Effect of the Dosing Interval on Myelotoxicity and Survival in Mice Treated by Cytarabine

Zvia Agur, Ruth Arnon and Bilha Schechter

Many antineoplastic drugs are cell-cycle-phase-specific. These drugs are often highly toxic to the host, as they have the potential to impair replication, not only in the cancer cells, but also in the normal tissues. Using mathematical models it has been shown how selectivity of these drugs can be increased by exploiting the relatively large variability in cell-cycle parameters of the neoplasia. These models predict that toxicity to the host of cell-cycle-phase-specific drugs can be minimised if the dosing interval is an integer multiple of the average intermitotic interval of the susceptible host cells. Experimental evidence supporting this prediction is presented in this work. Our results show that a constant duration of the dosing interval yields higher survival rates in mice treated by cytarabine, as compared with random dosing intervals. Minimal myelotoxicity is exerted when the dosing interval is an exact multiple of the intermitotic time of bone marrow stem cells and erythroid progenitors (i.e. 7 h). Survival is significantly lower in mice treated every 8 h, or its multiple, as compared with that of mice treated at a 7 h or 10 h dosing interval.


**INTRODUCTION**

In the heyday of cell kinetics, scientists attempted to improve the rationale of cancer chemotherapy by showing that synchronisation of dosage with cell division phase could allow greater tumour cell kill, while minimising the damage to the normal tissues. To prove the theory they used highly transplanted murine tumour cells, where there was quite a narrow distribution of intermitotic intervals, so that beneficial effects could be shown. However, as spontaneous tumours that arise in patients have a very large distribution of intermitotic intervals [1], the experimental results could not be reproduced in clinical trials. The inevitable conclusion from this experience is that variation in cell-cycle duration should be taken into account in models of cell dynamics. However, rather than drawing this conclusion and improving the theory, the quantitative approach to cancer chemotherapy has been almost completely abandoned [2, 3].

In previous work a mathematical model of cell population dynamics has been developed, incorporating realistic assumptions about the variation in cell-cycle parameters of host and cancer cells [4–6]. This work suggests that the differences in the distribution (average or variation) of the intermitotic interval between normal and cancer cells can be exploited for increasing treatment selectivity. Based on this model the Z-method was put forward, suggesting that host toxicity of cell-cycle-phase-specific drugs may be reduced without hampering efficacy, by using short, high dose, drug pulses; the dosing interval should be an integer multiple of the intermitotic interval in the most susceptible host cells [7]. The mathematical proof of this assertion is presented elsewhere [8], but its essence can be captured if we consider a normal cell population whose average intermitotic interval is 7 h. When the cells are not naturally synchronised, only a small fraction of this population will be in the S-phase of the cell-cycle (the susceptible phase for many anticancer drugs) at, say, time 0 h, and their daughter cells at around 7 h, 14 h, 21 h, etc. Now, if a cell-cycle-phase-specific drug is applied exactly at 6, 7, 14, 21 h etc., as the Z-method suggests, it will effectively eliminate only this small fraction of the cell population. In another population, e.g. cancer, where cells have a much larger variation in the intermitotic interval [1], a much larger fraction will be eliminated by this protocol. This will be the case also if the cell's intermitotic interval has a limited variation but its average differs from the dosing interval. In theory the method will not be effective only in the unlikely case in which the host susceptible cells and the cancer cells have exactly the same distribution in the intermitotic interval.

We have previously tested the efficacy of the Z-method in _vivo_ and _in vitro_. In _vivo_ results show that cytarabine is least toxic when applied in a fixed dosing interval, similar to the intermitotic interval of the treated cells [6]. In the _in vitro_ experiments mice were treated by short duration zidovudine protocols and toxicity was measured by spleen weight, differential peripheral blood cell measurements, and the proportion of bone marrow (BM) cells in the S-phase gate of the DNA-content distributions. Results support the model's predictions in showing that zidovudine dosing interval per se affects the drug's toxicity to murine BM and that this drug is least toxic when the dosing interval is similar to the average BM intermitotic interval [9].

The present work investigates the effect of cytarabine dosing interval on treatment efficacy. Cytarabine is an S-phase-specific cytotoxic agent, with a half-life of about 20 min in the serum of mice [10, 11]. This drug causes severe depletion to murine bone marrow pluripotent stem cells, committed cells and erythrocyte progenitors [12, 13] whose cycle time, as measured from growth curves and from flow cytometry data is roughly 7 h [14–16].

Our results, showing out that a smaller BM toxicity is related to a dosing interval of 7 h, indicate the need for a careful estimation of cell-cycle parameters in the human BM.

**MATERIALS AND METHODS**

**Drug**

Cytarabine was obtained from Sigma and dissolved in 0.01 mol/l phosphate buffer containing 0.15 mol/l NaCl, pH 1085.

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7.3 (PBS) at the working concentration immediately before use. Doses were as indicated for the individual experiments. Control mice were injected with PBS.

In vivo experiments

Unless specified otherwise C3H.HJu mice, 6–10 weeks old, were given intraperitoneal inoculations of 10^5 38C-13B lymphoma cells [17], and treatment with periodic intraperitoneal injections of cytarabine was initiated 1 or 2 days following tumour inoculation. All protocols involved interval drug dosing with a drug-free episode between successive dosings. Dosing intervals in a given protocol were either fully periodic or random. Fully periodic protocols were applied in intervals of 7 h or an exact multiple of 7 h (denoted × 7 h), or every 8 h or 16 h (× 8 h), or every 10 h or 20 h (× 10 h), or every 11.5 h, or every 13 h. Random protocols involved dosing intervals generated by the random number generator of the IBM/NAG routine. The individual and total drug doses and the number of injections were identical, while the total treatment period was roughly the same for all mice in a given experiment. Treatment initiation and termination, as well as blood collection, were performed simultaneously in all mice in a given experiment. In order to neutralise the possible effect of circadian rhythms on cellular sensitivity and cytarabine pharmacokinetics, the number of night/day injections and the timing of blood tests were roughly similar for all mice in a given experiment.

Toxicity measurement

Peripheral blood assay was performed by collecting samples from the tail vein of mice approximately 30 h following termination of treatment. The effect of cytarabine on peripheral blood was evaluated by determining various blood parameters, such as white blood cell (WBC) count, or by differential blood counts, employing light scattering at different angles (Technicon H * 1™ [18]). Mice were observed for survival for 2 months. SAS/GLM pairwise t test was employed for measuring significance of differences in peripheral blood counts while the χ^2 test was employed for measuring significance of differences in survival and nuclear lobularity cytograms (NLC).

Bone marrow analysis

Mice were treated with cytarabine, as described above, and BM aspirates were obtained 48 h after the last injection, simultaneously from all mice. Propidium iodide labeled cells were analysed for DNA content distribution and cell size, using the fluorescence-activated cell sorter (FACS; Becton Dickinson). In addition, viscosity vs. cell size FACS BM analysis (right angle/forward light scatter), was performed for detecting differential damage to different BM compartments. SAS/GLM pairwise t test was employed for measuring significance of differences between schedules in the BM analysis.

**RESULTS**

**Effect of dosing interval on myelotoxicity**

The first three experiments were aimed at checking the effect of the dosing interval on host toxicity alone. In each experiment several groups of healthy mice were given the same number and same total dose of intraperitoneal cytarabine injections over the same period of time. The groups differed in the dosing interval alone, which was either random or fully periodic of × 7 h, or × 8 h, or × 10 h, etc. Since these were short duration experiments, it was expected that meaningful toxic effects will be manifested in WBC counts, rather than in red blood cells (RBC) counts, due to a relatively short turnover time of the former. Indeed, WBC counts showed lower toxicity in mice treated by cytarabine exactly every × 7 h, as compared to all other tested protocols (Fig. 1). Note, however, that the difference between WBC counts of mice treated by this protocol and those treated every × 10 h was not statistically significant.

In the experiment presented in Tables 1 and 2 healthy mice were given 6 × 1.15 mg cytarabine injections, every × 7 h, or every × 10 h, during a period of 3 days. From the samples presented in Table 1 it appears that the average loss of body weight was higher in mice treated every × 10 h, as compared with mice treated every × 7 h. Moreover, there was a statistically significant reduction in both treated groups in spleen weights and the counts of WBC, platelets, and mean platelet volume (MPV), but this reduction (and also that in RBC counts, not shown) was significantly larger in mice treated every × 10 h. An instructive result appears in the NLC of the Technicon H * 1™ peripheral blood analysis of this experiment. This cytogram displays granulocytes according to their size and nuclear lobularity [18]. Interestingly, 100% of the mice treated every × 10 h, displayed an atypical NLC, as compared with 0% in the control group, and 36% of the mice treated every × 7 h. The atypical NLC is characterised by a visually distinct cluster of cells with increased lobularity and reduced size (Fig. 2). Statistical analysis shows that mice with atypical cytograms have significantly lower
Table 1. Effect of the dosing interval on cytarabine toxicity

<table>
<thead>
<tr>
<th>Body Dosing weight* (g)</th>
<th>Spleen weight (g)</th>
<th>PLT (10⁴/µl)</th>
<th>MPV (fl)</th>
<th>AT NLC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>77.58 (8.0)</td>
<td>713 (174)</td>
<td>6.28 (0.52)</td>
<td>0</td>
</tr>
<tr>
<td>× 7 h</td>
<td>8.60 (14.3)</td>
<td>408.2 (150)</td>
<td>4.92 (0.42)</td>
<td>36</td>
</tr>
<tr>
<td>× 10 h</td>
<td>15.38 (5.2)</td>
<td>279.6 (98)</td>
<td>4.37 (0.44)</td>
<td>100</td>
</tr>
</tbody>
</table>

Mice were given 6 × 1.15 mg cytarabine injections (total dose 319 mg/kg) at different dosing intervals. Last injection was done simultaneously in all mice. Differential blood measurements were performed by Technicon H*1™ haematology analyser.

Mean (S.D.).
* Total group changes in body weight; statistical analysis was not performed.
† Means are significantly different from the control (P < 0.01).
‡ Means are significantly different from those of the group treated every 7 h (P < 0.05).
§ Means are significantly different from those of the group treated every 7 h (P < 0.005).

Table 2. Effect of cytarabine on DNA content distribution of BM cells

<table>
<thead>
<tr>
<th>Dosing interval</th>
<th>Cell-cycle compartment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G₂/G₁</td>
</tr>
<tr>
<td>Control</td>
<td>64.83 (3.88)</td>
</tr>
<tr>
<td>× 7 h</td>
<td>82.36 (4.3) *</td>
</tr>
<tr>
<td>× 10 h</td>
<td>88.19 (2.92) *‡</td>
</tr>
</tbody>
</table>

Entries are the group average percentages (S.E.) of BM cells, categorised into three cell-cycle compartments according to FACS evaluation of DNA content of individual cells (for experimental details see Table 1).
* Results are significantly different from control (P < 0.0001).
† Results are significantly different from control (P < 0.0002).
‡ Results are significantly different from the group treated every 7 h (P < 0.0002).
§ Results are significantly different from the group treated every 7 h (P < 0.02).

Effect of dosing interval on survival and tumour elimination

The next step was to check the dosing interval effect on treatment efficacy. To this end mice were inoculated with 38G-13B lymphoma cells and treatment with various cytarabine protocols was initiated 2 days later. The average inter-mitotic interval of this lymphoma cell line was previously determined WBC, platelet and MPV, as well as a higher lobularity index (LI) (P < 0.01). It may be concluded that an atypical lobularity cytogram, characterised by a significant number of small highly nucleated cells, is correlated with increased blood toxicity, and that such an effect is significantly more prevalent in mice treated every × 10 h. In zidovudine experiments of the same nature an atypical NLC is also shown to be significantly more prevalent in mice treated every × 10 h. Here such cytograms are characterised by a distinct cluster of cells with reduced lobularity and increased size (Fig. 2) and are correlated with decreased WBC, platelet, MPV, LI and mean red blood cell volume [9].
Effect of Cytarabine Dosing Interval on Treatment Selectivity

However, even for a relatively well characterised drug, such as cytarabine, the current level of knowledge is insufficient to predict optimal therapeutic schedules. It has been noted that the cytotoxic cascade's characteristic of the population, the dose and the timing interval, determine the proportion of cells killed by multiple doses of cytarabine [10, 19] and host tolerance [20]. However, only recently a mathematical model has been provided, which enables a logical and systematic prediction of treatment efficacy for cell-cycle-phase-specific drugs, such as cytarabine. This method defines the exact relation between the drug pharmacokinetics, the dynamic properties of a given cell or viral population and the prospects of its elimination [6-8].

Using this method it has been suggested that drug selectivity can be increased by manipulating the duration of the dosing interval. Thus a “resonance” effect can be created for the normal cells, minimising their mortality, and a lack of “resonance” for the cancer cells, whose frequency of replication differs from that of the normal cells. According to this theory continuous drug infusion should be less selective than certain protocols of interval drug dosing. For this reason we have focused attention in this work on protocols involving strictly positive drug-free intervals. Moreover, the above method suggests that minimal toxicity is exerted when the dosing interval is equal to or an integer multiple of the average inter-mitotic interval of the susceptible host cells. The aim of the present study was to verify this prediction: Our results show that in a short duration treatment, a dosing interval of exactly 7 h or its multiple is less damaging to murine BM, peripheral blood, as well as body and spleen weight, than other treatment protocols. This effect is strictly associated with the basic drug periodicity, since the total drug dose, the treatment duration and circadian effects were similar for all protocols. If the average inter-mitotic interval of murine BM progenitors is indeed 7 h, as is indicated in the literature (see above), then the present results support our prediction that a protocol whose dosing interval is similar to the average inter-mitotic interval of susceptible host cells, is less toxic to these cells than other periodic treatments.

In order to show that the effects reported here were due to direct BM toxicity, a DNA content distribution analysis was performed on BM aspirates of mice treated at different dosing intervals. Results of these experiments show a clear direct effect of cytarabine on the BM granulocytes, and a complete elimination of the BM S-phase compartment when the drug doses were very high (results not shown). It should be noted, though, that the possibility that mice mortality was due to other toxic effects, e.g. gastrointestinal toxicity [19], cannot be ruled out.

Further experiments are warranted for examining our conclusions in greater detail. Thus, the putative effects of factors, such as the circadian rhythms [21], or drug effect on cell-cycle synchronisation and on shortening cycle duration [22], can be incorporated in our model. Note, however, that significant differences between protocols in our experiments, imply that in a short duration treatment such effects are not a dominant factor in determining treatment efficacy.

The implications of our results for chemotherapy should be interpreted with some caution, bearing in mind that the average inter-mitotic interval of BM cells (about 24 h [23]) and cytarabine half-life are longer in humans. Our theoretical results [8, 6] suggest that treatment efficacy is affected by the relation between the dosing interval and the inter-mitotic interval of the susceptible cells. Based on the observation, reported here, that a 1 h difference in cytarabine dosing interval affects murine BM toxicity, we expect a 3–4 h difference in this interval to have a similar effect on the human BM. A theoretical analysis of interval drug dosing in human patients is provided elsewhere [8, 24].

This and previous studies [25] suggest that a single daily dosing of cell-cycle phase specific drugs whose half-life is 1–3 h (e.g. cytarabine or zidovudine) will be less toxic to human BM than schedules involving the same total dose divided into several daily dosings. However, the latter schedules are expected to be somewhat faster in virus or neoplasia elimination. The potential clinical importance of these predictions indicates the need for further investigation.

Immunohistochemical and Biochemical Characterisation of the Expression of a Human Embryonal Carcinoma Cell Proteoglycan Antigen in Human Germ Cell Tumours and other Tissues

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In the embryonal carcinoma (EC) cell line GCT 27, monoclonal antibody GCTM-2 recognises an epitope on a 200 kD pericellular matrix keratan sulphate proteoglycan. Immunohistochemical analyses demonstrated staining of tissue sections from 21 out of 22 human non-seminomatous germ cell tumours, and from 22 out of 28 sections of seminomas. In normal human fetal tissues gut epithelium and muscle stained strongly, and certain other epithelia stained moderately. In adult tissues, the distribution of the epitope was similar, but staining intensity was weaker. Neoplastic tissues showed reactivity with embryonal rhabdomyosarcoma and colorectal carcinoma, but no other non-germ cell tumours. Immunofluorescence microscopy showed that GCTM-2 also stained cell lines from human colorectal carcinoma, embryonal rhabdomyosarcoma and choriocarcinoma. In contrast to EC cells the epitope in these other cell types required permeabilisation of the cells to be visualised, and the protein bands in immunoblots lacked extensive modification with keratan sulphate and were smaller. Thus, GCTM-2 reacts with an epitope which has a previously unrecognised tissue distribution; its expression as a pericellular matrix proteoglycan is predominantly a characteristic of human EC cells.


INTRODUCTION

The serum markers α-fetoprotein and human chorionic gonadotropin are useful in monitoring patients with testicular non-seminomatous germ cell tumours (NSGCT) [1]. These secreted polypeptides are products of differentiated yolk sac cells and trophoblastic lineage respectively, which appear in a proportion of testicular teratomas [2]. Despite the utility of these markers, it would be desirable for cell biological studies and for certain clinical applications to define markers expressed on embryonal carcinoma stem cells themselves, rather than on their differentiated derivatives. A preliminary study has suggested that the GCTM-2 antigen merits investigation as one such clinical marker [3].

A number of cell surface antigens defined by monoclonal antibodies have been described in association with embryonal carcinoma. Many of these monoclonals react with carbohydrate antigens and are of the IgM class [4]. Monoclonal antibody GCTM-2 was shown to recognise a pericellular matrix EC proteoglycan which was susceptible to degradation by keratanase but not other glycohydrolases or lysates [5]. More recent studies on the purified GCTM-2 antigen confirmed that it is a keratan sulphate proteoglycan. The further evidence supporting this conclusion included aminoacid and sugar analyses of the antigen; the reactivity of the antigen with Alcian blue dye; high affinity of the antigen for anion exchange resins; metabolic labelling of