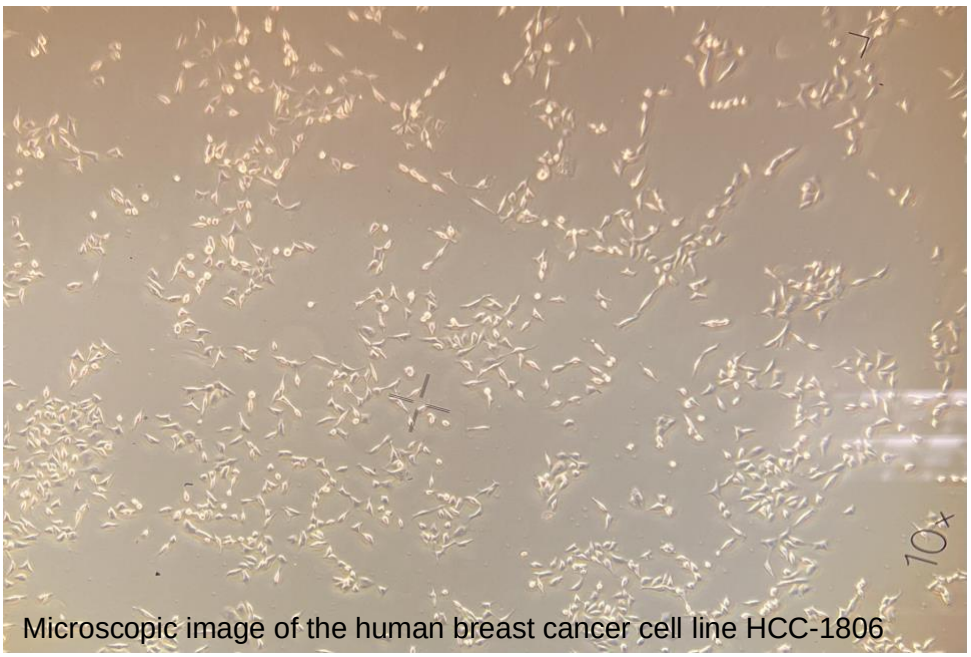
UNIVERSITY OF GOTHENBURG

DEPARTMENT OF MEDICAL RADIATION SCIENCES
SAHLGRENSKA ACADEMY



Microscopic image of the human breast cancer cell line HCC-1806

# Apoptosis induction in breast cancer cells after radiotherapy and potential radiosensitizers

**M.Sc. Thesis**
**Klara Simonsson**

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| Essay/Thesis: | 30 hp |
| Program and/or course: | Medical physics |
| Level: | Second Cycle |
| Term/year: | Spring 2023 |
| Supervisors: | Johan Spetz, Eva Forssell-Aronsson |
| Examiner: | Magnus Båth |

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| Keywords: | Breast cancer cells, radiotherapy, systemic therapy, combination therapy, apoptosis, BH3 profiling, flow cytometry |

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# Abstract

**Background.** Today breast cancer is the foremost cancer death type amongst women around the world. At present some of the toughest challenges in the clinic is recurrent, radioresistant breast cancer, and metastatic breast cancer where low cure rates are observed after surgery and radiotherapy. One way to possibly increase survival rates, is combining radiotherapy and systemic therapy that doubles as a radiosensitizer. The aim of this thesis was to identify pathways that increase radiosensitivity in breast cancer cells after combination treatment with external irradiation and anti-cancer drugs, specifically through increased apoptosis.

**Method.** Three different human breast cancer cell lines were cultured: MCF-7, MDA-MB-453 and HCC-1806. A dose-response study with irradiation was performed on the cells to determine a radiation absorbed dose and time-point after irradiation for the following combination treatment. Lastly, cells were treated with anti-cancer drugs +/– irradiation, and then stained with the fluorescent dyes Annexin V-DyLight 650 (apoptosis marker) and Hoechst 33342 (cell viability marker) whereafter flow cytometry was performed.

**Results.** A dose and time dependency was observed in the dose-response study. In total, 29 of 31 anti-cancer drugs exhibited a synergistic effect with radiation on any cell line at any concentration. The drugs birinapant (with target IAP) and ganetespib (with target HSP90) showed statistically significant radiosensitizing effects on all three cell lines.

**Conclusions.** Possible radiosensitizing targets for all three cell types included in this study are HSP90 and IAP. In addition, BH3 mimetics are potential radiosensitizing targets for cell lines MDA-MB-453 and HCC-1806, and the DNA repair machinery was a radiosensitizing target for cell line HCC-1806.

# Sammanfattning

**Bakgrund.** Idag är bröstcancer den enskilt största orsaken till cancerdöd för kvinnor runt om i världen. Kvinnor med återkommen, strålningsresistent bröstcancer och metastaserad bröstcancer utgör för närvarande en mycket svår klinisk utmaning, då få patienter med dessa tillstånd botas efter operation och strålbehandling. En metod att försöka öka överlevnaden skulle kunna vara att kombinera strålterapi med en systemisk behandling som ökar strålkänsligheten. Målet med denna studie var att identifiera sätt att öka strålkänsligheten i bröstcancerceller efter kombinationsbehandling med extern strålterapi och anticancer läkemedel, specifikt genom en ökad induktion av apoptos.

**Metod.** Tre olika humana bröstcancercellinjer odlades: MCF-7, MDA-MB-453 och HCC-1806. En dos-responsstudie med strålning gjordes med cellerna för att bestämma en absorberad dos och tidpunkt efter bestrålning att använda i den följande kombinationsbehandlingen. Slutligen behandlades celler med anticancerdroger +/– strålning, för att sedan färgas in med de fluorescerande färgerna Annexin V-DyLight 650 (en apoptosmarkör) och Hoechst 33342 (en cellviabilitetsmarkör) varefter flödescytometri utfördes.

**Resultat.** Ett dos- och tidsberoende observerades i dos-responsstudien. Totalt visade 29 av 31 anti-cancer droger en synergistisk effekt med strålning för alla kombinationer av cellinjer och drogkoncentrationer. Drogerna birinapant (med IAP som target) och ganetespib (med HSP90 som target) ökade strålkänsligheten statistiskt signifikant för alla cellinjer.

**Slutsatser.** Möjliga sätt att öka strålkänsligheten för alla cellinjer skulle enligt denna studie kunna vara inhibering av HSP90 och IAP. Även den inre apoptotiska signalvägen kan vara en potentiell väg att öka strålkänsligheten för cellinjer MDA-MB-453 och HCC-1806, och DNA-reparationen kan vara en annan möjlig måltavla för att öka strålkänsligheten för cellinjen HCC-1806.

# Acknowledgements

I would like to express my deepest gratitude to my supervisors: Eva, for this incredible opportunity and your endless support. Johan, for your brilliant ideas and guidance throughout this thesis.

John, thank you for generously giving us your time to help us irradiate the cells.

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Lastly, I would like to thank Nicholas, my family and friends for believing and inspiring me.

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# 1 Introduction

## 1.1 Background

Breast cancer is the most frequent form of cancer and causes the highest number of cancer fatalities in the world amongst women [1]. Treatment often consists of a combination of surgery, radiotherapy, and pharmaceuticals such as chemotherapy or targeted therapy [2]. Radiotherapy is mainly used following surgery to decrease the possibility of recurrence, or as pain relief if the disease is spread to other parts of the body [3]. One of the major cellular responses to irradiation is apoptosis, a type of programmed cell death. Apoptosis occurs when radiation causes microenvironmental perturbations within a cell, such as DNA or microtubular damage, endoplasmic reticulum stress, or an increase of reactive oxygen species. The ability of cells to undergo apoptosis is often impaired in many cancers, which may lead to radioresistance [4]. Today, one of the biggest clinical challenges is treating radioresistant breast cancer that has relapsed [5, 6]. Another clinical impediment is metastatic breast cancer, where surgery and radiotherapy have a low cure rate. Thus, a combination of radiotherapy and a systemic treatment, that doubles as a radiosensitizer of the cancer cells, could be a possible way to kill more cancer cells and improve therapeutic efficacy [6]. An additional benefit of killing more cancer cells with combination therapy is the prospect of lowering radiation doses to decrease side effects on normal tissues [7]. Although many new studies of potential breast cancer drugs in combination with standard chemotherapy have showed promising results [8], few of them have been tried in combination with radiotherapy.

## 1.2 Aim

The aim of this study was to identify pathways that increase the radiosensitivity in breast cancer cells after combination therapy with external irradiation and anti-cancer drugs, using functional measurements of apoptosis.

# 2 Theory

## 2.1 Breast cancer

Breast cancer is divided into four different groups according to expression analyses of estrogen and progesterone hormone receptors, human epidermal growth factor 2 (HER2) expression, cancer stage and growth rate: luminal A, luminal B, HER2-positive, or triple negative [2, 9]. See characteristics in Table 1. Luminal A accounts for 40% of all breast cancer diagnoses. It has a low growth rate and malignancy. Therefore, it has the best prognosis of the four breast cancer types. About 35-40% of the patients have type luminal B. It has a higher growth rate and malignancy grade than luminal A, resulting in a worse prognosis. HER2 type occurs in almost 15% of the patients. It has a higher growth rate than both the luminal types due to the expression of the HER2 oncoprotein, yielding an even lower survival rate. Triple negative breast cancer is the least common type, occurring in almost 10% of the patients. It is the most aggressive breast

cancer type, leading to more recurrences and the lowest survival rate of all breast cancer types [2, 10]. The five year relative survival spans between 94.4% for type luminal A, and 77.1% for triple negative, confirming that triple negative breast cancer has the worst prognosis [11].

**Table 1.** Characteristics of the four breast cancer types.

| Breast cancer type | Luminal A | Luminal B | HER2-positive | Triple negative |
|---|---|---|---|---|
| ***Estrogen receptors*** | + | + | - | - |
| ***Progesterone receptors*** | + | +/- | - | - |
| ***HER2*** | - | + | + | - |

### 2.1.1 Systemic treatments in breast cancer

Systemic therapy is treatment with drugs circulating in the blood, enabling treatment of cells throughout the body [12]. It is a common cancer treatment, and most women treated for breast cancer receive systemic treatment. For women diagnosed with metastatic breast cancer it is often the only treatment received [13], although the five year relative survival is only 30% [11]. This indicates that systemic treatment is insufficient as single treatment of advanced disease. There are four main types of systemic treatments: chemotherapy, hormone therapy, targeted therapy, and immunotherapy. Chemotherapy kills cells in specific cell cycle phases. Because cancer cells proliferate faster than normal cells, cancer cells are more prone to die. Albeit normal cells are killed as well, making the therapeutic window an important factor. Hormone therapy prevents estrogen and progesterone from binding to the receptors on hormone positive breast cancer cells to inhibit their growth. Targeted therapy employs pharmaceuticals that target specific receptors on cancer cells, leading to cell death or suppressed growth. Immunotherapy drugs enhance the immune system to identify and kill more cancer cells [14]. Luminal breast cancer types are often treated with hormones due to the expression of estrogen and progesterone receptors, and HER2-positive breast cancer types are often treated with targeted therapy with HER2 receptors as target. Since triple negative breast cancer cells lack expression of estrogen receptors, progesterone receptors and HER2 altogether, it is not possible to treat them with hormone therapy or HER2 antibodies. Thus, fewer treatment options exist for patients diagnosed with triple negative breast cancer [2, 9].

### 2.1.2 Radiotherapy in breast cancer

External beam radiotherapy (EBRT) is not a new treatment modality. In 1896, only a year after the discovery of x-rays, the first irradiation treatment of a breast cancer patient took place. Since then, radiotherapy has progressed substantially. Modernization of radiotherapy during the last 30 years has resulted in increased survival rates and less dramatic side effects for patients diagnosed with breast cancer. For women receiving radiotherapy following breast conservative surgery, the recurrence rate decreases by half and the mortality decreases with approximately one sixth, compared with women not receiving radiotherapy [15]. More than half of all breast cancer patients receive radiotherapy during treatment [16]. Although, patients with metastatic breast cancer receive less radiotherapy than patients without metastases, where only specific lesions are irradiated for palliation or pain relief [17, 18]. Consequently, there is a need for better treatment options. One potential alternative is the use of systemic drugs that also function as radiosensitizers, substances that heightens the effects of radiotherapy. If a potential

radiosensitizer should be interesting in clinical settings, it should only radiosensitize cancer cells. The ideal radiosensitizer for cancer cells would also function as a radioprotector of normal cells [19].

## 2.2 p53

Tumor suppressor genes regulate DNA reparation, cell division and apoptosis. One of the most important tumor suppressor genes is p53, often mutated in cancer cells. When a cell is damaged from irradiation, the p53 gene will initiate reparation of the DNA, or make the cell undergo apoptosis if the DNA damage is too extensive. If the p53 gene is mutated it can lose its function. This can lead to uncontrolled cell division and cells refusing to undergo apoptosis despite unrepairable DNA damage, both hallmarks of cancer [20, 21]. Also, studies have showed that cells expressing an altered variant of p53 are more radioresistant than cells expressing wild-type p53 [4]. For example, p53 is mutated in 30-35 % of all breast cancers, and in 80 % of all triple negative breast cancers, making the p53 pathway a possible target for breast cancer treatment [22].

## 2.3 Apoptosis

Apoptosis is a highly regulated and energy-dependent type of programmed cell death [4]. It is a central process for normal tissue cell turnover, development, and homeostasis. Hence, anomalous regulation of apoptosis is often associated with development and treatment outcome of several diseases [23]. For example, neurodegenerative disease and coronary heart disease are linked to an excessive apoptosis rate, whereas cancer is related to an insufficient apoptosis rate [23, 24]. Anti-cancer drugs can therefore target the apoptotic pathways by blocking important signaling pathways or damaging cellular components, such as DNA or microtubules, leading to stimulation of the apoptotic pathways [4]. There are two main apoptotic pathways, the intrinsic and the extrinsic pathway. The intrinsic pathway, also known as the mitochondrial pathway, is activated when the cell is damaged, for example after irradiation, and it is regulated by the BCL-2 protein family. The BCL-2 family consists of proteins that all have BCL-2 Homology (BH) domains, and can be divided into the pro-survival proteins and the pro-apoptotic proteins, which are further divided into effectors and the BH3-only proteins: activators and sensitizers (Figure 1). Activators and sensitizers are called BH3-only proteins because they only have the BH3 domain, which is the domain the BCL-2 protein family interacts with. After irradiation of a cell, the pro-apoptotic proteins are activated. The effector proteins, BAX and BAK, form pores in the mitochondrial outer membrane. Activator proteins, *e.g.* BIM and BID, activate BAX and BAK, but cannot form pores themselves. The pro-survival proteins, such as BCL-2, BCL-$X_L$, and MCL-1, can inhibit and sequester effector and activator proteins, thus acting as a buffer for endogenous or low-level pro-death signals, and promoting cell survival. Sensitizer proteins, for example BAD, HRK, and NOXA, indirectly promote apoptosis by inhibiting specific pro-survival proteins. See Figure 2 for the binding selectivity of the BCL-2 family. This releases effector or activator BCL-2 family proteins from the anti-apoptotic proteins, enabling them to cause mitochondrial outer membrane pores. The pore-formation leads to mitochondrial outer membrane permeabilization (MOMP), where the protein cytochrome C is released from inside the mitochondria. Cytochrome C will form the "apoptosome" with the proteins APAF1 and caspase-9, where caspase-9 turns into its activated

form. Active caspase-9 cleaves caspase-3, caspase-6 and caspase-7 to their active form, which in turn leads to cleavage and degradation of multiple cellular proteins including DNA. The extrinsic pathway, also known as the death receptor pathway, is initiated when death receptors (*e.g.* TNFR, TRAIL receptors) on the cell surface are activated by ligands (*e.g.* TNF, FAS, TRAIL) from surrounding cells. This leads to activation of caspase-8 and caspase-10, which in turn activates caspase-3, caspase-6 and caspase-7, and apoptosis occurs. Caspase-8 and caspase-10 can also activate BID, which activates the intrinsic pathway. Caspase-3 can also activate caspase-8, leading to activation of BID and the intrinsic pathway, meaning if only a few mitochondria go through MOMP it can be amplified by this pathway (Figure 3). Overexpression of the TNF death receptors can be seen on irradiated cells [21, 25]. Mitotic catastrophe or other forms of cell death can also occur, but generally requires a higher radiation dose. Consequently, cells which have an intact apoptosis signaling pathway as well as functioning upstream signaling (through *e.g.* p53), will generally undergo apoptosis rather than mitotic catastrophe [4].

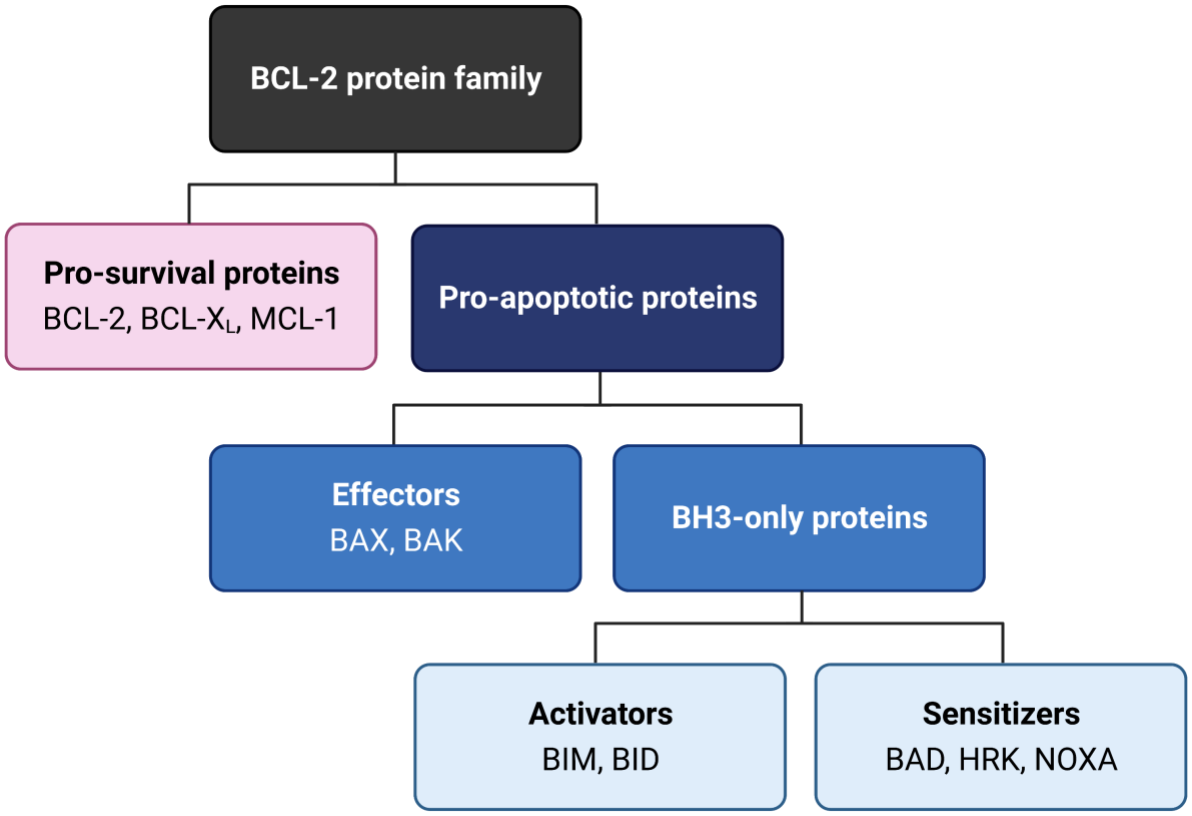


**Figure 1.** Examples of pro-survival and pro-apoptotic proteins of the BCL-2 protein family [25].

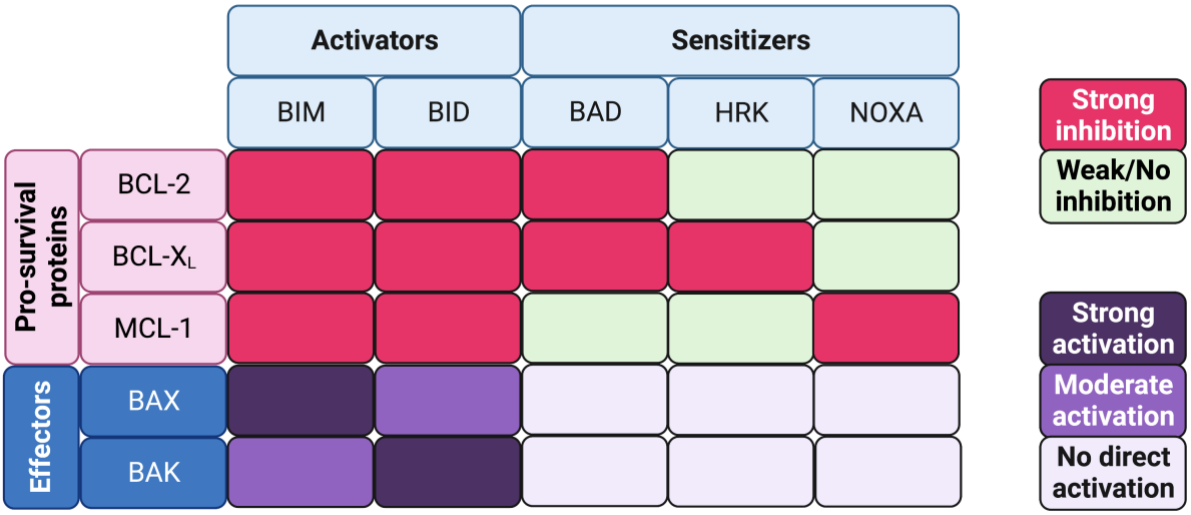


**Figure 2.** The binding selectivity of activators and sensitizers in the BCL-2 protein family [26].

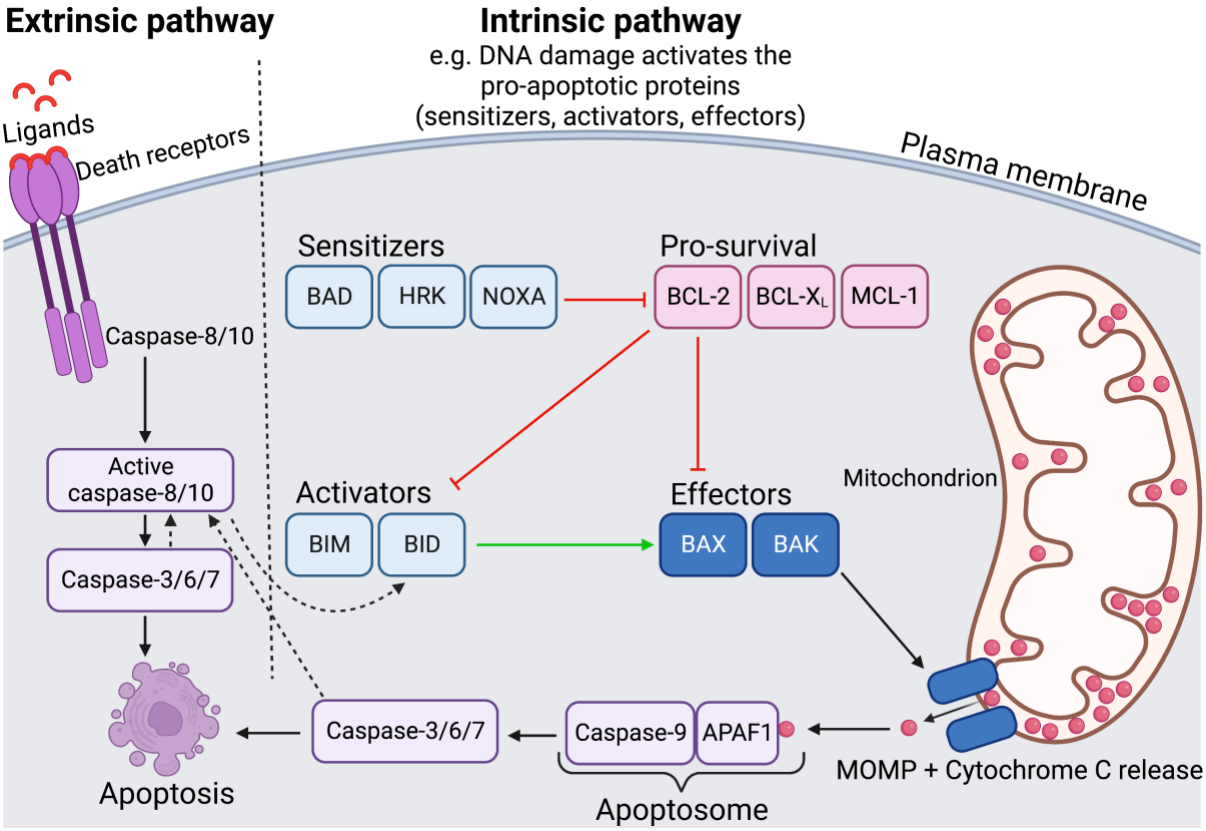


**Figure 3.** The extrinsic and intrinsic apoptosis pathways [21, 25].

## 2.4 Flow cytometry

Flow cytometry is a method to analyze cells, and is commonly used to study apoptosis [27]. Flow cytometry builds on the principles of light scatter and fluorescent emission. Cells are stained with fluorescent dye, and then scanned by a laser. The fluorescent substances can bind to specific components of a cell, *e.g.* DNA. Therefore, a specific dye can be chosen to identify different types of cells, or cells in specific phases or conditions. When a cell is scanned by the laser, the light will scatter depending on wavelength of the laser, size of the cell, light collection angle, and the refractive index of the cell and the medium it travels in. Light emitted from the cell depends on the fluorescent substance bound to the cell. The scattered and emitted light is detected by either a photodiode or a photomultiplier (Figure 4) [28, 29]. Annexin V fluorescent staining is used as a marker of apoptotic cells. Annexin V is a protein that binds to phosphatidylserine, a phospholipid normally located on the inner leaflet of the cell membrane but transferred to the outside of the cell membrane during apoptosis. This allows Annexin V to stain apoptotic cells at a relatively early stage [30, 31]. By using Annexin V conjugated to a fluorophore with specific excitation and emission wavelengths, the Annexin V–phosphatidylserine binding can be detected by a flow cytometer. Hoechst 33342 (subsequently shortened Hoechst) fluorescent staining is used as a marker of cell viability. Hoechst permeates intact cell membranes and binds to DNA. This staining persists into early apoptosis but is lost when DNA is degraded by caspases in late apoptosis. If a cell is alive, the Hoechst fluorescent intensity will be high [32]. The output of a flow cytometer is a scatter plot with forward scatter (FSC) on the x-axis and side scatter (SSC) on the y-axis, where each data point is a detected event. Not every recorded event is a cell, thus the collected data must be reduced. A strategy for this is gating. A gate is a graphic demarcation in the scatter plot that determines which events should be included for further analysis [33]. One gate discriminates which events are cells, and one gate discriminates which events are single cells. Additional gates are used to determine if an event is fluorescent positive or negative of a specific dye [34].

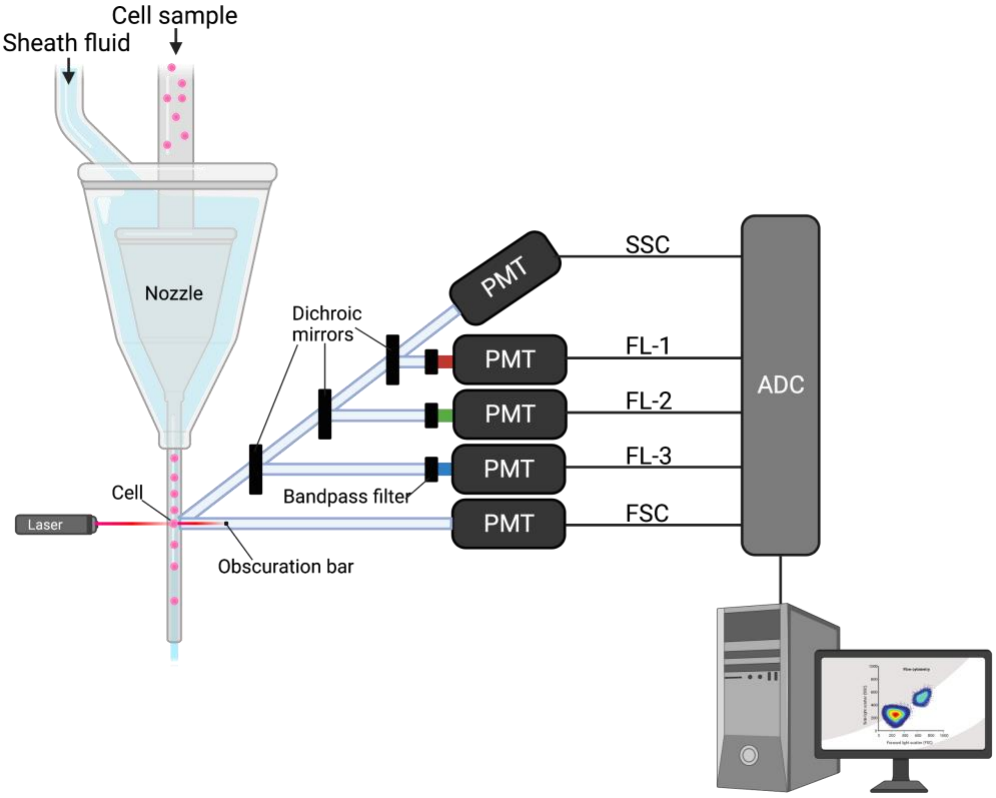


**Figure 4.** A schematic illustration of flow cytometry [35]. A single cell suspension passes through a nozzle together with sheath fluid. The cells then pass a scanning laser. Forward scattered light (FSC), side scattered light (SSC), and fluorescent light (FL) are detected by photomultiplier tubes (PMT). The detected signals then pass an ADC, and results are viewed on a computer.

# 3 Materials and Methods

## 3.1 Experimental design

In short, the experimental design was as follows. Human breast cancer cells were cultured. To optimize conditions for the fixed Annexin V and Hoechst staining assay, a test experiment was performed using cells treated with staurosporine (an ATP-competitive pan-kinase inhibitor and potent apoptosis inducer). To determine a suitable radiation absorbed dose and time point following irradiation, for the combination study, human breast cancer cells were also subjected to irradiation with EBRT and the dose-responses were examined over time. The combination study compared cells treated with anti-cancer drugs to cells treated with both anti-cancer drugs and irradiation. The cells in both the dose-response study and the combination study were fixated and stained with Annexin V and Hoechst and analyzed with flow cytometry. Lastly, statistical analyses were done. An illustration of the workflow can be viewed in Figure 5.

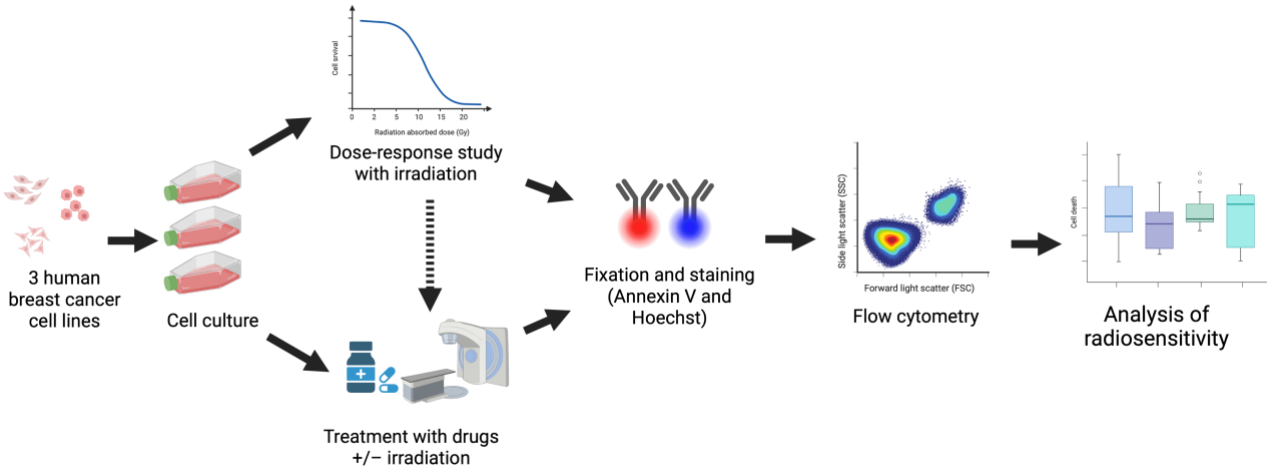


**Figure 5.** The workflow of this study.

## 3.2 Cell lines and cell culture

Human breast cancer cell lines MCF-7, MDA-MB-453, and HCC-1806 (Table 2) were used in this study. The cells were cultured with sterile filtered RPMI 1640 medium with L-glutamine (Life Technologies, the Netherlands) with 10% fetal bovine serum (FBS, Life Technologies, the Netherlands) in flasks (VWR, Sweden), and kept in a humidified incubator (Thermo) at 37°C with 5 % $CO_2$. The cells were always handled in an LAF-bench, and a mycoplasma test (MycoFluor™ Mycoplasma Detection Kit, Thermo Fisher Scientific) was performed on live cells with negative results. The cell lines were a generous gift from Dr. Toshima Parris and Dr. Khalil Helou, but originally purchased from ATCC.

**Table 2.** Cell lines used in this study.

| Cell line | Breast cancer type | p53 status |
|---|---|---|
| MCF-7 | Luminal A | Wild type |
| MDA-MB-453 | HER2 | Mutant |
| HCC-1806 | Triple negative | Mutant |

## 3.3 EBRT Dose-response

A dose-response study was performed to determine a suitable radiation absorbed dose to the cells in the following combination study, and at which time point after irradiation apoptosis assays should be performed. The day before irradiation, 10 000 cells/well were seeded in three 96-well flat bottom plates (VWR) per irradiation dose, with RPMI medium + 10% FBS. In each plate, there were 4 wells/cell type and time point. The cells were irradiated by a linear accelerator (VARIAN TrueBeam) with 6 MV photons in a 20x20 $cm^2$ static field and SSD 90 cm with radiation absorbed doses 0, 2, 5, 10, 15 or 20 Gy. The cell plates were placed in a polystyrene phantom during irradiation to include backscatter and build-up. The phantom can be viewed in Figure 6. An additional bolus was placed under the plate inside the phantom to avoid an air gap. Also, the wells were filled with 200 uL medium to increase the amount of backscatter. At time points 24 h, 48 h and 72 h after irradiation the cells were fixated and stained with Annexin V and Hoechst as described below [36]. The dose and time point where around 30 % of the cells were dead was chosen to be used in the combination study.

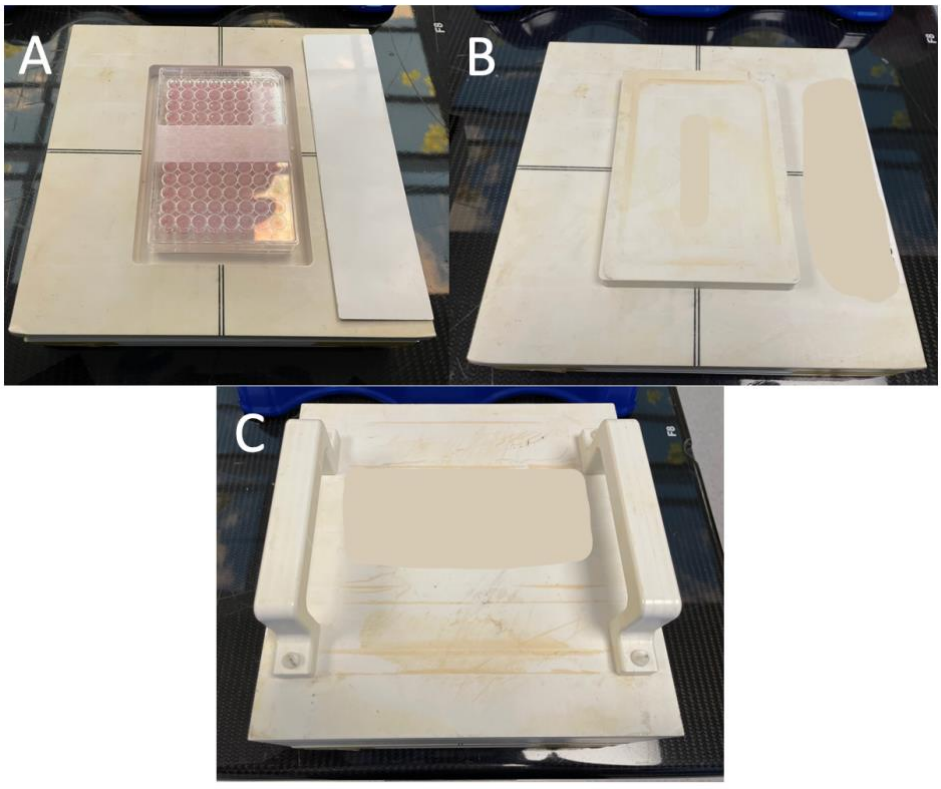


**Figure 6.** Set-up of the phantom used at the dose-response study and the combination treatment. Cell plate placed on a polystyrene block (A), a smaller polystyrene block placed on top of the plate (B), and an additional polystyrene block placed on top (C).

### 3.3.1 Fixed Annexin V/Hoechst

The medium in the wells of the plates was transferred to new 96-well V-bottom plates (Sarstedt) 24, 48, or 72 hours after irradiation. Thereafter, 25 uL PBS (Gibco)/well was added and removed in the original plates. The original plates were then placed in the incubator for five minutes with 25 uL of 0.0025% trypsin (Gibco)/well. The trypsinized cells were then transferred to the new V-bottom plates and stained with Annexin V and Hoechst according to the following protocol. A staining solution with 10x annexin binding buffer (0.1 M Hepes (Sigma-Aldrich), 40 mM KCl (Sigma-Aldrich), 1.4 M NaCl (Sigma-Aldrich), 7.5 mM MgCl2 (Sigma-Aldrich), and 25 mM CaCl2 (Scharlab) solution, sterile-filtered, pH 7.4), 1:500 DyLight 650-conjugated Annexin V, and 1:500 Hoechst (Abcam) was prepared and added at 1:10 dilution to the cells. The cells were stained for 15 minutes at room temperature, then fixed with 30uL/well of Annexin V Fix buffer (4% formaldehyde (Thermo Fisher Scientific), 0.5% glutaraldehyde (Merck Life Science) in 1x Annexin V binding buffer) for 10 minutes at room temperature, and then neutralizing (N2) buffer (1.7 M Tris base (Fisher Scientific) and 1.25 M glycine (Fisher Scientific) (pH 9.1)) was added. The plates were sealed, wrapped in aluminum foil, and stored on a rocker at 4°C until analysis. The flow cytometer BD LSRFortessa$^{TM}$ Cell Analyzer was used to measure Annexin V and Hoechst positivity. Populations were scored as live (Hoechst +, Annexin V –), early apoptotic (Hoechst +, Annexin V +), late apoptotic (Hoechst –, Annexin V +), and degraded (Hoechst –, Annexin V –). The gating strategy can be viewed in Figure 7. To optimize gating and ensure that results from the fixed Annexin V/Hoechst remain comparable regardless of when after fixation the measurement and analysis was performed, a test using staurosporine (an ATP-competitive kinase inhibitor and potent apoptosis inducer) was performed. HCC-1806 cells were treated with 1 % DMSO or 1 µM staurosporine as described above, and fixed Annexin V/Hoechst staining was performed after 48 h. After fixation, Annexin V/Hoechst staining intensity was measured in the flow cytometer at 0, 1, 3, 7 and 28 days after fixation.

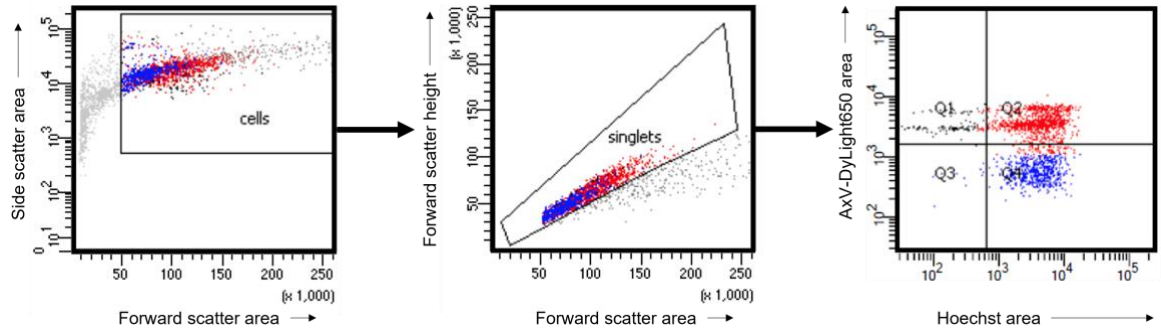


**Figure 7.** The gating strategy of the dose-response study and the combination treatment study. The first gate discriminates which events are cells. The second gate discriminates which events are single cells. The last gate determines which cells are live (Q1), early apoptotic (Q2), late apoptotic (Q4), and degraded (Q3).

## 3.4 Combination treatment

Following the dose-response study, a combination treatment study was conducted using irradiation and treatment with drugs on the cancer cells. Two groups per cell line were included: cells treated with irradiation and drugs, and cells treated with drugs only. The plates contained

negative control wells (drug-only plate: 1 % DMSO; drug + irradiation plate: 1 % DMSO + irradiation) to enable direct comparison with irradiation alone. To select drug candidates for the combination experiment, 3 different strategies were used; 1) FDA/EMA-approved and standard of care agents of different classes were chosen, possibly enabling a fast-track for clinical application if radiosensitization is observed; 2) a literature search was performed to identify anti-cancer agents previously tested in combination with radiation or chemotherapy for breast cancer; and 3) drugs targeting signaling pathways which are implicated in cellular responses to ionizing radiation (*e.g.* p53, apoptosis, ER stress and DNA repair) were chosen. In total, 31 different drugs were used in this study, and they can be viewed in Table 3, including targets, clinical stage of drug development for breast cancer, and references. To determine drug concentrations to treat the cells, maximal plasma concentration ($C_{max}$) observed in patients was searched for in literature as a reference. Three different concentrations of each drug were given to the cells (Table A1, Appendix). All drugs were purchased at MedChemExpress, except Berzosertib which was purchased from RayBiotech, and hydrogen peroxide that was purchased from Histolab. Cells in the combination group were treated with drugs within an hour before irradiation. The radiation dose was 10 Gy for all cell lines. Apoptosis and cell viability assays were performed 72 h after treatment as described in 3.3.1.

**Table 3.** Drugs used in this study, their target, clinical stage of drug development for breast cancer and other cancers, and references. * In combination with radiotherapy. ** In combination with chemotherapy. *** In combination with hormone therapy.

| Drug | Target | Breast cancer treatment | Reference |
|---|---|---|---|
| **Doxorubicin** | Topoisomerase II | Standard | FASS [37] |
| **Paclitaxel** | Microtubules | Standard | FASS [38] |
| **Lapatinib** | HER2 | HER2-positive BC | FASS [39] |
| **Abemaciclib** | CDK4/6 | Metastatic BC *** | EMA [40] |
| **Olaparib** | PARP | BRCA-positive BC | EMA [41] |
| **Hydrogen Peroxide** | Oxidation | Clinical radiosensitizer (Japan) * | Shimbo, Nakata [42] |
| **Buparlisib** | PI3K | Phase III | Baselga, Im [43] |
| **Ganetespib** | HSP90 | Phase II | Jhaveri and Modi [44] |
| **Melatonin** | ATF-6 | Phase II ** | Sadoughi, Dana [45] |
| **Adavosertib** | WEE1 | Phase II ** | Keenan, Li [46] |
| **Carboplatin** | DNA damage | Phase I-II * | Formenti, Golden [47] |
| **Berzosertib** | ATR | Phase Ib ** | Telli, Tolaney [48] |
| **Navtemadlin** | MDM2 | Phase I | Gluck, Gounder [49] |
| **Panobinostat** | HDAC | Phase I *** | Tan, Allred [50] |
| **GSK461364** | PLK1 | Phase I | Olmos, Barker [51] |
| **ABT-199** | BCL-2 | Phase II *** | Lindeman, Fernando [52] |
| **A-1331852** | BCL-$X_L$ | In vivo * | Pesch, Chandler [53] |
| **S63845** | MCL-1 | In vivo ** | Merino, Whittle [54] |
| **JNK-IN-8** | JNK | In vivo | Soleimani, Somma [55] |
| **Birinapant** | IAP | In vivo | Lalaoui, Merino [56] |
| **SR-4835** | CDK12/13 | In vivo | Quereda, Bayle [57] |
| **Empesertib** | TTK | In vivo | Chandler, Moubadder [58] |
| **UC2288** | p21 | In vivo | Hany, Zoetemelk [59] |
| **Birabresib** | BET | In vitro | Vázquez, Riveiro [60] |
| **KU-60019** | ATM | In vitro | Li, Yan [61] |
| **N-Butylidenephthalide** | COX | In vitro | Su, Huang [62] |
| **Niclosamide** | JAK/STAT | In vitro * | Lu, Dong [63] |
| **KU57788** | DNA-PK | In vitro * | Ciszewski, Tavecchio [64] |
| **KDOAM-25** | KDM5 | In vitro * | Pippa, Mannironi [65] |
| **Bortezomib** | Proteasome | In vitro | Mehdizadeh, Ataei [66] |
| **ISRIB** | eIF2α | In vitro | Lee, Seo [67] |

## 3.5 Statistical methods

To analyze the dose-response study a two-way variance (ANOVA) test with correction for multiple testing was executed, to see if there were any differences among the means of cell viability at different doses and time-points. A Tukey's range test was also performed to see if any specific means significantly differed from one another. To analyze the combined effects of irradiation and anti-cancer drugs, the Bliss independence model was used. It states that if two treatments are independent in terms of effect, then the effect after a combination of the treatments is equal to the product of the effect after single treatments:

$$E_{rad+drug} = E_{rad} \times E_{drug} \quad (1)$$

where $E_{rad+drug}$ is the calculated effect after combination treatment, $E_{rad}$ is the observed effect after treatment with *e.g.* radiation only, and $E_{drug}$ is the observed effect after treatment with *e.g.* drugs only [68]. To calculate the independent effect, equation 2 below was used.

$$E_{rad+drug} = E_{rad} + E_{drug} - E_{rad} \times E_{drug} \quad (2)$$

The difference between the calculated independent effect and the observed combination effect ($E_{combination}$) was then computed, called the excess over Bliss score (I, equation 3).

$$I = E_{combination} - E_{rad+drug} \quad (3)$$

Propagation of uncertainty was performed on the standard deviations from the single treatments (drug only ($\sigma_{drug}$) or radiation only ($\sigma_{rad}$)) to use for the standard deviation of the calculated independent effect ($\sigma_{rad+drug}$, equation 4) [69].

$$\sigma_{rad+drug}^2 = (1 - E_{rad})^2 \times \sigma_{drug}^2 + (1 - E_{drug})^2 \times \sigma_{rad}^2 + \sigma_{drug}^2 \times \sigma_{rad}^2 \quad (4)$$

The observed effect from the combination treatments ($E_{combination}$) was compared to the calculated independent effect ($E_{rad+drug}$) from the single treatments with a Welch's t-test (using $E_{rad+drug}$, $\sigma_{rad+drug}$, $E_{combination}$ and $\sigma_{combination}$), and correction for false discovery rate was performed according to Benjamini, Krieger and Yekutieli [70]. A synergistic or antagonistic effect was determined to be statistically significant with $p < 0.05$ and with and with $|I| \geq 0.2$.

# 4 Results

## 4.1 Fixed Annexin V/Hoechst test

Results from the 48 h staurosporine treatment on HCC-1806 cells, measured at different times after fixation is shown in Figure 8. The results indicate that the staining is stable up to a month after fixation, and going forward the assay was run at different times after fixation ranging from 0 to 28 d.

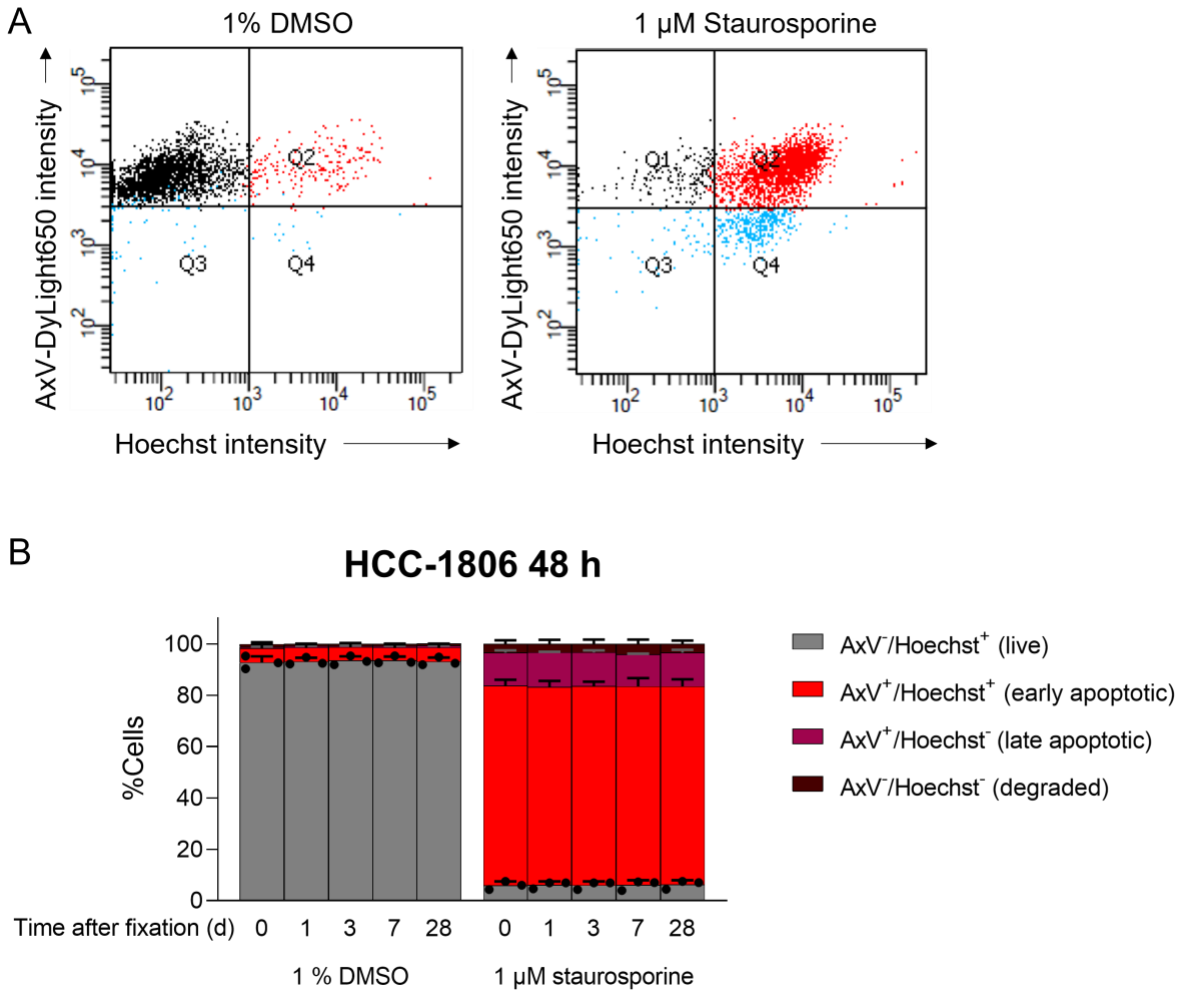


**Figure 8.** Annexin V/Hoechst measurements in HCC-1806 cells treated with staurosporine for 48 h. (A) Representative flow cytometry plots showing fluorescence intensity of cells for AxV-DyLight 650 and Hoechst. Q1, Q2, Q3 and Q4 gates correspond to cells staining Hoechst +/AxV –, Hoechst +/AxV +, Hoechst -/AxV + and Hoechst -/AxV –, respectively. (B) quantification of % cells in each of the populations gated in (A) measured at different times after fixation, n=4 technical replicates and n=3 biological replicates per time point.

## 4.2 Dose-response

Results from the irradiation dose-response study can be viewed in Figure 9. The percentage of cells undergoing apoptosis increases with time and dose for all three cell lines, except for the dose-response at 24 h after irradiation, which does not show a statistically significant dose-dependency for cell lines MCF-7 and MDA-MB-453.

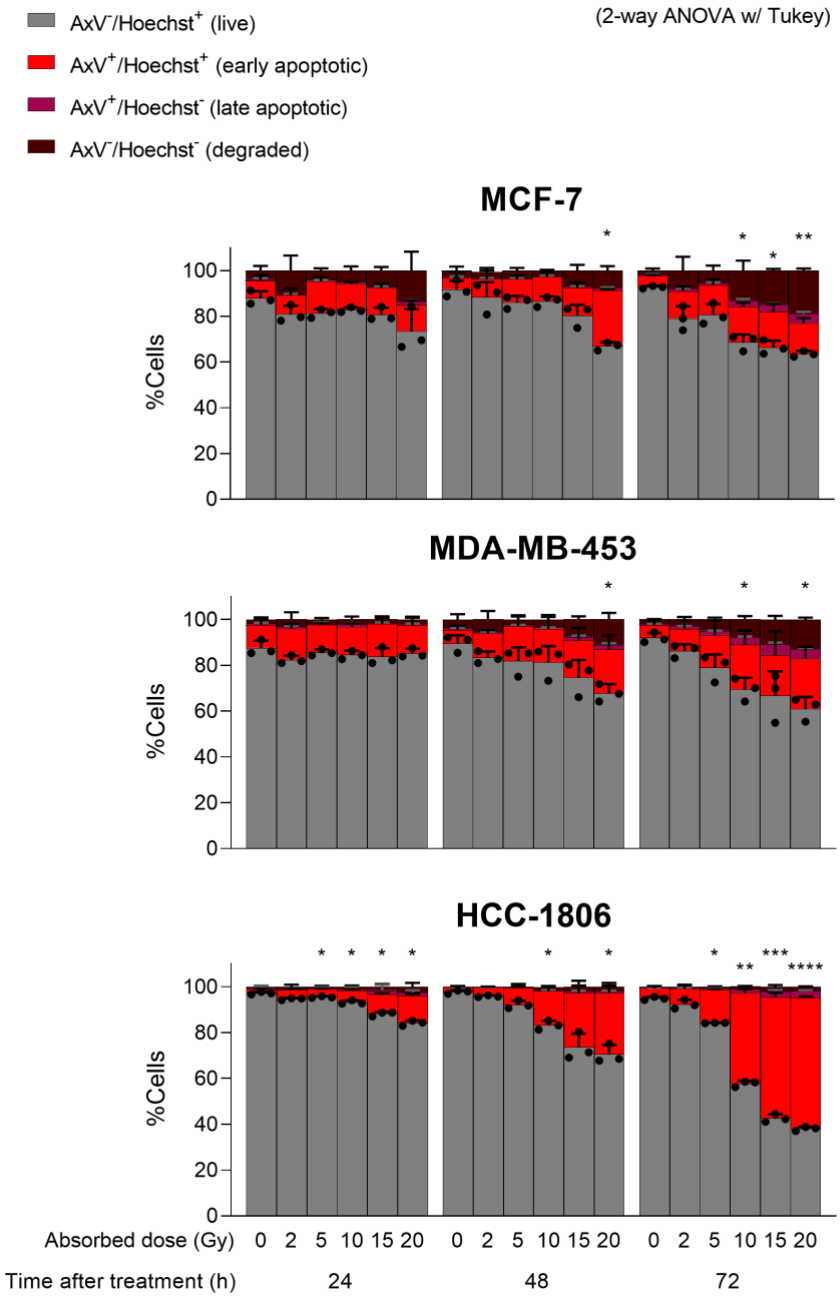


**Figure 9.** Percentage of live (gray), early apoptotic (light red), late apoptotic (dark red), and degraded (black) cells after radiation absorbed doses 0-20 Gy at time-points 24, 48 and 72 hours after irradiation. *: p<0.05, **: p<0.01, ***: p<0.001, ****: p<0.0001. Statistical comparisons were performed between the indicated absorbed dose and 0

Gy at the same time point, n=4 technical replicates and n=3 biological replicates per condition.

## 4.3 Combination treatment

The percentage of live cells (Annexin V –, Hoechst +) decreased with increasing concentration for most drugs (Figure 10). This can also be observed for the cells treated with drugs and irradiation (Figure 11). Dose-response curves for each drug with and without irradiation for the three cell lines is shown in Figures A1-A3.

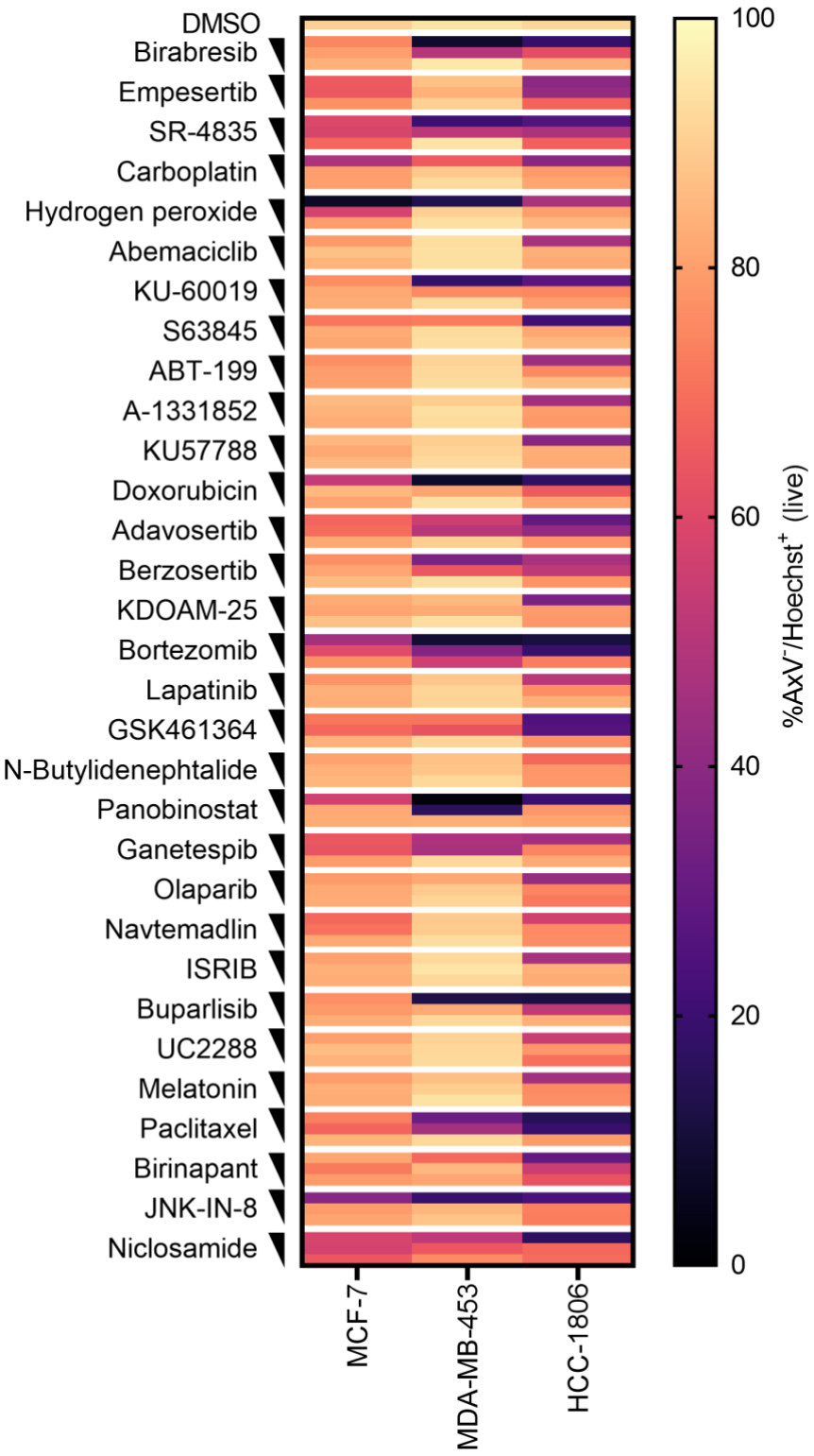


**Figure 10.** Heatmaps showing percentage of live cells after treatment with drugs at three different concentrations (Table A1) for 72 hours, n=3 biological replicates per condition.

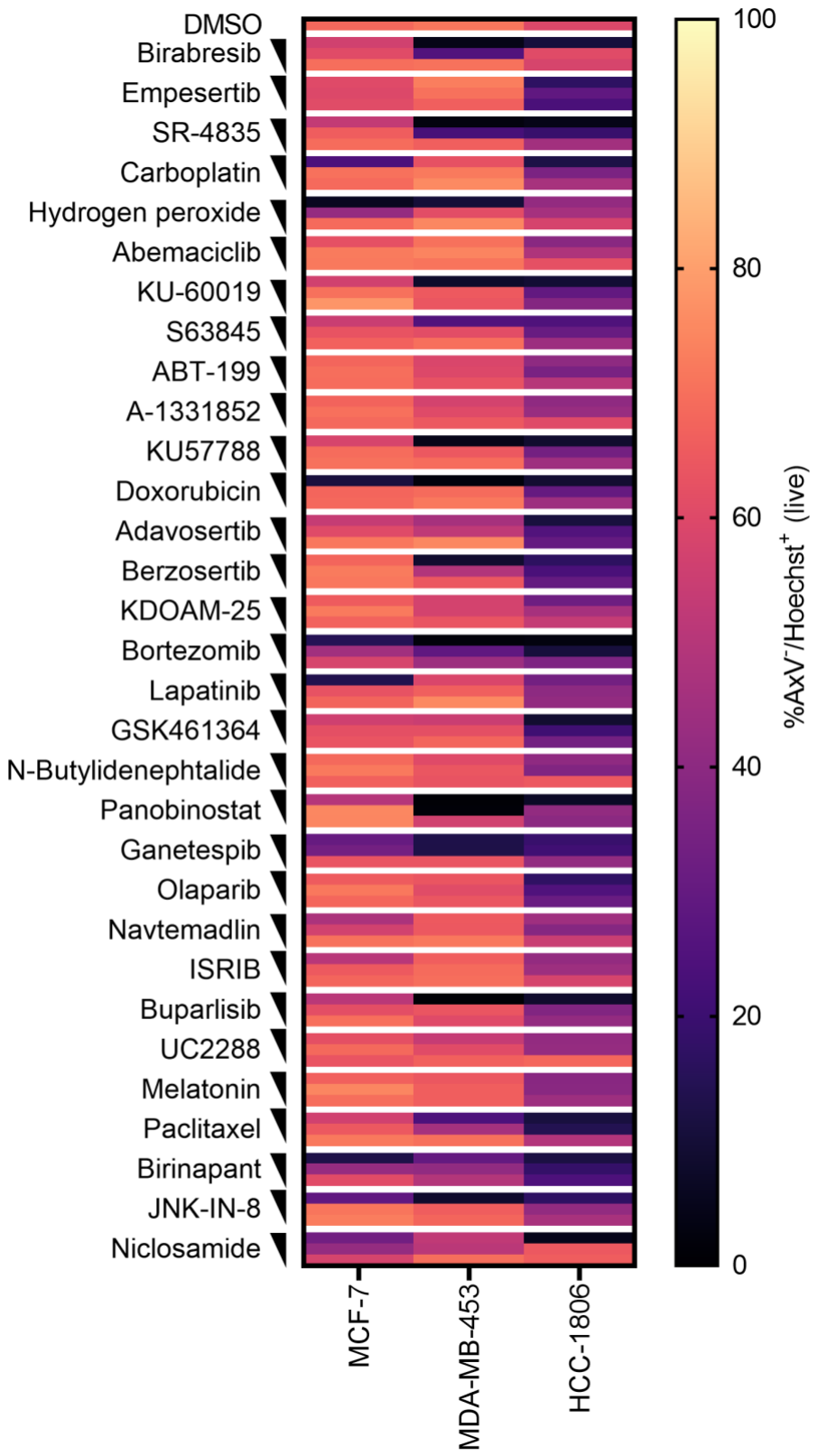


**Figure 11.** Heatmaps showing percentage of live cells after treatment with drugs at three different concentrations (Table A1) and a radiation absorbed dose of 10 Gy after 72 hours, n=3 biological replicates per condition.

In Figure 12 where the excess over Bliss score (I) can be viewed, many drugs have an antagonistic effect in combination with radiation (blue color in the figure), but many drugs also have a synergistic effect in combination with radiation (red color in the figure). A synergistic effect (I>0) was observed for 9 drugs in MCF-7, 18 drugs in MDA-MB-453, and 26 drugs in HCC-1806. All synergistic effects in MCF-7 were at the highest drugs concentration (except birinapant and ganetespib, where also the second highest concentration showed synergism). For MDA-MB-453, the drugs ABT-199, panobinostat and birinapant had a synergistic effect with radiation at all concentrations. An additional 9 drugs had synergistic effects at two concentrations. For HCC-1806, the drugs KU-60019, KU57788, berzosertib, panobinostat, ganetespib, olaparib, and birinapant had a synergistic effect with radiation at all three concentrations. The drugs birinapant, ganetespib, buparlisib, bortezomib, doxorubicin, and lapatinib had a synergistic effect for all three cell lines at some concentrations, where birinapant and ganetespib had synergism with radiation at most concentrations of all drugs.

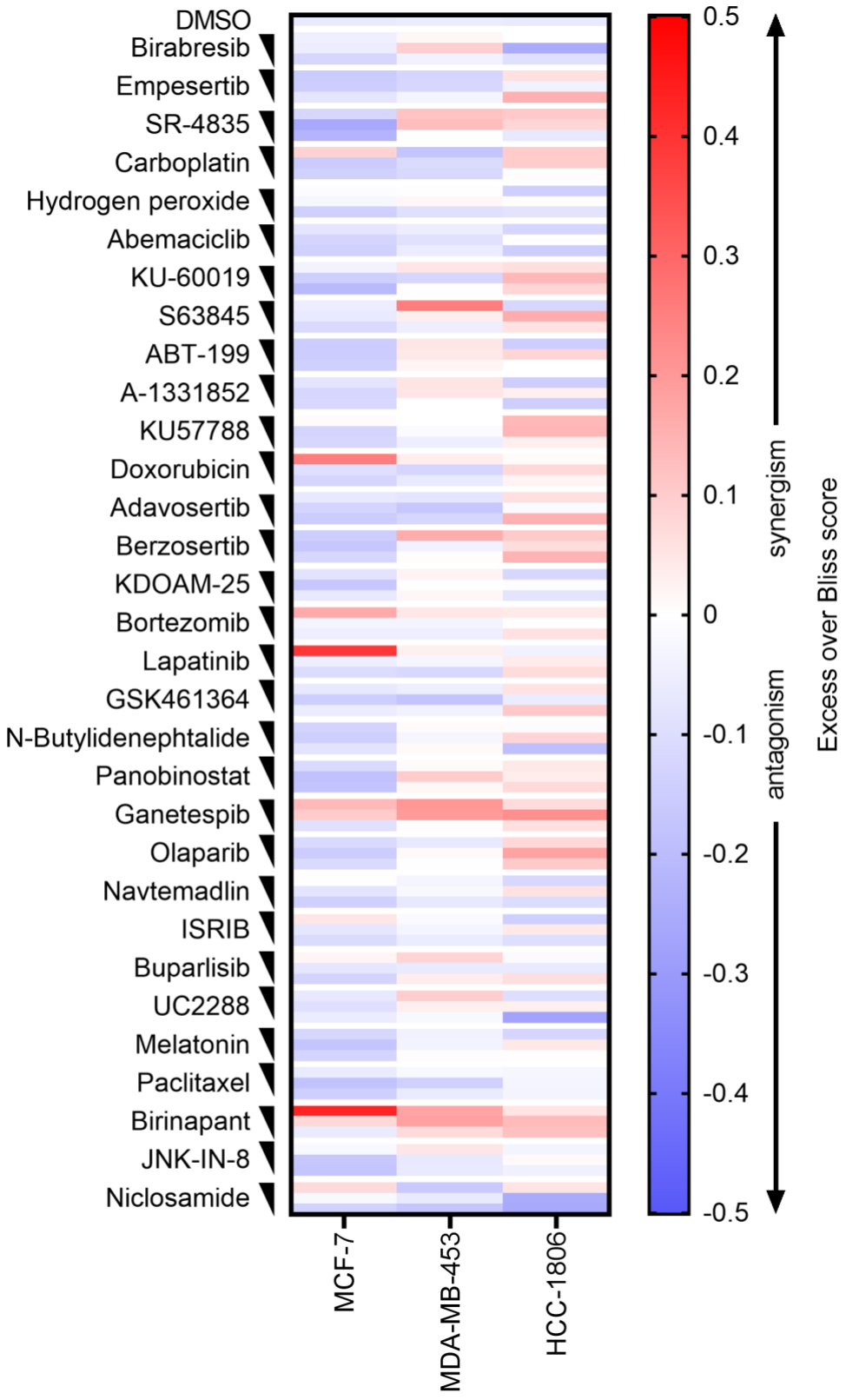


**Figure 12.** Heatmap of excess over Bliss score (equation( 2 )) reveal antagonistic and synergistic effects of drugs at different concentrations (Table A1) in combination with a radiation absorbed dose of 10 Gy after 72 hours.

Figure 13 shows a volcano plot of the excess over Bliss score for each respective cell line. The drugs birinapant and ganetespib exerted statistically significant synergistic effects with radiation for all cell lines. Additionally, the drugs bortezomib, doxorubicin and lapatinib had statistically significant synergistic effects on MCF-7, whereas the drug KU-60019 had a statistically significant antagonistic effect. No antagonistic effects were observed for MDA-MB-453, but the drugs KU57788 and S63845 displayed synergistic effects. For HCC-1806 the drugs KU-60019, KU57788, olaparib and S63845 had synergistic effects, whereas the drug niclosamide exhibited an antagonistic effect.

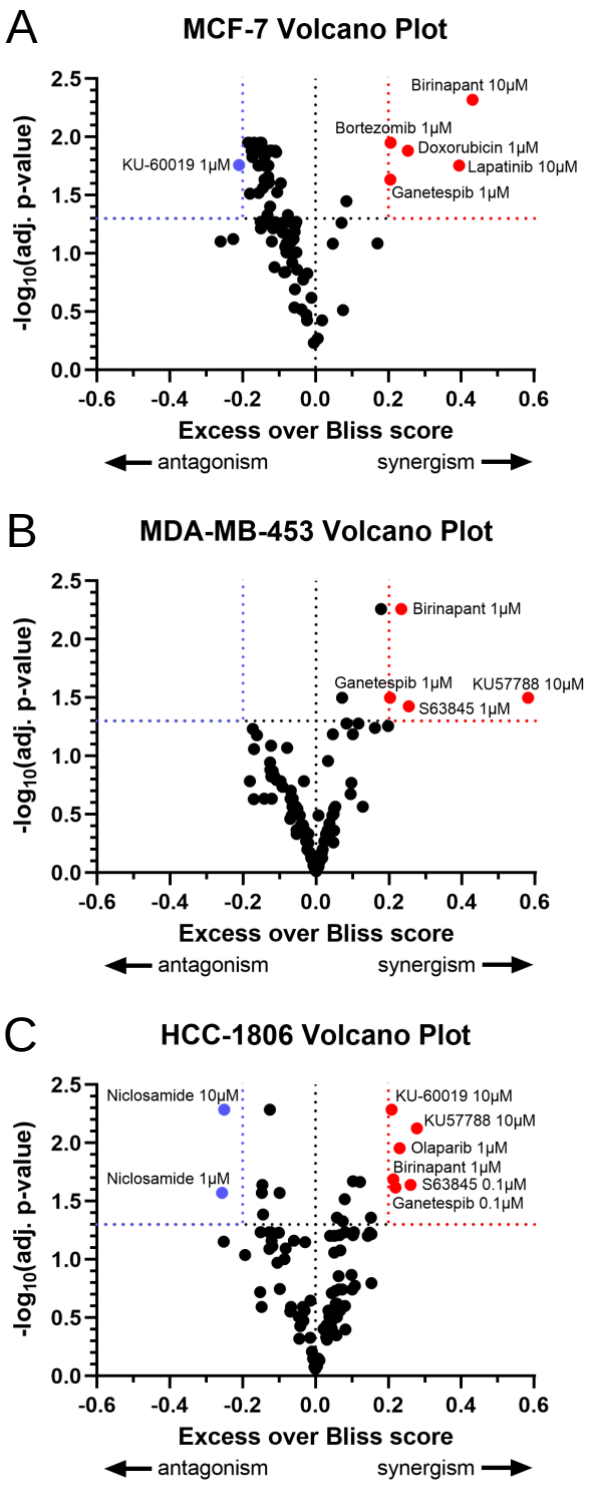


**Figure 13.** Volcano plots for respective cell line. The x-axis presents the excess over Bliss score, values below -0.2 and above 0.2 were considered antagonistic and synergistic, respectively. The y-axis presents negative log value of the adjusted p-value from the Welch's t-test, $p<0.05$ was considered statistically significant, n=3 biological replicates per condition.

# 5 Discussion

Despite continuous advances in treatment of breast cancer during the last decades [71], there is great need for better survival outcomes, especially for patients with triple negative breast cancer [72], metastatic breast cancer [73], and recurrent breast cancer [5]. Women diagnosed with metastatic triple negative breast cancer have a 5-year relative survival of merely 12 % [11], demonstrating the lack of efficient treatments. Radiotherapy is a non-invasive and common treatment of breast cancer, leading to lower mortality rates when used after breast conservative surgery [15]. Albeit only specific sites are irradiated for pain relief when the disease is spread [3], and triple negative breast cancer types and relapsed cancer are generally more radioresistant than other breast cancer types [6, 74]. One way to tackle these challenges is to combine radiotherapy with systemic therapy. If a systemic drug also functions as a radiosensitizer, more cancer cells are prone to undergo apoptosis, thereby improving the therapeutic effect [6]. This study aims to identify pathways that increase the radiosensitivity in three different human breast cancer cell lines after combination therapy with anti-cancer drugs and irradiation. Following treatment of the cells, apoptosis and cell viability were assessed by functional measurements with flow cytometry.

## 5.1 Dose-response

Apparent dose-dependent cell death was observed, where the effects increased with time (Figure 9). A more prominent effect was observed for the HCC-1806 cell line at all time-points compared with cell lines MCF-7 and MDA-MB-453. This could be due to the short time-points after the cells were irradiated, and due to the observation that HCC-1806 has a faster proliferation rate more HCC-1806 cells may undergo apoptosis. Moreover, the cell line MCF-7 does not express caspase-3 [75]. This can affect the staining with Annexin V to yield an apparent lower apoptotic response.

The dose 10 Gy at time-point 72 h killed approximately 30 % of the cells for all three cell lines, which motivated the use of these values in the following combination treatment study.

## 5.2 Drug and combination treatment

In total, 29 of 31 drugs exhibited a synergistic effect with radiation on any cell line at any concentration, indicating that it might be possible to interfere with a variety of cellular pathways to increase radiosensitivity in breast cancer cells. Many of the drugs also killed a lot of cells as a single treatment at the highest concentration (Figure 10). A drug that efficiently kills cancer cells may be preferred, but also necessitates detailed studies of its effects on normal cells. In general, the HCC-1806 cell line was more sensitive to treatment compared with the other two cell lines MCF-7 and MDA-MB-453. This could, at least in part, be due to a notably higher proliferation rate of triple negative breast cancer cells [2], which could potentially cause more cells to undergo apoptosis during the 72 hours after treatment.

Many drugs show an apparent antagonistic effect when used in combination with radiation, especially for cell line MCF-7 (Figure 12). This could be because MCF-7 does not express caspase-3, leading to less cell death by apoptosis (Figure 3). The MCF-7 cell line also had a

higher passage number at the time of combination treatment, likely making the cells more resistant to treatment. In some cases, the antagonistic effect may however be explained by the fact that a high percentage of the cancer cells die when treated with the drug alone, leaving only few additional cells to kill with radiation. Thereby the observed difference between single treatment with a drug, and combination treatment with the drug and radiation, may appear deceptively low. For example, the drug paclitaxel showed no synergistic effects with radiation at any concentration for any of the three cell lines (Figure 12), but it killed a lot of cells on its own at the two highest concentrations for cell lines MDA-MB-453 and HCC-1806 (Figure 10). The only other drug that did not exhibit any synergistic effect at any concentrations for any cell line was abemaciclib, although it did not kill as many cells on its own compared with other drugs, apart from the highest concentration for cell line HCC-1806.

Statistically significant synergistic effects with radiation were observed for five of the drugs for MCF-7, four of the drugs for MDA-MB-453, and six of the drugs for HCC-1806 (Figure 13). Birinapant and ganetespib had statistically significant synergistic effects with radiation for all three cell lines. Birinapant targets inhibitor of apoptosis protein (IAP). As the name suggests IAP is an anti-apoptotic agent for cells and acts by inhibiting caspases. Upon inhibition by birinapant cells are therefore more likely to undergo apoptosis [56]. Birinapant possibly acts in synergy with radiation through release of caspases. Caspases cleave BID, which in turn activates the intrinsic apoptotic pathway and MOMP (Figure 3). Ganetespib targets heat shock protein 90 (HSP90) [44]. HSP90 is often overexpressed in cancer cells and have an important role in regulation of apoptosis and cell proliferation [76]. When a cell is stressed, for example by ER stress through irradiation [77], HSP90 regulates protein homeostasis. Inhibition of HSP90 thus impairs the stress response of a cell, possibly leading to more cell death after treatment [78].

For cell lines MDA-MB-453 and HCC-1806 statistically significant synergistic effects were observed for the drug S63845 (a BH3 mimetic). It mimics the actions of NOXA [26], and targets MCL-1 [26] which is part of the intrinsic apoptotic pathway. This indicates that MDA-MB-453 and HCC-1806 are sensitive to intrinsic apoptotic stimuli when irradiated, perhaps suggesting that the intrinsic apoptotic pathway could constitute a possible radiosensitizing target. For MCF-7, S63845 was also the most potent BH3 mimetic tested (the other two being ABT-199 and A-1331852), indicating that breast cancers in general may be dependent on MCL-1 to buffer endogenous pro-apoptotic signals and evade apoptosis, and suggesting a potential vulnerability for MCL-1 inhibition. This has also been observed previously in other triple-negative breast cancers [79].

For the triple negative cell line HCC-1806, a synergistic effect was also observed for the drugs KU-60019, KU57788, olaparib, and S63845. The former three drugs targets the DNA repair machinery by inhibiting ATM, DNA-PK and PARP respectively [64, 80, 81]. These results thus suggest that the cell line HCC-1806 is more sensitive to radiation when DNA repair mechanisms are impaired. The synergistic effect of KU57788 was also observed for the cell line MDA-MB-453, suggesting that the DNA repair machinery is a possible target, even though not as apparent as for HCC-1806. On the other hand, the drug KU-60019 had an antagonistic effect on cell line MCF-7. This highlights the importance of individualized treatment planning based on functional precision medicine.

Lapatinib targets HER2 and is already in clinical use to treat HER2 positive breast cancer [39]. Lapatinib displays a synergistic effect at the highest concentration for the luminal A type MCF-

7 (Figure 13). Although there is typically a lack of expression of HER2 in luminal A breast cancer, studies have shown an expression of HER2 in MCF-7 cells [2]. Doxorubicin is also a clinical chemotherapy drug [37] that could be put to immediate clinical use as a radiosensitizer for breast cancer type luminal A, possibly due to its action on the DNA repair protein topoisomerase II, but it is likely to also radiosensitize normal cells due to its additional DNA damaging properties, which can lead to severe side effects on normal tissue. Rigorous toxicology studies would need to be performed before attempting a combined radiotherapy/doxorubicin treatment schedule in patients. Bortezomib also displayed a synergistic effect on cell line MCF-7. Bortezomib inhibits proteasomes, which leads to decreased capability of removing degraded proteins [66]. When cells are irradiated, proteins can get degraded [82]. The synergistic effect observed with bortezomib and radiation may therefore be attributed to a decreased ability to remove degraded proteins.

Niclosamide is an inhibitor of the proteins JAK and STAT. In this study, niclosamide showed an antagonistic effect with radiation at the two lowest concentrations for cell line HCC-1806 (Figure 13). The reason for this is unclear, since it was previously shown to radiosensitize breast cancer cells [63].

## 5.3 Future prospects

One obvious extension of the current study is to identify precise biological pathways and/or mechanisms that cause *e.g.* the drugs birinapant and ganetespib to radiosensitize breast cancer cells. Another essential line of future work would be to repeat the current study on normal cells to examine the general toxicity of potential radiosensitizers. Moreover, *in vivo* studies with the most promising drugs should be conducted. It is important to assess how the drugs function *in vivo* before starting clinical trials. Finally, this study could be repeated using several other types of breast cancer cell lines, *e.g.* luminal B breast cancer.

## 5.4 Limits of the study

There are divided opinions on whether MDA-MB-453 constitutes a HER2 or triple negative breast cancer type. Since the cell line has been shown to express HER2 [83], it was viewed as a HER2 breast cancer type in the current study. Thus, HER2 expression in these cells should be classified with *e.g.* western blot or ELISA.

Hydrogen peroxide is not a systemic treatment, it is injected intratumorally. This makes it difficult to treat metastatic breast cancer, since the tumor need to be well localized before treatment. Despite this, it is included in this study because of the usage as a clinical radiosensitizer of breast cancer in Japan [42].

Cell culture conditions were designed to enable comparison between cell lines, and so each cell line was cultured in the same medium and not necessarily in the medium recommended by ATCC. The cell lines used in this study had relatively high passage numbers, which itself may affect the results. Furthermore, the use of in vitro cell lines to evaluate cancer therapies is limited by the lack of supporting cells and an active immune system. Future studies should focus on evaluating these effects in an in vivo system.

# 6 Conclusions

Birinapant and ganetespib radiosensitized all three cell lines, suggesting that HSP90 and IAP may constitute targets for the development of drugs that radiosensitize breast cancer cells.

Cell lines HCC-1806 and MDA-MB-453 seem to be sensitive to intrinsic apoptotic stimuli in combination with radiation. Cell line HCC-1806 was also radiosensitized when the DNA repair machinery was targeted.

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# Appendix

## A1 Combination treatment

**Table A1.** Drugs used in this study, the three concentrations used to treat the cells in the combination study, and their respective catalogue number.

| Drug | Treatment concentrations [uM] | Catalogue number |
|---|---|---|
| **Doxorubicin** | 1, 0.1, 0.01 | HY-15142A |
| **Paclitaxel** | 1, 0.1, 0.01 | HY-B0015 |
| **Lapatinib** | 10, 1, 0.1 | HY-50898 |
| **Abemaciclib** | 1, 0.1, 0.01 | HY-16297A |
| **Olaparib** | 10, 1, 0.1 | HY-10162 |
| **Hydrogen Peroxide** | 1000, 100, 10 | HL10860.1000 |
| **Buparlisib** | 10, 1, 0.1 | HY-70063 |
| **Ganetespib** | 1, 0.1, 0.01 | HY-10389 |
| **Melatonin** | 100, 10, 1 | HY-B0075 |
| **Adavosertib** | 10, 1, 0.1 | HY-10993 |
| **Carboplatin** | 100, 10, 1 | HY-17393 |
| **Berzosertib** | 10, 1, 0.1 | 331-20203-1 |
| **Navtemadlin** | 10, 1, 0.1 | HY-12296 |
| **Panobinostat** | 0.1, 0.01, 0.001 | HY-10224 |
| **GSK461364** | 1, 0.1, 0.01 | HY-50877 |
| **ABT-199** | 1, 0.1, 0.01 | HY-15531 |
| **A-1331852** | 1, 0.1, 0.01 | HY-19741 |
| **S63845** | 1, 0.1, 0.01 | HY-100741 |
| **JNK-IN-8** | 10, 1, 0.1 | HY-13319 |
| **Birinapant** | 10, 1, 0.1 | HY-16591 |
| **SR-4835** | 10, 1, 0.1 | HY-130250 |
| **Empesertib** | 10, 1, 0.1 | HY-12858 |
| **UC2288** | 10, 1, 0.1 | HY-112780 |
| **Birabresib** | 10, 1, 0.1 | HY-15743 |
| **KU-60019** | 100, 10, 1 | HY-12061 |
| **N-Butylidenephthalide** | 100, 10, 1 | HY-N0336 |
| **Niclosamide** | 100, 10, 1 | HY-B0497 |
| **KU57788** | 10, 1, 0.1 | HY-11006 |
| **KDOAM-25** | 100, 10, 1 | HY-102047 |
| **Bortezomib** | 1, 0.1, 0.01 | HY-10227 |
| **ISRIB** | 1, 0.1, 0.01 | HY-12495 |

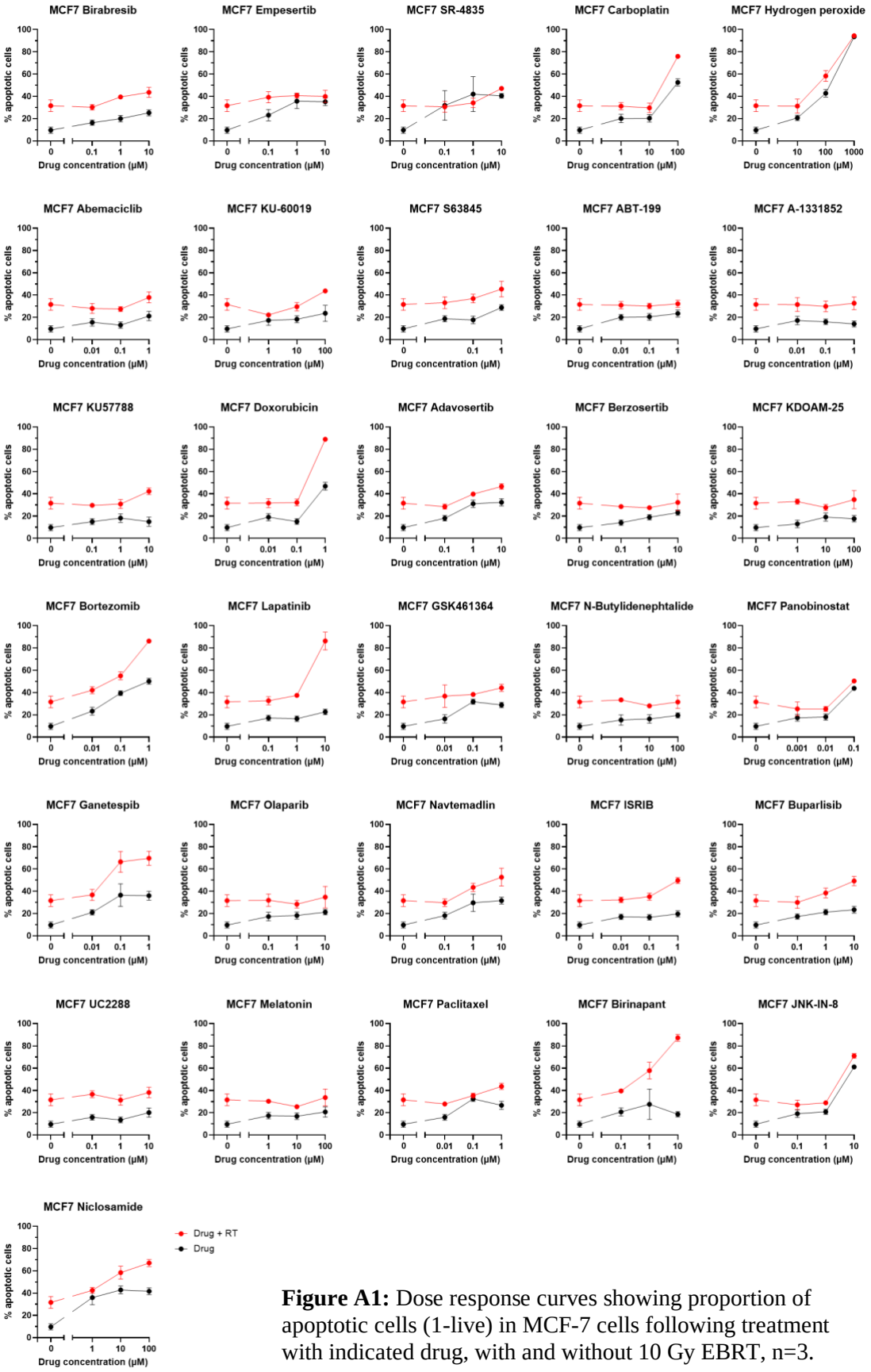


**Figure A1:** Dose response curves showing proportion of apoptotic cells (1-live) in MCF-7 cells following treatment with indicated drug, with and without 10 Gy EBRT, n=3.

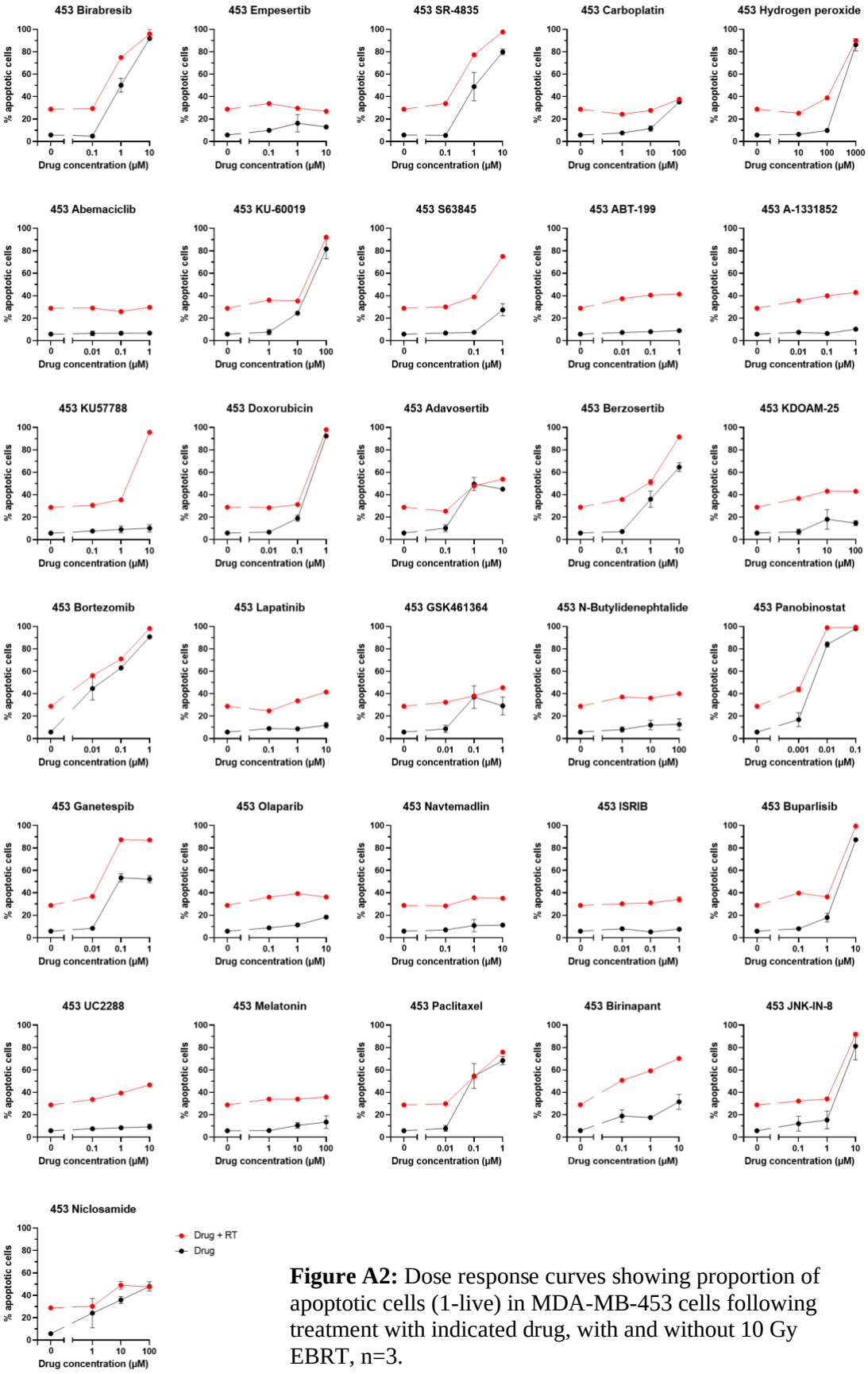


**Figure A2:** Dose response curves showing proportion of apoptotic cells (1-live) in MDA-MB-453 cells following treatment with indicated drug, with and without 10 Gy EBRT, n=3.

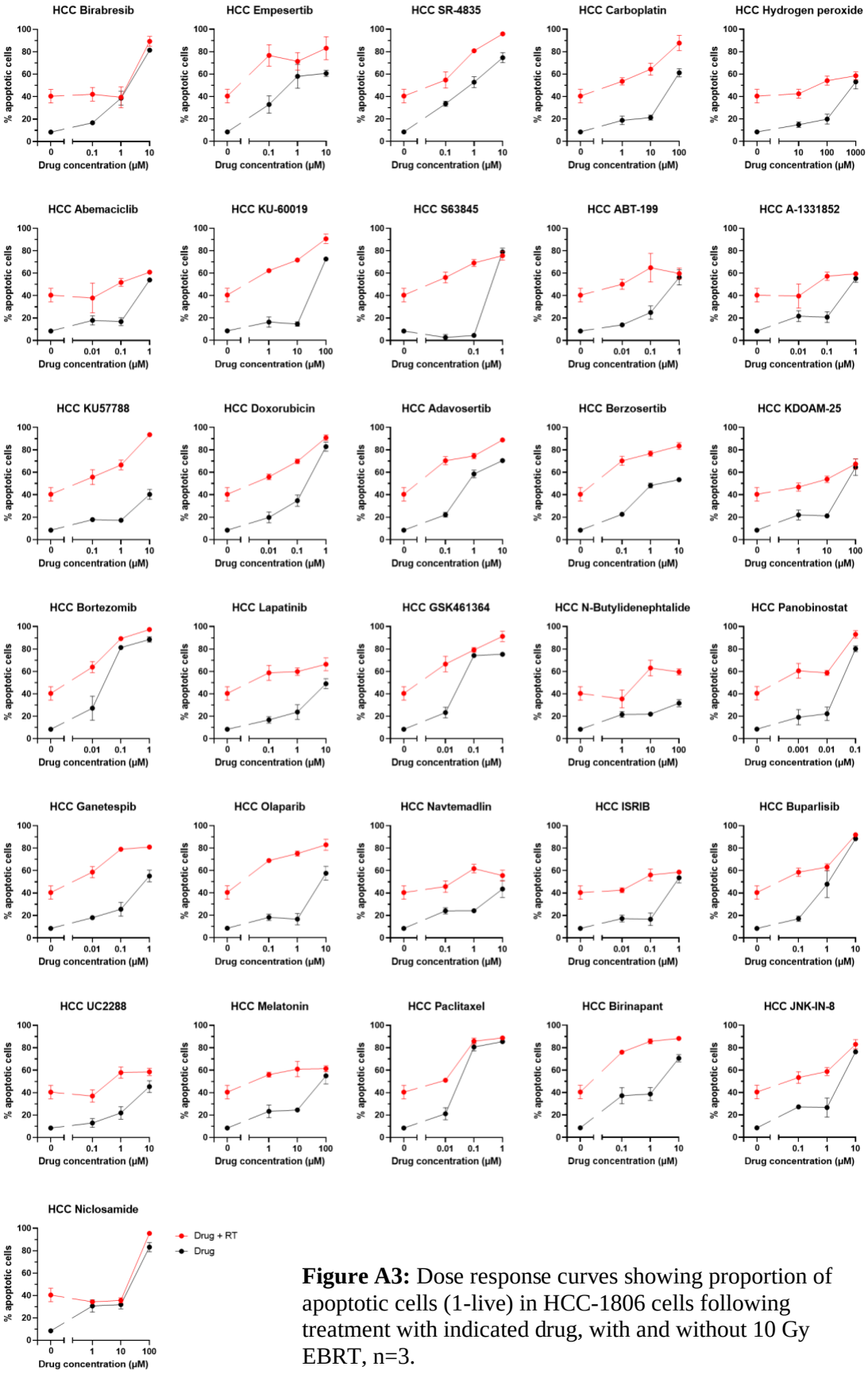


**Figure A3:** Dose response curves showing proportion of apoptotic cells (1-live) in HCC-1806 cells following treatment with indicated drug, with and without 10 Gy EBRT, n=3.

## A2 Flow cytometer settings

Plate (High throughput sampler)
FSC: 300 V
SSC: 150 V
APC: 270 V
BV421: 250 V