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F. Mignot, Y. Kirova, P. Verrelle, M.-P. Teulade-Fichou, F. Megnin-Chanet. In vitro effects of Trastuzumab Emtansine (T-DM1) and concurrent irradiation on HER2-positive breast cancer cells. *Cancer/Radiothérapie*, 2021, 25 (2), pp.126-134. 10.1016/j.canrad.2020.06.028 . hal-04425194

HAL Id: hal-04425194

<https://hal.science/hal-04425194>

Submitted on 22 Jul 2024

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1 **Title page**

2 *In vitro* effects of Trastuzumab Emtansine (T-DM1) and concurrent irradiation on HER2-
3 positive breast cancer cells

4

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19 Conflicts of interest: None for all authors

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27 **Abstract**

28 **Background:** To determine the effects of concurrent irradiation and T-DM1 on HER2-
29 positive breast cancer cell lines.

30 **Methods:** Five human breast cancer cell lines (*in vitro* study) presenting various levels of
31 HER2 expression were used to determine the potential therapeutic effect of T-DM1 combined
32 with radiation. The toxicity of T-DM1 was assessed using viability assay and cell cycle
33 analysis was performed by flow cytometry after BrdU incorporation. HER2 cells were
34 irradiated at different dose levels after exposure to T-DM1. Survival curves were determined
35 by cell survival assays (after 5 population doubling times).

36 **Results:** The results revealed that T-DM1 induced significant lethality due to the intracellular
37 action of DM1 on the cell cycle with significant G2/M phase blocking. Even after a short time
38 incubation, the potency of T-DM1 was maintained and even enhanced over time, with a
39 higher rate of cell death. After irradiation alone, the D₁₀ (dose required to achieve 10% cell
40 survival) was significantly higher for high HER2-expressing cell lines than for low HER2-
41 expressing cells, with a linearly increasing relationship. In combination with irradiation, using
42 conditions that allow cell survival, T-DM1 does not induce a radiosensitivity.

43 **Conclusions:** Although there is a linear correlation between intrinsic HER2 expression and
44 radioresistance, the results indicated that T-DM1 is not a radiation-sensitizer under the

45 experimental conditions of this study that allowed cell survival. However, further
46 investigations are needed, in particular *in vivo* studies before reaching a final conclusion.

47 **Résumé**

48 **Objectif de l'étude : Déterminer les effets du T-DM1 avec une irradiation concomitante**
49 **sur des lignées cellulaires de cancer du sein HER2-positives.**

50 **Matériel et Méthodes : Cinq lignées cellulaires humaines de cancer du sein (étude in**
51 **vitro) présentant différents niveaux d'expression de HER2 ont été utilisées pour**
52 **déterminer l'effet thérapeutique potentiel du T-DM1 combiné à l'irradiation. La toxicité**
53 **du T-DM1 a été évaluée à l'aide d'un test de viabilité et une analyse du cycle cellulaire a**
54 **été effectuée par cytométrie de flux après incorporation de BrdU. Les cellules HER2 ont**
55 **été irradiées à différents niveaux de dose après exposition au T-DM1. Les fractions de**
56 **survie ont été déterminées par des tests de croissance cellulaire (après 5 temps de**
57 **doublent de la population).**

58 **Résultats : Les résultats ont révélé que le T-DM1 induisait une létalité significative due à**
59 **l'action intracellulaire de la DM1 sur le cycle cellulaire avec un blocage significatif de la**
60 **phase G2/M. Même après une courte période d'incubation, la toxicité du T-DM1 a été**
61 **maintenue et même renforcée au fil du temps, avec un taux de mort cellulaire plus élevé.**
62 **Après irradiation seule, la D10 (dose nécessaire pour obtenir une survie cellulaire de 10**
63 **%) était significativement plus élevée pour les lignées cellulaires à forte expression de**
64 **HER2 que pour les cellules à faible expression de HER2, avec une relation linéairement**
65 **croissante. En combinaison avec l'irradiation, dans des conditions qui permettent la**
66 **survie cellulaire, le T-DM1 n'induit pas de radiosensibilité.**

67 **Conclusion : Bien qu'il existe une corrélation linéaire entre l'expression intrinsèque de**
68 **HER2 et la radorésistance, les résultats indiquent que le T-DM1 n'est pas un**
69 **sensibilisateur au rayonnement dans les conditions expérimentales de cette étude qui**

70 **permettent la survie cellulaire. Cependant, des recherches supplémentaires sont**
71 **nécessaires, en particulier des études in vivo avant de parvenir à une conclusion finale.**

72

73 **Manuscript:**

74

75 **Introduction**

76

77 Anti-HER2 (Human Epidermal Growth factor Receptor 2) drugs have become standard
78 treatment for HER2-positive breast cancer patients and are routinely used in adjuvant and
79 neoadjuvant therapy, and for metastatic disease [1,2]. Trastuzumab emtansine (T-DM1,
80 Kadcyła[®]), is an ADC (Antibody-drug conjugate) which combines trastuzumab (an anti-
81 HER2 monoclonal antibody) covalently linked via a non-reducible linker to the maytansinoid
82 DM1, a powerful mitotic spindle inhibitor [3]. T-DM1 improves overall survival in patients
83 with HER2-positive metastatic breast cancer [4-6]. T-DM1 binds the extracellular domain of
84 HER2 via the trastuzumab component and enters into the cell. The HER2-T-DM1 complex is
85 then internalized and cleaved by lysosomal degradation, allowing intracytoplasmic release of
86 the cytotoxic agent DM1, a potent inhibitor of tubulin polymerization [7].

87 *In vitro*, down-regulation or over-expression of HER2 in breast cancer cells induces changes
88 in the radio-response. In HER2 transfected cells, the mechanisms of radioresistance are
89 complex and have not been fully elucidated, but the PI3-K/Akt (Phosphatidylinositol-3-
90 Kinase/Protein kinase B) pathway appears to play a major role in radioresistance by
91 deregulating the cell cycle, accelerating DNA repair mechanisms, leading to resistance to
92 apoptosis [8-12].

93 It has been previously reported that anti-HER2 therapies, and more specifically Trastuzumab
94 and Lapatinib (inhibitor of tyrosine kinase activity in the intracellular domain of HER2), are
95 specific radiosensitizing agents for HER2-positive cells [8,9,13]. These findings have led
96 clinicians to deliver radiation concurrently with these anti-HER2 drugs [14].

97 We therefore conducted an *in vitro* preclinical study in HER2-positive breast cell lines to
98 evaluate the potential radiosensitizing effect of T-DM1.

99 **Materials and Methods:**

100

101 ***Cell lines***

102 Five HER2-positive cell lines expressing different levels of HER2 amplification and one
103 triple-negative (MDA-MB-231) human breast cancer cell line (TNBC) were used in this study
104 (see characteristics of these cell lines in supplementary table TS1). All cell lines, originally
105 derived from the ATCC[®], were kindly provided by Drs L. De Koning and T. Dubois (Institut
106 Curie). Cells were routinely subcultured every 5 days and grown (37°C, 5% CO₂) as
107 monolayers in RPMI (HCC1954 and ZR-75-1), DMEM/F12 (MDA-MB-453, MDA-MB-
108 231), DMEM (BT474) and Mc Coy's (SKBr3) media supplemented with antibiotics and 10%
109 fetal calf serum.

110 Cell line authentication was performed on all cell lines by the determination of Short Tandem
111 Repeat (STR) DNA sequencing (Institut Curie, Genomics platform) and correspond exactly to
112 those described by ATCC[®] (supplementary table TS2).

113 T-DM1 was provided by Genentech Inc. (South San Francisco, CA, USA) as a lyophilized
114 powder through a Material Transfer Agreement. A 20 mg/ml stock solution of T-DM1 was
115 prepared in water and kept in aliquots at 4°C.

116

117 ***Western blots analysis***

118 Total cell extracts were prepared using the Cell Lysis Buffer reagent (CST, #9803) containing
119 a cocktail of protease and phosphatase inhibitors (Roche®), and quantified (BCA protein
120 Assay, Thermo Scientific). 10 µg of total proteins were loaded onto precast NuPAGE®
121 Novex® 4–12% Bis–Tris gels (Invitrogen). After transfer (Trans Blot® Turbo™, BioRad),
122 nitrocellulose membranes were blocked with 5% non-fat milk for 1 h at room temperature and
123 then incubated with primary antibodies directed against HER2 (CST, clone 29D8) or Actin
124 (Abcam, #ab49900) overnight at 4°C then with secondary antibodies coupled to HRP
125 (Jackson ImmunoResearch) for 1 h at room temperature. Membranes were probed with an
126 ECL reagent (Clarity™, BioRad) and quantified using the ChemiDoc™ Imaging system
127 (BioRad).

128

129 ***Cell viability assay***

130 Cell viability was detected using the CellTiterGlo® Luminescent assay (Promega). Cells (from
131 6000 to 20,000 cells/well) were seeded in 96-well plates and treated with concentrations of T-
132 DM1 ranging from 0.33 ng/ml to 20 µg/ml for 72 hours. All measurements were performed in
133 triplicate. ED₅₀ (effective dose to achieve 50% cell death) were calculated after fitting the
134 experimental points according to the classical four parameter Hill equation using
135 KaleidaGraph software (Synergy Software, Pennsylvania).

136

137 ***Cell survival assays***

138 Among the HER2 expressing cell lines, only HCC1954 and BT474 were able to form
139 colonies. To ensure homogeneous analysis, the effect of concurrent radiation in combination
140 with T-DM1 was assessed using cell survival assays on all the cell lines used in this study.
141 For this, cells from mid-log growing subcultures were plated in triplicate in 6-well plates at

142 2×10^4 cells/ well, allowed to attach overnight and then treated with T-DM1 for 6 (SKBr3) or
143 12 h (other cell lines). As the sensitivity of the cell lines to T-DM1 was found to vary over a
144 3-log range and in order to compare the cell lines within a same “iso-effect”, we chose for
145 each cell line a concentration of T-DM1 leading to around 50% of cell survival for the non-
146 irradiated controls by the end of the experiment. These concentrations were: 0.006 (SKBr3),
147 0.01 (HCC1954), 0.03 (MDA-MB-453), 0.185 (BT474) and 5 (ZR-75-1 and MDA-MB-231)
148 $\mu\text{g/ml}$. The sensitivity of SKBr3 to T-DM1 was such that the incubation time also had to be
149 reduced to maintain sufficient cell survival. Cells were irradiated after T-DM1 treatment at
150 room temperature using a GSR-D1 (^{137}Cs) γ -ray irradiator at a dose rate of 1.3 Gy/min and
151 the medium was then rinsed (free-drug medium). As the main radiation-induced cell death
152 mechanism in solid tumors is mitotic cell death, we compared all the survival assays after the
153 same number of mitosis following irradiation. Cells were incubated after irradiation for 5
154 (HCC1954), 8 (MDA-MB-453, ZR-75-1), 9 (SKBr3) and 14 (BT474) days, representing
155 respectively 5 population doubling times (5-PDT). Mock-irradiated blanks were still far from
156 confluent at these times. Cells were then harvested and counted using Moxi™ Z automated
157 cell counter (ORFLO Techn.). Surviving fractions (S) were calculated by dividing the number
158 of surviving irradiated cells over non-irradiated.

159 In order to validate the cell survival assay used, classical clonogenic assays were also
160 performed on HCC1954 and BT474 cell lines. In this case, 800 to 2000 cells were seeded in
161 6-well plates for 8 h, T-DM1 treated and/or irradiated in the same conditions as for cell
162 survival assays. Colonies were allowed to grow for 10 days (HCC1954) or 21 days (BT474)
163 before fixation (ethanol), staining (Blue Coomassie) and manually scoring. Small colonies
164 (less than 50 cells) were disregarded. Surviving fractions (S) were calculated by dividing the
165 plating efficiency (PE) of the treated cells by the PE of the controls (unirradiated cells).

166 The cell/colony count relative to mock-treated cells (S) was adjusted for best fit to the

167 classical linear-quadratic equation ($\text{Ln } S = -\alpha D - \beta D^2$), where D is the radiation dose and α and
168 β are adjustable parameters characterizing the response. Calculations were performed by non-
169 linear least-squares regression using KaleidaGraph software. For each experiment, the D_{10}
170 (dose to achieve 10% cell survival) was calculated using these parameters.

171

172 *Cell cycle analysis*

173 Cells were incubated for 18 h with increasing concentrations of T-DM1 (0.01 to 20 $\mu\text{g/ml}$).
174 Before harvest, cells were pulse-labeled with 10 μM bromodeoxyuridine (BrdU) for 30 min
175 and frozen in medium with 10% DMSO. BrdU incorporation allows precise analysis of the
176 replication and mitotic phases of treated cells, with quantification of the fraction of cells in
177 sub-G1 (or sub-diploid population with a DNA content $< 2n$), G0/G1, S, sub-S (arrested cells
178 in S phase with a $2n < \text{DNA content} < 4n$ with no BrdU incorporation) and G2/M
179 (supplementary Figure S1 and S2). Before cytometry analysis, cells were thawed, fixed with
180 70% ice-cold ethanol and kept at 4°C overnight until further processing. Preparation of nuclei,
181 enzymatic digestion, propidium iodide staining, labeling of BrdUrd in DNA using a
182 fluorescein monoclonal antibody (e-Biosciences, clone BU20A), bivariate data acquisition
183 (FACSCanto II cytometer, Becton-Dickinson) and processing (FlowJo software) were carried
184 out as previously described [15]. Depending on the cell line, the coefficient of variation (CV)
185 for the G0/G1 control cell population was between 2.80 to 4.32%

186

187 *Statistical analysis*

188 Statistical analysis was performed using Prism-6 (GraphPad software Inc., La Jolla, CA).

189

190

191 **Results**

192

193 *T-DM1: a long and major cytotoxic effect depending on the level of HER2 expression*
194 *(except for BT474)*

195 The HER2 protein expression of cells lines was first demonstrated by western blots,
196 confirming different levels of HER2 expression for the various cell lines (Figure 1A and B).
197 Cell lines were classified as follows: high (HER2 3+: HCC1954, SKBr3, BT474), moderate
198 (HER2 2+: MDA-MB-453) and low (HER2 1+: ZR-75-1) HER2 expression. The triple-
199 negative cell line MDA-MB-231 was used as negative control. The three HER2 3+ cell lines
200 showed approximately the same levels of HER2. HER2 expression by MDA-MB-453 and
201 ZR-75-1, relative to the HCC1954 cell line, was decreased by 65% ($p=0.0019$, $n=3$) and 82%,
202 respectively ($p<0.0001$, $n=3$) (Figure 1B).

203 The ED₅₀ (effective dose for 50% cell death) determined from viability assays (CellTiterGlo®
204 luminescent assay) after 72h-exposure to T-DM1 were calculated after fitting the
205 experimental data with the classical four parameter Hill equation and were found to vary over
206 a 3-log range (from 0.006 to 5.16 $\mu\text{g/ml}$) (Figure 2A). As expected, these ED₅₀ values varied
207 as a function of the level of HER2 expression of the cell line used, except for BT474. The
208 most sensitive were the HER2 3+ SKBr3 (0.006 $\mu\text{g/ml}$) and HCC1954 (0.010 $\mu\text{g/ml}$) cell
209 lines. The third HER2 3+ cell line BT474 needed a 30-fold higher T-DM1 concentration
210 (0.185 $\mu\text{g/ml}$) to achieve 50% cell death compared to SKBr3 (Figure 2B). MDA-MB-453 and
211 ZR-75-1 showed a moderate (0.024 $\mu\text{g/ml}$) and a low toxicity (5.17 $\mu\text{g/ml}$) respectively, upon
212 T-DM1 treatment. However, high concentrations of T-DM1 (ED₅₀ > 10 $\mu\text{g/ml}$) exhibited
213 some potency against the triple-negative cell line MDA-MB-231 used as negative control.

214 The survival of breast cancer cell lines was then assessed over a longer time period after a
215 short-term exposure to T-DM1. The potency of T-DM1 increased and remained effective over

216 time, leading to cell death even after cell growth in T-DM1-free medium for 5-PDT (ranging
217 from 5 to 14 days). The effect of 12-hour incubation of T-DM1 after 5-PDT varied as a
218 function of the HER2 expression of the cell line studied, except for BT474 and was dependent
219 on the initial T-DM1 incubation time (Figures 3A and B).

220 For cell cycle analysis, cells were treated with increasing concentrations of T-DM1 for 18 h
221 and then pulse-labeled with BrdU before fixation (Figure 4). T-DM1 induced a concentration-
222 dependent G2/M arrest of treated cells except for BT474 cell line (Fig. 4C). Yet, we observed
223 different responses according to the cell line: while the maximum G2/M arrest in the two
224 HER2 3+ cell lines was reached with 5 µg/ml of T-DM1 with a very high blockade ($66.05\% \pm$
225 3.05% , n=2) for HCC1954 (Fig. 4A) and a more modest one ($29.7\% \pm 2.2\%$, n=2) for SKBr3
226 (Fig. 4B), the G2/M arrest still increased over T-DM1 concentration range for MDA-MB-453
227 (Fig. 4D) (from $25.35\% \pm 1.65$, n=2 at 5 µg/ml to $40.45\% \pm 3.45\%$, n=2 at 20 µg/ml) and ZR-
228 75-1 (Fig. 4E) (from $11.88\% \pm 2.015$, n=2 at 5 µg/ml to $23.7\% \pm 0.2\%$, n=2 at 20 µg/ml) cell
229 lines. The TNBC cell line MDA-MB-231 (Fig. 4F) showed also a G2/M block over 18h-T-
230 DM1 treatment in high concentration. For ZR-75-1 (HER2 1+) and MDA-MB-231 (TNBC)
231 cell lines, T-DM1 treatment induced also an increased Sub-G1 population.

232

233 ***Intrinsic radioresistance and HER2 expression: a linear correlation***

234 The intrinsic radiation response of the HER2 expressing cell lines was determined (Figure
235 5A). In order to validate our cell survival assay, comparative classical clonogenic and cell
236 survival assays after 5-PDT were performed on HCC1954 and BT474 cell lines. The
237 radiosensitivity parameters (D_{10}) were not statistically different between the two techniques
238 (Figure S3). Following these results, we used cell survival (after 5 population doubling times)
239 assays for all the cell lines. Cell lines were ranked into two categories: HCC1954, BT474 and
240 SKBr3 expressing high HER2 levels and MDA-MB-453 and ZR-75-1 expressing lower

241 HER2 levels, which presented significantly lower D_{10} from that of the HCC1954 cell line
242 ($p=0.0008$ for MDA-MB-453; $p=0.002$ for ZR-75-1; $n=3$ independent experiments) (Figure
243 5B). Moreover, a significantly linear correlation was observed between the level of the
244 intrinsic HER2 expression of each cell line and their corresponding D_{10} , (linear regression;
245 $p<0.0001$) showing that HER2 status influence the radiation response of these breast cancer
246 cells (Figure 6).

247

248 ***Concurrent combination of T-DM1 with radiation does not radiosensitize HER2-positive***
249 ***cells***

250 The combination of T-DM1 with radiation was evaluated by cell survival assays after 5-PDT.
251 Cells were treated with T-DM1 for 12 h (or 6 h for SKBr3) before irradiation and T-DM1 was
252 then removed to allow cells to grow. It is noteworthy that higher exposure times or higher T-
253 DM1 concentrations did not allow sufficient cell survival without irradiation to assess the
254 impact of graded doses of photons.

255 Using these conditions and after irradiation, the radiation parameters and the D_{10} calculated
256 from experimental data ($n=3$) were not statistically different with or without concurrent T-
257 DM1 incubation, as shown in Figure 7 (t-test statistical analysis; $0.367<p<0.786$, not
258 significant) and in supplementary Figure S4. Under experimental conditions using drug
259 concentrations allowing cell survival, the T-DM1 did not have a radiosensitizing effect on
260 HER2-positive cell lines, including HER2 3+ expressing lines.

261

262 **Discussion**

263 This *in vitro* study provides three take home messages: The first is that T-DM1 presents a
264 delayed toxic effect on HER-2 positive cell lines. The second is the linear correlation between
265 intrinsic HER2-positive status and radioresistance (D_{10}). The third is that concurrent

266 combination of T-DM1 allowing cell survival with radiation does not radiosensitize HER2-
267 positive breast cancer cells.

268 Despite improvement of the outcome associated with the use of anti-HER2 drugs, between 10
269 and 15% of patients with early HER2-positive breast cancer will develop distant metastases at
270 8-10 years, some of which require radiotherapy [16,17]. In addition, the recent results of
271 phase III KATHERINE trial evaluating T-DM1 (versus trastuzumab) in adjuvant situation for
272 residual invasive HER2-positive breast cancer could extend the use of radiotherapy in
273 combination with T-DM1 to a common practice. In this trial, T-DM1 significantly improved
274 the invasive disease-free survival and the radiation skin injury was similar between
275 trastuzumab and T-DM1 [18]. However, some others studies suggest that the combination of
276 T-DM1 and radiation may increase radionecrosis in the case of brain irradiation using
277 stereotactic radiosurgery technique, but with a good local control rate [19, 20]. Thus, there is a
278 real need for preclinical studies to evaluate the combination of T-DM1 and radiation.

279 The *in vitro* radioresistance status of HER2-overexpressing cells has been established for a
280 long time. In most studies, radioresistance was assessed using HER2-negative breast cancer
281 cells (like MCF7) overexpressing or not HER2 by transfection [8-11]. However, there is no
282 report on the intrinsic HER2 expression and the innate radio-response of breast cancer cell
283 lines. To our knowledge, we have demonstrated for the first time a clear linear correlation
284 between radioresistance (D_{10}) and the intrinsic expression level of HER2 receptor in breast
285 cancer cells.

286 Kim et al. used a similar approach to our study by comparing the survival curves of several
287 breast cancer cell lines including two HER2-positive ones (SKBr3 and BT474). Relatively to
288 the other cell lines, they showed a radioresistance of the two HER2-positive cell lines but
289 without possible correlation with the HER2 level due to the lack of moderate or low-
290 expressing cell lines [21].

291 In this work, combination of T-DM1 with radiation, using drug concentrations that allow cell
292 survival leads to a strictly additive effect whatever the cell line considered and no more
293 radiosensitizing effects were found. In another study, Edwards A. et al also found that the
294 combinatorial effect of maytansinol isobutyrate and radiation is additive in both *Drosophila*
295 and human cancer cells [22]. However, the experimental modalities have a significant impact
296 on the outcomes and the ADC can make the subject more complex. Adams R.T. et al. studied
297 the radiosensitivity of ADC containing anti-ErbB antibodies and anti-tubulin drugs, including
298 T-DM1 on esophageal and gastric models. The authors demonstrated, as we do, that T-DM1
299 selectively blocks two HER2-positive cancer cell lines in the G2/M phase. Using relative
300 neutral comet assay, they showed that mertansine (20 nM) in combination with radiation
301 (single dose of 6 Gy) induces an excess of double-strand breaks when compared with
302 radiation alone, regardless of HER2 status. However, this effect was restricted to HER2-
303 positive cells when using T-DM1 at the same concentration (IC₅₀ on their cell lines < 1nM for
304 72-96h incubation time). In this study, typical *in vitro* radiation survival curves were not
305 shown to assess radiosensitization and we believe were not possible using such high doses of
306 T-DM1. However, on mice bearing HER2-positive xenografts (esophageal and gastric) and
307 using T-DM1 concentrations ten times lower, combination of T-DM1 and radiation prolonged
308 tumor control [23].

309 A strong correlation between HER2 receptor amplification and increased T-DM1 toxicity was
310 demonstrated for all cell lines studied, except BT474. No G2/M block could be observed in
311 BT474 cell line after 18 h of incubation with T-DM1, even at high concentrations. This was
312 the only cell line to present this phenotype, as even cells with low or negative HER2
313 expression exhibited lesser degrees of G2/M block. In our study, only HCC1954 had a G2/M
314 blockage more than 50% of the total population with a plateau observed from 5 µg/ml of T-
315 DM1. A plateau was also observed for the SKBr3 cell line using 0.01 µg/ml of T-DM1 but

316 with only 20% of the cells blocked in G2/M. In the first Genentech's study which led to the
317 development of T-DM1, the authors also observed a G2/M blockade but without showing the
318 data [3]. In the Adams' study, the cell fraction blocked in G2/M was more than 50% when the
319 cells were exposed to T-DM1 overnight and reached also a plateau. However, the cell models
320 used were one esophageal (OE19; HER2 positive) and one colon (HCT116; HER2 negative)
321 cancer cells and not breast cancer cells [23]. Thus, high HER2 expression in cells is not
322 enough to predict T-DM1 potency, and other parameters not yet fully elucidated influence the
323 T-DM1 response.

324 Other authors have reported the lower responsiveness of BT474 to T-DM1 [7,24] and several
325 mechanisms have been proposed: poor internalization of the HER2-T-DM1 complex due to a
326 deficit of endophilin A2 (a protein promoting the internalization of HER2) [25]; formation of
327 HER2/HER3 heterodimers inhibiting the mechanism of T-DM1 [3,24]; defective cell cycle
328 regulation machinery [26,27]. The possibility of a poor HER2-T-DM1 complex
329 internalization on BT-474 has been explored. It is established that the HER2-T-DM1 complex
330 enters cancer cells via the clathrin-dependent endocytosis pathway. However, a clathrin-
331 independent mechanism, such as caveolae membranes composed mainly by caveolin-1 has
332 also been demonstrated [28]. Chung Y. C. et al. [28] showed that BT-474 cells overexpressing
333 caveolin-1 protein were more sensitive to T-DM1 treatment than mock-transfected cells. We
334 looked at the caveolin-1 (CAV-1) expression in the breast cancer cell lines used in this study
335 from the Cancer Cell Line Encyclopedia (CCLE) database
336 (<http://www.broadinstitute.org/ccle/id283537>), and found that CAV-1 expression in BT474 is
337 much more lower compared to the other HER2-positive cell lines used in this study (Figure
338 8A), leading to the conclusion that T-DM1 might be poorly internalized in BT-474 cells. It is
339 interesting to note that we found also a high CAV-1 mRNA expression in MDA-MB-231
340 cells, confirmed at the protein level by Chung Y. C. et al. [28] that could explain some T-

341 DM1 uptake on this cell line via HER-2 independent Caveolin-1 dependent trapping. Using
342 the same method, we found that the expression of Endophilin-A2 is not different in BT-474
343 cells compared to the other cell lines (data not shown).

344 Once internalized, T-DM1 is reduced inside the lysosome mostly to the lysine-MCC-DM1
345 catabolite that does not diffuse easily across cell membranes [29]. SLC46A3, a specific
346 transporter, then intervenes to transfer this catabolite to the cytoplasm [30]. It has been shown
347 that the loss of SLC46A3 expression is a mechanism of innate and acquired resistance to non-
348 cleavable ADCs bearing DM1 [31]. After analyzing data from the CCLE database we found
349 that the expression of SLC46A3 in BT474 cell line is very low compared to the other cell
350 lines used in this study (Figure 8 B). Moreover, loss of SLC46A3 expression has been found
351 in acquired T-DM1 resistance [32]. Apart from the high HER2 expression, SLC46A3 could
352 be a potential patient selection biomarker for T-DM1 treatment. Recently, Tsui et al. using
353 CRISPR-Cas9 screens, identified lysosomal regulators as modulators of ADC toxicity [33].
354 Data analysis of the expression of late endosomal trafficking regulators such as RAB7,
355 C18ORF8/RMC1, WDR81 and WDR91 using the CCLE database showed no significant
356 difference of these regulators on the cell lines used in this study (data not shown).

357 We also showed that the potency of T-DM1 increased and was still effective over time,
358 leading to cell death even after cell growth in T-DM1-free medium for 5-PDT (ranging from 5
359 to 14 days). This effect was dependent on HER2 expression and the initial T-DM1 incubation
360 time. Lewis Phillips G. et al. also reported that brief exposure of SKBr3 cells to T-DM1
361 followed by a 3-day incubation in T-DM1-free culture medium resulted in growth inhibition
362 (data not shown) but with no further explanation [3]. Another T-DM1 mechanism of action
363 has been described mediated by exosomes derived from HER2-positive cancer cells. These
364 exosomes can carry T-DM1 (and may be DM1) and when purified can induced growth
365 inhibition to non-exposed cells [34]. Indeed, more studies showed that some toxic effect of T-

366 DM1 is not only due to the HER2 expressing cells but also to the surrounding tissue.
367 Recently, Stumpf P. et al reported on a clinically significant alarming rates of radionecrosis
368 with the combination of stereotactic radiosurgery in 39.1% of patients with brain metastases
369 from breast cancers who received T-DM1 [35]. This radionecrosis was due to an additional
370 unintended targeting effect of T-DM1 as they observed a swelling of the astrocytic cell
371 population surrounding the tumour via upregulation of Aquaporin-4. This effect is specific to
372 T-DM1 and has not been observed with trastuzumab or others chemotherapeutic agents. The
373 authors hypothesized that this effect could result from the direct uptake of T-DM1 in
374 astrocytes (expressing normal levels of HER2), however, it could be also result from late
375 toxicity mediated by exosomes derived from HER2 positive tumour.

376

377 **Conclusion**

378

379 Our work indicated that i) within the first 24 hour-incubation time, the main determining
380 factor is the presence of HER-2 receptors: the higher, the better (except for BT-474). For the
381 moderate and low HER-2 expressing cell lines, T-DM1 uptake increased more slowly over
382 time; ii) the G2/M blockade observed on the triple-negative (HER-2 negative) cell line leads
383 us to think about a HER-2 independent trapping for T-DM1, even if we have no clear
384 evidence of this mechanism. iii) The effects of T-DM1 on BT-474 cells seem to be plural,
385 with a possible defect of the receptor internalization on this cell line due to low expression of
386 caveolin-1 and a low expression of the specific lysosomal transporter SLC46A3 to transport
387 the payload on his target.

388 *In vitro*, on HER2-positive breast cancer cells, we demonstrated that T-DM1 has a high and
389 prolonged over time toxicity and that concurrent irradiation induces strictly additive effects.
390 The results indicated that T-DM1 is not a radiation-sensitizer under the experimental

391 conditions of this study. These results are the first step in the investigation of the combination
392 of T-DM1 and radiation on HER2-positive breast cancer cells, even if *in vivo* studies are
393 needed.

394 **List of abbreviation**

395 ^{137}Cs : Cesium 137

396 5-PDT: 5 population doubling times

397 ADC: Antibody-Drug Conjugate

398 BrdU: bromodeoxyuridine

399 CV: Coefficient of Variation

400 D_{10} : dose to achieve 10% cell survival

401 DNA: Deoxyribonucleic Acid

402 HER2: Human Epidermal Growth factor Receptor 2

403 PE: Plating Efficiency

404 PI3-K/Akt: Phosphatidylinositol-3-Kinase/Protein kinase B

405 SF: Surviving Fractions

406 STR: Short Tandem Repeat

407 T-DM1: Trastuzumab Emtansine

408 TNBC: Triple-Negative human Breast Cancer

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519 **Declarations**

520 **Ethics approval and consent to participate:** Not applicable

521 **Consent for publication:** Not applicable

522 **Availability of data and materials:** The datasets used and/or analysed during the current
523 study are available from the corresponding author on reasonable request.

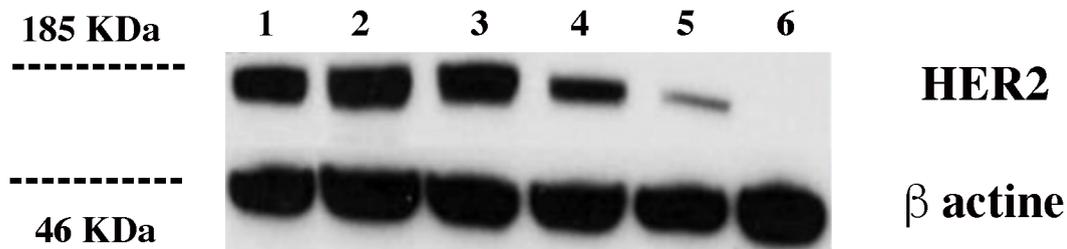
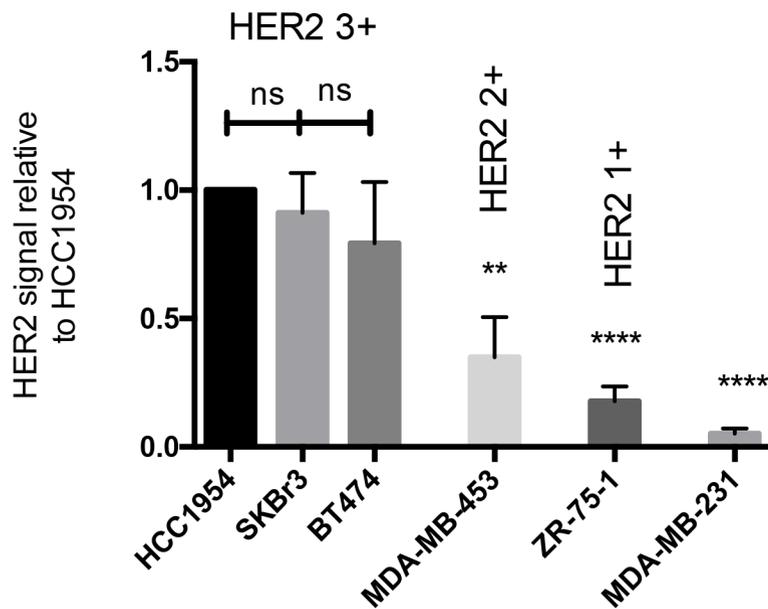
524 **Competing interests:** The authors declare that they have no competing interests.

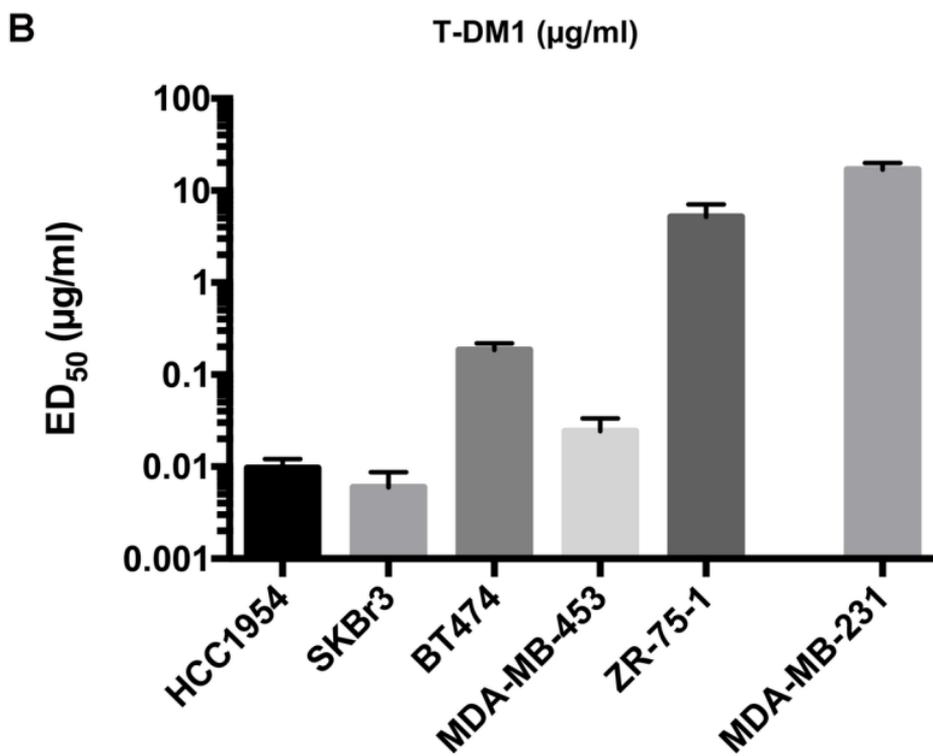
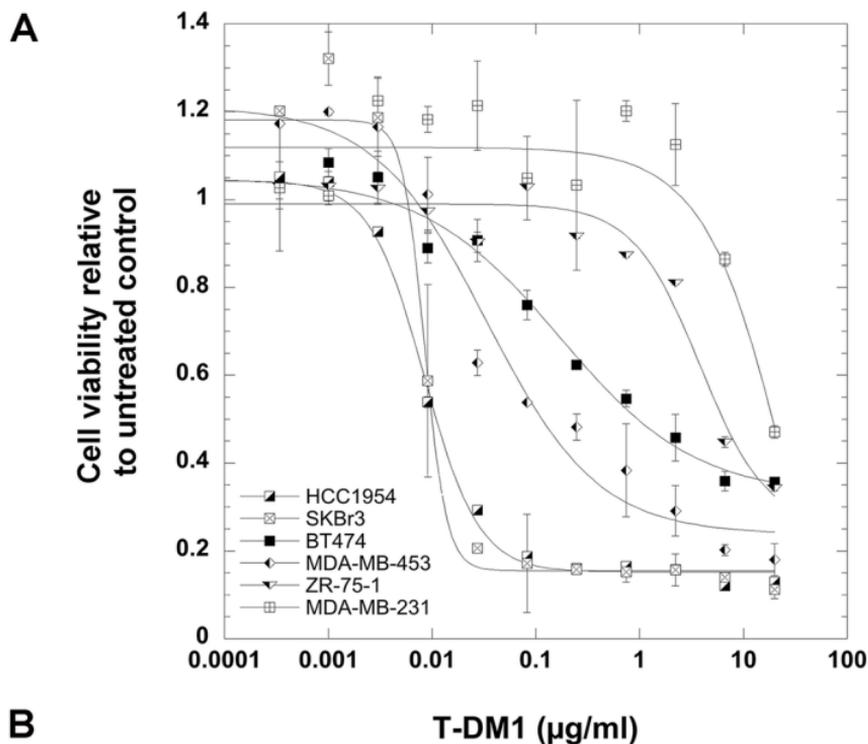
525 **Funding:** Academic study. This work was supported by financial aid from the Institut Curie,
526 INSERM and CNRS.

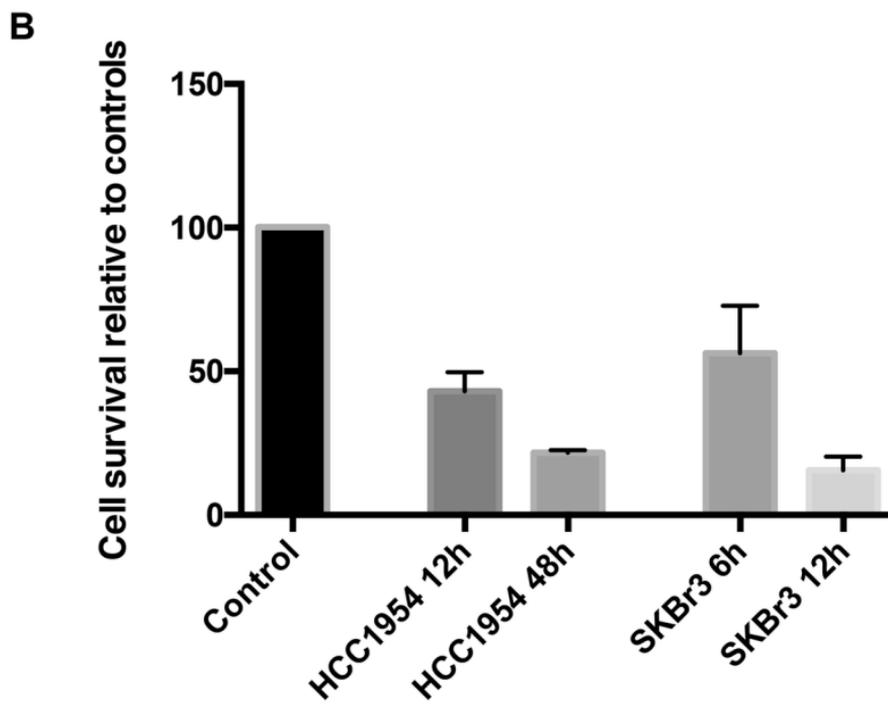
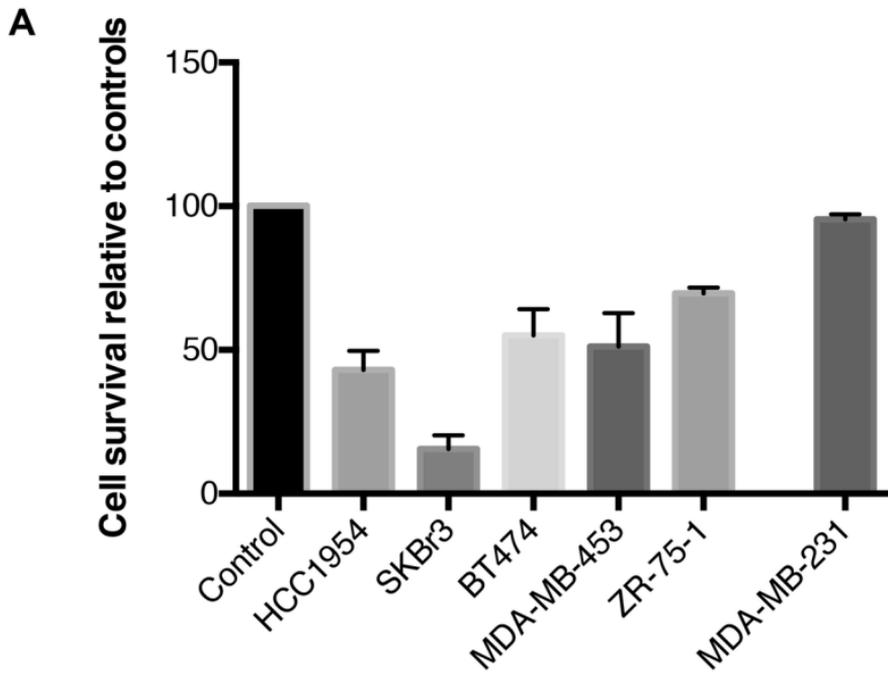
527 **Authors' contributions:** FM and FMC conducted the experiments. FM and FMC analyzed
528 the data. PV, MPTF and YK read and approved the final manuscript.

529 **Acknowledgements:**

530 The authors thank Genetech, USA Inc. (San Francisco, CA) for providing T-DM1 under
531 Material Transfer Agreement (# OR-215963) and Drs Nora Ady-Vago and Fanny Bouquet
532 (Roche Institute, France) for their help and continuous support. Members of the Institut Curie
533 RadeXp platform is acknowledged. We want to thank Emilie Brun (Institut Curie, Genomics
534 Platform) for DNA sequencing, Leanne de Koning (Institut Curie, RPPA Platform) and
535 Thierry Dubois (Institut Curie, Breast Cancer Biology Group, Translational Research
536 Department) for providing us the cell lines used in this study.

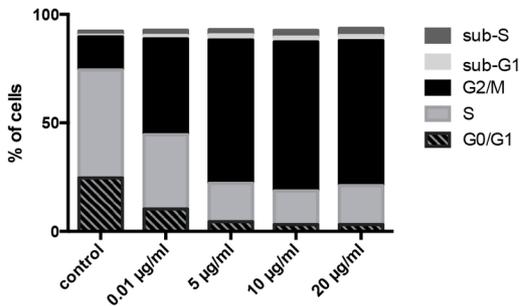
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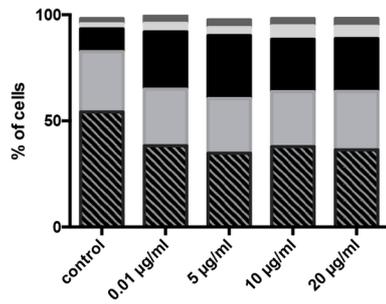
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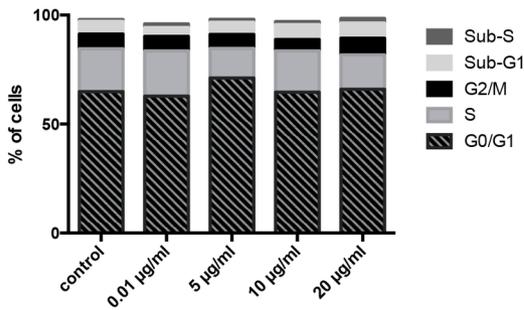
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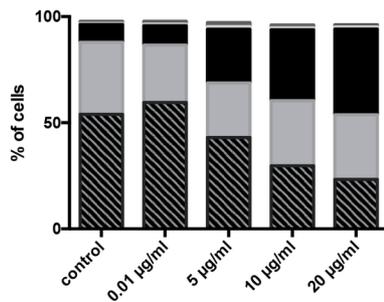
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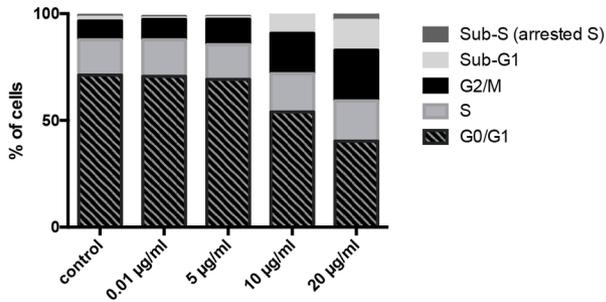
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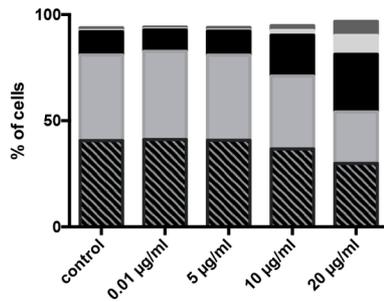
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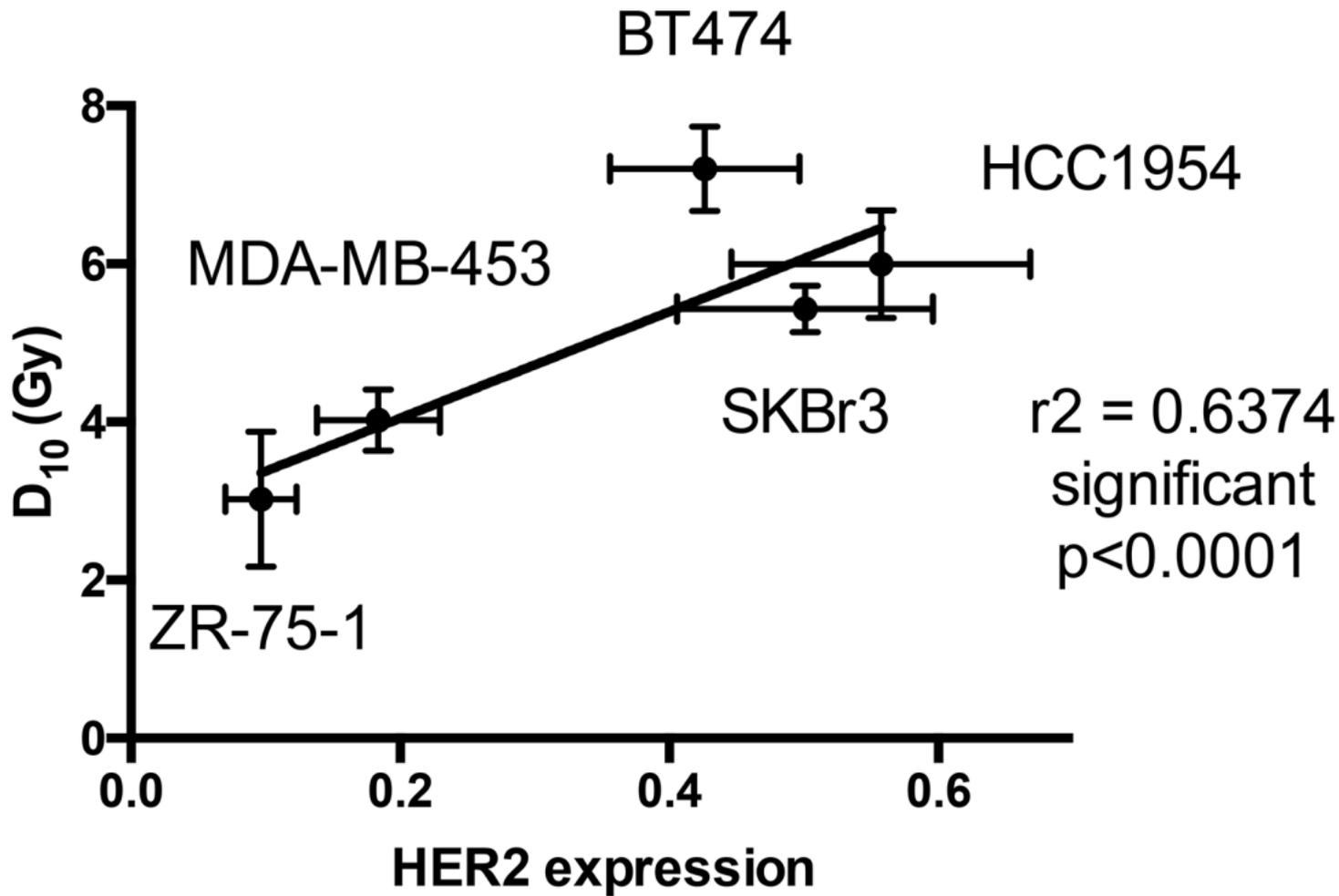
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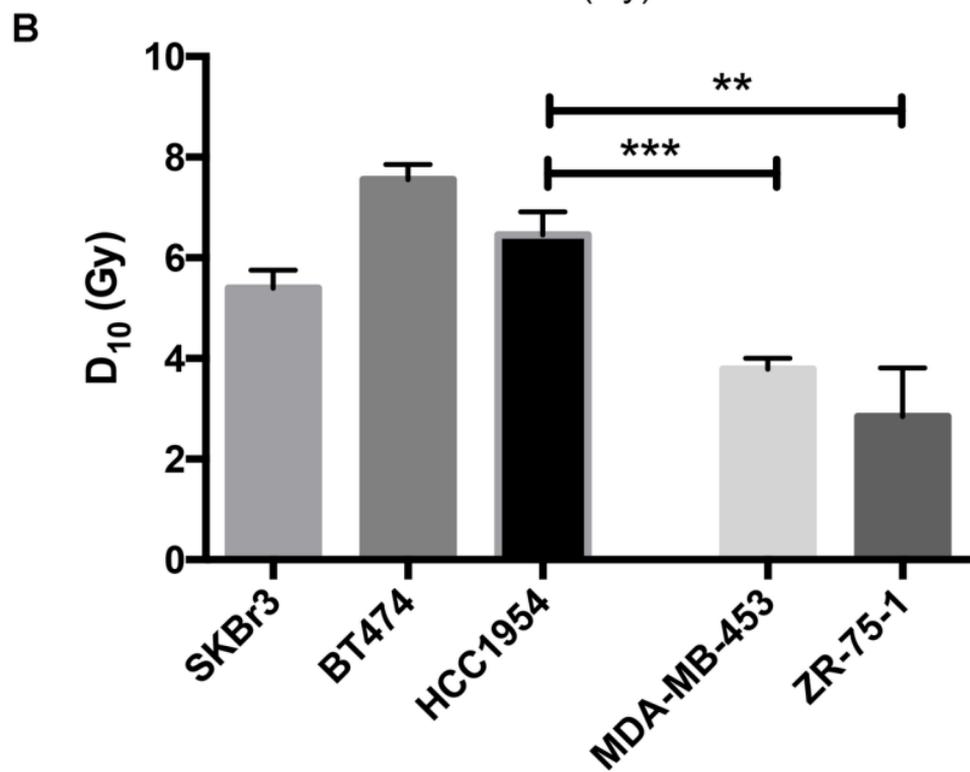
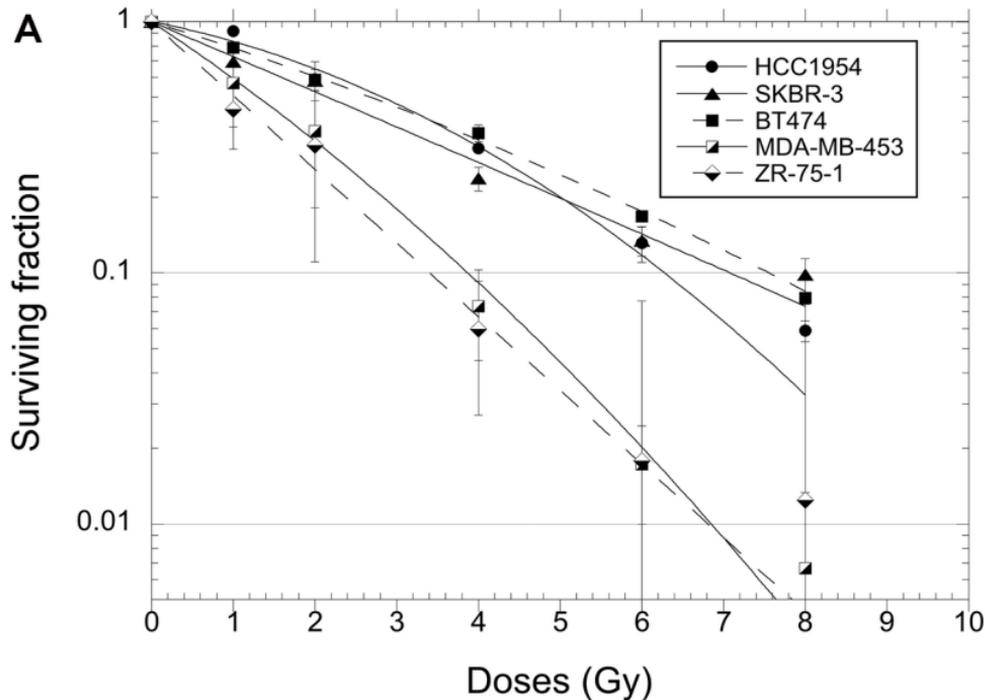


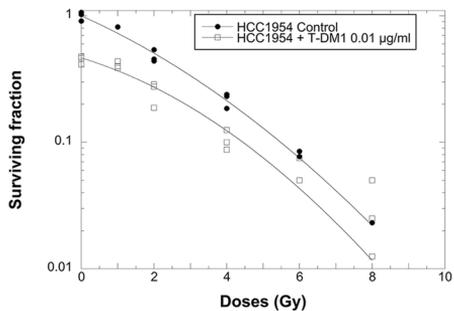
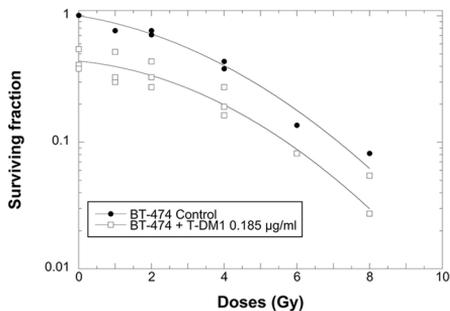
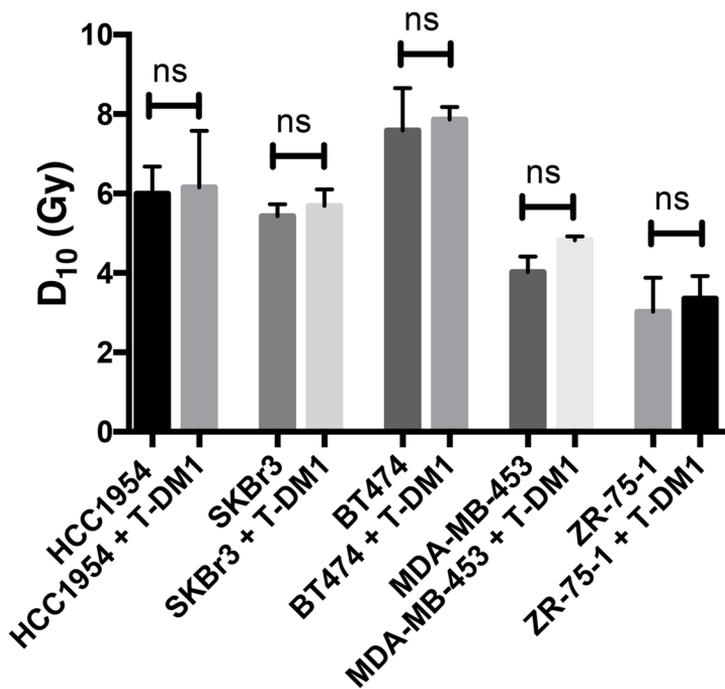
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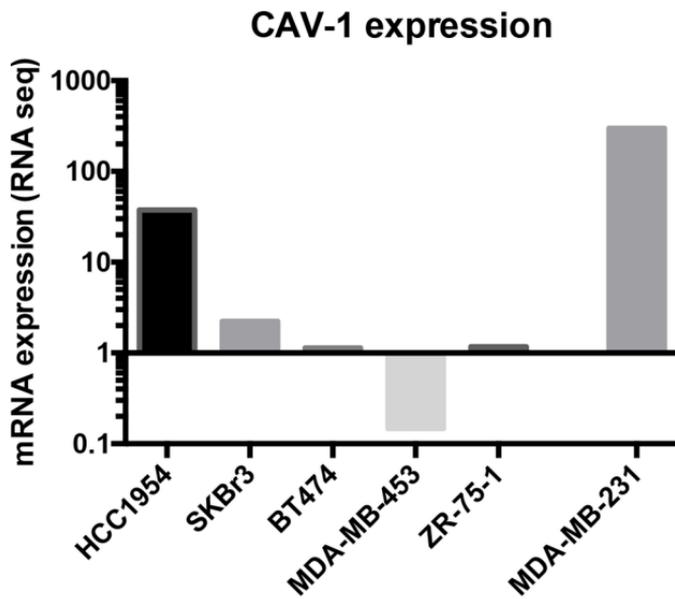
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A**B****C**

A**B**