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Synergy theory for murine Harderian gland tumorigenesis after irradiation by mixtures of high-energy ionized atomic nuclei

Edward Huang, Yimin Lin, Mark Ebert, Dae Woong Ham, Claire Yunzhi Zhang, others???, Rainer K. Sachs[1,2]

Coauthors: choose your name carefully. For continuity you should probably use the same name for the rest of your life, and a nearly unique name is quite useful if someone is searching for you on the web.

[1] Department of Mathematics, University of California at Berkeley

[2] Corresponding Author, Rainer K. Sachs, sachs@math.berkeley.edu, 510-658-5790

[3] Department of Statistics, University of California at Berkeley

Abstract [264 words]

Murine Harderian gland (HG) tumorigenesis found in one-ion accelerator experiments for simulated galactic cosmic ray spectrum ions has been a NASA concern for many years. We describe synergy theory applicable to corresponding mixed-field experiments. The "obvious" approach of comparing an observed mixture dose-effect relationship (DER) to the sum of the components' DERs is known from other fields of biology to be unreliable when the components' DERs are highly curvilinear. Such curvilinearity may be present at low fluxes in the HG experiments due to non-targeted ('bystander') effects, in which case a replacement for simple effect additivity synergy theory is needed.

This paper studies a recently introduced, arguably optimal replacement, incremental effect additivity, which is based on computer integration of non-linear ordinary differential equations. Customized open-source software is used. Possible one-ion or mixture experiments illustrate the main aspects, including calculations of 95% confidence intervals and also including a "mixture of mixtures principle" important because even nominally one-ion radiations are usually mixtures when they strike the HG, due to mouse self shielding or other matter in the beam. No new experimental results are presented.

In addition to discussing synergy theory, we argue that mixed field experiments whose components are high linear energy transfer (LET) should be emphasized more than "representative" mixtures where most of the total dose (in Gy) entering the beam is contributed by comparatively well understood low LET radiation qualities.

Whether mixing galactic cosmic ray components sometimes leads to statistically significant synergy for tumorigenesis is not yet clear. Significant synergy would increase risks for prolonged astronaut voyages in interplanetary space but hopefully does not occur.

1. Introduction

1.1. Scope of the paper

Murine Harderian gland (HG) tumorigenesis induced, in accelerator experiments, by ions in the galactic cosmic ray (GCR) spectrum has long been a NASA concern {Fry, 1985 #135;Curtis, 1992 #18;Alpen, 1993 #134;Alpen, 1994 #17;Chang, 2016 #115;Norbury, 2016 #123}. We here describe synergy theory applicable to corresponding mixed-field experiments.

The data considered consist of already published results. *In vitro* calculations are tailored to analyze the first relevant experimental mixed beam results (which will become available soon), but are also used to discuss hypothetical mixed beams that illustrate some key points about synergy theory. Some of our methods are borrowed from synergy theory developed in pharmacometrics, toxicology, evolutionary ecology and other fields.

Dose-effect relations (DERs) will play a central role. Typically the main information on mixed beam components comes from their individual one-ion DERs. The paper uses new DERs for the one-ion data. These are more parsimonious (i.e. have fewer adjustable parameters) than other recent models {Cucinotta, 2013 #138;Chang, 2016 #115;Cucinotta, 2017 #257} for the same data. Because radiation biologists often have a strong preference for parsimony, the new models are of possible interest in their own right. However they are used in the present paper mainly because their relative simplicity facilitates synergy analysis of mixed radiation fields whose components have these one-ion IDERs.

Given one-ion DERs, synergy theory results can be calculated. The question to be answered is whether mixture data manifest synergy, antagonism, or neither. This is usually quantified by comparing an observed mixture DER with a calculated baseline mixture DER defining the absence of synergy and absence of antagonism. Researchers in pharmacology and toxicology have known for a very long time {Fraser, 1872 #83;Loewe, 1926 #32} that the “obvious” method of comparing mixture effects with simply adding component effects is unreliable unless each mixture component one-ion DER is approximately linear-no-threshold (LNT). This difficulty is reviewed, e.g., in {Zaider, 1980 #30;Berenbaum, 1989 #41;Geary, 2013 #81;Foucquier, 2015 #225;Piggott, 2015 #77;Tang, 2015 #89}.

As a simple example of this unreliability, consider two agents with respective one-ion DERs $E_1 = \beta d_1^2$ and $E_2 = 2\beta d_2^2$, where β is a positive constant. These one-ion DERs, shown in Fig. 1.1.1, are curvilinear, since the second derivative (i.e. 2β or 4β respectively) is positive. Suppose we have a 50-50 mixture of the two agents, so that $d_1 = d/2$ and $d_2 = d/2$, where d is the total mixture dose. Then, since $(d/2)^2 = d^2/4$ the simple effect additivity baseline no-synergy/antagonism effect, instead being

approximately the average of the two one-ion DER heights is only half that average (Fig. 1.1.1). [when we submit the paper, figure numbering and equation numbering will be simplified; for the moment a messy system that facilitates later global search and replacement is being used].

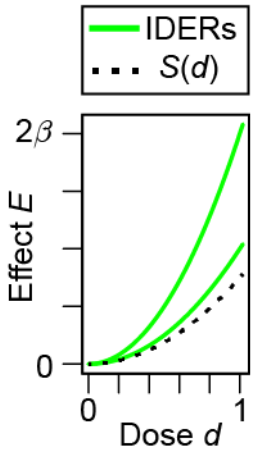

Fig. 1.1.1. Simple effect additivity synergy theory often gives absurd criteria when component one-ion DERs are highly curvilinear. The dashed black line is the baseline no-synergy/antagonism mixture DER *S(d)* specified by the simple effect additivity theory for a 50-50 mixture of two agents. The two green lines show the DERs that would result if one or the other mixture component supplied the total mixture dose *d* instead of just *d*/2. Any sensible synergy criterion would consider the dashed black curve, which specifies unexpectedly small mixture effects, as evidence for antagonism rather than using it as the baseline definition of neither synergy nor antagonism. Such discrepancies often arise when mixture component one-ion DERs are curvilinear, like the green one-ion DERs in the figure. Consequently, many different replacements for simple effect additivity synergy theory are now in use to plan and interpret mixture experiments.

At sufficiently small radiation doses and high LETs, only a small fraction of all cell nuclei suffer a direct hit by a radiation track {Curtis, 1992 #18;Hanin, 2014 #2792}. Non-targeted effects (NTE) are then sometimes important {Cucinotta, 2010 #44;Cucinotta, 2013 #76;Hada, 2014 #24;Chang, 2016 #115;Cacao, 2016 #116;Shuryak, 2017 #262;Cucinotta, 2017 #257}, with cells directly hit by an ion influencing nearby cells through intercellular signaling {Hatzi, 2015 #269}. The question of whether NTE are significantly carcinogenic at very low HZE doses remains open {Piotrowski, 2017 #3204}. Models of NTE action that are smooth (i.e. have continuous derivatives of all orders) use one-ion DERs that are very curvilinear, with negative second derivative, at low doses {Brenner, 2001 #48}. So for small doses and high LETs replacements for the simple effect additivity synergy theory are needed to investigate mixtures whose component one-ion DERs take NTE into account.

The paper will use a replacement for simple effect additivity theory called incremental effect additivity (IEA). IEA theory was introduced in two recent papers {Siranart, 2016 #1746;Ham, 2017 #507}. “Incremental” refers to the fact that one-ion DER slopes play an essential role in the theory. The

underlying idea was suggested by G.K. Lam in 1987 {Lam, 1987 #212}. A one-ion DER slope defines a linear relation between a sufficiently small dose increment and the corresponding effect increment; thus by analyzing sufficiently small increments one can circumvent the curvilinearities that plague simple effect additivity synergy theory. A systematic analysis of slopes requires using ordinary differential equations (ODEs) but Lam did not take this additional step. Implementing his insight has since become practical because computers have become adept at integrating non-linear ODEs.

The two recent references {Siranart, 2016 #1746;Ham, 2017 #507}, after reviewing many other synergy theories, give evidence that IEA synergy theory is probably the optimal substitute for simple effect additivity synergy theory. The present paper will concentrate on calculating the implications of IEA.

A potential source of confusion for all synergy calculations is that 3 conceptually different kinds of DERs must be considered. The three kinds are: 1) individual one-ion DERs; 2) mixture baseline DERs that define absence of synergy and absence of antagonism; and 3), experimental mixture DERs, which may indicate synergy, or antagonism, or neither.

Because papers on synergy theory are sometimes over-optimistic as regards the usefulness of some specific synergy theory, we will also discuss drawbacks of IEA. For example, many (but not all) synergy theories do not even try to predict whether mixed-agent synergy will occur; they merely try to define what synergy is {Zaider, 1980 #30;Berenbaum, 1989 #41;Geary, 2013 #81;Kim, 2015 #226}. Like simple effect additivity, IEA is among the synergy theories that have this drawback.

In addition to discussing synergy theory, the paper will consider NASA guidelines for mixed radiation field experiments. We will argue that mixed field experiments all of whose components are high LET should be emphasized more than “representative” mixtures where most of the total dose (in Gy) entering the beam is contributed by comparatively well understood low LET radiation qualities.

1.2. Terminology

There will be a number of acronyms in this paper. Table 1 lists the main ones, with less familiar but here often used ones, such as DER and IEA, in bold-face and underlined. The table also lists some of our most frequently used mathematical symbols. Online Resource 1 (part 1) gives more detailed lists.

Table 1. Main acronyms and mathematical notation used.

DER	dose-effect relation, for a single agent or a mixture; sometimes denoted by $E(d)$
$\mathbf{d}E/\mathbf{d}d$	A derivative. The slope of the one-ion DER $E(d)$
$D(E)$	compositional inverse function of a monotonic one-ion DER: $D(E(d)) = d$
$d_j = r_j d$	dose of the j^{th} mixture component as a fraction r_j of total mixture dose d
$E_j(d_j)$	one-ion DER for the j^{th} component of a mixture
$E(d)$	one-ion DER
$E(d; \boldsymbol{P})$	DER for an ion which is identified by its atomic charge, LET, and kinetic energy
GCR	galactic cosmic rays. The mixed radiation field in interplanetary space.
HG	Harderian gland. An organ found in many rodents
HZE	high-Z (charge) and high energy atomic nuclei, almost fully ionized
IEA	incremental effect additivity. A synergy theory based on using ODEs
$I(d)$	IEA baseline no synergy no-antagonism mixture DER
L=LET	linear energy transfer, stopping power, LET_∞
LNT	linear-no-threshold one-ion DER. A straight line with $E(0) = 0$
NTE	non-targeted effect(s) due to inter-cellular signaling. 'Bystander' effect(s)
ODE	ordinary differential equation
r_j	ratio of mixture component dose to total mixture dose, $r_j = d_j / d, 0 < r_j < 1$
$S(d)$	simple effect additivity baseline no-synergy/antagonism mixture DER
TE	targeted effect(s). Standard radiobiology action due to a direct hit or near miss
β^*	ion speed relative to the speed of light. $0 < \beta^* < 1$.
Y_0	background zero-dose HG prevalence for sham-irradiated controls.

1.3. Summary

The main purpose of this paper is to explain how IEA synergy theory can be applied to experiments on murine HG tumorigenesis induced by mixed radiation fields some of whose components are one-ion HZE beams. No new experimental data are presented.

The paper focusses on synergy theory techniques, emphasizing mathematical methods and customized computer programming more than biophysical insights. For example all our one-ion DERs for HZE ions will include terms that can model NTE in addition to terms for TE. Competing HZE one-ion DERs that assume TE-action only are here omitted because the TE-NTE one-ion DERs can illustrate synergy theory techniques adequately. The question of whether the data in fact favor NTE significance is addressed only partially, by noting that the NTE terms in the HZE TE-NTE one-ion DERs are significantly different from zero, with the p-values being very small. More systematic head-to-head comparisons between TE-NTE one-ion DERs vs. TE-only one-ion DERs are beyond the scope of the paper.

Similarly, recent one-ion DERs for the data in {Fry, 1985 #135;Curtis, 1992 #18;Alpen, 1993 #134;Alpen, 1994 #17;Chang, 2016 #115} are based on modifications of Katz' amorphous track structure approach {Katz, 1988. Quantitative Mathematical Models in Radiation Biology`, ed. J. Kiefer. #235;Cucinotta, 1999 #230;Goodhead, 2006 #231;Cucinotta, 2010 #44;Cucinotta, 2013 #138;Chang, 2016 #115;Cucinotta, 2017 #257}. Our one-ion DERs in the present paper for the same data take full advantage of a hazard function equation , favored by Cucinotta and coworkers and reviewed in {Cucinotta, 2017 #257}. As a result they are, as discussed above, more parsimonious than the one-ion DERs based on the amorphous track structure approach. However the present paper, due to its emphasis on mathematical synergy theory rather than biophysical insights, does not attempt a balanced comparison that would take into account goodness of fit of the different one-ion DERs as well as parsimony of the different one-ion DERs.

2. Methods

2.1. Customized Software

We use the open-source computer language R {Matloff, 2011 #7}, initially designed for statistical calculations but now rapidly gaining acceptance among modelers {IEE, 2014 #8}. Our customized programs are available at https://github.com/rainersachs/SynergyREBP/. Readers can freely download, use and modify them to evaluate our conclusions critically.

2.2. one-ion DERs

A mixed radiation field consists of $N \geq 2$ components. Each component has a one-ion DER. Synergy theory starts with these one-ion DERs, and trying to devise appropriate one-ion DERs was the most challenging part of our calculations.

2.2.1. Standard one-ion DER properties

Fig. 2.2.1.1 illustrates "standard" properties that the one-ion DERs considered in this paper will be assumed to have unless explicitly stated to the contrary; it also illustrates convexity/concavity terminology.

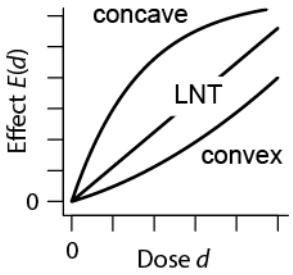

Fig. 2.2.1.1. one-ion DERs. Some relevant properties are here shown in a schematic graph that does not specify numerical values. The graph also serves to illustrate the terms "convex" and "concave".

The standard properties are the following.

1). For each mixture component when acting by itself, the data have background plus radiogenic contributions. We define the component's one-ion DER as the radiogenic contribution. Thus by definition one-ion DERs are zero when there is no dose above background. In figure 2.1 all 3 curves start at the origin $d = 0$, $E = 0$

2). one-ion DERs are continuous, continuously differentiable curves with a first derivative $\mathbf{d}E/\mathbf{d}d > 0$ for all non-negative doses d from zero up to the largest dose of interest. It follows that standard one-ion DERs are monotonic increasing for all doses of interest. Here "doses of interest" may be mixture doses. For example, even if an ion participating in a mixture contributes only half of

the total mixture dose d and the maximum value of interest for total mixture dose d is 1 Gy, the ion's one-ion DER is still required to have positive first derivative all the way up to 1 Gy, not just up to 0.5 Gy.

3). The one-ion DERs have continuous second derivatives.

Non-standard one-ion DERs are often useful but are not needed for this paper. An example of a DER that violates properties 2) and 3) is a linear DER with threshold. At the threshold there is a kink where the first derivative is discontinuous and the second derivative is in effect a Dirac delta function so that, roughly speaking, it is $+\infty$ there.

As regards convexity and concavity: if the second derivative of a standard one-ion DER is respectively (positive, zero, or negative) for all doses of interest the one-ion DER is (strictly convex, LNT, or strictly concave) respectively, as shown in Fig 2.1.

Standard one-ion DERs can have shapes more complicated than those shown. Allowed, for example, are sigmoidal curves where the second derivative is positive at all small doses, is zero at one point of inflection, and is negative on up to the largest dose of interest [where, however, the slope is still required to be positive by property 2)].

2.2.2. Scope of the data

The data includes experimental observations on the fraction of female B6CF1/Anl mice that develop at least one radiogenic HG tumor after exposure at various doses to various one-ion HZE beams {Fry, 1985 #135;Curtis, 1992 #18;Alpen, 1993 #134;Alpen, 1994 #17;Chang, 2016 #115}. This fraction, the tumor prevalence, is by definition ≤ 1. The HZE ions' parameter ranges are: atomic charge $10 \leq Z \leq 57$; approximate LET $25 \leq L$ (keV/μm) ≤ 950; approximate speed relative to speed of light $0.61 \leq \beta^* \leq 0.81$. Additional data in the same data set is for fast, low LET protons and alpha particles. All this data is included in customized open-source software freely downloadable from GitHub.

Ongoing experiments involve additional one-ion beams or involve, for the first time, corresponding mixed-beam exposures. The data from these ongoing experiments has not been published and will not be used in this paper. However, some of our synergy theory examples here were selected with ongoing or planning-stage mixed-beam experiments in mind.

2.2.3. HZE one-ion DERs: notation for physics parameters that specify an HZE ion entering the beam

We will characterize different HZE ions by their charge Z, LET L, and, instead of ion speed β^* relative to the speed of light, kinetic energy KE. The units for KE will usually be MeV/u, where u is the atomic mass number. These parameters will be assembled into an ion-characterizing parameter vector with 3 components and used to label one-ion DERs as follows:

$$E(d; \boldsymbol{P}) \text{ where } \boldsymbol{P} = (Z, L, KE). \qquad (2.2.3.1)$$

Sometimes $E(d; \boldsymbol{P})$ will be abbreviated as simply $E(d)$.

2.2.4. HZE one-ion DERs: preliminary remarks

The one-ion DERs we use here for the HZE data modify some of the tumor prevalence models in {Chang, 2016 #115} and {Cucinotta, 2017 #257}. All relevant one-ion DERs, in the literature and in the present paper, assume TE dominate at high doses. The HZE one-ion DERs we will use assume in addition that NTE dominate at low doses. This NTE assumption was not tested stringently. It was adopted mainly because recent modeling, e.g. {Chang, 2016 #115;Cucinotta, 2017 #257}, somewhat favors it and the resulting one-ion DERs suffice to illustrate synergy theory methodology. As discussed later in the Methods section and in the Results section, we found some evidence in its favor.

As a preview, Fig. 2.2.4.1 illustrates the shape of the HZE one-ion DERs that result from our assumption that NTE dominate at very low doses. The calculations, including regression, that lead to these shapes will be described only in subsequent sub-sections but the figure's information on the shapes may help guide the reader through those sub-sections. The ion is Fe56 with $Z = 26$, $L = 195$ keV/μm, and $KE = 600$ MeV/u.

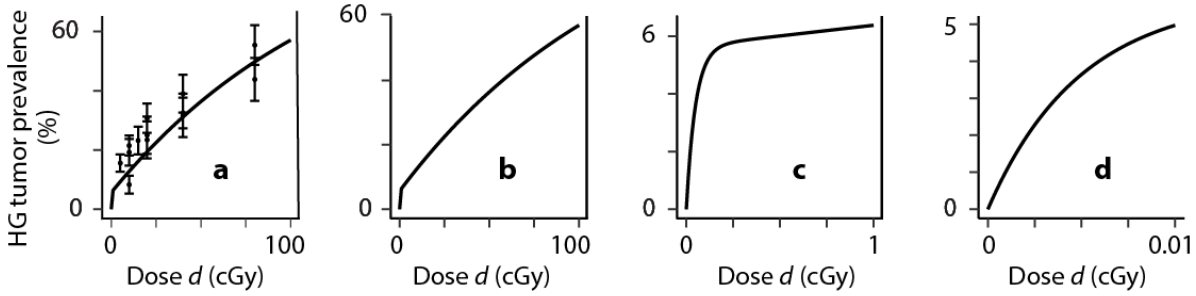

Fig. 2.2.4.1. HZE one-ion DER shapes. Panel A shows data and a DER obtained by regression. Panel B shows just the DER. Panels C and D zoom in on panel B to give details on the very low dose region where NTE putatively dominate. Online Resource 1 (part 2) gives figures for all one-ion data and DERs; it includes panel **a** with a larger dose-range. Edward: when making figures, look at the table in online resource 1, part 2 for information you will need.

Our HZE one-ion DERs will be standard as defined in subsection 2.2.1. However, in panels A and B of Fig.2.2.4.1 it looks as if standard smoothness conditions might be violated. The slope at $d = 0$ looks like it might be infinite; in addition it appears as if at one point there is a kink where the first derivate is discontinuous. Panel C zooms in to show that actually there is no kink, just a region of very high concavity. Panel D zooms in again to show that the slope at the origin is finite.

The overall shape (panel B) changes as dose increases. At very low doses there is very high concavity. Then there is a region where slight concavity and/or slight convexity can occur. Then at high doses there must be concavity, despite the fact that in radiobiology high LET TE are often linear, due

merely to the fact that prevalence, defined as presentation of at least one HG tumor, can never go above 100% no matter how large the dose.

Our HZE DER calculations will cover dose ranges larger than $0 \le d < 50$ cGy despite the fact that HZE doses of > 50 cGy are vigorously deprecated in some recent NASA calls. There are two reasons. First, our modeling emphasizes the following idea. If an HZE ion is a component of a mixture, its relevant damage need not be the just the damage it itself would inflict if acting alone. More likely to be relevant is the incremental damage the ion inflicts when it adds its incremental dose to all the other incremental doses contributed by all the other mixture components. In this context, one-ion DER shapes at doses > 50 cGy remain relevant even if the policy of deprecating all high-dose HZE experiments continues. The second reason is that we believe, and will in the Discussion section argue, that continuing experiments with HZE doses considerably larger than those encountered even during several years above low earth orbit should be a major part of efforts at the Brookhaven (NY) NASA space radiation laboratory (NSRL).

2.2.5. HZE one-ion DERs: the hazard function approach

The starting point for our models is a very useful hazard function equation [reviewed in {Cucinotta, 2017 #257}] suggested by Cucinotta and coworkers:

$$E(d) = 1 - \exp[-H(d)]. \qquad (2.2.5.1)$$

Here $E(d)$ is a one-ion DER and $H(d)$ is a non-negative hazard function, which can be chosen by biophysical modeling and then defines $E(d)$ via Eq. (2.2.5.1). Short calculations show that if $H(d)$ is a standard DER as defined above in connection with Fig. 2.2.2.1, then the one-ion DER $E(d)$ in Eq. (2.2.5.1) is also standard. We will use hazard functions which are standard DERs. Eq. (2.2.5.1) is an important improvement over earlier models of the HZE HG data because, without needing to add any extra adjustable parameters, it incorporates the limitation that $E(d) \le 1$, which holds since prevalence is defined as having at least one HG tumor.

2.2.6. HZE one-ion DERs: the NTE term

Our hazard functions will be taken to have additive NTE and TE contributions:

$$H(d; \boldsymbol{P}) = N(d; \boldsymbol{P}) + T(d; \boldsymbol{P}), \text{ where } \boldsymbol{P} = (Z, L, KE). \qquad (2.2.6.1)$$

Here the NTE and TE contributions are denoted by N and T respectively.

We first consider the NTE contribution, N. This was taken to have the equation

$$N(d) = \eta\left[1 - \exp(-d / d_0\right]. \qquad (2.2.6.2)$$

In Eq. (2.2.6.2) η is an adjustable parameter, interpreted as the prevalence at which NTE for any HZE ion saturates so that, for prevalences larger than η, NTE, if any, are so small compared to TE that they are negligible (or already included in TE models and in measured prevalences).

The other quantity, d_0, is a nominal dose chosen to be much smaller than 0.01 Gy; in our calculations we used 0.00001 Gy. There are no HZE data points in the region $0 < d < 0.033$ Gy. Data at higher doses do (perhaps) suggest NTE which lead to a large average positive slope, in the region $0 < d < 0.033$ Gy, whose cumulative influence builds up a prevalence large enough to be detectable above background and noise at doses ≥ 0.033 Gy. To take into account NTE dose dependence in a way consistent with the concavity found in mechanistic models for NTE for other endpoints {Brenner, 2001 #48} we used the factor $[1-\exp(-d/d_0)]$ and used $d_0 = 10^{-5}$ Gy. Numerical explorations show that the final results are insensitive to d_0 as long as $d_0 \ll 0.01$ Gy.

2.2.7. HZE one-ion DERs: TE term and newDERs

For the other term, *T*, in the HZE hazard Eq. (2.2.61) we devised new equations. There were six motives for using new models; these motives are detailed in Online Resource 1 (part 3). After many attempts, we hit upon an algebraic combination of two adjustable parameters in earlier models that is, for the dose range of main interest, nearly dose-independent in those earlier models. Choosing this combination as one adjustable parameter allowed us to reduce the number of adjustable parameters from 4 to 3."Parsimonious" models, with a minimal number of adjustable parameters in the spirit of Occam's razor, are often especially emphasized in radiobiology.

Specifically, we used for *H* a TE term linear-no-threshold in dose with a coefficient involving a standard two-parametric *L* dependence that typically peaks at an LET of several hundred keV/μm:

$$T(d;L) = a_1 L \exp\left[-a_2 L\right] d. \qquad (2.2.7.1)$$

Combining Eqs. (2.2.6.2) and (2.2.7.1) gives the comparatively simple equation for our new HZE model:

$$E(d;L) = 1 - \exp[-H(d;L)] \text{ where } H(d;L) = a_1 L \exp\left[-a_2 L\right] d + \eta\left[1 - \exp(-d / d_0\right]. \quad (2.2.7.2)$$

The 3 adjustable parameters are a_1, a_2, and η.

2.2.8. HZE one-ion DERs: calibration methods and variance-covariance matrices

The background value, Y_0, for sham-irradiated controls was taken from {Chang, 2016 #115} to be Y_0 = 2.7 % prevalence, as determined from all the zero-dose data, including older data not acquired at NSRL. Y_0 was regarded as an exact value. If one is attempting to compare a TE-only model with a model that allows for both TE and NTE, the value of Y_0 and its variance are very important (because at the very low doses where NTE putatively dominate TE, Y_0 and NTE have approximately the same magnitude). For reasons discussed above we did not consider TE-only models at all in this paper, where the emphasis is on synergy theory methodology rather than optimizing one-ion DERs, so taking Y_0 as fixed was adequate for our purposes here.

Given Y_0, the 3 adjustable parameters in the HZE one-ion DER of Eq. (2.2.7.2) were obtained by inverse-variance-weighted non-linear regression. The nls() function of the open-source computer

language R determined the variance-covariance matrix during the regression calculation, and this matrix was used in subsequent 95% confidence interval (CI) estimates for mixture baseline no-synergy/antagonism DERs.

After calibration, our one-ion HZE DERs were considered applicable to all ions in the *Z*, LET, and kinetic energy ranges covered by the data, i.e. applicable even to one-ion beams not in the data set. The relevant *Z* and *L* ranges were given above: $10 \leq Z \leq 57$; approximate LET $25 \leq L$ (keV/μm) ≤ 950. The kinetic energy range was $260 \leq KE$/u (MeV) ≤ 1000.

2.2.9. One-ion DERs for low LET proton and alpha particle beams

The one-ion DER for data on $Z = 1$ or 2 ions was taken to be

$$E(d) = 1 - \exp[-H(d)] \quad \text{where} \quad H(d) = ad, \qquad (2.2.9.1)$$

where *a* is the only adjustable parameter, whose statistical distribution was determined by inverse-variance-weighted non-linear regression. We originally assumed a linear-quadratic form for the hazard function *H*, but the quadratic term was found to be not significantly different from zero so it was omitted from the model. As for HZE DERs, the hazard function approach thus facilitated use of parsimonious models.

When one-ion data for $2 < Z < 10$ is added to this HG data set, it would be reasonable to treat *L* as having a continuous spectrum. The one-ion DERs used to date, including ours, in effect neglect the existence of biophysical similarities between the case $L \leq 1.6$ keV/μm for $Z \leq 2$ and the case $L \geq 25$ keV/μm for $Z \geq 10$.

2.3. Synergy theory calculations

2.3.1. Notation

Consider acute irradiation with a mixed beam of $N \geq 2$ different radiation qualities. The dose proportions r_j that the different qualities contribute to total dose $d = \sum_{j=1}^{N} d_j$ obey the equations

$$d_j = r_j d; \quad r_j > 0; \quad \sum_{j=1}^{N} r_j = 1. \qquad (2.3.1.1)$$

In our subsequent calculations r_j will always, for convenience, be independent of dose. Dose independent proportions r_j model one typical pattern for irradiation. The assumption of dose-independent proportions does not affect the final results. It implies that any one of the d_j can be considered a control variable on essentially the same footing as the total dose d since d_j determines d, via $d = d_j/r_j$ with $r_j > 0$, and thereby determines each $d_i = r_i d_j/r_j$. However we will distinguish sharply between the dose control variables d and d_j vs. total mixture effect considered as a control variable. In our analyses effect magnitude is sometimes used to determine d and d_j, instead of being determined by one of them.

2.3.2. Simple Effect Additivity S(d)

Using the notations specified above, the baseline no-synergy/no-antagonism mixture DER of the simple effect additivity theory, denoted by $S(d)$, is:

$$S(d) = \sum_{j=1}^{N} E_j(d_j). \qquad (2.3.2.1)$$

We shall sometimes use $S(d)$ as shorthand to indicate the simple effect additivity approach.

2.3.3. Inverse Functions

Inverse functions (sometimes called compositional inverse functions) play a prominent role in various synergy theories, including IEA. Inverse functions are needed when using effect, rather than dose, as the independent variable. A familiar radiobiology example of inverse functions occurs when calculating the relative biological effectiveness (RBE) of two different radiations.

The inverse of a monotonically increasing function undoes the action of the function. For example, for x > 0, $\sqrt{x^2} = x$ so the positive square root function is the inverse of the squaring function; note that the inverse of x^2 is not x^{-2}. As another example $\exp[\ln(x)] = x$ for $x > 0$, and $\ln[\exp(y)] = y$ so the functions exp and ln are inverses of each other.

2.3.4. The IEA equation that defines absence of synergy and absence of antagonism

When simple effect additivity theory *S(d)* is inappropriate, an IEA baseline no-synergy/antagonism mixture DER $I(d)$ has a number of conceptual and practical advantages over other known replacements

for $S(d)$. {Siranart, 2016 #1746}. This subsection defines the elementary version of IEA, which suffices for the present paper; there is a much more general version {Ham, 2017 #507} that we do not need here.

Suppose we have a mixture of N components with each component one-ion DER 'standard' as defined when discussing Fig. 2.2.2.1. It follows that each component one-ion DER has a compositional inverse function D_j, defined for all doses of interest and thus for all sufficiently small non-negative effects E. As discussed in sub-section 2.3.3 this means $D_j(E) = d$ when $E = E(d)$. The baseline IEA no-synergy no-antagonism mixture DER $I(d)$ is defined as the solution of the following initial value problem for a first order, typically non-linear, ODE:

$$\mathbf{d}I / \mathbf{d}d = \sum_{j=1}^{N} r_j \left[\mathbf{d}E_j / \mathbf{d}d_j \right]_{d_j = D_j(I)}; \;\; d = 0 \Leftrightarrow I = 0, \qquad (2.3.4.1)$$

with r_j = constant > 0 being again the fraction of the total mixture dose contributed by the j^{th} component. Thus the k^{th} one-ion DER also obeys Eq. (2.3.4.1) but with $r_k = 1$ and all the other $r_j = 0$. Under our assumptions there is a unique, monotonically increasing solution $I(d)$ for all doses of interest {Ham, 2017 #507}.

In Eq. (2.3.4.1), the square bracket with its subscript indicates the following calculations. First find the slope of the j^{th} one-ion DER curve as a function of individual dose d_j. Then evaluate d_j using the inverse function D_j with the argument of D_j being the effect I already present due to the influence of all the components acting jointly. Using $d_j = D_j(I)$ in Eq. (3) instead of the seemingly more natural $d_j = D_j(E_j)$ is the key assumption made. Using $d_j = D_j(E_j)$ would merely lead back to simple effect additivity $S(d)$ {Ham, 2017 #507}.

Eq. (2.3.4.1) can be interpreted as follows. As the total mixture dose d increases slightly, every individual component dose d_j has a slight proportional increase since $\mathbf{d}d_j / \mathbf{d}d = r_j > 0$. Therefore every mixture component contributes some incremental effect. The size of the incremental effect is determined by the state of the biological target, specifically by the total effect already contributed by all the components collectively (and <u>not</u> by the dose the individual component has already contributed). In this way different components appropriately track changes of slope both in their own one-ion DER and in the other one-ion DERs.

2.3.5. Calibrating Background and Radiogenic Effects Separately

Synergy is typically considered as due to interactions among agents. Our mathematical synergy analysis applies to radiogenic effects. In calibrating one-ion DERs $E_j(d_j)$ from data we always, as discussed above, used only data at non-zero doses, $E_j(0)$ being 0 by definition. Background, designated by Y_0, was based on the zero-dose data for sham irradiated controls. Y_0 is needed when calibrating one-ion DERs from data or

comparing baseline mixture DERs to data. However the main synergy calculations involve only one-ion DERs, not background plus radiogenic, effects.

2.4. Uncertainties in mixture effects

Synergy theory requires not only a way to calculate a baseline mixture DER defining no-synergy/no-antagonism but also a method of estimating uncertainties for the baseline mixture DER from mixture component one-ion DER uncertainties. Taken together these two elements constitute a default hypothesis useful for statistical significance tests on mixture observations. Without such tests, it is sometimes unclear if an unexpectedly large or small observed result does or doesn't call for a follow-up experiment. We used Monte Carlo simulations {Binder, 1995 #1227} to calculate 95% CI for $I(d)$. Because it is known that neglecting correlations between calibrated parameters tends to overestimate how large CI are {Ham, 2017 #507}, we used sampling techniques guided by variance-covariance matrices.

2.5. Summary of *in-silico* synergy theory methodology

Fig. 2.5.1 summarizes our synergy calculations in a flow chart that with minor rewording would also apply to many other synergy theory papers. The flow chart re-emphasizes that three conceptually different kinds of DERs are involved.

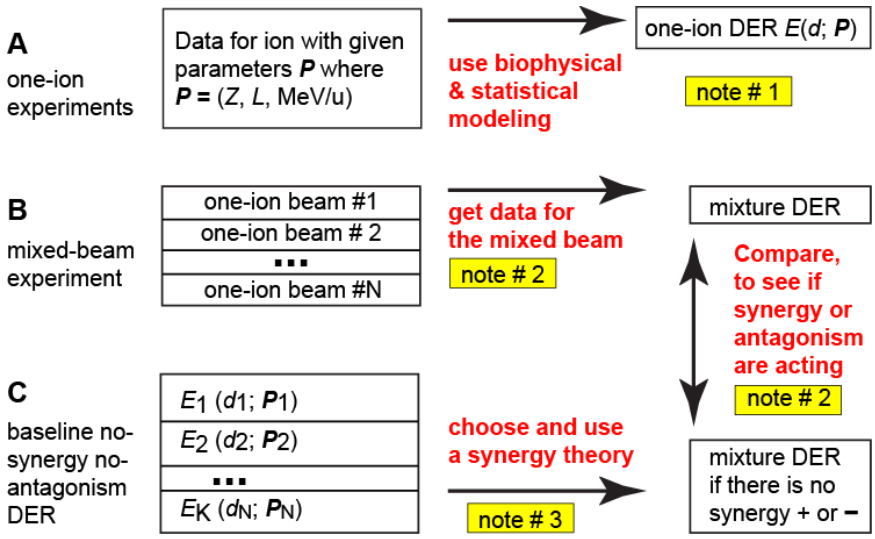

Fig. 2.5.1. Synergy modeling. Notes explaining some details are given in the text.

Note # 1. In our hands the biophysical and statistical modeling of one-ion DERs is not systematic. It uses educated guesswork trying to assemble many different kinds of biophysical, mathematical, and statistical information into equations.

Note # 2 (applies to both spots where it is shown on Fig. 2.5.1). No published results on mixed beams are yet available in this data set. We will here discuss instead mixed beams for which data will soon be available, mixed beams tentatively planned, mixed beam experiments we believe should be carried out but most probably will not be carried out, and purely hypothetical mixed beams discussed only because they illustrate some aspect of synergy theory.

Note # 3. For brevity, $S(d)$, defined in Eq. 2.3.2.1, and IEA are the only synergy theories for which this paper gives any graphs, tables, or numerical results.

Online Resource 1 (part 4) gives a detailed description of the freely downloadable open-source R script that implements the methods shown in Fig. 2.5.1.

3. Results

3.1. One-ion DERs

Contains the following for NTE HZE one-ion DERs and for the low LET DER:

Summary of regression results, with p-values.

Correlation and covariance matrices for the HZE one-ion DER

Refers to Online Resource 1 (part 3). This will be for a file on the Journal's web site that we get to submit.

3.2. Mixture baseline no-synergy/antagonism DERs

All mixtures used in the present grant for HG tumorigenesis

p,Si: 60,40;

H, Fe600, 40,30

Si, Fe600, 20,20

51.9cGyH250+5.2cGyHe228+1.5cGyO350+3.9cGyTi1,000+7.5cGyFe600=9.8cGyFe300??

.5. Limitations of Synergy Theories

All known synergy theories have substantial limitations. Because such limitations are sometimes soft-pedaled, we give details on some of the major ones. For example, every published

alternative to simple effect additivity requires some restrictions on one-ion DERs that limit its scope. Often monotonic increasing one-ion DERs are explicitly required or implicitly assumed.

Another problem, already mentioned above, is that synergy theory produces only a baseline mixture DER. Mixture component interactions can produce synergy or antagonism, i.e. deviations from the baseline. Mathematical manipulations of one-ion DERs are needed to define synergy but cannot predict it {Lam, 1994 #56}. If there is significant synergy or antagonism, biophysical insights and multiple mixture experiments or observations, not just mathematical

Table 3. Calibrated Parameters

	Units	Value	±[b]	*p*	Comments
Y_0	dimensionless	7.0e-5 [a]	NA	NA	Background. See sub-section 2.6.2
η_0	dimensionless	2.5e-4	4.60e-5	2.0e-06	Both parameters help specify how NTE magnitude depends on LET *L*
η_1	μm(keV)$^{-1}$	9.0e-3	1.61e-3	1.1e-6	
σ_0	μm^2	5.96	1.96	3.8e-3	Both parameters help specify TE slope as a function of *d*, *L*, and Z_{eff}^2/β^{*2}
κ	dimensionless	695	263	1.1e-2	

[a]Powers of 10 are indicated by 'e', for example 3.5e-3=0.0035.
[b]Standard errors are indicated by ± .

2.6.7 one-ion DER Calibration

The one-ion DERs were calibrated by using non-linear least squares inverse variance weighted regression with the Levenberg-Marquand algorithm to determine the four adjustable parameters from combined data of all six ions at all non-zero doses. The auxiliary parameter α_γ and the background value Y_0 were held fixed at their central values during the calibration. The results for the four adjustable parameters were shown above, in Table 3.

The regression also determined a variance-covariance matrix, shown in Online Resource 1 (part), which we used during synergy analyses in calculating 95% CI for the baseline IEA mixture

DER $I(d)$. The variance-variance matrix in turn determined a parameter correlation matrix, shown in Table 4.

	η_0	η_1	σ_0	κ
η_0	1	0.96	0.43	0.41
η_1	0.96	1	0.44	0.39
σ_0	0.43	0.44	1	0.96
κ	0.41	0.39	0.96	1

Table 4. Pairwise Correlations. In this case, all correlations are > 0. An intuitive argument for the strong positive correlation between η_0 and η_1 is given in Online Resource 1 (part).

3.2. HZE Murine Harderian Gland (HG) Results for $I(d)$

For the data set on murine HG prevalence after exposure to one-ion HZE irradiation (sub-section 2.7) we chose one-ion DERs with 3 adjustable parameters, and again calibrated the one-ion DERs using inverse variance weighted non-linear least squares regression (sub-section 2.6 above) for all ions at the same time. We will graph mixture DER results using average values for the adjustable parameters. 95% CI for $I(d)$ have been sufficiently exemplified in Figs. 7 and 8.

Fig. 9 shows an example where simple effect additivity $S(d)$ again unrealistically specifies that a mixture effect larger than that of any component defines absence of synergy and antagonism. Similar calculations show that if more than about 20 HZE ions are involved $S(d)$ makes the absurd claim that absence of synergy or antagonism at 0.5 Gy means the fraction of mice that have at least one tumor is greater than 1. In contrast $I(d)$ in Fig. 9 is close to the average of the four one-ion DERs at all doses larger than the nominal value of 10^{-5} Gy.

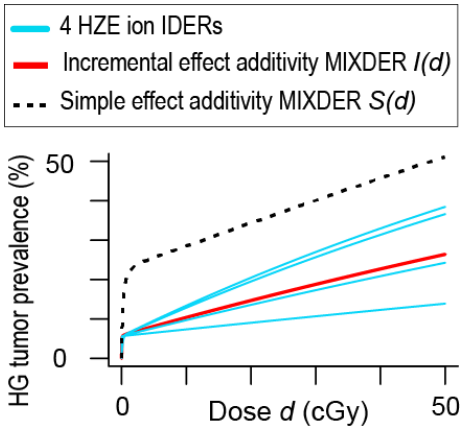

Fig. 9. A 4-ion Mixture. The figure shows results for ions of respective LETs (bottom to top) L = 25, 70, 190, and 250 keV/μm. For HG prevalence (unlike WGE simple CA) higher LETs are more damaging. Each ion contributes ¼ of the total mixture dose d. Blue lines are again the one-ion DERs that would result if one of the ions contributed the entire dose d.

[reviewed in {Shuryak, 2017 #1745}] that occur in trying to compute RBEs when a high LET radiation can produce effects larger than any effect the low LET reference radiation can produce. Eq. (18) solves this problem.

3.5. Summary of Results

To illustrate the aspects of synergy theory likely to be most important in radiobiology we gave results exemplifying the following: the importance of having high-quality one-ion DERs available; the use of alternatives, such as IEA, to simple effect additivity $S(d)$ when one-ion DER curvilinearity requires an alternative; calculation of baseline mixture DER 95% CI taking into account correlations between one-ion DER adjustable parameters; and the extraordinarily rapid increase in the number of possible mixtures needed to determine synergy patterns for N-component mixtures as N increases.

4. Discussion

4.1 Synergy Theory

For the foreseeable future radiobiologists studying mixed radiation field effects will almost inevitably emphasize possible synergy and antagonism among the different radiation qualities in the mixture. Therefore trying to find a systematic quantification of synergy, general enough to cover most cases of radiobiological interest and precise enough to enable credible estimates of statistical significance when synergy is indicated by a mixture experiment, is worthwhile. But it is not easy.

One main problem is the following. The common belief that synergy can always be defined as an effect greater than the simple effect additivity baseline mixture DER $S(d)$ is wrong. $S(d)$ can almost always be calculated, but in some important cases is clearly inappropriate. There are a number of published alternatives which seem appropriate whenever they can be calculated, but are not well defined unless all one-ion DERs in a mixture are monotonic in the same direction. The monotonicity requirement restricts their scope unduly.

Among the alternatives to $S(d)$, the IEA baseline mixture DER $I(d)$ seems preferable. We have here illustrated it with detailed examples of mixtures of ions in the GCR spectrum, since more experimental information on such mixtures will soon become available.

4.2. Summary

- Synergy theory will continue to be used to plan experiments involving mixed radiation fields and interpret the results of such experiments. It can and should include calculations that give IEA theory confidence intervals based on variance-covariance matrices.
- If non-targeted effects are important the simple effect additivity no-synergy/no-antagonism baseline mixture DERs should be ignored or used only cautiously.
- IEA theory is in our opinion the preferred replacement for simple effect additivity theory.
- When individual dose-effect relations for components of a mixture are all monotonically increasing there are many other synergy theories that have been developed over many years in many different fields of biology to supplement or replace simple effect additivity.
- In any case, all synergy theories have more limitations than is generally realized.

- Whether mixing GCR components ever leads to statistically significant synergy for animal tumorigenesis is not clear. Upcoming mixture experiments will help clarify this question.

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WEB Online Resource 1.

Parts.

1. Glossaries
2. Ion characteristics
3. New one-ion DERs
4. Implementation of script

Online Resource 1, part 2

ion	*L*	*Z*	*β**	MeV/u	comments
H1	0.4	1	0.614	250	Chang
He4	1.6	2	0.595	228	Alpen
Ne20	25	10	0.813	670	Chang
Si28	70	14	0.623	260	Chang
Ti48	100	22	0.876	1000	Chang
Fe56	195	26	0.793	600	Alpen
Fe56	195	26	0.793	600	Chang
Fe56	250	26	0.654	300	Alpen
Nb93	464	43	0.793	600	Chang
La139	953	57	0.791	593	Alpen

Chang LET at mouse, Alpen at entering beam. Exception Alpen Entering beam changed to 195 since the data sets were found to be combinable, and were combined, in

Online Resource 1, part 3.

New one-ion models were used instead of recent models based on modifications of Katz' amorphous track structure approach {Katz, 1988. Quantitative Mathematical Models in Radiation Biology`, ed. J. Kiefer. #235;Cucinotta, 1999 #230;Goodhead, 2006 #231;Cucinotta, 2010 #44;Cucinotta, 2013 #138;Chang, 2016 #115;Cucinotta, 2017 #257}. There were a number of reasons we considered new models, as follows.

First, it has often been argued, e.g. in {Goodhead, 2006 #231}, that when applied to experiments with complex biological targets the amorphous track structure approach loses some of the simplicity, elegant

agreement with data, and biophysical credibility which it has when applied to experiments where the targets are emulsions or viruses.

Second, NTE are conceptually completely different from delta rays as regards transmitting influences from a directly hit cell to neighboring cells {Hatzi, 2015 #269}. NTE involve endogenous cell signaling. We saw no reason why the biophysical reasoning that leads to the amorphous track structure models should be relevant to NTE.

Third, the HZE models in {Cucinotta, 2017 #257} contain a factor interpreted as due to cell killing; we felt that the factor implicitly assumes cell repopulation after cell killing is strongly biased against repopulation of tumorigenic cells whereas unbiased repopulation or repopulation with the opposite bias is more likely to occur {Sachs, 2005 #70}.

Fourth, some models in {Cucinotta, 2017 #257} use an adjustable parameter that regression found to be not significantly different from zero even at the largest p-level, $p \leq 0.1$ usually considered. We reasoned that since the hazard function formalism automatically takes into account the constraint that prevalence $\leq 100\%$, one might, in the interests of parsimony (i.e. Occam's razor), be able to find models with fewer adjustable parameters, all significantly different from zero.

Fifth, we felt models which used discontinuous jumps in effect level should either be replaced by stochastic process models or by non-stochastic models with continuous values, first derivatives and second derivatives – postulating that an infinitesimal dose increment can produce a finite jump in effect conflates stochastic process models and deterministic models in an unusual way.

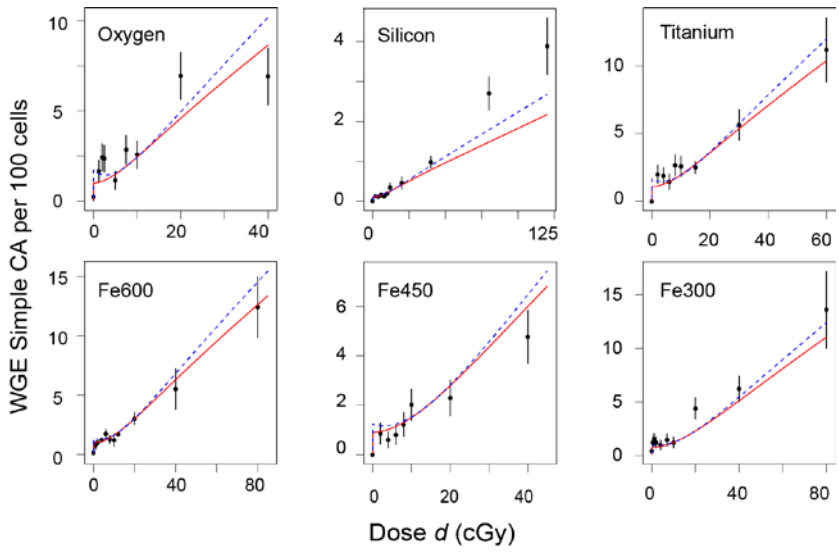
Sixth, the comparatively elementary version of IEA used in this paper assumes monotonic increasing one-ion DERs, while some previous models allow dose regions where the one-ion DERs are decreasing instead. Using one-ion DERs that are not monotonic increasing at all doses of interest would have required a far-reaching generalization of the elementary IEA formalism {Ham, 2017 #507}, more challenging computationally than necessary for the present paper's explanation of synergy theory.

However, all these arguments do not rule out the possibility that the amorphous track structure approach may have important advantages over the one-ion DERs we used here, for example perhaps much better goodness of fit for published and upcoming murine HG tumorigenesis data. Balanced comparisons have not yet been carried out.

Fig. 3.1. One-ion DERs. 8-panels with data and error bars

The figure will look similar to the following but will refer to HG prevalence instead of the chromosome aberration data shown in this placeholder and will contain 8 panels for the 7 HZE

ions in our present database instead and our low LET DER instead of the 6 panels shown in the place holder.



Online Resource 1, part 4.

The data are sourced from Chang et al. (2016) and Alpen et al. (1993, 1994) and implemented as R dataframes throughout the calculations. A number of R packages from the CRAN repository were used, notably stats for non-linear regression, deSolve for solving differential equations, mvtnorm for Monte Carlo simulations, and ggplot2 for plotting.

Our computational workflow with respect to R methods and functions is as follows. Various datasets on Harderian gland tumorgenesis are first implemented as R dataframe structures. Inverse variance weighted non-linear least square models are fitted over these dataframes using the Gauss-Newton algorithm inside the function nls from the package stats. Coefficients extracted from the models with coef are used to construct hazard functions in the form of a user-written R function. Standardized one-ion DERs are initialized from these hazard functions as user-written functions following the hazard function equation Eq. (2.2.4.1). These resulting one-ion DERs encompass various 1-ion beam variants (HZE, low-LET) and effect models (TE, NTE + TE).

Computing $I(d)$ involves calling a user-written R function calculate_complex_id that applies IEA to mixtures of $N \geq 2$ one-ion DERs, with at most one low-LET DER. calculate_complex_id takes an argument to specify use of either the NTE+TE or the TE-alone model. Calculation of $I(d)$ requires construction of an R vector dE with elements corresponding to the derivative of each one-ion DER curve

as a function of dose. A one-dimensional root finder uniroot is used to find the incremental effect of each one-ion DER. We construct dI, a vector corresponding to the numerical derivative of $I(d)$ with respect to mixture dose d by applying Eq. (2.3.4.1) to each element of **d**E. A numerical ODE integrator from deSolve is used to integrate dI with a Radau method to return a R list of mixture DER entries.

Confidence intervals for the calculated IEA baseline mixture DER $I(d)$ are found through Monte Carlo (MC) simulations. A vector of total-mixture dose points is chosen. For each MC iteration, a user-written function generate_ci initializes a vector of random parameter value samples for a particular dose from multivariate distributions determined during one-ion DER fitting. Our MC simulations use 500 total parameter samples over each selected dose point. These samples are drawn with the rmvnorm function from the mvtnorm package. An IEA dose effect relation is calculated at each selected dose point with calculate_complex_id and the sample parameters.

When the MC step is completed a 95% confidence interval is constructed at each dose point sorted by effect size. The naive confidence intervals are also computed within generate_ci by choosing parameters using each parameter marginal distribution instead of using variance-covariance matrices.

Bibliography