Suicide Enzyme Inhibition as a Chemotherapeutic Strategy For Controlling Metastases Derived From Intraocular Melanomas

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The anti-metastatic effect of two chemotherapeutic agents was analyzed in a murine melanoma model. Difluoromethylornithine (DFMO), a specific irreversible inhibitor of ornithine decarboxylase, was administered as a 2% aqueous solution in the drinking water. A second drug, dacarbazine (DTIC) was administered intravenously in single bolus injections. Each drug produced significant anti-metastatic effects that were manifested by a reduction in the number of pulmonary metastases and in the prolongation of host survival times. Maximal chemotherapy was achieved when both drugs were combined. The specificity, low toxicity, ease of administration, infrequent side effects, and therapeutic effectiveness of DFMO make it an attractive candidate for clinical use in human subjects being treated for uveal melanoma. The effectiveness of DTIC against blood-borne melanoma cells suggests that this drug may prove useful as a prophylactic adjunct in patients undergoingenucleation of a melanoma-containing eye. Invest Ophthalmol Vis Sci 28:1844-1850, 1987

Although there have been several recent advances in the treatment of primary intraocular neoplasms, there is no effective therapeutic modality for controlling metastases arising from intraocular melanomas. This is especially troublesome because metastatic disease is the leading cause of death in patients with intraocular melanoma.1

Recently a new category of anti-neoplastic agents has captured the attention of oncologists. Suicide enzyme inhibitors act by impairing the enzymatic conversion of crucial compounds needed for tumor growth. In particular, the inhibition of polyamine biosynthesis has been targeted for suicide enzyme inhibition therapy.

Polyamines are polycationic compounds that have been implicated in a wide variety of biological functions, including the synthesis of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), as well as protein synthesis.2 Recent evidence indicates that polyamines play an important role in the growth and differentiation of murine melanomas.3,4 The first and rate-limiting step in polyamine synthesis is the conversion of ornithine to putrescine—a reaction catalyzed by ornithine decarboxylase.5 Impairment of this synthetic pathway by alpha-difluoromethylornithine (DFMO)—a specific irreversible inhibitor of ornithine decarboxylase (ODC)—causes a rapid depletion of intracellular polyamines in a variety of cells including melanomas.6 In vivo inhibition of polyamine biosynthesis by orally administered DFMO has been shown to retard the growth and metastasis of several transplanted animal tumors.3,4,6–8

Although there is no chemotherapeutic agent that is known to be effective in the treatment of intraocular melanoma, the purine analogue, dacarbazine (DTIC) has been used with some degree of success in the management of cutaneous melanoma.9,10 DTIC exerts its anti-neoplastic effect by inhibiting the synthesis of DNA, RNA, and protein. The drug appears to kill a broad spectrum of tumor cells in all phases of the cell cycle.11 Thus, of the chemotherapeutic agents in general clinical use, DTIC is the best candidate for use as an anti-metastatic drug.

In the present study we examine the therapeutic effectiveness of DTIC and DFMO in controlling metastases arising from intraocular melanomas or intra-venously inoculated tumor cells in mice.
Materials and Methods

Experimental Animals

Adult female C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) and used as experimental subjects when they were between 3 and 5 months of age. Athymic nude BALB/c mice were purchased from Life Sciences, Inc. (St. Petersburg, FL) and used when they were between 3 and 4 months of age. The present investigations conform to the ARVO Resolution on the Use of Animals in Research. All surgical procedures were performed using ketamine hydrochloride anesthesia. There were 5–14 animals for each experimental and control group.

Tumor Cells

B16F10 melanoma cells (C57BL/6 origin) were grown in monolayer cultures in Falcon 75 cm² tissue culture flasks (Falcon Plastics, Oxnard, CA) using Dulbecco’s modified Eagle’s minimal essential medium (MEM; GIBCO Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS; GIBCO), gentamicin (0.05 mg/ml; Schering Corp., Kenilworth, NJ), and vitamin solution (GIBCO). The origin and properties of the B16F10 melanoma subline of B16 melanoma have been described in detail previously. B16F10 melanoma cells were harvested from tissue culture flasks by gentle trypsinization, washed in Hank’s balanced salt solution (HBSS), and resuspended in HBSS for the various inoculations.

Intracameral Inoculations

A modified quantitative technique for depositing a definite number of tumor cells into the anterior chamber of the mouse eye has been described in detail elsewhere. Mice were anesthetized deeply with 0.66 mg of ketamine hydrochloride (Vetalar; Parke, Davis and Co., Detroit, MI) given intramuscularly. B16F10 melanoma cells, at a concentration of 10⁵ cells/5 μl, were injected intracameral (IC) into panels of mice. The eyes were examined three times per week with a dissecting microscope (X8).

Metastases Models

Two metastases models were used in the present study: (1) intravenously injected melanoma cells = “simulated metastases”; and (2) tumor cells disseminated from intraocular melanomas = “enucleation-induced metastases”.

Intravenous Tumor Inoculation: “Simulated Metastases”

B16F10 cells were injected intravenously at a concentration of 5 × 10⁴ cells per 0.1 ml. Two weeks later experimental mice and untreated controls were killed and their lungs removed. The number of pulmonary tumor colonies was determined by direct inspection of the lungs under a dissecting microscope. The lung colony assay is a simple and accurate method for evaluating metastases of B16F10 melanoma because these tumor cells localize on the lung surface following intravenous inoculation or after spontaneous metastasis from primary intraocular tumors. In some experiments, survival studies were performed in which treated and untreated mice received intravenous inocula containing 5 × 10⁵ B16F10 melanoma cells. In such studies, mice were observed daily and the time of death recorded for each experimental mouse. In some cases mice were sacrificed when they were clearly moribund and cachectic. The observer in these coded experiments was unaware of the identity of the individual mice.

Tumor Cells Disseminated From Intraocular Melanomas: “Enucleation-Induced Metastases”

Tumor-containing eyes were enucleated 10–12 days after IC tumor inoculation. Mice were anesthetized deeply with ketamine hydrochloride and the tumor-containing eye subjected to traumatic enucleation as previously described. Briefly, the globes were squeezed 10 times in rapid succession using wide blade forceps. The globes were subjected to the same degree of pressure without visibly rupturing the external surface of the eye. Mice were allowed to rest for 30–60 minutes and the traumatized tumor-containing eyes were enucleated with sterile curved scissors. All mice were observed three times per week and necropsied when morbidity indicated imminent death (usually day 30 post tumor inoculation).

Immunosuppressed Hosts

In some experiments the effect of an intact immune system on chemotherapy was examined by using immunoincompetent mice as hosts. Athymic nude mice: T cell-deficient, athymic, nude mice fail to mount cytotoxic T lymphocyte, delayed type hypersensitivity, and antibody responses against tumor-specific antigens. Moreover, intraocular melanomas metastasize extensively in such hosts. Gamma irradiation of mice: Mice were subjected to sublethal whole-body irradiation (500 rads), at a rate of 109 rads/minute, in a Gammacell 40 (Atomic...
**Fig. 1.** Effect of DFMO on the development of pulmonary B16F10 melanoma formation. Panels of mice received intravenous inocula containing $5 \times 10^4$ B16F10 melanoma cells on day 0 and were killed on day 14. Experimental mice were provided a 2% aqueous solution of DFMO in their drinking water throughout the entire course of the experiment. The number of lung tumors was determined by direct inspection of lungs under a dissecting microscope. There were 5-9 mice per group. * = $P < 0.01$, ** = $P = 0.02$.

Energy of Canada, Ltd., Ottawa, Canada) containing a $^{136}$Cs source. This dose of gamma irradiation has been shown to induce lymphopenia and abolish the capacity of mice to mount a primary anti-tumor response.12

**Therapeutic Agents**

**DFMO treatment:** Difluoromethylornithine (DFMO) was a gift from Merrell Dow Research Center (Cincinnati, OH) and was administered in the drinking water as a 2% aqueous solution. Unless otherwise noted, the experimental mice were provided DFMO-containing water ad libitum throughout the entire course of each experiment. The mean daily intake of the drug was approximately 3 g/kg body weight.

**Dacarbazine (DTIC) treatment:** Dacarbazine (DTIC) was purchased from Miles Laboratories, Inc. (Elkhart, IN) and injected intravenously at a dose of 25 mg/kg, 75 mg/kg, or 150 mg/kg body weight. DTIC was always given as a single bolus injection via the tail vein.

**Statistical Analysis**

The number of lung metastases in control and experimental groups was compared using student t-test and the rank-sum test. Survival studies were analyzed by the Mantel-Haenszel tests.

**Results**

The goal of the present investigation was to examine the efficacy of two chemotherapeutic agents in controlling melanoma metastases. Two metastases models were used to evaluate the effectiveness of the therapeutic protocols. In the first model, metastases were produced by the intravenous inoculation of B16F10 melanoma cells. This method has been used extensively by numerous investigators involved in metastases studies.12-14 A second model is one we have used previously and involves enucleation-induced metastasis of intraocular B16F10 melanoma.12 Therapeutic success of each anti-metastatic agent was evaluated by two criteria: (1) a reduction in the number of distinct metastatic tumor colonies in the lungs of the various experimental mice; and (2) significant prolongation of survival time in treated mice bearing metastases.

**DFMO Protects Against Blood-Borne Melanoma Cells (“Simulated Metastases”)**

The first series of experiments was designed to determine if DFMO would protect mice against blood-borne melanoma cells (ie, “simulated metastases”). Panels of C57BL/6 mice received intravenous inocula containing $5 \times 10^4$ B16F10 melanoma cells suspended in 0.1 ml of sterile HBSS. Fourteen days later mice were killed and the number of pulmonary tumors determined by direct inspection of the lungs under a dissecting microscope. The results, shown in Figure 1, indicate that DFMO had a profound protective effect when compared to untreated controls. Although DFMO treatment did not significantly reduce the number of mice harboring pulmonary tumors (ie, 75% versus 88% tumor frequency), it did result in a marked reduction (ie, 94%) in the average number of lung tumors compared to untreated controls. This possibility was examined in additional experiments in which C57BL/6 mice were rendered lymphopenic by sublethal whole-body gamma irradiation (500 rads) one day prior to intravenous tumor challenge. As before, immunocompetent mice treated with DFMO developed significantly ($P < 0.01$) fewer pulmonary tumors than their untreated counterparts (Fig. 1). Immunologically impaired lymphopenic C57BL/6 mice were also protected from intravenously injected B16F10 mela-
noma cells; however, this protective effect was significantly ($P < 0.01$) less than that observed with immunologically intact hosts. Similar experiments were performed with athymic nude BALB/c mice as a means of determining if the presence of a normal T cell repertoire influenced the therapeutic efficacy of DFMO. Once again, the anti-neoplastic effect of DFMO was demonstrated (ie, 65% reduction in the number of pulmonary melanomas). However, the data also indicate that both untreated and DFMO-treated nude mice had significantly higher numbers of pulmonary tumors compared to euthymic hosts (Fig. 1). Thus, the therapeutic effect of DFMO in controlling blood-borne melanoma cells is maximized by the presence of an intact immune system.

**DFMO Treatment Prolongs Survival Time of Mice Harboring Pulmonary Melanomas**

The marked reduction in the number of pulmonary tumors in DFMO-treated mice suggested that this treatment protocol would result in a prolongation of survival time of similar pulmonary tumor-bearing hosts. This hypothesis was tested by challenging panels of treated and untreated C57BL/6 mice and athymic nude BALB/c mice with $5 \times 10^4$ B16F10 melanoma cells administered intravenously. Although DFMO treatment did not result in a permanent cure for any of the experimental mice, significant prolongation of survival time was clearly evident in all panels of treated mice (Table 1).

**DFMO Protects Against Metastases From Intraocular Melanomas**

The results shown above indicated that DFMO was effective in controlling blood-borne melanoma cells. This is an important consideration since the metastatic spread of intraocular melanoma occurs by the blood vascular route. Accordingly, one would predict that metastases derived from intraocular melanomas would be vulnerable to the anti-neoplastic effects of DFMO. This was tested in the next series of experiments. Panels of C57BL/6 mice received intracameral inocula of B16F10 on day 0. Ten days later the hosts were subjected to sublethal whole-body gamma irradiation (500 R) and within 2 to 3 hr the melanoma-containing eyes were enucleated. A 2% aqueous solution of DFMO was provided ad libitum beginning either on day 0 or day 11. Mice were necropsied when moribund and the number of pulmonary metastases calculated. There were 14 animals in each group. Both experimental groups were significantly different from the untreated controls ($P < 0.02$).

| Table 1. DFMO treatment prolongs survival of mice bearing pulmonary melanoma metastases |
|----------------------------------|--------------------|----------------|--------------|
| Hosts*                          | Treatment†         | Number | Medial survival time (days) | $P^*$ |
| Nude mice                       | DFMO               | 5      | 35 | <0.02 |
| Nude mice                       | Control            | 5      | 25 | — |
| Normal mice                     | DFMO               | 10     | 27 | <0.01 |
| Normal mice                     | Control            | 10     | 24 | — |

* Mice received intravenous inocular containing $5 \times 10^4$ B16F10 melanoma cells on day 0.
† DFMO was provided ad libitum as a 2% aqueous solution in the drinking water.
‡ $P$ value determined by Mantel-Haenszel test comparing the survival of DFMO-treated with the survival of the appropriate control group.

As might be expected, DFMO treatment had a profound effect in reducing the number of melanoma metastases (Fig. 2). Mice treated with DFMO throughout the entire experiment (ie, days 0 through 32) demonstrated the most striking therapeutic bene-

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**Fig. 2. DFMO protects against metastases originating from intraocular melanomas.** Panels of C57BL/6 mice received intracameral inocula containing $1 \times 10^5$ B16F10 melanoma cells. Ten days later the hosts were subjected to sublethal whole-body gamma irradiation (500 R) and within 2 to 3 hr the melanoma-containing eyes were enucleated. A 2% aqueous solution of DFMO was provided ad libitum beginning either on day 0 or day 11. Mice were necropsied when moribund and the number of pulmonary metastases calculated. There were 14 animals in each group. Both experimental groups were significantly different from the untreated controls ($P < 0.02$).
Table 2. Effect of dacarbazine (DTIC) against intravenously injected B16F10 melanoma cells

<table>
<thead>
<tr>
<th>Dose of DTIC*</th>
<th>Number of pulmonary melanomas† (mean ± SD)</th>
<th>P‡</th>
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<tbody>
<tr>
<td>None</td>
<td>93.5 ± 54.8</td>
<td>—</td>
</tr>
<tr>
<td>25 mg/kg</td>
<td>45.2 ± 37.1</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>75 mg/kg</td>
<td>62.6 ± 34.0</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>150 mg/kg</td>
<td>26.7 ± 17.3</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

* DTIC given as a single intravenous bolus 24 hr after intravenous inoculation of B16F10 melanoma cells.
† 5 × 10⁴ B16F10 melanoma cells injected intravenously on day 0. Mice were necropsied on day 14 and the number of pulmonary melanoma colonies determined by direct inspection under a dissecting microscope. There were 8–10 mice per group.
‡ P value determined by student t-test. All significant results confirmed by the rank-sum test.

fit. Only 42% of these hosts harbored one or more metastatic foci. Delaying DFMO treatment until one day after enucleation (ie, day +11) did not appreciably impair the drug’s anti-neoplastic effectiveness. These hosts developed an average of six metastatic foci compared to a mean of 39.5 for their untreated counterparts (Fig. 2). Thus, DFMO can exert significant anti-neoplastic effects even if treatment is delayed until shortly after enucleation.

Efficacy of DTIC in Controlling Blood-Borne Melanomas

Others have reported a therapeutic response rate of 25% in cutaneous melanoma patients treated with DTIC.⁹ Accordingly, it was important to determine if this drug would be effective against blood-borne B16F10 melanoma cells and therefore serve as a candidate for use as an anti-metastatic agent. This was tested in the next series of experiments in which various doses of DTIC were administered as single intravenous bolus injections 24 hr after the intravenous inoculation of 5 × 10⁴ B16F10 melanoma cells. Mice were killed 14 days later and the number of pulmonary melanomas determined. The results shown in Table 2 confirm the anti-melanoma effect of DTIC and indicate that the most effective dose was in the range of 150 mg/kg body weight.

Combining DFMO and DTIC For Optimal Chemotherapeutic Efficacy

Since DTIC and DFMO exert their anti-neoplastic effects by different biochemical pathways, it would seem logical to combine these two drugs in an effort to optimize the therapeutic effectiveness. Accordingly, panels of mice were treated with either DFMO, DTIC, or a combination of these two drugs. All mice received 5 × 10⁴ B16F10 melanoma cells injected intravenously on day 0. DFMO was provided ad libitum in the drinking water beginning on day 0 and continuing throughout the entire experiment. DTIC was given at a dose of 150 mg/kg either 1 day or 7 days after enucleation. Panels received DTIC only, DFMO only, DTIC and DFMO, or no treatment.

Fig. 3. Maximal anti-melanoma therapy by combined treatment with DTIC and DFMO. C57BL/6 mice received intravenous inocula of 5 × 10⁴ B16F10 melanoma cells on day 0. DFMO was provided ad libitum in the drinking water beginning on day 0 and continuing throughout the entire experiment. DTIC (150 mg/kg) was administered intravenously as a single bolus injection either 1 or 7 days after intravenous melanoma injection. There were 8–10 mice per group.
Each panel was coded and the observer was unaware of the identity of the various mice. Survival times were determined by daily observation of the experimental mice. As shown before, DTIC and DFMO are effective when given independently (Fig. 3). Treatment with either drug resulted in a significant prolongation of the hosts’ survival times. However, the most profound prolongation was found in animals treated with both drugs (Table 3). Thus, combined therapy with the purine analogue, DTIC, and the suicide enzyme inhibitor, DFMO, produces an additive therapeutic effect.

**Discussion**

Previous studies have shown that either in vitro or in vivo treatment with DFMO profoundly inhibited the growth of B16 melanoma. Combined therapy of DFMO and mouse fibroblast interferon resulted in a 96% inhibition of subcutaneous B16 melanomas in mice. Thus, B16 melanoma has been shown to be exquisitely sensitive to the antiproliferative effects of DFMO. The present study examined the therapeutic effects of DFMO in controlling blood-borne B16 melanoma cells as well as spontaneous metastases derived from primary intraocular tumors. This study also served to evaluate the anti-metastatic efficacy of an anti-melanoma drug, DTIC, that is commonly used in the treatment of primary cutaneous melanoma.

Our interest in exploring the efficacy of DFMO in controlling metastatic melanoma is three-fold: (1) metastatic disease remains the leading cause of death in cancer patients; (2) malignant melanoma is the most common primary intraocular malignancy in humans; and (3) there are currently no known effective chemotherapeutic agents for treating metastases arising from intraocular melanomas.

Since there is no known lymphatic drainage of the internal compartments of the eye, metastasis of intraocular melanomas occurs via a hematogenous route. Thus, therapeutic modalities that are effective against intravenously injected (ie, blood-borne) melanoma cells should exert similar effects against metastases derived from intraocular tumors. Although our primary interest lies in the treatment and prevention of metastases arising from intraocular melanomas, many of the present experiments used intravenously injected melanoma cells as a model since this method is less cumbersome and has several technical advantages over enucleation-induced metastasis of intraocular melanomas. For example, one measure of therapeutic efficacy is the prolongation of survival time. Due to the invariable development of recurrent orbital tumors in mice subjected to enucleation, survival studies are not feasible in the enucleation-induced metastasis model because many of the hosts die from local orbital tumors before metastatic lung tumors attain a mass that is capable of causing death. Nonetheless, both metastasis models possess unique advantages that permit the evaluation of the therapeutic efficacy of the two anti-metastatic drugs under study.

The results demonstrate that orally administered DFMO exerts a profound therapeutic effect against blood-borne melanoma cells as well as metastases originating from primary intraocular melanomas. Not only was there a marked reduction in the number of metastases but the host’s survival time was significantly prolonged as a result of DFMO therapy. It is noteworthy that a therapeutic effect was present even when DFMO treatment was delayed until shortly after enucleation of the tumor-containing eye. This is particularly important when one considers the possible conditions for DFMO treatment of humans for primary intraocular melanoma—in the clinical setting, it is not always possible to administer an anti-neoplastic drug at the first appearance of the primary intraocular melanoma. The ability to delay DFMO treatment until the time of enucleation without jeopardizing its therapeutic effectiveness is a valuable feature of this drug therapy which has important clinical applications in the treatment of humans.

The results of the experiments addressing the anti-metastatic effect of DTIC are promising. Although DTIC can be highly toxic when delivered by the intravenous route, its anti-metastatic effect is maximal when the drug is given shortly after the melanoma cells are blood-borne. This feature of DTIC treatment could serve as a prophylactic strategy for sterilizing the blood stream of blood-borne melanoma emboli that might be liberated at the time of enucleation. As with DFMO therapy, DTIC treatment results in a

### Table 3. Effect of DFMO and DTIC on survival in mice challenged intravenously with B16F10 melanoma

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>Median survival time (days)</th>
<th>P†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>24</td>
<td>—</td>
</tr>
<tr>
<td>DTIC: day 1</td>
<td>9</td>
<td>33</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DTIC: day 7</td>
<td>9</td>
<td>32</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DFMO</td>
<td>10</td>
<td>34</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DTIC: day 1 + DFMO</td>
<td>8</td>
<td>35</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>DTIC: day 7 + DFMO</td>
<td>9</td>
<td>37</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* C57BL/6 mice received 5 x 10⁵ B16F10 melanoma cells injected intravenously on day 0. DTIC was injected intravenously at a dose of 150 mg/kg on the day indicated. DFMO was provided ad libitum as a 2% solution in the drinking water beginning on day 1 and continuously thereafter.

† P value derived by Mantel-Haenszel test comparing survival of each group with the untreated control (line 1).
significant prolongation of survival time in melanoma-bearing hosts. The most profound therapeutic effect is seen when both drugs are combined. Since DTIC and DFMO exert their anti-neoplastic effects by different biochemical pathways, the combination of these drugs probably results in multiple impairment of tumor cell metabolism.

The prospect of employing DFMO as a chemotherapeutic agent in the prevention and treatment of melanoma metastases is exciting for several reasons. DFMO exerts anti-neoplastic effects by “suicide enzyme inhibition” and is therefore specifically directed at tumor cells which have only limited enzymatic pathways for polyamine biosynthesis. By contrast, normal mammalian cells are significantly less vulnerable to the action of “suicide enzyme inhibitors.” In fact, an attractive feature of DFMO is its exceedingly low toxicity. Unlike most cancer chemotherapeutic agents, DFMO is not cytotoxic. Human subjects have been treated with doses as high as 24 gm/day with only minor complications. Another advantage of DFMO is that it does not adversely affect the host’s immune apparatus. Sustained treatment with 2% DFMO drinking water does not impair natural killer (NK) cell activity in mice. Likewise, we have shown that neither NK nor natural cellular (NC) cytotoxicity is affected by oral administration of 2% DFMO (unpublished data). By contrast, DTIC is known to have immunosuppressive effects.

The findings reported here, although promising, should be viewed with caution. The B16 melanoma model differs from human intraocular melanoma in several significant ways. B16F10 melanoma cells are transplanted into the anterior segment of the mouse eye and therefore are not the result of in situ transformation within the uveal tract. Moreover, it is not known if human uveal melanomas are vulnerable to DFMO or other “suicide enzyme inhibitors.” However, in light of the numerous advantages of DFMO and the broad clinical experience that oncologists have had with DTIC, it seems plausible to cautiously consider similar therapeutic regimens for use in human patients.

Key words: melanoma, metastases, suicide enzyme inhibition, difluoromethylornithine, dacarbazine, DTIC, DFMO

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References