Hypoxia-Selective Antitumor Agents. 8. Bis(nitroimidazolyl)alkanecarboxamides: A New Class of Hypoxia-Selective Cytotoxins and Hypoxic Cell Radiosensitisers

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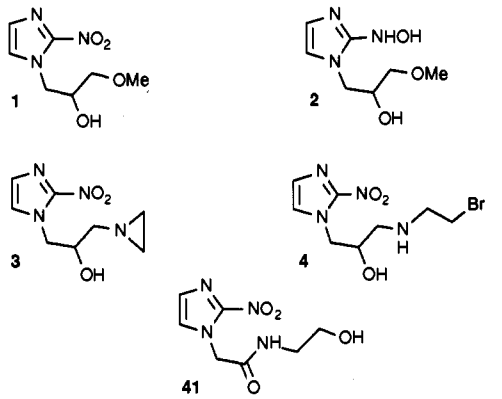
A series of novel bis(nitroimidazolyl)alkanecarboxamides has been prepared and evaluated for hypoxia-selective cytotoxicity and hypoxic cell radiosensitisation *in vitro* and *in vivo*. The compounds were prepared by direct coupling of preformed side chain acid and amine components, using diethyl phosphorocyanidate at room temperature. Although designed to be bis-bioreductive prodrugs of DNA cross-linking agents, none of the compounds showed evidence of DNA cross-linking activity, being equally potent against cell lines deficient and proficient in repair of cross-links. However, one of these compounds, *N*-[2-(2-methyl-5-nitro-1*H*-imidazolyl)ethyl]-4-(2-nitro-1*H*-imidazolyl)butanamide (**10**; SN 24699), showed high hypoxic selectivity as a cytotoxin (rising to 200-fold after exposure to the drug for several hours) in the repair-proficient Chinese hamster cell line AA8. This selectivity was greater than observed for the alkylating 2-nitroimidazole (**4**; RB 6145) (40-fold) or simple mononitroimidazoles (5–25-fold). Investigation of structure–activity relationships for hypoxic selectivity of bis(nitroimidazoles) was restricted by their low aqueous solubility, but a certain minimum separation of the two nitroimidazole units (by more than five atoms) appears desirable. All the compounds radiosensitized hypoxic cells *in vitro* but were little more potent as radiosensitizers than the corresponding monomeric nitroimidazoles. Compound **10** caused additional cell killing in the KHT tumor when multiple drug doses were administered in combination with a single dose of radiation. It is not yet clear whether this activity reflects hypoxic cell radiosensitization or cytotoxicity toward hypoxic cells, but this new class of bis-bioreductive agent clearly warrants further investigation.

The existence of hypoxic cells in tumors[1] has long been recognized as a major problem in radiotherapy[2] and is also a potential problem in the chemotherapy[3] of cancer. The development of drugs which combat this problem, using hypoxia as a basis for achieving tumor selectivity,[4] focused first on compounds (oxygen-mimetic radiosensitisers) capable of sensitising hypoxic cells to radiation.[5] More recently, there has been an increasing interest[4,6,7] in the development of drugs (hypoxia-selective cytotoxins, HSCs) which act indirectly as radiosensitizers by themselves killing radioresistant hypoxic cells.

To be effective, such drugs need to penetrate to the hypoxic regions of solid tumors by extravascular diffusion, to undergo selective activation in these regions, and to generate cytotoxic metabolites following activation. This activation is usually by metabolic reduction, and most HSCs can thus be described as bioreductive drugs. One major class of HSCs are the 2-nitroimidazoles, which undergo enzymatic reduction of the nitro group via the one-electron reduction product. This initial step in nitroreduction can be efficiently reversed by oxygen, resulting in preferential metabolism in hypoxic cells.[8]

Thus the 2-nitroimidazole misonidazole (**1**) shows an hypoxic selectivity of ca. 20-fold in AA8 cells in culture,[10,30] and is known to undergo 4-electron reduction to the unstable[11] hydroxylamine **2**, but has low cytotoxic potency. In comparison, the 2-nitroimidazole aziridine analogue (**3**; RSU-1069)[12] not only has much higher cytotoxicity than misonidazole, but also shows enhanced selectivity for hypoxic cells (ca. 50–100-fold).[10,13] While the predominant mechanism of cytotoxicity of this compound under aerobic conditions is DNA monoalkylation by the aziridine, reductive metabolism converts it into a much more toxic bifunctional alkylating agent capable of cross-linking DNA.[14] This compound received clinical trial, but severe emetic side effects (attributed to the aziridine moiety) were found.[15] More recently, interest has centered on a prodrug of **3**, the 2-bromoethyl compound (**4**; RB 6145).[16] This compound is rapidly converted to **3** *in vitro* and *in vivo* and shows equivalent activity as a radiosensitizer and an HSC, but is less emetic and has pronounced oral activity.[17]



However, neither aziridines nor 2-haloethyl precursor moieties have selectivity for hypoxic cells, being direct DNA-alkylating functions. Further gains in hypoxic selectivity might, in principle, be achieved by replacing this oxygen-insensitive DNA alkylating moiety with a bioreductive electrophile, thus restricting activation of the electrophilic center to hypoxic cells. Such a compound could be termed a "bis-bioreductive" agent, in that oxygen-

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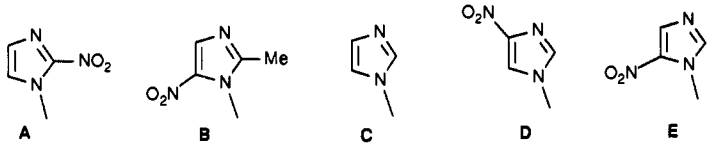
Table 1. Structural and Physicochemical Data for Bis(nitroimidazolyl) Carboxamides

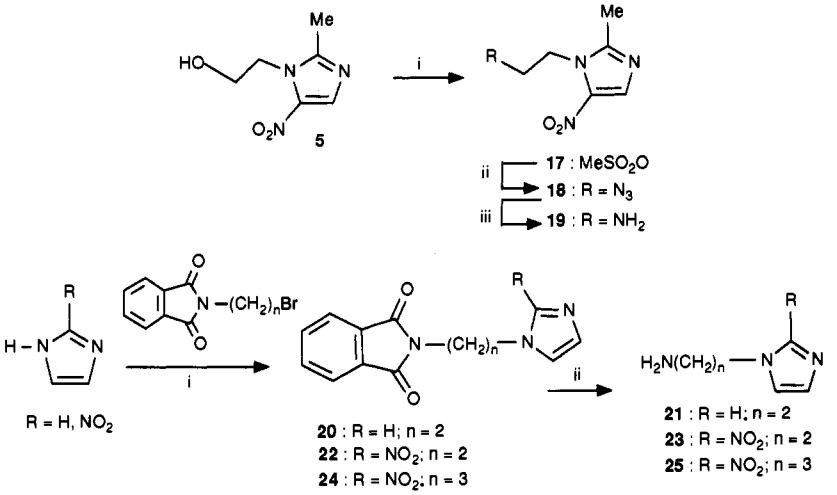
R_1-$(CH_2)_n$CONH$(CH_2)_m$-R_2



no.	R_1	n	m	R_2	mp, °C	formula	anal.	log P^a	sol[b]
1		misonidazole							>120
4		RB 6145							-0.79
5		metronidazole							-0.80
6	A	3	2	A	149.5-150.5	$C_{12}H_{15}N_7O_5$	C,H;N[d]	-1.55	4.2
7	A	2	3	A	124-125	$C_{12}H_{15}N_7O_5$	C,H,N	-1.55	21
8	A	1	2	B	nc[e]	$C_{11}H_{13}N_7O_5$	C,H,N	-1.03	59
9	A	2	2	B	144.5-145.5	$C_{12}H_{15}N_7O_5$	C,H;N[f]	-0.99	13
10	A	3	2	B	129	$C_{13}H_{17}N_7O_5$	C,H;N[g]	-1.28	20
11	A	4	2	B	136-137.5	$C_{14}H_{19}N_7O_5$	C,H,N[h]	-0.79	8.6
12	A	5	2	B	133-134	$C_{15}H_{21}N_7O_5$	C,H,N	-0.22	2.1
13	A	3	2	C	nc[e]	$C_{12}H_{17}N_6O_3$	HRMS[i]	1.32	408
14	A	3	2	D	175.5-177	$C_{12}H_{15}N_7O_5$	C,H;N[i]	-1.55	2.3
15	A	3	2	E	152.5-153	$C_{12}H_{15}N_7O_5$	C,H,N	-1.55	5.5
16	B	1	2	B	nc[e]	$C_{12}H_{15}N_7O_5$	C,H,N	-0.76	36

[a] Calculated using the program CLOGP (version 3.51, BioByte Corp., Claremont, CA 91711). [b] Solubility in millimolar in αMEM + 5% FCS, measured by UV spectrophotometry. [d] N: calcd, 29.07; found, 29.74. [e] Noncrystalline. [f] N: calcd 29.07; found, 29.90. [g] N: calcd 27.91; found 28.46. [h] N: calcd 26.84; found 27.43. [i] High-resolution mass spectrometry. [j] N: calcd 29.07; found 28.32.

Scheme 1



[a] (i) MsCl/CH_2Cl_2/20 °C; (ii) NaN_3/DMF/100 °C; (iii) Ph_3P; concentrated HCl/100 °C.[b] (i) NaH/DMF (**20**) or K_2CO_3/DMF (**22, 24**); (ii) NH_2NH_2.

inhibitable reduction of two centers would be required for full cytotoxicity. We have recently shown[18] that another bis-bioreductive agent can achieve very high levels of hypoxic selectivity (>1000-fold).

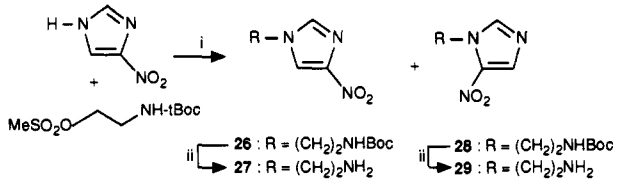
In this paper we report the synthesis and evaluation as HSCs and hypoxic cell radiosensitizers of a series of bis(nitroimidazolyl) compounds (**6–16**) (Table 1), linked by an alkanecarboxamide chain. Nitroimidazoles were selected as the nitroarene units, since examples of 2- and 5-nitroimidazoles have been shown to have hypoxic selectivity due to the generation of reactive intermediates following nitro reduction in cells.

Despite ring fragmentation during nitro reduction under some conditions,[19] the N-1 side chain of misonidazole is known to be retained during covalent binding to macromolecules.[20] This suggests that linkage of bis(nitroimidazoles) via the N-1 positions might be stable to reduction, at least in the case of bis(2-nitroimidazoles), and that reactive bifunctional molecules could thus be generated under hypoxic conditions. In the present study, linkage of the two nitroarene units was effected at the 1-positions through *N*-alkyl chains. These linkers ensure electronic isolation of the two nitroimidazole units, avoiding significant perturbation of nitro group reduction potentials. A carboxamide linking functionality was chosen for its high hydrophilicity (fragment constant f = -2.71)[21] and for synthetic flexibility.

Chemistry

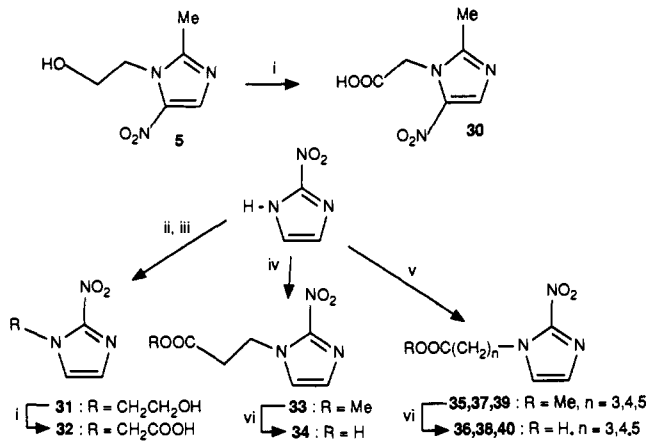
The carboxamide-linked bis(nitroimidazoles) were prepared by coupling of preformed side chain acid and amine components. A key amine intermediate, 2-(2-methyl-5-nitro-1*H*-imidazolyl)ethylamine (**19**), was prepared by nucleophilic displacement of metronidazole mesylate **17** by NaN_3, and subsequent reduction of the azide **18** to the amine **19** (Scheme 1a). Alkylation of imidazole with (2-bromoethyl)phthalimide gave the phthalimide **20**, and phthalimide exchange with hydrazine monohydrate gave 2-(1*H*-imidazolyl)ethylamine (**21**) (Scheme 1b). 2-Nitroimidazole was similarly elaborated with (2-bromoethyl)-phthalimide and (3-bromopropyl)phthalimide to the amines (**23** and **25**). Amines **27** and **29** were prepared

Scheme 2[a]



[a] (i) K_2CO_3/DMF/110 °C; (ii) HCl/MeOH/20 °C.

Scheme 3[a]



[a] (i) CrO_3/Me_2CO; (ii) $I(CH_2)_2OTHP/K_2CO_3$/DMF/110 °C; (iii) HCl/MeOH/20 °C; (iv) CH_2=CHCOOMe/KF-alumina/100 °C/18 h; (v) $Br(CH_2)_nCO_2Me/K_2CO_3$/DMF/110 °C; (vi) HCl/20 °C/18 h.

more conveniently by alkylation of 4-nitroimidazole with *N*-[(*tert*-butoxycarbonyl)mesyl]ethylamine under basic conditions (Scheme 2). This procedure afforded a 6:1 mixture of the 4-nitro and 5-nitro isomers of *N*-Boc-2-(nitro-1*H*-imidazolyl)ethylamine, **26** and **28**, which were separated by flash chromatography, and hydrolyzed to the amines under acid conditions.

2-(2-Methyl-5-nitro-1*H*-imidazolyl)acetic acid (**30**) was prepared by Jones' oxidation of metronidazole (**5**) (Scheme 3). 2-(2-Nitro-1*H*-imidazolyl)acetic acid (**32**) was prepared by alkylation of 2-nitroimidazole with the tetrahydropyranyl ether of 2-iodoethanol under basic conditions, followed by deprotection to the alcohol **31** and Jones' oxidation. 4-(2-Nitro-1*H*-imidazolyl)butanoic acid (**36**) and the higher homologues (**38** and **40**) were prepared by alkylation of 2-nitroimidazole with the appropriate ω-haloalkyl esters, followed by mild acid hydrolysis of the resulting esters (**35**, **37**, **39**). Attempts to hydrolyze corresponding nitriles to the acids under more vigorous conditions (AcOH/HCl mixtures) resulted in partial substitution of the imidazole nitro group by chlorine.

3-(2-Nitro-1*H*-imidazolyl)propionic acid (**34**) could not be prepared by alkylation of 2-nitroimidazole with 3-halopropionic acid esters under basic conditions. Coupling of the preformed 2-nitroimidazole salt with either 3-halopropionic acid esters or methyl acrylate, using 18-crown-6 in acetonitrile, was also unsuccessful. Presumably the nitroimidazolyl anion is sufficiently basic to dehydrohalogenate the reagents, giving the acrylic acid ester. Reaction of 2-nitroimidazole with a large excess of methyl acrylate with 18-crown-6 and K_2CO_3[22] gave mostly starting material and only poor yields (5–10%) of the ester **33**, which was hydrolyzed to give the desired propionic acid **34**. The yield of ester could be raised to 35% by using KF/alumina[23] as a catalyst.

Coupling of the various amine and acid fragments was best achieved using diethyl phosphorocyanidate (DEPC)[24] (Scheme 4). Reaction at room temperature for 16 h, followed by chromatographic purification and crystallization, gave moderate to good yields of the amides, free of starting materials. Several other condensation methods, including carbonyldiimidazole, acyl halide, and mixed anhydride, were significantly inferior.

Biological Studies

The cytotoxicities of the compounds against a panel of four cell lines (the Chinese hamster lines AA8 and UV4, the murine mammary carcinoma EMT6, and the human melanoma FME) were determined under aerobic conditions using a growth inhibition microassay which has been described in detail previously.[25] The UV4 cell line, a repair-defective mutant derived from AA8, is hypersensitive to agents whose cytotoxicity is due to bulky DNA adducts or cross-links[26] and was thus used to provide initial information on mechanisms of cytotoxicity.

The cytotoxic potencies of the compounds were compared under aerobic and hypoxic conditions by clonogenic assay of stirred plateau-phase AA8 cultures, continuously gassed with 5% CO_2 in air or N_2, as described previously.[27,28] Detailed studies of selected compounds in this series show a complex relationship between cell killing, drug concentration, and duration of exposure.[29] While the parameter CT_{10} (concentration of drug × time for 10% survival) was essentially constant under aerobic conditions, for some drugs this parameter decreased progressively under hypoxia (see Figure 2 for an example) so that hypoxic selectivity was greater using low drug concentrations and long exposure times. Hypoxic selectivity was assessed as the ratio (aerobic CT_{10}/hypoxic CT_{10}) at the same time, and ranges of this ratio over the drug exposure period of 1–8 h are shown in Table 3.

Radiosensitization was also examined in stirred AA8 cultures, under the same conditions as for the toxicity assays. Briefly, suspensions of hypoxic AA8 cells were exposed to drug for 30 min and then irradiated with a ^{60}Co γ source at 37 °C. Radiation dose–response curves were generated at a range of drug concentrations to define the concentration required to give a sensitizer enhancement ratio (SER; ratio of radiation doses for 10% survival with and without drug) of 1.6 ($C_{1.6}$), and these results are given in Table 3. The *in vitro* therapeutic index (IVTI = aerobic IC_{50}/hypoxic $C_{1.6}$) was calculated as a measure of hypoxic cell radiosensitizing potency relative to aerobic cytotoxicity (Table 3).

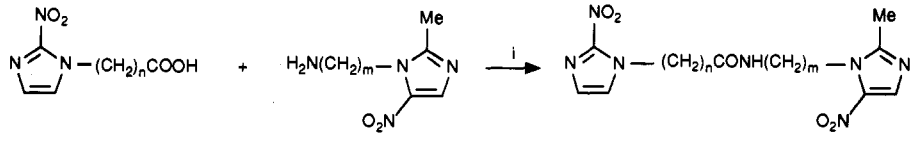
Drug uptake was assessed by HPLC, after exposing the cells to drugs at the $C_{1.6}$ concentration for 30 min at 37 °C. Efficiencies of drug recovery from the cellular and extracellular compartments were determined in separate experiments by spiking cell pellets and medium at concentrations close to those estimated in the uptake experiments, and average intracellular concentrations at the $C_{1.6}$ ($C_{1.6(i)}$) were calculated.

Compound **10** was compared with misonidazole (**1**) in its ability to kill or radiosensitize hypoxic cells in KHT tumors growing subcutaneously in C_3H/HeN mice. The compounds were administered using single or multiple doses of 2.5 mmol/kg, this being the maximum ip dose of **10** which could be administered due to solubility limitations.

Results and Discussion

Physicochemical Properties. The structures and physicochemical properties of the bis(nitroimidazoles)

Scheme 4[a]



compounds of Table 1

[a] (i) DEPC/Et_3N/DMF/20 °C/18 h.

Table 2. Aerobic Toxicity of the Bis(nitroimidazole) Derivatives of Table 1 As Determined by Growth Inhibition in Four Cell Lines

no.	IC_{50}[a] (mM) AA8	hypersensitivity factor[b] UV4	EMT6	FME
1	12.1 ± 1.1[c]	1.4 ± 0.1	3.6 ± 0.9	1.9 ± 0.2
4	0.15 ± 0.02	5.9[d] ± 0.9	1.6 ± 0.1	1.0 ± 0.1
5	23 ± 2	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.2
6	ca. 3[e]			
7	6.1 ± 0.7	1.2 ± 0.1	3.5 ± 0.1	3.9 ± 0.1
8	4.7 ± 0.4	1.0 ± 0.1	2.2 ± 0.5	2.1 ± 0.2
9	5.5 ± 0.1	0.9 ± 0.2	2.5 ± 0.4	2.9
10	7.2 ± 0.8	1.4 ± 0.2	1.3 ± 0.2	4.6 ± 1.2
11	3.8 ± 0.2	1.2 ± 0.1	1.7 ± 0.1	3.5 ± 0.4
12	ca. 1[e]	ca. 1	ca. 1	ca. 1
13	24 ± 5	1.3 ± 0.1	4.2 ± 0.7	3.0 ± 1.3
14	ca. 2[e]			
15	>4.2[f]		>1.7	>2.1
16	7.7 ± 1.5	1.0 ± 0.1	4.4	2.3

[a] IC_{50}: the concentration of drug required to reduce cell numbers to 50% of controls in a growth inhibition assay (see text). [b] Hypersensitivity factor = IC_{50} for AA8/IC_{50} for the indicated cell line. [c] Errors are SEM for two to eight independent determinations. [d] Hypersensitivity factor 30 ± 8 after 18-h exposure under hypoxic conditions. [e] Tested near the solubility limit with variable results. [f] Nontoxic at the solubility limit.

Table 3. Hypoxia-Selective Cytotoxicity, Cellular Uptake, and Radiosensitization by Bis(nitroimidazole) Derivatives in AA8 Cell Cultures

	cytotoxicity			radiosensitization		
no.	hypoxic CT_{10}[a] (mM-h)	air/N_2[b]	uptake C_i/C_e[c]	$C_{1.6}$[d] (mM)	$C_{1.6(i)}$[e] (mM)	IVTI[f]
1	8.3–16[g]	18–25	0.90	0.43	0.40	28
4	0.18–0.38	20–40		0.13		1.1
5	100–135	5–7	1.3	13.3	17.3	1.7
6	3.4	>6[h]	0.50	1.54	0.77	1.7
7	2.5–5.2	6.5–14				
8	11	2.4	0.50	1.08	0.54	4.3
9	12	>7[h]	0.60	0.70	0.42	7.9
10	0.37–12	8–200	0.33	0.63	0.21	11
11	6	>7[h]	0.52	0.93	0.48	4.0
12	1.5	>17[h]	0.55	0.65	0.36	1.2
13	26–39	8–11	0.33	4.00	1.32	6.0
14	>15[i]		0.33	>1.1	>0.36	<1.6
15	0.8–4.2	>30[h]	0.30	0.90	0.27	>4.7
16	50	2.0	1.5	2.7	4.1	2.8

[a] Drug concentration × time to reduce cell survival to 10% of control values under hypoxic conditions. [b] Ratio aerobic CT_{10}/hypoxic CT_{10}. [c] Ratio of mean intracellular to extracellular drug concentration, determined under hypoxic conditions 30 min after exposure to the drug at the $C_{1.6}$ concentration. [d] Drug concentration at which the sensitizer enhancement ratio (SER) = 1.6 under hypoxia. [e] Average intracellular drug concentration when the hypoxic SER = 1.6. [f] IVTI (in vitro therapeutic index) = aerobic IC_{50}/hypoxic $C_{1.6}$. [g] Ranges indicate that values changed with time (see text). Where ranges are not given, toxicity was not assessed over a sufficient range of concentrations to estimate changes in CT_{10} with time. [h] Not cytotoxic under oxic conditions at the solubility limit. [i] Not cytotoxic under hypoxic or oxic conditions at the solubility limit.

6–16 are shown in Table 1. The majority of the compounds possess a misonidazole-like (2-nitroimidazole) unit (R_1), linked through the N-1 position via an alkanecarboxamide chain to a second nitroimidazole unit (R_2) of varying reduction potential. The $E(1)$ values for these various nitroimidazoles are estimated from literature values[30–32] as –390 mV for *N*-alkyl-2-nitro, –490 mV for *N*-alkyl-2-methyl-5-nitro, –560 mV for *N*-methyl-4-nitro, and –470 mV for *N*-methyl-5-nitro isomer and assume complete electronic isolation by the linker chain. The solubilities of the compounds in the buffer used for *in vitro* studies (αMEM with 5% fetal calf serum) were determined by spectrophotometry.

Relative lipophilicities (as log $P_{octanol}$) were computed using the program CLOGP.[33] Previous work[34,35] has shown clear relationships between the therapeutic indices of 2-nitroimidazole radiosensitisers and measured log *P* values, with an optimal value for log *P* of ca. –1.4. While the calculated log *P* for misonidazole (–1.25; Table 1) is seriously at variance with the measured value (–0.37),[34] possibly due to unallowed interactions of the side chain OH group, the correspondence is much better for compounds bearing alkylamide side chains of the type discussed here (e.g., for etanidazole (41); measured[34] –1.34; calculated –1.57).

Over the whole dataset, no statistically-significant correlation was observed between CLOGP and aqueous solubility, suggesting that other factors (possibly crystal packing forces) are more important determinants of solubility than is lipophilicity. While the isomeric bis-nitro compounds **6**, **14**, and **15** all had similar solubilities, addition of a 2-methyl group to **15** to give **10** increased solubility 4-fold, while removal of the (hydrophilic) nitro group altogether (compound **13**) increased solubility enormously. However, within the homologous series **8–12**, aqueous solubilities did trend as expected with chain length and showed a correlation with CLOGP values.

Aerobic Cytotoxicity *in Vitro*. The cytotoxicities of the compounds against AA8 cells, measured as IC_{50} values following an 18-h exposure, are given in Table 2. For the bis(2-nitroimidazoles) (**6** and **7**), IC_{50} values were 3–6 mM, indicating cytotoxic potencies 2–4-fold greater than for misonidazole (**1**), and about 4–8-fold greater than that of the more closely analogous mononitro bisimidazole **13**. The mixed bis(nitroimidazoles) with both 2-nitro and 5-nitroimidazole units had IC_{50} values in the range 1–7.2 mM, similar to those of the bis(2-nitroimidazoles). There was no obvious dependence on carboxamide chain length within the homologous series **8–12**, although the two more lipophilic compounds (**11**, **12**) appeared to be the most cytotoxic. The one example (**14**) of a 2-nitroimidazole linked to a 4-nitroimidazole was not sufficiently soluble to determine an IC_{50} value. The bis(5-nitroimidazole) **16** was about 3-fold more potent than the mono-5-nitroimidazole metronidazole (**5**). Overall, the carboxamide-linked bis(nitroimidazoles) are therefore not greatly more cytotoxic under aerobic conditions than would be expected for the molar equivalent of the mononitro compounds.

All of the bis(nitroimidazoles) were much less cytotoxic than the alkylating 2-nitroimidazole **4**. The latter compound was more active against UV4 than AA8 cells, with

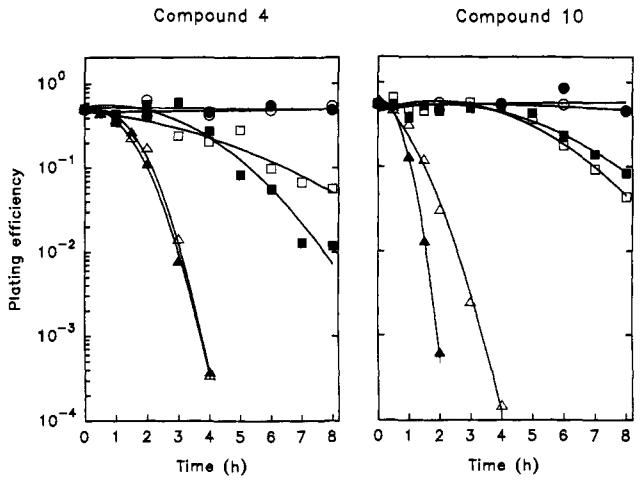


Figure 1. Rate of killing of AA8 cells by compound 4 and 10 under aerobic (open symbols) and hypoxic (filled symbols) conditions. Left panel: drug concentrations 0 (○, ●), 0.03 (■), 0.13 (▲), 0.75 (□), and 3.0 (△) mM. Right panel: drug concentrations 0 (○, ●), 0.05 (■), 1.0 (▲), 10 (□), and 30 (△) mM.

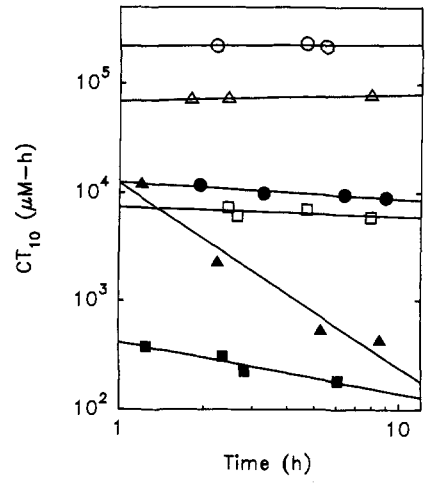


Figure 2. Cytotoxic potency of nitroimidazoles (CT_{10} values) assessed at a range of drug exposure times. Point were derived by interpolating the time for 10% survival from curves of the type shown in Figure 1. Open symbols: aerobic. Filled symbols: hypoxic. Circles: misonidazole (1). Squares: RB 6145 (4). Triangles: compound 10.

a hypersensitivity factor (HF = IC_{50}(AA8)/IC_{50}(UV4)) of 5.9, consistent with a major mechanism of cytotoxicity being DNA interstrand cross-links, as previously suggested for the active aziridinyl form of this agent.[10,14] In contrast, the bis(nitroimidazoles), like the nonalkylating mononitro compounds (1, 5, 13), gave HF values close to unity. This observation suggests that the bis(nitroimidazoles) do not act as DNA cross-linking agents, at least under aerobic conditions. Subsequent comparisons of AA8 and UV4 cells under hypoxic conditions have shown larger HF values for 4 (ca. 20-fold), consistent with increased DNA cross-linking due to nitroreduction. However, 10 was no more toxic to UV4 than AA8 cells, suggesting that it is not a DNA cross-linking agent, even under hypoxic conditions.[29]

All of the compounds tested showed similar aerobic cytotoxic potencies in the AA8, EMT6, and FME cell lines (Table 2). Thus there do not appear to be major species or cell line differences in aerobic cytotoxic potency in this series.

Hypoxia-Selective Cytotoxicity *in Vitro*. The hypoxia-selective cytotoxicities of the compounds were determined by clonogenic assay, using stirred suspensions of AA8 cells (Table 3). In each case, the kinetics of killing were assessed at several different drug concentrations which gave widely different rates of killing. Representative data (for compounds 4 and 10) are illustrated in Figure 1. CT_{10} values were calculated for each survival curve and were in all cases essentially independent of time under aerobic conditions (Figure 2). However, under hypoxia CT_{10} values often decreased with time. This trend was very marked for compound 10 (Figure 2), and apparent hypoxic selectivities were thus time-dependent. Of the mononitro compounds, 4 was the most selective for hypoxic conditions, with a CT_{10}air/$CT_{10}N_2$ ratio which increased from 20 to 40 between 1 and 8 h. Hypoxic selectivity was less for misonidazole (1) (18–25-fold), metronidazole (5) (5–7-fold) and the mononitro bis(imidazolyl) carboxamide bearing only one nitro group (13) (8–11-fold).

Evaluation of the hypoxic selectivity of the bis(nitroimidazoles) was limited by the low aqueous solubility of many of these compounds, which often precluded measurement of aerobic cell killing under the conditions of the clonogenic assay. However, hypoxic cell killing could be quantitated for all compounds except the bis(nitroimidazole) with a 4-nitro unit 14, which had both a low reduction potential and low solubility. The bis(2-nitroimidazole) 6 and its reversed amide isomer 7 had potencies under hypoxic conditions which were only about 3-fold greater than that of misonidazole (1). The solubility of 7 allowed determination of its hypoxic selectivity, which was less than that of misonidazole (1). Similar results were obtained for the bis(5-nitroimidazole) 16 which was 2–3-fold more potent than metronidazole (5) under hypoxia, but was less hypoxia-selective.

All of the members of the homologous series of mixed 2-nitroimidazole/5-nitroimidazole compounds 8–11 were also selectively toxic under hypoxic conditions, but only two were sufficiently soluble for their aerobic toxicities to be determined in the clonogenic assay. The compound of shortest chain length (8) showed only weak selectivity (2.4-fold). Its higher homologue 9 was more selective (>7-fold), but the C-3 homologue 10 was sufficiently potent and soluble to allow measurement of both aerobic and hypoxic cytotoxicity over a wide time range. Its hypoxic selectivity increased markedly from 8-fold at 1 h to 200-fold by 8 h (Figure 2 and Table 3). At the early times its hypoxic potency was similar to that of misonidazole (1), while at late times its potency approached that of the alkylating analogue 4 (Figure 2). This is consistent with its slow metabolic conversion to a bis-functional species. The corresponding compound without the 2-methyl substituent on the 5-nitroimidazole ring (15) showed biological properties similar to 10, with a potency under hypoxia which also increased markedly with time (Table 3). While this compound was 3.6-fold less soluble than compound 10, and cell killing could not be detected under aerobic conditions, its hypoxic selectivity was >30-fold at late drug exposure times.

Radiosensitization *in Vitro*. All of the nitroimidazoles, including the bis compounds, radiosensitized hypoxic AA8 cells in culture as expected. Representative data showing the relationship between SER and concentration are illustrated for misonidazole (1), the aziridine (4), and the bis(nitroimidazole) (10) in Figure 3. The drug concentrations required to provide SER values of 1.6 ($C_{1.6}$ values) are given in Table 3. The values for the bis(nitroimidazoles) 6–15, containing one or more 2-nitroim-

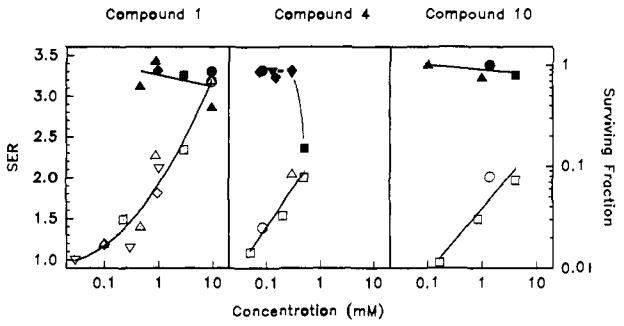


Figure 3. Sensitizer enhancement ratios (SER) for radiosensitization of hypoxic AA8 cells by compounds 1, 4, and 10 following incubation at 37 °C for 30 min (open symbols). Surviving fractions for unirradiated cultures exposed to drugs under identical conditions are shown as filled symbols.

idazole units, were at least 2-fold lower than the value of 430 μM for misonidazole, with no obvious structure–activity relationships. Compound **16**, containing two 5-nitroimidzole units, was also more potent (by a factor of 5) than the mono-5-nitroimidazole **5** (metronidazole). Uptake factors (the ratio of average intracellular to extracellular drug concentrations, C_i/C_e) were measured at the $C_{1.6}$ after incubation with the drug for 30 min under hypoxic conditions. The value of 0.9 for misonidazole agrees well with that reported previously.[36] Uptake factors for the bis(nitroimidazoles) **6–15** were significantly lower than this, ranging from 0.33 to 0.6. Again, no relationships could be discerned between uptake and either drug structure or overall lipophilicity, although the compounds span a range of log *P* values from −0.22 to −1.55. The bis(5-nitroimidazole) **16** had a somewhat higher uptake factor of 1.5.

When these uptake factors are taken into account, some of the bis(nitroimidazoles) appear to be marginally more efficient radiosensitizers than their monomeric counterparts. Comparing $C_{1.6(i)}$ values (Table 3), most members of the homologous series **8–11** were more efficient than misonidazole, with the best compound being **10** (shown above to also have the greatest hypoxia-selectivity). The higher potency of the bis(5-nitroimidazole) **16** than metronidazole (**5**) was also still observed when cellular uptake was taken into account ($C_{1.6(i)}$ values of 4.1 and 17.3 mM respectively; Table 3). All of the bis(nitroimidazoles) had in vitro therapeutic indices (IVTI = aerobic IC_{50}/hypoxic $C_{1.6}$) greater than that of the very toxic alkylating analogue **4**, with the highest index (11) being obtained with compound **10**. However, even this is only about half of the IVTI of misonidazole (**1**) (although interpretation of these data is complicated by the observation that the IVTI for the other mononitro imidazolylcarboxamide **13** is less than that of misonidazole). Overall, the bis compounds appear to be broadly similar to monomeric nitroimidazoles as hypoxic cell radiosensitizers with respect to potency and radiosensitizing selectivity relative to aerobic toxicity.

***In Vivo* Studies.** Because of its high hypoxia-selective cytotoxicity *in vitro*, and its efficient radiosensitising properties, **10** was selected for *in vivo* evaluation against KHT tumors growing subcutaneously in C_3H/HeN mice. The maximum dose which could be administered (because of solubility limitations) was 2.5 mmol/kg, using solution in either phosphate-buffered saline (PBS) (50 mM, 0.05 mL/g body weight) or in dimethylacetamide/polyethylene glycol 400/water, 1:3:6 v/v/v (250 mM, 0.01 mL/g body weight). Initial studies with the latter formulation indicated no additional cell killing in irradiated KHT tumors when the drug was administered 90, 60, or 30 min before radiation or 5 min after irradiation (data not shown). Subsequent studies confirmed that **10** is inactive and **1** is active at this dose when given in a large volume of PBS 45 min before irradiation. However, four such doses of **10** at 3-h intervals (with irradiation 45 min after the last dose) gave greater tumor cell killing than radiation alone (Figure 4). No killing was observed with either drug or schedule in the absence of radiation (data not shown). In contrast to the bis(nitroimidazole), misonidazole (**1**) was no more active in the multidose schedule than in a single dose (Figure 4). This is as expected for "electron-affinic" radiosensitization dependent only on the concentration in the tumor at the time of irradiation, since **1** is cleared with a half-life of 1.5 h at a dose of 2.5 mmol/kg in the mouse,[37] indicating that little accumulation would be expected in tumors with this dosing schedule. The superiority of **10** when administered as multiple doses suggests that its activity in combination with radiation is primarily due to hypoxic cell toxicity rather than direct radiosensitization, although pharmacokinetic studies are required to exclude the possibility that parent drug might accumulate in tumors with the q3h × 4 schedule. No toxicity was observed in non-tumor-bearing C_3H/HeN mice treated with **10** using the 4 × 2.5 mmol/kg dose schedule (observation time 30 days). Recent studies have also shown **10** to be active in combination with radiation against the MDAH-MCa-4 mouse mammary tumor in growth delay assays when administered using the above q3h × 4 schedule (but not as a single dose), while misonidazole (**1**) was no more active in the multidose schedule.[29]

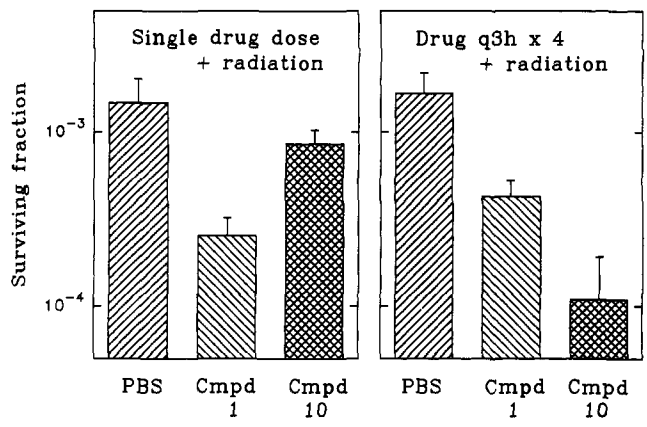


Figure 4. Activity of compounds **1** and **10** against the KHT tumor in combination with radiation (15 Gy). Mice were treated with either a single dose of 2.5 mmol/kg (left panel) or four doses of 2.5 mmol/kg at 3 hourly intervals (right panel) and were irradiated whole-body 45 min after the last drug dose. The injection volume was 0.05 mL/g per dose for both compounds and for the controls, which were irradiated 45 min after the last injection of PBS. Each value is the geometric mean ± range for two experiments with two to four pooled tumors in each experimental group.

Conclusions

The bis(nitroimidazoles) investigated here showed aerobic toxicities quite similar to those of the related mononitro compounds. The potencies of these compounds as hypoxic cell radiosensitizers was also only a little greater than those of analogous mononitroimidazoles. However, compounds **10** and **15**, both with a 2-nitro- and a 5-nitroimidazole linked by a seven-atom carboxamide

linker chain, were highly potent cytotoxins under hypoxic conditions using drug exposure times of several hours. The increase in cytotoxic potency with time under hypoxia suggests these compounds may be "bis-bioreductive" agents, with slow reduction at the redox center of lower potential (5-nitroimidazole) generating a bis-reactive molecule under hypoxia. The more soluble of these two compounds, **10**, was active as a radiosensitizer of KHT tumors when administered is multiple doses, presumably because of its selective toxicity to hypoxic radioresistant cells. However, the relative contributions of bioreductive toxicity and direct ("electron affinic") radiosensitization to the observed activity have yet to be determined. Evaluation of **10** in combination with fractionated radiation will be of interest; if it is acting primarily as a bioreductive drug there are theoretical reasons to expect it to be more active in a protracted schedule.[4]

One of the possible mechanisms of cytotoxicity considered in the design of bis(nitroimidazoles) was that they might generate DNA cross-linking agents when both nitro groups are reduced to reactive species. The lack of hypersensitivity of the UV4 cell line, which is defective in DNA cross-link repair, suggests that this is not the mechanism of toxicity of these agents under aerobic conditions. Subsequent studies have shown that UV4 is also not hypersensitive to **10** under hypoxic conditions.[29] In addition, alkaline elution studies provide no evidence of interstrand cross-links by **10** under hypoxia using concentrations and exposure times well above those required for cytotoxicity.[29] The lack of cross-linking by **10** is consistent with recent studies showing that metabolic reduction of metronidazole in *E. coli* does not result in significant DNA alkylation, with an upper limit for covalent binding under anoxic conditions of <1 molecule/10^6 base pairs.[38] However, the generation of a highly toxic lesion under hypoxia need not be limited to crosslinking events since other duplex lesions (e.g. a single strand break combined with a monoalkylation site) might also be highly cytotoxic. We have suggested elsewhere that bis(nitroimidazoles) such as **10** might act as bis-bioreductive drugs by generating such locally doubly damaged sites when both nitro groups are reduced.[29]

Despite the use of water-soluble carboxamide linkers, aqueous solubility has limited the evaluation of the present series and structure-activity relationships for hypoxia-selective toxicity are not well defined. The low hypoxic selectivity of **8** suggests that a linker chain length of more than five atoms may be required between the nitroimidazole moieties, but development of more soluble analogues will be required to explore structural requirements further.

Experimental Section

Analyses indicated by symbols of the elements were within ±0.4% of theoretical. Analyses were carried out in the Microchemical Laboratory, University of Otago, Dunedin, New Zealand. Melting points were determined on an Electrothermal 2300 melting point apparatus. NMR spectra were obtained on a Bruker AC-200 or AM-400 spectrometer, and are referenced to Me_4Si for organic solvents, or DSS (2,2-dimethyl-2-silapentane-5-sulfonate) for aqueous solvents. Mass spectra were determined on a Varian VG-70SE mass spectrometer using an ionizing potential of 70 eV at a nominal resolution of 1000. High-resolution spectra were obtained at nominal resolutions of 3000, 5000, or 10000 as appropriate. Spectra were obtained using the ionization mode specified, with PFK as the reference unless otherwise stated. Thin-layer chromatography was carried out on aluminum-backed silica gel plates (Merck 60 F_{254}). Column chromatography was carried out on silica gel (Merck 230–400 mesh). All compounds designated for biological testing were analyzed at >99% purity by reverse-phase HPLC using a Philips PU4100 liquid chromatograph, a Phenomenex BondClone 10-C18 stainless steel column (300 mm × 3.9-mm i.d.), and a Philips PU4120 diode array detector. Chromatograms were run using various gradients of aqueous (1 M NaH_2PO_4, 0.75 M heptanesulfonic acid, 0.5 M dibutylammonium phosphate, and MilliQ water in a 1:1:1:97 ratio) and organic (80% MeOH/MilliQ water) phases.

2-(2-Methyl-5-nitro-1*H*-imidazolyl)ethylamine (19). A solution of methanesulfonyl chloride (5.4 mL, 70 mmol) in CH_2Cl_2 (10 mL) was added dropwise to a stirred suspension of 2-(2-methyl-5-nitro-1*H*-imidazolyl)ethanol (**5**; metronidazole) (10.0 g, 58 mmol) and Et_3N (12.2 mL, 88 mmol) in CH_2Cl_2 (200 mL) at 20 °C under N_2. After being stirred for 4 h, the mixture was filtered, and the filtrate was washed with water (2 × 60 mL), evaporated, and crystallized from $CHCl_3$ to give 2-(2-nitro-5-nitro-1*H*-imidazolyl)ethyl methanesulfonate (**17**) (13.5 g, 92%): mp 153–154 °C; ^{1}H NMR ($CDCl_3$) δ 8.06 (s, 1H, H-4′), 4.67 (dd, *J* = 5.1, 4.7 Hz, 2 H, H-2), 4.56 (dd, *J* = 5.1, 4.7 Hz, 2 H, H-1), 3.15 (s, 3H, SO_2CH_3), 2.46 (s, 3 H, CH_3); ^{13}C NMR ($CDCl_3$) δ 151.7 (C-2′), 138.5 (C-5′), 133.0 (C-4′), 45.2 (C-1), 45.0 (C-2), 36.7 (SO_2CH_3), 8.3 (CH_3). Anal. ($C_7H_{11}N_3O_5S$) C, H, N, S. A solution of the mesylate (13.5 g, 54 mmol) and NaN_3 (3.8 g, 59 mmol) in DMF (150 mL) was stirred at 100 °C for 1 h, cooled, poured into brine, and extracted with EtOAc (3 × 200 mL). The combined organic extracts were washed with brine and evaporated to give crude 2-(2-nitro-5-nitro-1*H*-imidazolyl)ethyl azide (**18**) (8.26 g, 78%): mp (EtOAc/petroleum ether) 56–58 °C; ^{1}H NMR ($CDCl_3$) δ 7.98 (s, 1H, H-4′), 4.45 (dd, *J* = 5.7, 5.3 Hz, 2 H, H-2), 3.79 (dd, *J* = 5.6, 5.3 Hz, 2 H, H-1), 2.44 (s, 3 H, CH_3); ^{13}C NMR δ 151.3 (C-2′), 136.7 (C-5′), 133.4 (C-4′), 50.8 (C-1), 45.5 (C-2), 14.5 (CH_3); HRMS calcd for $C_6H_8N_6O_2$ 196.0709 (M^+), found 196.0707. Anal. ($C_6H_6N_6O_2$) C, H.

A stirred solution of **18** in dry THF (100 mL) was treated dropwise with a solution of Ph_3P (14.2 g, 54 mmol) in dry THF (20 mL) at 20 °C and stirred for a further 3 h. Concentrated HCl (150 mL) was then added, and the mixture was heated under reflux for 5 h before being evaporated to dryness. The residue was partitioned between EtOAc and water, and the aqueous layer was evaporated to give 2-(2-methyl-5-nitro-1*H*-imidazolyl)ethylamine (**19**) as the dihydrochloride salt (11.0 g, 84%): mp (MeOH) 194–194.5 °C dec; ^{1}H NMR (D_2O) δ 8.50 (s, 1 H, H-4′), 4.86 (t, *J* = 6.9 Hz, 2 H, H-2), 3.58 (t, *J* = 6.9 Hz, 2 H, H-1), 2.78 (s, 3 H, CH_3); ^{13}C NMR (D_2O) δ 157.2 (C-2′), 140.9 (C-5′), 127.1 (C-4′), 46.5 (C-1), 40.3 (C-2), 14.3 (CH_3). Anal. ($C_6H_{10}N_4O_2$·2HCl) C, H, N.

2-(1*H*-Imidazolyl)ethylamine Dihydrochloride (21·2HCl). A solution of imidazole (3.0 g, 44.1 mmol) in DMF (30 mL) was added dropwise to a stirred suspension of NaH (1.94 g, 48.5 mmol) in DMF (30 mL) at 0 °C, and the mixture was stirred until homogeneous. A solution of *N*-(2-bromoethyl)phthalimide (11.8 g, 46.3 mmol) in THF/DMF (1:1, 25 mL) was then added, and the mixture was stirred at 100 °C for 2 h, cooled, and poured into brine (200 mL). Extraction with EtOAc, workup of the organic layer, and crystallization from EtOAc/petroleum ether gave *N*-[2-(1*H*-imidazolyl)ethyl]phthalimide (**20**) (4.88 g, 46%): mp 162–164 °C; ^{1}H NMR ($CDCl_3$) δ 7.81–7.84 (m, 2 H, ArH), 7.72–7.75 (m, 2 H, ArH), 7.42 (s, 1H, H-2′), 7.03 (t, *J* = 1.2 Hz, 1 H, H-5′), 6.96 (t, *J* = 1.2 Hz, 1 H, H-4′), 4.29 (t, *J* = 6.5 Hz, 2 H, H-2), 4.05 (t, *J* = 6.5 Hz, 2 H, H-1); ^{13}C NMR ($CDCl_3$) δ 167.8 (2CO), 137.3 (C-2′), 134.4 ($2C_{arom}$), 131.6 ($2C_{arom}$), 130.0 (C-5′), 123.6 ($2C_{arom}$), 118.9 (C-4′), 44.4 (C-2), 38.3 (C-1). Anal. ($C_{13}H_{11}N_3O_2$) C, H, N.

A stirred solution of **20** (2.8 g, 11.6 mmol) and hydrazine monohydrate (1.13 mL, 23.2 mmol) in EtOH (100 mL) was heated under reflux for 4 h. The resulting suspension was cooled to 0 °C and filtered, and the filtrate was evaporated to dryness under reduced pressure. The residue was dissolved in 1 N HCl (50 mL) and filtered, and the solvent was removed under reduced pressure to give 2-(1*H*-imidazolyl)ethylamine dihydrochloride (**21**·2HCl) (1.49 g, 70%): mp (MeOH/EtOAc) 217–220 °C; ^{1}H NMR (D_2O) δ 8.92 (s, 1 H, H-2′), 7.65 (s, 1 H, H-5′), 7.57 (s, 1 H, H-4′), 4.66 (t, *J* = 6.3 Hz, 2 H, H-2), 3.61 (t, *J* = 6.3 Hz, 2 H, H-1); ^{13}C NMR (D_2O) δ 138.1 (C-2′), 124.5 (C-5′), 123.3 (C-4′), 48.8 (C-1), 4 1.5 (C-1). Anal. ($C_5H_8N_3$·2HCl) C, H, N, Cl.

2-(2-Nitro-1*H*-imidazolyl)ethylamine Hydrochloride (23·HCl) and 3-(2-Nitro-1*H*-imidazolyl)propylamine Hydrochloride (25·HCl). A stirred solution of 2-nitroimidazole (3.0 g, 26.5 mmol), 2-bromoethylphthalimide (7.08 g, 27.9 mmol), and K_2CO_3 (3.85 g, 27.9 mmol) in DMF (75 mL) was heated at 110 °C for 2 h. The solvent was removed under reduced pressure, and the residue was poured into water (200 mL). The precipitate was collected, washed with water (100 mL), and dried to give 2-(2-nitro-1*H*-imidazolyl)ethylphthalimide (**22**) (5.28 g, 78%): mp 208.5–209 °C (lit.[39] mp 208–210 °C); ^{1}H NMR ($(CD_3)_2SO$) δ 7.82–7.84 (m, 4 H, ArH), 7.60 (s, 1 H, H-5′), 7.07 (s, 1 H, H-4′), 4.64 (br t, 2 H, H-2), 4.06 (br t, 2 H, H-1); ^{13}C NMR δ 167.3 (2CO), 144.7 (C-2′), 134.5 ($2C_{arom}$), 131.2 (C-5′), 128.4 ($2C_{arom}$), 127.9 (C-4′), 123.1 ($2C_{arom}$), 48.3 (C-1), 37.1 (C-2). A stirred solution of this phthalimide (3.65 g, 12.8 mmol) and hydrazine monohydrate (1.24 mL, 25.5 mmol) in EtOH (70 mL) was heated under reflux for 2 h. The resulting suspension was cooled to 0 °C and filtered, and the filtrate was evaporated to dryness under reduced pressure. The residue was dissolved in 1 N HCl (50 mL) and filtered, the solvent was removed under reduced pressure, and the residue was crystallized from MeOH/EtOAc to give 2-(2-nitro-1*H*-imidazolyl)ethylamine hydrochloride (**23**·HCl) (1.49 g, 70%): mp 188.5–189 °C (lit.[40] mp 188–190 °C); ^{1}H NMR (D_2O) δ 7.54 (d, J = 1.0 Hz, 1 H, H-5′), 7.25 (d, J = 1.1 Hz, 1 H, H-4′), 4.82 (t, J = 6.1 Hz, 2 H, H-2), 3.58 (t, J = 6.1 Hz, 2 H, H-1); ^{13}C NMR (D_2O) δ 147.4 (C-2′), 131.1 (C-5′), 130.6 (C-4′), 49.4 (C-2), 41.2 (C-1).

Similar reaction of 2-nitroimidazole with (3-bromopropyl)-phthalimide gave 3-(2-nitro-1*H*-imidazolyl)propylphthalimide (**24**) (98% yield): mp 203–204 °C; ^{1}H NMR (CD_3Cl) δ 7.85–7.89 (M, 2 H, ArH), 7.74–7.78 (m, 2 H, ArH), 7.37 (s, 1 H, H-4′), 7.16 (s, 1 H, H-5′), 4.48 (t, J = 7.0 Hz, 2 H, H-3), 3.78 (t, J = 6.3 Hz, 2 H, H-1), 2.25–2.32 (m, 2 H, H-2); ^{13}C NMR δ 168.4 (2CO), 144.0 (C-2′), 134.3 (2C-3″), 131.8 (2C-2″), 128.4 (C-4′), 126.3 (C-5′), 123.5 (2C-4″), 47.7 (C-3), 34.7 (C-1), 29.8 (C-2). Anal. ($C_{14}H_{12}N_4O_4$) C, H, N. Reaction of this with hydrazine monohydrate as above gave 3-(2-nitro-1*H*-imidazolyl)propylamine hydrochloride (**25**·HCl) (23.8 g, 66%): mp (MeOH) 214–214.5 °C; ^{1}H NMR ($(CD_3)_2SO$) δ 8.26 (br s, 3 H, NH_2·HCl), 7.87 (s, 1 H, H-5′), 7.22 (s, 1 H, H-4′), 4.53 (t, J = 6.8 Hz, 2 H, H-3), 2.78 (br s, 2 H, H-1), 2.10–2.17 (m, 2 H, H-2); ^{13}C NMR δ 144.6 (C-2′), 127.9 (C-4′), 127.8 (C-5′), 46.6 (C-3), 35.7 (C-1), 27.5 (C-2). Anal. ($C_5H_{10}N_4O_2$·HCl) C, H, N, Cl.

2-(4-Nitro-1*H*-imidazolyl)ethylamine Hydrochloride (27·HCl) and 2-(5-Nitro-1*H*-imidazolyl)ethylamine Hydrochloride (29·HCl). A stirred solution of 4-nitroimidazole (5.0 g, 44.2 mmol), *N*-(*tert*-butyloxycarbonyl)-2-(methylsulfonyl)ethylamine (10.76 g, 48.6 mmol) and K_2CO_3 (6.72 g, 48.6 mmol) in DMF (250 mL) was heated at 110 °C for 4 h. The solvent was removed under reduced pressure, and the residue was partitioned between EtOAc and water (500 mL). The aqueous fraction was washed with EtOAc (2 × 50 mL), and the combined organic fractions were washed with water (2 × 100 mL), brine (150 mL), dried, and evaporated. Chromatography of the residue on silica gel with a gradient (60–100%) of EtOAc/petroleum ether first gave *N*-(*tert*-butyloxycarbonyl)-2-(5-nitro-1*H*-imidazolyl)ethylamine (**28**) (0.78 g, 7%): mp (EtOAc/petroleum ether) 1.48–149.5 °C; ^{1}H NMR ($CDCl_3$) δ 8.00 (d, J = 1.0 Hz, 1 H, H-4′), 7.53 (s, 1 H, H-2′), 4.80 (br t, J = 5.6 Hz, 1 H, NH), 4.52 (t, J = 5.6 Hz, 2 H, H-2), 3.51 (dd, J = 6.0, 5.6 Hz, 2 H, H-1), 1.41 (s, 9 H, t-Bu); ^{13}C NMR ($CDCl_3$) δ 155.9 ($NHCO_2$), 142.1 (C-5′), 142.1 (C-2′), 133.8 (C-4′), 80.3 (C-(CH_3)$_3$), 47.4 (C-2), 40.6 (C-1), 28.3 (C(CH_3)$_3$). Anal. ($C_{10}H_{16}N_4O_4$) C, H, N. Further elution gave *N*-(*tert*-butyloxycarbonyl)-2-(4-nitro-1*H*-imidazolyl)ethylamine (**26**) (4.65 g, 41%): mp 106–107 °C; ^{1}H NMR ($CDCl_3$) δ 7.80 (d, J = 1.4 Hz, 1 H, H-5′), 7.41 (d, J = 1.4 Hz, 1 H, H-2′), 5.33 (br s, 1 H, NH), 4.19 (dd, J = 5.9, 5.5 Hz, 2 H, H-2), 3.52 (dd, J = 5.9, 5.6 Hz, 2 H, H-1), 1.43 (s, 9 H, t-Bu); ^{13}C NMR ($CDCl_3$) δ 155.9 ($NHCO_2$), 148.0 (C-4′), 136.4 (C-2′), 119.6 (C-5′), 80.4 (C(CH_3)$_3$), 47.9 (C- 2), 41.2 (C-1), 28.3 (C(CH_3)$_3$). Anal. ($C_{10}H_{16}N_4O_4$) C, H, N.

A solution of **28** (0.76 g, 3.0 mmol) in MeOH (20 mL) and 5 N HCl (20 mL) was stirred at 20 °C for 16 h and then evaporated under reduced pressure to give 2-(5-nitro-1*H*-imidazolyl)ethylamine hydrochloride (**29**·HCl) (0.62 g, 91%): mp (MeOH) 199.5–200 °C dec; ^{1}H NMR ($(CD_3)_2SO$) δ 8.31 (s, 1 H, H-4′), 8.12 (s, 1 H, H-2′), 7.04 (br s, 1 H, NH), 4.57 (t, J = 5.8 Hz, 2 H, H-2), 3.15 (q, J = 5.7 Hz, 2 H, H-1); ^{13}C NMR ($(CD_3)_2SO$) δ 142.8 (C-2′), 138.6 (C-5′), 132.0 (C-4′), 44.8 (C-2), 38.1 (C-1). Anal. ($C_5H_{10}Cl_2N_4O_2$) C, H, N. Similar treatment of **26** gave 2-(4-nitro-1*H*-imidazolyl)ethylamine hydrochloride (**27**·HCl) (58%): mp (MeOH) 240–241 °C; ^{1}H NMR ($(CD_3)_2SO$) δ 8.53 (d, J = 1.4 Hz, 1 H, H-5′), 8.03 (br s, 2 H, NH), 7.95 (d, J = 1.4 Hz, 1 H, H-2′), 4.44 (d, J = 6.0, 5.8 Hz, 2 H, H-2), 3.31 (dd, J = 6.0, 5.8 Hz, 2 H, H-1); ^{13}C NMR ($(CD_3)_2SO$) δ 147.0 (C-4′), 137.7 (C-2′), 121.9 (C-5′), 44.8 (C-2), 38.6 (C-1). Anal. ($C_5H_{10}Cl_2N_4O_2$) C, H, N.

2-(2-Methyl-5-nitro-1*H*-imidazolyl)acetic Acid (30). A stirred solution of metronidazole (**5**) (2.0 g, 11.7 mmol) in Me_2CO (30 mL) at 0 °C was treated dropwise with CrO_3/Me_2CO until an orange color persisted. The solvent was removed under reduced pressure and the residue chromatographed on silica. Elution with MeOH/EtOAc (0–10% MeOH) gave 2-(2-methyl-5-nitro-1*H*-imidazolyl)acetic acid (**30**) (1.05 g, 49%): mp (MeOH/EtOAc) 175–177 °C (lit.[41] mp 179–180 °C); ^{1}H NMR (D_2O) δ 7.96 (s, 1 H, H-4′), 5.05 (s, 2 H, H-2), 2.45 (s, 3 H, CH_3).

2-(2-Nitro-1*H*-imidazolyl)acetic Acid (32). A stirred suspension of 2-nitroimidazole (2.0 g, 17.1 mmol), 2-iodoethyl tetrahydropyranyl ether (5.0 g, 19.5 mmol), and K_2CO_3 (2.7 g, 19.5 mmol) in DMF (50 mL) was heated at 110 °C for 2 h. The solvent was removed under reduced pressure, and the residue was partitioned between EtOAc and water. The residue from the organic phase was dissolved in MeOH (30 mL) and 6 M HCl (5 mL) and stirred at 20 °C for 18 h and then evaporated under reduced pressure to give 2-(2-nitro-1*H*-imidazolyl)ethanol (**31**) (1.55 g, 57%): mp (MeOH/EtOAc) 148–150 °C (lit.[42] mp 157 °C); ^{1}H NMR (D_2O) δ 7.47 (s, 1 H, H-5′), 7.19 (s, 1 H, H-4′), 4.62 (t, J = 5.2 Hz, 2 H, H-2), 3.96 (t, J = 5.2 Hz, 2 H, H-1); ^{13}C NMR (D_2O) δ 147.2 (C-2′), 131.1 (C-5 ′), 130.4 (C-4′), 62.8 (C-1), 54.6 (C-2).

Oxidation of **31** with Jones' reagent as above, followed by chromatography on silica and elution with a gradient (0–10%) of MeOH/EtOAc gave 2-(2-nitro-1*H*-imidazolyl)acetic acid (**32**) (0.75 g, 37%): mp (MeOH) 158–159 °C (lit.[43] mp 159–160 °C); ^{1}H NMR ($(CD_3)_2SO$) δ 7.63 (s, 1 H, H-5′), 7.20 (s, 1 H, H-4′), 5.16 (s, 2 H, H-2); ^{13}C NMR ($(CD_3)_2SO$) δ 168.5 (CO_2H), 144.7 (C-2′), 128.3 (C-5′), 127.4 (C-4′), 50.9 (C-2).

3-(2-Nitro-1*H*-imidazolyl)propionic Acid (34). A stirred suspension of 2-nitroimidazole (5.0 g, 44.2 mmol), methyl acrylate (8 mL, 88.4 mmol), and KF/alumina (0.25 g) was heated at 100 °C for 18 h. The suspension was cooled to 20 °C, the solvent was removed under reduced pressure, and the residue was chromatographed on silica gel. Elution with EtOAc/petroleum ether (1:1) gave methyl 3-(2-nitro-1*H*-imidazolyl)propionate (**33**) as an oil (3.08 g, 35%) (lit.[22] mp 68–69 °C): ^{1}H NMR ($CDCl_3$) δ 7.24 (s, 1 H, H-5′), 7.13 (s, 1 H, H-4′), 4.71 (t, J = 6.2 Hz, 2 H, H-3), 3.69 (s, 3 H, OCH_3), 2.93 (t, J = 6.2 Hz, 2 H, H-2); ^{13}C NMR ($CDCl_3$) δ 170.8 (CO_2H), 144.7 (C-2′), 128.4 (C-5′), 127.1 (C-4′), 52.2 (OCH_3), 45.6 (C-3), 34.6 (C-2). Hydrolysis of **33** (concentrated HCl/20 °C/4 h) gave 3-(2-nitro-1*H*-imidazolyl)propionic acid (**34**) (170 mg, 96%): mp (H_2O) 162–164 °C; ^{1}H NMR ($(CD_3)_2SO$) δ 7.66 (s, 1 H, H-5′), 7.16 (s, 1 H, H-4′), 6.33 (br s, 1 H, CO_2H), 4.59 (t, J = 6.9 Hz, 2 H, H-3), 2.85 (t, J = 6.9 Hz, 2 H, H-2); ^{13}C NMR ($(CD_3)_2SO$) δ 171.1 (CO_2H), 144.7 (C-2′), 127.7 (C-5′), 127.6 (C-4′), 44.9 (C-3), 34.2 (C-2). Anal. ($C_6H_7N_3O_4$) C, H, N.

4-(2-Nitro-1*H*-imidazolyl)butanoic Acid (36): Example of General Method. A stirred suspension of 2-nitroimidazole (1 equiv), methyl 4-bromobutanoate (1.2 equiv), and K_2CO_3 (1.1 equiv) in DMF (1 mL/3 mmol) was heated at 110 °C for 2 h. Solvent was removed under reduced pressure, and the residue was partitioned between EtOAc and water. The organic layer was worked up, and the residue was chromatographed on silica gel to yield methyl 4-(2-nitro-1*H*-imidazolyl)butanoate (**35**) as an oil: ^{1}H NMR ($CDCl_3$) δ 7.16 (s, 1 H, H-5′), 7.15 (s, 1 H, H-4′), 4.53 (t, J = 7.1 Hz, 2 H, H-4), 3.69 (s, 3 H, OCH_3), 2.41 (t, J = 7.0 Hz, 2 H, H-2), 2.17–2.24 (m, 2 H, H-3); ^{13}C NMR ($CDCl_3$) δ 172.4 (CO_2-), 144.6 (C-2′), 128.2 (C-5′)*, 126.1 (C-4′)*, 51.7 (OCH_3), 49.0 (C-4), 30.1 (C-2), 25.3 (C-3). Hydrolysis of **35** (conc. HCl/20 °C/16 h) gave a quantitative yield of 4-(2-nitroimidazol-1*H*-yl)-butanoic acid (**36**): mp (water) 114–115 °C; ^{1}H NMR (D_2O) δ 7.50 (s, 1 H, H-5′), 7.22 (s, 1 H, H-4′), 4.54 (t, J = 7.0 Hz, 2 H, H-4), 2.48 (t, J = 7.1 Hz, 2 H, H-2), 2.15–2.22 (m, 2 H, H-3); ^{13}C NMR (D_2O) δ 179.8 (CO_2H), 147.5 (C-2′), 130.7 (C-5′)*, 130.2

(C-4')*, 52.0 (C-4), 33.1 (C-2), 27.2 (C-3). (* Assignments interchangeable.) Anal. ($C_7H_9N_3O_4$) C, H, N.

A similar reaction using methyl 5-bromopentanoate gave methyl 5-(2-nitro-1*H*-imidazolyl)pentanoate (**37**) (87%) as an oil: ^{1}H NMR ($CDCl_3$) δ 7.16 (d, J = 1.0 Hz, 1 H, H-5'), 7.15 (d, J = 1.0 Hz, 1 H, H-4'), 4.45 (t, J = 7.3 Hz, 2 H, H-5), 3.68 (s, 3 H, OCH_3), 2.39 (t, J = 7.2 Hz, 2 H, H-2), 1.88–1.96 (m, 2 H, H-4), 1.66–1.73 (m, 2 H, H-3); ^{13}C NMR ($CDCl_3$) δ 173.2 (CO_2), 140.5 (C-2'), 128.3 (C-5')*, 125.9 (C-4')*, 51.6 (OCH_3), 49.8 (C-5), 33.0 (C-4), 29.7 (C-3), 21.5 (C-2). Hydrolysis as above gave 5-(2-nitroimidazol-1*H*-yl)pentanoic acid (**38**), mp (water) 125–126.5 °C. ^{1}H NMR ($(CD_3)_2SO$) δ 7.71 (s, 1 H, H-5'), 7.19 (s, 1 H, H-4'), 6.25 (br s, 1 H, CO_2H), 4.40 (t, J = 7.0 Hz, 2 H, H-5), 2.26 (t, J = 7.3 Hz, 2 H, H-2), 1.75–1.83 (m, 2 H, H-4), 1.54–1.53 (m, 2 H, H-3); ^{13}C NMR ($(CD_3)_2SO$) δ 174.1 (CO_2H), 144.6 (C-2'), 127.9 (C-5')*, 127.8 (C-4')*, 49.2 (C-5), 33.0 (C-4), 29.1 (C-3), 21.3 (C-2). (* Assignments interchangeable.) Anal. ($C_8H_{11}N_3O_4$) C, H, N.

A similar reaction using methyl 6-bromohexanoate gave methyl 6-(2-nitro-1*H*-imidazolyl)hexanoate (**39**) as an oil (92%): ^{1}H NMR ($CDCl_3$) δ 7.14 (s, 2 H, H-4', H-5'), 4.43 (t, J = 7.3 Hz, 2 H, H-6), 3.67 (s, 3 H, OCH_3), 2.39 (t, J = 7.3 Hz, 2 H, H-2), 1.85–1.93 (m, 2 H, H-5), 1.65–1.73 (m, 2 H, H-3), 1.37–1.44 (m, 2 H, H-4); ^{13}C NMR ($CDCl_3$) δ 173.6 (CO_2-), 144.7 (C-2'), 128.4 (C-5')*, 125.9 (C-4')*, 51.5 (OCH_3), 49.9 (C-6), 33.5 (C-2), 30.1 (C-4), 25.7 (C-5), 24.0 (C-3). (* Assignments interchangeable.) Hydrolysis as above gave 6-(2-nitro-1*H*-imidazolyl)hexanoic acid (**40**): mp (water) 106–108 °C; ^{1}H NMR ($CDCl_3$) δ 7.95 (br s, 1 H, CO_2H), 7.14 (d, J = 0.9 Hz, 1 H, H-5'), 7.10 (d, J = 0.9 Hz, 1 H, H-4'), 4.42 (t, J = 7.3 Hz, 2 H, H-6), 2.37 (t, J = 7.2 Hz, 2 H, H-2), 1.84–1.92 (m, 2 H, H-5), 1.65–1.72 (m, 2 H, H-3), 1.39–1.45 (m, 2 H, H-4); ^{13}C NMR ($CDCl_3$) δ 178.2 (CO_2H), 144.5 (C-2'), 128.3 (C-5')*, 125.9 (C-4')*, 50.0 (C-6), 33.4 (C-2), 30.1 (C-4), 25.7 (C-5), 23.9 (C-3). (* Assignments interchangeable.) Anal. ($C_9H_{13}N_3O_4$) C, H, N.

***N*-[2-(2-Methyl-5-nitro-1*H*-imidazolyl)ethyl]-4-(2-nitro-1*H*-imidazolyl)butanamide (10): Example of General Method.** A stirred suspension of the acid (**36**) (5.0 g, 25.1 mmol), the amine dihydrochloride (**19**·2HCl) (6.41 g, 26.3 mmol), and Et_3N (10.5 mL, 75.3 mmol) in DMF (100 mL) was treated dropwise at 0 °C under N_2 with diethyl phosphorocyanidate (4.57 mL, 30.1 mmol). The suspension was stirred for 2 h at 0 °C and for a further 18 h at 20 °C and then evaporated to dryness under reduced pressure. The residue was chromatographed directly on silica gel, elution with a gradient (0–20%) of MeOH/EtOAc giving *N*-[2-(2-methyl-5-nitro-1*H*-imidazolyl)ethyl]-4-(2-nitro-1*H*-imidazolyl)butanamide (**10**) (5.5 g, 76%): mp ($CHCl_3$/petroleum ether) 129 °C; ^{1}H NMR (CD_3CN) δ 7.86 (s, 1 H, H-4'''), 7.28 (d, J = 1.0 Hz, 1 H, H-5'), 7.07 (d, J = 1.0 Hz, 1 H, H-4'), 6.61 (br s, 1 H, CONH), 4.31–4.37 (m, 4 H, H-4, H-2''), 3.47 (2 t, J = 6.2 Hz, 2 H, H-1''), 2.38 (s, 3 H, CH_3), 2.06–2.10 (m, 2 H, H-2), 1.99–2.02 (m, 2 H, H-3); ^{13}C NMR (CD_3CN) δ 173.0 (CONH), 152.3 (C-2'''), 140.1 (C-2'''), 139.1 (C-5'), 133.8 (C-4'''), 128.8 (C-5')*, 128.1 (C-4')*, 50.2 (C-2''), 46.5 (C-4), 39.1 (C-1''), 32.8 (C-2), 26.5 (C-3), 14.5 (CH_3). (* Assignments interchangeable.) Anal. ($C_{13}H_{17}N_7O_5$) C, H; N: calcd 27.91; found 28.46.

Similar reaction of **36** and **23** gave *N*-[2-(2-nitro-1*H*-imidazolyl)ethyl]-4-(2-nitro-1*H*-imidazolyl)butanamide (**6**) (67%): mp (MeOH/iPr_2O) 149.5–150.5 °C; ^{1}H NMR ($(CD_3)_2SO$) δ 7.97 (br t, J = 5.8 Hz, 1 H, CONH), 7.62 (d, J = 1.0 Hz, 1 H, H-5'''), 7.52 (d, J = 1.0 Hz, 1 H, H-5'), 7.18 (d, J = 1.0 Hz, 1 H, H-4'''), 7.14 (d, J = 1.0 Hz, 1 H, H-4'), 4.43 (t, J = 5.8 Hz, 2 H, H-2''), 4.35 (t, J = 6.7 Hz, 2 H, H-4), 3.46 (q, J = 5.8 Hz, 2 H, H-1''), 1.89–2.08 (m, 4 H, H-2, H-3)), 2.35 (s, 3 H, CH_3); ^{13}C NMR ($(CD_3)_2SO$) δ 171.4 (CONH), 144.6 (C-2'''), 144.5 (C-2'), 128.3, 127.7, 127.6 (C-4', C-4''', C-5', C-5'''), 49.0, 48.8 (C-4, C-2''), 38.1 (C-1''), 31.6 (C-2), 25.3 (C-3). Anal. ($C_{12}H_{15}N_7O_5$) C, H; N: calcd 29.07; found 29.74.

Similar reaction of **34** and **25** gave *N*-[3-(2-nitro-1*H*-imidazolyl)propyl]-3-(2-nitro-1*H*-imidazolyl)propanamide (**7**) (49% yield): mp (MeOH/iPr_2O) 124–125 °C; ^{1}H NMR ($(CD_3)_2SO$) δ 8.02 (br t, J = 5.5 Hz, 1 H, CONH), 7.65 (s, 1 H, H-5')*, 7.54 (s, 1 H, H-5''')*, 7.19 (s, 1 H, H-4')*, 7.13 (s, 1 H, H-4''')*, 4.60 (t, J = 6.5 Hz, 2 H, H-3), 4.32 (t, J = 7.0 Hz, 2 H, H-3''), 3.04 (dt, J = 6.5, 6.1 Hz, 2 H, H-1''), 2.67 (t, J = 6.6 Hz, 2 H, H-2), 1.57 (quintet, J = 6.9 Hz, 2 H, H-2''). (* Assignments interchangeable.) ^{13}C NMR δ 169.1 (CONH), 144.6 (C-2'), 127.9, 127.8, 127.7, 127.5 (C-4', C-5', C-4''', C-5'''), 47.2 (C-3), 45.8 (C-3''), 35.6 (C-2), 35.5 (C-1''), 29.5 (C-2''). Anal. ($C_{12}H_{15}N_7O_5$) C, H, N.

Similar reaction of **32** and **19** gave *N*-[2-(2-methyl-5-nitro-1*H*-imidazolyl)ethyl]-2-(2-nitro-1*H*-imidazolyl)ethanamide (**8**) as a hygroscopic foam (35%): ^{1}H NMR (CD_3CN) δ 7.87 (s, 1 H, H-4'''), 7.23 (d, J = 1.0 Hz, 1 H, H-5'), 7.11 (d, J = 1.0 Hz, 1 H, H-4'), 6.96 (br s, 1 H, CONH), 4.97 (s, 2 H, H-1), 4.36 (t, J = 6.1 Hz, 2 H, H-2''), 3.58 (2 t, J = 6.2 Hz, 2 H, H-1''), 2.41 (s, 3 H, CH_3); ^{13}C NMR (CD_3CN) δ 167.4 (CONH), 152.5 (C-2''), 144.2 (C-2'), 139.9 (C-5'''), 133.9 (C-4'''), 128.9 (C-5'), 128.8 (C-4'), 57.2 (C-2''), 46.3 (C-2), 39.6 (C-1''), 14.6 (CH_3); MS (DEI) m/z 323 (M^+, 3), 277 (50), 231 (85), 97 (100); HRMS (DEI) m/z calcd for $C_{11}H_{16}N_5O_7$ 323.0978 (M^+), found 323.0981. Anal. ($C_{11}H_{16}N_7O_5$) C, H, N.

Similar reaction of **34** and **19** gave *N*-[2-(2-methyl-5-nitro-1*H*-imidazolyl)ethyl]-3-(2-nitro-1*H*-imidazolyl)propanamide (**9**) (42%): mp (MeOH/iPr_2O) 144.5–145.5 °C; ^{1}H NMR ($(CD_3)_2SO$) δ 8.18 (br t, J = 5.7 Hz, 1 H, CONH), 8.00 (s, 1 H, H-4'''), 7.51 (s, 1 H, H-5'), 7.13 (d, J = 1.0 Hz, 1 H, H-4'), 4.53 (t, J = 6.6 Hz, 2 H, H-2), 4.27 (t, J = 5.9 Hz, 2 H, H-2''), 3.40 (q, J = 5.9 Hz, 2 H, H-1''), 2.62 (t, J = 6.6 Hz, 2 H, H-2), 2.35 (s, 3 H, CH_3); ^{13}C NMR ($(CD_3)_2SO$) δ 159.5 (CONH), 151.2 (C-2'''), 144.5 (C-2'), 138.4 (C-5'''), 133.0 (C-4'''), 127.9 (C-4')*, 127.6 (C-5')*, 45.5 (C-3), 45.2 (C-2''), 37.8 (C-1''), 35.3 (C-2), 13.6 (CH_3). (* Assignments interchangeable.) Anal. ($C_{12}H_{16}N_7O_5$) C, H; N: calcd, 29.07; found 29.91.

Similar reaction of **38** and **19** gave *N*-[2-(2-methyl-5-nitro-1*H*-imidazolyl)ethyl]-5-(2-nitro-1*H*-imidazolyl)pentanamide (**11**) (54%): mp (MeOH/iPr_2O) 136–137.5 °C; ^{1}H NMR (CD_3CN) δ 7.85 (s, 1 H, H-4'''), 7.32 (d, J = 0.9 Hz, 1 H, H-5'), 7.09 (d, J = 0.9 Hz, 1 H, H-4'), 6.55 (br s, 1 H, CONH), 4.32–4.37 (m, 4 H, H-5, H-2''), 3.50 (2 t, J = 6.2 Hz, 2 H, H-1''), 2.39 (s, 3 H, CH_3), 2.04–2.09 (m, 2 H, H-2), 1.72–1.80 (m, 2 H, H-4), 1.45–1.53 (m, 2 H, H-3); ^{13}C NMR (CD_3CN) δ 173.7 (CONH), 152.4 (C-2'''), 140.1 (C-2')*, 139.9 (C-5''')*, 133.8 (C-4'''), 128.8 (C-4'), 128.1 (C-5'), 50.6 (C-2''), 46.6 (C-5), 39.1 (C-1''), 35.8 (C-2), 30.4 (C-4), 22.9 (C-3), 14.6 (CH_3). (* Assignments interchangeable.) HRMS m/z calcd for $C_{14}H_{19}N_7O_5$ 365.1448 (M^+), found 365.1452. Anal. ($C_{14}H_{19}N_7O_5$) C, H; N: calcd 26.84; found 27.43.

Similar reaction of **40** and **19** gave *N*-[2-(2-methyl-5-nitro-1*H*-imidazolyl)ethyl]-6-(2-nitro-1*H*-imidazolyl)hexanamide (**12**) (53%): mp (MeOH/iPr_2O) 133–134 °C; ^{1}H NMR (CD_3CN) δ 7.88 (s, 1 H, H-4'''), 7.33 (d, J = 1.0 Hz, 1 H, H-5'), 7.08 (d, J = 1.0 Hz, 1 H, H-4'), 6.50 (br s, 1 H, CONH), 4.33–4.38 (m, 4 H, H-6, H-2''), 3.50 (2t, J = 6.2 Hz, 2 H, H-1''), 2.39 (s, 3 H, CH_3), 2.03 (t, J = 7.4 Hz, 1 H, H-2), 1.75–1.83 (m, 2 H, H-5), 1.46–1.54 (m, 2 H, H-4), 1.22–1.28 (m, 2 H, H-3); ^{13}C NMR (CD_3CN) δ 173.9 (CONH), 152.4 (C-2'''), 140.1 (C-2'), 139.9 (C-5'''), 133.9 (C-4'''), 128.8 (C-4'), 128.1 (C-5'), 50.7 (C-2''), 46.6 (C-6), 39.1 (C-1''), 36.2 (C-2), 30.7 (C-5), 26.5 (C-4), 25.5 (C-3), 14.6 (CH_3); HRMS m/z calcd for $C_{15}H_{21}N_7O_4$ 379.1604 (M^+), found 379.1613. Anal. ($C_{15}H_{21}N_7O_5$) C, H, N.

Similar reaction of **36** and **21** gave *N*-[2-(1*H*-imidazolyl)ethyl]-4-(2-nitro-1*H*-imidazolyl)butanamide (**13**) (70%) as a hygroscopic gum; ^{1}H NMR (CD_3CN) δ 7.42 (s, 1 H, H-2'''), 7.29 (d, J = 1.0 Hz, 1 H, H-5'), 7.08 (d, J = 1.0 Hz, 1 H, H-4'), 7.00 (t, J = 1.1 Hz, 1 H, H-5'''), 6.90 (s, 1 H, H-4'''), 6.62 (br. s, 1 H, CONH), 4.37 (t, J = 6.9 Hz, 2 H, H-4), 4.02 (t, J = 6.1 Hz, 1 H, H-2''), 3.44 (2t, J = 6.0 Hz, 2 H, H-1''), 2.10–2.14 (m, 2 H, H-2), 2.01–2.08 (m, 2 H, H-3); ^{13}C NMR (CD_3CN) δ 172.8 (CONH), 141.0 (C-2'), 138.2 (C-2'''), 129.7 (C-5'''), 128.2 (C-5'), 128.2 (C-4'), 120.4 (C-4'''), 50.3 (C-2''), 46.9 (C-4), 40.8 (C-1''), 33.0 (C-2), 26.7 (C-3); MS m/z 246 ($M^+ - NO_2$, 100); HRMS m/z calcd for $C_{12}H_{17}N_6O_3$ 293.1362 (M^+), found 293.1363.

Similar reaction of **36** and **27** gave *N*-[2-(4-nitro-1*H*-imidazolyl)ethyl]-4-(2-nitro-1*H*-imidazolyl)butanamide (**14**) (56%): mp (MeOH/iPr_2O) 175.5–177 °C; ^{1}H NMR ($(CD_3)_2SO$) δ 8.40 (d, J = 1.4 Hz, 1 H, H-5'''), 8.08 (t, J = 5.6 Hz, 1 H, CONH), 7.81 (d, J = 1.4 Hz, 1 H, H-2'''), 7.64 (d, J = 0.9 Hz, 1 H, H-5'), 7.18 (d, J = 0.9 Hz, 1 H, H-4'), 4.35 (t, J = 6.7 Hz, 2 H, H-4), 4.12 (dd, J = 6.0, 5.2 Hz, 2 H, H-2''), 3.43 (dd, J = 6.0, 5.7 Hz, 2 H, H-1''), 1.97–2.09 (m, 4 H, H-2, H-3); ^{13}C NMR ($(CD_3)_2SO$) δ 171.4 (CONH), 146.9 (C-4'), 144.5 (C-2'), 137.5 (C-2'''), 127.7 (C-4', C-5'), 121.7 (C-5'''), 48.8 (C-4), 47.0 (C-2''), 45.3 (C-1''), 31.7 (C-2), 25.6 (C-3); MS (DCI) m/z 338 ($MH^{\cdot+}$, 40), 332 (10), 225 (35); HRMS (DCI) m/z calcd for $C_{12}H_{16}N_7O_5$ 338.1213 (MH^+), found 338.1203. Anal. ($C_{12}H_{15}N_7O_5$) C, H; N: calcd, 29.07; found 28.32.

Similar reaction of **36** and **29** gave *N*-[2-(5-nitro-1*H*-imidazolyl)ethyl]-4-(2-nitro-1*H*-imidazolyl)butanamide (**15**) (59%): mp (MeOH/iPr_2O) 152.5–153 °C; ^1H NMR ($(CD_3)_2SO$) δ 8.07 (s, 1 H, H-4‴), 7.07 (br t, *J* = 5.9 Hz, 1 H, CONH), 7.94 (d, *J* = 1.4 Hz, 1 H, H-2‴), 7.63 (d, *J* = 0.8 Hz, 1 H, H-5′), 7.18 (d, *J* = 0.8 Hz, 1 H, H-4′), 4.31–4.40 (m, 4 H, H-2″, H-4), 3.43 (dd, *J* = 5.8, 5.3 Hz, 2 H, H-1″), 1.90–2.09 (m, 4 H, H-2, H-3); ^{13}C NMR ($(CD_3)_2SO$) δ 171.4 (CONH), 144.5 (C-2′), 143.4 (C-2‴), 138.5 (C-5‴), 133.6 (C-4‴), 127.8 (C-5′)*, 127.7 (C-4′)*, 48.8 (C-4), 47.0 (C-2″), 38.0 (C-1″), 31.6 (C-2), 25.5 (C-3). (* Assignments interchangeable.) MS (DCI) *m/z* 338 (MH^+, 75), 332 (15), 225 (100); HRMS (DCI) *m/z* calcd for $C_{12}H_{16}N_7O_5$ 338.1213 (MH^+), found 338.1208. Anal. ($C_{12}H_{15}N_7O_5$) C, H, N.

Similar reaction of **30** and **19** gave *N*-[2-(2-methyl-5-nitro-1*H*-imidazolyl)ethyl]-2-(2-methyl-5-nitro-1*H*-imidazolyl)-ethanamide (**16**) (55%) as a hygroscopic foam: ^1H NMR ($CDCl_3$) δ 7.96 (s, 1 H, H-4‴), 7.39 (s, 1 H, H-4′), 6.90 (br s, 1 H, CONH), 4.92 (s, 2 H, H-1), 4.48 (t, *J* = 6.5 Hz, 2 H, H-2″), 3.67 (q, *J* = 6.3 Hz, 2 H, H-1″), 2.50 (s, 6 H, $2CH_3$); ^{13}C NMR ($CDCl_3$) δ 166.2 (CONH), 151.5 (C-2‴)*, 151.2 (C-2′)*, 142.5 (C-5‴)*, 139.9 (C-5′)*, 133.6 (C-4‴)*, 133.1 (C-4′)*, 48.4, 44.6, 39.5 ($3CH_2$), 14.2, 14.1 ($2CH_3$). (* Assignments interchangeable.) MS (DEI) *m/z* 337, (M^+, 4), 291 (35), 245 (20), 211 (45), 53 (100); HRMS (DEI) *m/z* calcd for $C_{12}H_{15}N_7O_5$ 337.1135 (M^+), found 337.1142. Anal. ($C_{12}H_{15}N_7O_5$) C, H, N.

***In Vitro* Cytotoxicity.** Cell lines were maintained as log-phase monolayers in tissue culture flasks using antibiotic-free Alpha MEM with 10% v/v heat-inactivated (56 °C, 40 min) fetal calf serum. Doubling times were approximately 14 h for AA8, 15 h for UV4, 9 h for EMT6, and 24 h for FME cells. Cultures were tested for mycoplasma contamination frequently, using a cytochemical staining method.[44] Bulk cultures of AA8 cells were prepared in spinner flasks, using the above growth medium plus penicillin (100 IU/mL) and streptomycin (100 μg/mL).

Growth inhibition studies were performed as described in detail elsewhere,[27,45] using 200 AA8, 300 UV4, 50 EMT6, or 1000 FME cells in 0.05 mL per well in 96-well tissue culture dishes. Drugs were added 24 h after initiation of cultures and removed 18 h later. The IC_{50} was determined as the drug concentration needed to reduce the cell mass (protein content, measured 72–78 h after drug washout by staining with methylene blue and determining absorbance in a microplate photometer) to 50% of the mean value for eight control cultures on the same 96-well plate.

Clonogenic assays with magnetically-stirred 10-mL suspension cultures (plateau-phase AA8 cells, 10^6/mL) were performed using continuous gassing with 5% CO_2 in air or N_2, as detailed elsewhere.[28] Both cell suspensions and drug solutions in growth medium were preequilibrated under the appropriate gas phase for 60 min prior to mixing, to ensure essentially complete anoxia throughout the period of drug contact in hypoxic cultures. Plating efficiency was determined at intervals of 0.5–1 h for up to 8 h, and the concentration × time required to reduce survival to 10% of control values (CT_{10}) was estimated at each drug concentration tested. Hypoxic selectivity was determined as the ratio of (interpolated) aerobic and hypoxic CT_{10} values at the same time.

***In Vitro* Radiosensitization.** Stirred suspensions of AA8 cells were made hypoxic by gassing with 5% CO_2 in N_2 for 60 min using the same conditions as in cytotoxicity assays. Cultures were irradiated at 37 °C (cobalt 60, dose rate ca. 2 Gy/min) 30 min after mixing drug and cells, with the cell cultures, waterbath, and stirrers positioned in the vertical radiation beam. Samples were withdrawn after each radiation dose (typically 6 Gy intervals), and plating efficiency (PE) was determined. The surviving fraction (SF) was determined with respect to unirradiated cells from the same culture, assayed immediately before irradiation. The data was fitted to the linear-quadratic model ($\ln SF = -\alpha D - \beta D^2$, where *D* is the radiation dose) to interpolate the dose for 10% survival (D_{10}). The sensitizer enhancement ratio (SER = D_{10} without drug/D_{10} with drug) was determined at a range of drug concentrations to estimate the concentration for an SER of 1.6 ($C_{1.6}$). The SF as determined above corrects for drug toxicity due to preirradiation drug treatment, but not for any additional killing due to drug toxicity during the irradiation period. This was determined separately, using cell suspensions treated identically with drug, in the same waterbath but outside the radiation field, and the SF was calculated relative to hypoxic controls. The $C_{1.6}$ was analyzed using only SER values for drug concentrations where this toxicity was minimal over the irradiation period.

Drug Uptake Studies. Intracellular and extracellular drug concentrations were determined under the same conditions as the radiosensitization assays, after a 30-min hypoxic incubation of AA8 cells at a drug concentration equal to the $C_{1.6}$. The culture (10^7 cells) was centrifuged at 140*g* for 5 min, and samples of the supernatant (50 μL) were diluted into 19 volumes of ice-cold MeCN for determination of extracellular drug concentration. The remaining supernatant was discarded, the tubes centrifuged (13000*g* for 2 min), aspirated, and centrifuged again to remove residual medium from the walls of the tube. Water (90 μL) was added to lyse the cell pellets, and 9 volumes of MeCN was added. The MeCN extracts were frozen at −80 °C. After thawing, samples were centrifuged (13000*g* × 5 min), and the supernatant was evaporated to dryness. The residues were dissolved in 0.2 mL of HPLC mobile phase and centrifuged again to remove any particulate matter, and samples (0.17 mL) were analyzed by HPLC using a Waters C18 μ-Bondapak column (8 × 100 mm) at a flow rate of 1.8 mL/min. The initial mobile phase was constructed by mixing an organic phase ($MeCN/H_2O$, 4:1, v/v) with formate buffer at pH 4.5 (28 g ammonium formate and 2.55 mL formic acid in 1 L) in the ratio 18:82. After 2 min the organic component was increased linearly to 40% by 12 min. The nitroimidazoles were detected by absorbance at 320 nm using a Hewlett-Packard diode array detector (HP 1040A). All drug concentrations were corrected for recovery (detection efficiency) by spiking culture medium (for extracellular recoveries) or lysed cell pellets (for intracellular recoveries) with known amounts of drug, using concentrations similar to those estimated in the unknown samples. Recoveries were in the range 0.70–0.99 for all compounds. Average intracellular concentrations in cells were calculated assuming a cellular water volume of 11.6 μL/10^7 AA8 cells and an extracellular water volume in the pellet of 1.1 μL in a pellet of 10^7 cells, using [^{14}C(U)]sucrose to mark the extracellular space and ^3H_2O to mark the total water volume as reported elsewhere.[46]

***In Vivo* Studies.** Single cell suspensions were prepared from KHT tumors by enzymatic dissociation (0.5 mg/mL, pronase, 0.2 mg/mL collagenase, 0.2 mg/mL DNAse I for 40 min). The tumor was passaged in C_3H/HeN mice by sc inoculation of 2 × 10^5 cells in the inguinal region. Tumors were prepared in the same manner for experiments, and treated when in the size range 0.5–1.0 mL as assessed by caliper measurements. Compounds **1** and **10** were administered ip as solutions in phosphate-buffered saline, using single or multiple doses of 2.5 mmol and an injection volume of 0.01 (**1**) or 0.04 (**10**) mL/g body weight per dose. Tumors were irradiated 45 min after the last drug dose, using whole body irradiation (cobalt 60) of unanaesthetised, unrestrained mice at a dose rate of ca 2.5 Gy/min. Tumors were excised 18 h after irradiation, dissociated as above, and the cell yield determined with an electronic cell counter. Clonogenic survival was then assessed, essentially as described,[47] by plating 10–20 replicate 1-mL samples in α MEM containing 0.3% agar, with lethally-irradiated (40 Gy) KHT cells added to give a total of 10^4 cells/mL, in 24-well culture dishes. After growth for 13 days, colonies were stained by adding 0.175 mL of (iodophenyl)(nitrophenyl)-tetrazolium chloride (0.5 mg/mL in H_2O) to each well. Cultures were incubated at 37 °C overnight, and colonies were counted with a binocular microscope.

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