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ELECTROCHEMICAL STUDIES AND DNA DAMAGING EFFECTS OF THE BENZOTRIAZINE-*N*-OXIDES

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Abstract—The electrochemical behaviour of eight benzotriazine 1,4 di-*N*-oxides has been examined and compared with the mono- and zero-*N*-oxides. The di-*N*-oxides all show two reduction steps, an irreversible followed by a quasi-reversible response assigned to the 4 electron reduction of both *N*-oxide groups, followed by the 2 electron reduction of the benzotriazine ring. Mono- and zero-*N*-oxides show only a single, quasi-reversible reduction step, similar in character to the second reduction of the di-*N*-oxides. This has been assigned to reduction of the benzotriazine ring, with the available, redox-active, *N*-oxide group of the mono-*N*-oxide complex being reduced at less negative potentials, but only after ring reduction, hence only a single electrode response. The importance of reductive activation of the *N*-oxide group has been examined using a ΦX174 double transfection technique which assays biologically relevant DNA damage. For the di-*N*-oxides, no effect on DNA was recorded under oxic conditions, however, DNA damage was marked under anoxic reduction conditions. The extent of DNA damage was found to increase with the acidity of the medium, suggesting the protonated form of the reduction product as being responsible for the cytotoxic action. The mono-*N*-oxide was shown to be biologically inactive under all conditions.

A series of benzotriazine di-*N*-oxides are currently attracting considerable attention as a new class of anti-tumour drugs which exhibit preferential toxicity towards hypoxic cells. The lead compound of the series, SR4233, (3-amino-1,2,4-benzotriazine-1,4-dioxide) has been reported to show a greater selectivity for killing hypoxic cells than both the bioreductive quinone alkylating agents and the nitroimidazole radiosensitizers [1]. The precise mode of action is unknown, although it has been suggested [2] that the mechanism may be oxidation of sugar residues in DNA (effectively H abstraction from carbon) by the protonated conjugate of the radical intermediate. Selectivity to hypoxic cells implies a drug activation by reductive metabolism which is O_2 sensitive [3]. It is proposed that the one-electron addition product is the most likely damage-causing species as neither of the two major reduction products, SR4317 (3-amino-1,2,4-benzotriazine-1-oxide) and SR4330 (3-amino-1,2,4-benzotriazine), two and four electron additions respectively, are toxic to hypoxic cells *in vitro*.

Previous studies on the reduction mechanism, using pulse radiolysis, electrochemical and enzymatic reduction techniques [2, 4] but also by *in vitro* metabolism [3], have focused on the identification and distribution of the electron addition products with time for SR4233. In an attempt to understand better the mode of action of these compounds and identify the cytotoxic agent(s) we have conducted a detailed investigation into the electrochemical reduction characteristics of eight benzotriazine di-*N*-oxides with the mono- and zero-*N*-oxides, SR4317 and SR4330, included for comparison. To study further the cytotoxic mechanism and the biological implications of the redox activation step we have examined the drug-induced DNA damage produced oxically and during anoxic controlled potential electrolytic reduction for SR4233 and SR4317 using a ΦX174 double transfection technique.

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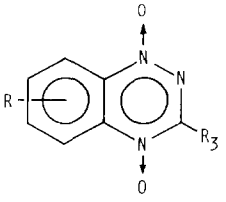
MATERIALS AND METHODS

All drugs were obtained from Prof. J. M. Brown, Stanford University, California. Single stranded (ss) ΦX174 was prepared according to Blok *et al.* [5] and was obtained from Prof. M. V. M. Lafleur, Vrije Universiteit, Amsterdam. The *Escherichia coli* strains, AB1157 and *E. coli* C (the natural host of ΦX174 DNA) were also obtained from Prof. Lafleur. DNA from *E. coli* was purchased from the Sigma Chemical Co. (Poole, U.K.) and further prepared as described previously [6]. All other chemicals were analytical grade and used as received without further purification.

Electrochemical methods. Voltammetric experiments were carried out in 1.5×10^{-2} mol/dm^3 NaCl and 1.5×10^{-3} mol/dm^3 trisodium citrate buffer (0.1 SSC), pH 7.4, purged with H_2O-saturated N_2. Cell solutions were typically 1×10^{-4} mol/dm^3 with respect to drug concentration. Measurements were performed using a PAR 264A polarographic analyser interfaced with a PAR 303 cell stand employing a 3-electrode cell configuration and a Bausch and Lomb RE 0088 recorder. An aqueous Ag/AgCl reference electrode and a Pt wire counter electrode were used in a 5–10-mL glass cell. Differential pulse and dc polarography employed a dropping mercury electrode (dme) with an electronically controlled drop-time (t_d) of 1 sec, and a routine scan rate of 5 and 2 mV/sec respectively. Cyclic voltammetry (CV) used a hanging drop mercury electrode (hdme) with scan rates from 10 to 500 mV/sec.



SR no	R	R_3	OXIDES
4233	H	NH_2	2
4286	6(7)-OMe	NH_2	?
4308	6(7)-Cl	NH_2	2
4317	H	NH_2	1
4318	7-Cl	ONH_4	2
4330	H	NH_2	0
4355	H	ONa	?
4452	H	NHCOMe	2
4453	H	$NHCOCH_2COMe$	2
4466	$7\text{-}OCH_2CH{=}CH_2$	NH_2	2

Fig. 1. Structures of the benzotriazine-*N*-oxides.

Bulk scale reduction was carried out at a constant potential approximately 200 mV negative of the overall redox process in 1.0 SSC buffer under an N_2 atmosphere. The two electrode cell consisted of a mercury pool as the cathode and Ag/AgCl as the anode. Coulometry was carried out at approximately neutral pH using an automatic integration of current and time with an integrating multivoltmeter (Time Electronics) connected across a 1 K ohm resistance in the reduction circuit as previously described [7].

Biological assay. For oxic and anoxic electrolytic reduction experiments, approximately 450 μL of a 5×10^{-3} mol/dm^3 stock solution of drug was incubated with *E. coli* DNA containing a small amount of (ss) ΦX174 DNA to give a drug:nucleotide ratio of one. The biological activity of the ss ΦX174 DNA, before and during incubation, was measured using an *E. coli* transfection assay as described by Blok *et al.* [5] and Lafleur *et al.* [8]. Essentially a 0.1-mL sample from the reaction vessel was mixed with an equal volume of freshly prepared *E. coli* AB1157 spheroplasts (part of the cell wall being removed by lysozyme and EDTA). After 10 min at room temperature, 0.8 mL of prewarmed (37°) liquid broth was added and incubated for at least 2 hr. Cold distilled water, 4 mL, was then added and the active phage released by osmotic shock. The phage were titrated using *E. coli* C as an indicator organism and the plates scored for plaques. Rates of survival followed an exponential relationship, indicative of single hit kinetics, giving a Poisson distribution of inactivating damage amongst the phage molecules.

RESULTS

The structures of the drugs available for study are illustrated in Fig. 1. The electrochemical characteristics of each have been investigated using dc and differential pulse polarographies and by cyclic voltammetry (CV). The reduction potentials obtained by each technique are listed in Table 1. For comparison, the data for misonidazole recorded under analogous conditions are also included. The bis-*N*-oxides generally show two reduction steps of varying degrees of resolution. By dc polarography, two reduction waves are observed, but it is frequently difficult to accurately determine the diffusion limiting current (i_d) of the first reduction (i.e. to less negative potentials) and consequently the baseline current for the second reduction step. However, the first reduction always has a greater current response than the second (for example, SR4233 shows i_d values of 0.51 and 0.14 μA, respectively). Logarithmic analysis plotting $\log i/(i_d - i)$ vs E(mV), where i is the current at potential E, yields a straight line relationship for both reduction steps, illustrating diffusion control. The gradient is $59/n$ mV (where n = the number of electrons in the charge-transfer step) for a fully reversible process, and $59/\alpha n$ for an irreversible process (where α = transfer coefficient). Analysis of SR4233 yields gradients of 31 and 85 mV, respectively, and in general the gradient of the first reduction is less than that of the second.

Using differential pulse polarography the resolution of the two reduction steps is generally poor. Where the separation between the stages is small (or the electrode response is particularly distended) the first reduction is frequently observed only as a shoulder on the second reduction step. Where resolution is sufficient that two steps are clearly seen, the first reduction shows a marked decline of approximately 50% on changing the scan direction from negative to positive potentials. (The second reduction shows a decrease of 10%.) Both reductions are broad in appearance, with peak widths at half-height of the order of 120 mV, which further broadens on changing the scan direction.

The CV mode gives the best resolution of the two redox stages and consequently the most information. The CV of SR4233 is shown in Fig. 2 as a typical example. Better separation of the two processes can be achieved at slower scan rates. As the scan rate (ν) is increased, a general shift to negative potentials is observed, which is more marked for the first reduction. In some instances, therefore, at scan rates of $\nu = 100$ mV/sec or greater, only one reduction step is observed, although at $\nu = 50$ mV/sec, two stages are clearly in evidence. Where the resolution is sufficiently good, we can see that the first reduction has approximately twice the current response of the second. For the first reduction, $ip_f/\nu^{\frac{1}{2}}$ is not constant, illustrating lack of diffusion control. On the reverse potential sweep, a return wave is found, associated with the second reduction step. The peak-to-peak separation, ΔEp, for SR4233 is 60 mV, but can be 150 mV where the CV is more distorted. Due to the

Table 1. Reduction potentials for the benzotriazine-*N*-oxides using various electrochemical techniques

Compound	Electrochemical technique		
	Cyclic voltammetry (V)	Differential pulse Ep(V)	dc polarography $E_{\frac{1}{2}}$(V)
SR4233	Ep_f = −0.61, −0.67	−0.60	−0.52
	Ep_r = −0.61	−0.64	−0.645
	Ep_f = −0.68, −0.73		−0.595
SR4286	Ep_r = −0.66	−0.695	−0.69
	Ep_f = −0.51, −0.63	−0.49	−0.43
SR4308	Ep_r = −0.57	−0.60	−0.59
	Ep_f = −0.68		
SR4317	Ep_r = −0.59	−0.64	−0.615
	Ep_f = −0.72		
SR4318	Ep_r = −0.50	−0.69	−0.635
	Ep_f = −0.67		
SR4330	Ep_r = −0.58	−0.605	−0.595
	Ep_f = −0.64, −0.77	−0.61	−0.58
SR4355	Ep_r = −0.48	−0.75	−0.70
	Ep_f = −0.47, −0.59	−0.47	−0.38
SR4452	Ep_r = −0.54	−0.57	−0.54
	Ep_f = −0.59, −0.62		−0.50
SR4453	Ep_r = −0.56	−0.59	−0.61
	Ep_f = −0.59, −0.66	−0.61	−0.51
SR4466	Ep_r = −0.60	−0.685	−0.65
Misonidazole	Ep_f = −0.54	−0.54	−0.51

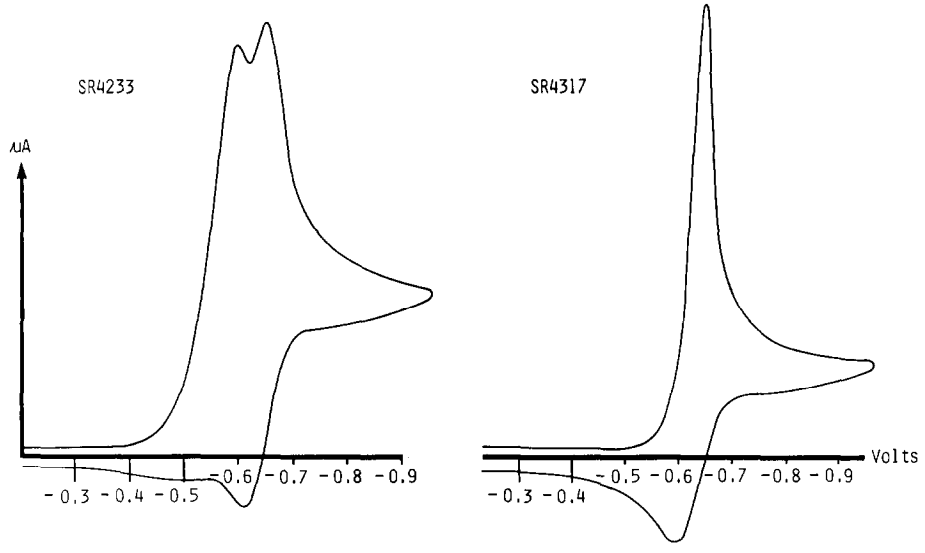


Fig. 2. Cyclic voltammetry of SR4233 and SR4317 (scan rate = 100 mV/sec).

closely overlapping nature of the reduction processes, it was impossible to accurately determine the return-to-forward peak current ratio, ip_r/if_f, but qualitatively, the return wave character becomes more predominant as ν is increased.

The mono-*N*-oxide, SR4317, shows only a single reduction step by all techniques (see Fig. 2). Logarithmic analysis of the dc polarographic wave yields a straight line of slope = 44 mV. By cyclic voltammetry, a quasi-reversible response is observed. The forward reduction wave, which is quite sharp in appearance, shifts (40 mV) to more negative potentials as ν is increased, but ΔEp = 95 mV does not change with increasing ν. The ip_r/ip_f ratio, however, increases from 0.42 to 0.52 as ν increases from 10 to 500 mV/sec. Even at ν = 10 V/sec (using an oscilloscopic display mode) ip_r/ip_f does not achieve unity.

Likewise, SR4330, the zero-*N*-oxide, shows only a single reduction wave. Differential pulse polarography shows only a single reduction wave and a peak with width at half-height of 110 mV, with a peak height which decreases approximately 10% on

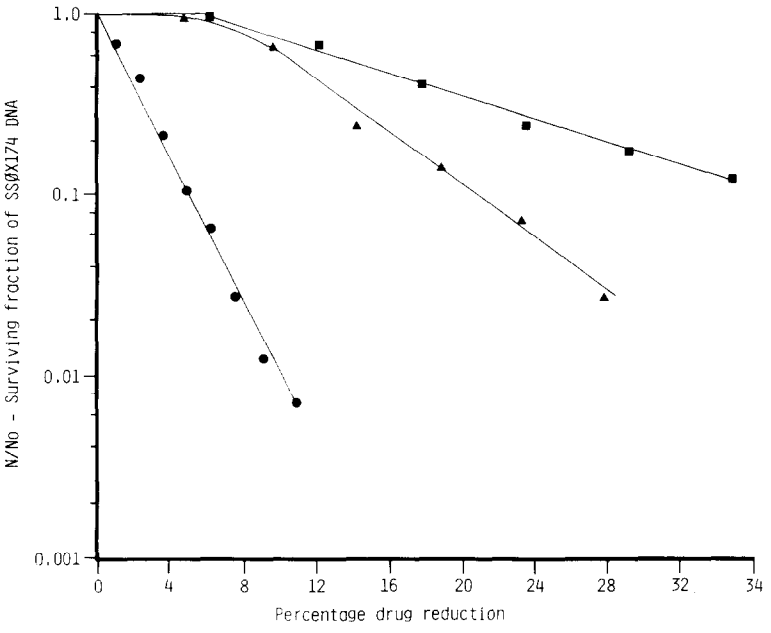


Fig. 3. Survival of single stranded ΦX174 DNA during anoxic electrolytic reduction of SR4233 and SR4317. (▲) SR4233, pH 7, R_{37} = 12.6%, (●) SR4233, pH 4, R_{37} = 2.3%; (■) SR4317, pH 4, R_{37} = 19.2%.

switching the scan direction from negative to positive potentials. Logarithmic analysis of the dc polarographic wave gives a slope of 35 mV. By CV, the response shows an overall 30 mV shift to negative potentials as ν is increased, giving ΔEp of 90 mV, which is invarient with ν. The ip_r/ip_f ratio increases from 0.55 to 0.68 with increase in ν from 10 to 200 mV/sec, but no further increase in ip_r/ip_f ratio was found beyond this point. The $ip_f/\nu^{\frac{1}{2}}$ value was constant.

To yield further information on the number of electrons involved in each reduction step, coulometry was performed on the series SR4233, SR4317 and SR4330 (the bis-, mono- and zero-*N*-oxides). At an approximate pH of 6.9 (± 0.3) the electron requirements for complete reduction of the three drugs were as follows: SR4233, 5.97 (±0.05); SR4317, 3.98 (±0.08); and SR4330, 2.22 (±0.09).

The importance of reductive activation for the di-*N*-oxides has been examined using SR4233, chosen for its reported high biological activity and its greater degree of solubility than many of the other analogues. Under oxic or anoxic, non-reductive incubation of the drug in the presence of DNA, no damage resulted with no change in the surviving fraction observed over a 24 hr period. Upon anoxic reduction, however, at neutral pH, the surviving fraction decreased exponentially by approximately an order of magnitude over a 7 hr period after a 2.5 hr lag phase (Fig. 3). At pH 4 (Fig. 3) damage to DNA was markedly greater. This can be quantitatively shown by the decrease in the R_{37} value (the % drug reduction required to give 37% survival). At a pH of 4, the R_{37} is 2.3% but at pH 7, the R_{37} is 12.6%. No lag phase was observed at acid pH.

The mono-*N*-oxide, SR4317, is reportedly biologically inactive. At a pH of 4, where SR4233 exhibited increased activity, SR4317 was found to show a small amount of DNA damaging capability on anoxic reduction (Fig. 3), but with an increased R_{37} of 19.2%.

DISCUSSION

All reduction potentials are achievable under biological conditions, but the values recorded in this study do not correlate with previous work [9]. This is not particularly surprising given the differences in the techniques and the conditions employed. Hirst *et al.* [9] used dc polarography exclusively, with a phosphate buffer system. We have used three display modes and 0.1 SSC as the supporting electrolyte. Given the irreversible nature of the redox steps and the distorted nature of the voltammetry encountered for many of these drugs, changes in the experimental conditions may well influence strongly the reduction potentials measured. We believe that the character of the electrode response to be of equal, if not greater, importance.

The complete range of bis-*N*-oxides studied show a two step reduction process of varying degrees of resolution caused by a slow charge-transfer, resulting in the electrode response being distended from the ideal behaviour. The first reduction (i.e. to less negative potentials) is highly irreversible as exemplified by a broad differential pulse response, which alters significantly depending on direction of scan, and a shift to more negative potentials with increasing ν and no return wave character by CV. The second reduction, however, is quasi-reversible, always being

clearly resolved by differential pulse polarography with a clear return wave on the reverse CV scan, although of smaller magnitude than the forward wave, and ΔEp increasing with ν. Where sufficiently good resolution was found, both dc polarography and CV implied a greater number of electrons involved in the first reduction step than in the second. However, such comparisons for non-reversible systems must be treated with caution.

Both SR4317 and SR4330 show only a single quasi-reversible redox step, which are both very similar in character, and also closely resemble the second reduction step of the bis-*N*-oxide series (compare the CVs of SR4317 and SR4233 in Fig. 2). Although both show an increase in ip_r/ip_f with ν, neither approaches unity.

Assignment of the various redox steps is difficult, as comparison of the current response data with coulometric studies on the electron requirements for complete reduction are not straightforward. The $n = 6$ value for SR4233 might suggest a 4 electron followed by a 2 electron addition mechanism (taking into account current response data), which would correspond to SR4233 → SR4330 conversion in a single step, followed by 2 electron reduction of the benzotriazine ring system itself. However, this would imply that two reductions should be seen for SR4317, which contains a single *N*-oxide group available for reduction. This can be interpreted if the initial 2 electron addition is to the benzotriazine. The reduction potential of the remaining *N*-oxide group then occurs at less negative potentials than are necessary for benzotriazine reduction, so a further two electrons are immediately added, thus yielding only a single redox response. This would explain why the general characteristics of the second reduction for the bis-*N*-oxides, and that found for SR4317 and SR4330 are similar, being dominated by the redox properties of the benzotriazine and why ip_r/ip_f is consistently greater for SR4330. The prior reduction of the benzotriazine in the mono-*N*-oxide may also explain why it is biologically inactive, although still containing a reducible *N*-oxide. The assignment of the benzotriazine ring in the mono-*N*-oxide as being the redox-active function is also supported by the study of similar heterocyclic ring systems, which show a redox response, particularly in the CV mode, analogous to those found in the present investigations (J. H. Tocher, unpublished work). It should also be pointed out that this reduction mechanism for SR4317 is in line with *in vitro* studies of the metabolism of these drugs, where SR4330 is only formed on incubation of SR4233 and not from SR4317 [3]. No mention of the benzotriazine reduction has been found from pulse radiolysis studies, which stated that the major products of the reduction of SR4233 were SR4317 and SR4330 and was confirmed by electrolytic and enzymatic reduction [2, 4]. A value of $n = 4$ was found in butanol. The importance of benzotriazine reduction to the overall biological activity is unknown.

The one-electron addition product, the radical anion, has been suggested as the species responsible for the cytotoxic activity of these drugs [2]. Electrochemically, under the conditions employed, we cannot detect an individual $SR/SR^{\cdot -}$ one electron couple. However, work is progressing to establish the complete redox pathway.

The importance of the reductive activation of SR4233 is well illustrated using the ΦX174 transfection technique. Under oxic conditions, virtually no damage to DNA was monitored. Under anoxic electrolytic reduction, however, damage to DNA was marked as exhibited by a decrease in the surviving fraction.

There is substantial evidence that it is the protonated form of the nitroimidazole reduction product which is responsible for its toxicity, as found by an increase in the DNA damage as the pH is lowered [10]. Results on the influence of pH on the action of SR4233 also showed an increase in the DNA damaging capability, with an increase in acidity, as observed by a fall in the R_{37} from 12.6 to 2.3% as the pH was lowered from 7 to 4. This would suggest that the biologically active reduction product for the benzotriazine di-*N*-oxides is also the protonated form. The delay time observed at pH 7 before a decrease in the surviving fraction was most likely due to a required accumulation of the protonated species. As the pK_a of the radical is 6 [2], only 10% of the radicals formed at neutral pH are protonated.

The biological inactivity of SR4317 was confirmed under electrolytic reduction conditions, despite the presence of the *N*-oxide group. At a pH of 4, where the damaging ability of SR4233 was most favourable, a slight decrease in the surviving fraction was observed, but with an increased R_{37} of 19.2%. This confirms the absence of biological activity from *in vitro* metabolism studies [3] and is in line with the electrochemical assignment of the benzotriazine ring being primarily involved in the charge-transfer step in the mono-*N*-oxides yielding a product with substantially less biological activity.

Studies are in progress to investigate the influence of pH more fully and to include the effects of reduction rate. This approach will also be extended to other drugs in the series to determine the specific features that make SR4233 so much more successful a specific hypoxic cytotoxic agent than other compounds in the series. The use of various mutant cell lines, deficient in certain repair enzymes will allow more specific identification of the mode of action and the type of DNA damage resulting. By coupling biological information with data on the redox mechanism we should obtain important evidence on the cytotoxic mode of action of this new class of drugs.

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