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# Testing additivity of anticancer agents in pre-clinical studies: A PK/PD modelling approach

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ABSTRACT

In clinical oncology, combination regimens may result in a synergistic, additive or antagonistic interaction (i.e. the effect of the combination is greater, similar or smaller than the sum of the effects of the individual compounds). For this reason, during the drug development process, *in vivo* pre-clinical studies are performed to assess the interaction of anticancer agents given in combination. Starting from a widely used single compound PK/PD model, a new additivity model able to predict the tumour growth inhibition in xenografted mice after the administration of compounds in combination was developed, under the assumption of a pharmacodynamic null interaction. By comparing the predicted curves with actual tumour weight data, possible departures from additivity can be immediately ascertained by visual inspection; a statistical procedure based on a $\chi^2$ test has also been developed for this aim. The advantages of the proposed approach in comparison to other modelling methodologies are discussed and its application to four combination studies is presented.



## 1. Introduction

In several circumstances, the use of combination regimens in oncology provides clear benefits in term of clinical responses compared to the use of single agent anti-tumour therapies.[1] As a consequence, the evaluation of the most promising combinations of a new compound with other anticancer agents, including those already available in the clinics, is a fundamental step in early drug development for obtaining a complete description of the compound properties and characteristics. For this purpose, *in vitro* and *in vivo* experiments, based on cell cultures and tumour-bearing animals, are performed to evaluate the anticancer activity of combination regimens. The aim of these experiments is to assess if a

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combination has a synergistic, additive or antagonistic interaction. (i.e. the effect is greater, similar or smaller than the sum of the effects of the individual compounds given in the same conditions).[2] Different statistical and mathematical approaches to assess the effect of a combination, such as combination index-isobolograms,[3–5] point-to-point curve comparison,[6,7] surface analysis,[8–10] tumour growth delay and log cell kill analyses,[11,12] are already available in the literature and are currently applied in the drug development process.[13–21] However, their application, especially to early drug development in oncology, is still cumbersome because these methodologies are based on metrics of efficacy, such as the percent tumour growth inhibition index or the tumour growth delay, that provide only a partial evaluation of compound efficacy. Moreover, these metrics may be strongly affected by the experimental conditions, making questionable the extrapolation to other settings (for instance, different doses of co-administered drugs or different treatment durations).[22,23] In addition, currently available methodologies require the execution of multiple experiments, administering the compounds at several dose levels both as single agents and in combination. This makes the process extremely expensive and time consuming, thus motivating the quest for new approaches based on modelling techniques that could make a better use of the data generated in combination studies.

More recently, model-based methodologies for evaluating *in vivo* pharmacodynamic drug–drug interactions using standard indirect PK/PD models,[24] or tumour growth PK/PD models,[25] have been proposed. In the first paper, 14 different models were used to describe all the possible interaction cases and the difficulties of distinguishing and identifying the appropriate model from the experimental data are highlighted through a series of simulations. In the second one, a unique interaction model based on a multiplicative factor on the action of one of the two drugs in a modified version of the TGI model,[22,26] is presented. Unfortunately, the proposed model can be applied only to drugs acting with the same mechanism of action in terms of tumour cell death rate and this may strongly restricts its applicability. This modelling approach to the problem can be very complex as it has to be considered that two compounds may interact with different modalities depending on their specific mechanism of action, which often is not completely known. In addition, the practical limitations intrinsic to the experimental designs and the variability in the data often hamper the typical model-building approach based on standard fitting procedures and/or statistical criteria. This constrains the degree of complexity of the interaction models and/or increases the identifiability problems in parameter estimation.

In the present paper a different approach for analysing the data from *in vivo* drug interaction studies is proposed. Instead of fitting different drug-specific interaction models, we developed a model able to predict the response of the tumour to a combination treatment in case of additivity of the effects (null-interaction hypothesis). In fact, the killing rate derives from the application of the Bliss independence criterion[2] in a dynamic context (see Appendix A.1 for more details). The null-interaction assumption requires that the death processes triggered by the two drugs are independent of each other. In turn, this calls for multiple transit compartments accounting for all possible combinations of stages of the two death processes, see again Appendix A.1. In spite of this, model complexity (in terms of number of parameters and consequent identifiability issues) is not critical because the transit compartments share the same micro-rate constants. Using this new model, the expected tumour growth curves in the combination regimens can be predicted from the data obtained from the single agent arms of the study (or previously performed experiments). Then, by comparing the predictions with the observed data, the presence of a possible interaction and its nature can be directly ascertained by visual inspection. In fact, tumour weights lying below or above the predicted additivity tumour growth curves would immediately indicate the presence of synergistic or antagonistic behaviours. In addition to the qualitative evaluation a statistical procedure for the assessment of possible departures from additivity was also developed. Different examples of the application of this approach to experimental data are shown.

## 2. Methods

### 2.1. PK/PD model structures

#### 2.1.1. The single agent TGI model

The main features and the formulas of the TGI model,[22,26,27] are summarised in Fig. 1. Tumour growth in non-treated animals is described by an exponential growth phase followed by a linear one; see Fig. 1, lower left. In the model, $w_0$ represents the tumour weight at the inoculation time (t = 0), while $\lambda_0$ and $\lambda_1$ are parameters characterising the rate of exponential and linear growth, respectively. In the treated animals, due to the action of the anticancer treatment, some cycling cells become non-proliferating and eventually die after passing through a compartmental transit system describing progressive stages of damage. For a given time t, the variable $Z_0(t)$ indicates the mass of proliferating cells and $c(t)$ the plasma concentration of the anticancer agent. The total tumour weight $W(t)$ is given by the sum of $Z_0(t)$ and $Z_i(t)$, $i = 1, 2, 3$, where $Z_i(t)$, $i = 1, 2, 3$ represent the weights of the tumour cells in the three transit compartments. The action of the drug on tumour growth is completely characterised by $k_1$, the micro-rate constant describing the kinetics of the transit compartments (which is inversely proportional to the mean time-to-death of tumour cells), and $k_2$, the proportionality factor linking drug concentration to the effect. Parameter $k_2$ describes the anti-tumour potency of the compound whilst $k_1$ is related to how rapidly the tumour cells are brought to death. It is important to underline that in the TGI model, the tumour cells, once damaged, when exposed again to the same drug, do not accelerate their death process. Further details regarding the tumour cell populations (e.g. cycling and quiescent cells) and the biochemical mechanism of action of the drugs were not included into the model. Nevertheless, possibly due to an appropriate compromise between its empirical and semi-physiological nature, the TGI model was able to successfully describe and predict the tumour growth curves following various administration of standard anticancer drugs,[22,29] and candidate compounds, including target-oriented agents,[30,31] and has been used as a reference or starting

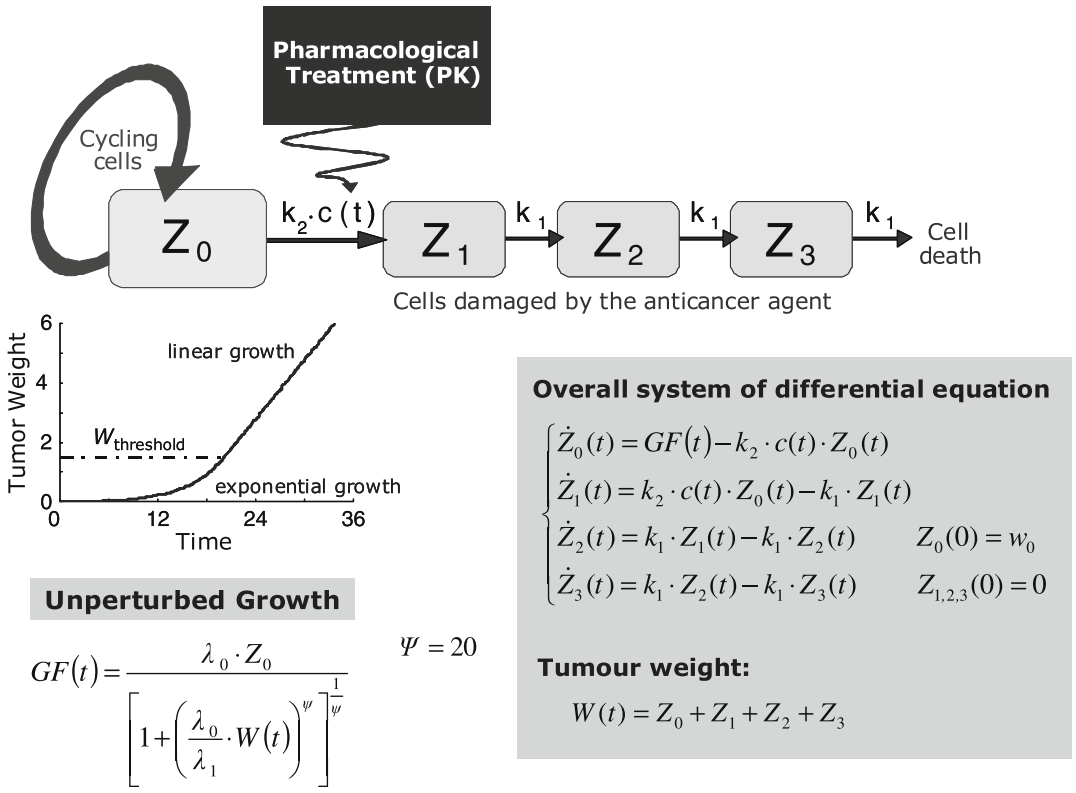



**Fig. 1 – Scheme and equations of the PK/PD tumour growth inhibition (TGI) model according to Simeoni et al.[22]**

point for the development of case-specific PK/PD models in this area.[28,32,33]

#### 2.1.2. *The additive TGI model, $TGI_{add}$*

When two drugs are considered, both drugs may act on the proliferating tumour cells, which, once damaged by either of the two drugs (A or B), enter the corresponding mortality chain each characterised by the micro-rate constant $k_{1A}$ or $k_{1B}$. Similarly to TGI model, parameters $k_{2A}$ and $k_{2B}$ describe the potencies of the two drugs, linking the drug concentration to the damage rate, but, in agreement with the null-interaction hypothesis for the additivity of the effects, the total number of proliferating cells hit by the two drugs is set proportional to $k_{2A}c_A(t) + k_{2B}c_B(t)$. As for the TGI model, the tumour cells damaged by one of the two drugs, when exposed again to the same drug, do not further accelerate their death process. On the other hand, it cannot be excluded that a cell already damaged by one of the two drugs could undergo a further damage due to the action of the other drug of the combination. In other words, at any time t, each of the two drugs (A and B) might interact not only with the proliferating tumour cells but also with cells previously hit by the other drug and still present in the corresponding mortality chain and this may create a further damage that can effectively anticipate their death. The mortality chains of the two drugs are still characterised by the micro-rate constants $k_{1A}$ and $k_{1B}$, but transitions along either direction of damage are allowed. Thus, the whole mortality process can be represented by a grid of transit compartments corresponding to all possible states of damage. These transitions are governed by the same $k_{2A}$ and $k_{2B}$ values, describing the drug effect on proliferating cells, but the final death time distribution of the cells is determined by both the $k_{1A}$ and $k_{1B}$ parameters. In particular, for cells damaged by both compounds, the death time is mainly governed by the larger between the two, determining in this way the final effect of the combination treatment. The scheme of the new $TGI_{add}$, model is shown in Fig. 2 and the corresponding equations are reported in Appendix A.1.

### 2.2. *Data analysis*

The PK/PD analyses for estimating the PK and PD parameters of each drug given as a single agent and the computation of the expected tumour growth curves predicted by the $TGI_{add}$ model were carried out using Winnonlin (version 3.1, Pharsight, CA, USA). Data fitting was performed on the mean tumour weight data in accordance with procedures described in the literature.[22,26]

### 2.3. *Assessing additivity of effects*

The method for assessing additivity of two anticancer agents A and B given in combination is based on the following procedure. The extension to three or more agents, using a generalisation of the $TGI_{add}$ model, is straightforward:

1. PK parameters of drugs A and B are estimated from single agent studies or taken from the literature. Assuming no PK interaction, the pharmacokinetic parameters obtained in the single agent studies can be used for describing $c_A(t)$

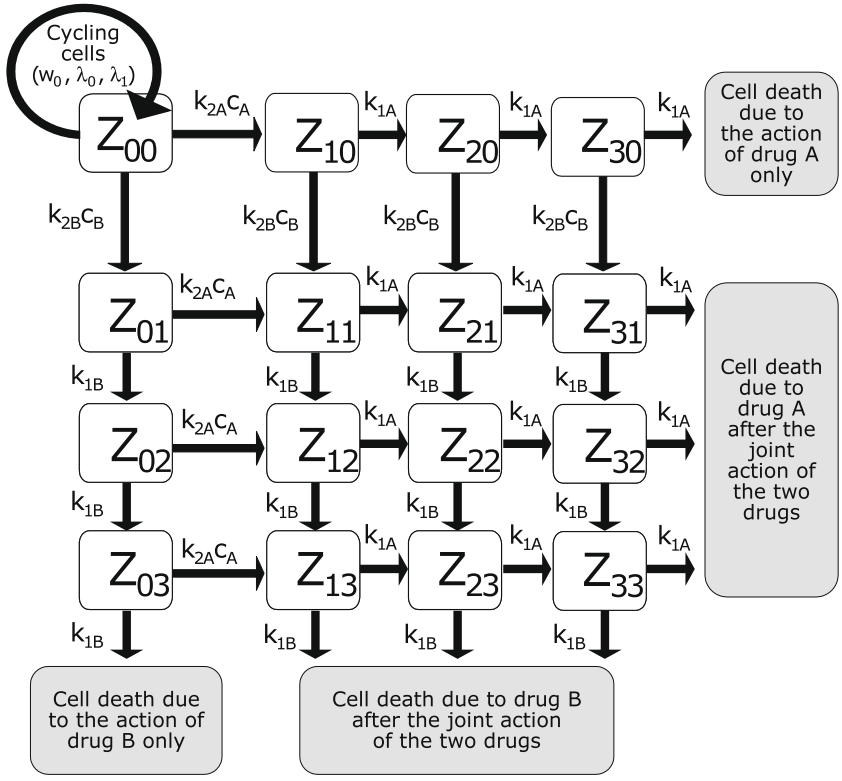



**Fig. 2 – Scheme of the $TGI_{add}$ model.**

and $c_B(t)$ in the combination experiments utilising the doses and schedules adopted for each drug given in combination. In case of PK interactions, the pharmacokinetics of the compounds should be experimentally evaluated in the same condition of the combination arms of the experiment.

2. The PD parameters $w_0$, $\lambda_0$, $\lambda_1$, $k_{1A}$, $k_{2A}$, $k_{1B}$, $k_{2B}$, relative to the single agent TGI models of the two drugs, are estimated from specific single agent arms included in the combination study or from the available single agent studies.
3. The plasma profiles $c_A(t)$ and $c_B(t)$ are used as inputs in the $TGI_{add}$ model, together with the previously derived PD parameters $w_0$, $\lambda_0$, $\lambda_1$, $k_{1A}$, $k_{2A}$, $k_{1B}$, $k_{2B}$, in order to compute predicted tumour growth curves (PTGCs) in the combination study in the absence of a pharmacodynamic interaction between the two drugs.
4. A first visual assessment is obtained by plotting the observed tumour weights over the PTGC. This allows an immediate evaluation of the anti-tumour effect of the drug combination:
   - Experimental tumour weights lying below the PTGC indicate a synergic interaction.
   - Experimental tumour weights lying above the PTGC indicate an antagonistic interaction.
   - Experimental tumour weights lying close the PTGC indicate no interaction.
5. In addition, a statistical evaluation based on a $\chi^2$ test can be performed. More precisely, under the null hypothesis that the additive $TGI_{add}$ model holds, the score

$$\tilde{X}^2 = n_{comb}\left(\frac{\hat{\sigma}^2_{comb} - \delta^2}{\hat{\sigma}^2_{s.ag}}\right), \quad \hat{\sigma}^2_{comb} = \frac{WSSR_{comb}}{n_{comb}} \quad \hat{\sigma}^2_{s.ag} = \frac{WSSR_{s.ag}}{n_{s.ag} - q}$$

is distributed as a $\chi^2$ random variable with $n_{comb} + q$ degrees of freedom; $n_{comb}$ is the number of tumour weight observations in the combination study, $WSSR_{comb}$ is the weighted sum of squared residuals associated with the predicted combination arm, $q = 7$ is the number of PD parameters, $n_{s.ag}$ is the number of tumour weight observations in the single agent study and $WSSR_{s.ag}$ is the weighted sum of squared residuals associated with the single agent arms used to estimate the PD parameters.

Responses belonging to a pre-defined acceptability range based on pharmacological assessment are regarded as equivalent by a proper choice of the parameter $\delta$. In this paper, $\delta = 0.2$ was selected, meaning that a 20% difference between the observed response and the PTGC does not rule out additivity. More details on the statistical analysis can be found in Appendix A.2.

### 2.4. *In vivo experiments*

In all the experiments, male Balb nude–*nu* mice of 23–38 g, 5–6 weeks of age (Harlan, S.Pietro al Natisone, Italy), were used with different tumour cell lines implanted s.c. into the left flank of mice on day 1. One week after inoculation, mice bearing a palpable tumour (approximately 100–300 $mm^3$) were randomised into control and treatment groups; usually eight animals for each group were considered. After treatment, mice were clinically evaluated daily and tumours were measured, usually every two or three days, using callipers.

Tumour masses (mg) were calculated as *length* (mm) · *width*$^2$ (mm$^2$)/2, assuming unit density.

#### 2.4.1. *Experiment 1*

Bx-pc3 human pancreas adenocarcinoma tumour-bearing mice were treated with a compound under development named drug C1, gemcitabine and a combination of both drugs. Drug C1 was given i.v. at 15 and 30 mg/kg twice a day (bid) for 9 days starting from day 9 to two groups of animals. Gemcitabine was given i.v. at 80 mg/kg, three injections with a 4-day interval (q4dx3), starting from day 9. In the combination arm gemcitabine was given i.v. at 80 mg/kg q4dx3 starting from day 9, whilst drug C1 was given at 15 mg/kg bid for 3 days for three cycles starting from days 10, 14 and 18 (one day later each administration of gemcitabine).

#### 2.4.2. *Experiments 2 and 3*

Within the same experimental plan, irinotecan (CPT-11) and 5-fluorouracil (5-FU) were given i.v. both alone and in combination with a compound under development (drug C2) to mice bearing HT29 human colon adenocarcinoma tumours. CPT-11 and 5-FU were given i.v. q4dx3 starting from day 9 at 45 mg/kg and 50 mg/kg, respectively. Drug C2 was given orally at 60 mg/kg on days 10, 11, 12, 14, 15 and 16. In the combination arms CPT-11 and drug C2, 5-FU and drug C2 were administered maintaining the same routes and dosage administrations. For the purpose of this study, the PK/PD analysis was performed considering separately the two combination arms. Control group, CPT-11, drug C2 and their combination data were considered as Experiment 2. The same control data and drug C2, but with 5-FU given alone and in combination with drug C2, were considered as Experiment 3.

#### 2.4.3. *Experiment 4*

KM-12 tumour-bearing mice were treated with a compound under development named drug C3, 5-FU and a combination of both drugs. Drug C3 was given orally at 15 mg/kg bidx6 for two cycles starting from days 14 and 21. 5-FU was given i.v. at 50 mg/kg once every week on days 13, 20 and 27 (q7dx3). In the combination arms, 5-FU and drug C3 were administered maintaining the same routes and dosage administrations.

#### 2.4.4. *Pharmacokinetic data*

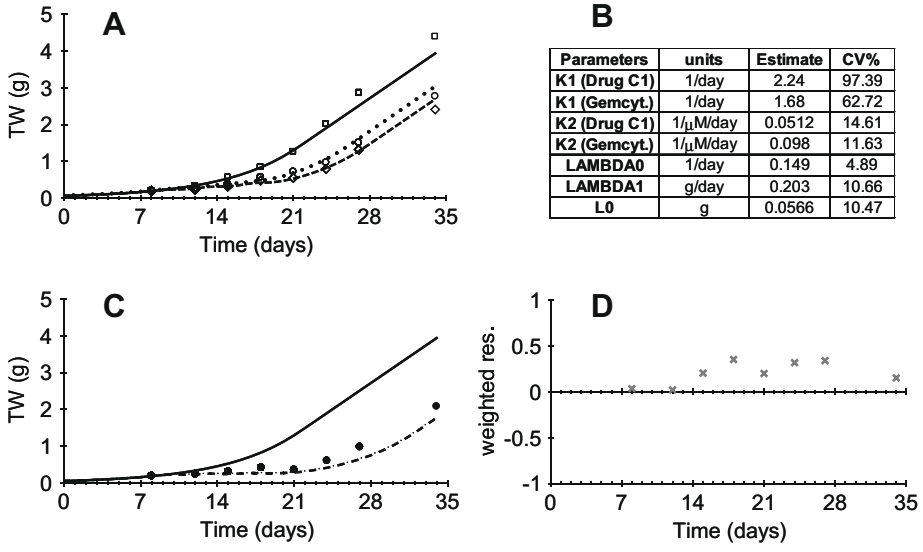
PK profiles of gemcitabine, CPT-11 and 5-FU at the corresponding doses and schedules were generated using PK parameters previously reported in Rocchetti et al.[29]

The pharmacokinetics of candidate drugs C1–C3 were investigated in separate groups of tumour-bearing mice. Blood samples for the pharmacokinetic assessment were collected and the drugs were assayed in plasma using LC-MS–MS techniques based on a generic procedure adopted for compounds in discovery phases.[34]

PK profiles of drug C1 were described by a two-compartment i.v. model ($V_1$ = 1.42 L/kg, $k_{10}$ = 28.14 day$^{-1}$, $k_{12}$ = 4.94 day$^{-1}$ and $k_{21}$ = 5.58 day$^{-1}$). Two compartments with first-order absorption were adopted to describe PK profile of drug C2 ($V_1$ = 2.13 L/kg, $k_{01}$ = 18.84 day$^{-1}$, $k_{10}$ = 49.2 day$^{-1}$, $k_{12}$ = 141.05 day$^{-1}$, $k_{21}$ = 10.39 day$^{-1}$). Finally one compartment with first-order absorption was adopted to describe PK profile of drug C3 ($V_1$ = 24.83 L/kg, $k_{01}$ = 48.41 day$^{-1}$, $k_{10}$ = 2.38 day$^{-1}$).

## 3. Results

All the experiments previously described were analysed using the procedure of Section 2.3. Results of Experiments 1–4 are




| Parameters | units | Estimate | CV% |
|---|---|---|---|
| K1 (Drug C1) | 1/day | 2.24 | 97.39 |
| K1 (Gemcyt.) | 1/day | 1.68 | 62.72 |
| K2 (Drug C1) | 1/μM/day | 0.0512 | 14.61 |
| K2 (Gemcyt.) | 1/μM/day | 0.098 | 11.63 |
| LAMBDA0 | 1/day | 0.149 | 4.89 |
| LAMBDA1 | g/day | 0.203 | 10.66 |
| L0 | g | 0.0566 | 10.47 |

**Fig. 3 – Experiment 1. (A) Observed and model-fitted tumour growth curves obtained in bx-pc3 tumour-bearing mice given either the vehicle (□) or drug C1 15 mg/kg i.v. bidx9 days treatment (○) or gemcytabine 80 mg/kg i.v. q4dx3 days treatment (◆), each administered as a single agent. (B) Pharmacodynamic parameters obtained simultaneously fitting the TGI model to the tumour weight data obtained after administration of the vehicle and the single agents. (C) Observed tumour growth data obtained in nude mice given drug C1 15 mg/kg i.v. bidx9 days treatment in combination with gemcytabine 80 mg/kg i.v. q4dx3 days treatment (●) overlapped to the predicted tumour growth curve obtained applying the additive $TGI_{add}$ model with parameters previously estimated. (D) Weighted residuals (i.e. (observed-estimated)/observed) between observed data in the combination arm and the predicted curve. The hypothesis of additivity cannot be rejected (p-value = 0.74).**

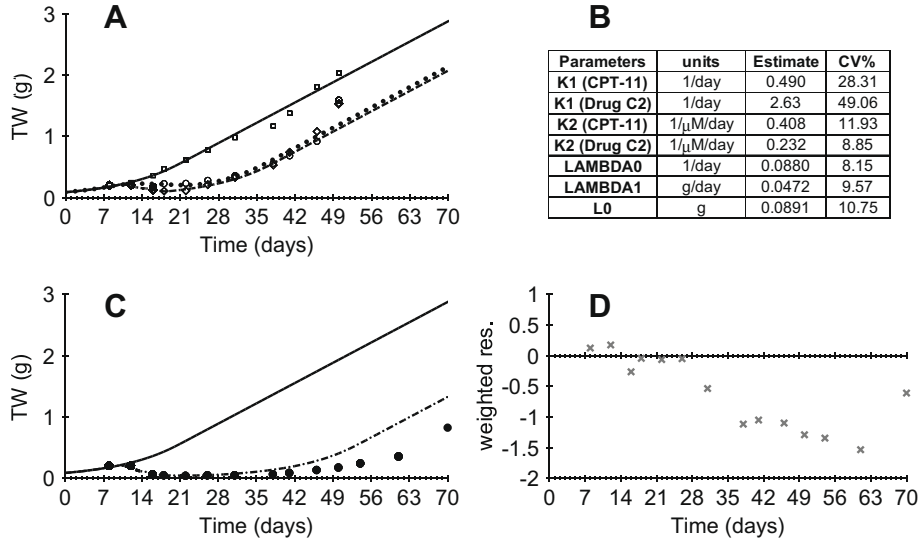
presented in Figs. 3–6, respectively. The plots in the upper left corner (A) shows the simultaneous fitting of the average tumour growth in control and treated animals with the two drugs administered as single agents, the tables in the upper right corner (B) report the corresponding PD parameters together with their coefficients of variation. In the lower left panels (C) the PTGCs assuming additive effect are over-imposed to the observed average tumour weights of the combination arm; the estimated control profiles are also plotted for comparison. The distributions of the weighted residuals are presented in the lower right panels (D).

Except Experiment 2, where CPT-11 and drug C2 showed a more than additive effect ($\delta = 0.2$, $p$-value < 0.01), all the other combinations (gemcitabine administered with drug C1, 5-FU with drug C2 and C3) did not show significant departures from additivity.

## 4. Discussion

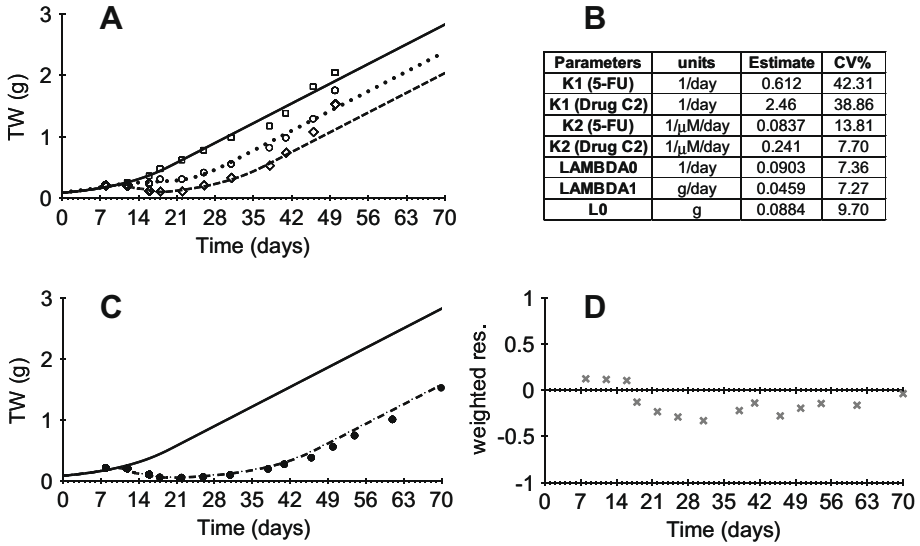
Starting from a well-established PK/PD model used for predicting the tumour growth inhibition after the administration of anticancer compounds in xenografted mice, a novel and simple approach for assessing additivity of the effects of anticancer agents given in *in vivo* combination studies is proposed. Control and single agent arms are used to estimate PK/PD parameters of the individual drugs. Then, based on their estimates, the additive combination model $TGI_{add}$ is used to predict the expected tumour growth curve in the combination experiment assuming a pharmacodynamic null interaction between drugs. By comparing the predicted curve with actual tumour data, possible departures from additivity can be easily ascertained by visual inspection; further, a statistical $\chi^2$ test, also accounting for a pharmacological equivalence factor based on user's choice, has been derived for this aim. Although PK/PD parameters of each drug may be retrospectively obtained from previous single agent experiments, the assessment of the effects of single agents should be preferably derived from arms of the same experiment, especially if significance testing is to be performed. Compared to other approaches, this procedure makes use of the entire TGI curves and not only of terminal values or growth delays measured at specific time points.

The proposed approach has been applied to the mean tumour weight curves. This choice was adopted as the model application requires information from different groups of animals: ancillary group (or historical data) for the pharmacokinetic assessment, control group for unperturbed tumour growth, active compound group for perturbed tumour growth of both agents in the combination. The individual data could be modelled using population pharmacokinetic approaches. These methodologies have already been used for fitting the individual tumour weight data in untreated[35] and in treated animals when, in a dedicated experiment, pharmacokinetics and tumour weight data were assessed in the same animals.[36] In these cases the model parameters were identifiable with sufficient accuracy, however, when pharmacokinetics and pharmacodynamic are assessed in different groups of animals with the design and sample size typically adopted in these studies, the population approach is becoming fairly complex, and the variability terms are difficult to be treated.[37] Considering that the fitting of average data is able to provide in any case meaningful and robust conclusions on the activity



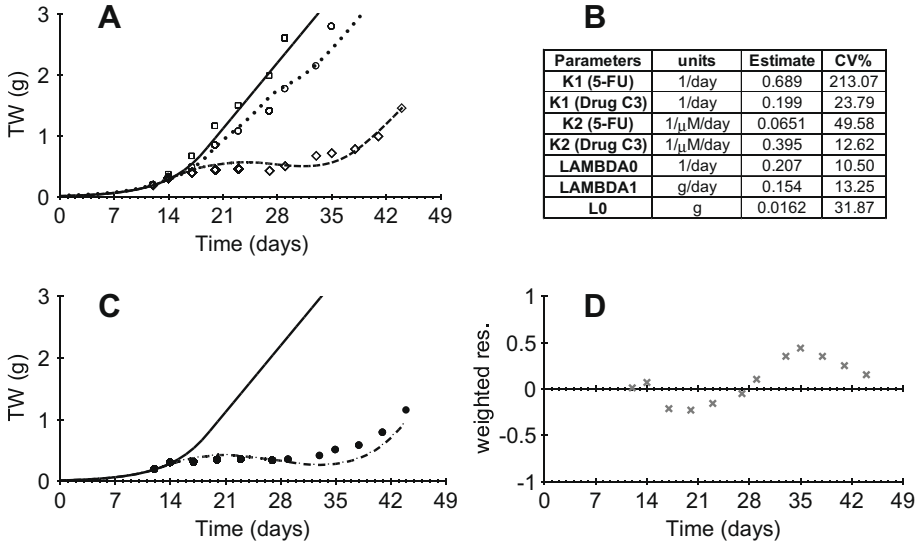

| Parameters | units | Estimate | CV% |
|---|---|---|---|
| K1 (CPT-11) | 1/day | 0.490 | 28.31 |
| K1 (Drug C2) | 1/day | 2.63 | 49.06 |
| K2 (CPT-11) | 1/μM/day | 0.408 | 11.93 |
| K2 (Drug C2) | 1/μM/day | 0.232 | 8.85 |
| LAMBDA0 | 1/day | 0.0880 | 8.15 |
| LAMBDA1 | g/day | 0.0472 | 9.57 |
| L0 | g | 0.0891 | 10.75 |

**Fig. 4 – Experiment 2. (A) Observed and model-fitted tumour growth curves obtained in HT29 tumour-bearing mice given either the vehicle (□) or CPT-11 45 mg/kg i.v. q4dx3 days treatment (○) or drug C2 60 mg/kg orally given on days 10, 11, 12, 14, 15, 16 (◆), each administered as a single agent. (B) Pharmacodynamic parameters obtained simultaneously fitting the TGI model to the tumour weight data obtained after administration of the vehicle and the single agents. Panel C: observed tumour growth data obtained in nude mice given CPT-11 45 mg/kg i.v. q4dx3 days treatment in combination with drug C2 60 mg/kg o.s. repeated doses treatment (●) overlapped to the predicted tumour growth curve obtained applying the additive $TGI_{add}$ model with parameters previously estimated. (D) Weighted residuals (i.e. (observed-estimated)/observed) between observed data in the combination arm and the predicted curve. The hypothesis of additivity is rejected ($p$-value < 0.01).**




| Parameters | units | Estimate | CV% |
|---|---|---|---|
| K1 (5-FU) | 1/day | 0.612 | 42.31 |
| K1 (Drug C2) | 1/day | 2.46 | 38.86 |
| K2 (5-FU) | 1/μM/day | 0.0837 | 13.81 |
| K2 (Drug C2) | 1/μM/day | 0.241 | 7.70 |
| LAMBDA0 | 1/day | 0.0903 | 7.36 |
| LAMBDA1 | g/day | 0.0459 | 7.27 |
| L0 | g | 0.0884 | 9.70 |

**Fig. 5 – Experiment 3. (A) Observed and model-fitted tumour growth curves obtained in HT29 tumour-bearing mice given either the vehicle (□) or 5-FU 50 mg/kg i.v. q4dx3 days treatment (○) or Drug C2 60 mg/kg orally given on days 10, 11, 12, 14, 15, 16 (◆), each administered as a single agent. (B) Pharmacodynamic parameters obtained simultaneously fitting the TGI model to the tumour weight data obtained after administration of the vehicle and the single agents. (C) Observed tumour growth data obtained in nude mice given 5-FU 50 mg/kg i.v. q4dx3 days treatment in combination with Drug C2 60 mg/kg o.s. repeated doses treatment (●) overlapped to the predicted tumour growth curve obtained applying the additive $TGI_{add}$ model with parameters previously estimated. (D) Weighted residuals (i.e. (observed-estimated)/observed) between observed data in the combination arm and the predicted curve. The hypothesis of additivity cannot be rejected ($p$-value ≈ 1).**




| Parameters | units | Estimate | CV% |
|---|---|---|---|
| K1 (5-FU) | 1/day | 0.689 | 213.07 |
| K1 (Drug C3) | 1/day | 0.199 | 23.79 |
| K2 (5-FU) | 1/μM/day | 0.0651 | 49.58 |
| K2 (Drug C3) | 1/μM/day | 0.395 | 12.62 |
| LAMBDA0 | 1/day | 0.207 | 10.50 |
| LAMBDA1 | g/day | 0.154 | 13.25 |
| L0 | g | 0.0162 | 31.87 |

**Fig. 6 – Experiment 4. (A) Observed and model-fitted tumour growth curves obtained in KM12 tumour-bearing mice given either the vehicle (□) or 5-FU 50 mg/kg i.v. q7dx3 days treatment (○) or drug C3 15 mg/kg orally at 15 mg/kg bidx6 on days 14 and 21 (◆), each administered as single agent. (B) Pharmacodynamic parameters obtained simultaneously fitting the TGI model to the vehicle and the single agents tumour weight data. (C) Observed tumour growth data obtained in nude mice given 5-FU 50 mg/kg i.v. q7dx3 days treatment in combination with drug C2 15 mg/kg orally at 15 mg/kg bidx6 on days 14 and 21 (●) overlapped to the predicted tumour growth curve obtained applying the additive $TGI_{add}$ model with parameters previously estimated. (D) Weighted residuals (i.e. (observed-estimated)/observed) between observed data in the combination arm and the predicted curve. The hypothesis of additivity cannot be rejected ($p$-value = 0.98).**

of the tested anticancer agents, this procedure was chosen for minimising the amount of time and resources employed for obtaining the necessary information, as it is typically requested in this phase of pre-clinical development.

The novel approach for interaction assessment was successfully tested in various pharmacological studies. As demonstrated in the experimental results, a further advantage is that no constraints are posed on dosing and schedules and, in principle, a single combination experiment may suffice to assess the presence of possible interactions. An additional tool based on a $\chi^2$ test that takes into account an acceptance additivity region has also been developed. In practice, this test considers as additive all the tumour growth curves that do not differ more than a given percent value ($\delta$ parameter) from the predicted additivity curve. As for the bioequivalence studies, $a \pm 20\%$ acceptance region was chosen in the analyses presented here ($\delta = 0.2$), meaning that differences below ±20% from additivity were considered as not clinically relevant. For the studies analysed here visual and statistical assessments were always in good agreement; however based on user choice this criterion may be set to any desired value.

From a modelling perspective, the assumption of a pharmacodynamic null interaction implies that the potency parameters of the two drugs given in combination, $k_{2A}$ and $k_{2B}$, maintain the same values as measured when the two drugs are given as single agents. If this is the case, the additivity of the effects can be modelled assuming that at each time t the total number of cells hit by the drugs and made non-proliferating is proportional to $k_{2A}c_A(t) + k_{2B}c_B(t)$. Hence, assuming $c_A(t) = c_B(t)$, it is immediate to verify that the effect of the two drugs given in combination is simply proportional to $k_{2A} + k_{2B}$ showing that the potency of the combination is exactly the sum of the potencies of the two drugs when given alone in the same conditions. The presence of the concentration terms $c_A(t)$ and $c_B(t)$ in the model equations extends the null-interaction assumption to any possible administration schedule in the combination arm of the study.

The grid of transit compartments introduced in $TGI_{add}$ is essential for correctly modelling the joint presence of two damage processes. A model without the grid (i.e. a model as proposed by Koch and colleagues in which the cell damage process is described using two distinct chains of transit compartments, one for each agent) applied to drugs with different tumour cell death rates ($k_{1A} \neq k_{1B}$) would yield paradoxical behaviours. For instance, combining a drug A with high $k_{1A}$ (i.e. a drug producing a fast death rate of tumour cells) with a drug B whose action is more delayed (low $k_{1B}$), this model would bring to the conclusion that the effect of drug B "protects" a fraction of the tumour cells by the action of drug A (i.e. cells already damaged by drug B cannot be further damaged by drug A). This would produce the paradox, in the null-interaction assumption, of a predicted tumour growth curve temporarily higher than that observed after the administration of drug A as a single agent, see Fig. 7 for an illustrative example.

Starting from our model, on the basis of different biological and pharmacological hypotheses, it would be possible to develop a gamut of interaction models, just by including interaction factors in different places of the equations. For example it may be hypothesised that the $k_1$ parameters may take different values when the drugs are given alone or in combination; also the $k_2$ parameter might be different when representing the drug action on proliferating cells or on those already damaged by the other drug. So, if a significant deviation from additivity is ascertained, the $TGI_{add}$ model can be taken as starting point for developing and testing more specific models including different synergistic or antagonistic interaction terms. For example, as proposed by Koch and colleagues,[25] a multiplicative interaction factor can be included in the equations to model the effect of the interaction of drug A on drug B or vice versa. However, it is important to notice, that different from Koch's model in our model the tumour cells are continuously exposed to the possible action of both drugs with their corresponding dynamics, which appears by far the most interesting case for the combination therapy, which typically uses drug with different mechanisms of action to optimise the therapeutic effects, while minimising the overlapping toxicity. In any case, as previously mentioned, the setting up of complex interaction models should be carefully evaluated in view of the inevitable identifiability problems stemming from a potential over-parameterisation.

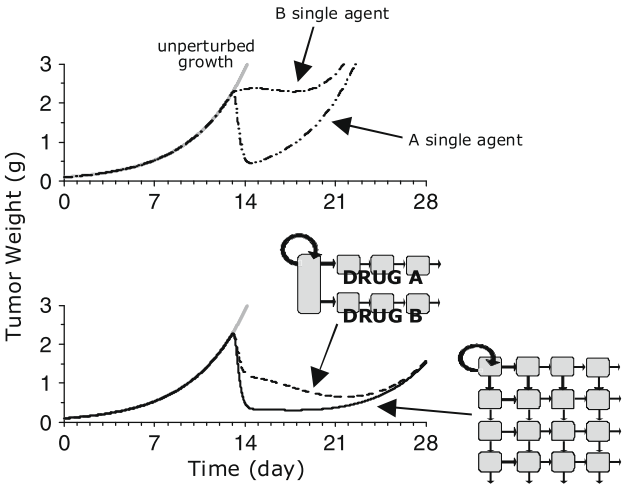



**Fig. 7 – In this figure, the need for a grid of transit compartments describing the cell damage process is illustrated through the comparison of $TGI_{add}$ with a model including two separate chains of transit compartments, one for each drug. Upper panel: predicted tumour growth curves in control and treated group after 200 mg, single 1 h infusion, of two compounds, A and B, given as single agents on day 13. The tumour growth parameter for the unperturbed growth was: $\lambda_0 = 0.01$ 1/h, $\lambda_1 = 0.04$ g/h, $w_0 = 0.1$ g. The compounds were characterised by the same pharmacokinetic behaviour (described using a two compartment model with $V = 1$ L, $k_{10} = 1$ 1/h, $k_{12} = 0.05$ 1/h, $k_{21} = 0.08$ 1/h) and by the same potency values ($k_{2A} = k_{2B} = 0.01$ mL/μg/h) but different average time-to-death ($k_{1A} = 0.25$ 1/h, $k_{1B} = 0.018$ 1/h). In the lower panel the predicted tumour growths after the combined treatment of the two drugs A and B given simultaneously on day 13 using the $TGI_{add}$ model (solid line) and the model with two distinct chains of transit compartment (dashed line) are compared. The latter plot highlights a paradox: there is a transient during which the combined administration has less effect that the administration of drug A as a single agent. In fact, tumour cells are captured by the transit chain B whose transit times are slower. The use of a grid of transit compartments completely avoids this paradox and is therefore to be preferred.**

For these reasons, the merit of the proposed approach stays in the simplicity of the suggested procedure: provided that the TGI model is applicable to the drugs given as single agents, the $TGI_{add}$ model provides an easy tool for assessing deviation from additivity without assuming any specific mechanistic drug interaction model, thus, avoiding all the difficulties related to validity range, data fitting, and model comparison procedures. This may prove particularly useful in the early drug development phase, when very limited knowledge of the pharmacological characteristics of the compounds is usually available. Gaining immediately information on the compounds under study about their possible future use in combination therapy may substantially impact on the subsequent phases of the drug development process.

## Conflict of Interest statement

None declared.

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## Appendix

### A.1. $TGI_{add}$ model equations (according to scheme of Fig. 2)

The $TGI_{add}$ model consists of the following 16 differential equations, indexed by all pairs $(i, j)$ with $0 \leqslant i, j \leqslant 3$, see Fig. 2:

$$\frac{dZ_{00}}{dt} = UGF(t) - (k_{2A}c_A(t) + k_{2B}c_B(t))Z_{00}$$

$$\frac{dZ_{ij}}{dt} = u_{Aij} + u_{Bij} - (k_{1A} + k_{1B})Z_{ij}, \quad i + j > 0$$

$$u_{Aij} = \begin{cases} 0, & i = 0 \\ k_{2A}c_A(t)z_{0j}, & i = 1 \\ k_{1A}z_{i-1,j}, & i = 2,3 \end{cases}$$

$$u_{Bij} = \begin{cases} 0, & j = 0 \\ k_{2B}c_B(t)z_{i0}, & j = 1 \\ k_{1B}z_{i,j-1}, & j = 2,3 \end{cases}$$

where UGF(t) is the same growth function as in the single agent TGI model (see Fig. 1); $c_A(t)$ and $c_B(t)$ are the plasma concentration profiles due to the administration of drug A and drug B, respectively; $w_0$, $\lambda_0$ and $\lambda_1$ are the parameters related to the proliferation process; $k_{1A}$ and $k_{1B}$ are the transit rate constants and $k_{2A}$ and $k_{2B}$ are the potency indexes of drug A and drug B, respectively. The total tumour weight time course is given by:

$$W(t) = \sum_{i=0}^{i=3} \sum_{j=0}^{j=3} Z_{ij}(t)$$

where $Z_{00}$ is (the weight of the proliferating tumour cells), $Z_{i0}$ and $Z_{0j}$ are (the weights of the tumour cells already hit only by drugs A and B, respectively, with $i$, $j = 1, 2, 3$ indicating increasing degree of damage), satisfy the system of differential equations and $Z_{ij}$, $1 \leqslant i$, $j \leqslant 3$ are the weights of cells already hit both drugs in the different stages of damage.

Note that the $TGI_{add}$, model stems directly from the Bliss criterion: letting dt represent an infinitesimal time interval, the probability of a tumour cell to be killed by drug A is $k_{2A}c_A(t)dt$. Accordingly, the overall survival probability will be $1 - k_{2A}c_A(t)dt - k_{2B}c_B(t)dt + k_{2A}c_A(t)k_{2B}c_B(t)dt^2$. In order to formulate the model in terms of differential equations, we have to take a limit for dt tending to zero: consequently, the last term, which depends on the square of dt, disappears because it is a higher order infinitesimal compared to the first two terms depending on dt. Note that the elimination of the last term is not an approximation but a rigorous result of infinitesimal calculus rules. A detailed derivation of $TGI_{add}$ in terms of survival probabilities can be obtained along the line of a recent paper[27], where an analogous derivation for the single agent TGI model is thoroughly discussed.

Note also that all the 15 transit compartments are required to describe the joint presence of two non-interacting damage processes depending on drugs A and B, respectively. The 15 compartments correspond to all possible damage states of tumour cells, taking into account that the single damage process evolves through three progressive stages. For instance, a cell, not damaged by drug A, that has reached the second damage state associated with drug B, will be in the state (0.2).

### A.2. $\chi^2$ additivity test

The aim of this section is to derive an appropriate $\chi^2$ score to be used for testing the additivity hypothesis. Under the null hypothesis that the additivity model holds, the observed tumour weights are given by:

$$y_i = W_{comb}(t_i) + e_i, \quad i = 1, \ldots, n_{comb}$$

where $e_i$ are assumed to be independent zero-mean normal errors with $Var[e_i] = \sigma^2_{comb} \cdot y_i$, and $W_{comb}(t)$ is the tumour weight computed according to the $TGI_{add}$ model. Let

$$WSSR_{comb} = \sum_{i=1}^{n_{comb}} \frac{(y_i - W_{comb}(t_i))^2}{y_i^2} = \sum_{i=1}^{n_{comb}} \frac{e_i^2}{y_i^2}$$

be the weighted sum of squared residuals between observed weights and simulated ones. Then, $X^{*2} = \frac{WSSR_{comb}}{\sigma^2_{comb}}$ is $\chi^2$ distributed with $n_{comb}$ degree of freedom.

This result would be useful in order to validate a specific additive model, with fixed parameter values that are assumed to be the correct ones. As a matter of fact, the $q = 7$ parameters are estimated from the single agent arms of the experiment. Let $\hat{W}_{comb}(t_i)$ denote the tumour weight in the combination experiment predicted on the basis of the additive model based on the estimated parameters. Moreover, let $\tilde{WSSR}_{comb} = \sum_{i=1}^{n_{comb}} \frac{(y_i - \hat{W}_{comb}(t_i))^2}{y_i^2}$. Then, $\tilde{X}^{*2} = \frac{\tilde{WSSR}_{comb}}{\sigma^2_{comb}}$ is approximately $\chi^2$ distributed with $n_{comb} + q$ degree of freedom.

In order to estimate $\sigma^2_{comb}$, it is assumed that it is equal to $\sigma^2_{s.ag}$ obtained from the single agent arms. Then, we have that

$$\tilde{X}^{*2} \cong \hat{X}^{*2} = \frac{\mathrm{WS\tilde{S}R_{comb}}}{\sigma^2_{\mathrm{s.ag}}} = n_{\mathrm{comb}}\left(\frac{\hat{\sigma}_{\mathrm{comb}}}{\hat{\sigma}_{\mathrm{s.ag}}}\right)^2$$

where $\hat{\sigma}^2_{\mathrm{comb}} = \frac{\mathrm{WS\tilde{S}R_{comb}}}{n_{\mathrm{comb}}}$.

However, this statistical test may reject the null hypothesis of additive effects also in case of differences that are not pharmacologically relevant. In order to avoid statistically significant differences that are not pharmacologically meaningful, a modified $\chi^2$ score is introduced allowing for a suitable acceptability region. Under the null hypothesis that the additivity model holds, the observed tumour weights in the combination arm are now written as:

$$y_i = W_{\mathrm{comb}}(t_i) + b_i + e_i, \quad i = 1, \ldots, n_{\mathrm{com.}}, \quad |b_i| \leqslant \delta|y_i|, \text{ with } \delta \geqslant 0$$

where $b_i$ is an additional error term, proportional to the measured tumour weight $y_i$, and $\delta$ is the parameter defining the width of the acceptability region around the predicted additive effect curve. Then, the weighted sum of squared residuals between observed weights and simulated ones is given by

$$\mathrm{WSSR_{comb}} = \sum_{i=1}^{n_{\mathrm{comb}}} \frac{(e_i + b_i)^2}{y_i^2} = \sum_{i=1}^{n_{\mathrm{comb}}} \frac{e_i^2}{y_i^2} + \sum_{i=1}^{n_{\mathrm{comb}}} \frac{b_i^2}{y_i^2} + 2\sum_{i=1}^{n_{\mathrm{comb}}} \frac{e_i b_i}{y_i^2}$$

Being the term $2\sum_{i=1}^{n_{\mathrm{comb}}} \frac{e_i b_i}{y_i^2}$ a sum of random zero-mean variables with variance less than $4n_{\mathrm{comb}}\delta^2\sigma^2_{\mathrm{comb}}$, it can be neglected with respect to other terms. Moreover, observing that $\sum_{i=1}^{n_{\mathrm{comb}}} \frac{b_i^2}{y_i^2} < n_{\mathrm{comb}}\delta^2$, we obtain $\mathrm{WSSR_{comb}} \leqslant \sum_{i=1}^{n_{\mathrm{comb}}} \frac{e_i^2}{y_i^2} + n_{\mathrm{comb}}\delta^2$. Then, in the worst case (i.e. all errors $b_i$ take their largest possible values),

$$X^2 = \frac{(\mathrm{WSSR_{comb}} - n_{\mathrm{comb}}\delta^2)}{\sigma^2_{\mathrm{comb}}}$$

is $\chi^2$ distributed with $n_{\mathrm{comb}}$ degree of freedom. Again, one has to keep into account that the model parameters are not perfectly known, but are estimated from the single agent arms. Then, following the same considerations made before, it is straightforward to see that

$$\tilde{X}^2 \cong \hat{X}^2 = \frac{\mathrm{WS\tilde{S}R_{comb}} - n_{\mathrm{comb}}\delta^2}{\hat{\sigma}^2_{\mathrm{s.ag}}} = n_{\mathrm{comb}}\left(\frac{\hat{\sigma}^2_{\mathrm{comb}} - \delta^2}{\hat{\sigma}^2_{\mathrm{s.ag}}}\right)$$

is approximately $\chi^2$ distributed with $n_{\mathrm{comb}} + q$ degree of freedom.

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