The Cytotoxic Effects of Gemtuzumab Ozogamicin (Mylotarg) in Combination with Conventional Antileukemic Agents by Isobologram Analysis *In Vitro*

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Abstract. *Background: The CD33 antigen is expressed on leukemia cells in most patients with acute myeloid leukemia (AML) and acute promyelocytic leukemia (APL), and in 20% of patients with acute lymphoblastic leukemia (ALL), while it is absent from pluripotent hematopoietic stem cells and nonhematopoietic cells. Gemtuzumab ozogamicin (GO) is an immunoconjugate of an anti-CD33 antibody linked to calicheamicin, which is a potent cytotoxic agent that causes double-strand DNA breaks, resulting in cell death. GO was developed against CD33 antigen-positive leukemias. The aim of this study was to investigate the cytotoxic effects of this agent in combination with conventional antileukemic agents. Materials and Methods: The cytotoxic effects of GO in combination with antileukemic agents were studied against human CD33 antigen-positive leukemia HL-60, U937, TCC-S and NALM20 cells. The leukemia cells were exposed simultaneously to GO and to the other agents for 4 days. Cell growth inhibition was determined using a MTT reduction assay. The isobologram method was used to evaluate the cytotoxic interaction. Results: GO produced synergistic effects with mitoxantrone, additive effects with cytarabine, daunorubicin, idarubicin, doxorubicin, etoposide and 6-mercaptopurine, and antagonistic effects with methotrexate and vincristine. Conclusion: Our findings suggest that the simultaneous administration of GO with most agents studied would be advantageous for antileukemic activity. The simultaneous administration of GO with methotrexate or vincristine would have little cytotoxic effect, and this combination may be inappropriate. These findings may be useful in clinical trials of combination chemotherapy including GO or other monoclonal antibodies linked to calicheamicin.*

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Gemtuzumab ozogamicin (GO) is a humanized anti-CD33 antibody conjugated with the cytotoxic antibiotic calicheamicin (1, 2), which is a potent chemotherapeutic agent with a low therapeutic index that requires targeting to tumor cells for clinical use. On binding to target cells, the antibody-antigen complex is internalized into the cells, and hydrolytic release of the toxic calicheamicin moiety occurs, which subsequently causes DNA double-strand breaks that lead to apoptosis (1, 3, 4).

Acute myeloid leukemia (AML) is a major target of GO, since the CD33 antigen is expressed on blast cells in most patients with AML, while it is absent from pluripotent hematopoietic stem cells and nonhematopoietic cells (5-8). In spite of positive expectations, GO only has a moderate antileukemic activity. It produces a complete response (CR) rate of 10-16% of cases, with another 7-15% achieving CR with inadequate platelet recovery in relapsed CD33-positive AML (9-16). The median survival of patients treated with GO alone is less than 6 months. GO in monotherapy at 9 mg/m^2 is complicated by hepatic veno-occlusive disease in 5-10% of patients. Acute promyelocytic leukemia (APL) cells express large amounts of CD33 and GO is also effective as a single agent with relapsed APL, including those cases with very advanced disease (17).

Around 20% of acute lymphoblastic leukemia (ALL) is also observed to express CD33 and is considered as a target of GO (5-8). Preclinical studies have shown that CD33-positive ALL cells are much more sensitive to GO than are AML cells (18). In clinical studies, several cases of relapsed CD33-positive ALL were reported to achieve complete remission following GO administration (19-20).

Combination of lower doses of GO with other agents is the next strategy for improving the response and avoiding toxicity, and clinical studies are in progress for fresh and relapsed AML, APL and CD33-positive ALL cases as remission induction and consolidation therapies with other agents with a variety of schedules (15, 16, 21-26). However, to our knowledge, there are no experimental data available about the cytotoxic effects of GO in combination with conventional antileukemic agents. In the present study, we investigated the *in vitro* effects of GO in combination with antileukemia agents against CD33-positive human leukemia cell lines.

Materials and Methods

Cell lines. Experiments were conducted with CD33-positive human acute myeloid leukemias, U937 and HL-60, and Philadelphia chromosome-positive myeloid leukemia TCC-S, and acute lymphoblastic leukemia NALM20 cells. HL-60 and U937 were obtained from Health Science Research Resources Bank (Osaka, Japan). TCC-S was established in our laboratory (27). NALM20 was kindly donated by Yoshinobu, Matsuo, Hayashibara Biochemical Laboratories Inc., Fujisaki Cell Centre (Okayama, Japan). Cells were maintained in 75-cm^3 plastic tissue culture flasks containing RPMI-1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Grand Island Biological Co. Grand Island, NY, USA) and antibiotics. The cell cycle times of rapidly growing U937, HL60 and TCC-S cells were around 24 h, while that of slowly growing NALM20 cells was 70-80 h.

Drugs. Anticancer agents used and their sources were: GO (Wyeth Laboratories, Philadelphia, PA, USA), cytarabine (Nihon Shinyaku Co. Ltd., Tokyo, Japan), daunorubicin (Meiji Co. Ltd., Tokyo, Japan), doxorubicin (Meiji Co. Ltd., Tokyo, Japan), idarubicin (Pfizer Japan Inc. Tokyo, Japan), etoposide (Nihon Kayaku Co. Ltd., Tokyo, Japan), 6-mercaptopurine (Takeda Co. Ltd., Tokyo, Japan), vincristine (Shionogi Co. Ltd., Tokyo, Japan), and methotrexate (Wyeth Lederle Japan Ltd., Tokyo, Japan). All drugs were dissolved in RPMI-1640. Appropriate drug concentrations were made by dilution with fresh medium immediately before each experiment.

Inhibition of cell growth by combination of GO and other agents. Two to four leukemia cell lines were used for the each study of GO in combination with other agents. Leukemia cells lines were harvested from the media and resuspended to a final density of 1×10^5 cells/ml for U937, HL-60, and TCC-S cells, and of 5×10^5 cells/ml for NALM20. Cell suspensions (100 μl) were dispensed into individual wells of 96-well tissue culture plates with lids (Falcon, Oxnard, CA, USA). Eight plates were prepared for the testing of each drug combination. Each plate had one 8-well control column containing medium alone and one 8-well control column containing cells but no drugs. Cells were incubated in a humidified atmosphere of 95% air/5% CO_2 at 37°C overnight. Drug solutions of GO and other drugs at different concentrations were then added (50 μl) to 8 wells containing cell suspensions and the plates were then incubated under the same conditions for 4 days for U937, HL-60 and TCC-S cells, and for 8 days for NALM20 cells.

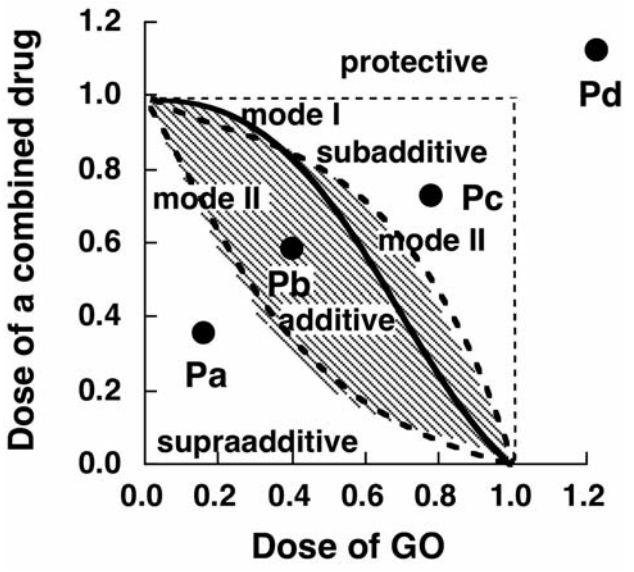

Figure 1. *Schematic representation of isobologram. Envelope of additivity (shaded area), surrounded by mode I (solid line) and mode II (dotted lines) isobologram lines, was constructed from the dose-response curves (shaded area) of GO and a combined drug. The concentrations that produced 80% cell growth inhibition were expressed as 1.0 on the ordinate and the abscissa of the isobolograms. Combined data points Pa, Pb, Pc, and Pd show supraadditive, additive, subadditive, and protective effects, respectively.*

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT assay). Viable cell growth was determined using a modified MTT assay as described previously (28).

Isobologram method of Steel and Peckham. Cytotoxic interactions of GO with other agents at the 80% inhibitory concentration (IC_{80}) level were evaluated by the isobologram method of Steel and Peckham (Figure 1) (29). The theoretical basis of the isobologram method and the procedure for making isobolograms have been described in detail previously (30, 31).

Based upon the dose-response curves of GO and the other agents, three isoeffect curves were constructed (Figure 1). If the agents were acting additively by independent mechanisms, the combined data points would lie near the mode I line (hetero-addition). If the agents were acting additively by similar mechanisms, the combined data points would lie near the mode II lines (iso-addition).

Since it is unknown in advance whether the combined effects of two agents will be hetero-additive, iso-additive or an effect intermediate between these extremes, all possibilities should be considered. Thus, when the data points of the drug combination fell within the area surrounded by three lines (envelope of additivity), the combination was regarded as additive. When the data points fell to the left of the envelope, *i.e.* the combined effect was caused by lower doses of the two agents than was predicted, we regarded the drug combination as having a supraadditive effect (synergism). When the points fell to the right of the envelope, *i.e.* the combined effect was caused by higher doses of the two agents than was predicted, but within the square or on the line of the square, we regarded the combination as having a subadditive effect, *i.e.* the combination was

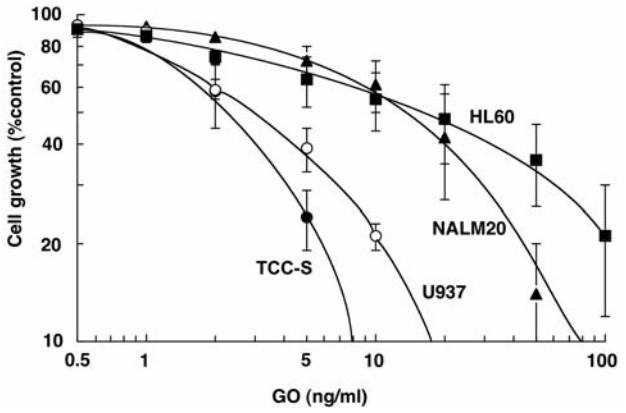

Figure 2. *The dose-response curves of GO in U937, HL-60, TCC-S and NALM20 cells. Cell growth inhibition was measured using the MTT assay and was plotted as a percentage of the control (cells not exposed to drugs). Each point represents the mean±SEM (n>10).*

superior or equal to the single agents but was less than additive. When the data points were outside the square, the combination was regarded as having a protective effect, *i.e.* the combination was inferior in cytotoxic action to the single agents. Bothsubadditive and protective interactions were regarded as antagonism.

Data analysis. To determine whether the condition of synergism (or antagonism) truly existed, statistical analysis was performed. The Wilcoxon signed-ranks test was used for comparing the observed data with the predicted minimum (or maximum) values for additive effects, which were closest to the observed data (*i.e.* the data on the boundary (mode I or mode II lines) between the additive area and supraadditive area (or subadditive and protective areas) (32). Probability (P) values ≤0.05 were considered to be significant. Combinations with $p>0.05$ were regarded as indicating additive/synergistic (or additive/antagonistic) effects. All statistical analyses were performed using the Stat View 4.01 software program (Abacus Concepts, Berkeley, CA, USA).

Results

Figure 2 shows the dose-response curves of GO in U937, HL-60, TCC-S and NALM20 cells. The IC_{80} values of GO alone against U937, HL-60, TCC-S, and NALM20 cells were 10.9±1.1 ng/ml, 100±36 ng/ml, 5.6±1.1 ng/ml, and 41±9 ng/ml, respectively (n>10). Figure 3 shows the dose-response curves for GO in combination with cytarabine, doxorubicin, and vincristine in U937 cells. Each isobologram was generated based on such dose-response curves.

Cytotoxic effects of GO in combination with cytarabine. U937, HL-60 and TCC-S cells were used for this combination study. Figure 4A-C shows the isobolograms of the combination of GO and cytarabine in these cells. In the U937 cells, the combined data points fell within the envelope of additivity (Figure 4A). The mean value of the data (0.55) was larger than that of the predicted minimum value (0.39) and smaller than that of the predicted maximum value for an additive effect (0.74) (Table I), indicating that the simultaneous exposure to GO and cytarabine produced an additive effect. In HL-60 and TCC-S cells, most data points for the combination also fell within the envelope of additivity (Figure 4B, and C). These findings suggest that the simultaneous administration of GO and cytarabine produced additive effects.

Cytotoxic effects of GO in combination with doxorubicin, daunorubicin, idarubicin, or etoposide. Figure 5A-C shows the isobolograms of the combination of GO with doxorubicin in U937, HL-60 and TCC-S cells, respectively. In all cell lines, all combined data points fell within the envelope of additivity, indicating that the simultaneous exposure to GO and doxorubicin produced additive effects (Table I). The simultaneous exposure to GO and daunorubicin, idarubicin, and etoposide showed quite similar effects (isobolograms not shown) in the cell lines studied (Table I)

Cytotoxic interaction between GO and mitoxantrone. U937 and HL60 cells were used for this study and showed similar effects. Most data points for the combination fell in the area of supraadditivity (isobolograms not shown). The mean values of the data were slightly smaller than those of the predicted minimum values for an additive effect (Table I). Statistical analysis showed that the difference was significant, indicating that the simultaneous exposure to GO and mitoxantrone produced marginally synergistic effects.

Cytotoxic effects of GO in combination with 6-mercaptopurine. U937, HL60 and TCC-S cells were used for this study. U937 and TCC-S cells were resistant to 6-mercaptopurine and the cytotoxic effects of this combination were evaluated at the IC_{50} level. In all three cell lines studied, most combined data points fell within the envelope of additivity, indicating that the simultaneous exposure to GO and 6-mercaptopurine produced additive effects (Table I).

Cytotoxic interaction between GO and methotrexate. In all four cell lines studied, most data points for the combination fell in the areas of sub-additivity and protection (isobolograms not shown). The mean values of the observed data were larger than those of the predicted maximum additive values (Table I). The difference was statistically significant, indicating antagonistic effects of the simultaneous exposure to these two agents.

Cytotoxic interaction between GO and vincristine. All four cell lines were used for this study. Figure 6A-C shows the isobolograms of this combination of this combination in U937, HL-60, and TCC-S cells, respectively. In U937, TCC-

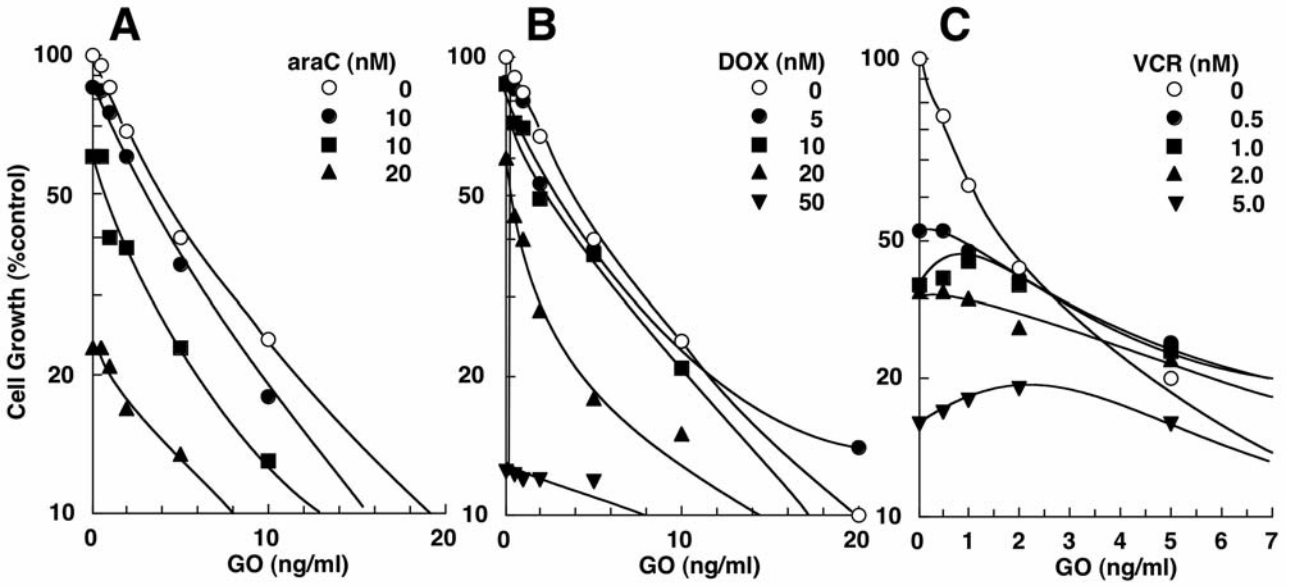

Figure 3. *Dose-response curves for GO in combination with cytarabine (ara-C) (A), doxorubicin (DOX) (B) and vincristine (VCR) (C) in U937 cells. Cell growth was measured using the MTT assay after 4 days and was plotted as a percentage of the control (cells not exposed to drugs). Each point represents the mean value for at least three independent experiments; the SEs of the means were less than 25% and are thus omitted.*

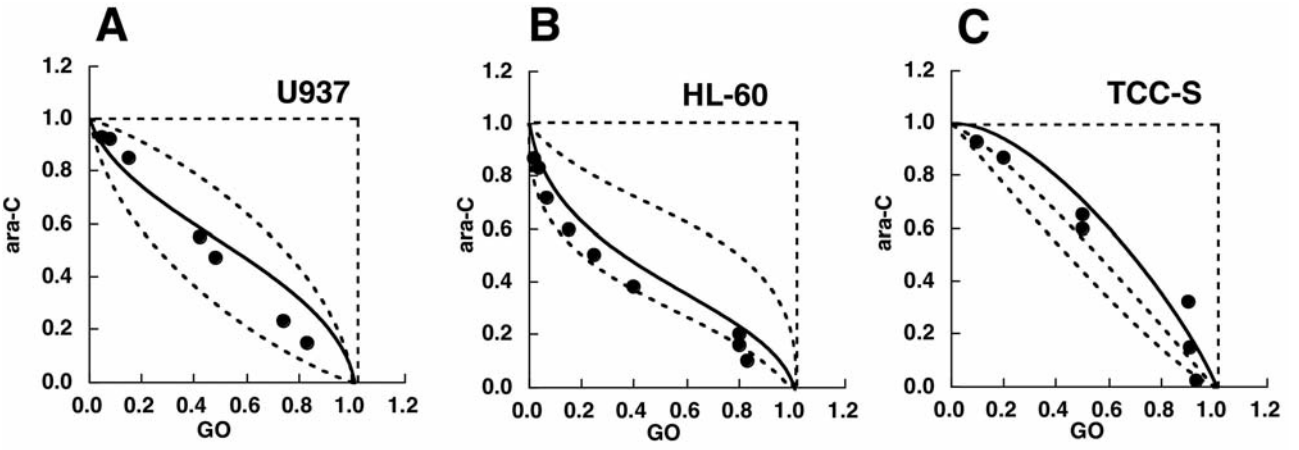

Figure 4. *Isobolograms of simultaneous exposure to GO and cytarabine (ara-C) in U937 (A), HL-60 (B) and TCC-S (C) cells. Data are presented as mean values of at least three independent experiments. In all three cell lines, all or most data points of the combinations fell within the envelope of additivity, suggesting additive interactions.*

S and NALM20 cells, the data points fell in the areas of subadditivity and protection. The mean values of the observed data were larger than those of the predicted maximum additive values. Statistical analysis showed that the difference was significant, indicating antagonistic effects (Table I). For HL60 cells, the data points fell within the envelope of additivity and in the area of subadditivity. The mean value of the observed data was slightly smaller than that of the predicted maximum additive value, indicating additive effects.

Discussion

Linking anticancer agents to an antibody that recognizes a tumor-associated antigen can improve the therapeutic index of the drug. The most promising results have been obtained with GO ozogamicin, a CD33 monoclonal antibody joined to the potent cytotoxin calicheamicin. The purpose of this study was to assess the cytotoxic effects of GO alone or in combination with commonly used antileukemic agents against CD33-positive leukemia cell lines.

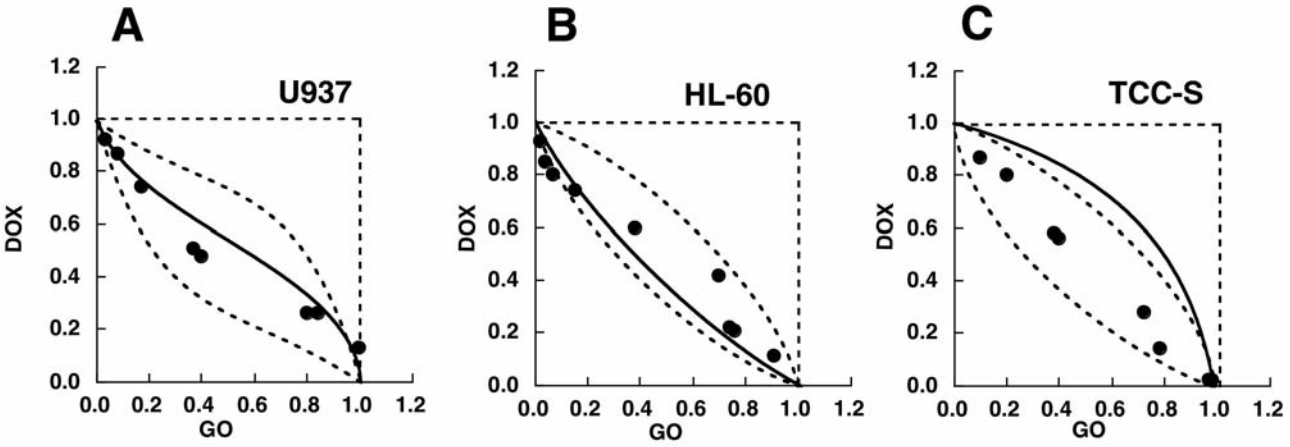

Figure 5. *Isobolograms of simultaneous exposure to GO and doxorubicin (DOX) in U937 (A), HL-60 (B) and TCC-S (C) cells. Data are presented as mean values of at least three independent experiments. In all three cell lines, all or most data points of the combinations fell within the envelope of additivity, suggesting additive interactions.*

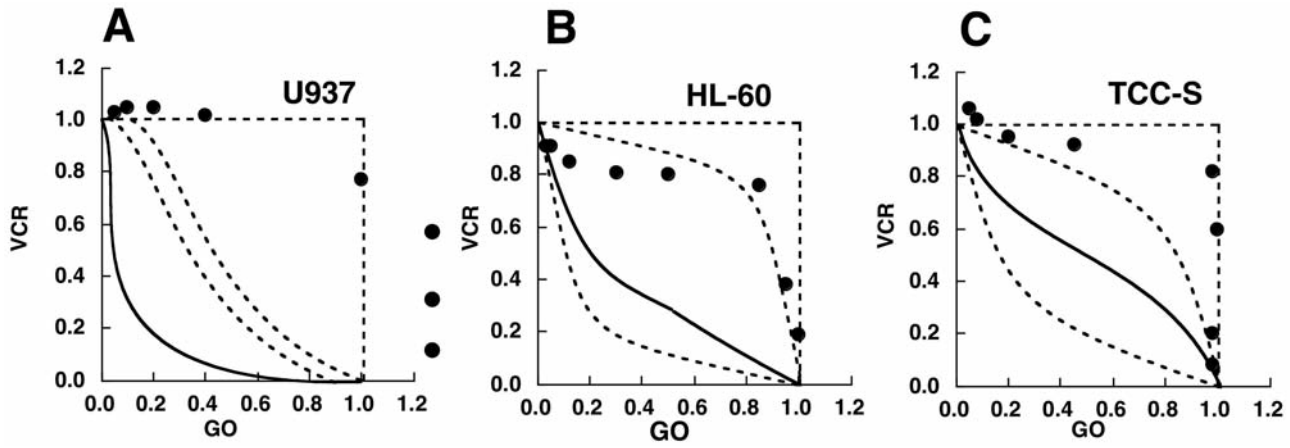

Figure 6. *Isobolograms of simultaneous exposure to GO and vincristine (VCR) in U937 (A), HL-60 (B) and TCC-S (C) cells. Data are presented as mean values of at least three independent experiments. In U937 and TCC-S lines, all or most data points fell in the areas of sub-additivity and protection, suggesting antagonistic interactions, while, in HL-60 cells, data points of the combinations fell within the envelope of additivity and in the area of subadditivity, suggesting additive interactions.*

The IC_{80} values of GO alone against U937, HL-60, TCC-S and NALM20 cells were approximately 10 ng/ml, 100 ng/ml, 5 ng/ml and 10 ng/ml, respectively. From the pharmacokinetic study, these concentrations are clinically achievable as the peak plasma concentration of GO was 2.86±1.35 mg/l and the half life of GO was 72.4±42.0 h after administration of the first 9 mg/m^2 dose of GO (33).

We studied the cytotoxic effects of GO in combination with conventionally used antileukemic agents. Cytarabine and anthracyclines such as daunorubicin and idarubicin are most widely used for remission induction or consolidation therapy of AML. At present, clinical trials of remission induction or consolidation therapy, with or without GO, are in progress. In our study, GO in combination with cytarabine and anthracyclines showed additive effects for all three cell lines studied.

The combination of cytarabine and an anthracenedione anticancer agent, mitoxantrone, is also used for the treatment of AML. Both anthracyclines and mitoxantrone inhibit topoisomerase-II and disrupt DNA synthesis and DNA repair in cancer cells. Mitoxantrone produced marginally synergistic effects with GO. These findings suggest that the simultaneous administration of GO with cytarabine or topoisomerase-II inhibitors could produce the expected (or more than expected) clinical activity. However, since the dose-limiting toxicity of GO, cytarabine, and topoisomerase-

Table I. *Mean values of observed data, predicted minimum, and predicted maximum of gemtuzumab ozogamicin in combination with other anticancer agents.*

Combined drug	Cell line	No. of data points	Observed data*	Predicted min.**	Predicted max.***	Effect
Cytarabine	U937	6	0.55	0.39	0.74	Additive
	HL60	9	0.67	0.49	0.85	Additive
	TCC-S	7	0.81	0.67	0.83	Additive
Doxorubicin	U937	8	0.68	0.50	0.84	Additive
	HL60	9	0.67	0.49	0.85	Additive
	TCC-S	8	0.74	0.58	0.91	Additive
	NALM-20	9	0.67	0.64	0.81	Additive
Daunorubicin	U937	6	0.66	0.49	0.86	Additive
	HL60	5	0.47	0.32	0.70	Additive
Idarubicin	U937	7	0.63	0.56	0.84	Additive
	HL60	6	0.51	0.37	0.81	Additive
Mitoxantrone	U937	6	0.54	0.60	0.82	Synergism ($p<0.05$)
	HL60	7	0.51	0.57	0.68	Synergism ($p<0.05$)
Etoposide	U937	7	0.53	0.51	0.63	Additive
	HL60	9	0.60	0.56	0.79	Additive
	TCC-S	7	0.53	0.49	0.75	Additive
6-Mercaptopurine	U937	7	0.66	0.60	0.69	Additive (IC_{50})
	HL60	7	0.54	0.46	0.66	Additive
	TCC-S	5	0.52	0.53	0.58	Additive (IC_{50})
Methotrexate	U937	6	>1.19	0.23	0.84	Antagonism ($p<0.01$)
	HL60	7	0.92	0.23	0.81	Antagonism ($p<0.05$)
	TCC-S	7	0.81	0.05	0.40	Antagonism ($p<0.02$)
	NALM-20	9	0.86	0.32	0.75	Antagonism ($p<0.01$)
Vincristine	U937	8	>1.09	0.26	0.66	Antagonism ($p<0.01$)
	HL60	8	0.90	0.36	0.93	Additive
	TCC-S	10	0.97	0.36	0.87	Antagonism ($p<0.01$)
	NALM-20	9	0.91	0.40	0.85	Antagonism ($p<0.05$)

*Mean value of observed data; **mean value of the predicted minimum values for an additive effect; ***mean value of predicted maximum values for an additive effect.

II inhibitors involves myelosuppression, there must be careful monitoring for myelosuppression during the combination treatment.

About 20% of ALL is observed to express CD33 and is considered as a target of GO (5-8) and encouraging data have been obtained from preclinical and clinical stuies (18-20). Recently, a CD22-targeted immunoconjugate of calicheamicin (CMC-544) has been developed for B-cell non-Hodgkin's lymphoma and ALL. CMC-544 has shown significant preclinical potential in studies in a mouse model (34-36).

We also studied the cytotoxic effects of GO in combination with methotrexate and vincristine, which are mainly used for lymphoid malignancies. GO showed definite antagonistic effects with methotrexate and vincristine in four out of four, and three out of four cell lines, respectively (Table I). The observed data values of GO in combination with methotrexate and vincristine were greater than 0.80 in all cell lines. These combinations also produced protective effects in the Philadelphia chromosome-positive leukemia cell line KU812 (data, not shown). Our findings suggest that the simultaneous administration of GO with methotrexate or vincristine may have almost no cytotoxic advantage over the administration of either agent alone, and thus may be inappropriate for the treatment of CD33-positive ALL. When CMC-544 is clinically available, the simultaneous administration of CMC-544 with methotrexate or vincristine would be also inappropriate.

There are a number of difficulties in the translation of results from *in vitro* to clinical therapy, and the pharmacokinetic profiles are significantly different between them. The toxic effects of the combination cannot be measured by *in vitro* systems, and the cell kinetics and cell biochemistry may be quite different. These differences between *in vitro* and clinical systems may influence the cytotoxic interaction of GO and other agents. In addition, we tested only simultaneous exposure to GO and other agents. Since cytotoxic effects are often schedule dependent, sequential exposure to GO followed by other agents or the reverse sequence may not show the same effects as simultaneous exposure to these agents. Continued preclinical and clinical studies would be necessary to assist in determining the optimal combination and schedule of GO in clinical use.

In conclusion, the present study suggests that the simultaneous administration of GO with most agents studied would be advantageous for antileukemic activity. The simultaneous administration of GO with methotrexate or vincristine would have little cytotoxic effect, and these combinations may be inappropriate. Our findings may be useful in clinical trials of combination chemotherapy including GO or other monoclonal antibodies linked to calicheamicin.

Disclosure

No disclosures.

Conflict of Interest

The authors declare that they have no potential conflicts of interest.

Acknowledgements

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