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Antineoplastic Drug Cytotoxicity in a Human Bladder Cancer Cell Line: Implications for Intravesical Chemotherapy

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Summary. The clonogenic survival of MGH-U1 human bladder carcinoma cells treated with melphalan, cisplatin, mitomycin-C, adriamycin, vincristine and 5-fluorouracil was measured to determine the relative contribution of drug concentration and duration of exposure to cytotoxicity and to measure the relative cytotoxic effects of these agents used in intravesical chemotherapy. The survival curves were plotted as a function of log (C x T) and were fitted using a linear least squares analysis. The survival was the same for any given C x T whether this was determined by varying concentration or by varying the duration of exposure in the cases of melphalan, cisplatin, adriamycin, mitomycin-C and 5-fluorouracil. However, duration of exposure was more important than was drug concentration in the case of vincristine cytotoxicity. By utilizing the slope of the log (survival fraction) as a function of log (C x T), the relative cytotoxicity of each agent was determined. Mitomycin C, melphalan, adriamycin and cisplatin had comparable activity in this cell line, whereas vincristine and 5-fluorouracil demonstrated much lower cytotoxicity. We conclude that: 1) mitomycin-C, adriamycin and melphalan were the agents with the greatest cytotoxic efficacy; 2) determination of survival as a function of C x T can be used to separate the relative importance of concentration and of duration of exposure. 3) the cytotoxicity of 5/6 drugs studied was equal when the C x T was kept constant but concentration and exposure times were varied.

Key words: Bladder cancer, Intravesical chemotherapy.

Introduction

Intravesical chemotherapy is useful in the treatment of human bladder carcinoma in various groups of patients with high grade tumours, invasion of the lamina propria, multifocal tumours, positive cytotology or large primary tumours [7]. These biological characteristics have been associated with a high incidence of recurrence [5, 19], morbidity and ultimately mortality. It is suggested that if chemotherapy is administered when the tumour burden is low, then the likelihood of eradicating all clonogenic tumour cells is much greater than when the tumour mass is large. Intravesical chemotherapy delivers high concentrations of drug with limited systemic exposure; this depends upon the regional exchange rate, total body clearance and varies between drugs [3].

Two important questions must be addressed to achieve the therapeutic advantage of intravesical chemotherapy in the population of patients at high risk of recurrence. What is the relative importance of drug concentration and instillation time in the treatment? Which is the most active agent? The use of various agents at varying doses and for variable periods of instillation has been reported [4, 6, 9, 12, 15, 17]. These choices appear to be dictated by drug toxicity and clinical experience. We determined the cytotoxicity of six antineoplastic agents in the MGH-U1 human bladder cancer cell line grown in monolayer using varying drug concentrations and exposure times. The drugs included agents active in the treatment of bladder cancer; both phase and cycle specific agents. The MGH-U1 human bladder cell line was selected because it was derived from a high grade tumour and has been extensively characterized [10, 14].

Materials and Methods

MGH-U1 cells were obtained from Dr. G. Prout and his colleagues (Boston, MA). Adriamycin was supplied by Adria Laboratories Inc. (Dublin, OH). Cisplatin was purchased from Bristol Laboratories (Syracuse, NY). Mitomycin-C was a gift from Bristol Laboratories (Syracuse, NY). 5-Fluorouracil and melphalan were purchased from Sigma Chemical Co. (St. Louis, MO). Trypsin and Noble agar were purchased from Difco (Detroit, MI). Solution, A, a calcium-free PBS was preapred as follows: 8 g sodium chloride, 0.2 g potassium

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Table 1. Linear least square analysis of survival curves

Drug	Fixed duration of exposure (h)	Fixed exposure concentration (g/ml)	m[a]
Melphalan	1	–	−3.76
	–	6	−4.16
Cisplatin	2	–	−2.37
	–	4.5	−3.30
Adriamycin	2	–	−3.84
	–	1	−3.47
Mitomycin C	1	–	−4.00
	–	0.5	−3.63
Vincristine	24	–	−0.71
	–	0.04	−1.42
5-fluorouracil	6	–	−1.67
	–	20	−1.87

[a] Slope of the linear least square fit equation

chloride, 2.17 g of disodium phosphate, 0.2 g potassium phosphate, 0.1 g glucose, 2.4 g phenol red, 0.606 mg penicillin-G and 1.35 mg streptomycin sulfate were added to 1 l of double distilled water. Tissue culture flasks were purchased from Costar (Cambridge, MA) and petri dishes from Falcon (Oxnard, CA).

Adriamycin and mitomycin-C were initially dissolved in normal saline, cisplatin was dissolved in double-distilled water, 5-fluorouracil was dissolved in PBS and melphalan was dissolved in ethanol:HCL solution prior to each experiment. Each drug was serially diluted in α-MEM (18) with 10% (v/v) FCS (Flow Laboratories Inc., Rockville, (MD). 5-Fluorouracil was diluted in α-MEM without nucleosides and with 10% (v/v) dialyzed FCS.

Cells were seeded into control and drug-treated flasks at a density of 6–7 x 10^3 cells/cm^2 48 h prior to drug treatment. On the day of treatment, during exponential cellular growth the media was removed and drug-containing media was added at the appropriate concentrations for the specified periods of time. Drug-containing media was removed and the cells were washed twice with equal volumes of PBS and then once with an equal volume of solution A at the end of the exposure time. The cells were trypsinized using 0.05% trypsin with 0.02% EDTA in solution A and the trypsin-EDTA solution was neutralized with an equal volume of α-MEM with 10% (v/v) FCS. The cells were inspected microscopically to ensure a mono-dispersed population and counted using a Model-F Coulter Counter, then diluted appropriately and placed in 60-mm petri dishes at varying dilutions in replicates of 6 for control and 3 for drug-treated cells. The dishes were incubated for 12 days at 37 °C with 5% CO_2 and then removed, fixed with methanol for 10 min and stained with a 1:1 (v/v) dilution of Giemsa (Fisher Scientific Co., Fairlawn, NJ) for 30 min. Colonies visible by eye were counted and survival in drug-treated dishes was expressed as a fraction to control survival. Each experiment was performed at least three times.

The in vitro C x T was calculated as the product of drug concentration and exposure time. The logarithm of the mean surviving fraction for each data point was plotted as a function of log (C x T). This data was fitted to a linear equation with the following form: log(mean surviving fraction) = m [log (C x T)] + b using linear least squares analysis, where m was the slope of the line and b was the intercept (Table 1).

Results

The survival of MGH-U1 cells exposed to melphalan, cisplatin, adriamycin, mitomycin-C, vincristine and 5-fluorouracil are plotted as a function of (C x T) in Figs. 1 and 2. The data points derived from experiments in which concentration was varied and time was constant and experiments in which time was varied and concentration was constant are

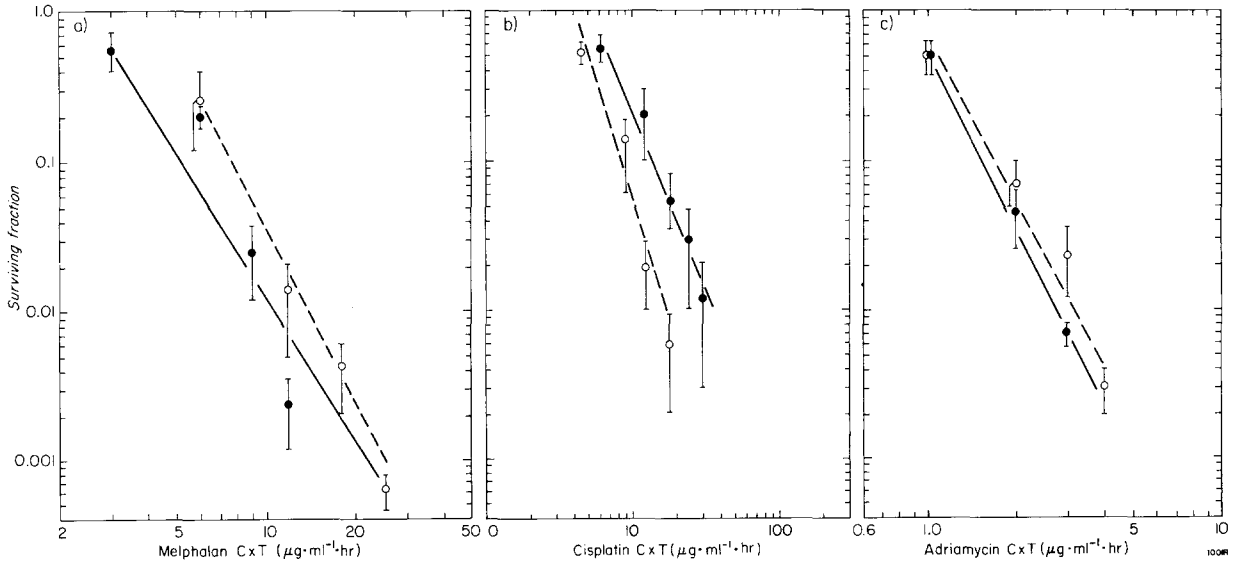

Fig. 1a–c. Clonogenic survival of MGH-U1 cells treated with melphalan as a function of C x T (a). Exposure time, 1 h (○); exposure concentration, 6 μg/ml (○). Error bars represent standard deviation (n =3). b Clonogenic survival of MGH-U1 cells treated with cisplatin as a function of C x T. Exposure time, 2 h (○); exposure concentration, 4.5 μg/ml (○). Error bars represent standard deviation (n = 4). c Clonogenic survival of MGH-U1 cells treated with adriamycin as a function of C x T. Exposure time, 1 h (○); exposure concentration, 1 μg/ml (○). Error bars represent standard deviation (n = 3)

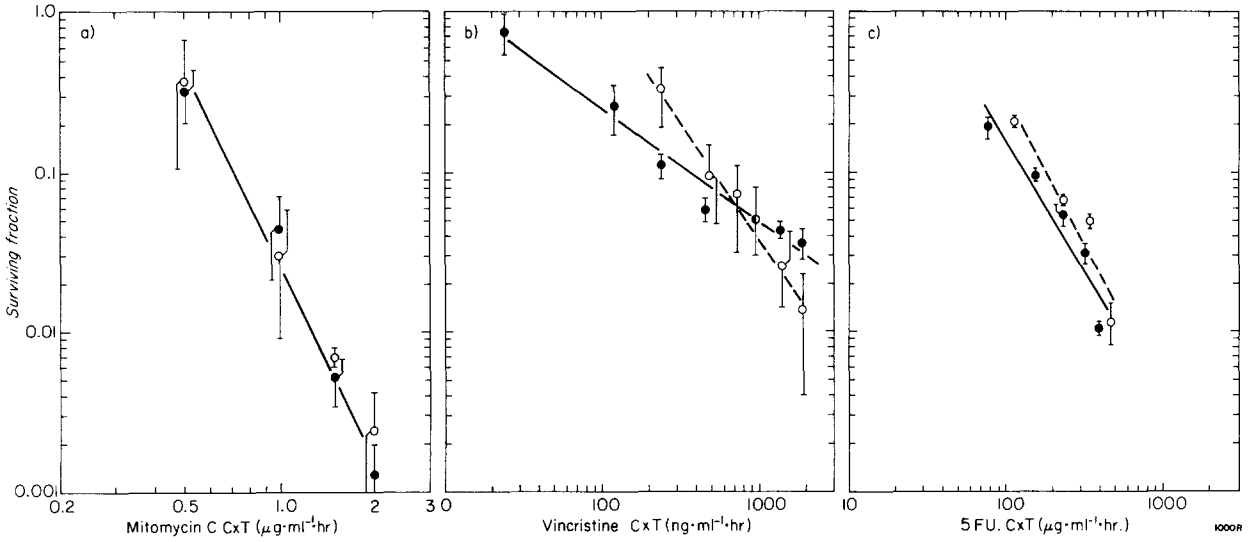

Fig. 2a–c. Clonogenic survival of MGH-U1 cells treated with mitomycin-C as a function of C x T (**a**). Exposure time, 1 h (○); exposure concentration, 0.5 μg/ml (○). Error bars represent standard deviation (n = 3). **b** Clonogenic survival of MGH-U1 cells treated with vincristine as a function of C x T. Exposure time, 24 h (○); exposure concentration, 40 ng/ml (○). Error bars represent standard deviation (n = 3). **c** Clonogenic survival of MGH-U1 cells treated with 5-fluorouracil as a function of C x T. Exposure time, 6 h (○); exposure concentration, 20 μg/ml (○). Error bars represent standard deviation (n = 5)

plotted in the same figure for each drug to allow a direct comparison of the effects of varying concentration and time. The correlation coefficient for each curve generated by the linear least square analysis was greater than 0.89.

The clonogenic survival of the MGH-U1 cells treated with varying concentrations of melphalan for a fixed exposure time was similar to their survival when the exposure time was varied and concentration was fixed (Fig. 1a). The survival curves of the MGH-U1 cells treated with cisplatin (Fig. 1b), adriamycin (Fig. 1c) and mitomycin-C (Fig. 2a) were the same whether the exposure time or the concentration time was varied. When MGH-U1 cells were exposed to vincristine for 24 h at concentrations varying between 1 ng/ml and 80 ng/ml and plotted on a semi-log plot, the survival was characteristically bi-exponential with a plateau being approached at C x T greater than 400 ng/ml/h^{-1} (not shown). When the concentration was kept constant at 40 ng/ml and the exposure time varied, the survival curve was bi-exponential also, but the terminal portion of the curve had a steep slope which did not plateau at the highest C x T assessed (not shown). This is reflected in the steeper survival curve observed when the time of exposure was varied (Fig. 2b). The survival of MGH-U1 cells as a function of C x T for 5-fluorouracil when cells were exposed for 6 h to varying concentrations of drug or to 20 μg/ml 5-fluorouracil for various exposure times is plotted in Fig. 2c. No difference in the survival curves could be demonstrated.

The activity of the six agents was ranked according to the slope to compare the cytotoxic efficacy of the drugs because it reflected the amount of cell kill as a function of drug exposure (C x T). In this analysis, a steeper slope indicated a greater cytotoxic effect. When this was done for values of m derived from the experiments with fixed exposure durations, mitomycin-C had the steepest slope and was ranked first. When the ranking was performed for values of m from experiments with fixed exposure concentration, melphalan was the most effective agent and, for the other 5 drugs, ranking was decreased by one accordingly.

Discussion

We undertook these studies to define the relationship between drug concentration and duration of exposure using a colony-forming assay. The C x T was chosen as the independent variable allowing us to compare the effects of varying exposure duration or concentration on cytotoxicity over a range of C x T values. The 6 agents selected – melphalan, cisplatin, adriamycin, mitomycin-C, vincristine and 5FU – represent drug categories with different mechanisms of action, act at different sites in the cell cycle, and some have been used in the treatment of bladder cancer in man.

The results of our studies suggest that survival of MGH-U1 cells as a function of C x T depends on the drug tested. For melphalan, cisplatin, adriamycin, mitomycin-C and 5-fluorouracil, cytotoxicity is independent of varying concentration or exposure time if the C x T remains constant. The studies of vincristine revealed that the manner in which the C x T is achieved can affect the cell survival. Prolonged exposures to low concentrations of vincristine resulted in greater cell kill than higher concentrations for short time periods. This may reflect the phase specificity of vincristine activity and the doubling time of MGH-U1 cells under the experimental conditions used (20 h). The continued decrease in survival of cells exposed to a constant concentration of drug for prolonged exposure times enables more cells to pass through mitosis and be exposed to the cytotoxic ef-

fects of vincristine. Hence, the survival decreased by log 1.42 for a 1 unit increase in C x T when the exposure duration was varied but only log 0.71 when the drug concentration varied. In contrast to vincristine, 5-fluorouracil cytotoxicity was similar when an exposure time of 6 h was used or when 20 g/ml 5-fluorouracil was employed to achieve similar C x T. This finding was surprising because 5-fluorouracil has a major effect on thymidylate synthetase which inhibits DNA synthesis [2]. The lack of dependence on exposure time by 5-fluorouracil may be due to factors which are independent of cell cycle and cause cell death. The incorporation of 5-fluoruracil into the RNA [1, 11, 16] may be one such factor with a prolonged effect, whereas the inhibition of thymidylate synthetase by 5-fluorouracil metabolites and the subsequent inhibition of DNA synthesis during S-phase would be related to cell cycle.

The relationship beween concentration and duration of exposure in causing cytotoxicity is complex. We attempted to simplify the analysis of this interaction by expressing the surviving fraction as a function of C x T. The C x T is a pharmacokinetic parameter which can be used as a measure of overall drug exposure. This approach lends itself to unweighted linear least square analysis of the log transformations of survival fraction and C x T. A good correlation between log (surviving fraction) and log (C x T) was observed in the case of each agent which we studied.

The ranking of drug activity was defined by determining the slope of the linear least square fit equation which fitted the survival data. The slope was used because it was derived independent of assumptions about mechanism of drug action. Since the mechanisms of action for each of these agents differ and may be multiple or poorly defined, this approach is unbiased by underlying assumptions. Furthermore, the slope gives a measure of clonogenic cell kill per log (C x T) allowing a comparison between drugs. The ranking from this analysis would suggest that mitomycin-C, adriamycin, cisplatin, and melphalan appear to be the most active agents, whereas 5-fluorouracil and vincristine demonstrate low activity. Hepburn et al. [8] reported the cytotoxicity of 13 agents in the RT112 cell line. However, these investigators studied one exposure duration and used the ID_{70} value to rank the drug activity. Although their results are compatible with the clinical activity of the agents in bladder cancer treatment, the importance of concentration and exposure duration were not assessed. Furthermore, the use of ID_{70} to rank the drugs is relatively arbitrary and does not take into consideration the shape of the survival curve over the complete drug range studied.

Our studies suggest that equivalent cytotoxic activity can be achieved by varying drug concentrations and exposure durations such that the C x T is constant for each drug tested except for vincristine. Mitomycin-C, adriamycin and melphalan are the most active agents against this cell line. The high growth fraction and direct access of drug to cells may not reflect the growth characteristics of bladder tumours in man but may contribute to greater cell kill than is possible in vivo. The use of a single cell line does not allow extrapolation of in vitro activity to the clinical setting and can be used only to guide the clinician regarding the agents. Nevertheless, the clinical implication of these studies is that the concentration of drug and exposure duration for intravesical chemotherapy may be varied to achieve minimal normal tissue toxicity if the C x T remains constant with the most active clinical agents.

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