Effect of Inhibitors of Plant Cell Division on Mammalian Tumor Cells

Shmuel Zilkah, Michael E. Osband, and Ronald McCaffrey

School of Medicine, Boston, Massachusetts 02118
Pediatrie Hematology-Onco/ogy, Department of Pediatrics, Boston City Hospital, Boston 02118, and Departments of Medicine and Pediatrics, Boston University School of Medicine, Boston, Massachusetts 02118

ABSTRACT

We studied the activity of 14 compounds, all of which have been shown to interfere in plant cell division, in two animal tumor cell cultures, EL-4 and L1210. Four compounds [propham, chlorpropham, bensulide S-(O,O-diisopropylphosphorodithioate) ester of N-(2-mercaptoethyl)benzenesulfonamide, and siduron] had a 50% inhibitory dose less than $10^{-4}$ M; six [2,3,5-triodobenzoic acid, (2,4-dichlorophenoxy)acetic acid, bromacil, (2,4,5-trichlorophenoxy)acetic acid, naphtalam, and (4-chloro-2-methylphenoxy)acetic acid] had a 50% inhibitory dose between $10^{-4}$ and $10^{-3}$ M, and the remaining four 2,3,4,6-di-O-isopropylidene-2-keto-L-gulonate, eptam, maleic hydrazide, and 4-(methylsulfonyl)-2,6-dinitro-N,N,N,dipropylaminoethyl sulfone] had a 50% inhibitory dose at higher than $10^{-3}$ M. There was a significant correlation between the effect on the two cell lines as well as between the inhibition of cell proliferation and that of thymidine and leucine uptake. More detailed study of cell proliferation and leucine and thymidine uptake for bensulide and 2,3,5-triodobenzoic acid revealed a dose-response pattern of inhibition starting shortly after exposure of the cells to the compounds. These results indicate that some inhibitors of plant cell division are capable of inhibiting the proliferation of animal tumor cells.

INTRODUCTION

The major sites of mitotic activity in plants are stem and root tips. The growth of these tissues, termed meristematic tissues, is inhibited by a variety of plant growth regulators and herbicides. Because both meristematic tissue and mammalian tumors contain dividing cells, we have asked whether those herbicides which are active against meristematic tissue might also inhibit the growth of mammalian tumor cells.

While there has been extensive research of herbicides as potential carcinogenic agents (9, 10, 12, 17), there have been very few reported attempts to study the potential of these compounds as inhibitors of mammalian cells (2, 6, 16, 19, 21). The obvious discrepancies between plant and animal life might lead to differences in drug responsiveness; nonetheless, significant correlation was found between the ability of 17 herbicides to inhibit the growth of mammalian tumor cells.

We report here the results of a study which examined the effect of 14 inhibitors of plant cell division on the proliferation of cultured EL-4 and L1210 cells.

MATERIALS AND METHODS

Chemical Compounds. The 14 compounds tested are listed in Table 1. All are commercially available herbicides or plant growth regulators. Compounds of high purity were purchased or provided by the manufacturers for use in these studies. Propham, chlorpropham, maleic hydrazide, (2,4-dichlorophenoxy)acetic acid, (2,4,5-trichlorophenoxy)acetic acid, and TIBA were purchased from Sigma Chemical Company (St. Louis, Mo.). Nitralin and (4-chloro-2-methylphenoxy)acetic acid were purchased from Chem Service (West Chester, Pa.). Naptalam was obtained from Uniroyal (Naugatuck, Conn.), dikegulac was obtained from Maag Agrochemicals (Vero Beach, Fla.). Bromacil and siduron were obtained from E. I. DuPont de Nemours & Co., Inc., (Wilmington, Del.), and eptam and bensulide were obtained from Stauffer Chemical Corporation (Richmond, Va.).

Stock solutions were prepared freshly for each experiment by dissolving the compounds in ethyl alcohol (final v/v concentration of the ethyl alcohol in growth medium, 0.15 to 0.4%) except for nitralin, which was dissolved in acetone (0.3% final v/v concentration), and maleic hydrazide and dikegulac, which were dissolved directly in growth medium.

Cell Culture. L1210 mouse leukemia cells and EL-4 mouse lymphoma cells were obtained from Drs. M. Bennet and V. Kumar at the Boston University School of Medicine. The cells were cultured routinely in growth medium consisting of Roswell Park Memorial Institute Medium 1640 (M. A. Bioproducts, Walkersville, Md.) containing 10% fetal bovine serum, streptomycin, penicillin, nonessential amino acids, sodium pyruvate, and glutamine (all from Microbiological Associates, Bethesda, Md.). The cells were incubated at 37°C in moist air containing 5% CO₂ in tissue culture flasks (25 sq cm/50 ml; Costar, Cambridge, Mass.).

Growth Inhibition Studies. The inhibition studies were conducted in tissue culture flasks containing 6 ml of growth medium and 2 x 10⁴ cells/ml.

The stock solutions of the plant inhibitors were diluted in the growth medium to achieve a variety of concentrations. Control experiments were performed using identical final concentrations of the various solvents. The effect of herbicide exposure...
on cell proliferation was calculated by the equation

\[
\text{Treated cultures (no. of cells at conclusion of incubation)} - \text{Control cultures (no. of cells at beginning)} \times 100
\]

Cell viability was determined by trypan blue dye exclusion. All experiments were performed at least in duplicate.

**[3H]Thymidine and [3H]Leucine Uptake.** Aliquots (0.2 ml) of cell suspension were removed from each flask at various times and placed in wells of a microtiter plate (Flow Laboratories, Hamden, Conn.). [3H]Thymidine, 0.15 μCi (2 Ci/mmole; New England Nuclear, Boston, Mass.), or [3H]leucine, 0.3 μCi (5 Ci/mmole; New England Nuclear), was added to the wells. After incubation at 37°C for 1.5 hr, the cells were harvested onto filler mats using Titertek Cell Harvester (Flow Laboratories), and the radioactivity was determined.

**RESULTS**

The 14 inhibitors of plant cell division used are listed in Table 1. As can be seen, they are of relatively low toxicity, as defined by the Environmental Protection Agency on the basis of the acute 50% lethal p.o. dose for animals (11). No compounds were in Category I, the highest toxicity; one compound was in Category II, 8 compounds were in Category III, and 5 compounds were in Category IV.

**Inhibition of Cell Proliferation.** All 14 compounds were tested at concentrations of 10⁻⁵, 10⁻⁴, and 10⁻³ M for their ability to inhibit in vitro proliferation of EL-4 and L1210 cells. From these experiments, the ID₅₀ was calculated (Chart 1). The compounds varied in their ID₅₀. Although dikegulac and nitralin had an inhibitory effect at lower concentrations, the ID₅₀ was higher than 10⁻³ M. Eptam and maleic hydrazide had virtually no effect at that concentration. The compounds related to the phenoxy acid group, such as (2,4-dichlorophenoxy)acetic acid, showed a moderate inhibition (ID₅₀ between 10⁻⁴ and 10⁻³ M). The carbamates (propham and chlorpropham) had an inhibitory effect at lower concentrations, the ID₅₀ was higher than 10⁻³ M. Eptam and maleic hydrazide had a relatively potent inhibitory effect, achieving an ID₅₀ around 10⁻⁴ M. Chart 1 also shows that there was an overall high correlation for all the compounds in their effects against the 2 different cell lines (p < 0.01).

**[3H]Thymidine and [3H]Leucine Uptake.** As in the experiments measuring cell proliferation, the compounds were tested at concentrations of 10⁻⁵, 10⁻⁴, and 10⁻³ M for their ability to inhibit in vitro proliferation of EL-4 and L1210 cells. From these experiments, the ID₅₀ was calculated (Chart 1). The compounds varied in their ID₅₀. Although dikegulac and nitralin had an inhibitory effect at lower concentrations, the ID₅₀ was higher than 10⁻³ M. Eptam and maleic hydrazide had virtually no effect at that concentration. The compounds related to the phenoxy acid group, such as (2,4-dichlorophenoxy)acetic acid, showed a moderate inhibition (ID₅₀ between 10⁻⁴ and 10⁻³ M). The carbamates (propham and chlorpropham) had an inhibitory effect, achieving an ID₅₀ around 10⁻⁴ M. Chart 1 also shows that there was a high correlation for all the compounds in their effects against the 2 different cell lines (p < 0.01).
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Chart 1. Inhibition of cell proliferation of L1210 and EL-4 tumor cells by various inhibitors of plant cell division. The code letters correspond to the compounds listed in Table 1, Column 1. The ID₅₀ was determined for each compound as explained in the text and is given on the abscissa for L1210 cells and on the ordinate for EL-4 cells. The correlation between the ID₅₀ for the 2 cell lines is significant at p < 0.01.

Chart 2. Inhibition of thymidine and leucine uptake in L1210 and EL-4 cells by inhibitors and correlation with the inhibition of cell proliferation. The code letters refer to the compound as listed in Table 1, Column 1. The ID₅₀ for cell proliferation is on the ordinate; the ID₅₀ for thymidine and leucine uptake is on the abscissa. The correlation between the ID₅₀ for cell proliferation and either thymidine or leucine uptake is p < 0.001.

compounds on thymidine and leucine uptake is similar to their effect on cell proliferation. This implies that the metabolic machinery of the remaining treated cells in culture is not significantly changed. This conclusion is supported by the finding that the viability of the cells, as measured by trypan blue, is not significantly decreased at intermediate levels of inhibition of cell proliferation (data not shown).

Dose Response and Kinetics of TIBA and Bensulide. In order to study more carefully the effect of such plant inhibitors on animal cells, detailed data for 2 such agents, TIBA and bensulide, are given in Charts 3 to 5. The dose response of these 2 inhibitors of plant cell division on proliferation of EL-4 and L1210 cells is given in Chart 3. As can be seen, the inhibition of growth is concentration dependent. Bensulide inhibits up to 100% over a concentration range of 5 to 100 μM; with TIBA, comparable inhibition is seen over a concentration range of 50 to 250 μM.

The kinetics of the TIBA and bensulide inhibition of proliferation and thymidine and leucine uptake of EL-4 and L1210 cells is presented in Charts 4 and 5. Cells were inoculated at the same density into a series of tissue culture flasks in control medium or in medium containing various concentrations of the 2 compounds. At various times, the cultures were checked for cell number, and thymidine and leucine uptake was determined.

In control cultures, the average doubling time for cell number was 20 hr. Thymidine and, to a lesser degree, leucine uptake did not correlate with cell number but occurred at a slower rate (Chart 4).

TIBA, at 3 × 10⁻⁴ M, caused immediate inhibition of thymidine and leucine uptake, although some cell proliferation continued. The greatest inhibitory effect of the compound occurred on the first day (Chart 4). A lower concentration of drug, 1 × 10⁻⁴ M, produced similar effects, although to a lesser extent. The viability of the 10⁻⁴ M TIBA-treated cells was not significantly different from that of the control cultures. The viability of 3 × 10⁻⁴ M TIBA-treated cells, although lower than control, was relatively high (72 to 83%) in view of the strong inhibition of cell proliferation (90%) seen at that concentration.

The effect of various concentrations of bensulide on cell proliferation and thymidine uptake of EL-4 cells is shown in Chart 5. The inhibition of cell proliferation is dose dependent,
being greatest at $3 \times 10^{-5}$ M. In addition, the viability of the cells treated with that concentration is very low (4 to 17%). There is a similar dose-dependent inhibition of total thymidine uptake. However, when the thymidine uptake is determined per viable cell, no inhibition is seen (data not given). While the 2 lowest concentrations of bensulide still produce demonstrable inhibition of cell proliferation, there was no corresponding inhibition of thymidine uptake.

**DISCUSSION**

Much data have been accumulated concerning the efficacy, mode of action, and toxicity of a variety of compounds active on plant life. Very little of this data has been applied to the study of animal tumors.

We report in this paper the results of in vitro testing of a variety of herbicides against EL-4 mouse lymphoma cells and L1210 mouse leukemia cells. The 14 compounds tested in these experiments were chosen for 2 reasons: (a) each of them has been identified as an inhibitor of plant cell division, making it possible that some of them would be capable of inhibiting the growth of dividing animal tumor cells; and (b) because the toxicity of these compounds (Table 1) is lower than that of other groups of herbicides (1, 4), it might be possible to test in vivo any compounds found to be active in vitro.

Initially, we have chosen to study these compounds in vitro in a manner similar to that done in plant cell cultures for the study of phytotoxicity (22–24). Animal cell cultures used in this way are a sterile and homogeneous system in which all cells are metabolically active, and there are fewer problems concerning the administration, inter- and intracellular transport, and absorption of compounds.

Also, although the testing of herbicides identified previously as inhibitors of plant cell division has not been conducted in any organized way, a few reports have discussed the results of studying the effect of isolated compounds on animal cells (2,
6, 16, 18, 19, 21). Interestingly, in all of these reports, the results indicated a biological effect by some of the compounds tested.

The compounds demonstrated marked variability in their inhibition of cell proliferation. Some compounds (dikegulac, eptam, maleic hydrazide, and nitralin) were active only in high concentrations (10^{-3} m), while besulide and the carbamates (propham and chlorpropam) achieved an ID_{50} at less than 10^{-5} m. There was good correlation in the effect of the various compounds on the 2 cell lines (Chart 1).

Another interesting compound is dikegulac. This compound is used primarily as a pinching agent (2) (a compound which inhibits specifically apical meristematic growth, thereby stimulating lateral growth). This compound never showed greater than 40% inhibition in any of the concentrations used.

Another compound that we tested, maleic hydrazide, was shown in another study to be protective against tumorigenesis (2) but was inactive in our experiments.

We were interested as well in the possible effect of the various compounds on cell metabolism, even if it did not result in actual inhibition of proliferation. We therefore studied the effect of these compounds on protein and DNA synthesis, using leucine and thymidine uptake, respectively.

In general, there was good correlation between the ID_{50} for inhibition of cell proliferation and that for inhibition of thymidine and leucine uptake (Chart 2). The most important exceptions to this are the carbamate compounds, which have an ID_{50} of about 10^{-5} m for cell proliferation but require a 10-fold higher concentration for comparable inhibition of leucine and thymidine uptake. The significance of this discrepancy is uncertain. One possible explanation is that these compounds are cycle specific and are thereby selectively enriching the proportion of cells with a high metabolic rate.

In order to better understand the kinetics of these compounds, we selected high activity, TIBA and besulide, for more detailed study.

Besulide demonstrates inhibition in a dose-responsive pattern, as does TIBA. The range of effect of both of these compounds is very narrow, being only approximately an order of magnitude between a concentration without any effect and that with maximal inhibition (Chart 3).

The viability of TIBA-treated cells is relatively high, given the strong inhibition of cell proliferation (Chart 4). This result might mean that the TIBA-treated tumor cells were viable until their destruction. In support of this hypothesis is the result that thymidine uptake per viable cell is not significantly different between TIBA-treated cells and control, implying that the remaining cells possess a relatively normal metabolic rate.

Very few of the remaining cells were viable after treatment with the highest concentration of besulide (3 \times 10^{-5} M) (Chart 5). However, when thymidine uptake per viable cell is determined, there is little difference between treated cells and control.

We believe that we have demonstrated clearly that some inhibitors of plant cell division are active in vitro against animal tumor cells. We are uncertain as to the significance of this. We are doing further work, both in vitro as well as in vivo, to gain a better understanding of the nature of these compounds and their possible potential as tumor chemotherapeutics.

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