DISCUSSION

The major metabolite of sulpiride in monkeys was obtained by isolation from urine followed by chromatographic purification. The chromatogram in Fig. 3 represents the sample that was submitted for identification by ^{1}H-NMR, chemical ionization mass spectroscopy, and HPLC. Comparison for the ^{1}H-NMR spectra of the metabolite and sulpiride showed that some biotransformation had occurred to the pyrrolidine ring, where it appeared that alpha oxidation had taken place similar to the model compounds, VI–VIII.

This conclusion was further supported by the fact that sulpiride is retained on a cation exchange column, while metabolite II can be eluted with an acidic solvent system. The different chemical behavior of these two species was attributed to the differences in the basicity of the sulpiride pyrrolidine ring nitrogen, which was a tertiary amine prior to biotransformation and subsequently was converted into a cyclic amide of lesser basicity. The synthesis of compound II by an unambiguous route (Scheme I) and analysis by ^{1}H-NMR of the synthetic material gave unequivocal evidence that alpha oxidation had occurred. In addition, a sample of the radiolabeled metabolite and the synthetically prepared sample were compared by HPLC and found to have identical retention times (Fig. 5).

Previous attempts to obtain a mass spectrum on II by electron impact had failed, probably due to the presence of impurities. Subsequently, chemical ionization mass spectral data were obtained on the same sample that had been subjected to ^{1}H-NMR. The spectrum was of good quality and demonstrated that II has a molecular weight of 355. A compound of molecular weight 369 ($\simeq$10%) was also observed. Although its structure has not been identified, it has undergone oxidation at two methylene groups and may be the compound shown by XVI.

Isolation of the metabolite by column chromatography and purification by HPLC with subsequent identification by Fourier transform-NMR, chemical ionization mass spectroscopy, HPLC, and unambiguous chemical synthesis has shown that the structure of the major metabolite of sulpiride is II.

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ACKNOWLEDGMENTS

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Potential Radiosensitizing Agents III: 2-Nitro-4-Acetylimidazole Analogs

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Abstract □ New analogs of 2-nitroimidazole have been synthesized in an effort to minimize the toxicity and increase selective sensitization of hypoxic mammalian cells toward lethal effects of ionizing radiation. 2-Nitro-4(5)-acetyl-5(4)-methylimidazole was synthesized from the corresponding 2-amino analog and then reacted with oxiranes to produce the corresponding 1-substituted 2-propanol and 3-methoxy-2-propanol derivatives. The biological results of radiosensitizing activity of these agents against Chinese hamster cells (V-79) indicated that the 3-methoxy-2-propanol derivative was a more effective radiosensitizer than misonidazole *in vitro*. Evaluation of the acute toxicity of these agents as determined by LD_{50} demonstrated no significant difference between these agents and misonidazole suggesting that the 3-methoxy-2-propanol analog may possess a therapeutic advantage over misonidazole.

Keyphrases □ Radiosensitizing agents, potential—2-nitro-4-acetylimidazole analogs □ 2-Nitro-4-acetylimidazole—analogs, potential radiosensitizing agents □ Analogs—2-nitro-4-acetylimidazole, potential radiosensitizing agents

In a continuing effort to develop new effective radiosensitizers to sensitize selectively the relatively resistant hypoxic tumor cells toward radiotherapy, an additional electron affinic acetyl function has been incorporated into the 2-nitroimidazole nucleus. A direct correlation between the sensitizing efficiency and electron affinity of the radiosensitizers has been demonstrated (1). Initially, a series of 2,4-dinitroimidazoles were synthesized in an effort to increase the electron affinity of the 2-nitroimidazole nucleus (2, 3). The 1-(2-hydroxy-3-methoxypropyl)-2,4-dinitroimidazole was found to be the most effective radiosensitizer of this series (4). However, the 2,4-dinitroimidazole derivatives were found to be generally more toxic than misonidazole (5), an agent currently under clinical trials for evaluation as a radiosensitizer. It was deemed desirable to study the effect of another electron affinic group other than the nitro function at the 4-position of the 2-nitroimidazole nucleus. Accordingly, 4-acetyl substituted 2-nitroimidazole analogs have been synthesized. This modification was thought to be of interest in view of the report that 1-methyl-2-nitroimidazole-5-carboxaldehyde sensitized the hypoxic Chinese hamster cells *in vitro* to ionizing radiation at much lower concentrations (25 μM) than misonidazole (1).

BACKGROUND

It is obvious that an aldehyde function is a metabolically unstable group, and perhaps in addition to high electron affinity, lack of an hydroxyl group in the side chain at the 1-position may have contributed toward enhanced cytotoxicity of this agent. Therefore, the synthesis of 4-acetyl analogs of 2-nitroimidazole with a 2-hydroxypropyl side chain at the 1-position was undertaken. The molecular design of agents described in this report was also related to the structure of metronidazole, a known but less potent radiosensitizer than misonidazole, in that the functional groups at 2- and 5-positions were reversed to provide a 2-nitro-5-methyl analog, since 2-nitroimidazoles have been reported to be more effective radiosensitizers (6).

The synthesis of the required intermediate 2-amino-4(5)-acetyl-5(4)-methylimidazole (III) was accomplished by the known mononuclear 1,2,4-oxadiazole imidazole rearrangement with minor modification of utilizing sodium methoxide in dimethylformamide rather than sodium ethoxide as a base (7). The starting material 3-amino-5-phenyl-1,2,4-oxadiazole, readily obtained from hydrolysis of the *N,O*-dibenzoyl hydroxyguanidine (8), was condensed with an equimolar amount of acetylacetone in anhydrous toluene in the presence of *p*-toluenesulfonic acid

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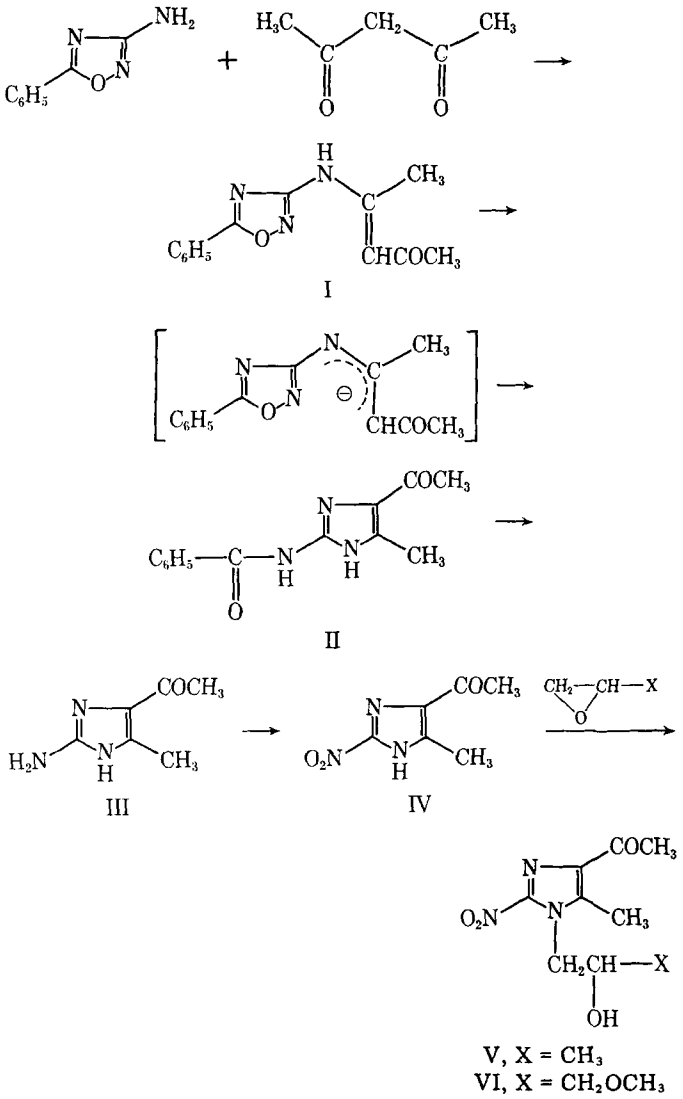

Scheme I

as a catalyst, removing azeotropically the reaction water to give the enaminoketone I (Scheme I). Treatment of I with a molar equivalent amount of sodium methoxide in anhydrous dimethylformamide caused isomerization to the anticipated 2-benzoylaminoimidazole (II). Acid hydrolysis of II afforded the 2-aminoimidazole analog (III). 2-Nitro-4(5)-acetyl-5(4)-methylimidazole (IV) was prepared from the corresponding 2-aminoimidazole derivative (III) by diazotization in fluoroboric acid followed by the reaction of the diazonium salt with nitrous acid in the presence of copper sulfate. Treatment of IV with propylene oxide in absolute ethanol, in the presence of a catalytic amount of sodium hydroxide, afforded Alcohol V. Reaction of IV with 1,2-epoxy-3-methoxypropane during 16 hr at 80° under reflux afforded alcohol VI. In each case the reaction with oxiranes produced the corresponding 4-acetyl analog and not the 5-acetyl isomer, suggesting that the reaction mechanism may be similar to the reaction of 2,4(5)-dinitroimidazoles with oxiranes as reported earlier (4).

EXPERIMENTAL

IR spectra were recorded on a spectrophotometer[1] as potassium bromide pellets. PMR spectra were recorded on a 90-MHz spectrometer[2] using tetramethylsilane as the internal reference. Electron-impact mass spectra were run on a spectrometer[3] at 70 eV ionization potential using direct inlet injection. The elemental analyses[4] were within ±0.4% of the theoretical values. Melting points were determined on glass cover slips[5] and are uncorrected. Analytical TLC was performed on plastic plates coated with a 0.25-mm layer of silica gel[6] GF_{254} and the compounds were detected by visual examination under UV light (254 nm).

[1] Beckman IR-10.
[2] JOEL.
[3] Hitachi Perkin-Elmer RMU-6E.
[4] Performed by the Integral Microanalytical Laboratories, Raleigh, N.C.
[5] Fisher-Johns melting point apparatus.

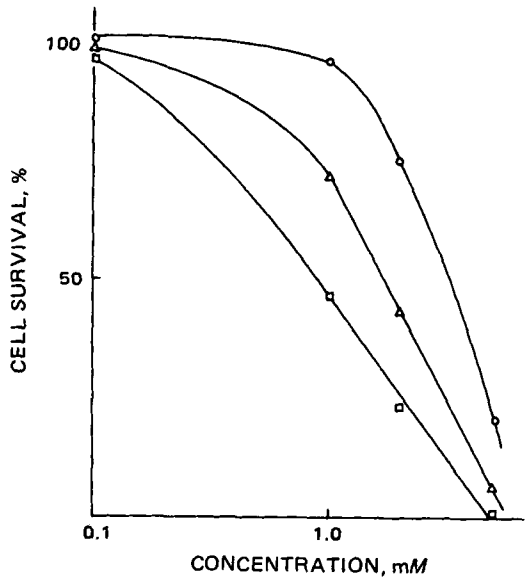

Figure 1—*The effect of Compound V at various time intervals on the survival of hypoxic Chinese hamster cells as a function of drug concentration.*

4(5)-Acetyl-5(4)-methyl-2-nitroimidazole (IV)—4(5)-Acetyl-5(4)-methyl-2-aminoimidazole (III) (0.556 g, 4 mmoles) (7) was dissolved in a mixture containing 10 ml of water, 0.5 ml of sulfuric acid, and 3.5 ml of 50% fluoroboric acid. The solution was then cooled to −20° in an ice–salt bath and a solution of sodium nitrite (2.76 g, 40 mmoles) in 5 ml of water was added dropwise to the cooled 2-aminoimidazolium sulfate solution. The mixture was stirred at −10° for 1 hr and then added to a solution of copper sulfate (19.97 g, 80 mmoles) in 150 ml of water. An additional 2.76 g of sodium nitrite was added to this mixture and stirred at room temperature overnight. The pH of the mixture was then adjusted to ~2.0 with dilute nitric acid. The mixture was repeatedly extracted with ethyl acetate (8 × 30 ml), dried over sodium sulfate, and the solvent removed under reduced pressure to leave a yellow residue, which was recrystallized (ethyl ether–hexane) to afford 210 mg (31%): mp 155–156°; IR (KBr) 1652 (CO) 1540 and 1342 (NO_2) cm^{-1}; NMR ($CDCl_3$) δ 2.68 [s, CH_3CO (CH_3)], 2.72 [s, $CH_3(CH_3CO)$]; MS, *m/z* 169 (M^+), 154 (M—CH_3), 122 [M—(NO_2+H)], 108 [M—(NO_2+CH_3)].

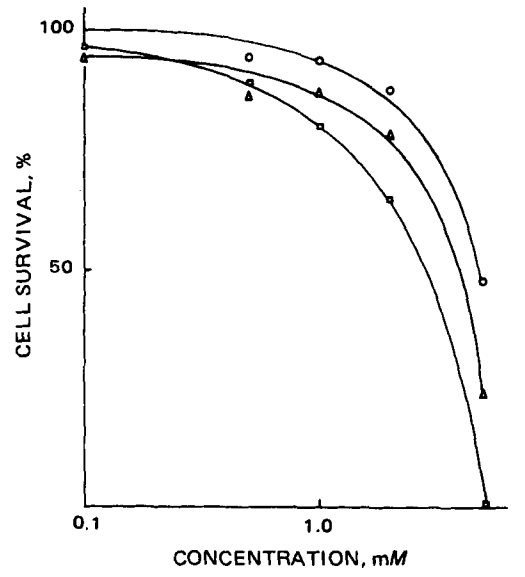

Figure 2—*The effect of Compound VI at various time intervals on the survival of hypoxic Chinese hamster cells as a function of drug concentration.*

[6] E-Merck AG, Darmstadt, Germany.

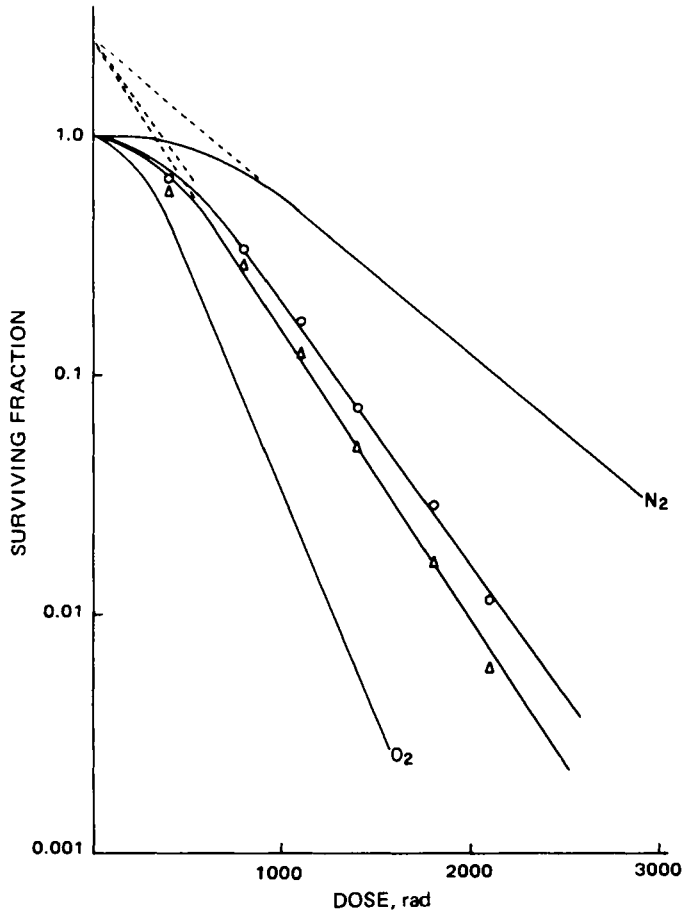

Figure 3—*The radiation survival curves of 2-nitro-4-acetylimidazole analogs by employing Chinese hamster cells (V-79). Key: N_2, cells irradiated in nitrogen; O_2, cells irradiated in air; (○) Compound VI, 1 mM; (△) Compound V, 1 mM. Each point represents the mean of three different experiments.*

Anal.—Calc. for $C_6H_7N_3O_3 \cdot H_2O$: C, 38.50; H, 4.81; N, 22.46. Found: C, 38.23; H, 4.75; N, 22.39.

Reaction of 4(5)-Acetyl-5(4)-methyl-2-nitroimidazole (IV) with Propylene Oxide—Propylene oxide (10 ml) and sodium hydroxide (15 mg) were added to a solution of IV (0.169 g, 1 mmole) in 25 ml of absolute ethanol. The reaction mixture was stirred at room temperature for 8 hr. However, the reaction was found to be negligible as followed by TLC in ethyl acetate. This reaction was completed upon heating the mixture to 40–45° under reflux for 60 hr. The solvent and excess oxirane were removed under reduced pressure to leave a residual oil which was recrystallized (ethyl ether–hexane) to afford 125 mg (55%) of alcohol (V): mp 134–135°; IR (KBr) 3360 (OH), 1650 (CO), 1540 and 1340 (NO_2) cm^{-1}; NMR ($CDCl_3$) δ 2.60 [s, $CH_3CO(CH_3)$], 2.72 [s, $CH_3(CH_3CO)$], 4.36 (m, NCH_2), 4.60 (m, CHO), 1.36 (d, CH_3): MS, *m/z* 227 (M^+), 212 (M—CH_3) 210 (M—OH), 180 [M—(NO_2+H)].

Anal.—Calc. for $C_9H_{13}N_3O_4$: C, 47.58; H, 5.73; N, 18.50. Found: C, 47.61; H, 5.68; N, 18.46.

Reaction of 4(5)-Acetyl-5(4)-methyl-2-nitroimidazole (IV) with 1,2-Epoxy-3-methoxypropane—A solution of IV (0.169 g, 1 mmole) in 2.5 ml of 1,2-epoxy-3-methoxypropane was initially stirred at room temperature for 8 hr. The reaction was found to be negligible as followed by TLC in ethyl acetate; however, it was completed upon further heating the solution to 80° under reflux for 16 hr. The excess oxirane was removed under diminished pressure to leave a residual oil, which was recrystallized (ethyl ether) to afford 148 mg (58%) of VI: mp 126–127°; IR (KBr) 3367 (OH), 1667 (CO), 1540 and 1350 (NO_2) cm^{-1}; NMR ($CDCl_3$) δ 2.64 [s, $CH_3CO(CH_3)$], 2.76 [s, $CH_3(CH_3CO)$], 4.28 (m, NCH_2), 4.64 (m, CHO), 3.60 (d), and 3.51 (s, CH_2OCH_3): MS, *m/z* 257 (M+), 242 (M—CH_3), 240 (M—OH), 211 (M—NO_2).

Anal.—Calc. for $C_{10}H_{15}N_3O_5$: C, 46.69; H, 5.84; N, 16.34. Found: C, 46.75; H, 5.62; N, 16.41.

Biological Studies—The cytotoxicity studies were carried out by employing asynchronous monolayer cultures of Chinese hamster cells (V-79). The techniques used for culturing and handling this cell line were followed as previously reported (2). To determine differential cytotoxicity between the oxic and hypoxic cells, ~200 cells were plated in petri dishes[7] (60 × 15 mm) containing 3 ml of minimum essential medium with 15% fetal calf serum and allowed to attach for 2 hr. The medium was then removed by aspiration and replaced by the medium containing an appropriate concentration of the compound under study. The cells were exposed to a range of concentrations of each compound for intervals of 2, 4, and 8 hr at 37° in air or in hypoxia. The hypoxia was achieved by placing the open petri dishes in sealed containers and then purging with nitrogen–carbon dioxide (95:5). The gas mixture was bubbled through a flask containing sterile water before entering the sealed containers to maintain humidity inside the chambers. At the end of the specific time interval, the medium containing the drug was removed and replaced with 3 ml of fresh medium. Cultures were incubated for 6 days at 37° in an atmosphere of air–carbon dioxide (95:5); the resulting colonies were fixed in absolute ethanol, stained with methylene blue, and counted.

The radiosensitization studies were carried out by irradiating the cells inside the sealed containers under hypoxia by employing a cobalt 60 source at a dose rate of ~240 rad/min. Complete survival curves were obtained for each compound at radiation doses of 400–3000 rad under oxic and hypoxic conditions. The D_0 values were calculated for the hypoxic control cells and for the hypoxic cells treated with the drug. The ratio of these two D_0 values provided the sensitizer enhancement ratio of the corresponding agent.

Partition Coefficients—The compounds were dissolved in phosphate buffer (0.1 *M*, pH 7.4) and were then stirred with an equal volume of octanol at room temperature for 1 hr. The concentration of the nitroimidazoles in the aqueous phase was determined spectrophotometrically at 320 nm.

Table I—Sensitizer Enhancement Ratios, LD_{50} Values, and Partition Coefficients of 2-Nitro-4-acetylimidazole Analogs

Compound	SER[a]	LD_{50}[b], mg/kg	PC[c]
V	2.1	1,200	2.46
VI	1.9	1,400	1.07
Misonidazole	1.9	1,300	0.43

[a] Sensitizer enhancement ratio determined by dividing the D_0 value for the control hypoxic cells with the D_0 value obtained for the cells irradiated in the presence of the sensitizer. [b] LD_{50} values were determined in C57BL mice by the standard procedures. [c] Partition coefficients.

RESULTS AND DISCUSSION

Both Compounds V and VI were essentially nontoxic to the Chinese hamster cells up to a concentration of 5 m*M* when exposed to a maximum period of 8 hr under oxic conditions. However, when the cells were exposed to various drug concentrations under nitrogen, Compounds V and VI were differentially more toxic to the hypoxic cells, and the toxicity increased as a function of time with increased concentration (Figs. 1 and 2, respectively). Similar differential cytotoxicity has been reported for other nitroimidazoles (9) and has been related to the metabolic reduction of the drug under hypoxia, causing the production of the reduced intermediates that are toxic to the cells (10). Compound V was comparatively more toxic to the hypoxic Chinese hamster cells than VI. The hypoxic toxicity increased with time and drug concentration. Compound V, although nontoxic, at 1.0 m*M* concentration upon 2 hr of exposure, caused >50% inhibition by 8 hr (Fig. 1). Compound VI did not show significant differential cytotoxicity up to a concentration of 1 m*M* and required higher concentrations and increasing incubation time for hypoxic cytotoxicity (Fig. 2).

The radiosensitizing efficiency of these agents was assessed from the radiation survival curves of hypoxic Chinese hamster cells (Fig. 3). These experiments were conducted at a nontoxic concentration of 1 m*M*, and the sensitizer enhancement ratios were calculated by dividing the D_0 value for the control hypoxic cells with the D_0 value obtained for the cells irradiated in the presence of the radiosensitizer under nitrogen. The enhancement ratios for Compounds V and VI were found to be 2.1 and 1.9, respectively (Table I). Misonidazole, under similar conditions, produced an enhancement ratio of 1.9 at 1 m*M* concentration, suggesting that V is, comparatively, a more effective radiosensitizer.

Although 2,4-dinitroimidazole derivatives have been shown to be more efficient radiosensitizers than 2-nitroimidazoles *in vitro* (4), the powerful electron withdrawing group at the 4-position also causes an increase in

[7] Permanox, Lux Scientific Corp.

cytotoxicity (5). In attempts to develop radiosensitizers relatively less toxic than misonidazole, the results obtained upon introducing an acetyl function at the 4-position of 2-nitroimidazole have been described in this report. The acetyl function, although relatively less powerful than the nitro group with respect to the electron withdrawing capacity, has been reported to possess radiosensitizing properties (11). It was contemplated that the insertion of an acetyl function in the 2-nitroimidazole molecule may provide analogs which are comparatively less toxic than the 2,4-dinitroimidazoles. The *in vivo* acute toxicity of these agents was assessed by determining the LD_{50} in C57BL mice. Compounds V and VI were found to have an LD_{50} of 1.2 and 1.4 g/kg, respectively (Table I). These values are similar to misonidazole, which had an LD_{50} of 1.3 g/kg but are greater than the LD_{50} of 2,4-dinitroimidazoles (5). These agents are relatively less toxic than the corresponding dinitroimidazoles as expressed by the doses required for acute toxicity. The partition coefficients of these agents were determined in an octanol–phosphate buffer (pH 7.4) mixture. Compounds V and VI are more lipophilic than misonidazole (Table I), a property that would be expected to be favorable for *in vivo* diffusion into the hypoxic region of the tumors. This work has demonstrated that an additional electron affinic group could be inserted in the misonidazole molecule without significantly increasing the acute toxicity and yet enhancing the sensitizing efficiency. Thus, V may possess a therapeutic advantage over misonidazole as a radiosensitizer.

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The Hydrolysis of Spirohydantoin Mustard

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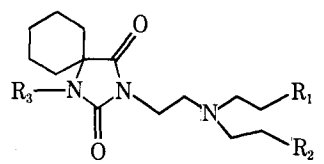
Received July 23, 1981, from the *Pharmaceutical Resources Branch, and the Laboratory of Medicinal Chemistry and Biology, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, MD 20205.* Accepted for publication January 13, 1982.

Abstract □ Spirohydantoin mustard (I) is a rationally designed antitumor agent with substantial *in vivo* activity against intracranially implanted tumors in mice. However, hydrolysis of I was much faster than that of mechlorethamine hydrochloride or melphalan, two parenterally administered mustards. The hydrolysis products of I were identified by GC–MS of their silylated derivatives. The decomposition of I (at 25° in 10% dimethylacetamide at pH 4–6), as monitored by GLC was pseudo first-order. The half-life of I ranged from 20 min at pH 4.0 to 14 min at pH 6.0. Nonionic surfactants enhanced the stability of I, but this effect was diminished at lower pH, presumably due to decreased solubility of I in the micelle as more drug was protonated. Several dilute parenterally suitable solvents exhibited no marked effect on the hydrolysis of I. The drug was most stable in a 10% fat emulsion system where the time for 10% decomposition of I was 49 ± 5 min. Plots of the concentration of I *versus* time were linear indicating the disappearance was zero order in the 10% fat emulsion system.

Keyphrases □ Spirohydantoin mustard—hydrolysis, antitumor agent, mechlorethamine hydrochloride, melphalan, mice □ Hydrolysis—spirohydantoin mustard, antitumor agent, mechlorethamine hydrochloride, melphalan, mice □ Antitumor agents—hydrolysis of spirohydantoin mustard, mice

Spirohydantoin mustard (I) (NSC-172112), 3-[2-[bis(2′- chloroethyl)amino] - ethyl] - 5,5 - pentamethylenehydantoin, is a potential antitumor agent that has been designed specifically for use against central nervous system tumors. Compound I has demonstrated significant antitumor activity against L-1210 leukemia, P-388 leukemia, B-16 melanoma, and ependymoblastoma when administered in an aqueous suspension by the intraperitoneal route (1). Compound I was designed to incorporate a mustard alkylating function coupled to a substituted hydantoin carrier to provide an overall optimum partition coefficient for crossing the blood–brain barrier (1). A preliminary pharmacological study in dogs indicates that I does enter the central nervous system, although actual concentrations in cerebrospinal fluid are low (2).

As is often the case with nitrogen mustard derivatives, solution decomposition problems are evident. The aqueous stability of I and related compounds is such that octanol–water partition coefficients could not be determined (1). Preliminary chloride titration data also indicate that the decomposition of I in aqueous solution is rapid. In fact, the decomposition of I is more rapid than the hydrolysis of either of two clinically useful mustards, melphalan or mechlorethamine hydrochloride. In addition, the aqueous solubility of I is limited (<10 μg/ml) (3). Dissolution in concentrated acid with subsequent dilution is possible (1.25 mg/ml); however, the upward adjustment of pH usually results in precipitation of the drug. Likewise, I has



I $R_1 = R_2 = -Cl$; $R_3 = -H$
Ia $R_1 = R_2 = -Cl$; $R_3 = -$Trimethylsilyl or $-Si(CH_3)_3$
II $R_1 = -Cl$; $R_2 = -OH$; $R_3 = -H$
IIa $R_1 = -Cl$; $R_2 = -OSi(CH_3)_3$; $R_3 = -Si(CH_3)_3$
III $R_1 = R_2 = -OH$; $R_3 = -H$
IIIa $R_1 = R_2 = -OSi(CH_3)_3$; $R_3 = -Si(CH_3)_3$
IV $R_1 = -OH$; $R_2 = -OCOCH_3$; $R_3 = -H$
IVa $R_1 = -OSi(CH_3)_3$; $R_2 = -OCOCH_3$; $R_3 = -Si(CH_3)_3$

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