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Enhancement of Cytotoxicity of Cisplatin *in vitro* by Recombinant Human Tumor Necrosis Factor and/or Recombinant Human Interferon-α, -β and -γ

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This study was conducted to investigate the modulatory effects of recombinant human tumor necrosis factor (rH-TNF) and recombinant human interferon (rH-IFN)-α, -β and -γ, either alone or in combination, on the cytotoxicity of cisplatin, using MTT assay, against MKN-45 (human stomach adenocarcinoma). MKN-45 was resistant to rH-TNF even at doses up to 10^3 U/ml. rH-IFN-γ inhibited the survival of MKN-45 dose-dependently, while rH-IFN-α and -β did not inhibit the survival of MKN-45 even at the highest concentrations tested (10^4 U/ml). Combination of rH-TNF with rH-IFN-α, -β or -γ did not significantly inhibit the survival of MKN-45, except for a combination of 10 U/ml of rH-TNF and 10^3 U/ml of rH-IFN-γ ($P<0.05$). Cisplatin inhibited the survival of MKN-45 dose-dependently. By the simultaneous combination of cisplatin with rH-TNF and/or rH-IFN-α, -β or -γ, cytotoxicity of cisplatin was enhanced and the combination effects were additive. The effects of rH-TNF and rH-IFN-α, -β and -γ on the modification of cytotoxicity of cisplatin were evaluated in terms of modification index (MI), demonstrating that rH-TNF, rH-IFN-α, -β and -γ all augmented the cytotoxicity of cisplatin: MI values at 10^3 U/ml of rH-IFN-α, -β and -γ were 1.4, 1.4 and 2.3, respectively; those at the same concentrations of rH-IFN-α, -β and -γ in the presence of 10 U/ml of rH-TNF were 3.6, 2.5 and 5.1, respectively. These results demonstrating that the cytotoxicity of cisplatin was enhanced by rH-TNF and/or rH-IFN-α, -β or -γ suggest that cancer may be more effectively treated with the combination of cisplatin with these biological response modifiers than with cisplatin alone.

Key words: Cytotoxicity — Cisplatin — rH-TNF — rH-IFNs — Human stomach adenocarcinoma cell line

Innate low sensitivity of cancer cells to chemotherapeutic agents is one of the major factors in chemotherapeutic failure in many solid tumors. To improve the response and survival in patients with cancer, it is of great importance to develop methods to increase the innate sensitivity of cancer cells to chemotherapeutic agents. Cisplatin (CDDP) is now an important chemotherapeutic agent widely used in the treatment of a variety of human tumors. Since CDDP entered clinical use, it has remarkably improved the response rate and survival in certain types of tumor, such as testicular and ovarian cancer.[1] However, in many solid tumors including stomach cancer, clinical usefulness of CDDP has frequently been limited by the innate resistance of cancer cells to CDDP.[2]

In recent years, much attention has been paid to the use of biological response modifiers (BRMs) in the treatment of cancer, because BRMs have been known to modify the host response to cancer cells directly and/or indirectly, resulting in therapeutic benefit.[3] Previous studies have demonstrated that BRMs can also enhance the sensitivity of cancer cells to chemotherapeutic agents, and tumor necrosis factor (TNF) and interferons (IFNs) are two of the most promising BRMs for this purpose.[4–10] Both TNF and IFNs have been reported to have a potent cytotoxicity against some kinds of cancer cells, the cytotoxicity being enhanced by the combined use of them.[11–15]

In this study, primary attention was given to the direct effects of recombinant human (rH)-TNF and/or rH-IFN-α, -β and -γ on the modification of cytotoxicity of CDDP. In the present experiments, we have demonstrated that the cytotoxicity of CDDP against a human stomach cancer cell line (MKN-45) was enhanced by the use of rH-TNF and rH-IFN-α, -β and -γ, being more enhanced by the combined use of rH-TNF with rH-IFN-α, -β or -γ.

MATERIALS AND METHODS

Cells and medium MKN-45 cells, human stomach adenocarcinoma, were kindly supplied by Dr. N. Saijo, National Cancer Center Hospital, Tokyo. The tumor cells were grown as a monolayer culture in RPMI-1640

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medium (Gibco, Grand Island, NY) supplemented with heat-inactivated 10% fetal bovine serum (Gibco), penicillin (100 U/ml) and streptomycin (100 μg/ml)(RPMI-FBS) at 37°C in a humidified 5% CO_2 atmosphere.

Drugs CDDP was obtained from Dong-A Pharm. Co., Ltd., Korea. rH-TNF, rH-IFN-α, -β and -γ, kindly gifts from Dr. N. Saijo, were produced by Asahi Chemical Industry Co., Ltd., Shizuoka, Nippon Roche K.K., Tokyo, Kyowa Hakko Co., Tokyo and Toray Co., Tokyo, respectively. rH-TNF, rH-IFN-α, -β and -γ had specific activities of 1×10^5, 3×10^6, 3×10^6 and 1×10^6 U protein/mg, respectively. The stock solutions of these drugs were made with distilled water and stored at −70°C. Just before experiments, stock solutions were dissolved in RPMI-1640 medium to the required concentrations.

Sensitivity test The assay system used in this study was essentially the one devised by Mosmann[16] and described in detail previously.[17] In brief, a single cell suspension was obtained by treatment with trypsin and EDTA and by mechanical disaggregation. After viability was confirmed to be more than 95% by trypan blue dye exclusion, cells were counted using a hemocytometer, diluted with RPMI-FBS to the final concentration of 1×10^4 cells/well, plated (135 μl/well in single-agent experiments and 120 μl/well in combination experiments) in 96-well microtest plates (Becton Dickinson, Oxnard, CA) and preincubated at 37°C in a humidified atmosphere of 5% CO_2 for 4 h. Seeding cell number and incubation time were determined after confirming the linear relationship between the absorbance and number of cells plated in standard and growth curves of MKN-45. They were then treated continuously with 15 μl of various concentrations of CDDP, rH-TNF, rH-IFN-α, -β and -γ. In experiments on combination treatment, 12% FBS was used, to minimize the dilution effect of serum by drug solution. After incubation at 37°C in a humidified incubator with 5% CO_2 for 4 days, 15 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Chemical Co., St. Louis, MO) dissolved in phosphate-buffered saline (5 mg/ml) was added to each well and the plates were further incubated for 4 h. To solubilize the intracellular crystals formed, 150 μl of acid isopropanol was added to each well and the contents of each well were subjected to repeated pipetting, about 20 times, resulting in good solubilization. The absorbance was measured at 570 nm using a spectrophotometer (Minireader II, Dynatech Lab, USA). Each experiment was performed in triplicate and repeated three times. The percent survival was determined by the formula: [(mean absorbance

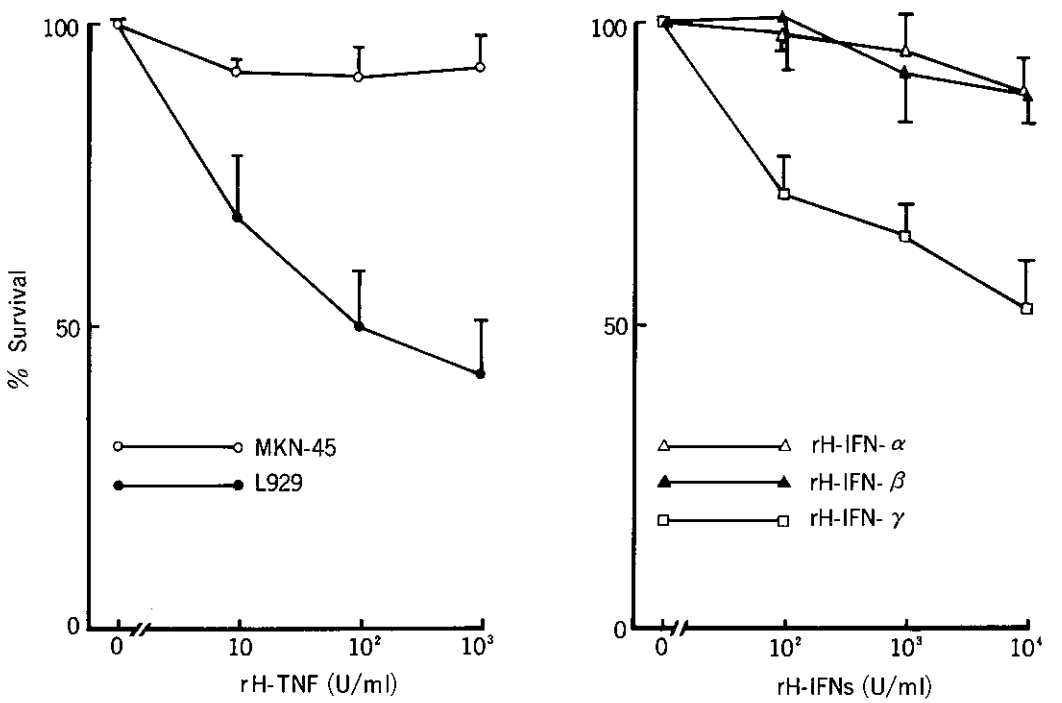

Fig. 1. Effects of rH-TNF and rH-IFN-α, -β and -γ on the survival of MKN-45 (human stomach adenocarcinoma). Additional experiments were performed with rH-TNF against L929 (murine transformed fibroblasts). Each point and bar represent the mean and SD of three experiments.

in three test wells − absorbance in background well)/(mean absorbance in three control wells − absorbance in background well)] × 100.

Statistics The significance of differences between percent survivals was determined by using Student's *t* test. The multiplicative model was used to evaluate the interaction between CDDP, rH-TNF and rH-IFNs. Briefly, if the product of percent survivals of cells treated with *a* or *b*, alone, (*Sa, Sb*) was higher than the observed percent survival of cells given the combined treatment (*Sa*+*b*), the interaction was considered to be synergistic: $Sa+b<Sa\times Sb$. If $Sa+b=Sa\times Sb$, the interaction was additive and if $Sa\times Sb<Sa+b$, the interaction was antagonistic. The range for additive interaction was taken to be the calculated value ±15%.[18]

RESULTS

Effects of rH-TNF and rH-IFN-α, -β and -γ, alone or in combination MKN-45 was evaluated for sensitivity to rH-TNF and rH-IFN-α, -β and -γ, alone or in combination, in MTT assay (Fig. 1). MKN-45 was insensitive to doses of up to 10^3 U/ml of rH-TNF. Additional experiments was performed with L929, because of its known high sensitivity to TNF, demonstrating that L929 was significantly inhibited by rH-TNF (% survival: 69, 50 and 42% at 10, 10^2 and 10^3 U/ml of rH-TNF, respec-

Table I. Combination Effects of rH-TNF with rH-IFN-α, -β or -γ on IC_{50} to Cisplatin

	Concentrations of rH-IFNs (U/ml)		
	0	10^2	10^3
rH-IFN-α			
TNF (−)[a]	0.94±0.19[b]	0.79±0.17	0.69±0.14
TNF (+)[c]	0.43±0.13[d]	0.44±0.21[d]	0.26±0.03[d]
rH-IFN-β			
TNF (−)	1.05±0.13	0.86±0.21	0.75±0.08[d]
TNF (+)	0.62±0.29	0.52±0.22[d]	0.42±0.17[e]
rH-IFN-γ			
TNF (−)	1.02±0.14	0.70±0.15	0.45±0.03[e]
TNF (+)	0.69±0.08[d]	0.49±0.18[d]	0.20±0.05[e]

a) Absence of rH-TNF.
b) Mean of IC_{50}s (μg/ml) ± SD of three experiments.
c) Presence of 10 U/ml of rH-TNF.
d) Significantly different from the controls: $P<0.05$.
e) $P<0.01$.

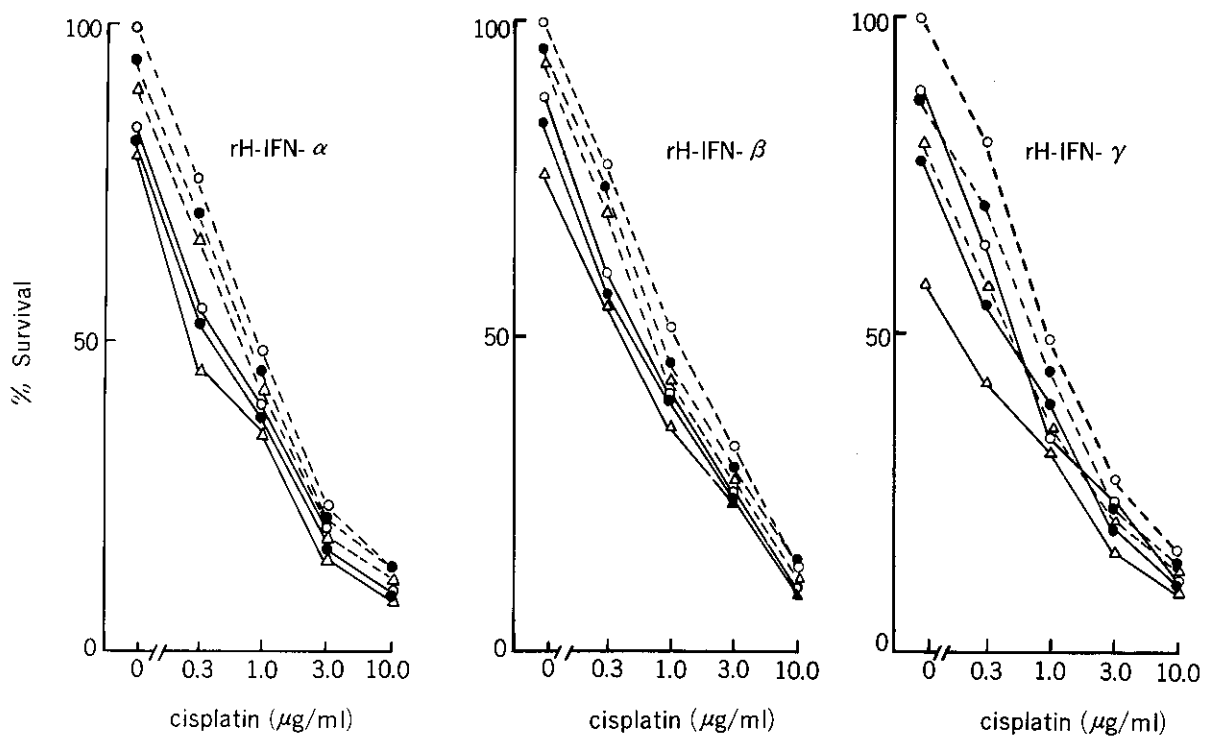

Fig. 2. Enhancement of cytotoxicity of cisplatin by rH-TNF and/or rH-IFN-α, -β or -γ in MKN-45 (human stomach adenocarcinoma). The dotted and solid lines represent the absence and presence of 10 U/ml of rH-TNF, respectively: (○), absence of rH-IFNs; (●), 10^2 U/ml of rH-IFNs; (△), 10^3 U/ml of rH-IFNs. Each point represents the mean of three experiments.

tively). rH-IFN-γ inhibited the survival of MKN-45, dose-dependently ($P<0.05$), while rH-IFN-α and -β did not significantly inhibit the survival of MKN-45.

The cytotoxicity of simultaneous combinations of rH-TNF and rH-IFN-α, -β or -γ was evaluated (data not shown). In combination experiments, the concentration of rH-TNF was 10 U/ml and those of rH-IFN-α, -β and -γ were 10^2 and 10^3 U/ml. The percent survival was not significantly inhibited by any combination treatment of rH-TNF with rH-IFN-α, -β or -γ, except for a combination of rH-TNF with 10^3 U/ml of rH-IFN-γ (% survival: 58%).

Effect of rH-TNF and rH-IFN-α, -β and -γ on cytotoxicity of cisplatin Enhancement of cytotoxicity of CDDP by rH-TNF and rH-IFN-α, -β and -γ, either alone or in combination, was evaluated. Combination of CDDP with 10 U/ml of rH-TNF and/or 10^2 and 10^3 U/ml of rH-IFN-α, -β or -γ showed a tendency to enhance the cytotoxicity of CDDP (Fig. 2). Combination effects of CDDP with rH-TNF and/or rH-IFN-α, -β or -γ were additive in all combinations tested. Enhancing effects of combined use of rH-TNF and rH-IFN-α, -β or -γ on the cytotoxicity of CDDP were evaluated using IC_{50}, demonstrating that 10 U/ml of rH-TNF together with rH-IFN-α, -β and -γ decreased IC_{50} (Table I). The enhancing effects of rH-TNF and rH-IFN-α, -β and -γ on the cytotoxicity of CDDP were evaluated in terms of MI: MI values at 10^3 U/ml of rH-IFN-α, -β and -γ were 1.4, 1.4 and 2.3, respectively; MI at the same concentrations of rH-IFN-α, -β and -γ in the presence of rH-TNF were 3.6, 2.5 and 5.1, respectively (Fig. 3). These results demonstrated that cytotoxicity of CDDP was enhanced 1.8 times by 10 U/ml of rH-TNF and further enhanced by the combined use of rH-TNF and rH-IFN-α, -β or -γ.

DISCUSSION

Despite marked improvement of chemotherapy in recent years, the response rate and prolongation of survival remain low in many solid tumors including stomach cancer, mainly because of the innate resistance of tumor cells to chemotherapeutic agents. For the improvement

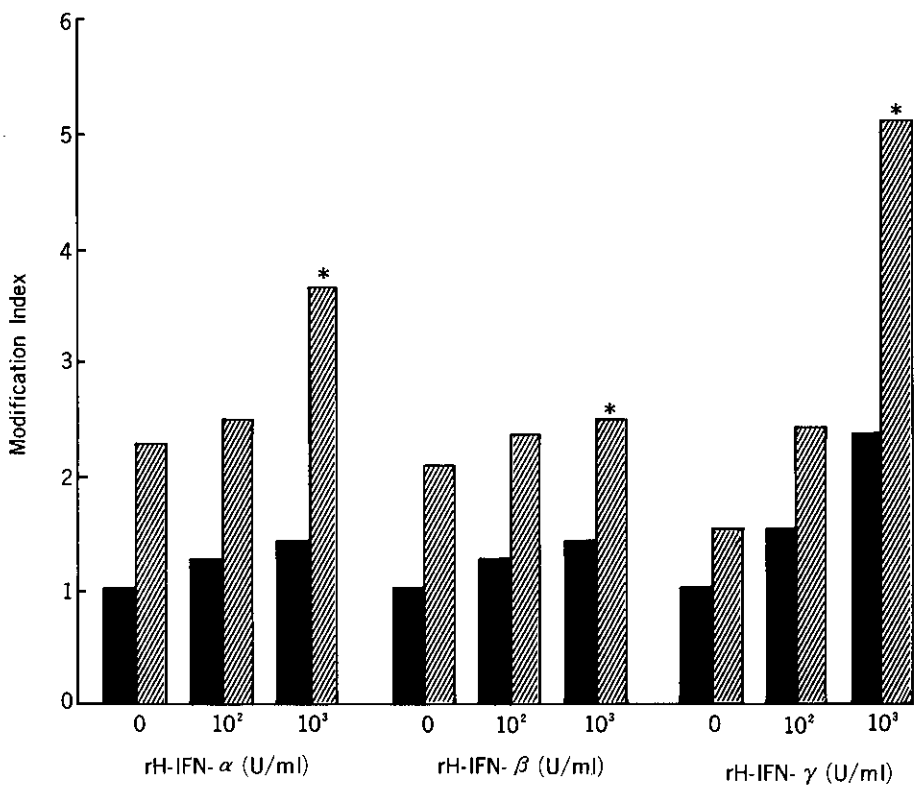

Fig. 3. Enhancement of cytotoxicity of cisplatin against MKN-45 (human stomach adenocarcinoma) by rH-TNF and/or rH-IFN-α, -β or -γ. Modification index was determined as the ratio of IC_{50} to cisplatin alone to IC_{50} to cisplatin in the presence of rH-TNF and/or rH-IFN-α, -β or -γ. ■, absence of rH-TNF; ▨, presence of 10 U/ml rH-TNF. *, significantly different from the controls: $P<0.05$.

of chemotherapeutic results, therefore, it is necessary to develop new, potent drugs to overcome innate resistance or to find methods to enhance the cytotoxicity of chemotherapeutic drugs.

CDDP is now generally accepted as one of the most active drugs for many solid tumors.[1] In patients with stomach cancer, responses have frequently been obtained by the administration of CDDP or CDDP-containing regimens, although the durations of response have usually been short.[2] In an attempt to investigate acquired resistance to CDDP, we have developed sublines resistant to CDDP from five human lung cancer cell lines and demonstrated that some biological characteristics were different from pleiotropic type resistance.[17] We have also reported that rH-TNF and rH-IFN-γ overcame the acquired resistance to CDDP in two human lung cancer sublines resistant to CDDP among four sublines tested and the combination effects were synergistic in one subline and additive in the other subline.[19] If acquired resistance develops as a result of selection for subclones innately resistant to CDDP and acquired resistance shares some of the mechanisms of innate resistance, rH-TNF and rH-IFN-γ may overcome innate resistance to CDDP. This study has been performed to investigate the modifying effects of rH-TNF and rH-IFNs on the innate resistance to CDDP.

TNF and IFNs are immune mediators which have potent antitumor activity against some kinds of cancer cells. TNF is a glycoprotein produced by macrophages which are primed by Bacillus Calmette Guérin (BCG) or other stimulating agents and triggered by endotoxin.[3, 20] IFNs are a group of glycoproteins which can be divided into three types, IFN-α, IFN-β and IFN-γ, on the basis of their antigenic, biologic and chemical properties. All three types of IFNs are known to have antiviral, immunomodulatory and antitumor activities.[3] The antitumor activity of TNF is primarily due to direct cell killing by entry of TNF into lysosomes after binding to TNF receptor.[21] The antitumor effect of IFNs is considered to be mainly due to indirect cytotoxicity by the activation of the immune system, while some direct cytotoxicity has also been reported in certain cancer cell lines.[3, 17, 19]

In this study, the direct cytotoxic activity of rH-TNF and/or rH-IFNs and the direct effect of rH-TNF and/or rH-IFNs on the modification of cytotoxicity of CDDP were investigated. As shown in Fig. 1, the inhibition of survival of L929 by rH-TNF was very low in this study, 42% even at the highest concentration tested (10^3 U/ml), because one unit of TNF activity was determined, in general, by the amount killing 50% of the L929 cells in the absence or presence of actinomycin-D (1 μg/ml) in the standard *in vitro* test.[20, 22] The mechanism of this phenomenon is unclear at present. In agreement with previous reports, rH-TNF did not suppress MKN-45.[23] On the other hand, rH-IFN-γ showed significant cytotoxicity although rH-IFN-α and -β did not show any cytotoxicity against MKN-45. MKN-45 was inhibited by CDDP dose-dependently and was more strongly inhibited when treated with CDDP combined with rH-TNF and/or rH-IFNs. It is an interesting finding that the cytotoxicity of CDDP, determined by MI, was enhanced approximately two times by rH-TNF regardless of the absence or the presence of IFN-α, -β or -γ. In these experiments, a relatively low concentration of rH-TNF (10 U/ml) was used, because one of the major limiting factors in clinical application of rH-TNF is known to be severe side effects, and 10 U/ml of rH-TNF is a clinically acceptable serum level, one-tenth of the peak plasma concentration in patients administered the maximum tolerated dose of rH-TNF.[23]

The mechanism by which cytotoxicity of CDDP is enhanced by rH-TNF and rH-IFNs is quite unclear at this time. IFNs have been demonstrated to enhance the cytotoxicity of chemotherapeutic agents, when IFNs were used in combination with various agents, such as actinomycin-D, mitomycin-C, adriamycin and CDDP, *in vitro* or in murine models.[4–8] The enhancement of *in vitro* cytotoxicity of chemotherapeutic agents by TNF was also reported.[9, 10] However, the combination effect of TNF and IFNs on the modification of cytotoxicity of CDDP has not been reported previously.

This study showed that the cytotoxicity of CDDP was greatly increased by the simultaneous use of rH-TNF and rH-IFN-γ (MI=5.1). These results were obtained using an *in vitro* test. The results obtained from *in vitro* test do not always indicate *in vivo* response, because *in vitro* culture conditions are different from *in vivo* conditions. However, an *in vitro* test has an advantage over an *in vivo* test, giving more precise results in general and making the analysis of the results easier. Therefore, we think that this preclinical study provides a basis for *in vivo* experiments with rH-TNF and rH-IFNs as modifiers of innate resistance to CDDP. From these results, it is also suggested that cancer may be more effectively treated by the combination of chemotherapeutic agents with BRMs.

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