA, at the second transplant generation. Cell suspensions were prepared from fourth generation s.c. tumors by mincing with scissors, stirring magnetically in CM for 30 min at room temperature, filtering through a 200 mesh screen, and collecting the cells in the filtrate by centrifugation. The pellet was resuspended in CM without serum and cells were given i.m. injections in the right gastrocnemius muscle (5 µl packed cell volume) to provide fifth passage isografts in female C3H/HeN mice for experiments. The tumor was not significantly immunogenic in this strain, with the packed cell volume required for 50% tumor takes being 0.08 µl (95% confidence limits, 0.055–0.11 µl by logistic regression) in naive mice and 0.23 µl (95% confidence limits, 0.08–0.62 µl) in mice immunized by i.p. injection of lethally irradiated cells (10 µl packed cell volume) weekly for 3 weeks before challenging with viable cells in both hind legs (7 mice/group).

For tumor growth delay experiments tumor size was measured by determining the smallest diameter circular hole through which the leg would pass. Mice were allocated to treatment groups (7 mice/group) when the tumor plus leg diameter reached 10 mm (0-5 g tumors). Tumors were irradiated (20 Gy; Philips RT 250; 175 kVp; filtration, 0.2 mm Cu; 1.05 Gy/min) by restraining unanesthetized mice in a Lucite-lined box, with the tumor-bearing leg drawn out and tethered in the radiation field using a wire clip around the ankle. The body was shielded by lining the lid of the restraining box with lead; the dose to the center of the body was 3% of that to the tumor as assessed by thermoluminescent chip dosimetry. Drugs were formulated in PBS and administered i.p. at 0.05 ml/g. Differences in the time required to reach a leg diameter of 13 mm were tested by ANOVA followed by Dunnett's test to assess significance of the growth delay in individual groups.

**RESULTS**

**Cytotoxicity of NNB.** The time and concentration dependence of cell killing by the bisnitroimidazole NNB was investigated using stirred, continuously gassed cultures of AA8 cells. Its cytotoxicity was markedly enhanced under hypoxic conditions, particularly when cells were exposed to low drug concentrations for long times (Fig. 2), CT₁₀ values, calculated as an inverse measure of cytotoxic potency, were constant with time under aerobic conditions but decreased progressively under hypoxia (Fig. 3). Hypoxic selectivity (determined as the ratio of drug concentrations required for 10% survival under air or N₂) thus increased markedly with time to give a ratio of about 200 by 8 h (Fig. 4). The potency and hypoxic selectivity of NNB against the AA8-derived repair-defective UV4 cell line, which is hypersensitive to agents causing DNA cross-links and bulky monoadducts (42, 43), was similar to that for AA8 cells (Figs. 3 and 4).

**Cytotoxicity of RB 6145.** A similar investigation of the hypoxic selectivity of RB 6145 was performed using the AA8 and UV4 cell
lines. $CT_{10}$ values were almost constant with time under aerobic conditions but decreased progressively under hypoxia in both cell lines (Fig. 3), although this change was less pronounced than with NNB. Hypoxic selectivity increased about 2-fold between 1 and 8 h in the AA8 cell line and 3-fold in the UV4 line (Fig. 4). In contrast to NNB, UV4 cells were much more sensitive to RB 6145 than were AA8 cells [differential 8-fold under aerobic conditions and 20-fold under hypoxia (Fig. 3)]; hypoxic selectivity was also much greater in the repair-deficient line (Fig. 4).

**Cytotoxicity of Mononitroimidazoles.** The mononitroimidazoles MISO, METRO, and INB were investigated using the AA8 cell line (Fig. 3). For MISO and INB the aerobic $CT_{10}$ was again constant with time; aerobic cell killing by the least potent compound (METRO) could be detected only using long exposure times and a concentration (72 mM) close to the solubility limit. Under hypoxia there was a slight trend towards lower $CT_{10}$ values at late times for MISO and INB and the hypoxic selectivity of these compounds thus increased with time, although much less dramatically than for NNB (Fig. 4). At late times (about 8 h) hypoxic selectivities were 12-fold for INB, 25-fold for MISO, and 5-fold for METRO. All three mononitro compounds were thus much less potent and selective than NNB as hypoxic cell toxins at this time.

**Variation of Pregassing Time.** The large increase in potency of NNB under hypoxia with time (and the smaller increases observed with RB 6145, MISO, and INB) raised concerns that residual oxygen concentrations as illustrated for NNB in Fig. 2. Ratios calculated from the data of the hypoxic selectivity of these compounds thus increased with time, although much less dramatically than for NNB (Fig. 4). At late times (about 8 h) hypoxic selectivities were 12-fold for INB, 25-fold for MISO, and 5-fold for METRO. All three mononitro compounds were thus much less potent and selective than NNB as hypoxic cell toxins at this time.

**DNA Elution Studies.** The DNA ssb frequencies induced by nitroimidazoles in hypoxic AA8 cells were determined by alkaline elution using 5-h hypoxic drug treatments and were compared with cell killing measured in the same experiments (Fig. 7). The elution curves for all compounds were approximately exponential (data not shown), except for RB 6145 which showed a distinct lag, presumably reflecting slow breakage at alkali-labile sites as reported for RSU 1069 (39). All four mononitroimidazoles induced ssb at broadly similar frequencies (equivalent to about 5 Gy radiation at drug concentrations giving 99% cell kill). In contrast, NNB failed to induce ssb significantly above that in hypoxic controls (which consistently reflect slow breakage at alkali-labile sites as reported for RSU 1069 (39). All four mononitroimidazoles induced ssb at broadly similar frequencies (equivalent to about 5 Gy radiation at drug concentrations giving 99% cell kill). In contrast, NNB failed to induce ssb significantly above that in hypoxic controls (which consistently showed slightly faster DNA elution than cells not incubated under hypoxia), even at concentrations up to 3-fold greater than the highest in Fig. 7.

Treatment of AA8 cells with NNB (up to 2.5 mM) under hypoxia for 5 h failed to prevent the increase in DNA elution rate caused by
significant antitumor activity at a dose of 2.5 mmol/kg and, unlike MISO, did not significantly increase growth delay when administered 30 min before radiation (Fig. 9). This was the highest single dose of NNB which could be administered because of solubility limitations, but the total dose could be increased without morbidity using a multidose schedule (every 3 h for 4 treatments). This schedule did not improve the activity of MISO (Fig. 9A), but irradiation 30 min after the last NNB dose provided a statistically significant \( P < 0.01 \) growth delay \([11.2 \pm 3.8 \text{ (SE) days}]\) additional to radiation alone (Fig. 9B). The activity of multiple doses of NNB (2.5 mmol/kg, every 3 h for 4 treatments) in combination with radiation was confirmed in a second experiment, which gave a mean growth delay of 1.0 \pm 0.9 \text{ days} in the absence of radiation but a larger and significant \( P < 0.01 \) growth delay (relative to radiation only) of 10.9 \pm 4.4 \text{ days} when combined with radiation.

**DISCUSSION**

**Hypoxia-selective Toxicity of NNB in Culture.** This study demonstrates that, under conditions of prolonged exposure (5–8 h), the bisnitroimidazole NNB has higher potency and selectivity as a hypoxic cell toxin than simple 2-nitro- or 5-nitroimidazoles. In comparison with its mononitro analogue, INB, the bisnitrityl compound is irradiation (Fig. 8A). Thus DNA interstrand cross-links could not be detected even at these highly cytotoxic drug concentrations. In parallel experiments using chlorambucil (5 h hypoxic exposure) as a positive control, extensive cross-linking was demonstrated (Fig. 8B) in the cytotoxic concentration range \( D_{10} = 32 \mu \text{M} \) under these conditions; data not shown. Thus at equivalent toxicity NNB was much less effective than chlorambucil in inducing DNA cross-links. Similar studies with the cross-link repair-defective UV4 line using 1 mm NNB under hypoxia for 5 h also failed to demonstrate cross-links (data not shown).

Nondenaturing elution experiments showed a DNA double strand break frequency after treatment of hypoxic AA8 cells with NNB (20 mm, 5 h) which was equivalent to irradiation of oxygenated cells to about 30 Gy (data not shown). These doses are 4 and 130 times those required for 90% cell kill with radiation and NNB, respectively, suggesting that the cytotoxic lesions induced by NNB are not radiation-like double strand breaks. However, the relevance of these DNA breakage data at such toxic drug concentrations and long exposure times are unclear.

**Activity against Hypoxic Cells in Tumors.** The activity of NNB against MDAH-MCa-4 mammary tumors was assessed in combination with radiation and compared with MISO. NNB alone had no significant growth delay at 2.5 mmol/kg dose, with only 9.4 ± 4.0 (SE) days growth delay compared to 11.2 ± 3.8 (SE) days for MISO. However, when given 30 min before irradiation, MISO had no significant effect on growth, whereas NNB provided a statistically significant \( P < 0.01 \) growth delay \([10.9 \pm 4.4 \text{ days}]\) when combined with radiation.

**Fig. 8.** Alkaline elution tests for DNA interstrand cross-links after treatment of hypoxic AA8 cells with drugs for 5 h. A, NNB: , control; , 1 mm; ▲, 1.75 mm; △, 2.5 mm. B, chlorambucil: , control; ▲, 18 µM; △, 34 µM; △, 72 µM. O, 144 µM. O, ●, ▲, △, ○, unirradiated; ●, ▲, △, □, irradiated (3 Gy).

**Fig. 9.** Activity of MISO (A) or NNB (B) against hypoxic cells in i.m. MDAH-MCa-4 tumors. Growth curves are for the median mouse in each group of 7 animals, ranked at a leg + tumor diameter of 13 mm, and are from a single experiment. O, control; , single dose drug; , four drug doses (every 3 h for 4 treatments); , single dose radiation (20 Gy); , single drug dose 30 min before radiation; , four drug doses (every 3 h for 4 treatments) irradiated 30 min after the last dose. All drug injections were i.p. at 2.5 mmol/kg/dose (0.05 ml/g body weight).
The present data are consistent with the action of RB 6145 as a bis-bioreductive drug: (a) activity of hypoxic UV4 cells (or other lines of the same complementation group) to RSU 1069, the active form of RB 6145, has been reported by others and is considered to reflect the increased contribution of cross-linking to cytotoxicity under hypoxic conditions (29, 45). The present data are consistent with the action of RB 6145 as an RSU 1069 prodrug in this system. RB 6145 is known to be unstable in culture medium with rapid conversion (t1/2 about 30 min at pH 7.4, 23°C) to the aziridine RSU 1069 and to the less toxic oxazolidin-2-one (34). Hence extensive conversion to RSU 1069 would have occurred by the time of addition of cells (60 min after addition of drug, 37°C) in the present experiments.

Is NNB a Bis-bioreductive Agent? Several lines of evidence are consistent with the hypothesis that NNB is a bis-bioreductive drug: (a) its hypoxic potency and selectivity is greater than that of simple mono-2-nitroimidazoles (Figs. 3, 4, and 7), including the close analogue INB. This high potency is not accounted for by enhanced cell uptake since the uptake factors (intracellular/extracellular concentration) for NNB and INB are identical (0.33) in hypoxic AA8 cultures (33). NNB thus resembles the nitroaridine N-oxides which appear to owe their high selectivity for hypoxic cells to their bis-bioreductive character (32); (b) the greater potency and selectivity of NNB appear not to be accounted for by interaction between 2- and 5-nitroimidazoles which are not linked in the same molecule, as demonstrated using equimolar combinations of MISO and METRO (Fig. 6); (c) concentration-time relationships for cell killing by NNB are different from the other nitroimidazoles tested in that the cytotoxic potency increases markedly with time under hypoxic conditions. This may reflect the slow activation of a second reducible center to generate a bifunctional molecule. The cytotoxic potency of NNB at 1 h is similar to those of Miso and INB suggesting that initial toxicity is due to the 2-nitroimidazole moiety and that the slow second activation is due to reduction of the lower reduction potential 5-nitro group.

The increase in hypoxic potency of NNB with time is not readily accounted for by slow consumption of residual oxygen since the increase in rate of killing is much greater than is observed after prolonged precessing; further, the mononitroimidazoles tested [including METRO, which is known to be highly oxygen sensitive in CHO cultures (44)] show a much smaller increase in activity with time than does NNB. The observation that the CT10 for NNB is constant under oxic conditions also implies that the time dependence under hypoxia is specifically a consequence of slow metabolic activation at a second center. This interpretation is supported by recent experiments showing that after exposure of hypoxic AA8 cells to NNB for 2 h followed by extensive washing (removing 99.95% of the parent drug), cells continue to die when incubated under hypoxic conditions (46). Similar results have been reported by Stratford et al. (24) for hypoxic exposure of V79 cells after aerobic treatment with RSU 1069 and were also interpreted as indicating metabolic activation of a covalently bound nitroimidazole at the target site.

A Locally Doubly Damaged Site Model. If NNB has the biological characteristics of a bis-bioreductive agent, then the possible formation of some highly cytotoxic bistranded DNA lesion should be considered. We propose a LDDS model analogous with the locally multiply damaged site model, as advanced by Ward (47, 48) to account for the cytotoxicity of ionizing radiation in terms of clustered DNA lesions. In the LDDS model, bisreduction of NNB is proposed to generate a species which is highly cytotoxic because the DNA lesions formed by the two functional domains of the molecule are closely linked spatially and can thus give rise to damage sites which are closely paired on opposite strands of the DNA. Such duplex lesions (e.g., pairs of ssb, a monoadduct or abasic site associated with a ssb, interstrand cross-link) may be highly cytotoxic because they are difficult to repair with fidelity. This LDDS model is analogous to that proposed for the cytotoxicity of bleomycin and the endolyne antibiotics, which generate bistranded DNA lesions (49). In the latter case such lesions result from a single oxidizing biradical species which can abstract H atoms from deoxyribose sugars in opposite strands (50, 51) to generate high yield (52, 53).

The lack of ssb formation by NNB in comparison with other nitroimidazoles at equitoxic concentrations (Fig. 7) is suggestive evidence for the generation of a more toxic duplex DNA lesion. The critical cytotoxic lesions do not appear to be DNA dsb analogous to those formed by ionizing radiation or DNA interstrand cross-links. That cross-links are not involved is strongly suggested by the lack of hypersensitivity of UV4 cells to NNB under either aerobic or hypoxic conditions (Fig. 3) since this line is sensitive (6–60-fold with respect to AA8) to all DNA cross-linkers tested (43, 45, 54). Alkaline elution assays in combination with radiation also failed to detect cross-links in either AA8 or UV4 cells at concentrations well above the cytotoxic range, while cross-linking by chlorambucil could be readily demonstrated under these conditions (Fig. 8). Reduction products of 2-nitroimidazoles can alkylate DNA (55, 56), but the lack of DNA alkylation on metabolic reduction of 5-nitroimidazoles (57) may preclude formation of a cross-link on bisnitroreduction of NNB. However, a variety of other types of nucleoprotein damage by 2- and 5-nitroimidazole reduction products might contribute to the formation of a lethal duplex lesion. Nitro(hetero)aromatic compounds are efficient inducers of DNA ssb (58–60) as illustrated for METRO and MISO in Fig. 7, and when reduced electrochemically METRO is even more effective than MISO in inactivating phage DNA (61). In addition, the nitroso reduction products of 2-nitroimidazoles can alkylate protein thiol groups (62). Further, reduced 2-nitroimidazoles are known to bind covalently to tissue macromolecules with retention of the N-1 side chain (63, 64); thus covalently bound products resulting from reduction of the 2-nitro group are expected to retain a 5-nitroimidazole moiety which might induce further local damage when subsequently reduced. Therefore duplex lesions such as a DNA monoadduct opposing a ssb, a protein thiol adduct in close association with a ssb, or an intrastrand cross-link might provide the enhancement of toxicity required for the bisbioreduction/LDDS model.

DNA lesion clustering may be important in the action of bioreductive drugs other than NNB. RB 6145 and RSU 1069 probably owe their high selectivity and potency to generation of LDSS in the form of DNA cross-links (29, 65). In addition, the high frequency of dsb in Chinese hamster ovary cells treated with the benzotiazidine-di-N-oxide tirapazamine (SR 4233) (66, 67) has been suggested to reflect the production of a high local concentration of oxidizing radicals because of the relative immobility and physical proximity to DNA of the critical reductase(s) responsible for SR 4233 activation (67, 68).

Therapeutic Potential of Binsnitroimidazoles. NNB is able to eliminate hypoxic cells in MDA-Mb-4 mammary tumors as judged by its ability to extend the tumor growth delay induced by ionizing radiation (Fig. 9). It is similarly active against hypoxic cells in the KHT tumor as assessed by clonogenic assay (33). In both cases, activity was observed only when multiple doses were used, with no significant activity after the highest single dose (2.5 mmol/kg) which could be administered because of solubility limitations. In contrast, the activity of MISO was not improved by repeated dosing. The latter
result was expected since enhancement of radiation response with MISO is due primarily to radiosensitization rather than killing of hypoxic cells (22), and its half-life in the mouse [1.5 h at 2.5 mmol/kg (69)] is too short to provide substantial accumulation in the tumor with three hourly dosing intervals. The improvement in activity of NNB with multiple doses suggests that activity is probably related to the concentration-time integral rather than peak concentration in the tumor at the time of irradiation and is thus likely to be due to hypoxic cell toxicity rather than direct (electron affinic) radiosensitization. However, pharmacokinetic studies are required to confirm this conclusion. The potency of NNB as a hypoxic cell radiosensitizer in vitro is no greater than that of simple mononitroimidazoles (33), but this property may play some part in its activity in vivo. Nitroimidazoles radiosensitizers have generally shown disappointing activity in clinical studies (5), and there are theoretical reasons for expecting that bioreductive drugs will be superior in radiotherapy (14-16). Further development of bisnitroimidazoles related to NNB would therefore warrant a high priority if their antitumor activity in combination with radiation is due primarily to hypoxic cell killing. Recent studies in this laboratory have identified a bis(nitroimidazole) derivative, NSC 661066, with improved water solubility and higher hypoxic potency in vitro at early exposure times, and which is highly active against KHT tumors when administered after irradiation. This postirradiation activity strongly implicates hypoxic cell killing as the major mechanism and indicates that the bisnitroimidazoles are a novel class of bioreductive agent which warrant further exploration.

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BIS-NITROIMIDAZOLE-BIOREDUCTIVE DRUGS
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