Pergamon

0006-2952(95)02010-A

Biochemical Pharmacology, Vol. 50, No. 9, 1367–1371, 1995.

0006-2952/95 $9.50 + 0.00

THE INTERACTION OF NITROAROMATIC DRUGS WITH AMINOTHIOLS

JOANNE H. TOCHER* and DAVID I. EDWARDS

Chemotherapy Research Unit, University of East London, Romford Road, London E15 4LZ, U.K.

Received 16 *January* 1995; *accepted* 12 *June* 1995

Abstract—The effect of cysteamine and glutathione addition on the redox behaviour of metronidazole, chloramphenicol, M&B 4998, nitrofurazone, and nifuroxime has been studied by electrochemical techniques. The presence of thiol influences the redox behaviour of the nitro compound in a number of ways. In aqueous media, the single-step nitro/hydroxylamine reduction shows a decrease in current and a shift to more positive potentials, which is assigned to the thiol acting as the reducing agent, but only after the formation of the nitro radical anion. In addition, the reversible RNO/RNHOH couple is greatly diminished or removed. In a dimethylformamide/H_2O solvent, the nitro radical anion can be selectively generated. The effect of thiol addition on the stability of the radical anion is strongly dependent on the drug, the identity of the thiol, and the concentration of the supporting electrolyte. The presence of thiol can result in an increase or a decrease in the lifetime of the radical with no apparent correlation with the redox couple of the nitro compound, or can act as an oxidizing agent and regenerate the original nitro compound. These disparate routes by which thiol can modify the redox characterisics of nitro compounds suggest that the traditional role of thiol as a radical scavenger needs to be extended.

Key words: nitro-aromatic compounds; voltammetry; cysteamine; glutathione; thiols

Aminothiols, such as GSH† and cysteamine, can protect biological systems from the cellular damage caused by ionizing radiation [1]. Thiols can also protect against the action of a number of chemotherapeutic agents, including the anaerobic cytotoxicity of nitro-aromatic compounds. Various mechanisms to explain this protection have been proposed when the sulfhydryl group acts as a radical scavenger, as a hydrogen donor repairing radical damage at the biological target (DNA), or inhibits the action of the biologically active species [2]. In previous studies on a range of nitro-aromatic compounds, a controlled potential electrolytic method has been used to examine the effect of thiol on the DNA damage caused by reductive activation of the nitro group. The degree of protection afforded by the thiol, as measured by the decreased amount of DNA damage, was shown to depend on the reduction potential of the drug [3].

Nevertheless, the mechanism of the aminothiol/drug interaction remains open to question. In an attempt to understand more fully the influence of aminothiols on the cytotoxic properties of the nitro-aromatic compounds, the reduction properties of a range of nitro-aromatic compounds in the presence of an aminothiol have been studied to establish whether the thiol acts as a radical scavenger, an oxidizing agent, or a reducing agent, or all three depending on the conditions. In addition, we wished to establish whether the thiol acted indiscriminately with any radical, or whether its action was dependent on the potential difference between the thiol and nitro group redox couples. We have used an *in situ* electrochemical model to examine the effect of cysteamine and glutathione addition on the voltammetric response of five nitro compounds, the nitro-pyrazole M&B 4998, metronidazole, CAP, nifuroxime, and nitrofurazone, chosen to represent a variety of ring structure and reduction potential. Four redox couples were examined to enable the role of individual nitro reduction products in the mechanism of thiol protection to be better understood; *viz.* the 4-electron RNO_2/RNHOH and the 2-electron RNO/RNHOH observed in an aqueous medium, and the 1-electron $RNO_2/RNO_2^{\cdot -}$ and the following $RNO_2^{\cdot -}$/RNHOH 3-electron addition step investigated in a mixed dimethylformamide/water solvent.

MATERIALS AND METHODS

Compounds

Metronidazole (1-β-hydroxyethyl-2-methyl-5-nitroimadazole), M&B 4998* (1-β-hydroxyethyl-nitropyrazole), chloramphenicol (D-*threo*-2,2-dichloro-*N*-[β-hydroxy-α-(hydroxymethyl)-4-nitrophenethyl]acetamide, nifuroxime† (*anti*-5-nitro-2-furaldoxime), and nitrofurazone‡ (5-nitro-2-furaldehyde semicarbazone) were used as received. The thiols cysteamine and glutathione (reduced form) and dimethylformamide (DMF, spectroscopic grade) were also obtained from a commercial source.§

Electrochemical measurements

Voltammetric experiments used a PAR 264A polarographic analyzer, interfaced with a PAR 303A cell stand and a Bausch and Lomb RE 0088 x-y recorder. A 3-electrode cell configuration was used with an aqueous Ag/AgCl reference electrode and a Pt wire counter electrode. Sampled dc and differential pulse polarographies used a dropping mercury electrode, with a drop time of

* Corresponding author.

† Abbreviations: CAP, chloramphenicol; GSH, reduced glutathione; DMF, dimethylformamide; and CV, cyclic voltammetry.

* Rhone-Poulenc Rorer, Dagenham, Essex, U.K.
† Sigma Chemical Co., Poole, Dorset, U.K.
‡ SmithKline Beecham, Beckenham, Kent, U.K.
§ Aldrich Chemical Co., Poole, Dorset, U.K.

1 second and a scan rate of 5 mV/sec. Cyclic voltammetry (CV) used a hanging drop mercury electrode, which was automatically renewed between scans. Scan rates ranged between 20 and 200 mV/sec but were typically 100 mV/sec. Cyclic voltammetry was used most extensively, as this technique gives the greatest information on the chemical stability and interaction of redox intermediates.

Solutions were purged with solvent-saturated N_2 prior to measurements. Studies were undertaken in water and 33.3% DMF/H_2O (v:v), with both using 0.15 M NaCl plus 0.015 M trisodium citrate buffer (1.0 SSC, pH 7.1) as the supporting electrolyte. The concentration of drug or thiol ranged from 25 μM to 1 mM, but was typically 0.2 and 0.4 mM for drug and thiol, respectively. Both cysteamine and GSH were prepared as 20 mM aqueous stock solutions immediately before use to prevent decomposition.

The analysis of the voltammetric response was complicated by the redox activity of both the thiol and the nitro compound. To assist in the interpretation, the effect of thiol addition on the electrochemical behaviour of the drug and the effect of drug addition to the thiol was studied. Some important voltammetric parameters for the nitro compounds in the absence of thiol are listed in Table 1.

RESULTS

In aqueous buffer

Cysteamine has received particular attention because it is the thiol that gives the most effective protection in *in vitro* systems, and because data is available on the decrease in DNA damage produced during electrolytic nitro drug reduction by its presence [3, 4]. Cysteamine shows a two-step oxidation, with $E_{1/2}$ values of –0.40 and –0.535 volts, assigned from concentration studies as free and Hg-adsorbed thiol, respectively. Cyclic voltammetry showed both steps to be quasi-reversible in nature, with peak-to-peak separations, ΔEp, of 95 and 70 mV, respectively, at a scan rate of 100 mV/sec, which increases with scan rate. A fully reversible CV response gives ΔEp = (60 mV)/n, where n is the number of electrons transferred, and ΔEp is invariant with scan rate.

Glutathione has a similar two-step oxidation, with $E_{1/2}$ values of –0.43 and –0.55 volts, and ΔEp of 160 and 50 mV.

The five nitro compounds studied show substantial overlap between the thiol and the 4-electron single-step reduction RNO_2/RNHOH by the three voltammetric techniques employed. The voltammetric behaviour was not additive. At a [cysteamine]:[drug] ratio of 20:1, the nitro reduction is seen only as a weak shoulder on the thiol oxidation. An estimation of the current response for the drug shows an approximate 70% decrease and a reduction potential 100 to 150 mV to more positive potentials from that found in the absence of cysteamine. As the drug concentration is increased, the drug response is seen to grow markedly at a 2:1 ratio with the effective masking of the second thiol oxidation step. The change in the voltammetry is illustrated for metronidazole in Fig. 1. There is also a shift in nitro reduction potential in the order of 50 mV. At a 1:1 ratio, the nitro group reduction dominates the voltammetry and the thiol oxidation is seen only as a relatively minor process. The second thiol oxidation step is no longer visible (Fig. 1c). The drug current response, however, is still less by 25 to 50%, with a positive shift of 15 to 40 mV in reduction potential from that found in the absence of thiol.

Preliminary studies with glutathione present the same difficulties in separating the individual thiol and drug responses, but the same general behaviour is found.

The reversible 2-electron RNO/RNHOH couple, formed as a consequence of nitro group reduction, can be identified by repeat CV for M&B 4998 and CAP [5]. For nitrofurazone and nifuroxime, only the oxidation of the hydroxylamine to the nitroso is observed (i.e. there is no corresponding reduction response on the second negative scan). Addition of cysteamine or glutathione at a [thiol]:[drug] ratio of 1:1 results in a 50 to 100% overall decrease in the current response for the RNO/RNHOH couple, accompanied by a 100 mV positive potential shift. At lower thiol concentrations for M&B 4998 and CAP, it is seen that the decrease in current for the RNO reduction wave (on the second negative potential sweep) is greater than for the RNHOH oxidation, indicating preferential reaction of the thiol with the nitroso rather than the hydroxylamine reduction products.

Table 1. Cyclic voltammetric data on the reduction of nitro aromatic compounds in different solvent systems

	H_2O	DMF/H_2O			
	RNO_2/RNHOH	$RNO_2/RNO_2^{\cdot -}$			$RNO_2^{\cdot -}$/RNHOH
Compound	E_{80}(V)*	$E_{1/2}$(V)†	ip_r/ip_f‡	ΔEp§	E_{80}(V)*
Nitrofurazone	–0.41	–0.49	0.683	85	–0.83
Nifuroxime	–0.51	–0.63	0.794	55	–0.99
Chloramphenicol	–0.615	–0.77	0.748	60	–1.245
Metronidazole	–0.65	–0.805	0.712	80	–1.35
M&B 4998	–0.74	–0.83	0.781	80	–1.45

All potentials quoted in Volts vs an Ag/AgCl(aq) reference electrode.

* An irreversible process, measured as 80% of the forward wave peak current.

† The mid-point potential between the forward and return waves.

‡ The return-to-forward peak current ratio at a scan rate of 100 mV/s; supporting electrolyte concentration of 0.1 SSC, the ratio increases by approximately 10% in 1.0 SSC electrolyte.

§ The difference (in mV) between the forward and return waves at a scan rate of 100 mV/s.

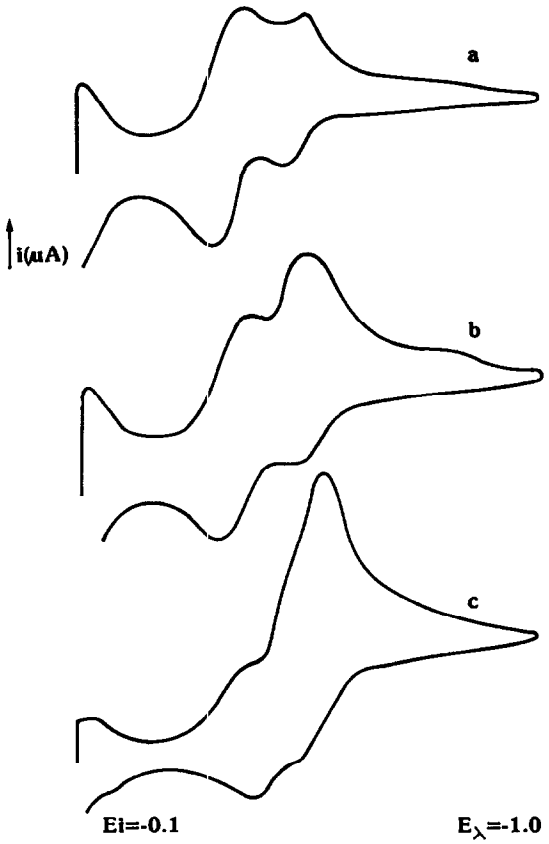

Fig. 1. Cyclic voltammetry of cysteamine plus metronidazole (a) cysteamine only; (b) plus metronidazole at a [thiol]:[drug] ratio of 10:1; and (c) plus metronidazole at a [thiol]:[drug] ratio of 2:1.

In DMF/H_2O

There are a number of advantages when using a mixed solvent system. The redox behaviour of the thiol is more 'compact', appearing as only a single wave in both scan directions. This is particularly true for GSH, where the forward wave is very sharp in appearance. The addition of DMF causes the nitro reduction to shift to more negative potentials (Table 1). In a mixed solvent, therefore, the individual voltammetry of the thiol and the drug are easier to distinguish. In addition, the nitro reduction is now separated into a two-stage process [6].

$$RNO_2 + e^- \longleftrightarrow RNO_2^{\cdot -} + 3e^- \rightarrow RNHOH.$$

The first reduction step is now reversible, with the reduction product, $RNO_2^{\cdot -}$, being stable on the voltammetric time-scale. This is shown by the return wave observed on the reverse potential sweep, corresponding to regeneration of the parent material. This can be quantified by the return-to-forward peak current ratio, ip_r/ip_f. A fully reversible couple where there is no tendency for the reduction product to undergo further reaction gives an ip_r/ip_f of 1. A decrease in the observed ip_r/ip_f in the presence of a biological target (e.g. thiol) indicates interaction between the reduction product and the target [7]. The mixed solvent therefore allows us to specifically examine the influence of thiol on the nitro radical anion. The effect of cysteamine on the $RNO_2/RNO_2^{\cdot -}$ couple for M&B 4998 by CV is shown in Fig. 2. Thiol and drug

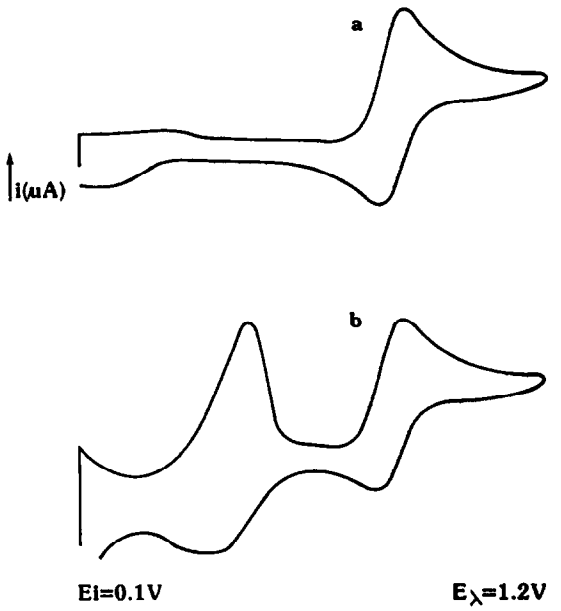

Fig. 2. Cyclic voltammetry of M&B 4998 in DMF/H_2O (1.0 SSC) plus cysteamine (a) drug only; and (b) plus cysteamine at a [thiol]:[drug] ratio of 1:1.

couples can be clearly distinguished, with a decrease in the return wave for the drug upon thiol addition. Table 2 shows the difference in $RNO_2^{\cdot -}$ stability for CAP, metronidazole, and M&B 4998, expressed as the percentage change, % Δ, in the ip_r/ip_f from that measured in the absence of thiol. The influence of the SSC supporting electrolyte concentration was also investigated (Table 2).

The voltammetric behaviour upon addition of cysteamine to nitrofurazone and nifuroxime is different. A large increase in the 1-electron RNO_2 reduction step and the removal of the return oxidation step are observed (Fig. 3). The alterations to the voltammetry are virtually complete after the first addition of cysteamine (at a [thiol]:[drug] ratio of 1:8).

DISCUSSION

In aqueous buffer

The current response of the 4-electron nitro reduction to the hydroxylamine is decreased and shifted to more positive potentials in the presence of thiols (Fig. 1). This can be explained if the reduction mechanism of the nitro group is considered. The formation of the nitro radical anion is the rate determining step, with further reduction steps to give the nitroso or hydroxylamine de-

Table 2. The effect of cysteamine and glutathione addition on the stability* of $RNO_2^{\cdot -}$ as determined by cyclic voltammetry

Thiol†	[SCC]	CAP	Metro	M&B 4998
Cysteamine	0.1	−23.2	−12.7	−18.3
Cysteamine	1.0	+3.1	+9.1	−6.0
Glutathione	0.1	−26.6	−12.2	−28.6
Glutathione	1.0	−10.0	−13.1	−12.8

* The effect on the stability of $RNO_2^{\cdot -}$ is expressed as the % change in the ip_r/ip_f value from the response measured in the absence of thiol.

† All data were recorded at a [thiol]:[drug] ratio of 1.

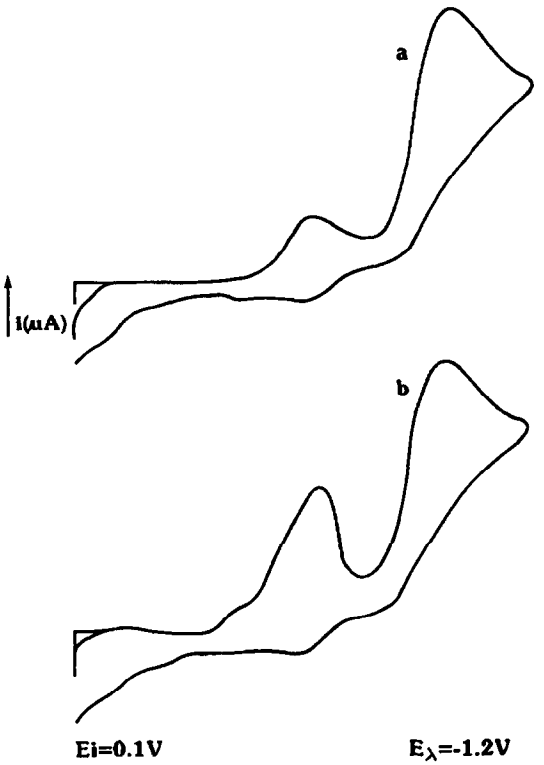

Fig. 3. Cyclic voltammetry of nifuroxime in DMF/H_2O (1.0 SSC) plus cysteamine (a) drug only; and (b) plus cysteamine at a [thiol]:[drug] ratio of 8:1.

rivatives occurring at more positive potentials [5]. In the presence of cysteamine, the first electron addition to give $RNO_2^{\cdot -}$ takes place at the Hg working electrode, but the thiol present in the bulk solution then acts as the reducing agent, resulting in the decreased electron requirement for the further reduction of the nitro radical anion. The typical voltammetry for nitro reduction is only observed as the drug concentration is increased and the contribution by thiol as the reducing agent diminishes.

The decrease or removal of the RNO/RNHOH couple upon cysteamine addition is in agreement with the known chemical reactivity of the nitroso and hydroxylamine derivatives with thiols [2, 8]. The larger decrease in the RNO reduction wave for CAP and M&B 4998 suggests greater or faster interaction with the nitroso derivative.

In DMF/H_2O

The selective generation of $RNO_2^{\cdot -}$ in the mixed solvent has enabled us to examine the change in stability of $RNO_2^{\cdot -}$ for CAP, metronidazole, and M&B 4998 in the presence of cysteamine and GSH by measuring the change in the ip_r/ip_f ratio for the $RNO_2/RNO_2^{\cdot -}$ couple. The inhibition of cytotoxicity by thiols is traditionally viewed as [3]:

$$RNO_2^{\cdot -} + R'SH \rightarrow RNO + R'S^{\cdot} + OH^-.$$

Cyclic voltammetrically we would predict a decrease in the ip_r/ip_f ratio due to less $RNO_2^{\cdot -}$ being available for oxidation on the return potential sweep and the formation of the RNO/RNHOH couple for CAP and M&B 4998. Table 2 shows that a decrease in $RNO_2^{\cdot -}$ stability is found for all three drugs with both thiols when using 0.1 SSC buffer. When the electrolyte concentration is increased to 1.0 SSC, the % Δ ip_r/ip_f is markedly less (i.e. the thiol is a less effective radical scavenger), and indeed the presence of cysteamine increases the lifetime of $RNO_2^{\cdot -}$ for CAP and metronidazole. The voltammetry of the nitro compounds, without the addition of thiol, shows an increased $RNO_2^{\cdot -}$ stability at higher electrolyte concentrations, detected as a 10% increase in the ip_r/ip_f ratio (Table 1). The decreased reactivity between drug and thiol with increased electrolyte concentration is in accord with an SN2 type reaction, where the initial charge on the substrate(s) is dispersed or decreased in the transition state. Increasing the ionic strength of the solvent also resulted in decreased drug activity as measured by the drug-induced DNA damage [9]. Overall, GSH is the more effective thiol at removal of $RNO_2^{\cdot -}$. Voltammetrically the formation of the RNO/RNHOH couple was not detected, but the reactivity of both derivatives with thiols has already been noted. There is no apparent trend in % Δ ip_r/ip_f with the $RNO_2/RNO_2^{\cdot -}$ redox couple.

Nitrofurazone and nifuroxime present a complex voltammetry due to the overlap of drug and thiol responses, despite using a mixed solvent system. The thiol and nitro couples are sufficiently close that $RNO_2^{\cdot -}$ formed at the electrode is immediately re-oxidized to the original material. The effect is essentially catalytic, with the voltammetric changes to the nitro compound being virtually complete after the first thiol addition. With use of bulk electrolytic reduction techniques, the protection of DNA damage by thiols has been shown to depend on the reduction potential of the nitro compound. Only drugs having a redox potential close to that of the thiol were found to be effectively protected [4], in agreement with the redox cycling observed voltammetrically. The thiol species responsible for redox cycling has not been identified, but it is possible that the –NH· or –S· radical (produced via the 1 electron oxidation of cysteamine) is the oxidizing agent for the $RNO_2^{\cdot -}$.

In conclusion, the addition of cysteamine and GSH has a distinct influence on the voltammetric behaviour of a range of nitro-aromatic compounds. The interaction of the thiol with the nitro group redox chemistry is dependent on the solvent conditions and the reduction potential of the nitro compound. In an aqueous buffer, the thiol present in the bulk solution acts as a reducing agent, following formation of the nitro radical anion. The thiol also strongly interacts with the nitroso (and hydroxylamine) reduction product, as shown by the removal of the RNO/RNHOH couple from the voltammetry. In a mixed DMF/H_2O solvent, the thiol acts as an oxidizing agent for nitrofurazone and nifuroxime, regenerating the starting material from the nitro radical anion. This is due to the close proximity of the thiol and $RNO_2/RNO_2^{\cdot -}$ redox couples. Where the nitro reduction potential is well removed from that of the thiol (metronidazole, CAP, and M&B 4998), the thiol interacts with the nitro radical anion, but the efficiency of the radical scavenging is highly dependent on the medium. It appears that no single role can be assigned to the action of thiols on the reductive activation of nitro-aromatic compounds. We are in the process of investigating how these voltammetric observations correspond with the biological effect of thiols.

Acknowledgements—We wish to thank the Wellcome Trust and the Cancer Research Campaign for their financial support.

REFERENCES

1. Mitchell JB and Russo A, The role of glutathione in radiation and drug induced cytotoxicity. *Br J Cancer* **55**(Suppl. VIII): 96–104, 1987.
2. Berube LR, Farah S, McClelland RA and Rauth AM, Depletion of intracellular glutathione by 1-methyl-2-nitrosoimidazole. *Int J Radiat Oncol Biol Phys* **22**: 817–820, 1992.
3. Edwards DI, Mechanisms of cytotoxicity of nitroimidazole drugs. *Progress in Medical Chemistry* **18**: 88–116, 1981.
4. Edwards DI, Knox RJ, Rowley DA, Skolimowski IM and Knight RC, Mechanism of cytotoxicity of nitroimidazoles. In: *Nitroimidazoles: Chemistry, Pharmacology, and Clinical Application* (Eds. Breccia A, Cavalleri B and Adams GE), pp. 105–114. Plenum Press, New York, 1982.
5. Tocher JH and Edwards DI, Electrochemical characteristics of nitroheterocyclic compounds of biological interest. III. Nitroso derivative formation. *Free Rad Res Commun* **5**: 327–332, 1989.
6. Tocher JH and Edwards DI, Electrochemical characteristics of nitroheterocyclic compounds of biological interest. I. The influence of solvent. *Free Rad Res Commun* **4**: 269–276, 1988.
7. Tocher JH and Edwards DI, Electrochemical characteristics of nitroheterocyclic compounds of biological interest. V. Measurement and comparison of nitro radical lifetimes. *Int J Radiat Biol* **57**: 45–53, 1990.
8. McClelland RA, Molecular interactions and biological effects of the products of reduction of nitroimidazoles. In: *Selective Activation of Drugs by Redox Processes.* (Eds. Adams GE, Breccia A, Fielden EM and Wardman P), pp. 125–136. Plenum Press, New York, 1990.
9. Knox RJ, Ph.D. Thesis, Studies on the Action of Nitroimidazole Drugs. CNAA, North East London Polytechnic, 1982.