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Synthesis of a novel nitroimidazole-spermidine derivative as a tumor-targeted hypoxia-selective cytotoxin

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Abstract—A four-step synthesis of (*R,S*)-N^4-[3-(2-nitro-1-imidazolyl)-2-hydroxypropyl]-spermidine trihydrochloride (**4**) is described and the utilization of the polyamine active transport system for the uptake of this compound in cells is demonstrated. Thus, V79 cells pretreated with an inhibitor of spermidine biosynthesis, α-difluoromethylornithine (DFMO), are ca. 2-fold more sensitive to **4** under hypoxic conditions, compared to untreated cells. Similarly, radiosensitization of hypoxic V79 cells by **4** is improved in DFMO-pretreated cells.

Polyamines such as N^1-(3-aminopropyl)-1,4-butanediamine (spermidine), 1,4-butanediamine (putrescine), and N^1,N^4-bis(3-aminopropyl)-1,4-butanediamine (spermine) are a requirement for the optimal growth and replication of various cell types and are present in higher concentrations in rapidly proliferating cells.[1–4] Thus, polyamine analogues that vary slightly in structure from natural polyamines might compete with naturally occurring polyamines for critical cellular binding sites. In addition, a wide range of mammalian and bacterial cells have an active, energy-dependent and carrier-mediated polyamine transport system which seems to be regulated by intracellular polyamine concentrations.[5–8] Porter et al. also demonstrated that the N^1 and N^8 primary amines of spermidine are the most critical determinants in transport specificity, leaving the central N^4 secondary amine for derivatization.[5] Utilization of the polyamine transport system can afford selective accumulation of polyamine analogues in neoplastic tissues and therefore, presents a very attractive anticancer chemotherapeutic strategy.[9–15] We tried to exploit this methodology in the development of novel bioreductive drugs as cytotoxins and radiosensitizers of hypoxic cells, because hypoxia in tumors is a limiting factor for success in cancer radio/chemotherapy[16–19] and because such an approach could increase effectiveness due to selective uptake and DNA-binding in tumor cells. In this paper we report the synthesis of N^4-[3-(2-nitro-1-imidazolyl)-2-hydroxypropyl]spermidine trihydrochloride (**4**) and its evaluation as a hypoxia-selective cytotoxin and radiosensitizer in V79 cells. The 2-nitroimidazole group was selected on the basis of its one-electron reduction potential, known to be in the 'correct' range of −330 to −450 mV, which allows for its exclusive reduction under hypoxic conditions and has been previously incorporated in a variety of bioreductive drugs.[20]

The synthesis of **4** is outlined in Scheme 1. Protection of the terminal amino groups of N^4-benzyl-spermidine (**1**, Aldrich) with di-*tert*-butyldicarbonate, followed by debenzylation through hydrogenation in dry MeOH with 5% Pd–C catalyst, at 55 psi (rt, overnight) led to N^1,N^8-diBoc-spermidine, (**2**), in 98% yield. Protected spermidine (**2**) was coupled with 1-(oxiranylmethyl)-2-nitroimidazole in dry EtOH, under reflux for 12 h under an inert atmosphere, to afford *(R,S)*-N^4-[3-(2-nitro-1-imidazolyl)-2-hydroxypropyl]-N^1,N^8-diBoc-spermidine,[21] (**3**), in 65% yield. Compound (**3**) was separated as an oil by preparative alumina TLC with 60:40 hexane:ethyl acetate eluent (R_f = 0.54). Finally, compound (**3**) was deprotected and converted to its trihydrochloride salt (**4**), in 97% yield, by dissolving it in dry ether and treating with a HCl/dioxane solution.[22] 1-(Oxiranylmethyl)-2-nitroimidazole was synthesized by alkylation of 2-nitroimidazole with (*R,S*)-1-bromo-2,3-

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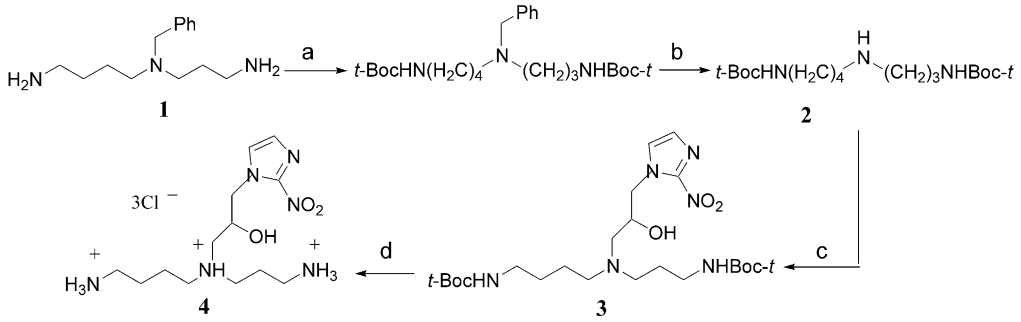

Scheme 1. Reagents and conditions: (a) $(t\text{-Boc})_2O$, ET_3N, THF, rt (85%); (b) Pt/C, H_2, MeOH (98%); (c) 1-(oxiranylmethyl)-2-nitroimidazole, EtOH, reflux (65%); (d) HCl gas in dioxane, THF (97%).

epoxypropane, followed by base-catalyzed ring closure, generally according to the method of Beaman et al.[23]

The cytotoxicity of **4** was assessed in suspended V79 Chinese hamster lung fibroblasts under aerobic or hypoxic exposures, by using the clonogenic assay.[24] Cytotoxicity of **4** was also determined after aerobic pretreatment of the same cells (while in exponential growth as monolayers) for 48 h with DL-α-difluoromethylornithine (DFMO), an inhibitor of ornithine decarboxylase (ODC), the first and rate limiting enzyme involved in the polyamine biosynthetic pathway.[25,26] The radiosensitizing ability of **4** was also tested under hypoxic or aerobic conditions in suspended V79 cells, previously pretreated or not with 100 μM DFMO for 48 h as monolayers.

The presence of an active polyamine transport system in V79 cells was tested by measuring the uptake of radiolabeled ^{14}C-spermidine (0.1–1.0 μCi, specific activity of ca. 100 μCi/mmol) before or after polyamine depletion by exposing the cells, while in monolayers, to 100 μM DFMO for 48 h and then measuring radioactivity by scintillation counting. Thus, the uptake of ^{14}C-spermidine was increased by 2.1 fold in DFMO-pretreated cells (Fig. 1).

Cells were relatively insensitive to compound **4**, since cytotoxicity was obtained at mM concentrations (Table 1) whereas the nitroimidazole-based bioreductive compound NLCQ-1, {4-[3-(2-nitro-1-imidazolyl)propylamino]-7-chloroquinoline hydrochloride} which enters cells through passive diffusion and targets DNA through weak intercalation, demonstrates CT_{50H} values at μM concentrations in various cell-lines.[27] However, **4** was still a more potent but less selective hypoxic cytotoxin than misonidazole [1-(2-nitro-1-imidazolyl)-2-hydroxy-3-methoxypropane], a bioreductive compound which also enters cells through passive diffusion but does not target DNA. Thus, misonidazole demonstrates a CT_{50H} of 7.5±0.5 mM and a CT_{50A} of 120.6±7.3 mM, in V79 cells. The cytotoxicity of **4** was statistically increased in DMFO-pretreated cells (1 mM, 48 h), to a similar degree under both aerobic or hypoxic conditions, consistent with an increased uptake of **4** through the polyamine transport system. Similar increases of the IC_{50} values have been observed before in DFMO-treated L1210 cells for several polyamine conjugates.[15] However, 4 μM of spermidine co-administered with **4** in cells not pretreated with DFMO, did not hinder the uptake of **4**, resulting thus in unaltered CT_{50} values (Table 1). On the other hand, 4 μM spermidine given in parallel with **4** in DFMO-pretreated cells, caused a slight but statistically significant increase in the aerobic toxicity of **4**, which was further increased by using 0.5 mM spermidine. To the contrary, the hypoxic toxicity of **4** was slightly decreased by 4 μM spermidine (Table 1). Therefore, the potentially antagonistic effect of spermidine is not clear at this moment and should be further clarified. However, one can speculate that perhaps different polyamine-binding proteins on the surface of V79 cells are responsible for the transport of compound **4** and spermidine. For instance, in the L1210 murine leukemia and A549 human lung carcinoma cells many polyamine-binding proteins have been found.[28]

Compound **4**, at 0.6 and 0.8 mM, was able to increase the responsiveness of hypoxic DFMO-pretreated V79 cells to ionizing radiation, by a factor of 1.5 and 2.0, respectively (Fig. 2). The corresponding factor in hypoxic cells not pretreated with DFMO was 1.0 and

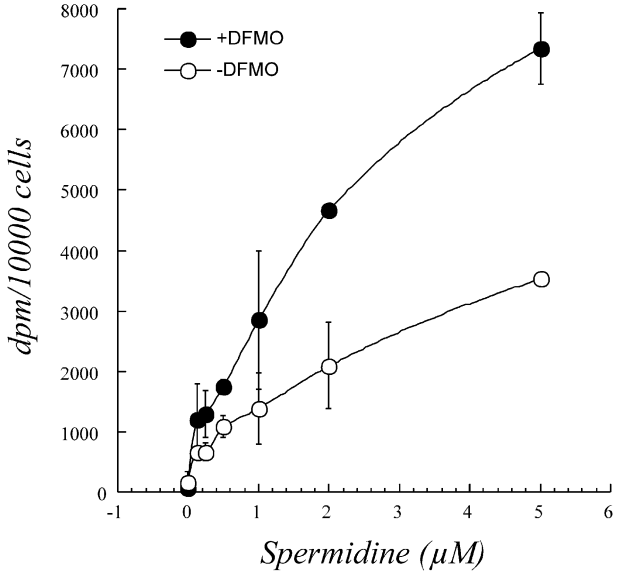

Figure 1. ^{14}C-Spermidine uptake by V79 cells. Suspensions of V79 cells (10^5 cells/mL, 1 mL) in duplicate were incubated at 37 °C for 30 min with various ^{14}C-spermidine concentrations (0.1–1.0 μCi, the bulk of it being cold spermidine). A second set of cells, pretreated as monolayers with DFMO (100 μM, 48 h) were similarly treated with ^{14}C-spermidine. Following incubation, the cells were prepared for scintillation.

Table 1. Aerobic and hypoxic toxicity of compound **4** in V79 cells

	CT_{50A}[a]	CT_{50H}[b]	Ratio[c]
−DFMO	2.60±0.24	1.28±0.20	2.0
+DFMO (1 mM)[d]	1.63±0.18 ($P<0.001$)	0.74±0.07 ($P<0.005$)	2.2
−DFMO+spermidine (4 μM)[e]	2.64±0.23 ($P>0.05$)	1.15±0.09 ($P>0.05$)	2.3
+DFMO (0.1 mM)+spermidine (4 μM)	1.15±0.06 ($P<0.005$)	0.88±0.07 ($P<0.05$)	1.3
+DFMO (1 mM)+ spermidine (0.5 mM)	0.63±0.02 ($P<0.001$)	ND	

[a,b] CT_{50} values determined under aerobic (A) or hypoxic (H) conditions in suspended cells and represent the drug concentration (mM) required to reduce clonogenicity to 50% of corresponding controls. Values are means ±SEM (4 determinations).
[c] Ratio of CT_{50} values in air and N_2 (CT_{50A}/CT_{50H}).
[d] Cells were exposed to DFMO under aerobic conditions for 48 h, while in monolayers.
[e] Spermidine was administered to the cells in parallel and under the same conditions with **4**. *P* values were determined by using the Student's *t*-test in comparisons with corresponding controls.

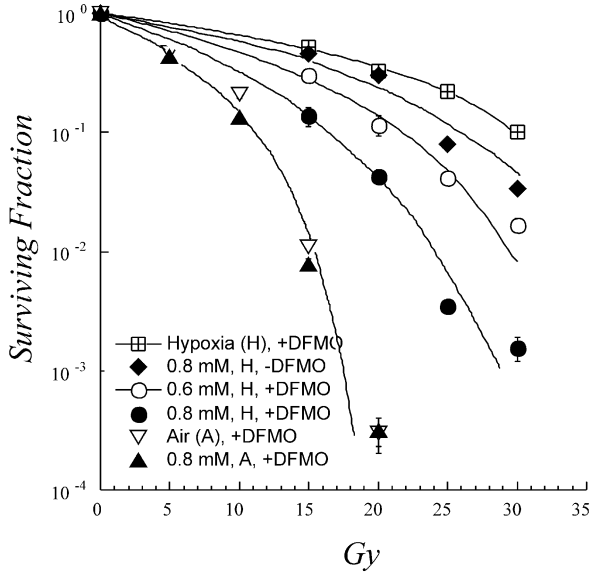

Figure 2. Radiosensitization of V79 cells by compound **4**. Cells were exposed or not to DFMO (1 mM, 48 h) as monolayers. Then, cells were exposed to 0.6 or 0.8 mM of **4** under aerobic or hypoxic conditions (37 °C, 4 h) in suspension, before irradiation at 25 °C. Cells were then processed for clonogenicity.

1.2 at 0.6 and 0.8 mM of **4**, respectively. No radiosensitization was observed by **4** in aerobic V79 cells regardless of pretreatment with DFMO (Fig. 2). Misonidazole at 0.8 mM increased the responsiveness of V79 cells by a factor of 1.8, regardless of pretreatment with DFMO, as was expected for compounds that do not utilize the active polyamine transport system to enter cells.

The above results suggest that optimized nitroimidazole-based polyamine derivatives, obtained perhaps through terminal amine alkylation, could be of interest as hypoxia-selective cytotoxins and radiosensitizers for their selective uptake by cancer cells through the polyamine transport system. Hypoxic potency rather than selectivity would be more meaningful in such systems.

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21. Compound **3**: Clear oil; yield 65%; ^{1}H NMR (500 MHz) ($CDCl_3$) δ 7.27 (s, 1H), 7.07 (s, 1H), 4.86 (s, broad, 1H), 4.74 (s, broad, 1H), 4.65 (d, $J=13.5$ Hz, 1H), 4.15–4.06 (m, 2H), 3.97 (m, 1H), 3.22–3.03 (m, 4H), 2.60–2.34 (m, 6H), 1.58 (m, 2H), 1.43–1.39 (m, 22H). HRMS (VG 70-250SE mass spectrometer): calcd for $C_{23}H_{43}N_6O_7$, $[M+H]^+$: *m/z* 515.3193. Found: 515.3191 (100%). Anal. ($C_{23}H_{42}N_6O_7$) C, H, N.
22. Compound **4**: White solid, very hygroscopic; yield 97%; ^{1}H NMR (500 MHz) (D_2O, 4.8 δ) δ 7.49 (s, 1H), 7.24 (s, 1H), 4.51 (t, broad, 1H), 4.37 (dd, $J=11.7$ Hz, 3.3 Hz, 1H), 3.38 (t, broad, 6H), 3.08 (m, 5H), 2.18 (m, 2H), 1.70–1.90 (m, 4H). FAB (VG 70-250SE mass spectrometer):

calcd for $C_{13}H_{29}Cl_2N_6O_3$ [M-Cl]$^+$: m/z 387; Found: 387. Anal. ($C_{13}H_{29}N_6Cl_3O_3$) C, H, N, Cl.
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