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Antiproliferative effects of glutathione *S*-transferase inhibitors on the K562 cell line

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Glutathione *S*-transferases (GSTs* EC 2.5.1.18) are enzymes which catalyze the reaction of reduced glutathione (GSH) with a variety of electrophilic compounds [1, 2]. They are distributed not only in mammalian tissues but also in microorganisms and even in plants [3]. In spite of its wide distribution, little is known about its physiologic function (e.g. detoxification of xenobiotics [4], leukotriene C_4 synthesis [5] and transport of heme [6] and bilirubin [7]). Recently, there have been a few reports suggesting the possible involvement of GST in cell proliferation. Senjo and Ishibashi [8] reported that GST activity in C6 astroglioma cells changes significantly during the cell cycle and that the cell growth is inhibited in a dose-dependent manner by the GST inhibitors ethacrynic acid (EA) and caffeic acid. Tew *et al.* [9] reported that in rat breast carcinoma cell lines and human colon carcinoma cell lines EA and piriprost inhibit their colony forming ability and cytosolic GST activity.

The K562 cell is from a human leukemia cell line which has been established from the blast cells in pleural effusion of a patient with chronic myelogenous leukemia in blast crisis [10]. To investigate whether GST may play a role in the proliferation of K562 cells, we examined the effects of GST inhibitors, EA, bromosulfophthalein (BSP) and ferulic acid (FA) [11, 12], on cell proliferation.

Materials and methods

Materials. EA, BSP, FA, DL-buthionine[*S*,*R*]-sulfoximine (BSO) and GSH were purchased from the Sigma Chemical Co. (St Louis, MO). RPMI 1640 medium, fetal calf serum and penicillin–streptomycin were obtained from Flow Laboratories (U.S.A.), and [^{3}H]thymidine (50 Ci/mmol) was obtained from New England Nuclear (Boston, MA). 1-Chloro-2,4-dinitrobenzene (CDNB) was purchased from Nakarai Chemicals, Ltd (Kyoto, Japan). All other chemicals were of analytical grade.

Cell culture. The K562 cell line was supplied by the Japanese Cancer Research Resources Bank. K562 cells were routinely grown in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, penicillin (50 units/mL) and streptomycin (50 μg/mL) at 37° in a humidified atmosphere with 5% CO_2.

Effects of GST inhibitors on cell proliferation. EA, BSP and FA were dissolved in RPMI 1640 medium by vigorous shaking. K562 cells were cultured in the medium containing various concentrations of a GST inhibitor. Cells were counted in a hemocytometer, and viability was estimated by the trypan blue exclusion assay.

To examine the effects of GST inhibitors on DNA synthesis, after cells were incubated for 24 hr with various concentrations of a GST inhibitor on 96-well microtiter plates, [^{3}H]thymidine was added at a final concentration of 5 μCi/mL. After a 2-hr incubation, the trichloroacetic acid insoluble counts were counted on filter paper (Labo Science Corp., Japan) in toluene-based scintillant.

GST assay. K562 cells were washed twice with ice-cold phosphate-buffered saline and sonicated in 10 mM potassium phosphate buffer (pH 7.0). After centrifugation by an Eppendorf centrifuge at 4000 *g* for 10 min, GST activity in the supernatant fraction was assayed by the method of Habig *et al.* [1] using CDNB as a substrate at 37°. The inhibitory effects of EA, BSP and FA were examined in the presence of 1 mM GSH and 1 mM CDNB.

Measurement of intracellular glutathione. Intracellular glutathione was measured by the method of Griffith [13], with concurrent standards being monitored.

* Abbreviations: GST, glutathione *S*-transferase; EA, ethacrynic acid; BSP, bromosulfophthalein; FA, ferulic acid; BSO, DL-buthionine[*S*,*R*]-sulfoximine; GSH, reduced glutathione; and CDNB, 1-chloro-2,4-dinitrobenzene.

Results and discussion

When K562 cells were incubated continuously with various concentrations of either EA or BSP, cell growth was inhibited in a dose-dependent manner and viability was moderately impaired by 3 days at the highest concentration used (Fig. 1). In contrast, FA had no effect on the cell growth and viability at concentrations below 0.8 mM. To test the reversibility of the cell growth inhibition by EA and BSP, cells treated with 0.1 mM EA or 0.2 mM BSP for 3 days were transferred to the inhibitor-free medium. In each group cells proliferated with the same growth rate as non-treated controls, suggesting that the effects of these agents were reversible (data not shown). As for DNA synthesis, [^{3}H]thymidine incorporation was inhibited in a dose-dependent manner when cells were incubated for 24 hr with various concentrations of either EA or BSP, whereas no inhibition was observed with FA (Fig. 2). These results were consistent with those on cell growth (Fig. 1).

Using CDNB as a substrate, K562 cells at logarithmic phase growth had GST activity of 0.292 ± 0.017 μmol/min/mg protein. Figure 3 shows the inhibitory effects of EA, BSP and FA on GST activity in crude extracts of K562 cells. The I_{50} values of EA and BSP were 0.3×10^{-5} M and 4×10^{-5} M, respectively, suggesting that EA is the most potent inhibitor among the agents used. GST activity was inhibited by only 16% at 0.8 mM FA. From these results, it appears that the inhibitory potencies of EA, BSP and FA

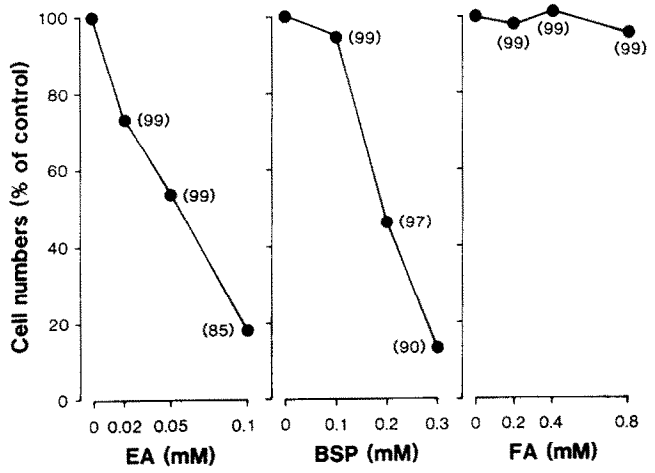

Fig. 1. Effects of EA, BSP and FA on the growth of K562 cells. Cells were plated (2×10^4/mL) in the medium containing various concentrations of either EA or BSP or FA. After a 3-day incubation, cells were counted in a hemocytometer. Cell numbers were expressed as percent of the control value (2.9×10^5/mL). The numbers in parentheses represent viability as estimated by the trypan blue exclusion assay. Values are the means of three determinations.

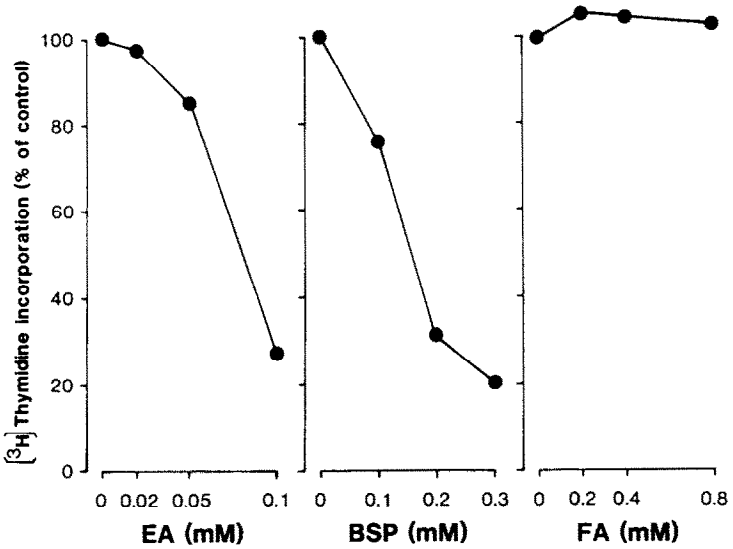

Fig. 2. Effects of EA, BSP and FA on [^{3}H]thymidine incorporation into K562 cells. Cells were plated (1×10^5/mL) in the medium containing various concentrations of either EA or BSP or FA on 96-well microtiter plates. After a 24-hr incubation, [^{3}H]thymidine was added at a final concentration of 5 μCi/mL. After 2 hr the radioactivity was measured. [^{3}H]Thymidine incorporation was expressed as percent of the control value (23,500 cpm/well). Values are the means of three determinations.

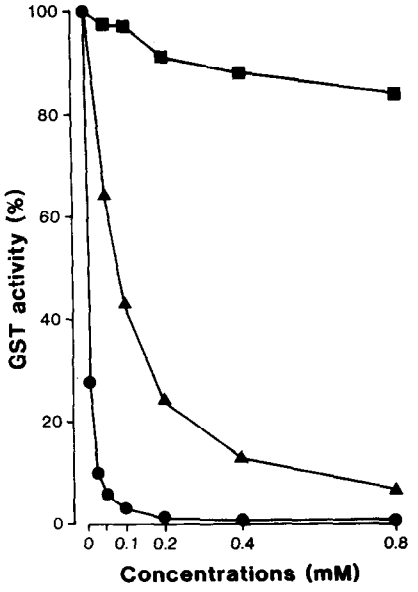

Fig. 3. *In vitro* inhibitory effects of EA, BSP and FA on GST activity in crude extracts of K562 cells. The inhibitory effects of EA (●), BSP (▲) and FA (■) were examined in the presence of 1 mM GSH and 1 mM CDNB. GST activity was expressed as percent of the control value (0.292 μmol/min/mg protein). Values are the means of three determinations.

on GST activity *in vitro* paralleled those on cell growth. It is likely that inhibition of GST activity by EA and BSP can account for the cell growth inhibition observed.

Cordeiro and Savarese [14] showed that formamides deplete intracellular glutathione and this reduction correlated well with growth inhibition, suggesting the relationship between glutathione content and cell growth. As EA and BSP are not only GST inhibitors but also substrates for GST [3], they may act by depleting intracellular glutathione. To exclude this possibility, we first examined the effect of BSO, an inhibitor for glutathione synthesis, on cell growth and intracellular glutathione content. K562 cells at logarithmic phase growth had intracellular glutathione of 10.2 ± 0.51 nmol/10^6 cells. As shown in Fig. 4, after continuous incubation for 3 days with BSO at various concentrations, cell growth and intracellular glutathione content were suppressed in a dose-dependent manner. On the contrary, after continuous incubation for 3 days with either EA or BSP at various concentrations, intracellular glutathione content increase dose dependently in spite of a decrease in cell number (Fig. 5). These results clearly demonstrate that glutathione depletion is not responsible for the growth inhibitory effects of EA and BSP.

Another possibility about the action of EA and BSP is that glutathione *S*-conjugates of these agents may act as cytostatic metabolites. Glutathione *S*-conjugates have been reported to affect several glutathione-related enzymes. Bilzer *et al.* [15] reported that glutathione reductase was competitively inhibited by the glutathione *S*-conjugate *in vitro*. Even if this inhibition may actually occur intracellularly, it would not yield an unfavorable alteration of the reduced glutathione/glutathione disulfide ratio for cell

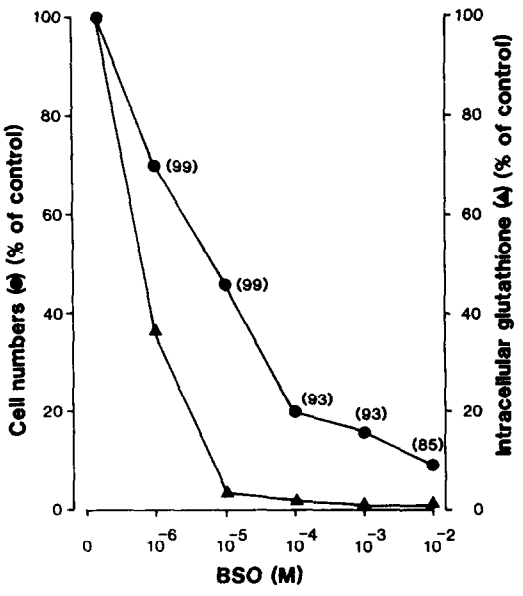

Fig. 4. Effects of BSO on cell growth and intracellular glutathione content. Cells were plated (2 × 10^4/mL) in the medium containing various concentrations of BSO. After a 3-day incubation, cells were counted and processed for total glutathione determination. Cell numbers and glutathione content were expressed as percent of the control value (2.5 × 10^5/mL and 10.2 nmol/10^6 cells respectively). The numbers in parentheses represent viability as estimated by trypan blue exclusion assay. Values are the means of two determinations.

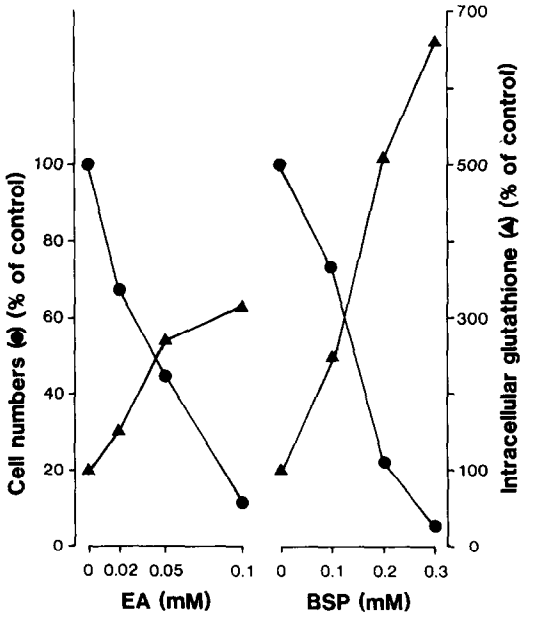

Fig. 5. Effects of EA and BSP on cell growth and intracellular glutathione content. Cells were plated (2 × 10^4/mL) in the medium containing various concentrations of either EA or BSP. After a 3-day incubation, cells were counted and processed for total glutathione determination. Cell numbers and glutathione content were expressed as percent of the control value (3.0 × 10^5/mL and 9.8 nmol/10^6 cells respectively). Values are the means of two determinations.

growth in the absence of the condition where glutathione disulfide formation is stimulated (e.g. oxidative challenge) [16]. Jakobson *et al.* [17] reported that glutathione *S*-conjugate binds to GST and that the dissociation constant K_d is 0.5 μM. However, since the physiological levels of GSH range between 5 and 10 mM, an inhibition by the glutathione *S*-conjugate may be overcome. Kondo *et al.* [18] reported that gamma-glutamylcysteine synthetase, a key enzyme for glutathione synthesis, is stimulated by the glutathione *S*-conjugate *in vitro* and in the cell system. This stimulation of glutathione synthesis would not affect negatively the cell growth or viability. In addition, there are several reports demonstrating that glutathione *S*-conjugates of various xenobiotics are transported rapidly outside of the cells [19, 20]. From these studies, it seems unlikely that glutathione *S*-conjugates of EA or BSP may accumulate inside the cells and act as cytostatic metabolites.

We demonstrated here the antiproliferative effects of GST inhibitors on K562 cells. Preliminarily, we observed that the results in HL60 cells were similar to those in K562 cells. Although not direct evidence, our results do not conflict with the intriguing hypothesis that GST may be involved in K562 cell proliferation.

Third Department of Internal Medicine
Yamaguchi University School of Medicine
Ube, Yamaguchi 755
Japan

YUTAKA SATO*
SHINYA FUJII
YASUHIKO FUJII
TOSHIO KANEKO

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* To whom correspondence should be addressed.