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Cytotoxicity of diethyldithiocarbamate in human versus rodent cell lines

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Summary

Diethyldithiocarbamate (DDTC) and other dithiocarbamates are currently receiving attention as potential adjuncts to traditional chemotherapy. *In vitro* studies with rodent cancer cell lines have consistently shown that DDTC concentrations of 0.1–1.0 μg/ml are highly cytotoxic. Paradoxically, however, concentrations of 10–100 μg/ml have been significantly less toxic.

In the present study, such a 'biphasic' pattern was reproduced when 3 rodent cell lines were exposed for 1 hour to 0.001 to 1000 μg DDTC/ml. In contrast, in 7 human cell lines survival decreased steadily with increasing DDTC concentration (in the same dose range) without evidence of a biphasic pattern. These data might have implications for studies in which rodent cell lines are used to model the effects of dithiocarbamates in human tissues.

Introduction

Dithiocarbamates are a family of highly reactive compounds with widespread applications in agriculture, industry, and medicine such as in the treatment of certain metal poisonings (e.g. Cd, Ni [1]), in the vulcanization of rubber, in aversion therapy for alcohol addiction as well as in other applications [1–3]. In the oncology setting there has been clinical interest in the complex and possibly useful "immune restorative" effects of diethyldithiocarbamate ("DDTC") and its oxidized form, disulfiram [4,5]. Because of its high affinity chelation of certain metallic ions (e.g. Zn, Cu, Ni) and inactivation of certain metalloenzymes [3], DDTC is also being actively investigated as an adjunctive oncologic therapy to reduce the normal tissue toxicities of cisplatin chemotherapy [5,6] or to enhance the efficacy of cyclophosphamide [7,8], bleomycin [9], and ionizing irradiation [10].

Unfortunately, DDTC never displayed potentially useful tumoricidal efficacy in its own right despite predictions based upon its potent inhibition of key cellular enzymes [2,4]. Instead, it was consistently shown in various murine, rat, and hamster cell lines that cell survival decreased profoundly after exposure to 0.1 to 1.0 μg DDTC/ml but cell survival was greater or even normal using higher concentrations of 10 to 100 μg/ml [e.g. 8,10]. Perhaps because of these findings, DDTC appears to have dropped from consideration as a potential antineoplastic agent.

It was the objective of the present investigation to test whether this traditional biphasic survival pattern also occurs in human cancer cell lines. Toward this end we evaluated the cytotoxicity of DDTC in 7 human cell lines and in 3 histologically analogous rodent cell lines.

Methods

Cell lines

Four human cell lines were of lymphoid origin including two human T cell acute lymphoblastic

leukemias (JM and MOLT3), a human Burkitt's lymphoma (CA46), and, to provide a contrast, a slowly growing B cell acute lymphoblastic leukemia with a very low growth fraction (CCRF SB). These cell lines were maintained in exponential growth and treated in RPMI 1640 with 20% fetal bovine serum ("FBS"; Hyclone Laboratories, Inc., Logan, UT). Survival was assessed by counting macroscopically visible colonies after 16–20 days growth in 0.30% Difco Bacto agar (Difco Laboratories, Detroit, MI) top layers (20% FBS) over 0.51% agar bottom layers (10% FBS) in 6 cm diameter petri dishes. For comparison, two murine leukemias of lymphoid origin (L1210 and P388D1) were grown and cloned in precisely the same manner except using 10% donor horse serum (GIBCO Laboratories, Grand Island, NY).

A human glioblastoma multiforme cell line (U87 MG) and two human transitional cell carcinomas (647V and T24) were grown in Eagle's minimum essential medium with 10% FBS. A rat glioblastoma cell line (C6) was grown in Ham's F10 medium supplemented with 2.5% FBS and 15% donor horse serum. Immediately before treatments, cells were harvested with trypsin-EDTA (GIBCO Laboratories, Grand Island, NY). T24 and 647V were also repeatedly pipetted through a finely drawn Pasteur pipet to decrease cell clumping. Survival was assessed by staining plates with methylene blue and counting macroscopic colonies after 14 days growth in 6 cm diameter tissue culture plates containing medium with 20% FBS (for U87 MG, T24, and 647V) or 5% FBS and 15% donor horse serum (for C6).

The possibility was also considered that a rodent cell line might not exhibit a biphasic response to DDTC if it were grown under precisely the same conditions as were the human cell lines. Accordingly, C6 cells were also grown, treated, and cloned to assess survival using Eagle's minimum essential medium with fetal bovine serum or with donor horse serum.

All media contained 100 units penicillin/ml and 100 μg streptomycin/ml (Sigma Chemical Co., St. Louis, MO) and all cell lines were incubated at 37.0°C with a 5% CO_2, 95% air atmosphere. Lymphoid cell suspensions were at least 99.99% single cells. U87 MG and C6 produced $\geq$ 94% single cell suspensions. T24 and 647V produced $\geq$ 90% single cell suspensions. Cloning and plating efficiencies under the described conditions were: JM 34.6 $\pm$ 3.1%; MOLT3 27.1 $\pm$ 4.6%; CA46 28.0 $\pm$ 0.4%; CCRF SB 0.48 $\pm$ 0.085%; L1210 63.8 $\pm$ 5.6%; P388D1 58.0 $\pm$ 1.2%; U87 MG 21.2 $\pm$ 4.8%; T24 57.0 $\pm$ 6.3%; 647V 14.5 $\pm$ 3.5%; C6 78.0 $\pm$ 6.0%. JM and MOLT3 were kindly provided by Dr. S.Z. Salahuddin, National Cancer Institute, while T24 and 647V were kindly provided by Dr. E.M. Messing, University of Wisconsin, Madison, WI. U87 MG (passage 125), P388D1, C6 (passage 39), CCRF SB, and CA46 were obtained from the American Type Culture Collection, Rockville, MD. Each of these cell lines was used in its first 4 passages in our laboratory.

Drug exposures

DDTC less than 6 months old and stored at −20°C and disulfiram (Sigma Chemical Co., St. Louis, MO) were freshly dissolved in medium and filter sterilized immediately prior to each use. Equal aliquots of cells in 1 ml medium were placed in 15 ml disposable centrifuge tubes to which 1 ml of drug-containing medium was added. For each cell line the DDTC concentration ranged from 0.001 to 1000 μg/ml in 10 fold increments. Samples were briefly and gently vortexed and placed immediately in 37.0°C water baths with continuous shaking. After 1 hour of incubation, centrifuge tubes were removed from water baths and washed three times with fresh drug-free medium. Cells were then diluted and plated or cloned in agar as noted above.

Short DDTC exposures were achieved for JM and MOLT3 cells by adding 0.1 ml of DDTC-containing medium to 0.1 ml aliquots of cells at the bottoms of centrifuge tubes. After precisely 2 minutes of DDTC exposure, 10 ml of drug-free medium was rapidly added to give an immediate 50 fold dilution of DDTC. Immediately thereafter the cells were washed twice with drug-free medium and cloned in agar.

Experiments were performed at least in duplicate and were repeated at least twice. Brackets in each

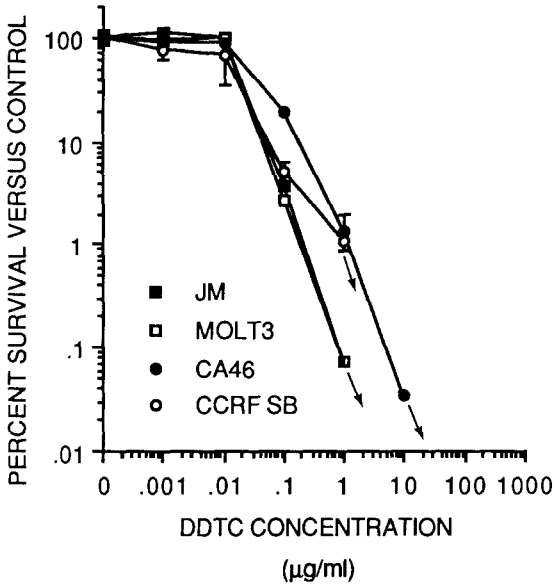

Fig. 1. Survival of exponentially growing JM, MOLT3, CA46, and CCRF SB lymphoid cell lines after 1 hour exposures to varying concentrations of diethyldithiocarbamate (DDTC). Brackets indicate the standard error of the mean except for small standard errors encompassed in the accompanying symbol. Arrows denote zero colonies per plate at higher drug concentrations.

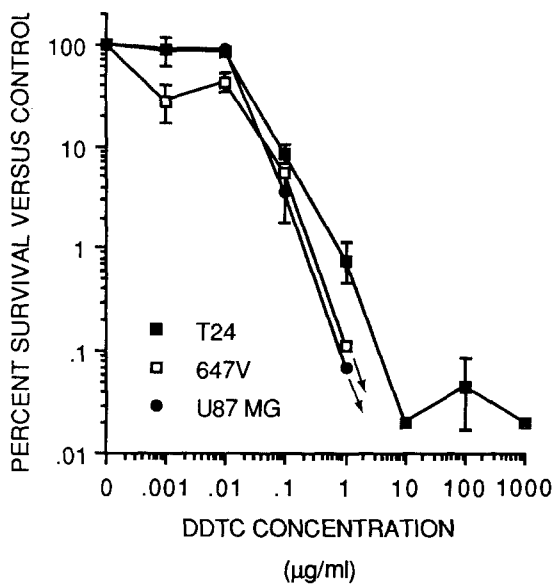

Fig. 2. Survival of exponentially growing T24, 647V, and U87 MG non-lymphoid cell lines after 1 hour exposures to varying concentrations of diethyldithiocarbamate (DDTC). Brackets indicate the standard error of the mean except for small standard errors encompassed in the accompanying symbol. Arrows denote zero colonies per plate at higher drug concentrations.

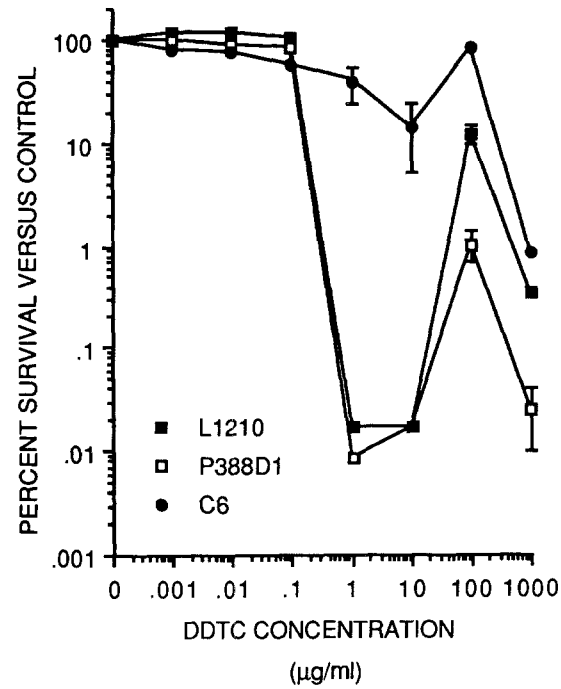

Fig. 3. Survival of exponentially growing L1210, P388D1, and C6 cell lines after 1 hour exposures to varying concentrations of diethyldithiocarbamate (DDTC). Brackets indicate the standard error of the mean except for small standard errors encompassed in the accompanying symbol.

figure indicate the standard error of the mean except when standard errors were small enough to be encompassed in the symbols.

Results

Figures 1 and 2 show that the 7 human cell lines were sensitive to DDTC at concentrations as low as 0.1 μg/ml (× 1 hour) without substantially greater survival at higher DDTC concentrations. In contrast, as is demonstrated in Fig. 3, L1210, P388D1, and C6 show a biphasic pattern of sensitivity to DDTC. Cell viability at 10 to 100 μg/ml can approach or equal the survival of untreated control cells and then decreases again at 1000 μg/ml. C6 cells showed the same biphasic pattern when grown using only FBS or donor horse serum in Eagle's minimum essential medium.

The lethal effect of DDTC occurs even following a very brief drug exposure. A two minute DDTC ex-

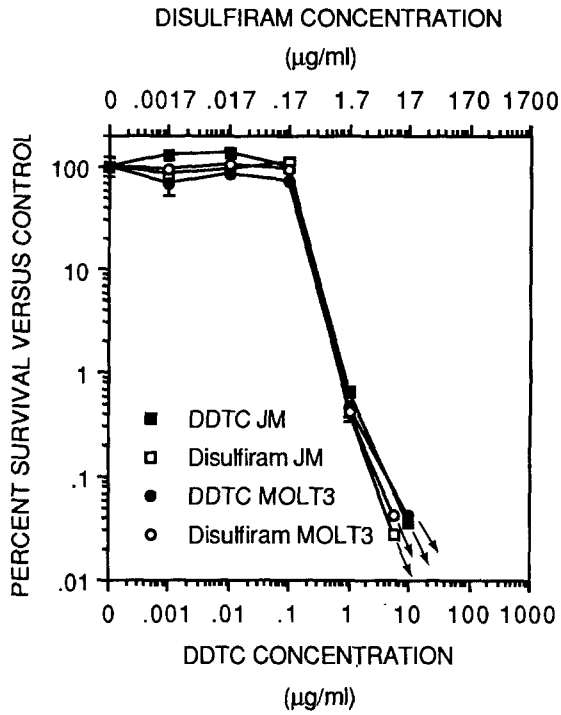

Fig. 4. Survival of exponentially growing JM and MOLT3 cells after 1 hour exposures to equimolar concentrations of diethyldithiocarbamate (DDTC) or disulfiram (1.73 μg disulfiram/ml = 1 μg DDTC/ml). The largest disulfiram concentration used was 10 μg/ml due to its lower solubility in medium. Arrows denote zero colonies per plate at higher drug concentrations. Brackets indicate the standard error of the mean except for small standard errors encompassed in the accompanying symbol.

posure still caused an abrupt decrease in JM and MOLT3 survival just as in Fig. 1 except that the first drop occured at 10 μg/ml (data not shown). Also as in Fig. 1, cell survival remained decreased for higher DDTC concentrations up to 1000 μg/ml.

We were also interested in the effects of disulfiram, the oxidized form of DDTC. Disulfiram was of interest as DDTC and disulfiram are interconvertible *in vivo* by interaction with intracellular oxidants/reductants [3]. Also, considerable clinical data exist regarding disulfiram's clinical pharmacology [e.g. 11]. As is shown in Fig. 4, equimolar disulfiram and DDTC exposures have similar effects on the survival of JM and MOLT3 cells.

Discussion

The results presented in Figs. 1 and 2 appear to be the first data regarding the cytotoxicity of DDTC and disulfiram in human cancer cells (It should be noted that the human cell lines were selected on the basis of their availability and the rodent cell lines were selected only for their similarity to some of the human cell lines). Interestingly, DDTC and disulfiram cause substantial cell killing at concentrations which are extremely low from a clinical perspective. For example, DDTC has been investigated in man as an adjunctive therapy to modulate the toxicity of cisplatin chemotherapy [5]. Relatively low DDTC doses up to 600 mg/m^2 have been used in this setting [5]. Doses in this range – which cause no detectable clinical toxicity – are equivalent to 195 mg DDTC/kg in the mouse using the methods of Freireich for interspecific dosage comparison [12]. In mice 250 mg DDTC/kg produces peak serum levels of 50 to 200 μg/ml with a plasma half life of 10–20 minutes [13].

It should also be noted that murine LD_{10} doses (10% lethality [13]) correspond to a dose of 4162 mg/m^2 in a 60 kg man. Moreover, in clinical phase I trials using disulfiram to decrease cisplatin nephrotoxicity, disulfiram was tolerated at oral doses up to 3000 mg/m^2 with two patients experiencing dose-limiting confusion without hematologic or other significant disulfiram toxicity [11]. Based on these considerations, one might reasonably suggest that clinical serum DDTC levels could safely be 10^3 to 10^4 fold greater than the 0.1 μg/ml DDTC concentrations which kill human cancer cells as in Figs. 1 and 2.

At DDTC concentrations of 10 to 100 μg/ml the three rodent cell lines in Fig. 3 appear to be less sensitive than are the analogous human cell lines (Glioblastomas: U87 MG versus C6. Lymphoid malignancies: JM, MOLT3, CA46, CCRF-SB versus L1210, P388D1). This unusual biphasic pattern, which has been noted previously [1,8,10], may complicate preclinical *in vivo* studies regarding the possible antineoplastic utility of DDTC and disulfiram. However, it is quite possible that other rodent (or other animal) cell lines will have survival patterns closer to the human ones in Fig. 1. Nude

mouse tumor xenografts might not be useful; DDTC has multiple "immune restorative" effects in rodents (and possibly in man) including restoring normal T cell maturation and function in nude mice [4]. As a result, xenograft tumor regression due to DDTC immune modulation might be incorrectly interpreted as cytotoxic efficacy.

It is not clear how DDTC causes its peculiar biphasic pattern of survival or how it kills cancer cells in the first place. Some 35 years ago DDTC's *in vitro* cytotoxicity was attributed to its high affinity chelation of metal cations effecting a covalent inactivation of key metalloenzymes [2]. Certainly, such a mechanism would be consistent with the rapid and apparently irreversible effect of DDTC on JM and MOLT3 cells when using just 2 minute exposures. Given the efficacy of 2 minute drug exposures, it is also unlikely that DDTC acts by removing important factors from the tissue culture medium.

Recent studies have focused on specific enzymes implicating those involved in nucleotide pool balance [1], detoxification of superoxide radicals [10] and of organic hydroperoxides [14]. Unfortunately, DDTC was eventually shown to inactivate a remarkable variety of enzymes including superoxide dismutase [10], glutathione transaminase [14], and aldehyde dehydrogenase [8] in addition to other enzymes. Presently there is no definitive evidence that one of these enzymes is the principal target of DDTC.

The dithiocarbamate literature also provides no clues regarding which molecular mechanism(s) could cause the differential sensitivities of human and rodent cell lines as in Figs. 1 to 3. For C6 cells, the biphasic cytotoxicity pattern was the same when cells were grown, treated, and cloned in FBS or in donor horse serum using Eagle's minimum essential medium or Ham's F10 medium. Thus, the biphasic DDTC cytotoxicity pattern in these cells appears to not be a tissue culture artifact related to the choice of serum or medium. However, it is still conceivable that the biphasic pattern of the rodent cell lines (or its absence in the human cells) might result from unknown tissue culture variables (e.g., the long cumulative durations for which these cell lines have been in culture etc.). It should be noted that the biphasic behavior of T24 in Fig. 2 supports the possibility that human and rodent cell lines might differ quantitatively rather than qualitatively with regard to DDTC sensitivity.

Overall, we conclude from these data that low DDTC or disulfiram concentrations can kill human tumor cells *in vitro* without decreased efficacy at higher drug concentrations. This tumoricidal action may deserve further investigation and should be considered in future studies combining DDTC or disulfiram with traditional chemotherapeutic drugs. Moreover, it is conceivable that rodents and rodent cell lines might not be appropriate for modelling some actions of dithiocarbamates in human tissues.

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