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PHYSICS CONTRIBUTION

Effect of Conventional and Ultrahigh Dose Rate FLASH Irradiations on Preclinical Tumor Models: A Systematic Analysis

Till Tobias Böhlen, PhD,[*] Jean-François Germond, PhD,[*] Kristoffer Petersson, PhD,[†,‡] Esat Mahmut Ozsahin, MD, PhD,[§] Fernanda G. Herrera, MD, PhD,[§] Claude Bailat, PhD,[*] François Bochud, PhD,[*] Jean Bourhis, MD, PhD,[§] Raphaël Moeckli, PhD,[*] and Gabriel Adrian, MD, PhD[†,‖]

[*]*Institute of Radiation Physics, Lausanne University Hospital and Lausanne University, Lausanne, Switzerland;* [†]*Department of Hematology, Oncology, and Radiation Physics, Skåne University Hospital, Lund, Sweden;* [‡]*MRC Oxford Institute for Radiation Oncology, Department of Oncology, University of Oxford, Oxford, United Kingdom;* [§]*Department of Radiation Oncology, Lausanne University Hospital and Lausanne University, Lausanne, Switzerland; and* [‖]*Division of Oncology and Pathology, Department of Clinical Sciences, Skåne University Hospital, Lund University, Lund, Sweden*

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Purpose: Compared with conventional dose rate irradiation (CONV), ultrahigh dose rate irradiation (UHDR) has shown superior normal tissue sparing. However, a clinically relevant widening of the therapeutic window by UHDR, termed "FLASH effect", also depends on the tumor toxicity obtained by UHDR. Based on a combined analysis of published literature, the current study examined the hypothesis of tumor isoefficacy for UHDR versus CONV and aimed to identify potential knowledge gaps to inspire future in vivo studies.

Methods and Materials: A systematic literature search identified publications assessing in vivo tumor responses comparing UHDR and CONV. Qualitative and quantitative analyses were performed, including combined analyses of tumor growth and survival data.

Results: We identified 66 data sets from 15 publications that compared UHDR and CONV for tumor efficacy. The median number of animals per group was 9 (range 3-15) and the median follow-up period was 30.5 days (range 11-230) after the first irradiation. Tumor growth assays were the predominant model used. Combined statistical analyses of tumor growth and survival data are consistent with UHDR isoefficacy compared with CONV. Only 1 study determined tumor-controlling dose (TCD_{50}) and reported statistically nonsignificant differences.

Conclusions: The combined quantitative analyses of tumor responses support the assumption of UHDR isoefficacy compared with CONV. However, the comparisons are primarily based on heterogeneous tumor growth assays with limited numbers of animals and short follow-up, and most studies do not assess long-term tumor control probability. Therefore, the assays may be

Corresponding author: Raphaël Moeckli, PhD; E-mail: raphael.moeckli@chuv.ch

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insensitive in resolving smaller response differences, such as responses of radioresistant tumor subclones. Hence, tumor cure experiments, including additional TCD_{50} experiments, are needed to confirm the assumption of isoeffectiveness in curative settings.

Introduction

In several preclinical models from different laboratories, ultrahigh dose rate (UHDR) irradiation has been reported to cause less damage to normal tissues than conventional dose rate (CONV) irradiation[1-11] while maintaining tumor growth control.[2-4,11-17] This selective sparing of normal tissues is termed "FLASH effect" and has the potential to broaden the therapeutic window in radiation therapy (RT). As a result, there has been a surge in scientific interest in the use of UHDR irradiation, and most studies thus far have focused on UHDR irradiations of normal tissues. However, for UHDR RT to broaden the therapeutic window, tumor control efficacy must not be compromised in clinically relevant settings. Although several mechanisms for the FLASH effect have been proposed, including radiolytic oxygen depletion and vascular, inflammatory, and immune responses, so far none of them could be sufficiently validated.[18-20]

There are several approaches to studying the efficacy of radiotherapeutic interventions in vivo. The most recognized preclinical method is the tumor control probability (TCP) design, which can determine a tumor-controlling dose (TCD_{50}, the dose at which 50% of the animals are cured).[21,22] To detect late recurrences, at least 3 months of follow-up, preferably longer for some tumor models, is required.[23] Hereby, the curable potential of a treatment can be evaluated because the assay is thought to reflect the killing of all clonogenic cells. The assay requires a large number of animals, is labor intensive and time consuming. Alternative assays assess tumor growth and offer more time-, cost-, and labor-efficient methods. Instead of determining the dose required to cure the animals, repeated tumor measurements are used to determine tumor growth/shrinkage and derived quantities, such as probability of growth, time to regrowth, tumor growth delay, or the doubling time of tumors.[24] Such growth assays are thereby useful to detect abilities of anticancer treatment to inhibit tumor growth.[25] However, their relevance in a curative setting is questionable. Part of the disadvantages could be referred to heterogeneity within tumors. A large bulk of tumor cells may be nonclonogenic and could be eliminated by irradiation resulting in tumor shrinkage. However, subclones of clonogenic cells may be less responsive to the treatment and cause treatment failure, even if the initial measured response was tumor shrinkage. Findings provided by tumor growth assays can thus be indicative for a certain response, but results should preferably be confirmed using other assays.[21]

UHDR has been shown to spare normal tissue in a variety of tissues, including the brain, skin, lung, and gastrointestinal tract.[18,26,27] These sparing effects have been demonstrated in a variety of animal models (including zebra fish, mouse, rat, cat, and mini pig) and for a variety of beam qualities (including electron, photon, proton, and carbon ion irradiation). The magnitude of the FLASH normal tissue sparing effect has been reported to range from ~5% to 50% (in terms of isoeffective doses),[18,26,27] and a recent meta-analysis of single-fraction irradiations reported that the magnitude of FLASH tissue sparing increases with higher doses and varies among tissues.[27] Remarkably, most studies report that UHDR maintains in vivo tumor efficacy compared with CONV.[18,28] There are exceptions, however, such as cell line dependent response to UHDR,[4] or even increased tumor efficacy for UHDR.[29,30] Of note, most current studies that assess in vivo tumor efficacy of UHDR have used tumor growth as the endpoint.

In this study, we present a comprehensive review and analysis of the current evidence on the tumor efficacy of UHDR versus CONV irradiation in preclinical in vivo models. For this purpose, we used the published literature to examine the hypothesis of UHDR tumor isoefficacy compared with CONV irradiation by conducting a qualitative review and combined quantitative analyses of available experimental data. Hereby, we aimed at identifying potential knowledge gaps to inspire future in vivo studies.

Materials and Methods

Record and report identification

We conducted a systematic review of published reports comparing the response of in vivo tumors (ie, preclinical animal tumor models) to UHDR and CONV irradiation. To do so, we searched PubMed (https://pubmed.ncbi.nlm.nih.gov/) for references published between January 2014 and October 2022 using the query: FLASH AND ("RADIOTHERAPY" OR "RADIATION" OR "IRRADIATION"). Our search query resulted in 1114 results. We screened the query results manually for data on in vivo tumor responses to UHDR irradiations in comparison to CONV irradiations and added other publications to the best of our knowledge. A record and report identification and screening flowchart based on PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses)[31] is provided in Table E1. Data sets from a report were included if they met the following selection criteria:

1. It contains in vivo tumor response data for both UHDR irradiations with a time-averaged dose rate (TADR) > 40 Gy/s and CONV irradiations (with TADR between 0.001 and 1 Gy/s).

2. It contains tumor response data for (at least) one of the following clinically relevant endpoints: probability of survival, TCP, probability of tumor regrowth, tumor growth data (this encompasses relative and absolute data of tumor size, volume, weight, and bioluminescence), number of solid tumors, metastatic tissue volume, and ascites volume.
3. It was obtained for comparable biologic system groups and comparable irradiation conditions (ie, same dose and fractionation scheme, same or similar energy spectra and field sizes/dose distributions for CONV or UHDR irradiations).

Data extraction, categorization, conversion, and combined quantitative analysis

The not-yet-well-understood mechanism of action of the FLASH effect is present for UHDR irradiations of normal tissues, resulting in their protection, but supposedly does not result in any response changes for tumors when irradiated with UHDR. It is generally assumed that this differential response is due to intrinsic biologic differences between tumors and normal tissues, involving an interplay between physics, chemistry and biology.[18-20] The overarching goal of this analysis is to present available experimental data in a concise quantitative manner and to examine pooled data for signs of systematic response differences of tumors to UHDR and CONV irradiation.

In most publications numerical experimental data were not available and we digitized them from the original graphs using WebPlotDigitizer.[32] The majority of the endpoints were reported as "time after irradiation" or "time after inoculation," often at multiple time points. To facilitate a combined analysis of these data sets, we converted all reporting times to "time after first irradiation" t in days.

Most experiments reported endpoints related to tumor growth to assess differences between UHDR and CONV irradiation. This included measured size or volume (ie, based on tumor extensions measured in 1 to 3 dimensions), weight, and bioluminescence. For the sake of brevity, we will refer hereafter to all of these endpoints as "tumor size" endpoints. We normalized all tumor size endpoints to relative tumor size (ie, $S_{\mathrm{rel}}^{\mathrm{UHDR}}(t)$ and $S_{\mathrm{rel}}^{\mathrm{CONV}}(t)$) with respect to the day of the first day of irradiation (ie, t = 0 d) to compute the tumor size ratio

$$r_S(t) = \frac{S_{\mathrm{rel}}^{\mathrm{UHDR}}(t)}{S_{\mathrm{rel}}^{\mathrm{CONV}}(t)} \tag{1}$$

Tumor sizes were interpolated linearly between the measured data points. For 2 studies,[14,33] tumor weight was measured only once after sacrificing the mice and weight at the first day of irradiation (t = 0 d) is unknown. For these data sets, we assigned the average weight measured for the CONV and the UHDR group at the time of sacrifice for t = 0 d. To obtain scalar and time-resolved measures of tumor sizes with reduced fluctuations for each individual data set for statistical tests, we also computed averages of $r_S(t)$ over the whole data set time span (r_S) and over times spans of 10 days, that is, $r_S(t, t+9)$. To the extent that experimental data were available, $r_S(t, t+9)$ was computed between 1 and 50 days, that is, averages for 1 to 10, 11 to 20, 21 to 30, 31 to 40, and 41 to 50 days.

Probability (or percentage) of survival of tumor-bearing animals at a given time t in days after UDHR and CONV irradiations ($P^{\mathrm{UHDR}}(t)$ and $P^{\mathrm{CONV}}(t)$) was also reported by multiple studies. According to the scope of our study, data sets in which the death cause was clearly specified to be due to radiation-induced side effects (and not tumor-related events) were not included (this resulted in the exclusion of 1 data set[34]). Using the reported Kaplan-Meier survival curves, we extracted data to estimate survival for the individual mice. Time-to-event was calculated from the first day of irradiation. We performed an analysis of the individual mice data, comparing UHDR with CONV irradiation, and the Kaplan-Meier method was used to visualize the combined survival probability. In addition, using these data we also computed the difference in survival probability as

$$d_P(t) = P^{\mathrm{UHDR}}(t) - P^{\mathrm{CONV}}(t) \tag{2}$$

If the $P^{\mathrm{UHDR}}(t)$ and $P^{\mathrm{CONV}}(t)$ are either both zero or both 1, the quantities are likely not sensitive to possible response differences between UHDR and CONV irradiations. Therefore, we computed $d_P(t)$ only for values of $P^{\mathrm{UHDR}}(t)$ and $P^{\mathrm{CONV}}(t)$ where at least 1 of them was different from zero ($\geq$0.01) and from 1 ($\leq$0.99). This led to the exclusion of 1 additional data set because both survival curves are at 100% over the whole data range.[34] We evaluated the integrated difference in survival probability as

$$D_P = 1\ \mathrm{d} \cdot \sum_i d_P(t_i) \tag{3}$$

where i runs from day 1 to the end of the follow-up $t_{\max}$, that is, $t_i = 1\ \mathrm{d}, 2\ \mathrm{d}, \ldots, t_{\max}$. D_P provides a scalar metric that assesses cumulative gain (>0) or loss (<0) in survival days for UHDR irradiation compared with CONV irradiation.

A combined analysis of data on TCP was not attempted because TCP was defined very heterogeneously for different studies (see Results section for details).

Survival differences from the combined Kaplan-Meier curve were compared by the log-rank test and by a Cox regression model to estimate an effect size. The assumption of proportional hazards was tested by the Schoenfeld residuals test. The assumption of normality is not generally supported for $\ln r_S$, $\ln r_S(t, t+9)$, and D_P (evaluated by Shapiro-Wilk test). Therefore, 2-sided Wilcoxon signed rank tests were used to test for differences from isoeffectiveness of UHDR and CONV irradiations (ie, null hypothesis is that UHDR and CONV samples come from the same distribution) for $\ln r_S$, $\ln r_S(t, t+9)$, and D_P. Furthermore, we performed bivariate correlation analyses to check for possible dependencies of r_S, $r_S(t, t+9)$, and D_P on the irradiation parameters: dose-per-fraction d, total dose D,

time-averaged dose rate (TADR), intrapulse dose rate (IPDR), and exposure time (ET). We used Pearson's R correlation coefficient to test for linear correlations and Spearman's ρ and Kendall's τ to test for rank correlations. P values below 5% were considered to be statistically significant. Data processing, data analysis, and visualization were performed using Python version 3.8 and its libraries (notably numpy, pandas, and scipy)[35] and R 3.6.3 (survival, survminer, ggplot2).[36]

We restricted the combined quantitative analyses of overall trends and statistical tests (described previously) to solid tumors without artificially altered tumor oxygenation state. This was done because liquid tumors are not necessarily representative for the gross of tumors treated with RT and require particular tumor models[4] and because the oxygenation state of a biologic system during irradiation is known to have a decisive effect on its response to UHDR irradiation.[9,30,37,38] In practice, this resulted in the exclusion of 6 data sets (1 vascular clamp, 1 carbogen breathing, and 4 leukemia; see Discussion section) from 2 publications.[4,30] We performed univariate analysis including data sets for all particle types and for electron and proton data set subgroups separately. The conducted data pooling resulted in heterogeneous animal and tumor models. To reduce data set heterogeneity and to examine the robustness of the results, we conducted subgroup analyses for data sets using glioblastoma models and for data sets using limb or flank tumor models. Subgroups for other tumor categories and tumor sites contain each less than 10 data sets (from 1 or 2 reports only) and are not reported.

Results

From a total of 15 reports, we identified 66 different experimental data sets (defined as direct comparisons of tumor responses to UHDR and CONV irradiation for a specific tumor model endpoint and using the same irradiation conditions) that fulfilled the selection criteria. A summary of the tumor models, endpoints, and irradiation parameters of the corresponding experiments is given in Table 1 and a more extensive overview is provided in Table E2. The majority of the data sets reported tumor responses by measurements of tumor growth using calipers or similar (n = 25), or via bioluminescence (n = 8), followed by data sets reporting survival probability (n = 19) (Table 1). Survival of solid tumor-bearing rodents was reported in 5 publications for in total 15 data sets, and survival ≥3 months was achieved for a subset of animals in 3 of the publications.[2,39,40] Long-term control of solid tumors (at around 3 months after irradiation) was assessed by 3 publications,[17,39,40] including a TCD_{50} determination in one of them (Table 2). Three additional publications reported TCP with unconventional definitions and/or shorter follow-

Table 1 Summary of the experimental tumor models, endpoints, and irradiation parameters of the 64 data sets included in this study that compare the response of in vivo tumor models between UHDR and CONV irradiations from 15 reports[2–4,11,13–17,29,30,33,34,39,40]

Property	Values
Rodent species (number of data sets)	Mice (54), rats (12)
Human tumor models (xenografts)	Human xenografts: breast cancer, glioblastoma, human T-cell acute lymphoblastic leukemia
Murine tumor models (syngeneic)	Lung carcinoma, glioblastoma, pancreas cancer, ovarian cancer, oral squamous cell carcinoma, (fibro-, osteo-) sarcoma, breast cancer
Animals per (control, CONV, or UHDR) group, median (range)*	9 (3-15)
Endpoints (number of data sets)	Size/volume/weight (25), bioluminescence (8), survival (19), TCP (7), "tumor regrowth probability" (2), ascites (3), number of solid tumors (1), metastatic tissue (1)
Last data point/maximum follow-up time after first irradiation (d), median (range)	30.5 (11-230)
Particle type (number of data sets)	Electrons (49), protons (15), carbon ions (2)
Total dose (Gy), median (range)	17.5 (4-60)
Dose per fraction (Gy), median (range)	14 (3.5-60)
Fraction number, median (range)	1 (1-5)
UHDR TADR (Gy/s), median (range)*	210 (60-7.8 × 10^6)
UHDR IPDR (MGy/s), median (range)*	0.59 (6.2 × 10^{-5}-7.8)

A more detailed overview of the data sets is given in Table E2.

Abbreviations: CONV = conventional dose rate; IPDR = intrapulse dose rate; TADR = time-averaged dose rate; TCP = tumor control probability; UHDR = ultrahigh dose rate.

* In the case that only a range of values was reported by the original publication, the mean value was used for computation of the median.

Table 2 Overview of different TCP and "tumor regrowth probability" definitions used in published preclinical comparisons of ultrahigh dose rate irradiations with conventional dose rate irradiations

Reference	Definition of tumor control
Bourhis et al (2019)[16]	TCP $= (V_{CTRL} - V_{RT})/V_{CTRL}$, where V is the tumor volumes measured 15 d after irradiation for different dose levels
Diffenderfer et al (2020)[15]	"Tumor regrowth probability" defined as tumor growth to 4 times the starting tumor volume at a given time
Velalopoulou et al (2021)[3]	Percentage of tumors with a volume <500 mm^3 at a given time
Konradsson et al (2022)[39]	No measurable tumor at 100 d after inoculation for different dose levels. In addition, "stable disease" was defined as tumor diameter >0 mm but less than or equal to the tumor diameter at day of first irradiation.
Liljedahl et al (2022)[40]	No sign of tumor growth at day 91
Sørensen et al (2022)[17]	No recurrent tumor at 90 d after irradiation for different doses; included TCD_{50}

Abbreviations: CTRL = control group; RT = irradiated group; TCD_{50} = the dose at which 50% of the animals are cured; TCP = tumor control probability.

up (details in Table 2). Reported endpoints also included occurrence of lung metastases, tumor number, and ascites volume (Table E2). Findings and conclusions of 12 of the 15 included reports support an isoefficacy hypothesis for UHDR irradiation of tumors (Table E3).

Tumor response after UHDR and CONV irradiations: General characteristics

Figure 1 provides an overview of relative tumor responses reported for UHDR compared with CONV irradiations for all endpoints included in this study (ie, tumor size, survival, TCP, etc), while indicating whether trend differences were reported to be statistically significant or not.

Tumor responses

The only study determining TCD_{50} reported a nonsignificant difference of 4% for UHDR and CONV.[17]

As the majority of the experiments assessed tumor size, we performed detailed analyses of the reported results. Most studies reported nonsignificantly different tumor sizes after UHDR and CONV irradiations. Exceptions are 3 studies that reported UHDR as significantly more effective in reducing tumor sizes of U87 glioblastoma (but not for H454 glioblastoma investigated in the same study), for hypoxic U87 glioblastoma (by clamping), and when irradiating osteosarcoma with carbon ions.[2,29,30] Figure 2 displays the development of UHDR over CONV tumor size ratios $r_S(t)$ after the first irradiation and Table E4 provides the corresponding averages and medians of $\overline{r_S}$ and $\overline{r_S}(t, t+9)$. Figure E1 presents $r_S(t)$ for data sets using glioblastoma models and for data sets using limb/flank tumor models. For tumor size ratios smaller than 1, UHDR irradiation was more effective in reducing tumor size (ie, in favor of UHDR irradiation) and vice versa. For times up to 50 days post irradiation, the median (and mean) of all tumor growth data is about 1 and compatible with an isoeffect of UHDR and CONV irradiations for tumors. Accordingly, these differences are statistically not significant between 1 and 50 days, as summarized in Table E4. For times longer than 50 days, there is only a single data set.

Survival

Two-hundred seventy-six animals with 178 events from 14 data sets were included (1 data set was excluded because it specified death due to radiation lethality[34]) in the survival analysis of reconstructed individual mice data. For glioblastoma tumor models, median survival was 55.5 (95% confidence interval, 44-77) and 46 (43-59) days for UHDR and CONV, respectively (hazard ratio, 0.84 [95% confidence interval, 0.62-1.14], $P = 0.26$) (Fig. 3a). Between 50 and 90 days there was a nonsignificant trend in favor of UHDR. Analysis for all included data and subgroup analysis of limb/flank tumor models are provided in Fig. E2. Differences in survival probabilities between UHDR and CONV irradiations $d_P(t)$ are displayed in Fig. 3b. Figure E3 presents $d_P(t)$ for data sets using glioblastoma models. For values of $d_P(t) > 0$, UHDR irradiation is more effective in prolonging survival compared with CONV irradiation (ie, in favor of UHDR irradiation) and vice versa. Median (and mean) of all $d_P(t)$ for the time region with more than 3 observations (up to about 80 days) is about 1 and compatible with an isoeffect of UHDR and CONV irradiations. The distribution of the corresponding D_P values was also statistically compatible with an isoeffect (Table E4).

Tumor response after UHDR and CONV irradiations: Correlations with irradiation parameters

No significant correlations with the irradiation parameters *d, D,* TADR, IPDR, and ET were indicated by bivariate correlation analysis testing for linear and rank correlations. Table E5 provides a comprehensive summary of the results from correlation analysis.

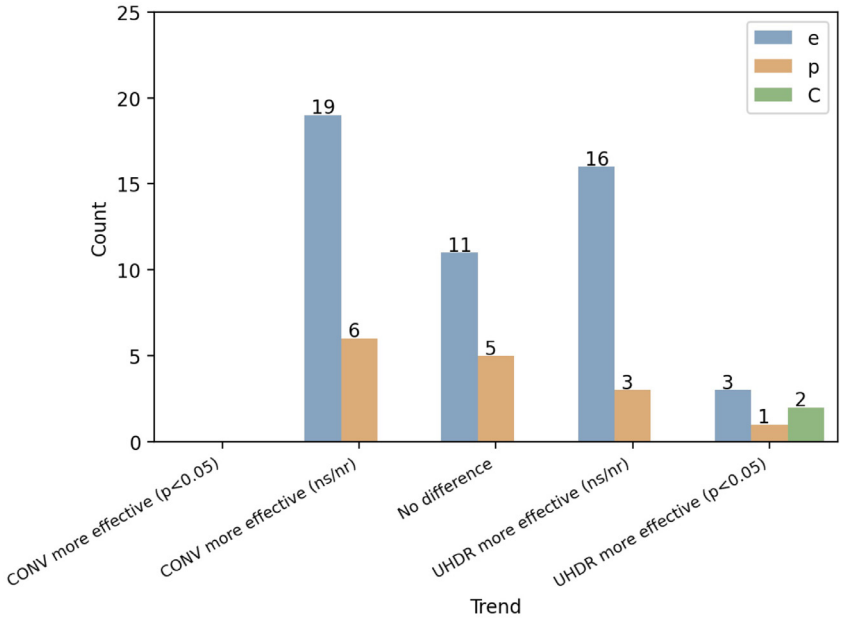

Fig. 1. Frequency distribution of trends of relative tumor responses reported for UHDR and CONV irradiations for all endpoints included in this study (ie, tumor size, survival, TCP, ascites, etc) grouped by beam particle type (see legend). Relative trends in endpoints were grouped as follows. For "UHDR more effective" the endpoint suggests a higher tumor toxicity/prolonged survival for UHDR compared with CONV irradiation (ie, in favor of UHDR irradiation) whereas "CONV more effective" suggests the opposite. These groups are further split indicating if a statistically significant difference at a significance level of *P* below 5% was reported or if no significant difference was reported (ns/nr) between CONV and UHDR irradiations. For data set and evaluation details, see Tables 1 and E2. *Abbreviations:* C = carbon ion beam; CONV = conventional dose rate; e = electron beam; nr = significance not reported; ns = not significant; p = proton beam; TCP = tumor control probability; UHDR = ultrahigh dose rate.

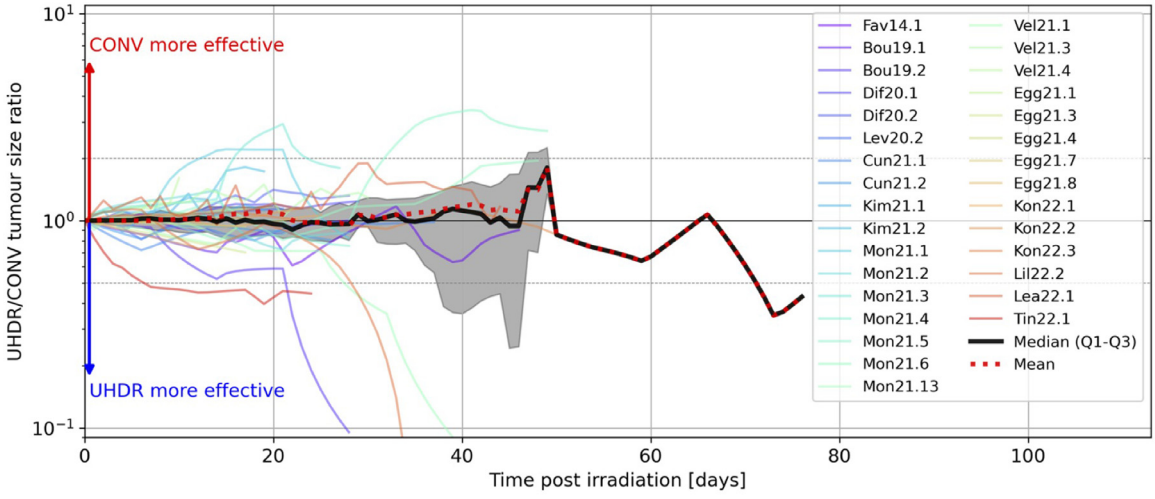

Fig. 2. Development of UHDR over CONV tumor size ratios $r_S(t)$ for all tumor growth data sets (n = 31) by data set identification (see Table E2) with the exclusion of data for which the tumor oxygenation state was artificially altered. Median (black solid line), quartiles (gray band), and mean (red dotted line) values of data are also displayed. For tumor size ratios smaller than 1, UHDR irradiation is more effective in reducing tumor size (ie, in favor of UHDR irradiation) and vice versa. Of note, data sets Lil22.2 and Mon21.13 continue with values $<1 \times 10^{-1}$ until day 46 and 56, respectively, but are no longer displayed due to the limited graph range. *Abbreviations:* CONV = conventional dose rate; Q1 = first quartile; Q3 = third quartile; UHDR = ultrahigh dose rate.

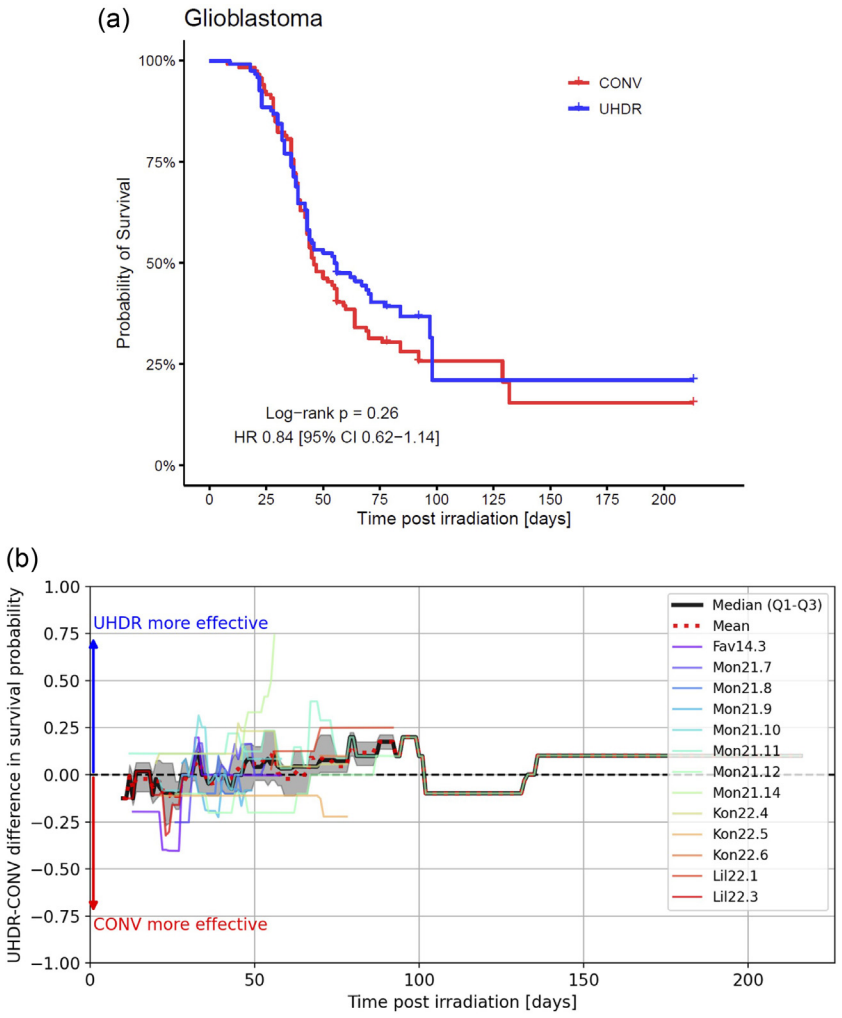

Fig. 3. Survival comparisons between UHDR and CONV irradiations with the exclusion of data for liquid tumors. (a) Combined survival analysis of individual glioblastoma-bearing rodents (data sets n = 12). (b) Development of UHDR-CONV difference in survival probability $d_P(t)$ by data set identification (data sets n = 13). Median (black solid line), quartiles (gray band), and mean (red dotted line) values of data are also displayed. For values of $d_P(t) > 0$, UHDR irradiation is more effective in prolonging survival compared with CONV irradiation (ie, in favor of UHDR irradiation) and vice versa. For data set details, see Table E2. *Abbreviations:* CI = confidence interval; CONV = conventional dose rate; HR = hazard ratio; Q1 = first quartile; Q3 = third quartile; UHDR = ultrahigh dose rate.

Discussion

Tumors are known to be decisively aberrant from normal tissues and it is generally assumed that some of these intrinsic biologic differences give rise to an improved therapeutic index of UHDR irradiation, notably by maintaining an isoefficacy to tumors. We conducted a systematic review and combined analyses of published experimental data comparing response differences of in vivo tumor models to UHDR and CONV irradiations. Twelve out of the 15 identified publications support an isoefficacy hypothesis for UHDR irradiation of tumors and report predominantly nonsignificant response differences. Based on these reports, the isoefficacy of UHDR and CONV irradiations for tumor tissues has become the commonly proclaimed working hypothesis when assessing the clinical potential of FLASH RT.[16,18,20,26,28]

Thirteen of the 15 identified publications used tumor growth assays to assess treatment efficacy. Only 3 publications reported tumor control after a 3-month follow-up. The isoefficacy hypothesis for tumors is supported by our combined analysis of tumor growth data. In addition, a combined analysis of survival data revealed no significant differences between UHDR and CONV irradiations. These findings imply that if a deviation from isoefficacy exists for tumors irradiated with UHDR, it is either below the detectability limit using currently available data and employed analysis techniques (notably, the pooled analyses may be hampered by heterogeneities among the studies, including different animal/tumor models and endpoints, varying irradiation conditions, and the overall limited amount of

available experimental data) or it may exist only for specific tumors, endpoints, and irradiation conditions. Supporting the second point, 4 recent studies found UHDR to be significantly more effective than CONV irradiation in tumor models, but mostly only for some of the investigated settings.[2,4,29,30] It would be of large therapeutic interest, in particular, if it is confirmed that UHDR irradiation is substantially more toxic than CONV irradiation in clinical settings for hypoxic tumors, as suggested by one of these studies.[30]

Even if the current analyses are consistent with UHDR isoefficacy, most of the data rely on tumor growth assays (or bioluminescence that correlates with tumor growth[41,42]) and a further discussion is warranted. Tumor growth assessed days up to a few months after irradiation is an endpoint sensitive to differences in effects for the more radiosensitive tumor cell subpopulation. TCP (and the corresponding TCD_{50}) measured after several months to years, on the other hand, are clinically more relevant endpoints that may allow to capture the response of radioresistant tumor cell subpopulations. Previous studies have revealed poor correlations between tumor growth delay and TCD_{50} in other radiobiological fields of research[23,43,44] and the importance of proper TCD_{50} experiments has been emphasized in particular for treatments with curative potential.[45] Additionally, we note that the current UHDR studies report direct comparisons of tumor volumes and not tumor growth delay (ie, the additional time needed for a treated tumor to reach a certain volume compared with nontreated tumors). In a recent paper it was found that the correlation between tumor volume measurements and TCD_{50} was worse than between tumor growth delay and TCD_{50}.[46] A discussion of the survival data is also warranted because death does not necessarily reflect the natural course of tumor progression. The decision to euthanize animals depends on the discretion of the experimentalist, introducing a potential bias.[47] Reasons for euthanization (or spontaneous death) are not explicitly specified for individual animals, and the criteria for euthanization differed among studies or were not specified. In terms of survival events, animals might die or be euthanized due to tumor progression or due to severe radiation side effects. All the analyzed animals with an event (n = 178) were found in publications using tumor growth assays and the corresponding group-averaged tumor growth curves displayed tumor progression. Although death from radiation-induced side effects cannot always be ruled out, the animals have likely been sacrificed due to tumor progression determined by the experimentalist. The previously discussed limitations of tumor growth assays will also propagate to the survival endpoints. Together, the currently available tumor growth results and survival data may thus be insufficient predictors of potential differences in TCP for UHDR versus CONV irradiation in curative clinical settings.

It is also worth noting that experimental studies that compare the normal tissue sparing of UHDR versus CONV irradiation are frequently designed to detect differences as small as $\lesssim$5%.[27,28,48,49] By contrast, tumor assays comparing the effects of UHDR versus CONV irradiation that we analyzed in this work may often not be sensitive enough to detect changes of this magnitude. A recent meta-analysis found that experiments reported average FLASH-modifying factors (ie, isoeffect dose ratios) of 0.95 $\pm$ 0.11, 0.92 $\pm$ 0.08, and 0.73 $\pm$ 0.11 for normal tissues exposed to single UHDR fractions of <10 Gy, 10 to 25 Gy, and >25 Gy, respectively.[27] This highlights that the majority of the available data evidence normal tissue sparing by FLASH of less than 10% for doses per fraction <25 Gy. Tumor data analyzed in this work were mostly acquired for doses per fraction <25 Gy (median, 14 Gy; third quartile: 17 Gy), and although large variations in tumor growth are to be expected, the number of included animals per group was relatively low (median, 9; third quartile: 11) (Table 1). Thus, one can speculate that any potential differences in tumor response with magnitudes <10% could not be detected by most studies examined in this work. Different sensitivities for detecting response differences between UHDR and CONV irradiations for tumors and for normal tissue may lead to a reporting bias. That is, studies examining differences in responses of normal tissues with a higher sensitivity may report statistically significant differences (which are often <10%) more frequently than studies examining differences in responses of tumors with a lower sensitivity.

The sparing of normal tissues by FLASH was generally found to be larger for large single fraction doses >25 Gy[27] and one can hypothesize that the same might be true for tumor tissues. In that case, experimental designs using large single fractions doses >25 Gy would be more sensitive and would need to be less powered to detect possible differences. Reassuringly, nonsignificant differences in TCD_{50} doses of 4% (49.1 [47.0-51.4] Gy and 51.3 [48.6-54.2] Gy for CONV and UHDR irradiation, respectively), were recently reported by Sørensen et al for large single-fraction doses.[17] A recent randomized phase 3 trial treated nasal squamous cell carcinoma of cat patients with UHDR RT (1 $\times$ 30 Gy, n = 7) and standard-of-care CONV RT (10 $\times$ 4.8 Gy, n = 9) and also reported comparable local control (all cats but 1 in each arm were tumor free after 1 year) and overall survival time,[12] although severe toxicity developed (probably due to local overdosage) in 3 cats in the UHDR arm. However, due to different UHDR and CONV RT arm schedules, the outcome does not facilitate any conclusions on isoefficacy of UHDR irradiations for tumors. The first-in-human UHDR RT treatment was administered in 2018 to a patient with cutaneous lymphoma.[50] Two distinct tumors were treated, each with a single-fraction dose of 15 Gy UHDR and CONV RT, respectively. At 2 years' follow-up, there were no differences in tumor control but also no differences in acute reactions and late effects.[51]

As previously mentioned, 2 recent works reported for in vivo tumor models that UHDR beams may be more effective than CONV beams for some conditions (U87 glioblastoma, hypoxia).[2,30] Another recent study reported that UHDR carbon ion irradiations were more effective compared with

CONV carbon ion irradiations in reducing tumor size and metastatic lung tissue,[29] and Chabi et al reported cell-line-specific responses occurring already at 4 Gy for T-cell acute lymphoblastic leukemia.[4] Also in vitro studies that evaluated clonogenic survival of tumor cells after UHDR and CONV irradiation have shown diverging results, with some responses in favor of UHDR, others in favor of CONV, and yet others compatible with an isoefficacy.[9,52-54] All this makes it plausible that response differences may be present for certain in vivo tumors and may depend on both dose delivery parameters (such as dose, exposure time, and irradiation type) as well as the biologic system, its condition, and endpoint. Because the underlying mechanism of action for the FLASH effect is not elucidated, it is an open question whether the differential UHDR sparing of normal tissues compared with tumors is generalizable in all scenarios and represents an intrinsic biologic difference between tumors and normal tissues.

There are several limitations in the current work. The quantitative analyses rely on data from published figures, with the accompanying uncertainties of extracting the data (typically $\lesssim$3% and $\lesssim$0.1 days). For some data sets, the reported average tumor size differed between the CONV and UHDR groups on the day of the first irradiation (Table E2). Further size differences between groups may exist for data sets where only relative size data were reported by the study. Tumor size may influence response to irradiation,[55] and such differences could therefore negatively affect the comparisons. The survival data should be interpreted cautiously due to lack of specified event reasons (spontaneous death, fulfillment of euthanization criteria due to tumor progression or side effect) and the propagation of the limitations in the tumor growth assays used. The pooled cohort is heterogeneous and consists of different animal models and tumor types, with varying endpoints, follow-up, euthanasia criteria, irradiation doses, and beam parameters, and experiments were performed in different laboratories. The heterogeneity hampers the accuracy and precision of the pooled analyses and should be considered when interpreting the results. Smaller differences in response, for instance in subgroups of tumors/models, could be blurred and not resolved in the analyses. At the same time, subgroup analyses may be of less relevance because they are affected by multiple covarying factors.

Based on the findings of the present work, we propose some leads for future studies to complement current experimental evidence on the response of tumors to UHDR irradiation and to address some of its current limitations:

- Investigate isoefficacy of UHDR irradiations using TCP endpoints (including TCD_{50}) and prolonged follow-up times. The correlation between TCD_{50} and tumor growth in UHDR studies could then also be investigated.
- Design studies to demonstrate an opening of the therapeutic window by simultaneously assessing effect differences after UHDR and CONV irradiation for both tumors and normal tissues and for endpoints and fractionation regimens of clinical relevance.
- Aim at achieving similar sensitivities to detect differences between UHDR and CONV irradiations for tumors and normal tissue to avoid reporting bias and to allow clinically relevant comparisons. A prerequisite for this is comparable quantities, such as TCP and normal tissue complication probability, or isoeffect dose ratios for both tumors and normal tissues.
- Include various tumor models, including transgenic and orthotopic models, representing different histology and radiobiological behaviors (eg, tumors with high and low α/β-ratios and primary human tumors that reflect population diversity encountered in the clinics).
- For possible future clinical implementation, it will also be relevant to study tumor efficacy and widening of the therapeutic window for multifractionated schedules using low doses per fraction (in the range of 2-4 Gy).[56]

Conclusion

Summarizing, the results of the present study are compatible with a tumor isoefficacy of UHDR irradiations on average. However, it does not seem unlikely that biologic variations among tumors could affect their response and suitability for UHDR. The underlying mechanism of action for the FLASH effect, defined as a differential response between normal tissues and tumors, remains to be elucidated. A cautious approach is therefore warranted before assuming tumor isoefficacy for all tumors and clinical scenarios.

References

1. Sørensen BS, Krzysztof Sitarz M, Ankjærgaard C, et al. In vivo validation and tissue sparing factor for acute damage of pencil beam scanning proton FLASH. *Radiother Oncol* 2022;167:109-115.
2. Montay-Gruel P, Acharya MM, Gonçalves Jorge P, et al. Hypofractionated FLASH-RT as an effective treatment against glioblastoma that reduces neurocognitive side effects in mice. *Clin Cancer Res* 2021;27:775-784.
3. Velalopoulou A, Karagounis IV, Cramer GM, et al. Flash proton radiotherapy spares normal epithelial and mesenchymal tissues while preserving sarcoma response. *Cancer Res* 2021;81:4808-4821.
4. Chabi S, To THV, Leavitt R, et al. Ultra-high-dose-rate FLASH and conventional-dose-rate irradiation differentially affect human acute lymphoblastic leukemia and normal hematopoiesis. *Int J Radiat Oncol Biol Phys* 2021;109:819-829.
5. Gao F, Yang Y, Zhu H, et al. First demonstration of the FLASH effect with ultrahigh dose rate high-energy X-rays. *Radiother Oncol* 2021;166:44-50.
6. Pawelke J, Brand M, Hans S, et al. Electron dose rate and oxygen depletion protect zebrafish embryos from radiation damage. *Radiother Oncol* 2021;158:7-12.
7. Fouillade C, Curras-Alonso S, Giuranno L, et al. FLASH irradiation spares lung progenitor cells and limits the incidence of radio-induced senescence. *Clin Cancer Res* 2020;26:1497-1506.
8. Vozenin M-C, De Fornel P, Petersson K, et al. The advantage of FLASH radiotherapy confirmed in mini-pig and cat-cancer patients. *Clin Cancer Res* 2019;25:35-42.

9. Montay-Gruel P, Acharya MM, Petersson K, et al. Long-term neurocognitive benefits of FLASH radiotherapy driven by reduced reactive oxygen species. *Proc Natl Acad Sci* 2019;116:10943-10951.
10. Montay-Gruel P, Petersson K, Jaccard M, et al. Irradiation in a flash: Unique sparing of memory in mice after whole brain irradiation with dose rates above 100 Gy/s. *Radiother Oncol* 2017;124:365-369.
11. Favaudon V, Caplier L, Monceau V, et al. Ultrahigh dose-rate FLASH irradiation increases the differential response between normal and tumor tissue in mice. *Sci Transl Med* 2014;6:245ra93.
12. Rohrer Bley C, Wolf F, Gonçalves Jorge P, et al. Dose- and volume-limiting late toxicity of FLASH radiotherapy in cats with squamous cell carcinoma of the nasal planum and in mini pigs. *Clin Cancer Res* 2022;28:3814-3823.
13. Cunningham S, McCauley S, Vairamani K, et al. FLASH proton pencil beam scanning irradiation minimizes radiation-induced leg contracture and skin toxicity in mice. *Cancers* 2021;13:1012.
14. Levy K, Natarajan S, Wang J, et al. Abdominal FLASH irradiation reduces radiation-induced gastrointestinal toxicity for the treatment of ovarian cancer in mice. *Sci Rep* 2020;10:21600.
15. Diffenderfer ES, Verginadis II, Kim MM, et al. Design, implementation, and in vivo validation of a novel proton FLASH radiation therapy system. *Int J Radiat Oncol Biol Phys* 2020;106:440-448.
16. Bourhis J, Montay-Gruel P, Gonçalves Jorge P, et al. Clinical translation of FLASH radiotherapy: Why and how? *Radiother Oncol* 2019;139:11-17.
17. Sørensen BS, Sitarz MK, Ankjærgaard C, et al. Pencil beam scanning proton FLASH maintains tumor control while normal tissue damage is reduced in a mouse model. *Radiother Oncol* 2022;175:178-184.
18. Wilson JD, Hammond EM, Higgins GS, Petersson K. Ultra-high dose rate (FLASH) radiotherapy: Silver bullet or fool's gold? *Front Oncol* 2020;9:1563.
19. Zhou G. Mechanisms underlying FLASH radiotherapy, a novel way to enlarge the differential responses to ionizing radiation between normal and tumor tissues. *Radiat Med Prot* 2020;1:35-40.
20. Vozenin M-C, Bourhis J, Durante M. Towards clinical translation of FLASH radiotherapy. *Nat Rev Clin Oncol* 2022;19:791-803.
21. Krause M, Zips D, Thames HD, Kummermehr J, Baumann M. Preclinical evaluation of molecular-targeted anticancer agents for radiotherapy. *Radiother Oncol* 2006;80:112-122.
22. Ciecior W, Ebert N, Borgeaud N, et al. Sample-size calculation for preclinical dose–response experiments using heterogeneous tumour models. *Radiother Oncol* 2021;158:262-267.
23. Budach W, Budach V, Stuschke M, Dinges S, Sack H. The TCD50 and regrowth delay assay in human tumor xenografts: Differences and implications. *Int J Radiat Oncol Biol Phys* 1993;25:259-268.
24. Demidenko E. Three endpoints of in vivo tumour radiobiology and their statistical estimation. *Int J Radiat Biol* 2010;86:164-173.
25. Zips D, Thames HD, Baumann M. New anticancer agents: In vitro and in vivo evaluation. *In Vivo* 2005;19:1-8.
26. Vozenin MC, Hendry JH, Limoli CL. Biological benefits of ultra-high dose rate FLASH radiotherapy: Sleeping Beauty awoken. *Clin Oncol* 2019;31:407-415.
27. Böhlen TT, Germond J-F, Bourhis J, et al. Normal tissue sparing by FLASH as a function of single-fraction dose: A quantitative analysis. *Int J Radiat Oncol* 2022;114:1032-1044.
28. Friedl AA, Prise KM, Butterworth KT, Montay-Gruel P, Favaudon V. Radiobiology of the FLASH effect. *Med Phys* 2021;49:1993-2013.
29. Tinganelli W, Weber U, Puspitasari A, et al. FLASH with carbon ions: Tumor control, normal tissue sparing, and distal metastasis in a mouse osteosarcoma model. *Radiother Oncol* 2022;175:185-190.
30. Leavitt R, Vozenin M-C, Kacem H, et al. Not just healthy tissue sparing: hypoxia does not impact FLASH-RT anti-tumor efficacy. *Physica Medica* 2022;94:S46-S47.
31. Page MJ, McKenzie JE, Bossuyt PM, et al. The PRISMA 2020 statement: An updated guideline for reporting systematic reviews. *BMJ* 2021;n71.
32. Rohatgi A. Webplotdigitizer: Version 4.5 2021. Available at: https://automeris.io/WebPlotDigitizer. Accessed April 12, 2022.
33. Eggold JT, Chow S, Melemenidis S, et al. Abdominopelvic FLASH irradiation improves PD-1 immune checkpoint inhibition in preclinical models of ovarian cancer. *Mol Cancer Ther* 2022;21:371-381.
34. Kim MM, Verginadis II, Goia D, et al. Comparison of FLASH proton entrance and the spread-out Bragg peak dose regions in the sparing of mouse intestinal crypts and in a pancreatic tumor model. *Cancers* 2021;13:4244.
35. Van Rossum G, Drake FL. *The Python Language Reference*. Hampton, NH: Python Software Foundation; 2010 Release 3.0.1 [Repr.].
36. R Core Team. *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing; 2020.
37. Hornsey S, Bewley DK. Hypoxia in mouse intestine induced by electron irradiation at high dose-rates. *Int J Radiat Biol Relat Stud Phys Chem Med* 1971;19:479-483.
38. Hendry JH, Moore JV, Hodgson BW, Keene JP. The constant low oxygen concentration in all the target cells for mouse tail radionecrosis. *Radiat Res* 1982;92:172.
39. Konradsson E, Liljedahl E, Gustafsson E, et al. Comparable long-term tumor control for hypofractionated FLASH versus conventional radiation therapy in an immunocompetent rat glioma model. *Adv Radiat Oncol* 2022;7 101011.
40. Liljedahl E, Konradsson E, Gustafsson E, et al. Long-term antitumor effects following both conventional radiotherapy and FLASH in fully immunocompetent animals with glioblastoma. *Sci Rep* 2022;12:12285.
41. Jenkins DE, Oei Y, Hornig YS, et al. Bioluminescent imaging (BLI) to improve and refine traditional murine models of tumor growth and metastasis. *Clin Exp Metastasis* 2003;20:733-744.
42. Jost SC, Collins L, Travers S, Piwnica-Worms D, Garbow JR. Measuring brain tumor growth: Combined bioluminescence imaging–magnetic resonance imaging strategy. *Mol Imaging* 2009;8:245-253.
43. Baumann M, Krause M, Zips D, et al. Selective inhibition of the epidermal growth factor receptor tyrosine kinase by BIBX1382BS and the improvement of growth delay, but not local control, after fractionated irradiation in human FaDu squamous cell carcinoma in the nude mouse. *Int J Radiat Biol* 2003;79:547-559.
44. McNally NJ, Sheldon PW. The effect of radiation on tumour growth delay, cell survival and cure of the animal using a single tumour system. *Br J Radiol* 1977;50:321-328.
45. Coleman CN, Higgins GS, Brown JM, et al. Improving the predictive value of preclinical studies in support of radiotherapy clinical trials. *Clin Cancer Res* 2016;22:3138-3147.
46. Kummer B, Löck S, Gurtner K, et al. Value of functional in-vivo endpoints in preclinical radiation research. *Radiother Oncol* 2021;158:155-161.
47. O'Connor AM, Sargeant JM. Critical appraisal of studies using laboratory animal models. *ILAR J* 2014;55:405-417.
48. Ruan J-L, Lee C, Wouters S, et al. Irradiation at ultra-high (FLASH) dose rates reduces acute normal tissue toxicity in the mouse gastrointestinal system. *Int J Radiat Oncol* 2021;111:1250-1261.
49. Evans T, Cooley J, Wagner M, Yu T, Zwart T. Demonstration of the FLASH effect within the spread-out Bragg peak after abdominal irradiation of mice. *Int J Part Ther* 2021;8:68-75.
50. Bourhis J, Sozzi WJ, Gonçalves Jorge P, et al. Treatment of a first patient with FLASH-radiotherapy. *Radiother Oncol* 2019;139:18-22.
51. Gaide O, Herrera F, Sozzi WJ, et al. Comparison of ultra-high versus conventional dose rate radiotherapy in a patient with cutaneous lymphoma. *Radiother Oncol* 2022;174:87-91.
52. Venkatesulu BP, Sharma A, Pollard-Larkin JM, et al. Ultra high dose rate (35 Gy/sec) radiation does not spare the normal tissue in cardiac and splenic models of lymphopenia and gastrointestinal syndrome. *Sci Rep* 2019;9:17180.
53. Adrian G, Konradsson E, Beyer S, et al. Cancer cells can exhibit a sparing FLASH effect at low doses under normoxic in vitro conditions. *Front Oncol* 2021;11 686142.

54. Adrian G, Ruan J-L, Paillas S, Cooper CR, Petersson K. In vitro assays for investigating the FLASH effect. *Expert Rev Mol Med* 2022;24:e10.
55. Blankenbaker F, Suit HD, Sedlacek RS, Thames HD. *Rodent Tumor Models in Experimental Cancer Therapy*. New York, NY: McGraw-Hill; 1987.
56. Böhlen TT, Germond J, Bourhis J, et al. The minimal FLASH sparing effect needed to compensate the increase of radiobiological damage due to hypofractionation for late−reacting tissues. *Medical Physics* 2022 2022. https://doi.org/10.1002/mp.15911.