Anti-Cancer Drugs 1994, **5**, pp. 367–372

Short communication

Cyclopropamitosenes: novel bioreductive anticancer agents—mechanism of action and enzymic reduction

Christopher J Moody,[1] Noeleen O'Sullivan,[1] Ian J Stratford,[2] Miriam A Stephens,[2] Paul Workman,[3] Sue M Bailey[3] and Alex Lewis[3]

[1]Department of Chemistry, Loughborough University of Technology, Loughborough LE11 3TU, UK. Tel: (+44) 509 222550; Fax: (+44) 509 233163. [2]Medical Research Council Radiobiology Unit, Chilton, Didcot OX11 0RD, UK. [3]CRC Department of Medical Oncology, Cancer Research Campaign Beatson Laboratories, University of Glasgow, Switchback Road, Bearsden, Glasgow G61 1BD, UK.

The mechanism of action of cyclopropamitosenes, novel bioreductive anticancer agents, has been investigated using a unique combination of chemical and biochemical techniques. The compounds 4 function as reductively activated alkylating agents under chemical reducing conditions, and the biochemical experiments establish that the methoxy- and aziridinyl-derivatives 4a and 4b behave differently upon bioreduction.

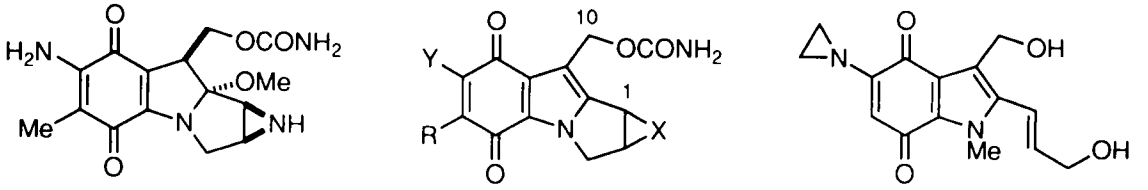
Key words: Alkylation, bioreduction, DT-diaphorase, mitosene.

Mitomycin C (MMC) **1**, a clinically useful antitumor antibiotic, is the archetypical quinone bioreductive alkylating agent.[1] The understanding of the reductive activation mechanism of MMC and related mitosenes, such as aziridinomitosenes **2** and the indolequinone EO9 **3** (Figure 1),[2] in which quinone reduction sequentially activates electrophilic sites in the drug molecules (C-1 and C-10 for MMC), has increased markedly in recent years.[3–6] Our own work was designed to investigate the role of C-10 in alkylation processes by preparing compounds in which electrophilicity at C-1 is much reduced by substituting a cyclopropane for the aziridine ring.[7] The resulting cyclopropamitosene **4** could on reductive activation, followed by elimination of the carbamate, generate a powerful electrophile capable of alkylating DNA (or other nucleophiles) at C-10 (Figure 2). However, ionic ring opening of the cyclopropane, analogous to that proposed for the corresponding 'natural' aziridine, is very unlikely, although radical induced ring opening of the cyclopropane[8] to give a highly reactive radical capable of abstracting the 4′-hydrogen from the deoxy-ribose ring of DNA (and hence causing strand cleavage)[9] is an alternative possibility (Figure 2). We now report the results of a detailed study of the properties of these novel 'unnatural' mitosenes **4** using a unique combination of chemical and biochemical methods designed to determine whether cyclopropamitosenes can act as reductively activated alkylating agents.

The 7-methoxycyclopropamitosene **4a**, prepared using the method previously described for the 6-methyl analog,[10] was converted into a range of 7-substituted derivatives by reaction with amine nucleophiles.[11] Of these, the 7-aziridinyl compound **4b** proved the most interesting. That cyclopropamitosenes can indeed function as reductively activated alkylating agents was demonstrated by the chemical reduction of **4a** in the presence of nucleophiles.[12] Thus a mixture of **4a** and potassium ethyl xanthate in deaerated dichloromethane : methanol : water (1:1:1) was treated with aqueous sodium dithionite. After 10 min, reoxidation with air and chromatography gave the C-10 adduct **5a** (46% yield) as the only observed product. Likewise, the adduct **5b** was isolated (53%) when the reduction was carried out in the presence of 4-toluidine (Figure 3). In neither experiment was there any evidence for the formation of products derived by ring opening the cyclopropane. Having established the reactivity of cyclopropamitosenes under chemical reducing conditions, we next investigated their biological properties.

Reductive activation of the mitosenes **4**, that will lead to toxicity towards cells in culture, can occur in two ways. Firstly, by *initial* 1-electron reducton to give a semiquinone. This would be carried out by enzymes such as cytochrome P450 reductase in a

Correspondence to CJ Moody



1 mitomycin C (MMC)

2 X = NH aziridinomitosene
4 X = CH_2 cyclopropamitosene
4a X = CH_2, R = H, Y = OMe
4b X = CH_2, R = H, Y = N

3 EO9

Figure 1. Reductive activation mechanism of MMC and related mitosenes.

process that is potentially reversible by oxygen,[13] i.e. O_2 will inhibit toxicity. Secondly, by an *initial* concerted 2-electron reduction to give a hydroquinone. This is generally carried out by the obligate 2-electron reductase DT-diaphorase in a process that is O_2-independent. The subsequent level of the ultimate alkylating species will then be governed by any disproportionation reaction between the semi- and hydro-quinones.[14,15]

Figure 4 shows results of experiments where Chinese hamster V79 cells are exposed to the novel cyclopropamitosenes **4a** and **4b** for 3 h at 37°C in aerobic or hypoxic conditions. Values of IC_{50}, the concentration required to kill 50% of the cells, are recorded in Table 1 and comparison made with results obtained with MMC and EO9. In air **4b** is 1000-fold more toxic than **4a**, with MMC and EO9 showing intermediate levels of toxicity. Under hypoxic conditions there is no change in the toxicity of **4b** whereas the potency of **4a** is increased 34-fold. These results indicate that under anaerobic conditions initial 1-electron reduction will contribute significantly to the toxicity of **4a** which is in line with the demonstrable alkylating ability of **4a** under the chemical reducing conditions described above. Similar processes may also occur with **4b**. However, no additional toxicity is observed in N_2 which, taken together with the far greater potency of **4b** in air suggests that O_2-independent 2-electron reductive activation may contribute significantly to the toxicity of this compound.

Evidence demonstrating the relative importance of initial 2-electron reduction for the activity of **4b** versus **4a** was obtained by repeating the assays in the presence of dicoumarol, an inhibitor of DT-diaphorase. The results (Table 1 and Figure 5) indicate that dicoumarol has no effect on the aerobic toxicity of **4a** whereas **4b** is considerably less potent under these conditions, thereby supporting the contention that O_2-independent 2-electron reduction processes are important in the activation of the aziridinyl cyclopropamitosene **4b**, and suggesting that DT-diaphorase is the enzyme involved.

Particular interest has focused on the role of DT-diaphorase or NAD(P)H : (quinone acceptor) oxidoreductase (EC 1.6.99.2) in the activation of bioreductive antitumor quinones.[15] This enzyme is known to activate MMC, particularly at acid pH.[16] It also bioactivates the indolequinone EO9 much more efficiently than MMC at physiological pH.[17] We therefore evaluated the ability of the cyclopropamitosenes **4a** and **4b** to act as substrates for

Table 1. Mechanistic investigation of cyclopropamitosenes **4** using biochemical techniques

Compound	Toxicity towards Chinese hamster V79 cells[23]					Enzyme studies[24]
	IC_{50} (μmol/dm³)		ratio	IC_{50} (μmol/dm³) air + dicoumarol	protection factor	DT-diaphorase reduction rate (nmol cytochrome *c* reduced/min/mg)
	air	N_2				
MMC 1	0.8	0.4	2.0	0.8	1.0	8.96×10^2
4a	4.8	0.14	34	4.8	1.0	1.60×10^3
4b	0.003	0.003	1.0	0.1	33	4.64×10^3
EO9 **3**	0.08	0.015	5.3	0.5	6.2	6.48×10^5
Menadione	—	—	—	—	—	4.96×10^6

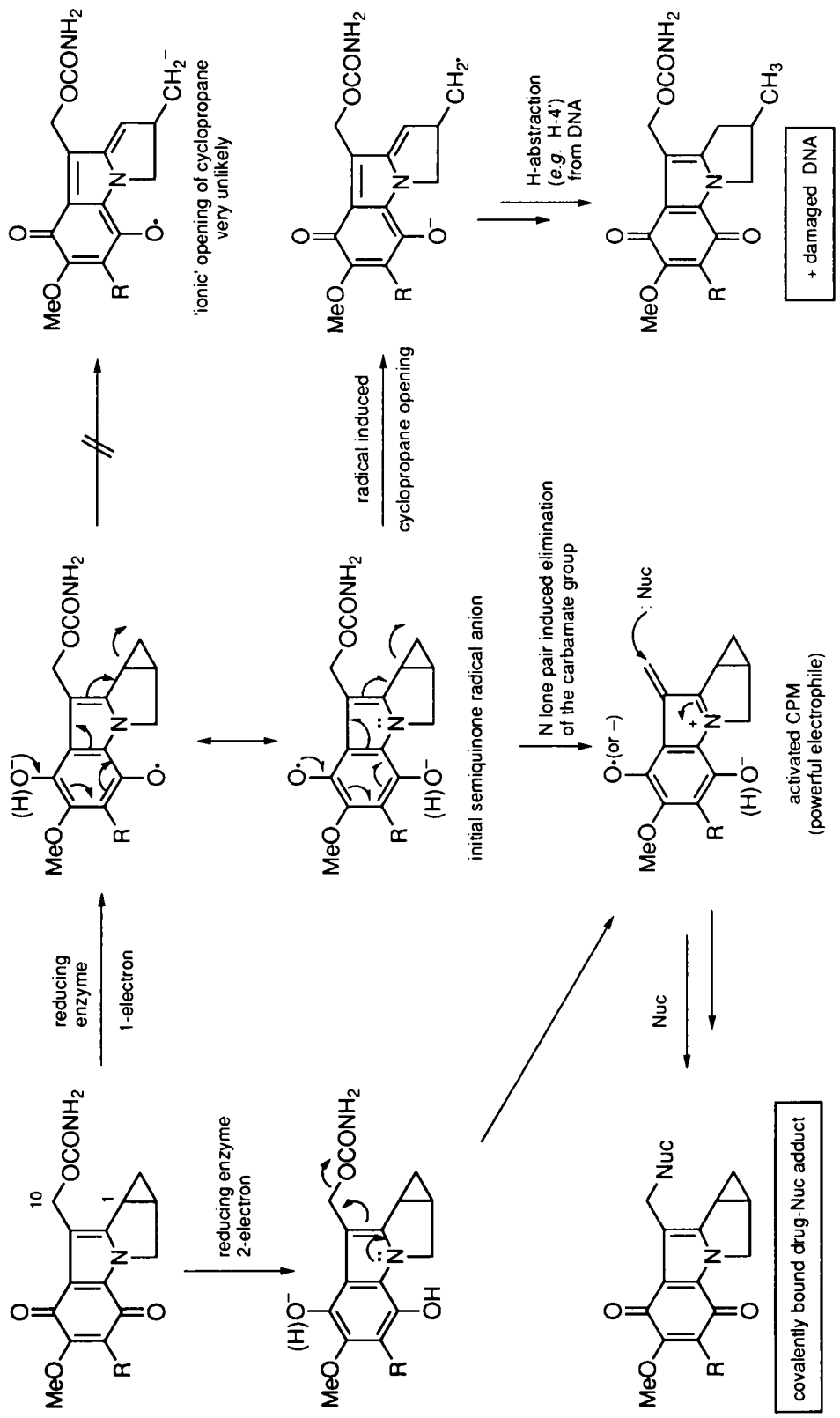


Figure 2. Postulated 'activation cascade' of unnatural cyclopropamitosenes.

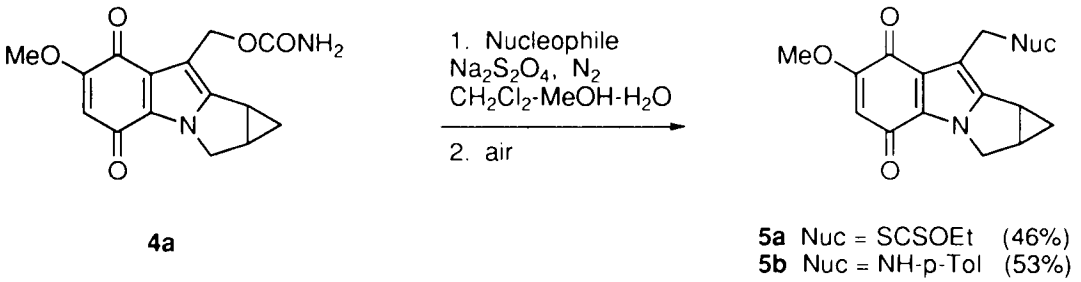


Figure 3. Reduction of 4a in the presence of 4-tolvidine.

reduction by purified Walker rat tumor DT-diaphorase.[18] The method used involved a modification[16b] of the classical assay in which reduction of the quinone to the hydroquinone is monitored spectrophotometrically by the subsequent reduction of cytochrome *c*.[19] Table 1 shows that both the 7-methoxy **4a** and the 7-aziridinyl analog **4b** did act as substrates for DT-diaphorase, with **4b** giving approximately 3-fold faster rates. Interestingly, an analog of EO9 in which a methoxy group replaced the aziridine moiety was also a poorer substrate for the same DT-diaphorase preparation, and exhibited lower cytotoxicity.[20] Reduction of **4a** and **4b** by DT-diaphorase was confirmed in experiments where the oxidation of the NADH cofactor was monitored spectrophotometrically. These supported the ordering of substrate efficiencies in Table 1. Both **4a** and **4b** are better substrates than MMC at physiological pH, although they are inferior to indolequinone EO9 and menadione in this respect. For example, **4b** was reduced 4 times more rapidly than MMC but 140-fold less efficiently than EO9.

It is clear that the 7-aziridinyl cyclopropamitosene **4b** is considerably more cytotoxic than the 7-methoxy compound **4a** towards V79 cells, under both hypoxic and aerobic conditions. However, no differential toxicity is seen for **4b**, and its aerobic cytotoxicity was reduced by dicoumarol; in contrast, a hypoxic cytotoxicity ratio of 34 was seen for **4a** with no modulation by dicoumarol. Although there are a number of caveats associated with the use of dicoumarol to probe for the importance of DT-diaphorase in bioreductive drug activation in whole cells,[21] the aforementioned cellular sensitivities to the two compounds, together with their differing abilities to act as substrates for DT-diaphorase, are consistent with a more predominant role for 2-electron reduction by this enzyme in the bioactivation in air of **4b** compared with **4a**.

In the final analysis, the relative importance of 1- versus 2-electron reduction processes in the expression of anticancer activity will depend on the relative expression of reducing enzymes in cells.[22] The potential importance of cyclopropamitosenes **4a** and **4b** as therapeutic agents (relative to MMC and EO9) is being actively investigated. These results, together with further chemical and electrochemical studies, will be reported elsewhere.

References

1. For reviews, see: Carter SK, Crooke ST. *Mitomycin C; current status and new developments*. New York: Academic Press 1979; Remers WA, Dorr RT. Chemistry,

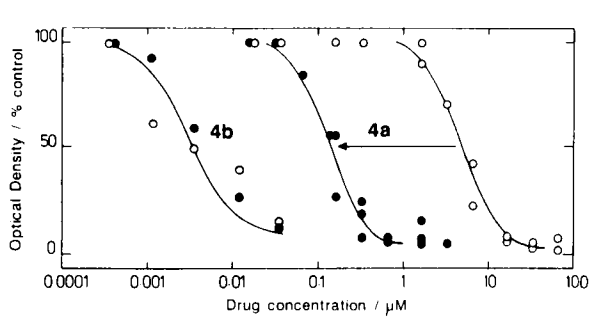


Figure 4. MTT assay of the toxicity of **4a** and **4b** in V79 cells following 3 h exposure at 37°C under aerobic (○) or hypoxic (●) conditions.

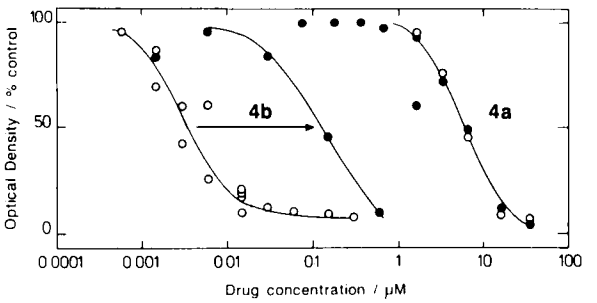


Figure 5. MTT assay of the toxicity of **4a** and **4b** in V79 cells following exposure for 3 h at 37°C under aerobic conditions in the presence (●) or absence (○) of 0.2 mmol/dm^3 dicoumarol.

biology, and therapeutics of the mitomycins. In: Pelletier SW, ed. *Alkaloids: chemical and biological perspectives;* New York: Wiley 1988; **6:** 1–74; Franck RW, Tomasz M. The chemistry of mitomycins. In: Wilman DEV, ed. *Chemistry of antitumor agents.* Glasgow: Blackie and Son 1990; 379–93; Remers WA, Iyengar BS. Synthesis of mitomycins. In: Lukacs G, Ohno M, eds. *Recent progress in the chemical synthesis of antibiotics.* Berlin: Springer-Verlag 1990; Kasi M, Kono M. Studies on the chemistry of mitomycins. *Synlett* 1992; 778–90.

2. Oostveen EA, Speckamp WN. Mitomycin analogs. I. Indolequinones as (potential) bis alkylating agents. *Tetrahedron* 1987; **43:** 255–62.
3. For the most recent contributions, see the following (and references 4–6) and the papers cited therein: Kohn H, Li VS, Schiltz P, *et al.* On the origins of the DNA sequence selectivity of mitomycin monoalkylation transformations. *J Am Chem Soc* 1992; **114:** 9218–9220; Schiltz P, Kohn H. Reductively activated mitomycin C; an efficient trapping reagent for electrophiles. *J Am Chem Soc* 1992; **114:** 7958–9; Schiltz P, Kohn H. Sodium dithionite mediated mitomycin C reductive activation processes. *Tetrahedron Lett* 1992; **33:** 4709–12; Han I, Russel DJ, Kohn H. Studies on the mechanism of mitomycin C(1) electrophilic transformations: structure–reactivity relationships. *J Org Chem* 1992; **57:** 1799–807.
4. McGuinness BF, Lipman R, Nakanishi K, *et al.* Reaction of sodium dithionite activated mitomycin C with guanine at non-cross linkable sequences of oligonucleotides. *J Org Chem* 1991; **56:** 4826-29.
5. Egbertson M, Danishefsky SJ. Modeling of the electrophilic activation of mitomycins: chemical evidence for the intermediacy of a mitosene semiquinone as the active electrophile. *J Am Chem Soc* 1987; **109:** 2204–5; Egbertson M, Danishefsky SJ, Schulte G. On the remarkable stability of derivatives of leucomitomycin F. Novel mitomycin analogs. *J Org Chem* 1987; **52:** 4424–6.
6. Hopkins PB, Millard JT, Woo J, *et al.* Sequence preferences of DNA interstrand cross-linking agents; importance of minimal DNA structural reorganisation in the cross linking reactions of mechlorethamine, cisplatin and mitomycin C. *Tetrahedron* 1991; **47:** 2475–89; Iyengar BS, Dorr RT, Shipp NG, *et al.* Alkylation of DNA by C-10 of 2,7-diaminomitosene. *J Med Chem* 1990; **33:** 253–7; Orlemans EOM, Verboom W, Scheltinga MW, *et al.* Synthesis, mechanism of action and biological evaluation of mitosenes. *J Med Chem* 1989; **32:** 1612–20.
7. Jones GB, Moody CJ. Structurally modified antitumour agents. Part 1. Synthesis of cyclopropapyrrolo[1,2-*a*]indoles related to mitosenes by intramolecular cycloaddition. *J Chem Soc, Perkin Trans 1* 1989; 2449–54.
8. Giese B. *Radicals in organic synthesis: formation of carbon–carbon bonds.* New York: Pergamon 1986; Motherwell WB, Crich D. *Free radical chain reactions in organic synthesis.* London: Academic Press 1991.
9. Strand breakage by agents such as bleomycin and the enediynes is well known. Blackburn GM, Gait MJ, eds. *Nucleic acids in chemistry and biology.* Oxford: IRL Press 1990.
10. Jones GB, Moody CJ. Structurally modified antitumour agents. Part 2. Total synthesis of a cyclopropamitosene. *J Chem Soc, Perkin Trans 1* 1989; 2455–62; O'Sullivan N. PhD Thesis, University of Loughborough, 1992; Moody CJ, O'Sullivan N. *UK Patent Application 9225799.7,* 10 December 1992.
11. *cf.* Iyengar BS, Remers WA, Bradner WT. Preparation and antitumor activity of 7-substituted 1,2-aziridinomitomycins. *J Med Chem* 1986; **29:** 1864–8.
12. The electrochemical reduction was also studied by carrying out measurements on **4** in DMF solution using tetra-*n*-butylammonium tetrafluoroborate as supporting electrolyte. The E_{redox} values obtained (referenced to ferrocene to avoid liquid junction potential) were: MMC, −1.421 V; **4a**, −1.395 V; **4b**, −1.360 V. We thank Dr RJ Mortimer for carrying out these experiments.
13. Wardman P. Reductive activation of quinones: redox properties and thiol reactivity. *Free Rad Res Commun* 1990; **8:** 219–30.
14. Hoey BM, Butler J, Swallow AJ. Reductive activation of mitomycin C. *Biochemistry* 1988; **27** 2608–14; Machtalére G, Houé-Levim C, Gardes-Albert M, *et al.* Pulse radiolysis study of the reduction mechanism of an antitumour antibiotic, mitomycin C. *CR Acad Sci Paris II* 1988; **307:** 17–22.
15. For a review, see: Riley RJ, Workman P. DT-Diaphorase and cancer chemotherapy. *Biochem Pharmacol* 1992; **43:** 1657–69.
16. For the most recent contributions, see the following: (a) Siegel D, Gibson NW, Preusch PC, *et al.* Metabolism of mitomycin C by DT-diaphorase: role in mitomycin C-induced DNA damage and cytotoxicity in human colon carcinoma cells. *Cancer Res* 1990; **50:** 7483–9; (b) Walton MI, Sugget N, Workman P. The role of human and rodent DT-diaphorase in the reductive metabolism of hypoxic cell cytotoxins. *Int J Radiat Oncol Biol Phys* 1992; **22:** 643–8.
17. Walton MI, Smith PJ, Workman P. The role of NAD(P)H: quinone reductase (EC 1.6.99.2, DT-diaphorase) in the reductive bioactivation of the novel indoloquinone antitumor agent EO9. *Cancer Commun* 1991; **3:** 199–206; Robertson N, Stratford IJ, Houlbrook S, *et al.* The sensitivity of human tumour cells to quinone bioreductive drugs: what role for DT-diaphorase? *Biochem Pharmacol* 1992; **44**: 409–12.
18. Knox RJ, Boland MP, Friedlos F, *et al.* The nitroreductase enzyme in Walker cells that activates 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB 1954) to 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide is a form of NAD(P)H dehydrogenase (quinone (EC 1.6.99.2). *Biochem Pharmacol* 1988; **37:** 4671–7.
19. Ernster L, Danielson L, Ljunggren M. DT-diaphorase. I. Purification from the soluble fraction of rat-liver cytoplasm, and properties. *Biochem Biophys Acta* 1962; **58**: 171–88.
20. Bailey SM, Sugget N, Walton MI, *et al.* Structure–activity relationships for DT-diaphorase reduction of hypoxic cell directed agents: indoloquinones and diaziridinyl benzoquinones. *Int J Radiat Oncol Biol Phys* 1992; **22:** 649–54.
21. Workman P, Walton MI, Powis G, *et al.* DT-Diaphorase: questionable role in mitomycin C resistance, but a target for novel bioreductive drugs. *Br J Cancer* 1989; **60:** 800–2; Preusch PC, Siegel D, Gibson NW, *et al.* A note on the inhibition of DT-diaphorase by dicoumarol. *Free Rad Biol Med* 1991; **11:** 77–80.
22. For recent reviews, see: Workman P, Walton MI. Enzyme-directed bioreductive drug development. In: Adams GE,

Breccia A, Fielden EM, *et al.*, eds. *Selective activation of drugs by redox processes*. New York: Plenum 1991: 1973–91; Workman P. Bioreductive mechanisms. *Int J Radiat Oncol Biol Phys* 1992; **22:** 631-7.

23. Stratford IJ, Stephens MA. The differential hypoxic toxicity of bioreductive agents determined *in vitro* using the MTT assay. *Int J Radiat Oncol Biol Phys* 1989; **16**: 973–6. Robertson N, Haigh A, Adams GE, *et al.* Factors affecting sensitivity to E09 in rodent and human tumour cells *in vitro*: DT-diaphorase activity and hypoxia. *Eur J Cancer* 1994; in press.
24. Incubations contained: purified rat Walker tumour DT-diaphorase, NADH (2 mM) as cofactor, quinone substrate (50 kM), and cytochrome *c* (77 kM) as electron acceptor in tris–HCl (50 mM) buffer, pH 7.4, containing 0.14% bovine serum albumin at 37°C. The reaction was monitored spectrophotometrically by the change in absorbance of cytochrome *c* at 550 nm.

(Received 31 January 1994; accepted 25 February 1994)