# Effects of lobaplatin as a single agent and in combination with TRAIL on the growth of triple-negative p53-mutated breast cancers *in vitro*

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**Lobaplatin as a single agent and in combination with tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) is investigated in in-vitro models of p53-negative triple-negative breast cancers (TNBCs) and compared with a model of oestrogen receptor-positive p53-positive breast cancer. In addition, the induction of programmed cell death by lobaplatin is further explored. By using cell viability assays and western blotting, the cytotoxic effects of lobaplatin alone and in combination with TRAIL are compared with cisplatin in HCC 1806, HCC 1937, and MCF 7 cells. The multicaspase inhibitor z-VAD-fmk and necrostatin, an inhibitor of necroptosis, are used to demonstrate the mechanism of cell death caused by lobaplatin. Lobaplatin displayed antitumour activity in all three cell lines, which increased time dependently. Cotreatment of lobaplatin and TRAIL induced an increase in cytotoxicity by 30–50% in the different cell lines. The pan-caspase inhibitor z-VAD-fmk as well as necrostatin could weaken but not abolish the cytotoxic effect of lobaplatin and cisplatin. Lobaplatin showed substantial cytotoxic effects in two in-vitro models of p53-mutated TNBC. Cotreatment with TRAIL and platinum agents resulted in increased antitumour activity in the TNBC cell lines investigated. Cell death subsequent to treatment with cisplatin and lobaplatin occurred because of apoptosis. However, caspase-independent mechanisms of programmed cell death were also involved. It was also demonstrated that platinum compounds could induce necroptosis, although to a minor extent.** ***Anti-Cancer Drugs*** **23:426–436** © **2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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## Introduction

Breast cancer is a heterogeneous disease that encompasses several distinct entities with remarkably different biological characteristics and clinical behaviour. Currently, breast cancer patients are managed according to different treatment algorithms based on a constellation of clinical parameters in conjunction with assessment of sex steroid receptor (oestrogen and progesterone receptor) status and overexpression of the human epidermal growth factor receptor 2 (HER2) receptor. Although effective tailored therapies have been developed for patients with hormone receptor-positive or HER2-positive disease, so far chemotherapy is the only modality of systemic therapy for patients with breast cancers lacking the expression of these three markers – that is, triple-negative cancers.

Thus, the term triple-negative breast cancer (TNBC) is used to refer to a group of tumours that do not express receptors for oestrogen, progesterone and HER2. This subgroup shows distinctive clinical features and accounts for 10–17% of all breast carcinomas [1,2]. TNBC tends to affect younger patients more frequently [3], are more prevalent in African Americans [4] and are clinically more aggressive than tumours belonging to the other known molecular subgroups [1,2,5,6]. Although triple-negative cancers are sensitive to chemotherapy [1], survival of patients with such tumours is poor. Thus, in patients with triple-negative cancers, disease recurrence takes place earlier and the majority of deaths occur in the first 5 years after diagnosis [2,7]. These clinical findings underline the importance of establishing effective treatment strategies for this disease, as so far no particular standard therapy for early-stage or late-stage TNBC has been established.

Some preclinical studies found good antitumour activity for alkylating agents such as cisplatin in TNBC, particularly in tumours with mutated BRCA 1 [8,9]. Likewise, three retrospective clinical studies carried out in a neoadjuvant setting found excellent response rates in patients with TNBC after platinum-based chemotherapy [10–12]. Another platinum-based chemotherapeutic

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agent is the recently developed third-generation platinum agent lobaplatin [1,2-diammino-methyl-cyclobutaneplatinum(II)-lactate], which has shown activity in phase I and phase II studies of advanced ovarian cancer [13], small cell lung cancer [14] and oesophageal cancer [15]. In these and other studies, lobaplatin was well tolerated, particularly with respect to ototoxicity, nephrotoxicity and neurotoxicity [16]. However, the efficacy of lobaplatin in TNBC has not been evaluated yet.

Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) activates apoptosis upon binding to its receptors in many tumour types. TRAIL and agonist antibodies are currently being studied in patients in phase I and phase II clinical trials [17]. In-vitro studies suggest that many breast cancer cell lines are resistant to TRAIL-induced apoptosis. However, recent data have shown that a subset of triple-negative breast cancer cell lines is sensitive to TRAIL as a single agent [18]. In addition, further studies have demonstrated that TRAIL can be favourably combined with chemotherapy, radiation and various targeted agents [19–22].

In multicellular organisms such as humans, the number of cells is a balance between the cell-generating effects of mitosis and cell death that is induced through apoptosis. A disruption of this delicate balance can lead to the development of cancer [23]. Apoptosis provides an important barrier against cancer; however, specific mutations enable some tumour cells to escape apoptotic death and become more malignant. Two signalling pathways initiate classical apoptosis; one acts through intracellular Bcl-2 proteins and the other through cell-surface proapoptotic receptors [24]. Apoptosis can be engaged by a range of cellular insults, and one of the major modes of action of chemotherapeutic drugs such as platinum derivatives may be through the activation of apoptosis [25]. In recent years, the coexistence of various apoptotic ways of cell death and the existence of caspase-independent apoptotic pathways have been described [26,27]. The clonogenic survival of cancer cells most probably relies on the simultaneous blockade of both apoptotic and nonapoptotic cell death mechanisms [28]. Furthermore, a form of programmed cell death (PCD) with features of necrosis was discovered [29] and referred to as necroptosis, meaning a cellular mechanism of necrotic cell death induced by apoptotic stimuli such as TNFα, Fas ligand and TRAIL [30]. Although occurring under regulated conditions, necroptotic cell death is characterized by the same morphological features as classical necrosis, so far regarded as an unregulated process [31]. The initiation of 'necroptosis' by death receptors such as the TNFα receptor requires the kinase activity of receptor-interacting protein (RIP)-1 and RIP-3, and its execution involves the active disintegration of mitochondrial, lysosomal and plasma membranes [32]. Necroptosis was found in diseases, such as ischaemic injury, neurodegeneration and viral infection [33]. Furthermore, in the development of higher vertebrates, necroptosis seems to be a backup mechanism when apoptosis is inhibited [34,35]. Recently, necroptosis has also been demonstrated in tumour cells subsequent to treatment with agents such as shikonin; thus, it was proposed to bypass cancer drug resistance by activating multiple death pathways using necroptosis-inducing agents [36].

In the current study, lobaplatin as a single agent and in combination with TRAIL was investigated in in-vitro models of p53-negative TNBC and compared with a model of oestrogen receptor (ER)-positive p53-positive breast cancer. In addition, the induction of PCD by lobaplatin was further investigated by using the multicaspase inhibitor z-VAD-fmk for inhibition of caspase-dependent apoptosis and necrostatin for inhibition of necroptosis.

## Materials and methods

### Reagents and cell lines

Lobaplatin was kindly provided by Aeterna Zentaris GmbH (Frankfurt, Germany). Human TRAIL was purchased from PeproTech (Rocky Hill, Connecticut, USA). The human TNBC cell lines HCC 1806, HCC 1937 and ER-positive HER2-negative MCF 7 human breast cancer cells were obtained from American Type Culture Collection (Manassas, Virginia, USA). All cells were cultured in Dulbecco's modified Eagle's medium (PAA, Cölbe, Germany) containing 10% fetal calf serum (Biochrom, Berlin, Germany), 2 mmol/l glutamine, 1% penicillin/streptomycin and 0.5% sodium pyruvate solution (PAA). The broad spectrum caspase inhibitor [benzyloxycarbonyl-val-ala-asp(OMe)-fluoromethylketone] z-VAD-fmk was from Bachem (Weil am Rhein, Germany), and Necrostatin-1 and Super-Fas-Ligand were from Enzo Life Sciences (Farmingdale, New York, USA). The FuGENE transfection reagent was obtained from Roche Applied Science (Indianapolis, Indiana, USA), and the plasmids used for tranfection were from Agilent (Santa Clara, California, USA) and Promega (Madison, Wisconsin, USA). The poly(ADP-ribose) polymerase (PARP) antibody was purchased from Cell Signaling (Danvers, Colorado, USA), the RIP-1 antibody was from BD Biosciences (Heidelberg, Germany), the caspase-3 antibody was from Cell Signaling (Frankfurt, Germany), the β-actin antibody was from Abcam (Cambridge, UK) and the secondary antibodies were from KPL (Gaithersburg, Maryland, USA). Propidium iodide and all other reagents, unless indicated otherwise, were purchased from Sigma (St Louis, Missouri, USA).

### Reporter gene fluorescence assay

The cells were seeded at 6000 cells per well in 96-well plates, adhered for 24 h and transfected with two

plasmids. A total of 2 µg of DNA and 6 µl of FuGENE transfection reagent were dissolved in 100 µl of serum-free medium. The DNA consisted of the reporter gene and a control plasmid at a ratio of 5 : 1. The control plasmid used in our experiments encoded for the renilla luciferase and contained its own promoter, indicating by the expression and activity of the luciferase that transfection into the cell had been successful. Furthermore, renilla luciferase expression was used for normalizing the firefly luciferase expression under control of the p53 promoter (p53luc, reporter-gene plasmid). The activity of the firefly luciferase correlated with the activity of the endogenous p53 gene. A volume of 5 µl of the transfection mix was added to each well and the plates were incubated for another 48 h. The cells were then lysed by a short incubation with a lysis buffer containing 40 mmol/l Tricine, 50 mmol/l NaCl, 2 mmol/l EDTA, 1 mmol/l $MgSO_4$, 5 mmol/l DTT and 1% Triton X-100. Solutions A (25 mmol/l glycylglycine, 15 mmol/l $K_2HPO_4$, 4 mmol/l EGTA, 2 mmol/l ATP, 1 mmol/l DTT, 15 mmol/l $MgSO_4$, 0.1 mmol/l CoA and 75 µmol/l luciferin) and B (1.1 mol/l NaCl, 2.2 mmol/l $Na_2$-EDTA, 220 mmol/l $KH_2PO_4$, 0.44 mg/ml BSA, 1.3 mmol/l $NaN_3$ and 1.43 µmol/l coelenterazine) were freshly prepared. Luciferin was the substrate for the firefly luciferase and coelenterazine was the substrate for the renilla luciferase. The light units produced in the course of the enzymatic reactions were measured on a luminometer (Orion Plate Luminometer; Berthold, Bad Wildbad, Germany).

### Viability assays

For acute cytotoxicity assays, the cells were seeded at $1 \times 10^4$ cells per well in 96-well plates, adhered for 24 h and exposed to the drugs for 24–72 h. The cell lines doubled twice and grew exponentially within the time frame of the experiment. Proliferation was assessed by crystal violet staining [37]. Briefly, the cell culture medium was removed and surviving cells were stained with 0.5% crystal violet in 20% methanol for 20 min at room temperature. The plates were washed extensively under running tap water, air dried and optical density values were read in an enzyme-linked immunosorbent assay reader at 550 nm wavelength. Effective concentration (EC) values were derived from graphic intrapolation.

### Immunoblot analysis

The general procedure has been described by Naumann *et al.* [38]. The cells were untreated or treated with lobaplatin or cisplatin as indicated and lysed. Soluble protein levels were analysed by immunoblot using 20 mg of protein per lane separated on 10–12% acrylamide gels (Biorad, Munich, Germany). After transferring to a nitrocellulose membrane, the blots were pretreated for 2 h with PBS containing 5% skim milk and 0.05% Tween 20 and then incubated overnight at 4°C with anti-PARP (Cell Signaling, Danvers, Colorado, USA), anti-RIP-1 (BD Biosciences) and anti-β-actin (Abcam). Visualization of protein bands was accomplished using horseradish peroxidase (HRP)-coupled IgG secondary antibody (Sigma) and enhanced chemiluminescence (Amersham, Braunschweig, Germany).

## Results

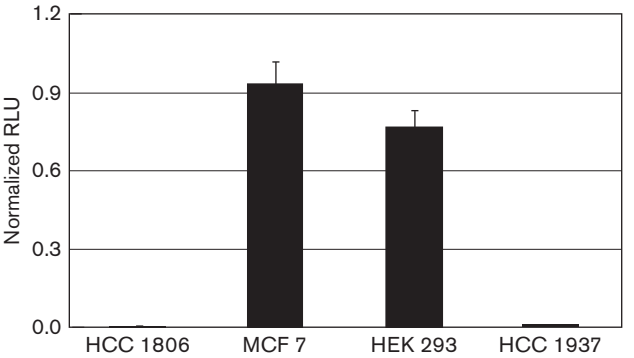
### p53 status and BRCA 1 status of investigated cells

To analyse the p53 status of the three human breast cancer cell lines used in this study, the luciferase activity under the control of the p53 promoter in transiently transfected cells was determined (Fig. 1). Luciferase activity in MCF 7 cells was comparable to that in HEK 293 cells used as positive controls, indicating wild-type p53. In HCC 1937 and 1806 cells, luciferase activity was decreased more than 100-fold as compared with the two control cell lines, indicating a very low p53 promoter activity in HCC 1937 and HCC 1806 cells. As a further verification, the *BRCA 1* gene was sequenced and the expected variations were found: in HCC 1937 cells a specific mutation (insertion of a C at position 5382) of *BRCA 1* was detected and in HCC 1806 cells the wild-type *BRCA 1* gene was found (data not shown).

### Effects of lobaplatin on the growth of human breast cancer cells

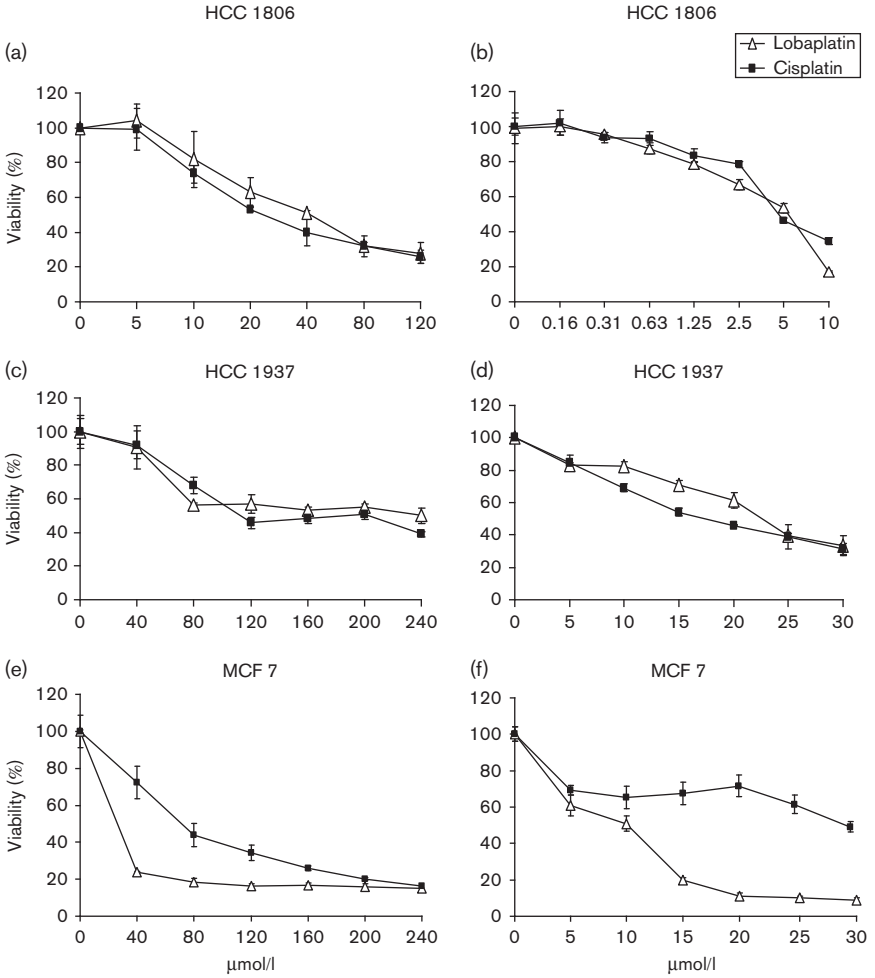
Figure 2 shows cytotoxic activity of cisplatin and lobaplatin as single agents in ER-positive MCF 7 and triple-negative HCC 1806 and HCC 1937 cells after treatment for 24 and 48 h, respectively. Cisplatin and lobaplatin demonstrated comparable activity in triple-negative cell lines HCC 1806 and HCC 1937. Cytotoxic effect subsequent to treatment with lobaplatin appeared to be more pronounced in ER-positive p53 wild-type MCF 7 cells as compared with cisplatin. The EC50 values are summarized in Table 1.

**Fig. 1**




Measurement of p53 promoter activity in HCC 1806, MCF 7, HEK 293 and HCC 1937. Cells were transiently cotransfected with a plasmid carrying renilla luciferase and a plasmid encoded for the firefly luciferase under the control of p53 promoter. At 48 h after transfection, cells were harvested and luciferase activities were measured. The mean value of five independent experiments and measurements is shown. RLU, relative light units.

**Fig. 2**




Cytotoxic activity of cisplatin and lobaplatin as single agents in triple-negative HCC 1806 (p53 mutation; a and b), HCC 1937 (p53 mutation; c and d) and oestrogen receptor-positive MCF 7 (p53 wild type; e and f) human breast cancer cells. Cells were treated with increasing concentrations of lobaplatin and cisplatin for 24 h (a, c and e) and 48 h (b, d and f). These experiments are representative of three independent experiments, each performed in triplicate wells. Relative cell number in treated and control wells was determined by crystal violet staining and expressed as percent *T*/*C* values, where *T* is the absorbance of treated cultures and *C* is the absorbance of control cultures.

### Cotreatment of human breast cancer cells with lobaplatin and TRAIL

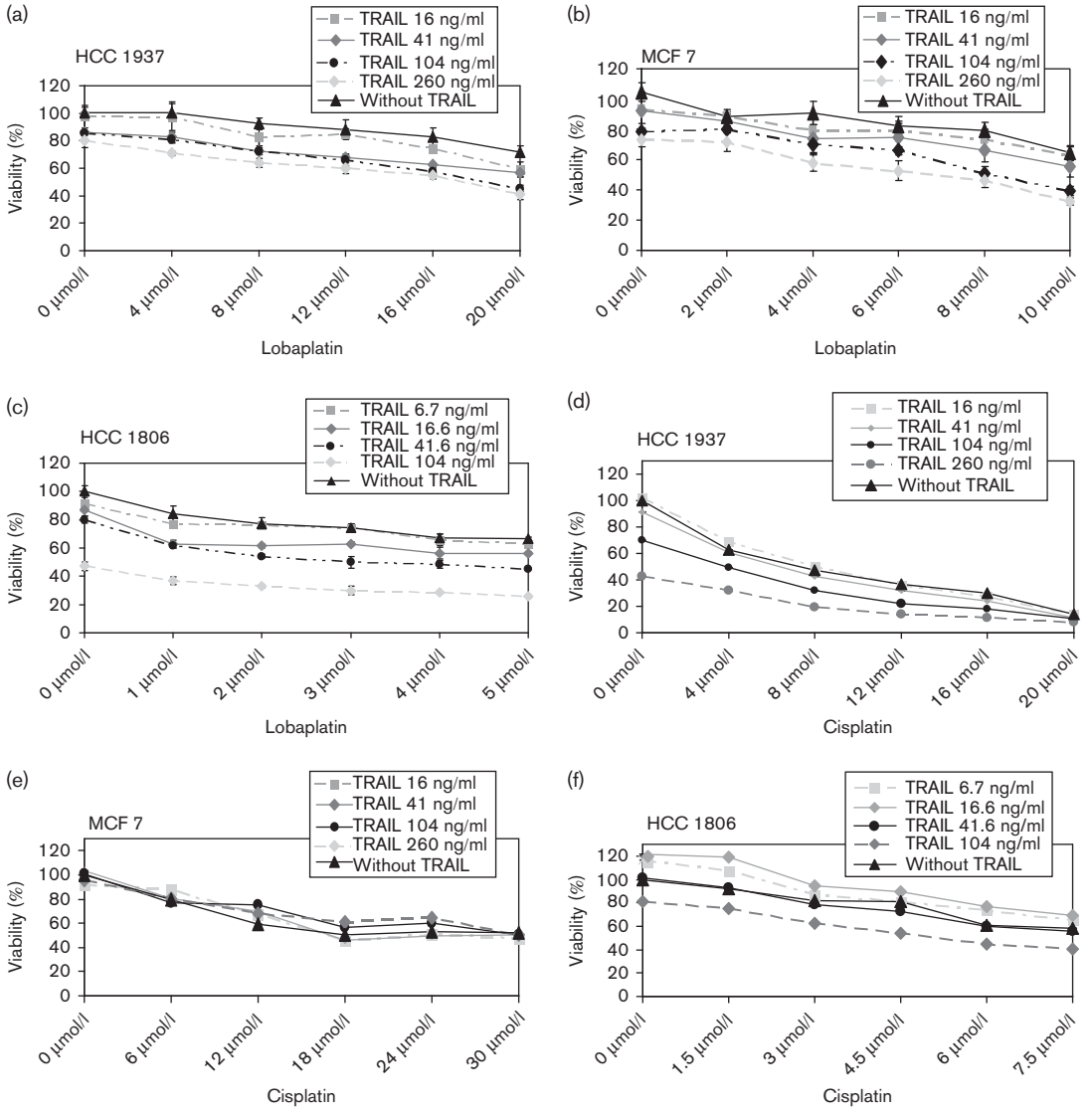
Cotreatment of lobaplatin and TRAIL was evaluated in HCC 1806, HCC 1937 and MCF 7 cells. Cells were treated with either lobaplatin or TRAIL alone and with a combination of both compounds at different concentrations for 48 h (Fig. 3a–c). Adding TRAIL to lobaplatin clearly increased its cytotoxic effect in all cell lines over the whole dose range. As can be extrapolated, HCC 1806 cells (Fig. 3c) were highly sensitive to TRAIL (with an EC50 of 104 ng/ml), whereas HCC 1937 (Fig. 3a) and MCF 7 cells (Fig. 3b) appeared to be less sensitive (with 260 ng/ml leading to growth inhibition by 20 and 30%, respectively). These effects are compared with cotreat-

**Table 1 EC50 (as graphically intrapolated) subsequent to treatment with lobaplatin and cisplatin in triple-negative cell lines HCC 1806 and HCC 1937 and oestrogen receptor-positive MCF 7 human breast cancer cells**

| | HCC 1806 | | HCC 1937 | | MCF 7 | |
|---|---|---|---|---|---|---|
| | Lobaplatin | Cisplatin | Lobaplatin | Cisplatin | Lobaplatin | Cisplatin |
| EC50 (24 h) | | | | | | |
| M | 42 | 25.4 | 190 | 188 | 18.5 | 63.4 |
| SD | 8.1 | 10.4 | 29.4 | 45.3 | 2.1 | 15.4 |
| EC50 (48 h) | | | | | | |
| M | 4.8 | 6.7 | 23.9 | 17.1 | 11.3 | 31 |
| SD | 0.9 | 2.6 | 1.7 | 4.3 | 1.9 | 5.5 |
| EC50 (72 h) | | | | | | |
| M | 1.4 | 2 | 13.6 | 11.9 | 5.2 | >20 |
| SD | 0.4 | 0.8 | 2.1 | 11.9 | 1.8 | ND |

M, mean.

**Fig. 3**



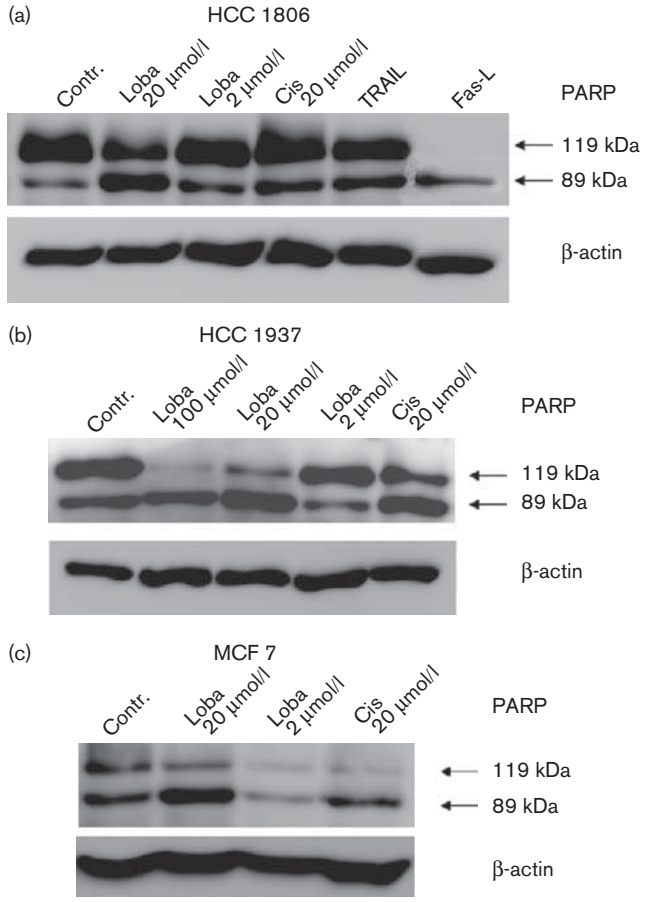

Cotreatment of lobaplatin and TRAIL (a, b, c) and cotreatment of cisplatin and TRAIL (d, e, f) were evaluated in HCC 1937 (a, d), MCF 7 (b, e) and HCC 1806 (c, f) cells. Cells were treated with lobaplatin, cisplatin or TRAIL alone and with combination of TRAIL with one platinum compound as indicated for 48 h at different concentrations. These experiments are representative of three independent experiments, each carried out in triplicate wells. Relative cell number in treated and control wells was determined by crystal violet staining and expressed as percent *T/C* values, where *T* is the absorbance of treated cultures and *C* is the absorbance of control cultures.

ment of cisplatin and TRAIL in HCC 1806, HCC 1937 and MCF 7 cells (Fig. 3d–f). Adding TRAIL to cisplatin strongly increased its cytotoxic effect in HCC 1937 cells (Fig. 3d), resulting in a growth inhibition by 30% with 260 ng/ml TRAIL. In contrast, addition of TRAIL to cisplatin had only a minor impact on cytotoxicity in MCF 7 cells (Fig. 3e), and in HCC 1806 cells (Fig. 3f) 104 ng/ml TRAIL resulted in a decreased viability of 20%. As Fig. 3 depicts, adding TRAIL to lobaplatin induced an increase in cytotoxicity by 30–50% in the three cell lines studied here, whereas only in HCC 1937 cells did the addition of TRAIL to cisplatin result in an increase in cytotoxicity in the same range.

### Induction of cleavage of poly(ADP-ribose) polymerase

Western blot analysis revealed a protein band at 89 kDa indicative of cleaved PARP after treatment with cisplatin and lobaplatin at doses of 20 µmol/l in MCF 7, HCC 1806 and HCC 1937 cells after 24 h of incubation (Fig. 4). At doses of 2 µmol/l, lobaplatin induced only minimal cleavage of PARP. Coincubation with TRAIL at doses of 31 ng/ml induced PARP cleavage in HCC 1806 cells.

**Fig. 4**




Effect of lobaplatin (Loba) and cisplatin (Cis) at different concentrations on the cleavage of PARP in HCC 1806 (a), HCC 1937 (b) and MCF 7 (c) human breast cancer cells as determined by western blotting in comparison with untreated cells (Contr.). The 119 kDa protein corresponds to the uncleaved form of PARP and the second protein running at 89 kDa is the cleaved form of PARP. In HCC 1806 cells mega-FAS-ligand (FAS-L), a known inductor of apoptosis through the extrinsic pathway, and TRAIL are used as positive controls. As internal control the housekeeping protein β-actin was used.

Furthermore, mega-FAS-ligand, a well-known inducer of PARP cleavage, was used as positive control for the antibody specificity in HCC 1806 cells.

### Cotreatment of breast cancer cells with lobaplatin, cisplatin, mega-FAS-ligand, TRAIL and either z-VAD-fmk or necrostatin

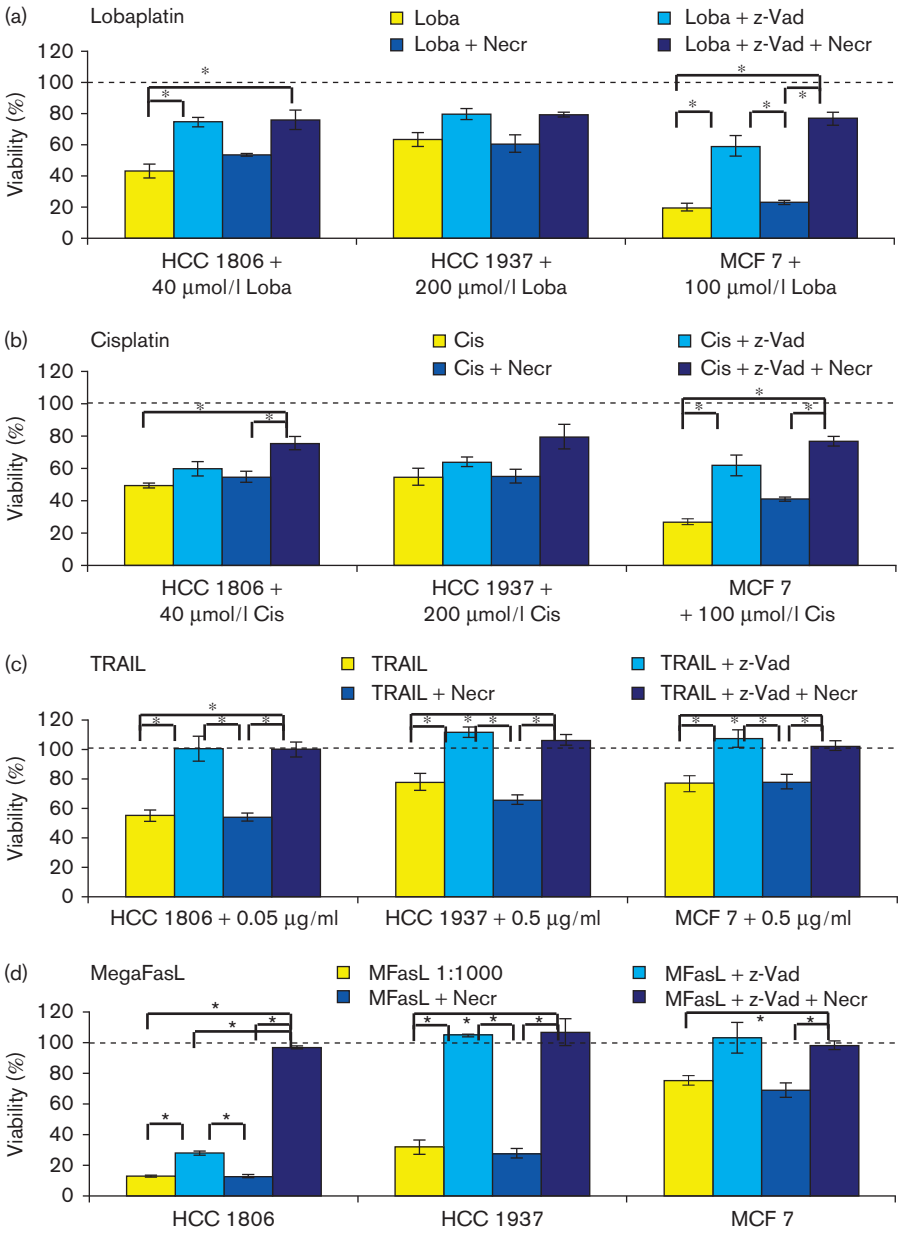
It was evaluated whether cotreatment with the multi-caspase inhibitor z-VAD-fmk decreases or abrogates the cytotoxic effect subsequent to treatment with lobaplatin, cisplatin, TRAIL and mega-FAS-ligand. Recently, an alternative method of PCD, necroptosis, was described. Therefore, a specific inhibitor of necroptosis, necrostatin, was applied to evaluate whether platinum derivatives induce necroptosis. Figure 5 shows that cotreatment with z-VAD-fmk at 30 µmol/l or with necrostatin at 50 µmol/l could weaken but not abolish the cytotoxic effect of lobaplatin and cisplatin. Simultaneous addition of z-VAD-fmk and necrostatin could counteract the toxic effects of the platinum derivatives more efficiently. The cytotoxic effect of TRAIL could be abrogated to a larger extent in all cell lines with z-VAD-fmk. In contrast, the addition of necrostatin has only marginal influence on the cytotoxic effects caused by TRAIL in the three cell lines. PCD induced by mega-FAS-ligand was abolished by z-VAD-fmk in HCC 1937 and MCF 7 cells. In HCC 1806 cells, only the combination of z-VAD-fmk and necrostatin was able to prevent the toxic effects of mega-FAS-ligand. In all the three cell lines, necrostatin alone was unable to weaken the mega-FAS-ligand-induced toxic effects.

To gain insight into the mechanism of cell death caused by lobaplatin and cisplatin in the different breast cancer cell lines, RIP-1 and PARP protein expressions were analysed, respectively, with specific antibodies by western blotting (Fig. 6). In untreated HCC 1806 (Fig. 6a, on the left) and HCC 1937 (Fig. 6b, on the left) cells (control), only the 74 kDa full-length RIP-1 protein could be detected. A protein band at 42 kDa indicative of cleaved RIP-1 after treatment with cisplatin and lobaplatin at doses of 40 µmol/l in HCC 1806 and 200 µmol/l in HCC 1937 cells after 24 h of incubation was detected together with the full-length protein. Addition of necrostatin prevented the cisplatin-induced RIP-1 cleavage. The lobaplatin-induced RIP-1 cleavage was also decreased by necrostatin addition but to a minor extent.

In untreated HCC 1806 (Fig. 6a, on the right) cells (control), only the 119 kDa full-length PARP protein could be detected, whereas in untreated HCC 1937 (Fig. 6b, on the right) cells (control) the full-length PARP protein (119 kDa) together with a small amount of cleaved PARP (89 kDa) was detected. Treatment with cisplatin and lobaplatin at doses of 40 µmol/l in HCC 1806 and 20 µmol/l in HCC 1937 cells after 24 h of incubation resulted in an increase in cleaved PARP and a reduction in full-length PARP. By coincubation of cisplatin and lobaplatin with z-VAD-fmk, the amount of uncleaved PARP was increased in both cell lines.

The influence of cisplatin and lobaplatin on procaspase-3 cleavage was further analysed by western blotting (Fig. 7). In untreated HCC 1806 (Fig. 7a) and HCC 1937 (Fig. 7b) cells (control) only the 35 kDa full-length caspase-3 protein could be detected. After treatment with cisplatin and lobaplatin, respectively, the cleaved 17 kDa and 12 kDa caspase-3 fragments could be detected in both cell lines. Caspase-3 cleavage was very faint in cisplatin-treated HCC 1937 cells. In HCC 1806 cells the 19 kDa precursor of the 12 kDa fragment was also detectable. Addition of z-VAD-fmk resulted in a decrease in the cleaved caspase-3 fragments.

**Fig. 5**



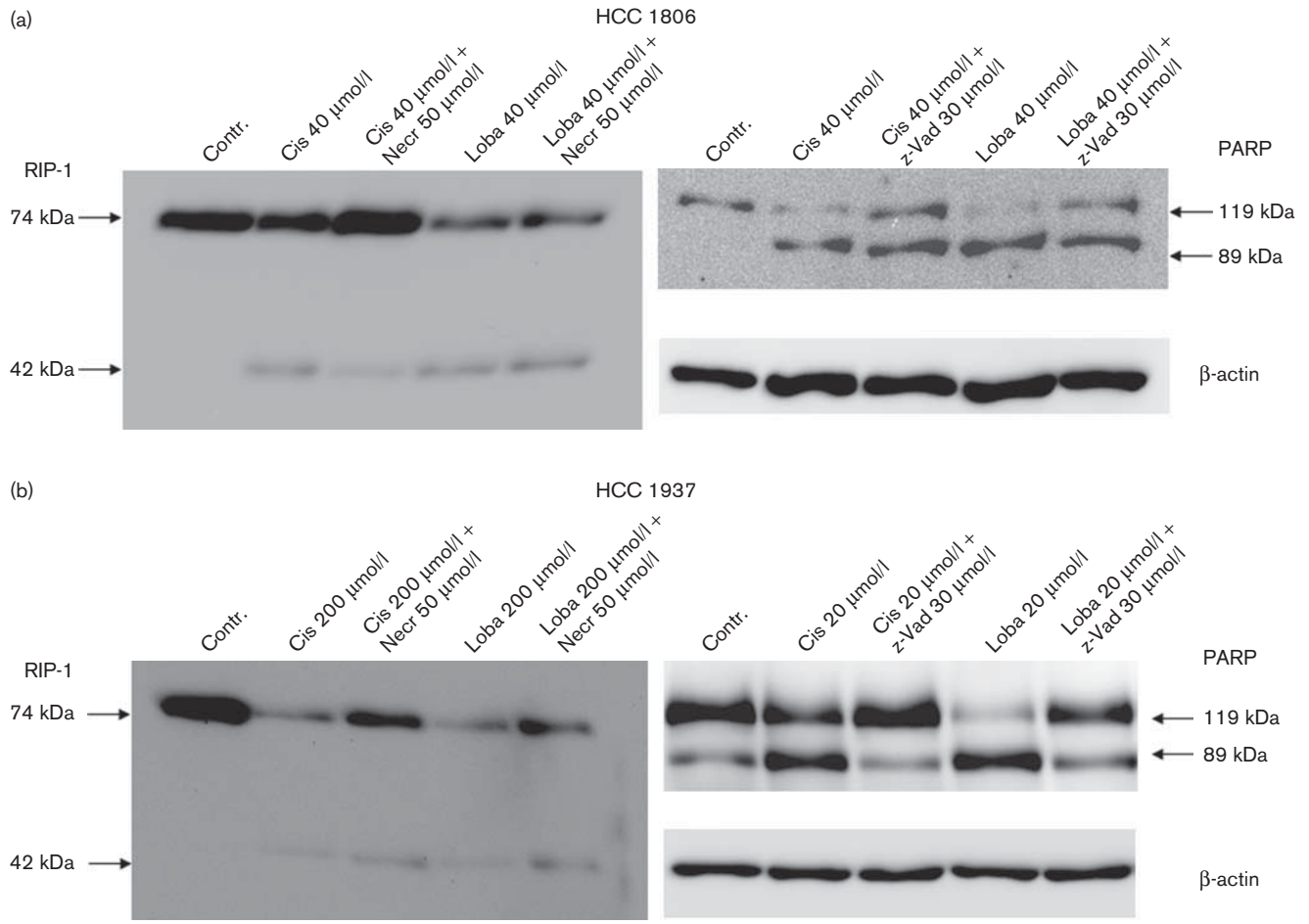

Cotreatment of HCC 1806, HCC 1937 and MCF 7 cells with lobaplatin (Loba) (a), cisplatin (Cis) (b), TRAIL (c), mega-FAS-ligand (MFasL) (d) and either multicaspase inhibitor z-VAD-fmk (z-Vad) or inhibitor of necroptosis necrostatin (Necr). These experiments are representative of three independent experiments, each carried out in triplicate wells. Relative cell number in treated and control wells was determined by crystal violet staining and expressed as per cent *T*/*C* values, where *T* is the absorbance of treated cultures and *C* is the absorbance of control cultures. Statistically significant differences ($P<0.005$) between columns are indicated by * in the figure.

## Discussion

The use of platinum-based chemotherapeutic agents is of special interest in TNBC as preclinical and early clinical evidence has suggested sensitivity to platinum derivatives [39]. Thus, Silver *et al.* [40] have recently reported a rate of partial or complete response of 64% using single-agent cisplatin as a neoadjuvant therapy in triple-negative patients.

Our experiments demonstrate good antitumour activity of lobaplatin, a third-generation platinum derivative with only moderate nephrotoxic side effects, at doses in micromolar range in two TNBC cell lines. In these cell lines, lobaplatin and cisplatin displayed comparable activity. As both TNBC cell lines were p53 negative as determined by p53-dependent luciferase activity, we also evaluated the antitumour effect of lobaplatin in p53

**Fig. 6**

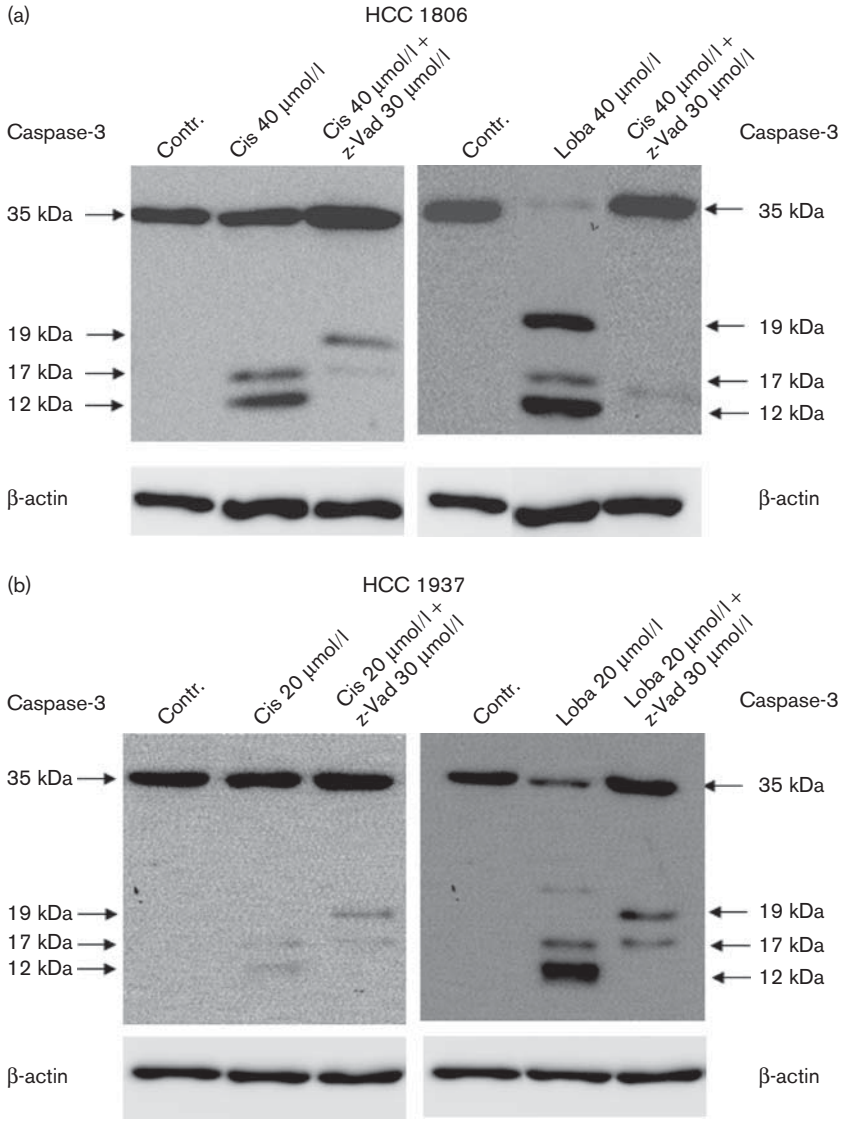



Effect of cisplatin (Cis) and lobaplatin (Loba) alone on the cleavage of receptor-interacting protein (RIP)-1 and PARP in HCC 1806 (a) and HCC 1937 (b) human breast cancer cells as determined by western blotting in comparison with untreated cells (Contr.). Cleavage of RIP-1 and PARP was also analysed in the presence of necrostatin (Necr.) and z-VAD-fmk (z-Vad), respectively. The signal with the apparent molecular weight (amw) of 74 kDa corresponds to the uncleaved form of RIP-1 and the signal with amw of 42 kDa correlates to the cleaved form of RIP-1. The signal with amw of 119 kDa corresponds to the uncleaved form of PARP and the signal with amw of 89 kDa correlates to the cleaved form of PARP.

wild-type ER-positive MCF 7 human breast cancer cells. Of note, in this cell line lobaplatin displayed good cytotoxic activity, which was more pronounced compared with cisplatin.

Recombinant human tumour necrosis factor-related apoptosis-inducing ligand (rhTRAIL) is a potential, novel anticancer agent because of its selective cytotoxicity to various cancer cells but not to nonmalignant cells [41]. TRAIL induces apoptosis through its death receptors (DR4 and DR5) independently of the p53 tumour suppressor gene, which is frequently inactivated in TNBC [17]. Preclinical studies have shown that TRAIL displays single-agent activity and cooperates with chemotherapy or radiotherapy in a variety of tumour xenograft mouse models [17]. Moreover, early clinical trial data suggest that rhTRAIL is generally safe [17]. As a recent study demonstrated sensitivity to TRAIL-mediated apoptosis in the majority of triple-negative breast cancer cell lines [18], we investigated whether cotreatment with TRAIL and lobaplatin increased the antitumour activity. Accordingly, one of the two triple-negative cell lines showed good sensitivity to TRAIL with an EC50 of 104 ng/ml. HCC 1937 triple-negative and ER-positive MCF 7 cells were less sensitive to TRAIL. For MCF 7 cells, semisensitivity to TRAIL has been previously described by Singh *et al.* [42], which is in good accordance with our results. Cotreatment with lobaplatin and TRAIL substantially increased the cytotoxic effect in all cell lines. Interestingly, one group found that cisplatin, but not other platin derivatives (e.g. carboplatin), could inhibit TRAIL-induced apoptosis in HeLa human cervical cancer cells by cisplatin-induced inactivation of caspase-8 [43]. Thus, effects may vary from cancer cell line to cell line and between the different platin

**Fig. 7**




Effect of cisplatin (Cis) and lobaplatin (Loba) on the cleavage of caspase-3 in HCC 1806 (a) and HCC 1937 (b) human breast cancer cells as determined by western blotting in comparison with untreated cells (Contr.). Cleavage of caspase-3 was also analysed in the presence of z-VAD-fmk (z-Vad). The signal with the apparent molecular weight (amw) of 35 kDa corresponds to the uncleaved form of caspase-3 and the signals with amw of 19 kDa, 17 kDa and 12 kDa correlate to the cleaved forms of caspase-3.

derivatives. As we could demonstrate that caspase inhibition with z-VAD-fmk does at least partly decrease the cytotoxic effect of cisplatin and lobaplatin, and we additionally saw increased antitumour activity of lobaplatin and TRAIL, our data do not support caspase inhibition by either cisplatin or lobaplatin in the cell lines we investigated.

In recent years, it has become evident that the classic dichotomy between apoptosis and necrosis is a simplification of a process that is much more complex [44]. Up to now three caspase-independent ways of apoptosis have been described [26]. Necroptosis is a cellular mechanism of necrotic cell death induced by apoptotic stimuli in the form of death domain receptor engagement by their respective ligands under conditions in which apoptotic execution is prevented [30]. Although it occurs under regulated conditions, necroptotic cell death is characterized by the same morphological features as unregulated necrotic death [31]. Necrostatin-1 is a selective inhibitor of necroptosis [30]. To elucidate how lobaplatin in comparison with cisplatin induces cell death, we demonstrated by western blot analysis that treatment with both platin derivatives in HCC 1806 and HCC 1937 cells

induces cleavage of PARP suggestive for classical apoptosis. Likewise, lobaplatin led to cleavage of caspase-3 in both cell lines. The inactive zymogen is cleaved into the p17 and p12 fragments, which dimerize to form the heterodimeric active caspase-3 enzyme that is at least partially responsible for the proteolytic cleavage of many key proteins – for example, PARP [45]. Furthermore, RIP-1, a protein known to be a key signalling molecule in necroptosis [30], was also analysed by western blot. RIP-1 cleavage was observed in both cisplatin-treated and lobaplatin-treated HCC 1806 and HCC 1937 cell lines. This observation argues for a participation of necroptosis in the induced cytotoxicity in both cell lines. As recent results by our group and others found that the cellular death response triggered by cytotoxic agents depends on the type and dose of chemotherapeutic stress and the cell system investigated and may involve classic apoptosis, as well as various types of apoptotic or necrotic PCD if classic apoptosis is inhibited [26,44,46], we evaluated the effect of coadministration of platin derivatives with the multicaspase inhibitor z-VAD-fmk and specific inhibitor of necroptosis necrostatin. Thus, cell death could somehow be decreased but not inhibited by both z-VAD-fmk and necrostatin. To test whether this effect was specific for platinum agents, we evaluated whether cell death after treatment with two known inductors of classic apoptosis, such as mega-Fas-ligand and TRAIL, could be decreased or abrogated by coadministration of z-VAD-fmk. However, PCD was decreased to a different extent but could not be completely abrogated. We additionally investigated whether a known inhibitor of necroptosis, necrostatin, had an influence on the cytotoxic effects of lobaplatin, mega-FAS-ligand and TRAIL. Again, PCD was decreased to a different extent in the cell lines investigated. Our data are in accord with previous results, which indicate that necroptosis occurs by external stimuli in the form of death domain receptor engagement by ligands such as TNFα, Fas ligand and TRAIL under conditions when apoptotic cell death is prevented [31]. Although it did not reach statistical significance, a proportion of the induced cytotoxicity by both platin compounds seems attributable to necroptosis.

In conclusion, our study could show for the first time that the platinum derivative lobaplatin has good antitumour activity in in-vitro models of p53-mutated triple-negative breast cancers. In particular, it was shown for the first time that cotreatment with TRAIL substantially increases the cytotoxic effect of lobaplatin, providing a rationale for clinical studies based on platinum derivatives and rhTRAIL or antibodies directed to TRAIL receptors in patients with triple-negative breast cancers. In addition, it could be demonstrated for the first time that platinum derivatives can induce caspase-independent forms of cell death if caspases are inhibited and also induce necroptosis, although to a small extent.

## Acknowledgements

### Conflicts of interest

There are no conflicts of interest.

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