Targeted inhibition of a novel MALT1 and MAPK signaling network synergistically suppresses aggressive B cell lymphoma growth

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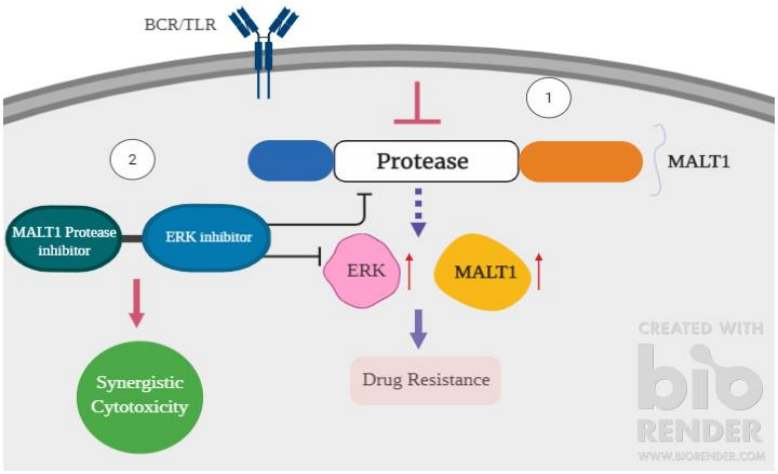
Abstract:

Mucosa associated lymphoid translocation protein 1 (MALT1) mediates pathogenesis of the Activated B cell subtype of Diffuse large B cell lymphoma (ABC DLBCL) via cleaving negative regulators of the Nuclear Factor kappa Beta pathway (Nf-κB) pathway. Targeted degradation of MALT1 proteolytic domain induces cytotoxicity among ABC DLBCLs but unknown resistance mechanisms have prevented clinical translation of MALT1 protease inhibitors. This study sought to identify novel targets on MALT1 by suppressing its caspase domain and analyzing MALT1 protein expression at varying time points. Treatment of ABC DLBCL cell lines HBL-1, OCI-ly3, and TMD8 with MALT1 protease inhibitors SCM-02-138, MI-2, and Mepazine did not affect the MALT1 expression at 1hr time point, but MALT1 expression upregulated after the 48 and 96 hr. timepoints. Western Blot analysis further suggested that MALT1 protease suppression induces upregulation of the MAPK signaling pathway by activating the ERK kinase, uncovering a novel Nf-κB and MAPK signaling crosstalk network. ABC DLBCLs were then administered with Trametinib in order to suppress ERK, which also led to attenuation of the MALT protein expression. To address phenotypical implications of this network, fixed ratio growth inhibition experiments were conducted, which elucidated that combinatorial inhibition of MALT1 and ERK suppresses ABC DLBCL progression. Chou-Talalay analysis further suggested that combinatorial inhibition induces synergistictic cytotoxicity (CI < 1) among lymphoma clones. Taken together, by diagnosing a novel signaling network and developing a therapeutic approach, this study has provided a rationale for exploring combination therapies for numerous MALT1-addicted tumors.

Graphical Abstract:



1.0: Introduction:

In addition to being the most common Non-Hodgkin lymphomas (NHLs), Diffuse large B cell lymphomas (DLBCLs) account for 30,000 annual cases in the United States (1). DLBCLs are characterized by a heterogeneous genetic background, resulting in clonal heterogeneity and an aggressive clinical behavior (2-4). Molecular classifications segregate DLBCLs into subtypes based on the cell origin: Germinal cell B cell (GCB) and Activated B cell (ABC) (5). These subtypes significantly differ in their spectrum of somatic mutation and overall survival in response to the multi-agent Rituximab-Cyclophosphamide-Doxorubicin-Vincristine-Prednisone (R-CHOP) therapy (6-8). While GCB-DLBCLs typically have favorable outcomes (7), ABC DLBCLs which account for 60% of overall DLBCL cases, (9) exhibit extensive genetic heterogeneity and poor survival rate, (2), necessitating the urgent development of novel targeted therapies.

A distinct molecular characteristic of ABC DLBCLs is their addiction to genetic and somatic mutations expressed in the B cell Receptor (BCR), Toll-like receptor (TLR), and the downstream Nuclear factor kappa Beta (Nf-κB) pathways (10, 11, 12). BCR and TLR linkage to the Nf- κB is mediated via the Mucosa-associated lymphoid translocation protein 1 (MALT1), which in itself constitutes a gain of function mutation located in 18q21 (13) in 70% of ABC DLBCLs. MALT1 forms a multimeric complex with the B Cell lymphoma 10 (BCL10) and Caspase-recruitment domain Protein 11 (CARD11) in response to BCR/TLR ligation (Figure 1). As the only human paracaspase, MALT1 constitutes a dual function where its scaffolding role consists of inducing the activation of the IkB kinase (IKK) by mediating IKK2 phosphorylation, resulting in nuclear translocation of the canonical Nf-κB subunits p65 and c-Rel which activate gene transcription (14, 15). Moreover, the MALT1 caspase region belongs to family of arginine-specific proteases and allows MALT1 to perform cleavage mediated inactivation of Nf- κB inhibitors such as A20, and activation of Nf- κB favoring proteins such as BCL10, among others (16,17). Nuclear Nf- κB is responsible for transcription of anti-apoptotic genes such as BCL2 and TRAF1/2, which counteract the cytotoxicity induced by R-CHOP, making ABC DLBCLs prone to resistance (18, 19).

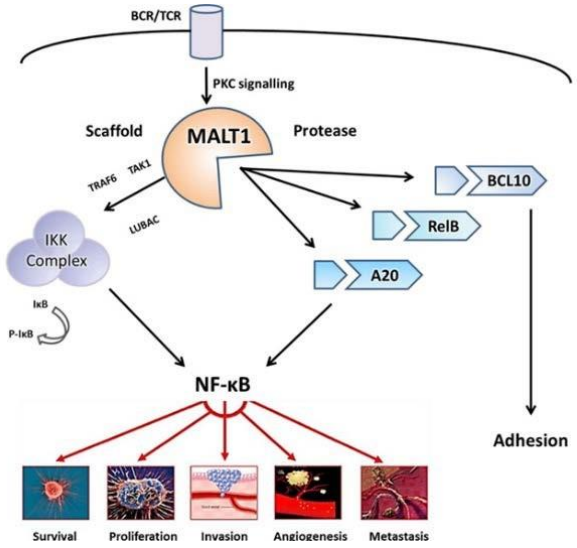


Figure 1. In response to BCR/TLR activation, MALT1 recruits protein complexes in order to degrade the inhibitory kappa B complex and allow Nf-κβ nuclear translocation. The protease domain simultaneously regulates MALT1-mediated cleavage of negative regulators of Nf-κB, for inducing Nf-κB nuclear localization.

MALT1 protease function is therefore crucial for regulating Nf-κB stability and has emerged as a therapeutic target in ABC DLBCLs. MALT1 inhibition suppress Nf-κB activation as manifested by extensive downregulation in production of Nf-κB serums such as IL-10 (20-21). Consequently, numerous inhibitors each with varying selectivity and pharmacological properties have been designed, with SCM-02-138 emerging as the most selective peptide (21,22). However, the signaling mechanisms underlying MALT1 function remain poorly understood as lymphomas are capable of rendering MALT1 protease suppression inefficacious and escaping cytotoxicity. A better understanding of mechanisms that regulate MALT1 suppression efficacy would provide a rationale for development of therapeutic agents that effectively target the most aggressive components in the critical ABC DLBCL survival pathways.

A plausible explanation for resistance to MALT1 protease suppression lies under the heterogeneity expressed in genetic background of ABC DLBCLs (3). Implication of numerous mechanisms that cumulatively regulate the pathology of lymphomas suggests that lymphomas escape MALT1 suppression induced cytotoxicity by adapting to new signaling mechanisms that are initially downregulated under physiological tumor conditions. To discover the mechanisms regulating MALT1 protease inhibition efficacy, we turned towards analyzing the genetic response of lymphomas after they're administered with MALT1 protease inhibitors. Indeed, it

was discovered that MALT1 proteases suppression potentiates an alternative signaling function in order to escape cytotoxicity. Furthermore, we also identified a therapeutic approach that is synergistic at being toxic to three different ABC DLBCL cell lines and suppresses B cell lymphomagenesis.

2.0 Methods:

2.1.1 Cell lines:

HBL-1 cell lines were purchased from Universidad de Navarra, Pamplona, Spain, OCI-Ly3 cells were obtained from Memorial Sloan Kettering Cancer Center, New York, New York, and TMD8 cells were acquired from the National Cancer Institute, Bethesda, Maryland. All cell lines were authenticated by the University of Arizona Genetic Core and cultured in the presence of penicillin G, streptomycin, and 2 mM L-glutamine at 37°C in a humidified atmosphere of 5% CO_2. In spite of exhibiting distinct phenotypical features due to differences in their gene expression signatures, all of these cell lines have previously shown to be sensitive to MALT1 protease inhibition (10, 25)**.**

2.1.2 MALT1 protease inhibitors:

To ensure inhibition specificity and produce consistent outcomes, three different inhibitors, each with different pharmacological properties were acquired for MALT1 protease suppression. MALT1 protease inhibitor, SCM-02-138 was developed by the Melnick laboratory, Mepazine was acquired from Milli Pore Sigma, and MI-2 was purchased from R&D systems

2.1.3 MAPK and mTOR inhibitors:

MEK inhibitors, Selumetinib and Trametinib and the mTORC1 inhibitor Rapamycin were acquired from Milli Pore Sigma.

2.1.4 Antibodies:

Both primary and secondary antibodies for MALT1, extracellular signal regulated kinase (ERK), and phosphorylated ERK (pERK) were acquired from cell signaling technology (CST).

2.2 *Cell culture and drug treatment:*

At day 1, 0.5×10^5 TMD8 and HBL-1 cells were cultured in Roswell Park Memorial Institute Medium (RPMI) 1640 supplemented with 10% Fetal Bovine Serum (FBS) and 10 mM 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES) solution. Each well was individually treated with 1uM concentration of SCM-02-138, 100 nM concentration of Mepazine,

100 nM concentration of MI-2, or 100 nM of DMSO as a vehicle control. For the later experiments, TMD8 and HBL-1 cells were treated with 1uM concentration of the MEK inhibitors, Selumetinib or Trametinib. All drug concentrations were prepared in DMSO, in order to ensure that if cells are affected by DMSO, those results are normalized during vehicle comparison.

For each experiment cells were incubated for 1hr, 48hrs, and 96hrs. These time points were chosen because it was expected that ABC DLBCLs escape the effects of MALT1 protease inhibition during a prolonged period of time.

2.3 *Western Blots:*

After the devoted incubation periods, cells were collected in a falcon tube and washed with 1x Dulbecco's Phosphate Buffered Saline (DPBS) via the centrifuge at 14,000 rpm. RIPA buffer was used for lysing along with the protease and phosphate inhibitors. Cells were then centrifuged at 14,000 rpm for 30 minutes and the supernatant was collected. Proteins were then quantified using the Gen5 program and normalized to 30ug for uploading into pre-made SDS gels. The proteins were initially transferred to the gel for 1hr and then transferred to a nitrocellulose membrane.

Membranes were incubated with primary antibodies, including anti-rabbit MALT1, anti-mouse ERK, and anti-mouse pERK, at 4°C overnight. Membranes were washed with 1x PBS three times for 15 minutes before being incubated with corresponding secondary antibodies: anti-Rabbit IgG-HRP conjugated for ERK and pERK, and IgG-mouse conjugated for MALT1. These secondary antibodies were prepared in 5% milk, with the dilution of 1:2000, constantly shaking at the room temperature at 2 hours. Actin or Tubulin were used as a loading control after stripping the previous antibodies.

2.4 *Growth inhibition experiments:*

HBL-1, OCI-ly3, and OCI-l0 cell lines were cultured in RPMI supplemented with 10% FBS and 10 mM HEPES. Cells were treated with nine exponentially increasing concentrations of DMSO, SCM-02-138, Selumetinib, or a combination. Cells were then treated with the cell titer Glo (cctGlo) which initially lysed the cells and then produced luminescence by attaching to the ATP molecules. This luminescence signal was detected using the protein quantification plate reader with the Gen5 program. These values were normalized to the DMSO control.

2.5 *Chou Talalay Analysis:*

Since growth inhibition experiments were performed in a fixed ratio manner, data was further imported into the Compusyn (29) software which created simulations based on the drug-drug interactions. Combination Index Values of less than 1 were considered to be synergistic.

2.6 *Statistics:*

Proteins were detected based on their molecular weight as following: 42kDa for MALT1, 92 kDa for pERK and actin, and ~42-44 kDa for ERK. These bands were then quantified using the Fiji Image J software. The data was imported to Microsoft Excel 2016 and each lane was normalized to actin with that ratio being divided by the ratio of protein to vehicle. These analyses were further imported into Graphpad Prism 8 for stastitical analysis.

3.0 Results

3.1 MALT1 expression increases in response to MALT1 protease suppression:

Western blots indicated that MALT1 protein expression increases over time in response to three different MALT1 protease inhibitors in both HBL-1 and TMD8 cells (Figure 2). MALT1 protein expression was stable after 1hr treatment, but there was a large peak observed at 48hrs and 96hrs in MALT1 protein expression. This manifests lymphomas' dependency upon MALT1 function, as degradation of MALT1 protease domain induces cytotoxicity among ABC DLBCL cell lines, lymphomas urgently signal new MALT1 synthesis in order to escape the cytotoxic arrest. Consequently, this upregulation makes ABC DLBCLs resistant to MALT1 protease inhibitors.

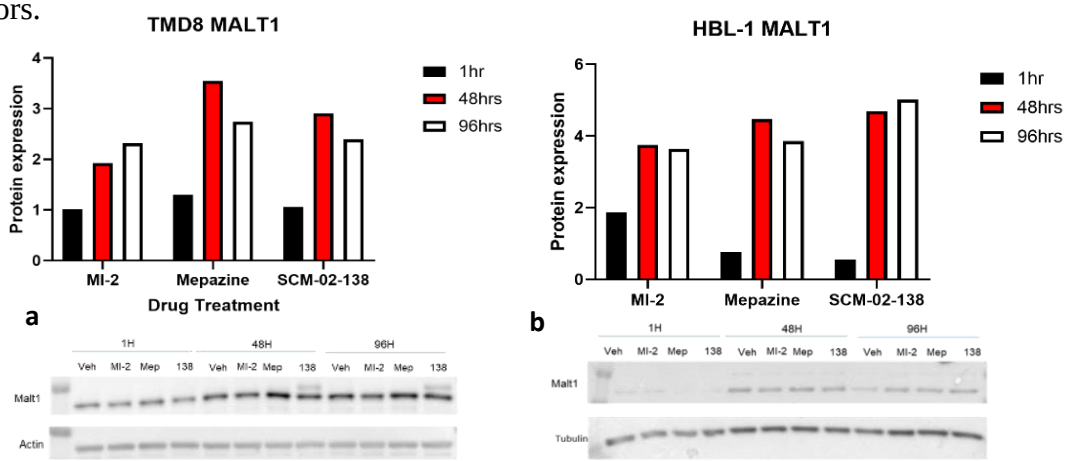


Figure 2: **MALT1 protease suppression induces new MALT1 synthesis.** All expressions have been normalized to the 1hr time point. **a)** In HBL-1 cells MALT1 expression is upregulated at 48 and 96hr time points, compared to the 1hr time point. **b)** Similarly, in TMD8 cells, MALT1 expression is also upregulated at 48hrs and 96hrs time points.

3.2 MAPK signaling activates to aid MALT1 upregulation and the cytotoxic escape

To discover the mechanisms that ABC DLBCLs employ in order to synthesize new MALT1s after proteolytic degradation of MALT1 protease domain, we turned towards identifying additional protein upregulations which could be observed in response to protease suppression. It was found that an alternative signaling pathway is activated in response to

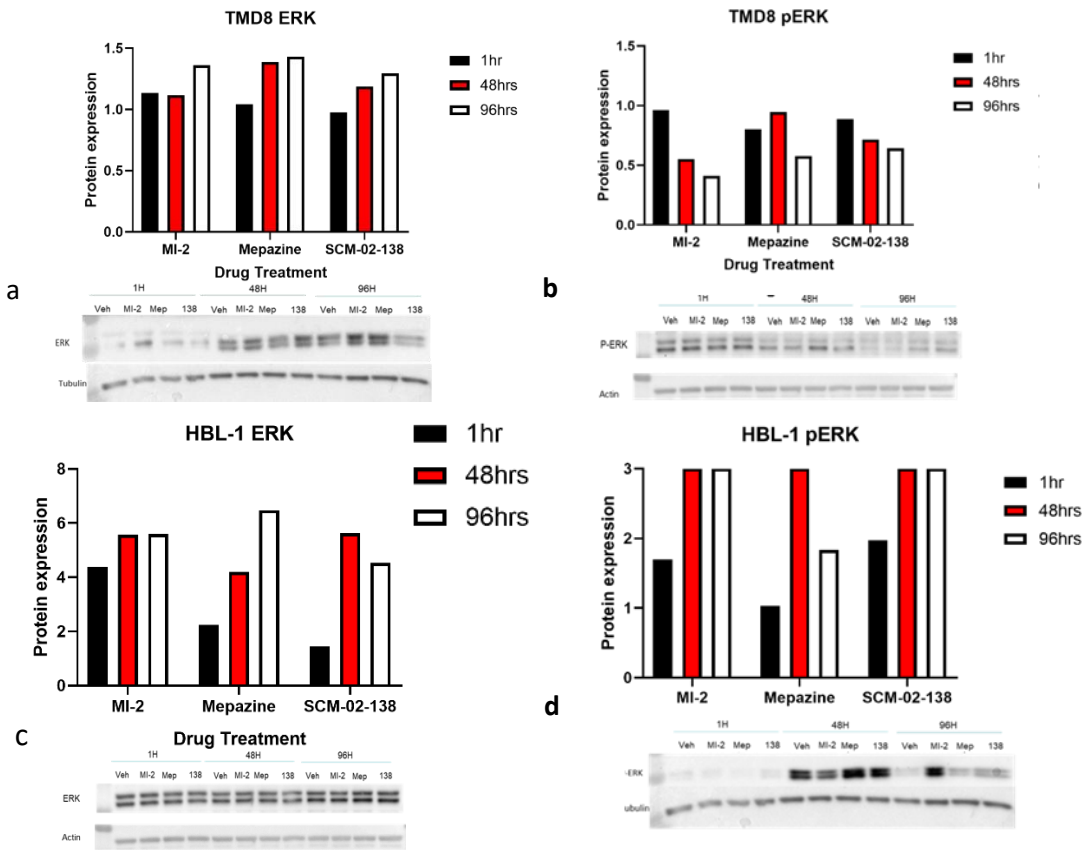


Figure 3: **MALT1 protease suppression upregulates MAPK signaling activation.** All expressions have been normalized to the 1hr time point. a) In HBL-1 cells, ERK expression is also upregulated at 48 and 96hr time points, compared to the 1hr time point. b) ERK phosphorylation does not follow any specific patterns., which enforces the need to perform phosphorylation specific experiments. C) ERK expression is upregulated in HBL1 cells at 48hrs and 96hrs time points. d) ERK phosphorylation also upregulates in HBL-1 cells after 48 and 96 hours of treatment.

MALT1 protease degradation (Figure 3). Blotting for ERK, a kinase downstream of the Mitogen Activated Protein Kinase (MAPK) signaling pathway, we discovered that ERK protein expression, as well as ERK phosphorylation increased in response to MALT1 protease inhibition at 48 and 96 hrs. This manifests that signaling cross-talk occurs between the Nf-κβ and MAPK signaling, through MALT1 and ERK respectively.

3.3 ERK inhibition downregulates MALT1 expression

HBL-1 cells were treated with DMSO, SCM-02-138 and the MAPK inhibitor Selumetinib for 4hrs, 24hrs, and 48hrs and blotted for ERK and MALT1. As expected, Selumetinib downregulated its target ERK in a time-oriented manner and more excitingly it also downregulated the MALT1 expression in the same time-oriented manner (Figure 4). Although ERK does not directly synthesize new MALT1s, our analysis suggests that ERK activation indirectly aids new MALT1 transcription in order to aid resistance.

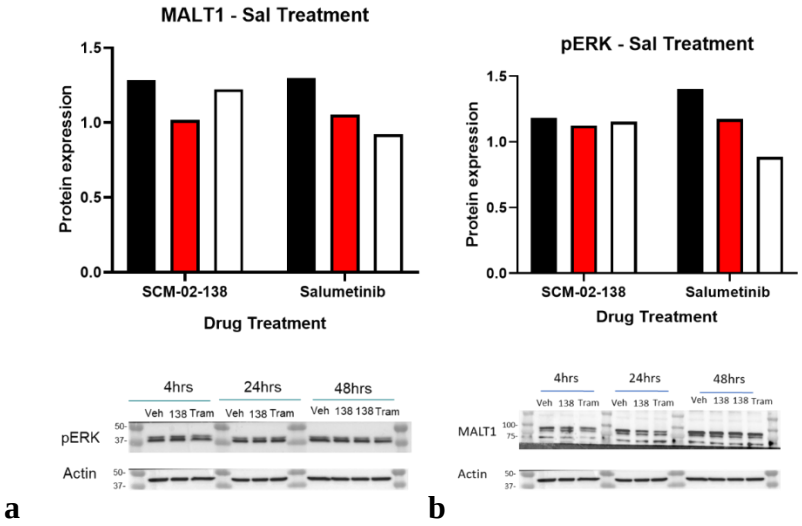


Figure 4: **Seluemetinib downregulates MALT1 protein expression.** All expressions have been normalized to the 4hrs time point. Selumetinib treatment downregulated its target p-ERK expression (b) as well as MALT1 (a), confirming the bridging of a network between MALT1 and ERK.

3.4 *Growth inhibition combinatorial inhibition suppresses B cell progression*

In order to discover if the novel MALT1 and MAPK signaling network has phenotypical implications in lymphoma's pathology, growth inhibitions were carried out. Indeed, we found that combinatorial inhibition resulted in greater apoptosis (80-85%) compared to sole treatment of Selumetinib or SCM-02-138 (Figure 5). SCM-02-138 provided ~70% inhibition, while Seumetinib provided ~35% inhibition, and the combination resulted in ~80-85% inhibition; it can thus be concluded that MALT1 protease inhibition in combination with Selumetinib strongly suppresses B cell pathogenesis.

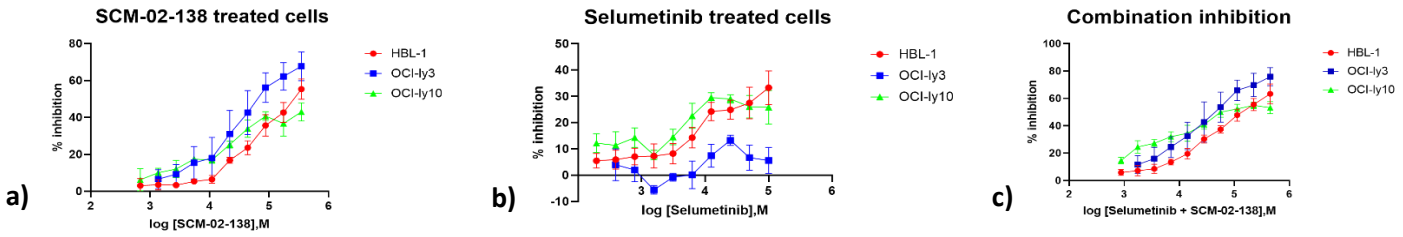
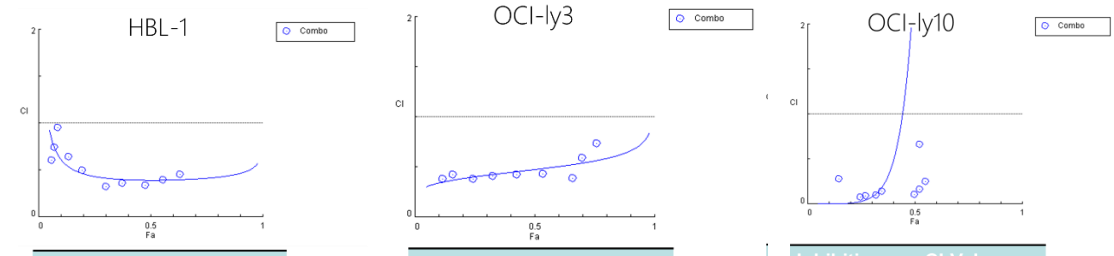


Figure 5: **Combinatorial suppression of MALT1 and ERK induces cytotoxicity.** Logarithmic measures were taken for each drug concentration and plotted against % inhibition; all values have been normalized to respective vehicle controls and represent ±SEM obtained from three experiments. **a)** SCM-02-138 treatment provided up to ~70% of inhibition, **b)** while Selumetinib treatment did not function well, providing non-specific inhibition of up to ~35%, whereas **c)** combinatory inhibition provided up to 80% inhibition.

3.5 Chou Talalay analysis suggest combinatorial inhibition is synergistic and reduces unfavorable toxicity

Fixed ratio Growth inhibition results were imported into the compusyn software which develop simulations based on drug to drug interactions. It was discovered that combinatorial inhibition of MALT1 via SCM-02-138 and MAPK via the Trametinib is significantly synergistic at being toxic to OCI-ly3 and HBL-1 cells but not the OCI-ly10 cells (Figure 6). This is a resultant of compusyn producing simulations based on drug-drug interactions and since ERK inhibitor did not have significant function in OCI-ly10 cells, this simulation was skewed.



a

Inhibition	CI-Value
97%	0.55131

b

Inhibition	CI-Value
97%	0.80777

c

Inhibition	CI-Value
97%	7199737

Figure 6: **Combinatorial suppression of MALT1 and is synergistically toxic.** CI values for both HBL1 **(a)** and OCI-ly3 **(b)** are below one which shows that combinatorial inhibition is synergistic. However, while the CI-values are below one for OCI-ly10 cells, lack of Selumetnib function skewed the comusyn produced simulation and thus the graphical representation does not match the data points.

4.0 Discussion:

This study set out to elucidate the mechanisms that drive ABC DLBCLs to escape the cytotoxic effects of MALT1 protease suppression. It was hypothesized that excessive genetic heterogeneity expressed among the ABC DLBCL clones allows MALT1 to deploy signaling

interactions which render its suppression inefficacious. The results elucidated that new MALT1s are synthesized in response to MALT1 protease domain suppression, allowing lymphomas to escape cytotoxicity and develop resistance to MALT1 protease inhibitors. Additionally, the uncovering of a novel signaling crosstalk interaction between the Nf-κβ and the MAPK pathways in response to MALT1 suppression suggests that MALT1 regulates lymphomagenesis through actively mediating ERK upregulation. These results are consistent with previous literature that recorded MALT1 and ERK interactions in oral carcinoma (23), but failed to elucidate the dynamics that regulate this signaling cross-talk. In addition to being the first study to define how lymphomas make MALT1 protease suppression inefficacious, this study is the first of its kind to observe the roles of MAPK pathway in aiding Nf-κβ oriented ABC DLBCL progression.

Reponses to MALT1 protease suppression extensively vary among ABC DLBCL cell lines *in vitro* and in primary patients, with samples harboring mutations downstream of MALT1 being the least sensitive (24). Hence, in this study we initially determined the genetic background of responders to MALT1 protease suppression and discovered that TMD8, HBL-1, and OCI-ly10 cells each demonstrate new MALT1 synthesis in response to MALT1 protease degradation. This led to our designation of a holistic approach that combined MALT1 and ERK inhibitors in order to simultaneously suppress the mechanisms ABC DLBCLs were deploying to escape therapy. Together, our data defines a novel and additively synergistic therapeutic benefit of MALT1 and MAPK oriented combination therapy.

Since the MAPK signaling pathway does not transcriptionally regulate MALT1 protein synthesis, it was vital that this study provides insight into mechanisms regulating new MALT1 production. MALT1 protein transcription is critically regulated via the mTOR pathway. Hence, in order to elucidate the mechanisms that are required for the mTOR pathway to obtain signaling for initiating MALT1 transcription, we hypothesized that MALT1 cleaves mTOR associated protein(s) that suppresses mTOR signaling under physiological tumor conditions.

Further interestingly, previous literature has established that MALT1 is an arginine specific kinase, as numerous MALT1 substrates have been discovered over the last years. In this study, we found that MALT1 cleavage site—the arginine—very often constitutes a serine before

the arginine and a glutamine after the arginine, suggesting that MALT1 cleaves at arginine sites with serine/glutamine interactions (Table 1).

Table 1. All sequencing data was collected from www.uniprot.org website and is publicly available. Among the above MALT1 substrates, while there is no single concrete trend, most MALT1 substrates tend to constitute a Serine before the Arginine and have a Glutamine after the Arginine, suggesting SRG might be the most common MALT1 cleavage site.

Protein Name	Known Cleavage Sequence
hBCL10	LPLR***SRT***VSRQ
mBCL10	LPLR***SRA***LSRQ
hRelB	PRLV***SRG***AASL
mRelB	PRLV***PRG***PASL
hA20	ALGA***SRG***EAYE
hCYLD	LAFM***SRG***VGDK
mCYLD	LAFM***SRG***VGDK
hRegnase-1	LPLV***PRG***GGTP
mRegnase-1	PPLV***PRG***GSTP
hRC3H1	TQLI***PRG***TDPS
mRC3H1	TQLI***PRG***TDPS
hRC3H2	SQLI***SR***STDST
mRC3H2	SQLI***PRG***TDSA
MALT1 (isoform 1)	SCHC***SR***TPDAF
LIMA alpha	LSPD***SR***ASSLS
hHOIL1	LTLQ***PRG***PLEP
mHOIL1	LTLQ***SRG***PLEP

We then turned towards identifying an amino acid site similar to that of Serine-Arginine-Glutamine (SRG) among the mTOR associated proteins and found three proteins with either SRG or an SR amino acid. These proteins were then chosen to be validated for being MALT1 substrates (Table 2).

Table 2. Potential MALT1 Substrates. mTORC1, S6 Kinase, and Ampk1 are proteins that are extensively associated with mTOR signaling. They were isolated because of expressing an SRG or SR amino acid site.

Protein Name	Potential Cleavage Sequence
hmTORC1	SISLSRGNNLQ
hs6k	RNAASRLGAGP
Ampk1	DEKESRRLFQQ

Western Blots were carried out by specifically suppressing the mTORC1, s6k, and Ampk1 and looking for the production ofc cleavage bands. However, none of the above proteins were discovered to be MALT1 substrates, asserting that ABC DLBCLs signal mTOR pathway via mechanisms other than the protease domain of MALT1 (Figure 7). It is therefore suggested that future research specifically suppresses the scaffolding domains of MALT1 in combination with the protease domain in order to define the biological interplay underlying new MALT1 synthesis in response to MALT1 protease degradation. Additionally, it is also suggested that future works explores biochemical interactions among the mTOR and MAPK signaling pathways, as B cells could be utilzing MAPK to signal mTOR and not protease domain of

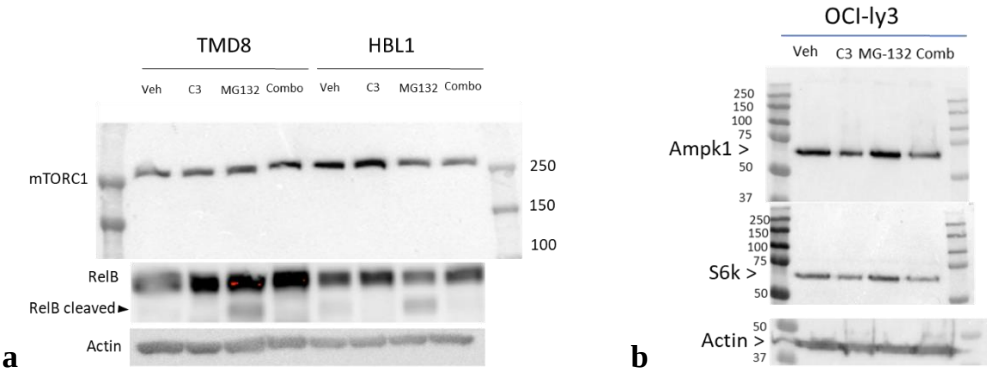


Figure 7. MALT1 protease does not cleave mTOR associated proteins. RelB is a known MALT1 substrate and was therefore used as a control. As seen in 7a, treatment with MG132 produced a cleavaed band for RelB. No other cleaved bands become became visible for other proteins, suggesting that MALT1 does not cleavae mTORC1, S6k, or Ampk1.

MALT1. Consequently, an extensive understanding of biology underlying MALT1 functions and a determination of the efficacy of combination therapies will provide basis for future studies to specifically determine the downstream pathways responsible for making ABC DLBCLs adapt MALT1 and ERK network and develop resistance.

In addition to ABC DLBCLs, BCR pathway and consequent MALT1 addiction is expressed among numerous subtypes of follicular lymphoma (27), mantle cell lymphoma (28) and chronic lymphocytic leukemia/lymphoma (29). It is plausible that certain neoplasm subtypes could be genetically isolated based on their MALT1 dependency and then be administered with combinatorial MALT1 and ERK inhibitors with hopes of eradicating chemotherapeutic agents and developing MALT1 oriented targeted therapies for some human malignancies.

Clinical translation of MALT1 inhibitors would be a vital step towards eradicating chemotherapeutic agents, but the efforts to stabilize MALT1 suppression via inhibiting the proteins that render its suppression inefficacious have yet been very few. It is imperative that mechanisms surrounding MALT1 functions are critically understood prior to investigation of MALT1 inhibitors with other modalities, including the emerging cancer immunotherapy.

5.0 Conclusion:

This study has shown that sensitivity of MALT1 protease inhibitors becomes inefficacious after ABC DLBCLs potentiate MALT1 protein synthesis in order to escape cytotoxicity. This escape is combined with upregulation of the MAPK signaling pathway which in-directly also aids the mechanisms responsible for new MALT1s, as demonstrated by attenuation in MALT1 expression in response to ERK inactivation. Combinatorial suppression of this network is additively synergistic at inducing cytotoxicity and provides a rationale to elaboratively develop combination therapies for MALT1-addicted tumors.

6.0 Acknowledgements:

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