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RAD-ADAPT: software for modelling clonogenic assay data in radiation biology

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Abstract

We present a comprehensive software program, RAD-ADAPT, for the quantitative analysis of clonogenic assays in radiation biology. Two commonly used models for clonogenic assay analysis, the linear-quadratic model and single-hit multi-target model, are built in the software. RAD-ADAPT uses maximum likelihood estimation method to obtain parameter estimates with the assumption that cell colony count data follow a Poisson distribution. The program has an intuitive interface, generates model prediction plots, tabulates model parameter estimates, and allows automatic statistical comparison of parameters between different groups. The RAD-ADAPT interface is written using the statistical software R and the underlying computations are accomplished by the ADAPT software system for pharmacokinetic/pharmacodynamic systems analysis. The use of RAD-ADAPT is demonstrated using an example that examines the impact of pharmacologic ATM and ATR kinase inhibition on human lung cancer cell line A549 after ionizing radiation.

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The authors declare no conflict of interests. Y. Zhang is currently employed at Genentech.

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Keywords

Clonogenic assays; clonogenic survival curves; radiation biology; linear quadratic model; single-hit multi-target model

1. Introduction

The first graphical representation of the replicative potential of single mammalian cells following exposure to increasing doses of ionizing radiation (IR) was published by Puck and Marcus in 1956 [1]. In the experiments described, interphase HeLa human cervical carcinoma cells were plated in petri dishes at concentrations conducive to colonial growth from single cells. Cells were allowed to attach to the dishes for 5 h to 24 h and dishes were then irradiated with one of 13 different doses of IR from 50 rads to 700 rads. At 9 to 17 days after irradiation, the number of colonies arising from single cells was enumerated. As such, the assay measured the potential of irradiated single cells to replicate and form a colony rather than single cell survival. When the logarithm of the replicating fraction of HeLa human cervical carcinoma cells was plotted against radiation dose, two distinct components were evident in the curve. At lower doses a shallow shoulder was observed and above approximately 3 Gray (Gy) IR the log of cell inactivation was proportional to the dose. The shallow shoulder in the survival curve indicates that the proportion of cells inactivated by a given dose increases with the dose already given. In essence, the clonogenic assay has not changed since its innovation sixty years ago [1, 2].

A number of mathematical models have been used to describe the relationship between replicative potential and radiation dose observed in clonogenic assays. The linear quadratic (LQ) model assumes that cell inactivation following IR can be described by an equation with two principal components, a component that is proportional to dose (Linear), and a component that is proportional to the square of the dose (Quadratic). The theoretical basis underlying the LQ model is that pairs of primary lesions (often associated with DNA double-strand breaks (DSBs)) are needed for cell inactivation and that these can be generated by either one (linear or α -type damage) or two different (quadratic or α -type damage) radiation tracks. The LQ model has a biological basis dating back to 1938 when the mitotic death of mammalian cells was observed to be strongly associated with chromosome aberrations that arise from two DSBs [3]. While the LQ model is widely used to describe the data generated in clonogenic assays, other models have been proposed and applied (as reviewed in [4, 5]), including the single-hit multi-target (SHMT) model. The SHMT model, which has no biological basis, assumes that that one or more targets of equal volume must receive a single hit from one radiation track to inactivate a cell [6, 7].

To implement the statistical methods used to fit survival models, a number of special purpose and general purpose programs can be used to estimate model parameters and their standard errors, and to make inferences about the estimated model. A widely used set of programs designed specifically for data analysis and model fitting involving cellular survival data was introduced by Albright in 1987 [8]. Other special purpose software for fitting survival models includes the DRFIT program reported in 1990 by Roberts [9]. More recent

special purpose programs include the clonogenic survival calculation program (CS-CAL) from the German Cancer Research Center [10]. In addition, general purpose statistical software systems can be used to estimate parameters of commonly used cell survival models, as illustrated by Franken et al. using the SPSS Statistics program [2]. These and other reported programs and methods, use weighted least squares regression with the logarithm of the survival models and data to estimate model parameters. Since cell colony formation data are counts, however, maximum likelihood estimation using a Poisson model allows for more formal and robust statistical inference. This was explored by Frome and Beauchamp in 1968 [11] and again recently by Braselmann et al [12].

In this report, the RAD-ADAPT software for modelling clonogenic assay data in radiation biology is presented. It uses maximum likelihood estimation based on a Poisson data model to fit widely used survival models to measured cell survival data. RAD-ADAPT is a standalone, integrated software system with a graphical user interface for convenient entry and display of clonogenic assay data and modelling results. It is freely available as a downloadable executable for Windows-based systems.

2. Materials and methods

2.1 Clonogenic Survival Models

The RAD-ADAPT software incorporates both the linear-quadratic (LQ) and the single-hit multi-target (SHMT) model equations used to describe clonogenic assay data. For the LQ model, the number of colonies relative to the number of cells seeded versus radiation dose is given as follows:

$$S(D_i) = PE \cdot e^{(-\alpha D_i - \beta D_i^2)}, \quad i=1, \dots, M \quad (1)$$

where $S(D_i)$ is the survival function (mean colonies formed/cells seeded) at a given dose, D_i represent each of the radiation doses (including the zero dose), PE is the plating efficiency which is the ratio of the number of colonies formed to the number of cells seeded when cells are untreated, $S(D_i)/PE$ is defined as survival fraction, α is interpreted as the probability of a single track of radiation generating non-repairable lesion, and β as the probability of multiple tracks of radiation generating repairable damage that combines to produce lethal event. The total number of doses used in the experiment including replicate doses is denoted as M . For the SHMT model, the survival function versus radiation dose is:

$$S(D_i) = PE \left(1 - \left(1 - e^{-D_i/D_0} \right)^n \right), \quad i=1, \dots, M \quad (2)$$

where n has been interpreted as the number of distinct and identical targets that must be all inactivated to kill the cell, and D_0 is the dose at which ~37% survival occurs. All other symbols are as defined above.

2.2 Model Parameter Estimation

The measurement of colony counts is represented by a Poisson process and all measurement errors are assumed to be independent. Under these assumptions, the likelihood function of the measured colony counts can be written as follows:

$$L = \prod_{i=1}^M \frac{e^{-N_i S(D_i)} (N_i S(D_i))^{y_i}}{y_i!} \quad (3)$$

where y_i represents the measured colony counts divided by the number of cells seeded for the corresponding dose exposure D_i , while N_i is the number of cells seeded. The model parameter values that maximize the likelihood can be obtained by minimizing the negative of the natural logarithm of the likelihood function (NLL) given below (ignoring constant terms involving data).

$$NLL(\theta) = \sum_{i=1}^M \{N_i S(D_i) - y_i \ln(S(D_i))\} \quad (4)$$

The symbol θ denotes the collection of model parameters: PE , α and β for the LQ model; PE , D_0 and n for the SHMT model. The maximum likelihood estimates of the model parameters that minimize the function in Eq. (3) (denoted θ_{ML}) are obtained using the computational algorithms in the ADAPT software system as detailed in [13], which involves direct minimization of Eq. (4) without log transformation of the measured colony count data. The standard errors and 95% confidence intervals of the maximum likelihood parameter estimates θ_{ML} are also calculated via ADAPT for the case of a Poisson distribution as presented in the Appendix of this paper. Goodness-of-fit measures are calculated and used to test both the Poisson and survival function model assumptions, and to make corrections to the calculated standard errors of the model parameters in the case of over dispersed Poisson variance (see Appendix). These quantities are also used to make inferences about differences in model parameters between two experiments (t test).

2.3 The RAD-ADAPT program

The RAD-ADAPT program is a stand-alone application, with the interface written in R [14], and the core computation performed by the general purpose ADAPT software [13]. R is a widely used software environment for statistical computing and graphics, and ADAPT has robust computation capabilities for advanced modeling, estimation and data analysis applications in pharmacokinetics and pharmacodynamics. However, the use of RAD-ADAPT does not require any experience with R or ADAPT.

RAD-ADAPT implements two commonly used clonogenic assay survival models, LQ model and SHMT model. Model estimation is based on maximum likelihood method and the assumption that cell colony counts follow a Poisson distribution, as discussed above. In addition to model parameter estimation, RAD-ADAPT also allows raw data exploration, and

statistical comparison of parameter estimates between different groups (multiple comparisons against a single control). Bonferroni corrected t test with degrees of freedom $M_1 + M_2 - 6$ (M_1, M_2 is the total number of observations in each of the two experimental groups) is used for between-group comparison of parameters α and β in the LQ model as well as parameters D_0 and n in the SHMT model. Results provided by RAD-ADAPT are either graphically displayed or tabulated. The flowchart of the RAD-ADAPT program is illustrated in Figure 1.

2.4 Tissue culture example

A549 cells were purchased from the American Type Culture Collection (ATCC) in November, 2013. Cells were periodically tested for mycoplasma (Lonza MycoAlert Mycoplasma Detection Kit). Experiments were conducted on cells with fewer than 20 passages after initial recovery. Cells were cultured in RPMI-1640 (containing 2 mM l-glutamine) supplemented with 10% FBS, penicillin/streptomycin in a humidified incubator at 37°C with 5% CO₂. ATM kinase inhibitor KU55933 [15] and ATR kinase inhibitor AZD6738 [16] (AstraZeneca, Cambridge, UK) were dissolved in DMSO at 30 mM and diluted in DMSO to desired working concentrations. The final KU55933, AZD6738 and DMSO concentrations in media were 10 μ M, 1 μ M and 0.025%, respectively, from -15 min to +4 h after IR. Cells were seeded at 1,000 cells per 6 cm dish 4 h prior to gamma-irradiation in a Shepherd Mark I Model 68 [¹³⁷Cs] irradiator (J.L. Shepherd & Associates) at a dose rate of 71.1 Rad/min up to the dose of 6 Gy. The experimental data can be found in the supplemental material in a format for RAD-ADAPT program.

3. Results

To describe the generation of clonogenic assay curves using RAD-ADAPT, we examined the impact of pharmacologic ATM and ATR kinase inhibitors on human lung cancer cells lines after IR, as we have done previously [15–18]. ATM and ATR were inhibited from -15 min to +4 h after IR using KU55933 and AZD6738, respectively. LQ and SHMT survival curves were generated using RAD-ADAPT.

Figure 2 shows a screen-shot of the RAD-ADAPT interface for this example. The left most panel shows that the selected LQ and SHMT models in the top and displays the data entry in the bottom. Required data entry includes dose levels (Dose), observed colony counts (Count), character string to identify groups (Group) and the number of cells plated (Plated). The middle panel shows the estimated survival function S(D) curves and survival fraction S(D)/PE curves based on each of the selected models, and below the plots are options available to modify the display of the plots. The right panel provides details of the estimated parameters, including initial guesses, final estimates, standard errors and 95% confidence intervals of parameters.

The plots in the middle panel of Figure 2 are extracted and displayed in Figure 3. The symbols in survival function S(D) plots represent the mean and standard deviation of the survival data, which is calculated as the observed number of colonies formed over the number of cells plated at each dose level, and the curves are the model predictions based on equations (1) and (2) for the LQ and SHMT models respectively. Survival fraction S(D)/PE

plots are derived from survival function plots. It similarly shows the mean and standard deviation of the survival fraction data and model predictions. Symbols are the survival data divided by the average of calculated PE at dose 0. Curves are the predicted S(D) curves divided by the estimate of parameter PE, therefore the survival fraction prediction is always 1 at dose 0.

The parameter estimates of LQ model for this example are listed in Table 1. The α constant that describes the initial slope of the cell kill curve is increased ~2 fold for each inhibitor alone and ~4 fold for the combination of inhibitors relative to DMSO alone. The β constant that describes the quadratic component of the cell killing is very similar for ATR kinase inhibition and vehicle, and increased ~4 fold for ATM kinase inhibitor and the combination of inhibitors. This suggests that ATR kinase inhibition does not increase the number of lethal lesions directly induced by IR. A statistical comparison of α and β parameter estimates between treatment and vehicle at the significance level of 0.05 is summarized in Table 2.

4 Discussion

RAD-ADAPT is a stand-alone application with a user-friendly interface that allows the mathematical and statistical analysis of clonogenic assays in radiation biology. This addresses an unmet need of scientists working in the DNA damage response field for an automated, comprehensive tool for quantitative analyses of clonogenic assays. Two commonly used clonogenic survival models, LQ model and SHMT model, are implemented in the program. RAD-ADAPT provides comprehensive model prediction plots, tabulated parameter estimates, and allows automatic statistical comparison of parameter estimates between groups. Model parameters are obtained by maximum likelihood estimation with the assumption that the cell colony count data are distributed as independent Poisson random variables. In addition, model comparisons can be performed (LQ versus SHMT) and dispersion corrected standard errors for estimated parameters are provided to allow for between group inferences. RAD-ADAPT is open source, thus allowing for incorporation of other clonogenic survival models and related statistical analysis.

Our example of the impact of pharmacologic ATM and ATR kinase inhibition after IR is instructive. It shows that mechanistic insight can be gained using RAD-ADAPT to analyse clonogenic assays. The probability of a single track of radiation generating non-repairable lesion is increased when ATM and ATR are concurrently inhibited after IR. These data support the hypothesis that ATM and ATR kinase inhibitors irreversibly inhibit the repair or “poison” certain DNA lesions [19, 20], and it may be that a larger analysis may separate dominant inhibitory functions of ATM kinase-inhibited and ATR kinase-inhibited at single lesions. The probability of multiple tracks of radiation generating repairable damage that combines to generate a cell inactivating event is increased when either ATM alone, or ATM and ATR are concurrently inhibited after IR. These data support the hypothesis that DNA repair is delayed and/or cell cycle checkpoints are inactivated such that binary misrepair can occur to generate a chromosome translocation that may inactivate the cell.

These analyses may have clinical implications for the use of pharmacologies including ATM and ATR kinase inhibitors in radiation oncology. A pharmacologic that increases the

probability of a single track of radiation generating non-repairable lesions may increase the efficacy of high LET (linear energy transfer radiation) radiotherapy such as $^{223}\text{Radium}$ dichloride, Xofigo, which generates more lesions in a cell along single radiation track than low LET radiation such as X-rays. Conversely, a pharmacologic that increases the probability of multiple tracks of radiation generating repairable damage that combines to generate a cell inactivating event may increase the efficacy of low LET X-rays.

A comprehensive software program, RAD-ADAPT, for the quantitative analysis of clonogenic assays in radiation biology is presented and illustrated using an example that examines the impact of pharmacologic ATM and ATR kinase inhibition on human lung cancer cell line A549 after ionizing radiation. This open source software is freely available through the Biomedical Simulations Resource Center at the University of Southern California and can be download via Center's web site (bmsr.usc.edu/software). The user's guide for the program can also be found in the supplemental material.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A Statistical Error Analysis and Inference

A.1. Maximum likelihood estimation method

The asymptotic covariance matrix for the maximum likelihood estimates of the model parameters ($\widehat{\text{cov}}(\theta_{ML})$) is calculated via ADAPT as follows:

$$\widehat{\text{cov}}(\theta_{ML}) = M_{\theta}^{-1} \text{ where } M_{\theta} = -E \left[\frac{\partial^2 \ln L(\theta)}{\partial \theta \partial \theta^T} \right]_{\theta=\theta_{ML}} \quad (\text{A.1})$$

The elements of M_{θ} , $\{m_{j,k}\}$, $j = 1, \dots, p$; $k = 1, \dots, p$, are then obtained as follows (p is the number of model parameters, which is three for both the LQ and SHMT models):

$$m_{j,k} = \sum_{i=1}^M \left\{ \frac{N_i}{S(D_i)} \frac{\partial S(D_i)}{\partial \theta_j} \frac{\partial S(D_i)}{\partial \theta_k} \right\}, \quad j=1, \dots, p; \quad k=1, \dots, p \quad (\text{A.2})$$

The required derivatives are calculated using the general methods for numerical partial derivatives incorporated in ADAPT [13]. The resulting standard errors for the estimated parameters obtained from ($\widehat{\text{cov}}(\theta_{ML})$) are then used for the inference calculations described in the Methods section.

A. 2. Dispersion correction

Following the approach presented in Frome and Beauchamp [11], a goodness-of-fit assessment testing the assumptions of Poisson variation and the selected form of the survival model is implemented in RAD-ADAPT. Let m be the total number of distinct doses ($m < M$) and assuming that the number of cells seeded is the same for each of the m distinct doses, then the statistic $T(T \sim \chi^2(df = M - p))$ for the case where y_i follow a Poisson distribution is:

$$T = \sum_{i=1}^M \frac{(y_i - N_i \hat{S}(D_i))^2}{N_i \hat{S}(D_i)}, \quad \text{where} \quad \hat{S}(D_i) = S(D_i) |_{\theta = \theta_{ML}}$$

Because we have replicated measurements, T can be partitioned into two components, $T = T_1 + T_2$, defined as follows:

$$T_1 = \sum_{i=1}^M \frac{(y_i - \bar{y}_i)^2}{N_i \hat{S}(D_i)} \quad \text{where} \quad T_1 \sim \chi^2(df_1 = M - m)$$

$$T_2 = T^2 - T_1, \quad \text{where} \quad T_2 \sim \chi^2(df_2 = m - p)$$

where $\bar{y}_i$ is the mean value of the measured colony counts divided by the number of cells seeded for each distinct dose value corresponding to the i^{th} dose. If it is determined that the residual variability is larger than would be expected under the Poisson assumption (i.e., $T_1 > \chi_{0.05}^2(df_1)$) and the selected form of the survival model is consistent with the data (i.e., $(T_1/df_1)/(T_2/df_2) < F_{0.05}(df_1, df_2)$), then the standard errors of the parameters estimated obtained from Eq. (A1) above are corrected using the dispersion parameter ϕ as follows:

$$\phi = T_1 / df_1, \quad \text{then} \quad \widehat{\text{cov}}(\theta_{ML}) = \phi M_{\theta}^{-1}$$

Appendix B Supplementary Material

Supplementary data associated with this article can be found in the online version.

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Highlights

In this report, the RAD-ADAPT software for modelling clonogenic assay data in radiation biology is presented. It uses maximum likelihood estimation based on a Poisson data model to fit widely used survival models to measured cell survival data. RAD-ADAPT is a standalone, integrated software system with a graphical user interface for convenient entry and display of clonogenic assay data and modelling results. It is freely available as a downloadable executable for Windows-based systems.

Figure 1.

Flowchart of the RAD-ADAPT program. Users first select one or two desired model(s) for the subsequent parametric analysis; then import an existing data file or create a new dataset using the interface. Users can do a quick data exploration or estimate parameters of the selected model(s). “ADAPT” in Figure 1 highlights the step where ADAPT is called. Fitted curves are displayed graphically and parameter estimates are tabulated. RAD-ADAPT also has the functionality of conducting statistical comparison for parameter estimates between groups.

Figure 2. RAD-ADAPT screen shot for the example. Selected models and data entry are shown in the left panel. After clicking the “Run” button below the data entry, RAD-ADAPT estimates parameters of selected models, and displays the fitted curves and parameter estimates for each group in the middle and right panels, respectively.

Figure 3.

Model prediction vs observation plots generated by RAD-ADAPT for the example, including survival function $S(D)$ plot for LQ model (top left), survival fraction $S(D)/PE$ plot for LQ model (top right), survival function $S(D)$ plot for SHMT model (bottom left) and survival fraction $S(D)/PE$ plot for SHMT model (bottom right).

Table 1

LQ model parameter estimates for the example generated by RAD-ADAPT.

Group	Parameters	Initial guesses	Estimates	SE (CV%)	95% CI	R squared
vehicle	PE	0.4310	0.4323	2.111	[0.4133, 0.4513]	0.9501
	α	0.04069	0.04316	45.94	[0.001917, 0.08439]	
	β	0.009577	0.009168	37.17	[0.002079, 0.01626]	
	α/β	4.249	4.707	82.29	[-3.349, 12.76]	
AZD6738	PE	0.4223	0.4259	2.158	[0.4068, 0.445]	0.9579
	α	0.08218	0.0861	24.72	[0.04182, 0.1304]	
	β	0.01255	0.01195	31.45	[0.004133, 0.01977]	
	α/β	6.548	7.204	55.58	[-1.123, 15.53]	
KU55933	PE	0.4427	0.4219	2.240	[0.4022, 0.4415]	0.9820
	α	0.1082	0.1122	22.56	[0.05956, 0.1649]	
	β	0.0454	0.04088	12.12	[0.03057, 0.05118]	
	α/β	2.383	2.745	34.25	[0.7895, 4.701]	
AZD6738+KU55933	PE	0.4370	0.4339	3.172	[0.4053, 0.4625]	0.9855
	α	0.1815	0.1751	22.29	[0.0939, 0.2562]	
	β	0.04891	0.04927	16.50	[0.03236, 0.06618]	
	α/β	3.711	3.553	38.20	[0.7298, 6.377]	

Statistical comparison of LQ model parameter α and β between treatment and vehicle at the significance level of 0.05

Table 2

Parameter	Comparison	t	df	P	Perit	P < Perit ?
α	AZD6738 vs Reference	1.476	42	0.1474	0.01667	No
	KU55933 vs Reference	2.147	42	0.0376	0.01667	No
	AZD6738+KU55933 vs Reference	3.014	42	0.00436	0.01667	Yes
β	AZD6738 vs Reference	0.5484	42	0.5863	0.01667	No
	KU55933 vs Reference	5.274	42	4.36E-06	0.01667	Yes
	AZD6738+KU55933 vs Reference	4.549	42	4.53E-05	0.01667	Yes