Identifying the optimal combination of hyperthermia with photon-based radiotherapy to produce the most effective cancer treatment

PhD project
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Background

Hyperthermia (heat temperatures of 39-43^{O}C) is an effective, non-toxic, therapy for enhancing the radiation treatment of cancers. This has been demonstrated in extensive pre-clinical single treatment regime studies and a Meta-analysis of randomized clinical trials [1, 2]. As such, hyperthermia is applied extensively in Europe and Asia. The importance of undertaking studies combining hyperthermia and radiation within Europe is shown by the European Union Horizon-2020 – Marie Sklodowska-Curie Actions – Innovative Training Networks (H2020-MSCA-ITN-2020) award to the project entitled "Hyperthermia boosting the effect of radiotherapy" (HYPERBOOST). That consortium includes 11 leading hyperthermia research groups within the European Union, and 7 partner organizations including the European Society for Radiotherapy & Oncology and the European Society for Hyperthermic Oncology. As a leading research group for undertaking pre-clinical studies related to the combination of hyperthermia and radiation, the Experimental Clinical Oncology (ECO) research group within the Department of Oncology at Aarhus University Hospital is one of the consortium members, responsible for one Work package (WP3) and two of the Early-Stage Researcher (ESR) projects; this PhD application is one of those funded ESR projects.

Despite the apparent success with combining hyperthermia and radiotherapy, many clinical trials failed to show any benefit. While this could be partially explained by insufficient number of patients, there are other factors that could be involved which may seriously influence future clinical studies. Firstly, there is the question of the importance of the time interval between radiation and heating. Pre-clinical studies using single treatments showed that the enhancement of radiation response by heat was greatest when these modalities were applied close together, and decreased as the interval increased [1, 2]. Two large positive clinical trials confirm this [3, 4], yet another clinical study suggests that timing is not important [5]. This is a critical point and pre-clinical studies using clinically relevant fractionated studies are needed to resolve this issue. Secondly, there is the question of what heat treatment is necessary to enhance radiation response. Clinical studies always use a 60-minute heating period, so that is unlikely to be altered. Early trials focused on achieving tumor temperatures of 42-43^{O}C [3]. However, it was later suggested that lower heat treatments (<42^{O}C) were the optimal temperatures [6] and now clinical trials focus on the lower temperature range with the average temperatures achieved being around 40^{O}C [4, 5]. However, pre-clinical studies using single dose treatments show that temperatures below 41.5^{O}C for 60 minutes are relatively ineffective, even when the interval between radiation and heat is short [1, 2]. The benefit seen in later clinical trials could simply be the result of "hot spots", where temperatures exceed 42^{O}C even though mean temperatures are only 40^{O}C. Aiming for lower temperatures, puts the emphasis on applying ineffective low temperature treatments rather than focusing on more effective higher temperatures. Again, relevant fractionated pre-clinical studies would resolve this issue. Thirdly, two phenomena may play a critical role when hyperthermia and radiation are combined in fractionated schedules. These are thermotolerance and step-down heating [7, 8]. Thermotolerance is where repeated heating leads to heat resistance. Conversely, clinical heat treatments are often associated with considerable temperature fluctuations during heating and if changes

from high to low temperatures (step-down heating) occurs it can result in thermo-sensitization. These two phenomena have been extensively studied when applying heat alone [9, 10], but their significance when heat and radiation are combined in fractionated schedules, especially at clinically relevant temperatures, is not known. The mechanism for the interaction between radiation and hyperthermia is also unclear. Suggested mechanisms include a decrease in radiation resistant tumor hypoxia, an inhibition of radiation-induced DNA damage repair, and heat induced tumor cell kill [1, 2]. Cell killing could also occur indirectly, via heat-induced damage to the tumor vascular supply thus starving tumor cells of essential oxygen and nutrients or by inducing an immune response to attack the tumor [1, 2]. Identifying these mechanisms will improve our ability to apply heat more effectively. The optimal heat treatment for the greatest anti-tumor effect will only be beneficial if similar effects do not occur in normal tissues, thus tumor studies on the interaction of hyperthermia with radiation must be compared with similar studies in clinically relevant normal tissues.

One area where the application of hyperthermia with radiation could be extremely clinically relevant is when patients need to undergo re-irradiation. Effective treatment of recurrent tumors is problematic because of the potential for the radiation to result in unacceptable normal tissue morbidity [11, 12]. Thus, there is a need to find approaches for sensitization of the tumor dose, without increasing that of the normal tissues. Recent studies suggest a role of hyperthermia in that context [12-14], but there is a lack of comprehensive pre-clinical data to support this approach, especially when using fractionated schedules.

Aims

This project will be to investigate the issues described above. Specifically, we will:

- Use tumors and normal tissues to determine the importance of the time interval between the application of radiation and heat, establish the minimal heat temperature needed to enhance radiation response, and understand the role of thermotolerance and step-down heating.
- Assess the pathophysiological parameter (i.e., blood flow, oxygenation/hypoxia) that primarily influences this heat-radiation interaction, and identify the mechanisms (i.e., oxygenation, DNA repair, direct/indirect killing, and immune effect) responsible.
- Investigate the potential of hyperthermia to be used as an adjuvant for re-irradiation treatment.

Our hypothesis is that using clinically equivalent fractionation schedules, rather than historical single treatment regimes, we will produce comprehensive pre-clinical data that determines the optimal application of hyperthermia and radiation. These results will be shared with the other Hyperboost consortium members, thus strengthening our international collaborations. More importantly, it will significantly improve ongoing research in the area, thereby impacting the application of hyperthermia and radiation in clinical settings where it is currently being applied. Furthermore, it should increase the likelihood of other cancers centers adopting this therapeutic approach, especially in Denmark.

Specific research plan

Project 1. The time interval between radiation and heat, the minimal heat temperature, and the role of thermotolerance and step-down heating for the optimal tumor effect.

Tumor studies will be carried out using the murine C3H mammary carcinoma (grown in CDF1 mice), one of the few pre-clinical models that allows for clinically relevant local tumor control assessment [15-17]. It is also one of the models in which pre-clinical studies performed at ECO [15, 16, 18] played a significant role in establishing clinical trials with hypoxic modifiers [19-21]. Experimental tumors will be produced by injecting 5-10 µl of tumor

material from large flank tumors, into the right rear foot of additional mice. This site allows for non-anaesthetised mice to be restrained in jigs and their tumor bearing legs exposed and loosely attached to the jig before leg immersion in a circulating water-bath (type TE 623; Heto, Birkerød, Denmark) for accurate tumor heating [22]. Tumors can also be locally irradiated in this set-up and by shielding the mice, irradiating critical normal tissues will be avoided [16, 17]. Treatments begin when experimental tumors reach a standard volume of 200 mm^3; this volume is typically achieved two-three weeks after inoculation and calculated from the formula D1 x D2 x D3 x π/6, where the D-values represent the three orthogonal diameters measured with calipers [22]. The treatments will involve using clinically relevant fractionated radiation schedules. These include conventional fractionation (30 x 2 Gy; given as 2 fractions/day with a 6-hour interval between fractions, 5 days/week) and stereotactic treatments (3 fractions of 5-25 Gy applied during a one-week period). Regardless of the radiation schedules, three days after the final irradiation, mice will be given single top-up doses of between 10-70 Gy under clamped (the tumor bearing leg clamped for 5 minutes before and during irradiation) conditions to produce full dose response curves [17]. Radiation will be delivered as photons using a pre-clinical 320 kV irradiator (YXLON International GmbH, Hamburg, Germany). Hyperthermia treatments involve heating tumors at different temperatures (40.5, 41.5 or 42.5^{O}C) for 60 minutes, with the heat applied either 30, 90 or 180 minutes after irradiating. These temperatures and times fit with the range of possible values from clinical studies [3-5]. The heat treatments will be applied once, twice, or three times each week in connection with the radiation treatment to allow us to determine the optimal application and investigate the role of thermotolerance on the interaction. For the step-down heating studies, similar single or multiple treatments per week will be applied, but with the temperature for the first 15 minutes of heating being at a higher temperature than that used for the remaining 45 minutes (the temperatures still being within the range described above). Treatment endpoint will be local tumor control (percentage of mice showing complete tumor control at 90 days after treatment) assays [16]. Following logit analysis of the radiation dose-response curves we will calculate the dose that produces a response in 50% of animals; the ratio of these values for radiation alone and radiation with heat will give an enhancement ratio. We will try to randomize the tumor bearing mice into the different treatment groups. However, since individual tumors grow at different rates, they will not all achieve the 200 mm^3 starting volume at the same time, so some selection will be necessary to ensure that tumors starting on the same day will be distributed among the different treatment groups. However, blinding of the assessment after treatment will be done.

Similar studies will also involve relevant normal tissue assays [23, 24], specifically, early responding foot skin damage and late responding leg fibrosis. For the skin response we will locally irradiate the right rear foot of restrained, non-anesthetized mice, in a similar fashion as explained for the tumor studies. However, the absence of a tumor will require a slightly modified approach to maintain the leg in the correct position for treatment, so we will expose the leg and attach it to the jig by applying tape to the toes of the treated foot. Mice will be observed daily from 11 to 30 days after treatment. A scoring system had previously been developed for defining different levels of damage in mouse foot skin [25] and for our current study we will use a reversible moist desquamation level affecting 75% of the skin area; the percentage of animals in each treatment group developing this level of damage will be recorded. Late radiation-induced fibrosis will be assessed in mice irradiated as for the skin studies, with response monitored using a modification of the leg contracture model described by Stone [26]. Based on the degree of extensibility of the irradiated leg, the endpoint for fibrosis is defined as a permanent reduction in extensibility of at least 75% relative to the untreated leg, with observations made every second week for at least 6-months after treatment. Following logit analysis of the clamped radiation dose-response

curves we will calculate the dose that produces a response in 50% of animals. The ratio of these values for radiation alone and radiation with heat will give an enhancement ratio, which can then be compared to the same values obtained in tumors to determine the therapeutic benefit. With the normal tissues, randomization and blinding are both possible.

Project 2. Assessing the pathophysiological parameters and identifying the mechanisms involved in the interaction between radiation and heat.

Determining the mechanisms responsible for the enhancement of radiation by hyperthermia will involve treating additional tumor or non-tumor bearing mice, as described above, but assaying at specific times before, during or after therapy, the exact time points depending on the parameter under investigation. Perfusion will be determined using a radioactive tracer uptake technique similar to that done previously using ^{86}RbCl [22, 27], but using 99mTechnicium (^{99m}Tc) labeled NaTcO4 instead [28]; this tracer is used routinely at the nearby Dept. Nuclear Medicine, Aarhus University Hospital. The technique involves intravenously (i.v.) injecting a PBS solution containing [^{99m}Tc]NaTcO4, and then five minutes later the animals are sacrificed and the tumor or normal tissue excised. This material is then weighed, the amount of radioactivity measured on a gamma counter, and the pbercentage injected dose per gram calculated [28]. Increased perfusion with this technique will give information on improved oxygenation, while reductions will be indicative of heat-induced vascular damage. Hypoxia will be estimated by measuring the binding of the hypoxia imaging agent, pimonidazole, as in our previous studies [29, 30]. Here mice will be i.v. injected with pimonidazole (60 mg/kg) 90 minutes before animal sacrifice, tissues excised, formalin fixed and paraffin embedded. Histological sections are then made and stained with an antibody to pimonidazole and digitalized using a Hamamatsu NanoZoomer tissue section scanner; the area of bound material expressed as a percentage of the total viable area. An inhibition of DNA damage repair will involve identifying the degree of DNA damage using immunohistochemical staining of γH2AX, the H2AX histone protein phosphorylated after DNA double-strand breaks [31, 32]. Basically, following irradiation, the animals are sacrificed, and histological sections prepared from formalin fixed, paraffin embedded, material as described above, before being exposed to an antibody for γH2AX and visualized using a fluorescent microscope [31, 32]. Immune modulation by hyperthermia will be assessed using immunohistochemistry measurements of the infiltration by immune cells using markers (i.e., CD4, CD8, & Foxp3 positive cells) similar to previous reported studies [33]. Again, measurements will be made on formalin-fixed, paraffin embedded, sections stained with antibodies to the relevant markers, and the sections digitalized as described for the pimonidazole studies. Measurements for all assays prior to the start of treatment will give us baseline values, while measurements during or after treatment will indicate how these parameters influence hyperthermia and how they are changed by hyperthermia and thereby influence radiation response.

Project 3. The potential of using hyperthermia to improve outcome with re-irradiation.

Normal mouse legs will be irradiated as described previously. They will be initially treated with a single dose of 38 Gy. This dose produces severe fibrosis in 50% of treated mice, and does so within 6 months after irradiation [24]. At that time the same legs will then be re-irradiated with fractionated doses of either 30 x 2 Gy or 3 x 5-25 Gy, followed by clamped top-up treatments with graded doses to produce full dose-response curves. Additional mice will receive the fractionated radiation treatments in combination with weekly hyperthermia treatments using the optimal time interval between radiation and heat and heating temperatures derived from the earlier studies. The animals will then be followed for a further 6-12 months and the degree of fibrosis recorded and analyzed as stated previously.

Statistical Considerations
According to the Biostatistical Advisory Service, Aarhus University (AU), the planned experiments are not suited to classical power calculations. Selection of the number of treatments and animals/group will be based on the departments experience with studies of this type, while keeping animal numbers to a minimum as with the principles of the 3Rs (replacement, reduction, and refinement) for animal welfare.

Feasibility
The studies will be carried-out at the ECO research group within the Dept. Oncology, Aarhus University Hospital. This is a fully equipped facility undertaking pre-clinical and clinical research. It has its own animal house, experimental photon radiation machine, heating set-up, and molecular and histology laboratories. The necessary models are established as are the assays for monitoring tumor and normal tissue response. That research group also has extensive experience with all the mechanistic techniques, except the γH2AX procedure, but will get advice from other members of the Hyperboost consortium that have experience with this technique [34, 35], It does not have in-house imaging/gamma counting facilities, but has a long history of collaborating with the Dept. Nuclear Medicine at Aarhus University Hospital, thus guaranteeing a successful completion of the entire study. The principal supervisor will be Michael R. Horsman (Professor of Experimental Radiotherapy), who has an international reputation for undertaking pre-clinical studies related to the combination of hyperthermia and radiation. This is demonstrated by his extensive publications in this area and for him receiving the 2011 ESHO-BSD award from the European Society of Hyperthermic Oncology; an annual award given to that person who had made significant contributions to the field of hyperthermia. The other supervisors will be Jens Overgaard (Professor and Head of ECO), who is considered one of the foremost authorities on pre-clinical and clinical studies combining hyperthermia and radiation; Morten Busk (Senior Scientist at ECO), an expert on the use of imaging and radioactive tracers, and immunohistochemical analysis of tumor sections; and Pernille B. Elming (Clinical Oncologist at the Dept. Oncology), who has a background in hyperthermia related studies and will act as clinical advisor.

Ethics
All animal studies will be conducted according to the animal welfare policy of Aarhus University (http://dyrefaciliteter.au.dk) and with the Danish Animal Experiments Inspectorate's approval. Approval has already been obtained for many of the planned experiments, but approval will be requested for those experiments not yet covered.

Relevance
The combination of hyperthermia and radiation is an effective therapy used extensively to treat cancer patients, especially in Europe and Asia. However, there are strong indications that this combination may not be applied optimally. This PhD project focuses on further understanding the interaction between hyperthermia and radiation to determine the best application for the greatest benefit. The study itself will be an integral part of the European Union Horizon-2020 HYPERBOOST (Hyperthermia boosting the effect of radiotherapy) consortium project as described earlier. The results of this study will be made available to the other consortium members to help define the optimal application of hyperthermia with radiation and although a purely pre-clinical study, it will help develop personalized treatment combinations of hyperthermia with radiation for cancer patients. The results will likely also help establish clinical trials with hyperthermia and radiation in other cancer centers not already using this effective treatment option.

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