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DNA DAMAGING EFFECTS AND VOLTAMMETRIC STUDIES ON THE HYPOXIC CELL TOXIN 3-AMINO-1,2,4-BENZOTRIAZINE-1,4-DIOXIDE, SR4233, AS A FUNCTION OF pH

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Abstract—The compound 3-amino-1,2,4-benzotriazine-1,4-dioxide, SR4233, has recently attracted considerable attention as a possible hypoxic cell radiation sensitizer and cytotoxic agent. The present study examines the influence of pH on the DNA damaging ability of SR4233 upon electrolytic reductive activation, and the corresponding changes in electrochemistry. A ΦX174 double transfection assay has been employed to assess the DNA damaging ability of SR4233 between pH 4 to 7. Upon electrolytic reduction the drug was found to be more effective in damaging DNA at acidic pH than at neutral conditions. This indicated that the damaging species was probably protonated. The DNA damaging ability of SR4233, as measured by a viral transfection assay, was linearly related to pH between the values of 4 and 7, and this feature has implications for its potential efficacy in the treatment of hypoxic tumors. The electrochemistry of SR4233 has been examined as a function of pH between the ranges 2 and 10.5. Three investigation techniques have been employed, cyclic voltammetry and differential pulse and dc polarographies. A general shift towards less negative potentials with increasing acidity was found between pH 2 and 8.5 giving a linear relationship. The behaviour was found to be relatively invariant at alkaline pH.

The initial promise of the nitroaromatic compounds as hypoxic cell radiation sensitizers and cytotoxic agents in cancer treatment has generally proved disappointing, with the compounds showing little or no effect when tested clinically, coupled in many instances with unacceptable side-effects such as neurotoxicity or peripheral toxicity [1]. The search for chemotherapeutic agents with an improved efficiency and a higher degree of selective activity has therefore continued and a new range of compounds currently attracting considerable attention is the benzotriazine di-*N*-oxides. The lead compound of the series, SR4233 (3-amino-1,2,4-benzotriazine-1,4-dioxide) has been shown to have a considerable selective toxicity and radiosensitization towards hypoxic mammalian cells both *in vitro* and murine tumors *in vivo* [2, 3]. Although studies into the mode of action of these drugs are at an early stage it is believed that bio-reductive activation is necessary, in common with the nitro-containing heterocyclic compounds, and provides the basis for their selectivity. It has been proposed that the one-electron addition product is the most likely damage-causing species, resulting in oxidation of the sugar residues in DNA [4]. Neither of the two major reduction products, the 2-electron 3-amino-1,2,4-benzotriazine-1-oxide or the 4-electron 3-amino-1,2,4-benzotriazine, have shown toxicity to hypoxic cells *in vitro*.

In our previous research on the mechanism of a range of drugs activated by reduction[5, 6] we have found that a particularly profitable approach was the combination of detailed electrochemical investigations with biological studies. From preliminary studies on the benzotriazine di-*N*-oxides, including SR4233 [7], we observed an increase in acidity resulted in an increased biological activity. We have therefore examined the influence of pH on the redox characteristics of SR4233 in detail using cyclic voltammetry and differential pulse and dc polarographies. The biological implications of the reductive activation step has been assessed using a ΦX174 double transfection technique in conjunction with the controlled anoxic electrolytic reduction of SR4233, to determine the drug-induced, biologically relevant, damage to DNA.

MATERIALS AND METHODS

The drug, SR4233, was obtained from Professor J. M. Brown, Stanford University, California. The *E. coli* strains, AB1157 (wild type) and *E. coli* C (natural host of ΦX174 DNA) were obtained from Professor M. V. M. Lafleur of the Vrije Universiteit, Amsterdam, The Netherlands, and were maintained on nutrient agar plates reinforced with 10% (w/v) glucose. Single stranded (ss) ΦX174 DNA was prepared according to Blok *et al.* [8] and was obtained from Professor Lafleur. *E. coli* DNA was purchased from the Sigma Chemical Co. (Poole, Dorset, U.K.), and was further prepared according to Rowley *et al.* [9]. All other chemicals were of analytical grade and used as received without further purification.

Electrochemical methods

Voltammetric investigations were carried out in

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1.5×10^{-1} mol/dm^3 NaCl and 1.5×10^{-2} mol/dm^3 trisodium citrate aqueous buffer (1.0 SSC) as the inert supporting electrolyte, purged with H_2O-saturated N_2, a positive pressure of N_2 being maintained at all times. The pH range was varied between approximately 2 and 10.5 and was monitored by a Whatman PHA 250 pH probe.

Cell solutions were 1×10^{-4} mol/dm^3 with respect to concentration of SR4233. Measurements were made using a PAR 264A polarographic analyser interfaced with a PAR 303 cell stand employing a 3-electrode configuration, and a Bausch and Lomb RE 0088 *x–y* 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 polarographies employed a dropping mercury electrode (dme) with an electronically controlled drop-time (t_d) of 1 second, and a routine scan rate of 5 mV/s. The pulse amplitude in the differential pulse mode was 25 mV. Cyclic voltammetry (CV) used a stationary hanging drop mercury electrode (hdme), automatically renewed after each voltammogram, with scan rates from 10 to 500 mV/s, but 100 mV/s being the typical rate employed.

Biological methods

Drug/DNA interaction. The reaction vessel contained between 3 and 4.25×10^{-4} mol/dm^3 nucleotides *E. coli* DNA, depending on the original molarity of the DNA solution. The vessel also contained a trace amount of ss ΦX174 DNA (7.6×10^{-11} mol/dm^3 nucleotides). The amount of drug used corresponded to the amount of *E. coli* DNA nucleotides present in the reaction vessel to give a drug to nucleotide ratio of 1. An approximate reduction rate of 5% per hr was employed. The drug was dissolved in 1.0 SSC and the pH adjusted to the required value. For the electrochemical reduction, the vessel was sealed and purged overnight with O_2-free (<10 ppm) N_2 prior to ss ΦX174 DNA addition. Electrolytic reduction of the drug in the presence of DNA was performed at room temperature under O_2-free N_2 at the potential which was at least 500 mV more negative than the $E_{1/2}$ of the drug. A mercury pool cathode and Ag/AgCl anode were used. The reduction vessel and electrochemical reduction procedure have been described previously [9, 10].

ΦX174 assay. The biological activity of ss ΦX174 DNA was monitored by transfection of *E. coli* spheroplasts as described by Guthrie and Sinsheimer [11] and later modified by Lafleur *et al.* [12]. The DNA and drug were allowed to react at a drug to nucleotide ratio of 1 at room temperature. In all the experiments, the DNA was a mixture of *E. coli* and ss ΦX174 DNA.

The spheroplasts were prepared by removing part of the cell wall with lysozyme and EDTA. At frequent intervals 0.1-mL samples of the DNA/drug mixture were removed from the reaction vessel and diluted 10-fold with 0.25 mol/dm^3 Tris buffer, pH 8.1, prior to determination of its biological activity. In brief, 0.1 mL of sample was mixed with 0.1-mL freshly prepared *E. coli* AB1157 spheroplasts. The mixture was allowed to stand at room temperature for 10 min and then 0.8 mL of prewarmed (37°) LBM (Luria broth medium) added. The mixture was then incubated at 37° for 1.5 to 2 hr. The active phage were then released by osmotic shock with the addition of 4-mL ice-cold sterile distilled water. The phage were then titrated using *E. coli* C as the indicator organism. The rate of survival was exponential and thus indicative of single-hit kinetics, giving a Poisson distribution of inactivating damage of the phage molecules. The parameter t_{37} was used as a measure of the average of one lethal inactivation per DNA molecule and was the time (t) at which 37% of the original phage DNA retained biological activity. The parameter R_{37} may also be used and was the percentage drug reduced at which 37% of the original phage DNA retains biological activity.

All experiments were done in duplicate and each sample transfected twice. Hence each point on the kill curve was the mean of two experiments and four transfections.

RESULTS

The pH of the electrolytic medium had a pronounced effect on the electrochemical behaviour of SR4233, not only in terms of a shift in the redox potential, but in the overall characteristics of the electrode response. At neutral and alkaline pH two reduction steps were observed by all techniques with varying degrees of resolution. By differential pulse polarography the second, or more negative, reduction could be seen as a prominent shoulder on the dominant first reduction step. The dc polarogram showed two waves, with an approximate diffusion limiting current ratio of 3 to 1. Upon logarithmic analysis of the response, both yielded straight-line relationships, illustrating diffusion control of the charge-transfer step, with gradients of 57 and 110 mV respectively. The CV mode showed two waves on the forward scan, but only a single wave on the reverse sweep, associated with the second reduction step. The peak-to-peak separation was typically 40 mV, which did not change with scan rate, ν. Despite the overlapping nature preventing absolute measurement, the return-to-forward peak current ratio, ip_r/ip_f, could be estimated as 0.60 at ν = 100 mV/s, but decreasing with slower scan rates. The first reduction always had the greater current response. The resolution of the two individual forward waves was clearer at scan rates less than 100 mV/s. Above $\nu = 200$ mV/s the second reduction appeared only as a shoulder. There was a shift towards more negative potentials for the first reduction as ν was increased, which was typical behaviour for an irreversible charge-transfer step. The actual redox potentials shifted only slightly to more negative values as the pH was increased, with the second reduction showing the greater negative shift, so that the separation between the two reduction steps became larger, i.e from 40 mV at pH 8 to 85 mV at pH at 10.5. These trends were observed by all investigation modes.

A change to acid pH, however, produced more dramatic alterations. Over a very limited pH range from 7 to 6.5 the response increased in complexity. This was particularly evident from the CV mode. On the forward scan two clear reduction waves were

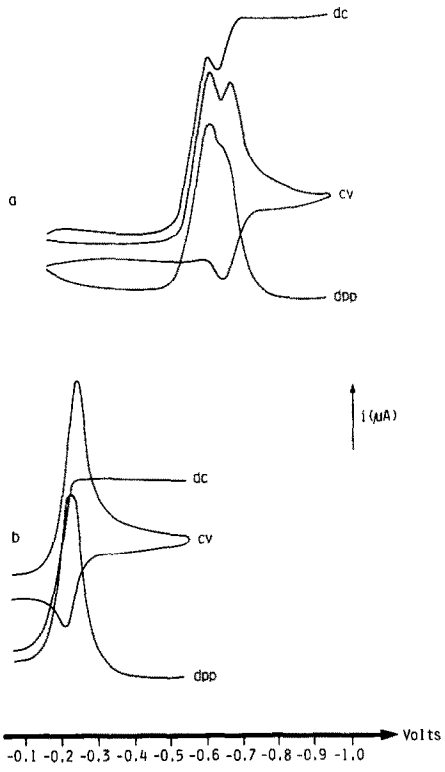

Fig. 1. Comparison of the voltammetric response of SR4233 at pH 9 (a) and at pH 3.5 (b).

seen, but, in addition, there was a definite shoulder towards positive potentials on the first reduction wave. The response generally was broader than found at alkaline pH. The second reduction wave retained its well-defined nature on the forward scan, but the return wave was slightly broadened, with ΔE_p of 100 mV. The ip_r/ip_f ratio could be estimated as 0.9. A shift towards less negative potentials was observed for the first reduction step.

As the pH was lowered, the response initially simplified to again show two reduction steps, but with a decreased potential separation. A further increase in acidity, below pH 5, resulted in only a single overall reduction step being observed by all techniques. (Figure 1 shows the comparative electrochemical behaviour obtained at pH 9 and 4.) The magnitude of the current response was approximately the sum of the individual values where a two stage reduction was observed. The differential pulse polarography showed a single, well-defined, symmetrical peak, with a peak separation of 30–40 mV on negative and positive scan directions. A logarithmic analysis of the dc polarographic wave gave a straight-line relationship, with a gradient of 50–60 mV. The CV showed a single response on both forward and return scans. The ΔE_p increased from 40 to 80 mV on increasing ν from 20 to 200 mV/s. The ip_r/ip_f ratio also increased with scan rate, although the ideal value of 1.0 was never approached. Typical values at ν = 100 mV/s were 0.45. The reduction potential continued to shift to less negative values as the pH was decreased.

The reduction stoichiometry showed some variation with pH. Over the pH range examined the electron requirements for the overall reduction process decreased in a stepwise fashion from 6.4 at pH 10 to 5.5 at pH 5 before rising again to a value of 6.0 at pH 3.

Table 1 lists typical data obtained throughout the pH range examined, but the trend in reduction potential with pH was best illustrated graphically (Fig. 2). The values used have been taken from the differential pulse mode, but the general trend was the same irrespective of the technique considered. As may be seen, a good linear relationship was found between E_p and pH if the first, or less negative, reduction was considered where two reduction steps were observed. This relationship could be described by the equation

$$E_p = -0.086\,\text{pH} + 0.11.$$

This expression was found to hold between pH 2 and 8.5 approximately.

Table 2 gives the reduction potentials ($E_{1/2}$) obtained for SR4233 at varying pH from which the values used for the bulk scale electrolytic reduction of the drug in the presence of DNA were chosen (see Materials and Methods).

There was no change observed in the survival of ΦX174 DNA with SR4233 under oxic or non-reductive anoxic conditions (results not shown). However, under anoxic reduction conditions, damage to DNA was observed.

At pH 7 there was a lag phase of about 1.5 hr before there was a significant decrease in the surviving fraction of ΦX174 DNA exposed to SR4233 (Fig. 3). Slight shoulders were also seen with the kill curves at pH 6 and 5, whereas at pH 4 there was no shoulder.

A similar pattern was seen with the kill curves when the percentage drug reduced was plotted against the surviving fraction of DNA (Fig. 4).

The t_{37} value increased from 0.33 hr ± 0.106 at pH 4 to 2.68 hr ± 0.016 at pH 7 indicating that the drug causes more damage at acidic pH. This was also confirmed by the R_{37} value where the amount of drug required to cause a fixed amount of damage increased from 2.35% ± 0.05 at pH 4 to 12.65% ± 0.05 at pH 7 (Fig. 5). The values obtained for the t_{37} and R_{37} at pH 5 and 6 are also shown in Table 2.

DISCUSSION

The results show that the effect of pH markedly influence both the electrochemical and biological behaviour of the reduction process of SR4233.

The influence of pH on the voltammetry of SR4233 divided into two distinct categories. At alkaline pH, above 8.5, a slight shift to more negative potentials and an increased separation of the two reduction steps was observed. At acid pH the potential separation between the two reduction steps decreased until only a single process was observed (Fig. 1). At alkaline pH the less negative process was irreversible in character, whereas the second step was quasi-reversible with a distinct return wave response in

Table 1. Reduction potentials for SR4233 as a function of pH

	Electrochemical technique				
		CV		Differential pulse	dc Polarography
pH	E_p (V)	ΔE_p (mV)	ip_r/ip_f	E_p (V)	$E_{\frac{1}{2}}$ (V)
9.0	$E_{pf} = -0.635$			−0.615	−0.58
	$E_{\frac{1}{2}} = -0.67$	40	0.60	−0.66	−0.685
6.8	$E_{pf} = -0.48$(sh)			−0.46	−0.45
	−0.55				
	$E_{\frac{1}{2}} = -0.58$	100	0.95	−0.575	−0.60
5.3	$E_{\frac{1}{2}} = -0.365$	60	0.33	−0.36	−0.35
3.0	$E_{\frac{1}{2}} = -0.16$	40	0.40	−0.15	−0.14

sh = shoulder.

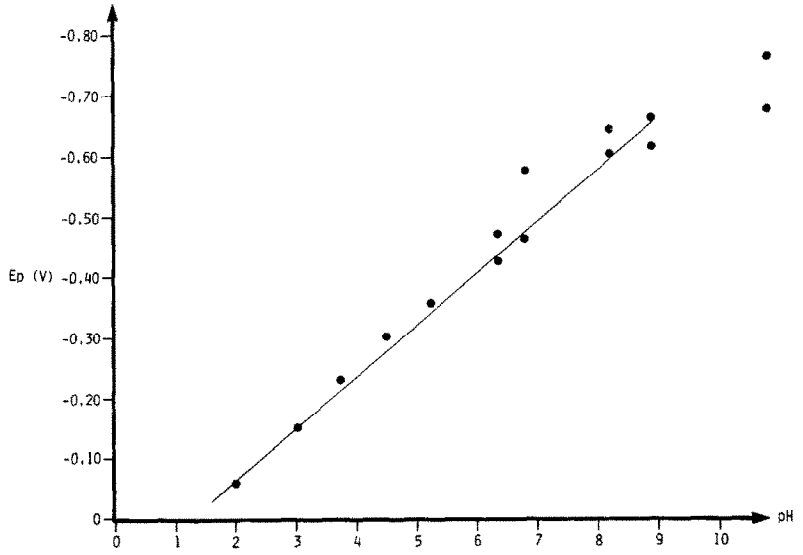

Fig. 2. The variation of reduction potential (in Volts) of SR4233 with pH of the electrolytic medium.

Table 2. Biological data for SR4233 obtained under electrolytic reduction

pH	Reduction potential $E_{\frac{1}{2}}$ (V)	t_{37} (hr)	R_{37} (%)
4	−0.235	0.33 (±0.106)	2.35 (±0.05)
5	−0.320	1.235 (±0.0)	5.0 (±0.4)
6	−0.405	1.65 (±0.125)	9.1 (±0.5)
7	−0.49 and −0.59	2.68 (±0.016)	12.65 (±0.05)

The reduction potentials ($E_{1/2}$) for SR4233 at varying pH are those applicable to determining the t_{37} and R_{37} values listed above. The data obtained are the mean of two experiments and figures in parentheses show the variance between the values.

the CV mode. From our previous studies on a number of benzotriazine di-*N*-oxides, together with comparison with the mono- and zero-*N*-oxides, we assigned the first electron addition step to the reduction and subsequent loss of the oxide groups, followed at more negative potentials by reduction of the benzotriazine heterocycle itself. This latter process yielded a product with some stability on the electrochemical time-scale, hence the return wave in the CV. As we observed a gradual decrease in the potential difference between the reduction steps before coalescence, and a current response for the single reduction step being the sum of the magnitudes for the two separate processes, the single step at

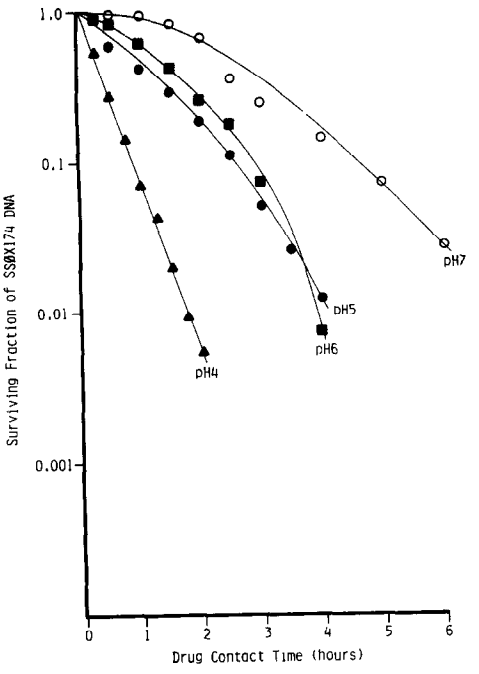

Fig. 3. Survival of ss ΦX174 DNA during anoxic reduction of SR4233 illustrating the effect of pH.

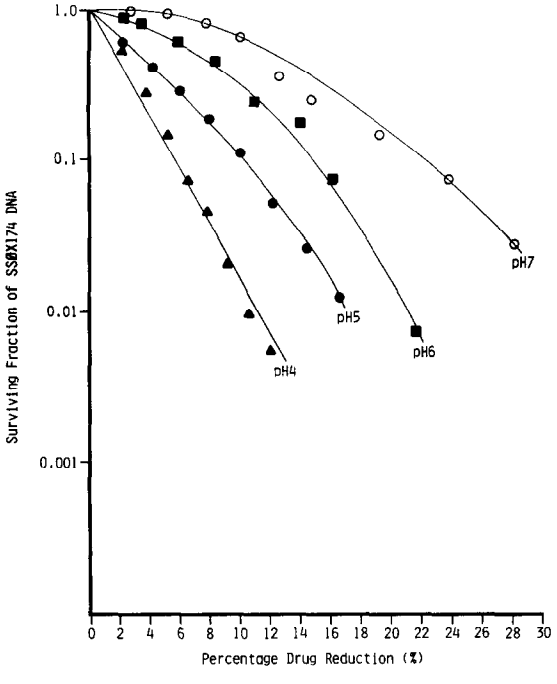

Fig. 4. Survival of ss ΦX174 DNA with percentage reduction of SR4233.

acidic pH would seem to contain both *N*-oxide and heterocycle reduction features. The assignment was substantiated by the consistently greater current response on the forward CV scan than on the return sweep, with a maximum ip_r/ip_f of 0.45. Repeat scan

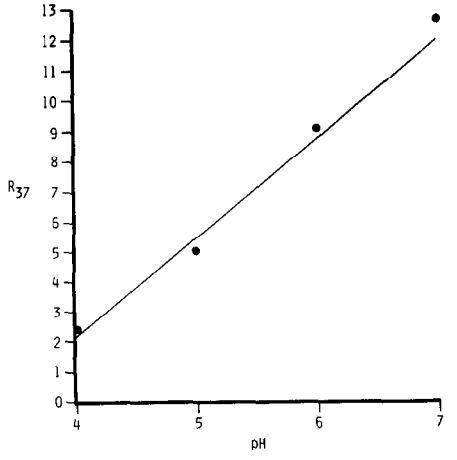

Fig. 5. The influence of pH of the electrolytic medium on the R_{37} value.

CV continued to show only a single response in both directions with an increased ip_r/ip_f. Reduction of the di-*N*-oxide resulted in the formation of the corresponding zero-*N*-oxide which was then concentrated in the vicinity of the working electrode (with the exclusion of a fresh supply of the di-*N*-oxide). The second and subsequent scans were therefore essentially the response of the heterocycle, and hence the increased ip_r/ip_f ratio. This was also confirmed by the coulometric studies, where the electron stoichiometry did not change markedly with pH. This was in contrast to pulse radiolysis studies [4], where the electron requirements changed from 4 at pH 10.9 to 0.5 at pH 5.8. Under these conditions the benzotriazine heterocycle was apparently redox inactive.

As the pH was decreased below 8.5 a progressive shift to less negative reduction potentials was observed (Table 1). The trend in reduction potential with pH could be described by a straight line relationship between the pH range 2 and 8.5 (Fig. 2). Similar electrochemical behaviour with pH has also been found with the nitroaromatic compounds [13].

The ΦX174 transfection assay has shown that upon reductive activation SR4233 was capable of causing biologically relevant DNA damage. However, the damage caused was substantially increased as the pH was decreased. This pattern was similar to that observed for the nitroimidazoles [14] where a decrease in pH resulted in increased damage, as observed by the decrease in specific viscosity of DNA. There was a linear relationship between the R_{37} value and the pH, which was also observed with metronidazole [14].

It has been suggested that the damaging species is the one-electron reduction product of SR4233, the radical anion, because the mono-*N*-oxide (2-electron reduction product, SR4317) does not display cytotoxicity or selective killing under hypoxic conditions [2]. Using the transfection assay, SR4317 did show some damaging ability at pH 4 although the R_{37} value was seven times greater than that for SR4233 [7].

The increase in activity of SR4233 with acidity might suggest that the damaging species was protonated, to give a more stable or active form at low pH, or that a proton assisted rearrangement of the active species was necessary. The increased rate of kill observed with a lowering of pH suggests that the effect was probably due to the increased activity of the drug rather than its stability. The enhanced oxidizing ability of the protonated radical has been previously postulated and compared to the enhanced reactivity of HO_2^- relative to the conjugate base O_2^- [4]. However, this does not necessarily explain the lag phase. A shoulder on the survival curve would be more in line with the required conversion of an intermediate into the active species or the formation of a DNA-adduct which subsequently decayed to give a strand-break. DNA-binding of the reduced drug has been postulated previously [4].

From Table 2 it can be seen that all the reduction potentials were attainable under biological conditions. The more negative the reduction potential the more difficult for anaerobic organisms or hypoxic tumours to reduce the drug. At pH 6 or below the reduction of the drug became steadily more favourable, as the redox potential shifted to less negative potentials. The contribution of changing redox potential and protonation of the active species are obviously of importance to the increased DNA damaging ability found with a decrease in pH.

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REFERENCES

1. Brown JM, Hypoxic cell radiosensitizers: where next? *Int J Radiat Oncol Biol Phys* **16**: 987–993, 1989.
2. Zeman EM, Brown JM, Lemmon MJ, Hirst VK and Lee WW, SR4233: a new bioreductive agent with high selective toxicity for hypoxic cells. *Int J Radiat Oncol Biol Phys* **12**: 1239–1242, 1986.
3. Zeman EM and Brown JM, Pre- and post-irradiation radiosensitization by SR4233. *Int J Radiat Oncol Biol Phys* **16**: 967–971, 1989.
4. Laderoute K, Wardman P and Rauth AM, Molecular mechanisms for the hypoxia dependent activation of 3-amino-1,2,4-benzotriazine-1,4-dioxide (SR4233). *Biochem Pharmacol* **37**: 1487–1495, 1988.
5. Dale LD, Tocher JH and Edwards DI, Comparative DNA damage induced by nitroimidazole-aziridine drugs: I. Effects of methyl substitution on drug action. *Anti-Cancer Drug Design* **3**: 169–175, 1988.
6. Zahoor A, Knight RC, Whitty P and Edwards DI, Satranidazole: mechanism of action on DNA and structure-activity correlations. *J Antimicrob Chemother* **18**: 17–25, 1986.
7. Tocher JH, Virk NS and Edwards DI, Electrochemical studies and DNA damaging effects of the benzotriazine-*N*-oxides. *Biochem Pharmacol* **39**: 781–786, 1990.
8. Blok J, Luthjens LH and Roos ALM, The radiosensitivity of bacteriophage DNA in aqueous solution. *Radiat Res* **30**: 468–482, 1967.
9. Rowley DA, Night RC, Skolimowski IM and Edwards DI, The effect of nitroheterocyclic drugs on DNA: an *in vitro* model of cytotoxicity. *Biochem Pharmacol* **28**: 3009–3013, 1979.
10. Knight RC, Rowley DA, Skolimowski IM and Edwards DI, Mechanism of action of nitroimidazole antimicrobial and antitumour radiosensitizing drugs. Effects of reduced misonidazole on DNA. *Int J Radiat Biol* **36**: 367–377, 1979.
11. Guthrie GD and Sinsheimer RL, Observations on the infection of bacterial protoplasts with the deoxyribonucleic acid of bacteriophage ΦX174. *Biochim Biophys Acta* **72**: 290–297, 1963.
12. Lafleur MVM, Pluijmackers-Westmijze EJ and Loman H, Contrasting effects of cytochrome C on the radiosensitivity of single-stranded ΦX174 DNA in the presence of misonidazole or phenol under anoxia. *Int J Radiat Biol Phys* **10**: 1195–1197, 1984.
13. Edwards DI, Mechanisms of cytotoxicity of nitroimidazole drugs. In: *Progress in Medical Chemistry* (Eds. Ellis GP and West GB), Vol. 18, pp. 87–116. Elsevier, North-Holland Biomedical Press, Amsterdam, 1981.
14. Edwards DI, Knight RC and Zahoor A, DNA damage induced by reductively activated nitroimidazoles—pH effects. *Int J Radiat Oncol Biol Phys* **12**: 1207–1209, 1986.