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Reinventing Radiobiology in the Light of FLASH Radiotherapy

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Abstract

Ultrahigh–dose rate FLASH radiotherapy (FLASH-RT) is a potentially paradigm-shifting treatment modality that holds the promise of expanding the therapeutic index for nearly any cancer. At the heart of this exciting technology comes the capability to ameliorate major normal tissue complications without compromising the efficacy of tumor killing. This combination of benefits has now been termed the FLASH effect and relies on an in vivo validation to rigorously demonstrate the absence of normal tissue toxicity. The FLASH effect occurs when the overall irradiation time is extremely short (<500 ms), and in this review we attempt to understand how FLASH-RT can kill tumors but spare normal tissues—likely the single most pressing question confronting the field today.

Keywords

FLASH radiotherapy; conventional radiotherapy; ultrahigh dose rate; normal tissue sparing; isoefficient tumor killing

INTRODUCTION TO THE FLASH EFFECT

Definition

The FLASH effect is an in vivo effect where normal tissue toxicities can be ameliorated while maintaining equal efficacy in tumor growth control, achieved by delivering radiation at ultrahigh dose rates above a prescribed threshold (mean dose rates ≥100 Gy/s).

Historical Context

Radiation oncology is one of the more interdisciplinary fields of medicine, where well-defined treatment protocols implementing ionizing radiation from a variety of sources to eradicate tumors have always been empirical and relied on expertise from physics, chemistry, and biology. Technological advancements in beam delivery and imaging have ushered in the modern era of conformal radiation therapy, where the therapeutic index can be enhanced through more precise targeting of tumor volumes while minimizing the collateral dose to the surrounding normal tissue. This fundamental tenet drives nearly all

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standard of care, where curative intent seeks to deliver the maximum toxic dose to a tumor bed while maintaining normal tissue tolerances. While precision in radiotherapy may be plateauing, chemists and biologists have more versatility to develop radiosensitizers and radioprotectors designed to differentiate the killing of cancer cells versus normal cells. More sophisticated treatments designed to co-opt the immune system, disrupt the tumor microenvironment (TME), or exploit metabolic differences between tumors and normal tissue are still handcuffed by the relative similarity of neoplastic tissue and the surrounding cellular milieu. Given this rather simplistic but factual outlook, cancer biologists continue to refine the holy grail of cancer therapies, where any chance to expand the therapeutic index stands to improve survivorship and quality of life.

A relatively recent groundbreaking paper peeled back decades of dogma in the radiobiology community and provided a novel but surprising avenue for increasing the therapeutic index through ultrahigh–dose rate FLASH radiotherapy (FLASH-RT) (Favaudon et al. 2014). The idea that dose rate modulation could be exploited for therapeutic gain (Harrington 2019) was not fundamentally new at the time, as it had been discovered decades prior but then abandoned. These past studies were limited to high–dose rate electron beams, focused largely on early reactions (skin and gut toxicities), and did not investigate the impact of high dose rate on tumor models (Field & Bewley 1974, Hendry et al. 1982, Hornsey & Alper 1966, Inada et al. 1980). Earlier efforts had found that high dose rates spared select skin and gut tissues, from which one might have inferred a similar sparing of tumors, so the presumption that this might provide little clinical utility was not a non sequitur. Favaudon et al. (2014) provided the first side-by-side in vivo evidence of normal tissue sparing and isoefficient tumor growth delay (i.e., a term meant to convey that radiation-induced reductions in the growth of tumors were observed to be dose rate independent), thereby revealing previously unrealized opportunities for enhancing the differential effect of radiotherapy. Subsequent work in the field was sporadic, limited largely by the availability of FLASH machines capable of delivering sufficiently high and uniform isodoses over subsecond timescales (Vozenin et al. 2019b). The lack of FLASH-enabled irradiator systems still hampers the field today, and while significant industry- and academic-based research is actively characterizing the beam parameters for optimizing the conditions necessary to elicit the in vivo FLASH effect, much work remains. Not unexpectedly, details regarding the specific beam requirements for delivering different FLASH modalities (e.g., electron, photon, proton) vary considerably and have been reviewed recently (Farr et al. 2022). To date the ubiquitous capability of FLASH-RT to spare normal tissue toxicities has been substantiated in nearly every normal tissue studied using multiple preclinical models of various genetic backgrounds (Figure 1).

RADIOBIOLOGY TENETS CHALLENGED

As the field of FLASH radiobiology gained momentum by 2019, it became increasingly apparent that a unifying hypothesis to account for both FLASH's sparing of normal tissue and its tumor killing efficacy was conspicuously lacking. Many of the long-standing tenets of radiobiology were unable to explain an effect that was defined in vivo by largely late-responding normal tissues (e.g., brain and lung—tissues that exhibit protracted toxicities many months after irradiation) devoid of rich stem cell populations, where tissue turnover rates were correspondingly low, which necessitated an analysis of more latent functional outcomes less dependent on overt, acute radiosensitivity. Our prior understanding of classical dose rate effects was clearly challenged by FLASH-RT, where chronic low–dose rate (cGy/h) exposures were routinely documented to ameliorate the adverse effects of irradiation of acutely responding (stem cell–rich) tissues and cancers. This effect has been accurately ascribed to the superposition of DNA repair processes transpiring over the course of chronic dose delivery (Hall & Giaccia 2019). The ability of DNA repair to restore genomic integrity over protracted irradiation has been largely accepted and experimentally validated, where recovery of tissues during low–dose rate exposures or during multifraction treatment plans is due to recovery from sublethal damage reliant on DNA double-strand break (DSB) repair (Steel et al. 1986). The reliance of clonogenic survival, chromosome aberrations, and mitotic cell death on residual DSBs has also been validated (Berthel et al. 2019) and suggests that when dose rates are elevated normal tissue damage and recovery should be similar, as exposure times are too short to allow for sublethal damage repair. This expectation has not been borne out in the FLASH literature, as the underlying assumption is likely flawed. It also suggests, but does not prove, that the FLASH effect will be less dependent (if at all) on DSB induction and repair kinetics. In normal tissues, lower levels of DSBs have been measured after isodoses of FLASH-RT compared to conventional radiotherapy (CONV-RT), which rapidly normalize at later time points (<24 h) (Buonanno et al. 2019, Dokic et al. 2022, Fouillade et al. 2020, Levy et al. 2020), whereas in tumors, DSB levels after FLASH-RT are similar to those after CONV-RT (Levy et al. 2020). These observations suggest that the gold standard of radiobiology—the clonogenic survival assay, which is intimately linked to the production and repair of DNA DSBs (Hill et al. 2004, McMillan et al. 1990)—will not provide any useful readouts of the FLASH effect. Interestingly, dose rate modulation has been found to have little impact for in vitro clonogenic assays at biologically relevant levels of exposure (≤10 Gy), and most changes reaching statistical significance have only been uncovered at total doses far exceeding those found to elicit robust normal tissue sparing in vivo and under hypoxic conditions (Adrian et al. 2020, 2021; Montay-Gruel et al. 2019).

THE RISE AND FALL OF MECHANISTIC HYPOTHESES

The field of FLASH-RT has been inundated with reviews, which serve as a tribute to the global interest in the field, but also as a detriment to a burgeoning area of research in need of more experimental data than untested models. We among others were quick to proffer certain hypotheses to rationalize the ability of dose rate modulation to spare normal tissue toxicities. While differences in the early physicochemical steps between FLASH-RT and CONV-RT had not yet been formally investigated, two of the early and most popular hypotheses at this early time discussed the higher rates of free radical recombination/diffusion and oxygen depletion. For the former, FLASH was theorized to elicit higher “instantaneous” free radical densities that could facilitate their recombination and alter diffusion of free radicals, the production of radiolytic species, and downstream damage; numerous models were then been put forth in support of or against this idea (Alanazi et al. 2021, Labarbe et al. 2020, Wardman 2020). The most compelling experimental evidence in favor of the diffusion/recombination hypothesis was demonstrated by the lower concentration of H_2O_2 measured in water exposed to FLASH-RT versus CONV-RT (Montay-Gruel et al. 2019). A similar delivery of FLASH could have the ability to elicit a transient, volume-dependent drop in oxygen tension, possibly sufficient to cause a radioprotective hypoxic state (Pratx & Kapp 2019, Spitz et al. 2019). This has been the topic of extensive reviews and modeling efforts (Favaudon et al. 2022, Wilson et al. 2019) based upon classical studies of the oxygen enhancement ratio.

In contrast, the most compelling experimental evidence in favor of the hypoxia hypothesis was generated by a series of in vivo experiments conducted using a reverse approach, where the FLASH effect found under normoxia was eliminated by carbogen breathing that essentially doubled the oxygen levels in the brain (Montay-Gruel et al. 2019). Thus, at that dose, sufficient levels of radioprotective hypoxia were not attained, thereby obviating the benefits of FLASH in the overly oxygenated brain. Interestingly, more recent and sensitive real-time measurements of oxygen tension, in silico, in vitro, and in vivo, have now provided convincing evidence that radiolytic hypoxia induced by FLASH was insufficient to provide any biological benefit (Boscolo et al. 2021, Cao et al. 2021, Jansen et al. 2021). These recent measurements also fit past data finding that doses approaching 100 Gy are typically required to elicit a 3% drop in oxygen levels (Michaels 1986, Weiss et al. 1974)—doses that are far above those necessary to observe the in vivo FLASH effect (~10 Gy).

Other hypotheses of the protective benefits of FLASH-RT that have found support in the literature (Diffenderfer et al. 2020, Velalopoulou et al. 2021) have posited a protection of the stem cell niche (Pratx & Kapp 2019), which may involve tissue-specific hypoxia. In the brain, electron FLASH-RT was reported to preserve the neurogenic niche where, compared to CONV-RT, increased numbers of proliferating [bromodeoxyuridine ($BrdU^+$)], immature [doublecortin-positive (DCX^+)], and mature neuronal [$BrdU^+$/neuronal nuclear antigen ($NeuN^+$)] cell populations were found in the hippocampal dentate gyrus (Alaghband et al. 2020, Montay-Gruel et al. 2017). Electron (lung) and proton (gut, skin) FLASH-RT also identified higher levels of EdU labeling and a preservation of regenerating crypts and skin stem cells ($Lgr6^+$) (Fouillade et al. 2020, Velalopoulou et al. 2021). This provides a logical explanation for the preservation of acute normal tissue functionality, but it fails to account for other protracted benefits such as neurocognitive sparing, which is not solely reliant on hippocampal neurogenesis, lymphedema, or tissue fibrosis. Stem cell sparing also fails to account for isoefficient tumor killing, and while the tendency to equate cancer stem cells to their normal tissue counterparts has received considerable attention in the literature, their equivalence is debatable and beyond the scope of this review. Suffice it to say, however, that if cancer stem cells were also spared by FLASH-RT, then the future clinical utility of this radiation modality would be brought into question.

The rapid dose delivery inherent to FLASH-RT has also triggered ideas and models related to the fraction of the total blood volume irradiated (Jin et al. 2020, Zhang et al. 2021). Under CONV-RT, one could argue that a large fraction of the total blood supply passes through the target volume and is irradiated at a mean low dose, whereas with FLASH-RT only a small proportion of blood is irradiated but at a higher dose. The possibility that the blood volume represents a critical radiolytic target itself is intriguing, and a wealth of past studies have argued about the merits of circulating cells and the constituents (DNA, exosomes, cytokines, etc.) for mediating classical radiation-induced toxicities (Chaudhuri et al. 2015, Leavitt et al. 2019, Marples & Welford 2018, Vilalta et al. 2016). It is also possible that FLASH depletes a critical compound impacting the radiation response or does not generate the radiolytic production of paracrine mediators (Wardman 2020). Consistently, reduction of inflammatory and fibrotic factors has been described in normal tissues exposed to FLASH (Favaudon et al. 2014, Simmons et al. 2019, Velalopoulou et al. 2021), and other mediators such as microRNA carried in the cargo of exosomes could also be involved (C.L. Limoli & M.-C. Vozenin, personal communication). If the blood and constituent cell populations were the site of this generation, or served to circulate such signals, then a natural consequence of FLASH-RT would be to reduce the levels of proinflammatory or other pathogenic signaling normally generated upon irradiation at conventional dose rates (Figure 2). While experiments are currently underway that are designed to test this more formally using cells cultured within microfluidic devices and whole-thorax/heart irradiation, data in support of this possibility are currently lacking.

It is remarkable that radiobiologists and clinicians both failed for decades to recognize the ability of FLASH-RT to increase the therapeutic index through such a relatively simple change in dose rate (at least in theory—not in practice). While much of the foregoing discussion has focused on select radiochemical, molecular, and cellular mechanisms to account in part for normal tissue sparing, few can account for the response of tumors to FLASH. Unfortunately, the simplest explanations have failed to provide an adequate explanation of the FLASH effect. This partly reflects the early stages of FLASH research. Mechanistic questions will invariably require deeper data sets across multiple disciplines implementing validated FLASH beam modalities. At this juncture it remains a challenge to account for how subsecond irradiation can discriminate between tumors and normal tissue. This is especially noteworthy when considering the marked molecular and structural diversity of the normal tissues involved, the early and late toxicities that are only spared at ultrahigh dose rates, and the inherent heterogeneity of tumors (16 experimental tumor models have studied to date) that equally succumb to irradiation, independent of dose rate. FLASH-RT is arguably the most topical area in the radiation sciences, as groups across the world are now grappling with the field's single most pressing question: Why does FLASH kill tumors?

WHY DOES FLASH KILL TUMORS?

To date the answer to this question is that FLASH kills tumors in the same way conventional dose rate radiotherapy does, as tumor cells appear insensitive to dose rates spanning the current FLASH literature. On the contrary, the dose rate–dependent responses reported in normal tissues suggest that there is a fundamental difference between normal cells and cancer cells that can be realized by delivering ionizing radiation over microsecond to millisecond timescales.

It is noteworthy that to date the responses of 23 tumor types have been compared using FLASH-RT and CONV-RT (Table 1). The cancers investigated have included mouse, rat, and human tumors from the brain (Montay-Gruel et al. 2021, Liljedahl et al. 2022), breast (Favaudon et al. 2014, Gao et al. 2022, Sorensen et al. 2022), blood (Chabi et al. 2021), bone

(Velalopoulou et al. 2021), head and neck (Cunningham et al. 2021, Favaudon et al. 2014), lung (Favaudon et al. 2014, Fouillade et al. 2020, Gao et al. 2022, Y.E. Kim et al. 2021), muscle (Velalopoulou et al. 2021), ovary (Eggold et al. 2022, Levy et al. 2020) and pancreas (Diffenderfer et al. 2020, M.M. Kim et al. 2021), spanning a range of carcinoma (breast, lung, ovary, pancreas, and skin), glioma, and sarcoma tumor models. These models have included either syngeneic or xenograft tumors implanted subcutaneously or orthotopically and studied in immune-competent or immune-compromised mouse models, as well as in one genetically engineered mouse model (GFAP-HRas^{v12}; GFAP-CRE; p53^{flox7wt}) (Maxim et al. 2020). The impact of FLASH-RT on spontaneous tumors has also been investigated in larger vertebrates (dog and cat) (Konradsson et al. 2021, Rohrer Bley et al. 2022, Velalopoulou et al. 2021, Vozenin et al. 2019a) and early human clinical trials (Bourhis et al. 2019, Gaide et al. 2022). In these studies, FLASH-RT has proven to be as efficacious in restraining tumor growth as CONV-RT, although direct comparison of isodoses in some of these situations was more difficult due to the clinical/ethical constraints. More formal tumor control studies using mouse mammary carcinoma cells implanted into the feet of mice confirmed that TCD_{50} (50% tumor control dose) values were similar between FLASH-RT (51 Gy) and CONV-RT (49 Gy) (Sorensen et al. 2022). In another study, Fischer 344 rats bearing subcutaneous or orthotopic rat glioblastoma tumors were given CONV-RT and FLASH-RT using 2 × 8 Gy or 2 × 12.5 Gy, respectively (Liljedahl et al. 2022). While isoefficient tumor cure was achieved in the majority of subcutaneous tumors, it was not in the orthotopic tumors, although increased survival was equivalent at both dose rates. In addition, similar long-term tumor cure assays are still ongoing; however, intermediate results at three months obtained with murine glioblastoma multi-forme also show FLASH-RT and CONV-RT to be isoefficient (M.-C. Vozenin et al., unpublished results).

However, there are a few exceptions to the foregoing findings, where in certain mouse models, including Lewis lung carcinoma and LM8 osteosarcoma, FLASH-RT was shown to be more efficacious than CONV-RT (albeit minimally) in slowing the growth of tumors (B.W. Loo Jr. et al., unpublished data) and preventing metastasis when delivered with carbon ions (Tinganelli et al. 2022). Also noteworthy are our recent unpublished findings demonstrating the curability of orthotopic U87 tumors in mice treated with hypofractionated FLASH-RT (P Montay-Gruel, B Petit & M-C Vozenin, unpublished data) (Figure 3). There is one other notable exception involving the response of three human T cell acute lymphoblastic leukemias (T-ALL) grafted into immunocompromised mice as patient-derived xenografts (Chabi et al. 2021). Following bone marrow transplantation and reconstitution, mice were given 4 Gy of whole-body FLASH-RT or CONV-RT; two of the primary T-ALL cases showed an enhanced response to FLASH-RT, whereas the other case was more responsive to CONV-RT. Thus, this represents the only reported instance where a specific cancer type showed resistance to FLASH-RT compared to CONV-RT. While the precise mechanisms underlying these atypical responses are under investigation, differences in expression of proteins in the GADD45, Wnt, metabolic, and p53 pathways have been found in the FLASH-sensitive primary T-ALL cancers (Chabi et al. 2021).

FLASH RADIOTHERAPY AND CONVENTIONAL RADIOTHERAPY KILL TUMOR CELLS EQUALLY

While cell death mechanisms induced in tumors after FLASH-RT versus CONV-RT remain to be fully characterized, tumor cells are equally sensitive after exposure to FLASH-RT and CONV-RT, unlike in normal tissues where less apoptosis and senescence have been reported after FLASH-RT than after CONV-RT (Allen et al. 2020, Favaudon et al. 2014, Fouillade et al. 2020, Y.E. Kim et al. 2021). In addition, based on the global benefits to vastly different normal tissue beds and the similar responses of equally disparate tumor types, the FLASH effect seems to be independent from the primary DNA sequence (genetically predetermined) or the damage and repair of the DNA backbone. Experimental results indeed show no major differences between normal and tumor cells in clonogenic assays known to be primarily dependent on lethal DSBs (Adrian et al. 2020, Schuler et al. 2022) at clinically relevant doses (<10 Gy), whereas higher doses (20 Gy) and hypoxia have been shown to promote resistance in both cases. Consistently, no in vivo difference in residual DSBs (Levy et al. 2020) was observed in ovarian tumors and normal intestinal crypts after exposure to FLASH-RT versus CONV-RT. Studies investigating the role of chromatin structure, compaction, and epigenetics, as well as those related to the fidelity of transcriptional and post-translational processes, are presently lacking. Other cellular components might also be dose rate responsive such as lipids or proteins (cytoskeleton), which are known to differ between normal and tumor cells. Lastly and for purposes of simplicity, we equate the efficacy of tumor killing obtained by different FLASH beam modalities (electron, photon, proton), recognizing that this is likely an oversimplification of forthcoming data sets.

DIFFERENTIAL EFFECTS ON THE TUMOR MICROENVIRONMENT

The complexities of the cross talk between the TME and tumor cells upon cancer treatment, such as radiotherapy, dictate interindividual variabilities in radiosensitivity and drive recurrence and metastasis. Differences in immune and vascular response, hypoxia, and metabolism are well-known factors that contribute to the success or failure of radiotherapy protocols designed to shrink and ultimately eradicate tumors. Detailed descriptions of the complexities of the TME have been reviewed elsewhere (Joyce & Fearon 2015), and here we focus on those factors projected to play more prominent roles in determining how FLASH-RT remains as effective as CONV-RT in killing tumors.

VASCULAR AND INFLAMMATORY HYPOTHESES

Most FLASH studies focused on the vasculature have been performed in the normal brain and have shown consistent preservation of vascular morphology in adult and juvenile animals that was associated with decreased inflammation (Alaghband et al. 2020; Allen et al. 2020; Montay-Gruel et al. 2019, 2020). Since intratumoral vessels are composed of normal stromal cells (endothelium, pericytes) (Allen & Limoli 2022), past findings might be generalized to approximate the response of tumor vessels to FLASH, but to date no formal investigations have been conducted. The impact of high single doses of FLASH-RT versus CONV-RT (10–25 Gy) was investigated at acute (1 week) and late (1 month) times. Vascular dilation and endothelial NOS (nitric oxide synthase) expression that colocalized with vessels were decreased in animals exposed to FLASH-RT, and tight junction integrity monitored by occludin and claudin-5 expression was preserved. FLASH exposure has also been associated with reduced GFAP and TLR-4 induction, which are surrogate markers of astrogliosis, whereas the expression levels of complement Clq and C3 were elevated regardless of dose rate, supporting the previous idea that differences between FLASH and CONV-RT may be relatively minor within the tumor vasculature and the blood–tumor barrier.

IMMUNE HYPOTHESIS

As quoted earlier, tumor studies have shown the isoefficacy of FLASH-RT and CONV-RT in restraining tumor growth in immunocompetent and compromised mice (Favaudon et al. 2014, Gao et al. 2022, Levy et al. 2020, Montay-Gruel et al. 2021). Consistently, immunologically cold mouse oral carcinoma (MOC2; i.e., tumors that are not infiltrated with immune cells) versus immunologically hot MOC1 (i.e., tumors that are infiltrated with immune cells) cells showed no difference in their response to FLASH-RT versus CONV-RT (Cunningham et al. 2021). Lastly, similar induction of intratumoral adaptive immune profiles ($CD8^+$) was found in ID8 and UPK10 tumors after the combination of anti-PD1 therapy with FLASH-RT and CONV-RT (Eggold et al. 2022). In the same paper, FLASH was reported to modify slightly monocyte infiltration and macrophage polarity. Combined with the fact that FLASH does not induce TGFβ1 in normal tissues (Cunningham et al. 2021, Favaudon et al. 2014, Velalopoulou et al. 2021), a growth factor known to promote fibrosis and act as a potent immunosuppressive signal, this finding might suggest a possible role for innate immunity, which has yet to be demonstrated. Furthermore, ongoing work (unpublished data) has evaluated the response of various cell lines grafted subcutaneously into wild-type (C57BL/6), nude, or immuno-deficient mice and found that antitumor efficacy between FLASH-RT and CONV-RT is independent of the host immune status. In summary, it remains difficult to support the hypotheses developed earlier (Jin et al. 2020, Zhang et al. 2021) that FLASH-RT protects adaptive immune cells and their potential to enhance tumor killing. Published and unpublished data (from our group) point to a relatively minor involvement of the immune response in modulating tumor growth delay, which is unlikely to account for the dose rate independence of FLASH-RT's tumor cell killing.

Thus, at this juncture we have discounted a fundamental role for (*a*) the primary sequence of DNA, (*b*) the induction and repair of DNA damage, (*c*) the TME, or (*d*) the immune response in discriminating between the changes in dose rate found between normal tissue (points *a* and *b*) and tumors (points *c* and *d*). However, vascular and inflammatory responses have shown differential modulation. So what other targets or processes might account for the FLASH effect? To speculate on the most likely alternatives, we need to consider lipid and protein targets, as well as the role of metabolism.

LIPIDS AS A TARGET

For organs rich in lipids like the brain and normal endothelial cells, where lipid membrane composition is specifically defined, chain reactions involving organic hydroperoxides that rely on high levels of polyunsaturated fatty acids to propagate chain reactions and consume oxygen have been proposed to explain the differential response of normal tissue and tumors to FLASH (Favaudon et al. 2022, Labarbe et al. 2020, Spitz et al. 2019). In this regard, inherent differences in the ability of normal tissues and tumors to detoxify organic hydroperoxides could be involved in the FLASH effect. Spitz et al. (2019) posited the half-life of organic hydroperoxides, which is prolonged in tumors relative to normal tissues, to account for the improved therapeutic ratio obtained with FLASH-RT. This idea also invoked the importance of redox chemistry and labile iron, which is known to be biologically sequestered, transported, and metabolized differently in tumors than it is normal tissues. While the relative importance of each of these factors remains to be experimentally determined, some emerging evidence does support the idea that lipids may be sensitive to changes in dose rate. P. Froidevaux et al. (submitted manuscript), recently used cell-free linoleic acid micelles and phosphatidylcholine liposomes as cell membrane models to probe the lipid peroxidation yields after FLASH-RT and CONV-RT. Remarkably, yields of lipid peroxidation end products increased linearly with CONV-RT dose but were conspicuously absent after FLASH-RT, which suggests that lipids may be a critical target in mediating the FLASH effect. While caution is warranted before translating these findings directly to living cell membranes, other data derived from lipidomic analyses of the FLASH- versus CONV-irradiated rodent brain have also uncovered dose rate–dependent differences. In these studies, brains were extracted two months after a 10-Gy dose of FLASH-RT or CONV-RT and then analyzed for the endocannabinoid ligands 2-arachidonoylglycerol (2-AG) and anandamide, along with the endogenous lipids oleoylethanolamide and palmitoylethanolamide (PEA). With the exception of anandamide, the levels of these lipid adducts were increased after CONV-RT but were similar in control and FLASH-irradiated cohorts (Figure 4). Endocannabinoid signaling in the brain is complex and beyond the scope of this review, but earlier work from our group has shown that proton irradiation decreased CB_1-dependent tonic inhibition of GABA (gamma-aminobutyric acid) release that was associated with reduced 2-AG levels (Lee et al. 2016). These data may also reflect the response of the brain to persistent inflammation triggered by CONV-RT, and several studies have implicated PEA in the regulation of neuroinflammation (Bandiera et al. 2014, Solorzano et al. 2009). While further work is clearly needed to substantiate the functional significance of these findings, it remains difficult to reconcile how tumor cells would not be protected as well. However, given the marked differential effect measured, lipids certainly constitute a previously unrecognized target that may be important to the FLASH effect, where lipidomic analyses provide an attractive route for evaluating the sensitivity of the lipid compartment to dose rate modulation.

A ROLE FOR MITOCHONDRIAL METABOLISM

The link between lipids and metabolism is complex, and it is unknown if or how differential damage to specific lipids caused by FLASH-RT versus CONV-RT might alter mitochondrial fatty acid transport and β-oxidation. Mitochondrial usage of fatty acids is highly tissue specific, where β-oxidation in tissues outside the central nervous system drives acetyl-coenzyme A production that feeds into the citric acid cycle (Houten et al. 2016). The brain prefers carbohydrates, a preference that has likely evolved to minimize oxygen consumption and ROS (reactive oxygen species) production and maintain ATP reserves (Schonfeld &

Reiser 2017). What is known, however, is that mitochondrial electron transport is highly sensitive to irradiation. Mitochondria regulate energy metabolism in cells and generate the vast majority of ATP through oxidative phosphorylation (OXPHOS). The resultant proton gradient drives ATP production but comes at a cost, as free radicals are produced at variable yields and with different reactivities. Superoxide ($O_2^{\bullet-}$) produced from the back-reaction of resident electrons with molecular oxygen (O_2) serve not only as signaling molecules but also as precursors and substrates for more toxic species. Diffusion-controlled reactions of superoxide with nitric oxide (NO) can lead to reactive peroxynitrite ($ONOO^-$), while select isoforms of SOD can convert superoxide into the toxic ROS H_2O_2. Hydrogen peroxide can diffuse across intracellular compartments and participate in 1 electron Fenton chemistry, oxidizing Fe^{2+} to Fe^{3+} and generating the highly reactive hydroxyl radical (OH·) (Wardman & Candeias 1996). Irradiation has long been known to disrupt OXPHOS, enhance mitochondrial-derived ROS, and elevate oxidative stress over protracted postirradiation times (Dayal et al. 2008, 2009; Leach et al. 2001; Spitz 2011). We have substantiated this concept and linked prior radiation exposure to the induction and persistence of genomic instability and an oxidative phenotype (Limoli & Giedzinski 2003; Limoli et al. 1998, 2003). At the mechanistic level, oxidative damage accumulated in cancer, but not in normal cells, where increased H_2O_2 levels in cancer cells were caused by dissociation of the subunits comprising electron transport chain (ETC) complex II (Dayal et al. 2008, 2009; Slane et al. 2006). Radiation exposure can elevate oxidative stress though other routes as well (Ameziane-El-Hassani et al. 2015, Collins-Underwood et al. 2008); however, based on significant past work (Coyle et al. 2006, Epperly et al. 2002, Fath et al. 2009, Zhu et al. 2018), the possibility that mitochondrial metabolism is differentially disrupted by dose rate modulation deserves further consideration.

At the level of the tumor, higher dose/fraction (hypofractionation) regimens afforded by FLASH-RT may potentiate mitochondrial dysfunction and electron leakage from the ETC. Resultant increases in the yields of superoxide could further drive intratumoral hydrogen peroxide production within the irradiation field. Some evidence in favor of this has recently been obtained by comparing ROS yields in Lewis lung carcinoma xenografts exposed to single doses of 15-Gy FLASH-RT or CONV-RT (Y.E. Kim et al. 2021). In this tumor model, FLASH-RT was found to produce significantly more ROS (via redox-sensitive fluorogenic dye measurements) than CONV-RT. More recent findings have been the first to measure mitochondrial complex I–IV activity in the normal brain following FLASH-RT and CONV-RT. In these studies, fractionated protocols (e.g., 2 × 10 Gy, 3 × 10 Gy) were used to deliver whole-brain irradiation, and following a comprehensive series of follow-up studies, brains were isolated four months later for biochemical determinations of mitochondrial function. Interestingly, while each radiation modality reduced the activity of mitochondrial complex I, II, and III significantly, FLASH-RT caused larger decreases, especially at complex II. Moreover, activity at complex IV was insensitive to either radiation modality, suggesting a preservation of downstream electron transport and proton flux for maintaining homeostatic levels of ATP. The persistent and elevated reductions in complex I–III activity found after FLASH may lower toxic ROS yields by minimizing electron flux and back-reactions with oxygen. While these very limited data sets need to be replicated in different tumors and normal tissue beds, such observations do suggest that changes in mitochondrial metabolism may lie at the heart of the FLASH effect. Interestingly, a recent paper by Spinelli et al. (2021) identified fumarate as a terminal electron acceptor (as opposed to molecular oxygen) in mammalian electron transport. In the context of FLASH-RT, this provides an intriguing mechanism whereby reverse electron flow favored under hypoxic conditions drives the accumulation of ubiquinol and succinate dehydrogenase (SDH) (complex II) to deposit electrons onto fumarate. Intriguingly, even at physiological oxygen levels, fumarate reduction could sustain electron flux through the ETC in a tissue-specific manner (high for brain, liver, and kidney; lower for lung, heart, and pancreas). The authors speculate that certain tissues might favor the forward (normal) SDH activity to maximize ATP production (such as heart and skeletal muscle) while other tissues (such as brain and kidney) may favor the reverse SDH activity (fumarate reduction) to minimize the electron leakage and toxic ROS production. While the normal physiological role of reverse electron transport remains to be understood, it has yet to be evaluated in tumors. However, in the context of FLASH-RT, mitochondria are bathed with an instantaneous pulse of electrons that could universally favor reverse electron flow in normal tissue—minimizing ROS production while maintaining requisite levels of ATP, an effect that will likely differ substantially in tumors (Figure 5).

METABOLIC HIBERNATION

Tumors have long been known to rely for energy production on glycolysis more than normal cells, which is less efficient than OXPHOS (this is known as the Warburg effect) (Warburg 1956). Correspondingly, disruption of OXPHOS in tumors should make them more reliant on glycolysis, triggering an upregulation of glycolytic enzymes to maintain energy reserves. The inherent inefficient energy production in tumors makes them far more susceptible to metabolic perturbation, whereas normal tissues can better tolerate similar disruptions, downregulating proliferative survival pathways to minimize stress to transcriptional and translational programs. Another explanation of the FLASH effect we proffer dovetails onto the preceding discussion and involves the concept of metabolic hibernation. If both FLASH-RT and CONV-RT are equally detrimental to OXPHOS, then disrupting electron transport preferentially will favor glycolysis, albeit to a varying extent across tissues and tumor types. Normal tissues may enter a relative state of metabolic quiescence, consuming lower levels of oxygen without producing overt amounts of ROS. Over protracted times, normal tissues can adapt and tolerate this insult better than tumors and do not succumb to toxic pathogenic processes activated by oxidative stress and injury. Here, reduced oxygen consumption is beneficial. For tumors, disruptions to OXPHOS are far less tolerated, where reductions in energy production trigger an upregulation of compensatory glycolysis and suboptimal ATP production. If sustained, macromolecular synthesis will be compromised and tumors may succumb, in part, to their inability to maintain adequate pools of ATP (Figure 6). Interestingly, this hypothesis is supported by our recent RNA sequencing analysis showing that hypoxic tumors remain sensitive to FLASH by upregulation of the glycolytic pathway (C.L. Limoli & M.-C. Vozenin, unpublished data). While real-time measurements of ATP in vivo after FLASH-RT still need to be performed, the idea that low metabolic activity is protective against radiation injury has an interesting backstory. For over 70 years it has been known that depression of body temperature and hibernation protect mammals from lethal doses of irradiation (Barr & Musacchia 1967, Hornsey 1956, Smith & Grenan 1951, Storer & Hempelmann 1952), spanning the major radiation syndromes (i.e., lethal syndromes resulting from excessive radiation exposure that cause gastrointestinal or hematopoietic failure). The expression of mortality is slowed significantly during hibernation, and a comparison of LD50/30 (lethal dose, 50%/30%) values for winter-hibernating (15–17.5 Gy) and winter-active squirrels (11–12.5 Gy) yields a dose-modifying factor (DMF) of 1.4 (Musacchia & Barr 1968), which is remarkably similar to the DMF values (1.2–1.4) derived across multiple normal tissues when compared to similar isodoses of FLASH-RT to CONV-RT.

SUMMARY: PROTEINS AND OTHER STRUCTURAL TARGETS

As alluded to above, the idea that proteins constitute critical targets for radiation effects has been met with considerable skepticism over the years, as their continual turnover discounted their importance in heritable changes or persistent functional effects transpiring over the lifespan of an organism. It comes as no surprise that certain organs (such as the brain or heart) contain cells (such as neurons and cardiomyocytes) that persist throughout the lifespan of an organism. While virtually no evidence exists to date that FLASH differentially effects certain protein classes, this longstanding dogma may need to be reevaluated to accurately capture how FLASH-RT produces different effects across tumors and normal tissues. Extending this logic, we propose that FLASH-RT be envisioned as a fundamental tool that can discriminate a structural predeterminant in cells or tissues at the microscopic, mesoscopic, or macroscopic level. In many respects, FLASH may now be able to probe more subtle nuances between cancer cells and normal cells than previous tools, akin to the discovery of restriction enzymes and monoclonal antibodies that refined modern day molecular biology. To this end, we propose that for the field to move forward in an innovative way, and to provide deeper mechanistic insights, additional targets (such as lipids and proteins) and metabolic processes should be considered with current technologies to help answer the pressing question of why FLASH kills tumors, but kills normal tissues to a much lesser extent (Figure 7).

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LITERATURE CITED

Adrian G, Konradsson E, Beyer S, Wittrup A, Butterworth KT, et al. 2021. Cancer cells can exhibit a sparing FLASH effect at low doses under normoxic *in vitro*-conditions. Front. Oncol 11:686142 [PubMed: 34395253]

Adrian G, Konradsson E, Lempart M, Back S, Ceberg C, Petersson K. 2020. The FLASH effect depends on oxygen concentration. Br. J. Radiol 93:20190702 [PubMed: 31825653]

Alaghband Y, Cheeks SN, Allen BD, Montay-Gruel P, Doan NL, et al. 2020. Neuroprotection of radiosensitive juvenile mice by ultra-high dose rate FLASH irradiation. Cancers 12(6):1671 [PubMed: 32599789]

Alanazi A, Meesungnoen J, Jay-Gerin JP. 2021. A computer modeling study of water radiolysis at high dose rates. Relevance to FLASH radiotherapy. Radiat. Res 195:149–62 [PubMed: 33300999]

Allen BD, Acharya MM, Montay-Gruel P, Jorge PG, Bailat C, et al. 2020. Maintenance of tight junction integrity in the absence of vascular dilation in the brain of mice exposed to ultra-high-dose-rate FLASH irradiation. Radiat. Res 194:625–35 [PubMed: 33348373]

Allen BD, Limoli CL. 2022. Breaking barriers: neurodegenerative repercussions of radiotherapy induced damage on the blood-brain and blood-tumor barrier. Free Radic. Biol. Med 178:189–201 [PubMed: 34875340]

Ameziane-El-Hassani R, Talbot M, de Souza Dos Santos MC, Al Ghuzlan A, Hartl D, et al. 2015. NADPH oxidase DUOX1 promotes long-term persistence of oxidative stress after an exposure to irradiation. PNAS 112:5051–56 [PubMed: 25848056]

Bandiera T, Ponzano S, Piomelli D. 2014. Advances in the discovery of *N*-acylethanolamine acid amidase inhibitors. Pharmacol. Res 86:11–17 [PubMed: 24798679]

Barr RE, Musacchia XJ. 1967. Radiation sensitivity of the hibernating ground squirrel, *Citellus tridecemlineatus*. Proc. Soc. Exp. Biol. Med 124:1204–7 [PubMed: 6024835]

Berthel E, Ferlazzo ML, Devic C, Bourguignon M, Foray N. 2019. What does the history of research on the repair of DNA double-strand breaks tell us? A comprehensive review of human radiosensitivity. Int. J. Mol. Sci 20(21):5339 [PubMed: 31717816]

Boscolo D, Scifoni E, Durante M, Kramer M, Fuss MC. 2021. May oxygen depletion explain the FLASH effect? A chemical track structure analysis. Radiother. Oncol 162:68–75 [PubMed: 34214612]

Bourhis J, Sozzi WJ, Jorge PG, Gaide O, Bailat C, et al. 2019. Treatment of a first patient with FLASH-radiotherapy. Radiother. Oncol 139:18–22 [PubMed: 31303340]

Buonanno M, Grilj V, Brenner DJ. 2019. Biological effects in normal cells exposed to FLASH dose rate protons. Radiother. Oncol 139:51–55 [PubMed: 30850209]

Cao X, Zhang R, Esipova TV, Allu SR, Ashraf R, et al. 2021. Quantification of oxygen depletion during FLASH irradiation in vitro and in vivo. Int. J. Radiat. Oncol. Biol. Phys 111(1):240–248 [PubMed: 33845146]

Chabi S, To THV, Leavitt R, Poglio S, Jorge PG, et al. 2021. 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 109:819–29 [PubMed: 33075474]

Chaudhuri AA, Binkley MS, Osmundson EC, Alizadeh AA, Diehn M. 2015. Predicting radiotherapy responses and treatment outcomes through analysis of circulating tumor DNA. Semin. Radiat. Oncol 25:305–12 [PubMed: 26384278]

Collins-Underwood JR, Zhao W, Sharpe JG, Robbins ME. 2008. NADPH oxidase mediates radiation-induced oxidative stress in rat brain microvascular endothelial cells. Free Radic. Biol. Med 45:929–38 [PubMed: 18640264]

Coyle CH, Martinez LJ, Coleman MC, Spitz DR, Weintraub NL, Kader KN. 2006. Mechanisms of H_2O_2- induced oxidative stress in endothelial cells. Free Radic. Biol. Med 40:2206–13 [PubMed: 16785034]

Cunningham S, McCauley S, Vairamani K, Speth J, Girdhani S, et al. 2021. FLASH proton pencil beam scanning irradiation minimizes radiation-induced leg contracture and skin toxicity in mice. Cancers 13(5):1012 [PubMed: 33804336]

Dayal D, Martin SM, Limoli CL, Spitz DR. 2008. Hydrogen peroxide mediates the radiation-induced mutator phenotype in mammalian cells. Biochem. J 413:185–91 [PubMed: 18352860]

Dayal D, Martin SM, Owens KM, Aykin-Burns N, Zhu Y, et al. 2009. Mitochondrial complex II dysfunction can contribute significantly to genomic instability after exposure to ionizing radiation. Radiat. Res 172:737–45 [PubMed: 19929420]

Diffenderfer ES, Verginadis II, Kim MM, Shoniyozov K, Velalopoulou A, et al. 2020. Design, implementation, and in vivo validation of a novel proton FLASH radiation therapy system. Int. J. Radiat. Oncol. Biol. Phys 106:440–48 [PubMed: 31928642]

Dokic I, Meister S, Bojcevski J, Tessonnier T, Walsh D, et al. 2022. Neuroprotective effects of ultra-high dose rate FLASH Bragg peak proton irradiation. Int. J. Radiat. Oncol. Biol. Phys 113:614–23 [PubMed: 35196536]

Eggold JT, Chow S, Melemenidis S, Wang J, Natarajan S, et al. 2022. Abdominopelvic FLASH irradiation improves PD-1 immune checkpoint inhibition in preclinical models of ovarian cancer. Mol. Cancer Ther 21:371–81 [PubMed: 34866044]

Epperly MW, Sikora CA, DeFilippi SJ, Gretton JA, Zhan Q, et al. 2002. Manganese superoxide dismutase (SOD2) inhibits radiation-induced apoptosis by stabilization of the mitochondrial membrane. Radiat. Res 157:568–77 [PubMed: 11966323]

Farr JV, Parodi K, Carlson DJ. 2022. FLASH: current status and the transition to clinical use. Med. Phys 49(3):1972–73 [PubMed: 35262219]

Fath MA, Diers AR, Aykin-Burns N, Simons AL, Hua L, Spitz DR. 2009. Mitochondrial electron transport chain blockers enhance 2-deoxy-D-glucose induced oxidative stress and cell killing in human colon carcinoma cells. Cancer Biol. Ther 8:1228–36 [PubMed: 19411865]

Favaudon V, Caplier L, Monceau V, Pouzoulet F, Sayarath M, et al. 2014. Ultrahigh dose-rate FLASH irradiation increases the differential response between normal and tumor tissue in mice. Sci. Transl. Med 6:245ra93

Favaudon V, Labarbe R, Limoli CL. 2022. Model studies of the role of oxygen in the FLASH effect. Med. Phys 49:2068–81 [PubMed: 34407219]

Field SB, Bewley DK. 1974. Effects of dose-rate on the radiation response of rat skin. Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med 26:259–67 [PubMed: 4547756]

Fouillade C, Curras-Alonso S, Giuranno L, Quelennec E, Heinrich S, et al. 2020. FLASH irradiation spares lung progenitor cells and limits the incidence of radio-induced senescence. Clin. Cancer Res 26:1497–506 [PubMed: 31796518]

Gaide O, Herrera F, Sozzi WJ, Gonçalves Jorge P, Kinj R, et al. 2022. Comparison of ultra-high versus conventional dose rate radiotherapy in a patient with cutaneous lymphoma. Radiother. Oncol 174:87–91 [PubMed: 34998899]

Gao F, Yang Y, Zhu H, Wang J, Xiao D, et al. 2022. First demonstration of the FLASH effect with ultrahigh dose rate high-energy X-rays. Radiother. Oncol 166:44–50 [PubMed: 34774651]

Hall EJ, Giaccia AJ. 2019. Radiobiology for the Radiologist. Philadelphia: Wolters Kluwer

Harrington KJ. 2019. Ultrahigh dose-rate radiotherapy: next steps for FLASH-RT. Clin. Cancer Res 25:3–5 [PubMed: 30093447]

Hendry JH, Moore JV, Hodgson BW, Keene JP. 1982. The constant low oxygen concentration in all the target cells for mouse tail radionecrosis. Radiat. Res 92:172–81 [PubMed: 7134382]

Hill MA, Herdman MT, Stevens DL, Jones NJ, Thacker J, Goodhead DT. 2004. Relative sensitivities of repair-deficient mammalian cells for clonogenic survival after α-particle irradiation. Radiat. Res 162:667–76 [PubMed: 15548117]

Hornsey S 1956. Protection from whole-body X-irradiation afforded to adult mice by reducing the body temperature. Nature 178:87

Hornsey S, Alper T. 1966. Unexpected dose-rate effect in the killing of mice by radiation. Nature 210:212–13 [PubMed: 5962093]

Houten SM, Violante S, Ventura FV, Wanders RJ. 2016. The biochemistry and physiology of mitochondrial fatty acid β-oxidation and its genetic disorders. Annu. Rev. Physiol 78:23–44 [PubMed: 26474213]

Inada T, Nishio H, Amino S, Abe K, Saito K. 1980. High dose-rate dependence of early skin reaction in mouse. Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med 38:139–45 [PubMed: 6968733]

Jansen J, Knoll J, Beyreuther E, Pawelke J, Skuza R, et al. 2021. Does FLASH deplete oxygen? Experimental evaluation for photons, protons, and carbon ions. Med. Phys 48(7):3982–90 [PubMed: 33948958]

Jin JY, Gu A, Wang W, Oleinick NL, Machtay M, Spring Kong FM. 2020. Ultra-high dose rate effect on circulating immune cells: a potential mechanism for FLASH effect? Radiother. Oncol 149:55–62 [PubMed: 32387486]

Joyce JA, Fearon DT. 2015. T cell exclusion, immune privilege, and the tumor microenvironment. Science 348:74–80 [PubMed: 25838376]

Kacem H, Psoroulas S, Boivin G, Folkerts M, Grilj V, et al. 2022. Comparing radiolytic production of H_2O_2 and development of Zebrafish embryos after ultra high dose rate exposure with electron and transmission proton beams. Radiother. Oncol 175:197–202 [PubMed: 35868604]

Karsch L, Pawelke J, Brand M, Hans S, Hideghéty K, et al. 2022. Beam pulse structure and dose rate as determinants for the flash effect observed in zebrafish embryo. Radiother. Oncol 175:49–54

Kim MM, Verginadis II, Goia D, Haertter A, Shoniyozov K, et al. 2021. 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 13(16):4244 [PubMed: 34439398]

Kim YE, Gwak SH, Hong BJ, Oh JM, Choi HS, et al. 2021. Effects of ultra-high doserate FLASH irradiation on the tumor microenvironment in Lewis lung carcinoma: role of myosin light chain. Int. J. Radiat. Oncol. Biol. Phys 109:1440–53 [PubMed: 33186615]

Konradsson E, Arendt ML, Bastholm Jensen K, Borresen B, Hansen AE, et al. 2021. Establishment and initial experience of clinical FLASH radiotherapy in canine cancer patients. Front. Oncol 11:658004 [PubMed: 34055624]

Labarbe R, Hotoiu L, Barbier J, Favaudon V. 2020. A physicochemical model of reaction kinetics supports peroxyl radical recombination as the main determinant of the FLASH effect. Radiother. Oncol 153:303–10 [PubMed: 32534957]

Leach JK, Van Tuyle G, Lin PS, Schmidt-Ullrich R, Mikkelsen RB. 2001. Ionizing radiation-induced, mitochondria-dependent generation of reactive oxygen/nitrogen. Cancer Res. 61:3894–901 [PubMed: 11358802]

Leavitt RJ, Limoli CL, Baulch JE. 2019. miRNA-based therapeutic potential of stem cell-derived extracellular vesicles: a safe cell-free treatment to ameliorate radiation-induced brain injury. Int. J. Radiat. Biol 95:427–35 [PubMed: 30252569]

Lee SH, Dudok B, Parihar VK, Jung KM, Zoldi M, et al. 2016. Neurophysiology of space travel: energetic solar particles cause cell type-specific plasticity of neurotransmission. Brain Struct. Funct 222:2345–57 [PubMed: 27905022]

Levy K, Natarajan S, Wang J, Chow S, Eggold JT, et al. 2020. Abdominal FLASH irradiation reduces radiation-induced gastrointestinal toxicity for the treatment of ovarian cancer in mice. Sci. Rep 10:21600 [PubMed: 33303827]

Liljedahl E, Konradsson E, Gustafsson E, Jonsson KF, Olofsson JK, et al. 2022. Long-term anti-tumor effects following both conventional radiotherapy and FLASH in fully immuocompetent animals with glioblastoma. Sci. Rep 12:12285 [PubMed: 35853933]

Limoli C, Giedzinski E. 2003. Induction of chromosomal instability by chronic oxidative stress. Neoplasia 5:339–46 [PubMed: 14511405]

Limoli C, Giedzinski E, Morgan W, Swarts S, Jones G, Hyun W. 2003. Persistent oxidative stress in chromosomally unstable cells. Cancer Res. 63:3107–11 [PubMed: 12810636]

Limoli CL, Hartmann A, Shephard L, Yang CR, Boothman DA, et al. 1998. Apoptosis, reproductive failure, and oxidative stress in Chinese hamster ovary cells with compromised genomic integrity. Cancer Res. 58:3712–18 [PubMed: 9721883]

Marples B, Welford SM. 2018. Radiation biology and circulating tumor cells. Int. J. Radiat. Oncol. Biol. Phys 100:813–15 [PubMed: 29485052]

Maxim PG, Loo BW, Bailat C, Montay-Gruel P, Limoli CL, Vozenin M-C. 2020. FLASH radiation therapy: a new treatment modality. In The Modern Technology of Radiation Oncology: A Compendium for Medical Physicists and Radiation Oncologists, ed. JV Dyk, pp. 488–500. Madison, WI: Med. Phys. Publ.

McMillan TJ, Cassoni AM, Edwards S, Holmes A, Peacock JH. 1990. The relationship of DNA double-strand break induction to radiosensitivity in human tumour cell lines. Int. J. Radiat. Biol 58:427–38 [PubMed: 1975605]

Michaels HB. 1986. Oxygen depletion in irradiated aqueous solutions containing electron affinic hypoxic cell radiosensitizers. Int. J. Radiat. Oncol. Biol. Phys 12:1055–58 [PubMed: 3744926]

Montay-Gruel P, Acharya MM, Gonçalves Jorge P, Petit B, Petridis IG, et al. 2021. Hypofractionated FLASH-RT as an effective treatment against glioblastoma that reduces neurocognitive side effects in mice. Clin. Cancer Res 27:775–84 [PubMed: 33060122]

Montay-Gruel P, Acharya MM, Petersson K, Alikhani L, Yakkala C, et al. 2019. Long-term neurocognitive benefits of FLASH radiotherapy driven by reduced reactive oxygen species. PNAS 116:10943–51 [PubMed: 31097580]

Montay-Gruel P, Bouchet A, Jaccard M, Patin D, Serduc R, et al. 2018. X-rays can trigger the FLASH effect: Ultra-high dose-rate synchrotron light source prevents normal brain injury after whole brain irradiation in mice. Radiother. Oncol 129(3):582–88 [PubMed: 30177374]

Montay-Gruel P, Markarian M, Allen BD, Baddour JD, Giedzinski E, et al. 2020. Ultra-high-dose-rate FLASH irradiation limits reactive gliosis in the brain. Radiat. Res 194:636–45 [PubMed: 32853387]

Montay-Gruel P, Petersson K, Jaccard M, Boivin G, Germond JF, et al. 2017. Irradiation in a flash: unique sparing of memory in mice after whole brain irradiation with dose rates above 100 Gy/s. Radiother. Oncol 124:365–69 [PubMed: 28545957]

Musacchia XJ, Barr RE. 1968. Survival of whole-body-irradiated hibernating and active ground squirrels; *Citellus tridecemlineatus*. Radiat. Res 33:348–56 [PubMed: 5637298]

Pratx G, Kapp DS. 2019. A computational model of radiolytic oxygen depletion during FLASH irradiation and its effect on the oxygen enhancement ratio. Phys. Med. Biol 64:185005 [PubMed: 31365907]

Rohrer Bley C, Wolf F, Gonçalves Jorge P, Grilj V, Petridis I, et al. 2022. 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 28(17):3814–23 [PubMed: 35421221]

Schonfeld P, Reiser G. 2017. Brain energy metabolism spurns fatty acids as fuel due to their inherent mitotoxicity and potential capacity to unleash neurodegeneration. Neurochem. Int 109:68–77 [PubMed: 28366720]

Schuler E, Acharya M, Montay-Gruel P, Loo BW Jr., Vozenin MC, Maxim PG. 2022. Ultra-high dose rate electron beams and the FLASH effect: from preclinical evidence to a new radiotherapy paradigm. Med. Phys 49:2082–95 [PubMed: 34997969]

Simmons DA, Lartey FM, Schuler E, Rafat M, King G, et al. 2019. Reduced cognitive deficits after FLASH irradiation of whole mouse brain are associated with less hippocampal dendritic spine loss and neuroinflammation. Radiother. Oncol 139:4–10 [PubMed: 31253467]

Slane BG, Aykin-Burns N, Smith BJ, Kalen AL, Goswami PC, et al. 2006. Mutation of succinate dehydrogenase subunit C results in increased $O_2{\bullet}^{-}$, oxidative stress, and genomic instability. Cancer Res. 66:7615–20 [PubMed: 16885361]

Smith F, Grenan MM. 1951. Effect of hibernation upon survival time following whole-body irradiation in the marmot (Marmota monax). Science 113:686–88 [PubMed: 14845719]

Solorzano C, Zhu C, Battista N, Astarita G, Lodola A, et al. 2009. Selective *N*-acylethanolamine-hydrolyzing acid amidase inhibition reveals a key role for endogenous palmitoylethanolamide in inflammation. PNAS 106:20966–71 [PubMed: 19926854]

Sorensen BS, Sitarz MK, Ankjaergaard C, Johansen JG, Andersen CE, et al. 2022. Pencil beam scanning proton FLASH maintains tumor control while normal tissue damage is reduced in a mouse model. Radiother. Oncol 175:178–84 [PubMed: 35595175]

Soto LA, Casey KM, Wang J, Blaney A, Manjappa R, et al. 2020. FLASH irradiation results in reduced severe skin toxicity compared to conventional-dose-rate irradiation. Radiat. Res 194(6):618–24 [PubMed: 32853385]

Spinelli JB, Rosen PC, Sprenger HG, Puszynska AM, Mann JL, et al. 2021. Fumarate is a terminal electron acceptor in the mammalian electron transport chain. Science 374:1227–37 [PubMed: 34855504]

Spitz DR. 2011. Metabolic oxidative stress and low dose radiation responses: Are mitochondria involved? Health Phys. 100:295 [PubMed: 21595073]

Spitz DR, Buettner GR, Petronek MS, St-Aubin JJ, Flynn RT, et al. 2019. An integrated physico-chemical approach for explaining the differential impact of FLASH versus conventional dose rate irradiation on cancer and normal tissue responses. Radiother. Oncol 139:23–27 [PubMed: 31010709]

Steel GG, Down JD, Peacock JH, Stephens TC. 1986. Dose-rate effects and the repair of radiation damage. Radiother. Oncol 5:321–31 [PubMed: 3726169]

Storer JB, Hempelmann LH. 1952. Hypothermia and increased survival rate of infant mice irradiated with X-rays. Am. J. Physiol 171:341–48 [PubMed: 13007799]

Tinganelli W, Weber U, Puspitasari A, Simoniello P, Abdollahi A, et al. 2022. FLASH with carbon ions: tumor control, normal tissue sparing, and distal metastasis in a mouse osteosarcoma model. Radiother. Oncol 175:185–90 [PubMed: 35537606]

Velalopoulou A, Karagounis IV, Cramer GM, Kim MM, Skoufos G, et al. 2021. FLASH proton radiotherapy spares normal epithelial and mesenchymal tissues while preserving sarcoma response. Cancer Res. 81:4808–21 [PubMed: 34321243]

Vilalta M, Rafat M, Graves EE. 2016. Effects of radiation on metastasis and tumor cell migration. Cell. Mol. Life Sci 73:2999–3007 [PubMed: 27022944]

Vozenin MC, De Fornel P, Petersson K, Favaudon V, Jaccard M, et al. 2019a. The advantage of FLASH radiotherapy confirmed in mini-pig and cat-cancer patients. Clin. Cancer Res 25:35–42 [PubMed: 29875213]

Vozenin MC, Hendry JH, Limoli CL. 2019b. Biological benefits of ultra-high dose rate FLASH radiotherapy: sleeping beauty awoken. Clin. Oncol 31(7):407–15

Warburg O 1956. On the origin of cancer cells. Science 132:309–14

Wardman P 2020. Radiotherapy using high-intensity pulsed radiation beams (FLASH): a radiation-chemical perspective. Radiat. Res 194:607–17 [PubMed: 33348369]

Wardman P, Candeias LP. 1996. Fenton chemistry: an introduction. Radiat. Res 145:523–31 [PubMed: 8619017]

Weiss H, Epp ER, Heslin JM, Ling CC, Santomasso A. 1974. Oxygen depletion in cells irradiated at ultra-high dose-rates and at conventional dose-rates. Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med 26:17–29 [PubMed: 4607987]

Wilson JD, Hammond EM, Higgins GS, Petersson K. 2019. Ultra-high dose rate (FLASH) radiotherapy: silver bullet or fool's gold? Front. Oncol 9:1563 [PubMed: 32010633]

Zhang Y, Ding Z, Perentesis JP, Khuntia D, Pfister SX, Sharma RA. 2021. Can rational combination of ultra-high dose rate FLASH radiotherapy with immunotherapy provide a novel approach to cancer treatment? Clin. Oncol 33:713–22

Zhu Y, Dean AE, Horikoshi N, Heer C, Spitz DR, Gius D. 2018. Emerging evidence for targeting mitochondrial metabolic dysfunction in cancer therapy. J. Clin. Investig 128:3682–91 [PubMed: 30168803]

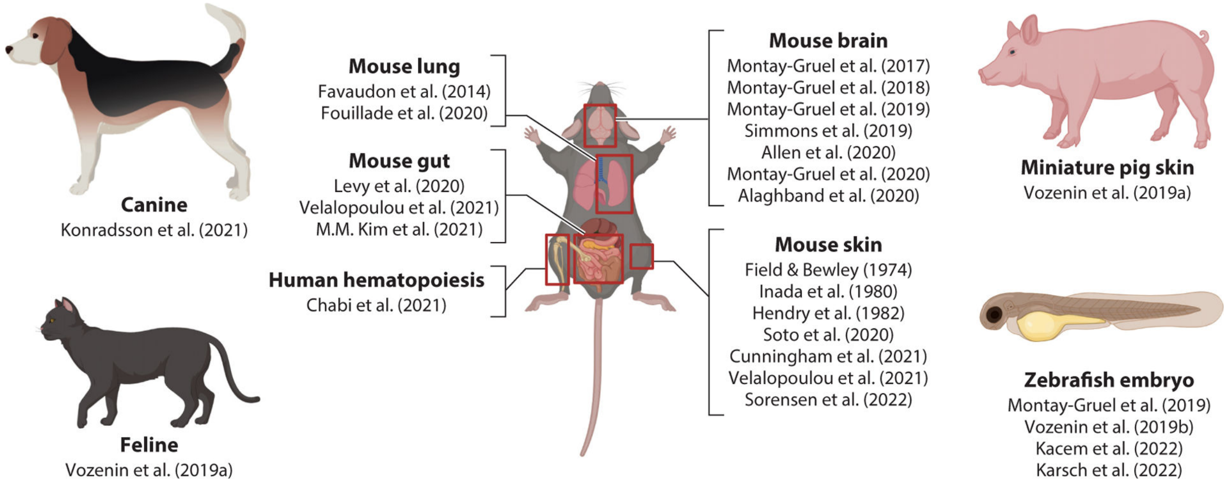

Figure 1.

Normal tissue sparing by FLASH radiotherapy. FLASH radiotherapy provides a unique opportunity to dose escalate while minimizing normal tissue toxicities throughout a variety of normal tissue beds. Reduced normal tissue complications have been found in nearly all normal tissues examined to date, using a variety of preclinical models. Figure adapted from images created with Biorender.com.

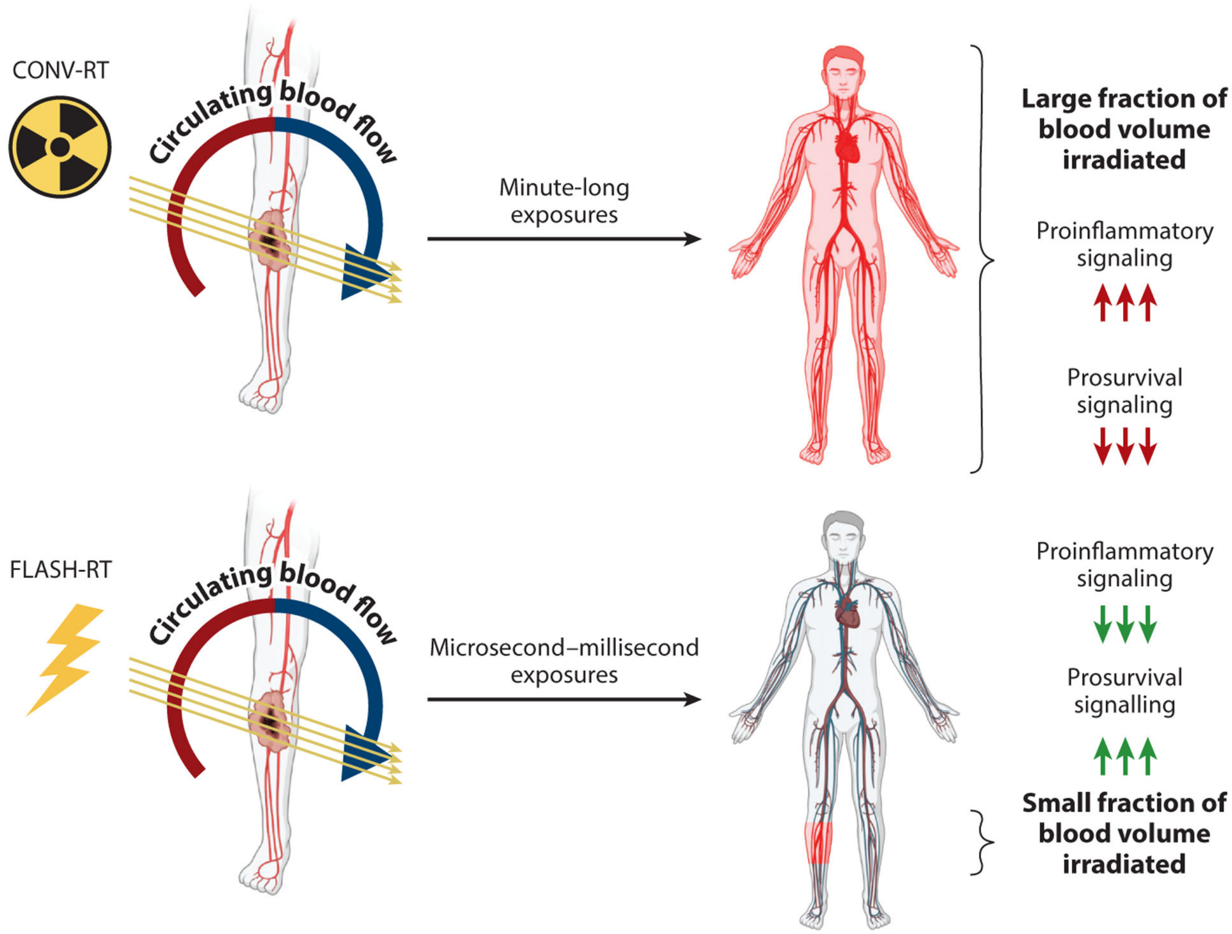

Figure 2.

Blood volume as a target for FLASH radiotherapy (FLASH-RT). (*Top*) Under conventional radiotherapy (CONV-RT), standard dose rates have the potential to expose a significant fraction of the total blood volume as circulation moves blood continuously through the target volume during a prescribed treatment. In this scenario, prolonged exposure of blood cell constituents may maximize proinflammatory signaling or deplete factors important for prosurvival signaling. Certain blood cell constituents or other paracrine mediators such as secreted exosomes are primed to trigger more widespread pathogenic responses. (*Bottom*) The short irradiation times of FLASH-RT minimize the fraction of blood irradiated. Since perfusion transpires on a much shorter timescale, only blood residing at the target volume is exposed, but at a high dose. Resultant inflammatory signals are reduced, and the depletion of prosurvival factors is not as extensive. While this model may account for a certain level of normal tissue sparing after FLASH-RT, it fails to account for isoefficient tumor killing. Figure adapted from images created with Biorender.com.

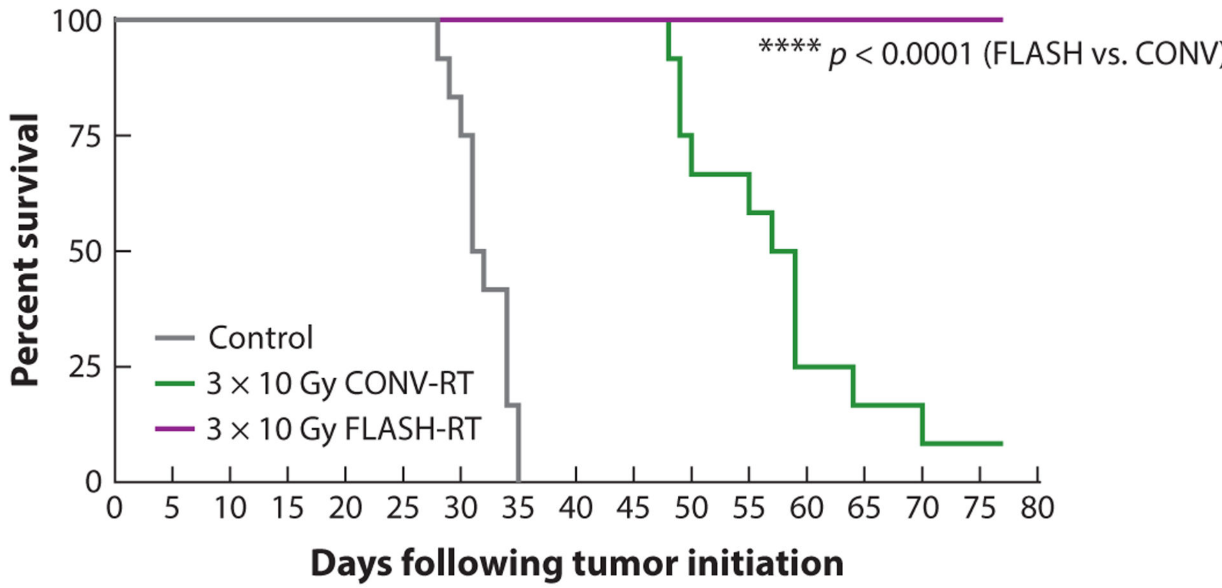

Figure 3.

U87 tumor cure after FLASH radiotherapy (FLASH-RT). Survival curves of U87 glioblastoma tumors orthotopically implanted in the striatum of female nude mice treated with 3 × 10 Gy whole-breast irradiation in 48-h intervals delivered with FLASH-RT or conventional radiotherapy (CONV-RT). There were $N = 12$ animals per group, and p-values were derived from the logrank test (**** $p < 0.0001$ for the FLASH-RT group compared with the CONV-RT group).

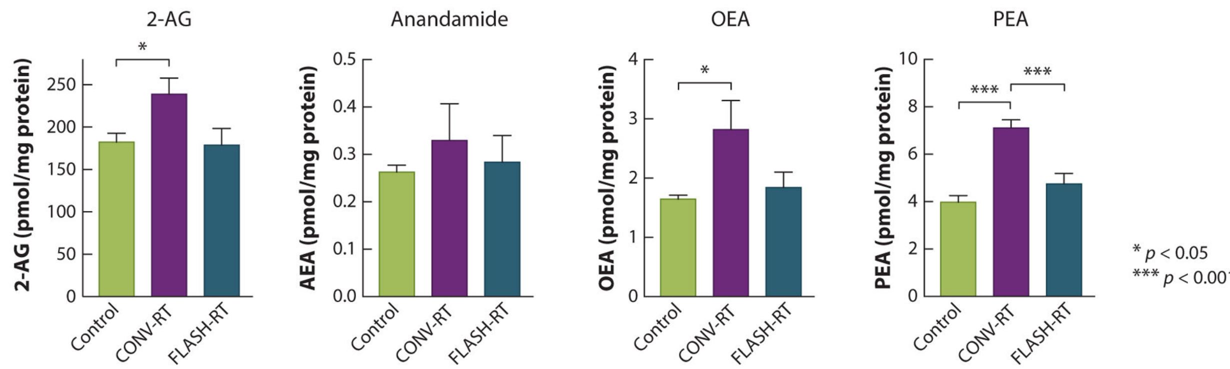

Figure 4.

Quantification of select lipids in the hippocampus of mice after CONV-RT and FLASH-RT. Endocannabinoids and endogenous lipids were analyzed four months after irradiation. The hippocampi of control (0 Gy), CONV-RT (10 Gy), and FLASH-RT (10 Gy) mice (n = 5–6/group) were homogenized in methanol. Lipids were extracted with chloroform and lipid levels were quantified using liquid chromatography tandem mass spectrometry (Agilent 6410 system). Compared to the control group, p-values indicated by asterisks were * $p < 0.05$ for 2-AG and OEA and *** $p < 0.001$ for PEA, and between CONV-RT and FLASH-RT, *** $p < 0.001$ by two-tailed t-test or one-way ANOVA (analysis of variance). Abbreviations: 2-AG, arachidonoylglycerol; CONV-RT, conventional radiotherapy; FLASH-RT, FLASH radiotherapy; OEA, oleoylethanolamide; PEA, palmitoylethanolamide.

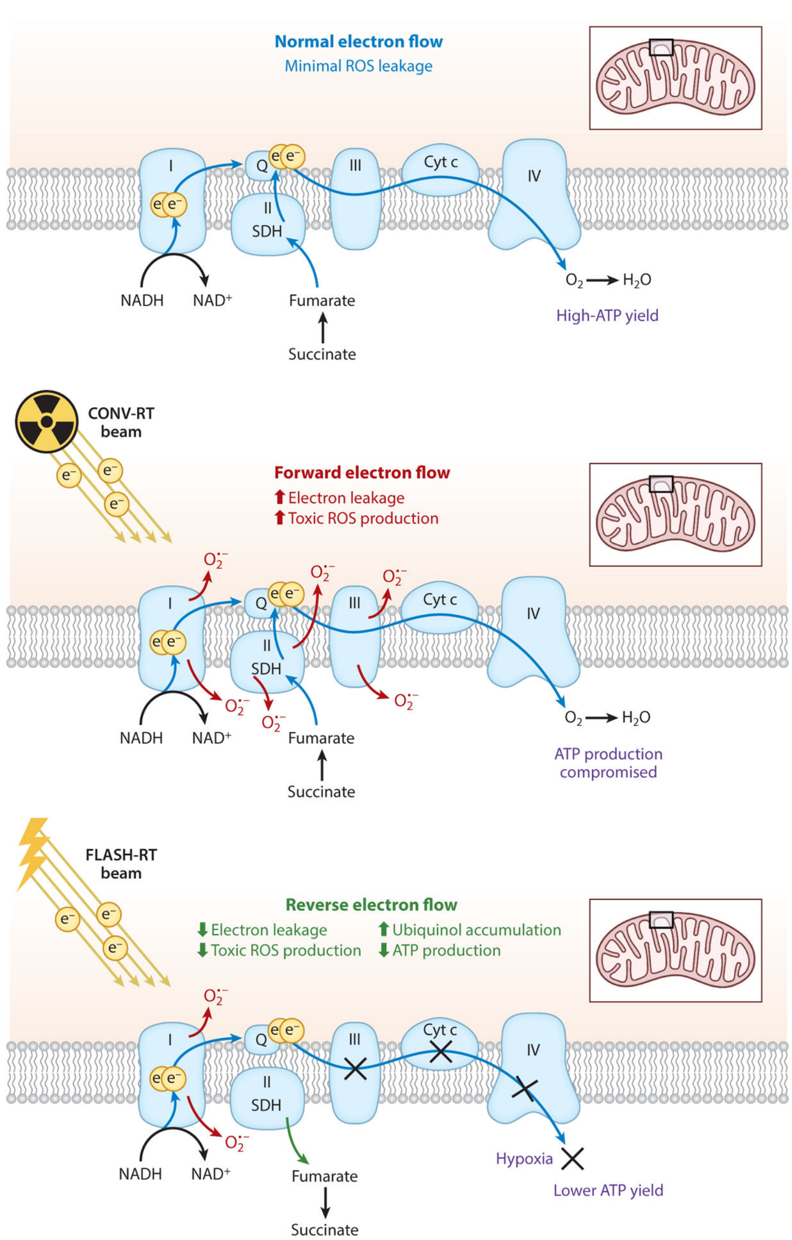

Figure 5.

Reverse electron flow and the FLASH effect. (*Top*) In normal situations mitochondrial OXPHOS mobilizes electrons through a series of electron donor and acceptor subunits embedded in ETCs I–IV. Resultant ATP production is relatively high with a minimal of ROS leakage into the mitochondrial matrix and intermembrane space. (*Middle*) CONV-RT compromises efficient electron transfer through the ETC by a variety of mechanisms, leading to compromised ATP production and elevated ROS production. In this scenario, forward electron flow is maintained with oxygen serving as the principal terminal electron acceptor. (*Bottom*) FLASH-RT saturates the intracellular and mitochondrial milieu with electrons, possibly favoring reverse electron flow where fumarate can act as a terminal electron acceptor from complex II. In this scenario, ATP is also relatively lower, but the production of toxic ROS may be minimized. Tissue hypoxia may promote reverse electron flow, but if or how this possibility might differ between normal tissues and tumors or between different FLASH modalities remains to be elucidated. Figure adapted from images created with Biorender.com. Abbreviations: Cyt c, cytochrome complex; CONV-RT, conventional radiotherapy; ETC, electron transport complex; FLASH-RT, FLASH radiotherapy; NAD^+, nicotinamide adenine dinucleotide; NADH, NAD + hydrogen; $O_2^{\bullet -}$, superoxide; OXPHOS, oxidative phosphorylation; ROS, reactive oxygen species; SDH, succinate dehydrogenase.

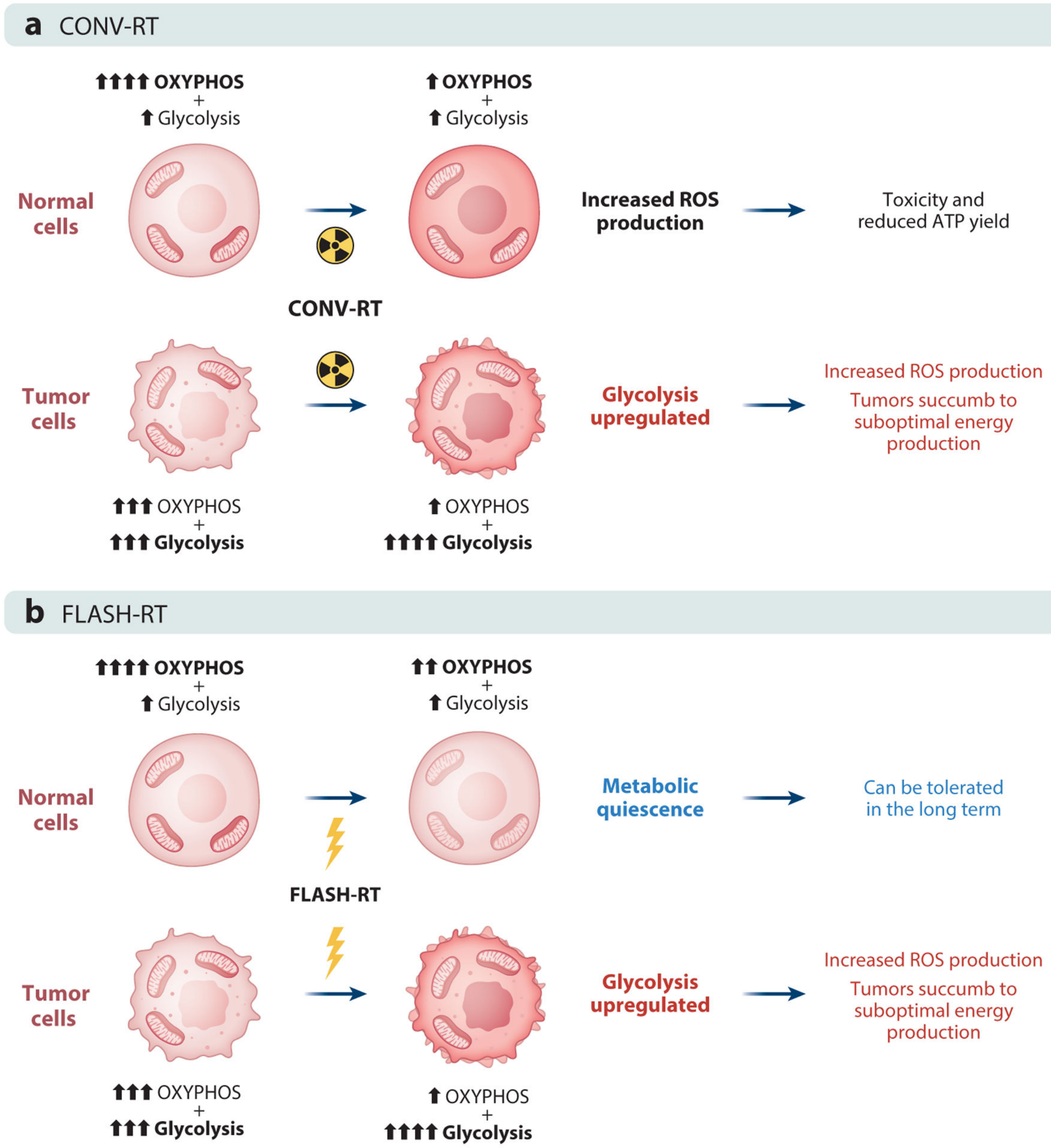

Figure 6.
Metabolic hibernation and the FLASH effect. Normal cells and tumors rely on oxidative phosphorylation (OXPHOS) and glycolysis at different levels to meet energy demands. (*a*) Following conventional radiotherapy (CONV-RT), OXPHOS is uniformly disrupted, leading to an increase in toxic reactive oxygen species (ROS) and an upregulation of glycolysis in tumors. Over the long term, normal tissue toxicity results and tumors succumb to suboptimal energy production and oxidative injury. (*b*) The situation differs following FLASH radiotherapy (FLASH-RT), where tumor cells change little, but for normal tissue reduced OXPHOS activity yields lower levels of ATP but also lower ROS accumulation and reduced oxidative injury. The result is a quiescent state of metabolic hibernation that can be

tolerated over protracted times and minimizes late normal tissue toxicities. Figure adapted from images created with Biorender.com.

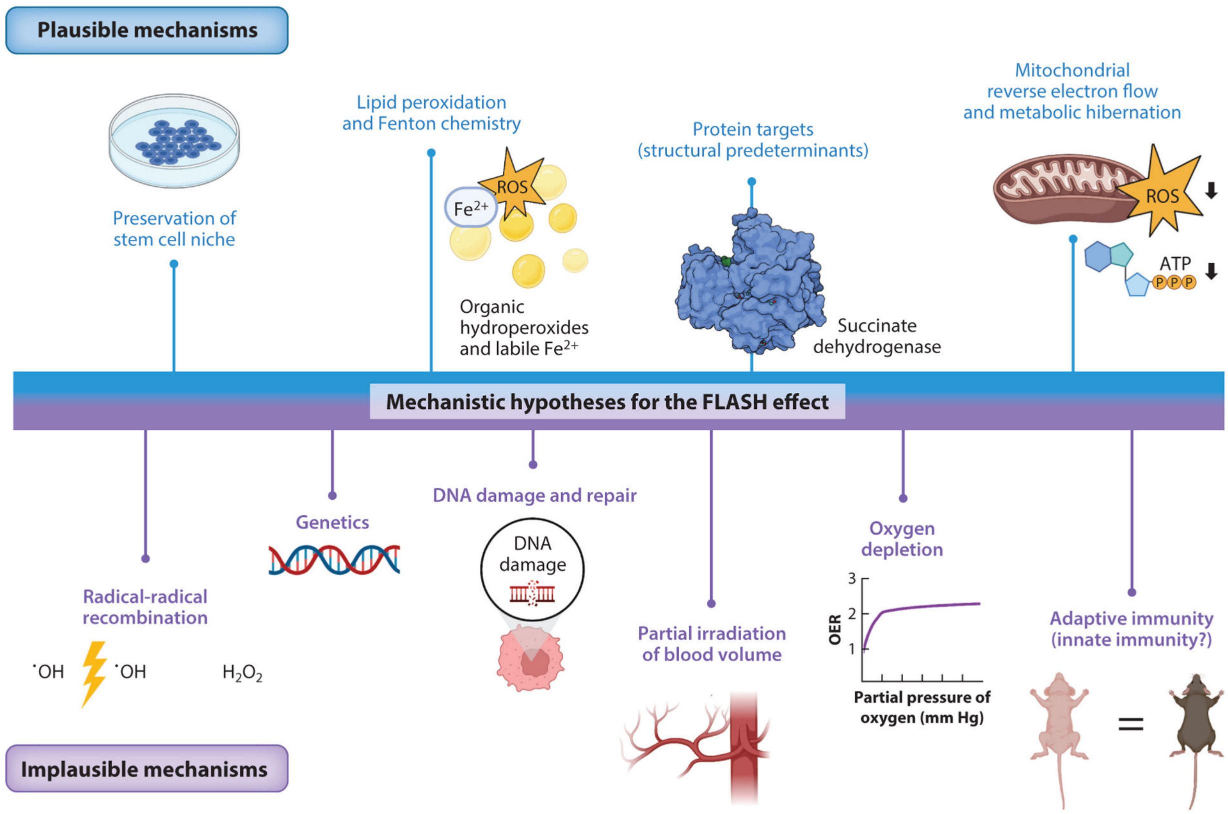

Figure 7.

Mechanistic summary: Why does FLASH kill tumors? (*Top*) Plausible mechanisms that can account for the FLASH effect include stem cell niche preservation, differential lipid peroxidation and Fenton chemistry, structural predeterminants in specific protein classes, and changes in mitochondrial metabolism such as reverse electron flow or metabolic hibernation. (*Bottom*) Implausible mechanisms include those involving radical-radical recombination, genetic predisposition, DNA damage and repair, partial blood volume irradiation, oxygen depletion, and adaptive immunity (not exclusive of innate immunity). Experimental evidence for and against these specific hypotheses varies tremendously, and while certain hypotheses may substantiate normal tissue sparing (i.e., oxygen depletion), few can fully account for both normal tissue sparing and isoeffective tumor killing. As the field advances and evidence accumulates, so too will the evolution of more hypotheses requiring experimental validation. Figure adapted from images created with Biorender.com; succinate dehydrogenate structure is from the Protein Data Bank (PDB ID: 6VAX; https://doi.org/10.2210/pdb6VAX/pdb), rendered with Biorender.com. Abbreviations: OER, oxygen enhancement ratio; ROS, reactive oxygen species.

Table 1

Tumor types subjected to FLASH radiotherapy

Tissue	Species	Tumor type	Radiation modality	Total dose(s) (Gy)	Mean dose rate (Gy/s)	Reference
Blood/lymph	Murine host	Patient-derived xenograft: $CD7^+$ and $CD45^+$ cells (human T-ALL)	Electron (6 MeV)	4	200	Chabi et al. 2021
	Human	Spontaneous: $CD30^+$ T cell cutaneous lymphoma	Electron (5.6 MeV)	15	166	Bourhis et al. 2019
Brain	Murine/murine host	Orthotopic: isogenic H454, human U87 glioblastoma	Electron (6 MeV)	10, 14, 25, 30	$2.5 \times 10^3 - 7.8 \times 10^6$	Montay-Gruel et al. 2021
	Rat	Orthotopic: xenograft NS1 glioblastoma	Electron (10 MeV)	16, 25	66–74	Liljedahl et al. 2022
Breast	Murine	Isogenic: EMT6 breast cancer in BALB/c mice	High-energy X-rays	18	1,000	Gao et al. 2022
	Murine	Isogenic: breast cancer in C3H mice	Proton (250 MeV)	40–60	71–89	Sorensen et al. 2022
	Murine host	Xenograft: HBCx-12A (human breast cancer)	Electron (4.5 MeV)	17	≥40	Favaudon et al. 2014
	Murine host	Xenograft: MDA-MB 231 (human breast cancer)	Electron (10 MeV)	10, 20, 1930	90, 180, 270	Cao et al. 2021
Gut	Murine	Isogenic: flank pancreatic tumor MH641905	Proton (230 MeV)	12, 15, 18	80	Diffenderfer et al. 2020
	Murine	Isogenic: flank pancreatic tumor MH641905	Proton (230 MeV)	18	106–118	M.M. Kim et al. 2021
Head & neck	Murine	Isogenic: oral carcinoma cell line	Proton (250 MeV)	15	50–115	Cunningham et al. 2021
	Murine host	Xenograft: Hep-2 (human head and neck carcinoma)	Electron (4.5 MeV)	15, 20, 25	≥40	Favaudon et al. 2014
	Feline	Spontaneous: squamous cell carcinoma	Electron (5.5 MeV)	25, 27, 28, 31, 34, 41	300	Vozenin et al. 2019a
	Feline	Spontaneous: squamous cell carcinoma	Electron (5.5 MeV)	30	1,500	Rohrer-Bley et al. 2022
Lung	Murine	Orthotopic: isogenic TC-1 (lung carcinoma)	Electron (4.5 MeV)	15, 23, 28	≥40	Favaudon et al. 2014
	Murine	Isogenic: Lewis lung carcinoma	Electron (16 MeV)	15	350	Y.E. Kim et al. 2021
Muscle/bone	Murine	Isogenic: orthotopic and subcutaenous fibrosarcoma	Proton (230 MeV)	12, 30	69–124	Velalоupoulou et al. 2021
	Canine	Spontaneous: osteosarcoma	Proton (230 MeV)	4, 8, 12	103 (range 61–128)	Velaloupoulou et al. 2021
Ovary	Murine	Orthotopic: isogenic ID8 or UPK10	Electron (16 MeV)	14	210	Eggold et al. 2022
	Murine	Orthotopic: isogenic ID8 ovarian cancer	Electron (16 MeV)	14	216	Levy et al. 2020
Various	Canine	Spontaneous: carcinoma, sarcoma, mast cell, melanoma	Electron (10 MeV)	15, 20, 25, 30, 35	430–500	Konradsson et al. 2021

Abbreviation: T-ALL, T cell acute lymphoblastic leukemia.