of the animals to exhibit clonic seizures.

Effects on the Uptake of Norepinephine and Serotonin by Crude Synaptosomes. Experiments were performed with crude preparations of synaptosomes from rat whole brain. The procedure of Thornberg and Moore[5] was followed with minor modifications. Incubations were carried out for 5 or 15 min with L-[^{3}H]norepinephrine (20 or 21.2 nM unless otherwise specified) or [^{3}H]serotonin (9.2–14 nM unless otherwise specified). The test compounds were dissolved in ethanol; the final concentration of ethanol in the assay mixture was 1% (v/v).

Effect on Apomorphine Inhibition of Tyrosine Hydroxylase in Synaptosomes. These experiments were conducted essentially according to the protocol described by Bronaugh and co-workers.[6] Percent prevention of apomorphine (Apo) inhibition was calculated according to Christiansen and Squires[8] as follows:

$$(\% \text{ of control act. with test compd and Apo} - \% \text{ of control act. with Apo}) / (\% \text{ of control act. with test compd in the absence of Apo} - \% \text{ of control act. with Apo}) \times 100$$

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Effects on Histamine-Activated Adenylate Cyclase Preparations. Guinea pig cerebrum or hippocampus particles were prepared, prelabeled with [2-^{3}H]adenine, and activated with histamine (50 μM), 2-methylhistamine (200 μM), or 4-methylhistamine (100 or 200 μM) essentially as described by Chasin et al.[9] The test drugs were added at concentrations ranging from 0.01 to 100 μM. Quantitation of cyclic AMP was based on the procedure of Krishna et al.[10] The percent inhibition by test compounds was calculated after subtraction of the basal value for formation of cyclic AMP.

Acknowledgment. We thank Drs. J. K. Saelens, R. Lovell, and S. Psychoyos for help in the biological evaluations and L. Dorfman and the staff of the Analytical Services group of CIBA-GEIGY Pharmaceuticals Division for the determination of spectral and analytical data.

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Nitrobenzyl Halides and Carbamates as Prototype Bioreductive Alkylating Agents

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o-Nitrobenzyl and *p*-nitrobenzyl alcohols, halides, and N-substituted carbamates were prepared as potential bioreductive alkylating agents, and first half-wave reduction potentials of these compounds were measured by differential pulse polarography. The cytotoxicities of these agents were examined by determining the colony-forming ability of EMT6 tumor cells following exposure to each agent at concentrations of 0.01 to 500 μM for 1 h at 37 °C under conditions of normal aeration and chronic hypoxia. The *o*-nitrobenzyl compounds were significantly more cytotoxic to hypoxic cells than to oxygenated cells. This selective cytotoxicity is hypothesized to result from alkylation following more efficacious bioreductive activation of these compounds by hypoxic cells. Nitrobenzyl compounds with such selective toxicity to hypoxic neoplastic cells may prove to be particularly valuable in combination therapy designed for the treatment of solid tumors.

The cellular subpopulations in solid tumors are physiologically more heterogeneous with respect to the degree of oxygenation and rate of proliferation than are the cellular components of hematological or particularly well-vascularized tumors. With respect to these considerations, a solid neoplastic mass may be envisioned to consist of at least four classes of malignant cells: cells which are well-oxygenated, relatively rapidly traversing the cell cycle and which may correspond in drug sensitivities to exponentially growing cells in culture; nonproliferating oxygenated cells which may correspond in susceptibility to chemotherapeutic agents to plateau phase cells in culture; and cells in various degrees of hypoxia. The hypoxic cell population may be composed of cells with either relatively normal or prolonged cell cycle times or may be blocked in their progression through the cell cycle. The hypoxic cell compartment contains viable neoplastic stem cells which are capable of reentering the rapidly proliferating cellular pool of the tumor. It is well-established that hypoxic cells are relatively resistant to the cytotoxic effects of ionizing radiation, and, since hypoxic cells may be either noncycling or slowly progressing through the cell cycle, they are also presumed to be relatively resistant to cell cycle specific chemotherapy. Thus, the hypoxic cell population has the capacity to limit the curability of solid tumors, and, for this reason, agents directed specifically against hypoxic cells would appear to have considerable potential in cancer therapy in combination with other agents or treatment modalities designed to erradicate the other cellular classes that may be present.[1]

The nitroheterocyclic radiosensitizers were first examined because of their ability to sensitize hypoxic cells to the lethal effects of ionizing radiation.[2] These compounds also have been shown to be selectively cytotoxic to hypoxic cells;[3-13] this differential toxicity appears to depend upon the preferential reductive activation of the nitro-

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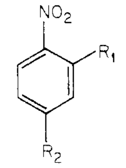
Table I. Physical Constants of *o*- and *p*-Nitrobenzyl Alcohols, Halides, and Carbamates



compd	R_1	R_2	mp, °C	anal.	$E_{1/2}$, V	ED_{50}, μg/mL aerobic	hypoxic	ratio (aerobic/hypoxic)
1	H	CH_2OH	92-94	C, H, N	−0.84	2.6	0.77	3.3
2	H	CH_2Br	97-99	C, H, N, Br	−0.75	0.10	0.06	1.7
3	H	CH_2Cl	71-73	C, H, N, Cl	−0.72	0.14	0.07	2.0
4	H	$CH_2OCONHCH_3$	120-122	C, H, N	−0.82	0.63	0.03	21
5	H	$CH_2OCONHC_6H_5$	125-126	C, H, N	−0.78	0.22	0.08	2.7
6	H	$CH_2OCONHC_2H_4Cl$	69-71	C, H, N, Cl	−0.77	31	10	3.1
7	CH_2OH	H	70-72	C, H, N	−0.83	3.0	1.5	2.0
8	CH_2Br	H	44-46	C, H, N, Br	−0.75	1.5	0.06	25
9	CH_2Cl	H	52-54	C, H, N, Cl	−0.72	1.2	0.1	12
10	$CH_2OCONHCH_3$	H	70-72	C, H, N	−0.79	3.57	0.006	595
11	$CH_2OCONHC_6H_5$	H	121-123	C, H, N	−0.77	6.8	0.22	31
12	$CH_2OCONHC_2H_4Cl$	H	66-68	C, H, N, Cl	−0.76	52	0.65	80
misonidazole					−0.68			
metronidazole					−0.74			

heterocyclic drugs by enzyme systems selectively expressed under conditions of hypoxia.[13,14] The selective cytotoxicity of the electron-affinic radiosensitizers to hypoxic cells in the absence of radiation requires prolonged contact times (i.e., several hours) and relatively high drug concentrations (e.g., 1–5 mM misonidazole).[8,15,16] Consequently, it may not be feasible to exploit effectively the preferential action of these agents on hypoxic cells in the treatment of human cancer.[17,18]

The postulate by this laboratory that hypoxia results in environmental conditions conducive to reductive processes led us to develop a series of quinone bioreductive alkylating agents.[19,20] The envisioned mechanism of activation involved reduction of these agents to the hydroquinone level and spontaneous degradation of the hydroquinone to generate a reactive quinonemethide capable of alkylating cellular molecules.

It was conceivable that this principle could be extended by employing a nitroaromatic moiety containing a functionality designed to be activated to a highly reactive alkylating species under the reducing conditions present in hypoxic neoplastic cells. Such agents should be selectively toxic to hypoxic cells, with the cytotoxicity to hypoxic neoplastic cells being significantly greater than the nitroheterocyclic radiosensitizing agents. To test this hypothesis, *o*- and *p*-nitrobenzyl halides and carbamates were prepared and evaluated in vitro for cytotoxicity to hypoxic and oxygenated EMT6 mammary tumor cells. These relatively simple molecules are regarded as prototypes of nitroreductase-catalyzed bioreductive alkylating agents; thus, the necessary structural features for bioreductive activation and selective cytotoxicity to hypoxic cancer cells can be incorporated into any chosen molecular nucleus.

Chemistry. Nitrobenzyl carbamates were prepared by reaction of anhydrous *o*-nitrobenzyl alcohol (**7**) or *p*-nitrobenzyl alcohol (**1**) at room temperature with methyl, phenyl, or chloroethyl isocyanate.

The first half-wave reduction potentials of compounds **1**–**12** were measured and are shown in Table I. The redox potentials of *o*- and *p*-nitrotoluene are −0.88 and −0.90 V, respectively, when measured in the buffer system used in this study. Substitution of electron-withdrawing substituents on the methyl group of nitrotoluene produced agents which are significantly more electron affinic than the parent compounds. The first half-wave potentials of the nitrobenzyl compounds investigated were similar to those of metronidazole, with misonidazole being slightly more electron affinic.

Biology. The survival of EMT6 tumor cells treated in culture for 1 h with various concentrations of *o*- (**7**) and *p*-nitrobenzyl alcohol (**1**), *o*- (**8**) and *p*-nitrobenzyl bromide (**2**), *o*- (**9**) and *p*-nitrobenzyl chloride (**3**), *o*- (**10**) and *p*-nitrobenzyl *N*-methylcarbamate (**4**), *o*- (**11**) and *p*-nitrobenzyl *N*-phenylcarbamate (**5**), and *o*- (**12**) and *p*-nitrobenzyl *N*-(chloroethyl)carbamate (**6**) is shown in Figures 1–6, respectively. These survival curves are plotted with the surviving fraction on a linear scale to emphasize the differential cytotoxicities of the ortho compounds to oxygenated and hypoxic cells at low drug concentrations. Inserts on the figures replot cellular survival using more traditional semilog plots for those cases in which the surviving fraction fell below 5%. The survival of chronically hypoxic EMT6 cells (i.e., cells maintained under 95% N_2/5% CO_2 for 4 h prior to the addition of the drug) exposed to the *o*-nitrobenzyl halides and carbamates for 1 h was significantly lower than that of normally aerated cells treated with these agents under similar conditions. In contrast, the corresponding *p*-nitrobenzyl halides and carbamates and the *o*- and *p*-nitrobenzyl alcohols showed no significant difference in their cytotoxicity toward aerobic and hypoxic cells.[21,22] Furthermore, nitrotoluenes,

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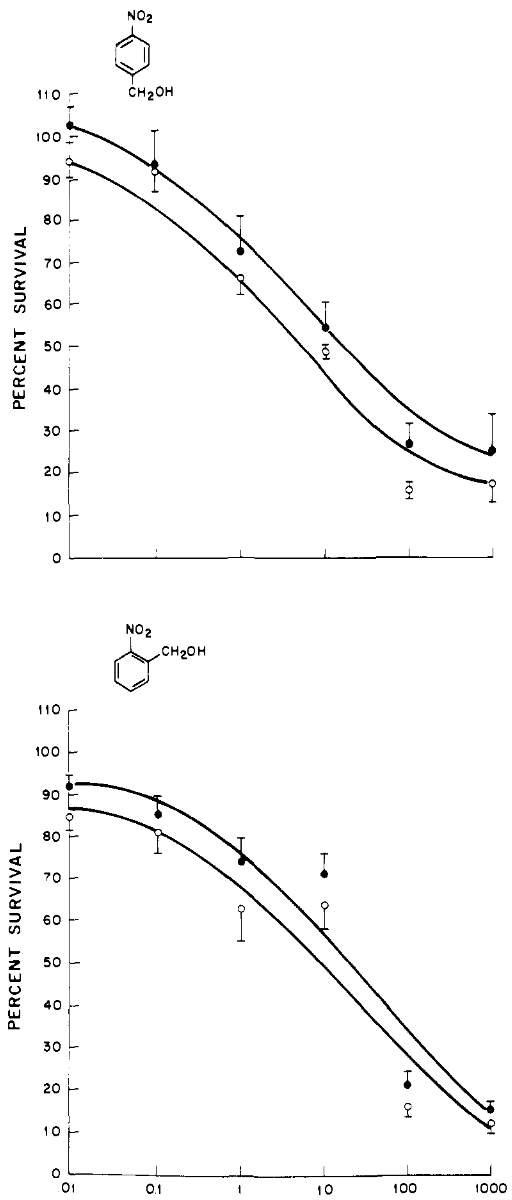

Figure 1. Survival of aerobic (●) and hypoxic (○) EMT6 cells treated for 1 h with various concentrations of *p*-nitrobenzyl alcohol (1) (top) or *o*-nitrobenzyl alcohol (7) (bottom) in cell culture.

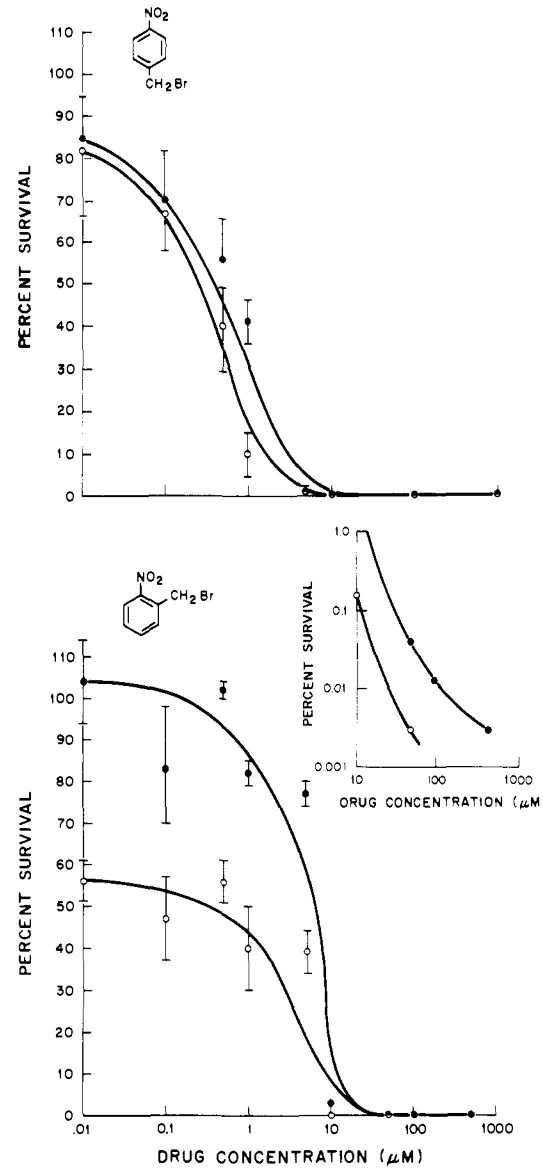

Figure 2. Survival of aerobic (●) and hypoxic (○) EMT6 cells treated for 1 h with various concentrations of *p*-nitrobenzyl bromide (2) (top) or *o*-nitrobenzyl bromide (8) (bottom) in cell culture.

benzyl halides, and benzyl carbamates were not selectively cytotoxic to chronically hypoxic cells.

As can be seen from the ED_{50} values shown in Table I, the ratios of the drug concentrations needed to achieve 50% kill of EMT6 cells under oxygenated and hypoxic conditions was 3.3 and 2.0 for the *p*- (1) and *o*-nitrobenzyl alcohols (7), respectively. For other para-substituted compounds, the ratio varied between 1.7 and 3.1, with the exception of *p*-nitrobenzyl *N*-methylcarbamate (4) for which the ratio of drug concentrations needed to obtain a 50% survival of EMT6 cells under aerobic and hypoxic conditions was 21. With ortho-substituted compounds, this ratio varied between 12 and 80, with the exception of *o*-nitrobenzyl *N*-methylcarbamate (10), for which the ratio of drug concentrations needed to obtain a 50% kill of EMT6 cells under aerobic and hypoxic conditions was 595. Figures 1–6 demonstrate that the nitrobenzyl halides were, in general, more cytotoxic than the corresponding nitrobenzyl carbamates, especially at concentrations above 10 μM. The greatest differential kill at any given concentration was obtained with *o*-nitrobenzyl chloride; at concentrations of 10 and 50 μM, this compound killed 100 times more hypoxic cells than aerobic cells (Figure 3).

The electron-affinic nitroimidazole radiosensitizers have been shown by a variety of laboratories to have a selective action on hypoxic cells in the absence of irradiation; however, this cytotoxicity requires prolonged exposure and relatively high concentrations.[15] The severity of the hypoxia determines the degree of susceptibility of cells to misonidazole-induced cytotoxicity[7] and appears to be a reflection of differences in the capacities of oxygenated and hypoxic cells to metabolize this agent. Thus, relatively large amounts of *N*-hydroxy and amine metabolites of misonidazole are produced by hypoxic cells, indicating that reduction of the nitro group occurs,[7,13,16] while few metabolites are found in cells maintained under aerobic conditions.[13]

Our data suggest that the presence of a nitro group is necessary and the ortho orientation of the nitro moiety and the halomethylene or carbamylmethylene group of the

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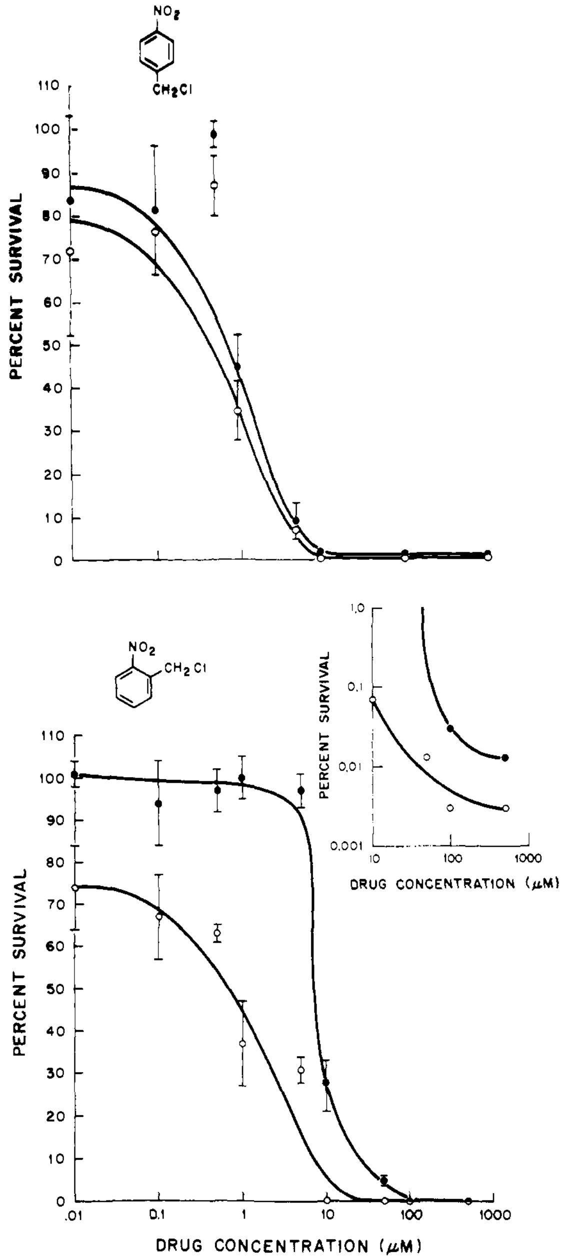

Figure 3. Survival of aerobic (●) and hypoxic (○) EMT6 cells treated for 1 h with various concentrations of *p*-nitrobenzyl chloride (**3**) (top) or *o*-nitrobenzyl chloride (**9**) (bottom) in cell culture.

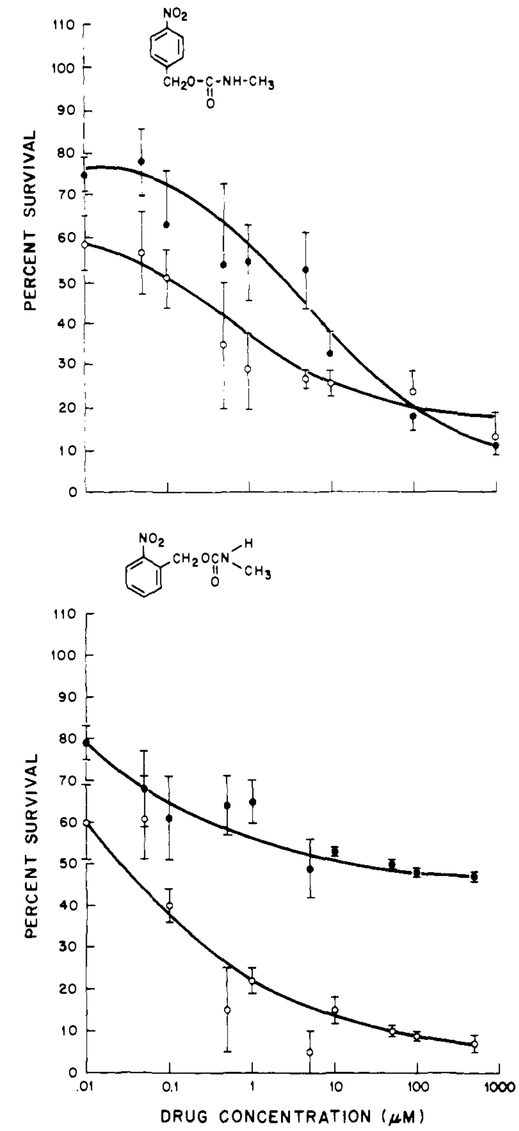

Figure 4. Survival of aerobic (●) and hypoxic (○) EMT6 cells treated for 1 h with various concentrations of *p*-nitrobenzyl *N*-methylcarbamate (**4**) (top) or *o*-nitrobenzyl *N*-methylcarbamate (**10**) (bottom) in cell culture.

nitroaromatic compounds tested is optimal for selective toxicity to hypoxic tumor cells. Mechanisms of cytotoxicity involving the quenching of a nitro radical anion formed by one-electron reduction by molecular oxygen with the subsequent formation of toxic species, such as superoxide anion, hydroxyl radical, and hydrogen peroxide, would appear to be nonoperative in hypoxic cells because of the absence of molecular oxygen. The formation of other products of nitro reduction, such as nitroso and the hydroxylamine intermediates, may account for some of the toxicity of these agents toward hypoxic cells. It is also possible that the greater toxicity of ortho-substituted derivatives to hypoxic EMT6 tumor cells is the result of the formation of a reactive methide-containing species following bioreduction. A mechanism consistent with this possibility is shown in Figure 7. Complete reduction of the nitro substituent of the *o*-nitrobenzyl halides and carbamates can be envisioned to result in an amino group adjacent to a methylene moiety bearing a good leaving group. The participation of the amino function in forming the postulated reactive methide intermediate may be necessary to overcome the energy barrier associated with the aromaticity of the benzene ring. Intramolecular rearrangement is hypothesized to lead to an anilinium ion and the reactive methide group; upon reaction of the methide with any available cellular nucleophile, the aromaticity of the system is restored. The greater toxicity of *o*-nitrobenzyl halides and carbamates to hypoxic cells than to their oxygenated counterparts may be due to an influence of the ortho substituent on the reductive process.

The *o*-nitrobenzyl halides and carbamates are considerably more cytotoxic to hypoxic cells than are the nitroimidazoles, misonidazole and metronidazole. One log of cell kill was achieved with the *o*-nitrobenzyl halides and carbamates when cells were exposed to a concentration of 10 μM drug for 1 h, whereas, in contrast, to produce a comparable degree of kill of hypoxic cells, concentrations of 1–5 mM of misonidazole and incubation times of 2–5 h must be employed.[10,11,16] Moreover, the selective cytotoxicity of *o*-nitrobenzyl halides and carbamates for hypoxic cells was evident at concentrations as low as 0.01 μM.

Although the simple aerobic/hypoxic cell in vitro screening methodology used in these studies has several

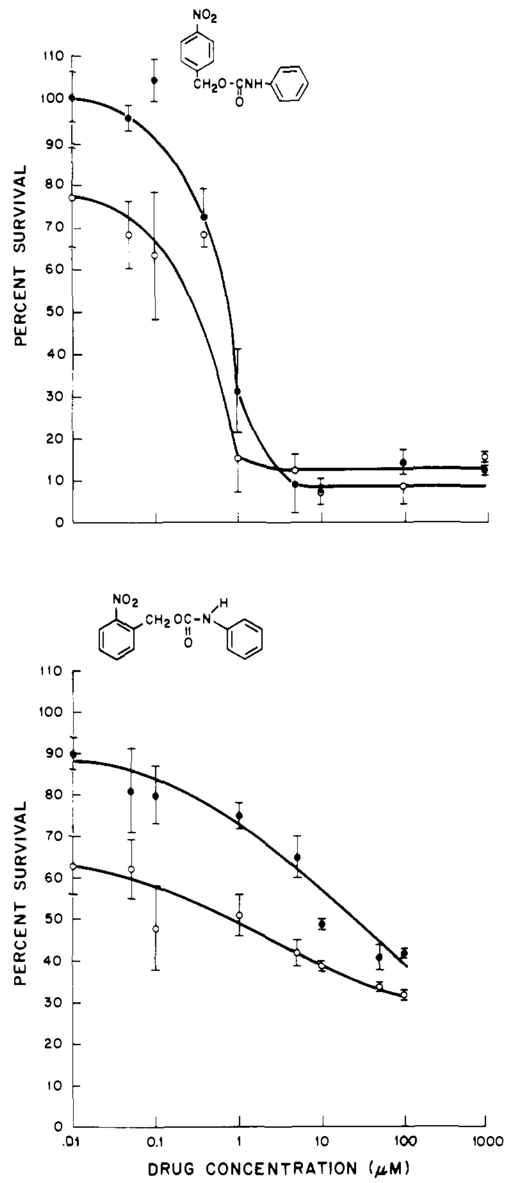

Figure 5. Survival of aerobic (●) and hypoxic (○) EMT6 cells treated for 1 h with various concentrations of *p*-nitrobenzyl *N*-phenylcarbamate (**5**) (top) or *o*-nitrobenzyl *N*-phenylcarbamate (**11**) (bottom) in cell culture.

major limitations, it provides a relatively rapid means of selecting agents with the potential to accomplish selective hypoxic cell kill. One property of major importance for hypoxic cell directed chemotherapeutic agents not detected by this methodology is the ability of the drug to penetrate to poorly vascularized regions of the tumor to reach target hypoxic cells in therapeutically useful concentrations. The ability to diffuse to hypoxic cells has clearly been demonstrated for the nitroimidazole radiosensitizers[2] and for mitomycin C, a naturally occurring bioreductive alkylating agent.[23] In contrast, molecular oxygen is rapidly metabolized and has a relatively short diffusion distance in tissue.[24] It is probable that additional nitroaromatic agents can be developed which retain the features necessary for specificity against hypoxic cells through selective metabolic reduction by this population of neoplastic cells but which do not possess the undesirable cytotoxicity of the existing

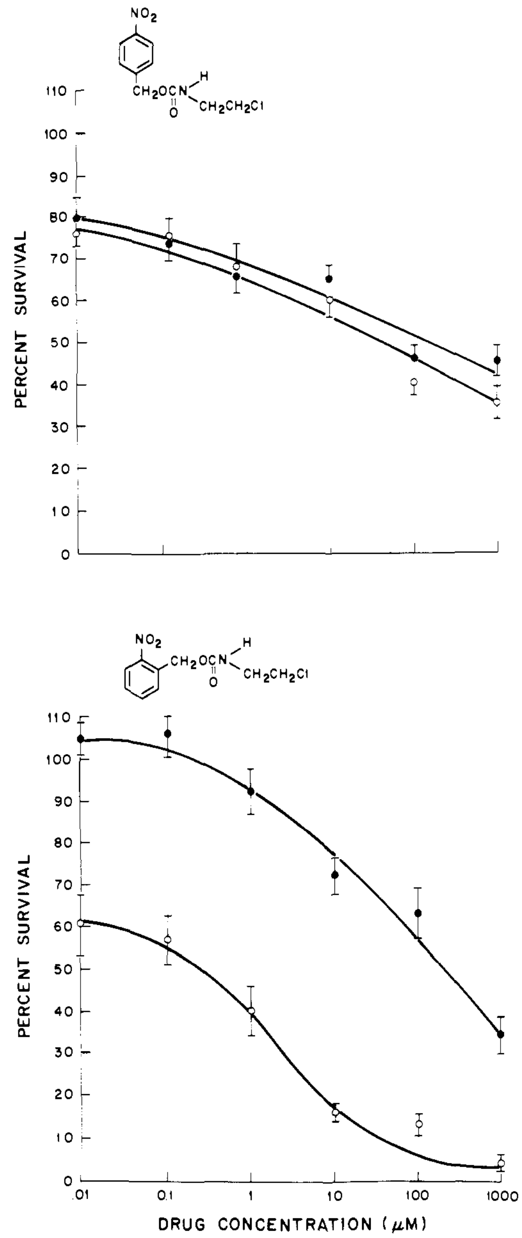

Figure 6. Survival of aerobic (●) and hypoxic (○) **EMT6** cells treated for 1 h with various concentrations of *p*-nitrobenzyl *N*-(chloroethyl)carbamate (**6**) (top) or *o*-nitrobenzyl *N*-(chloroethyl)carbamate (**12**) (bottom) in cell culture.

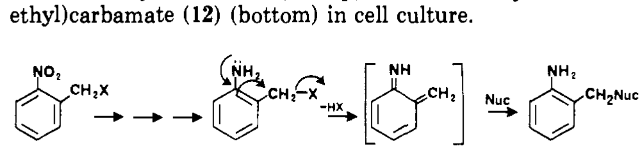

Figure 7. Proposed mechanism for bioreductive activation of o-nitrobenzyl compounds. Nuc represents any available biological nucleophile.

compounds to oxygenated normal cells of tissues. Such drugs would be expected to be of major value in combination with other chemotherapeutic agents in the treatment of solid tumors.

Experimental Section

Biological Methods. The techniques used for propagating and handling of EMT6 tumor cells and for measuring the survival of treated cells in culture have been detailed previously.[25,26] Cells

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were grown as monolayers in 25-cm^2 plastic culture flasks (Corning) in Waymouth's medium supplemented with 15% fetal calf serum. To produce chronic hypoxia, flasks were fitted with sterile rubber sleeve serum stoppers and exposed to an atmosphere of 95% nitrogen/5% CO_2 for 4 h at 37 °C before drug treatment. Parallel flasks were maintained in 95% air/5% CO_2. Drugs were added to each flask in 25 μL of acetone without breaking the hypoxia, and cells were exposed to each agent for 1 h under conditions of chronic hypoxia or normal aeration. At the end of this period, the medium was removed and the cells were washed with 3 mL of sterile phosphate-buffered saline, suspended by treatment with 0.05% trypsin, and plated and cultured in replicate dishes in Waymouth's medium plus 15% fetal calf serum under 95% air/5% CO_2; after 8 to 10 days, cell survival was measured by colony formation. No difference existed between the survival of untreated or vehicle only treated cells maintained under either the aerobic or hypoxic conditions employed; the plating efficiency for these control cultures was 65-80% depending upon the individual experiment. Cells exposed to hypoxic conditions appeared to continue traversing the cell cycle during the course of these experiments, since no decrease in the rate of [^{3}H]thymidine incorporation into acid-insoluble material or in mitotic index was observed after 4 h of incubation in the hypoxic atmosphere (data not shown). Each drug was tested in at least three separate experiments, and the bars on the figures represent the standard error of the mean. Using a two between group analysis of variance (i.e., factorial design: two factors), the aerobic/hypoxic differential exhibited by the *o*-nitrobenzyl compounds was significant at the $p < 0.001$ level.[27] The factors in this test were aerobic vs. hypoxic surviving cell fractions and drug concentration.

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Chemical Methods. All melting points were measured on a calibrated Thomas-Hoover capillary melting point apparatus. Analyses were performed by the Baron Consulting Co., Orange, CT. Analytical results were within ±0.4% of theoretical values. NMR data were obtained using a Varian T-60A spectrometer with Me_4Si as an internal standard. *o*-Nitrobenzyl and *p*-nitrobenzyl alcohols, bromides, and chlorides were obtained from commercial sources and recrystallized from ethanol prior to use in tissue culture.

General Procedure for the Preparation of N-Substituted Carbamates. *o*-Nitrobenzyl alcohol (**7**) or *p*-nitrobenzyl alcohol (**1**) (0.01 mol) was dissolved in a slight molar excess of methyl, phenyl, or chloroethyl isocyanate. Two or three drops of anhydrous pyridine or triethylamine were added to catalyze the reaction, and solutions were warmed for about 5 min. The reaction mixtures were then cooled in a beaker of ice to effect crystallization. All of the carbamates were purified for use in tissue culture by recrystallization from ethanol.

Polarography. First half-wave reduction potentials were measured in 0.05 M potassium phosphate buffer prepared in water-ethanol (1:1, v/v), pH 7.0, by differential pulse polarography (PAR 174A polarographic analyzer). The values were obtained in volts vs. a saturated calomel reference electrode using 0.1 M potassium chloride as the supporting electrolyte.

Acknowledgment. This research was supported by U.S. Public Health Service Grants CA-02817 and CA-16359 from the National Cancer Institute and a grant from the Bristol-Myers Co. B. A. Teicher is a recipient of postdoctoral fellowship (CA-06365) from the National Cancer Institute.

Synthesis and Preliminary Binding Studies of 4,4-Ditritio-(−)-nicotine of High Specific Activity

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4,4-Ditritio-(−)-nicotine (**5**) of high specific activity (4.7 Ci/mmol) has been synthesized from (−)-nicotine via the readily prepared 4,4-dibromocotinine (**3**). Scatchard analysis of the binding of **5** to the crude mitochondrial fraction of whole rat brain revealed a K_a of 4.7×10^6 M^{-1} and 13 fmol of binding sites/mg of protein.

Current interest in nicotine receptors in the central nervous system has created a need for radiolabeled nicotine with high specific activity.[2-4] Commercially available (−)-nicotine-*t* is randomly labeled and varies in both quality and specific activity from batch to batch, while (−)-nicotine-^{14}C is of very low specific activity. We now report the synthesis and some preliminary binding data of 4,4-ditritio-(−)-nicotine (**5**). The distinct advantages of **5** synthesized by our method over commercially available radiolabeled (−)-nicotine are higher specific activity, good stability, and location of the tritium atoms at a specific carbon (4).

Chemistry. A prerequisite of the synthesis of (−)-nicotine (**1**) tritiated at a definite position was the accessibility of a stable intermediate which could be readily hydrogenated (tritiated). 4,4-Dibromocotinine (**3**), first reported by Pinner[5] in 1893 and isolated by Bowman and McKennis[6] as the crystalline HBr·Br_2 complex **2** in an improved procedure, appeared to be such a compound. Conversion of **2** to **3** (Scheme I) in two stages, although more time-consuming than previously reported procedures,[7] was effected in superior yield (~90%).

In "pilot runs", hydrogenolysis of **3** to cotinine (5-oxonicotine)[7] and reduction of cotinine to (−)-nicotine (**1**) was accomplished with H_2 (Pd-$CaCO_3$) in EtOH and $LiAlH_4$, respectively. The resultant **1** could be isolated as the di-*l*-tartrate[8] and was identical with authentic **1**. Furthermore, $LiAlH_4$, reduction of 4,4-dideuteriocotinine,[7] prepared from cotinine and D_2O as described by Glenn and

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