Sir – Marshall et al. (1989) reported recently that a human fibroblast cell line from a member of a cancer-prone family (343TT) was six times more resistant than an equivalent cell line from a normal donor (GM38) to the bioreductive antitumour antibiotic mitomycin C. Interestingly, this resistance was seen only when the drug exposure was carried out under well oxygenated conditions, but not under hypoxic conditions (<10 p.p.m. O2). Similarly, a Chinese hamster ovary cell line with induced resistance to mitomycin C under oxic conditions did not exhibit comparable resistance under hypoxic conditions (Hoban et al., 1989).

Compared to the normal human fibroblast line GM38, Marshall et al. (1989) reported that the resistant 343TT cell line exhibited substantially reduced levels of the enzyme DT-diaphorase or NADPH: (quinone-acceptor) oxidoreductase (E.C.1.6.99.2) (Ernster et al., 1987). Also, the DT-diaphorase inhibitor dicoumarol (3,3'-methylene-bis [4-hydroxycoumarin]) was shown to decrease the aerobic sensitivity of the normal GM38 line but not the resistant 343TT line. The conclusion was drawn that DT-diaphorase may play an important role in the bioreductive activation of mitomycin C under oxic conditions, and that deficient expression of this enzyme by the 343TT cell line leads (at least in part) to aerobic resistance to the drug. Similarly, this resistance is mimicked to some extent in the normal GM38 line by dicoumarol inhibition of the enzyme.

A number of studies on the mechanism of action of mitomycin C have relied heavily on the use of dicoumarol as a specific inhibitor of DT-diaphorase to probe for the functional role of this enzyme (e.g. Keyes et al., 1984, 1985a, b; Dhul Hannah et al., 1989). In one series of studies, the intriguing result was obtained that dicoumarol was able to increase the sensitivity of EMT6 mouse mammary tumour cells to mitomycin C under hypoxic conditions, yet decreased the sensitivity of the same cells when exposed to the drug under oxic conditions (Keyes et al., 1984, 1985a, b). The increased toxicity under hypoxic conditions was related to the stimulation by dicoumarol of the amounts of alkylating species generated (Keyes et al., 1984). The clear and reasonable implication at the time the studies were conducted was that DT-diaphorase activates mitomycin C in air but serves to detoxify the same drug under hypoxia.

The proposed role for DT-diaphorase in the toxification of mitomycin C under aerobic conditions is contradictory to that which is generally thought to apply for simple quinones such as menadione (Lind et al., 1982; Thor et al., 1982; Morrison et al., 1984; Ernster et al., 1987). The aerobic toxicity of such compounds arises via one-electron reduction to the semiquinone free radical by enzymes such as NADPH:cytochrome P-450 reductase, leading to production of toxic oxygen species by auto-oxidation in a futile cycle (Figure 1). These radicals cause DNA and membrane damage. Since DT-diaphorase is an obligatory two-electron donor (Iyanagi & Yamazaki, 1970), reduction of quinones by this enzyme bypasses the toxic semiquinone radical by direct formation of the relatively stable hydroquinone, which can then undergo conjugation (Figure 1). Thus under aerobic conditions DT-diaphorase plays an important role in the cellular defense against oxygen-acceptor) oxygen species caused by simple quinones, and inhibition by dicoumarol gives rise to increased toxicity (see Ernster et al., 1987).

The picture is of course more complex with mitomycin C since toxicity under oxic conditions will arise not only via oxidative stress (Bachur et al., 1979; Pritsos & Sartorelli, 1986) but also through bioreductive activation to DNA-alkylating species (Pan et al., 1984; Tomasz et al., 1987). Under hypoxic conditions DNA adducts, including cross-links, will predominate. It has been argued that both one-electron and two-electron reduction will result in bioreductive alkylation (Tomasz et al., 1987; Hoey et al., 1988). Nevertheless, the precise role of these two reduction mechanisms in this toxic pathway remains unclear.

Further complications to the ongoing controversy are suggested by correlations between DT-diaphorase activity and cytotoxicity obtained in two other recent studies. Pritsos et al. (1987) compared DT-diaphorase in three xenograft tumours grown in nude mice (human, equine and canine neoplasms) and found the lowest enzyme activity in the tumour with the greatest mitomycin C sensitivity. In addition, in vivo treatment of the sensitive tumour with mitomycin C resulted in a higher level of enzyme activity in the subsequently regrowing tumours. These correlations are consistent with a detoxifying function for DT-diaphorase in vivo. On the other hand, in the previously mentioned study of a Chinese hamster ovary cell line with induced resistance through exposure to mitomycin C under aerobic conditions in vitro, there was no measurable DT-diaphorase activity but a decrease in NADPH:cytochrome P-450 reductase (Hoban et al., 1989; Walton et al., 1989). These results indicate a more predominant role for cytochrome P-450 reductase in governing mitomycin C sensitivity. This enzyme is known to metabolise mitomycin C (Bachur et al., 1979).

Much of the evidence in the continuing debate of the role of DT-diaphorase in mitomycin C bioactivation comes from studies such as those discussed earlier utilising dicoumarol as an inhibitor of the enzyme. There has, however, always been some concern regarding the over-reliance on dicoumarol as a

Figure 1 Predominant roles of enzymatic one-electron versus two-electron transfer in the toxification versus detoxification reactions of simple quinones such as menadione. According to this scheme one-electron transferring enzymes such as NADPH:cytochrome P-450 reductase catalyse the formation of the semi-quinone free radical, leading to the generation of the toxic species superoxide in the presence of oxygen. This pathway is bypassed as a result of direct two-electron reduction via DT-diaphorase, forming the more stable hydroquinone which can be further detoxified via conversion by conjugating enzymes to water soluble glucuronides and sulphates for excretion.
putative inhibitor of mitomycin C metabolism by DT-diaphorase where the measured end-point is cytotoxicity and not modulation of bioreductive metabolism. The potential for dicoumarol to modify cytotoxicity via additional mechanisms is clear (Keyes et al., 1987; Akman et al., 1985). In particular, dicoumarol was shown to increase hypoxic mitomycin C toxicity (but not reduce oxic toxicity) in L1210 cells with measurable DT-diaphorase activity (Keyes et al., 1987). Potentiation of menadione toxicity in L1210 cells by a method not involving DT-diaphorase has also been proposed (Akman et al., 1985). Indeed the authors of the various papers cited above, including Marshall et al. (1989), have been appropriately cautious in pointing out the potential artefacts of this approach.

In view of the contentious role of DT-diaphorase in mitomycin C resistance, it is surprising that until recently there has been no published attempt to demonstrate metabolism of the drug by the purified enzyme. Such studies have now been carried out with several enzyme preparations and the results directly contradict a role for the enzyme in modulating mitomycin C sensitivity and resistance. The drug acts not as a substrate but in fact as an inhibitor of DT-diaphorase purified from human kidney by affinity and hydroxypapitate chromatography. This was demonstrated initially by two of us (Schlager & Pows, 1988) and confirmed independently by the others (Walton & Workman, unpublished). Mitomycin C is also a substrate for DT-diaphorase purified from rat liver (Pows & Schlager, unpublished). In addition, no dicoumarol-inhibitable metabolism of mitomycin C could be identified in DT-diaphorase-rich preparations obtained from the Chester Beatty strain of the rat Walker tumour (liver) or the HT29 human colon carcinoma (Walton & Workman, unpublished). In all cases the most direct assay involved analysis of mitomycin C using a sensitive and specific high-performance liquid chromatography assay, and we can now define the lower limit of measurable activity as <30 pmol min⁻¹ Unit enzyme at 50 μM mitomycin C (1<br>unit of activity = 1 pmol of NADH produced per unit time) or 1 pmol of NADPH produced per unit time). (2) An alternative group including tert-butylhydroquinone and redox-labile diphenols capable of inducing phase 2 enzymes, such as UDPG-glucuronyl transferases, which normally play a detoxication role (see De Long et al., 1987). DT-diaphorase has been shown to exhibit co-ordinately increased level of mRNA expression with glutathione-S-transferase Ya and Yb genes in rat hepatic preneoplastic nodules induced during chemical carcinogenesis in the Solt – Farber model, apparently as a result of hypomethylation of the gene (see Pickett, 1987).

Exciting new possibilities have been revealed by the recognition of close similarities in the biochemical profiles of rat preneoplastic and neoplastic hepatocytes and of in vitro derived multidrug resistant cells (Moscow & Cowan, 1988; Burt & Thorgersson, 1988). In both situations resistance to a range of toxins is seen; toxin accumulation is reduced; expression of the P-170 drug efflux membrane glycoprotein decreases; protective phase 2 and related enzyme activities rise; and phase 1 enzymes may fall or rise. Thus expression of these various genes may depend on overlapping regulatory elements.

The role of DT-diaphorase in multidrug resistance is unknown. No up-regulation was observed in a multidrug-resistant MCF-7 human breast cancer line showing increased expression of glutathione-S-transferase π and expression of hydrocarbon hydroxylase activity; in fact a decrease in DT-diaphorase was seen (Vickers et al., 1989). However, protein changes, including cytochrome P-450s, glutathione-S-transferases and even P-glycoprotein, are by no means consistent across all multidrug-resistant cell lines (neither is the cross-resistance profile), and a range of lines should now be examined to clarify this situation.

Increased expression of DT-diaphorase in tumour cells, including human breast and colon tumours, may be quite widespread (Koudstaal et al., 1975; Schor & Cornelisse, 1983; Schor, 1987; Schlager & Pows, 1987, 1988). In view of these general findings, the presence of high DT-diaphorase levels in the HT29 human colon carcinoma and Walker 256 tumour cell lines makes these tumours especially appropriate models. It is indeed possible that the Walker 256 rat tumour, though now an undifferentiated carcinosarcoma, originally arose as a mammary carcinoma of typical adenomatous structure (Earle, 1935; Rosenoer et al., 1966).

Despite the apparent inability to metabolise mitomycin C, DT-diaphorase may represent an important target for drug bioactivation. In support of this, the elegant recent work of Knox et al. (1988a, b) shows that the rat liver DT-diaphorase from the Chester Beatty Walker 256 rat accounts for the extreme sensitivity of this tumour to CB 1954 (5-aziridinyl-1-yI)-2,4-dinitrobenz-1-amide). DT-diaphorase reduces this agent in vitro to a highly toxic 4-hydroxylamine derivative. Moreover, we have recently shown similar activity for purified human kidney DT-diaphorase, and the Walker enzyme to have the ability to reduce the novel benzotriazine di-N-oxide hypoxic cell cytotoxic SR 4223 (3-amin0-1,2,4-benzotriazine-1,4-dioxide) (unpublished data).

In summary, DT-diaphorase appears to play a questionable role in mitomycin C resistance, and further work is required to resolve this issue. DT-diaphorase may, however, provide an attractive target for the design of novel bioreductive drugs for the treatment of human tumours shown specifically to express high levels or an unusual form of this intriguing enzyme.
References

AKMAN, S.A., DIETRICH, M., CHLEBOWSKI, R., DOROSHOW, J. & BLOCK, J.B. (1985). Menadione (K3) and dicumarol (D) synergy vs leukaemia L1210. Proc. Am. Assoc. Cancer Res., 26, 325.

BACHUR, N.R., GORDON, S.L., GEE, M.V. & KON, H. (1979). NADPH-cytochrome P-450 reductase activation of quinone anticancer agents to free radicals. Proc. Natl. Acad. Sci. USA, 76, 954.

BURT, R.K. & THORGERSSON, S.S. (1988). Coinduction of MDR-1 multidrug-resistance and cytochrome P-450 genes in rat liver by xenobiotics. J. Natl. Cancer Inst., 80, 1383.

DE LONG, M.J., SANTAMARIA, A.B. & TALALAY, P. (1987). Role of cytochrome P-450 in the induction of NAD(P)H: quinone reductase in a murine hepatoma cell line and its mutants. Carcinogenesis, 8, 1549.

DULHANTY, A.M., LI, M. & WHITMORE, G.F. (1989). Isolation of Chinese hamster ovary cell mutants deficient in excision repair and mitomycin C bioactivation. Cancer Res., 49, 117.

EALR, W.R. (1935). A study of the Walker rat mammary carcinoma 256, in vivo and in vitro. Am. J. Cancer, 24, 566.

ERNSTER, L., ESTABROOK, R.W., HOCHSTEIN, P. & ORRENIUS, S. (eds) (1987). DT-diaphorase: a quinone reductase with special functions in cell metabolism and detoxication. Chim. Scripta, 27A.

HOBAN, P.R., WALTON, M.I., ROBSON, C.N. & 5 others (1989). Mitomycin C resistance under aerobic but not hypoxic conditions in a mammalian cell line: Association with impaired drug activation and decreased NADPH: cytochrome P-450 reductive activity. Cancer Res.

HOEY, B.M., BUTLER, J. & SWALLOW, A.J. (1988). Reductive activation of mitomycin C. Biochemistry, 27, 2608.

IYANAGI, T. & YAMAZAKI, I. (1970). One-electron transfer reactions in biochemical systems. V. Difference in the mechanism of quinone reduction by the NADH dehydrogenase and the NAD(P)H dehydrogenase (DT-diaphorase). Biochim. Biophys. Acta, 216, 282.

KEYES, S.R., FRACASSO, P.M., HEIMBROOK, D.C., ROCKWELL, S., SLIGAR, S.G. & SARTORELLI, A.C. (1984). Role of cytochrome c reductase and DT-diaphorase in the biotransformation of mitomycin C. Cancer Res., 44, 5628.

KEYES, S.R., ROCKWELL, S. & SARTORELLI, A.C. (1985a). Enhancement of mitomycin C cytotoxicity to hypoxic cells by dicumarol in vivo and in vitro. Cancer Res., 45, 213.

KEYES, S.R., ROCKWELL, S. & SARTORELLI, A.C. (1985b). Porphyrin as a bioreductive alkylating agent with selective toxicity to hypoxic EM/56 tumor cells in vivo and in vitro. Cancer Res., 45, 3642.

KEYES, S.R., ROCKWELL, S. & SARTORELLI, A.C. (1987). Studies on the modulation of mitomycin C cytotoxicity by dicumarol. Proc. Am. Assoc. Cancer Res., 28, 411.

LIND, C., HOCHSTEIN, P. & ERNSTER, L. (1982). DT-diaphorase as a quinone reductase: a cellular device against semiquinone and superoxide formation. Arch. Biochem. Biophys., 216, 178.

MARSHALL, R.S. & RAUTH, A.M. (1989). Deficient activation by a human cell strain leads to mitomycin resistance under aerobic but not hypoxic conditions. Br. J. Cancer, 59, 341.

MORRISON, H., JERNSTROM, B., NORDENSKJOLD, M., THOR, H. & ORRENIUS, S. (1984). Induction of DNA damage by menadione (2-methyl-1,4-naphthoquinone) in primary cultures of rat hepatocytes. Biochem. Pharmacol., 33, 1763.

MOSKOW, J.A. & COWAN, K.H. (1988). Multidrug resistance. J. Natl Cancer Inst., 80, 14.

PAN, S.S., ANDREWS, P.A. & GLOVER, C.J. (1984). Reductive activation of mitomycin C and mitomycin C metabolites catalyzed by NADPH-cytochrome P-450 reductase and xanthine oxidase. J. Biol. Chem., 259, 959.

PICKETT, C.B. (1987). Structure and regulation of glutathione S-transferase genes. Essays Biochem., 23, 116.

PRITSON, C.A., PARDINI, L.L., ELLIOT, A.J. & PARDINI, R.S. (1987). Relationship between the antioxidant enzyme DT-diaphorase and tumour response to mitomycin C treatment. In Oxygen Radicals in Biology and Medicine, Simic, M.G. & Taylor, K.A. (eds) p. 713. Plenum Press: New York.

PRITSON, C.A. & SARTORELLI, A.C. (1986). Generation of reactive oxygen radicals through bioactivation of mitomycin C antibiotics. Cancer Res., 46, 3528.

ROSENOER, V.M., MITCHELY, B.C.V., ROE, F.J.C. & CONNORS, T.A. (1986). Walker carcinosarcoma 256 in a study of anticancer agents. I. Method for simultaneous assessment of therapeutic value and toxicity. Cancer Res., 46, suppl. 2, 937.

SCHLAGER, J.J. & POWIS, G. (1987). NAD(P)H:quinone-acceptor oxidoreductase (QAO, E.C.1.6.99.2) activity in human normal and tumour tissues. Pharmacologist, 29, 177.

SCHLAGER, J.J. & POWIS, G. (1988). The effect of smoking on human cytosolic DT-diaphorase (DT) (E.C.1.6.99.2) activity in normal and tumour tissues. Proc. Am. Assoc. Cancer Res., 29, 8.

SCHOR, N.A. (1987). DT-diaphorase and the cancer cell. Chem. Scripta, 27A, 135.

SIEGEL, D., PACHECO, D.Y., GIBSON, N.W. & ROSS, D. (1989). Mechanisms of cytotoxicity associated with the two electron reduction of 2,5-diaziridinyl-3,6-bis (carboethoxyamino)-1,4-benzoquinone (AZQ) in human colon carcinoma cells. Proc. Am. Assoc. Cancer Res., 30, 558.

THOR, H., SMITH, M.T., HARTZELL, F., BELLAMO, G., JEWELL, S.A. & ORRENIUS, S. (1982). The metabolism of menadione by isolated hepatocytes. J. Biol. Chem., 257, 12419.

TOMASZ, M., LIPMAN, R., DONDATI, C., PAWLAK, J., VERDINE, G.L. & NAKANISHI, K. (1987). Isolation and structure of a covalent cross-link adduct between mitomycin C and DNA. Science, 235, 1204.

VICKERS, P.J., TOWNSEND, A.J. & COWAN, K.H. (1989). Mechanisms of resistance to antineoplastic drugs. In Developments in Cancer Chemotherapy, Glazer, R.I. (ed) p. 117. CRC Press: Boca Raton.

WALLIN, J.R. (1986). Adriamycin and DT-diaphorase. Cancer Lett., 36, 97.

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