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Higher anti-tumor efficacy of the dual HER3-EGFR antibody MEHD7945A combined with ionizing irradiation in cervical cancer cells

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Running title: MEHD7945A enhances response to ionizing radiation in preclinical cervix cancer models

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SUMMARY

Targeting EGFR and HER3 with a specific dual antibody (MEHD7945A) enhanced irradiation efficacy *in vitro* (cervical cancer cell lines) and *in vivo* (xenografted mice) in an additive way. Moreover, our study suggests that co-expression of EGFR and HER3 could be a candidate prognostic biomarker of poor overall survival in patients with cervical cancer. These preliminary results should be confirmed in a study with a larger sample.

ABSTRACT

Purpose: The outcome of locally advanced cervical cancer (LACC) is dismal. Biomarkers are needed to individualize treatments and to improve patient outcome. Here, we investigated whether co-expression of EGFR and HER3 could be an outcome prognostic biomarker, and whether targeting both EGFR and HER3 with a dual antibody (MEHD7945A) enhanced ionizing radiation (IR) efficacy.

Methods and Materials: Expression of EGFR and HER3 was evaluated by immunohistochemistry in cancer biopsies (n=72 patients with LACC). The antitumor effects of the MEHD7945A and IR combotherapy were assessed in two EGFR/HER3-positive cervical cancer cell lines (A431 and CaSki), and in A431 cell xenografts. The mechanisms involved in tumor cell radiosensitization were also studied. The interaction of MEHD7945A, IR and cisplatin was evaluated using dose-response matrix data.

Results: EGFR and HER3 were co-expressed in only in 7 of the 22 biopsies of FIGO IVB cervix cancer. The median overall survival was 14.6 months and 23.1 months in patients with FIGO IVB tumors that co-expressed or not EGFR and HER3, respectively. In mice xenografted with A431 (squamous cell carcinoma) cells, MEHD7945A significantly increased IR response by reducing tumor growth and by increasing cleaved caspase 3 expression. In A431 and CaSki cells, the combotherapy increased DNA damage and cell death, particularly immunogenic cell death, and decreased survival by inhibiting the MAPK and AKT pathways. An additive effect was observed when IR, MEHD7945A and cisplatin were combined.

Conclusions: Targeting EGFR and HER3 with a specific dual antibody enhanced IR efficacy. These preliminary results should be confirmed in a larger sample, as well as the hypothesis that EGFR/HER3 co-expression might be a negative prognostic marker.

INTRODUCTION

Cervical cancer represents the second most frequent malignancy in women worldwide, especially in developing countries. Radiotherapy in combination with cisplatin (as radiosensitizer) is the gold-standard therapy for the locoregional management of cervical cancer, and has significantly improved patient outcome. However, a high relapse risk still persists and leads to poor overall survival (OS) (5-year OS rate: 70%) (1-3).

Many publications have suggested the implication of the epidermal growth factor receptor (EGFR) pathway in cervical cancer via EGFR overexpression or amplification, often in association with other EGFR family members, such as HER2, 3 and 4. EGFR or HER3 overexpression in locally advanced cervical cancer (LACC) is correlated with poor prognosis (4-8). Emerging evidences indicate that HER3 overexpression contributes to the acquired resistance to EGFR and HER2 targeted therapy by activating downstream pathways (9,10), and also to carcinoma cell survival following radiation (11,12). Preclinical studies on the association of MEHD7945A, a dual humanized IgG1 antibody targeting HER3 and EGFR, with ionizing radiation (IR) showed radiosensitization in head and neck (HN) and non-small cell lung cancer models (13,14). MEHD7945A safety, pharmacokinetics, pharmacodynamics and antitumor activity were assessed in a phase I study in which no dose limiting toxicity (DLT) was observed (15). When combined with chemotherapy (cisplatin/5-fluorouracil or carboplatin/paclitaxel) in patients with HN recurrent/metastatic squamous cell cancer (16), DLT was recorded in four patients, and the chemotherapy dose was reduced in 19 of 24 patients.

Here, we first assessed whether EGFR and HER3 are co-expressed in cervical cancer biopsy samples, and then we used *in vitro* and *in vivo* cervical cancer models to determine whether the IR-MEHD7945A combination could be a relevant strategy for patients with cervical cancer showing EGFR-HER3 co-expression.

METHODS AND MATERIALS

Clinical and biological database

A clinical database was retrospectively generated using the data of all patients (n=75) with cervical epidermoid carcinoma referred to xxx University Hospital from October 2001 to October 2014. Expression of EGFR and HER3 was analyzed by immunohistochemistry on all biopsies at diagnosis (n=72) and scored using the H-score. Additional details are in Supplementary Materials and Methods. The patients' and tumor characteristics are listed in Table 1. This study was approved by the Hospital Institutional Review Board.

All patients treated by radiochemotherapy (followed or not by pelvic surgery) were eligible. Most patients (87%) underwent cisplatin-based (40mg/m^2/week) chemoradiotherapy with a total dose of 45 to 50.4 Gy to the whole pelvis, and to the entire para-aortic lymph node chain for patients with para-aortic lymph node involvement. After whole pelvis external beam radiation therapy, all patients received pulsed dose rate brachytherapy, according to the GEC-ESTRO recommendations (17), and 32 patients underwent radical hysterectomy and pelvic lymphadenectomy. Pathological complete response was defined as the absence of any residual tumor after treatment. Follow-up visits were planned every 3 months after brachytherapy or definitive surgery for the first 2 years, then every 4 months for 1 year, every 6 months for the next 2 years, and annually after 5 years (median follow-up: 3.8 years; range: 3-162 months).

Cell lines and reagents

The A431 (squamous cell carcinoma) and CaSki (metastatic cervical cancer) human cell lines were from ATCC (Rockville, MD, USA) and cultured following ATCC recommendations. The MEHD7945A antibody was provided by Genentech, cisplatin was purchased from the hospital (xxx).

Ionizing radiation

A linear particle accelerator was used (Varian Medical Systems; 6MV-photons; dose rates of 200UM/min and 400UM/min for *in vitro* and *in vivo* experiments, respectively) at the xxx Radiation Oncology Department. For combotherapy, MEHD7945A was added *in vitro* 4h before, and *in vivo* 24h before IR.

Clonogenic survival assay

The ability of MEHD7945A to radiosensitize A431 and CaSki cells was assessed using a standard colony-forming assay and growth curve analysis. Cells were trypsinized, washed, and seeded in triplicate in 6-well plates one day before IR. After exposure to IR (from 0 to 8Gy) and/or MEHD7945A (1μg/ml and 100μg/ml), cells were fixed with acetic acid: methanol (1:3) at different time-points (from 9 to 12 days post-treatment) and stained with Giemsa (Sigma Chemical Co., St Louis, MO, USA). MEHD7945A concentration was chosen on the basis of the results of the cell proliferation assay (see below). The survival fraction was calculated by counting the number of colonies containing at least 50 cells relative to the number of seeded cells, and multiplied by plating efficacy (number of counted cells relative to plating cells in untreated condition).

Human tumor xenografts

All *in vivo* experiments (minimun of 7 animals per group)were performed in compliance with the xxx regulations and ethical guidelines for experimental animal studies in an accredited establishment (Agreement No. C34-172-27). A431 cells ($1x10^6$) were injected subcutaneously in the right flank of 6-week-old female athymic nude Hsd mice (Envigo, Huntingdon, UK). Tumor-bearing mice were treated with 2.5 or 10 mg/kg of MEHD7945 by intraperitoneally injection with or without IR (2 or 4 Gy), twice per week for 4 weeks. Tumor volumes were calculated with the formula: $D_1 \times D_2 \times D_3 / 2$.

Additional details about cleaved caspase 3 and Ki67 expression analysis in xenografts are in Supplementary Materials.

Cell death analysis

The Annexin A5 FITC/7-AAD Apoptosis Detection Kit (Beckman coulter, IM3614) was used according to the manufacturer's instructions to quantify apoptosis at 24h and 48h post-incubation with MEHD7945 (10 μg/ml), IR (one single dose of 2, 5 or 10Gy), or IR and MEHD7945. Data were acquired on a Gallios Flow Cytometer and analyzed with the Kaluza software (Beckman Coulter, Brea, USA).

Calreticulin membrane exposure was measured at 24h and 48h post-treatment (IR, MEHD1945A or combotherapy) using an anti-calreticulin antibody (Abcam, AlexaFluor® 647/ab196159, 1:200) and a Cytoflex cytometer (Beckman) with FlowJo 10 (Tristar). Additional details are in Supplementary Materials.

Supernatants were collected 24h, 48h and 72h after treatment (IR, MEHD1945A or combotherapy), centrifuged and pellets frozen. ATP release was detected using the ATPlite Luminescence Assay System (PerkinElmer®), according to the manufacturer's recommendations.

Assessment of γH2AX by immunofluorescence

Briefly, fixed cells were incubated with an anti-phosphorylated H2A.X (γH2AX; Ser139) antibody (1:200 in PBS-BSA, Millipore, 05-636). γH2AX foci were visualized using an epifluorescence Zeiss Imager 2 (Zeiss, Germany). The number of foci was counted in at least 100 cells. Additional details are in Supplementary Materials.

Western blotting

A431 and CaSki cells were lysed and expression of EGFR, HER3, ERK1/2, AKT, phosphorylated EGFR, HER3, ERK1/2 and AKT (antibodies from Cell Signaling Technology, Beverly, MA) assessed, as previously described (18). Equal loading was assessed with an antibody against GAPDH (Cell Signaling Technology). Band intensity was analyzed with a PXi imager (Syngene, Cambridge, UK).

Cell proliferation assay and treatments' interactions

The effect of the combination of IR, MEHD7945 and cisplatin on cell proliferation was evaluated using a sulforhodamine B (SRB) colorimetric assay, as already described (19). Growth inhibition was calculated based on the percentage of proliferating cells in treated samples relative to untreated cultures. Treatments' interactions were analyzed using dose-response matrices, according to the Bliss equation (20). Additional details are in Supplementary Materials.

Statistical analysis

Analyses of the correlation of EGFR/HER3 expression with survival included only patients with available data (N=72 patients). For clinical results, continuous variables were described using medians and ranges, and categorical variables with frequencies and percentages. The Fisher's exact test was used to compare the distribution of categorical variables. All tests were two-sided. OS and disease-free survival (DFS) were estimated using the Kaplan-Meier method, and median follow-up durations using the reverse Kaplan–Meier method. Survival curves were generated with their log-rank tests. Variables, including prognostic factors, were selected for univariable analysis. Hazard ratios (HR) were presented with 95% confidence intervals (CI).

For preclinical results, data were presented as the mean ± standard error of the mean (SEM) of triplicate experiments, if not mentioned otherwise. Groups were compared using the non-parametric Kruskal–Wallis test. Clonogenic survival was analyzed using a linear mixed model to explore the relationship between the surviving fraction and IR doses. IR doses, treatment group and their

interaction were the variables included in the fixed part of the model. Random intercepts were considered to take into account the replicate effect inside the IR dose groups.

A p-value <0.05 was considered statistically significant; multiplicity of testing was not considered due to the exploratory nature of this study. Statistical analyses were performed using STATA 13.0 (StataCorp, College Station, TX, USA).

RESULTS

EGFR and HER3 as biological targets in cervical cancer

We first analyzed EGFR and HER3 expression in cervical cancer biopsies at diagnosis (n=72; one for each patient). EGFR was overexpressed (H-scores higher than 150) in 46 samples (63.0%): 4.1% of FIGO stage IA2/IB1, 42.5% of FIGO stage IB2-IVA, and 16.4% of FIGO stage IVB tumors (p=0.018). HER3 was overexpressed (H-scores higher than 50) in 18 samples (25.0%): 5.5% of FIGO stage IA2/IB1, 4.2% of FIGO stage IB2-IVA, and 15.3% of FIGO stage IVB tumors (p<0.001). EGFR and HER3 were concomitantly overexpressed only in seven of the 22 FIGO IVB cancer specimens (32%) (Table 1). These seven patients had pelvic and para-aortic node involvement (p<0.001; Table 1). We did not find any correlation between tumor response or relapse and EGFR, HER3, or EGFR/HER3 co-expression.

Univariable analysis of common clinical prognostic factors showed that lymph node involvement, metastatic disease, and FIGO stage were significantly correlated with the patients' outcome (DFS and OS, Supplementary Table 1). As expected, due to the fact that all patients with EGFR/HER3 co-expression had a FIGO IV cancer, this co-expression was also significantly correlated with the 3-year OS (HR=4.27; 95%CI=1.38 – 13.19; p=0.006. Fig. 1; Supplementary Table 1) and DFS (HR=4.23; 95%CI=1.37 – 13.00; p=0.006) (Supplementary Table 1). However, in multivariable analysis, only para-aortic lymph node involvement remained significantly correlated with outcome (DFS and OS, Supplementary Table 2). Nevertheless, in the FIGO IVB subgroup, the median OS

was 14.6 and 23.1 months (p=0.317, Log-rank test) and the median DFS was 9.5 and 11.3 months (p=0.662, Log-rank test) in patients with and without EGFR/HER3 co-expression, respectively.

MEHD7945 increases the response to radiation in A431 cell tumor xenografts

Then, to investigate whether the IR-MEHD7945A combination could be a relevant strategy, particularly for cervical cancer showing EGFR-HER3 co-expression, we used A431 and CaSki cells. Immunocytochemistry and cytometry (Supplementary figure 1) showed that HER3 was mildly expressed in both cells lines, whereas EGFR expression was stronger in A431 than in CaSki cells.

In mice xenografted with A431 cells (at least 7 mice/group), tumor volume was significantly reduced and the median survival increased after combotherapy (IR=4Gy; MEHD7945=10 mg/kg) compared with IR alone (63 versus 34 days; p=0.079) (Fig. 2A and supplementary figure 2A). Due to the high rate of radiation-induced toxicity (weight loss), we performed a second experiment using lower IR dose and antibody concentration (IR=2 Gy; MEHD7945A=2.5 mg/kg). Survival was significantly higher after combotherapy than IR alone (42 vs 28 days; p=0.0408) (Fig. 2B and supplementary figure 2B). Moreover, intra-tumor apoptosis (percentage of cleaved caspase 3-positive cells) was higher in the combotherapy group compared with IR alone and untreated controls (Fig. 2C). Conversely, the fraction of KI67-positive cells was not significantly different (supplementary figure 2C).

Effect of combotherapy on cell survival, cell proliferation, DNA damage and EGFR/HER3 signaling

We then evaluated *in vitro* the combotherapy effects by measuring the clonogenic survival in A431 and CaSki cells after 10 days of exposure to MEHD7945A and/or IR (Fig. 3A). Compared with IR, the combination of MEHD7945A (10 μg/ml) with IR significantly reduced the surviving fraction (p= 0.004 for A431; p<0.001 for CaSki) while 1μg/ml of MEHD7945A with IR did not have any

effect (p=0.334 for A431; p=0.854 for CaSki cells). In addition, the MEHD7945A (1µg/ml) and IR (10Gy) combination had the strongest inhibitory effect on A431 and CaSki cell proliferation (cell number after 72h of treatment) (Fig. 3B).

Analysis of DNA damage (γH2AX foci) (Fig. 3C) indicated that the number of γH2AX foci per cell was significantly increased at 1h after combotherapy compared with IR alone (p=0.0015 for A431; p=0.0001 for CaSki) or MEHD7945A alone (p<0.0001).

Finally, analysis of EGFR and HER3 signaling by western blotting (Fig. 3D and supplementary figure 3) showed that in untreated cultures, EGFR and HER3 phosphorylation levels were low in A431 cells and high in CaSki cells. In A431 cells, IR alone increased EGFR and HER3 phosphorylation starting at 15min and until 240min post-IR. Conversely, after co-exposure to IR and MEHD7945A, EGFR phosphorylation level was lower than in untreated cells, and HER3 phosphorylation was completely inhibited as well as MAPK and AKT phosphorylation. In CaSki cells, IR did not modulate EGFR and HER3 phosphorylation compared with untreated cells. Conversely, co-exposure to IR and MEHD7945A inhibited HER3, AKT and MAPK phosphorylation, but had no effect on EGFR phosphorylation. Finally, differently from the IR-MEHD7945A combination that inhibited both the EGFR and HER3 pathways, the association of cetuximab and IR had only a slight effect on HER3 and AKT phosphorylation (supplementary figure 4).

Combotherapy increases tumor cell death

Analysis of early and late apoptosis in A431 cells exposed to IR (2, 5, or 10 Gy) and/or MEHD7945A indicated that at 24h post-exposure, apoptosis was higher in cells incubated with MEHD7945A (mean=19.5%) than in untreated cells (p= 0.0209) and irradiated cells, regardless of the IR dose (from 6.5 to 10.5%) (Fig. 4A). Conversely, at 48h, the apoptosis rates induced by 10Gy and MEHD7945A were comparable (27%). Compared to IR, combotherapy increased both early and late apoptosis/necrosis rates, especially at 24h and 48h (Fig. 4A). We obtained similar results in

CaSki cells (supplementary figure 5A). This effect was associated with an increase of cleaved caspase 3 expression in IR (4 Gy) and MEHD7945A (10μg/mL)-treated cells (Fig. 4B).

Then, to determine the immunogenic cell death role, we measured calreticulin exposure on the cell surface and ATP release. At 48h, calreticulin translocation was increased in A431 cells incubated with MEHD7945A alone (1μg/mL), but not with IR alone (different doses) (Fig. 4C). The MEHD7945A and IR combination significantly increased calreticulin exposure, but only at high IR dose (10Gy). We obtained similar results in CaSki cells (supplementary figure 5B).

Compared with untreated cells, IR alone (but not MEHD7945A alone) significantly increased ATP release in a dose-dependent manner (Fig. 4D). Compared with IR alone, ATP release was increased at 24h post-combotherapy, but not at later time points. These results suggest that IR and the combotherapy could induce immunogenic cell death.

Association of irradiation, MEHD7945A and cisplatin

As cisplatin-based chemotherapy combined with radiotherapy is the gold standard for cervical cancer, we assessed whether the combination of IR, MEHD7945A and cisplatin had a synergic or additive effect. After incubation of A431 and CaSki cells with increasing doses of IR, MEHD7945A and cisplatin for 5 days (Fig. 5), their combination had mainly an additive effect.

DISCUSSION

Our study shows that EGFR and HER3 were co-expressed only in aggressive and advanced cervical cancer (FIGO stage IVB tumors). The too small number of EGFR- and HER-positive tumor samples did not allow determining whether their co-expression is a candidate prognostic marker, and in our series only para-aortic nodal involvement was an independent prognostic factor. In a recent large cohort of patients with cervical cancer (n=401) and mainly treated by surgery alone (74%), HER2 amplification (tissue micro-array analysis) was associated with poor patients outcome, but only tumor size, vascular space invasion and histologic type were independent prognostic factors (21). In the study by Fuchs and coworkers in which 50% of patients were treated by surgery alone and 33% by radiotherapy alone (n=78) (6), 63% of tumor samples overexpressed EGFR and 74.4% HER3 (HER1-4 co-expression was not studied). EGFR overexpression was associated with higher survival, and HER3 overexpression with poor survival. Conversely, a recent translational study within a phase II clinical trial found that tumor EGFR expression was not correlated with the treatment response or patient outcome (22). In our cohort, EGFR/HER3 co-expression was only observed in FIGO IVB cancer samples that are known to have a poor outcome. Yet, in the FIGO IVB subgroup, outcome seemed to be worse in patients with EGFR/HER3 co-expressing tumors. A larger cohort is required to confirm this finding and to limit the possible selection bias.

Besides the balance between HER1-4 receptors (6), patient outcome is influenced also by the presence of Human Papillomavirus (HPV) that regulates HER3 expression (23,24). Fahkry and colleagues recently showed that persistent oral HPV DNA detection is associated with poor outcome in patients with HN cancer (25). The lack of data on HPV status is another limitation of our study in addition to the very small number of patients with tumors showing HER3 and EGFR co-expression.

Our preclinical results on the ability of MEHD7945A to radiosensitize EGFR/HER3-co-expressing cervical cancer cells lines are consistent with those previously reported in lung and HN cancers

(13,14). Compared with single treatments, MEHD7945A with IR induced efficiently calreticulin exposure and ATP release, suggesting that this combotherapy might trigger immunogenic cell death. Similarly, inhibition of EGFR interaction with cetuximab (alone or in combination with chemotherapy) induces calreticulin exposure (26), and anti-EGFR antibodies induce immunogenic apoptosis of tumor cells (27). In cervical cancer, radiochemotherapy has a significant role in immune response activation through immune cell activation within the local tumor microenvironment (28), and cisplatin also induces apoptotic tumor cell death in a JNK-dependent manner (29). As our results showed a synergic effect of the combination of a small dose of MEHD7945A, cisplatin and IR, their association could improve patients' outcome by increasing tumor control and by activating the immune response.

Although in the MEGHAN trial, the outcome of patients with recurrent/metastatic HN cancer who progressed after at least one line of cisplatin-based chemotherapy was not improved by MEHD7945A compared with cetuximab (30), McKnight and colleagues (31) suggested that EGFR and HER3 inhibition should be considered in patients resistant to EGFR treatment. As our results showed a synergic effect of the combination of MEHD7945A, cisplatin and radiotherapy, we propose to assess MEHD7945A as a radiosensitizer. Moreover, our data indicate that MEHD7945A could be effective at low dose. This might reduce the occurrence of adverse events when combined with radiochemotherapy, compared with the MEGHAN trial. Furthermore, it is well described that blocking one HER receptor can be compensated by activation of another HER family member, particularly HER3 that has been identified as a key contributor to resistance to anti-EGFR or -HER2 therapy (32). MEHD7945A capacity to overcome this type of resistance has been already reported for non-small lung carcinoma (14). We found that MEHD7945A combined with IR can inhibit HER3 signaling, particularly in CaSki cells that are derived from HPV-positive cervical cancer and in which basal HER3 phosphorylation is very high, probably due to a PI3KCA mutation that strongly induces HER3 and AKT activity. Conversely, EGFR phosphorylation was inhibited only in A431 cells, but not in CaSki cells that show high basal EGFR phosphorylation and harbor super-

enhancers in the proto-oncogene *EGFR* (33). Despite this difference, the MEHD7945A-IR combination had a strong effect on cell viability and proliferation in both cell lines, confirming the importance of HER3 in MEHD7945A anti-proliferative effect.

The MEHD7945A-IR combination also increased cell apoptosis (both *in vitro* and *in vivo*) and γH2AX expression in A431 and CaSki cells. These results indicate that MEHD7945A promotes the radiation response by modulating DNA damage-induced cell death.

Finally, in HPV-positive HN cancer, HER3 overexpression is associated with worse prognosis, and HER3 targeting inhibits growth of HPV-positive cell lines (24). Furthermore, as the development of cervical malignant squamous cell lesions is commonly related to HPV infection, our preclinical and clinical results suggest the possible use of the MEHD7945A-IR combination in cervical cancer. These preliminary results should be confirmed in a larger sample before transfer to clinical development.

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FIGURE LEGENDS

Figure 1: EGFR and HER3 are relevant targets in cervical cancer.

(A) Analysis of EGFR and HER3 expression by immunohistochemistry in LACC tumor biopsies collected at diagnosis; and (B) overall survival in patienst with LACC classified according to the tumor EGFR and/or HER3 expression (n=72).

Figure 2: **The association of MEHD7945A with irradiation increases survival of mice xenografted with A431 cancer cells.**

(A and B) Nude mice were xenografted subcutaneously with A431 cells and treated with MEHD7945A (MEHD), irradiation (IR) or combotherapy (MEHD+IR) twice per week for 4 weeks. Different doses of each treatment were tested (A and B). Tumor volumes are presented as the mean ± SEM for each group (n=12 animal per group) and survival as Kaplan Meier curves. The number at risk at each time-point is shown. (C) After 4 weeks of treatment (MEHD7945A 2.5mg/kg; IR 2Gy), 4 mice were killed and tumors collected for cleaved caspase 3 analysis by immunohistochemistry.

Figure 3: Effect of the MEHD7945A and irradiation combination on the cell surviving fraction, cell proliferation, DNA damage and HER signaling in A431 and CaSki cells. (A) Clonogenic assay: A431 and CaSki cells were exposed to IR (from 0 to 6Gy) and to MEHD7945A (1 and 10 µg/ml), and 10 days later, the surviving fraction was determined. (B) Cell proliferation assay: A431 and CaSki cells were exposed to MEHD7945A (1µg/ml) and/or IR as indicated, and 3 days later they were counted. Data are the mean±SEM. (C) DNA damage in A431 and CaSki cells after exposure to MEHD7945A (10µg/ml) and/or IR (2Gy) was assessed by quantifying the number of γH2Ax foci per cell. (D) Western blot analysis of the EGFR and HER3 pathways in A431 and CaSki after IR (4 Gy) alone or with MEHD7945A (10µg/ml).

Figure 4: **The association of MEHD7945A with irradiation induces apoptosis and immunogenic cell death.**

(A) Early and late apoptosis were analyzed by Annexin A5/7AAD staining in A431 cells at 24h and 48h post-treatment with MEHD7945A (1µg/ml) and/or irradiation. (B) Cleaved and total caspase 3 expression by western blotting in A431 cells after exposure to MEHD7945A (10µg/ml) and/or irradiation (4 Gy). GAPDH was used as loading control. Quantification of each protein band was normalized to GAPDH and then represented as fold-change relative to untreatd cells. (C and D) Immunogenic cell death analysis by quantifying calreticulin exposure on the cell surface (C) and ATP release (D) in A431 cells exposed to MEHD7945A (1µg/ml) and/or irradiation. Results are presented as the fold-change relative to untreated cells.

Figure 5: Synergy of the association of irradiation, cisplatin and MEHD7945A.

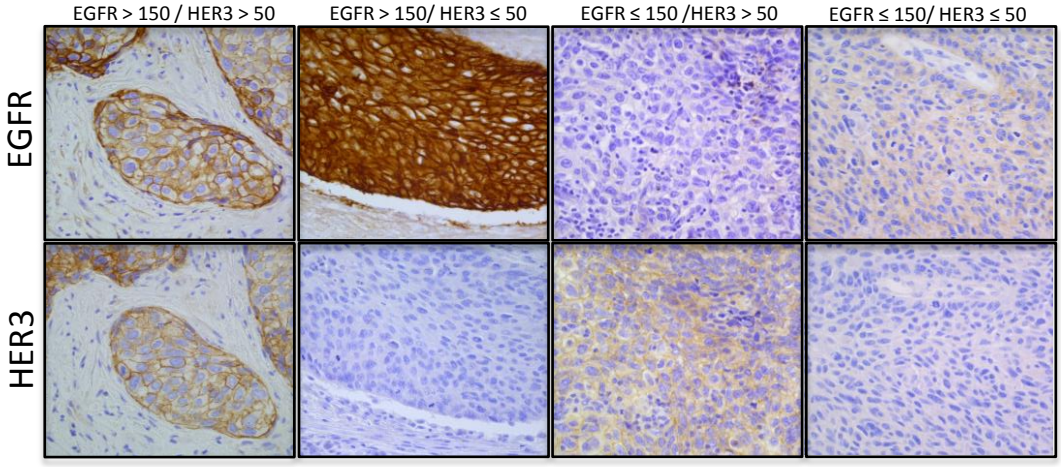
A431 and CaSki cells were exposed to the association at the indicated concentrations for five days and then cell proliferation was measured using the SRB colorimetric assay (n=3). Results are presented as the percentage relative to untreated cells. The interactions between the tested drugs were addressed using dose-response matrices.

Table 1. Patients' characteristics

		No EGFR/HER3 co-expression (n=65)	EGFR/HER3 co-expression (n=7)	*p*-value
Age (years)	Median, [Min-Max]	48 [27-82]	61 [35-69]	0.22
Tumor FIGO stage	IA2	1 (1.5)	0 (0)	0.04
	IB1	9 (13.9)	0 (0)	
	IB2	1 (1.5)	0 (0)	
	IIA	5 (7.7)	0 (0)	
	IIB	25 (38.5)	0 (0)	
	IIIA	2 (3.1)	0 (0)	
	IIIB	3 (4.6)	0 (0)	
	IVA	4 (6.2)	0 (0)	
	IVB	15 (23.1)	7 (100)	
Lymph node staging	N0	30	0	<.001
	Pelvic N+/para-aortic N0	16	0	
	Pelvic N+/para-aortic N+	14	7	
	Missing	5	0	
Chemoradiotherapy	Exclusive	26 (40.0)	3 (42.9)	0.003
	Followed by definitive surgery	31 (47.7)	1 (14.3)	
	After surgery	1 (1.5)	3 (42.9)	
Surgery first		7 (10.8)	0 (0)	
Missing		0	0	
Pathological response to chemoradiotherapy (histology on surgery specimen) N=32	Complete	15 (46.9)	0 (0)	1.000
	Partial	17 (53.1)	1 (100)	

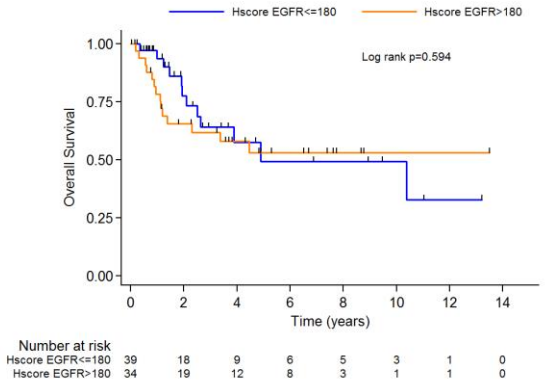
A.

Co-expression EGFR / HER3	N=75	%
EGFR ≤ 150 / HER3 ≤ 50	16	22.22
EGFR ≤ 150 / HER3 > 50	11	15.28
EGFR > 150 / HER3 ≤ 50	38	52.78
EGFR > 150 / HER3 > 50	7	9.72
Missing	3	

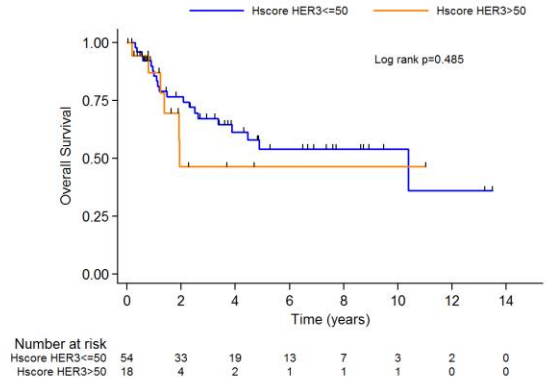


B.

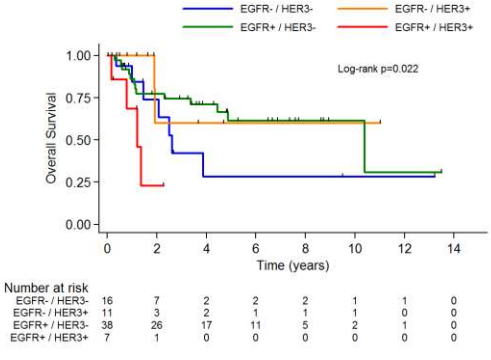
EGFR



HER3

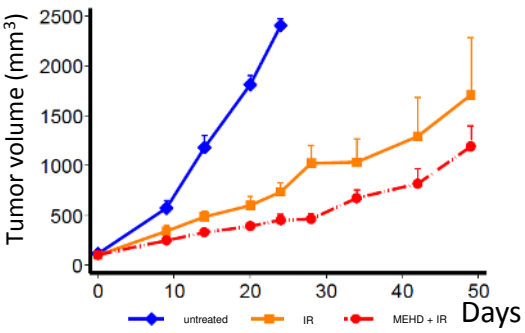


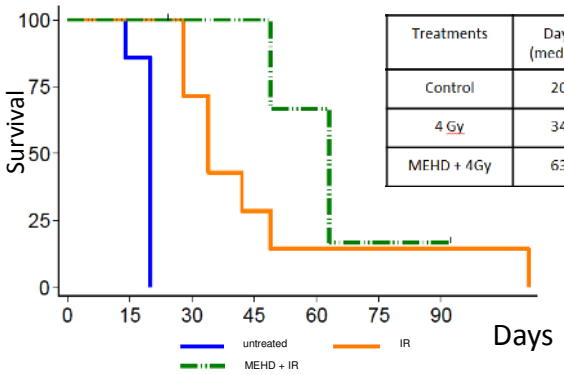
Co-expression



	Event	OS rate at 3 years	Hazard ratio	95% CI
EGFR / HER3 Co-expression				
EGFR- / HER3-	7/16	42.2	1	
EGFR- / HER3+	2/11	60.0	0.40	[0.08;1.91]
EGFR+ / HER3-	13/38	74.4	0.53	[0.21;1.33]
EGFR+ / HER3+	4/7	22.9	2.60	[0.73;9.24]
			p* = 0.022	
EGFR / HER3 Co-expression				
NEGATIVE	22/65	67.5	1	
POSITIVE	4/7	22.9	4.27	[1.38;13.19]
			p* = 0.006	

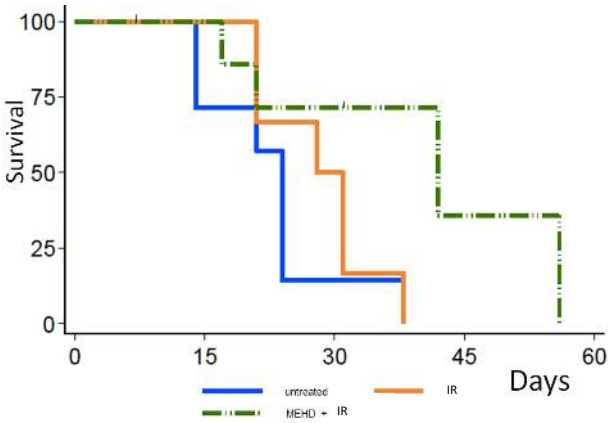
(A) MEHD7945A (10mg/kg) and IR (4Gy)





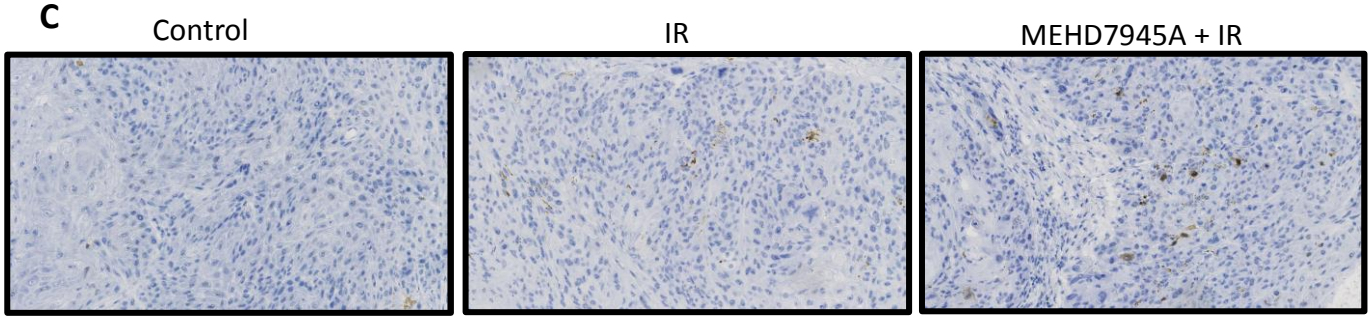
Treatments	Days (median)	Gain (%)
Control	20	
4 Gy	34	+14
MEHD + 4Gy	63	+43

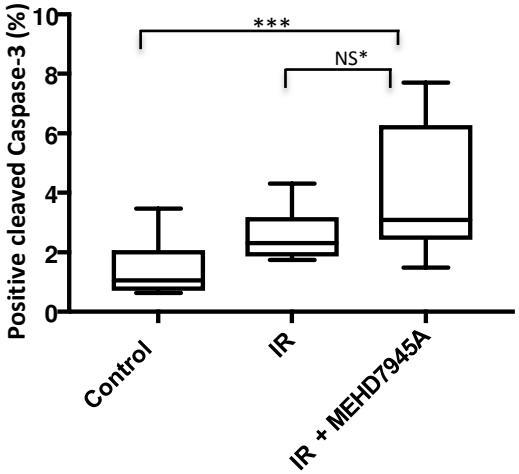
(B) MEHD7945A (2.5mg/kg) and IR (2Gy)



Treatments	Days (median)	Gain (%)
Control	24	
2 Gy	28	+4
MEHD + 2Gy	42	+18

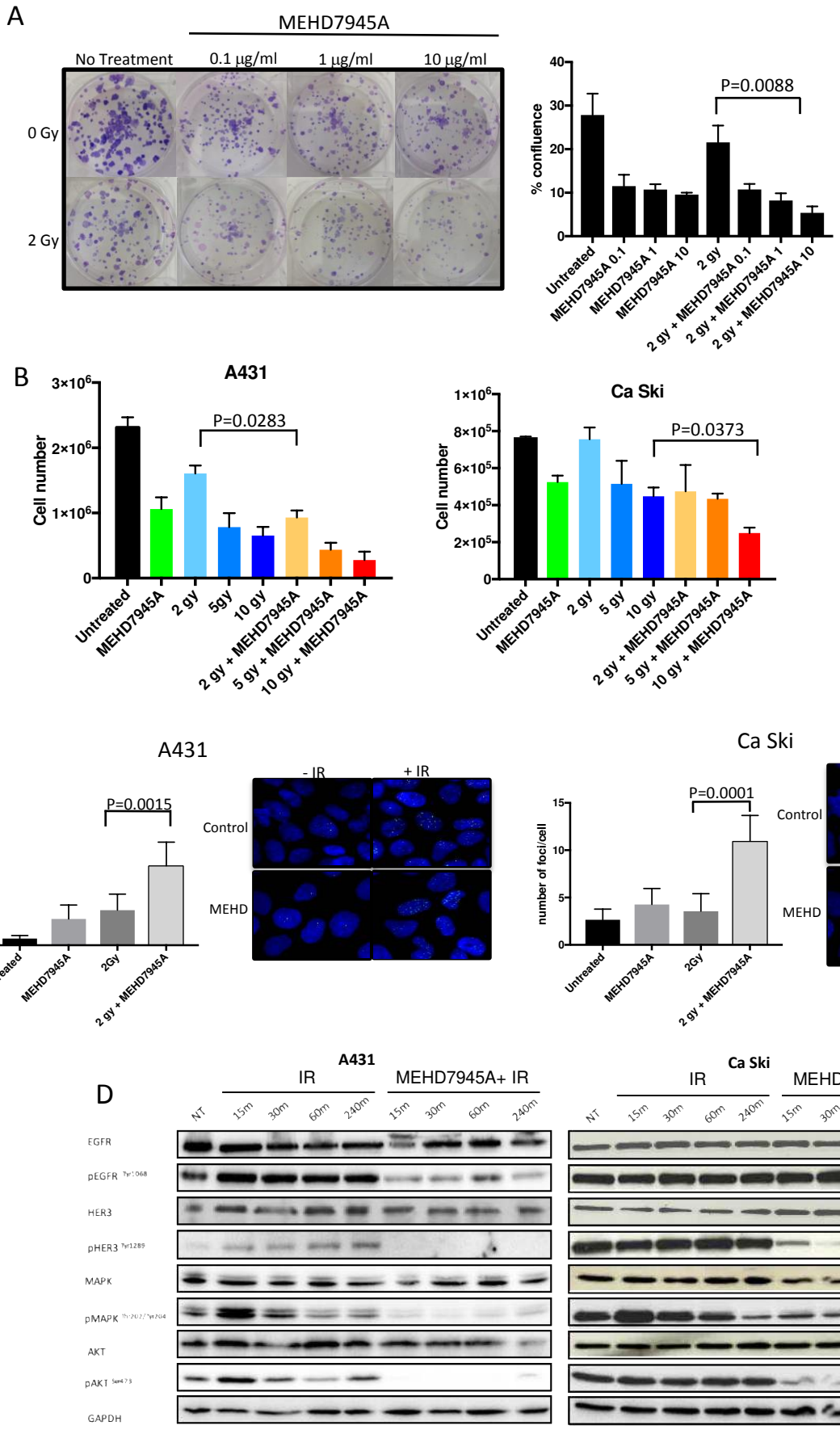
C

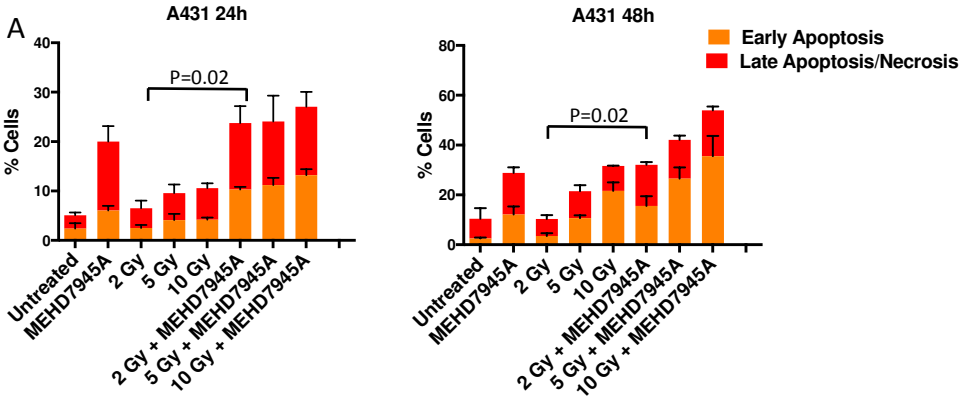


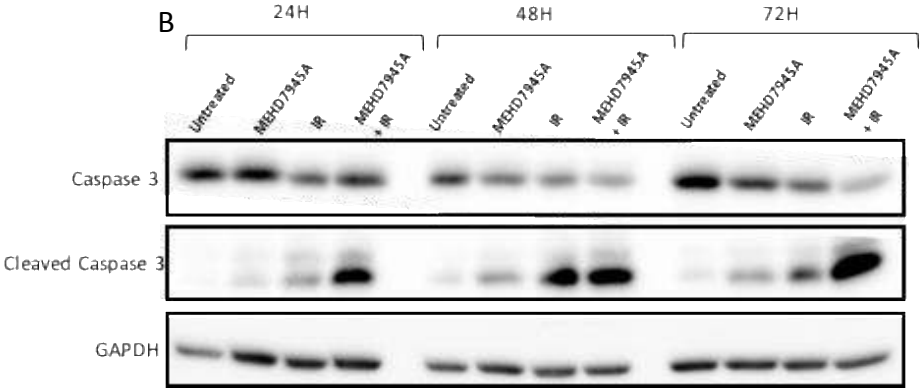


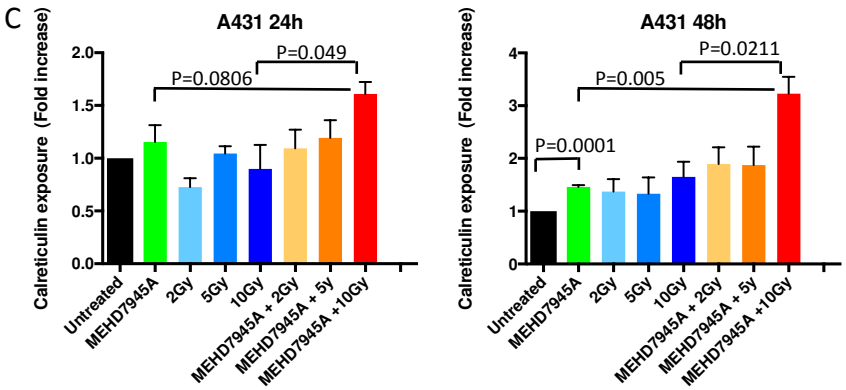
*** p=0.0001

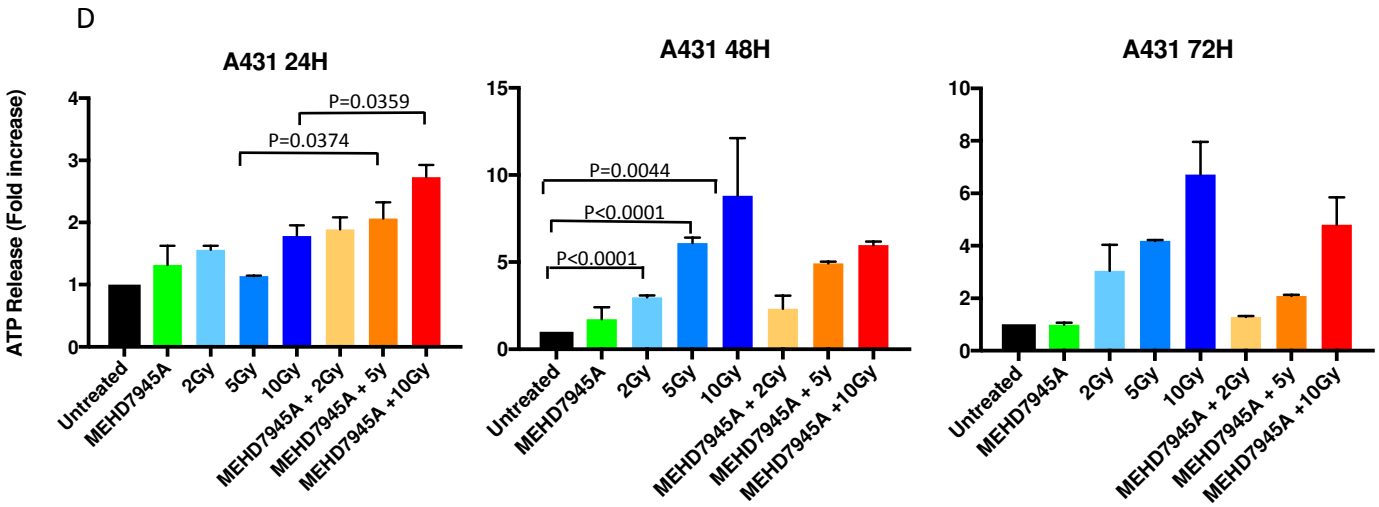
* p=0.0550


A
MEHD7945A
No Treatment
0.1 µg/ml
1 µg/ml
10 µg/ml
0 Gy
2 Gy
% confluence
P=0.0088
Untreated
MEHD7945A 0.1
MEHD7945A 1
MEHD7945A 10
2 gy
2 gy + MEHD7945A 0.1
2 gy + MEHD7945A 1
2 gy + MEHD7945A 10
B
A431
Cell number
P=0.0283
Untreated
MEHD7945A
2 gy
5gy
10 gy
2 gy + MEHD7945A
5 gy + MEHD7945A
10 gy + MEHD7945A
Ca Ski
P=0.0373
C
A431
number of foci/cell
P=0.0015
Untreated
MEHD7945A
2Gy
2 gy + MEHD7945A
- IR
+ IR
Control
MEHD
Ca Ski
P=0.0001
D
A431
IR
MEHD7945A+ IR
Ca Ski
NT
15m
30m
60m
240m
EGFR
pEGFR
HER3
pHER3
MAPK
pMAPK
AKT
pAKT
GAPDH

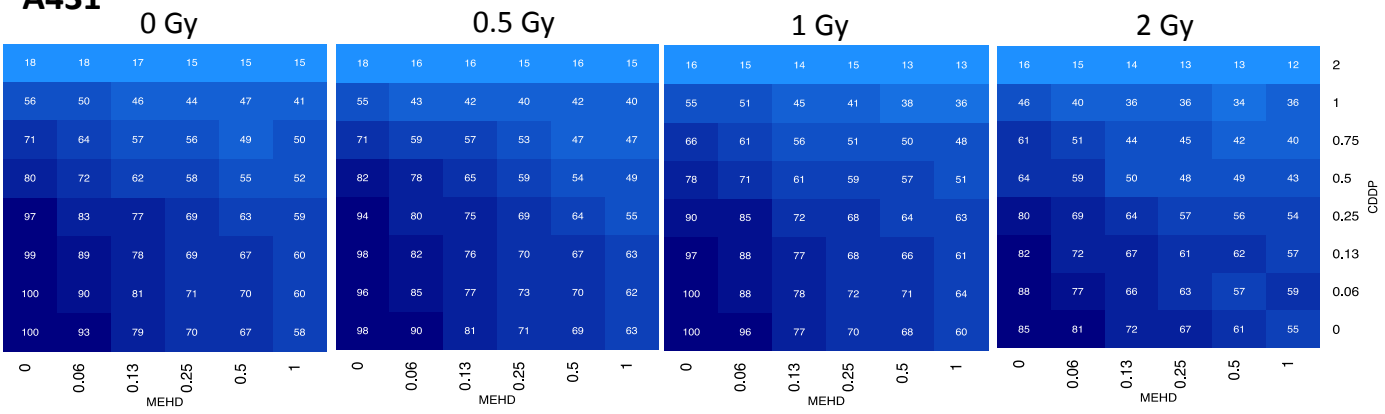

A
A431 24h
A431 48h
Early Apoptosis
Late Apoptosis/Necrosis
% Cells
P=0.02
P=0.02
Untreated
MEHD7945A
2 Gy
5 Gy
10 Gy
2 Gy + MEHD7945A
5 Gy + MEHD7945A
10 Gy + MEHD7945A


B
24H
48H
72H
Untreated
MEHD7945A
IR
MEHD7945A + IR
Caspase 3
Cleaved Caspase 3
GAPDH


C
A431 24h
A431 48h
Calreticulin exposure (Fold increase)
P=0.0806
P=0.049
P=0.005
P=0.0211
P=0.0001
Untreated
MEHD7945A
2Gy
5Gy
10Gy
MEHD7945A + 2Gy
MEHD7945A + 5y
MEHD7945A +10Gy


D
A431 24H
A431 48H
A431 72H
ATP Release (Fold increase)
P=0.0374
P=0.0359
P=0.0044
P<0.0001
P<0.0001
Untreated
MEHD7945A
2Gy
5Gy
10Gy
MEHD7945A + 2Gy
MEHD7945A + 5y
MEHD7945A +10Gy

A431


0 Gy
0.5 Gy
1 Gy
2 Gy
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56 50 46 44 47 41
71 64 57 56 49 50
80 72 62 58 55 52
97 83 77 69 63 59
99 89 78 69 67 60
100 90 81 71 70 60
100 93 79 70 67 58
18 16 16 15 16 15
55 43 42 40 42 40
71 59 57 53 47 47
82 78 65 59 54 49
94 80 75 69 64 55
98 82 76 70 67 63
96 85 77 73 70 62
98 90 81 71 69 63
16 15 14 15 13 13
55 51 45 41 38 36
66 61 56 51 50 48
78 71 61 59 57 51
90 85 72 68 64 63
97 88 77 68 66 61
100 88 78 72 71 64
100 96 77 70 68 60
16 15 14 13 13 12
46 40 36 36 34 36
61 51 44 45 42 40
64 59 50 48 49 43
80 69 64 57 56 54
82 72 67 61 62 57
88 77 66 63 57 59
85 81 72 67 61 55
2
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0.25
0.13
0.06
0
CDDP
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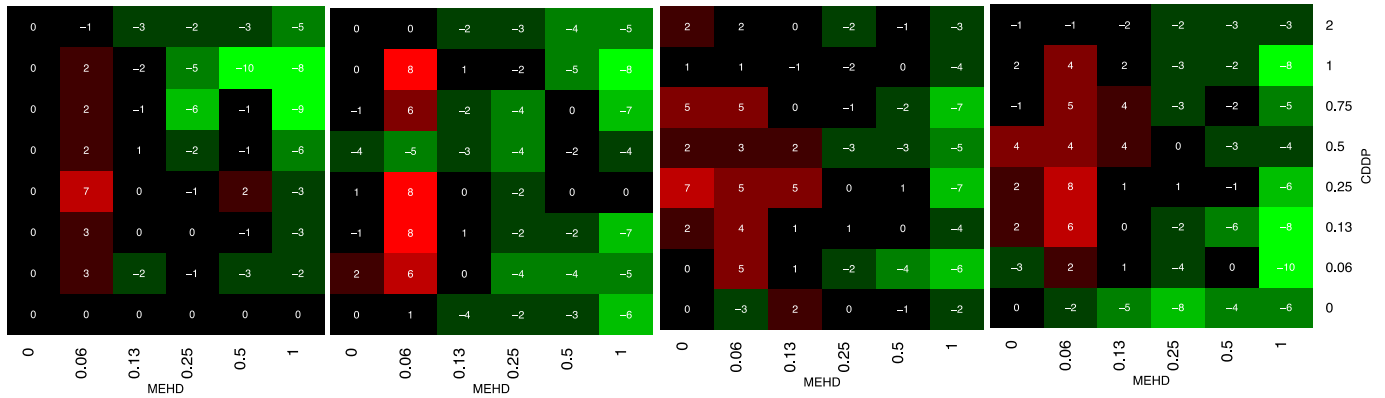
MEHD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0 3 −2 −1 −3 −2
0 0 0 0 0 0
0 0 −2 −3 −4 −5
0 8 1 −2 −5 −8
−1 6 −2 −4 0 −7
−4 −5 −3 −4 −2 −4
1 8 0 −2 0 0
−1 8 1 −2 −2 −7
2 6 0 −4 −4 −5
0 1 −4 −2 −3 −6
2 2 0 −2 −1 −3
1 1 −1 −2 0 −4
5 5 0 −1 −2 −7
2 3 2 −3 −3 −5
7 5 5 0 1 −7
2 4 1 1 0 −4
0 5 1 −2 −4 −6
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4 4 4 0 −3 −4
2 8 1 1 −1 −6
2 6 0 −2 −6 −8
−3 2 1 −4 0 −10
0 −2 −5 −8 −4 −6
2
1
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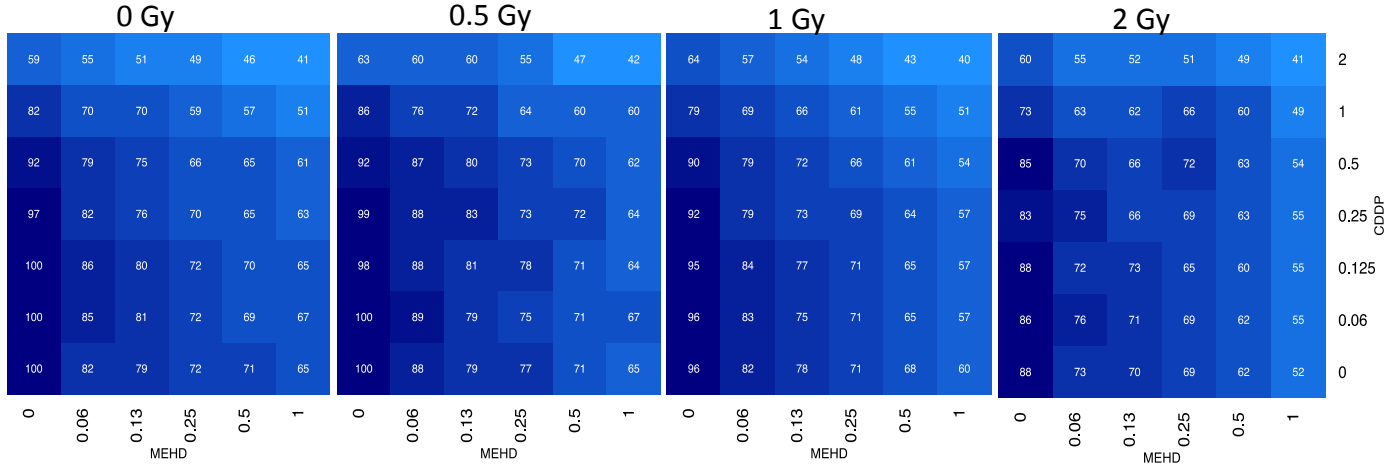
0.13
0.06
0
CDDP
0 0.06 0.13 0.25 0.5 1
MEHD

Ca Ski


0 Gy
0.5 Gy
1 Gy
2 Gy
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82 70 70 59 57 51
92 79 75 66 65 61
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100 86 80 72 70 65
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100 82 79 72 71 65
63 60 60 55 47 42
86 76 72 64 60 60
92 87 80 73 70 62
99 88 83 73 72 64
98 88 81 78 71 64
100 89 79 75 71 67
100 88 79 77 71 65
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73 63 62 66 60 49
85 70 66 72 63 54
83 75 66 69 63 55
88 72 73 65 60 55
86 76 71 69 62 55
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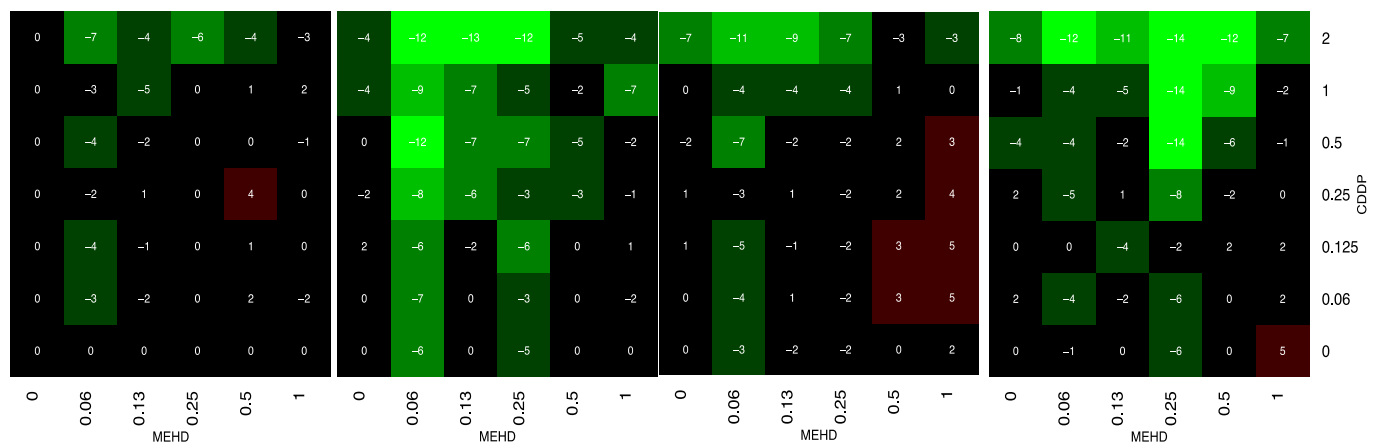
0
CDDP
0 0.06 0.13 0.25 0.5 1
MEHD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0 0 0 0 0 0
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−4 −9 −7 −5 −2 −7
0 −12 −7 −7 −5 −2
−2 −6 −6 −3 −3 −1
2 −6 −2 −6 0 1
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0 −6 0 −5 0 0
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0 −4 −4 −4 1 0
−2 −7 −2 −2 2 3
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0 −4 1 −2 3 5
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2
1
0.5
0.25
0.125
0.06
0
CDDP
0 0.06 0.13 0.25 0.5 1
MEHD