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ORIGINAL RESEARCH

Receptors for luteinizing hormone-releasing hormone (GnRH) as therapeutic targets in triple negative breast cancers (TNBC)

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Abstract Triple negative breast cancers express receptors for gonadotropin-releasing hormone (GnRH) in more than 50 % of the cases, which can be targeted with peptidic analogs of GnRH, such as triptorelin. The current study investigates cytotoxic activity of triptorelin as a monotherapy and in treatment combinations with chemotherapeutic agents and inhibitors of the PI3K and the ERK pathways in in vitro models of triple negative breast cancers (TNBC). GnRH receptor expression of TNBC cell lines MDA-MB-231 and HCC1806 was investigated. Cells were treated with triptorelin, chemotherapeutic agents (cisplatin, docetaxel, AEZS-112), PI3K/AKT inhibitors (perifosine, AEZS-129), an ERK inhibitor (AEZS-134), and dual PI3K/ERK inhibitor AEZS-136 applied as single agent therapies and in combinations. MDA-MB-231 and HCC1806 TNBC cells both expressed receptors for GnRH on messenger (m)RNA and protein level and were found sensitive to triptorelin with a respective median effective concentration (EC_{50}) of 31.21±0.21 and 58.50±19.50. Synergistic effects occurred when triptorelin was combined with cisplatin. In HCC1806 cells, synergy occurred when triptorelin was applied with PI3K/AKT inhibitors perifosine and AEZS-129. In MDA-MB-231 cells, synergy was observed after co-treatment with triptorelin and ERK inhibitor AEZS-134 and dual PI3K/ERK inhibitor AEZS-136. GnRH receptors on TNBC cells can be used for targeted therapy of these cancers with GnRH agonist triptorelin. Treatment combinations based on triptorelin and PI3K and ERK inhibitors and chemotherapeutic agent cisplatin have synergistic effects in in vitro models of TNBC. If confirmed in vivo, clinical trials based on triptorelin and cisplatin could be quickly carried out, as triptorelin is FDA approved for other indications and known to be well tolerated.

Keywords Targeted therapy · Triple negative breast cancer · LHRH receptor · Triptorelin · Combination therapy

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Introduction

Breast cancer is the most common female cancer and a heterogeneous disease consisting of distinct entities with different biological features and clinical behaviors. Among the various molecular entities of breast cancer, triple negative breast cancer (TNBC) refers to a distinct subgroup of breast tumors, which neither express receptors for estrogen or progesterone, nor overexpress the human epidermal growth factor receptor 2 (HER2/neu). TNBC accounts for approximately 15 % of all breast cancers [1], but exhibits a disproportional mortality rate. It is clinically more aggressive than breast cancers belonging to the other known subgroups [1–5] and demonstrates a higher incidence in African-American populations and younger premenopausal patients [2, 5]. As TNBC cells do not express the established therapeutic targets (i.e., receptors for estrogen, progesterone) and do not overexpress HER2/neu receptors, they cannot be treated with established targeted therapies. Thus, chemotherapy is the only therapeutic option, so far, for patients suffering from this subgroup of breast cancer. Although TNBCs are sensitive to chemotherapy [2], they are still burdened with the least favorable prognosis of all known subtypes of breast cancer [3, 5]. Thus, in TNBC patients, recurrence takes place earlier and most deaths occur in the first 5 years after diagnosis [3, 6]. As in other subtypes of breast cancers, therapies targeted to estrogen and HER2 receptors, have substantially improved the patients' prognosis, it is of paramount importance to identify therapeutic targets for specific treatment of patients with TNBC.

Receptors for gonadotropin-releasing hormone (GnRHR) were originally detected in the pituitary gland [7]. However, GnRHR have been also described in tissues of male and female reproductive organs and, strikingly, in a variety of human malignancies, such as breast, prostate, ovarian, and endometrial cancers [7, 8]. They were found to be expressed at low levels or not at all by other benign human tissues. These observations suggest that tumoral receptors for GnRH are potential targets for a specific therapy. There are two therapeutic strategies utilizing tumoral GnRH receptors as targets in various cancers. Peptidic analogs of GnRH may be used as targeted therapeutics, disrupting an autocrine stimulatory loop, based on locally produced GnRH and its respective receptor, thus inhibiting tumor growth [7, 8]. Accordingly, in GnRHR expressing endometrial, prostatic, colorectal, lung, and ovarian tumors, the proliferation of cancer cells was inhibited by agonistic or antagonistic analogs of GnRH [8, 9]. The advantage of this therapeutic strategy is the clinical availability of various analogs of GnRH, as they are FDA approved for other indications and clinical studies could be rapidly carried out [10]. The second strategy consisted in the establishment of a new class of antitumor compounds based on a GnRH analog carrier and a cytotoxic compound, such as doxorubicin, for targeted chemotherapy of GnRHR-positive cancers [11-13]. Recent work by our group and others have shown that more than 50 % of TNBC specimens express receptors for GnRH [14, 15], which may represent a suitable therapeutic target in this subgroup of breast cancers.

It has become clear, recently, that the therapeutic potential of novel anti-cancer drugs, which act as growth factor inhibitors, can be potentiated when they are used in combination with other anti-cancer drugs. However, only few combination therapies show real synergy and consequently yield beneficial results. For instance, mTOR-inhibitor everolimus was only associated with significantly increased PFS when applied in combination with an aromatase inhibitor [16], but not with a chemotherapeutic agent [17].

Thus, the current study explored the anti-proliferative potential of GnRH agonist triptorelin as a single agent and in combination with chemotherapeutic agents and novel growth factor inhibitors in in vitro models of TNBC.

Materials and methods

Cell culture

The TNBC cell lines HCC1806 and MDA-MB-231 were cultivated in Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco, Darmstadt, Germany) supplemented with 10 % fetal bovine serum (FBS) (PAA, Cölbe, Germany) in a humidified atmosphere with 5 % CO_2/95 % air at 37 °C.

The fibroblast cell line LTK(-), which does not express GnRH receptor, was cultured in DMEM (Gibco, Darmstadt, Germany) supplemented with 10 % heat-inactivated FBS. Eight hundred micrograms per milliliter G418 was applied freshly to L3.5/78, a LTK(-) originated cell line stably transfected with the human GnRH receptor gene, in addition to the complete medium (both cell lines were kindly provided by Dr. M. Teifel, Aeterna/Zentaris GmBH, Frankfurt, Germany).

Depending on the confluency, all cells were passaged regularly or medium was refreshed twice a week.

RNA extraction and reverse transcription

Extraction of RNA from the cell lines was conducted with the Total RNA and Protein Isolation kit (Macherey-Nagel, Düren, Germany) with reference to the manufacturer's user manual. The yield and quality of RNA were determined spectrophotometrically. RNA (0.3 μg) in a total volume of 10 μl was reverse transcribed with the AffinityScript Multiple Temperature cDNA Synthesis kit (Agilent, La Jolla, CA, USA).

Real-time RT-PCR for GnRH receptor

Messenger (m)RNA expression of GnRHR was detected by real-time RT-PCR. Primers for the GnRH receptor were designed according to the previous publication by Choi's group and were obtained from Eurofins MWG Synthesis GmbH (Ebersberg, Germany). Primer sequences and size of the DNA fragment amplified are shown in Table 1.

Primers for the house-keeping gene β-actin were kindly provided by Dr. Oliver Treeck (Department of Gynecology and Obstetrics, University of Regensburg, Germany). β-Actin was used as internal controls and amplified to check the integrity of cDNA.

PCR reaction mixtures were prepared using the LightCycler® FastStart DNA Master SYBR Green I kit (Roche, Penzberg, Germany) with reference to instructions in the manufacturer's user manual. Reactions were carried out in LightCycler® 2.0 Instrument (Roche, Germany). After denaturing at 94 °C for 15 min, 45 cycles of reactions were done under the following

Table 1 Primers used in real-time RT-PCR for the detection of mRNA expression of GnRH receptor in TNBC cell lines

Name of primer pair	Sequence	Size (bp)
GnRHR-5_413F	5′– ACCGCTCCCTGGCTATCAC – 3′	60
GnRHR-6_472R	5′– ACTGTCCGACTTTGCTGTTGCT – 3′	

conditions: denaturation at 94 °C for 10 s, annealing at 62 °C for 5 s, and extension at 72 °C for 12 s. At last, an extension at 65 °C for 10 min was performed.

The PCR products were resolved in a 1.5 % agarose gel by gel electrophoresis, and the sizes were confirmed. The relative signal intensities of the PCR products to β-actin were analyzed by the manufacturer-provided software.

Protein extraction

Protein was extracted from crude cell lysate with the Total RNA and Protein Isolation kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's user manual. Concentration of protein was determined by Bradford assay (Bio-Rad, Munich, Germany).

Western blot for GnRH receptor

Protein expression of GnRH receptor was detected by Western blot. Ten micrograms proteins extracted from the cell lines were separated in a 10 % SDS-polyacrylamide gel and transferred onto a PVDF membrane. Membrane with transferred proteins was blocked with 5 % skim milk/phosphate-buffered saline (PBS) with 0.1 % Tween (PBST) for 1 h at room temperature and was exposed to 10 μg/ml GHR-106 antibody, which was kindly provided by Dr. G. Lee (UBC, Vancouver, Canada), for GnRH receptor binding. The membrane was then rocked at room temperature for 1 h.

The membrane was washed in PBST and further incubated with ECL anti-mouse-horseradish peroxidase (HRP) antibody (1:10,000) for 1 h at room temperature. Western blot signals were detected by Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, Uppsala, Sweden) with reference to the manufacturer's protocol, and were recorded by either film autoradiography or CCD camera photography with ChemiDoc™ XRS+ System (Bio-Rad, Munich, Germany).

Protein disulfide isomerase (PDI), which is expressed extensively in the endoplasmic reticulum, served as the house-keeping protein in Western blot analysis. After washing in PBST, the GHR-106-blotted membrane was exposed to the PDI antibody, which was a courtesy from Dr. Oliver Treeck (Departme nt of Gynecology and Obstetrics, University of Regensburg, Germany), at 1:5000 for 1 h and then ECL anti-rabbit-HRP antibody at 1:50,000 for another hour at room temperature before signal detection.

In vitro cell proliferation assay

Anti-proliferation effect of the GnRH agonist triptorelin, disorazol-Z, and its GnRH conjugate AEZS-125, inhibitors of the PI3K/AKT pathway (perifosine, AEZS-129), the ERK inhibitor AEZS-134, the PI3K/ERK dual inhibitor AEZS-136, and selected chemotherapeutic agents (cisplatin, docetaxel, AEZS-112) were investigated in the TNBC cell lines HCC1806 and MDA-MB-231. Triptorelin, disorazol-Z, AEZS-125, perifosine, AEZS-129, AEZS-134, AEZS-136, and AEZS-112 were kindly provided by Æterna Zentaris GmBH. (Frankfurt, Germany). Cisplatin was purchased from Sigma-Aldrich (Taufkirchen, Germany). Docetaxel was the courtesies from Dr. Oliver Treeck (Department of Gynecology and Obstetrics, University of Regensburg, Germany).

Cells were starved in 1 % FBS containing DMEM/F12 2 days before treatment of the GnRH analogs, then were trypsinized and counted 24 h before treatment. Seven thousand five hundred HCC1806 or 3000 MDA-MB-231 cells were seeded in each well of a 96-well microplate with 100 μl serum free DMEM/F12. Three cultures of the same cell type were tested for each concentration and replicates were done for at least three times.

Stock solutions of the compounds were made according to the provider's instructions and were stored in 10 μl aliquots at −20 °C. On the day of treatment, 100 μM working solutions in serum and phenol red-free DMEM/F12 medium (Gibco, Darmstadt, Germany) were prepared from the stock solutions. Twelve half-log dilutions were done to produce a series of working solutions with concentrations from 0.0001 to 100 μM. For the cells in each well of the 96-well microplates, media were exchanged to 150 μl serum and phenol red-free DMEM/F12 supplemented with different concentrations of the drugs, or with DMSO, H_2O, or PBS used as the solvent for the drugs. In the case of triptorelin, for instance, up to 1 μM of the compound, 0.01 % DMSO was used as the solvent control. 0.032, 0.1, 0.32, and 1 % DMSO were used as the solvent control for 3.16 10 μM, 31.62, and 100 μM of the compound.

Forty eight hours later, cell titer blue (CTB) assay was performed by addition of 15 μl CTB reagent (Promega, Mannheim, Germany) to each well. The HCC1806 and MDA-MB-231 cells were then incubated under growth conditions for 4 and 1 h, respectively. The color change and intensity of the CTB reagent was quantified with the Wallac Victor™3 1420 Multilabel Counter (Perkin Elmer, Rodgau, Germany) at wavelengths of 530 nm Ex/590 nm Em. The measured absorbance is proportionate to the number of viable cells. median effective concentration (EC_{50}) was determined by the GraphPad Prism software (GraphPad, La Jolla, CA, USA). Experiments were performed in triplicate and repeated at least three times.

Drug synergism studies

In order to verify the synergistic anti-proliferation effects of triptorelin with other drugs in TNBC treatment, the GnRH agonist was individually mixed with perifosine, AEZS-129, AEZS-134, AEZS-136, cisplatin, docetaxel, or AEZS-112 in corresponding ratios and applied to HCC1806 and MDA-MB-231 cells for in vitro cell proliferation assay.

EC_{50} values of individual drug treatments were first determined by the GraphPad Prism software (GraphPad, La Jolla, CA, USA) as described. An equipotent ratio of each drug was then decided with regard to their individual EC_{50} values. After the ratio was set, a mixture of the two compounds at twofold of their EC_{50}s was made. The mixture was serially diluted with serum and phenol red-free DMEM/F12 to onefold, 0.5-fold, 0.25-fold, and 0.25-fold to obtain a dosage range and dose density. The drug mixtures were applied to the pre-seeded cells in the 96-well plates and the treated cells were kept at 37 °C with 5 % CO_2/95 % air for 48 h.

CTB assay was performed in the Wallace Victor™3 1420 Microlabel Counter. Results were analyzed by the CalcuSyn software (Biosoft, Cambridge, UK) according to the method proposed by Chou and Talalay [18], which is at present the best established model to explore the effect of combination therapies. A combination index (CI) value smaller than 1 indicates synergism, while CI values equalling to or larger than 1 represent an additional or antagonistic effect of the two combined drugs, respectively.

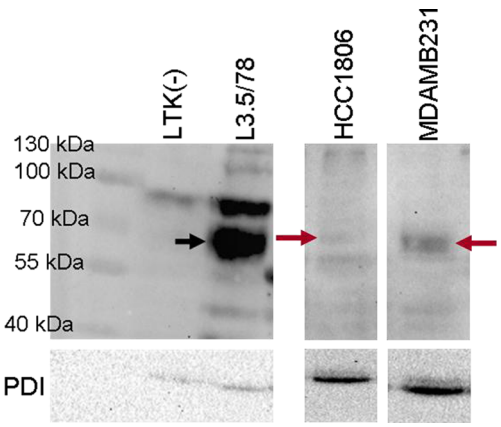

Fig. 2 GHR-106 antibody detection for the protein expression of GnRH receptor in TNBC cell lines. No expression was detected in the negative control cell line LTK(-), whereas signal for GnRH receptor was observed for the positive control cells L3.5/78 (*black arrow*) and the two TNBC cell lines: HCC1806 and MDA-MB-231 (*red arrows*). *PDI* protein disulfide isomerise that is expressed extensively in the endoplasmic reticulum, served as the house-keeping protein

Mutation analysis

The cell lines used were evaluated for somatic mutations using the COSMIC database. COSMIC (an

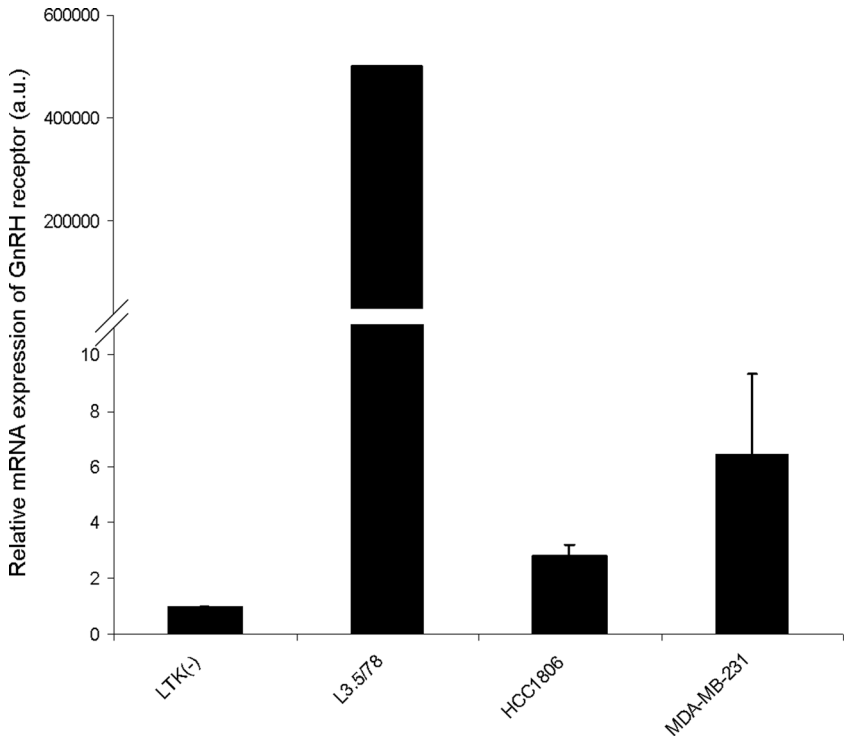

Fig. 1 mRNA expression levels of GnRH receptor in TNBC cell lines. LTK(-) was the negative control, whereas L3.5/78 served as the positive control of GnRH receptor

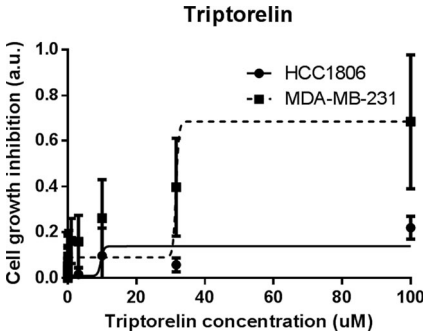

Fig. 3 In vitro cell proliferation assays for the determination of anti-proliferation activities of triptorelin in HCC1806 and MDA-MB-231

acronym of *Catalogue Of Somatic Mutations In Cancer*) is an online database of somatically acquired mutations found in human cancer [19]. Somatic mutations are those that occur in non-germ line cells that are not inherited by children. COSMIC curates data from papers in the scientific literature and large scale experimental screens from the Cancer Genome Project at the Sanger Institute. The database is freely available without restriction via its website.

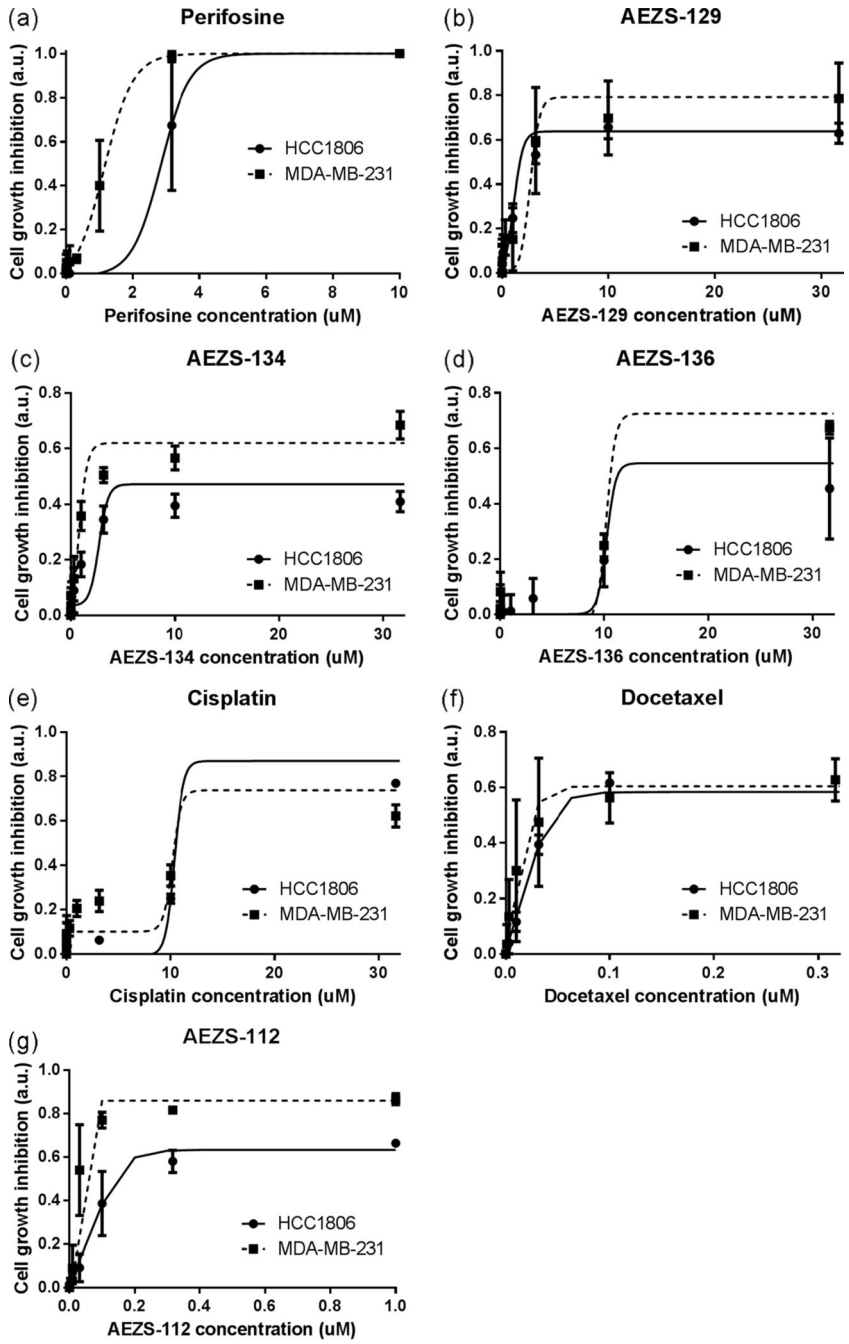

Fig. 4 The anti-proliferation activities of PI3K/AKT/ERK inhibitors and selected cytotoxic compounds on HCC1806 and MDA-MB-231

Results

mRNA expression of GnRHR in MDA-MB-231 and HCC1806 human TNBC lines

LTK(-), which does not express the GnRHR, served as the negative control in the PCR reactions. The expression levels of all cell lines were relative to that of LTK(-).

L3.5/78, on the contrary, was the positive control of GnRHR expression as it stably expresses the gene. Any detected signal represented successful amplification of the GnRH receptor by the primers in the PCR reactions.

mRNA expression of GnRHR was detected in both of HCC1806 and MDA-MB-231. The GnRHR expression level in HCC1806 was about three times of that in LTK(-), while the latter one was approximately six times of that of the negative control. Relative mRNA expression levels of the GnRH receptor in the TNBC cell lines are shown in Fig. 1.

Protein expression of GnRHR in MDA-MB-231 and HCC1806 human TNBC lines

Two antibodies were tested for the detection of protein expression of the GnRHR. GnRHR antibody C-18: sc-8681 was found to have non-specific binding to proteins other than GnRH receptor. GHR-106 was therefore used for Western blot. Detected signals of the GnRHR protein are shown in Fig. 2.

The targeted band representing GnRH receptor protein expression was about 60 kDa. LTK(-) was the negative control, whereas L3.5/78 served as the positive control for GnRHR protein expression (indicated by a black arrow). Mild protein expression of GnRHR was detected in HCC1806 and MDA-MB-231 (indicated by red arrows).

The Western blot result went along with the RT-PCR one that HCC1806 and MDA-MB-231 expressed detectable levels of GnRH receptor, demonstrating that both approaches were reliable in determining the expression of GnRH receptor.

Cytotoxic effect of GnRH agonist triptorelin on MDA-MB-231 and HCC1806 human TNBC cells

The GnRH agonist triptorelin was studied for its anti-proliferation effect in HCC1806 and MDA-MB-231 and found to be potent to reduce proliferation of the TNBC cells (Fig. 3). Triptorelin dose dependently inhibited the proliferation of GnRHR-positive MDA-MB-231 and HCC1806 TNBC cells.

Inhibition of MDA-MB-231 and HCC1806 TNBC cell proliferation by PI3K/AKT/ERK inhibitors and selected chemotherapeutic agents

The PI3K/AKT inhibitor perifosine, PI3K inhibitor AEZS-129, ERK inhibitor AEZS-134, PI3K/ERK dual inhibitor AEZS-136, the DNA-damaging compound cisplatin, and the tubulin inhibitors docetaxel and AEZS-112 were all found to effectively inhibit cell proliferation of HCC1806 and MDA-MB-231 (Fig. 4).

With reference to the EC_{50} values obtained (Table 2), the above tested compounds were used to treat HCC1806 and MDA-MB-231 in combination with triptorelin to determine their synergism of treatments.

Cytotoxic effects of treatment combinations of triptorelin and selected chemotherapeutic drugs or pathway inhibitors on MDA-MB-231 and HCC1806 TNBC cells

Treatments on HCC1806 and MDA-MB-231 indicated that triptorelin, when combined with certain compounds in their corresponding ratios, exhibited a synergistic effect in inhibiting cell proliferation (Tables 3 and 4).

Table 2 EC_{50} values of GnRH analogs and selected compounds in HCC1806 and MDA-MB-231 cells

	HCC1806			MDA-MB		
	EC_{50}		*n*	EC_{50}		*n*
	μm	nM		μm	nM	
Triptorelin	58.29±19.59	–	11	31.59±0.21	–	6
Perifosine	2.84±0.07	–	6	1.13±0.07	–	3
AEZS-129	1.10±0.16	–	3	2.63±0.18	–	6
AEZS-134	2.17±0.21	–	6	–	643.7±134.3	5
AEZS-136	10.26±0.13	–	6	10.23±0.10	–	5
Cisplatin	10.38±0.08	–	3	10.18±0.10	–	5
Docetaxel	–	18.96±1.15	3	–	10.28±2.72	4
AEZS-112	–	71.50±17.49	5	–	23.01±4.87	3

Table 3 Synergism determination of triptorelin and selected chemotherapeutic agents or pathway inhibitors in HCC1806

		Ratio	CI at EC_{50}	CI at EC_{75}	CI at EC_{90}	*n*
Triptorelin	Perifosine	20:1	0.92±0.07	0.91±0.01	0.93±0.05	3
Triptorelin	AEZS-129	60:1	0.77±0.10	0.76±0.13	0.77±0.16	3
Triptorelin	AEZS-134	20:1	>1	>1	>1	3
Triptorelin	AEZS-136	6:1	>1	>1	>1	3
Triptorelin	Cisplatin	6:1	0.45±0.14	0.37±0.14	0.31±0.14	3
Triptorelin	Docetaxel	3000:1	1.18±0.11	1.68±0.37	2.74±1.11	3
Triptorelin	AEZS-112	857:1	1.04±0.12	0.98±0.15	0.95±0.23	3

Among the compounds tested, the DNA-damaging compound cisplatin and the PI3K inhibitors AEZS-129 and perifosine had CI values smaller than 1 and demonstrated the largest degree of synergism in inhibiting cell growth of HCC1806 when combined with triptorelin. The tubulin inhibitor AEZS-112 had an average CI value very close to 1 and likely demonstrated mild synergistic or additional effect with triptorelin in anti-proliferation. Another tubulin inhibitor docetaxel, the PI3K/ERK dual inhibitor AEZS-136, and the ERK inhibitor AEZS-134 were observed to have CI values larger than 1, which represented antagonistic effects with triptorelin when they were applied together to the cells.

In the case of MDA-MB-231, cisplatin, AEZS-134, and AEZS-136 showed average CI values smaller than 1 and demonstrated the largest degree of synergism in inhibiting cell growth of MDA-MB-231 when combined with triptorelin. AEZS-129, which had an average CI value close to but still smaller than 1, showed a milder synergistic effect. Perifosine and docetaxel, the CI values of which were marginal, were likely to possess additional effect in inhibiting cell proliferation in the presence of triptorelin. AEZS-112 was observed to act antagonistically with triptorelin while CI values larger than 1 were noted.

Genomic mutations as found in the COSMIC database

A thorough search of the COSMIC database revealed that breast cancer cell line MDA-MB-231 was positive for a BRAF and K-Ras mutation whereas breast cancer cell line HCC1806 did not exhibit any mutation of the ERK pathway. Both cell lines had mutated CDKN2A and mutated p53; no mutations in the AKT pathway have been described in both cell lines.

Discussion

Tumoral GnRHR are expressed by more than 50 % of human specimens of TNBC [14, 15, 20]. Accordingly, in the TNBC cell lines HCC1806 and MDA-MB-231, expression of receptors for GnRH was also detected by RT-PCR and Western blot analysis. The EC_{50} values, which represent the cytotoxic efficacy, of the GnRH agonist triptorelin were found to lie in the micromolar range in both cell lines and were consistent with the expression level of the GnRH receptors. Thus, EC_{50} was lower in MDA-MB-231 cells, which showed a higher expression of the GnRH receptors, as compared to HCC1806 cells, which expressed GnRH receptors at a lower level.

Specific receptors for GnRH were detected in a variety of human cancers [7, 8]. The evidence for the production of a GnRH-like peptide and/or the expression of mRNA for LHRH was also demonstrated in human breast, prostate, ovarian, and endometrial cancer cell lines [7, 8]. These findings suggest that locally produced GnRH may be involved in the growth of these tumors, forming an autocrine stimulatory loop. This concept is supported by an inhibitory action of GnRH agonists and antagonists on human mammary, prostate, ovarian, and endometrial cancer cell lines through specific GnRH receptors on tumor cells in vitro, when effects

Table 4 Synergism determination of Triptorelin and selected chemotherapeutic agents or pathway inhibitors in MDA-MB-231

		Ratio	CI at EC_{50}	CI at EC_{75}	CI at EC_{90}	*n*
Triptorelin	Perifosine	20:1	1.10±011	1.03±0.08	0.98±0.06	3
Triptorelin	AEZS-129	15:1	1.04±0.18	0.90±0.14	0.83±0.19	3
Triptorelin	AEZS-134	40:1	0.77±0.18	0.74±0.27	1.01±0.69	3
Triptorelin	AEZS-136	2:1	0.99±0.08	0.89±0.14	0.81±0.21	3
Triptorelin	Cisplatin	3:1	0.64±0.34	0.63±0.36	0.74±0.42	3
Triptorelin	Docetaxel	3000:1	1.06±0.04	1.00±0.12	0.98±0.21	3
Triptorelin	AEZS-112	1200:1	1.14±0.22	1.17±0.27	1.21±0.38	3

through the pituitary and gonads can be excluded [7, 8, 21]. An interesting study by Emons and coworkers demonstrated that knockdown of the GnRH receptors could increase chemosensitivity of endometrial, ovarian, and breast cancers in vitro, suggesting synergy of chemotherapeutic agents and GnRH analogs [22].

As GnRH agonists are FDA approved for other indications and known to be very well tolerated [10], they have already been studied in phase II trials in advanced, mostly platinum-resistant ovarian cancer, which is known to be positive for GnRH receptors in 70 % of the cases. Out of 245 patients, which were treated with GnRH agonists, 23 had a partial remission (RR 9 %) and 64 had a disease stabilization (SD 26 %) [8, 23]. The observed clinical benefit was most likely due to direct inhibitory action of the GnRH agonists at tumoral GnRH receptors. The clear, yet moderate clinical effect might be increased by using treatment combinations based on GnRH agonists [24].

Recently, an increasing number of treatment protocols based on combinations of monoclonal antibodies, growth factor inhibitors, and chemotherapeutic agents are being established in clinical oncology. The ultimate goal of combination therapy is synergy, i.e., a superior efficacy of the compounds given as combined treatment as compared to the sum of their effects as single agent therapy. To define synergy mathematically is harder than it may seem at first glance, but has been successfully attempted by Chou and Talalay [18]. They have created a system of equations, which allows the distinction between synergistic, additive, and antagonistic effects of drug combinations over the whole dose range [18]. Their mathematical approach is the most valid method to evaluate synergy so far. This is mirrored by an increasing number of publications using this approach, which is accepted by major journals, such as *Cancer Research*. Accordingly, we investigated treatment combinations based on GnRH agonist triptorelin and chemotherapeutic agents and growth factor inhibitors using the CalcuSyn software, which is based on the equation system by Chou and Talalay, and found synergy with cisplatin and inhibitors of PI3K and ERK..

TNBCs have been evidenced from both preclinical and clinical practices to be sensitive to platinum-based chemotherapeutic agents [25]. Silver et al. have reported a rate of partial or complete response of 64 % using single agent cisplatin as a neoadjuvant therapy in TNBC patients [26]. Though being a potent antitumor agent, treatment using cisplatin is limited by side effects including nephrotoxicity, emetogenesis, and neurotoxicity [27]. Synergy of triptorelin and cisplatin over the whole dose range in both in vitro models of TNBC suggests this combination for clinical trials, if our results can be confirmed in vivo.

The PI3K/AKT pathway has been indicated to be frequently activated in TNBC and to possess a key regulatory function in tumor cell survival, proliferation, migration, metabolism, angiogenesis, and apoptosis. [28-30]. Accordingly, the PI3K/AKT inhibitors AEZS-129 and perifosine not only showed EC_{50} values in low micromolar range when applied individually to the two TNBC cell lines, but also demonstrated good synergistic effects with triptorelin in inhibiting HCC1806 proliferation.

There is increasing evidence that mutation in or hyperactivation of the Ras-Raf-MEK-ERK pathway is relevant for oncogenesis in humans. Thus, Ras is activated in 30 % of human cancers [31] and B-Raf mutations were found in 60 % of melanomas, 20 % of colorectal cancers, and 30 % of ovarian cancers [32, 33]. Activation of the ERK pathway promotes cell proliferation, cell survival, epithelial–mesenchymal transition, angiogenesis, and metastasis [34]. Accordingly, Bartholomeusz et al. [35] could correlate high ERK expression with a shorter survival time in TNBC patients.

Individual application of the ERK inhibitor AEZS-134 and PI3K/ERK dual inhibitor AEZS-136 to the two TNBC cell line models exhibited an excellent inhibitory effect of tumor cell proliferation, suggesting disabling ERK signal transduction may contribute to the treatment of TNBC. MDA-MB-231, but not HCC1806 TNBC, cells are known to harbor a k-ras and BRAF mutation, resulting in an overactivation of the ERK pathway. Accordingly, in our study, the PI3K/ERK dual inhibitor AEZS-136 and the ERK inhibitor AEZS-134, when combined with triptorelin, demonstrated synergism in inhibiting MDA-MB-231 cell proliferation.

The current study demonstrates that GnRH receptors on TNBC cells can be used for targeted therapy of these cancers with GnRH agonist triptorelin. Furthermore, treatment combinations based on triptorelin and PI3K and ERK inhibitors and most importantly chemotherapeutic agent cisplatin are identified as having synergistic effects in in vitro models of TNBC. If confirmed in vivo, clinical trials based on triptorelin and cisplatin could be quickly carried out, as triptorelin is FDA approved for other indications and known to be very well tolerated [10].

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Conflict of interest The authors declare that they have no conflict of interest.

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