Biochemical Pharmacology, Vol. 52, pp. 1631–1638, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00570-9

Inhibition of the Efflux of Glutathione S-Conjugates by Plant Polyphenols

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ABSTRACT. The formation of dinitrophenylglutathione (DNP-SG) in human colon adenocarcinoma cells was identified and quantified by an HPLC-UV method, following exposure to 1-chloro-2,4-dinitrobenzene (CDNB) at 10° for 40 min. The rate of efflux of DNP-SG at 37° likewise, was measured by monitoring the DNP-SG content in the extracellular medium. Among the polyphenols examined for their action on DNP-SG export, butein was the most potent inhibitor with an IC_{50} value of 15 μM. The others, in order of decreasing potencies, were quercetin, tannic acid, 2′-hydroxychalcone, 2-hydroxychalcone and morin, all of which have IC_{50} values in the micromolar range. These polyphenols did not affect the ATP or the glutathione content of the cells. Mg^{2+}-ATPase extracted from the plasma membrane of the cells was activated by DNP-SG in a concentration-dependent manner, and the reaction showed saturation kinetics with K_m and V_{max} values of 110 μM and 12.3 nmol/min/mg protein, respectively. However, the six polyphenols mentioned above had negligible effects on the Mg^{2+}-ATPase activity, suggesting that this was probably not the target of their inhibitory action. Probenecid, *p*-trifluoromethoxy-phenylhydrazone (FCCP) and chlorambucil also showed varying degrees of inhibition of the export of DNP-SG. BIOCHEM PHARMACOL 52;10:1631–1638, 1996. *Copyright © 1996 Elsevier Science Inc.*

KEY WORDS. glutathione S-conjugate; export; human colon cancer cells, plant polyphenols; butein; quercetin

Glutathione (GSH) and glutathione-S-transferases (GSTs) participate in the detoxification of a variety of electrophilic compounds, which include anticancer drugs such as chlorambucil and melphalan [1, 2]. Increased cellular level of GSH and overexpression of GST have been found in many tumor cells, and these parameters appear to be associated with the resistance of tumor cells to anticancer drugs [3, 4] such as chlorambucil and cisplatin [5, 6]. The intracellular accumulation of their GSH conjugates may inhibit GST and glutathione reductase activities [7]. Fortuitously, there exists a GS-X† pump on the plasma membrane, which is a transporter of GSH-conjugates [8]. In this context, the MRP, which functions as a drug efflux pump [9] has been reported to be overexpressed in non-Pgp multidrug-resistant cells [10]. It is involved in the transport of glutathione conjugates [11–13]. MRP was therefore proposed to be identical to or associated with the GS-X pump [13]. MDR mediated by Pgp could be reversed by chemosensitizers such as verapamil, cyclosporin A and its analogue PSC 833 by blocking the Pgp-linked efflux of drugs [14, 15]. They are, however, not effective in reversing resistance in non-Pgp multidrug-resistant cells [16]. To date, there are only a few resistance modifiers of MDR mediated by MRP. Among these, the isoflavonoid genistein and the flavonoid quercetin (both are tyrosine kinase inhibitors) could modulate non-Pgp MDR but at a high cytotoxic concentration [17, 18]. The other example was a protein kinase C inhibitor, GF 109203X, which showed partial reversal of resistance to adriamycin and vincristine [19].

The present study is an extension of our previous observations of the effects of plant polyphenols on glutathione metabolism. These polyphenols were found to be potent inhibitors of rat liver GST *in vitro* [20]. At low nontoxic concentrations, they could sensitize cancer cells to chlorambucil.‡ Although inhibition of GST could contribute to this phenomenon, it is conceivable that inhibition of the GS-X pump would elicit a similar response. The existence of such a pump in these colon cancer cells has been demonstrated in kinetic studies using monochlorobimane.§

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Received 18 March 1996; accepted 18 June 1996.

† *Abbreviations:* CDNB, 1-chloro-2,4-dinitrobenzene; CMB, chlorambucil; DNP-SG, dinitrophenylglutathione; FCCP, *p*-trichloromethoxyphenylhydrazone; GSH, glutathione; GST, glutathione S-transferase; GS-X pump, glutathione-S-conjugate export pump; HBSS, Hanks' balanced salt solution; MDR, multidrug resistance; MRP, multidrug resistance-associated protein; P_i, inorganic phosphate; and Pgp, P-glycoprotein.

‡ Zhang K and Wong KP, Modulation of sensitivity of human adenocarcinoma cells to chlorambucil by plant polyphenols. 3rd IUBMB Conference, Singapore, P-148, 1995.

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MATERIALS AND METHODS

Chemical

Tannic acid, butein, quercetin, morin, 2-hydroxychalcone and 2′-hydroxychalcone were obtained from Extrasynthesis-Genay (Genay, France). GSH, CDNB, FCCP, chlorambucil, probenecid, ammonium molybdate, polyvinyl alcohol, malachite green and the bioluminescent somatic cell ATP assay kit were purchased from the Sigma Chemical Co. (St Louis, MO, USA). The GSH colorimetric assay kit was bought from Bioxytech, SA (Marne, France). RPMI 1640, sodium pyruvate, nonessential amino acid and antibiotic/antimycotic were obtained from the Gibco Co. (Grand Island, NY, U.S.A.). Tissue culture flasks and multi-well plates were obtained from Nunclon (Roskilde, Denmark). Fetal bovine serum was obtained from Commonwealth Serum Laboratory (Australia). GST was purified from rat liver cytosol by *S*-hexylglutathione affinity chromatography as described by Reddy *et al.* [21]. Other chemicals of analytical grade were from the usual commercial suppliers.

Culture of Cells

The human colon adenocarcinoma cell line ATCC. CLL. 220.1 was a gift from the Institute of Molecular and Cell Biology, National University of Singapore. The cells were maintained in RPMI 1640 medium containing 1 mM sodium pyruvate, 2 mM L-glutamine, 10 mM HEPES, 1% non-essential amino acids, antibiotic/antimycotic and 10% fetal bovine serum. The cells were grown in a humidified atmosphere of 95% air and 5% CO_2 and subcultured every two days.

Identification and Measurement of DNP-SG Formation and Efflux

The cells were cultured on six-well plates at a density of 1.0 × 10^6 cells/well/2.5 mL in a CO_2 incubator for 24 hr. They were then incubated with 0.5 mM CDNB for 40 min at 10°. After loading with CDNB, some wells were washed three times with 1 mL HBSS containing 5.8 mM K^+, 143 mM Na^+, 0.3 mM Ca^{2+}, 0.8 mM Mg^{2+}, 146 mM Cl^-, 0.8 mM SO_4^{2-}, 0.8 mM P_i, 4.2 mM HCO_3, 5.6 mM glucose and 10 mM HEPES, pH 7.4, and the cells were then scraped with a rubber policeman and resuspended in 1 mL HBSS containing 10% perchloric acid. They were disrupted with a sonicator at 4° and then centrifuged for 2 min in a microfuge at 14,000 rpm. The resultant supernatant was passed through a 0.45 μm filter and aliquots were injected for HPLC-UV analysis using an HP 1050 liquid chromatographic system and a UV detector (Waters 490), set at 340 nm. The column (100 × 4.6 mm) was packed with C_{18} Hypersil ODS, and elution was carried out with a gradient of 40–90% (v/v) methanol in 0.01 M sodium phosphate, pH 6.0, for 6 min, following the solvent system reported by Oude Elferink *et al.* [22]. The flow rate was 1 mL/min. The remaining wells were also washed with HBSS, and the cells were then incubated in HBSS at 37°. The DNP-SG content in the extracellular HBSS was quantified at intervals up to 60 min by the HPLC-UV method as described above. The rate of DNP-SG efflux was calculated from a standard curve corresponding to DNP-SG formed by partially purified GST of rat liver using 1–40 nmol CDNB/mL in the presence of excess GSH.

Measurement of Cellular ATP and GSH

The ATP content of the cells was determined by using the Sigma bioluminescent somatic cell ATP assay kit as described in Sigma Technical Bulletin No. BSCA-1. GSH was determined by the colorimetric GSH assay kit from Bioxytech, SA (France). Three hundred microliters of the cell lysate was added to 600 μL buffer (200 mM potassium phosphate, pH 7.8, containing 0.2 mM diethylenetriaminepentaacetic acid and 0.025% lubrol) and then 50 μL reagent A (0.012 M chromogenic reagent in 0.2 M HCl) and 50 μL reagent B (30% aqueous sodium hydroxide) were added sequentially. The reaction mixture was incubated at ambient temperature for 10 min and the absorbance was measured at 400 nm.

Action of Polyphenols on DNP-SG Efflux

The cells were loaded with CDNB at 10° for 40 min as described above and washed with 3 × 1 mL HBSS. They were then exposed for 20 min at 37° to HBSS containing 5 to 160 μM polyphenols, which were freshly prepared in absolute alcohol just before use. The rate of DNP-SG efflux from the cells was measured by the HPLC-UV method described above.

Preparation of Plasma Membranes from Tumor Cells

Plasma membranes were prepared according to the method reported by Thom *et al.* [23]. Approximately 1 × 10^9 cells were harvested in 300 mL HBSS by scraping with a rubber policeman. They were collected by centrifugation and resuspended in 40 mL HBSS. The cells were then disrupted with a sonicator at 4° and centrifuged at 450 *g* for 10 min at 4°. The pellet, which contained whole cells and nuclei, was discarded, and the supernatant was recentrifuged at 12,000 *g* for 30 min at 4°. The plasma membrane-rich pellet was suspended gently in 5 mL HBSS and layered on top of a 35% (w/w) sucrose solution in HBSS and centrifuged at 24,000 *g* for 1 hr at 4°. The plasma membrane fraction, collected at the sucrose-HBSS interface, was removed with a pasteur pipette and resuspended in 5 mL HBSS and again centrifuged at 100,000 *g* for 10 min at 4°. The pellet (containing about 3 mg protein) was resuspended in 1.5 mL HBSS and stored frozen.

Solubilization of Mg^{2+}-ATPase and Measurement of its Activity

The plasma membrane fraction obtained above was centrifuged at 100,000 g for 10 min at 4°. The pellet was washed in buffer A containing 20 mM HEPES, 5 mM $MgCl_2$, 200 mM KCl, 20% glycerol (v/v) and 2 mM dithiothreitol, pH 7.4. This was repeated three times to ensure the removal of P_i present in the HBSS. The pellet was then resuspended in Buffer S, which is essentially buffer A with the addition of 0.55% Triton X-100, w/v, and stirred for 30 min at 4°. The insoluble fraction was removed by centrifugation for 10 min at 30,000 g and the ATPase activity in the supernatant was measured as follows: inorganic phosphate liberated from ATP by Mg^{2+}-ATPase was determined by a colorimetric assay according to the methods reported [24, 25]. An aliquot of 30 μL of the supernatant was mixed with 5 μL of 10 mM ouabain, 5 μL of 1.5 mM DNP-SG and 5 μL of Buffer S in a 96-well microtiter plate. The addition of ouabain and the exclusion of calcium in the reaction incubate allowed for the measurement of the DNP-SG activated Mg^{2+}-ATPase, which is known to be ouabain-insensitive and calcium-independent [25].

DNP-SG used in the ATPase assay was prepared by incubating 2 mM each of CDNB and GSH with partially purified rat liver GST (containing 50 μg protein) in 5 mL 20 mM HEPES, pH 7.0, at 37° for 5 min. The reaction was stopped by adding 0.5 mL perchloric acid. DNP-SG was isolated by collecting the effluent from the outlet of the HPLC-UV system described above, using a mobile solvent system containing 0.01 M sodium citrate-citric acid instead of 0.01 M sodium phosphate, and its concentration was determined by measuring its absorbance at 340 nm. Subsequently, the volume was reduced by using the speed-vacuum concentrator.

To examine the effects of polyphenols on the activity of Mg^{2+}-ATPase, 5 μL of Buffer S in the assay incubate was replaced by polyphenols at final concentrations ranging from 20 to 320 μM. The reaction was started by adding 5 μL of 20 mM ATP (final concentration of 2 mM) to the wells and the plates were incubated at 37° for 30 min with constant agitation. It was stopped by adding 150 μL malachite green reagent [24]. The plates were left at ambient temperature for 10 min and read at 630 nm on a Dynatech MR 5000 microplate reader.

RESULTS

Identification and Measurement of DNP-SG Export by the Cells

Under the chromatographic conditions employed, a standard of DNP-SG (Fig. 1A), prepared by incubating CDNB and GSH with rat liver GST partially purified by affinity chromatography, had a retention time of 2.1 min. Likewise, DNP-SG formed in the cells and DNP-SG exported to the

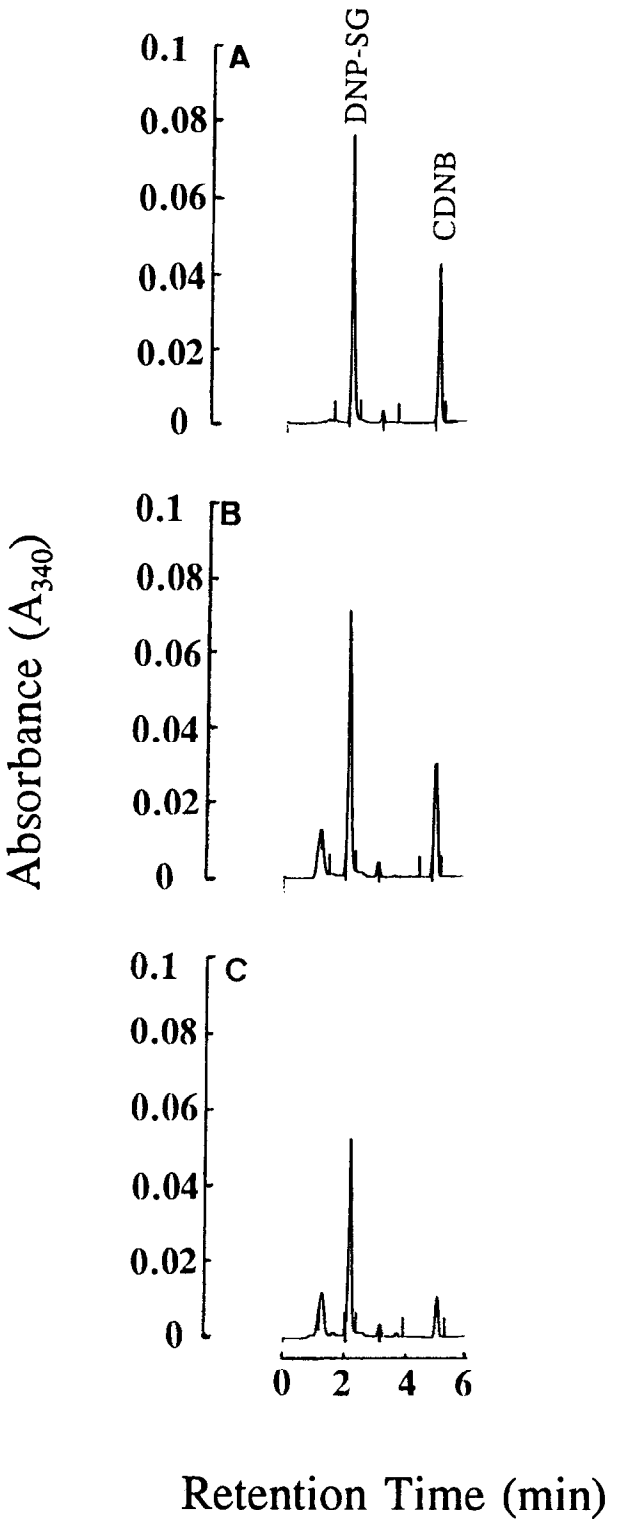

FIG. 1. Identification of DNP-SG by HPLC-UV detection. HPLC chromatograms of (A) a standard of DNP-SG biosynthesized using affinity-purified rat liver GST, (B) intracellular DNP-SG extracted from human colon cancer cells after loading with 0.5 mM CDNB at 10° for 40 min, and (C) DNP-SG in the extracellular HBSS, following incubation at 37° for 30 min.

extracellular HBSS had similar chromatographic profiles (Fig. 1B and C, respectively).

Upon warming to 37°, cells preloaded with CDNB at 10° for 40 min showed an efflux of the DNP-SG conjugate to the extracellular HBSS. A rapid transport was observed in the initial 20 min followed by a slow progress up to 60 min (Fig. 2). It was also observed that there was an inverse relationship between intracellular and extracellular DNP-SG (data not shown).

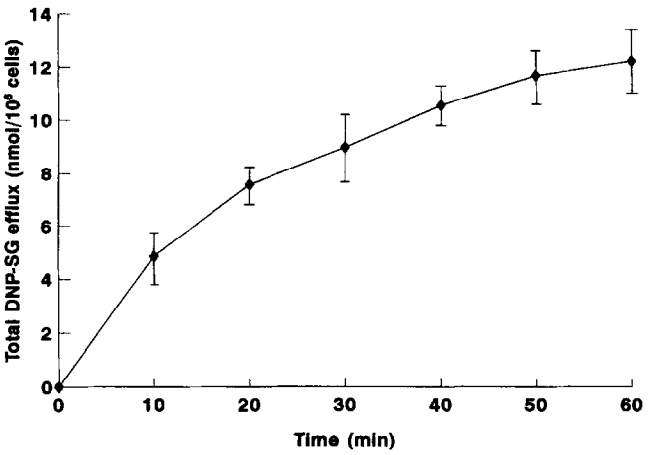

FIG. 2. Export of DNP-SG by human colon adenocarcinoma cells. Cells initially were loaded with 0.5 mM CDNB at 10° for 40 min. The efflux of the DNP-SG conjugate was measured in the extracellular HBSS at 37° for 60 min. Data points were mean values of triplicate experiments.

Effects of Polyphenols on:

(a) DNP-SG EXPORT BY THE CELLS. Nine polyphenols were tested for their effects on DNP-SG export by human colon tumor cells. They showed variable inhibitory potencies (Table 1), among which butein was the most potent with an IC_{50} value of 15 μM. Quercetin, tannic acid, 2′-hydroxychalcone, 2-hydroxychalcone and morin, in decreasing order of inhibitory potencies, had IC_{50} values of 43, 85, 132, 139 and 151 μM, respectively. These values were calculated from linear regression of five points in the range of 10–80% inhibition with correlation coefficient values ranging from 0.95 to 0.99. In addition, probenecid, an inhibitor of organic anion transport [26], also inhibited DNP-SG transport by the cells. The inhibition of DNP-SG transport by chlorambucil may be due to its competition for the GS-X pump by its glutathione S-conjugate. FCCP, an uncoupler of oxidative phosphorylation, also showed inhibition of DNP-SG export at low micromolar concentration; this was possibly a result of its effect on ATP production.

(b) CELLULAR ATP AND GSH. As transport of DNP-SG is ATP-dependent, factors that affect the ATP content of the cells would conceivably influence the export of DNP-SG. Under our experimental conditions, the polyphenols, introduced at concentrations up to 40 μM, did not alter the ATP content of the cells (Fig. 3a; $P > 0.2$). 2-Hydroxychalcone and 2′-hydroxychalcone showed some but insignificant effect ($P > 0.1$). This corroborates a similar observation on small cell lung cancer cells by genistein, an isoflavonoid; a decrease in the intracellular ATP content was, however, observed at a higher concentration [18]. Likewise, the GSH content of the cells was also not affected by the polyphenols (Fig. 3b; $P > 0.2$).

(c) MG^{2+}-ATPASE ACTIVITY OF THE PLASMA MEMBRANE. The DNP-SG-ATPase has been implicated in the transport of GSH conjugates and some anticancer drugs in tumor cells [27, 28]. Our study showed that the Mg^{2+}-ATPase prepared from the plasma membrane of the colon cancer cells was activated by DNP-SG in a concentration-dependent manner, and the reaction exhibited saturation kinetics (Fig. 4). The K_m and V_{max} values calculated from

TABLE 1. Effects of polyphenols and other inhibitors on DNP-SG efflux in human colon adenocarcinoma cells

	DNP-SG Efflux (nmol/min/10^6 cells)						
Inhibitors	Control	5 μM	10 μM	20 μM	40 μM	80 μM	160 μM
Tannic acid	0.43		0.30 (30.3)*	0.28 (34.9)	0.24 (44.2)	0.19 (53.8)	0.16 (62.8)
Butein	0.43	0.27 (37.3)	0.22 (48.9)	0.18 (58.1)	0.15 (65.1)	0.11 (74.4)	
Quercetin	0.43	0.32 (25.6)	0.28 (34.9)	0.24 (44.2)	0.21 (51.2)	0.15 (65.1)	
Morin	0.41		0.36 (12.2)	0.33 (19.6)	0.30 (26.8)	0.25 (39.1)	0.21 (48.8)
2-Hydroxychalone	0.41		0.34 (17.1)	0.33 (19.6)	0.29 (29.3)	0.23 (43.9)	0.20 (51.2)
2′-Hydroxychalcone	0.41		0.37 (9.8)	0.32 (21.2)	0.28 (31.7)	0.24 (41.5)	0.19 (53.7)
Quercitrin	0.40				0.33 (17.7)	0.27 (32.5)	
Naringenin	0.40				0.25 (37.5)	0.16 (60.0)	
Naringen	0.40				0.29 (27.5)	0.24 (41.5)	
Probenecid	0.42				0.25 (40.5)	0.17 (59.5)	
Chlorambucil	0.42				0.30 (28.6)	0.21 (50.0)	
FCCP	0.42		0.17 (60.5)				

Cells loaded with 0.5 mM CDNB at 10° for 40 min were exposed to HBSS containing each of the polyphenols and other inhibitors at the concentration range of 5–160 μM at 37° for 20 min. The rates of DNP-SG export were determined as described in Materials and Methods. The values are means of three separate experiments.

* Percent inhibition.

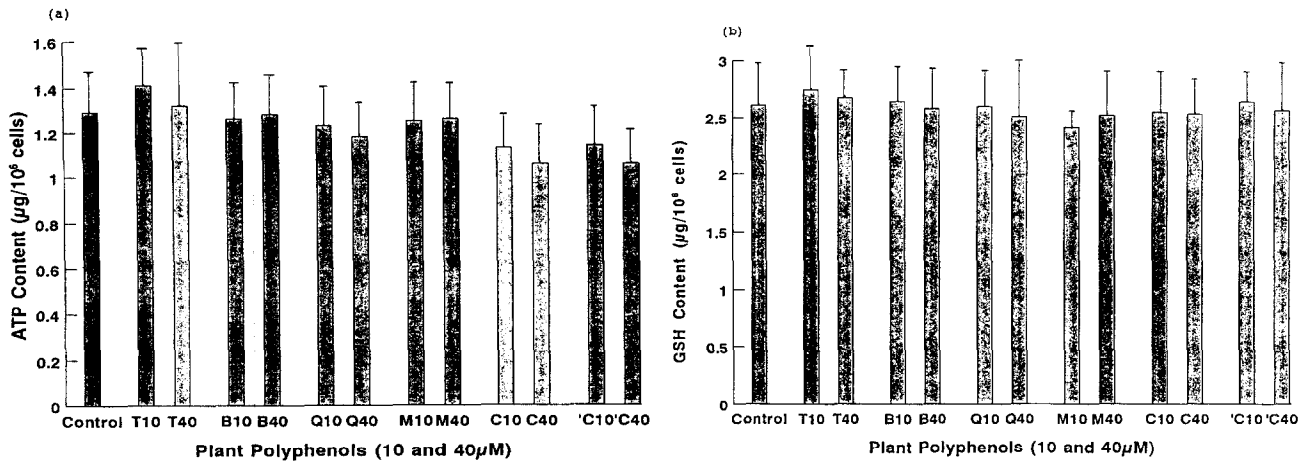

FIG. 3. Effects of polyphenols on intracellular concentrations of (a) ATP and (b) GSH. Following loading with 0.5 mM CDNB at 10° for 40 min, the cells were exposed to each of the polyphenols at concentrations of 10 and 40 μM for 20 min at 37°. Values are means (± SD) of three separate experiments. T, B, Q, M, C and 'C represent tannic acid, butein, quercetin, morin, 2-hydroxychalcone and 2'-hydroxychalcone, respectively. 10 and 40 denote 10 and 40 μM concentration.

the Lineweaver–Burk plot were 110.3 μM and 12.3 nmol/min/mg protein, respectively. However, the Mg^{2+}-ATPase activity measured in the presence of 150 μM DNP-SG was not affected by the six polyphenols introduced at concentrations up to 320 μM (Table 2, $P > 0.2$). These results demonstrated the activation of Mg^{2+}-ATPase of the plasma membrane by DNP-SG, suggesting that this enzyme may be involved in the transport of glutathione conjugates. However, it did not appear to be the site of action of the polyphenols examined.

DISCUSSION

Two proteins have been implicated in the transport of glutathione S-conjugates: they are the multidrug resistance-associated protein (MRP) and Mg^{2+}-ATPase. The MRP is a 190-kDa glycoprotein that contributes to the active transport of DNP-SG and LTC_4 by plasma membrane vesicles prepared from HeLa cells transfected with the MRP gene [29]. Studies with other glutathione conjugates supported the transport function of this glycoprotein [11–13]. The uptake of DNP-SG in plasma membrane vesicles prepared from human erythrocytes appeared to be catalyzed by a Mg^{2+}-dependent ATPase, as its GS-X pump activity was diminished by coating the vesicles with antibodies against DNP-SG-ATPase [28]. This Mg^{2+}-ATPase has been purified from human erythrocytes, muscle and lung [27, 28, 30] and was characterized as an 80-kDa protein composed of two similar subunits with molecular mass of 38 kDa [27]. To reconcile these two observations, it is conceivable that DNP-SG-ATPase is an integral part of the MRP, and that its activity is crucial to the operation of the GS-X pump. This is indeed a simplistic interpretation based on molecular size alone. Alternatively, the Mg^{2+}-ATPase and DNP-SG-transporter are separate independent entities that do not interact. Our data suggested that the Mg^{2+}-ATPase may be involved in the transport of DNP-SG since its activity is increased in a concentration-dependent manner in the presence of DNP-SG (Fig. 4). The polyphenols did not affect the Mg^{2+}-ATPase activity, but they inhibited the efflux of DNP-SG. These observations did not exclude a possible interplay of the Mg^{2+}-ATPase and DNP-SG transporter in the functioning of the GS-X pump, but they delineate the Mg^{2+}-ATPase as the site of action of polyphenols. Furthermore, the ATP content of the cells was not affected by these polyphenols (Fig. 3a).

The MRP has been demonstrated recently to be highly phosphorylated, and this phosphorylation could be blocked completely by inhibitors of PKC [31]. PKC inhibitors could also induce drug accumulation, concomitant with inhibition of drug efflux in drug-resistant cells. In this context, genistein, an isoflavonoid, is reported to be a PKC inhibitor, and it was able to modulate drug resistance in non-Pgp MDR cells [17, 18]. It is therefore, tempting to propose that the mechanism of action of the polyphenols used in our study could be on PKC activity, resulting in decreased phosphorylation of MRP and inhibition of DNP-SG transport. Indeed, quercetin and tannic acid inhibited PKC activity in other systems [32, 33], and quercetin decreased the efflux of daunorubicin in GLC4/ADR cells, which resulted in drug accumulation [17].

Hitherto, modifiers of MDR mediated by Pgp were not effective or less effective in non-Pgp MDR systems [16, 34]. Our studies have shown that butein and quercetin are relatively potent inhibitors of DNP-SG transport (Table 1). These polyphenols quench the fluorescence of GSH-bimane and could not be tested in our system developed earlier. The polyphenols also inhibited the activity of partially purified GST of rat liver [20]. Their combined inhibitory action on GST and the GS-X pump may provide an

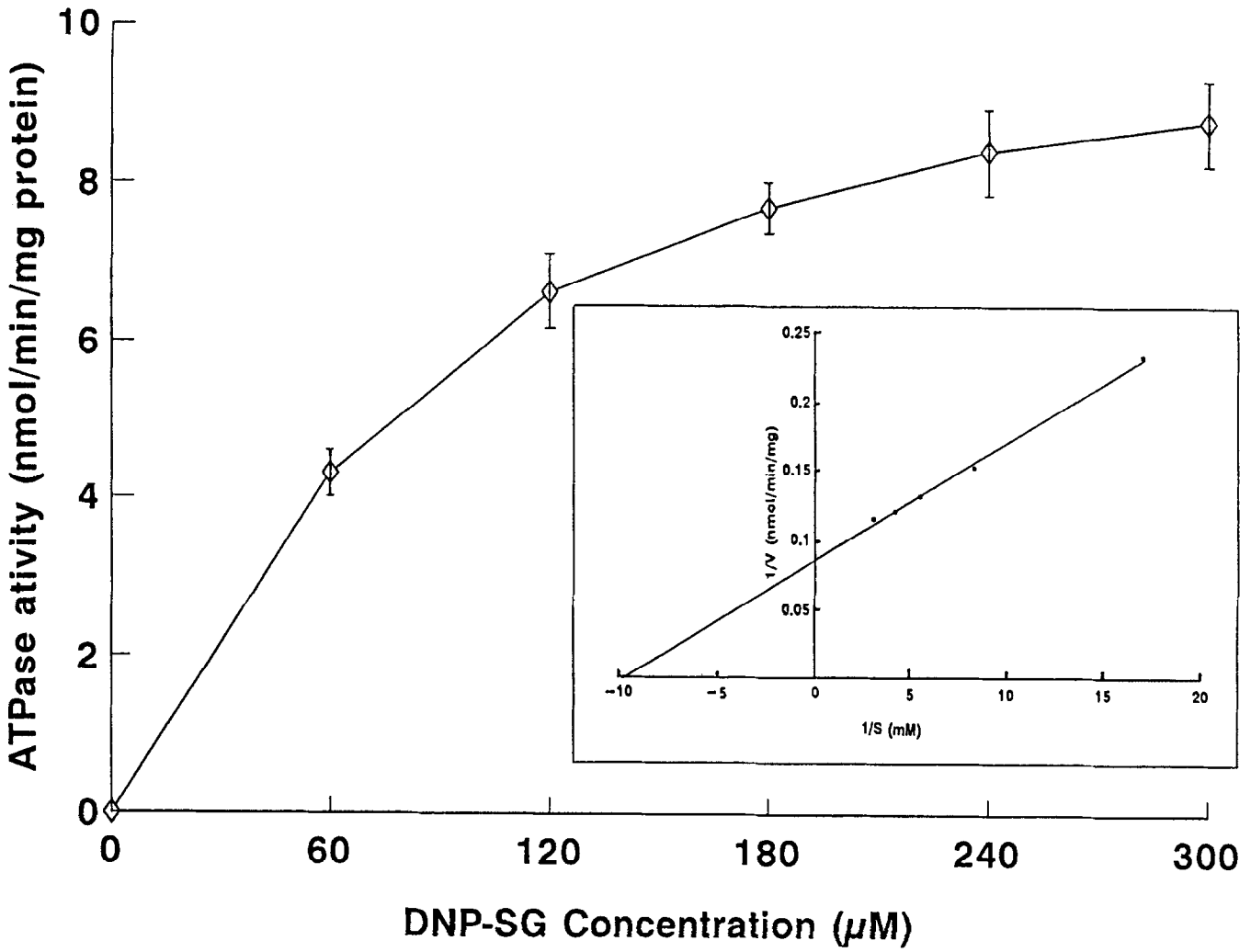

FIG. 4. Activation of Mg^{2+}-ATPase by DNP-SG. Mg^{2+}-ATPase was prepared from the plasma membranes, and its activity was determined by the procedure of Henkel *et al.* [24]. Activation by DNP-SG was calculated as a difference in Mg^{2+}-ATPase activity in the presence and absence of DNP-SG. Each data point represents the mean (± SD) of triplicates. Analysis of data by the Lineweaver–Burk plot is shown in the inset.

additive modulating effect in combating multidrug resistance in non-Pgp cells. Several reports have shown that overexpression of GST and MRP could confer resistance in non-Pgp multidrug-resistant cells to chemotherapeutic agents [4, 5]. As potential chemosensitizers, it is interesting to note that these polyphenols are generally considered to be non-toxic, as they are found in the normal human diet [35] and are constituents of herbal medicine [36].

TABLE 2. Effects of polyphenols on DNP-SG-Mg^{2+}-ATPase prepared from the plasma membranes of human colon adenocarcinoma cells

Concentration (μM)	DNP-SG-Mg^{2+}-ATPase activity (nmol/min/mg protein)						
	Control	Tannic acid	Butein	Quercetin	Morin	2-Hydroxy chalcone	2'-Hydroxy chalcone
20	6.38 ± 0.71	6.51 ± 0.24	6.74 ± 0.44	5.67 ± 0.66	5.48 ± 0.53	5.96 ± 0.65	5.85 ± 0.76
40	5.93 ± 0.45	5.66 ± 0.84	5.57 ± 0.50	5.53 ± 0.43	5.48 ± 0.48	5.93 ± 0.22	5.57 ± 0.36
80	6.23 ± 0.30	5.96 ± 0.42	5.94 ± 1.26	6.11 ± 0.22	5.65 ± 0.30	5.93 ± 0.36	6.37 ± 1.07
160	6.15 ± 1.15	6.47 ± 0.79	5.75 ± 0.55	5.67 ± 0.57	5.48 ± 1.14	5.72 ± 0.52	6.29 ± 0.36
320	6.38 ± 1.03	6.65 ± 0.78	5.95 ± 0.64	5.46 ± 0.76	6.31 ± 0.39	6.30 ± 0.94	6.23 ± 0.89

Inhibition studies were carried out in the presence of the six polyphenols in the concentration range of 20–320 μM. Results are means ± SD of three experiments (DNP-SG-Mg^{2+}-ATPase activities of test groups were compared to controls using Student's *t*-test. The differences were not significant; $P > 0.2$).

This work was supported by research grants RP870349 and RP900316 of the National University of Singapore. Zhang Kai is a recipient of a NUS research scholarship.

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