In summary, a sensitive and relatively specific method employing reverse-phase high pressure liquid chromatography and radioimmunoassay has been used to quantify both methionine-enkephalin and leucine-enkephalin in monkey brain. Chronic morphine treatment resulted in a statistically significant decrease in methionine-enkephalin concentration, which was reversed by acute naltrexone treatment.

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Identification of two major reduction products of the hypoxic cell toxin 3-amino-1,2,4-benzotriazine-1,4-dioxide

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Recently, it has been shown that 3-amino-1,2,4-benzotriazine-1,4-dioxide (SR-4233) exhibits a 50- to 200-fold greater toxicity for hypoxic relative to aerobic mammalian cells *in vitro* (E. M. Zeman and J. M. Brown, personal communication)*. Since the presence of hypoxic cells in solid tumors is believed to be a problem for the local control of tumors by radiation therapy, compounds that are selectively toxic for such radiation resistant cells have been proposed as adjuncts to radiation [1]. While two classes of compounds, the mitomycins [2] and the nitroimidazoles [3], are established as agents with selective toxicity toward hypoxic mammalian cells, SR-4233 represents a new class of these agents. Although the selectivity of SR-4233 implies that it is activated by reductive metabolism which is oxygen sensitive, little is known about the reduction chemistry of this molecule aside from applications to synthetic chemistry [4]. In an attempt to elucidate this chemistry, SR-4233 has been reduced in the absence of oxygen using radiation chemical, electrochemical and enzymatic systems. Two major reaction products of these systems have been isolated and characterized with the hope that this information may help in determining the nature of the cytotoxic agent or agents.

* Permission received from J. M. Brown, Stanford University, Palo Alto, CA, to cite this unpublished data.

Materials and methods

The compounds SR-4233, 3-amino-1,2,4-benzotriazine-1-oxide (SR-4317), and 3-amino-1,2,4-benzotriazine (SR-4330) were obtained from Dr. W. Lee of SRI International. Xanthine oxidase (grade III) and xanthine were purchased from the Sigma Chemical Co., St. Louis, MO, and DMSO-d_6 and trifluoroacetic acid-d_1 were obtained from MSD Isotopes, Montreal, P.Q.

Ultraviolet-visible spectra were recorded with a Varian 219 UV-visible spectrophotometer, and proton magnetic resonance (PMR) spectra were acquired with a Nicolet 360 MHz NMR spectrometer at the University of Toronto Biomedical NMR Centre. Analytical and preparative high pressure liquid chromatography (HPLC) were performed isocratically using Waters μBondapak C_{18} columns (3.9 mm × 30 cm; 7.8 mm × 30 cm) with a mobile phase of 20% methanol/water or 20% methanol/10% acetonitrile/water. A flow rate of 2 ml/min was used, and the eluant was passed through a u.v. detector set at 254 nm.

Reducing radicals were produced radiolytically using an AECL ^{60}Co gamma-cell (Atomic Energy of Canada Ltd., Chalk River, Ont.) (~3250 rads·min^{-1}). Solutions for irradiation were typically 0.5 mM in SR-4233, 100 mM in sodium formate, and 10 mM in sodium phosphate (pH 6.9) [5]. Small volumes (~1.5 ml) of the solutions were placed

in glass vials and deoxygenated by bubbling with pre-purified N_2 prior to sealing. The progress of the reduction was monitored by u.v.-visible spectroscopy (λ_{max} in H_2O at 263 nm; $\varepsilon \sim 38{,}000$). For preparative work, 100-ml 0.5 mM solutions of SR-4233 containing 0.1 M isopropanol/water [6] acidified to pH 2 were irradiated until spectra showed that the parent compound was completely reduced, and then the solutions were frozen and lyophilized to dryness. Concentrated, neutralized solutions were prepared and then fractionated by preparative HPLC. Subsequent experiments showed that the same products were formed in the pH 2–8 range. Appropriate fractions were pooled, frozen, and lyophilized, and the dry solids were dissolved in ~1 ml of DMSO-d_6 for PMR spectroscopy.

Electrochemical reductions were carried out under N_2 with 0.5 mM solutions of SR-4233 in 1 M KCl and 10 mM sodium phosphate (pH 6.9) over a mercury pool cathode held at −800 mV relative to a standard calomel electrode [7].

Enzymatic reduction employed xanthine oxidase. Solutions contained 10 mg of SR-4233 (5.6×10^{-5} moles) and 43 mg of xanthine (28×10^{-5} moles). The xanthine was dissolved in 16 ml of 0.1 N NaOH and the solution was diluted to 200 ml with 10 mM sodium phosphate (pH 7.8) [8]. The solutions were kept essentially anoxic by continuous bubbling with pre-purified N_2 which had been further deoxygenated by passage through a solution of 0.1 M $Na_2S_2O_4$/0.1 M Na_2CO_3. In each reduction system, 3.4 units (75 μl) of xanthine oxidase were present, and each system was protected from light for 24–48 hr.

The products of the various reductions were analyzed by HPLC. The isopropanol radiation reduction and the xanthine oxidase reduction products were purified by HPLC for characterization by PMR spectroscopy.

* This ignores absorption at the solvent front.

Results and discussion

Figure 1 illustrates the progress of a typical radiation reduction of SR-4233 which was monitored by u.v.-visible spectroscopy. The absorption maxima of the parent compound at ~263 and ~460 nm initially declined linearly with time, and product absorptions appeared and increased concurrently at ~234 and ~410 nm. No reduction was detectable in the presence of air. Figure 2A summarizes the time dependence of a radiation chemical reduction that was monitored by HPLC analysis at 254 nm. On the basis of the chromatographic mobilities of these major* reduction products and of the triazine 1-*N*-oxide SR-4317 and the triazine SR-4330, the first of these products was 3-amino-1,2,4-benzotriazine-1-oxide (SR-4317), and the second was 3-amino-1,2,4-benzotriazine (SR-4330). The retention volumes were 29 ml and 26 ml respectively. These assignments were confirmed by PMR spectroscopy as described below.

Assuming a steady-state production of reducing species of 6.1 μmoles of one-electron-donors per krad [5], the data for the loss of the chromatographic peak for SR-4233 (retention volume of 13 ml) in the formate radiation chemical reduction indicate that the compound was reduced with an apparent stoichiometry of 0.52 ± 0.04 reducing species per molecule (average of four separate experiments).

The progress of a typical electrochemical reduction of SR-4233 is shown in Fig. 2B. While the observed time dependence was different from that of the radiation chemical reduction system, a comparison of the chromatograms for SR-4317 and SR-4330 with those for the electrochemical reduction system shows that the same two major products were formed. The chromatograms for the radiation chemical and the xanthine oxidase reduction systems showed the same major products as that for the electrochemical reduction of SR-4233. The stoichiometry of reduction of SR-4233 could not be simply determined in the latter two

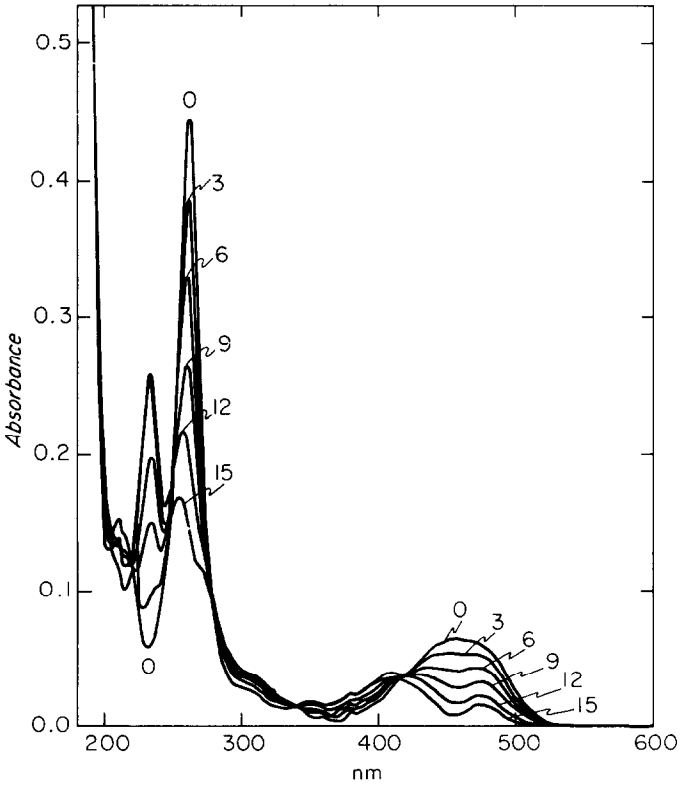

Fig. 1. Ultraviolet-visible spectrum of a radiation chemical reduction of SR-4233 in the formate system (0.5 mM; pH 7). Samples were diluted 10-fold and spectra were measured in a 0.2-cm path length cell.

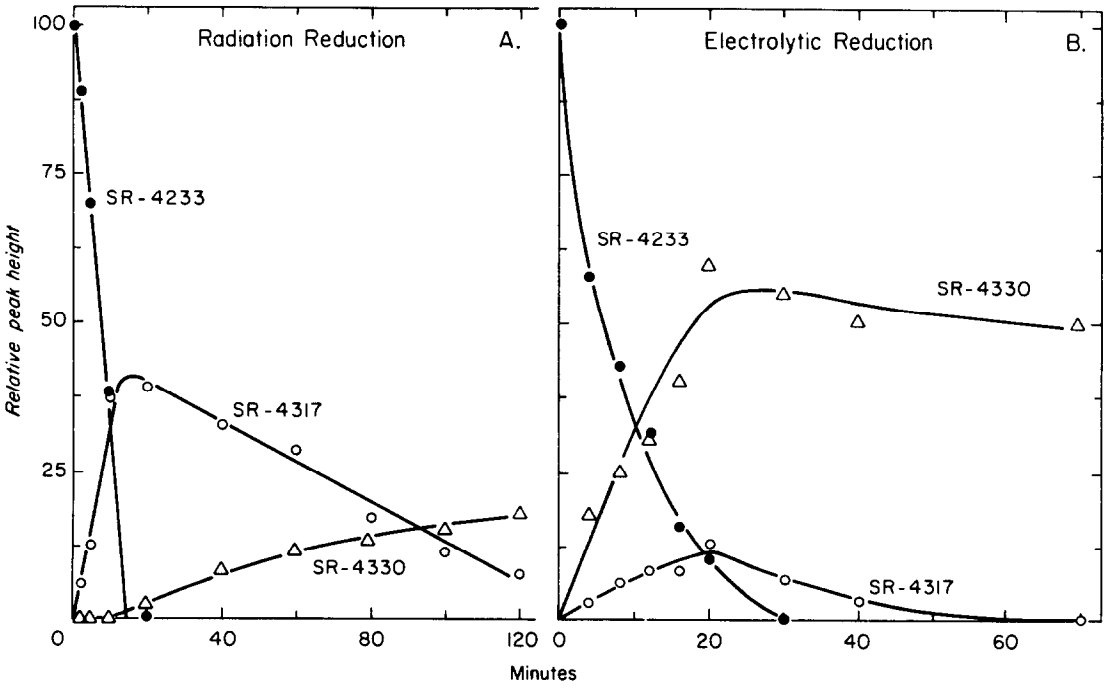

Fig. 2. Time–course of the reduction of SR-4233 as monitored by relative HPLC peak height. (A) Radiation chemical reduction. (B) Electrochemical reduction.

systems since the two major products are formed simultaneously with the loss of the parent compound.

The PMR chemical shifts and multiplicities of the authentic reduction products of SR-4233 are presented in Table 1. If the chemical shift values for the synthesized compounds are compared with those for the two products of the radiation chemical reduction, it is clear that the first product was the triazine 1-*N*-oxide SR-4317, and the second product was the triazine SR-4330. A comparison of the data for the final product of the enzymatic reduction of SR-4233 (xanthine oxidase reduction: second product) with that for SR-4330 establishes the identity of this product as the triazine. The triazine 1-*N*-oxide SR-4317, which is also formed enzymatically, can be isolated but its abundance relative to that of SR-4330 decreases with increasing reduction time.

The proton assignments of the PMR spectra of SR-4317 and SR-4330 are based upon earlier studies of SR-4233 and some of its derivatives [9]. In particular, it is assumed that positions 5 and 7 in both SR-4317 and SR-4330 are electron-rich due to electron-donation by the conjugated 3-amino group. The previous study showed that, in trifluoroacetic acid-d_1, the presence of the *N*-oxide group at position 1 in SR-4317 causes a downfield shift of the peak for H_a (position 8) relative to the corresponding peak in SR-4330, and this has been confirmed (data not shown). However, in DMSO-d_6, this relative order of peaks is reversed.

The apparent stoichiometry (~0.5 reducing species) of the radiation chemical reduction of SR-4233 is reproducible. The HPLC peak for the parent compound is well-resolved and thus can be used for a direct calculation of the stoichiometry. A plausible physical interpretation of this result is the operation of an autocatalytic or chain mechanism for the reduction of SR-4233 to the triazine 1-*N*-oxide SR-4317 (a two-electron reduction) via a radical anion. Hypothetically, following protonation of the oxygen at position 4 of the radical anion, and subsequent homolysis of the (N—O) bond, a hydroxyl radical could be formed which could generate $CO_2^{\cdot}$ from a formate ion in solution. Presumably, the oxygen inhibition of the overall process involves the removal of the added electron by O_2 to form $O_2^{\cdot}$. Preliminary studies indicate that very low levels of oxygen (~100 ppm) are able to inhibit the radiation reduction significantly.

The triazine 1-*N*-oxide SR-4317 and the triazine SR-4330 were the major products of all three reduction systems, although the time dependencies of their formation differed from system to system, as might be expected. However, attempts to quantitate their production relative to loss of the parent compound using u.v.-visible absorption indicate that only ~50% of the reduction products of SR-4233 was SR-4317 and SR-4330. A more complete analysis will require a radioactively tagged parent compound and this is planned using SR-4233 labeled with ^{14}C at the 3 position [9, 10].

Evidence that SR-4317 is the only metabolite which is produced *in vitro*, and that this compound is not selectively toxic for hypoxic cells (E. M. Zeman and J. M. Brown, personal communication)*, suggests that the toxic species is a radical form. An early study of an analogous class of molecules, the quinoxaline di-*N*-oxides, showed that radical species may be involved in the toxicity of these compounds towards hypoxic bacteria [11]. It is possible that the proposed analysis of the initial reduction chemistry of SR-4233 could uncover evidence for such species.

It is clear that the benzotriazine-di-*N*-oxides represent a potentially novel class of compounds whose mechanism of selective toxicity towards hypoxic cells may well differ from previously identified hypoxic cell toxins [2, 3]. Further studies of SR-4233 and related analogues will be required to determine the potential usefulness of these compounds in studies of tumor biology and in possible clinical applications.

In summary, the triazine di-*N*-oxide 3-amino-1,2,4-benzotriazine-1,4-dioxide (SR-4233) is a potent hypoxic cell toxic agent. The reduction chemistry of SR-4233 has been investigated using radiation chemical, electrochemical

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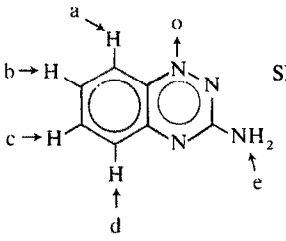
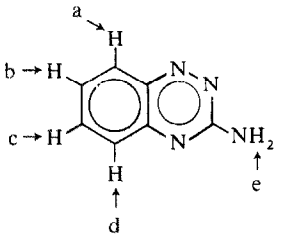
Table 1. Chemical shifts of SR-4317 and SR-4330 (relative to DMSO at δ2.490)





	H_a (1H, d of d)	$H_b + H_c$ (3H, m)*	H_c (1H, m)	H_d (1H, d)*
SR-4317	8.131, 8.128	7.347, 7.344	7.788, 7.784	7.527
	8.106, 8.104	7.329, 7.325	7.768, 7.764, 7.760	7.504
			7.745, 7.741	
RC first	8.130, 8.128	7.351, 7.347	7.791, 7.767	7.532, 7.529
product	8.108, 8.104	7.332, 7.328, 7.324	7.772, 7.768, 7.764	7.508, 7.505
		7.307, 7.303	7.746, 7.744	

	H_a (1H, d of d)*	H_b (1H, m)	H_c (1H, m)	H_d (1H, d)*	H_e (2H, broad s)
SR-4330	8.191, 8.188, 8.187	7.460, 7.465	7.805, 7.801	7.530	7.607
	8.167, 8.164	7.449, 7.445, 7.442	7.786, 7.782, 7.778	7.506	
		7.426, 7.423	7.763, 7.759		
RC second	8.194, 8.190, 8.189	7.472, 7.468	7.809, 7.805	7.534, 7.531	7.607
product	8.171, 8.169, 8.166	7.453, 7.449, 7.445	7.791, 7.786, 7.782	7.510, 7.507	
		7.430, 7.426	7.767, 7.762		
XO second	8.193, 8.190	7.472, 7.460	7.809, 7.805	7.533, 7.531	7.606
product	8.170, 8.167	7.452, 7.449, 7.445	7.790, 7.786, 7.782	7.510, 7.508	
		7.429, 7.426	7.767, 7.763		

Abbreviations: RC, radiation chemical reduction; and XO, xanthine oxidase reduction.

* Fine structure was identified by computer in dilute samples. Nonetheless, all corresponding spectra are superimposable.

and enzymatic techniques. Two major reduction products of these systems are the 1-*N*-oxide and the fully reduced triazine. The reduction stoichiometry (~0.5 reducing species) of SR-4233 suggests a unique process for the formation of the 1-*N*-oxide which may relate to the toxicity of the parent compound.

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