Title: Triple negative breast cancers express receptors for LHRH and are potential therapeutic targets for cytotoxic LHRH-analogs AEZS 108 and AEZS 125

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Author’s response to reviews: see over
Triple negative breast cancers express receptors for LHRH and are potential therapeutic targets for cytotoxic LHRH-analogs AEZS 108 and AEZS 125.

Reviewer 1 had nothing to object.

Reviewer 2:
1. "No RT/PCR data is provided in the manuscript to show expression of LHRH receptor."

We provide data on LHRH receptor expression for both cell lines used in the paper. Furthermore we additionally demonstrate using an siRNA approach that the LHRH receptor is a prerequisite for the targeted mechanism of action of AEZS-108.

2. "In addition, no pharmacokinetics or pharmodynamics, and accumulation of drug in heart as well as in other tissues is demonstrated."

We agree with the referee, however pharmacokinetics have already been determined in humans (see paragraph below). Therefore our main focus was directed towards the efficacy of AEZS-108 in a tumor entity, for which still no targeted therapy approaches exist.

In a phase I dose-escalation study in 17 patients with advanced LHRH-receptor positive, breast, ovarian or endometrial cancers, AEZS-108 was administered at doses up to 267 mg/m². The measured plasma concentrations of DOX and AEZS-108 showed a high variability. Following AEZS-108 doses of 160 and 267 mg/m², maximum plasma concentrations (Cmax) ranged from 728 to 6661 ng/ml. The high variability influenced all other pharmacokinetic parameters. Therefore, only a weak dose dependency was found in Cmax and AUC. The calculated $t_{1/2}$ and clearance of AEZS-108 were approximately 2 h and 1 l/min m², respectively. At the dose levels of 160 and 267 mg/m2, average Cmax values of DOX ranged from 600 to 700 ng/ml. As expected, average Cmax and AUC of DOX were closely correlated to the AEZS-108 levels. The $t_{1/2}$ of DOX was slightly above that calculated for AESZ-108, and the respective clearance was slightly lower. There was no evidence for accumulation of AESZ-108, and the respective clearance was slightly lower. There was no evidence for accumulation of AESZ-108 or DOX after repeated dosing cycles [1].

In order to give the reader the relevant information on the pharmacokinetics we included a phrase in the method section, which reads as follows:

"In the phase I study the calculated $t_{1/2}$ and clearance of AEZS-108 were approximately 2 h and 1 l/min m², respectively. At the dose levels of 160 and 267 mg/m², average Cmax values of DOX ranged from 600 to 700 ng/ml. As expected, average Cmax and AUC of DOX were closely correlated to the AEZS-108 levels."
3. "No toxicity data is included showing if the dose used in mice is safe and does not cause abnormal heart function."

We certainly agree with the referee, however the excellent safety profile, also with respect to absence of cardiac side effects of AEZS-108 was already shown in various nude mice experiments and also toxicity studies (see paragraph below) in rats and dogs. Additionally, in the phase I and the 2 phase II studies, no cardiac side effects in patients, who were heavily pretreated (see paragraph below).

Toxicity data in animal studies:

In nude mice models AEZS-108 displayed lesser toxic side effects than equimolar doses of DOX. In particular no apparent toxic side effects to the pituitary, the heart, or other organs were observed. This excellent safety profile was further enhanced in pharmacologic safety studies evaluating the effects of AEZS-108 on respiratory and cardiovascular parameters in the dog, as well as in the Irwin and Rotarod test and in a hexobarbital interaction study. In these studies no test-item related effects were observed. In the cardiovascular safety study in beagle dogs, no evidence of QT prolongation was seen at any administered dose of AEZS-108. No adverse findings were observed in a local tolerability study in rabbits after intravenous and intra-arterial infusions of AEZS-108. Perivascular application of AEZS-108 induced moderate local inflammatory reactions. Superior tolerability of AEZS-108 as compared to DOX was further confirmed in acute and subchronic toxicity studies in mice, rats and dogs, respectively. In contrast to DOX, where lymphohistiocytic myocarditis with intramuscular fibrosis was observed, AEZS-108 did not induce any cardiotoxicity [2].

Toxicity data in clinical studies:

Side effects observed in clinical studies were in accordance with safety data from the animal models and were, in general, moderate and could be easily managed. In the phase I study dose-limiting hematoxicity occurred at 267 mg/m² and was thus used as a treatment dose in the phase II studies. In patients with heavily pretreated platinum/taxane resistant ovarian cancer AEZS-108 at doses of 267 mg/m² was well tolerated [3]. Hematologic toxicities of G3/4 were: leukopenia 44% (G4: 9.2%), anemia 4.6%, thrombocytopenia 2.3%. Febrile neutropenia occurred in 3 pts (7%). Hematotoxicity was usually rapidly reversible; 6 patients (14%) received G-CSF, 2 patients (4.6%) RBC transfusion. In one patient (2.3%) dose reduction (160 mg/m²) was accomplished. Non-hematologic toxicities of G2/3 (no G4 toxicity was reported) were: alopecia 32.6%, nausea 20.9% (G3:2.3%), emesis 14%, diarrhea 4.6%, mucositis 4.6%, allergic reaction 4.6%.

In patients with advanced endometrial cancers, adverse events, possibly drug related, except for hematologic toxicity Grade 3/4 (rapidly reversible neutropenia: 60%, anemia: 5%), were commonly limited to CTCAE grade 1 or 2. There was 1 patient each with febrile neutropenia and a Grade 2 hand-foot-syndrome. Only one patient stopped therapy due to toxicity (recurrent anemia).

In the phase I and both phase II studies, there was no evidence of cardiotoxicity in serial controls of LVEF [3-5]. As the pituitary has receptors for LHRH, pituitary toxicity of AEZS-108 was evaluated in the phase I study [5]. No relevant effect of AEZS-108 on cortisol levels was observed in the ACTH stimulation test. Similarly, there was no effect of AEZS-108 on baseline serum levels of TSH, T3, and T4 and on the increase in TSH 30 min after stimulation.
with 200 µg TRH. Thus, at doses of 267 mg/m² AES 108 has a favorable safety profile with manageable toxicity. [1, 6, 7]

To give the reader relevant information on toxicity of AEZS-108 we included the following paragraph in the discussion:

"In nude mice models AEZS-108 displayed lesser toxic side effects than equimolar doses of DOX. In particular no apparent toxic side effects to the pituitary, the heart, or other organs were observed. This excellent safety profile was further enhanced in pharmacologic safety studies evaluating the effects of AEZS-108 on respiratory and cardiovascular parameters in the dog, as well as in the Irwin and Rotarod test and in a hexobarbital interaction study. In these studies no test-item related effects were observed. In the cardiovascular safety study in beagle dogs, no evidence of QT prolongation was seen at any administered dose of AEZS-108. No adverse findings were observed in a local tolerability study in rabbits after intravenous and intra-arterial infusions of AEZS-108. Perivascular application of AEZS-108 induced moderate local inflammatory reactions. Superior tolerability of AEZS-108 as compared to DOX was further confirmed in acute and subchronic toxicity studies in mice, rats and dogs, respectively. In contrast to DOX, where lymphohistiocytic myocarditis with intramuscular fibrosis was observed, AEZS-108 did not induce any cardiotoxicity."

Accordingly, in the phase I and both phase II studies, there was no evidence of cardiotoxicity in serial controls of LVEF. As the pituitary has receptors for LHRH, pituitary toxicity of AEZS-108 was evaluated in the phase I study [5]. No relevant effect of AEZS-108 on cortisol levels was observed in the ACTH stimulation test. Similarly, there was no effect of AEZS-108 on baseline serum levels of TSH, T3, and T4 and on the increase in TSH 30 min after stimulation with 200 µg TRH. Thus, at doses of 267 mg/m² AES 108 has a favorable safety profile with manageable toxicity.

3. "It may be more appropriate to use mouse mammary pads to develop breast tumor than s.c., since it will mimic the tumor micro-environment."

We agree with the referee, however in previous experiments we carried out the tumor take-rate was significantly decreased when choosing the mammary fat pad model, even though it represents the tumor microenvironment better than injecting the tumor cells in the flank of the animals does. We therefore chose the latter tumor model in the current study. In one of our previous publication however, using the targeted cytotoxic bombesin analog AN-215 we could demonstrate, that targeted therapy of MDA-MB-231 breast cancers was effective irrespective of the location the tumors were injected (flank or mammary fat pad) [8].

EDITORS COMMENTS:

Requires major revision addressing reviewer comments and also requires significant rewriting of the manuscript especially results section of the manuscript. In addition, revised version should also include (1) clear documentation of expression of receptors for LHRH in TNBC models, (2) should include representative IHC images used for generation of human tumor data in Table 2 , (3) should include further IHC analysis of xenograft tissues (4) should
include additional mechanistic studies and (5) siRNA studies to demonstrate the specificity of drugs on TNBC need to be included.

We agree with the editor and have therefore carried out additional experiments providing the following data.

1. LHRH receptors are now clearly demonstrated in HCC 1806 and MDA-MB-231 cells by real time RT-PCR analysis.

2. Representative IHC images of TNBC specimens are presented.

3. LHRH-receptors on MDA-MB-231 and HCC-1806 cells are now shown by fluorescent labeling.

4+5. siRNA studies showing the specific action of AEZS-108 in both cell lines used are provided.


