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Efficacy of a leptin receptor antagonist peptide in a mouse model of triple-negative breast cancer

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ABSTRACT

Triple-negative breast cancers, which represent 10–20% of all mammary tumours, are characterised by the aggressive phenotype, are often found in younger women and have been associated with poor prognosis. Obesity increases the risk for triple-negative breast cancer development. Because triple-negative breast cancer patients are unresponsive to current targeted therapies and other treatment options are only partially effective, new pharmacological modalities are urgently needed. Here we examined if the leptin (obesity hormone) receptor is a viable target for the treatment of this cancer subtype. In human triple-negative breast cancer tissues, the leptin receptor was expressed in 92% (64/69) and leptin in 86% (59/69) of cases. In a model triple-negative breast cancer cell line MDA-MB-231, the leptin receptor antagonist peptide Allo-aca inhibited leptin-induced proliferation at 50 pM concentration. In an MDA-MB-231 orthotopic mouse xenograft model, Allo-aca administered subcutaneously significantly extended the average survival time from 15.4 days (untreated controls) to 24 and 28.1 days at 0.1 and 1 mg/kg/day doses, respectively. In parallel, conventional treatment with 1 mg/kg/day intraperitoneal cisplatin prolonged the average survival time to 18.6 days, while administration of 20 mg/kg/day oral Tamoxifen (negative control) had no significant survival effects relative to controls. In normal CD-1 mice, Allo-aca produced no systemic toxicity up to the highest studied subcutaneous bolus dose of 50 mg/kg, while, as expected, it induced a modest 6–10% body weight increase. Our results indicate that leptin receptor antagonists could become attractive options for triple-negative breast cancer treatment, especially in the obese patient population.

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1. Introduction

Approximately 10–20% of breast cancers are considered triple-negative (TNBC) as they lack oestrogen and progesterone receptors (ER, PgR) and do not express or express low levels of the oncogenic receptor HER2.[1] TNBC are associated with shorter relapse-free survival and increased lymphovascular invasion.[2] The disease affects more frequently younger patients and it is more prevalent in African Americans and Hispanics.[3] Currently no targeted therapies exist for TNBC, while conventional chemotherapy is only partially effective.[4] Until recently, high dose combination chemotherapy was the preferred treatment option for TNBC.[5] Current clinical trials with cisplatin show promising results,[6] although the response rates with a combined surgery, cisplatin and radiotherapy regimen remain at 50–60%.[7] Consequently, the development of novel targeted therapies for TNBC is of paramount importance.

The risk of TNBC is increased by obesity, especially upper-body obesity.[8–10] Among obesity-related factors that are known to impact breast cancer development and progression, the prominent place is occupied by the adipokine leptin.[11] Leptin (obesity hormone) is produced mainly by the fat tissue, but it can also be synthesised by breast cancer cells in response to obesity-related stimuli.[12–15] Leptin induces breast cancer cell growth, transformation, and survival and reduces the efficacy of breast cancer treatments.[11,16,17] Both leptin and its receptor (ObR) are overexpressed in breast cancer, especially in higher grade tumours, but are absent in normal epithelial breast tissues.[11,12,18] The expression of the leptin system is found in 70–80% of breast cancer cases, including hormone receptor-and HER2-positive and negative cancers.[12,18] Consequently, in recent years, the leptin/ObR system has emerged as a new and promising therapeutic target for breast cancer therapy.[19] The data suggest that ObR is an independent biomarker, not correlating with ER/PgR or HER2, thus ObR antagonists could be useful for management of different breast cancer subtypes.[17,20]

In the course of development of specific, selective and clinically relevant ObR antagonists for cancer treatment,[21] we generated Allo-aca (H-alloThr-Glu-Nva-Val-Ala-Leu-Ser-Arg-Aca-NH_2), a 9-residue peptidomimetic.[22] The Allo-aca sequence is based on the C-terminal ObR-binding leptin site III.[23] Allo-aca inhibits leptin-induced proliferation and oncogenic signalling of MCF-7 cells and various glioblastoma cell lines at high pM concentrations. Furthermore, in an orthotopic mouse model of human hormone responsive breast cancer, the peptide added intraperitoneally (ip) or subcutaneously (sc) at 0.1 mg/kg/day doses reduces the growth of MCF-7 cell xenografts by approximately 50%. In the current report, we investigated the expression levels of ObR in tissues of human TNBC and assessed the potential of targeting ObR in *in vitro* and *in vivo* models of TNBC.

2. Materials and methods

2.1. Drugs

Allo-aca was synthesised by solid-phase methods and purified by reversed-phase high performance liquid chromatography (RP-HPLC).[22] The final product was lyophilised from 2% acetic acid and characterised by RP-HPLC and matrix-assisted laser ionisation/desorption mass spectrometry (MALDI-MS). Tamoxifen pills and cisplatin solution were manufactured by Teva Pharmaceutical Industries Ltd., Israel.

2.2. Cellular assays

2.2.1. Cell proliferation

Human leptin (R&D Systems, Minneapolis, Minnesota, USA) was used at the concentration of 6 or 12 nM. The MDA-MB-231 breast cancer cell line was purchased from the American Type Tissue Collection. The cells were grown in Dulbecco's modified Eagle's medium (DMEM):F12 plus 10% foetal bovine serum. Seventy percent confluent cultures were synchronised in serum-free medium (SFM) (DMEM plus 10 μM $FeSO_4$, plus 0.5% bovine serum albumin) for 24–72 h and treated with leptin and/or 1 pM–100 nM Allo-aca. Cell numbers were determined by direct counting after trypan blue exclusion. All assays were done in triplicate and repeated at least three times.

2.2.2. Western-blotting (WB)

MDA-MB-231 cells were lysed as described earlier[12] and 100 μg of total cell proteins were resolved on a 4–15% polyacrylamide gel. The expression of ObR was detected by WB using an anti-ObR primary antibody (Ab) H-300 (R&D Systems).[12]

2.2.3. HER2 fluorescence in situ hybridisation

HER2 fluorescence in situ hybridisation (FISH) was carried out according to the protocol of the manufacturer (Panpath Rembrandt Series Amsterdam, The Netherlands). Briefly, single layer cells were deposited on coated glass slides and air dried. Following RNase free protease digestion and denaturation, tissue sections were hybridised for 2 hours at 37 °C with prewarmed probes for the *HER2* gene and chromosome 17 centromere (CEP17). The nucleus was stained light blue with 4′-6-diamidino-2-phenyl indole. Ten section fields were examined and scored for HER:CEP17 ratio determination.

2.3. Breast cancer biopsy samples and immunohistochemistry (IHC)

Tissue samples were obtained from women who underwent surgery for primary breast cancer at the University and Public Hospitals in Verona between January 1, 1992 and November 15, 2006. All tissue samples were anonymised and the study protocol was approved by the local Institutional Review Board.

Serial-step 5 μm sections were cut from paraffin-embedded tissues and examined for ER and PgR levels and other routine biomarkers.[17] HER2 levels were determined with the HercepTest (DAKO) with FISH using PathVysion assay (Abbott Diagnostics, Rome, Italy). Sixty-nine tissues that were classified as negative for the expression of ER, PgR, and HER2 were selected for this study.

The expression of leptin and ObR was investigated by IHC with specific Abs, as described before.[11,17] For leptin staining, we used the A20 leptin polyclonal Ab (pAb) (Santa Cruz Bio-

technology, Santa Cruz, USA) at 1:100 dilution and for ObR, the M18 ObR pAb (Santa Cruz Biotechnology) at 1:50 dilution. Breast cancer specimens previously classified as positive for leptin and ObR were used as positive controls, while in negative controls the primary Abs were omitted. Assessment of immunoreactivity was performed in at least 10 different section fields by two independent evaluators. The expression of leptin and ObR in cancer samples was classified using a four-point scale: 0, <10% stained cells; 1+, 10–50% cells with weak staining; 2+, >50% cells with weak staining; 3+, >50% cells with strong staining. Tumours with the score of at least 1+ were considered positive for the expression of leptin or ObR.

Statistical analysis of ObR and leptin correlations was done with Chi-square and Spearman tests with a significance level of 0.05.

2.4. Orthotopic breast cancer model

Animals were maintained and handled in accordance with the recommendations of the Guidelines for the Care and Use of Laboratory Animals and were approved by the Animal Care Committee of Semmelweis University (permission No.:399/003/2005). Twenty-six home-bred 8-week-old female severe immunocompromised (*scid*) mice (genetic background CB17/Icr) were anesthetised to allow the implantation of 2×10^6 MDA-MB-231 cells into the two inguinal mammary glands. When tumours were palpable in all animals (65 days after cell transplantation), 23 mice were split into four groups. The groups contained mice with a comparable distribution of tumour sizes. One group of six mice was treated daily with 0.1 mg/kg peptide Allo-aca sc (between the two inguinal mammary glands), the second group of 6 mice received 1 mg/kg/day Allo-aca sc, and the third group of 6 mice was treated with 1 mg/kg/day cisplatin ip (Table 1). Five mice remained untreated. The three remaining mice received 20 mg/kg/day Tamoxifen orally (po). Tumour volume was measured by a caliper along two orthogonal axes and calculated as $a^2 \times b \times 0.52$ (mm^2). Values in Table 1 represent the larger and better measurable lesion volumes in each mouse. Survival time was recorded daily during 0–38 days after treatment initiation. Statistical analysis of animal survival time was performed with an independent Student's *t*-test (SlideWrite, Encinitas, California, USA). The statistical significance levels were $p = 0.05$ (95% confidence) and $p = 0.01$ (99% confidence).

2.5. In vivo toxicity and preliminary pharmacokinetics

Peptide Allo-aca was dissolved in water and given as bolus injections at 1, 5, 10, 25 and 50 mg/kg sc into healthy CD-1 mice (Charles River Laboratories, Budapest, Hungary). Mice were observed for signs of systemic toxicity. Three days later the animals were sacrificed by CO_2 inhalation, weighed and their brains, lungs, livers and spleens were removed and weighed. For the pharmacokinetic analysis 100 μg (5 mg/kg) Allo-aca was dissolved in 200 μL sterile phosphate-buffered saline (PBS) (pH 7.2) and was injected sc into healthy CD-1 mice using 4 mice for each time point. About 100 μL blood was taken from the eye at 0, 5, 15, 45, 90 and 120 minutes. The blood cells were centrifuged and 20 μL aqueous 15% trichloroacetic acid was added per 60 μL of plasma. After repeated centrifugation, 0.5 μL supernatant was loaded to a MALDI-MS instrument, and a combined volume of 210 μL supernatant was loaded to a C18 HPLC column that had been calibrated with 10 ng–10 μg Allo-aca.

3. Results and discussion

3.1. ObR antagonist target identification in TNBC

MDA-MB-231 cells are considered prototypical preclinical models for studying the effects of target drugs on aggressive and metastatic breast cancers.[24,25] When implanted into *scid* mice MDA-MD-231 xenografts can rapidly metastasise and usually kill the animals within 2 months.[24] In contrast with the slower growing MCF-7 mouse xenografts where tumour volume is the primary readout for drug efficacy,[22] in mice carrying MDA-MB-231 xenografts the time of survival is a more reliable outcome measure.[26]

Since leptin response, ObR expression as well as HER2 levels appear to vary among MDA-MB-231 cell sub-lines cultured in different laboratories,[24,25,27] we examined HER2 expression and ObR levels in a newly purchased MDA-MB-231 batch. Using FISH, which is a standard technique to discriminate between HER2-positive and HER2-negative tumours, we found that in our MDA-MB-231 cell line, the *HER2* gene was expressed in 3 copies on chromosome 17 while CEP17 was found

Table 1 – Summary of *in vivo* efficacy measurements.

Treatment groups	Average survival (days)	Average increase of tumour volume by day 11 (% ± SD)	Number of surviving animals after day 0/11/22 of treatment
Untreated	15.4	491 ± 183	5/4/1
0.1 mg/kg/day Allo-aca sc	24*	500 ± 211	6/5/4
1 mg/kg/day Allo-aca sc	28.1*	352 ± 194	6 /5/5
1 mg/kg/day cisplatin ip	18.6	282 ± 125	6/6/0
40 mg/kg/day Tamoxifen po	18.0	519 ± 0	3/2/1

The tumour volume was measured 11 days after treatment initiation in the larger or more accurately measurable mammary lesion in each animal. No statistical difference was found (*t*-test, $p > 0.1$) between any tumour volume groups. The asterisks indicate statistically significant survival differences compared to untreated controls and are calculated for the entire 38-day of the treatment/observation period. Administration routes: sc, subcutaneous; ip, intraperitoneal; po, oral. SD, standard deviation.

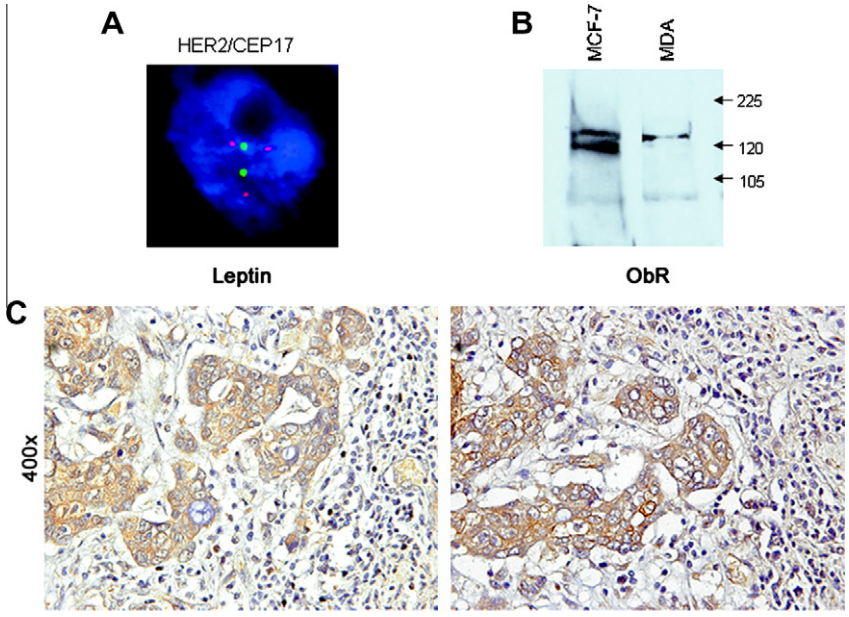

Fig. 1 – Validation of the leptin receptor (ObR) as a potential pharmaceutical target in an *in vitro* model of triple negative breast cancers (TNBC) and in human tissues. Panel A: *HER2* expression was assessed in MDA-MB-231 cells using fluorescence *in situ* hybridisation, as described in Section 2. Chromosome 17 markers are shown in green and *HER2* genes in red. The cell nucleus is stained blue. Panel B: Western-blot detection of the long signalling isoform of ObR (130–180 kDa) in MDA-MB-231 cells and MCF-7 cells (positive control) was performed as described in Section 2. The arrows indicate the alignment of molecular weight markers. Panel C: Immunohistochemistry assessment of leptin and ObR in TNBC. Representative images depicting leptin and ObR staining are shown at 400× magnification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

at 2 copies, identifying the HER2:CEP17 ratio as 1.5 (Fig. 1A). Currently a HER2:CEP17 ratio of less than 2.2 categorises tumours as HER2-negative and is not suitable for HER2-targeted therapies.[28] In parallel, we examined if MDA-MB-231 cells express ObR. Using Western-blot, we detected ObR in MDA-MB-231 cells but at levels slightly lower than in the triple-positive MCF-7 cells (Fig. 1B).

Next, we studied ObR and leptin expression levels in tissues of TNBC (Fig. 1C). The presence of leptin/ObR in this subgroup of breast cancers has never been quantitatively assessed before. ObR was detectable in 92% (64/69) and leptin in 86% (59/69) of TNBC biopsies examined. While the expression of leptin was found at 1 + (29%), 2 + (42%), and 3 + (29%) levels, ObR was scored at 1 + (18%), 2 + (62%), and 3 + (20%). The presence of ObR was highly correlated with the expression of leptin ($p = 0.01$). The remarkable correlation between leptin and ObR in other breast cancer types as well as in some other tumour types has been described before.[17,18,29,30] As noted before in human breast cancer[17,18] and other cancer biopsies,[29] the expression of leptin and ObR in adjacent non-cancer tissue was not detectable or present at minimal (less than +) levels.

3.2. *Peptide Allo-aca inhibits leptin-induced proliferation of MDA-MB-231 cells in vitro*

In MDA-MB-231 cells, leptin stimulates proliferation, activates ObR and its downstream signalling and induces the expression of certain cell cycle/survival proteins.[27] Stimulation of MDA-MB-231 cells with 6 nM leptin for 24 h increased cell proliferation by approximately 37% (Fig. 2), indicating that the receptor transmits mitogenic signals. Increasing the incubation time or leptin concentration did not improve this response. Addition of Allo-aca at 1 pM–100 nM concentrations antagonised the mitogenic effects of leptin in a concentration-dependent manner. The IC_{50} value was estimated to 50 pM, while full inhibition was observed at 1 nM (Fig. 2). The same peptide inhibits leptin-induced proliferation of MCF-7 cells with an IC_{50} of 200 pM.[22] Improved activity of Allo-aca in MDA-MB-231 cells relative to that seen in MCF-7 cells might be due to a lower level of ObR expression in cell cultures of TNBC (Fig. 1B).

Notably, the peptide did not interfere with MDA-MB-231 proliferation in the absence of leptin, except for 100 nM concentration that produced minimal cytotoxicity (Fig. 2). This is an important feature distinguishing Allo-aca from other potential ObR inhibitors that can display inverse pharmacology and act as ObR agonists in the absence of leptin, thus are not suitable for pharmaceutical development.[21]

3.3. *Allo-aca toxicity, off-tumour effects and pharmacokinetics in vivo*

To identify a safe dose of Allo-aca, we investigated its toxicity in normal mice. Dose-limiting toxic effects could not be observed up to 50 mg/kg, the highest sc bolus dose studied. The mice did not show any characteristic sign of systemic toxicity such as, reduced activity, head tilt or squinting. The average weights of the brains, livers, spleens and kidneys

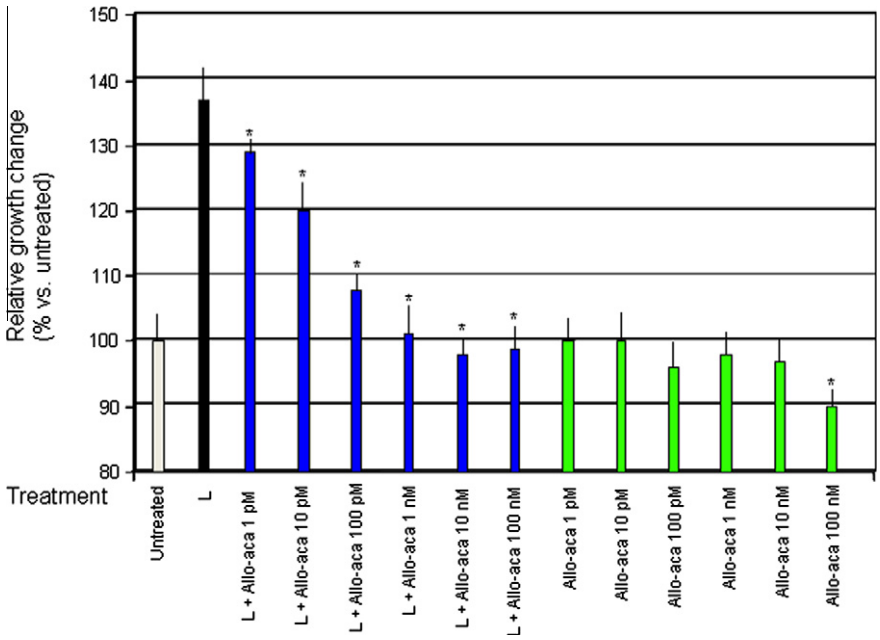

Fig. 2 – Effects of peptide Allo-aca on leptin-induced proliferation of MDA-MB-231 cells. The peptide was tested at 1 pM–100 nM concentrations in the presence or absence of 6 nM leptin (L) as described in Section 2. The number of cells in untreated cultures was taken as 100%. The relative% change in cell number under different conditions versus untreated controls was calculated as mean ± standard error. Statistically significant differences ($p \leqslant 0.05$) between L and L + Allo-aca, or control and Allo-aca are indicated by asterisks.

hovered around 0.3, 1.0, 0.08 and 0.22 grams, respectively, and were not associated with specific peptide doses. These organ weights were identical to those of untreated mice.

The mice subjected to the toxicity protocol gained weight, a phenomenon that was expected from ObR inhibition. The average total weight increases were 10.0%, 9.5%, 8.2% and 6.5% for the 1, 5, 10 and 25 mg/kg doses, respectively. We noted a similar inverse relationship between peptide dose and weight increase upon bolus administration of other leptin site III ObR antagonists.[22] At the highest 50 mg/kg dose the mice lost 1% of body weight, which was potentially associated with a reversal of the pharmacology and should be considered in clinical regimen design. Nevertheless, the weight increases seen with the anticipated low therapeutic doses of Allo-aca further validated ObR as its specific *in vivo* target.

We failed to detect any free peptide or degradation product in the mouse blood by MS or chromatography in the 5 min–2 h time period after sc Allo-aca administration. Based on calibration of the bioanalytical methods used, the sensitivity of our techniques was in the high ng/mL concentration range in mouse blood, a level that is somewhat higher than the 10 ng/mL level validated for HPLC-MS pharmacokinetic studies of peptide drugs.[31] The free Allo-aca concentration in the blood or that of any single metabolite remained under 1 μg/mL. Knowing the excellent biodistribution pattern of labelled Allo-aca upon ip administration,[22] it is likely that after sc administration the peptide was bound to a carrier biomolecule and could not be detected in free form by the methods and instrumentation used. Binding to carrier proteins is a likely reason for the even biodistribution and lack of systemic toxicity of a peptide-based antibacterial drug when it is administered intramuscularly.[32]

3.4. *Efficacy of Allo-aca treatment in an orthotopic mouse xenotransplant model of TNBC*

We assessed the *in vivo* efficacy of Allo-aca in an orthotopic MDA-MB-231 mouse xenograft model. The treatment success with the peptide and control compounds was evaluated in animals bearing established, palpable tumours. As MDA-MB-231 cells can produce endogenous leptin[13] and MCF-7 cells grow when xenotransplanted into *scid* mice without leptin addition,[22] exogenous leptin was not inoculated into the mice. To ascertain tumour establishment and fast killing, MDA-MB-231 cells were implanted into both inguinal mammary glands.

The average survival time of untreated mice after treatment initiation was 15.4 days (Table 1 and Fig. 3). While it cannot be considered as a main cause of death, we observed a metastatic lesion on the neck of one of the animals. Tumour volume was measured from the first day of treatment (day 0). However, as 2 untreated mice died by day 12 (and treated animals started to die immediately after) the last day when tumour volume comparison could be made was day 11 after treatment initiation. By day 11, cisplatin treatment reduced the size of the lesions to 57% of those measured in untreated animals. Overall, 1 mg/kg/day cisplatin added ip extended the survival time to 18.6 days. This increase of the survival time was not statistically significant relative to untreated controls ($p = 0.405$, Table 1 and Fig. 3). In contrast, Allo-aca administered at 0.1 and 1 mg/kg/day sc doses prolonged the average survival period of the animals to 24.0 and 28.1 days, respectively (Table 1 and Fig. 3). This outcome represented a statistically significant improvement relative to untreated controls ($p = 0.012$ and 0.0004, respectively). In addition,

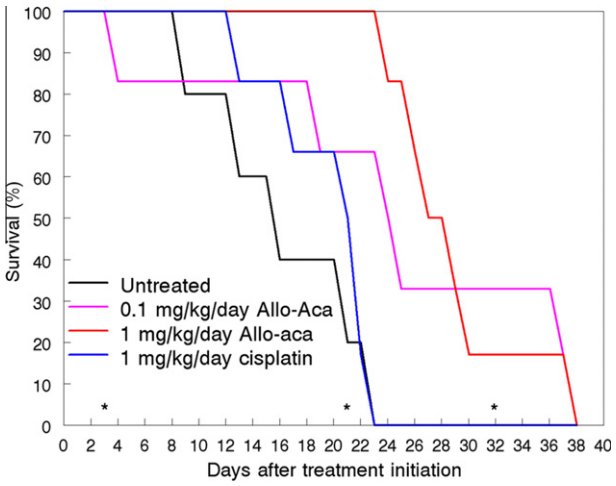

Fig. 3 – Subcutaneously added Allo-aca improves survival of *scid* mice xenotransplanted with MDA-MB-231 cells. The figure shows the survival times starting at treatment initiation (which took place 65 days after cell implantation) and continuing until all experimental animals succumbed to metastatic cancer. The Allo-aca peptide was administered sc as described in Section 2. In control experiments, mice were treated with ip cisplatin or po Tamoxifen. The death time points of Tamoxifen-treated mice are indicated by asterisks.

treatment with 1 mg/kg/day Allo-aca provided a significantly better result compared with the cisplatin regimen ($p = 0.012$). While the 11-day tumour volume after 1 mg/kg/day Allo-aca treatment was 71% of that observed in untreated animals, 0.1 mg/kg/day Allo-aca did not reduce the average size of the larger implanted tumour lesions (Table 1). Considering these data, one can hypothesise that peptide Allo-aca extends survival time only in part by inhibiting the growth of primary cancers. Other mechanisms that counteract known leptin functions, such as reduction of systemic oncogenic signalling including distant metastases and anti-angiogenic effects[16,33] could potentially be involved. We speculate that targeting ObR could be justified for aggressive and metastatic breast cancer treatment because leptin is a known angiogenic factor and clinical data associated ObR expression in breast cancer with distant metastases and shorter time of survival.[18] Noteworthy, anti-angiogenic therapy is currently being explored as a potential treatment option for TNBC.[34]

As expected, Tamoxifen treatment did not improve the survival of the animals in any significant manner (average survival time: 18.0 days) and did not reduce the volume of the larger mammary nodules in the two animals that survived by day 11 after treatment initiation (Table 1 and Fig. 3).

4. Conclusions

In conclusion, Allo-aca at a 1 mg/kg/day sc dose extended the average survival time of mice carrying TNBC xenografts by 80%. If developed as a drug, the peptide with its advantageous administration route and safety profile can be a useful addition to the existing oncology drug repertoire against various forms of cancers characterised by ObR overexpression such as breast, brain, prostate and colon cancers. While not a sole solution for the management of TNBC, an ObR antagonist such as Allo-aca or its improved derivative exhibiting peripheral activity only might become an attractive addition to current treatment protocols of these aggressive tumours.

Role of the funding source

Providing financial assistance only.

Conflict of interest statement

Laszlo Otvos and Eva Surmacz are inventors on a PCT patent application covering peptide Allo-aca and analogues for the treatment of various cancer, arthritis and autoimmune disease forms. The patent is owned by Temple University.

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