A MATHEMATICAL MODEL FOR TUMOR DRUG RESISTANCE BASED ON SALVAGE METABOLISM[1]

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ABSTRACT

The development of tumor resistance to cytotoxic agents has important implications in the treatment of cancer. Mathematical models of resistance, if supported by experimental data, can provide useful information on mechanisms and aid in the design of therapeutic regimens. We report here the development of a model of tumor growth kinetics which assumes that the rate of cell growth in a tumor are normally distributed. We have further assumed that the growth rate of each cell is proportional to its rate of total pyrimidine synthesis (*de novo* plus salvage). Using an ovarian carcinoma cell line (2008) and resistant variants selected from chronic exposure to PALA, we have derived a simple and specific analytical form describing the survival curves of 72–hour growth assays and clonogenic assays.

The model assumes that the rate of *de novo* pyrimidine synthesis, denoted α, is shifted down by an amount proportional to the $\log_{10}$ of PALA exposure and that cells whose rate of pyrimidine synthesis falls below a critical level, denoted α_0, the cell can no longer survive. This is described by the equation:

$$\text{Probability (survival)} = \text{Probability } (\alpha_0 < \alpha - \text{ constant } \log_{10} [\text{PALA}]).$$

This model predicts that survival curves, when plotted on probit paper, will produce straight lines. This prediction is in agreement with our data obtained from the 2008 cells. A second prediction of this model is that the same probit plots for resistant variants should shift to the right due to uridine salvage activity. Probit plots of dose response data of each resistant 2008 line again confirmed this prediction. Advantages in using this model for cytotoxic data analysis include: straight lines from the probit plots allow for the use of standard regression techniques for data analysis; shifts of probit plots predict tumor cell survival at different levels of resistance; and the slope of the regression lines allows the detection of synergy, such as that observed between dipyridamole and PALA. The rate–normal model may be widely applicable to other cytotoxic drugs, especially antimetabolites.

Introduction

The susceptibility of a population of tumor cells to a cytotoxic treatment is typically defined as the inhibition of growth of the population in the presence of the treatment, which may also have a residual effect, by which we mean the treatment may cause damage to a cell which is transmitted to its offspring, such as sublethal DNA damage. This inhibition may be taken to be some reduction in a parameter designed to measure growth rate or it may be a measure of cell death post–treatment. An example of the former may be to count the number of live cells in two flasks after a specified incubation period, one treated and the other not, assuming equal cell density prior to treatment. An example of the latter may be to count the number of single–cell–derived colonies formed in the presence or absence of the treatment (clonogenic assay), assuming equal initial plating density. In both cases the measure of effectiveness is the percent reduction relative to control.

An important aspect of cytotoxic treatments to a population of tumor cells is often the development of resistance to the treatment. By resistance, we mean the ability of some target cells to proliferate as the treatment progresses. During the development of acquired resistance, later applications of the treatment are overall less effective than earlier applications. There have been attempts to mathematically model tumor drug resistance (1) by applying the same concepts used to model bacteriophage resistance, i.e. the somatic mutation theory of Luria and Delbruck (2). There are two problems with this line of approach to tumor resistance modeling. One problem is that the fluctuation analysis underlying the theory of somatic mutation has never been successfully done in the context of drug resistance in tumor cells, although it has been attempted (3). Another problem is that predictions of the model were not well supported by experimental and clinical data (4, 5, 6, 7, 8). This points to a problem with the assumptions of the model, which were based upon a hypothetical mechanism (the occurrence of somatic mutation). It would seem to be a more fruitful approach to base the assumptions of a model on observed mechanisms of drug action and documented mode of resistance. In this communication, the target population is a line of human ovarian tumor cells (2008) and the treatment is exposure to the antipyrimidine drug PALA, a potent inhibitor of aspartate transcarbamylase (9). It is

the development of resistance to PALA that is of interest to us because this drug is a very specific and potent inhibitor of pyrimidine biosynthesis (9). Our approach is to model the effect of PALA on cell kinetics based upon its known effect on *de novo* pyrimidine nucleotide synthesis (9) and the mechanism of resistance based upon the amplification of tumor salvage activity (10, 11, 12).

Drug Susceptibility and Resistance to PALA.

In order to model the response of cells exposed to various concentrations of PALA, it is necessary to make assumptions regarding:

1) the kinetics of cell growth

2) the action of the drug

3) the dose response to the drug, and

4) the resistance mechanism.

The following are the assumptions made in constructing our model.

Assumption 1. Growth rate of cells is approximately normally distributed.

One measure of growth rate has been taken as the inverse of interdivision time (IDT) of individual cells as described by Kubitschek (13). He plotted growth rates from several data sets of interdivisional times for a variety of organisms on probit or normal paper and obtained straight lines, indicating that the data plotted followed a normal distribution.

Assumption 2. The rate of total pyrimidine nucleotide synthesis within a clonal line is normally distributed and it is directly proportional to growth rate.

Assumption 3. The rate of total pyrimidine nucleotide synthesis has two components

1) *de novo* synthetic activity

2) salvage metabolism (11).

Assumption 4. If the rate of total pyrimidine synthesis falls below some critical level, denoted α_0, then cell starvation and death occurs.

Assumption 5. For any clonal line, the rate of *de novo* pyrimidine synthesis is decreased by an amount = constant $\times$ log ([PALA]).

There is really a planted axiom here within Assumption 5. We assumed that PALA diffuses equally well into each cell so that at a particular concentration of PALA, each cell will have the same amount of drug in the cytosol, and therefore each line will shift down its rate of *de novo* pyrimidine synthesis, α, by the same amount. These assumptions allow us to make predictions of survival probability (Prob($\cdot$) denotes probability) as a function of PALA exposure.

$$\begin{aligned} &\text{Prob(survival)} \\ =&\text{Prob}(\alpha_0 < \alpha - \text{ constant } \times \log([\text{PALA}])). \\ =&\text{Prob}(\alpha_0 + \text{ constant } \times \log([\text{PALA}]) < \alpha). \end{aligned}$$

Implicitly we assume that growth rate is proportional to the residual rate (i.e. unaffected by drug) of pyrimidine nucleotide synthesis. If the assumptions are correct, the following prediction can be made.

Prediction 1: Survival data for 72 hour growth data and clonogenic assays in cells with varying levels of resistance should provide straight lines when plotted on normal paper.

Action of PALA on 2008 cells maintained in media with varying concentration of PALA. Cells which survive a specific dosage of PALA are those whose rates of pyrimidine production are not dropped below α_0. Cells which are maintained in chronic PALA have a portion of their pyrimidine synthesis blocked. As the chronic level of PALA increases, the blocked portion of *de novo* pyrimidine synthesis increases. The surviving cells adapt as follows.

Assumption 6 In order to compensate for pyrimidine production which has been blocked by PALA, cells exposed to chronic PALA augment unaffected *de novo* pyrimidine synthesis by increasing the salvage pyrimidine synthesis.

Prediction 2: Survival curves for 72 hour growth data and clonogenic assays for cells chronically exposed to PALA will shift in a parallel fashion to the right, when plotted on normal paper as the chronic exposure level to PALA increases.

PALA + Dipyridamole: The Meaning of Synergy. Shifts in survival curves may be obtained by a variety of means. One way is to expose cells chronically to PALA so that resistant cells are selected and the curves shift to the right (toward greater survival) in a parallel fashion. Another way is to enhance the effectiveness of PALA by combining PALA with another drug so that the curve shifts in the other direction toward less survival. Suppose, for the sake of argument, that a specific concentration of Drug X doubled the effect of PALA in the sense that 100 μm of PALA with Drug X had the effectiveness of 200 μm of PALA alone. Using the assumptions outlined previously, it is not hard to see that the survival curve for Drug X + PALA is parallel to the survival curve for PALA alone. Since this means that one curve is a constant plus the other, we would say that the effect of Drug X is additive, i.e., the slope remains the same. If the slope becomes steeper, i.e. becomes more negative, we say that the drugs are synergistic.

Prediction 3: Since dipyridamole has been shown to act snyergistically with PALA, in addition to the left shift the slope of the survival curve for PALA + dypiridamole will be steeper, i.e. more negative, than that for PALA alone.

PALA + Uridine in the presence of dipyridamole: Elucidating the effect of salvage in resistance to PALA. One way to elucidate the effect of the salvage mechanism with regard to resistance to PALA would be to relate survival to some measure of salvage activity. One such measure is uridine uptake. To this end we assume the following.

Assumption 7. Salvage activity is proportional to uridine uptake.

Assumption 8. In the presence of dipyridamole, uptake of uridine is proportional to the logarithm of exogenous uridine concentration, since dipyridamole has a much greater affinity for the nucleoside transporter than uridine (14).

Assumption 8 can be valid only when dipyridamole is present to block the receptor without which uridine may pass freely through. Now consider a culture of cells exposed to a fixed level of PALA + dipyridamole. According to the rate normal model, the rate of pyrimidine synthesis is $\alpha - k$ where k is a function of PALA + dipyridamole concentration.

Now in the presence of exogeneous uridine, the rate of pyrimidine synthesis is given by

$$\alpha - k + \text{ constant } \times \log([\text{uridine}])$$

as a result of assumptions 7 and 8. Therefore the model predicts the survival curve as a function of uridine concentration is given by

$$\text{Prob}(\alpha_0 < \alpha - k + \text{ constant } \times \log([\text{uridine}])).$$

Prediction 4: The survival curve for 2008 cells exposed to a fixed concentration of PALA + dypiridamole will form a straight line when plotted against the logarithm of uridine concentration.

As demonstrated in the Results section, all four predictions of this model are supported by data generated in the laboratory independently.

Materials and Methods

Drugs and Reagents. PALA (100 mg/ml) was obtained from the Division of Cancer Treatment, National Cancer Institute (Bethesda, MD), and Dipyridamole (Persantine, 5 mg/ml) was supplied by Boehringer Ingelheim, Ltd. (Ridgefield, CT). All nucleosides and nucleotide standards were purchased from Sigma Chemical Co. (St. Louis, MO), and all other chemicals used were obtained from Fisher Scientific (Fairlawn, NJ). All tissue culture media and sera were purchased from GIBCO (Grand Island, NY).

Human ovarian carcinoma. Cells (2008) growing in monolayer in T–75 flasks (Falcon Plastics, Cockeysville, MD) were harvested at 70–80% confluence after trypsin/EDTA treatment. They were washed twice in fresh medium and seeded at a cell concentration of 2×10^4/ml into Linbro 24–well culture plates (Flow Laboratories, McLean, VA) containing RPM 1640 medium plus 10% fetal bovine serum with varying concentrations of drugs and nucleosides already pipeted into the wells in triplicate. The plates were incubated under 5% CO_2 at 37°C for 72 h, and the cell number in each well was quantified with an electronic cell counter (Coulter Electronics, Hialeah, FL) after trypsinization. (9)

Clonogenic Assay. Cells growing in log–phase were harvested, washed, dispersed, and plated onto 60–mm plastic Petri dishes (Corning Glass Works, NY) in triplicate at a density of 400 cells/dish. Varying amounts of drugs and nucleosides were added to the dishes, and the cells were incubated under 7% CO_2 at 37°C for 10 days. Clusters of > 50 cells were counted as one colony, and usually the control dishes contained 100 to 120 colonies (10)

Selection of resistant cells. In the chronic sublethal stepwise exposure protocol, exponentially growing cells were used in a 72 hour growth assay to establish an averaged dose-response to PALA ($n = 4$). They were then exposed initially to 10 μM of PALA in their growth medium continuously for 2 weeks. After a second growth assay to document any changes in drug sensitivity, the cells were exposed to 50 μM of PALA in growth medium for the next two weeks, while one flask of cells were carried continuously in the 10 μM PALA medium (CS-0.01 cells). This process was repeated for media containing 100 μM (CS-0.1), 500 μM (CS-0.5), 2 mM (CS-2) of PALA; until we have cells that can grow exponentially in 5 mM PALA (CS-5). This series of resistant cells represents a model of acquired resistance at different drug exposure levels.

In the single exposure and cloning protocol, wild type 2008 cells were exposed to varying concentrations for PALA in a clonogenic assay. Clones of cells growing out at the higher PALA concentrations were picked using a sterile 8 mm cloning cylinder (Belco Glass, NJ) and released by Trypsin/EDTA treatment. Each harvested clone were split into 2 cultures, one growing in normal growth medium and one growing in medium contain the same concentration of PALA that the cells were isolated at. This series of resistant cells represents a model of natural resistance.

Nucleoside uptake and initial transport rates in 2008 cells. Freshly harvested cells were suspended in "uptake medium" consisting of normal RPMI 1640 medium supplemented with 10% dialysed fetal bovine serum. Uridine (5, 6 - H, 30 Ci/mM) was added to the cell suspensions to achieve a final concentration of 10 μM (10 μCi/ml) and the suspensions (5×10^6 cells in 2.0 ml) were incubated at 37°C with constant shaking. Aliquots of cells were removed at 1, 5, 10, 20, 30 and 60 minutes and diluted with 10 volumes

of chilled PBS and centrifuged at 4°C for 5 minutes at 1,000 × g. The cell pellets were washed twice with 1 ml chilled PBS and then resuspended in 0.9 ml of 0.1 M NaOH. After 10 minutes of alkaline digestion on ice, a 500 μl aliquot from each sample was removed and the radioactivity quantified using liquid scintillation counting (10). The intracellular radioactivity represents the amount of free radioactive uridine and its metabolites that are incorporated into cellular metabolites and macromolecules and give estimates of the contribution of salvage metabolism in the different resistant cell types.

Uridine transport into 2008 cells was measured using a modified oil-stop method (15). Briefly, 100 μl of the uptake medium containing radiolabeled uridine was layered carefully onto 100 μl of an oil mixture of 9:1 silicon oil (Aldrich 17563-3):parafin oil (Fisher 0-119) in a 1.5 ml eppendorf microcentrifuge tube. Transport measurements were initiated by the addition of 100 μl of cell suspension and the reactions were stopped at timed intervals between 0 to 60 seconds by pelleting the cells through the oil cushion at 12,000 × g for 30 seconds. The oil was then aspirated and the cell pellet digested with 0.1 M NaOH and the radioactivity quantified using liquid scintillation counting. The amount of radioactivity in the cell pellet not associated with transport was estimated by pre-incubating cells in medium containing 10 μM dipyridamole and initiating the reaction with the transport inhibitor present. The intracellular radioactivity in the different resistant cells represents the contribution of the membrane nucleoside transporter before significant intracellular phosphorylation has taken place.

Normal Plots

The normal plots were made as follows. The data points consist of the mean percent survival relative to control plotted against the $\log_{10}$ of PALA concentration. The concentration of PALA was in micromolar units. The percent survival is plotted on the vertical scale while the log–concentration is on the horizontal. Note that on normal or probit paper, the scale of the vertical axis is not linear for percents. The scale for the percents is as the inverse of the cumulative distribution function of the standard normal (i.e. the probit function). The computations were made using the probit function of SAS software (16), while the graphs were made by using the GPLOT procedure of SAS GRAPH

(17). Statistical comparisons of the survival curves with respect to slopes and intercepts were made by using the general linear model procedure, PROC GLM, with SAS software (18). Estimates of IC50 and their standard deviations for the 72 hour data were made by computation of individual IC50's for each of the three replications, then computing the mean and its standard deviation for the wild type and each chronically exposed line.

Results

The first predictions of the model were that both wild type and chronically exposed 2008 cells should have dose response curves which, when plotted on probit or normal paper, should produce straight lines. In Figure 1, 72 hour growth rate data are plotted on normal paper for 2008 cells which are: wild type, chronically exposed to 50μM PALA, chronically exposed to 100μM PALA and chronically exposed to 500μM PALA. The R^2 values of the fit of the least squares regression lines to these data are, respectively: .847, .950, .966 and .974. The slopes of the dose response curves were compared statistically and there were no significant differences ($p = .22$), while the intercepts were compared and were significantly different ($p < .0001$) due to the rightward shift of the survival curves for the chronically exposed 2008 cells. Table 1 contains estimates of IC_{50} values and their corresponding standard deviations for the parent and resistant 2008 lines. As expected the IC_{50} values increase as the cells are exposed on a chronic basis to increasing concentrations of PALA.

The data for the clonogenic assay is shown in Figure 2 for 2008 cells which are: wild type, chronically exposed to 100μM PALA and chronically exposed to 500μM PALA. The R^2 values of the fit of the least squares regression lines to these data are, respectively: .991, .878 and .991. The slopes of the plots from chronically exposed 2008 cells were not significantly different for the two different chronic levels of PALA ($p = .70$), while the slope of the wild type was significantly different from the slope of the two chronically exposed cells ($p = .03$).

In Figure 3, the dose response of 2008 cells to PALA in 72 hour growth rate assay with and without 1μM dipyridamole are plotted on normal paper (data from (11)), with least squares lines overlaid. The R^2 values of the fit of the least squares regression lines to

the data are .921 for PALA alone and .953 for PALA and dipyridamole. This is a graphic illustration of the synergy of PALA and dipyridamole since the slope of the survival curve changes downward in the presence of dipyridamole ($p = .056$).

In Figure 4, the 72 hour growth rate response of 2008 cell exposed to PALA plus 1μM dipyridamole in the presence of increasing levels of uridine are plotted. The straight line indicates that rate of pyrimidine synthesis shifts upward as a function of the log of the concentration of uridine in the presence of dipyridamole. The R^2 value of the fit of the least squares regression line to this data is .942. Table 2 contains mean transport rates of uridine for the chronically resistant lines along with their standard deviations. The increase in uridine transport is consistent with the hypothesis that it is by salvage that cells compensate for the portion of *de novo* pyrimidine which is blocked by chronic PALA exposure, although other mechanisms of resistance cannot be excluded.

Discussion

We have developed a model to describe the response of a population of ovarian cancer cells to the antipyrimidine metabolite PALA. The "rate normal" model allowed us to predict a specific form of the survival curves of cells in both 72–hour and clonogenic assays, as well as the shift in the survival curves for cells chronically exposed to PALA . The rate normal model implies that survival curves, when plotted on normal paper, will form straight lines. This then gives us a parametric form for survival curves to which regression techniques may be applied in their analysis. An essential aspect of the model is that it explicitly assumes that the population has a distribution of sensitivities to the drug. This contrasts with models for survival which are based on mass action although the two differing models can give very similar predictions (see figure 16 of (19)). The model of tumor susceptibility and/or resistance to the pyrimidine antimetabolite PALA presented here differs from the Goldie–Coldman model (1) in two major respects. First of all, it is based upon observed mechanisms of drug susceptibility/resistance, where susceptibility is the effect of PALA in inhibiting de-novo pyrimidine synthesis and resistance is based on the amplification in salvage activity although the model does not exclude other mechanisms of resistance such as CAD gene amplification. The Goldie-Coldman model, on the other

hand, is based solely upon somatic mutation which has not been experimentally verified in tumor cell lines. The second difference is that in our model we assume that all cells are affected to some degree by the drug, and that the range of susceptibility to the drug has a broad distribution in the population and is related to the kinetics of cell proliferation. The Goldie-Coldman model assumes that cells are either susceptible or resistant to a particular treatment with no finer distinctions.

Another benefit of the model is that it provides a specific interpretation of the synergy of PALA and dipyridamole. This is consistent with but stricter than the notion that drugs are synergistic if the effect of the combination has a greater effect than the sum of the individual effects (dipyridamole has no effect by itself). The notion of synergy presented here is also tailored for a population with a distribution of sensitivities as opposed to the isobole and median effect notion which are tailored for situations where laws of mass action hold (19, 20).

One application of the model would be to use it for single or combination drug screening of anticancer agents, since the simple form of the survival curves allows for easy interpretation and comparison. A second application would be to use the model to screen for synergy between drugs. The method presented here is simpler than that of constructing an isobole, for example (19).

This first step of our modeling efforts is "static" in the sense that it is related to describing properties of a population in equilibrium. Future directions of our modeling will be directed toward describing how the population of tumor cells will evolve over time with respect to drug response. We will need to describe how single cell derived populations of tumor cells generate such a broad range of susceptibilities over time and then, to become clinically relevant, to model the evolution of the population of tumor cells over time under selective pressure of drug exposure. At this point one can then make and compare predictions of clinical response under different treatment regimens. At each step the laboratory data will be our guide.

The model has only been, so far, applied to one cell line and one antimetabolite. Since

rate normality is a common feature of cell populations, it would be of great interest to know if the model is applicable to other tumor cell lines and other antimetabolites as well. Much data along these lines exist and statistical software packages which can do normal plots are widespread. The model may or may not apply to drugs which are not antimetabolites by nature such as cis–platinum or melphalan. We encourage other researchers to check the fit of the model on their data sets and are willing to provide guidance if so requested.

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Table 1. IC_{50} of chronic resistant lines to PALA $\pm$ SD

Cell line	$IC_{50}^{a}(\mu M)$
2008	266.3 ± 103.34
CS 0.05	818.0 ± 264.48
CS 0.1	940.0 ± 284.20
CS 0.5	2595.3 ± 795.01

[a] Values are mean $\pm$ SD of 3 experiments.

Table 2. Uridine transport rates of chronic resistant lines

Cell line	Transport rate[a] (pmole/min-10^6 cells)
2008	16.4 ± 2.7
CS 0.05	20.8 ± 2.2
CS 0.1	29.2 ± 1.8^b
CS 0.5	51.3 ± 5.4^b

[a] Values are mean $\pm$ SD of 5 experiments

[b] Significantly different from wild–type 2008 values, $p < 0.05$ Student's t test.