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Synergistic cytotoxicity of combinations of dimethyl sulfoxide and antineoplastic agents against P388 leukemia in CD-F1 mice

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We have reported that dimethyl sulfoxide (DMSO) and antineoplastic agents exhibit synergistic cytotoxicity against human tumors *in vitro*. This study was undertaken to investigate this effect *in vivo*. Groups of mice were given intraperitoneal (i.p.) injections of P388 leukemia cells. Groups were treated with i.p. injections of either saline, DMSO alone, mitoxantrone hydrochloride (DHAD) alone, methotrexate alone, DHAD in DMSO or methotrexate in DMSO. Combinations of DMSO and DHAD produced 46–61% increases above expected survival, demonstrating synergistic cytotoxicity *in vivo*. Following confirmatory animal studies, trials utilizing i.p. delivery of antineoplastics in DMSO as treatment for peritoneal tumors should be undertaken.

Key words: Chemotherapy, dimethyl sulfoxide, synergism.

Introduction

Dimethyl sulfoxide (DMSO) is a polar solvent with many interesting biological properties. Jacob, in 1964,[1] was the first to suggest potential therapeutic use of this compound and it has subsequently been administered topically and intravenously to treat musculoskeletal disorders. Despite the notoriety that DMSO has obtained from this application, the only approved clinical use for DMSO is for intravesical irrigation with 50% DMSO as a treatment for interstitial cystitis. Intravenous administration of up to 1 l of 10% DMSO has been performed safely in an investigational setting as a treatment for intractable cerebral edema with promising results.[2]

A new therapeutic application of DMSO is emerging in the field of oncology. We have previously reported that combinations of 10% DMSO and antineoplastic agents exhibit synergistic cytotoxicity against several human malignant ovarian tumors *in vitro*.[3] Subsequently, we reported that combinations of 5 and 10% DMSO and antineoplastic agents exhibit synergistic cytotoxicity against five human tumor reference cell lines *in vitro*.[4] Based on these results, we suggested that intraperitoneal (i.p.) delivery of antineoplastic agents in DMSO may be useful in the treatment of ovarian cancer and that regional delivery of antineoplastic agents in DMSO may be useful in the treatment of other human cancers.

Before clinical trials utilizing combinations of DMSO and antineoplastic agents can be undertaken, it must be demonstrated that synergistic cytotoxicity between DMSO and antineoplastic agents also occurs *in vivo*. Therefore, we tested combinations of DMSO and antineoplastic agents in a murine model against the P388 leukemia tumor. This murine tumor model was selected for two reasons. First, it is a model frequently used by the National Cancer Institute to test investigational antineoplastic agents for efficacy. Second, it is an excellent model for peritoneal malignancies because it produces peritoneal implants and massive ascites.

This experiment provided us with an excellent opportunity to investigate the usefulness of *in vitro* testing of combinations of DMSO and antineoplastic agents at predicting a clinical response. Therefore, prior to conducting the animal study, we elected to test the P388 leukemia tumor against seven antineoplastic agents, both alone and

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combined with 10% DMSO, *in vitro*. Based on the results of the *in vitro* tests, we would select two antineoplastic agents for use *in vivo*. One would be an antineoplastic predicted to be effective and to exhibit synergism with DMSO against the P388 leukemia tumor in mice. The other would be an agent predicted not to exhibit synergism with DMSO against the P388 leukemia tumor in mice. This would enable us to compare our *in vitro* predictions with our *in vivo* results.

Materials and methods

Two 7 day old mice with peritoneal implants of the P388 leukemia tumor were obtained from Battelle Laboratories (Columbus, OH). The mice were immediately sacrificed and a total of 3.0 ml of ascitic fluid was harvested from both mice under sterile conditions. This fluid was mixed with 40 ml of minimum essential medium (MEM) and 10% fetal bovine serum, both of which were obtained from KD Biological (Lenexa, KS). This solution was centrifuged at 750 *g* and the tumor cells were separated from red blood cells. The tumor cells were resuspended and washed twice in 40 ml of MEM. The resultant single cell suspension was adjusted to a density of 150 000 cells/ml using MEM and had greater than 95% cell viability.

In vitro testing

Triplicate tubes containing 1.00 ml of P388 cell suspension and 0.35 ml of MEM were incubated in 10% DMSO alone, each of seven antineoplastic agents alone or 10% DMSO plus each antineoplastic agent. The seven antineoplastic agents utilized were doxorubicin, mitoxantrone hydrochloride (DHAD), cisplatin, methotrexate, etoposide, vinblastine and 5-fluorouracil. Each tube of cell–antineoplastic, cell–DMSO and cell–DMSO–antineoplastic mixture was incubated for 1 h at 37°C in a 5% CO_2 and 95% air atmosphere. Six tubes containing cells and 0.35 ml of phosphate-buffered saline solution were incubated as negative controls.

Bacto-agar (Difco, Detroit, MI) was washed four times in demineralized water and was brought into solution. Feeder layer and plating media were prepared according to the protocol of the Southwest Oncology Group, except that the ratio used was 2:1.[5]

At the conclusion of the 1 h incubation period, the cells were washed by the addition of 5.0 ml of MEM to each incubation tube and the cells were pelleted by centrifugation at 750 *g*. The supernatants were aspirated and replaced with 0.3% agar-plating mixture. The cells were dispersed by trituration and were layered on top of the 0.5% agar feeding layer in 60 mm plastic Petri dishes. Each experiment was performed in triplicate.

Cultures were examined on day 0 and all cell clumps were identified for subtraction from the final colony count. Plates were incubated for 12 days at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. After 12 days, plates were counted at 25× magnification. Colonies with diameters of 50 μm or greater were considered viable.

The results of the *in vitro* tests are shown in Table 1. Results are expressed as percent cell kill relative to the negative control. Negative numbers represent decreased cell kill, i.e. an increase in colony counts. DHAD, which produced 100% cell kill, both alone and in the presence of 10% DMSO, was clearly the most effective agent against the P388 leukemia tumor *in vitro*. Therefore DHAD was selected for *in vivo* testing as an agent predicted to be effective and to possibly exhibit synergistic cytotoxicity with DMSO against the P388 leukemia tumor. Conversely, methotrexate alone was effective against the P388 leukemia tumor, but exhibited decreased cell kill in the presence of 10% DMSO. Accordingly, methotrexate was selected for *in vivo* testing as an agent predicted to not exhibit synergistic cytotoxicity with DMSO against the P388 leukemia tumor. We were particularly

Table 1. Results of *in vitro* testing of combinations of DMSO and antineoplastic agents against P388 leukemia cells

	Percent cell kill due to[a]							
	CONT	DOX	DHAD	CIS-P	MTX	ETO	VBL	5-FU
Alone	0	26	100	36	41	47	36	15
With 10% DMSO	17	32	100	62	−42	45	39	44

[a] CONT, control; DOX, doxorubicin; DHAD, mitoxantrone hydrochloride; CIS-P, cisplatin; MTX, methotrexate; ETO, etoposide; VBL, vinblastine; 5-FU, 5-fluorouracil. Negative numbers indicate decreased cell kill, i.e. increased colony counts relative to control.

interested in learning whether the negative synergism between methotrexate and DMSO that occurred *in vitro* would also occur *in vivo*, since that would have important negative implications for the clinical use of combinations of DMSO and antineoplastic agents.

In vivo testing

A total of 120 CD-F1 mice, aged 2 weeks, were obtained from Battelle Laboratories. The mice were randomly allocated into 12 treatment groups of 10 mice each. The mice were weighed prior to group allocation to insure even weight distribution among groups. The guidelines for the care and use of animals approved for our institutions were followed throughout the experiment.

Each mouse was given an i.p. injection of 150 000 P388 leukemia cells. After 2 days, groups of mice were treated with 10 daily i.p. injections as follows: Group 1, saline alone (control group); Group 2, 10% DMSO alone; Group 3, 15% DMSO alone; Group 4, 20% DMSO alone; Group 5, DHAD alone; Group 6, methotrexate alone; Groups 7–9, DHAD in 10, 15 and 20% DMSO, respectively; Groups 10–12, methotrexate in 10, 15 and 20% DMSO, respectively. Total antineoplastic doses in the treatment groups were 1.0 mg/kg for DHAD and 2.0 mg/kg for methotrexate.

The mean survival period, in days, after completion of treatment was calculated for all groups. The observed mean survival period for the control group, groups treated with DMSO alone and groups treated with an antineoplastic agent alone were used to calculate expected mean survival periods for groups treated with combinations of DMSO and antineoplastic agents according to the following equation:

Expected mean survival period for group treated with DMSO at concentration A and antineoplastic agent at total dose Z =

$$C \times \frac{\text{DMSO}_A}{C} \times \frac{\text{Antineoplastic}_Z}{C}$$

In this equation, C is equal to the mean survival period for the control group, DMSO_A is the mean survival period for the group treated with DMSO at concentration A and antineoplastic_Z is the mean survival period for the group treated with an antineoplastic agent at total dose Z. This equation assumes that the side effects of DMSO and the antineoplastic agent on survival are additive.

The expected mean survival period was compared with the observed mean survival period for groups treated with combinations of DMSO and an antineoplastic agent. The percent increase above the expected mean survival period for each group treated with a combination of DMSO and an antineoplastic agent was calculated using the equation:

Percent increase above expected mean survival period =

$$\frac{\text{observed mean survival period}}{\text{expected mean survival period}} \times 100$$

Results

The observed mean survival period, in days after completion of treatment, for each treatment group is shown in Table 2. The calculated expected mean survival period for groups treated with combinations of DMSO and an antineoplastic agent is

Table 2. Observed and expected mean survival periods (in days) for groups of P388 leukemia-bearing CD-F1 mice treated with combinations of DMSO and antineoplastic agents

Antineoplastic agent and total dose		Mean survival period (days) for group treated with DMSO			
		0%	10%	15%	20%
Saline (Control)		11.90	11.70	12.30	12.70
DHAD (1 mg/kg)	observed	14.00	20.13*	22.60*	24.00*
	expected	—	13.76	14.47	14.95
Methotrexate (2 mg/kg)	observed	15.50	16.25	17.20	16.90
	expected	—	15.24	16.02	16.54

* Indicated $p \leq 0.05$ for increase in observed mean survival period over expected mean survival period.

shown below the observed mean survival period for that group.

Mice treated with i.p. saline had a mean survival period of 11.90 days. Mice treated with 10% DMSO alone had a similar mean survival period, but mice treated with 15 or 20% DMSO had slightly increased mean survival periods of 12.30 and 12.70 days, respectively.

Mice treated with DHAD alone had an increased mean survival period of 14.00 days and mice treated with methotrexate alone had an increased mean survival period of 15.50 days. However, mice treated with combinations of 10, 15 or 20% DMSO and DHAD had markedly increased mean survival periods of 20.13, 22.60 and 24.00 days, respectively (Table 2). Furthermore, the observed mean survival periods for combinations of DMSO and DHAD were much greater than the mean survival periods that would be expected if their effects on survival were purely additive. The differences between observed and expected survival periods were statistically significant ($p < 0.05$, using analysis of variance) in every case.

This effect was not seen with combinations of 10, 15 or 20% DMSO and methotrexate, for which the observed mean survival periods were found to be very similar to the mean survival periods that are expected if their effects on survival were purely additive.

The percent increase above the expected mean survival period for each group treated with a combination of DMSO and an antineoplastic agent is shown in Table 3. Combinations of DMSO and DHAD produced 46–61% increases above expected mean survival periods. Combinations of DMSO and methotrexate produced only 2–7% increases above expected mean survival periods.

Discussion

We have previously demonstrated that combinations of 5–10% DMSO and antineoplastic agents exhibit synergistic cytotoxicity against human ovarian, breast, colon, kidney, endometrial and squamous cell carcinomas *in vitro*.[3,4] In one study,[4] the magnitude of the synergistic interaction was such that the dose of antineoplastic agent in DMSO could be reduced by a factor of 10 and still produce a cell kill equivalent to that produced by the antineoplastic agent in the absence of DMSO. Based on the results of those reports, we proposed that i.p. delivery of antineoplastic agents in DMSO may be useful in the treatment of human ovarian cancer and that regional delivery of antineoplastic agents in DMSO may be useful in the treatment of other human cancers.

Table 3. Percent increase above expected mean survival periods for P388 leukemia-bearing mice treated with combinations of DMSO and antineoplastic agents

Antineoplastic agent and total dose	DMSO		
	10%	15%	20%
DHAD (1 mg/kg)	46	56	61
Methotrexate (2 mg/kg)	7	6	2

Before clinical trials utilizing antineoplastic agents delivered in DMSO can be undertaken, synergistic cytotoxicity between DMSO and antineoplastic agents must be demonstrated *in vivo*. It is possible that the apparently synergistic effect which occurs *in vitro* is merely an artifact. For example, combinations of DMSO and antineoplastic agents may merely interfere with the ability of tumor cells to grow in an anchorage independent environment, as is present in the soft agar assay that was utilized in those studies. This would result in low tumor colony counts even if tumor cells remained viable after treatment. However, this effect was not seen in the DMSO control groups used in those studies and the potential for this effect was taken into account in the experimental equations used in those studies.

In this study, mice treated with i.p. DHAD or methotrexate alone had 18 and 30% increases in their mean survival periods, respectively. This indicates that these drugs alone were effective against the P388 leukemia tumor in mice. Treatment with 20% DMSO alone resulted in a 7% increase in the mean survival period. Similar mild inhibitory effects of DMSO alone on tumors have been demonstrated *in vitro*.[4] However, treatment with combinations of 10–20% DMSO and DHAD resulted in mean survival periods that were superior to those obtained with either DMSO alone or DHAD alone. Furthermore, the observed mean survival period for mice treated with combinations of DMSO and DHAD were 46–61% greater than would be expected if the effects of DMSO and DHAD on survival were purely additive. We attribute this marked increase in cytotoxicity to synergism between DMSO and DHAD, and take this to be strong evidence that synergistic cytotoxicity between DMSO and antineoplastic agents also occurs *in vivo*.

Mice treated with combinations of 10–20% DMSO and methotrexate had mean survival

periods that were very similar to those that would be expected if their effects on survival were purely additive. This makes it unlikely that synergistic cytotoxicity between DMSO and methotrexate occurred *in vivo* against the P388 leukemia tumor. We have frequently failed to demonstrate synergistic cytotoxicity against a given tumor *in vitro*, while other agents readily exhibit synergistic cytotoxicity with DMSO against the same tumor. The synergistic effect may be drug-specific for a given tumor. This underscores the importance of *in vitro* testing of various combinations of DMSO and antineoplastic agents against a patient's tumor before therapy is begun.

The results of the *in vitro* testing in this study proved to be fairly accurate predictors of *in vivo* responses. Both DHAD and methotrexate were predicted to be effective against the P388 leukemia tumor, and both were found to be effective. A specific prediction about synergistic cytotoxicity between DMSO and DHAD could not be made because DHAD produce 100% cell kill both alone and in the presence of DMSO. Despite this, we still selected DHAD for testing with and without DMSO in the experiment, just as we would in a human clinical setting, because it appeared to be the most effective agent. However, the negative synergism between DMSO and methotrexate that occurred *in vitro* did not occur *in vivo*. Such an observation would have important negative implications for the clinical use of combinations of DMSO and antineoplastic agents.

While synergistic cytotoxicity of antineoplastic agents combined with DMSO may be clinically useful, there is the potential for combinations of DMSO and antineoplastic agents to increase the toxicity to normal cell populations. There is evidence to suggest that combinations of DMSO and antineoplastic agents do not have increased toxicity to normal cell populations. Thunning *et al.*[5] did not find any difference in the mortality rates or in the incidence of chemical peritonitis in groups of rats who had i.p. injections of antineoplastic agents delivered in DMSO or in saline. Similarly, no increased mortality rates were seen in mice treated with combinations of DMSO and antineoplastic agents in the present study. Garrido *et al.*[6] have demonstrated an increased drug effect without increased normal cell toxicity using intravenous combinations of DMSO and cyclophosphamide in humans. These results support the concept that a reduced dose of an antineoplastic agent could be delivered in DMSO with the same tumoricidal effect as a full dose, but with less systemic toxicity. This could improve the quality of life of patients receiving chemotherapy. Alternatively, full dose intracavitary chemotherapy delivered in 10% DMSO may offer substantially greater tumor cell kill, and thus a greater chance of a complete remission, without a significant increase in toxicity above that which is produced by conventional intraperitoneal chemotherapy.

Conclusion

Based on the results of this study, we conclude that synergistic cytotoxicity between DMSO and antineoplastic agents does occur *in vivo*. This finding should be confirmed by additional animal studies. These studies should include monitoring of toxicity to normal cell populations. If these results are verified, then we suggest that a randomized, prospective clinical trial utilizing combinations of DMSO and antineoplastic agents as treatment for peritoneal tumors be undertaken. The benefit of increased tumoricidal effects without substantial increases in toxicity to normal cell populations may represent a significant advance in the therapy for some human tumors, particularly carcinomatosis from ovarian and colon adenocarcinomas.

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