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Article

Pharmacologic Inhibition of MALT1 Protease by Phenothiazines as a Therapeutic Approach for the Treatment of Aggressive ABC-DLBCL

Daniel Nagel,[1] Stefani Spranger,[3] Michelle Vincendeau,[1] Michael Grau,[4] Silke Raffegerst,[3] Bernhard Kloo,[1] Daniela Hlahla,[1] Martin Neuenschwander,[5] Jens Peter von Kries,[5] Kamyar Hadian,[2] Bernd Dörken,[6] Peter Lenz,[4] Georg Lenz,[6] Dolores J. Schendel,[3] and Daniel Krappmann[1,*]

[1]Research Unit Cellular Signal Integration
[2]Assay Development and Screening Platform
Helmholtz Zentrum München–German Research Center for Environmental Health, Institute of Molecular Toxicology and Pharmacology, Ingolstädter Landstrasse. 1, 85764 Neuherberg, Germany
[3]Helmholtz Zentrum München–German Research Center for Environmental Health, Institute of Molecular Immunology and Immune Monitoring Platform, Marchioninistrasse 25, 81377 Munich, Germany
[4]Department of Physics, Philipps-University Marburg, Renthof 5, 35032 Marburg, Germany
[5]Leibniz-Institut für Molekulare Pharmakologie, Robert-Roessle-Strasse 10, 13125 Berlin, Germany
[6]Department of Hematology, Oncology and Tumorimmunology, Molecular Cancer Research Center, Charité–University Medicine Berlin, Augustenburger Platz 1, Forum 4, 13353 Berlin, Germany
*Correspondence: daniel.krappmann@helmholtz-muenchen.de
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SUMMARY

Proteolytic activity of the mucosa-associated lymphoid tissue lymphoma translocation protein-1 (MALT1) paracaspase is required for survival of the activated B cell subtype of diffuse large B cell lymphoma (ABC-DLBCL). We have identified distinct derivatives of medicinal active phenothiazines, namely mepazine, thioridazine, and promazine, as small molecule inhibitors of the MALT1 protease. These phenothiazines selectively inhibit cleavage activity of recombinant and cellular MALT1 by a noncompetitive mechanism. Consequently, the compounds inhibit anti-apoptotic NF-κB signaling and elicit toxic effects selectively on MALT1-dependent ABC-DLBCL cells in vitro and in vivo. Our data provide a conceptual proof for a clinical application of distinct phenothiazines in the treatment of ABC-DLBCL.

INTRODUCTION

Upon antigenic stimulation, mucosa-associated lymphoid tissue lymphoma translocation protein-1 (MALT1) is a key mediator of upstream NF-κB signaling to control lymphocyte activation, survival, and differentiation (Hailfinger et al., 2009a; Thome, 2008). Together with CARMA1 (also known as CARD11) and BCL10, MALT1 assembles the CBM complex that bridges proximal antigen receptor signaling events to the IκB kinase (IKK) complex, the gatekeeper of the canonical NF-κB pathway (Scheidereit, 2006). Upon T cell antigen receptor (TCR)/CD28 costimulation, MALT1 acts as a protein scaffold that recruits other critical signaling molecules like TRAF6, caspase 8, and A20 to the CBM complex (Thome, 2008). Furthermore, covalent ubiquitin modifications in MALT1 catalyzed by the E3 ligase TRAF6 facilitate the association of two downstream protein kinase complexes, TAB2-TAK1 and NEMO-IKKα/β, which ultimately leads to IKK activation (Oeckinghaus et al., 2007).

In addition to its scaffolding function, MALT1 contains a paracaspase domain that displays high homology to caspases from mammals and metacaspases from plants and fungi (Uren et al., 2000). Like metacaspases, MALT1 cleaves substrates

Significance

Cleavage activity of the mucosa-associated lymphoid tissue lymphoma translocation protein-1 (MALT1) paracaspase contributes to the oncogenic potential of MALT1 in MALT lymphoma and ABC-DLBCL, the latter representing one of the most aggressive lymphoma entities. We identified small molecular compounds belonging to the class of phenothiazines as potent and selective inhibitors of the MALT1 protease. MALT1 inhibition by phenothiazines selectively kills ABC-DLBCL in vitro and in vivo, revealing their potency in achieving target-directed treatment of MALT1-dependent lymphoma. Phenothiazines have been widely used as antipsychotic drugs even in long-term treatment of schizophrenia and psychosis. Thus, well-defined toxicity, pharmacokinetics, and pharmacodynamics can facilitate clinical trials for an off-label use of these compounds in ABC-DLBCL therapy.

after arginine residues, indicating that the enzymatic cleavage activity is quite distinct from caspases, which in general require an aspartate at the P1 position (Vercammen et al., 2004). MALT1 proteolytic activity is induced upon TCR/CD28 costimulation, which promotes cleavage of the substrates BCL10, A20, CYLD, and RelB (Coornaert et al., 2008; Hailfinger et al., 2011; Rebeaud et al., 2008; Staal et al., 2011). Inhibition of MALT1 protease activity by the antagonistic tetrapeptide Z-VRPR-FMK, which was originally designed as an inhibitor of metacaspases in plants, impairs optimal NF-κB activation and interleukin-2 (IL-2) production in T cells (Düwel et al., 2009; Rebeaud et al., 2008). Similarly, mutation of the catalytic cysteine 464 renders MALT1 proteolytically inactive and also impairs IL-2 production after complementation of MALT1-deficient T cells (Düwel et al., 2009). These results indicate that small molecule inhibitors that selectively target MALT1 protease activity may be promising candidates for clinical use in T cell-dependent immune diseases, such as allergic inflammation or autoimmunity.

A tumor-promoting role of MALT1 has been found in a subset of diffuse large B cell lymphoma (DLBCL) and MALT lymphoma (Ngo et al., 2006). By gene expression profiling, DLBCL can be classified into distinct entities and the most abundant subtypes are the ABC-DLBCL and the germinal center B cell-like (GCB-) DLBCL (Alizadeh et al., 2000; Rosenwald and Staudt, 2003; Rosenwald et al., 2002; Savage et al., 2003; Wright et al., 2003). Based on the gene expression signature, the ABC-DLBCL subtype originates from B lymphocytes stimulated through their B cell antigen receptor (BCR). With a 5-year survival rate of 35%, patients with ABC-DLBCL have the worst prognosis, reflecting the aggressive clinical behavior of ABC-DLBCL cells (Lenz et al., 2008b). The hallmark of ABC-DLBCL, but not GCB-DLBCL, cells is the constitutive activation of the NF-κB signaling pathway (Alizadeh et al., 2000; Davis et al., 2001). Congruent with this, a recent genetic study of the DLBCL revealed the preferential association of gene alterations in the NF-κB and BCL6-BLIMP1 axis in ABC-DLBCL and in BCL2 and MYC in GCB-DLBCL, suggesting that anti-apoptotic NF-κB signaling is indeed critical for ABC-DLBCL survival (Pasqualucci et al., 2011). While some ABC-DLBCL patients carry oncogenic CARMA1 mutations (Lenz et al., 2008a), the majority of ABC-DLBCL is characterized by chronic active BCR signaling and in ~20% of the cases activating mutations in the BCR proximal regulator CD79A and CD79B are found (Davis et al., 2010). Consistent with a requirement for BCR signaling, an RNA interference screen identified CARMA1, BCL10, or MALT1 as critical regulators of NF-κB activation, and survival and growth of ABC-DLBCL (Ngo et al., 2006). Furthermore, inhibition of MALT1 proteolytic activity by Z-VRPR-FMK inhibits NF-κB-dependent gene expression and exerts toxic effects specifically in ABC-DLBCL cells (Ferch et al., 2009; Hailfinger et al., 2009b). MALT1 paracaspase activity also contributes to the pathogenesis of MALT lymphoma that is characterized by the translocation t(11;18)(q21;q21), which creates a fusion between the C terminus of MALT1, including the paracaspase domain, and the N terminus of IAP2 (API2-MALT1) (Isaacson and Du, 2004). The paracaspase domain of API2-MALT1 fusion protein catalyzes the cleavage of NIK and thereby enhances noncanonical NF-κB activation, which confers apoptosis resistance (Rosebeck et al., 2011). Thus, specific small molecule inhibitors against the MALT1 paracaspase could be beneficial for the treatment of lymphoma associated with deregulated MALT1 activity, such as ABC-DLBCL and MALT lymphoma.

The peptide Z-VRPR-FMK provided an initial hint that MALT1 inhibition is a feasible strategy for interfering with survival of ABC-DLBCL cells in vitro. However, due to poor pharmacologic properties, Z-VRPR-FMK needs to be administered in very high concentrations to exert effects on cells and antagonistic peptides in general are not adequate for clinical applications. The goal of this study was to identify small molecule inhibitors for pharmacologic inhibition of the MALT1 protease and to determine the effects of these MALT1 inhibitors on ABC-DLBCL proliferation and survival in vitro and in vivo.

RESULTS

Identification of Phenothiazine Derivatives as Selective MALT1 Protease Inhibitors

To identify small molecular weight compounds that could inhibit MALT1 protease activity, we purified recombinant GSTMALT1 from *Escherichia coli* to establish an in vitro protease cleavage assay suitable for high throughput screening (HTS). GSTMALT1 was incubated for 1 hr at 30°C in the presence of 50 μM of the tetrapeptide substrate Ac-LRSR-AMC, which is derived from the MALT1 cleavage site in the C terminus of BCL10 (Rebeaud et al., 2008). Proteolytic activity was determined by measuring the increase of fluorescence that is emitted after cleavage and the accompanying release of the fluorophore AMC (Figures 1A and 1B). MALT1-catalyzed cleavage of Ac-LRSR-AMC is evident from a robust increase in fluorescence intensity over time. As expected, mutation of the conserved cysteine (C453A) in the paracaspase domain of MALT1 (Isoform B) completely abolished MALT1 catalytic activity (Figure 1B). As expected, the tetrapeptide Z-VRPR-FMK blocked MALT1 cleavage activity at low nanomolar concentrations (Figure 1B; Figure S1A available online). In contrast, the potent caspase inhibitory peptide Ac-DEVD-CHO effectively blocked caspase 8 activity at picomolar concentrations, but only marginally reduced MALT1 activity when used at concentrations up to 200 μM (Figures S1B–S1D).

We screened approx. 18,000 compounds of the ChemBioNet collection (Lisurek et al., 2010) using the assay format depicted in Table S1. The primary screen was conducted by measuring the increase in AMC fluorescence in a 384 half-well format over an assay time of 20 min in the presence of 10 μM of each compound (see Supplemental Experimental Procedures). Dose-response assays were performed for secondary hit validation and selectivity on MALT1 was determined by comparing the influence on caspase 8 activity. These analyses yielded 15 hits, corresponding to ~0.08% of the primary screen. When examining the structure of the 15 hits, we noticed that three of the most efficient and selective compounds (Figure S1E, compounds A, B, and C) are derivatives of the tricyclic phenothiazine that contains two outer benzene rings linked by nitrogen and sulfur atoms in the inner ring. Also the heterocyclic core found in compound D displays high structural similarities to phenothiazine, whereby the nitrogen is replaced by carbon. These initial results suggested that certain phenothiazine derivatives (PD) may act as MALT1 inhibitors. Moreover, at 50 μM, all four compounds were reducing MALT1 protease activity to less than 10%, but

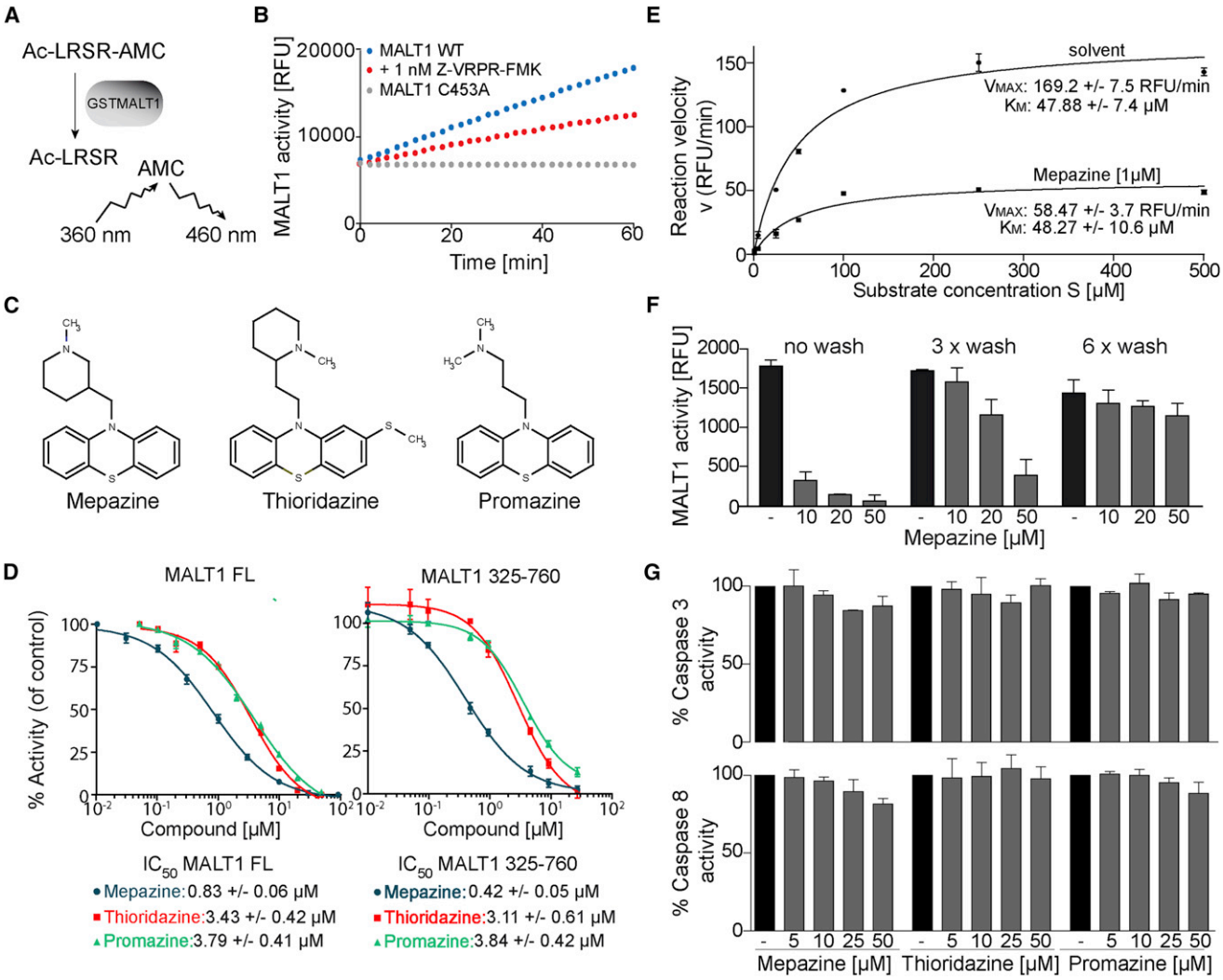

Figure 1. Selective MALT1 Inhibition by Mepazine, Thioridazine, and Promazine

(A) A schematic representation of the MALT1 protease assay for HTS. Cleavage of the fluorophore AMC by GSTMALT1 from the fluorogenic substrate Ac-LRSR-AMC results in an increase of fluorescence.

(B) Purified recombinant GSTMALT1 from bacterial expression was incubated for 1 hr at 30°C with 50 µM of Ac-LRSR-AMC and the proteolytic activity was determined by measuring the increase of AMC fluorescence. Graph shows a representative of at least five independent experiments.

(C) Chemical structures of the three inhibitory compounds.

(D) Dose-response curves and IC_{50} values for mepazine, thioridazine, and promazine.

(E) Michaelis-Menten kinetics was determined by increasing the concentration of Ac-LRSR-AMC substrate in the absence or presence of 1 µM mepazine.

(F) GSTMALT1 coupled to glutathione sepharose beads was treated with mepazine (10, 20, or 50 µM) for 30 min. MALT1 activity was assayed after washing the beads for 0, 3, or 6 times before cleavage reaction was started (mean ± SD; n = 3).

(G) Cleavage of human recombinant caspase 3 and caspase 8 against Ac-DEVD-AMC as substrate (mean ± SD; n = 3).

See also Figure S1 and Table S1.

caspase 8 activity was hardly affected even at the highest inhibitor concentration, indicating that the four PD display a high selectively for MALT1 (Figure S1F). We also tested the core phenothiazine scaffold and found that it inhibited MALT1 activity in a dose-dependent manner (Figure S1G). Notably, our initial results implied that only the modifications of compound A seemed to significantly improve the inhibitory potential of the phenothiazine backbone toward MALT1, suggesting that distinct phenothiazines could be promising candidates as selective MALT1 inhibitors.

Mepazine, Thioridazine, and Promazine Act as Potent and Selective MALT1 Paracaspase Inhibitors

We obtained 26 commercially available PD, to test their inhibitory potential (Figure S1H). Whereas most compounds (12–26) had no or only very weak inhibitory potential (IC_{50} > 20 µM), eight compounds (4–11) inhibited MALT1 activity with an IC_{50} roughly between 5 and 20 µM. Only three PD had an IC_{50} below 5 µM. Thus, only a small subset of PD was capable of efficiently inhibiting MALT1. The three most potent compounds represent promazine, thioridazine, and mepazine, the

latter initially identified in the screening (Figure 1C). To define the inhibitory potential, we determined exact IC_{50} values for each compound on recombinant full length (FL) GSTMALT1 and an enzymatically active MALT1 fragment encompassing amino acids 325 to 760 that contained the paracaspase and C-terminal Ig-like (Ig3) domains (Figure 1D). Mepazine was most effective in inhibiting GSTMALT1 FL and GSTMALT1 325-760 with IC_{50} values of 0.83 and 0.42 μM, respectively. Also thioridazine and promazine showed a dose-dependent inhibition of GSTMALT1 FL and GSTMALT1 325-760, but the IC_{50} values were approximately 4-fold (GSTMALT1 FL) or 8-fold (GSTMALT1 325-760) higher when compared to mepazine. In contrast, promethazine (compound 18 in Figure S1H), a drug still being used in the treatment of certain psychiatric disorders and highly related to the three active PD, did not cause any significant MALT1 inhibition at concentrations up to 20 μM. These results indicate a high degree of specificity in MALT inhibition even within the group of PD.

To test the mode of action, we determined the effect of mepazine in Michaelis-Menten kinetics on the basis of the fluorogenic MALT1 cleavage assay (Figure 1E). GSTMALT1 FL displayed a V_{MAX} of ~170 RFU/min and the Michaelis-Menten constant (K_M) was calculated to ~48 μM, which is in the range of what has been determined previously (Hachmann et al., 2012). Addition of mepazine at a concentration around the IC_{50} (1 μM) strongly decreased the V_{MAX} to ~58 RFU/min while the K_M of 48 μM was not altered. Mepazine and other phenothiazines do not contain reactive groups. However, to confirm that mepazine acts as a noncovalent reversible inhibitor, we performed washout experiments using GSTMALT1 attached to glutathione sepharose beads (Figure 1F). Again, mepazine inhibited MALT1 cleavage activity, but several cycles of washing the GSTMALT1 beads resulted in complete loss of inhibition even at the highest concentration of the compound (50 μM). Thus, the effects of mepazine on MALT1 enzymatic activity revealed a noncompetitive and reversible mode of MALT1 inhibition by phenothiazines.

Next we assayed the effects of PD on caspases, which are structurally the closest relatives of MALT1 in mammals (Uren et al., 2000). Importantly, all three PD did not significantly inhibit caspase 3 or caspase 8 activity, even at concentrations up to 50 μM (Figure 1G), reflecting the selectivity of the compounds as MALT1 inhibitors.

Phenothiazines Inhibit MALT1 Activity and IL-2 Induction in T Cells

As MALT1 protease activity is required for T cell responses, we determined the effects of PD on MALT1 activity and IL-2 production in T cells. We performed a MALT1 cleavage assay after immunoprecipitation (IP) of the protein from Jurkat T cells. Cells were left untreated or incubated for 3 hr with 10 μM of mepazine or thioridazine and subsequently left unstimulated or stimulated with anti-CD3/CD28. MALT1 protease activity was almost undetectable in the absence of stimulation and peaked at 30–60 min after CD3/CD28 treatment. Addition of either mepazine or thioridazine resulted in a strong reduction of MALT1 protease activity in stimulated Jurkat T cells at all time points (Figure 2A). Mepazine and thioridazine incubation also caused a severe reduction in PMA/Ionomycin (P/I)-stimulated MALT1 protease activity in Jurkat T cells (Figure S2A). To confirm that both phenothiazines were inhibiting MALT1 activity inside the cells, we monitored MALT1 cleavage of RelB after stimulation of Jurkat T cells (Figure 2B). RelB cleavage product RelBΔ could be detected when Jurkat T cells were incubated with proteasome inhibitor MG132 prior to P/I stimulation to prevent degradation of the unstable RelB truncation (Hailfinger et al., 2011). As evident from decreased RelBΔ levels and a parallel increased expression of full-length RelB, mepazine and thioridazine impaired RelB cleavage in a dose-dependent manner (Figure 2B). Similar to the situation with recombinant MALT1, mepazine was more efficient in inhibiting cellular MALT1 cleavage activity and significantly reduced the appearance of RelBΔ between 2 and 5 μM, whereas thioridazine was effective above 5 μM.

To determine the impact of MALT1 inhibition by PD on T cell activation, we measured secreted IL-2 amounts by ELISA after P/I or anti-CD3/CD28 stimulation of Jurkat T cells in the presence or absence of mepazine or thioridazine. Both compounds led to a decrease of IL-2 levels in the media of PD-treated cells after T cell activation (Figure 2C). To verify that the inhibitory potential of PD was also detectable in primary T cells, we isolated and purified murine CD4- positive Th1 T cells and measured *IL-2* mRNA induction by qPCR and IL-2 protein levels by ELISA after anti-CD3/CD28 coligation in the presence or absence of 5 and 10 μM of mepazine or thioridazine (Figures 2D and S2B). Both *IL-2* mRNA induction and protein expression were reduced in a dose-dependent manner. Finally, we used primary human peripheral blood mononuclear cells (PBMCs) from three donors to evaluate whether inhibition of MALT1 activity also promoted a decreased IL-2 production in primary human T cells (Figure 2E). Congruent with the previous results, mepazine and thioridazine treatment led to a significant decrease of IL-2 secretion in PBMCs of all three donors.

Phenothiazines Inhibit MALT1 Activity and Induction of NF-κB Target Genes in ABC-DLBCL Cells

Coinciding with a constitutive cleavage of MALT1 substrates, we previously showed enhanced MALT1 protease activity as a characteristic feature of all ABC-DLBCL cells (Kloo et al., 2011). To determine the effect of phenothiazines on cellular MALT1 activity, ABC-DLBCL cells were treated for 4 hr with 5 or 10 μM of mepazine, thioridazine, and promazine. We performed an anti-MALT1 IP and MALT1 protease activity was determined by adding the substrate AC-LRSR-AMC to the precipitates. All three PD inhibited MALT1 protease activity from ABC-DLBCL cells in a dose-dependent manner (Figure 3A). The inhibition after partial MALT1 IP purification suggested that binding of PD promotes MALT1 to adopt an inactive conformation inside the cells. Even though inhibition of cellular MALT1 activity varied depending on the individual cell lines and the compounds, in general, mepazine had the strongest effects and MALT1 activity was reduced by at least 75% in all ABC-DLBCL cells at 10 μM. Treatment of thioridazine and promazine significantly inhibited cellular MALT1 activity and in agreement with in vitro IC_{50} data the effects of both inhibitors were in general weaker compared to mepazine.

Next, we asked whether MALT1 inhibition by the two strongest compounds, mepazine and thioridazine, would also prevent MALT1 substrate cleavage in ABC-DLBCL cells. RelB was shown to counteract anti-apoptotic activity of the NF-κB factors RelA and c-Rel and its cleavage to RelBΔ by MALT1 releases

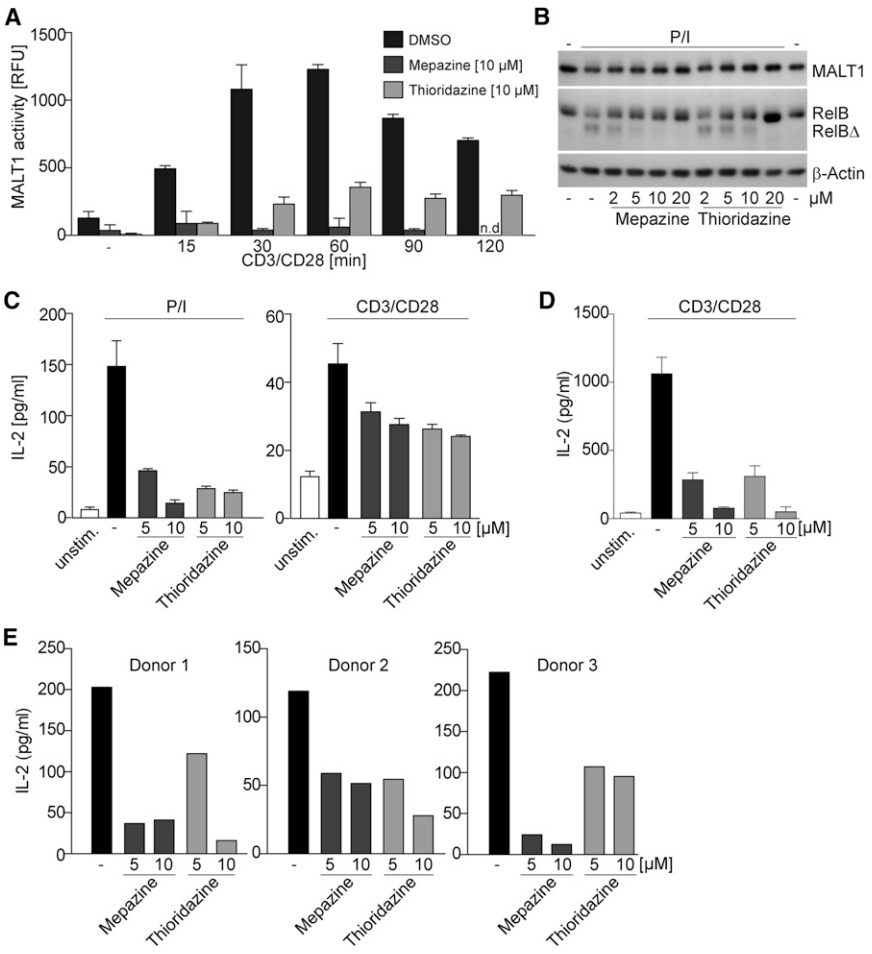

Figure 2. Mepazine and Thioridazine Impair MALT1-Mediated T Cell Activation

(A) Jurkat T cells were untreated or incubated for 3 hr with 10 μM of mepazine or thioridazine and then treated with anti-CD3/CD28 as indicated. Proteolytic activity after immunoprecipitation of MALT1 was determined by fluorogenic protease assay (mean ± SD; n = 3).

(B) Jurkat T cells were treated with solvent, mepazine, or thioridazine for 3 hr prior to 1 hr MG132 incubation. Afterward, cells were stimulated with P/I for 30 min. RelB and RelBΔ were analyzed by western blot. Blots show a representative of at least three independent experiments.

(C) IL-2 secretion was measured by ELISA after P/I or anti-CD3/CD28 stimulation of Jurkat T cells for 20 hr in the presence or absence of mepazine or thioridazine (mean ± SD; n = 3).

(D) Primary murine $CD4^+$ T cells were treated with mepazine or thioridazine and stimulated with anti-CD3/CD28 antibodies for 20 hr and then analyzed for IL-2 secretion (mean ± SD; n = 3).

(E) Primary human PBMCs from three donors were treated with mepazine and thioridazine for 3 hr before induction with anti-CD3/CD28 for 20 hr. Extracellular IL-2 levels were measured by ELISA (mean ± SD; n = 3).

See also Figure S2.

ABC-DLBCL cells from this negative regulation (Hailfinger et al., 2011). The inherently unstable RelB cleavage product (RelBΔ) was detected in ABC-DLBCL cells HBL1, OCI-Ly3, and TMD8 after addition of the proteasome inhibitor MG132 (Figure 3B). When the cells were treated for 4 hr with 2, 5, or 10 μM of mepazine or thioridazine, the appearance of RelBΔ cleavage product was inhibited and full-length RelB was stabilized in a dose-dependent manner. Correlating with the previous results on recombinant and cellular MALT1 inhibition, mepazine was more effective than thioridazine in inhibiting RelB cleavage. These results provided clear evidence that mepazine and thioridazine were effectively inhibiting constitutive cellular MALT1 cleavage activity in ABC-DLBCL cells.

In the ABC-DLBCL cell line HBL1, a MALT1 expression signature was generated using the covalently attached antagonistic tetrapeptide inhibitor Z-VRPR-FMK (Hailfinger et al., 2009b). To gain insight into whether PD exerted similar cellular effects, we determined gene expression profiles in HBL1 cells after different incubation times with mepazine, which most strongly inhibited MALT1 in previous assays (Figure 3C). When we compared the expression signature after administration of mepazine to the expression profile after Z-VRPR-FMK incubation, we observed a significant overlap in the genes that are downregulated in response to both treatments. In fact both the antagonistic tetrapeptide and the pharmacologic compound mepazine inhibited many genes quite rapidly and showed significant effects already after 6 hr of incubation, which further indicated that both inhibitors were exerting these cellular effects by acting through MALT1 inhibition. The weaker effects noted with mepazine after 12 and 24 hr are most likely due to the fact that, in contrast to Z-VRPR-FMK, the PD is inhibiting MALT1 in a noncovalent and reversible manner. As expected, we also observed differences in the two expression profiles, which were most likely related to MALT1-independent off-target effects of either Z-VRPR-FMK or mepazine.

Because MALT1 activity was shown to contribute to NF-κB activation and target gene expression in ABC-DLBCL cells (Ferch et al., 2009; Hailfinger et al., 2009a), we also determined if mepazine treatment impaired NF-κB target gene expression in HBL1 cells (Figure 4A). For this analysis, we compared relative changes in the gene expression after increasing times of treatment with mepazine in HBL1 cells by genome-wide expression arrays to an NF-κB expression signature generated after incubation with the highly specific IKKβ inhibitor MLN120B (Kloo et al., 2011). Mepazine significantly decreased the overall expression as well as the proportion of NF-κB target genes in the signature, clearly indicating that MALT1 inhibition by mepazine, just like Z-VRPR-FMK, suppressed activation of the NF-κB pathway in ABC-DLBCL cells.

To verify the effects of mepazine in other ABC-DLBCL cells, we monitored protein expression of selected NF-κB target genes that are known to possess critical functions for ABC-DLBCL biology (Figures 4B and 4C). One-time mepazine treatment led to a dose-dependent decrease of anti-apoptotic BCL-XL and FLIP-L proteins (Figure 4B). Furthermore, ABC- or GCB-DLBCL

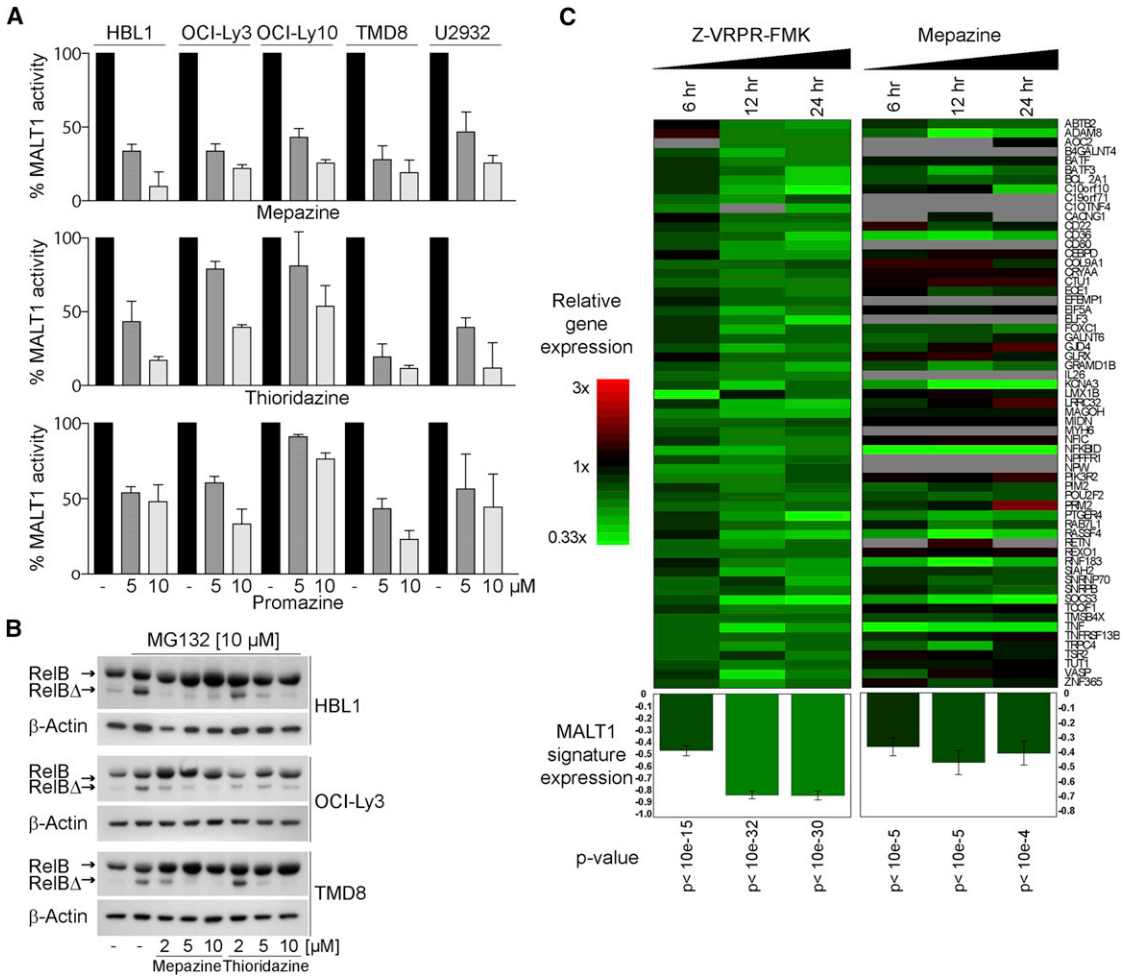

Figure 3. PD Treatment Impairs MALT1 Activity in ABC-DLBCL Cells

(A) Cellular MALT1 activity in ABC-DLBCL was analyzed after 4 hr incubation with mepazine, thioridazine, or promazine (mean ± SD; n = 3).

(B) Cells were treated with solvent, mepazine, or thioridazine for 4 hr prior to 1 hr MG132 incubation. RelB and RelBΔ were analyzed via western blot. Blots show a representative of at least three independent experiments.

(C) Gene expression profiling of the ABC-DLBCL cell line HBL1 after treatment with peptide Z-VRPR-FMK or mepazine (20 μM) for 6, 12, and 24 hr. Gene expression changes were assessed by DNA microarrays and are depicted according to the color scale shown. A gene was selected as dependent on MALT1 activity if Z-VRPR-FMK decreased gene expression by at least 33% after all three inhibition time points. This gene expression signature was subsequently applied to expression data following treatment with mepazine. Error bars depict standard error of the mean. Statistical significance of the decrease in the MALT1 signature average in each treated sample was verified by paired t test.

cells were treated with 10 μM mepazine for 20 hr and secretion of the NF-κB-regulated cytokines IL-6 and IL-10 was determined by ELISA (Figure 4C). Whereas GCB-DLBCL cells expressed low amounts of IL-6 or IL-10, ABC-DLBCL cells secreted both cytokines albeit to variable extents, reflecting heterogeneity among the different cell lines. Mepazine did not affect expression of IL-6 and IL-10 in GCB-DLBCL cells.

Selective Toxicity and Induction of Apoptosis by Phenothiazines in ABC-DLBCL Cells

Because the three PD efficiently inhibited MALT1 protease activity in vitro and in vivo, we tested their effect on the viability of ABC-DLBCL cells. As a control we used three GCB-DLBCL cell lines, BJAB, Su-DHL-6, and Su-DHL-4 that were previously shown to be independent of MALT1 proteolytic activity for their growth and survival (Ferch et al., 2009). We measured cytotoxic effects by MTT assays after 2 days of incubation after one-time compound treatment using increasing concentrations of mepazine, thioridazine, and promazine (Figure 5A). All compounds caused a decrease of cell viability in the ABC-DLBCL cells HBL1, OCI-Ly3, U2932, and TMD8, without significantly affecting GCB-DLBCL cells. Furthermore, we determined cell viability by cell counting after 4 days of incubation in response to a singular compound treatment (Figure 5B). Congruent with the MTT assay, the PD also decreased the overall number of viable ABC-DLBCL cells. Again, the reduced viability was much more pronounced in ABC-DLBCL cells, while GCB-DLBCL cell viability was only slightly impaired at the highest concentration of the compounds. Consistent with the results obtained in the cellular MALT1 cleavage assay, in general promazine had the

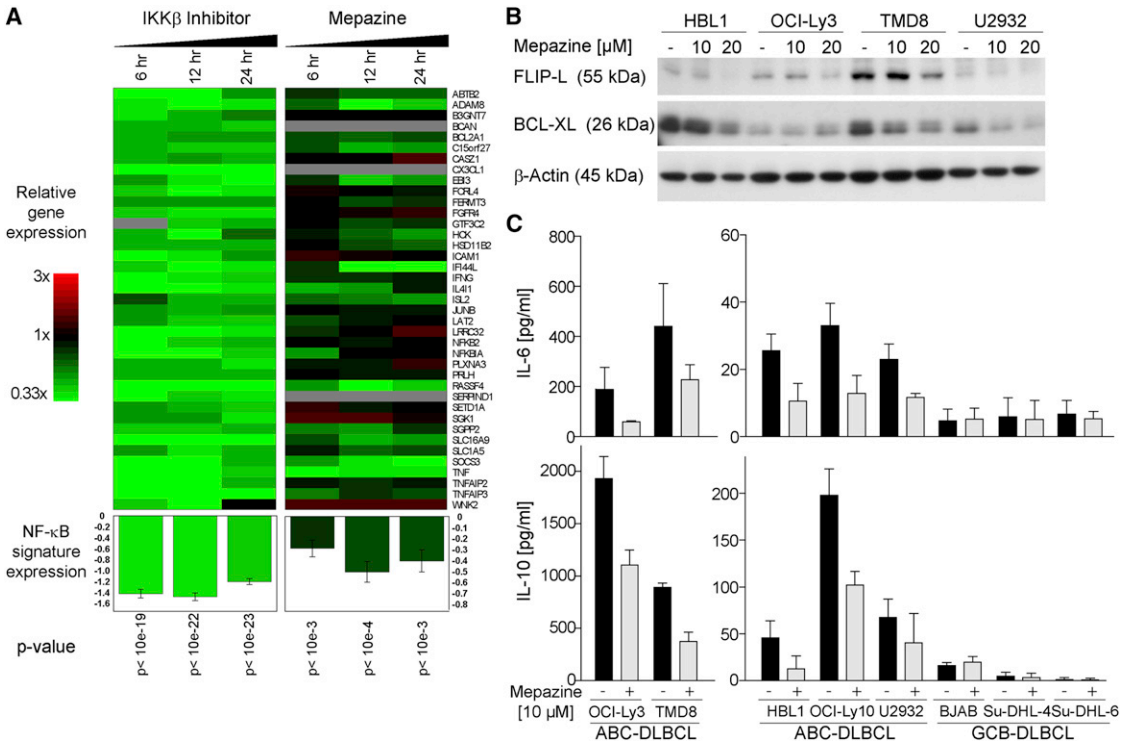

Figure 4. Mepazine Treatment Impairs NF-κB Target Gene Expression in ABC-DLBCL Cells

(A) Gene expression profiling of the ABC-DLBCL cell line HBL1 after treatment with mepazine (see Figure 3) was compared to the NF-κB gene signature generated after administration of the specific IKKβ inhibitor MLN120B. The significance of the decrease in the NF-κB signature average in each treated sample was verified by paired t test.

(B) Expression of NF-κB target genes BCL-XL and FLIP-L in ABC-DLBCL cells after mepazine treatment. Protein amounts were examined after 20 hr of treatment using the indicated concentrations by western blot. Data show a representative of three independent experiments.

(C) ABC- and GCB-DLBCL cells were treated with mepazine for 20 hr and the levels of secreted IL-6 and IL-10 were analyzed via ELISA (mean ± SD; n = 3).

mildest effects on the viability of the ABC-DLBCL cells. To validate that the decrease in viability of ABC-DLBCL cells is linked to the administration of distinct PD that act as MALT1 inhibitors, we treated DLBCL cells with promethazine (Figure S3A). Despite its close structural relation to promazine, promethazine did not inhibit MALT1 protease activity at concentrations up to 20 μM (see compound 18 in Figure S1H). Indeed, promethazine did not significantly inhibit viability of ABC- or GCB-DLBCL cells after 4 days of treatment, providing further evidence that the cellular effects of mepazine, thioridazine, and promazine were dependent on their ability to inhibit MALT1.

To demonstrate that decreased viability of ABC-DLBCL cells after PD treatment is linked to MALT1-dependent NF-κB inhibition, we infected HBL1 cells with a lentivirus expressing the constitutively active mutant IKKβ SS176/180EE (caIKKβ) and simultaneously GFP (Figure 5C). GFP-expressing cells were sorted by FACS, yielding caIKKβ or mock-infected populations of more than 95% GFP-positive cells. caIKKβ expression was verified by western blotting (Figure S3B). As expected, the viability of mock-infected HBL1 cells was strongly reduced in response to increasing doses of mepazine or thioridazine. In contrast, caIKKβ−expressing cells were resistant to the incubation of the PD. Thus, augmented IKKβ signaling counteracts the toxicity of PD, providing further evidence that PD are acting upstream of IKKβ through MALT1 inhibition.

For efficient anticancer drugs it is required that they exert toxic effects by enhancing cell death to eradicate the tumor cells. Therefore, we determined whether mepazine, as the most potent MALT1 inhibitor, affected viability of ABC-DLBCL cells by enhancing apoptosis (Figure 5D). To this end, DLBCL cells were treated for 5 days with 15 μM mepazine and apoptotic cells were identified by FACS as AnnexinV-PE positive and 7-AAD-negative cells. Mepazine provoked an enhanced apoptotic rate in all ABC-DLBCL cells, while apoptosis was not increased in the two GCB-DLBCL control cells. Thus, PD are selectively toxic to ABC-DLBCL cells and toxicity is at least partially due to enhanced apoptosis in the affected lymphoma cells, revealing a potential use of mepazine and structurally related compounds for ABC-DLBCL therapy.

Mepazine and Thioridazine Impede Growth of ABC-DLBCL In Vivo

We determined whether mepazine and thioridazine could also exert effects on lymphoma growth in vivo in a murine DLBCL xenogeneic tumor model. For this purpose, the ABC-DLBCL cell line OCI-Ly10 and the GCB-DLBCL cell line Su-DHL-6 were injected as subcutaneous xenografts into NOD/scid IL-2Rg^{null} (NSG) mice (Figure 6A). Both tumor cell lines were engrafted simultaneously on opposite flanks of individual mice. Starting 1 day after injection, the mice were treated by

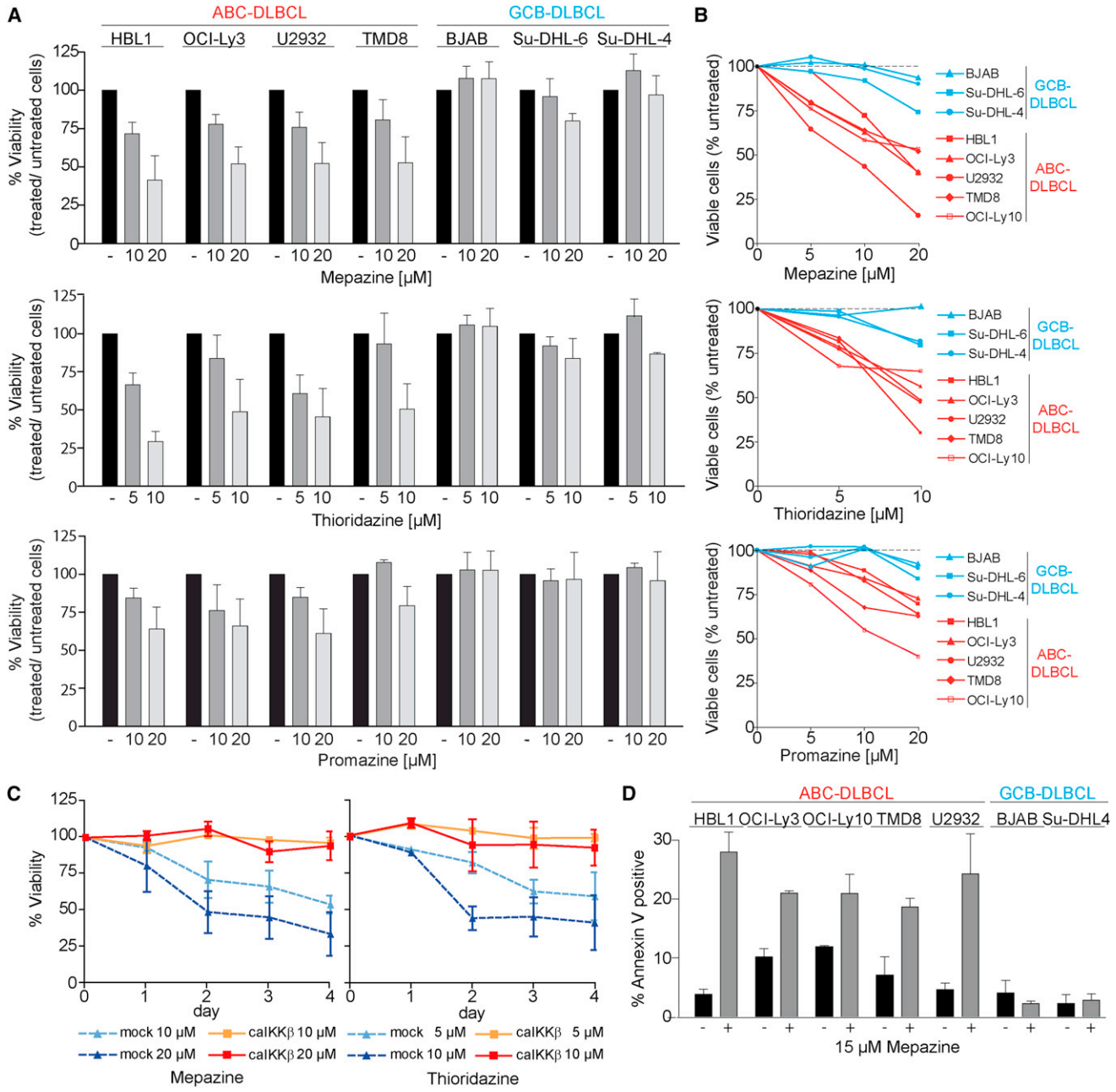

Figure 5. PD Are Selectively Toxic to ABC-DLBCL Cells

(A) ABC-DLBCL cell lines (HBL1, OCI-Ly3, U2932, TMD8) and GCB-DLBCL cell lines (BJAB, Su-DHL-6, Su-DHL-4) were treated with indicated concentrations of mepazine, thioridazine, or promazine (single treatment) for 2 days and their viability was subsequently analyzed with a MTT cytotoxicity test (mean ± SD; n = 3).

(B) Viability of cells treated with mepazine, thioridazine, or promazine (single dose treatment) for 4 days was determined by trypan blue exclusion counting of living cells. Data represent the mean of three independent experiments.

(C) HBL1 cells were lentivirally transduced to co-express calKKβ along with GFP or GFP alone (mock). GFP-positive cells were sorted via FACS and treated at day 0 with the indicated concentrations of mepazine or thioridazine. Cells were treated for 4 days and viable cells were counted by trypan blue exclusion every day during the treatment. Values represent the relative cell numbers compared to the untreated controls (mean ± SD; n = 3).

(D) ABC-DLBCL and GCB-DLBCL cell lines were treated for 5 days with 15 μM mepazine. Apoptotic cells were identified by FACS analysis as AnnexinV-PE positive and 7-AAD negative cells (mean ± SD; n = 3).

See also Figure S3.

intraperitoneal (i.p.) administration of solvent or either mepazine (16 mg/kg) or thioridazine (12 mg/kg). In control-treated mice, massive tumors grew from both DLBCL cell lines within 3 weeks of transplantation. Daily administration of mepazine or thioridazine strongly impaired the expansion of the ABC-DLBCL cell line OCI-Ly10. In contrast, both PD completely failed to exert

any inhibitory effects on the progression of the GCB-DLBCL cell line Su-DHL-6 in the same animals. To ascertain that mepazine and thioridazine are not only impeding growth in the initiation phase of xenografted OCI-Ly10 cells, but are also affecting grown tumors, we allowed OCI-Ly10 and Su-DHL-6 xenografted tumors to grow to ~75 mm^2 before treatment (Figure 6B). Again, mepazine and thioridazine strongly reduced the progression of OCI-Ly10 tumors while having no effect on the growth of Su-DHL-6 tumors in this setting. Thus, PD are in fact able to inhibit growth of a preformed ABC-DLBCL tumor.

Thioridazine is orally available in humans and pharmacokinetic (PK) studies led to a consensus therapeutic range between 200 and 2000 ng/ml in the blood of patients (Baumann et al., 2004). To determine if the concentrations of thioridazine in the blood of NSG mice after i.p. injection are not exceeding this therapeutic window, we measured serum concentrations in NSG mice after 0, 1, 2, 6, and 24 hr in response to acute dosing of 12 mg/kg thioridazine. Thioridazine concentrations between 170 and 270 ng/ml were measured 1 hr after dosing and the levels rapidly decreased thereafter (Figure 6C). After 24 hr, thioridazine levels were below the detection limit (20 ng/ml).

To show that mepazine and thioridazine were acting directly on the tumor cells, we determined the induction of apoptosis in the tumor tissue. Transplanted tumors were removed at the end of the treatment period (21 days; see Figure 6A) and apoptotic cells were visualized by TUNEL staining on sections of the tumor tissue (Figure 6D). Congruent with the selective in vivo toxicity, mepazine or thioridazine treatment increased the number of apoptotic cells in the xenografted ABC-DLBCL cell line OCI-Ly10, while no induction of apoptosis was observed in the in GCB-DLBCL cell line Su-DHL-6. Furthermore, constitutive cleavage of the MALT1 substrate RelB was impaired after mepazine and thioridazine treatment in specimens of xenografted OCI-Ly10 tumors, revealing that also in mice the compounds were indeed acting by inhibiting MALT1 activity in the tumor cells (Figure 6E). Thus, the murine tumor model provided evidence that MALT1 inhibition by phenothiazines selectively kills MALT1-dependent DLBCL in vivo and indicates a potential therapeutic benefit for use of the known compounds in ABC-DLBCL therapy.

DISCUSSION

In this study, we have identified PD as small molecule inhibitors that effectively and selectively inhibit proteolytic activity of recombinant and cellular MALT1. Importantly, only a distinct subset of PD inhibited MALT1, with the best inhibitory activity found for mepazine, thioridazine, and promazine, three known organic compounds that have been in clinical use as antipsychotic or sedative drugs. All three PD interfered with inducible MALT1 activity in activated T cells and constitutive activity in ABC-DLBCL cells. Furthermore, mepazine and thioridazine caused impaired T cell activation as well as reduced viability that was selective for the ABC subtype of DLBCL, processes that were shown to be critically dependent on MALT1 activity (Düwel et al., 2009; Ferch et al., 2009; Hailfinger et al., 2009b). In addition, both compounds were inhibiting MALT1 activity and selectively inducing apoptosis and thereby reducing growth of ABC-DLBCL in a preclinical DLBCL xenotransplantation model. Thus, cellular and in vivo data underscore the effectiveness of PD as pharmacologic MALT1 inhibitors and suggest their potential clinical application for ABC-DLBCL therapy.

MALT1 is the only human paracaspase with very distinct properties when compared to mammalian caspases (Hachmann et al., 2012), revealing that specific inhibitors are clearly promising drug candidates for selective inactivation of the MALT1 oncogenic activity. Selectivity is critical, because impairment of apoptosis through caspase inhibition would certainly trigger adverse effects detrimental for lymphoma therapy. Indeed, all PD tested displayed a high preference for MALT1 and did not impair the initiator caspase 8 and the executioner caspase 3. Congruently, mepazine and thioridazine were able to induce apoptotic cell death in MALT1-dependent ABC-DLBCL.

Michaelis-Menten kinetics and wash-out experiments revealed that mepazine does not directly affect substrate binding to the catalytic center of MALT1 in a competitive manner, but rather acts as a noncompetitive and reversible inhibitor. Because all PD inhibit activity of a catalytically active MALT1 fragment that spans the paracaspase and C-terminal Ig3 domain to a similar extent as the full length protein, they apparently exert allosteric effects within the active paracaspase domain. The recent elucidation of the crystal structure of the MALT1 paracaspase and Ig3 domains in its free form and bound to the tetrapeptide inhibitor indicates that substrate binding causes a substantial structural reorganization to achieve an active protease (Wiesmann et al., 2012; Yu et al., 2011). Mepazine and thioridazine inhibit constitutive activity of recombinant MALT1, suggesting that PD bind within the paracaspase-Ig3 region of MALT1 and thereby possibly counteract the structural rearrangement required to maintain the active confirmation. Also inside the cells, PD act directly on the MALT1 paracaspase, as indicated by impaired cleavage of the substrate RelB. Furthermore, the inhibitory effect on MALT1 is also evident from the inhibition of MALT1 activity in in vitro protease assays after IP of cellular MALT1. Thus, in T cells PD are apparently maintaining MALT1 in an inactive conformation that is resistant to activation by upstream pathways. Furthermore, the inhibition of constitutive MALT1 activity in ABC-DLBCL cells even after IP purification suggests that in the ABC-DLBCL cells PD are in fact forcing the active MALT1 protease into an inactive conformation. Mepazine and thioridazine are most likely causing cellular MALT1 to adopt a structure reminiscent to the inactive enzyme prior to stimulation. In fact, these results on cellular MALT1 inhibition are compatible with the noncompetitive, allosteric mode of inhibition that PD exert on recombinant MALT1. Taken together, our recombinant and cellular data underscore the efficacy and selectivity of PD as small molecule MALT1 inhibitors.

In cells, the strong inhibition of cellular MALT1 activity after relatively short PD incubation times clearly indicated that the substances were directly affecting MALT1 protease. Mepazine was ~4- to 6-fold more effective than thioridazine in inhibiting recombinant MALT1 and it was also more effective in cells, indicating that cellular uptake, compound stability, and cellular concentration may be in a similar range. Different PD have been or are still in clinical use, reflecting that the phenothiazine scaffold seems to be a privileged structure to obtain in vivo effects. Interestingly, toxicity tests on ABC-DLBCL cells

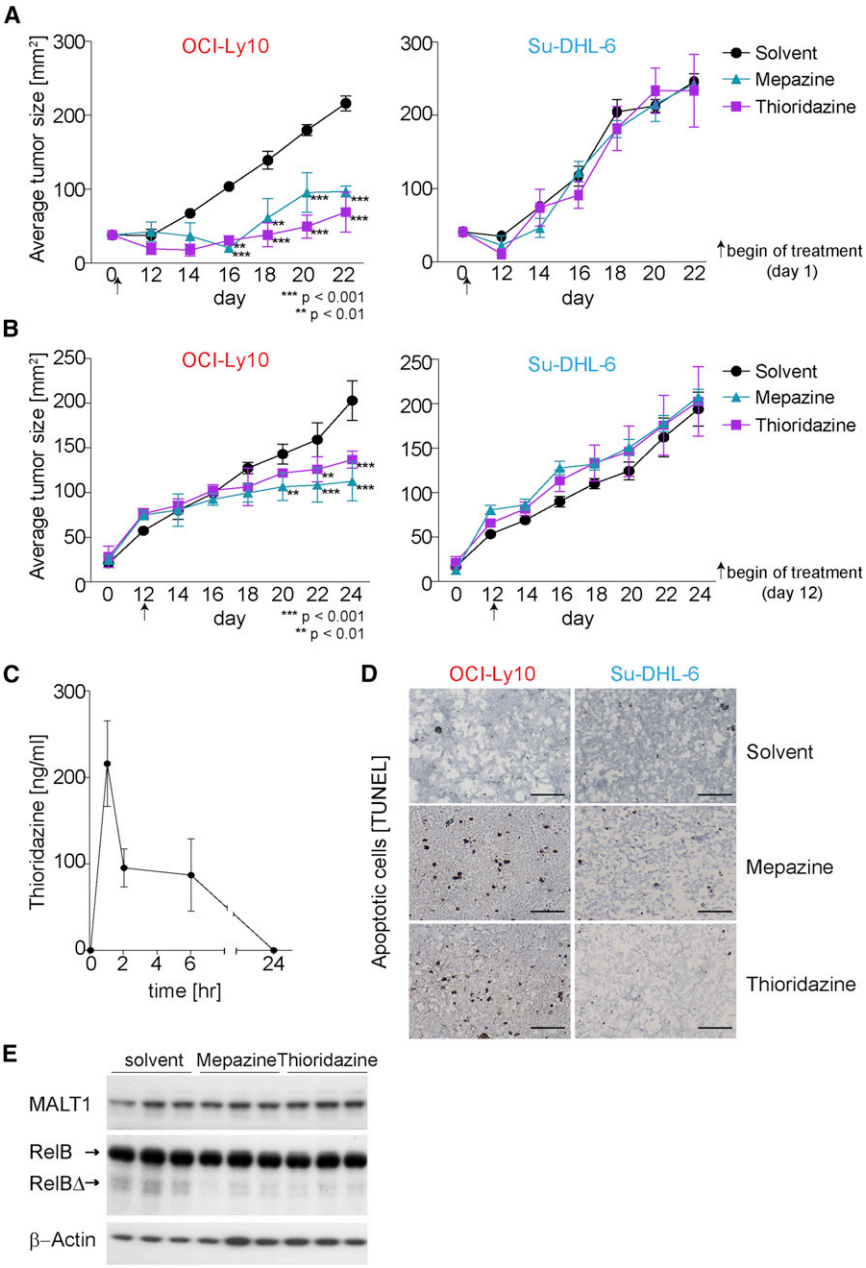

Figure 6. Mepazine and Thioridazine Interfere with Growth and Induce Apoptosis in ABC-DLBCL Cell Line OCI-Ly10 In Vivo

(A) Transplantation of OCI-Ly10 or Su-DHL-6 cells resuspended in matrigel into the flanks of NSG mice was carried out on day 0. Tumor size was determined by caliper measurement. Intraperitoneal administration of solvent, mepazine (400 μg/day) or thioridazine (300 μg/day) was started 24 hr after transplantation and given continuously every 24 hr for the entire treatment period. Statistical analysis was performed using a two-way Anova test (mean ± SD; n = 3).

(B) Mice were treated as in (A) but administration of compounds was started on day 12 after xenotransplantation when tumors were grown to ~75 mm^2 (mean ± SD; n = 3).

(C) Thioridazine concentrations were measured in the serum 0–24 hr after i.p. injection (12 mg/kg) by HPLC (mean ± SD; n = 3).

(D) Apoptosis was determined on tumor sections (experiment in A) by TUNEL staining after 22 days of treatment. Pictures show staining of representative tumor sections (scale bar 100 μm).

(E) RelB and RelBΔ were detected in extracts of OCI-Ly10 tumor specimens by western blotting 22 days after transplantation (experiment in A). Blots show results from three mice treated with solvent, mepazine, or thioridazine.

revealed some differences in the effects of thioridazine to mepazine. In fact, differential side effects of PD may synergize with MALT1 inhibition to exert the toxic effect on ABC-DLBCL cells. In this context, thioridazine has been reported to inhibit PI3K-PDK1-AKT signaling in ovarian cancer cells (Choi et al., 2008; Rho et al., 2011). Constitutive AKT phosphorylation is a common feature of DLBCL cells, independent of their classification. We observed some effects of thioridazine on AKT phosphorylation (data not shown), which may account for an augmentation of thioridazine toxicity. However, as GCB-DLBCL cells, which also show constitutive PI3K-AKT signaling (Kloo et al., 2011) and are resistant to thioridazine, MALT1 inhibition is clearly the primary cause for the cellular toxicity in this setting. We recently demonstrated that PI3K-PDK1 contributes to enhanced MALT1 activity in the two ABC-DLBCL cell lines HBL1 and TMD8 that carry oncogenic mutations in the BCR signaling adaptor CD79B (Kloo et al., 2011). Clearly, thioridazine did not only affect survival of HBL1 and TMD8 cells, but also was toxic to all MALT1-dependent ABC-DLBCL, again indicating that its primary target is MALT1. Nevertheless, concomitant inhibition of PI3K-AKT may add to the effects of thioridazine on MALT1.

The important clinical potential of the three PD identified and analyzed in this study stems from the fact that all have been used formerly in trials or in the clinic as antipsychotic or sedative drugs. This activity in this setting is thought to be based primarily on their ability to function as dopamine D2 receptor antagonists (Seeman et al., 1976). Mepazine, the most effective MALT1 inhibitor, has been evaluated as an antipsychotic and tranquilizing drug under the brand name Pacatal in the late 1950s and early 1960s. Whereas some clinical investigations provided evidence for antipsychotic effects, other studies failed to do so (Sarwer-Foner and Koranyi, 1957; Whittier et al., 1960). Some side effects were reported after mepazine treatment, including a reduction of asthma attacks, indicating potential immunosuppressive activity (Sarwer-Foner and Koranyi, 1957). Thioridazine (brand name Mellaril/Melleril) is still commercially available, but is reserved for the treatment of schizophrenic patients who do not respond to other antipsychotic drugs. Thioridazine is also

considered to be beneficial for other medical applications because it exerts toxic effects on different cancer cell lines (Rho et al., 2011; Zhelev et al., 2004). In addition, thioridazine is considered as a candidate drug for the treatment of tuberculosis or malaria, but the reasons for its antimicrobial and antiparasitic actions are currently unknown (van Soolingen et al., 2010; Weisman et al., 2006). Promazine (brand name Sparine), which displayed the weakest toxicity for MALT1-dependent ABC-DLBCL, is still used to treat restless behavior.

Comparative analysis of pharmacokinetic (PK) profiles in rodents and humans further support the possibility for a potential off-label clinical use of thioridazine to treat ABC-DLBCL. We measured thioridazine levels of 170–270 ng/ml in the serum of NSG mice 1 hr after treatment, which is congruent with previous single dose PK studies after i.p. injection (10 mg/kg) in rats (Daniel et al., 1997). The concentrations obtained in rodents are within the range of peak concentrations reported in humans (130–650 ng/ml) 1–4 hr after oral uptake of 100 mg thioridazine (Chakraborty et al., 1989; Shvartsburd et al., 1984). However, whereas in humans considerable concentrations of thioridazine are still present even after 1 day, the drug is undetectable in rodents after 24 hr (see Figure 6C; Daniel et al., 1997). A dose of 10–12 mg/kg in rodents corresponds to ~800 mg for an adult. Given these relatively high doses, PK studies clearly demonstrate a much faster metabolism of thioridazine in rodents compared to humans. Congruently, in rats 2 weeks of chronic i.p. injection (10 mg/kg twice daily) led to plasma concentrations of thioridazine that peaked at ~750 ng/ml and declined to ~90 ng/ml over 24 hr (Daniel et al., 1997). The recommended therapeutic range of thioridazine in humans has been set to steady state levels of 200–2,000 ng/ml in the plasma of patients treated for psychiatric disorders (Baumann et al., 2004). These data reveal that the thioridazine levels that are effective in killing xenografted ABC-DLBCL tumors after i.p. injection in mice are within the range or even below the therapeutic window that can be achieved after oral administration of thioridazine in humans. Thus, the PK profile lends support to a possible clinical off-label use of PD in ABC-DLBCL therapy.

The identification of mepazine, thioridazine, and promazine as three small molecule inhibitors of MALT1 opens potential additional therapeutic uses. The inhibitory action of PD on T cell activation indicates a potential immunosuppressive effect of MALT1 inhibition that needs to be further explored, but adverse effects of PD that have mostly terminated a long-term antipsychotic use are certainly not tolerated for an application as immunosuppressants. However, a restricted short-term usage of mepazine or thioridazine for treatment of patients with aggressive lymphoma that depends on MALT1 proteolytic activity and is resistant to current therapeutic protocols can be envisioned. The overall 5-year survival rate of 35% for patients with ABC-DLBCL emphasizes the urgent clinical need for alternative treatment options (Lenz et al., 2008b). The increase in apoptosis as well as the decrease in tumor growth of the ABC-DLBCL cell line OCI-Ly10 in response to mepazine or thioridazine treatment in mice underscores the potency of these drugs. Thioridazine is still on the market and the xenotransplantation model as well as PK profile suggests that a daily administration of up to 800 mg, which is the maximal permitted oral dosage in psychotic patients, could have beneficial effects in patients with ABC-DLBCL. A combinatorial therapy could be possible, because the reduced expression of prosurvival factors after MALT1 inhibition by PD could sensitize ABC-DLBCL tumor cells for chemotherapy-induced apoptosis. Besides the differential ABC- and GCB-DLBCL classification, further diagnostic markers to identify patients with lymphoma who would most likely benefit from MALT1 inhibition by PD are certainly required. In addition, effectivity and selectivity of MALT1 inhibition by PD can be optimized by medicinal chemistry programs to yield a more potent and possibly selective drug for the treatment of MALT lymphoma or ABC-DLBCL.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents

DLBCL cell lines (except OCI-Ly10) were cultured in RPMI 1640 medium (Invitrogen) supplemented with 20% FCS and 100 U/ml penicillin/streptomycin. OCI-Ly10 was cultured in IMDM (Invitrogen) with 20% human plasma, penicillin/streptomycin and 50 μM β-mercaptoethanol. Jurkat T cells were cultured like DLBCL cells with 10% FCS. The isolation of PBMCs from heparin-treated (1000 U/ml) whole blood was done with Lymphoprep according to manufacturer (Axis-shield). Isolation of murine $CD4^+$ T cells was performed with T-cell-specific Dynabeads (Invitrogen) and they were cultured in Jurkat medium and 50 μM β-mercaptoethanol. Stimulation of Jurkat T cells, PBMCs, and mouse $CD4^+$ T cells was either initiated by the addition of phorbol 12-myristate 13-acetate (PMA; 200 ng/ml) and ionomycin (I; 300 ng/ml) (both Calbiochem) or by hCD3/hCD28 and mIgG1/mIgG2a antibodies (BD Biosciences). The use of PBMC from healthy donors in this study was approved by the Ethics Board of the Medical Faculty of the Ludwig-Maximilians-University Munich, Germany and donors gave informed consent.

Z-VRPR-FMK (Alexis Biochemicals), mepazine acetate (Chembridge), promazine hydrochloride, thioridazine hydrochloride, promethazine hydrochloride (all Sigma Aldrich), and all other PD tested (Chembridge or Sigma) were solved in DMSO. Antibodies used were BCL-XL (Cell Signaling), MALT1 (H300, Santa Cruz), c-FLIP (Alexis Biochemicals), β-Actin (I-19, Santa Cruz), and RelB (Cell Signaling).

Recombinant and Endogenous Protease Cleavage Assays

Recombinant GSTMALT1 was purified from *E. coli* and all cleavage assays were in 384-well microplates format using 200 ng of GSTMALT1 and 50 μM of the substrate Ac-LRSR-AMC. Cleavage activity was determined by the increase of AMC fluorescence measured in Synergy 2 Microplate Reader (Biotek). Reversible inhibition was determined after coupling of 300 ng GSTMALT1 to glutathione sepharose and subsequent wash out of the compounds. Cleavage of human caspase 3 (BioVision) and caspase 8 (Cayman Chemical) was assayed accordingly against Ac-DEVD-AMC as substrate using 50 and 250 pg of protein, respectively. Activity of endogenous MALT1 in the absence or presence of inhibitors was determined after MALT1 IP as described earlier (Kloo et al., 2011).

Real-Time RT-PCR and ELISA

Synthesis of cDNA was performed with DNA-free RNA samples (RNeasy Mini Kit, QIAGEN) by reverse transcription with random hexamers and Superscript II (Invitrogen) according to the manufacturer's protocol and real-time PCR was performed using LC 480 SybrGreen PCR mix on a LC 480 Lightcycler system (Roche). RNA was normalized by β-Actin mRNA. Primers are shown in Supplemental Experimental Procedures.

Human and murine IL-2 (BenderMed Systems), IL-6, and IL-10 ELISAs (Immunotools) were performed according to the manufacturer's protocols.

Gene Expression Profiling

Gene expression profiling was performed for the ABC-DLBCL cell line HBL1 after treatment with DMSO or 20 μM of mepazine for the indicated time points. RNA extraction using RNeasy Mini Kit (QIAGEN) and analysis of whole-genome Agilent 4 × 44K gene expression arrays (Agilent Technologies) was performed following the manufacturer's protocol. Signals from either

DMSO-treated (labeled with Cy3) or mepazine-treated (labeled with Cy5) HBL1 cells were compared. We applied a previously developed MALT1 and NF-κB target gene signature to the gene expression data (Hailfinger et al., 2009b; Kloo et al., 2011). Genes that were significantly downregulated were determined by paired t test ($p < 0.05$).

Viability, MTT, and Apoptosis Assays

Viability of DLBCL cell lines was analyzed with a cell count assay of trypan blue-stained cells after 4 days and by MTT (3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazoliumbromid) cytotoxicity test after 2 days of inhibitor treatment in comparison to DMSO-treated cells. Reduction of MTT to formazan was measured at 450 nm with a μQuant microplate spectrophotometer (Biotek). Apoptosis rates were determined with PE-Annexin V staining of $7AAD^-$ cells (BD PharMingen) by FACS (LSRII, BD). Data were analyzed using FlowJo software (Treestar). TUNEL assay was performed on frozen 5 μm DLBCL tumor tissue sections with the DEAD End colorimetric TUNEL system kit according to the manufacturer's protocol (Promega). Tissue sections were stained with H&E and images were taken on a Zeiss Axioplan microscope.

Lentiviral Transduction

Constitutively active Flag-IKKβ (caIKKβ) mutant (SS176, 180EE) and GFP were linked by a cotranslational processing site T2A (Hadian et al., 2011) and introduced into pLVTHM plasmid. Infection into HBL1 cells was performed as described earlier (Wiznerowicz and Trono, 2003). After 1 week in culture, HBL1 cells were sorted by FACS yielding more than 95% GFP-positive HBL1 cells. Viability of cells in response to phenothiazine treatment was determined by counting of trypan blue staining.

In Vivo Tumor Cell Engraftment, Treatment of Mice, and PK Thioridazine

All experiments were approved by the Bavarian State authorities (file no. 115-09) according to the legal regulations for animal experimentation. Tumors were engrafted in 6- to 8-week-old female NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice by subcutaneous injection of 4×10^6 tumor cells (OCI-Ly10 or SU-DHL-6) resuspended in matrigel (BD). Both tumors were engrafted simultaneously on opposite flanks of individual mice, with three mice for each treatment group. Intraperitoneal administration of solvent, mepazine, or thioridazine was started 1 or 12 days after transplantation and given continuously every 24 hr thereafter. We used a daily application dose of 300 μg thioridazine and 400 μg mepazine per animal (25 g), corresponding to approximately 12 mg/kg and 16 mg/kg, respectively. The tumor size was measured every other day after visual appearance using a caliper and calculated as square millimeters (length × width). Mice were sacrificed when the SU-DHL-tumor size was above 250 mm^2. Statistical data were analyzed with a two-way Anova test.

For the pharmacokinetic study of thioridazine, NSG mice were i.p. injected with a single dose of 300 μg. For each indicated time-point three mice were treated and blood serum concentrations of thioridazine were determined via HPLC by standardized diagnostic protocol (MVZ Dortmund).

ACCESSION NUMBERS

The GEO database accession number for the raw data for expression profiling is GSE39741.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.ccr.2012.11.002.

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