Pharmaceutical Research, Vol. 3, No. 5, 1986

Report

In Vitro Cytotoxic Effects of Dibromodulcitol in 9L Rat Brain Tumor Cells

Stefan-M. Pulst,[1] Victor A. Levin,[1] and Dennis F. Deen[1-3]

Received March 31, 1986; accepted June 9, 1986

Dibromodulcitol (DBD) is a halogenated hexitol active against animal and human brain tumors *in vivo*. In aqueous solution, DBD is transformed to products with different cytotoxicities. We studied the *in vitro* activation and inactivation of DBD with a bioassay in 9L rat brain tumor cells. We developed a mathematical model to calculate the rate constants of activation and inactivation. The kinetics of the activation and inactivation of DBD transformation products in cell culture medium were exponential, with rate constants of 0.139 and 0.0189 hr^{-1}, respectively. The maximum cell kill was caused by DBD that had been preincubated in medium for 13–16 hr. Thus cell kill is not caused by parent drug but by active transformation products.

KEY WORDS: dibromodulcitol; bioassay; cytotoxicity.

INTRODUCTION

Despite the known cytotoxic effects of dibromodulcitol (1,6-dibromo-1,6-dideoxydulcitol) (DBD) against animal and human brain tumors, relatively few studies of the effects of the drug against cultured tumor cells have been conducted (1). The *in vitro* pharmacodynamic transformation of DBD has not been studied, even though it is known that some of the transformation products have different cytotoxicities (2).

The major products of DBD transformation in weakly acidic (pH 6.5–7) aqueous solution are 3,6-anhydro-1-bromo-1-deoxy-DL-galactitol and 1,2:3,6-dianhydro-DL-galactitol (3) (Fig. 1). In weakly alkaline medium (pH 7.5–8.0), mono- and diepoxyhexitols such as bromoepoxygalactitol (1,2-anhydro-6-bromo-6-deoxy-DL-galactitol) (BrEpG) and dianhydrogalactitol (1,2,5,6-dianhydrogalactitol) (DAG) are formed by elimination of HBr (3). Using a transplanted animal tumor model it has been shown that, on a molar basis, DAG is 30 times more cytotoxic than DBD and that BrEpG has an intermediate cytotoxicity (2). In preliminary studies of the *in vitro* cytotoxic effects of DAG, Levin and Wheeler (4) found that similar levels of cell kill were caused by treating 9L cells for 1 and 24 hr, if the concentration × time (exposure integral) product was the same for both treatment periods. The cytotoxic effects of other transformation products are not known. Because these products have different cytotoxicities, simple exposure integral relationships for the parent compound cannot be used to determine the dose effect relationships.

In this paper we describe the effects of DBD on the growth and survival of 9L monolayer cells. Using a bioassay developed in our laboratory, we measured the combined cell killing effects of DBD and its transformation products at increasing times during the transformation of DBD in medium. The results suggest that transformation products and not parent DBD are responsible for cell kill.

MATERIALS AND METHODS

Cell Culture Conditions

9L cells were grown in complete medium (CMEM) consisting of Eagle's minimum essential medium supplemented with nonessential amino acids, fetal calf serum (10%, v/v), and gentamicin (50 μg/ml). Cells had a doubling time of 18–19 hr and had a plating efficiency (PE) of 35 to 65% when maintained in this medium at 37°C in a humidified 95% air–5% CO_2 environment.

Drug Treatment

DBD was dissolved in dimethyl sulfoxide (1:20, w/v) and diluted with 100% ethanol. Solutions were prepared immediately before use. The volume of ethanol added to treatment flasks was always less than 1% of the final treatment volume and had no effect on the PE of cells.

Growth Curves

Cells (2×10^5) were seeded into 25-cm^2 tissue culture flasks. Various volumes of stock DBD solution were added and flasks were incubated under the conditions described

[1] Brain Tumor Research Center of the Department of Neurological Surgery, School of Medicine, University of California, San Francisco, California 94143.

[2] Department of Radiation Oncology, School of Medicine, University of California, San Francisco, California 94143.

[3] To whom correspondence should be addressed at The Brain Tumor Research Center, 783 HSW, University of California, San Francisco, California 94143.

0724-8741/86/1000-0302$05.00/0 © 1986 Plenum Publishing Corporation

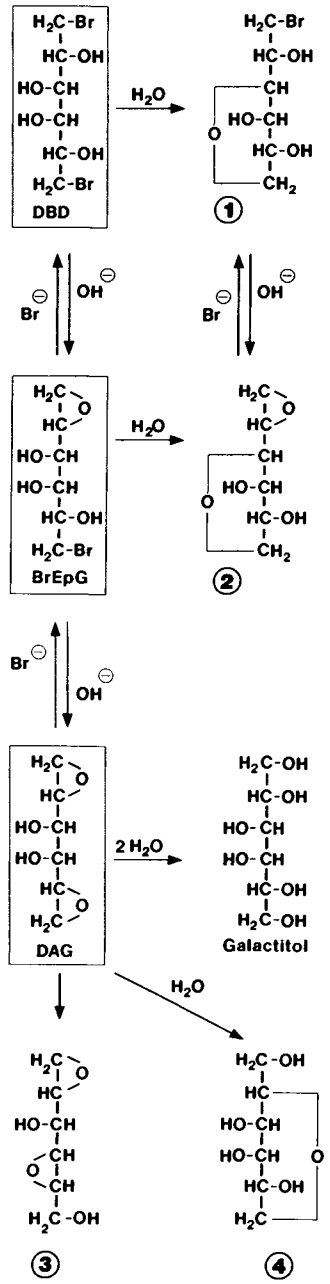

Fig. 1. Breakdown of DBD in aqueous solution, modified from Vidra *et al.* (3). (1) 3,6-Anhydro-1-bromo-1-deoxy-DL-galactitol; (2) 1,2:3,6-dianhydro-DL-galactitol; (3) 1,2:4,5-dianhydro-DL-altritol; (4) 2,6-anhydro-DL-galactitol.

above. At various times, cells were trypsinized with saline A containing 0.05% trypsin and 0.02% versene and counted electronically. Some of the cells used for the growth curves were plated for the colony-forming efficiency (CFE) assay to determine whether cell kill was involved in growth inhibition.

Cell Survival Studies

Cells ($0.5–1 \times 10^6$) were seeded into 75-cm^2 tissue culture flasks and incubated for 24 hr until early log-phase growth was established. After treatment with various concentrations of DBD or ethanol (control) for various times, cells were rinsed, trypsinized, counted, diluted, and plated into 60-mm petri dishes containing 5×10^4 irradiated (40 Gy) 9L feeder cells for the CFE assay (5). After incubation for 13 days at 37°C in a 5% CO_2–95% air atmosphere, colonies were fixed with methanol, stained with crystal violet, and counted. PE was calculated as the ratio of colonies observed to the number of cells plated. The surviving fraction (SF) was calculated as the ratio of the PE of treated cells to the PE of untreated cells.

Optimization of the CFE Assay

For most cytotoxic agents tested in the 9L CFE assay, maximum colony formation occurs when cells are plated into petri dishes with 5×10^4 irradiated feeder cells and incubated for 12–14 days. For DAG, a product of DBD, the optimum incubation time depended on the drug dose and was as long as 19 days for drug doses producing a 2 log cell kill (4). Therefore, we compared PEs at different incubation times using a concentration of DBD that produced a 2 log cell kill. Maximum colony information occurred by 11 days of incubation and the number of colonies formed did not increase further with incubation of up to 17 days. At feeder cell numbers between 1×10^4 and 1×10^5 per petri dish, colony formation was maximal for the serum batch used in these experiments (data not shown). Therefore, all experiments were carried out with 5×10^4 feeder cells per petri dish and an incubation time of 13 days.

Bioassay

Because DBD breaks down into several transformation products, only some of which are cytotoxic, we used a bioassay to determine the total "biological activity" of DBD and its transformation products. DBD was added to cell-free CMEM and incubated at 37°C in a 5% CO_2–95% air atmosphere (pH 7.2–7.4). At various times after incubation was begun, aliquots of DBD-containing medium were withdrawn and added to cell cultures for a 1-hr treatment period. The CFE assay was then performed as described above.

Mathematical Model

To obtain rate constants and halftimes for the activation and inactivation of cytotoxic products, data were fit to a simple mathematical model. It was assumed that DBD and the final reaction products (D_f) were nontoxic, that the intermediate product(s) D^* was toxic, and that product(s) forms by two first-order reactions with rate constants of k_1 and k_2.

$$\text{DBD} \xrightarrow{k_1} D^* \xrightarrow{k_2} D_f \qquad (1)$$

From Eq. (1),

$$D^* = C_0 k_1 (e^{-k_2 t} - e^{-k_1 t})/(k_1 - k_2) \qquad (2)$$

where C_0 is the concentration of DBD added to culture medium. It was further assumed that the SF of cells decreased exponentially with increasing concentrations of D^* such that

$$SF = e^{-\alpha D^*} \quad \text{or}$$
$$D^* = (-\ln SF)/\alpha \qquad (3)$$

Then Eq. (2) can be rewritten as

$$\ln SF = -\alpha C_o k_1 (e^{-k_2 t} - e^{-k_1 t})/(k_1 - k_2) \qquad (4)$$

By varying the values of α, k_1, and k_2, Eq. (4) was fit by computer to the experimental data using a least-squares procedure.

RESULTS

Growth Curves

Continuous treatment with 0.5 μM DBD did not affect the growth of cells, but marked inhibition of growth was caused by treatment with 5 μM DBD (Fig. 2A). Growth was completely inhibited by concentrations of 10 μM or more of DBD (Fig. 2B). Survival of cells treated with 5 μM DBD in this experiment was measured; the SF progressively declined with increasing treatment times and was 7.2×10^{-4} after 87 hr (Table I).

Cell Survival

Treatment for short periods with various concentrations of DBD did not kill many cells; for instance, treatment for 1 hr with 600 μM DBD produced only a 28% cell kill (Table II). Treatment for 24 hr with lower concentrations, however, produced significant cell kill (Fig. 3); the dose–response curve had an initial shoulder followed by an exponential region of cell killing between 10 and 20 μM DBD.

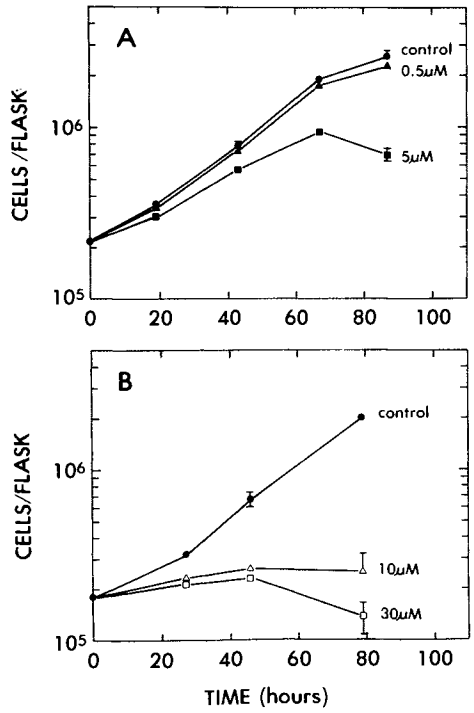

Fig. 2. (A) Growth curve of 9L cells treated with 0, 0.5 and 5 μM DBD. (B) Growth curve after treatment with 0, 10, and 30 μM DBD in a separate experiment. Symbols represent the mean ± SD of three flasks. If the SD is smaller than the data point, bars are not shown.

Table I. Cell Kill as a Function of Time of Treatment with 5 μM DBD[a]

Time (hr)	Surviving fraction
23	0.36 ± 0.07
43	0.023 ± 0.008
67	0.0065 ± 0.0020
87	0.00072 ± 0.00019

[a] Values are means ± SD for four to eight dishes.

Bioassay

Cell-free medium containing DBD was preincubated for various times before it was added to cells. The cell kill caused by a 1-hr treatment with preincubated DBD-containing medium increased during the first 11 to 16 hr of preincubation (Fig. 4), then decreased slowly thereafter. The dose–response curve for cells treated for 1 hr with 50–200 μM DBD in medium that had been preincubated for 13 hr had an initial shoulder followed by an exponential region (Fig. 5).

We used the mathematical model derived above, which assumes that DBD is not cytotoxic (Table II) and that the dose–response curve for the active products is exponential (Fig. 5), to calculate the rate constants involved in the formation and decay of active products. The rate constant for the activation to toxic products (k_1) was 0.139 ± 0.025 hr^{-1} (mean ± SE), and the rate constant for inactivation of toxic products (k_2) was 0.0189 ± 0.0028 hr^{-1}. The value for the constant α [Eq. (3)] was calculated to be 0.0308 ± 0.0027 μM^{-1}.

DISCUSSION

Treatment of 9L cells with 0.5 μM DBD for 87 hr had little effect on cell growth, but treatment with 5 to 30 μM DBD for up to 87 hr progressively inhibited the growth of 9L cells. The SFs of cells used in these growth studies showed that growth delay was caused, for the most part, by cell death (Table I). Several relatively high concentrations of DBD did not cause significant cell kill during a 1-hr treatment; even a dose as high as 600 μM produced only a 28% cell kill after a 1-hr treatment (Table II).

Olah *et al.* (1) found that treatment of CHO cells for 1 hr with 600 μM DBD gave an SF of 0.04. This apparent discrepancy between their data and ours might be explained by different cell-line sensitivities to parent DBD or, possibly, by different methods used to prepare the drug for experiments. Their stock solutions and dilutions were made with saline, in

Table II. Cell Survival After a 1-hr Treatment with Various Concentrations of DBD[a]

Concentration (μM)	Surviving fraction
0	1.00 ± 0.27
200	0.72 ± 0.13
500	0.66 ± 0.08
600	0.72 ± 0.12

[a] Values are means ± SD for four to eight dishes.

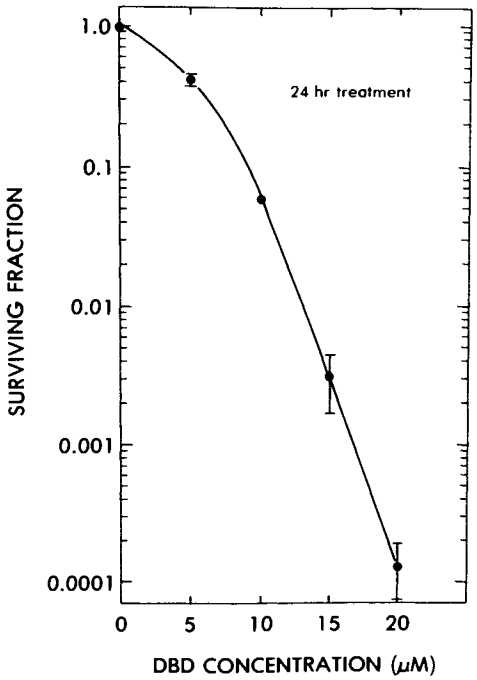

Fig. 3. Twenty-four-hour dose–response curve (●) Mean ± SD of four to eight petri dishes. The portion of the curve from 10 to 20 μM was fit by a least-squares regression analysis. The shoulder was fit by eye.

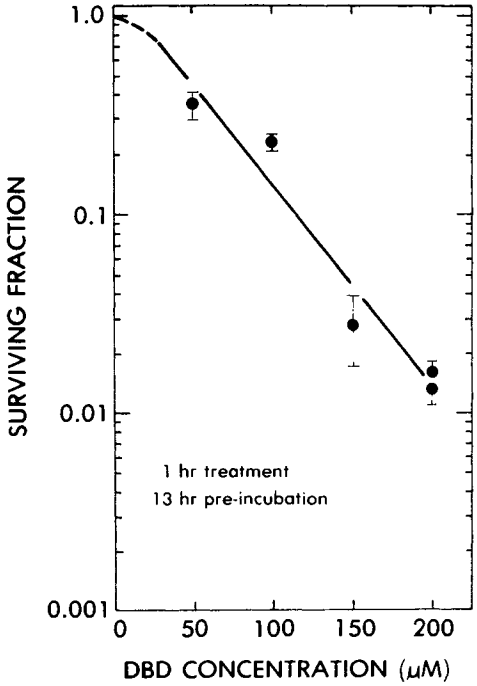

Fig. 5. One-hour dose–response curve after 13 hr of preincubation of DBD. The portion of the curve between 50 and 200 μM was fit by a least-squares analysis.

which DBD could break down into products. We made stock solutions and dilutions with a mixture of dimethyl sulfoxide and alcohol immediately before treatment. DBD is stable in this mixture for up to 24 hr (6).

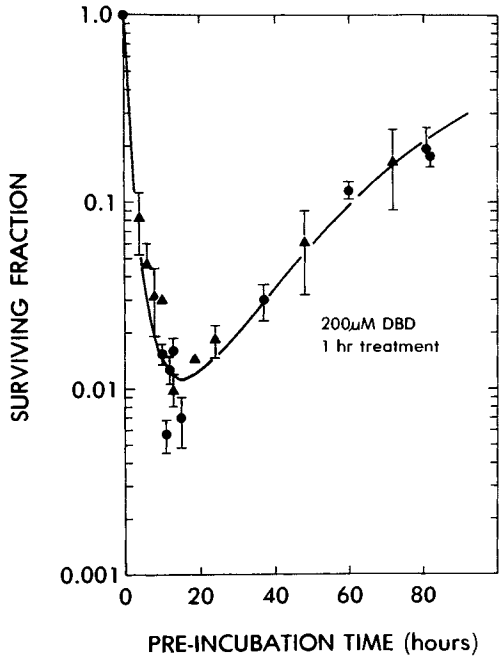

Fig. 4. Formation and decay of cytotoxic products of 200 μM DBD as determined with the bioassay. Symbols represent the mean ± SD for two experiments with four to eight petri dishes each. The curve was fit to the mathematical model described in Materials and Methods.

The hydrolysis and transformation reactions of DBD are complex (Fig. 1) and lead to a number of end products that form from several intermediates. Of the products that have been isolated and characterized, only BrEpG and DAG have been tested in animals and found to be cytotoxic (2). In addition to these two compounds, Horvath *et al.* (7) isolated anhydrogalactitol (1,4-anhydro-DL-galactitol) (AG), dulcitol, and five products of unknown structure from the urine of humans treated with DBD; these authors reported that several of the uncharacterized compounds contained bromine and/or had some alkylating activity.

Rate constants for the various reactions of DBD and its transformation products in aqueous solution or in plasma are not known. The halftime in aqueous solution for the elimination of one bromide from DBD is 3.5 hr (8). Data for excretion of DBD in urine indicate that DBD is almost completely converted to other compounds within 8 hr after oral administration, while BrEpG and DAG are almost completely excreted by 24 hr; other products are found in urine for at least 48 hr after administration (7,9).

The assumption of the mathematical model that formation and decay of toxic products occur with only one rate constant for each process is an obvious oversimplification of the sequence of reactions of transformation of DBD (Fig. 1). Nonetheless, the model provides a good graphic fit of the data, possibly because in this simple model some products such as DAG are so cytotoxic that rates of reaction leading to less toxic intermediates such as BrEpG do not influence greatly the shape of the survival plot. The same may be true for the cytotoxicity and rate constants for the decay products, which we assumed to be nontoxic. These possibilities could be examined by a detailed study of the early and late parts of the curve in Fig. 4 using various concentrations of DBD.

Results obtained with the bioassay cannot be used to measure the concentrations of single products or the rate constants of their formation but can be used to measure the total cytotoxic activity of all products. The advantage of the *in vitro* bioassay for the study of DBD and its products is that the combined cytotoxic effect of all compounds can be studied without the interference of the complex processes of oral absorption, renal clearance, tissue uptake, and alkylation of plasma proteins (7). In the studies reported here, cytotoxicity increased with a halftime of 5 hr, and the maximum cell kill occurred between 13 and 16 hr of preincubation. Cytotoxicity decreased slowly thereafter with a halftime of 37 hr.

The results of this study show that the maximum cytotoxic effect of DBD against 9L cells is obtained after preincubation in medium for 13–16 hr. Based on this and other results, it appears that parent DBD is not cytotoxic and that intermediate products, which form over relatively long incubation periods, are responsible for the cytotoxic effects observed.

ACKNOWLEDGMENTS

This work was supported in part by NIH Grants CA-31867 and CA-31868 and Program Project Grant CA-13525, and the Aaron Silvera Fund. S.-M.P. was supported in part by a training grant from the Deutsche Krebshilfe. We thank Herbert D. Landahl, Ph.D., for the derivation of the mathematical model and Neil Buckley for editing the manuscript.

REFERENCES

1. E. Olah, I. Palyi, and J. Sugar. *Eur. J. Cancer* 14:895–900 (1978).
2. I. P. Horvath, S. Somfai-Relle, L. Hegedus, and M. Jarman. *Eur. J. Cancer Clin. Oncol.* 18:573–577 (1982).
3. I. Vidra, K. Simon, and L. Institoris. *Carbohydrate Res.* 111:41–57 (1982).
4. V. A. Levin and K. T. Wheeler. *Cancer Chemother. Pharmacol.* 8:125–131 (1982).
5. D. F. Deen, P. M. Bartle, and M. E. Williams. *Int. J. Radiat. Oncol. Biol. Phys.* 5:1663–1667 (1979).
6. Product specification insert, Chicoin Chemical and Pharmaceutical Works, Budapest, Hungary.
7. I. P. Horvath, J. Csetenyl, I. Hindy, S. Kerpel-Fronius, L. Institoris, and S. Eckhardt. *Eur. J. Cancer Clin. Oncol.* 15:337–344 (1979).
8. L. Institoris, J. Kacmarek, I. Vidra, and K. Szebeni. In *Mitolactol, Chinoin Chemical and Pharmaceutical Works,* Budapest, Hungary, 1983, p. 16.
9. I. P. Horvath, J. Csetenyl, I. Hindy, S. Kerpel-Fronius, L. Institoris, L. Hegedus, and S. Eckhardt. *Eur. J. Cancer Clin. Oncol.* 18:1211–1219 (1982).