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Dual HER 1-2 targeting of hormone-refractory prostate cancer by ZD1839 and trastuzumab

Patricia Formento [a], Jean-Michel Hannoun-Levi [a,b], Jean-Louis Fischel [a], Nicolas Magné [a], Marie-Christine Etienne-Grimaldi [a], Gérard Milano [a,*]

[a] *Oncopharmacology Unit, Centre Antoine Lacassagne 33, Avenue de Valombrose, 06189 Nice Cedex 2, France*
[b] *Radiotherapy Unit, Centre Antoine Lacassagne, Nice, France*

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Abstract

Epidermal growth factor receptor (EGFR) and HER-2 are associated with a poor prognosis in various cancers, including prostate cancer. Inhibition of these receptors may provide a treatment for hormone-refractory prostate cancer. The presence of HER-2 (Western blot) and EGFR (5830 fmol/mg protein, ligand-binding assay) was assessed in the hormone-refractory human prostate cancer cell line, DU-145. Cells were exposed to the selective EGFR-TKI (EGFR tyrosine kinase inhibitor) gefitinib ('Iressa™; ZD1839) and/or the HER-2-targeted monoclonal antibody trastuzumab ('Herceptin®'), for 96 h. Irradiation (RX) at 6 Gy the dose causing 50% growth inhibition, was applied 48 h after the start of drug treatment. There was a dose-related effect on cell survival for both ZD1839 and trastuzumab treatments. Combining ZD1839 and trastuzumab led to less than additive effects on cell survival. Chou and Talalay representations further characterised this less than additive effect on cell survival. The application of ZD1839 led to a marked elevation in the level of the negative regulator of cell division, p27. The ZD1839-trastuzumab combination had less of an impact on p27 expression compared with the effect of ZD1839 treatment alone. The lowest expression of the apoptotic-related protein, Bax, was observed in the presence of the drug combination. There was a significant interaction (synergism) between RX and either ZD1839 or trastuzumab treatments. In contrast, the drug combination with RX resulted in antagonistic cytotoxic effects. These results indicate an antagonistic interaction between EGFR and HER-2 targeting and provide molecular mechanisms supporting this observation. Data from DU-145 cells suggest that dual targeting of EGFR and HER-2 may be inappropriate for the treatment of hormone-refractory prostate cancer, especially in the context of their combination with RX.

Keywords: Prostate cancer; *Trastuzumab*; ZD1839; Iressa™; Gefitinib; Herceptin®; Drug treatment; *erbB-1, erbB-2*

1. Introduction

Prostate cancer remains one of the leading causes of cancer-related deaths in the United States of America (USA) and Europe [1]. In prostate cancer, a pejorative evolution follows a typical course of metastatic disease that is refractory to androgen ablation [2]. The progressive growth of many human carcinomas, including prostate [3], has been associated with expression of epidermal growth factor receptor (EGFR). The progression of prostate cancer to androgen-independence has been shown to be linked with the tumoral overexpression of EGFR [4]. In addition, it has been shown that androgen-independent prostate cancers express increased levels of the ErbB2 receptor protein [5–7]. Thus, both ErbB1 (EGFR) and ErbB2 inhibitors may play an important role in the therapeutic targeting of hormone-refractory prostate cancer. Accordingly, experimental

* Corresponding author. Tel.: +33 4 92 03 15 53; fax: +33 4 93 81 71 31.
E-mail address: gerard.milano@nice.fnclcc.fr (G. Milano).

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data have shown that the EGFR tyrosine kinase inhibitor gefitinib (ZD1839; Iressa™) inhibited prostate cancer cell growth [8]. In addition, antitumour effects were observed in xenografts derived from androgen-independent prostate cancer cells treated with an anti-HER recombinant humanised monoclonal antibody [9]. However, another study by Agus and colleagues [10] pointed to the inefficacy of trastuzumab (Herceptin®) on androgen-independent prostate xenograft tumour growth. Phase I clinical studies suggest a promising clinical activity of ZD1839 in advanced prostate cancer patients [11]. A combination therapy of docetaxel, estramustine and trastuzumab has led to a promising biological response (decrease in prostate-specific antigen (PSA)) in metastatic androgen-independent prostate cancer patients [12]. Based on the above findings, we felt it would be interesting to combine both EGFR and HER-2 targeting approaches in the treatment of androgen-independent prostate cancer. This led us to study the impact of a combined application of ZD1839 and trastuzumab on the androgen-refractory prostate cancer cell line, DU145, that expresses both EGFR and HER-2. The impact on cell survival when coupling irradiation with anti-HER receptor therapy was also examined, since irradiation remains one of the primary therapeutic approaches in prostate cancer.

2. Materials and methods

2.1. Chemicals

ZD1839 (Gefinitib®, Iressa™) was kindly provided by AstraZeneca. A 50 mM working solution in dimethylsulphoxide (DMSO) was prepared before use. Trastuzumab (Herceptin®) was kindly provided by the pharmaceutical unit of our institution. Dulbecco's modification of Eagles medium (DMEM), and glutamine were purchased from Whittaker (Verviers, Belgium). Foetal bovine serum (FBS) was obtained from Dutscher (Brumath, France). Penicillin and streptomycin were obtained from Whittaker. 3-(4-5-Dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium.bromide (MTT) and DMSO were purchased from Sigma (St. Quentin Fallavier, France).

2.2. DU-145 cell line

The human prostatic cancer cell line, DU-145, is devoid of androgen receptors and was originally obtained from the American Type Culture Collection (Rockville, MD). This cell line is routinely grown in our laboratory and screened for the presence of Mycoplasma (Mycoplasma detection kit-Roche Diagnostics). DU-145 cells were found to show marked expression of EGFR: 5830 fmol/mg protein, ligand-binding assay; previously published in [13]. The presence of HER-2 was confirmed by Western blotting. The DU-145 cells were maintained as a monolayer culture in DMEM supplemented with 10% FBS, 2 mM glutamic acid, 50 000 units/l penicillin and 80 μM streptomycin in a fully humidified incubator (Sanyo, Japon) at 37 °C in an atmosphere containing 8% CO_2.

2.3. In vitro cytotoxicity measurements

Growth inhibition was assessed using the MTT assay [14] as described below. Cells were washed with fresh medium and incubated with MTT for 2 h, formazan was released and fixation was revealed by the addition of 100 μl of DMSO. Absorbance at 450 nm was measured using a microplate reader (Labsystems, Helsinki Finland). Results were expressed as the relative percentage of absorbance compared with controls without drug. Experimental conditions were tested in quintuplicate (5 wells of the 96-well plate per experimental condition), and independent experiments were performed in triplicate. The dose-effect curves were analysed using Prism software (GraphPad Software, San Diego, USA.) The antiproliferative activity was expressed as IC_{50} values (concentrations leading to 50% cell survival).

2.4. Determination of the cytotoxic effects of the agents alone

Cells were seeded in 96-well microtitration plates (100 μl/well) in order to maintain exponential growth for the control without the drug during the whole experiment: initial cell density was 3000 cells/well for DU-145. Forty-eight hours after seeding, cells were exposed for 96 h to the agents alone (ZD1839, trastuzumab) with 11 concentrations ranging as follows: 5×10^{-7} M < [ZD1839] < 5×10^{-5} M, and 0.2 μg/ml < [Trastuzumab] < 20 μg/ml. These doses were selected so as to cover the range from 'no effect' to 'full effect' with a fixed ratio.

Cells were irradiated with γ-rays (RX) 96 h after seeding as monolayers in 96-well microtitration using a ^{60}Co unit at a dose rate of 1 Gy/min. Dose effect curves were established using DU-145 cancer cells and a total of 9 doses: 0.5;1;2;4;6;8;10;15;20 Gy. Cells were maintained in DMEM supplemented with 10% FBS during all radiation exposures, which were performed at room temperature.

Cytotoxicity assays were performed at the end of drug (ZD1839 and trastuzumab alone) exposure using MTT and 48 h after RX application using the MTT assay. Non-irradiated cells (controls) were in the same microtitration plates as the irradiated cells.

2.5. Determination of cytotoxicity using ZD1839 and/or Trastuzumab in combination with radiation (RX)

Cells were seeded in 96-well microtitration plates (100 μl/well) to obtain exponential growth for the whole

duration of the experiment (initial cell density as indicated above) Fig. 1. Forty-eight hours after seeding, DU-145 cells line were treated for 96 h with different concentrations of drugs. Thus, ZD1839 was applied simultaneously or not with trastuzumab (concentrations were: 5×10^{-6}, 10^{-5} M and 8, 12 μg/ml for ZD1839 and trastuzumab, respectively). These drug concentrations were located close to their respective IC50 values as single drug treatments. Two doses of RX were applied (3 and 6 Gy, doses that are around the values leading to 50% cell survival). RX was delivered 48 h after the start of drug exposure to ZD1839 and/or trastuzumab (in one plate, a single concentration of a drug combination and a single RX dose were tested with concomitant controls).

Growth inhibition was assessed 144 h after cell seeding using the MTT assay as described above. After renewal of the medium, cells were incubated with MTT. Cell sensitivity to the radiation dose was expressed by IC30, IC50 and IC75 (concentrations leading to 30%, 50% and 75% cell survival, respectively). Experiments were done in triplicate.

2.6. Assessment of the effect of drug combinations and/or radiation

2.6.1. Combination Index calculations for the drug combinations (ZD1839 and trastuzumab)

The cytotoxic effect obtained with the zd1839-trastuzumab combinations was analysed according to the Chou and Talalay method using Calcusyn software (Biosoft Cambridge, UK) [15]. Interactions between ZD1839 and trastuzumab were assessed by means of an automatically computed combination (CI). CI was determined at 50%, 75% and 90% cell death and was defined as follows:

$$CI_{A-B} = [(D_{A/A+B})/D_A] + [(D_{B/A+B})/D_B] + [\alpha(D_{A/A+B} \times D_{B/A+B})/D_A D_B],$$

where CI_{A+B} is the CI for a fixed effect (F) for the combination of cytotoxic A and cytotoxic B. $D_{A/A+B}$ is the concentration of drug A in the combination A + B giving an effect F. $D_{B/A+B}$ is the concentration of drug B in the combination A + B giving an effect F. D_A is the concentration of drug A alone giving an effect F. D_B is the concentration of drug B alone giving an effect F. α is the parameter with value at 0 when A and B are mutually exclusive and at 1 when A and B are mutually non-exclusive. In the present application, ZD1839 and trastuzumab are mutually non-exclusive therefore $\alpha = 1$.

The Combination Index indicated: synergism <0.8; additivity >0.8 and <1.2; antagonism >1.2; slight synergistic and additive cytotoxic activity for values of 0.8 and 1.2, respectively.

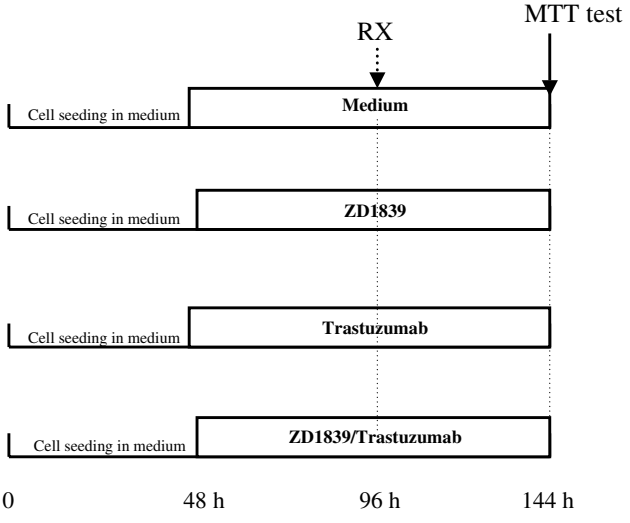

Fig. 1. Drug and radiation combinations tested in the DU-145 cell line. RX: γ ray irradiation (3 and 6 Gy). MTT, 3-(4-5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide.

2.7. Isobolographic method for drug combinations (ZD1839 and trastuzumab) and radiation

Dose-response interactions between RX and ZD1839 and/or trastuzumab at 30%, 50% and 75% cell growth inhibition ((IC30), (IC50) and (IC75)) were evaluated using the classical isobolographic method described by Steel and Peckham [16]. The theoretical basis and procedures of the isobologram method have been described in detail elsewhere in [17]. For a given level of efficacy (% survival), an "envelope of additivity" curve was calculated from the dose-effect curves of each drug alone (or drug combination) and from the dose effect-curves of RX alone (2 doses, 3 and 6 Gy). The coordinates of the experimental point are the drug concentration and the radiation dose which, when combined, result in the given level of efficacy. If the experimental point falls above, within or under the limits of the envelope of additivity, drugs and radiation combinations give rise to antagonistic, additive or synergistic effects, respectively.

2.8. Preparation of samples for cellular factor analysis

DU-145 1.5×10^6 cells were seeded in a 175-cm^2 flask with 30 ml of culture medium on day 0. On day 2, drugs were added to a final concentration of ZD1839 at 10^{-5} M and trastuzumab at 40 μg/ml. RX (3 Gy) was applied on day 4.

Cells were collected at 30, 60 and 90 min after irradiation and were also collected and counted on day 3 (24 h following drug exposure), day 4 (48 h following drug exposure), day 5 (72 h following drug exposure and 24 h after RX), and centrifuged. Cell pellets were washed

twice with phosphate-buffered saline (PBS) and processed for Western blotting by lysis. Protein content was measured using bicinchoninic acid (BCA).

The following parameters were measured by Western blotting analysis: Bax, Phospho-AKT, Phospho-mitogen activated protein (phospho-MAP) Kinases (p-P41-42), p21 and p27. Samples containing an equal amount of protein (50 μg) from DU145 lysates were resolved on a sodium dodecyl sulphate (SDS) polyacrylamide gel and transferred to nitrocellulose membranes overnight. The blots were first blocked for 2 h at room temperature in a buffer containing 200 mM NaCl, Tris 10 mM, pH 7.4, and 5% non-fat milk powder and then incubated in the same buffer with primary antibody at 4 °C overnight (range of dilutions 1/100 to 1/5000) according to manufacturer's instructions. After washing with TTBS (TS + Tween 80 0.1%), filters were incubated for 60 min with horseradish peroxidase-labelled antimouse or antirabbit secondary antibody at room temperature; signals were detected using the enhanced chemoluminescence assay (Amersham Life Science Inc.) according to the manufacturer's instructions. Results of investigated parameters were expressed as a relative intensity as compared with the internal control (p42 protein).

3. Results

3.1. Impact on cell survival

There was a dose-related effect on cell survival for both ZD1839 and trastuzumab (Fig. 2). Combining ZD1839 and trastuzumab led to a less than additive effect on cell survival. Chou and Talalay representations (Fig. 3 and Table 1) further characterised this less than additive effect on cell survival resulting from the ZD1839-trastuzumab combination. The application of ZD1839 led to a marked elevation in p27, a negative regulator of cell division (Fig. 4). The ZD1839-trastuzumab combination had slightly less of an impact on p27 expression compared with the effect of ZD1839 alone. This was observed in particular at 24 and 72 h following drug exposure. Similar effects generated by ZD1839 treatment alone and the drug combination were noted for the expression of p21, another negative cell cycle regulator (data not shown). The impact on the apoptotic-related protein, Bax, was also examined. The lowest expression of this protein was observed in the presence of the drug combination compared with the changes induced by treatments with the single drugs (Fig. 5).

3.2. Effects of irradiation

The combination with RX of the drugs alone or in combination was examined with regard to their effects on cell survival and RX-induced proteins. There was a marked increase in phospho-p42/44 shortly after irradiation (Fig. 6(a)). Irradiation also induced a marked elevation in phospho-AKT, but this change occurred later than that observed for phospho p42/44 (Fig. 6(b)).

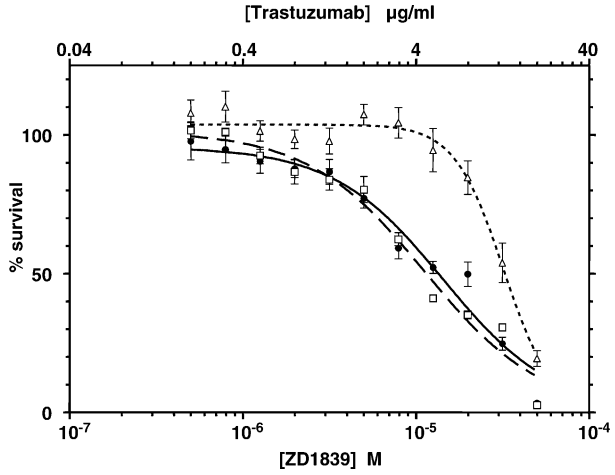

Fig. 2. Antiproliferative effects on DU-145 cells of ZD1839 (open squares), trastuzumab (open triangles) and the drug combination (closed circles).

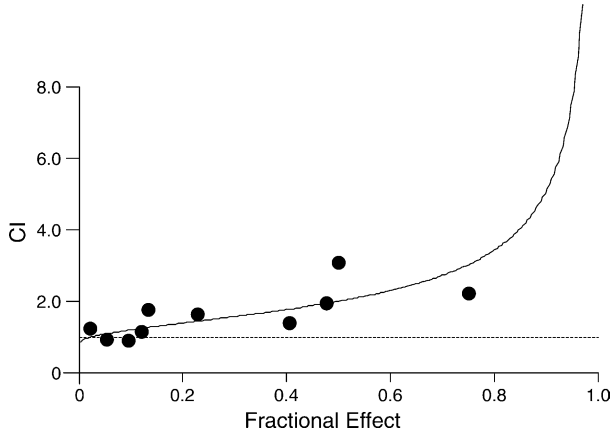

Fig. 3. Chou and Talalay analysis for the combined effects of ZD1839 and trastuzumab: The curve illustrates the Combination Index (CI) values for the different partial cytotoxic effects.

Table 1
Analysis of drug–drug interactions on cell survival

	Drug–drug interactions (ZD1839 + Trastuzumab) Combination Index-values		
	At 10% survival	At 25% survival	At 50% survival
First experiment	5.1	3.0	2.0
Second experiment	2.4	1.8	1.4
Third experiment	1.5	1.3	1.2

Drug–drug interactions were tested by the Chou and Talalay method with a Combination Index value $\geqslant 1.2$ denoting an antagonistic drug–drug interaction.

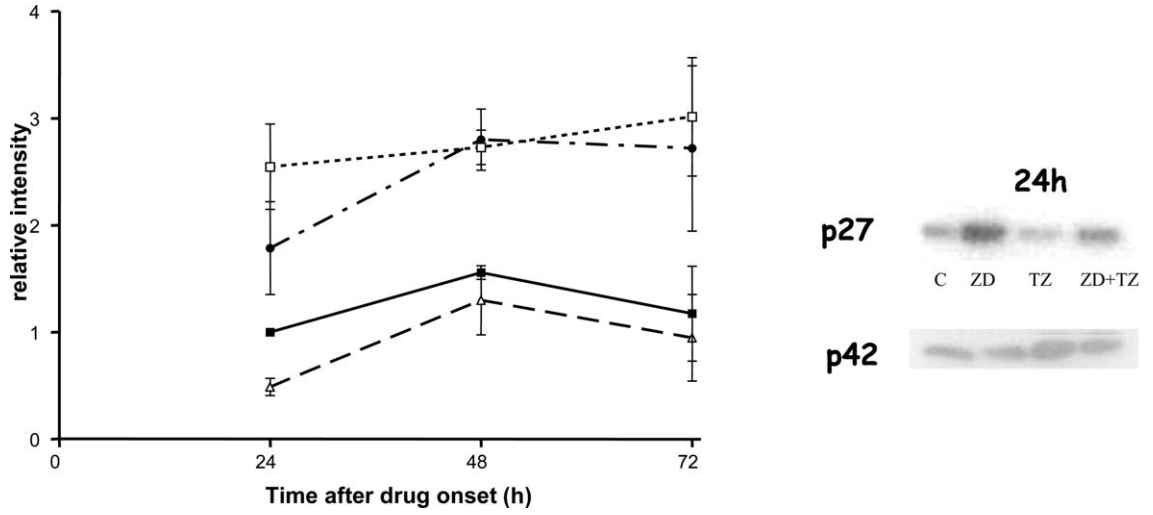

Fig. 4. Effects of ZD1839 (ZD, open squares), trastuzumab (TZ, open triangles) and drug combination (ZD + TZ, closed circles) on the relative intensity of p27 expression. Closed squares represent the control without drug (C). p42 was the control for the Western blotting analysis. A typical Western blotting analysis at 24 h is shown.

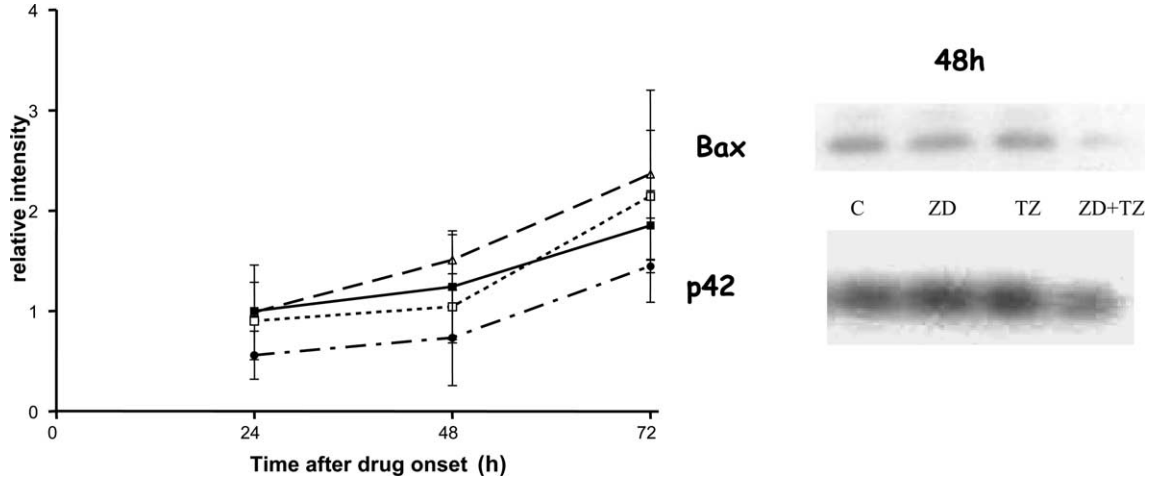

Fig. 5. Effects of ZD1839 (ZD, open squares), trastuzumab (TZ, open triangles) and drug the combination (ZD + TZ, closed circles) on the relative intensity of Bax; closed squares represent the control without drug (C). p42 was the control for the Western blotting analysis. A typical Western blotting analysis at 48 h is shown.

These RX-induced effects on the MAPK and phosphatidyl inositol (PI)-3 kinase pathways were markedly reduced by the presence of ZD1839 and much less so by trastuzumab. Cell survival after irradiation alone and irradiation combined with either drug alone or in combination was examined. There was a significant interaction (synergism) between RX and either ZD1839 or trastuzumab. By contrast, the combination (ZD1839-trastuzumab with RX) resulted in antagonistic cytotoxic effects (Table 2).

4. Discussion

Recent studies have demonstrated that ErB1 (EGFR) and ErbB2 (HER-2) both contribute to the growth of human prostate cancer [18]. EGFR and HER-2 are therefore relevant biological targets for innovative treatments in hormone-refractory prostate cancer [12,13]. The recent study by Melhinghoff and colleagues [18] showed marked growth inhibitory effects on human prostate cancer xenografts when using the dual ErbB1/ErbB2 tyrosine kinase inhibitor, PKI-166. However, it was not clearly established whether the dual ErbB1/ErbB2 approach was superior or not to single receptor targeting. The current study attempted to answer this question by using the DU-145 hormone-refractory prostate cancer cell line, which expresses both EGFR and HER-2. Our results show that the combination of ZD1839 with trastuzumab results in less than additive effects on human prostate cancer cell survival. Molecular factors of cell proliferative and apoptotic pathways

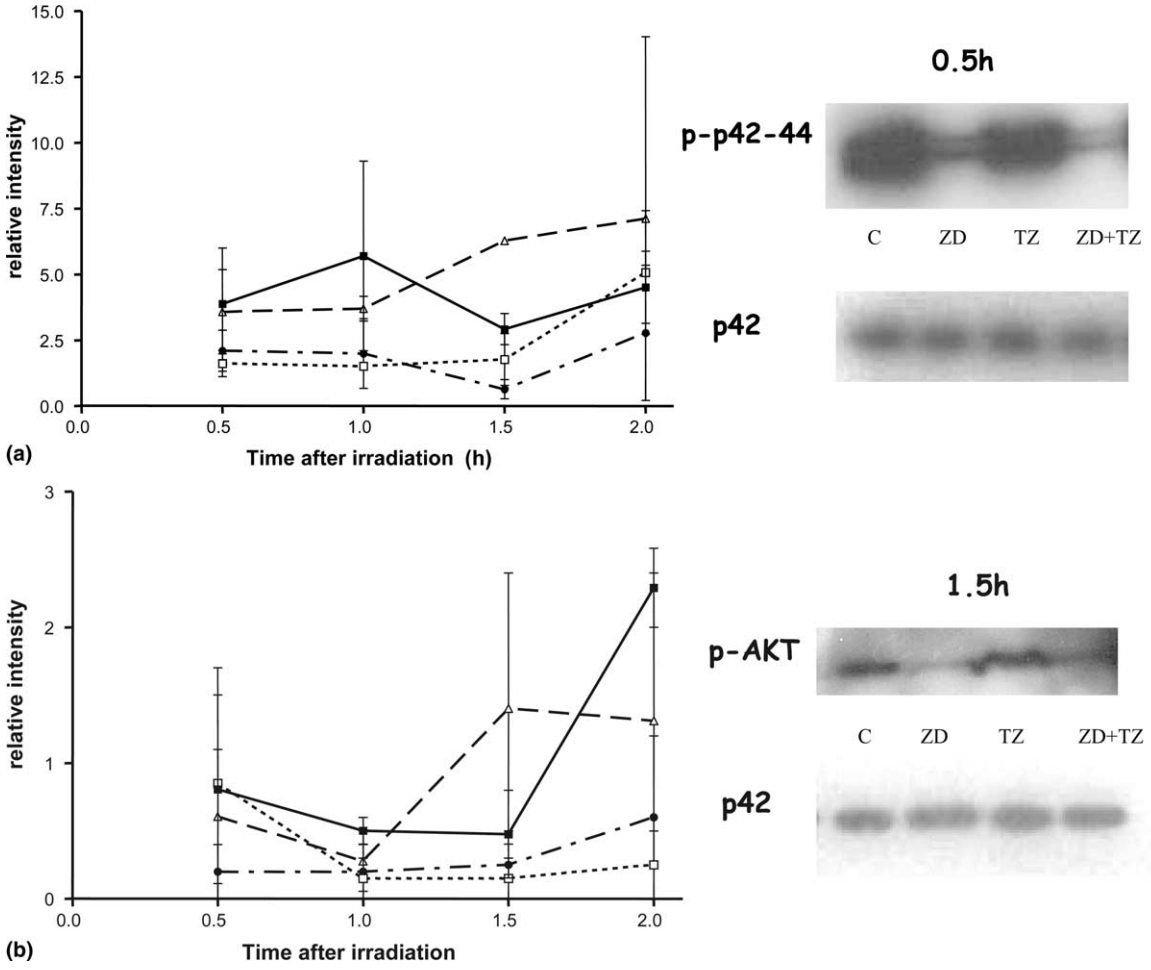

Fig. 6. (a) Effects of ZD1839 (ZD, open squares), trastuzumab (TZ, open triangles) and drug combination (ZD + TZ, closed circles) on the relative intensity of phospho-p42-44; closed squares represent the control without drug (C). p42 was the control for the Western blotting analysis. A typical Western blotting analysis at 0.5 h is shown. (b) Effects of ZD1839 (ZD, open squares), trastuzumab (TZ, open triangles) and drug combination (ZD + TZ, closed circles) on the relative intensity of phospho-AKT; closed squares represent the control without drug (C). p42 was the control for the Western blot analysis. A typical Western blotting analysis at 1.5 h is shown.

Table 2
Results of isobolographic analyses of radio-chemotherapy interactions on DU-145 cells

Drugs	RX Gy	30% Survival	50% Survival	75% Survival	Mean
ZD1839	3	Syn	Syn	+	Syn
	6	Syn	+	+	Syn
Trastuzumab	3	+	+	+	+
	6	Syn	Syn	+	Syn
ZD1839/Trastuzumab	3	Ant	Ant	Ant	Ant
	6	Ant	Ant	Ant	Ant

Isobolographic interpretation at 30%, 50% and 75% growth inhibition (results from 3 separate experiments). Means were calculated from isobolographic values obtained at IC30, IC40, IC50, IC60 and IC75. Syn, synergistic effect; Ant, antagonistic effect; +, additive effects.

were examined in parallel to the cell survival analyses. The changes observed in these cellular factors mirror the findings for cell survival. It was shown that an increase in the negative regulators of cell division, p27 and p21, was less marked with the combined drug application compared with the effects observed with ZD1839 treatment alone. A similar conclusion was drawn for the Bax results, one of the key regulators of apoptosis, whose time-related increase was the lowest when applying ZD1839 plus Trastuzumab compared with the changes induced by the individual drug treatments. The exact reason for the relatively unfavourable influence of the drug combination on cell survival is difficult to elucidate. The results of the present *in vitro* experiments have been confirmed it in vivo, using a DU-145 xenograft model in immunodeficient mice (data not shown, investigations still in progress). Various hypotheses can be drawn for the less than additive effects on cell survival observed when combining ZD1839 and trastuzumab. First, HER-2 is the preferred dimerisation partner for other members of the ErbB family [19] and EGFR-HER-2 heterodimers are a functionally potent signalling combination [20]. Thus, targeting HER-2

may also impact upon the EGFR pathway. By contrast, it has been shown that the growth of HER-2-overexpressing tumour cells could be inhibited by ZD1839 [21]. Thus, the effect of each individual drug presently tested may have overlapping effects on a single target and involve the dual EGFR-HER-2 pathways and this may explain, at least in part, why the combination of the two drugs results in less additive effects compared with the single drugs alone. However, a dual targeting by ZD1839 and trastuzumab on human breast cancer cells has been shown to result in synergistic inhibitory effects [22,23]. Experimental data reported by Christensen and colleagues [24] suggest that it is more difficult to inhibit EGFR phosphorylation in cells which express particularly high HER-2 levels. In addition, a recent study by Hendricks and coworkers [25] indicates that HER-2 overexpression reduces the EGFR internalisation rate, thereby increasing the fraction of EGFR that is recycled. Consequently, the relative abundance of EGFR and HER-2 may play a key role in the final effects on the cell. This EGFR/HER-2 ratio could explain, at least in part, the discrepancy between the present data and those reported for breast cancer cells [22,23], where the cellular models used had an overexpression of HER-2 compared EGFR expression. This contrasts to our cell model-DU-145 cells where EGFR is markedly expressed (5830 fmol/mg prot).

Dent and colleagues [26] recently made the interesting observation that irradiation of A431 squamous cell carcinoma and MDA-MB-231 mammary carcinoma cells caused rapid primary (0–10 min) and secondary (90–240 min) activation of the EGFR and MAPK pathways and these effects were abolished by the molecular inhibition of either EGFR or Ras function. This finding could explain the well-known phenomenon of cell survival after irradiation. The present data confirm these previously reported observations, with a marked elevation in phospho-AKT and phospho-p42/p44 (phospho-MAP kinase). These RX-related changes in key molecular protagonists of HER pathways were reduced by the presence of ZD1839, but much less so by trastuzumab (Figs. 6(a) and (b)). Yacoub and coworkers [27] recently reported the activation of DNA repair pathways shortly after irradiation of DU-145 cells. This phenomenon was suppressed by the application of the MEK inhibitor, PD90859 [27]. In the present study, the presence of either ZD1839, trastuzumab or the drugs combined did not significantly modify the time-related changes in some of the major DNA-repair-associated proteins (DNA-PK, ATM, ERCC1, XRCC1; data not shown). This absence of a significant impact of ZD1839 and trastuzumab on the expression patterns of these DNA-repair related enzymes therefore does not help to explain why their combination with irradiation resulted in synergistic cytotoxic effects (Table 2); such a synergistic cytotoxic interaction was highlighted in recent reports by others [28,29] and by us [30]. Of potential importance, and in line with the lack of an augmentation in cell death when ZD1839 and trastuzumab were combined, the drug combination plus irradiation gave rise to antagonistic cytotoxic effects (Table 2).

One of the main messages from our study is that the combination of ZD1839 and trastuzumab leads to less than additive effects on cell survival in DU-145 cells, a hormone- refractory prostate cancer cell line. This observation was corroborated and strengthened by the changes we observed in some molecular factors of cell division and apoptosis. In addition, there were clear antagonistic cytotoxic effects resulting from the combination of the two drugs plus irradiation. Thus, these data suggest this drug combination may be inappropriate in targeted treatments of hormone-refractory prostate cancer.

Conflict of interest statement

None declared.

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