Biperiden and mepazine effectively inhibit MALT1 activity and tumor growth in pancreatic cancer

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MALT1 is a key mediator of NF-κB signaling and a main driver of B-cell lymphomas. Remarkably, MALT1 is expressed in the majority of pancreatic ductal adenocarcinomas (PDACs) as well, but absent from normal exocrine pancreatic tissue. Following, MALT1 shows off to be a specific target in cancer cells of PDAC without affecting regular pancreatic cells. Therefore, we studied the impact of pharmacological MALT1 inhibition in pancreatic cancer and showed promising effects on tumor progression. Mepazine (Mep), a phenothiazine derivative, is a known potent MALT1 inhibitor. Newly, we described that biperiden (Bip) is a potent MALT1 inhibitor with even less pharmacological side effects. Thus, Bip is a promising drug leading to reduced proliferation and increased apoptosis in PDAC cells in vitro and in vivo. By compromising MALT1 activity, nuclear translocation of c-Rel is prevented. c-Rel is critical for NF-κB-dependent inhibition of apoptosis. Hence, off-label use of Bip or Mep represents a promising new therapeutic approach to PDAC treatment. Regularly, the Anticholinergicum Bip is used to treat neurological side effects of Phenothiazines, like extrapyramidal symptoms.

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Abbreviations: ABC-DLBCL: activated B-cell subtype of diffuse large B-cell lymphoma; BCL10: B-cell lymphoma/leukemia 10; Bip: biperiden; CARMA3: caspase recruitment membrane-associated protein 3; DMSO: dimethylsulfoxide; EGFR: epidermal growth factor receptor; FBS: fetal bovine serum; HPDE: human pancreatic ductal epithelial cell line; MALT1: Mucosa-associated lymphoid tissue lymphoma translocation protein 1; Mep: mepazine; MTT: (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay; NF-κB: nuclear factor κB; P/S: penicillin/streptomycin; PDAC: pancreatic ductal adenocarcinoma; PMA: phorbol 12-myristate 13-acetate; qRT-PCR: quantitative real-time polymerase chain reaction; TMA: tissue microarray; TNM: tumor, lymph node, metastasis TNM Classification of Malignant Tumors

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What's new?
Pancreatic cancer is the fifth leading cause of cancer-related deaths worldwide, with a five-year survival rate of just 6%. Thus, new therapeutic approaches are urgently needed. Here, targeting of the protein MALT1 with either biperiden or mepazine inhibited the growth of pancreatic ductal adenocarcinoma (PDAC) cells and increased PDAC cell apoptosis in vitro and in vivo. Analyses showed MALT1 to be expressed in the majority of pancreatic cancer cells, while lacking in healthy tissue. The data identify MALT1 as a novel therapeutic target in PDAC and identify biperiden and mepazine as promising therapeutic agents for the disease.

Introduction
Treatment of pancreatic ductal adenocarcinoma (PDAC) remains a major challenge because chemotherapeutic therapy after surgical resection is not sufficient to prevent tumor growth. Consequently, alternative treatments are urgently needed. Nuclear Factor κB (NF-κB) activation plays a critical role in tumor progression and inflammation and suppresses the apoptotic potential of chemotherapeutics. NF-κB is a complex of five transcriptional factors: NF-κB1, NF-κB2, RelA, RelB and c-rel. After activation, c-rel is translocated to the nucleus, degrades NF-κB inhibitors and translates proinflammatory cytokines. Additionally, NF-κB interacts with signaling molecules of the mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1) caspase cascade like protein kinase A and the IkBα complex which favors c-rel translocation and leads to transcription of pro-oncogenic NF-κB genes and inhibits NF-κB-dependent apoptosis.

MALT1 contains a paracaspase domain. This domain catalyzes arginine-specific protein cleavage and is a key mediator of upstream NF-κB signaling in lymphocyte activation, survival, and differentiation and multiple signaling pathways in non-immune cells.

Recently, MALT1 was identified as a potential therapeutic target for the activated B-cell subtype of diffuse large B-cell lymphoma (ABC-DLBCL). Nagel et al. demonstrated that mepazine (Mep) inhibits MALT1 activity enabling efficient treatment of MALT1-dependent ABC-DLBCL in vitro and in vivo by allosteric inhibition.

Biperiden (Bip) is an antiparkinsonian drug of the anticholinergic type that has been in clinical use for decades. It binds to muscarinic receptors (M1) leading to repression of acetylcholine and enhances dopamine signaling in the central nervous system. Therefore, the Anticholinergicum Bip is used to treat neurological side effects like extrapyramidal symptoms of phenothiazines, which antagonize the dopaminergic/alpha adrenergic pathway.

After detection of MALT1 in PDAC, we aimed to understand the effect of MALT1 inhibition and its potential inhibitors. Moreover, we were seeking to reveal a new well-tolerated approach which only affects a cancer cell-specific target.

We hypothesize that enhanced MALT1 activity leads to increased NF-κB activity and ultimately promotes a protumorigenic benefit for PDAC cells. Subsequently, activity reduction leads to impairment of tumor growth. Moreover, we propose that this specific inhibition of MALT1 can be achieved by Mep and Bip. Therefore, Bip and Mep shall be examined as new potent therapeutic agents for PDAC treatment.

Materials and Methods
Pancreatic cancer patients and tissue microarrays
A tissue microarray (TMA) containing tissue samples of 213 primary PDACs (including histopathological data), as well as tissue samples of 129 corresponding lymph node metastases, 22 distant metastases and 24 recurrences, was utilized for tissue analysis, as previously described. The tissues were retrieved from the UKE tumor bank. Informed consent was obtained from all patients.

Nonmalignant pancreatic tissue samples
After approval of the Medical Ethical Committee, Hamburg, Germany, nonmalignant exocrine pancreatic tissue samples were obtained from 30 postmortem autopsies of healthy decedents by the Department of Legal Medicine of the University Medical Center Hamburg-Eppendorf. Further histological examination is described in Supporting Information Experimental Procedures.

Cell lines
Panc-1, Panc-2 and BxPC3 human PDAC cells were cultivated in Dulbecco's Modified Eagle's Medium (Sigma, St. Louis, MO) supplemented with 1% penicillin/streptomycin (P/S; Life Technologies/GIBCO, New York, NY, 15140-122) and 10% fetal bovine serum (FBS; Life Technologies/GIBCO 100500-064). The human pancreatic ductal epithelial cell line (HPDE) was cultivated in Defined Keratinocyte SFM (1x; Life Technologies/GIBCO 170050-42) supplemented with the same supplements. L3.6pl wild-type cells (L3.6pl wt) were cultivated in RPMI 1640 Medium (Life Technologies/GIBCO 72400-21) supplemented with 1% P/S and 10% FBS. L3.6pl-Res, a gemcitabine-resistant subclone of the L3.6pl cell line previously established in our laboratory, was cultivated in RPMI 1640 Medium supplemented with 1% P/S, 10% FBS and 2 μM gemcitabine (GEMZAR®, Lilly, Indianapolis, IN).

All cell lines were authenticated by Eurofins Medigenomix Forsenkis GmbH (Ebersberg, Germany) in 2018 and 2019. The following synonyms were used according to the authenticated cell lines: Panc-1 is synonym for PANC-1 (CVCL_0480), Panc-2 for MIA PaCa-2 (CVCL_0428), BxPC-3 for BxPC-3 (CVCL_0186), HPDE (CVCL_4376), L3.6pl wt cells refer to L3.6pl (CVCL_0384), while...
the L3.6pl Res line is not listed in the ExPASy Cellosaurus database.Jurkat cells are synonyms for Jurkat E6.1 (CVCL_0367).

Animals
All animal experiments were conducted according to the German Animal Welfare Act (BGBl. I p. 1206, 1313, paragraph 1, appendix Article 3 from July 28th, 2014, BGBl. I p. 1308) and approved by the local authority (Behörde für Soziales, Gesundheit, Familie, Verbraucherschutz; Amt für Gesundheit und Verbraucherschutz, Hamburg, Germany, project no. G 102/13). Forty PfpRag2−/−/Rag2−/− double-knockout mice were used for the xenograft model. This mouse model lacks NK cell function, as well as mature T and B lymphocytes. Generation of the mouse model is described in the Supporting Information Experimental Procedures.

Methods

Immunohistochemistry. TMA sections were immunostained with primary antibody specific for MALT1 (polyclonal; rabbit, Sigma cat. # HPA003865; at 1/50 dilution). Slides were deparaffinized and antigen retrieval performed for 5 min at 121°C. Bound antibody was visualized by a single experienced pathologist using the EnVision Kit (Dako, Glostrup, Denmark). Human spleen tissue was used as positive control. The detailed method and the scoring system are described in the Supporting Information Experimental Procedures.

Docking studies of MALT1. To identify a preferred orientation and to compare binding energies of the compounds within the putative binding pocket, we performed protein-ligand docking studies in silico (Figs. 2b–2d). X-ray structure of MALT1 in complex with Thioridazine (PDB code: 411R) refined to 2.7 Å was applied as template structure and the software package SYBYL-X 2.1 (Tripos International, St. Louis, MO) was used for all docking studies. Structure of MALT1 without ligand and solvent water was examined before calculating charges and minimizing the energy over 500 iterations up to a value of 843.2 kcal/mol. The MOL2-files of the ligands Bip hydrochloride (PubChem CID: 92151) and mepazine chloride (PubChem CID: 102907) were applied for following calculations and their minimized energies. Thioridazine was used as reference. The details are described in Supporting Information Experimental Procedures.

Expression and purification of human MALT1 (339–719). Expression of human MALT1 (339–719) was performed using the E. coli Rosetta (DE3) strain. The recombinant hMALT1 (339–719) was purified via Ni-NTA affinity chromatography. See Supporting Information Experimental Procedures for details.

hMALT1 (339–719) activity assay. The MALT1 activity assay was performed according to Nagel et al. and adapted for application of purified proteins.

Western blotting. Cells were lysed in RIPA buffer (Sigma) containing 1x complete protease inhibitor cocktail (Roche, Branchburg, NJ) and proteins separated by SDS-PAGE followed by immunoblotting as previously described. The used antibodies are listed in Supporting Information Experimental Procedures.

Quantitative real-time reverse transcription PCR. Total RNA was isolated using Trizol Reagent (Invitrogen, Waltham, MA) followed by RNA cleanup using RNaseasy Plus (Qiagen, Germantown, MD). cDNA was synthesized using Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Penzberg, Germany). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed on a Light Cycler 480 (Roche Applied Science) with SYBR Green qPCR Master Mix (Thermo Scientific, Waltham, MA) as recommended by the manufacturer. The primers and conditions are listed in the Supporting Information Experimental Procedures.

Immunocytochemistry. Panc-1 cells were grown on sterile coverslips (50–60% confluent) and fixed in 4% formaldehyde (10 min). Cells were permeabilized and incubated with appropriate primary and secondary antibodies prior to mounting. See Supporting Information Experimental Procedures for details. Images were acquired on a Zeiss Axiostar 200M with ApoTome for structured illumination.

MALT1 activity cell assay. Constitutive MALT1 activity was measured in the pancreatic cancer cells; Jurkat T cells were used as a positive control. After 24 hr of incubation with Mep or Bip, cellular MALT1 protease activity was measured using a modified version of the protocol of Nagel et al. (see Supporting Information Experimental Procedures for details).

Apoptosis assay. Apoptosis was measured by Cleaved Caspase-3 Sandwich ELISA (Cell Signaling, Danvers, MA) according to the manufacturer’s instructions.

Ki67 assay. Fixed cells on coverslips were incubated in blocking buffer (1% BSA/TBS-T). Followed by an incubation with anti-Ki67 antibody o.n. and secondary antibody for 2 hr. Coverslips were washed and mounted with mounting solution (F6057, Fluoroshield with DAPI, Sigma-Aldrich, St. Louis, MO).

Cell proliferation assays. The proliferation of pancreatic cancer cells stimulated with Bip or Mep was measured in a (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction (MTT) assay as described in the Supporting Information Experimental Procedures.

Lentiviral-mediated knockdown of MALT1. pLKO.1-puro vector encoding MALT1 and nontarget (scrambled, SCR) shRNA were purchased from Sigma-Aldrich (Germany). Generation of pseudotyped lentiviruses and transduction were performed as previously described. Cells transduced with MALT1 shRNA were selected by addition of puromycin (Sigma-Aldrich) to culture medium with a final concentration of 2 mM for at least 1 week. Knockdown quality was checked using western blot analysis.
**Cell fractionation.** For cell fractionation, Ne-PER® Nuclear and Cytoplasmic Extraction Reagents (Prod #78833, Thermo Scientific) were used according to the manufacturer’s instructions.

**Xenograft mouse model.** We generated a subcutaneous xenograft mouse model using Panc-1 human PDAC cells obtained from ATCC (American Type Culture Collection, Manassas, VA).

Cells were cultivated in RPMI-1640 supplemented with 10% fetal bovine serum, 1% L-glutamate and 1% P/S (10,000 IU/ml from ATCC (American Type Culture Collection, Manassas, VA)).

A total of 10⁶ viable Panc-1 cells suspended in 200 μl of RPMI-1640 medium were injected subcutaneously between the mouse scapulae with CO₂/O₂ anesthesia. Twelve days after injection of tumor cells, mice were randomized and treated daily with either 16 mg/kg Mep i.p. (n = 10) or 10 mg/kg Bip i.p. (n = 10). The control group (n = 5) was not treated at all.

Daily treatment was conducted under the same standardized conditions (after a 3-day cycle): Day 1, drug injection and determination of body weight; Day 2, drug injection and measurement of subcutaneous tumor growth with a caliper; and Day 3, drug injection and neurological scoring.

**Tumor engraftment and growth rate.** When tumors in the control group reached ~10 mm in diameter, began to ulcerate or break-off criteria were achieved, all mice in the Bip and Mep treatment groups, as well as the control group, were sacrificed and dissected.

**Neurologic scoring.** Mice were screened for neurologic side effects every third day with a test battery described by Fleming et al.29: challenging beam transversal,29–31 spontaneous activity in the cylinder29,32,33 and adhesive removal.34 All video recording was performed with an iPad 4 (Apple, Cupertino, CA) using the iOS-App SloPro (Sand Mountain Studios, Provo, UT). See Supporting Information Experimental Procedures for details.

**Statistical analysis.** All analyses were exploratory, so no adjustment for multiple testing was used. Values of p < 5% were considered significant. Missing values in the data were not imputed. Analyses were conducted using Stata SE 14.1 and higher.

**Statistical analysis of in vitro data.** Association between MALT1 expression and tumor type, grade and Classification of Malignant Tumors (TNM)-staging was analyzed using an extended Fisher’s exact test for r x c tables (Figs. 1b and 1c, Supporting Information Tables S1 and S2).

Constitutive MALT1 paracaspase activity was analyzed by mixed linear regression. The model contained a random effect (with independent covariance structure) to account for repeated measurements and fixed categorical effects for cell line, measurement time point and the interaction between the two. Robust variance estimates (sandwich estimator) were used for fixed effects. No adjustment was made for activity at baseline, allowing differences in the starting value to be retained. Shown are the marginal means over time (Supporting Information Fig. S2d) and after 10 min (Fig. 3d) with 95% confidence intervals (CIs) and corresponding p values.

The effects of treatment in the MTT proliferation assay (Figs. 4b and 4c, Supporting Information Fig. S2a–S2c), cleaved caspase-3 activity assay (log₁₀-transformed; Fig. 3f, Supporting Information Fig. S2c) and MALT1 paracaspase activity assay (log₁₀-transformed; Fig. 3g, Supporting Information Fig. S2d) were analyzed by mixed linear regression. The models contained a random effect to account for repeated measurements and fixed categorical effects for measurement time point, treatment and, if significant (based on LR test), the interaction between the two. Robust variance estimates (sandwich estimator) were used for fixed effects.

For the MTT proliferation assay, cell viability at baseline was defined as 100% for all treatments. For the cleaved caspase-3 activity assay and MALT1 paracaspase activity assay, treatments were applied to identical replicates of cells. Therefore, no adjustment for baseline values was applied in the analyses.

Results are presented as predicted marginal means (as mean over observations) according to treatment group and time point with corresponding 95% CI, assuming the random effect is zero. In case of significant interaction between treatment group and time, the LR p-value of the interaction is given. If the group effect (but not the interaction) is significant, the Wald p values of pairwise comparisons to dimethylsulfoxide (DMSO) are specified. If neither the interaction nor the group effect is significant, the p-value for group effect is given. For Panc-1 MALT1 paracaspase activity assay, estimated marginal means after 10 min are displayed together with corresponding p values.

**Statistical analysis of in vivo data.** The influence of treatment on tumor volume (log₁₀-transformed) in mice was analyzed by simple (unadjusted) linear regression.

Mouse experiments were analyzed by mixed linear regression (forelimb step count, hindlimb step count, log₁₀-transformed beam traversal time), mixed Poisson regression (beam step count, beam errors), mixed negative binomial regression (grooming time, rear count) and a Cox model with shared frailty (adhesive sticker removal). Repeated measurements were accounted for by random effects (linear, negative binomial and Poisson regression) or shared frailty (Cox regression). For each outcome, the starting model included fixed categorical effects for treatment group, measurement day, the interaction between the two, and sex. If the group-by-time interaction is significant, the LR p-value of interaction is given. Without interaction, the LR p-value of the group effect is given if it is not significant, otherwise, the Wald p values of pairwise comparisons are given. To simplify the model, the categorical effect of measurement day was tested against a linear, quadratic, cubic or quartic effect after variable selection.

For shared frailty Cox regression, the estimated relative hazard is given with 95% CI. Results of the other regression models are presented as predicted marginal means (mixed linear regression) or predicted marginal number of events (mixed Poisson and negative binomial regression) according to group and time point, with corresponding 95% CIs. Predicted margins were calculated as means over subjects, assuming that the random effect was zero.
Data availability
The data generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Results
MALT1 is frequently overexpressed in PDAC tissue specimens and absent in normal exocrine pancreatic tissue
MALT1 expression in PDAC tissue specimens in comparison to healthy pancreatic tissue was investigated using immunohistochemically staining of a tissue microarray (TMA) (Fig. 1a; Supporting Information Tables S1 and S2). The TMA contained tissue samples from 177 primary PDACs, 103 lymph node metastases, 21 distant metastases and 9 local recurrences (Figs. 1a and 1b; Supporting Information Table S1). MALT1 expression was detected in 75.1% of primary PDAC tumors (Fig. 1a; Supporting Information Table S1) and the majority of lymph node metastases (87.4%). MALT1 expression levels ranged from absent to high and differed significantly between primary tumor cells, lymph node metastases, distant metastases and local recurrences ($p < 0.001$; Fig. 1b; Supporting Information Table S1). We identified an association between MALT1 expression and grade of primary tumors; in particular, poorly differentiated tumors (G3) were less likely to express MALT1 ($p < 0.001$; Fig. 1c; Supporting Information Table 2).

Figure 1. In contrast to normal cells MALT1 is expressed in pancreatic cancer tissue specimens. (a) Percentage of MALT1 expression levels in primary pancreatic cancer tissue samples ($n = 177$). MALT1 was detected in 133/177 (75.1%) primary PDAC tumors and exhibited high, moderate and weak expression in 49/177 (27.7%), 40/177 (22.6%) and 44/177 (24.9%) samples, respectively. MALT1 expression was absent in 44/177 (24.9%) tumor samples. (b) Differential MALT1 expression in primary tumors, locoregional lymph node metastases, distant metastases and local recurrences ($p < 0.001$). Out of 103 lymph node metastases, high, moderate, weak and absent MALT1 protein expression was observed in 34/103 (33.0%), 35/103 (34.0%), 21/103 (20.4%) and 13/103 (12.6%) specimens, respectively. Of 21 distant metastases, high, moderate, weak and absent protein expression was observed in 1/21 (4.8%), 2/21 (9.5%), 6/21 (28.6%) and 12/21 (57.1%) samples, respectively. We detected a statistically significant difference between MALT1 expression of primary tumors, lymph node metastases, distant metastases and local recurrences ($p < 0.001$). (c) MALT1 expression is associated with tumor grade ($p < 0.001$). Poorly differentiated tumors (G3) were less likely to express MALT1 protein. TNM staging was not significantly associated with MALT1 expression. (d) Immunohistochemistry of MALT1 in normal human exocrine pancreatic tissue ($n = 30$, left), in corresponding hematoxylin-/eosin-stained normal exocrine pancreas (middle); and in spleen tissue, used as a positive control (right). None of the nonmalignant exocrine pancreatic tissue samples tested were positive for MALT1 protein expression.
pancreatic tissues, whereas splenic control tissue stained positively for MALT1 (Fig. 1).

Bip and Mep bind the allosteric-inhibitory MALT1 binding pocket in a similar manner and lead to decreased activity of human MALT1

MALT1 inhibitors exert selective toxic effects on MALT1-dependent leukemia cells by binding inside a hydrophobic pocket located opposite the caspase active site, which lies within the interface between the caspase domain and the Ig3 domain of MALT1. Hence, Mep is an allosteric inhibitor of MALT1 paracaspase. Bip and Mep share structural similarities (Fig. 2a). Both compounds share an N-methyl-piperidine nitrogen site accompanied by three carbon atoms. This essential feature increases the electrophilic nature of nitrogen to the oxygen atom (OE1) of glutamic acid E397 (Fig. 2a) leading to stabilized H-bond interaction between the pharmacological agent and MALT1 protein. Moreover, the bicyclic ring system and aromatic component of Bip form ideal van der Waals interactions within the binding pocket (Fig. 2c). Therefore, we propose that both Mep and Bip are capable of binding the putative allosteric-inhibitory pocket of MALT1.

To identify the preferred orientation and compare the binding energies of the compounds within this pocket, we performed in silico protein–ligand docking studies (Figs. 2b and 2c). The binding energies were calculated using the equation, 

$$E_{\text{binding}} = E_{\text{complex}} - (E_{\text{ligand}} + E_{\text{protein}})$$

(Fig. 2c). Bip (orientation 3) shows superior interaction to MALT1 providing ideal binding energy, highest number of hydrophobic interactions in comparison to Mep and control substance Thioridazine (PDB code: 4I1R; Figs. 2c and 2d).

For proof of concept, on-target effect was demonstrated by performing a fluorogenic MALT1 activity assay, following recombinant expression and purification of the bioactive dimeric human MALT1 (339–719) protein fragment (Supporting Information).
Figs. S1a–S1d). The recombinant protein is present in the bioactive dimeric structure. MALT1 activity was measured upon incubation with generally used concentrations of Mep, Bip, reference substance Thioridazine in its known ED50 and control substance Z-VRPR-fmk. Z-VRPR-fmk is a synthetic tetramer, known as a selective and covalent irreversible-binding MALT1 inhibitor, while Phenothiazines are reversible and allosteric inhibitors.35 All substances significantly decreased MALT1 activity of recombinant human MALT1 (339–719). Compared to the positive control Z-VRPR-fmk (set as 100%), the MALT1 activity inhibited by Bip was reduced by 33% and 42% for Mep, respectively. For reference substance, Thioridazine, 61% inhibition of activity could be shown (Fig. 2d).

MALT1 expression and activity is elevated in human pancreatic cancer cells. Moreover, its pharmacological inhibition by Mep or Bip elicit toxic effects in PDAC cells. MALT1 expression was assessed in human PDAC cell lines by cytochemistry, western blot and qRT-PCR. The following cell lines were used: Panc-1, Panc-2, BxPC3 and L3.6pl wt, L3.6pl Res, and L3.6pl-Res (n = 10) cells. Differences significantly changed over time (p<0.001), but remained to be significant (Supporting Information Fig. S2b). All values were adjusted by baseline. (e) Loss of cell adherence and shift of cell morphology in L3.6pl-Res and Panc-1 after treatment with 25 μM Mep or 29.6 μg/ml Bip for 24 hr. (f) Cleaved Caspase-3 Sandwich ELISA to determine the effect of 29.6 μg/ml Bip (n = 3) or 25 μM Mep (n = 3) on apoptosis in L3.6pl-Res and Panc-1 cells. Estimated means with 95% confidence intervals are shown. Mep and Bip both resulted in significantly reduced MALT1 activity (p < 0.001). Differences significantly changed over time (p<0.001), but remained to be significant (Supporting Information Fig. S2b).

Figure 3. MALT1 is expressed and highly active in human pancreatic cancer cells; MALT1 inhibition by biperiden (Bip) or mepazine (Mep) elicit toxic effects. (a) Western blot analysis of MALT1 protein expression. HSC70 served as a loading control. The respective cell lines used are indicated (b) mRNA expression analysis by qRT-PCR. Normalized with GAPDH (n = 3). (c) Immunocytochemistry of Panc-1 cells. MALT1 is located in the cytosol and nucleus. Nuclei and actin were counterstained using DAPI and phalloidin, respectively. (d) Estimated mean MALT1 activity levels after 10 min (with 95% confidence interval and p-value to Control) in Panc-1 (n = 10), Bip (n = 10), BxPC3 (n = 6), L3.6pl wt (n = 10) and L3.6pl-Res (n = 10) cells. Jurkat T-cells in steady state (Control; n = 14) and after PMA/ionomycin-stimulation (Jurkat; n = 14) served as negative and positive control. All PDAC cells exhibited significantly higher constitutive MALT1 paracaspase activity than nonactivated Jurkat cells. Differences significantly changed over time (p<0.001), but remained to be significant (Supporting Information Fig. S2b). All values were adjusted by baseline. (e) Loss of cell adherence and shift of cell morphology in L3.6pl-Res and Panc-1 after treatment with 25 μM Mep or 29.6 μg/ml Bip for 24 hr. (f) Cleaved Caspase-3 Sandwich ELISA to determine the effect of 29.6 μg/ml Bip (n = 3) or 25 μM Mep (n = 3) on apoptosis in L3.6pl-Res and Panc-1 cells. Estimated means with 95% confidence intervals are shown. Mep and Bip both resulted in significantly reduced MALT1 activity (p < 0.001). Differences significantly changed over time (p<0.001), but remained to be significant (Supporting Information Fig. S2b).
along with a gemcitabine-resistant subclone of L3.6pl wt that was previously established at our laboratory (L3.6pl-Res).\textsuperscript{25,26} All cell lines expressed MALT1 at mRNA and protein level (Figs. 3a and 3b). MALT1 protein was predominantly localized in the cytosol and perinuclear regions (Fig. 3c).

Furthermore, fluorogenic MALT1 paracaspase assay was performed to value this expression in human PDAC cell lines:\textsuperscript{27} constitutive high MALT1 activity was detected in all PDAC cell lines. It showed to be significantly higher in PDAC cells than in unstimulated Jurkat T-cells or phorbol 12-myristate 13-acetate (PMA)/Ionomycin stimulated Jurkat T-cells which were used as control\textsuperscript{27} (Fig. 3d, Supporting Information Fig. S2e).

As previously reported, suppression of MALT1 activity elicits toxic effects in leukemia cells.\textsuperscript{15} To determine whether a similar effect arises in PDAC cells, we treated all PDAC cell lines with 25 μM Mep or 29.6 μg/ml Bip. The dosage of both substances was adapted to the known bioavailability and therapeutic range. Surprisingly, after 24 hr incubation, most of the cells exhibited apoptotic features and were detached from formerly adherent cell groups (Fig. 3e). After reseeding in fresh medium, the cells could neither readhere nor proliferate strengthening the idea of induced apoptosis. Following, we performed caspase 3-dependent apoptosis assays to quantify the effects. However, in this assay, Mep enhances apoptosis, but only Bip treatment induced significant apoptosis rates relatively to untreated cells (Fig. 3f; Supporting Information Fig. S2d).

We hypothesize that the observed effect of Mep and Bip is mediated by inhibition of MALT1 activity. Following, we measured MALT1 activity changes after pharmacological treatment: cellular MALT1 activity was assessed upon 24 hr incubation with either Mep or Bip and untreated cells (DMSO) as control. In Panc-1 and Panc-2 cells, difference in MALT1 activity between treatments significantly changed with time (ρ\textsuperscript{group-by-time} ≤ 0.001 and ρ = 0.003, respectively; Supporting Information Fig. S2e). Representative 10 min measurements of MALT1 activity after 24 hr of pharmacological inhibition were shown. Comprising, we showed that in Panc-1 (Fig. 3g) and Panc-2 cells treated with Mep or Bip, MALT1 activity was significantly lower than in untreated (DMSO) cells (ρ ≤ 0.001). For L3.6pl-Res, L3.6pl wt and BxPC3 activity reduction could be observed but without significant difference between treatment groups (Supporting Information Fig. S2e).

**Mep and Bip inhibit proliferation of MALT1-positive PDAC cells via MALT1 dependent c-Rel translocation**

To explore the effects of Mep and Bip on PDAC cells in greater detail, we assessed the impact on cell proliferation rates. First, we assessed proliferation rates by Ki-67 staining after 24 hr of treatment in comparison to DMSO control. Staining revealed diminished proliferation after pharmacological treatment (Fig. 4a). Following, MTT proliferation assays were performed with different doses of each drug to monitor metabolic activity and proliferation rates of viable cells over 5 days (Figs. 4b and 4c; Supporting Information Fig. S2a). Overall, differences between DMSO and treatment (Mep and Bip, respectively) tended to increase with time and dose level (Supporting Information Fig. S2a). We could detect different sensitivities of the cell lines to the treatment: Panc-1 and Panc-2 cells exhibited early significant reduction in proliferation rate at even lower dose levels. This matches the significant reduction of MALT1 activity after 24 hr incubation in these cell lines. Other cell lines showed significant reduction in proliferation rate with high-dose level incubation (29.6 μg/ml Bip; 25 μM Mep) after 72 hr at the latest.

All in all, after 72 hr of incubation cell proliferation was nearly completely impeded for highest doses of Bip and Mep and tumor cell growth stopped (Figs. 4b and 4c; Supporting Information Fig. S2a). Remarkably, cells of a human pancreatic ductal epithelial cell line (HPDE) which were treated in parallel with the cancer cells exhibited only minor reduction in cell proliferation by highest dose levels (29.6 μg/ml Bip; 25 μM Mep) after 72 hr. These results let us conclude that healthy pancreatic tissue should not be that affected by the drug as cancer cells did in our experiments (Supporting Information Fig. S2d).

To assure that the effects described above were based on MALT1 inhibition, MALT1 knockdown was established using short hairpin RNA (shRNA)-expressing puromycin-resistant lentiviruses to reduce accessible Malt1mRNA levels. As control, nontarget shRNA (SCR) was used in parallel. Subsequently, proliferation rates of MALT1 knockdown and SCR cells were measured. Here, inhibition with either Mep or Bip revealed only minor changes in proliferation (Figs. 4b and 4c, Supporting Information Fig. S2c). Without treatment, cell viability of knockdown cells was already reduced and proliferation rates showed to be remote suggesting that MALT1 plays a pivotal role in cell viability and proliferation. Knockdown quality was checked using western blot (Fig. 4d).

However, Mep or Bip treatment did not influence proliferation rates or viability of knockdown cells at a significant level in contrast to MALT1 wt cells. Leading to conclusion that as well Mep as Bip act through the interaction with MALT1 and do not lead to the described effects by another signaling.

Previous studies showed that MALT1 deactivation reduces nuclear c-Rel translocation in lymphocytes,\textsuperscript{5,35} which leads to decreased transcription of NF-κB responsive genes (Fig. 4e). Consequently, MALT1 inhibition should lead to concordant reduction of NF-κB activity in PDAC cells as well. Therefore, we assessed nuclear c-Rel translocation in PDAC cells before and after pharmacological MALT1 inhibition by cell compartment fractioning. Indeed, we could show decreased nuclear c-Rel translocation in Panc-1 cells after Mep and Bip treatment (Fig. 4d). Subsequently, Mep and Bip lead to reduced NF-κB activity in PDAC cells resulting in less inflammatory cytokines and antiapoptotic signals which are regularly enhanced by c-Rel. Nevertheless, shortly after pharmacological blockade, rest-activity of c-Rel can be detected in the cancer cells and it needs still to be elucidated if c-Rel can be deleted by either Bip or Mep entirely.
Mep and Bip inhibit PDAC tumor growth in vivo

For proof-of-principle, Panc-1 cells were injected subcutaneously into immune-insufficient mice. After tumor implementation, mice were randomized by tumor size into two treatment groups (Mep/Bip) and one control group. The Mep-treated group received 16 mg/kg Mep i.p. daily and the Bip-treated group received 10 mg/kg Bip i.p. daily, comparable to the high dose levels of in vitro experiments. Control mice received no treatment at all. Finally, tumor volumes of Bip and Mep groups revealed to be smaller than control group after 3 weeks of treatment. In the Mep-treated group, average tumor size was reduced by 95% relative to the control group (factor 0.05; 95% CI [0, 0.54]; p = 0.017).

During the treatment period, three mice of the Mep treatment group died due to intraabdominal hemorrhages. Neurological side effects were monitored by several mice assessments: spontaneous

Figure 4. Mepazine (Mep) and biperiden (Bip) inhibit MALT1 paracaspase activity significantly and lead to significant reduction of PDAC cell proliferation by reducing nuclear c-Rel translocation. (a) Immunocytochemical staining detecting Ki67 (FITC) and nuclear staining (DAPI) of Panc-1 cells after treatment with DMSO (vehicle), 25 μM Mep and 29.6 μg/ml Bip. (b, c) MTT proliferation assay of Panc-1 cells and MALT1 knockdown cells (Panc-1-MALT1KD) using increasing concentrations of Bip (c) and Mep (d). Vehicle (DMSO)-treated cells served as a control (n = 6 per group). Estimated means with 95% confidence intervals are shown. In wild-type cells, proliferation changed significantly over time (p < 0.001, p = p_group-by-time). In contrast, MALT1 knockdown cells were inhibited in growth per se and did not respond to pharmacological treatment (p > 0.05, p = p_group). (d) Western blot analysis of MALT1 knockdown. Comparison of MALT1 protein expression in Panc-1 cells transfected with MALT1KD or SCR plasmids and Panc-1 wild-type cells. HSC70 served as loading control. (e) Potential role of MALT1 in pancreatic cancer cells. MALT1 positively promotes NF-κB activity by nuclear c-Rel translocation leading to increased proliferation and apoptosis resistance in cancer cells. Mep and Bip impair MALT1 activity. Consequently, nuclear translocation of c-Rel is diminished, leading to inhibition of proliferation and repression of antiapoptotic gene transcription. (f) Western blot analysis showed reduced nuclear translocation of c-Rel after Mep or Bip treatment. Lamin B served as loading control. The ratio of c-rel/Lamin B in the nucleus showed to be diminished by at least 0.5 after pharmacological treatment.
activity, motor function and coordination, fine motor skills and somatosensory coordination of the mice were assessed using a standardized test set every third day. This set included the following tests: balance beam traversal count, adhesive removal and spontaneous activity in the cylinder.29,31,34,38

Bip and Mep had tolerable side effects on general activity, motor coordination and balance. A significant group effect was shown for beam traversal time and step count in the Bip and Mep group vs. the control group (both \( p < 0.001 \)). Additionally, significant differences between the Mep and control group were observed for spontaneous activity (\( p = 0.008 \)). Sticker removal test revealed that Bip and Mep treatment had no adverse effects on fine motor coordination (Figs. 5b–5d). Nevertheless, taking all these tests into account, treated mice showed only minor neurological instability compared to the control group without greater adverse effects on their condition.

**Discussion**

With our study, we aimed to make oncological treatment of pancreatic cancer more precise, seeking for a cancer-specific target to attack tumor cells without doing great harm to the rest of the human body. For the first time, we report that MALT1 is expressed in a large proportion of human pancreatic adenocarcinomas whereas expression in normal pancreatic tissue is absent. Moreover, constitutively high activity of MALT1 paracaspase was detected in PDAC cancer cells. The...
in vitro and in vivo data underscore the crucial role of MALT1 in pancreatic cancer, showing decreased proliferation and increased apoptosis of cancer cells by pharmacological inhibition of MALT1 paracaspase activity. However, the treatment efficacy varied between cell lines and pharmacological agents.

The drug compounds Bip and Mep most likely engage in structurally similar interactions with an allosteric hydrophobic binding site on MALT1. The binding results in inhibition of MALT1 paracaspase activity and reduction of tumor growth in vitro and in vivo. Additionally, nuclear c-Rel translocation into the nucleus was diminished and consequently, NF-κB activity was decreased upon Bip and Mep treatment in PDAC cells.

Previously, MALT1 has been associated with lymphoma and leukemia. However, earlier work implied already a role of MALT1 in pancreatic cancer; Dai et al. reported a twofold increase of MALT1 gene transcription in pancreatic cancer cells cocultured with mouse dorsal root ganglia. In addition, caspase recruitment membrane-associated protein 3 (CARMA3) and B-cell lymphoma/leukemia 10 (BCL10), essential components of the CARMA3/BCL10/MALT1 complex that initiates NF-κB activation, are expressed in PDAC cells. NF-κB is known to be constitutively activated in human pancreatic cancer cells.

We showed that MALT1 inhibition by Bip and Mep led to inhibition of nuclear c-Rel translocation in vitro. c-Rel gene expression is associated with antiapoptotic signaling in pancreatic cancer and is a key player in B-cell proliferation and survival. The effect of diminished nuclear c-Rel translocation upon MALT1 inhibition has been reported in lymphoma cells as well. Therefore, we hypothesized that inhibition of MALT1 paracaspase activity by Mep and Bip leads to attenuation of proliferation and promotion of apoptosis in PDAC cells via inhibition of nuclear c-Rel translocation.

Concordantly, all PDAC cells exhibited reduced c-Rel translocation and NF-κB activity, as well as reduced proliferation rates upon pharmacological treatment with high doses of MALT1 inhibitors.

As a proof-of-principle, MALT1 knockdown was performed. The knockdown cells featured reduced proliferation rates in comparison to Panc-1 wt cells and showed hardly any reaction to pharmacological treatment with neither Mep nor Bip. These results underline the potential of MALT1 as therapy target in PDAC and its crucial role in the cancer cell metabolism. Moreover, it bears a close resemblance to findings of Pan et al. who showed the evidence of MALT1 driven tumor progression in epidermal growth factor receptor (EGFR)-associated solid tumors.

Our in vivo data revealed a great difference between tumor mass of treated and nontreated mice. Both treatment groups of mice were injected with dosages of Bip or Mep in clinically approved range and showed a reduction of tumor mass of 95% by Mep (p = 0.017) and 83% by Bip (p = 0.112). All in all, tumor growth in vivo was significantly attenuated by Mep treatment. However, the reduction in tumor size upon Bip treatment was not statistically significant, even though the tumors were smaller on average. We attribute this lack of statistical significance to the small number of mice (n = 9) and large variability in the measurement of subcutaneous tumor. Moreover, only palpable tumors were included, after the effect on smaller tumor masses is not detectable by the given model.

Our in vitro data revealed that Mep did not affect apoptosis of PDAC cells, whereas high dose of Bip did induce apoptosis. This effect is consistent with in silico analysis, which identified Bip as the preferred binding partner for the allosteric MALT1 binding site due to its structural binding abilities and binding energy. Furthermore, the proapoptotic effect might be triggered by an additional not yet discovered mechanism. Since we detected lots of apoptotic cells after cell culture incubation and reduced viability in the proliferation assays but no consistent increase of caspase 3 activation, it is likely that apoptosis is induced via different signal pathways. Eitelhuber et al. proposed a RelB driven apoptosis by MALT1 inhibition in lymphoma cells instead of the caspase cascade. Moreover, Bip is capable of antagonizing muscarinic receptors, which are frequently expressed in cancer cells. Activation of these receptors triggers cell proliferation and cancer progression, whereas receptor blocking promotes upregulation of proapoptotic factors as Bax.

Currently, pancreatic cancer treatment outcomes remain poor, with an average 5-year survivorship of approximately 6%. Consequently, novel therapeutic approaches are urgently needed. Mep was withdrawn from the market in the early 1960s due to insufficient antipsychotic efficiency. However, three mice of the Mep treatment group died of intraperitoneal bleeding. This could be explained by an adverse effect of Mep causing neutropenia and agranulocytosis, suggesting a myelo-compressing effect of the substance. Holt et al. also observed that phenothiazine-treated patients showed significant decreases in platelet counts (Holt 1984). This thrombocytopenia might have resulted in a cumulative favoring of bleeding complications with lethal outcome, especially in an intraperitoneal drug application. Further experiments are needed to verify if there is a higher risk of bleeding complications, especially in regard to postoperative usage in patients. Nevertheless, Mep and especially Bip have less and also less severe adverse effects than commonly used chemotherapy regimes. This makes Bip to a safe and well-tolerated drug in the field of anticaner therapy. While common adverse effects of chemotherapeutic drugs lead to symptoms of immune system deficiency with high infection susceptibility and impaired cell regeneration.

In contrast, Bip, an anticholinergic drug, which is in clinical use for more than 60 years, is even in high dosages a well-tolerated drug with only mild adverse effects, especially when compared to conventional chemotherapeutics. In vitro assays revealed significant reduction of tumor growth and significant in decrease of apoptosis rates. In vivo, treatment with either Mep or Bip led to reduced tumor mass and only minor motor side effects that were acceptable in light of the severity of the illness and the common adverse effects of chemotherapy. The sensitivity of the chosen neurological tests has been proven in numerous studies.
Therefore, a therapeutic approach using Bip to inhibit MALT1 activity represents a new promising and most likely safe, therapeutic option for pancreatic cancer treatment in humans. MALT1 represents a cancer-specific target as it is only expressed in pancreatic cancer tissue but absent from healthy exocrine pancreatic tissue. Potential effects of MALT1 inhibition using an immunocompetent model and its role for chemo sensitivity still remains open and should be subject of future studies.

Nevertheless, by reporting our data, we can conclude that MALT1 represents a promising new target for pancreatic cancer therapy and can be inhibited by either Mep or Bip.

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