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BH3-mimetic small molecule inhibits the growth and recurrence of adenoid cystic carcinoma

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Abstract

Objectives—To evaluate the anti-tumor effect of BM-1197, a new potent and highly specific small molecule inhibitor of Bcl-2/Bcl-x_L, in preclinical models of human adenoid cystic carcinoma (ACC).

Methods—Low passage primary human adenoid cystic carcinoma cells (UM-HACC-2A,-2B,-5,-6) and patient-derived xenograft (PDX) models (UM-PDX-HACC) were developed from surgical specimens obtained from 4 patients. The effect of BM-1197 on cell viability and cell cycle were evaluated *in vitro* using this panel of low passage ACC cells. The effect of BM-1197 on tumor growth, recurrence and tumor cell apoptosis *in vivo* was evaluated with the PDX model of ACC (UM-PDX-HACC-5).

Results—Exposure of low passage primary human ACC cells to BM-1197 mediated an IC₅₀ of 0.92–2.82 μM. This correlated with an increase in the fraction of apoptotic cells (p<0.0001) and an increase in caspase-3 activity (p<0.0001), but no noticeable differences in cell cycle (p>0.05). *In vivo*, BM-1197 inhibited tumor growth (p=0.0256) and induced tumor cell apoptosis (p=0.0165).

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Conflict of interest statement

Ascentage Pharma has licensed the Bcl-2/Bcl-x_L inhibitor technology from the University of Michigan. Shaomeng Wang receives compensation and owns stock in Ascentage. All other authors declare no conflict of interest.

without causing significant systemic toxicities, as determined by mouse weight over time. Surprisingly, weekly BM-1197 decreased the incidence of tumor recurrence ($p=0.0297$), as determined by Kaplan-Meier analysis.

Conclusion—These data demonstrated that single agent BM-1197 induces apoptosis and inhibits tumor growth in preclinical models of adenoid cystic carcinoma. Notably, single agent BM-1197 inhibited tumor recurrence, which is considered a major clinical challenge in the clinical management of adenoid cystic carcinoma. Collectively, these results suggest that patients with adenoid cystic carcinoma might benefit from therapy with a BH3-mimetic small molecule.

Keywords

Salivary gland cancer; Resistance to therapy; Targeted therapy; Bcl-2; Bcl-x_L; Apoptosis; Tumor recurrence

Introduction

The treatment options for salivary gland adenoid cystic carcinoma (ACC) are primarily limited to surgery and radiation therapy, since these tumors are largely unresponsive to chemotherapy. Salivary gland cancer represents 10-15% of all head and neck tumors [1]. Adenoid cystic carcinomas are, together with mucoepidermoid carcinomas, the two most common malignant salivary tumors. The clinical features of adenoid cystic carcinomas include invasive local growth, frequent local recurrence, metastasis, and predisposition for perineural and perimuscular invasion [2,3]. Histologically, adenoid cystic carcinomas are composed of a blend of epithelial and myoepithelial cells with distinctive phenotypic features [2]. Adenoid cystic carcinomas display three morphologic patterns: tubular, cribriform and solid, with the latter being associated with the worst prognosis [4-6]. The average survival after surgical treatment is approximately 10 years. But, in presence of metastases, the average survival decreases to 3 years [7]. Approximately 45% of the ACC patients experience either recurrence or metastatic disease within 10 years [8]. In this scenario, the first-line of treatment is surgical, despite its limited effectiveness and high morbidity in patients with highly aggressive or invasive cancers. The second-line of treatment is radiotherapy with chemotherapy, despite the fact that these tumors are largely unresponsive to these treatment modalities [9]. Considering these facts, the search for safer and more effective systemic therapies for adenoid cystic carcinoma is warranted.

The anti-apoptotic B-cell lymphoma (Bcl)-2 and Bcl-x_L proteins are overexpressed in adenoid cystic carcinomas [10]. Notably, Bcl-2 has been linked to increased resistance to radiotherapy and chemotherapy in head and neck cancer [11, 12]. Members of the Bcl-2 family share conserved sequences in regions known as Bcl-2 homology (BH) domains [13, 14]. Bcl-2 family includes more than 20 anti-apoptotic and pro-apoptotic regulators that share at least one conserved BH domain [15-17]. Anti-apoptotic proteins inhibit cell death by binding to the pro-apoptotic proteins and inhibiting their function [15-17]. For example, when Bcl-2 is bound to Bax, the complex maintains stability of the mitochondrial membrane, inhibits cytochrome-c release, and prevents downstream activation of caspases and initiation of apoptosis [18, 19]. Knowledge of the mechanisms underlying the anti-apoptotic function of Bcl-2/Bcl-x_L in cancer has supported the development of BH3-

mimetic small molecules (*e.g.* BM-1197) that interfere with this effect and trigger cell death, as described below.

It is known that angiogenesis plays an important role in the pathobiology of cancer [20, 21]. We have demonstrated that Bcl-2 acts as a pro-angiogenic signaling molecule in endothelial cells through a pathway that involves activation of NF- κ B and upregulation of the angiogenic chemokines CXCL1 and CXCL8 [22]. While vascular endothelial cells support tumor growth by forming conduits of oxygen and nutrients for the tumor cells, the tumor cells in turn support angiogenesis by releasing vascular endothelial growth factor (VEGF) and other pro-angiogenic factors [23]. Interestingly, VEGF induces Bcl-2 expression and enhances the survival of both endothelial cells [24, 25] and tumor cells [26, 27] in Bcl-2 in several tumor types [28-30, 31]. Collectively, Bcl-2 has multiple effects in tumor cells and endothelial cells that contribute to tumor growth and dissemination, making it an excellent candidate molecule for the development of new anti-tumor therapies [32].

BM-1197 represents the 3rd generation of a novel class of targeted drugs that we developed using a structure-based design [33, 34]. It binds to Bcl-2 and Bcl-x_L proteins (K_i<1 nM) and has a >1,000-fold selectivity over Mcl-1 [34]. BM-1197 binds to the BH3 binding groove with high affinity and competes with pro-apoptotic BH3-containing proteins (*e.g.* Bax, Bak, Bid), preventing their heterodimerization with Bcl-2/Bcl-x_L, and allowing these proteins to induce apoptosis [33, 34]. Adenoid cystic carcinomas typically show overexpression of Bcl-2 [10]. However, the anti-tumor effect and underlying mechanism of action of BM-1197 in ACC remain unclear. Here, we tested the hypothesis that BM-1197 has an anti-tumor effect in adenoid cystic carcinoma. We observed that indeed, the BH3-mimetic small molecule BM-1197 slows down the growth and prevents the recurrence of adenoid cystic carcinoma in preclinical models.

Materials and Methods

Tumor specimens and generation of UM-HACC cells

Cells were generated from salivary gland adenoid cystic carcinomas that were surgically resected between March/2010 and August/2012 at the University of Michigan Hospital. The cell lines were named the University of Michigan Human Adenoid Cystic Carcinoma (UM-HACC) series, as follows: UM-HACC-2A (53 years-old; Caucasian female; minor salivary gland at base of the tongue, T3N1M0), UM-HACC-5 (45 years-old; Caucasian female; minor salivary gland at palate) and UM-HACC-6 (58 years-old; Caucasian male; minor salivary gland at buccal space; T3N0M0) were primary tumors, while UM-HACC-2B was generated from a lymph node metastasis found in the UM-HACC-2A patient. The cells retrieved from these tumors were used for up to 20 passages, *i.e.* they should be considered low-passage primary ACC cells and not established cell lines. The patient-derived xenograft model of ACC used here (UM-PDX-HACC-5) was generated from the same tumor specimen used to generate the UM-HACC-5 cells, and presented a solid histological pattern. Briefly, tumors were minced in small pieces, passed through a 25 ml pipette and centrifuged at 1,000 RPM, 4°C for 5 minutes. Tumor fragments were digested in 1× collagenase-hyaluronidase (Stem Cell Technologies, Vancouver, BC, Canada) at 37°C for 1 hour. Tumors were pipetted up-and-down every 15 minutes with a 25 ml pipette (1×) followed by

a 10 ml pipette (3×) to mechanically disrupt tissues. Single cell suspensions were generated with a 40-µm sieve (Fisher) placed in a Falcon tube containing 4 ml Fetal Bovine Serum (FBS; Invitrogen, Carlsbad, CA, USA). Red blood cells were lysed with the AKC lysis buffer (Invitrogen) according to manufacturer's instructions. Resulting primary cell cultures were grown in a salivary gland culture medium consisting of high glucose Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) supplemented with 2 mM/L L-glutamine (Invitrogen), 1% antibiotic (AAA; Sigma-Aldrich, St. Louis, MO, USA), 10% FBS, 20 ng/ml epidermal growth factor (Sigma-Aldrich), 400 ng/ml hydrocortisone (Sigma-Aldrich), 5 µg/ml insulin (Sigma-Aldrich), 50 ng/ml nystatin (Sigma-Aldrich), and 1% amphotericin B (Sigma-Aldrich), as shown [35]. Patients signed an IRB-approved informed consent authorizing the use tumor specimens for research.

Western blots

HACC cells (UM-HACC-2A,-2B,-5,-6) lysates were resolved by electrophoresis and membranes were probed overnight at 4°C with a 1:1,000 dilution of hamster anti-human Bcl-2 monoclonal antibody (BD Biosciences, San Jose, CA, USA), rabbit anti-human Mcl-1 monoclonal antibody (Cell Signaling, Beverly, MA, USA) or mouse anti-human Bcl-x_L monoclonal antibody (BD Biosciences) for 1 hour at room temperature. Membranes were exposed to appropriate peroxidase-coupled secondary antibodies and proteins were visualized with enhanced chemiluminescence (Amersham, Pittsburgh, PA, USA).

Cytotoxicity assays

Sulforhodamine B (SRB) cytotoxicity assays were done as described [25]. Briefly, UM-HACC cells were seeded at 3×10^3 cells per well of 96-well plates and allowed to attach overnight. BM-1197 or vehicle control was diluted in culture medium to treat cells for 24-96 hours. Cells were fixed onto the plates by addition of 10% cold trichloroacetic acid (final concentration) for 1 hour at 4°C. Cellular protein was stained by addition of 0.4% SRB (Sigma) in 1% acetic acid and incubation at room temperature for 30 minutes. Unbound SRB was removed by washing with 1% acetic acid and plates were air-dried. Bound SRB was resolubilized in 10 mmol/L unbuffered Tris base and absorbance was determined on a microplate reader at 560 nm (Genios Tecan). Test results were normalized against drug-free controls. Data were obtained from triplicate wells per condition and representative of at least three independent experiments.

Flow cytometry

UM-HACC cells were seeded at 2×10^5 per well in a 6-well plate and allowed to attach overnight. BM-1197 or vehicle control were diluted in culture medium and added to the cells. Cells were incubated for 48-96 hours and assessed for cell cycle and apoptosis by hypotonic lysis and staining of DNA with propidium iodide (PI), as described [36]. Cell cycle phase was determined by analysis of G₀/G₁, S and G₂/M fractions. Apoptotic levels were determined by the sub-G₀/G₁ fraction. For active caspase-3 analysis, UM-HACC cells were seeded and treated with BM-1197 or vehicle control under the same conditions described above. After 48-96 hour incubation, cells were washed twice and then resuspended in BD Cytofix/Cytoperm™ solution at a concentration of 5×10^5 cells per 250 µL, and incubated for 20 min on ice. Then, cells were washed twice with BD Perm/Wash™

buffer (1×) at room temperature. Finally, cells were incubated in BD Perm/Wash™ buffer (1×) plus anti-active caspase-3 for 30 min at room temperature and then analyzed by flow cytometry (FACSCalibur; BD Biosciences). Data were obtained from triplicate wells per condition and are representative of at least 3 independent experiments. Flow cytometry data were analyzed using FlowJo® software.

In Vivo Studies

A patient-derived xenograft model of adenoid cystic carcinoma (UM-PDX-HACC-5) at *in vivo* passage 4 or 5 was used for the studies presented here. UM-PDX-HACC-5 tumor fragments were implanted subcutaneously in the dorsal region of 5-7-week-old severe combined immunodeficient mice (CB.17.SCID; Charles River, Wilmington, MA, USA), as described [37]. Twenty-one days after implantation, mice were randomized into two groups (n=10) and adjusted to equalize the mean tumor volume (200 mm³) in each group. Mice received weekly tail vein injections of either 10 mg/kg BM-1197 or vehicle control (Polyethylene glycol/ Kolliphor® EL in PBS). Tumor volume was calculated using the formula: volume (mm³) = L × W² /2 (L, length; W, width). At termination of the experiment, mice were euthanized and tumors were harvested, fixed, and processed for hematoxylin and eosin (HE) staining. For tumor recurrence studies, mice were anesthetized with ketamine and xylazine, and a fragment of UM-PDX-HACC-5 xenograft tumor was implanted in the subcutaneous space of the dorsal region of each mouse. Twenty-four days after implantation, mice were randomized into two groups (n=10) and adjusted to equalize the mean tumor volume (500 mm³) in each group. Twenty-seven days after implantation the tumors were retrieved, surgical wounds were sutured, and mice were kept alive. Mice received either weekly tail vein injection of 10 mg/kg BM-1197 or vehicle control. Treatment started 3 days before surgical removal of the primary tumor, and continued thereafter. Kaplan-Meier curves were generated using as criteria for failure the presence of a palpable tumor. After resected, the tumor tissues were fixed with 10% buffered formalin for 24 hours, embedded in paraffin, and prepared for histology. Tissue slides were stained with HE and analyzed under light microscope. The care and treatment of experimental animals was in accordance with University of Michigan institutional guidelines.

In situ TUNEL

For terminal deoxyribonucleotide transferase-mediated nick-end labeling (TUNEL), tissues harvested from the xenograft tumors were permeabilized by incubation with 0.1% Triton X-100, 0.1% sodium citrate solution for 30 min. Subsequently, tissues were incubated with terminal deoxyribonucleotide transferase and fluorescein-dUTP (*In Situ* Cell Death Detection Kit Fluorescein; Roche, Basel, Switzerland), according to manufacturer's instructions. The number of TUNEL-positive cells was determined under fluorescence microscopy (Leica DM 5,000B) as the average (cell/field) of 9 high power fields from 3 specimens per experimental condition.

Statistical analyses

Data were analyzed by Student's t-test or by one-way ANOVA, followed by post-hoc tests (Tukey) for multiple comparisons. For analysis of tumor growth over time, after log-

transforming tumor volume data to linearize the data, we fitted a linear random effects model to assess the growth rate differences among the two treatments (*i.e.* vehicle versus BM-1197). We included mouse as a random effect in order to perform our repeated measures regression. We assumed an auto-regression correlation structure such that temporally proximate observations have a higher correlation, and we controlled for the initial tumor size. In the recurrence study, data were analyzed by Kaplan-Meier using the Gehan-Breslow-Wilcoxon test. Data were analyzed using GraphPad Software (San Diego, CA, USA). Significance was determined at $p < 0.05$.

Results

Effect of BM-1197 on low passage human adenoid cystic carcinoma cells

A mechanism frequently used by cancer cells to evade apoptosis is via overexpression of pro-survival proteins (*e.g.* anti-apoptotic members of the Bcl-2 family) [38]. Here, we performed an initial screening for expression of anti-apoptotic Bcl-2-family proteins using a panel of low passage, primary adenoid cystic carcinoma cells (UM-HACC) by Western Blot analysis. The 4 primary cell cultures evaluated here (UM-HACC-2A,-2B,-5,-6) showed strong expression of Bcl-2, Mcl-1, and Bcl-x_L (Fig. 1A,B). Then, we evaluated the effect of the small molecule inhibitor of Bcl-2/Bcl-x_L (BM-1197) on the viability of UM-HACC cells using the SRB assay. We observed a dose- and time-dependent cytotoxic response in all ACC cells evaluated, with IC₅₀ in the low micromolar range particularly for the 72-hour and 96-hour time points (Fig. 1C). As expected, the effect of BM-1197 on ACC cell survival did not involve a decrease in expression levels of Bcl-2 (Fig. 1D). This class of small molecule inhibitors functions by preventing the interaction of Bcl-2 with its pro-apoptotic partners (*e.g.* Bax) through the BH3-binding domain, and not by inhibiting the Bcl-2 expression levels (33,34).

BM-1197 induces apoptosis of primary human adenoid cystic carcinoma cells

To understand the mechanism(s) underlying the effect of BM-1197 on cell density observed in Figure 1, we performed analyses for apoptosis and cell cycle. Primary human adenoid cystic carcinoma cells (UM-HACC-5) exposed to BM-1197 showed visible nuclear fragmentation and apoptotic bodies, particularly after 72 or 96 hours of treatment (Fig. 2A). We performed two additional analyses to verify if BM-1197-induced cell death was indeed mediated by apoptosis. Identification of cells in sub-G₀/G₁ by propidium iodide staining followed by flow cytometry revealed a significant increase in the fraction of apoptotic cells ($p < 0.0001$) upon treatment with BM-1197 for 48 or 96 hours (Fig. 2B). Further, we observed a time- and dose-dependent increase in the percentage of cells expressing the pro-apoptotic active caspase-3 protein upon treatment with BM-1197 (Fig. 2C). To determine the effect of BM-1197 on cell proliferation, we performed cell cycle analyses by flow cytometry, and observed that BM-1197 had no measurable effect on cell cycle after 48 or 96 hours (Fig. 2D,E). Similar trends were observed with the other UM-HACC cells studied here (data not shown). Collectively, these data demonstrate that the anti-tumor cell effect of BM-1197 (Fig. 1) is mediated by apoptosis, and not simply by an anti-proliferative effect on these cells.

BM-1197 inhibits tumor growth and recurrence in a PDX model of adenoid cystic carcinoma

To determine the effect of BM-1197 in a preclinical model of adenoid cystic carcinoma, we generated and characterized a patient-derived xenograft (PDX) model from a 45 year-old Caucasian female (UM-PDX-HACC-5). The identity of these PDX tumors was determined by short tandem repeat (STR) profiling that confirmed the match with the human surgical specimen used to generate this PDX model (data not shown). These tumors grow readily in mice, making this PDX model amenable to drug screening studies (Fig. 3A). Histologically, the surgical specimen presented with bicellular layer structures configuring a tubular pattern characteristic of human adenoid cystic carcinomas (Fig. 3B). We observed that the PDX model assumed a more solid, less differentiated morphology, with fewer cystic regions and less stromal cells. The tumor cells exhibited high pleomorphism, some of them showing nuclear hyperchromasia and altered nuclear-cytoplasmic ratio (Fig. 3C). Such change in morphology upon serial passage in mice is expected [39]. Notably, the PDX model presented both perimuscular and perineural invasion (Fig. 3D,E), which are frequently observed in human adenoid cystic carcinomas and are associated with poor prognosis. PDX tumors were allowed to grow to an average volume of approximately 200 mm³ before beginning treatment with BM-1197. We found that treatment with BM-1197 decreased ($p=0.034$) the rate of tumor volume increase compared to vehicle (Fig. 3F). Here, we plotted the predicted regression output across the experimental time with the mean initial tumor size in each group. After 15 days of treatment, the tumor volume in the BM-1197-treated group was lower ($p=0.0256$) than in the vehicle control group (Fig. 3G). BM-1197 did not cause systemic toxicity in mice, as demonstrated by the maintenance of animal weight at levels comparable to vehicle control mice (Fig. 3H).

For the tumor recurrence study, we transplanted the UM-PDX-HACC-5 into mice ($n=10$), waited 21 days until the tumors reached the average of 600 mm³ and then surgically removed the tumors. Kaplan-Meier analyses demonstrated that mice treated with BM-1197 showed less recurrence ($p=0.0297$) than vehicle-treated mice (Fig. 3I). Notably, recurrent tumors showed similar histopathology as the primary tumors (Fig. 3J). Taken together, these data showed that the BM-1197 slows down tumor growth and prevents recurrence of a patient-derived xenograft model of adenoid cystic carcinoma, even in presence of a more aggressive histological subtype.

BM-1197 induces apoptosis of adenoid cystic carcinoma cells in vivo

To understand mechanism(s) mediating the anti-tumor responses observed with BM-1197 *in vivo*, we performed TUNEL staining of histological tumor sections from mice treated with BM-1197 or vehicle controls (Fig. 4A). We observed that the percentage of TUNEL-positive cells in the group treated with BM-1197 was higher ($p=0.0165$) than in the vehicle control group (Fig. 4B).

Discussion

Members of the Bcl-2 family play a major role in the cellular turnover observed in normal tissue homeostasis. Tumors frequently exploit Bcl-2-mediated anti-apoptotic effects to favor

cell accumulation [40, 41]. It is well known that adenoid cystic carcinoma cells overexpress anti-apoptotic Bcl-2 family proteins [10], providing a survival advantage for these cells that might correlate, or be functionally responsible, for the relentless growth and tenuous resistance to therapy observed in patients [42, 43]. These clinical observations provide strong rationale for the search of new therapies targeting anti-apoptotic Bcl-2 family members as a potential treatment for patients with adenoid cystic carcinoma.

Using a structure-based design approach, we have designed BM-1197 as a potent and efficacious dual inhibitor of Bcl-2 and Bcl-x_L, as described (34). The mechanism underlying the pro-apoptotic effect of this small molecule inhibitor is based on the following steps (34): (1) BM-1197 binds to Bcl-2 and Bcl-x_L; (2) it dissociates the heterodimeric interactions between pro-apoptotic (*e.g.* Bax) and anti-apoptotic Bcl-2 family proteins; (3) it induces conformational changes in the Bax protein, causing cytochrome-c release, caspase-9/caspase-3 activation and cell death by apoptosis. Therefore, the mechanism for BM-1197-induced apoptosis does not involve downregulation of Bcl-2 proteins. Rather, BM-1197 works by preventing the protective effect of the binding of Bcl-2/Bcl-x_L to Bax. When Bax is free, it induces cell death via mediating mitochondrial membrane permeability changes, cytochrome-c release, and activation of caspase-mediated apoptosis.

Here, we observed that inhibition of Bcl-2/Bcl-x_L with BM-1197 activated caspase-3 and induced apoptotic death of adenoid cystic carcinoma cells *in vitro*. We also observed that BM-1197 slowed down tumor growth and induced apoptotic cell death in patient-derived xenograft model of adenoid cystic carcinoma. In parallel studies, the Wang laboratory demonstrated that BM-1197 induced apoptosis in five colorectal cancer cell lines [44]. The same group has also demonstrated that BM-1197 induces apoptosis and mediates potent regression of small cell lung cancer xenograft tumors [34]. ACC is notoriously unresponsive to chemotherapy, as demonstrated by the fact that this cancer has no FDA-approved drug. We propose that any decrease in the rate of tumor growth in ACC is a significant step towards the development of an effective therapy. Notably, we did not observe any signs of systemic toxicities with BM-1197, suggesting that it could be used in combination with conventional chemotherapy or as a sensitizer to radiotherapy.

A rather surprising result of our study was that single agent BM-1197 prevented the recurrence of adenoid cystic carcinoma. Here, we developed a preclinical experimental design that mimics the clinical scenario in which a patient is treated surgically for the removal of the primary tumor, and then is observed for recurrences. Recurrences are frequently observed in this cancer due, at least in part, to the fact that these tumor cells have strong predisposition for perineural and perimuscular invasion [2, 3]. Notably, we observed both types of invasion in our PDX model of adenoid cystic carcinoma. These results suggest that long-term therapeutic inhibition of Bcl-2/Bcl-x_L with a BH3-mimetics approach might prevent ACC recurrence and metastatic spread. This hypothesis will need to be verified using large animal experimentation and eventually clinical trials. Nevertheless, it is encouraging to observe significant and reproducible effects in the preclinical studies presented here.

Collectively, our results demonstrate that single agent BM-1197 slows down tumor growth and prevents recurrence in preclinical models of adenoid cystic carcinoma. These results suggest that patients with adenoid cystic carcinoma may benefit from BH3-mimetics (*e.g.* BM-1197), particularly in a neoadjuvant setting as a single agent, or in combination with chemoradiotherapy. The development of experimental models, such as the low passage primary ACC cells and related PDX models, are enabling developmental therapeutics studies that have as ultimate goal the discovery of a mechanism-based therapy to improve the survival and quality of life of patients with adenoid cystic carcinoma.

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Highlights

- BM-1197 is a new, potent, BH3 mimetics small molecule inhibitor of Bcl-2/Bcl-x_L
- BM-1197 potently induces caspase-3 and apoptosis of adenoid cystic carcinoma cells
- BM-1197 slows down tumor growth in PDX models of adenoid cystic carcinoma
- BM-1197 prevents local recurrence of PDX tumors
- These data unveil therapeutic potential of BH3-mimetics in adenoid cystic carcinoma

Figure 1.

BH3-mimetics small molecule inhibitor of Bcl-2/Bcl-x_L (BM-1197) induces death of low passage primary human adenoid cystic carcinoma cells. A. Western blot analysis of pro-survival proteins from the Bcl-2 family in a panel of low passage primary human adenoid cystic carcinoma cells (UM-HACC-2A,-2B,-5,-6). B. Photomicrographs of low passage primary human UM-HACC cells cultured *in vitro*. C. Graphs depicting dose-dependence assays for the effect of BM-1197 treatment on the viability of HACC cells, as determined by the SRB assay. Assays were performed in quadruplicate wells/condition, and verified in 3

independent experiments. D. Western blot analysis of Bcl-2 expression in low passage primary human adenoid cystic carcinoma cells (UM-HACC-6) exposed for 24-96 hours to 10 μ M BM-1197 or vehicle control.

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Figure 2.

BM-1197 activates caspase-3 and induces apoptosis of low passage primary human adenoid cystic carcinoma cells. A. Photomicrographs of UM-HACC-5 cultured *in vitro* in presence of 0-10 μ M BM-1197 for 48-96 hours. B. Graph depicting the fraction of apoptotic cells after 48 or 96 hours of BM-1197 treatment. Apoptosis was determined by sub-G₀/G₁ fraction upon staining with propidium iodide followed by flow cytometry. C. Graph depicting the fraction of positive cells for active caspase-3, as determined by flow cytometry after 48 and 96 hours of BM-1197 treatment. Statistical analysis was performed with one-

way ANOVA followed by post-hoc tests. Asterisks indicate significance, as follows: * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$. D,E. Cell cycle analysis after 48 or 96 hours of BM-1197 treatment, as determined by propidium iodide staining followed by flow cytometry.

Figure 3. BM-1197 slows down growth and prevents recurrence of a patient-derived xenograft (PDX) model of adenoid cystic carcinoma. **A.** Macroscopic view of mice harboring UM-PDX-HACC-5 tumors. Top row shows all mice from the vehicle control group, and bottom row the mice included in the BM-1197-treated group. **B.** Photomicrograph of histological section (HE) of the human tumor specimen showing typical morphology of adenoid cystic carcinomas. **C,D,E.** Photomicrographs of histological sections of UM-PDX-HACC-5 tumors from the vehicle control group. The red arrows indicate (D) perimuscular and (E) perineural

invasion. F. Graph depicting tumor growth (UM-PDX-HACC-5) over time. Mice were treated either with vehicle or with 10 mg/kg BM-1197, weekly, via tail vein injection (n=10). G. Graph depicting tumor weight immediately after surgical resection. H. Graph depicting the mouse weight over time, as a measurement of systemic toxicity of BM-1197 as compared to vehicle control. I. Kaplan-Meier curve depicting recurrence-free survival in mice treated with 10 mg/kg BM-1197 or vehicle control (n=10). Recurrence was defined as the presence of a palpable tumor, as confirmed by histological evaluation. J. Photomicrographs of histological sections (HE) of the primary xenograft tumor and a recurrent tumor, indicating similar morphologies.

Figure 4. Treatment with BM-1197 induces apoptosis *in vivo*. A. Photomicrographs of histological sections stained for TUNEL (green) and DAPI (nuclei, blue) from mice transplanted with UM-PDX-HACC-5 tumors and that were treated with weekly 10 mg/kg BM-1197 or vehicle (n=10). B. Graph depicting the number of TUNEL-positive (apoptotic) cells per high power field (200x).