Selective inhibition of HER2 inhibits AKT signal transduction and prolongs disease-free survival in a micrometastasis model of ovarian carcinoma

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Although first-line chemotherapy induces complete clinical remission in many cases of epithelial ovarian cancer, relapse usually occurs 18–28 months from diagnosis owing to micrometastases. The present study aimed to evaluate the effect of trastuzumab on disease-free and overall survival in a specially designed murine model of ovarian cancer (OVCAR-3), which mimicked the natural history of human micrometastatic disease. Trastuzumab can cure the mice if started soon after induction chemotherapy. It can modestly inhibit the proliferation through mitogen-activated protein kinase signal transduction and clearly inhibit AKT phosphorylation, which is involved in survival pathway. As OVCAR-3 cell lines show no HER2 amplification or overexpression, these results warrant further studies to assess the efficacy of trastuzumab in the early stage of relapse in cancer models other than those overexpressing HER2.

Key words: antineoplastic drugs, disease-free, experimental animal models, ovarian carcinoma, survival, trastuzumab

Introduction

Death from epithelial ovarian cancer is generally due to early dissemination of cancer cells to secondary intra-abdominal sites [1]. Although first-line chemotherapy induces complete clinical remission in many patients with this cancer, relapse usually occurs 18–28 months from diagnosis [2, 3]. Relapse is documented by an increase in serum tumour markers or by the reappearance of tumour mass. Once relapse occurs, chemotherapy has little effect.

Several radiotherapeutic and chemotherapeutic techniques have been developed to treat the micrometastatic disease remaining after chemotherapy [4]. Unfortunately, the unfavourable therapeutic indices of such treatments are incompatible with their long-term use. In contrast, several new anticancer drugs, particularly inhibitors of the human epidermal growth factor receptor (HER), have shown promising results in epithelial cancers [5, 6]. These agents have very low toxicity, suggesting potential long-term administration. However, because they are cytostatic rather than cytotoxic, they have only modest effects in advanced metastatic disease. Potentially greater efficacy might be achieved in micrometastatic disease.

Trastuzumab (Herceptin®) is one of these new biological agents. Trastuzumab targets the HER2 receptor and has shown efficacy in clinical studies of HER2-positive breast cancer and activity in HER2-positive preclinical models of ovarian carcinoma [7]. HER2 gene amplification or protein overexpression is found in approximately one-third of ovarian cancers and is associated with a poor prognosis in many studies of advanced disease [8, 9]. The HER2 gene encodes a 185 kDa transmembrane receptor, which acts as a co-receptor for other members of the HER family. HER2 can be activated by secretory polypeptides such as heregulin. However, if HER2 is overexpressed, it may be activated by homodimerisation. HER2 plays a major regulatory role in the signalling network involved in many cellular processes, including the p21Ras/Mitogen-Activated Protein Kinase (MAPK), pS6 kinase, phospholipase C-γ and PI3K/AKT pathways. Trastuzumab inhibits HER2 signalling and downregulates the HER2 receptor [10, 11]. In vitro HER2 activation results in the cleavage of its extracellular domain and in the production of a truncated membrane-bound fragment p95 with kinase activity [12, 13].

The present study aimed to evaluate the effect of trastuzumab on disease-free interval and overall survival of mice with micrometastatic ovarian cancer after treatment with a cytotoxic agent (topotecan). The murine model mimics the natural history of the disease in humans. OVCAR-3 cells do not show HER2 amplification or overexpression, suggesting that biological effects of trastuzumab may occur in early relapse.
Materials and methods

Animals and agents

Female Swiss athymic nude mice, 4–5 weeks old (Charles River Laboratories, Wilmington, MA, USA), were housed in filter-capped cages and kept in a sterile facility, which was maintained in accordance with the standards of the Federation of European Laboratory Animal Science Associations. A 2-week quarantine was imposed on all animals before starting the study. Topotecan (GlaxoSmithKline, Marly le Roi, France) and trastuzumab (TZ: Herceptin®; Hoffmann-La Roche Ltd, Basel, Switzerland) were diluted in normal saline and administered in a volume of 10 μl/g body weight.

OVCAR-3 animal model

The study used the OVCAR-3 tumour model as originally established and described previously [14, 15]. A xenograft of OVCAR-3 tumour cells was produced in the mice by intraperitoneal (i.p.) implantation. The xenograft was fixed by irrigating the peritoneal cavity with normal saline and combining the wash and ascites. The cells were washed twice in phosphate-buffered saline (PBS), the pellet resuspended and the suspension diluted 1:3 in normal saline. Each mouse received 1 ml of the cell suspension i.p., representing 10–12 × 10⁶ cells.

Survival experiment

The OVCAR-3 ascites were allowed to grow for 10 days. Mice were randomly assigned to treatment as follows: saline 5 days a week for four consecutive weeks (control; n = 5); topotecan i.p. 0.625 mg/kg/day 5 days a week for three consecutive weeks (experiments were performed three times with five mice per group) and topotecan i.p. 0.625 mg/kg/day 5 days a week for four consecutive weeks (experiments were performed three times with five mice per group). The efficacy of treatment was evaluated by animal survival expressed as the increased life span, i.e.

\[
\text{median survival time of the treated group} - \text{median survival time of controls} / \text{median survival time of control group}
\]

The mice with peritoneal ascites were inspected once or twice daily for assessment of overall clinical status and food and water intake. Those in extremis were sacrificed immediately.

Histological characterisation of minimal residual disease

Mice bearing OVCAR-3 ascites were treated after 10 days with topotecan 0.625 mg/kg/day 5 days a week for three consecutive weeks. Pathological response was assessed by morphological study of formalin-fixed, paraffin-embedded tissue. Histological diagnosis was based on sections (5-μm thick) from each block stained with hemalun-eosin. The peritoneal cavity wash was microscopically examined for liver and diaphragmatic metastases and tumour cells every 7 days until death.

Determination of CA 125 plasma level

CA 125 blood level was assessed by an immunofluorometric method (Tracel®) with Brahms kit (n°K-CA 125II075; Brahms, Saint Ouen, France). Blood was obtained by weekly puncture of the caudal vein, recovered in heparinised microcapillaries and diluted in normal saline. After centrifugation at 2000 g for 10 min at room temperature, the plasma was collected and analysed based on the instructions on the kit. The level of detection was 5 U/ml. CA 125 blood levels were evaluated between the time of implantation and the development of macroscopic disease.

Trastuzumab treatment of minimal residual disease model

A four-arm design was used. A control group received either topotecan or trastuzumab; a topotecan-only group received topotecan 0.625 mg/kg/day from days 10–14, 17–21 and 24–28; a topotecan and early trastuzumab group received topotecan 0.625 mg/kg/day from days 10–14, 17–21 and 24–28, followed by trastuzumab weekly for 10 weeks starting on day 31, late enough to observe a specific effect of trastuzumab; a topotecan and late trastuzumab group received topotecan 0.625 mg/kg/day from days 10–14, 17–21 and 24–28, with trastuzumab started when the CA 125 blood level exceeded 50 U/ml. Trastuzumab was administered weekly by i.p. injection at a loading dose of 8 mg/kg and then at a maintenance dose of 4 mg/kg in a volume of 10 μl/g body weight.

Cell culture

NIH-OVCAR-3 (ATCC) cells were cultured routinely in RPMI-1640 medium containing 10% heat-decomplemented serum, supplemented with 20 ng/ml epidermal growth factor (EGF) (Roche, Meylan, France), 10 μg/ml insulin (Roche) and 2 mM l-glutamine (Cambrex biosciences, Emerainville, France) at 37°C and 5% CO₂. However, oestriadiol interferes with the effects of trastuzumab on HER2 signalling and phenol red acts as an oestriadiol-like factor [16]; therefore, before any in vitro study, OVCAR-3 cells were cultured for a week in an otherwise identical medium lacking phenol red.

Determination of cell proliferation

OVCAR-3 cells were seeded in a 24-well plate (25 000 cells/well) four times on day 0 in phenol red-free RPMI-1640 medium, which contained 2.5% heat-decomplemented serum supplemented with 2 mM l-glutamine. On day 1 and every day thereafter, cells were treated with either vehicle or trastuzumab 75, 150 or 300 μg/ml. Cell numbers were evaluated on day 1 and every 48 h thereafter by a sulforhodamine B test, a colorimetric assay that directly correlates protein content with cell number [17].

Flow cytometry analysis

Cells were plated in 100 mm dishes (6 × 10⁵/dish) in phenol red-free RPMI-1640 medium, which contained 2.5% heat-decomplemented serum supplemented with 2 mM l-glutamine. Twenty-four hours later, cells were treated from 24 to 122 h with either vehicle or trastuzumab 150 μg/ml. Media and treatment were repeated every 24 h and all kinetic and control points were stopped the same day. Media were then collected and the cells trypsinised, pelleted by low-speed centrifugation (800 g for 5 min), washed twice in 0.5 ml cold PBS, fixed in 1.5 ml of ice-cold absolute ethanol for 1 h at 4°C and then stained with propidium iodide (1 mg/ml). The DNA content was determined with a FACS-Calibur flow cytometer (Becton Dickinson, Le-Pont-de-Claix, France). Unless otherwise stated, the proportion of cells in G0/G1, S and G2/M phases of the cell cycle was calculated from their DNA histogram using the ModFit TL version 3.0 software (Becton Dickinson).

Western blot analysis

OVCAR-3 cells. On day 1, 1.5 × 10⁶ OVCAR-3 cells were plated in phenol red-free RPMI-1640 medium containing 10% heat-decomplemented serum supplemented with 20 ng/ml EGF (Roche), 10 μg/ml insulin (Roche) and 2 mM l-glutamine (Cambrex) in 60 mm Petri dishes. Forty-eight hours later, cells were serum-starved and treated with either vehicle or trastuzumab 150 μg/ml for 24–122 h. Before the end of the experiment and when necessary, cells were treated with either EGF 20 ng/ml for 15 min or heregulin (HRG) 100 nM for 20 min. The cells were harvested and lysed in lysis buffer [Tris 50 mM pH 8, NaCl 150 mM, 0.1% NP40, 5 mg/ml sodium deoxycholate, 6.4 mg/ml phosphatase substrate (Sigma 104®), Sigma, Saint Quentin Fallavier, France]. For analysis of HER2, MAPK, AKT or p95 and...
β-tubulin (loading control), 70 μg of the cleared lysates were separated on a 7.5% or 12.5% SDS-polyacrylamide gel, blotted to PVDF membranes (Amersham, Orsay, France), and incubated with the relevant antibodies. For the evaluation the basal levels of phosphorylated HER2 in OVCAR-3, 70 μg protein from OVCAR-3 whole-cell lysates were compared with 5 μg of whole cell lysates from SKOV3 and resolved by a 7.5% SDS–PAGE gel. Detection was performed using peroxidase-conjugated secondary antibodies (Bio-Rad, Marnes la Coquette, France) and an ECL chemiluminescence detection kit (Amersham). The blots were scanned and analysed with the Molecular Dynamics densitometer and ImageQuant software. Each value is shown as an arbitrary unit and representative of at least two independent experiments.

Murine OVCAR-3 xenograft model. Mice bearing OVCAR-3 ascites were treated every day with vehicle or trastuzumab 4 mg/kg/day from day 1 to day 4. OVCAR-3 ascites were collected from day 1 to day 4, washed twice in PBS and lysed in lysis buffer. The amount of total and phosphorylated HER2, total and phosphorylated MAPK, AKT or p95 and β-tubulin were evaluated by western blot analysis as described above.

Antibodies

Antibodies used were: Anti-phospho-Erb B2/HER2 (Y1248) (Upstate Ab, Euromedex, Mundolsheim, France); anti-total HER2 (c-erbB-2/HER2/neu Ab-12, clone CB11) and anti-β-tubulin (NeoMarkers Ab, Interchim, Montluçon, France); anti-ACTIVE MAPK pAb, rabbit (pTyr)(P) (Promega, Charbonnières-les-bains, France); phospho AKT antibody (CR 473 Lot-6) and total AKT antibody (Lot-6) (Ozyme, Saint Quentin Yvelines, France); anti-ERK (c-16), rabbit (Santa Cruz Biotech, Tebu-Bio SA, Le Perray en Yvelines, France); peroxidase-conjugated secondary mouse and rabbit antibodies (Bio-Rad).

Results

Tumour model and histology of micrometastatic disease

Effects of topotecan treatment on survival of mouse tumour model. Optimal survival is achieved with a topotecan dose of 0.625 mg/kg/day taken 5 days a week for 4 weeks (Figure 1). In contrast, the same schedule administered over 3 rather than 4 weeks reproducibly induces a clinical remission followed by a relapse at 60 days after that of the control group.

Pattern of pathological relapse in mice after intraperitoneal implantation of OVCAR-3. The pathological pattern of tumour growth after OVCAR-3 implantation was compared between treated animals and the control group. Five mice were dissected to assess the pattern of relapse at each step of the experiment. The animals receiving topotecan remained free of macroscopic disease for several weeks (Table 1) and signs of macroscopic peritoneal carcinomatosis completely disappeared (Figure 2). However, all these animals displayed persistent micrometastatic invasion of the peritoneum and liver. Consequently, they relapsed after a disease-free period of 6–8 weeks (Table 1).

Characterisation of blood CA 125 variations with time in topotecan-treated mice. Biological relapse (increase in CA 125 levels) preceded the clinical relapse and occurred after day 70 following implantation (Figure 3). This increase in CA 125 blood level preceded the macroscopic peritoneal clinical relapse.

Effect of trastuzumab on ovarian micrometastatic disease

Validation of the tumour model. The biological characteristics of our tumour model and its response to topotecan therapy mimic the natural history of human ovarian carcinoma after treatment (Figure 4). This indicates that the model is appropriate for investigating the effect of trastuzumab treatment on survival following the development of micrometastatic disease.

Survival effect of trastuzumab in OVCAR-3 model of residual disease. The survival rate was similar for animals treated with topotecan alone and topotecan combined with late trastuzumab, i.e. administered when biological relapse had occurred (Figure 5). However, no relapse was observed in mice treated with topotecan and early trastuzumab.

Effect of trastuzumab on HER2 on proliferation and apoptosis of treated OVCAR-3 ascites and cultured cells. In mice bearing OVCAR-3 ascites and in the OVCAR-3 cell line, levels of both phosphorylated and total HER2 were altered significantly with 24–96 h of trastuzumab treatment (Figure 6). In the cell line, this effect was particularly evident on EGF or heregulin (HRG) stimulation (Figure 6B). The same response pattern was observed up to 122 h (data not shown). HER2 activation has been described to result in the cleavage of its extracellular domain and in the production of a truncated membrane-bound fragment p95 with kinase activity as described by Christianson et al. [12] and Molina et al. [13]. This p95 membrane-bound fragment was detected using the c-erbB-2/HER2/neu Ab-12 (clone CB11, Neomarker) antibody directed against an epitope of the cytoplamic domain. Trastuzumab treatment also inhibited p95 levels in both the ascites and the cell line (Figure 6) consistent with the inhibition of the expression of both total and phosphorylated HER2.

Role of trastuzumab in OVCAR-3 cell proliferation and cell cycle repartition. When OVCAR-3 cells were maintained in RPMI-1640 medium containing 10% heat-decomplemented serum, trastuzumab had no notable anti-proliferative effect (data not shown). However, when cultured in a lower concentration (2.5%) of heat-decomplemented serum and in phenol red-free
RPMI-1640 medium, trastuzumab moderately inhibited cell proliferation in a dose-dependent manner (Figure 7A). Trastuzumab induced a weak (10%) and transitory increase in the number of cells in the G0/G1 phase (from 48–96 h, maximum at 72 h; Figure 7B and C). These results are consistent with the previous data demonstrating modest growth inhibition, and are unlikely to fully account for the in vivo remission, particularly in a cellular model not overexpressing HER2.

Table 1. Pattern of relapse after 3 weeks’ topotecan treatment

<table>
<thead>
<tr>
<th>Days after implantation</th>
<th>Control Ascites</th>
<th>Hepatic metastasis</th>
<th>Peritoneal metastasis</th>
<th>Treated Ascites</th>
<th>Peritoneal washing</th>
<th>Hepatic metastasis</th>
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Mice bearing OVCAR-3 ascites were treated after 10 days with topotecan 0.625 mg/kg/day 5 days a week for three consecutive weeks. Pathological response was assessed as described in Materials and methods. Mice in the control group developed peritoneal ascites and were sacrificed on day 45. Results represent three separate experiments (five mice per group).

+ +++, supramillimetric metastases (macro-nodules); ++, millimetric metastases; +, micrometastasis (inframillimetric or detected only on immunohistochemical analysis).

Figure 2. Pattern of microscopic relapse. (A) Two micrometastasis (non-infiltrating, <1 mm) on the surface of the liver (arrows) (hemalum-eosin, ×25). (B) Necrotic macrometastasis (arrows) widely infiltrating the peritoneum.
trastuzumab treatment from 24–96 h. In cell lines, trastuzumab inhibited MAPK phosphorylation but only on stimulation by EGF or heregulin and only when the cells were serum-deprived (Figure 8B, Table 3). These results suggest that regulation of MAPK phosphorylation is not the major mechanism of action of trastuzumab in this model. In contrast, trastuzumab markedly inhibited AKT phosphorylation in both ascites and cell lines as shown in Table 2 and 3. This effect was not observed with an HER1 tyrosine kinase inhibitor (data not shown). The reduction in AKT phosphorylation therefore seems to be an important mechanism by which trastuzumab modifies OVCAR-3 cell proliferation and survival.

Discussion

Cytotoxic chemotherapy is indicated for most patients with ovarian cancer; the combination of carboplatin and paclitaxel has become the standard of care [2, 18]. However, unanswered questions remain about the optimum schedule, duration, treatment intensity and the benefit of adding other drugs. The role of consolidation treatment after first-line chemotherapy is also incompletely defined. However, the initial response rate to chemotherapy and the disease-free interval between first-line treatment and relapse strongly predict prognosis [4]. Consequently, patients with a complete response to first-line chemotherapy might benefit from consolidation treatment. However, the unfavourable therapeutic indices of long-term cytotoxic chemotherapy or radiotherapy are incompatible with their prolonged use, and hence they have not been definitively evaluated in the setting of microscopic residual disease.

The development of new, rationally based, targeted anticancer agents such as trastuzumab provides a new opportunity in clinical research. Although the primary therapeutic benefit of these agents is expected to be inhibition of tumour growth, they target all facets of cell proliferation: signal transduction, angiogenesis, metastasis and cell cycle regulation. Clinical trials have established the efficacy of trastuzumab in patients with HER2-positive metastatic breast cancer [11, 19, 20]. HER2 gene amplification or overexpression has also been found in 30–50% of ovarian carcinomas [21–23]. In the one clinical trial in ovarian carcinoma conducted so far, in patients with relapsing disease, single-agent trastuzumab was slightly active [24]. However, this is not surprising, given the generally poor sensitivity of relapsed cancer to anticancer agents. To optimise the effect of trastuzumab in HER2-positive ovarian carcinomas, drug development...
Figure 6. Effect of trastuzumab (TZ) on HER2 and p95 expression in treated OVCAR-3 ascites (A) and in OVCAR-3 cultured and serum-staved cells (B). Comparison of HER2-P basal level in OVCAR-3 and SKOV-3 cell lines (C). EGF, epidermal growth factor; HRG, heregulin.

Figure 7. Effect of trastuzumab treatment on OVCAR-3 cell proliferation (A) and cycle repartition (B, G0/G1 and C, G2/M). Results represent (A) three separate experiments, each in triplicate or (B and C) two separate experiments.
should be directed to increasing disease-free survival and achieving maximal cytoreduction after induction chemotherapy.

No correlation has been demonstrated between HER2 overexpression and the effect of trastuzumab in ovarian cancer, unlike in breast cancer. Therefore, preclinical evidence is needed to verify the activity of trastuzumab in ovarian tumours without HER2 overexpression and during consolidation treatment rather than relapse.

Our model mimics the clinical situation in patients who have an apparent macroscopic remission, followed by biological relapse (as shown by increasing CA 125 levels) and then macroscopic (clinical) relapse. Mice treated systematically with trastuzumab after chemotherapy did not relapse, whereas survival in mice with increased CA 125 level was similar to that of control animals.

**Effect of trastuzumab on HER2 phosphorylation and expression**

The reduction of HER2 phosphorylation and expression in OVCAR-3 cell ascites and cell lines treated with trastuzumab suggest that HER2 is the biological target in this model. Decreased HER2 phosphorylation is associated with p95 [12, 13, 25], providing further evidence that trastuzumab mediates its antitumour activity via HER2. Moreover, this effect was observed in a cell line expressing normal levels of HER2, suggesting that HER2 overexpression may not be a prerequisite for these antitumoural effects [25]. In fact, trastuzumab transiently inhibited the proliferative pathway, inducing a biphasic response in cell cycle repartition; the G0/G1-phase fraction increased following 48–96 h of treatment with 150 μg/ml trastuzumab. This result is consistent with previous findings in ovarian and other cancer cell lines, showing a modest but detectable reduction of cell growth with trastuzumab [5, 26, 27].

**Effect of trastuzumab on the signalling pathway**

The trastuzumab-associated reductions in HER2 phosphorylation and HER2 expression in OVCAR-3 ascites and cultured cells are clearly linked to inhibition of AKT phosphorylation in vivo and in vitro [16]. The patterns of trastuzumab-induced inhibition of AKT phosphorylation were identical in ascites cells and cell lines. Therefore, the inhibition of the AKT pathway appears to be the major mechanism contributing to reduce HER2-mediated oncogenesis and prolonged survival in this model.

**Postulated mechanisms of action of trastuzumab in the model**

Trastuzumab demonstrated antitumour activity and prolonged survival in our model, which shows no HER2 overexpression or amplification. Several mechanisms could be involved. The first is an increased pro-apoptotic effect after topotecan-induced cytotoxic damage. The pro-apoptotic effect of trastuzumab is well documented [28]. In the current study, trastuzumab inhibited AKT phosphorylation in vitro and in vivo in the

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**Table 2.** Measure of phosphorylated AKT by densitometric scan (arbitrary unit) in ascitis

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<th>ACT phospho/total (% of inhibition)</th>
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<td>48 h</td>
<td>36.47</td>
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<tr>
<td>72 h</td>
<td>46.83</td>
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<td>96 h</td>
<td>100</td>
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**Table 3.** Measure of phosphorylated AKT and MAPK by densitometric scan (arbitrary unit) in serum-starved cell lines

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<tr>
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<th>MAPK phospho/total</th>
<th>AKT phospho/total</th>
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<tbody>
<tr>
<td>Control</td>
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</tr>
<tr>
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<tr>
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<tr>
<td>TZ + EGF</td>
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<tr>
<td>HRG</td>
<td>14</td>
<td>3</td>
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<td>TZ + HRG</td>
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TZ, trastuzumab; EGF, epidermal growth factor; HRG, heregulin.
OVCAR-3 cell line, an effect not observed with a HER1 inhibitor. AKT inhibition therefore appears to be a necessary but insufficient prerequisite for *in vivo* inhibition of tumour proliferation.

Alternatively, trastuzumab might modify the tumour micro-environment. Although the tumour grew in immunodeficient mice, they still possessed immune-activated effector cells, and trastuzumab is known to induce a cellular immune response [29]. However, animals who received late trastuzumab treatment relapsed at a similar timepoint to the control animals. Thus, if an immune response is involved in the mechanism of action of trastuzumab, it is weak in this model. Trastuzumab might also modify other microenvironmental parameters affecting early stages of tumour growth and invasion. An enhanced pro-apoptotic effect due to HER2 inhibition might be linked to other biological effectors. The role of AKT in the invasion, migration and adhesion of tumour cells has been described previously and is currently under investigation in our laboratory [30, 31].

In conclusion, the present study provides insights into the biological properties of trastuzumab in early-stage relapse of ovarian carcinoma. These findings may help design better trials to focus on the disease-free interval in human patients with this disease.

**References**


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