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Lentinan Enhances Sensitivity of Mouse Colon 26 Tumor to *cis*-Diamminedichloroplatinum(II) and Decreases Glutathione Transferase Expression

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We investigated the influence of a combination of lentinan, a biological response modifier, and *cis*-diamminedichloroplatinum(II) (CDDP) on the growth and glutathione *S*-transferase (GST) content of colon 26 tumor to examine whether lentinan represses GST expression and enhances the therapeutic effects of CDDP. Female CDF_1 mice inoculated subcutaneously with transplantable colon 26 adenocarcinoma cells (1×10^6/mouse) received intraperitoneal administrations of lentinan, CDDP, or the two drugs in combination, on days 10, 14, 17 and 21 after the inoculation. On day 24, tumor weights (estimated from their length and width) were significantly lower in the CDDP+lentinan group (2.7 ± 1.3 g) than in the CDDP alone group (4.3 ± 0.7 g, $P<0.05$), both values being less than in the nontreated control group (7.2 ± 1.5 g). The major GST form of colon 26 tumor was identified as GST-II, the Pi class form, and a minor form as GST-III belonging to the Mu class. Both GST-II and GST-III values on day 24 were significantly decreased in the lentinan alone (0.90 ± 0.29 and 0.26 ± 0.11 μg/mg protein, respectively) and lentinan+CDDP groups (0.98 ± 0.22 and 0.29 ± 0.07 μg/mg protein), as compared with the control levels (1.39 ± 0.20 and 0.52 ± 0.11 μg/mg protein). However, these values were not different between the CDDP alone and lentinan+CDDP groups. Neither tissue interleukin (IL)-6, glutathione nor platinum values were different between the two groups. IL-6 values were elevated in about half of the samples treated with lentinan or CDDP and exhibited a modest inverse correlation with GST-II levels ($r=-0.46$). A GST inhibitor, ethacrynic acid, enhanced the sensitivity of cultured colon 26 cells to CDDP, suggesting the possible involvement of GST in modulating the cytotoxicity of CDDP to this cell line. These results indicated that lentinan administration decreases tissue GST-II and GST-III contents and enhances the sensitivity of colon 26 tumor to CDDP.

Key words: Glutathione transferase — Colon tumor — Drug resistance — *cis*-Diamminedichloroplatinum(II) — Biological response modifier

cis-Diamminedichloroplatinum(II) (CDDP) has a broad range of activity against several malignancies. However, its efficacy as an anti-neoplastic agent is often hampered by inherent or acquired resistance of tumor cells to its cytotoxicity.[1] Cellular defense mechanisms that protect cells from CDDP-induced DNA damage include a decrease in CDDP accumulation, repair of DNA damage, and increased inactivation of the drug.[2,3] The glutathione *S*-transferases (GSTs) (EC 2.5.1.18) are a family of multifunctional proteins that act as enzymes and also as binding proteins in various detoxication processes. Many molecular forms of cytosolic GST are grouped into four classes, Alpha, Mu, Pi, and Theta.[4,5] Pi class GST forms (human GST P1-1, rat GST-P and mouse GST-II) are strongly expressed in many cancer tissues, including colon cancers.[6,7] Glutathione and GST forms have been shown to be increased in some CDDP-resistant cell lines.[2,8,9] Thus, these molecules are possible targets for attempts to enhance CDDP efficacy, and inhibitors of GST or glutathione synthesis have been used for this purpose.[10–12]

Since GST-P and GST-II genes possess 12-*O*-tetradecanoylphorbol 13-acetate-responsive element (TRE)-like sequences in their enhancer regions,[13–15] their expression has been suggested to be partly regulated by the oncogene products c-*Jun* and c-*Fos*.[16–18] In addition to positive enhancers, the *GST-P* gene has silencers in the upstream region. A *trans*-acting factor binding to the silencer has been reported to be identical to the interleukin (IL)-6-dependent DNA binding protein (IL-6 DBP),[19] a factor involved in IL-6 gene expression.[20] Nucleotide sequences similar to the silencer have also been found in the *GST-II* gene.[15]

A murine transplantable adenocarcinoma, colon 26 tumor, possesses the ability to synthesize IL-6, and IL-6 production has been reported to be induced by IL-1 or activated macrophages.[21] Lentinan, a biological response modifier, is a polysaccharide composed of a β-1,3-glucopyranoside main chain with β-1,6 branches[22,23] which inhibits the growth of some tumors through stimulation

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of host defense mechanisms.[24] It is known to activate macrophages, resulting in the production of acute-phase proteins in mouse liver in an IL-6-dependent manner.[25, 26]

In the present study, we investigated the influence of a combination of lentinan and CDDP on the growth and GST content of colon 26 tumor to examine whether lentinan represses GST expression and enhances the therapeutic effects of CDDP.

MATERIALS AND METHODS

Reagents 1-Chloro-2,4-dinitrobenzene (CDNB) was purchased from Wako Pure Chemicals (Osaka). Lentinan and CDDP were obtained from Roussel-Morishita Co. (Osaka) and Nihon Kayaku Co. (Tokyo), respectively. Collagenase and DNase were purchased from Sigma (St. Louis, MO). All other chemicals were of analytical grade.

Mice Female Balb/c×DBA/2 F_1 (CDF_1) mice obtained from Charles River Japan Inc. (Kanagawa) were used at 10 to 12 weeks of age. The mice were housed under conventional conditions and had free access to water and food.

Transplantation of colon 26 tumors and drug treatment Colon 26 carcinoma was kindly donated by Dr. T. Suga, Institute for Biological Sciences, Ajinomoto Co. (Yokohama). The tumor was minced into small fragments and a single cell suspension was prepared by treatment with collagenase (250 μg/ml) and DNase (1 μg/ml) for 30 min at room temperature. The cells (1×10^6/mouse) were inoculated subcutaneously into the back of CDF_1 mice. These mice were divided into 4 groups. Four to 5 mice in each group were injected intraperitoneally with lentinan (5 mg/kg of body weight), CDDP (4 mg/kg of body weight), or the two drugs in combination on days 10, 14, 17 and 21 after the inoculation. Four mice were injected with PBS, instead of the drugs, as a control group. Before the injection of drugs or PBS, tumor weight was estimated from the following equation:

$$\text{tumor weight (g)} = ab^2/2$$

where a and b are the length and width (cm) of the tumor, respectively, as reported by Tanaka *et al.*[27] On day 24 after the inoculation, mice were killed and the tumors were quickly excised, weighed and stored at −80°C until use.

Sensitivity of cultured colon 26 cells to CDDP Using 96-well plates, 1.25×10^3 colon 26 cells per well were cultured with 100 μl of RPMI 1640 medium containing 10% fetal calf serum at 37°C for 24 h in a 5% CO_2 humidified atmosphere. Then the cells were incubated for an additional 72 h with CDDP in the absence or presence of 10 μM ethacrynic acid. Viable cell numbers were measured by use of the alamar blue assay.[28] Ten μl of alamar blue solution (Iwaki Glass, Tokyo) was added to each well and the plates were incubated at 37°C for 3 h. Fluorescence was measured with excitation at 544 nm and emission at 590 nm using a fluorometer (Fluoroskan II, Labsystems Japan, Tokyo). The reaction proceeded linearly within the range of 3×10–4×10^3, corresponding to 3×10^2–4×10^4 viable cells.

Assay of GST activity Colon 26 tumors minced into small fragments were homogenized with 9 vol. of 10 mM Tris-HCl, pH 7.8, containing 0.2 M NaCl, and centrifuged at 105,000g for 45 min. Samples of the resulting supernatants were used for the assay of GST activity and for immunoblotting. GST activity was assayed using CDNB as the substrate by the method of Habig *et al.*[29] One unit of GST activity is the amount of enzyme catalyzing the conjugation of 1 μmol of substrate/min at 25°C. Protein amounts were determined by the method of Lowry *et al.*[30] Total glutathione (GSH together with GSSG) was determined according to the method of Owens and Belcher.[31]

Purification of GST forms from colon 26 tumors The supernatant fractions as described above were applied to an *S*-hexylglutathione-Sepharose column equilibrated with 10 mM Tris-HCl, pH 7.8, containing 0.2 M NaCl. After washing of the column, GST forms were eluted with 5 mM *S*-hexylglutathione in the buffer.[32]

Two-dimensional gel electrophoresis Two-dimensional gel electrophoresis was performed according to the method of O'Farrell[33] with slight modifications.[34]

Quantitation of individual GST forms The activity of individual GST forms (GST-I, II and III)[35] was determined by activity inhibition tests with the respective antibodies, as reported previously.[36, 37] Protein amounts of GST forms were calculated as follows. The inhibited activities were divided by the known specific activity of GST-II (0.10 unit/μg protein) or GST-III (0.13 unit/μg protein).[35] GST-II was purified from male mouse liver as reported previously.[35]

Immunoblot analysis Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed with 12.5% polyacrylamide gels by the method of Laemmli[38] and immunoblotting was carried out using anti-GST-II antibody by the method of Towbin *et al.*[39] The anti-GST-II and anti-GST-III antibodies were raised in rabbits as described previously.[35]

Quantitation of IL-6 IL-6 content in colon 26 tumors was determined using a murine IL-6 ELISA kit (Endogen, Boston, MA) according to the manufacturer's instructions.

Determination of platinum content Tissue platinum content of colon 26 tumors was determined using a flameless atomic absorption spectrophotometer (AA-8500 MKII, Nippon Jarrell-Ash Co., Tokyo) according to the method of Siddik *et al.*[40]

Statistics The statistical significance of differences in data was analyzed using Student's t test. In some cases, Mann-Whitney's U test was used.

RESULTS

Effects of lentinan and CDDP on colon 26 tumor growth Female CDF_1 mice inoculated with colon 26 cells were treated with lentinan, CDDP, the two drugs in combination or PBS, as described in "Materials and Methods." Tumor weights were estimated from the length and width and compared among these groups (Fig. 1). On day 24, tumor weights in the CDDP alone (4.3 ± 0.6 g, mean$\pm$SD) and CDDP+lentinan (2.7 ± 1.3 g) groups were significantly lower than in the control group (7.2 ± 1.5 g, both $P<0.01$). Similar results were also observed on day 21. Tumor weights in the CDDP+lentinan group were also lower than in the controls on days 14 and 17. The values for CDDP+lentinan were less than for CDDP alone on days 21 and 24 (both $P<0.05$). Treatment with lentinan alone resulted in essentially the same growth curve as in the controls. These results demonstrated more significant repression of colon 26 tumor growth by the combination of CDDP and lentinan than by CDDP alone.

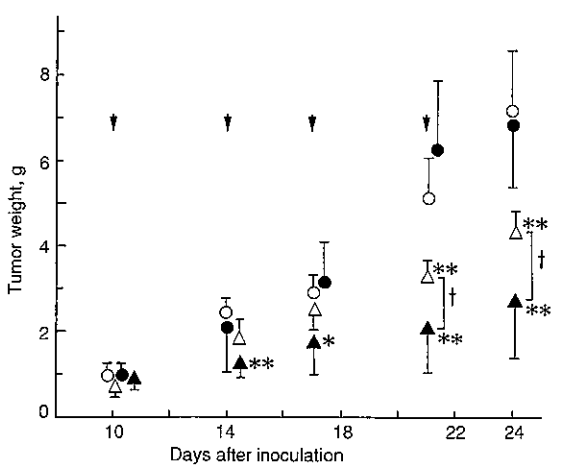

Fig. 1. Growth of colon 26 tumors in lentinan- or CDDP-treated female CDF_1 mice. Animals inoculated subcutaneously with 1×10^6 colon 26 cells were divided into 4 groups of 4 or 5 mice and given lentinan (●), CDDP (△), lentinan+CDDP (▲) or PBS as the control (○) on days 10, 14, 17 and 21 as indicated by arrows. The averages of their tumor weights$\pm$SD are presented as a function of time. Tumor weight was estimated from the length and width of each tumor as described in the text. *, $P<0.05$ versus control; **, $P<0.01$ versus control; †, $P<0.05$ CDDP versus CDDP+lentinan.

Decrease of GST in colon 26 tumors by lentinan GST forms were purified from nontreated colon 26 tumor by affinity chromatography and individual forms were resolved by two-dimensional gel electrophoresis. As shown in Fig. 2, the dominant form was identified as GST-II and a minor form as GST-III belonging to the Mu class, based on their isoelectric points and subunit molecular mass. Their identities were confirmed by immunoblot analysis using the respective antibodies (data not shown). Alpha-class forms were not detected in these experiments. Protein amounts of individual GST forms in tumor tissues obtained on day 24 were quantitated by immunological activity inhibition tests and their changes following lentinan or CDDP treatment were examined. The results for the four groups, together with glutathione, platinum and IL-6 contents, are summarized in Table I. Both GST-II and GST-III values were significantly decreased in the groups receiving lentinan alone (0.90 ± 0.29 and 0.26 ± 0.11 μg/mg protein, respectively) and lentinan+CDDP (0.98 ± 0.22 and 0.29 ± 0.07 μg/mg protein), as compared with control values (1.39 ± 0.20 and 0.52 ± 0.11 μg/mg protein, $P<0.05$ for GST-II; $P<0.02$ for GST-III). The GST-III value in the CDDP

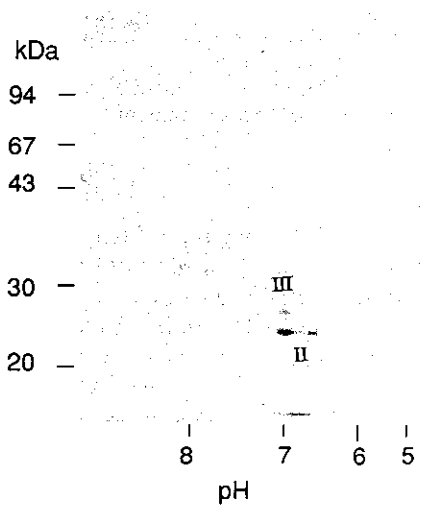

Fig. 2. Two-dimensional gel electrophoresis of GST purified from a non-treated colon 26 tumor. GST (5 μg) purified by S-hexylglutathione-Sepharose affinity chromatography was resolved by two-dimensional gel electrophoresis. As the first dimension, isoelectric focusing was performed in a polyacrylamide gel using 2% Ampholine, pH 3.5–10. SDS-polyacrylamide gel electrophoresis as the second dimension was performed in 12.5% acrylamide. II and III indicate the subunits of GST-II and GST-III, respectively. Molecular mass markers were phosphorylase (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa) and soybean trypsin inhibitor (20 kDa).

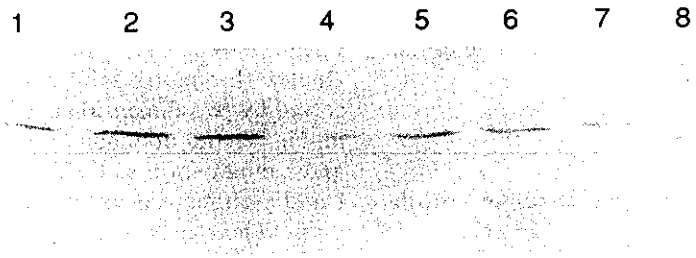

Fig. 3. Immunoblot analysis of GST-II in colon 26 tumors from mice treated with lentinan or lentinan+CDDP. Protein samples from 105,000*g* supernatants were subjected to SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose sheets. Immunoblotting was performed with anti-GST-II antibody. Aliquots of 100 μg protein were applied to lanes 2–8. Lane 1, purified GST-II (0.10 μg); lanes 2 and 3, control supernatants; lanes 4–6, lentinan group samples; lanes 7 and 8, lentinan+CDDP group.

Table I. Alterations in Glutathione Transferase (GST)-II, GST-III, Glutathione and Interleukin-6 in Colon 26 Tumors from Mice Treated with *cis*-Diamminedichloroplatinum(II) and Lentinan

Group		Content in tumor tissue				
		GST-II μg/mg protein	GST-III μg/mg protein	Glutathione nmol/mg protein	Platinum nmol/mg protein	Interleukin-6 pg/mg protein
Control	(4)[a]	1.39±0.20	0.52±0.11	20.8±3.8	n.d.[b]	82±47
Lentinan	(4)	0.90±0.29*	0.26±0.11*	14.6±4.4	n.d.	320±254
CDDP	(5)	1.01±0.29	0.28±0.10**	23.6±5.2	17.0±7.4	280±259
CDDP+Lentinan	(5)	0.98±0.22*	0.29±0.07**	17.3±4.4	18.9±5.7	350±399

Data are means±SD.
a) Number of samples examined.
b) Not determined.
*, $P<0.05$ versus control.
**, $P<0.01$ versus control.

alone group (0.28±0.10 μg/mg protein) was lower than in the controls ($P<0.01$), while GST-II was not ($P=0.06$). GST-II or GST-III values were not significantly different between the CDDP alone and lentinan+CDDP groups, indicating that the combination of the two drugs did not further decrease the levels. About half of the samples in the lentinan alone and lentinan+CDDP groups showed GST-II values less than 0.99 μg/mg protein (control mean −2SD). The decrease of GST-II in these samples was confirmed by immunoblot analysis, with weaker bands being exhibited as compared with those for the controls (Fig. 3). The total glutathione value in the lentinan alone group (14.6±4.4 nmol/mg protein) was not significantly lower than the control value (20.8±3.8 nmol/mg protein, $P=0.08$, Table I), and there was no significant difference between the lentinan+CDDP and CDDP alone groups ($P=0.07$). Tissue platinum content was not significantly different between the latter two groups, but was comparable to the glutathione value. IL-6 values in animals of the lentinan, CDDP, and lentinan+CDDP groups were distributed over a wide range (30–840 pg/mg protein), and were not significantly different from those in the controls (82±47 pg/mg protein). However, about half of the samples in these three groups exhibited elevated values (>180 pg/mg protein). Examination of the relation between GST-II and IL-6 levels in all samples (Fig. 4) revealed a weak inverse correlation (correlation coefficient, $r=-0.46$, $P<0.05$). For the lentinan and control groups, the coefficient was −0.74. No clear relation was observed between GST-III and IL-6 ($r=-0.24$).

Enhanced sensitivity of cultured colon 26 cells to CDDP induced by GST inhibitor Since addition of lentinan to cultured colon 26 cells did not alter the GST content or sensitivity to CDDP (data not shown), we examined the possible involvement of GST in modulating the sensitivity to CDDP by using a GST inhibitor, ethacrynic acid. The GST activity of cytosol fraction from colon 26 cells was dose-dependently inhibited by ethacrynic acid, the concentration giving 50% inhibition being 10 μM (data

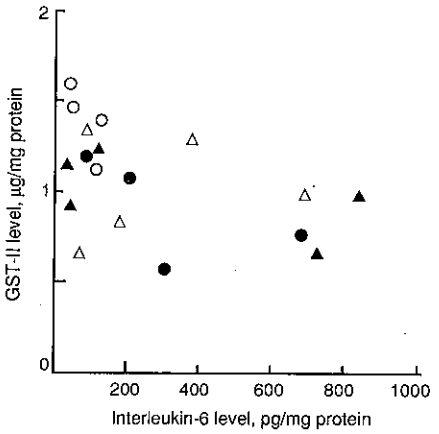

Fig. 4. Relation between GST-II and IL-6 values in individual samples of colon 26 tumors from mice treated with lentinan, CDDP, lentinan+CDDP or PBS. GST-II and IL-6 values were determined by immunological activity inhibition testing and enzyme-linked immunosorbent assay, respectively, as described in the text. Symbols are the same as in Fig. 1. The correlation coefficient for all samples is −0.46.

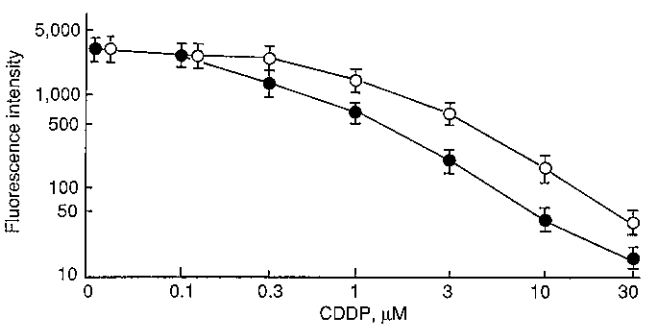

Fig. 5. Log survival curves of cultured colon 26 cells with CDDP alone and CDDP+ethacrynic acid. Colon 26 cells (1.25×10^3 per well) were cultured at 37°C for 24 h with 0.1 ml of RPMI 1640 medium containing 10% fetal calf serum. Then the cells were incubated for an additional 72 h with up to 30 μM CDDP in the absence (○) or presence of 10 μM ethacrynic acid (●). Viable cell numbers were determined and represented as fluorescence intensity using the alamar blue assay. Fluorescence was measured with excitation at 544 nm and emission at 590 nm. The results presented are means and SD calculated from triplicate determinations in three separate experiments.

not shown). An alamar blue cytotoxicity assay was used to determine the sensitivity of colon 26 cells to CDDP alone and CDDP+ethacrynic acid. The addition of 10 μM ethacrynic acid caused the CDDP concentration giving 90% reduction of surviving cells to be decreased to about a half of the value for CDDP alone (1.8 vs. 4.2 μM) (Fig. 5). Ethacrynic acid alone did not show cytotoxicity at this concentration. These results suggested the possible involvement of GST in modulating the sensitivity of colon 26 cells to CDDP. Since ethacrynic acid alone exerted cytotoxic effects at 20 μM and more, the effects of higher concentrations on CDDP cytotoxicity could not be evaluated.

DISCUSSION

The present study revealed that lentinan administration to CDF_1 mice results in significant decreases of both GST-II and GST-III values in colon 26 tumor (Table I). About half of the samples from animals treated with lentinan showed enhanced IL-6 values, which exhibited a low but significant negative correlation with GST-II levels. Since the GST-II gene possesses sequences similar to the silencer of rat GST-P gene that responds to IL-6 DBP, the low correlation may reflect a possible indirect relationship between the expressions of GST-II and IL-6. GST-III was also decreased in most samples with lower GST-II values, with a close relation being observed between the two ($r=0.83$). Lentinan enhanced the growth retardation of colon 26 tumors by CDDP (Fig. 1). This raised the possibility that decreased GST values may sensitize the tumors to CDDP. A GST inhibitor, ethacrynic acid, enhanced the sensitivity of cultured colon 26 cells to CDDP (Fig. 5), suggesting the possible involvement of GST in the modulation of CDDP cytotoxicity to this cell line. However, since tissue GST-II or GST-III contents were not different between the groups given CDDP alone and CDDP+lentinan (Table I), it remains possible that the enhanced therapeutic effect of lentinan *in vivo* may be due to factors other than GST content.

Recently Mizutani *et al.*[41] reported sensitization of human renal carcinoma cells to CDDP and repression of GST by anti-IL-6 antibody. Our finding of possibly enhanced IL-6 levels in lentinan-treated colon 26 tumors with decreased GST content are thus in contrast to their results. IL-6 DBP or CCAAT-enhancer binding protein β is known to function as either an activator or repressor of transcription, depending on post-translational modification.[42] In the present study, IL-6 levels were increased in about half of the samples with CDDP as well as lentinan, and the values were distributed over a wide range (Table I), indicating marked inter-individual variation in response to these drugs. Such variations, as well as strain differences, have been reported in the induction of acute-phase proteins in mouse liver by lentinan.[26] IL-6 values did not exhibit a relationship with tumor weight ($r=-0.18$), and thus, IL-6 does not seem to be a direct determinant of the sensitivity of colon 26 tumors to CDDP. Since tissue platinum and glutathione content were also similar between the two groups (Table I), they

were unlikely to be involved in the enhanced sensitivity to CDDP.

Tissue platinum values did not show any link with GST-II ($r=-0.15$), GST-III ($r=-0.08$) or glutathione ($r=-0.03$) values. Ishikawa and Ali-Osman have reported that the conjugation of CDDP with glutathione occurs nonenzymatically *in vitro* and the conjugates are pumped out by a glutathione-conjugate-dependent ATPase,[3] which is overexpressed in a CDDP-resistant cell line.[43] Since platinum level was determined without distinction between CDDP and its metabolites in our study, the possibility that their ratio might be altered by lentinan administration could not be ruled out. Further study is needed. Other factors, such as DNA repair[44,45] and glutathione-conjugate pump activities,[43,46] may also deserve consideration as possible mechanisms. Because addition of lentinan to cultured colon 26 cells did not alter the sensitivity to CDDP, the influence of lentinan observed *in vivo* seemed to be mediated through host mechanisms.

Our results indicated that lentinan administration to mice bearing colon 26 tumor results in decreased tissue GST-II and GST-III values and enhanced sensitivity to CDDP. GST inhibitors such as ethacrynic acid have been used to augment the therapeutic effects of anti-cancer drugs.[11,12] Such enhancement was observed in cultured colon 26 cells (Fig. 5). However, the efficacy of such GST inhibitors is reported to be weakened by induction of GST in cancer cells following exposure to chemotherapeutic agents.[47] Lentinan did not inhibit GST activity in *in vitro* experiments. Thus, combinations of lentinan or drugs to repress GST and anti-cancer agents may deserve further consideration as an approach to enhance therapeutic effects.

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