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Immunological Quantitation of DT-Diaphorase in Carcinoma Cell Lines and Clinical Colon Cancers: Advanced Tumors Express Greater Levels of DT-Diaphorase

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NAD(P)H:quinone oxidoreductase (DT-diaphorase; DTD) plays a major role in activating mitomycin C (MMC) in human colon and gastric carcinoma cell lines. Thus, measurement of DTD in clinical tumor samples could be beneficial in designing adjuvant chemotherapy. We explored immunological quantitation of DTD protein using a monoclonal antibody against DTD, demonstrating a close correlation between protein expression and enzyme activity of DTD in colon and gastric carcinoma cell lines and in colorectal tumor samples. This indicates that such immunoblot analysis is a simple alternative method for quantitating DTD in clinically excised samples. In most colorectal tumor samples, the tumors expressed larger amounts of DTD than did the peripheral normal tissues, suggesting a selective toxicity of MMC toward tumor cells. Also tumors with nodal metastases showed significantly higher DTD levels than did tumors without metastasis. These results raise the possibility that DTD expression is related to tumorigenesis and malignant progression of colorectal tumors. Measurement of DTD by the immunological method described here could be beneficial in designing a rational adjuvant chemotherapy with MMC.

Key words: Mitomycin C — DT-diaphorase — Immunoblot — Metastasis — Colon carcinoma

Mitomycin C (MMC) is an antitumor antibiotic effective against solid tumors, including colorectal tumors. MMC requires bioreductive activation in tumor cells to show its antitumor activity. The bioreductive activation of MMC can occur via two pathways. The first is one-electron reduction, mediated by NADPH:cytochrome P450 reductase,[1,2] xanthine oxidase,[2] cytochrome b_5 reductase[3] and xanthine dehydrogenase.[4] The second is two-electron reduction. A major two-electron reductase is NAD(P)H: quinone oxidoreductase, which is known as DT-diaphorase (DTD), encoded by the *NQO1* gene.[5]

DTD plays a major role in the activation of MMC in carcinoma cells because (i) MMC-resistant cell lines show no or only marginal DTD activity[6–14]; (ii) introduction of the *NQO1* gene into St-4 cells, which express no DTD, renders the cells several times more sensitive to MMC[15]; and (iii) the amount of DNA-bound MMC is increased in the *NQO1* transfectants over the parental St-4 cells.[15] Therefore, clinical measurement of DTD in carcinoma samples could be beneficial in designing adjuvant chemotherapy involving MMC.

DTD activity is usually examined spectroscopically by measuring dicoumarol-sensitive reduction of 2,6-dichlorophenol indophenol (DCPIP).[16] This procedure is complicated and time-consuming. In clinical tumor samples, enzyme activity is relatively unstable and can be affected by contaminants such as antioxidants and related enzymes. Therefore, an alternative method is needed to quantitate DTD effectively in large numbers of clinical tumor samples.

We previously developed an anti-human DTD monoclonal antibody.[14] Using this antibody in immunoblot analyses, we measured DTD protein expression and compared it with DTD enzyme activity. We found that the protein expression was closely correlated with the enzyme activity in the carcinoma cell lines and in the colorectal tumor samples. We also found that colorectal tumors with metastasis showed higher DTD levels than did tumors without metastasis.

MATERIALS AND METHODS

Chemicals MMC was generously donated by Kyowa Hakko Kogyo Co., Ltd. (Tokyo). NADPH was obtained from Nacalai Tesque Co., Ltd. (Tokyo). DIPCP was

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obtained from Sigma Chemical Company, and dicoumarol from Wako Pure Chemicals, Inc. (Osaka). All other reagents were of analytical grade.

Cell lines Seven colon carcinoma cell lines (HT-29, HCC2998, KM12, KM20L2, WiDr, HCT15 and HCT116), a variant of HT-29 that is resistant to MMC (HT-29/MMC),[14] and five gastric carcinoma cell lines (St-4, MKN7, MKN28, MKN45 and MKN74) were used in this study. All cell lines were cultured in RPMI 1640 medium (Nissui, Tokyo) containing 10% fetal bovine serum (Life Technologies, Inc., Grand Island, NY) and 100 μg/ml of kanamycin (Meiji Seika, Tokyo) and were maintained in a humidified atmosphere of 95% air-5% CO_2 at 37°C.

Growth inhibition by MMC The growth inhibition assay was performed as previously described.[17] In brief, exponentially growing cells were plated into 6-well plates in 2 ml of growth medium. The colon cell lines were plated at a density of 2×10^4 cells/ml, and the gastric cell lines were plated at a density of 4×10^4 cells/ml. After an overnight culture at 37°C, graded concentrations of MMC were added to the medium. After 2 days, cells were trypsinized and counted with a Coulter Counter. The drug concentrations that inhibit cell growth by 50% (IC_{50} values) were determined as described previously.[18]

Preparation of cell lysates The cultured cell lines were grown to subconfluence under standard conditions. The cell layer was washed with phosphate-buffered saline (136.9 m*M* NaCl, 2.7 m*M* KCl, 8.1 m*M* Na_2HPO_4 and 1.5 m*M* KH_2PO_4) and harvested. The pelleted cells were then suspended in ice-cold reticulocyte standard buffer (10 m*M* Tris-HCl, pH 7.4, 10 m*M* NaCl and 1.5 m*M* $MgCl_2$) containing 0.1 m*M* phenylmethylsulfonyl fluoride, lysed by sonication for 10 s and centrifuged at 1,500*g* for 10 min at 4°C to obtain the supernatant lysate. When clinically excised samples were used, the tumors and surrounding normal tissues were immediately trimmed to 0.5 cm^3 fragments after tumor resection. The samples were homogenized using a Dounce-type homogenizer (30 strokes) in ice-cold reticulocyte standard buffer and centrifuged at 1,500*g* for 10 min at 4°C.

Enzyme activity analysis The lysates were further centrifuged at 105,000*g* for 30 min at 4°C to obtain clear cytosol as the enzyme source. Protein concentrations were determined with a bicinchoninic acid protein assay kit (Pierce Chemical Co., Rockford, IL). DTD activity was assayed according to the method of Ernster,[16] as modified by Benson *et al.*[19]

Immunoblot analysis Whole lysates were subjected to immunoblot analysis without further fractionation. The immunoblot analysis of DTD was performed using a mouse anti-human DTD monoclonal antibody developed in our laboratory.[14] The DTD protein bands were detected by enhanced chemiluminescence with the western blotting protocol (Amersham Japan, Tokyo), and the density of each protein band was measured using an Ultra Scan XL (Pharmacia, Uppsala, Sweden).

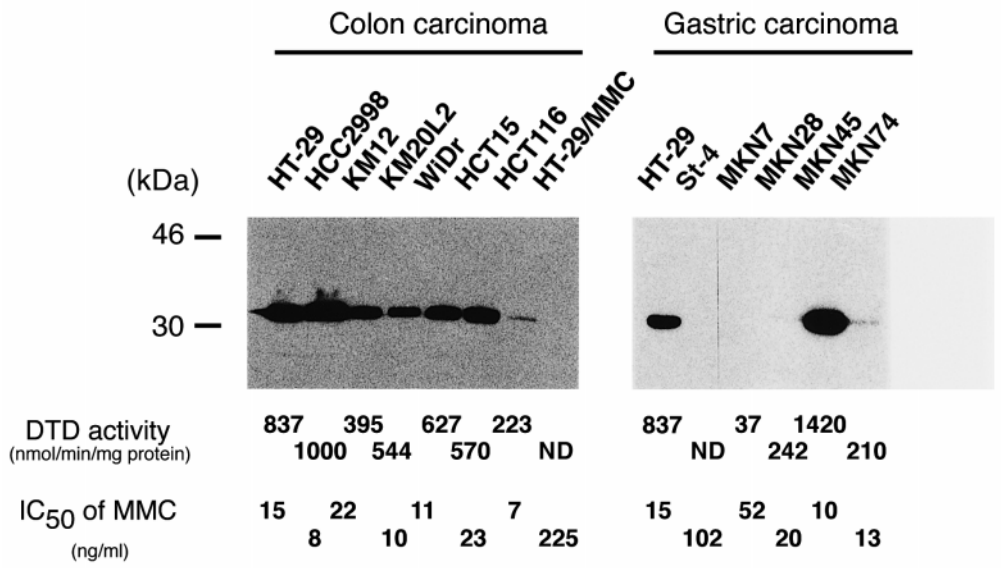

Fig. 1. Immunoblot analysis of DTD in human colon and gastric cell lines. Cell lysates (20 μg) from human cell lines were subjected to immunoblot analysis. DTD enzyme activity and IC_{50} of MMC were measured as described in "Materials and Methods." ND: <5 nmol/min/mg protein.

RESULTS

Correlation between DTD protein expression and enzyme activities in cell lines The amount of DTD protein was examined by immunoblot analysis in eight colon and five gastric carcinoma cell lines (Fig. 1). Most of the cell lines showed a 32-kDa protein band, depending on their expression levels. Among the colon cell lines, HCC2998 showed the highest level of DTD protein band, while HCT116 showed only a faint band. HT-29/MMC cells, which are resistant to MMC due to deficient DTD activity, exhibited no DTD protein band, as reported previously.[14] In gastric cell lines, MKN45 cells expressed the highest level of DTD. MKN7 cells and St-4 cells did not express DTD.

The DTD expression levels were normalized by taking HT-29 cells as the 100% control value. We also measured the DTD enzyme activity in the cell lines and compared the DTD protein levels with the enzyme activity. There was a good correlation (r=0.939) between expression and activity (Fig. 2). We previously reported that cell lines with zero or marginal DTD activity showed resistance to MMC.[15] In accordance with this, cell lines with no DTD protein expression were resistant to MMC (Fig. 1).

Expression of DTD in surgically excised tumor samples To examine the correlation between DTD protein expression and enzyme activity in clinical tumor samples, we measured DTD expression and activity in 20 surgically excised samples from colorectal tumors. In ten out of 20 cases, normal peripheral tissues were isolated from surgically resected tumor samples and compared with the tumors for expression of DTD (Fig. 3). We could detect the 32-kDa DTD protein band in the samples, as we had in the carcinoma cell lines. In five cases, tumors expressed higher levels of DTD than did the associated normal tissues (patients 1, 5, 6, 9, 10), as reported previously.[20–23] This indicates selective toxicity of MMC to tumors.

We then compared DTD activity in the 20 tumor samples, including the 10 samples mentioned above, with their DTD protein levels. Again, the level of DTD protein expression correlated closely with DTD activity (r=0.938) (Fig. 4). This indicates that DTD activity in clinical tumor samples could be measured quantitatively by examining DTD protein expression on immunoblots.

Table I summarizes the clinicopathological features and DTD expression of the tumors tested. There were 13 male and 7 female patients; the mean age was 67.2 years, ranging from 51 to 78 years. Histologically, all tumors were adenocarcinomas. Interestingly, when the tumors were classified according to the presence or absence of nodal metastasis, tumors with metastases (Dukes C tumors) expressed larger amounts of DTD protein than did tumors without metastases (Dukes A and B tumors). The difference in DTD enzyme activity between these tumors was statistically significant (P<0.05, by Mann-Whitney U-

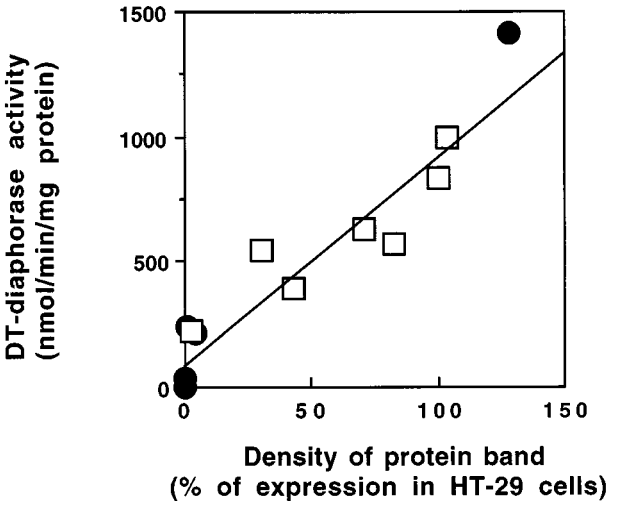

Fig. 2. Relationship between DTD protein expression and enzyme activity in human cell lines. Closed circles and open squares represent gastric and colon carcinoma cell lines, respectively. The density of each protein band is expressed as a percentage of the HT-29 expression level. r=0.939.

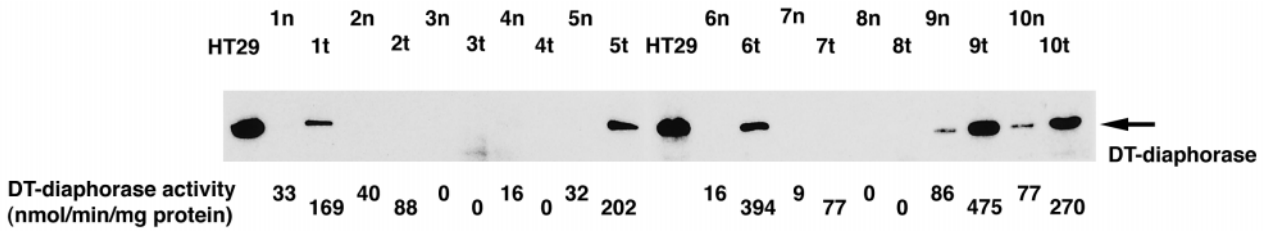

Fig. 3. Immunoblot analysis of DTD in human excisional samples. Tumors (t) and normal peripheral tissues (n) from the same individual were examined. Cell lysates (20 μg) from excisional samples were subjected to immunoblot analysis. DTD enzyme activity was measured as described in "Materials and Methods." ND: <5 nmol/min/mg protein.

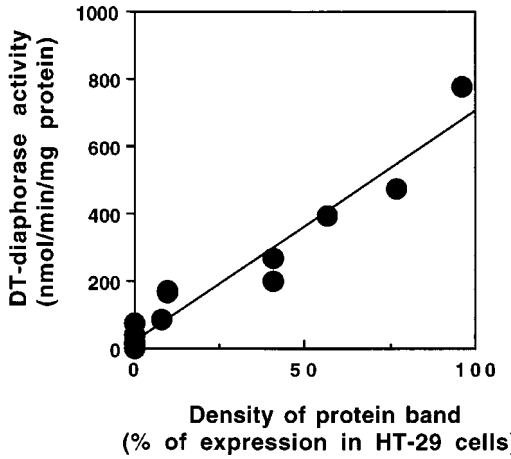

Fig. 4. Relationship between DTD protein expression and enzyme activity in clinical tumor samples. The density of each protein band is expressed as a percentage of the HT-29 expression level. r=0.938.

test). No correlation was found between DTD and tumor size or histology.

DISCUSSION

Since DTD activity is required for cytotoxicity of MMC *in vitro*, as we reported previously,[14, 15] measuring DTD in surgical tumor samples could be beneficial for predicting the sensitivity of remaining or metastasized tumors to adjuvant chemotherapy with MMC. Clinically speaking, a practical and consistent method of measuring DTD activity in tumor specimens from patients is needed. In this study, we demonstrated that immunological quantitation of DTD protein is an alternative and easy method for measuring DTD.

In comparing this method with the classical enzymatic method, we found the following advantages in the immunological approach: (i) many tumors can be examined at one time without concern for enzyme inactivation; (ii)

Table I. Tumor Samples

Staging	DTD expression[a]		DTD activity[b]		Patient		Tumor	
	Tumor	Normal	Tumor	Normal	Age	Sex[c]	Size[d]	Histology[e]
Dukes C	96.2		779		78	M	40	w
	76.9	8	475	86	62	M	85	m
	40.9	0	202	32	77	M	60	p
	0	0	88	40	77	F	45	w
	0	0	77	9	67	F	90	p
	0		40		58	M	40	w
	0		10		66	F	50	m
Mean	30.6		238.7[f]					
Dukes B	56.4	0	394	16	72	F	40	m
	9.7	0	169	33	57	M	45	m
	9.5		164		53	M	55	p
	0		17		78	F	40	w
	0	0	0	16	73	M	35	m
	0	0	0	0	57	F	80	w
	0		0		75	M	20	m
	0		0		51	M	50	w
	0		0		69	M	80	m
Mean	8.4		82.7					
Dukes A	40.9	8	270	77	70	M	25	m
	0	0	0	0	69	M	30	m
	0		0		54	F	30	w
	0		0		70	M	30	w
Mean	10.2		65.7					

a) The density of each protein band was compared with that of HT-29 and expressed as a percentage.
b) DT-diaphorase activity (nmol/min/mg protein).
c) M, male; F, female.
d) Tumor diameter (mm).
e) w, well-differentiated adenocarcinoma; m, moderately differentiated adenocarcinoma; p, poorly differentiated adenocarcinoma.
f) DTD activity of Dukes C tumors was significantly higher than that of Dukes B and A tumors according to the Mann-Whitney U-test, $P<0.05$.

preparation of tumor lysates for immunological detection is simplified because ultracentrifugation is not required; and (iii) detection of DTD protein is not affected by antioxidants, which influence DTD enzyme activity. Immunoblotting, however, can give only a relative level of DTD expression, while enzymatic examination provides absolute activity. The sensitivity of immunoblotting is also inferior, in that in our study the DTD protein band was barely detectable in tumor samples with DTD activity below 100 nmol/min/mg protein (Fig. 3 and Table I).

However, the DTD expression can be normalized by comparing it with expression in a standard cell line, as we did in HT-29. It is important to discriminate between tumors with minimal DTD expression and DTD-expressing tumors, since carcinoma cells with little or no DTD are resistant to MMC (Fig. 1). Immunoblot analysis can distinguish such tumors. Taking these observations into consideration, we believe that the immunological method is useful to quantitate DTD expression for large numbers of clinical cases.

When tumors were classified according to the presence or absence of nodal metastasis, those with metastases (Dukes C) expressed higher levels of DTD than did nonmetastatic tumors (Dukes A and B). Since the enzymatic method is more sensitive than immunoblot analysis, we performed statistical comparison with DTD enzyme activity. We found that tumors with nodal metastases showed significantly higher DTD activity than did nonmetastatic tumors ($P<0.05$, by Mann-Whitney U-test; Table I). Thus, it is likely that the increased expression of DTD is associated with malignancy in colorectal tumors, although the role of DTD in the malignant progression remains to be determined.

The therapeutic implications of such DTD measurements suggest that chemotherapy involving MMC may be ineffective for patients with tumors that do not express DTD because they can not activate MMC. Conversely, patients with advanced colorectal tumors that express DTD could have a positive response to MMC treatment. However, it has not been demonstrated in clinical tumors that DTD deficiency correlates with poor treatment outcome in chemotherapy involving MMC. We expect advanced colorectal carcinoma with nodal metastasis to be sensitive to MMC, but we did not test for sensitivity to MMC in clinically resected tumors. Further studies are required to elucidate the clinical significance of DTD in carcinomas.

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REFERENCES

1) Tomasz, M. and Lipman, R. Reductive mechanism and alkylating activity of mitomycin C induced by rat liver microsomes. *Biochemistry*, **20**, 5056–5061 (1981).
2) Pan, S. S., Andrews, P. A., Glover, C. J. and Bachur, N. R. Reductive activation of mitomycin C and mitomycin C metabolites catalyzed by NADPH-cytochrome P-450 reductase and xanthine oxidase. *J. Biol. Chem.*, **259**, 959–966 (1984).
3) Hodnick, W. F. and Sartorelli, A. C. Reductive activation of mitomycin C by NADH:cytochrome b5 reductase. *Cancer Res.*, **53**, 4907–4912 (1993).
4) Gustafson, D. L. and Pritsos, C. A. Enhancement of xanthine dehydrogenase mediated mitomycin C metabolism by dicoumarol. *Cancer Res.*, **52**, 6936–6939 (1992).
5) Jaiswal, A. K., McBride, O. W., Adesnik, M. and Nebert, D. W. Human dioxin inducible cytosolic NAD(P)H:menadione oxidoreductase. cDNA sequence and localization of gene to chromosome 16. *J. Biol. Chem.*, **263**, 13572–13578 (1988).
6) Pan, S. S., Forrest, G. L., Akman, S. A. and Hu, L. T. NAD(P)H:quinone oxidoreductase expression and mitomycin C resistance developed by human colon cancer HCT 116 cells. *Cancer Res.*, **55**, 330–335 (1995).
7) Traver, R. D., Horikoshi, T., Danenberg, K. D., Stadlbauer, T. H., Danenberg, P. V., Ross, D. and Gibson, N. W. NAD(P)H:quinone oxidoreductase gene expression in human colon carcinoma cells: characterization of a mutation which modulates DT-diaphorase activity and mitomycin sensitivity. *Cancer Res.*, **52**, 797–802 (1992).
8) Marshall, R. S., Paterson, M. C. and Rauth, A. M. Deficient activation by a human cell strain leads to mitomycin resistance under aerobic but not hypoxic conditions. *Br. J. Cancer*, **59**, 341–346 (1989).
9) Keyes, S. R., Rockwell, S. and Sartorelli, A. C. Enhancement of mitomycin C cytotoxicity to hypoxic tumor cells by dicoumarol *in vivo* and *in vitro*. *Cancer Res.*, **45**, 213–216 (1985).
10) Siegel, D., Gibson, N. W., Preusch, P. C. and Ross, D. Metabolism of mitomycin C by DT-diaphorase: role in mitomycin C-induced DNA damage and cytotoxicity in human colon carcinoma cells. *Cancer Res.*, **50**, 7483–7489 (1990).
11) Begleiter, A., Robotham, E., Lacey, G. and Leith, M. K. Increased sensitivity of quinone resistant cells to mitomycin

C. *Cancer Lett.*, **45**, 173–176 (1989).
12) Rockwell, S., Keyes, S. R. and Sartorelli, A. C. Modulation of the cytotoxicity of mitomycin C to EMT6 mouse mammary tumor cells by dicoumarol *in vitro*. *Cancer Res.*, **48**, 5471–5474 (1988).
13) Fitzsimmons, S. A., Workman, P., Grever, M., Paull, K., Camalier, R. and Lewis, A. D. Reductase enzyme expression across the National Cancer Institute tumor cell line panel; correlation with sensitivity to mitomycin C and EO9. *J. Natl. Cancer Inst.*, **88**, 259–269 (1996).
14) Lee, J. H., Naito, M., Nakajima, M. and Tsuruo, T. Isolation and characterization of a mitomycin C-resistant variant of human colon carcinoma HT-29 cells. *Cancer Chemother. Pharmacol.*, **33**, 215–220 (1993).
15) Mikami, K., Naito, M., Tomida, A., Yamada, M., Sirakusa, T. and Tsuruo, T. DT-diaphorase as a critical determinant of sensitivity to mitomycin C in human colon and gastric carcinoma cell lines. *Cancer Res.*, **56**, 2823–2826 (1996).
16) Ernster, L. DT-diaphorase. *Methods Enzymol.*, **10**, 309–317 (1967).
17) Watanabe, M., Komeshima, N., Naito, M., Isoe, T., Otake, N. and Tsuruo, T. Cellular pharmacology of MX2, a new morpholino anthracycline, in human pleiotropic drug-resistant cells. *Cancer Res.*, **51**, 157–161 (1991).
18) Tsuruo, T., Iida, H., Tsukagoshi, S. and Sakurai, Y. Overcoming of vincristine resistance in P388 leukemia *in vivo* and *in vitro* through enhanced cytotoxicity of vincristine and vinblastine by verapamil. *Cancer Res.*, **41**, 1967–1972 (1981).
19) Benson, A. M., Hunkeler, M. J., and Talalay, P. Increase of NAD(P)H:quinone reductase by dietary antioxidants: possible role in protection against carcinogenesis and toxicity. *Proc. Natl. Acad. Sci. USA*, **77**, 5216–5220 (1980).
20) Schor, N. A. and Cornelisse, C. J. Biochemical and quantitative histochemical study of reduced pyridine nucleotide dehydrogenation by human colonic carcinoma. *Cancer Res.*, **43**, 4850–4855 (1983).
21) Schlager, J. J. and Powis, G. Cytosolic NAD(P)H:(quinone-acceptor) oxidoreductase in human normal and tumor tissue: effects of cigarette smoking and alcohol. *Int. J. Cancer*, **45**, 403–409 (1990).
22) de Waziers, I., Cugnenc, P. H., Berger, A., Leroux, J. P. and Beaune, P. H. Drug-metabolizing enzyme expression in human normal peritumoral and tumoral colorectal tissue samples. *Carcinogenesis*, **12**, 905–909 (1991).
23) Cresteil, T. and Jaiswal, A. K. High levels of expression of the NAD(P)H:quinone oxidoreductase (NQO1) gene in tumor cells compared to normal cells of the same origin. *Biochem. Pharmacol.*, **42**, 1021–1027 (1991).