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Gefitinib–trastuzumab combination on hormone-refractory prostate cancer xenograft

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Abstract

New drugs and new combinations of drugs have recently shown promising clinical activity in hormone refractory prostate cancer. We studied the association of gefitinib with trastuzumab on the androgen-refractory prostate cancer cell line DU145 expressing both epidermal growth factor receptor (EGFR) and HER-2. Drug combinations with radiotherapy (RT) were considered along with the analysis of factors linked to cell proliferation and apoptosis. The antitumour effects of gefitinib were more pronounced than those observed with trastuzumab. In mice receiving the gefitinib–trastuzumab combination, reduction in tumour volume was inferior to that predicted by the observed impact of the agents alone. The presence of trastuzumab markedly attenuated the relative increase on p27 expression and the Bax:Bcl2 ratio induced by gefitinib. The combination gefitinib-RT had similar antitumour effects as those predicted by the impact of the individual treatments, whereas the effect of the trastuzumab-RT combination was inferior to that predicted by the individual effects. The present data should be borne in mind when designing new clinical schedules for treatment of hormone-refractory prostate cancer including the use of HER inhibitors.

Keywords: Prostate cancer; Gefitinib; Trastuzumab

1. Introduction

In prostate cancer, a pejorative evolution follows a typical course of metastatic disease refractory to androgen ablation [1]. Prostate cancer remains one of the leading causes of cancer related deaths in the USA and Europe [2] and thus new treatment options are needed especially for androgen independent disease. New drugs and new combinations have recently shown a promising clinical activity in hormone refractory prostate cancer. This is the case for drugs that target the microtubule network with interesting results reported for docetaxel [3,4]. It has been shown that androgen-independent prostate cancers express increased levels of ErbB2 receptor protein [5–9]. Recent studies reveal that tumoural expression of epidermal growth factor receptor (EGFR) increases during the natural history of prostate cancer and correlates with disease progression and hormone refractory status [10,11]. Thus, both ErbB1 (EGFR) and ErbB2 inhibitors can play an important role in the therapeutic targeting of hormone-refractory prostate cancer. Pre-clinical data have shown that the EGFR tyrosine kinase inhibitor gefitinib (Iressa®) inhibited prostate cancer cell growth [12]. Further, antitumour

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effects were observed in xenografts derived from androgen-independent prostate cancer cells treated with an anti-HER-2 recombinant humanised monoclonal antibody [13]. Indeed, early clinical studies suggest a promising clinical activity of gefitinib in advanced prostate cancer patients [14]. An association between docetaxel, estramustine and trastuzumab–Herceptin® (Tra) has led to an encouraging biological response such as a decrease in prostate specific antigen (PSA) in metastatic androgen-independent prostate cancer patients [15]. It was thus felt that an approach combining both EGFR and HER-2 targeting would be interesting to consider in androgen-independent prostate cancer. This led us to study the association of gefitinib with trastuzumab (Herceptin®) on the androgen-refractory prostate cancer cell line DU145 expressing both EGFR and HER-2. We first addressed this question by an *in vitro* investigation which demonstrated antagonistic cytotoxic effects when combining gefitinib and trastuzumab to DU145 cells [16]. In the present study, we analysed the antitumour effects of this dual HER targeting on DU145 cells xenografted in the nude mice. Various drug combinations with radiotherapy (RT) were considered in term of their impact on cell proliferation and apoptosis. Tumour angiogenesis was also specifically examined.

2. Materials and methods

2.1. Chemicals

Gefinitib, was kindly provided by AstraZeneca. Trastuzumab was kindly provided by the pharmaceutical unit of our Institute. Dulbecco's modification of Eagle's medium (DMEM), and glutamine were purchased from Whittaker (Verviers, Belgium). Fetal bovine serum (FBS) was obtained from Dutscher (Brumath, France). Penicillin and streptomycin were from Whittaker.

2.2. DU145 cell line

The human prostate cancer cell line DU145 is devoid of androgen receptors and was originally obtained from the American Type Culture Collection (Rockville, MD). This cell line was routinely grown in our laboratory and screened for the presence of mycoplasma (Mycoplasma detection kit-Roche Diagnostics). DU145 cells were found to express EGFR at 5830 fmol/mg protein measured by a specific ligand-binding assay as we have previously published [16]. The presence of HER-2 was established by western blotting. The DU145 cells were maintained as a monolayer culture in DMEM supplemented with 10% FBS v/v, 2 mM glutamic acid 50 000 units/l penicillin and 80 μM streptomycin in a fully humidified incubator (Sanyo, Japan) at 37 °C in an atmosphere containing 8% CO_2. Batches of 15×10^6 cells frozen in FBS supplemented with 5% DMSO v/v were prepared in advance for injection into mice. Shortly before injection, cells were thawed and suspended in Ringer lactate.

2.3. Mice

Animal experiments were performed in accordance to the regulations of the institutional ethical commission. Six-week-old male swiss nude mice were purchased from Charles River (L'Arbresle, France) and received subcutaneous (s.c.) inoculation in the right flank with 2×10^6 DU145 cells dissolved in 100 μl of Ringer lactate (six animals per treatment condition).

2.4. Treatment

Doses and regimen:gefitinib at a dose of 120 mg/kg/day was administered 5 days/week for 2 weeks by oral gavage. Trastuzumab was given intraperitoneally (i.p.) at a dose of 10 mg/kg/day 5 days/week for 2 weeks. RT at a dose of 3 Gy/day was given 3 days/week for 2 weeks, on tumour only. Two different and complementary experimental regimens were conducted:

1. Assessment of treatment effects and agent interaction (Fig. 1(a)): mice were treated with vehicle only (control), gefitinib alone, trastuzumab alone, RT alone and combinations: gefitinib + trastuzumab, gefitinib + RT and trastuzumab + RT (seven treatment groups).
2. Assessment of the influence of drug sequence: mice were treated with vehicle only (control) or with three different gefitinib and trastuzumab combinations according to schedules described in Fig. 1(b) (four treatment groups).

The treatment regimens started when tumours reached 8 mm in diameter (Day 0) according to the schedule shown in Fig. 1(a) until Day 14 for Group 1 and according to the schedule shown in Fig. 1(b) until Day 28 for Group 2; in this group the follow-up of "controls" was stopped after day 21 for ethical reason.

2.5. Tumours

Tumour lengths and widths were measured weekly using a caliper and tumour volume was calculated as $\pi/6 \times \text{length} \times \text{width}^2$ until animal sacrifice, which was Day 21 for Group 1 and Day 28 for Group 2 (Fig. 1(a) and (b)).

Animals were sacrificed by spinal cord dislocation and tumours were subsequently removed surgically and weighed; one half of the tumour was directly frozen in liquid nitrogen for protein analysis and the other half

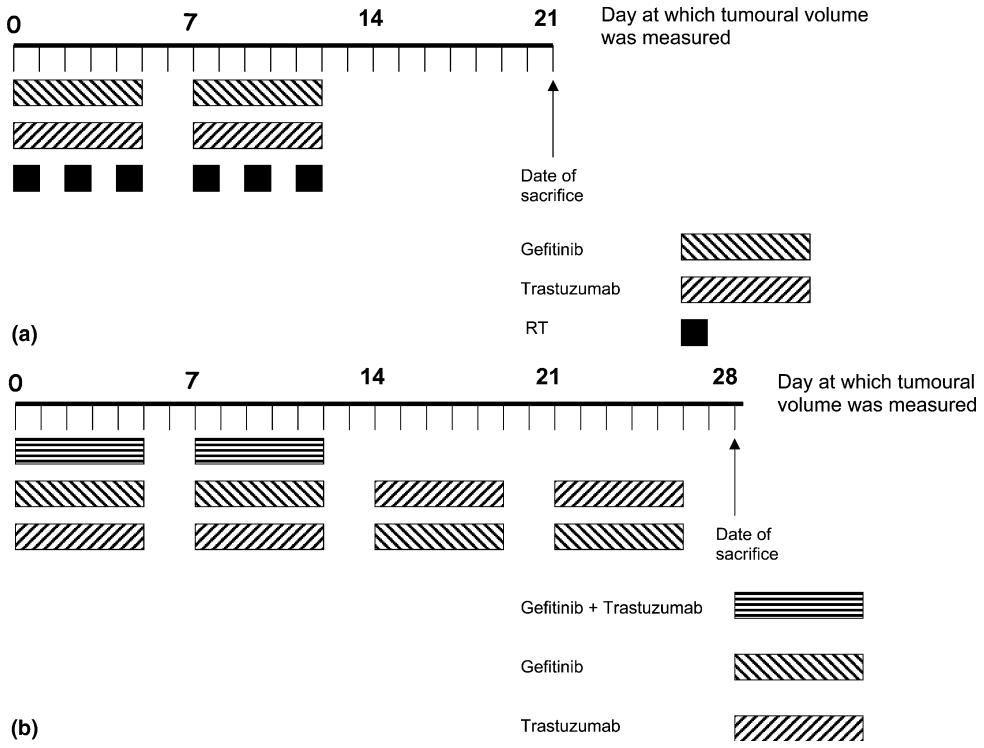

Fig. 1. (a) Schematic representation of the treatment schedule for assessment of treatment effects of agents and RT alone or in combination. See Section 2 for more details. (b) Schematic representation of the treatment schedule for assessment of the influence of drug sequence of gefitinib and trastuzumab. See Section 2 for more details.

fixed in paraformaldehyde overnight for vasculature examination.

2.6. Preparation of samples for cellular factor analysis

Frozen tumours were pulverised in a liquid nitrogen-cooled Thermovac pulveriser. The resulting powders were homogenised in 10 vol. of 10 mM Tris–HCl buffer pH 7.4, containing 1 mM EDTA, 0.5 mM dithiothreitol and 10 mM sodium molybdate. The homogenates were centrifuged for 1 h at 105 000 g (+4 °C). The supernatants (cytosol) was used for protein determination by western blotting. Total protein content was measured using bicinchoninic acid (BCA). Expression of proteins linked to apoptosis (Bax, Bcl2) or to cell proliferation (p27) was measured by Western blot analyses of tumours obtained on the day of animal sacrifice. Samples containing an equal amount of protein (50 μg) from homogenates of DU145 were resolved by SDS polyacrylamide gel electrophoresis 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% w/v non-fat milk powder and then incubated in the same buffer with primary antibody at 4 °C overnight (range of dilution 1/100–1/5000). After washing with TTBS buffer (Tris 10 mM, NaCl 200 mM, Tween 80 0.05% v/v), filters were incubated for 60 min with horseradish peroxydase-labeled anti-mouse or anti-rabbit secondary antibody at room temperature. Signals were detected using the enhanced chemoluminescence assay (Amersham Life Science, Inc.) according to manufacturer's instructions. Results of investigated tumoural parameters were expressed as a relative intensity using automatic integration, arbitrary units, on ImageMaster (PharmaciaBiotech) as compared to an internal control (p42 Map kinase).

2.7. Vessel density (peritumour and intratumour)

Blood vessel density was examined by pathologic examination of the blood vessels on deparaffinised tissue sections from tumours obtained on the day of animal sacrifice. Quantification of peritumour and intratumour vascularisation was based on blood vessel (BV) enumeration performed by a pathologist in our institute. BV was identified by the presence of endothelial cells and red blood cells. For each specimen 10 fields at the 400× magnification were examined, BV containing fields (BVCF) and scored as 0 (no BVCF), 1 (less than 3 BVCF), 2 (less than 7 BVCF) and 3 (more than 6 BVCF).

2.8. Statistical assessment of treatment effects

The effects of the treatments were evaluated using the non-parametric Mann–Whitney test by comparing

"predicted" tumour volumes with "observed" tumour volumes. We defined predicted tumour volume (PTV_{AB}) = [(tumour volume A alone) × (tumour volume B alone)]/[tumour volume for control]. The effects on vascularisation were evaluated using ANOVA. The impact of treatment on molecular factors was examined by using the Mann–Whitney test.

3. Results

3.1. Drugs and irradiation as single treatments

As compared to controls, gefitinib, trastuzumab, and RT alone reduced tumour growth by 30–60% (Figs. 2, 3(a) and (b), and Table 1). The antitumour effects of gefitinib were more marked than those observed with trastuzumab and gefitinib only induced a significant reduction in tumour volume as compared to controls without treatment (Table 1). It must be underlined that the better antitumour effect of gefitinib as compared to trastuzumab was observable during the treatment period only (day 0–day 14, Fig. 2). In contrast, trastuzumab had a weaker but a longer lasting effect than gefitinib. The effects of gefitinib alone and RT alone on tumour growth coincided with a significant increase in the expression of cell-cycle regulator p27 relative to controls (Fig. 4(a)). Apoptosis was evaluated by the determination of Bax and Bcl2. Single-agent administration of gefitinib induced a significant increase in the Bax:Bcl2 ratio relative to controls (Fig. 4(b)). In contrast, expression of p27 and the Bax:Bcl2 ratio was not significantly modified in tumours treated with single-agent trastuzumab (Fig. 4(a) and (b), and Table 2). Both peritumour and intratumour vascularisation was significantly reduced by single-agent trastuzumab, whereas it was not influenced by single-agent gefitinib (Table 1). RT induced a decrease in intratumour vascularity only (Table 1).

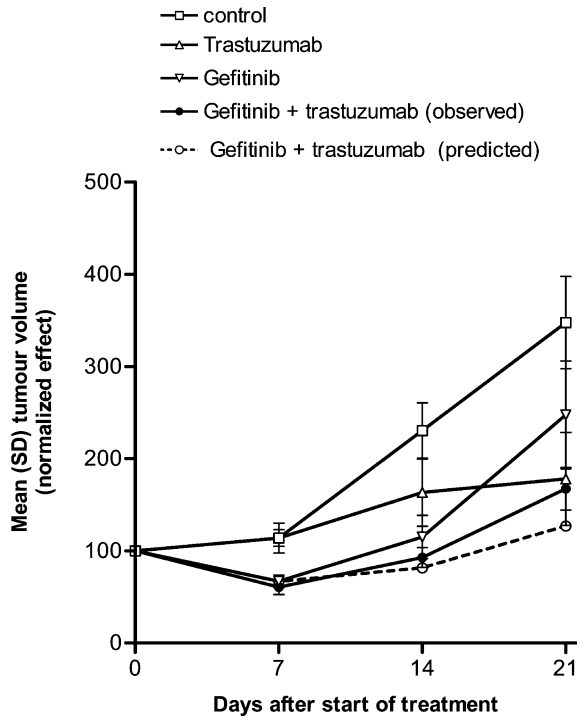

Fig. 2. Activity of gefitinib and trastuzumab, alone and in combination, on DU145 xenograft tumour volume in mice.

3.2. Treatment combinations

In mice receiving the gefitinib–trastuzumab combination, reduction in tumour volumes was inferior to that predicted by the observed impact of the agents alone (Fig. 2). In line with this observation, the presence of trastuzumab markedly attenuated the relative increase in p27 expression and the Bax:Bcl2 ratio induced by gefitinib (Fig. 4(a) and (b), and Table 2). It was noted that sequence of drug administration did not influence the effect of the gefitinib–trastuzumab combination on tumour growth (Fig. 5). Thus, the tumour growth curves were superimposable for the effect of gefitinib plus trastuzumab given together and those of the two drugs applied sequentially in either order. The combination gefitinib-RT had similar antitumour effects to those predicted by the impact of the individual treatments, whereas the effect of the trastuzumab-RT combination was significantly ($P = 0.006$) inferior to that predicted by the individual effects (Fig. 3(a) and (b)). This latter observation was corroborated by a significant decrease in the Bax:Bcl2 ratio for the condition trastuzumab-RT as compared to single treatments (Fig. 4(b) and Table 2).

4. Discussion

EGFR and HER-2 both constitute biological targets for innovative treatment in hormone refractory prostate cancer [14,15]. Thus testing a combination therapy hitting these two receptors is of potential interest in prostate cancer. Such an approach has been at the basis of an experimental study [17] showing the marked growth inhibition of the dual EGFR/HER-2 tyr kinase inhibitor PKI-166 on human prostate cancer xenografts. The results obtained with a single drug does not allow us to anticipate the effects of a combination of two drugs acting, respectively, and specifically on EGFR and HER-2. We first addressed this question by an *in vitro* study using the DU145 hormone refractory prostate cancer cell line which expresses both EGFR and HER-2 [16]. The results demonstrated that the gefitinib–trastuzumab combination led to antagonistic cytostatic/cytotoxic effects. The present study was designed as a necessary *in vivo* complement to the previous *in vitro* study. Based on the

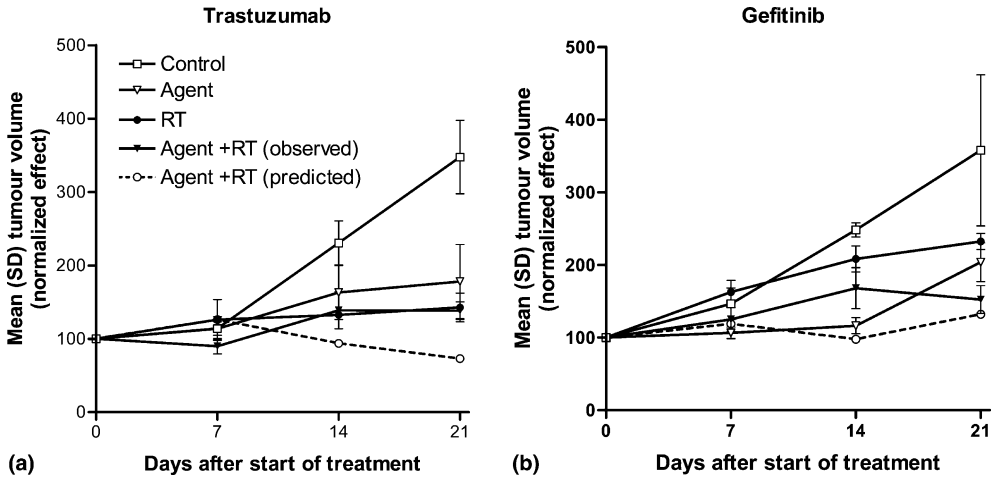

Fig. 3. Activity of (a) trastuzumab, (b) gefitinib alone or in combination with radiotherapy on DU145 xenograft tumour volume in mice.

Table 1
Effects of the different treatment schedules on the reduction of tumour volume and vascularity in DU145 (EGFR+, HER2+) prostate cancer xenografts in mice

Treatment	Reduction in tumour volume	Reduction in peritumour vascularity	Reduction in intratumour vascularity
Gefitinib	0.048	NS	NS
Trastuzumab	NS	0.021	0.021
RT	0.01	NS	0.004
Gefitinib + Trastuzumab	0.042	0.017	0.006
Gefitinib + RT	<0.001	0.059	0.001
Trastuzumab + RT	0.020	0.020	0.003

P values (*vs.* control), derived using the Mann–Whitney test.
NS, not significant.

results from both studies it is now possible to conclude that the association of gefitinib with trastuzumab is antagonistic for the antitumour effects on the DU145 hormone refractory prostate cancer cell line. We paid particular attention to the sequence effect when combining the two drugs and found similar impacts on tumour growth when applying the drugs together or sequentially in either order. Thus the antagonistic effect on growth inhibition cannot be attributed to a given sequence effect. Two studies similar to the present one have been recently reported in which human breast cancer xenografts were analysed [18,19]. Contrasting results were obtained since one study concluded that gefitinib plus trastuzumab induced a greater apoptotic effect than either inhibitor alone [18] and the other failed to reveal such synergistic effects leading the authors to recommend against the association of the two drugs [19]. It must be remembered that these two latter studies were conducted on ErbB2-overexpressing breast cancer cells. The relative abundance of EGFR and HER-2 may play a role in the final effects of dual receptor targeting since EGFR-HER-2 heterodimers are a functionally potent signaling combination [20]. In addition, HER-2 overexpressing tumour cells can be inhibited by gefitinib [21] and HER-2 overexpression reduces the EGFR internalisation rate thus increasing the fraction of EGFR recycled to the cell surface [22].

The present study provides molecular arguments to strengthen the observations on the growth inhibitory effects induced by trastuzumab and gefitinib. It was thus shown from the analysis of one of the main cell cycle regulators, p27, and the apoptosis-linked Bax-Bcl2 ratio, that the combined presence of trastuzumab and gefitinib minimised the impact on these factors generated by drugs alone. This was particularly stricking for the Bax–Bcl2 ratio when comparing the results following treatment with gefitinib alone to those after gefitinib + trastuzumab administration (Fig. 4(b)). It must be emphasised that similar unfavorable effects of this drug combination on p21, p27 and Bax were also noted following the *in vitro* study [16].

RT is an intrinsic component of the treatment of prostate cancer. Both our previous *in vitro* study [16] and the present investigation have included the application of RT. Both studies reveal an absence of beneficial cytotoxic effects when combining either gefitinib and RT or trastuzumab and RT, this conclusion is particularly well illustrated in Fig. 3 with antagonistic cytotoxic effects between trastuzumab

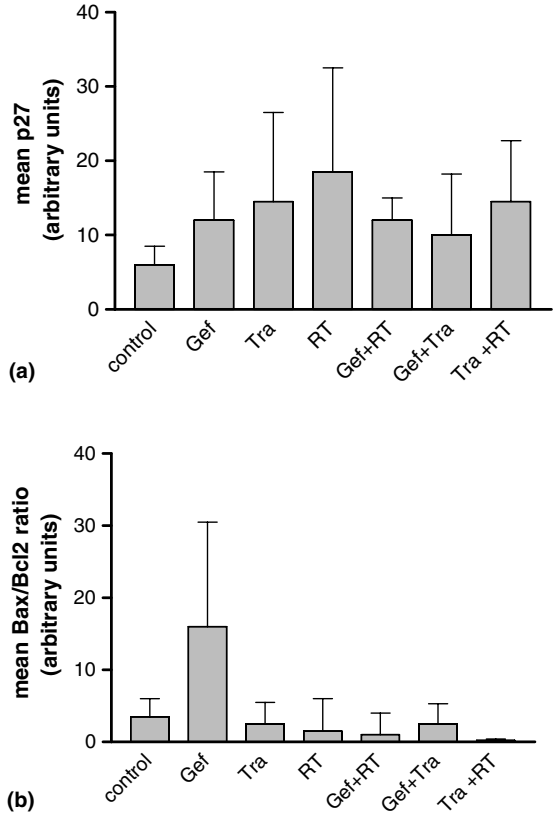

Fig. 4. Effect of gefitinib, trastuzumab, and RT, alone and in combination, on (a) the expression of marker p27 (cell proliferation) and (b) the Bax:Bcl2 ratio (apoptosis) in DU145 xenografts in mice. These experiments were done in triplicates using three different mice. Gef: gefitinib and Tra: trastuzumab.

Table 2
Impact of the different treatment schedules on the expression of p27 and the Bax:Bcl2 ratio in DU145 (EGFR+, HER2+) prostate cancer xenografts in mice

Treatment	Effect on expression of p27	Effect on Bax:Bcl2 ratio
Tested vs. control		
Gefitinib	↑0.037	↑0.014
Trastuzumab	NS	NS
RT	↑0.025	NS
Gefitinib + trastuzumab	NS	NS
Trastuzumab + RT	↑0.027	↓0.025
Gefitinib + RT	↑0.017	NS
Tested vs. gefitinib		
Gefitinib + trastuzumab	NS	↓0.011
Gefitinib + RT	NS	↓0.01
Tested vs. trastuzumab		
Gefitinib + trastuzumab	NS	NS
Trastuzumab + RT	NS	↓0.025
Tested vs. RT		
Trastuzumab + RT	NS	↓0.05
Gefitinib + RT	NS	NS

NS, not significant; ↑, augmented effect; ↓, diminished effect.
P values (*vs.* control), derived using the Mann–Whitney test.

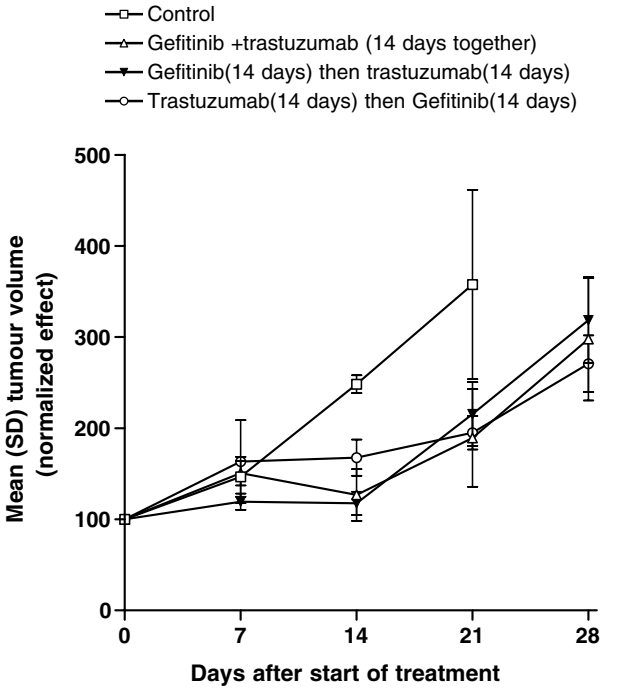

Fig. 5. Influence of sequencing on the activity of gefitinib and trastuzumab combination on DU145 xenograft tumour volume in mice. Controls had to be sacrificed after day 21 due to unethically large tumour volume.

and RT. These effects were sustained by the observation of the respective treatment impacts on p27 and on Bax:Bcl2 (Fig. 4 and Table 2).

HER targeting may concern not only the tumoural cell itself but also, and interestingly, the endothelial cell. This point has been recently brought to light for anti-EGFR therapy with gefitinib [23]. In the same way, trastuzumab has been shown to induce normalisation and regression of the vasculature in an experimental human breast tumour overexpressing HER-2 [24]. The present data are in line with the antiangiogenic effect of trastuzumab (Table 1). In contrast, we did not observe that gefitinib was able to significantly reduce intratumoural or peritumoural blood vessel density as effectively as did trastuzumab (Table 1). It must be stressed that the antitumoural efficacy and the antiangiogenic effects were dissociated since gefitinib, without marked antivascular impact, did influence tumour growth and the inverse was found for trastuzumab. It is possible that the partial destruction of the tumour vasculature by trastuzumab was not sufficient to significantly alter tumour growth but could reduce the access of gefitinib to the tumour. This would explain in part the antagonistic antitumoural effect of the drug combination on DU145 prostate cancer cells. Altogether, the present data should be borne in mind when designing new clinical schedules for treatment of hormone refractory prostate cancer including the use of HER inhibitors.

Conflict of interest statement

None declared.

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