Selective Toxicity of 5-(3,3-Dimethyl-1-triazeno)imidazole-4-carboxamide toward Hypoxic Mammalian Cells

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ABSTRACT

The chemotherapeutic agent 5-(3,3-dimethyl-1-triazeno)-imidazole-4-carboxamide (DTIC) is used in the treatment of malignant melanoma where response rates of 15 to 30% have been reported. Some current interest exists in combining DTIC chemotherapy with localized high-dose (800 rads)-per-fraction radiotherapy in the treatment of unresectable metastatic melanoma. The present work investigates the radiosensitizing and chemotherapeutic properties of DTIC in an in vitro system using Chinese hamster ovary or HeLa cells and in vivo using the KHT transplantable murine tumor. No evidence of a radiosensitizing effect of DTIC was found towards hypoxic or aerobic cells either in vitro or in vivo. In vitro, high drug concentrations (1 mg/ml) were approximately 5 times more effective in killing hypoxic Chinese hamster ovary or HeLa cells than in killing aerobic cells over exposure times of 0 to 12 hr. The degree of toxicity was drug dose and temperature dependent but was not highly dependent on cell number or cell type. In vivo plasma levels of DTIC were measured with high-pressure liquid chromatography after i.p. injection of drug into C3H mice. At the highest drug doses tested, near the 50% lethal dose in mice for DTIC (0.5 mg/g), the drug was toxic to both aerobic and hypoxic tumor cells with some evidence of increased toxicity towards hypoxic cells. The present work suggests that DTIC may be more efficiently activated under hypoxic conditions as compared to aerobic conditions. The increased toxicity of DTIC under hypoxic versus aerobic conditions may prove to be a feature of this drug that can be exploited in its clinical use and in the design of new analogs of DTIC.

INTRODUCTION

The antineoplastic agent DTIC has demonstrated clinical effectiveness against malignant melanoma in which response rates of 15 to 30% have been reported (5). The present work was initiated because of results of a pilot study with unresectable metastatic malignant melanoma treated with DTIC and radiotherapy. Preliminary results indicated that an improved response was seen in patients given DTIC at 850 mg/sq m i.v. on Days 1 and 22 plus radiotherapy at 800 rads/day on Days 2, 9, and 23 (20). Some nitroimidazoles have been shown to be hypoxic cell radiosensitizers and hypoxic cell toxic agents (4, 7), and DTIC is an imidazole derivative, although not a nitroimidazole. The one-electron oxidation-reduction potential of DTIC (−571 mV) is in the range of some 4-nitroimidazoles that have shown hypoxic cell-radiosensitizing ability (34). Recent studies have shown that a variety of commonly used chemotherapeutic agents can have selective toxicity towards either oxygenated or hypoxic tumor cells (28, 30). In the present work, a series of in vitro and in vivo experimental studies were carried out on DTIC alone and in combination with ionizing radiation. The questions asked were (a) is DTIC a hypoxic cell radiosensitizer and/or (b) does it have increased toxicity towards hypoxic as compared to aerobic cells?

MATERIALS AND METHODS

In Vitro Studies

Cells. For in vitro studies, CHO cells and human HeLa cells were routinely cultured in suspension in complete α-medium (26) supplemented with 10% FCS (v/v) (Flow Laboratories, Inc., McLean, Va.). Doubling times were typically 13 to 15 hr for CHO cells and 20 to 22 hr for HeLa cells at 37°C. All experiments made use of asynchronous populations of cells in exponential growth.

Drugs. DTIC was obtained from Dome Laboratories (Division of Miles Laboratories Ltd., Rexdale, Ontario, Canada). Each vial contained DTIC and citric acid which, when reconstituted with α-medium resulted in a 20-ml drug suspension of DTIC at 10 mg/ml in 1% citric acid. After medium was added, the pH was adjusted by NaOH addition to pH 7.2. The maximum aqueous solubility of the drug under these conditions was 1 mg/ml. The drug was made up immediately before use and was protected from light at all times.

Hypoxic Cell Radiosensitization. Ten-ml aliquots of a stock suspension culture at 5 to 7 × 10⁶ cells/ml in α-medium plus 10% FCS were placed in glass vials. The cells were in exponential growth at the time of aliquot removal. The cell suspensions were stirred in the glass vials which were closed with a silicone rubber stopper through which an inlet and outlet tube passed to allow continual gassing of the cell suspensions. Cells were irradiated aerobically by passing 5% CO₂:balance air mixtures over them before and during irradiation. Hypoxic irradiations were carried out with cell suspensions which were gassed with 5% CO₂ balance air mixtures over them before and during irradiation. Hypoxic radiations were carried out with cell suspensions which were gassed with 5% CO₂ 10 ppm O₂:balance nitrogen mixtures. Forty min of gassing time were allowed before the start of irradiation to ensure that the cells were in equilibrium with the gas mixture (33). All irradiations were carried out at room temperature using a ⁶⁰Co source. The dose rate in the vial was measured by Fricke dosimetry (25) to be 150 rads/min. Samples of cells were removed through the gas outlet tube as a function of radiation dose, diluted, and assayed for colony-forming ability.

Toxicity Tests. Chronic toxicity tests were done by plating cells in 60-mm dishes (Falcon Plastics, Oxnard, Calif.) containing various concentrations of DTIC in 5 ml of α-medium plus 10% FCS. Plates were incubated at 37°C in a humidified atmosphere at 95% air:5% CO₂. Plating efficiencies were determined after 8 days for CHO cells and after 10 days for HeLa cells. All gas mixtures used were obtained from Gas Dynamics, Ltd. (Toronto, Ontario, Canada.) Acute toxicity tests were carried out in glass vials containing 10 ml of cells 5 to 7 × 10⁶/ml in α-medium plus 10% FCS. Vials were closed with silicone rubber stoppers through which a gas inlet and outlet tube passed. Cells were

1 This work was supported by grants from the National Cancer Institute, the Medical Research Council of Canada, and the Ontario Cancer Treatment and Research Foundation.
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3 The abbreviations used are: DTIC, 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (NSC 45388); CHO, Chinese hamster ovary; FCS, fetal calf serum; HPLC, high-pressure liquid chromatography; LD₅₀, 50% lethal dose.

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stirred while gas flowed continuously over the surface of the cell suspension. For aerobic experiments, 5% CO₂:balance air mixtures were used. For acute hypoxia experiments, 5% CO₂ <10 ppm O₂:balance nitrogen was flowed. Cell samples could be removed as a function of time with a micropipet inserted through the gas outlet tube. Details of this procedure have been given previously (29). Temperature was controlled by placing the vials in a circulating water bath.

In Vivo Studies

Cells. The transplantable KHT sarcoma in C3H mice was used (13). It is transplanted routinely by injecting 2 × 10⁵ cells prepared as described previously (31) into both flanks of 8- to 10-week-old male C3H/HeDub mice. In 10 days, tumors approximately 10 mm in diameter were obtained. Tumors in passages 120 to 170 were used, and their tumor biology and radiobiology were identical to those reported previously (12, 21).

Drugs. The DTIC from Dome Laboratories was made up in phosphate-buffered saline for the in vivo studies. Dilutions were made in phosphate-buffered saline (21). All drug injections were made i.p.

Radiosensitization Tests. To test for the hypoxic cell radiosensitizing ability of DTIC, groups of 3 to 5 tumor-bearing mice were given various doses of DTIC, i.p., 30 min before irradiation. Mice were irradiated unanesthetized with 137Cs γ-rays at a dose rate of approximately 90 rads/min whole body (6). Five to 30 min after radiation, mice were sacrificed, tumors were removed and pooled for each group, and single-cell suspensions were prepared as described previously (31). Cell viability was assayed using the in vivo lung colony assay procedure of Hill and Bush (11) with minor modifications.

Toxicity Testing. To test for aerobic and hypoxic cell toxicity in vivo, groups of 3 to 5 KHT tumor-bearing mice were set up. Some groups were given 2500 rads of 137Cs γ-rays whole body, and others were not irradiated. Various doses of DTIC were given i.p. to both unirradiated and irradiated groups of animals immediately after radiation, and the animals were left undisturbed for 24 hr before tumor cell survival was assayed via the lung colony assay. Thus, in unirradiated animals, the toxicity of the drug was measured towards a tumor cell population in which the majority of cells were aerobic. In the irradiated population, the vast majority of cells were hypoxic, at least immediately after radiation.

Drug Levels in Plasma. Groups of 2 to 3 non-tumor-bearing mice were given various doses of DTIC i.p. At various times after injection, the mice were given injections of 0.2 ml of heparin solution 10 min before sacrifice. Blood samples were collected after opening the thoracic cavity and incision of the major blood vessels from the heart. Blood samples were centrifuged at 4°C at 240 × g for 15 min, and the plasma was removed. Plasma samples were precipitated with methanol at a ratio of 1:9, respectively, and the supernatant was run on a HPLC apparatus (Waters Instruments, Toronto, Ontario, Canada) at 1200 psi on a C18-Bondapak column in 0.05 M phosphate buffer:methanol, pH 7.2 (4:1). Column effluents were monitored at 314 nm. Standard samples were run to calibrate the column. Using HPLC analysis, plasma levels of DTIC in mice could be monitored as a function of time after injection.

RESULTS

Initial studies of the action of DTIC were carried out in vitro to determine the chronic toxicity of the drug towards CHO or HeLa cells. Cells in exponential growth were plated in a medium plus 10% FCS containing different concentrations of DTIC. Cells were incubated continuously for 8 to 10 days in the presence of the drug under aerobic conditions; the plates were then stained, and colonies were counted. The percentage of the colony-forming ability of the cells, relative to control cells which were not exposed to drug, is plotted versus the drug concentration in the Petri dish in Chart 1. Between 20 and 200 μg/ml DTIC, the survival of the cells dropped from essentially 100 to 0% survival. Fluctuations in survival below 10 μg/ml were not significant, and essentially 100% survival was seen for both CHO and HeLa cells. These results indicated that in subsequent experiments where the radiosensitizing or acute toxicity of DTIC was to be measured, it would be necessary to dilute the cells adequately to ensure that drug carry over was less than 10 μg/ml.

The radiosensitivity of aerobic and hypoxic CHO cells was measured in the presence and absence of DTIC to see if the drug altered survival of cells under either or both of these conditions. Irradiations with DTIC present were carried out at room temperature at 1000 μg/ml, the highest concentration of drug obtainable due to the relatively low solubility of the drug in aqueous solution at pH 7. The results seen in Chart 2 indicate an oxygen enhancement ratio of 3 for these cells, but no evidence of any radiosensitizing action of DTIC on either aerobic or hypoxic cells was seen. Thus, DTIC does not appear to be a radiation sensitizer.

Next, the toxicity of DTIC was measured for shorter incubation times under aerobic and hypoxic conditions. Cells were taken from suspension culture in the exponential phase of growth at 5 to 7 × 10⁵ cells/ml and plated in glass vials with 5% CO₂:balance air mixtures or 5% CO₂:<10 ppm O₂:balance nitrogen gas mixtures flowing over them as described in "Materials and Methods." Control aerobic and hypoxic cell cultures were compared to aerobic and hypoxic cell cultures containing DTIC at its solubility limit, 1000 μg/ml, as well as cell cultures with 500 or 250 μg/ml. Cultures were incubated at 37°C. Samples were removed as a function of time, diluted, and assayed for their colony-forming ability. Results of a number of separate experiments are shown in Chart 3. Hypoxic cell cultures were more sensitive to all concentrations of DTIC than were aerobic cell cultures, at least over the first 2 logs of cell-killing. The percentage of survival of the no-drug 250 and 500 μg/ml DTIC aerobic and the no-drug hypoxic cell cultures fell between the data for no effect (dashed line) and the air plus DTIC (1000 μg/ml) results. They are omitted for clarity. At the

![Chart 1](chart1.png)
The hypoxic cell toxicity of DTIC would appear to be linearly related to drug concentration; i.e., with 500 μg/ml or 250 μg/ml, approximately 2 or 4 times the time for the same degree of cell killing is required, respectively. No toxicity of DTIC towards aerobic cells was demonstrable at the lower concentrations over this time period (data not shown).

Previous studies with the nitroimidazoles, metronidazole (17) and misonidazole (29), had shown a strong temperature dependency for increased toxicity towards hypoxic compared to aerobic cells. Thus, the effect of temperature on the hypoxic cell toxicity of DTIC was measured at 20 and 0°C. As can be seen in Chart 4, most of the cell killing ability of DTIC (1000 μg/ml) towards hypoxic cells is lost at 20°C, and even less occurs at 0°C. The data for the toxicity of DTIC (1000 μg/ml) at 37°C towards hypoxic and aerobic cells from Chart 3 are included in Chart 4 as dashed lines.

The toxicity of DTIC towards hypoxic cells was not strongly dependent on cell number, since 10-fold greater cell concentrations, 7 × 10^6 cells/ml, did not appreciably change the rate at which hypoxic cells were killed by DTIC at 37°C (data not shown). Previous studies have indicated that hypoxic HeLa cells were 2- to 3-fold more sensitive to misonidazole toxicity than were CHO cells (29). Thus, HeLa cells were exposed to DTIC (1000 μg/ml) under hypoxic and aerobic conditions in the same fashion as CHO cells. The results that are shown in Chart 5, although somewhat scattered, indicate that HeLa cells may be slightly more sensitive to DTIC under both aerobic and hypoxic conditions, but the difference in response between the 2 cell lines is not great. The dashed lines again are the data for hypoxic and aerobic CHO cells at 1000 μg/ml, taken from Chart 3. The limited data indicate that HeLa cells do not have a resistant subpopulation below 1% survival, as was apparent with CHO cells.

Next, the effects of DTIC towards the KHT tumor in vivo were measured. Initial studies indicated that the LD₅₀ for 24-hr survival for C3H mice given DTIC i.p. was about 0.5 mg/g. Thus, testing of mouse plasma for the presence of DTIC was
carried out at this dose and 0.25 mg/g. Groups of 2 to 3 mice were given these doses of DTIC and then sacrificed as a function of time after injection and plasma samples were prepared. Over the time range assayed (0 to 8 hr), no animals died from DTIC. The plasma samples were assayed for the presence of DTIC. The results are shown in Chart 6. The results for the 0.25 mg/g indicate that the plasma level was highest at the earliest time measured (15 min), and an exponential decay of the drug occurred with a half-life of about 20 min. At 0.5 mg/g, the DTIC level is 4 times that after 0.25 mg/g at 15 min. It decays to a plateau level at 1 hr that extends out to at least 4 hr. There was a large scatter in these measurements, and measurements made at longer times (data not shown) indicated a large variability in the rate of decline from this plateau. What is clear is that DTIC is present at a maximal level in the plasma shortly after injection. Preliminary in vitro studies with KHT cells indicated that they were similar to CHO cells in their sensitivity to DTIC (data not shown). Thus, it was questionable whether adequate levels of drug would be present in vivo to give toxicity. Nevertheless, in vivo testing was carried out on the radiosensitizing and hypoxic cell toxicity effects of these doses of DTIC.

DTIC was tested by a procedure used previously to screen for hypoxic cell radiosensitizers in vivo (21). In this experiment, groups of KHT tumor-bearing mice were given i.p. injections of graded doses of DTIC, left undisturbed for 30 min, and then given whole-body irradiation at a dose of 2500 rads of 137Cs γ-rays. Animals were sacrificed immediately, and tumors were assayed for the percentage of survival of KHT tumor cells using the lung colony assay (see "Materials and Methods"). At this dose of radiation, cell survival is controlled by the hypoxic cell population (21). As seen in Chart 7, no evidence of radiosensitization of hypoxic cells was seen.

Next, an in vivo test of the toxicity of DTIC towards aerobic and hypoxic cells was carried out. One group of KHT tumor-bearing mice was irradiated (whole body) with 2500 rads, and a second group was left unirradiated. Immediately after irradiation, both groups were given injections of graded doses of DTIC. The animals were left undisturbed for 24 hr, then they were sacrificed, and the tumors were removed and assayed for tumor cell survival. The results are shown in Chart 8. There is little effect of DTIC towards either unirradiated or irradiated cells, except at the highest dose tested, 0.375 and 0.500 mg/g. There appears to be slightly more toxicity toward the irradiated cells (hypoxic survivors) than toward the unirradiated cells (predominantly aerobic cells). The interpretation of the 0.500-mg/g data is complicated since this dose is the LD50 dose for 24-hr survival for both the unirradiated and irradiated mouse groups.

**DISCUSSION**

The present results indicate that DTIC is neither a hypoxic nor an aerobic cell radiosensitizer in vitro (Chart 2) or in vivo (Chart 7). This is perhaps not unexpected since, although DTIC is an imidazole derivative, it is not a nitroimidazole and is not strongly electron affinic (34). The results on the toxicity of DTIC towards CHO cells under aerobic conditions are in relative agreement with the work of Gerulath et al. (9). Their work, like...
the present work, indicated that aerobic CHO cells are relatively resistant to the action of DTIC. They found that CHO cells incubated under fluorescent light in the presence of DTIC were much more sensitive to the drug, approximately equal to the increased toxicity seen in the present experiments with hypoxic cells (Chart 3).

Proposed mechanisms of action of DTIC both for its light-mediated toxicity and toxicity in the dark require drug activation. In the presence of light, DTIC in water gives rise to a hydroxyimidazolecarboxamide and, at high DTIC concentrations, azoimidazoles (27). Thus, the presence of citric acid can divert the photolysis of DTIC to new products. In the presence of light, DTIC in water gives rise to a hydroxyimidazolecarboxamide and, at high DTIC concentrations, azoimidazoles (27). Thus, the presence of citric acid can divert the photolysis of DTIC to new products.

Integration of the area under the mouse plasma level curves of Chart 6 yields values of 750 µg-hr/ml for 0.5-mg/g doses and 3000 to 4000 µg-hr/ml for 0.25-mg/g doses. Despite this reduced drug exposure, appreciable killing of both aerobic and hypoxic tumor cells appears to occur, as shown in Chart 8. Whether this represents a greater sensitivity of KHT tumor cells in vivo to the drug compared to in vitro or a different mechanism of action in vivo is not clear at the present time. Certainly, the cell killing seen in Chart 8 does not indicate a large selective toxicity towards hypoxic cells.

The present results for the LD50 of DTIC towards C3H mice (0.5 mg/g) and moderate activity towards the KHT tumor is consistent with reports in other mouse-tumor systems (32). Investigations of the sensitivity of human melanoma cells grown in vivo in nude mice have indicated, as in the present study, that the drug is effective only near its LD50 (19, 22). Rofstad et al. (22) have investigated the effects of DTIC alone or DTIC combined with 60Co γ-rays on 2 human malignant melanomas transplanted in nude mice. Their data for combining daily DTIC with radiation given 1 to 2 hr after the animals were given DTIC showed some evidence of increased effectiveness in delaying tumor regrowth compared to radiation alone. If radiation is most efficient in killing aerobic cells compared to hypoxic cells, while DTIC is most effective in killing hypoxic cells, some advantage in the combination of these 2 agents might be expected.

Clinical studies have indicated that radiotherapy may be of value in the treatment of inoperable malignant melanomas (18, 24). It has been suggested that high daily doses of radiotherapy combined with chemotherapy might give better results than standard fractionated radiation dose procedures (15). Studies are presently in progress combining DTIC with radiotherapy for unresectable malignant melanoma (20). Although, qualitatively, one can anticipate differential effects of DTIC against hypoxic and aerobic cells based on experimental studies, attempts to quantitatively relate such results to the human situation are difficult. It can be estimated that DTIC (0.1 mg/g) in the mouse is equivalent to 425 mg/sq m in humans (8). Thus, some doses of DTIC used in the present experiments are, on this basis, in the range used clinically. On the other hand, human plasma levels of DTIC, after i.v. administration of 250 mg/sq m, may be only as high as 15 µg/ml, declining with a terminal half-life of 5 hr (2, 16). The total µg-hr/ml of drug in the plasma would be at least an order of magnitude less than in the present in vivo or in vitro experimental studies. Of course, the lifetime of DTIC may be different than the lifetime of the effective species responsible for cell killing, there may be differences in the degree and type of drug activation in these systems, and there may be large differences in the toxicity of the drugs towards different cell types.

Thus, the role of the hypoxic cell toxicity of DTIC in the clinical situation is not clear. However, the mechanism by which DTIC has increased toxicity towards hypoxic compared to
aerobic cells remains of interest both from the standpoint of understanding the nature of this toxicity at a basic level and from that of trying to select and/or design chemotherapeutic agents with increased toxicity for hypoxic cells (14, 30).

REFERENCES


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