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Effects of kaempferol on the mechanisms of drug resistance in the human glioblastoma cell line T98G

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Food contains components that may either increase or decrease the bioavailability of a drug. In particular, it is known that grapefruit juice and St. John's Wort induce drug interactions via an effect on the drug-metabolizing enzyme cytochrome P450 (CYP). However, interactions with membrane transporters, such as P-glycoprotein and multidrug resistance-related protein (MRP), may also influence drug bioavailability. The objective of the present study was to investigate the effects of kaempferol, a flavonoid present in food, on the cytotoxicity of anticancer drugs and the mechanisms of drug resistance in the human glioblastoma cell line T98G. Acute exposure to kaempferol inhibited the efflux of calcein, a substrate of MRP; however, chronic exposure caused no apparent effect on calcein efflux. The cytotoxicity of doxorubicin was not influenced by chronic exposure of cells to kaempferol, although that of cisplatin was significantly reduced. Multidrug resistance is often associated with increased levels of MRP1, glutathione *S*-transferase (GST) and activity by chronic exposure to kaempferol, although MRP2 protein levels are decreased. Accordingly, we hypothesized that the cytotoxicity of anticancer drugs that conjugate with glutathione and the substrate of MRPs may be influenced by long-term intake of drugs such as kaempferol, which are substrates of MRPs and GST.

1. Introduction

In recent years, food-drug interactions have become a major concern. Drug interactions have been reported with some herbal medicines and supplements, such as St. John's Wort (SJW). There have been a relatively large number of studies investigating SJW and chronic intake has been shown to reduce the oral bioavailability of some drugs through upregulation of the 3A subfamily of cytochrome P450 (CYP3A) and permeability glycoprotein (P-gp) levels (Perloff et al. 2001; Bray et al. 2002; Gurley et al. 2002). It is known that hypericin in SJW induces drug interactions. Furanocoumarin derivatives, bergamottin and 6′,7′-dihydroxybergamottin in grapefruit juice also induce drug interactions (Takanaga et al. 1998; Ohnishi et al. 2000; Uesawa and Mohri 2006).

The flavonoids are a major group of polyphenolic antioxidants found in vegetables, fruits and herbal functional food. In addition to their known antioxidant effects, flavonoids have a broad range of biologic activities that include inhibition of the β-catenin/Tcf signal, telomerase, and CYP3A4 (Ha et al. 1995; Schubert et al. 1995; Naasani et al. 2003; Park et al. 2005). Furthermore, clinical trials have been conducted to evaluate the efficacy of supplements such as soy isoflavones and Ginkgo biloba (Arjmandi et al. 2005; McCarney et al. 2007).

Frequently, large quantities of flavonoids are ingested by healthy individuals as antioxidant supplements and by cancer patients as a form of alternative or complementary therapy. However, the flavonoids are important modulators or substrates of P-gp and multidrug resistance-related proteins (MRPs), and inhibitors of glutathione *S*-transferase (GST) activity (Leslie et al. 2001; Limtrakul et al. 2004; van Zanden et al. 2004, 2005; Nieri et al. 2006; Schutte et al. 2006; Hayeshi et al. 2007). There is also some evidence of a possible inhibition of ATP-dependent transporters by flavonoids, although the relationship between chemical structure and inhibitory potency remains unclear. Consequently, it is important to know whether these components affect the functions of proteins involved in drug metabolism and/or transport because this may have a significant impact on the physiologic functions of the proteins. In addition, the pharmacokinetics or toxicokinetics may also be affected and may impact on the efficacy of chemotherapy.

In tumor cell lines, multidrug resistance is often associated with an ATP-dependent decrease in cellular drug accumulation attributable to overexpression of the efflux transporter proteins P-gp, MRP, and breast cancer resistance protein (BCRP) (Conrad et al. 2002). MRPs have broad substrate specificity and among their substrates are glutathione, glucuronide, and sulphate conjugates, and some drugs (Zaman et al. 1995; Borst et al. 2000; Depeille et al. 2004). T98G cells of a human glioblastoma cell line do not express P-gp, and multidrug resistance is associated with overexpression of MRPs and BCRP. Kaempferol is one of many flavonoids found in vegetables, such as leek and broccoli, as well as in antioxidant supplements. Therefore, with many opportunities for kaempferol to be part of the daily diet, it is important to consider its influence on prescribed medication. We investigated the effects of

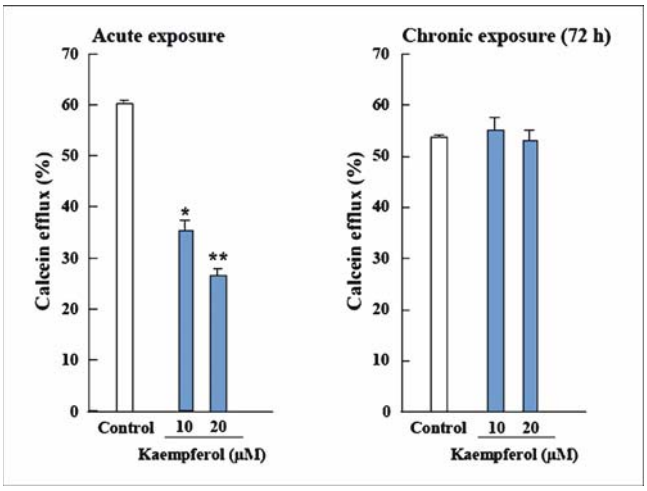

Fig. 1: Effect of kaempferol on the efflux of calcein in T98G cells. The efflux of calcein, a fluorescent substrate of MRPs, was measured in T98G cells. Acute exposure measures the influence on the efflux of calcein in the presence of keampferol. Chronic exposure measures the efflux of calcein in T98G cells exposed to kempferol for 72 h. Data are expressed as mean ± S.E.M. from six experiments. *$p < 0.05$, **$p < 0.01$ vs control

kaempferol on anticancer drug transport by MRPs and GST in T98G cells.

2. Investigations and results

2.1. Effect of kaempferol on calcein efflux

The efflux of calcein, which is a fluorescent substrate of MRPs, was measured in T98G cells. Acute exposure to kaempferol caused a concentration-dependent decrease in the extracellular efflux of calcein compared with control (Fig. 1). However, after chronic exposure to kaempferol (72 h), the efflux of calcein did not differ significantly from control values. These results were similar to those of another study investigating the overexpression of MRPs in other cell lines (van Zanden et al. 2005).

2.2. Effect of kaempferol on the cytotoxicity of anticancer drugs

We first observed the effects of chronic kaempferol exposure on the cytotoxicity of the MRP substrates doxorubicin and cisplatin. The Table shows the concentration required to kill 50% of cells (IC_{50}) for doxorubicin and cisplatin. Chronic exposure to kaempferol increased the IC_{50} and significantly decreased the cytotoxicity of cisplatin. However, the cytotoxicity of doxorubicin did not significantly differ from controls with chronic exposure to kaempferol.

Table 1: Effect of 72-h incubation with kaempferol on cytotoxicity of doxorubicin and cisplatin in T98G cells

	IC_{50} (μM)	
	Control	Kaempferol 20 μM
Doxorubicin	18.2 ± 1.3	13.0 ± 1.2
Cisplatin	93.9 ± 1.1	282.2 ± 1.3**

The number of viable cells was determined by sulforhodamin B assay. Data are expressed as mean ± S.E.M. from n = 4–5 experiments
** $p < 0.01$ vs control

2.3. Effect of kaempferol on the expression of MRPs and GST

The expression of MRP1, MRP2 and GST-π mRNA was determined by real-time reverse transcription-polymerase chain reaction (RT-PCR). Expression of GST-π mRNA (after normalization to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression) in cells after chronic exposure to kaempferol was significantly increased, by approximately twofold compared with the control. No significant difference was found in the levels of mRNA of MRP1 and MRP2 expressed by T98G cells (Fig. 2).
The expression level of MRP2 protein decreased in T98G cells after chronic exposure to kaempferol in a concentration-dependent manner, as determined by Western blotting. Conversely, MRP1 and GST-π protein levels increased in T98G cells after chronic exposure (Fig. 3).

2.4. Effect of kaempferol on GST activity

The effect of chronic exposure to kaempferol on GST activity was investigated next (Fig. 4). GST activity increased in T98G cells after chronic exposure to kaempferol in a concentration-dependent manner, and was significantly increased by 20 μM kaempferol, compared with the control.

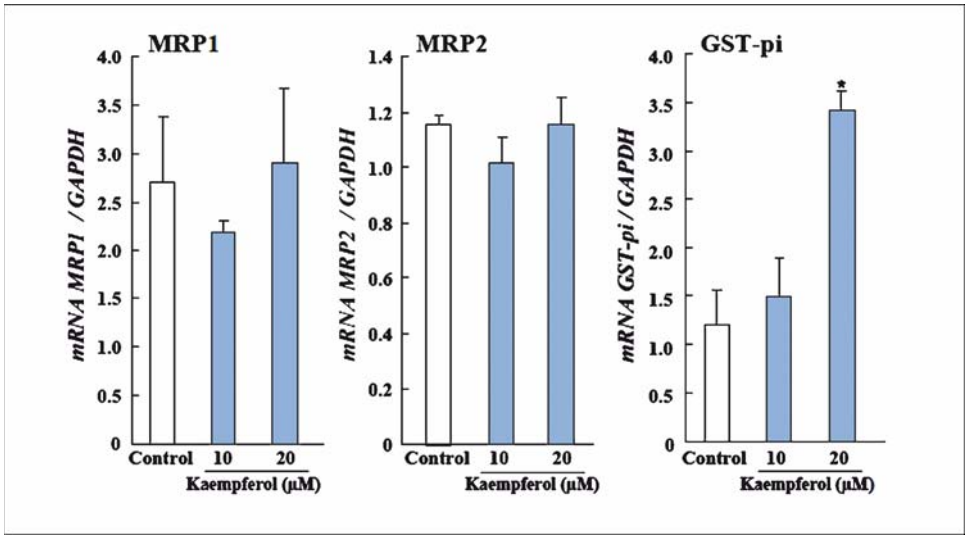

Fig. 2: Effects of 72-h incubation with kaempferol on the expression of MRP1, MRP2 and GST-π mRNA in T98G cells. MRP1, MRP2 and GST-π mRNA levels were determined by real-time RT-PCR. GAPDH was used for normalization of the results. Data are expressed as mean ± S.E.M. from three experiments. *$p < 0.05$ vs control

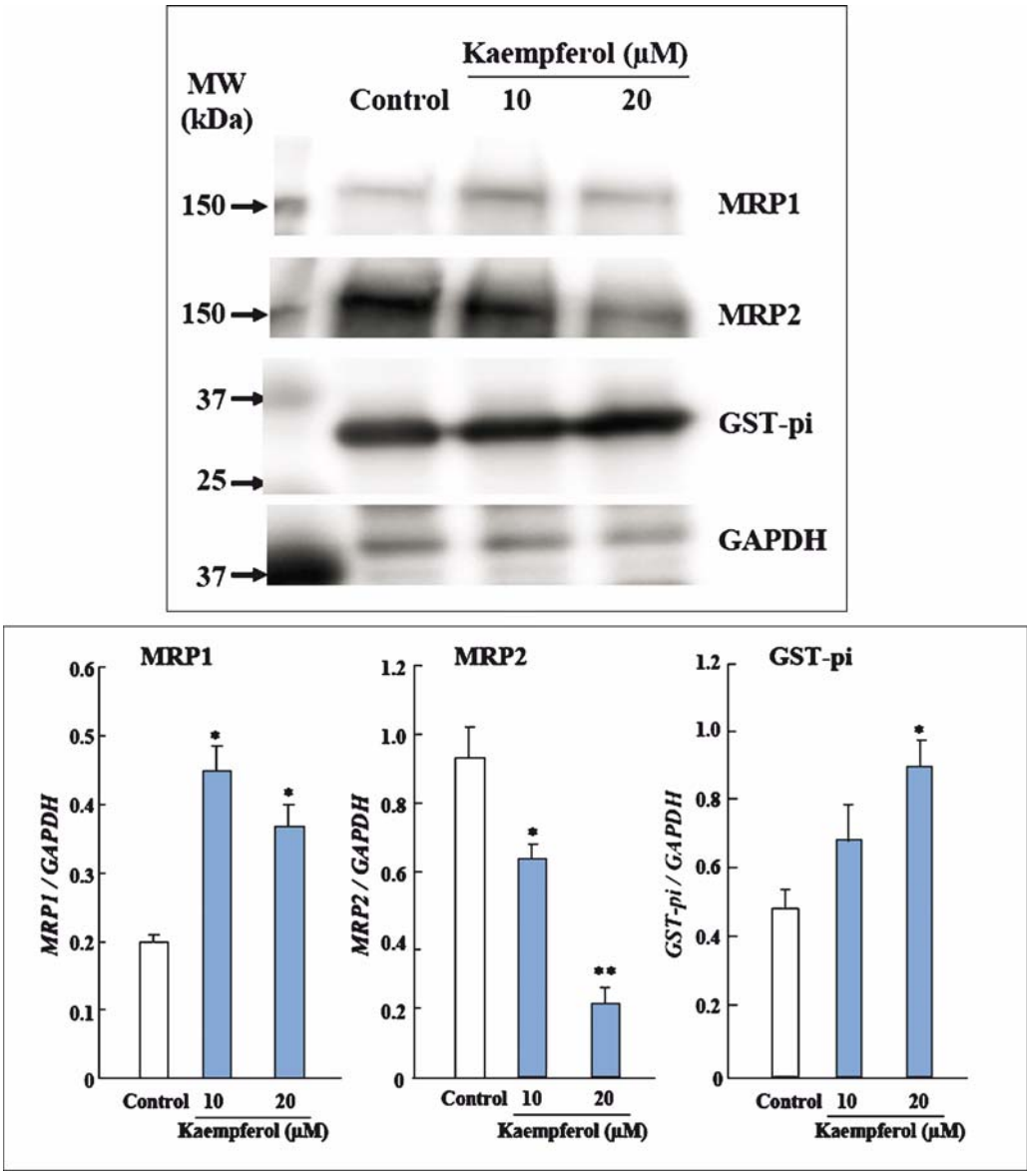

Fig. 3: Effects of 72-h incubation with kaempferol on the amount of MRP1, MRP2, and GST-π in T98G cells. MRP1, MRP2 and GST-π protein levels were determined by Western blotting (top), and quantified by densitometry (below). GAPDH was used for normalization of the results. Data are expressed as mean ± S.E.M. from three experiments. *$p < 0.05$, **$p < 0.01$ vs control

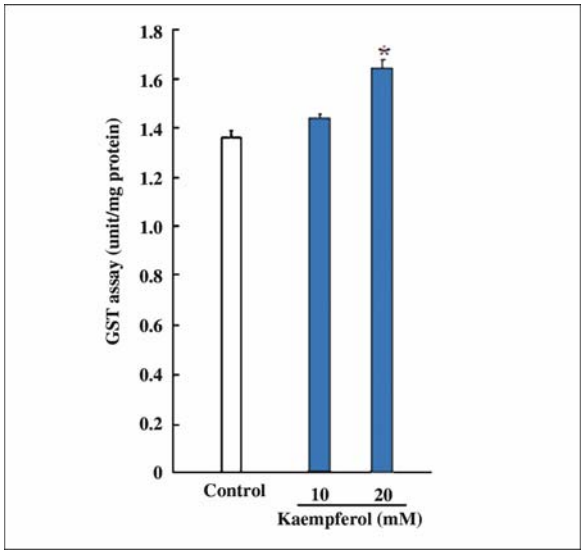

Fig. 4: Effects of 72-h incubation with kaempferol on GST activity in T98G cells. Data are expressed as mean ± S.E.M. from three experiments. *$p < 0.05$ vs control

3. Discussion

Acquired drug resistance follows selection, or induction by therapy, of diverse molecular events and is the principal unresolved cause of cancer treatment failure. The relationship between efflux mechanisms and drug resistance is well established (Marchetti et al. 2007). There have been a number of reports of MRP and GST inhibition by flavonoids that relate to drug resistance and, in our study, we have confirmed that the efflux of calcein, as the MRP substrate, is inhibited by kaempferol. However, there have been few studies of the influence of kaempferol on drug resistance per se. We would have expected that simultaneous exposure to kaempferol and cisplatin would increase the cytotoxicity of cisplatin because MRP and GST are both inhibited by kaempferol. However, the cytotoxicity of cisplatin decreased. This discrepancy was not noted when calcein was used as a substrate of MRPs. When thinking about this result, it is necessary to consider the influence of a contribution rate each MRP1/2 on efflux of calcein. It is difficult to evaluate the levels of endogenous MRP using calcein, because it is a substrate of both MRP1 and MRP2 (Doyle et al. 1998; Litman et al. 2000). In future studies, it may be necessary to measure this by

selective inhibition or by MRP1/2 expression analysis using small interfering RNAs.

Western blotting revealed that chronic exposure to kaempferol led to an increase in MRP1 expression. By contrast, MRP2 expression decreased in a concentration dependent manner. This is thought to be mainly related to the efflux of glutathione-drug conjugates by MRP1, and MRP1 was more potently inhibited by kaempferol than MRP2 (van Zanden et al. 2005). In many cases, mRNA and protein expression levels are correlated, and it was reported that both P-gp mRNA and protein decrease following exposure to curcumin (Limtrakul et al. 2004). However, expression was not affected by the modulation of MRP1/2 mRNA. Chronic exposure to kaempferol is thought to influence mRNA expression levels of MRP1 and MRP2 during splicing, translation, and protein turnover and not during the regulation of mRNA transcription. It is necessary to study the effects of kaempferol on the enzymes, MRP degradation or stabilization, and mRNA related to these processes.

Western blotting and real-time RT-PCR assays demonstrated that kaempferol induces a concentration-dependent increase in GST-π expression. This may be related to a decrease in the cytotoxicity of cisplatin metabolized by GST-π and to the efflux of glutathione conjugates by MRP1.

The influence of kaempferol on doxorubicin cytotoxicity is more complicated. Doxorubicin resistance is due to efflux pathways other than MRPs, such as P-gp and BCRP, and Pgp-mediated efflux of unconjugated doxorubicin is efficient, although MRP1 supports ATP-dependent efflux of glutathione-drug conjugates (Doyle et al. 1998). However, it is known that multidrug resistance in T98G cells is associated with the overexpression of MRPs and BCRP, but not with the expression of P-gp (Abe et al. 1994; Matsumoto et al. 2005). Furthermore, GST-dependent metabolism of doxorubicin is thought to contribute to the increase in cytotoxicity of doxorubicin in T98G cells after treatment with L-buthionine-sulfoximine, through inhibition of GST (data not shown). In T98G cells exposed to kaempferol, the cytotoxicity of doxorubicin, was expected to decrease. However, there was no influence on doxorubicin cytotoxicity, although GST activity and MRP1 mRNA increased. The cytotoxic effects of doxorubicin are known to be caused by cell injury through free radical generation, as well as by the inhibition of DNA and RNA polymerase (Ogura et al. 1991; Asmis et al. 2005). Furthermore, it has been reported that kaempferol reduces the ability to remove radicals, although oxidant-scavenging agents, such as superoxide dismutase and thioredoxin, are available to suppress cell injury induced by oxidative stress (Sharma et al. 2007). Because cell injury is increased through oxidative stress, it is possible that the cytotoxic effects were not noticeable, although the efflux of doxorubicin increased during chronic exposure to kaempferol.

In the present study, we investigated the interaction of kaempferol with MRP and the interplay between kaempferol-dependent MRP inhibition and its modulation of GST and MRPs.

The results of the study point to the possibility of a kaempferol-drug interaction, especially when the cytotoxicity of an anticancer drug is dependent on GSTs and MRP-mediated transport processes. These possible effects need to be examined further in detail under *in vivo* conditions.

4. Experimental

4.1. Cell culture and treatment

The human cell line T98G (glioblastoma) was obtained from the Cell Resource Center for Biomedical Research, The Institute of Development, Aging and Cancer, Tohoku University. The cells were grown in 90 mm dishes in RPMI 1640 medium supplemented with 10% fetal bovine serum.

In this study, T98G cells were grown to confluency in 60 mm cell culture dishes or 96-well microplates. Before exposure to kaempferol, the medium was removed and cells were washed with phosphate-buffered saline (PBS). The 'exposure medium', consisting of RPMI 1640 supplemented with 10 or 20 μM kaempferol (Wako Pure Chemical, Japan) from a 2,500-fold concentrated stock solution in ethanol, was then added to the cells. The exposure medium was replaced every 24 h, and cells subjected to chronic exposure were incubated for 3 days (Limtrakul et al. 2004; Nieri et al. 2006).

4.2. Measurement of the cellular accumulation and efflux of calcein

Cells (5,000 cells/well) were seeded into 96-well plates in 100 μL of culture medium. Cells were then incubated with 2 μM calcein-AM, (the acetoxymethyl ester form of calcein), in the absence or presence of 100 μM Hanks' balanced salt solution (HBSS) for 1 h at 37 °C. The fluorescence intensity of the calcein converted from calcein-AM in cells was measured from the underside of the 96-well plate. The excitation and emission wavelengths were 485 and 520 nm, respectively. After incubation, the cells were washed with PBS, and the first cellular accumulation of calcein was measured. Cells were then incubated with HBSS, or HBSS plus kaempferol, and then subjected to acute exposure for 2 h at 37 °C. The 50% efflux of calcein was determined in the non-treated cells at 2 h from measurements taken at 0.5, 1, 2, and 4 h. After incubation, the cells were washed with PBS, and cellular accumulation of calcein was measured a second time. The amount of calcein efflux was calculated from the difference between the results of the first and second measurements.

4.3. Cytotoxicity assays

Cytotoxicity was measured by a colorimetric assay using sulforhodamin B (Sigma, Missouri USA) for the detection of living cells. Cells (1,000 cells/well) were seeded into 96-well plates in 100 μL of culture medium for 24 h, and then the culture medium was exchanged for medium containing kaempferol. After incubation for 3 days at 37 °C, the culture medium was replaced by medium containing various concentrations of doxorubicin or cisplatin, followed by further incubation for 4 h. Thereafter, the cells were washed with PBS and incubated in culture medium alone for 2 days. The optical density was determined in a 96-well plate reader at 564 nm. The cytotoxic effects of doxorubicin and cisplatin were expressed quantitatively as the IC_{50}.

4.4. Real-time RT-PCR assays

Total RNA was isolated from peritoneal macrophages using a High Pure RNA Tissue kit (Roche, Germany). Complementary DNA (cDNA) was synthesized from total RNA with a PrimeScript RT^{TM} reagent kit reverse transcriptase (TaKaRa, Japan). The coding regions of the respective cDNA species were amplified by RT-PCR with oligonucleotide primers designed by DNASIS Pro (Hitachi soft, Japan). The Mastercycler® ep realplex 2 (Eppendorf, Germany) was used for amplification and specific sequence detection and the SYBR® Premix Ex Taq kit (TaKaRa) was used. The primer sequences used in establishing the amount of MRP1, MRP2, or GST-π were as follows: MRP1 forward primer, 5′-GGTCCTTAAACAAGGAGGAC-3′; MRP1 reverse primer, 5′-TCCTTGGAGGAGTACACAAC-3′; MRP2 forward primer, 5′-ATCTTCACGTTTGTGAGTCC-3′; MRP2 reverse primer, 5′-GAGGCAGAAAGACTGAATG-3′; GST-π forward primer, 5′-GACCTTCATTGTGGGAGAC-3′; GST-π reverse primer, 5′-CCACATATGCTGAGCAG-3′. The primer sequences of the endogenous reference GAPDH were as follows: forward primer, 5′-GTCAAGCTCATTTCCTGGTA- 3′; reverse primer, 5′-CTCTTCCTCTTGTGCTCTTG-3′.

4.5. Western blot analysis of MRPs and GST

Cells grown in 60 mm dishes were washed with PBS, and then detached by scraping. Total cell lysates were prepared by lysing the harvested cells in 1% NP-40, 150 mM NaCl, 0.5% deoxycholate, and 20 mM Tris-HCl (pH 7.5), with 1 mM phenylmethylsulfonyl fluoride and a protease inhibitor cocktail, Compleat® (Roche). Protein concentration was determined with a Bio-Rad protein assay using bovine serum albumin as a standard. Proteins were electrophoresed on 7.5% or 12% sodium dodecyl sulfate-polyacrylamide gels and electroblotted on a polyvinylidene difluoride membrane. The membrane was blocked for 1 h at room temperature in PBS containing 0.05% Tween-20 and 3% gelatin. It was then incubated with the primary antibodies MRP1 (Santa Cruz, California USA) and MRP2 (Sigma) at 1:1000 in Can Get signal solution (TOYOBO, Japan), or with antibody GST-π (Alexis, California USA) at 1:200, overnight at 4 °C. The membrane was then rinsed with washing buffer (PBS (pH 7.4) with 0.05% Tween-20) and incubated for 30 min with a secondary antibody, anti-rabbit IgG–horseradish peroxidase (HRP) (Santa Cruz), at 1:2000 in Can Get signal solution (for MRP1 and

MRP2) or anti-mouse IgG–HRP secondary antibody (Zymed, California USA) at 1:2000 (for GST-π). After washing, the protein was detected using a chemiluminescent detection reagent (Super signal West Pico® Thermo, Massachusetts USA). Fujifilm image analysis software, Multi Gauge, was used to analyze the Western blot results.

4.6. Enzymatic assay of GST

Enzyme activity with the aromatic substrate 1-chloro-2.4-dinitrobenzene (CDNB) was usually determined by monitoring changes in absorbance in a 96-well plate reader at 340 nm (Habig et al. 1974). Cells grown in 60-mm dishes were washed with PBS, and then detached by scraping. Total cell lysates were prepared by lysing the harvested cells in PBS with 1% Triton X-100. A complete assay mixture without cells was used as a control. Assays were conducted in a thermostat-controlled plate reader chamber at 25 °C in reaction mixture containing 100 mM potassium phosphate buffer (pH 6.5), 1 mM EDTA, 2.5 mM glutathione, 1 mM CDNB, and 3.3% (v/v) cell lysates. Activity of GST for each amount of protein was obtained from the result of measurement of the increase in absorbance at 340 nm for 5 min.

4.7. Statistical analyses

Data were expressed as mean ± standard error of the mean (S.E.M.). Student's t-test and one-way analysis of variance followed by Dunnett's test were used to determine the significance of differences between the control and exposure groups. A value of $P < 0.05$ was considered significant.

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