Glutathione conjugation of chlorambucil: measurement and modulation by plant polyphenols

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Chlorambucil (CMB), an anticancer drug, was cytotoxic at concentrations of 5–20 μ M to human colon adenocarcinoma cells. It inhibited [14 C]thymidine uptake in a dose-dependent manner. Both effects were potentiated by simultaneous exposure of the cells to 10 μ M plant polyphenols. In an attempt to explain the possible mechanism of action of the polyphenols in relation to these observations, an HPLC-radiometric method was developed to measure the conjugation of CMB with glutathione in these cells and to monitor the export of monochloromonogluthionyl CMB (MG-CMB), its main glutathione conjugate.

At micromolar concentrations, five polyphenols, namely quercetin, butein, tannic acid, 2'-hydroxychalcone and morin, inhibited the efflux of CMB significantly; an inhibition of 40 % was observed with 10 μ M quercetin. The glutathione S-transferase (GST) activity of the cancer cells, measured with 1-chloro-2,4-dinitrobenzene, was also inhibited by the polyphenols. Their combined action on GST and on the efflux of MG-CMB conjugate could provide an enhanced positive modulation of sensitivity of the tumour cells to CMB.

INTRODUCTION

Glutathione S-transferases (GSTs; EC 2.5.1.18) are a group of important drug-metabolizing enzymes. They participate in the detoxification of many anticancer drugs including chlorambucil (CMB) [1]. An overexpression of GSTs has been reported in many drug-resistant cells, a phenomenon suggested to confer drug resistance against anticancer drugs such as CMB [2], cisplatin [3], thiopeta [4] and mitomycin [5]. Ethacrynic acid has been used to inhibit GST activity in drug-resistant tumour cells for the modulation of drug resistance [6,7]; more than 85 % inhibition of GST activity was observed in resistant MCF/HC (human breast cancer) cells, resulting in sensitization to 4-hydroperoxycyclophosphamide [8].

The multidrug resistance-associated protein (MRP) has been found to be overexpressed in many non-P-glycoprotein multidrug-resistant cells and this is believed to be associated with resistance of the tumour cells to cytotoxic drugs [9–11]. MRP is an ATP-dependent transporter of glutathione conjugates of anticancer drugs [12,13]. It was therefore considered to be identical with or associated with the glutathione S-conjugate export pump [14–16]. It is, however, different from Pgp-170, the gene product of MDR1. Inhibitors of Pgp-170 such as verapamil, cyclosporin A and its analogue PSC 833 do not necessarily inhibit MRP [17]. However, inhibitors of MRP might be of clinical value because the inhibition of glutathione conjugate transport by polyethoxylated surfactants has previously been suggested to be potentially useful in reversing multidrug resistance in some tumours [18].

MATERIALS AND METHODS

Chemicals

Quercetin, morin, tannic acid, butein, 2-hydroxychalcone and 2'-hydroxychalcone were obtained from Extrasynthese (Genay, France). CMB, 1-chloro-2,4-dinitrobenzene (CDNB), buthionine sulphoximine (BSO), *N*-ethylmaleimide (NEM) and carbonyl

cyanide *m*-chlorophenylhydrazine were bought from Sigma Chemical Co. (St Louis, MO, U.S.A.). RPMI 1640 medium containing 1 mM sodium pyruvate, 2 mM L-glutamine, 10 mM Hepes, 1 % non-essential amino acids, 10 % (v/v) fetal bovine serum (FBS) and 1 % antibiotic/antimycotic were obtained from Gibco (Grand Island, NY, U.S.A.); 1 ml of antibiotic/antimycotic contained 10⁴ i.u. of penicillin, 10 mg of streptomycin and 25 μ g of amphotericin. Tissue culture flasks and multi-well plates were from Nunclon (Roskilde, Denmark) and FBS was purchased from Commonwealth Serum Laboratory (Australia). [14 C]Thymidine of specific radioactivity 52 mCi/mmol was purchased from Amersham International (Little Chalfont, Bucks., U.K.) and [35 S]cysteine (of specific radioactivity 1075 Ci/mmol and concentration 10.2 nmol/ml) was from DuPont–NEN (Boston, MA, U.S.A.). Other chemicals of analytical grade were from the usual commercial suppliers.

Culture of human colon adenocarcinoma cells

The human colon adenocarcinoma cell line (ATCC CCL 220.1; COLO 320HSR) was a gift from the Institute of Molecular and Cell Biology, National University of Singapore. The cells were maintained in RPMI 1640 medium as described above in a humidified atmosphere of air/CO₂ (19:1) and were subcultured every 2 days.

GST activity of colon cancer cells: assay conditions and effects of polyphenols

Approx. 2×10^7 cells were harvested in 10 ml of PBS by scraping with a rubber 'policeman'. The cells were disrupted by sonication (sonicator XL; Heat Systems, Farmingdale, New York, U.S.A.) and centrifuged at 105 000 *g* for 45 min at 4 °C. The GST activity in the high-speed supernatant fraction was measured with CDNB as substrate by the method of Habig et al. [19]. The reaction mixture contained 125 mM potassium phosphate buffer, pH 6.5, 1 mM glutathione and 1 mM CDNB. The reaction was started by the addition of GST purified from colon cancer cells by S-

Abbreviations used: BSO, buthionine sulphoximine; CDNB, 1-chloro-2,4-dinitrobenzene; CMB, chlorambucil; DNP-SG, dinitrophenyl-S-glutathione; FBS, fetal bovine serum; GST, glutathione S-transferase; HBSS, Hanks balanced salt solution; MG-CMB, monochloromonogluthionyl chlorambucil; MRP, multidrug resistance-associated protein; NEM, *N*-ethylmaleimide.

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Figure 1 Inhibitory effects of plant polyphenols on the GST activity of human colon cancer cells, measured with CDNB

The GST activity of the controls, represented as 100%, was 296.5 nmol of DNP-SG/min per mg of protein.

hexylglutathione affinity chromatography as described by Reddy et al. [20]. The rate of formation of CDNB-GS conjugate was determined spectrophotometrically by monitoring the absorbance at 340 nm. The GST activity was expressed as μmol of dinitrophenyl-S-glutathione (DNP-SG) formed/min per mg of protein at 37 °C, taking the molar absorption coefficient as $9.6 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ [19]. The effect on GST activity of polyphenols at final concentrations of 5–200 μM were examined. Fresh stock solutions of polyphenols were prepared in less than 2% (v/v) ethanol, a concentration that did not have an effect on GST activity.

Colony formation assays

Cells were seeded into each well of six-well plates in 2 ml of RPMI 1640 medium and were left overnight for attachment. After incubation for 24 h, the cells were exposed to the plant polyphenols (10 μM each) for 15 min and to 5–20 μM CMB for 30 min in the medium without FBS. The cells were then grown in a drug-free medium for 12 days to produce colonies of appropriate size (of diameter greater than 1 mm) for counting. The plates were rinsed with PBS, fixed with methanol and stained with Giemsa. The cell survival for each dose of drug was calculated as the ratio of colony formation in wells containing drug-treated cells to that in control wells with untreated cells. The plating efficiency of the latter ranged from 30% to 38%.

[¹⁴C]Thymidine incorporation studies

Cultures of human colon adenocarcinoma cells in 24-well plates were plated at a cell density of 6×10^4 in 1 ml of medium per well. After 24 h the medium in each well was removed and replaced with 1 ml of fresh medium. Each plant polyphenol was added to

Table 1 Effects of polyphenols on [¹⁴C]thymidine uptake of human colon adenocarcinoma cells treated with CMB

Abbreviations: TA, tannic acid; B, butein; Q, quercetin; M, morin; 2'-OH C, 2'-hydroxychalcone; 2-OH C, 2-hydroxychalcone. * $P < 0.05$ ($n = 3$), ** $P < 0.005$ ($n = 3$) compared with control without polyphenols; † $P < 0.05$ ($n = 3$), †† $P < 0.005$ ($n = 3$) compared with control without CMB

CMB (μ M)	[¹⁴ C]Thymidine uptake (c.p.m./ μ g of protein)						
	Control	10 μ M TA	5 μ M B	10 μ M Q	10 μ M M	5 μ M 2'-OH C	5 μ M 2-OH C
Control	333.4 $\pm$ 45.3	311.2 $\pm$ 17.5	344.6 $\pm$ 61.9	326.4 $\pm$ 20.2	358.8 $\pm$ 25.2	326.9 $\pm$ 14.3	314.4 $\pm$ 10.6
10	289.7 $\pm$ 18.8	208.0 $\pm$ 13.7*	277.4 $\pm$ 59.8	232.1 $\pm$ 34.2	274.9 $\pm$ 15.1	257.5 $\pm$ 16.7	244.2 $\pm$ 48.3
20	239.2 $\pm$ 23.7†	187.8 $\pm$ 9.9*	205.3 $\pm$ 32.4	202.5 $\pm$ 4.6	228.7 $\pm$ 20.3	221.9 $\pm$ 23.1	220.1 $\pm$ 10.2
40	221.8 $\pm$ 7.9††	164.0 $\pm$ 15.3**	190.1 $\pm$ 25.6	181.3 $\pm$ 23.1*	201.9 $\pm$ 50.9	194.0 $\pm$ 18.2*	210.5 $\pm$ 20.3
80	182.0 $\pm$ 18.1††	91.8 $\pm$ 10.8**	94.2 $\pm$ 16.2**	111.3 $\pm$ 6.5**	137.1 $\pm$ 12.1*	125.5 $\pm$ 17.3**	114.5 $\pm$ 9.4**

a final concentration of 5–10 μ M and the cells were incubated at 37 °C for 20 min. CMB at final concentrations of 10–80 μ M was then introduced and the cells were incubated at 37 °C for 60 min. Finally, after the addition of 20 μ M (0.5 μ Ci) of [¹⁴C]thymidine to each of the wells, the plates were incubated at 37 °C for 6 or 18 h. The medium was decanted and the cells were washed twice with PBS, once with methanol, twice with ice-cold 5% (w/v) trichloroacetic acid and finally with ethanol. The cells were air-dried and then solubilized in 0.3 ml of 0.3 M NaOH. Aliquots of 0.15 ml of the digests were transferred to Ready Caps scintillation discs (Beckman, Fullerton, CA, U.S.A.) and oven-dried at 60 °C; the radioactivity was then measured in a liquid-scintillation counter. Data were expressed in c.p.m. per 10⁶ cells or c.p.m. per μ g of protein.

Protein determination

The protein contents of the cell lysates were measured by the method of Bradford [21] with Bio-Rad reagents, using BSA as a standard.

Measurement of formation and export of monochloromonogluthionyl-CMB (MG-CMB) by human colon cancer cells

Approx. 2×10^6 cells were seeded in each well of six-well tissue culture plates. They were placed in a CO₂ incubator for 24 h. The medium was removed and replaced by 0.5 ml of Hanks balanced salt solution (HBSS) containing 5.8 mM K⁺, 143 mM Na⁺, 1.3 mM Ca²⁺, 0.8 mM Mg²⁺, 146 mM Cl⁻, 0.8 mM SO₄²⁻, 0.8 mM P_i, 4.2 mM HCO₃⁻, 5.6 mM glucose and 10 mM Hepes, pH 7.4. [³⁵S]Cysteine was added to each of the wells at a final concentration of 10 μ Ci/ml (equivalent to 9.3 pmol/ml) and incubated at 37 °C for 30 min. CMB at a final concentration of 1 mM was then added, followed by incubation at 37 °C for 30 min. The cells were washed twice with HBSS and collected in 0.3 ml of HBSS by scraping with a rubber 'policeman'. Trichloro-

acetic acid [50% (w/v); 50 μ l] was added before the cells were disrupted by sonication. The cell lysate was centrifuged at 14000 rev./min for 2 min. The supernatant was passed through a Millipore filter (pore size 0.45 μ m) and injected for HPLC-radiometric analysis of MG-CMB into a Hewlett-Packard 1090 liquid chromatograph connected to a detector of radioactivity (Flo-one/Beta, Packard Instrument Company). MG-CMB present in the extracellular HBS was also determined in the same way at intervals up to 80 min of incubation at 37 °C. The column (300 mm $\times$ 3.9 mm) was packed with Nova-Pak and eluted at a flow rate of 1 ml/min with a solvent system modified from that reported by Ciaccio et al. [22]: 0.1 M ammonium acetate buffer, pH 5.0, was introduced in the first 4 min followed by two step gradients of 40% and 90% methanol at 5 and 18 min respectively.

Effects of plant polyphenols on the export of MG-CMB in the cells

In studies of the inhibition of efflux of MG-CMB, the cells were first loaded with this conjugate by sequential exposure to [³⁵S]cysteine and CMB as described above. They were washed twice with HBSS and incubated for 20 min in HBSS containing the polyphenols (10 or 40 μ M each). MG-CMB exported to the extracellular HBSS was determined by the HPLC-radiometric method described above. The rate of efflux of MG-CMB was quantified as radioactivity (c.p.m.) incorporated into MG-CMB/min per 10⁶ cells. Standards of [³⁵S]cysteine containing 93–740 nmol/ml (corresponding to 1–8 nCi/ml) were measured at the same time; a linear detection response was observed for 9.3–74 nmol [³⁵S]cysteine injected.

RESULTS

Inhibition of GST of colon cancer cells by plant polyphenols

Five polyphenols, namely tannic acid, butein, quercetin, morin and 2'-hydroxychalcone, inhibited the GST activity of colon cancer cells measured with CDNB in a concentration-dependent manner (Figure 1); IC₅₀ values calculated by linear regression were 90.5, 71.4, 72.1, 111.8 and 57.0 μ M respectively. These values were higher than those obtained with rat liver GST, for which the respective IC₅₀ values were 1–20 μ M [23]. At 10 μ M concentrations of these polyphenols, the degrees of inhibition of GST were 7.5–11.1%.

Effects of polyphenols on colon cancer cells treated with CMB

Cell survival

Previous studies from our laboratory had shown that flavonoids and other related natural polyphenols were cytotoxic to HeLa

Figure 2 Effects of plant polyphenols on colon cancer cells treated with CMB on cell survival (a), cell growth (b) and [¹⁴C]thymidine uptake (c)

(a) Cells were preincubated for 15 min with each polyphenol at 10 μ M followed by 5–20 μ M CMB for 30 min. They were then grown in drug-free medium for 12 days, after which an average of 50 ± 4 colonies were observed in control wells originating from 150 seeded cells. * $P = 0.0001$ to 0.007 compared with the corresponding controls. (b) The protein contents of cells in the wells containing drug-treated and untreated cells were compared; the average value for the latter was 4.5 ± 0.4 μ g from 10⁴ seeded cells. * $P = 0.0001$ to 0.001 compared with the corresponding controls. (c) The average [¹⁴C]thymidine uptake of controls was 1226 ± 98 c.p.m./10⁴ cells. * $P = 0.0001$ to 0.006 compared with the corresponding controls. All data points in the above figures are means $\pm$ S.D. for no less than three separate experiments.

Figure 3 HPLC-radiometric profiles of [^{35}S]cysteine and its metabolites formed in tumour cells exposed to CMB

Cells were exposed at 37 °C to (a) [^{35}S]cysteine for 40 min followed by (b) 1 mM CMB for 30 min. Similarly, cells were pretreated with (c) 1 mM BSO for 2 h and (d) 0.2 mM NEM for 10 min, followed by sequential exposure to [^{35}S]cysteine and CMB as in (b). Peaks 2 and 3 are possibly metabolic products of [^{35}S]cysteine (peak 1). Peak 2 was tentatively identified as glutathione as it was decreased by pretreatment with BSO (c) and NEM (d). Peaks 4 and 5 were identified as diglutathionyl-CMB and MG-CMB respectively. The complete disappearance of peaks 4 and 5 was apparent on pretreatment of cells with BSO and NEM (c, d). Peaks 3 and U were unidentified.

cells [24,25]. Thus low, non-toxic concentrations (5–10 μM) were used in this study to examine their modulating effects on the cytotoxicity of CMB in these cells. At 10 μM the polyphenols had negligible action on the survival of these cells compared with the controls (Figure 2a). CMB, in contrast, was cytotoxic in a concentration-dependent manner from 5 to 20 μM . The polyphenols at 10 μM potentiated the cytotoxicity of CMB when added at 5 μM concentration, as reflected by a decrease in colony formation by 18–26 % compared with controls exposed to CMB alone (Figure 2a).

Figure 4 Efflux of MG-CMB from human colon adenocarcinoma cells

Cells loaded with radioactive MG-CMB by exposure to [^{35}S]cysteine and CMB were incubated in HBSS for intervals of up to 80 min at 37 °C. The MG-CMB exported to the extracellular medium was determined by HPLC–radiometry as described in the text. Data points are means $\pm$ S.D. for three separate experiments.

Cell growth

When cell growth was quantified by determining the protein content of the cells after incubation with CMB for 18 h, the inhibition of cell growth by CMB was apparent (Figure 2b). This effect on cell growth was again potentiated by plant polyphenols at 5–10 μM . At 20 μM CMB the polyphenols potentiated the CMB-induced inhibition of cell growth by 9–48 %.

[^{14}C]Thymidine uptake

CMB also inhibited [^{14}C]thymidine uptake in a concentration-dependent manner; these effects were likewise enhanced by simultaneous exposure of the cells to non-toxic concentrations of the polyphenols tested. When 20 μM CMB was used, the polyphenols potentiated the CMB-induced inhibition of [^{14}C]thymidine uptake by 12–37 % although by themselves there was a negligible effect at the concentrations used (Figure 2c).

When the incorporation of [^{14}C]thymidine into the cells (expressed as c.p.m./ μg of protein) was determined after 6 h of exposure, CMB showed a concentration-dependent inhibition of thymidine uptake (Table 1). Polyphenols at 5–10 μM potentiated this effect of CMB significantly at a higher concentration of CMB (80 μM).

Measurement of MG-CMB conjugate

On exposure to [^{35}S]cysteine, this amino acid and its radiolabelled metabolites were demonstrated inside the tumour cells (Figure 3a). Peaks 2 and 3 were presumably metabolic products of [^{35}S]cysteine (peak 1). Two additional radioactive products corresponding to peaks 4 and 5 were apparent in the presence of 1 mM CMB (Figure 3b). Preincubation of the cells with BSO (Figure 3c) and NEM (Figure 3d) resulted in the complete disappearance of these two peaks and a decrease in peak 2. As BSO and NEM are glutathione depleters [26,27], it was concluded that peak 2 corresponded to glutathione. On the basis of the HPLC data of Ciaccio et al. [22], and our own studies on HPLC–UV analysis of CMB conjugates (K. Zhang and K. P. Wong, unpublished work), peaks 4 and 5 were tentatively identified as diglutathionyl-CMB and MG-CMB respectively. After a 30 min incubation with CMB at 37 °C, MG-CMB seemed to be the major conjugate detected (Figure 3b). Both peak 3 (present in all

Table 2 Inhibitory effects of polyphenols on the export of MG-CMB^a

Cells loaded with radioactive MG-CMB by incubation with [³⁵S]cysteine and 1 mM CMB were exposed to various inhibitors. MG-CMB exported to the extracellular medium was determined by HPLC–radiometry. Rates of export are expressed as c.p.m. of MG-CMB/min per 10⁶ cells; values are means ± S.D. Controls treated in the same way but without test inhibitors had an export rate of 439 ± 41 c.p.m./min per 10⁶ cells. Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; MCB, monochlorobimane. * *P* < 0.05, ** *P* < 0.01 compared with control.

Inhibitor	Inhibitor concentration (μM) ...	Rate of export of MG-CMB (c.p.m./min per 10 ⁶ cells) (% inhibition)		
		10	40	200
Tannic acid		387 ± 52 (12)	240 ± 35 (45)**	—
Butein		357 ± 32 (19)*	222 ± 21 (49)**	—
Quercetin		255 ± 35 (40)**	128 ± 16 (71)**	—
Morin		387 ± 46 (12)	290 ± 24 (34)*	—
2'-Hydroxychalcone		385 ± 32 (12)	268 ± 30 (39)**	—
CCCP		305 ± 25 (31)**	—	—
MCB		—	—	302 ± 47 (30)*
CDNB		—	—	158 ± 21 (64)**

the chromatograms) and peak U (found only in Figure 3d) were unidentified; presumably peak U was a [³⁵S]cysteine or glutathione conjugate of NEM or of its product. No attempt was made to characterize these two unknown peaks.

Export of MG-CMB by colon cancer cells and effects of polyphenols

Radiolabelled MG-CMB conjugate formed by incubation with [³⁵S]cysteine and CMB was exported to the extracellular HBSS in a time-dependent manner, with linearity up to 20 min (Figure 4). The rate of efflux was influenced by the polyphenols (and other compounds listed in Table 2) at 10–200 μM. All the polyphenols at 40 μM inhibited the export of MG-CMB significantly; the order of inhibitory potencies was quercetin > butein > tannic acid > 2'-hydroxychalcone > morin. An uncoupler of oxidative phosphorylation, carbonyl cyanide *m*-chlorophenylhydrazone (10 μM), also significantly inhibited MG-CMB export; this might have been a secondary process resulting from a decrease in ATP production. Monochlorobimane and CDBN (other substrates of GST), when introduced at 200 μM, competed significantly in the export of MG-CMB. Their inhibitory action on MG-CMB export might be due to the competition of their glutathione conjugates with MG-CMB for the glutathione S-conjugate export pump.

DISCUSSION

The identification of chemosensitizers modulating MRP-associated multidrug resistance is valuable in developing new strategies to overcome multidrug resistance of tumour cells during cancer chemotherapy. The present study focused on the possible modulating effects of selected plant polyphenols on the sensitivity of human adenocarcinoma cells to CMB; previous observations showed that they are potent inhibitors of GST of rat liver [23]. In this study, five of these polyphenols, at 5–10 μM, significantly potentiated CMB cytotoxicity, as assayed by colony formation, cell growth and [¹⁴C]thymidine uptake. These concentrations were non-toxic to HeLa cells [24,25]; this was confirmed in our study on colon cancer cells (Figures 2a–2c and Table 1). The thymidine uptake measured at 18 h after exposure to CMB and polyphenols reflected essentially the number of growing cells and was taken as an index of cell growth. CMB has been reported to act as a DNA cross-linker that decreases its ability to function as a template for DNA synthesis [28]. Inhibition of DNA synthesis by nitrogen mustard in cultured mammalian cells was reported to be more rapid than its inhibition

of RNA and protein synthesis [29]. In view of this, incorporation of [¹⁴C]thymidine was subsequently measured at 6 h and the CMB-induced inhibition of DNA synthesis was found to be potentiated significantly by non-toxic concentrations of the five polyphenols (Table 1).

To address the mechanism of action of polyphenols in sensitizing the colon cancer cells to CMB, two aspects of its metabolism were examined, namely its enzymic conjugation with glutathione and the export of its MG-CMB conjugate from the cells. The inhibitory effects of the polyphenols on GST of colon cancer cells measured with CDBN was modest in magnitude compared with rat liver GST as indicated by their IC₅₀ values. In another study, ethacrynic acid, a GST inhibitor, showed significant potentiation of the cytotoxic effect of CMB in HT-29 human colon adenocarcinoma cells [6]. This might be explained by the different isoenzyme compositions of the various tissues/cell lines, by virtue of their different substrate specificities and characteristics [1]. Measured at polyphenol concentrations of 10 μM, the inhibition of GST of colon cancer cells assayed with CDBN was only 7.5–11.1 % (Figure 1), whereas the polyphenols' potentiation of CMB cytotoxicity ranged from 9 % to 48 % in the three different assays employed in this study (Figures 2a–2c), suggesting that besides inhibition of GST, other mechanism/s might be involved in their potentiation of CMB cytotoxicity.

Detoxification of CMB with glutathione has been proposed to confer resistance to CMB on tumour cells [30,31]. In our previous report on the effects of polyphenols on the export of glutathione conjugates, CDBN was employed as the substrate [32]. The results might not be representative of CMB. A radiometric assay of CMB conjugation with glutathione was therefore developed. The formation of MG-CMB was demonstrated in human colon adenocarcinoma cells exposed to [³⁵S]cysteine and CMB (Figure 3b). The incorporation of radiolabelled cysteine into glutathione was also evident on the chromatogram (Figures 3a and 3b). [³⁵S]MG-CMB biosynthesized was actively exported by the cells (Figure 4). This transport was inhibited by monochlorobimane and CDBN (Table 2). The converse has also been shown: the export of DNP-SG and glutathione-bimane were inhibited by CMB [32,33], suggesting that the same transporter might be responsible for the efflux of all three glutathione S-conjugates. These kinetic data provided further evidence that the glutathione conjugate transporter could recognize the glutathione moiety in the conjugates with little specificity for the xenobiotic moiety. Plant polyphenols inhibited the export of the glutathione conjugates of CDBN and CMB in these human colon tumour cells ([32], Table 2). Thus their potentiation of CMB cytotoxicity as

demonstrated by studies of colony formation, [^{14}C]thymidine uptake and cell growth could be explained partly by their inhibition of the efflux of MG-CMB, the major glutathione conjugate of CMB in these colon cancer cells. The mechanism of action of these polyphenols is as yet unknown. Our earlier report showed that they have no significant effect on the intracellular concentrations of ATP and glutathione, or on DNP-SG Mg^{2+} -ATPase activity [32].

In summary, plant polyphenols inhibited GST activity as well as the transport of the major glutathione conjugates of CMB out of human colon cancer cells. The relative contribution of each of these processes has not been assessed but together they could conceivably provide additive effects as they represent two separate metabolic events in the overall detoxification of CMB. Ethacrynic acid, piroprost and indomethacin are inhibitors of GST that have been employed to modulate drug resistance in cells with high GST activity [5,34]. However, so far, modulators of the transport of drug-glutathione conjugates by the glutathione S-conjugate export pump and/or MRP are limited. Our study indicates that plant polyphenols are potential candidates as chemosensitizers of CMB, an anticancer drug whose glutathione conjugate/s utilize MRP for their transport. These polyphenols are found in the normal diet and in herbal medicine [35,36] and are likely to be more acceptable in terms of patient compliance.

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