Radiobiology Experiments With Ultra-high Dose Rate Laser-Driven Protons: Methodology and State-of-the-Art

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The use of particle accelerators in radiotherapy has significantly changed the therapeutic outcomes for many types of solid tumours. In particular, protons are well known for sparing normal tissues and increasing the overall therapeutic index. Recent studies show that normal tissue sparing can be further enhanced through proton delivery at 100 Gy/s and above, in the so-called FLASH regime. This has generated very significant interest in assessing the biological effects of proton pulses delivered at very high dose rates. Laser-accelerated proton beams have unique temporal emission properties, which can be exploited to deliver Gy level doses in single or multiple pulses at dose rates exceeding by many orders of magnitude those currently used in FLASH approaches. An extensive investigation of the radiobiology of laser-driven protons is therefore not only necessary for future clinical application, but also offers the opportunity of accessing yet untested regimes of radiobiology. This paper provides an updated review of the recent progress achieved in ultra-high dose rate radiobiology experiments employing laser-driven protons, including a brief discussion of the relevant methodology and dosimetry approaches.

Keywords: protontherapy, cancer, radiobiology, laser-driven ions, particle accelerator, ultra-high dose rate

INTRODUCTION

Radiotherapy is delivered to over 50% of cancer patients with curative intent for solid localized tumours [1]. Most radiotherapy facilities across the world still rely on high energy photon or electron sources [1]. With recent technological advances, modalities such as Intensity Modulated Radiotherapy (IMRT), Stereotactic Body Radiotherapy (SBRT) or Volumetric Modulated Arc Therapy (VMAT) can conform doses to tumours more precisely than possible a few decades ago sparing the normal tissue to a larger extent. However, the risk of exposure of the surrounding normal tissues remains a concern for patient outcomes [2], with the potential for late tissue damage and escalating the risk of initiating secondary cancers in a patient's lifetime, especially for paediatric patients [3]. Proton therapy has been proposed as the most effective treatment of solid tumours in critical locations including the brain, medulloblastoma and other central nervous system tumours [4, 5]. The dose deposition profile of protons in the form of a "Bragg peak" imparts a unique normal tissue sparing ability [6] while depositing maximal dose within tumours [4, 7]. Due to this higher

normal tissue sparing effect relative to any of the photon based modalities, proton therapy has drawn an increasing interest with globally over 150 proton therapy centres being operational and under construction or at the planning stage [8]. Several clinical trials have confirmed the benefit of proton therapy in patients with localized solid tumours giving enhanced quality of life and better tumour control [9–11].

While proton therapy is clearly envisaged to significantly benefit patients, the underlying operational and construction costs pose a significant obstacle for widespread access to this form of treatment [12–14]. Alternative strategies for ion acceleration to clinically relevant energies with a smaller footprint technology and at reduced costs have been pursued for a long time and in this direction high power lasers have been suggested as a potentially transformative technology (see [15–17] and references within). Thanks to the Chirped Pulse Amplification (CPA) technique, it is now possible to amplify ultra-short laser pulses to the Petawatt level, an approach which led to the award of 2018 Physics Nobel Prize to Strickland and Mourou [18]. Based on the application of CPA, several investigators have demonstrated the generation of high-energy laser-accelerated ions and developed an understanding of their physical properties as well as their upscaling through different approaches potentially enabling radiological and radiobiological application [15, 17, 19, 20]. The unique properties of laser-driven protons [15, 17] include ultra-high field acceleration gradient, high brightness with $\sim 10^{12}$ ions in picosecond-scale bunches, high laminarity, ultra-low emittance and scalable energy cut-off, ultra-short pulse duration, energy-dependent collimation which increases with proton energy. Furthermore, laser acceleration potentially allows controlling the accelerated ion species by changing the target material, as well as producing multispecies ion beams, a capability that could be valuable for mixed field irradiations, currently not possible even with the more advanced RF accelerators.

The vision of laser-driven ion acceleration for hadrontherapy was first proposed by Bulanov et al. [21] and further supported by Fourkal et al. and Malka et al. [22, 23]. This stimulated significant interest in the biomedical application of laser-accelerated ions, and has led to the demonstration of methods for handling and irradiating cell culture models in order to develop a radiobiological understanding of ion irradiations at the ultra-high dose rates deliverable with this acceleration technique (up to $\sim 10^9$ Gray per second (Gy/s)).
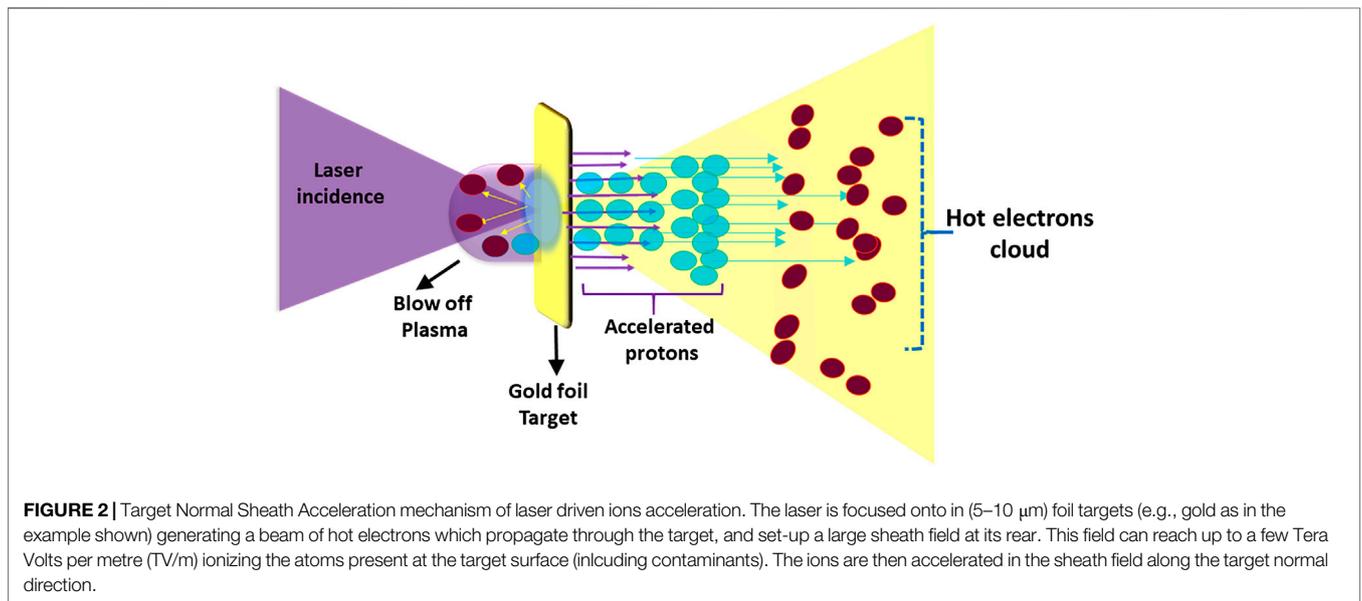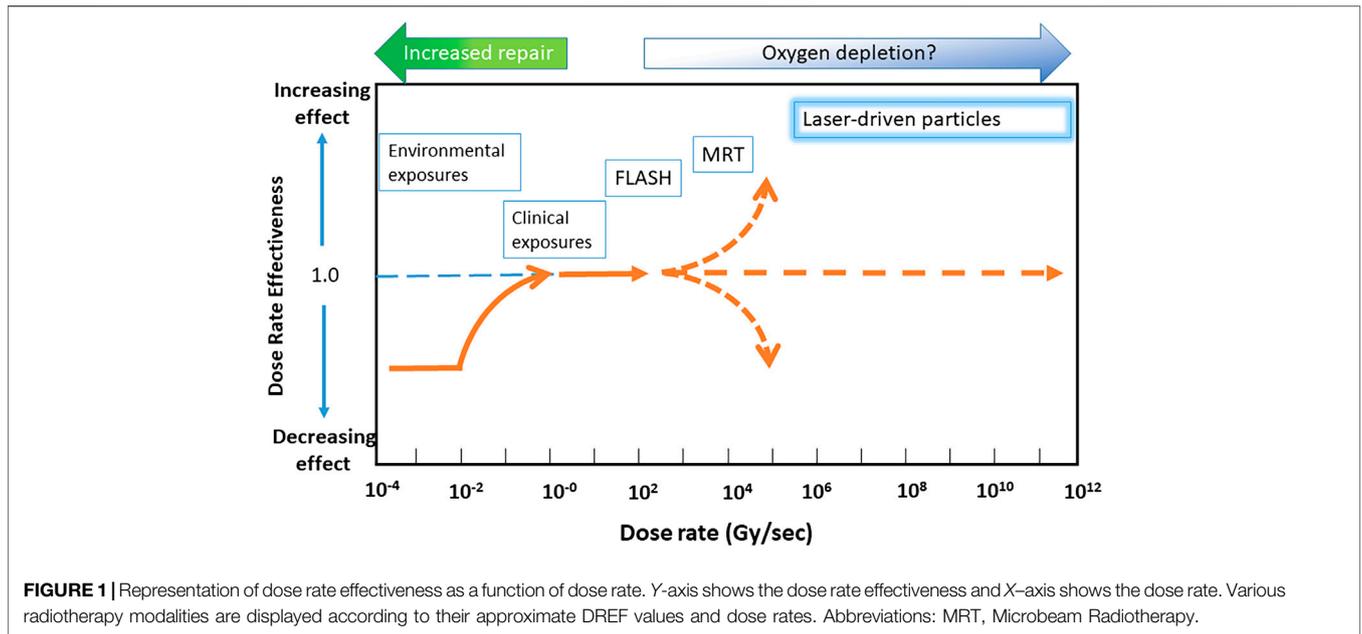
The impact of dose rate on the biological effects and clinical response of radiation exposure has been studied for many years and several clinical modalities such as hypo- and hyper-fractionation have emerged based on the dose rate concept [24, 25]. Hall and Brenner reviewed the clinical importance of dose rate effects about 30 years ago, where a dose rate of 10^{11}–10^{13} cGy/min was specifically defined as ultra-high dose rate [26] and a dose rate of 0.1–0.2 Gy/s was considered as a radiotherapy relevant dose rate or, in other words, a "conventional" dose rate. Since then, these conventional dose rates have been widely used in various modalities of external beam radiotherapy (reviewed by Ling et al. in Ref. 25).

Recently, numerous investigators have used dose rates significantly higher than the conventional ones, often in the context of FLASH radiotherapy where the dose rate usually ranges between 40 and 1000 Gy/s [27–30]. The term "ultra-high dose rate" is often used to indicate these "FLASH" dose rates, which is confusing and can lead to a misinterpretation of ultra-high dose-rate radiobiology. It is important to understand the differences between these various regimes, as the biological effects at each dose rate may involve a different mechanism of action. Recently, Vozenin et al. [31] have emphasized the importance of properly reporting beam parameters for the characterization of the biological effects of FLASH irradiations. They also point out that not all irradiations at ultra-high dose rate may lead to FLASH effects and thus to understand the differences between the two regimes is important. FLASH dose rate effects have been shown to involve the differential response of normal and cancer cells, in the removal and decay of hydroperoxide and other free radicals [32] and oxygen saturation during the irradiation mainly observed *in-vivo* [33]. This results in significant sparing of the normal cells and thus provides a larger therapeutic window compared to conventional dose rate irradiation [34]. Durante et al. [35] introduced a Dose Rate Effectiveness Factor (DREF), and defined the radiobiological effectiveness of exposures ranging from low to Flash dose rates. However the behaviour of DREF at ultra-high dose rates of 10^9–10^{10} Gy/s has not yet been clearly defined, as indicated in **Figure 1**. In order to capitalize on any future radiobiological applications of laser-driven protons it is imperative to properly understand the effects arising at such dose rates. This manuscript aims to present an update on recent radiobiological research in this area with an overview of the methodology, mechanisms, dosimetry approaches and radiobiological assays employed to study the radiobiology of laser-accelerated protons.

LASER-DRIVEN PROTON ACCELERATION

Research on the acceleration of ions by ultra-intense laser pulses has been pursued over the last 2 decades, and has driven continual progress in delivering higher laser intensities on target, creating new target types and improving diagnostic systems underpinning new scientific results.

Research on the acceleration of ions by ultra-intense laser pulses is mostly based on interactions with solid targets. In a typical experimental setting, as shown in **Figure 2**, an intense laser pulse, typically at intensities above 10^{19} W/cm^2, interacts with a thin foil with thickness in the order of µms to 10s of µm and, during this interaction, a significant amount of laser energy is transferred to a population of relativistic electrons. These energetic electrons propagate through the target and create a strong sheath electric field at its rear surface, leading to the acceleration of protons originally present as impurities on the target surfaces. This Target Normal Sheath Acceleration (TNSA) process [36], is currently the most explored and robust mechanism of laser-driven proton acceleration [15, 17, 20, 37, 38]. Nevertheless, further improvements in cut-off energy, spectral and angular properties and repetition rate are

FIGURE 1 | Representation of dose rate effectiveness as a function of dose rate. Y-axis shows the dose rate effectiveness and X–axis shows the dose rate. Various radiotherapy modalities are displayed according to their approximate DREF values and dose rates. Abbreviations: MRT, Microbeam Radiotherapy.

FIGURE 2 | Target Normal Sheath Acceleration mechanism of laser driven ions acceleration. The laser is focused onto in (5–10 μm) foil targets (e.g., gold as in the example shown) generating a beam of hot electrons which propagate through the target, and set-up a large sheath field at its rear. This field can reach up to a few Tera Volts per metre (TV/m) ionizing the atoms present at the target surface (inlcuding contaminants). The ions are then accelerated in the sheath field along the target normal direction.

required for effective application of laser-accelerated beams. For instance, applications in cancer therapy would require the delivery of high-energy protons (60–250 MeV) with a narrow energy spread and sufficient particle flux at appropriate distances from the interaction targets, so that any extraneous radiation produced during the intense laser interaction can be shielded adequately.

While control of TNSA beams has been a topic of active research over the last decade, significant attention has also been devoted to the exploration of a number of new acceleration mechanisms, including, for example, radiation pressure

acceleration (RPA), which can in principle, produce beams with smaller divergence and narrow energy spread, particularly in the Light-Sail (LS) regime [15, 39]. RPA approaches are particularly suited to the acceleration of bulk ions in the irradiated target (e.g., carbon ions as opposed to contaminant protons [40]). There has recently been significant progress in increasing the maximum proton energies delivered through laser-driven processes, with reports of the acceleration of near 100 MeV protons by hybrid acceleration schemes [41] where acceleration is initiated by the TNSA mechanism followed by RPA until the target becomes transparent, with a

FIGURE 3 | Experimental set up demonstrating laser driven proton acceleration. **(A)** Schematic of the arrangement of the target, aperture, magnet, beam exit and cells. **(B)** Actual image showing the laser target interaction point setup along with the beam exit and sample irradiation position. **(C)** Overview of the Laser Interaction chamber of the Vulcan Target Area Pettawatt Laser of the Central Laser Facility at the Rutherford Appleton laboratory, Didcot, Oxford, England.

further energy boost provided in a relativistic transparency regime.

Main Centres Involved in the Radiobiology Study of Laser-Driven Proton

Motivated by its potential applications in research, technology and medicine, laser-driven proton acceleration research has gained significant support from funding agencies and academia in many countries. A number of laboratories across Europe have a specific interest and focus on the radiobiological and clinical applications of laser-driven proton. In Germany, Ludwig Maximilian University (LMU) Munich (currently starting activities at the new Centre for Advanced Laser Applications, CALA) and Helmholtz-Zentrum Dresden-Rossendorf (HZDR) have been very actively involved in radiobiological applications of laser-driven protons and several publications have resulted from the work performed at these facilities (discussed later). Investigators in France, are also actively engaged in radiobiology research with laser-driven beams, particularly at Laboratoire d'Optique (LOA), Palaiseau [42, 43]. A focus for future activities in this area across Europe will be at the facilities of the Extreme Light Infrastructure (ELI), particularly at ELI Beamlines (Czech Republic), where the Extreme Light Infrastructure Multidisciplinary Applications of Laser-Ion Acceleration (ELIMAIA) beam lines in Prague are being commissioned [44]. ELI Nuclear Physics (ELI NP) Romania, is also planning an involvement in laser-driven ion radiobiology research [45]. In the United Kingdom, the main facilities used for this research are located at the Central Laser Facility of the Rutherford Appleton Laboratory (RAL) (GEMINI and VULCAN lasers), the University of Strathclyde (SCAPA) and Queen's University Belfast (TARANIS), with activities in this area carried out so far within the EPSRC-funded A-SAIL consortium. In Asia, pioneering activities in this area were carried out in Japan

at APRC-JAEA (now QST) [46, 47], while a proton beamline for radiobiology applications (CLAPA) has recently been developed at Peking University [48].

Typical Set Up for Laser-Driven Proton Irradiation

An example of an arrangement for radiobiology experiments employing laser-driven protons is shown in **Figures 3A–C** (based on the set-up employed for several experiments on the VULCAN and ASTRA GEMINI laser systems [49, 50]). Amplified laser light enters the evacuated interaction chamber and, after reflection from a plasma mirror (used for contrast enhancement), the laser is focused within a spot of a few microns onto a thin target, as shown in **Figure 3A**. The ions accelerated from the target are spatially selected by a narrow slit (of a few hundred μm) mounted in front of a ~1 T magnet (**Figure 3B**), which deflects the positively charged protons and other ions at an angle from the target normal separating them from other emitted radiation (electrons, X-rays). The deflected ions finally pass through a 50-200 μm-thick Kapton exit window (**Figure 3C**) and irradiate the cell sample positioned immediately after the window inside a sample holder. The sample holder is often a stainless steel dish covered with a thin Mylar foil (0.9–4 μm) which allows the ions to pass through with minimal beam attenuation, even at moderate energies.

DIAGNOSTICS AND DOSIMETRY

The monitoring of laser-accelerated ion parameters, such as beam composition, energy spectrum and dose distribution at the irradiation point is fundamental to achieving a controlled and accurate cell irradiation. Bolton et al. have provided an extensive

update on the instrumentation for diagnosis and control of laser-accelerated proton beams in Ref. 51. The beam characteristics are typically diagnosed using a range of techniques and detectors such as Thomson Parabola Spectrometers (TPS), Radiochromic Films (RCF) and Time of Flight (TOF) detectors [51]. The TPS and the RCF are usually placed along the target-normal direction to measure the higher-energy component and used for the full beam characterization in advance of the cell irradiations. In contrast, TOF detectors (typically diamond and silicon carbide) [52, 53] can be placed at various angles both in the forward and backward directions to monitor the particle flux and energy on-line during the irradiation, and detect any shot-to-shot variations [54]. A crucial task in view of meaningful radiobiological investigations is a precise measurement of the dose delivered to the cells. For conventional clinical beams, since the 2000s, a protocol for proton/ion dose measurement has been established where ionization chambers and calorimeters are defined as the absolute reference dosimeters [55]. In contrast, novel features of laser-driven protons such as ultra-high dose-rate, short pulse duration, as well as the presence of large electromagnetic pulses [56] generated by the laser-target interaction, require the development of new approaches and protocols for dose measurements [57].

The use of ionization chambers with pulsed beams at high dose rates is complicated due to an increase in ion recombination. Several authors have measured correction factors for Markus or Roos chambers developing new calibration procedures and models for both electrons and protons under different conditions relevant to FLASH radiotherapy [58–60]. They mainly used radiofrequency cavity (RF) accelerated protons up to 26 Gy/min and electrons up to 10^7 Gy/s. Extending the use of ionization chambers as absolute dosimeters for laser-driven protons at dose-rates near 10^9 Gy/s and at therapeutic doses (1–10 Gy) delivered per pulse, as typical in single-shot radiobiology experiments, is however a significant challenge, as it requires a large correction factor for the ion collection efficiency, which may affect the reliability of the dose measurement, as pointed out by McManus et al. [59].

The use of Faraday cups as suitable online dosimeters for laser-driven protons has also been proposed, e.g., by Richter et al. [61], who developed an integrated dosimetry and cell irradiation system (IDOCIS) that had about 13% dose uncertainty based on the quadratic summation of various contributing factors. An absolute dosimetry approach based on the use of Faraday cups is also being pursued on the ELIMED beamline at ELI Beamlines [62, 63].

In the attempt to further reduce dose uncertainties with laser-driven protons, an alternative approach employing graphite calorimeters as absolute dosimeters for laser-driven protons is under investigation at the National Physical Laboratory (NPL) in the framework of the European- Joint Research Project "UHDPulse" [57], aimed at establishing protocols and procedures for the dosimetry of ultra-high-pulse electron and proton beams [57, 64]. The measurement of the temperature increase in the calorimeter core upon irradiation is a direct measurement of the energy and dose deposited [65]. A first proof-of-principle experiment employing the high-energy

laser-driven protons accelerated with the Vulcan PW laser at Rutherford Appleton Laboratory (RAL) has been reported in Ref. 66.

The use of passive detectors such as radiochromic films (Gafchromic EBT2 or EBT3), previously calibrated with beams generated by RF accelerators is a simple and widespread technique for dose measurements in laser-based radiobiology experiments [67, 68]. A single layer of the film can be used for 2D (transverse) monitoring of the proton dose, while stacks of multiple layers can be used for 3D dose mapping and reconstruction of the local proton spectrum by using deconvolution procedures. In a set-up as shown in **Figure 3A**, depending on the energy of the protons and cell dish arrangement, the RCF films can be placed either behind or in front of the cell dish. On a single-shot radiobiology experiment, this provides shot-to-shot monitoring of the dose, although this is available only after scanning of the film, using a suitably calibrated scanner. Recently RCF dosimetry (employing customized, unlaminated EBT3 films) has also been demonstrated with laser-accelerated carbon ions [50].

Real time dose monitoring during an irradiation is also crucial for an accurate characterization of the radiobiological effects of ultra-high dose rate irradiations. In radiobiology experiments at conventional accelerators, large area transmission ionization chambers are typically calibrated against the absolute dosimeter and then used as current monitors during the irradiation. In a laser-driven context, this is particularly important in multi-shot radiobiological experiments (as well as in future *in-vivo* experiments) to control the dose delivered in every single laser shot. Commercially available standard transmission ionization chambers have been used to monitor dose stability and reproducibility, e.g., on experiments on the Draco laser facility at HZDR, where standard transmission chambers cross-calibrated against radiochromic film at the cell irradiation position have been employed effectively (as described in Refs. 69 and 70). Modified transmission ionization chambers based on a double-gap structure [63] have been developed for dose monitoring along the ELIMED beam line, where the second gap is used to quantify, on each shot, the ion recombination occurring in the first gap at high dose-rates and to correct the collection efficiency.

Other approaches based on modified ionization chambers, such as the DOSION beam monitoring equipment [71], have been applied successfully in FLASH regimes [57], and may have scope for application with laser-driven sources. Several real-time approaches employing volumetric scintillation detectors [72], fibre optic dosimeters [73, 74], radioluminescence and Cherenkov emission based dosimeters have also been used for conventional dose rate proton beams as well as FLASH protons. In a laser-driven context, the use of scintillator blocks for approximated online monitoring of the lateral and depth dose distribution of the proton beam has been reported in Ref. 70, while approaches based on optical fibre arrays have not yet been applied to laser-driven protons. Finally, an approach for depth dose reconstruction employing acoustic traces generated by laser-driven protons stopping in a water phantom has been demonstrated and reported in Ref. 75.
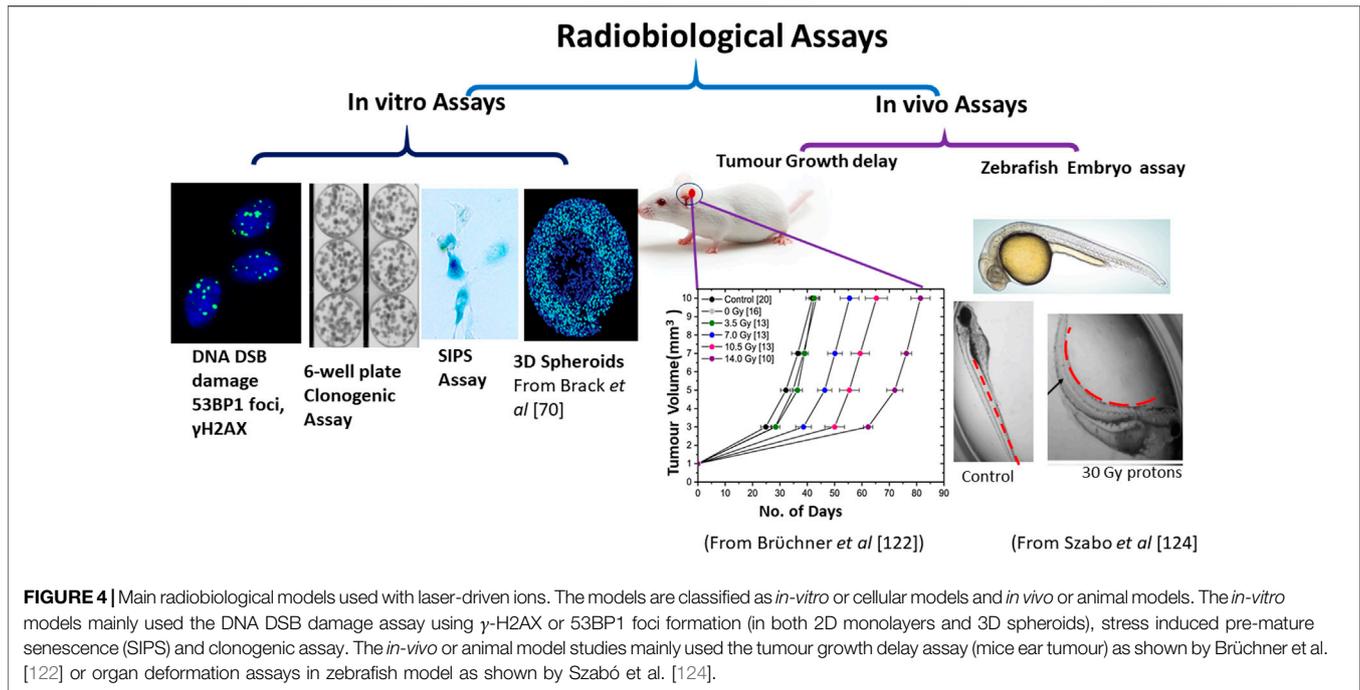
FIGURE 4 | Main radiobiological models used with laser-driven ions. The models are classified as *in-vitro* or cellular models and *in vivo* or animal models. The *in-vitro* models mainly used the DNA DSB damage assay using γ-H2AX or 53BP1 foci formation (in both 2D monolayers and 3D spheroids), stress induced pre-mature senescence (SIPS) and clonogenic assay. The *in-vivo* or animal model studies mainly used the tumour growth delay assay (mice ear tumour) as shown by Brüchner et al. [122] or organ deformation assays in zebrafish model as shown by Szabó et al. [124].

RADIOBIOLOGICAL APPROACHES

Radiation effects were first observed as a function of dose rate in the 1950s by Brasch et al. [76]. In 1969, Prempree et al. [77] studied the effects on the repair time of chromosome breaks induced by X-rays delivered at a dose rate of 4.5×10^8 Gy/s. Later, Berry in 1972–73 compared the effects of radiation dose rate from protracted, continuous irradiation to ultra-high dose rates of 10^9 Gy/s from pulsed accelerators [78, 79], noticed some non-predictable effects and discussed the impact of these dose rates on responses under hypoxia. Weiss et al. [80] demonstrated the depletion of oxygen in mammalian cells after ultra-high dose rate exposure. In all these studies, pulsed electron beams were mainly used and the pulse duration was of the order of a microsecond to a nanosecond. It was another two to 3 decades before studies were possible with laser-driven protons at comparable dose rates. During the early years of laser-driven proton radiobiology research, most experiments relied on multi-shot dose delivery, where several shots were required to deliver Gy level doses, and detect biological changes [81, 82]. Yogo et al. [81] delivered 200 shots to deposit a dose of 20 Gy in A549 lung adenocarcinoma cells that translates to an effective dose rate of 0.1 Gy/s with a single bunch dose rate of 1×10^7 Gy/s. Similarly, Kraft et al. [82] irradiated SKX squamous carcinoma cells using 12, 20, and 29 pulses, respectively, for low (1.5 Gy), medium (2.7 Gy) and high (4.1 Gy) doses of laser driven protons, demonstrating the feasibility of irradiating human cells with laser driven ions. These studies all employed femtosecond Titanium: Sapphire systems with limited energy per pulse, but were able to operate at sufficiently high repetition rates to make these multiple shot irradiations feasible.

A different approach, used by other groups, has been that of delivering Gy level doses in a single short burst of ~ns duration, therefore reaching dose rates of ~10^9 Gy/s. This has been possible on higher energy systems, typically delivering 10s–100s J per pulse [49, 83] or by focusing tightly the protons into a small spot [84]. Arguably, the approach employing single-shot irradiation, where the dose is not averaged over time, could in principle be better suited to test any biological effect related to the ultra-high dose rate.

The radiobiological assays that were applied in the above studies and also used more recently in laser-driven proton radiobiology research are briefly summarised in **Figure 4**, which includes endpoints for both the lethal and sub-lethal effects of radiation described in the following sub-sections.

Cell Based *In-Vitro* Studies
DNA Double Strand Break (DSB) Damage
The first experiments aimed at understanding the radiobiological effects of laser-driven protons quantified the yields of DNA damage in the form of DNA DSB using the foci formation assay. This assay uses immunofluorescent detection of the phosphorylated form (γ-H2AX) of the histone variant H2AX. The phosphorylation of the serine 139 amino acid residue on the H2AX protein is an early cellular response to DNA double strand break damage. The role of γ-H2AX in the formation of DNA DSB was first proposed by Rogakou et al. [85]. Sedelnikova et al. [86] found a direct correlation between the number of γ-H2AX foci and the number of radioiodine decay induced DNA DSBs providing a quantitative relationship for DNA DSB damage in human cell lines. Since then the γ-H2AX assay has been widely used in radiobiology and for over a decade has been an important biomarker of radiation induced DNA DSB damage from various

type of radiations including low linear energy transfer (LET) X-rays [87], protons and high LET ions such as silicon, iron [88] and carbon ions [89, 90]. Subsequently, additional biomarkers related to γ-H2AX were also identified which phosphorylate in the close proximity of DNA DSB. Among these, the-p53 binding protein-1(53BP1) protein which recruits to the nuclear structures at the site of DNA damage and forms readily visualized ionizing radiation induced foci [91, 92] showed a good correlation with γ-H2AX [93, 94]. This has been used by numerous investigators, including in our own work quantifying proton induced DNA DSB damage in human cells [95].

Laser-driven proton-induced DNA DSB damage in mammalian cells were first detected by using the γ-H2AX foci formation assay by Yogo et al. [81] in A549 lung adenocarcinoma cells monolayers irradiated with 2.4 MeV protons delivered in multiple 15 nanosecond pulses. Using a similar set up for cell handling Kraft et al. [82] also used a multi-shot approach to study DNA DSB damage induced by variable doses. In their study, Kraft et al. reached a dose rate of 4×10^7 Gy/s and irradiated cells with doses ranging from 1.4–4.5 Gy in about 12–29 pulses. Bin et al. [84] used a single shot approach and showed the induction of γ-H2AX foci in cervical cancer cells (Hela cells) exposed to 1 Gy dose of 4.45 MeV protons. In this study, the proton beam was generated using nanometre-thin, diamond-like carbon targets and delivered at dose rates in the range of 10^9–10^{10} Gy/s, reporting 7 Gy as the highest dose [84]. Overall, in these studies, laser-driven protons induced more DNA DSB damage than conventional X-rays but no comparison with cyclotron-accelerated protons, delivered at conventional dose-rates, was made.

Such a comparison was first made by Zeil et al. [96] who compared the yields of γ-H2AX foci induced by laser-driven protons to conventionally accelerated protons and found no significant differences. A few years later Raschke et al. [97] also found that laser-accelerated protons induce similar DSB damage to that induced by cyclotron-accelerated protons measured by γ-H2AX foci formation. In both of these studies, multi-shot irradiation methodology was used to deliver the desired doses.

Our group at QUB also performed single shot irradiation of human skin fibroblast cells (AG01522B) with 10 MeV laser-accelerated protons at a dose rate of 10^9 Gy/s. We compared the 53BP1 foci per cell per track induced by the laser driven ions to results obtained with cyclotron-accelerated protons at the same energy but at a dose rate of 0.06 Gy/s and also, in this case, did not observe any significant differences between the two dose-rates [49].

Clonogenic Cell Survival

The clonogenic assay has been used since the 1950s to study the radiation survival dose-response relationship for mammalian cell lines. Markus and Puck were the first to develop this assay [98] and since then it is considered a gold standard in radiation biology. Using this assay, the relative biological effectiveness (RBE), which is defined as the ratio of the doses required to cause the same biological effect using protons or other ions [99, 100] compared to reference X-ray radiation, can be calculated.

RBE has contributed to a better understanding of radiotherapy dose treatment plans by providing the modeling parameters for various cell lines derived from tumour and normal tissues. Yogo et al. [46] for the first time used the clonogenic assay to determine the RBE of laser-driven protons using human salivary gland tumour cells and reported it as 1.2 ± 0.11 with reference to X-rays. In this study, the cells were irradiated with 2.25 MeV protons with a single bunch dose rate of 10^7 Gy/s. The cumulative doses were deposited using a multi-shot approach reducing the effective dose rate to 0.2 Gy/s on the cells. Later Doria et al. [83] also reported the cell killing effects of laser driven protons at a dose rate exceeding 10^9 Gy/s in V79 cells. This was the first study that employed the single shot delivery of 1–5 MeV protons at Gy level doses. Doses as high as 5 Gy were delivered in single pulses of picosecond duration. In this study an RBE of 1.4 ± 0.2 at a surviving fraction of 10% was reported.

Zeil et al. [96] compared the cell killing ability of pulsed laser-driven protons to a continuous proton beam when the squamous cell carcinoma cell line (SKX) was irradiated with energies above 6.5 MeV at a peak dose rate of 4×10^7 Gy/s. The authors did not explicitly quantify the RBE value in this paper, although the cell killing effects of the laser driven protons were reported to be similar to those delivered continuously from a Tandem accelerator at conventional dose-rates. Overall, based on the results from these papers, the RBE values for laser-driven protons ranged between 1.2 and 1.4, similar to those reported for RF-accelerated protons in the same energy range.

In summary, none of the studies discussed above highlighted significant deviations in biological response associated with the use of ultra-high dose rates provided by a laser-driven approach. Only recently, Bayart et al. [43] from the LOA group in France have reported that temporal delivery of laser-driven protons at an ultra-high dose rate of 1.5×10^8 Gy/s, may change the biological response of the human cells to synthetic lethality or cell killing induced by inhibiting the DNA damage response protein, PARP-1.

Several investigators have suggested that ultra-high dose rate exposure with electrons leads to oxygen depletion, inducing radioresistance in cells by reducing the reactive oxygen species-mediated indirect DNA DSB damage [33, 80]. When normoxic cells were irradiated with ultra-high dose rate electrons an increase in cell survival was noticed. However, there is limited information available on the response of hypoxic cells irradiated at ultra-high dose rates with protons, e.g., whether any additional DNA DSB induced are additive or synergistic in nature. We have tested this hypothesis experimentally by irradiating normal human skin fibroblast cells under hypoxic as well as normoxic conditions and we detected a significant increase in residual DNA DSB damage in hypoxic cells at 24 h post irradiation compared to the normoxic cells [101].

Sub-lethal Effects

Although a key advantage of hadrontherapy is that healthy tissues can be spared much more effectively than in X-ray radiotherapy, the sub-lethal effects induced by low dose deposition in the healthy cells surrounding a solid tumour may eventually result in the initiation of secondary cancers or other complications and

are arguably as important as lethal effects for an effective radiotherapy treatment outcome. Sub-lethal effects have been demonstrated in the promotion of migration and invasion in brain tumours and liver cancer [102, 103]. Among various sub-lethal effects, cell senescence has been studied for a long time [104] and has been associated with an onset of inflammation [105], secondary tumours and cancer relapse [106] in patients undergoing radiotherapy [107–109]. Stress induced pre-mature senescence (SIPS), a process in which a sub-lethally damaged cell enters a permanent state of inactivity, has attracted interest for its possible long-term health implications after radiation exposures [110, 111]. It is different from the phenomenon of *in-vitro* replicative senescence, where a cell loses its ability to proliferate after a finite number of cell divisions. Normal tissue sparing and differential sub-lethal effects induction have been reported in several FLASH radiotherapy studies, and highlighted as the main advantage of this approach (e.g. in Ref. 30). Investigating whether these advantages extend to the ultra-high dose regime delivered by laser-driven ions is one of the current research objectives of laser-driven radiobiology. Manti et al. [112] irradiated human vein endothelial cells with laser-driven protons and found that laser-driven proton beams, at a dose rate of 10^9 Gy/s, yielded a higher number of senescent cells at quasi therapeutic doses, while causing a lower percentage of cells to enter pre-mature senescence at doses typical of healthy tissues. This work therefore provided indication of sparing effects at ultra-high dose rate. Raschke et al. [97] have shown that, while laser-driven protons induce similar levels of DNA DSB damage to cyclotron–accelerated protons, the levels of protein nitroxidation (a marker of non-targeted effects) as studied through 3- nitrotyrosine generation was lower with laser-driven proton as compared to cyclotron-accelerated protons or X-rays.

3D Cell Culture Models

3D spherical or organoid model systems have been suggested as a link between cellular *in-vitro* models and *in vivo* animal tumours [113] as they recapitulate cancer drug effects more closely to the effects observed in animal models [114, 115] and especially in Glioblastoma tumours [116, 117]. These 3D neurosphere models are highly relevant for laser-driven ion studies as the current energies achieved with laser driven ions may not be suitable for the uniform irradiation of mouse *in-vivo* tumours which have dimensions of a few millimetres to a centimetre. In contrast, these 3D spheres are about 500–700 μm (1 week after seeding) in diameter which enables complete irradiation of these neurospheres using laser driven ions beams with energies above 20 MeV. Recently, Brack et al. [70] have demonstrated the uniform irradiation of 3D spheroids of human tongue cancer cells with a solenoid focused laser-driven proton beam. They evaluated the impact of laser-driven protons on DNA DSB damage in 3D spheroids after a total deposited dose of 15.3 Gy delivered in multiple shots.

Animal Based *In-Vivo* Studies

Mice have been used in many biological studies ranging from pharmacology, drug development, and as disease models [118]. Radiation oncology has also benefitted from pre-clinical studies using the large variety of genetically modified mouse models [119, 120]. Using conventionally accelerated protons or external beam radiotherapy for *in vivo* experiments is however much easier than doing similar experiments with laser-driven proton beams since, as mentioned earlier, the beam energy attained with laser-driven protons is generally not yet sufficient to fully irradiate deep-seated tumours with the required doses.

Due to this limitation, one has to carefully choose the type of tumour suitable for studies with laser driven ions. A mouse ear tumor model has been recently proposed, which has been proven suitable for experiments with ~20 MeV protons [121]. Laser-driven electrons have been used to irradiate the mouse ear tumour model and the tumour growth delay after irradiation was found comparable to X-ray induced growth delay [122]. To date no mouse based *in-vivo* irradiation has been reported with laser-driven protons, although they can be readily used with the above-mentioned models. The only published evidence of *in-vivo* research comes from a recent study published by Rösch et al. [123]. Due to a good match of the beam spot dimensions of laser-driven protons and the body size of zebrafish embryos, they can be potentially used as suitable *in vivo* models. 24 h after fertilization, these embryos can be easily handled, irradiated, and monitored for various radiobiological endpoints. One of the most accessible endpoints is the shape of the embryo's spine; whereas the un-irradiated controls are lengthy and have a straight spine, their irradiated counterparts are shorter in length and show a curved spine along with pericardial swelling and inhibition of yolk sac resorption (as described by Szabó et al. [124]).

CONCLUSION

While hadrontherapy was highlighted as a key application for laser-driven protons at an early stage of the development of laser acceleration, it is clear that direct application of laser-driven beams remains challenging, and significant progress is still needed to match the parameters required for clinical particle therapy. Efforts to upscale the energies, control the dose and improve the reliability of beam production are continuing, and there has been significant recent progress especially in developing advanced acceleration mechanisms, new techniques for beam transport and delivery, as well as novel beam diagnostics and dosimetry approaches. While further progress is being pursued, alternative applications of laser-driven protons can be exploited [125]. The answer to whether laser-driven protons at ultra-high dose rate can elicit a different biological response than conventional dose rate protons is still uncertain, but emerging indications do warrant continuation of this research, coupled to improvements in the dose delivery, dosimetry and energy range investigated. Although the maximum observed energy of laser-driven protons have recently approached 100 MeV, significant doses allowing radiobiology investigations have only been demonstrated up to ~30 MeV. RF-accelerated proton beams at such energies may not have robust dosimetry, as the primary beam is typically degraded, which introduces uncertainties in the beam composition and range, and makes accurate comparisons challenging. For a better understanding of the radiobiological mechanisms at ultra-high dose rates there is therefore an urgent

need to enhance the energy output of laser-driven proton beams, as well as to advance techniques for the uniform irradiation of large surface areas of cells in adherent monolayers or tissues within larger volumes (3D). Ultra-high dose rate radiobiology is a relatively new field and still evolving but certainly has great potential, as suggested by the recent advances of FLASH radiotherapy which has shown unique normal tissue sparing effects [30, 126, 127] that can lead to a higher therapeutic index in cancer radiotherapy [128–131]. There is no doubt that a close collaboration between the cyclotron accelerator, high power laser and FLASH radiotherapy communities will be beneficial for advancing the prospects of ultra-high dose rate proton radiobiology.

AUTHOR CONTRIBUTIONS

PC, MB and KMP conceptualized the article. PC, GM, HA, BO and AM collected the literature, wrote various subsections. PC, KMP and MB finalized the manuscript. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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