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### Title

Targeting mTOR for the treatment of B cell malignancies

### Permalink

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### Journal

British Journal of Clinical Pharmacology, 82(5)

### ISSN

0306-5251

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### Publication Date

2016-11-01

### DOI

10.1111/bcp.12888

Peer reviewed



## Targeting mTOR for the treatment of B cell malignancies

Journal:	<i>British Journal of Clinical Pharmacology</i>
Manuscript ID	RT-00761-15.R1
Manuscript Type:	Review - Themed Issue
Date Submitted by the Author:	n/a
Complete List of Authors:	Lee, Jong-Hoon; UC Irvine, Molecular Biol Biochem Vo, Thanh-Trang; UC Irvine, Molecular Biol Biochem Fruman, David; UC Irvine, Molecular Biol Biochem
Key Words:	mTOR Protein, mTORC1, mTORC2, Leukemia, B-Cell, Lymphoma, B-Cell, Lymphoma, Non-Hodgkin, Molecular Targeted Therapy
Abstract:	<p>Mechanistic target of rapamycin (mTOR) is a serine/threonine kinase that functions as a key regulator of cell growth, division, and survival. Many hematologic malignancies exhibit elevated or aberrant mTOR activation, supporting the launch of numerous clinical trials aimed at evaluating the potential of single-agent mTOR-targeted therapies. While promising early clinical data using allosteric mTOR inhibitors (rapamycin and its derivatives, rapalogs) have suggested activity in a subset of hematologic malignancies, these agents have shown limited efficacy in most contexts. Whether the efficacy of these partial mTOR inhibitors might be enhanced by more complete target inhibition is being actively addressed with second generation ATP-competitive mTOR kinase inhibitors (TOR-KIs), which have only recently entered clinical trials. However, emerging preclinical data suggest that despite their biochemical advantage over rapalogs, TOR-KIs may retain a primarily cytostatic response. Rather, combinations of mTOR inhibition with other targeted therapies have demonstrated promising efficacy in several preclinical models. This review investigates the current status of rapalogs and TOR-KIs in B cell malignancies, with an emphasis on emerging preclinical evidence of synergistic combinations involving mTOR inhibition.</p>

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January 19, 2016

Editorial Office  
*British Journal of Clinical Pharmacology*

Dear Editor,

Thank you for providing reviewer comments and inviting a revised version of our review article, entitled "Targeting mTOR for the treatment of B cell malignancies". We are grateful for the positive feedback and the helpful suggestions. We have prepared a point-by-point reply that addresses all of the concerns raised by the referees as well as the comments of the editor. We have also prepared the revised figures according to the instructions. We hope that these changes are acceptable to the journal.

As requested by the Editorial Office, the name of the Principal Investigator is David Fruman.

Sincerely,

A handwritten signature in black ink that reads 'David Fruman'.

**Point by Point Response to Reviewers and Editor**

Lee, Vo and Fruman

British Journal of Clinical Pharmacology

RT-00761-15

Referee: 1

Minor suggestions:

1. Page 8: The authors describe studies of mTOR inhibitors (MTIs) with an emphasis upon studies performed in pediatric ALL. Consider citation addition & brief update for everolimus + chemo trials (AE Place ASH 2015 abstract #3765) for most current information.

*This has now been updated on page 6, lines 196-199.*

2. Reference #167 appears incomplete in citation and should be updated.

*This is now reference #172 and has been updated.*

3. Figure number is generous. Consider condensing into smaller number of figures, as they are very similar and individually highlight relatively minor points/data. In particular, consider deletion of Figure 5, which adds minimal information above that described in the main text.

*To reduce the overall figure number, we chose to remove former Figure 4. We felt that this was largely redundant with previous figures and the new points about PIM and MNK kinases were described sufficiently in the main text. Former Figure 5, now Figure 4, contains conceptual information that we feel is important for the reader to view in Figure format.*

4. Table 1: please define ORR in footnote and also on page 18/line 369 if not previously done. Consider changing Table 1 column title "Notes" to "Outcomes" or "Results." Consider adding column for class of inhibitor (e.g., MTI, TOR-Ki, etc.) after the drug names and updating title name.

*ORR has been defined in a footnote. It is also defined in the main text on line 213.*

*Table 1 column 6 title has been changed to "outcomes".*

*A column has been added for drug class.*

*We did not alter the main title of the Table, which seems to adequately describe the content:*

*"Published trials of mTOR-targeted therapies in ALL and NHL"*

Typographical corrections:

1. page 6, line 19: change "relapse" to "relapsed"

*Fixed. This is now on page 5, line 167.*

2. Please remove erroneous commas placed before non-independent clauses in compound sentences.

*We have checked the text thoroughly and attempted to remove unnecessary commas.*

Referee: 2

Minor points:

1. Highlighting or circling the mTORC1 and mTORC2 complexes in Figure 1 will make the figure more understandable.

*Boxes have been placed around mTORC1 and mTORC2 in a revised version of Figure 1.*

Provide a brief background explanation of how mTOR senses ATP and amino acids to maintain cellular homeostasis. Explain in detail the regulation sequence between mTORC1, 4EBP1 and eIF4E. Add an explanation of PDCD4 function in mTOR signaling.

*These requests are all addressed in the revised text, page 3, lines 59-72. We also briefly expanded the description of mTORC2 regulation on page 2, lines 45-48.*

2. Include FKBP12 along with rapalogs in Figure 2 and add an inhibition arrow directed at mTORC1.

*This has been added.*

3. The title "Rapalogs: partial mTORC1 inhibitors" (line 91) should be replaced with "Rapamycin and Rapalogs: partial mTORC1 inhibitors"; the subtitle "Rapalogs in B-ALL" (line 103) should be replaced with "Rapamycin and Rapalogs in B-ALL".

*Done.*

4. Add reference after "...in vitro or in xenograft models" (line 110).

*Three new references have been added here. Now line 157.*

Executive Editor's comments:

Executive Editor

Comments to the Author:

Please revise your manuscript according to the comments of the reviewers. Thank you.

*Done, please see above.*

Comments Regarding Format from the Editorial Office:

1) The submission guidelines for the British Journal of Clinical Pharmacology have changed slightly. We now request a brief statement in the cover letter which clearly states the name of the Principal Investigator.

*This statement have been added to the cover letter.*

2) Abstract: A structured summary must appear before the Introduction and include the following headings: Aim(s), Methods, Results (some numerical data, including confidence intervals on differences, when appropriate, must be included), Conclusions

The summary should be a maximum of 250 words. Please ensure the summary within the manuscript matches the one requested in the separate box during submission.

*This submission is a review article, not a research study. Therefore there were no aims, methods or results to include in a structured summary. We have included an abstract of 176 words. We believe this might have been overlooked because it was labeled “summary” rather than “abstract”, and because there was no header for the next section. We have now added “Introduction” as a header to the section immediately following.*

3) Please amend your conflict of Interest Statement. The statement should follow the format used by the British Medical Journal (BMJ) and must contain all three of the statements included below:

“All authors have completed the Unified Competing Interest form at [www.icmje.org/coi\\_disclosure.pdf](http://www.icmje.org/coi_disclosure.pdf) (available on request from the corresponding author) and declare: no support from any organisation for the submitted work OR [author initials] had support from [name of organisation] for the submitted work; no financial relationships with any organisations that might have an interest in the submitted work in the previous 3 years OR [author initials] [had specified relationship] with [name of organisation] in the previous 3 years; no other relationships or activities that could appear to have influenced the submitted work OR [initials of relevant authors] [had specified relationships or activities of this type]”  
*An appropriate Conflict of Interest statement has been added after the acknowledgements, before the References. All authors have completed the Unified Competing Interest form.*

4) Title Page:

– title should give an informative and accurate indication of the content of the paper. It should be no longer than 150 characters (including spaces);  
*This was already present; no changes have been made. The title is 55 characters, including spaces.*

– a running head of no more than 75 characters, including spaces

*The title page now provides a running title of 38 characters:*

*“mTOR inhibitors in B cell malignancies”*

*Please note that the main title is less than 75 characters so this could be used as well.*

– keywords (these are used to identify potential referees and as indexing terms)

*These were already present; no changes have been made.*

– the word count, excluding the title page, summary, references, tables, and figures

– the numbers of tables and figures.

*These have been added.*

5) Figure Files: Please upload files as GIF, JPEG, TIFF or PICT files [images >300dpi and graphs >600dpi]. PDFs and PPTs are not accepted. This is because should your manuscript be accepted for publication the Production Editor will need to edit the files in order to prepare them for print. *Figures in the request resolution have been provided as .jpg files*

Tables: Please upload tables as DOC or EXCEL files which are editable. Do not embed the tables as pictures. *The Tables are uploaded as .docx files in landscape view.*

1                                   **Targeting mTOR for the treatment of B cell malignancies**

2  
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15  
16                                  Running head: mTOR inhibitors in B cell malignancies

17  
18                                  Keywords: mTOR, rapamycin, rapalogs, TOR-KIs, leukemia, lymphoma

19  
20                                  Word count: 4,574

21                                  Figure count: 4

22                                  Table count: 2

## 23 Abstract

24 Mechanistic target of rapamycin (mTOR) is a serine/threonine kinase that functions as a key  
25 regulator of cell growth, division, and survival. Many hematologic malignancies exhibit elevated  
26 or aberrant mTOR activation, supporting the launch of numerous clinical trials aimed at  
27 evaluating the potential of single-agent mTOR-targeted therapies. While promising early clinical  
28 data using allosteric mTOR inhibitors (rapamycin and its derivatives, rapalogs) have suggested  
29 activity in a subset of hematologic malignancies, these agents have shown limited efficacy in  
30 most contexts. Whether the efficacy of these partial mTOR inhibitors might be enhanced by  
31 more complete target inhibition is being actively addressed with second generation ATP-  
32 competitive mTOR kinase inhibitors (TOR-KIs), which have only recently entered clinical trials.  
33 However, emerging preclinical data suggest that despite their biochemical advantage over  
34 rapalogs, TOR-KIs may retain a primarily cytostatic response. Rather, combinations of mTOR  
35 inhibition with other targeted therapies have demonstrated promising efficacy in several  
36 preclinical models. This review investigates the current status of rapalogs and TOR-KIs in B cell  
37 malignancies, with an emphasis on emerging preclinical evidence of synergistic combinations  
38 involving mTOR inhibition.

## 40 Introduction

### 41 *The mTOR Signaling Pathway*

42 mTOR is a serine/threonine kinase that functions as a master regulator of cell growth,  
43 proliferation, metabolism, and survival. mTOR is active in two distinct multi-protein complexes  
44 (mTORC1 and mTORC2) that are characterized by the defining subunits RAPTOR and  
45 RICTOR respectively [1,2]. Each complex is differentially regulated and has a distinct set of  
46 substrates (Figure 1). Activation of mTORC2 is incompletely understood, but has recently been  
47 shown to be dependent on the generation of PI(3,4,5)P<sub>3</sub> by phosphoinositide 3-kinase (PI3K)  
48 [3]. Upon activation mTORC2 functions to amplify the activity of AKT, a key oncogene involved

49 in cell survival and metabolism [4,5]. On the other hand, mTORC1 activation is coordinately  
50 regulated by growth factor signals (i.e. from the PI3K/AKT pathway), nutrient availability (amino  
51 acids), and cellular energy status (ATP levels). Under conditions of low nutrients, amino acid  
52 sensors (such as SLC38A9 [6,7]) suppress mTORC1 activation. Similarly, under conditions of  
53 low energy (low ATP), 5' AMP-activated protein kinase (AMPK) can also suppress mTORC1  
54 activation [8]. This multifaceted regulation ensures that the cell is at an appropriate bioenergetic  
55 state to support cell growth and division [9,10] (Figure 1).

56 Upon activation, mTORC1 promotes key biosynthetic pathways including translation,  
57 transcription, and lipogenesis, while suppressing apoptotic and autophagic processes [11,12].  
58 The most well-characterized downstream targets of mTORC1 are the p70 ribosomal-S6 kinases  
59 (S6Ks) and eukaryotic initiation factor 4E (eIF4E) binding proteins (4E-BPs). Phosphorylation of  
60 S6Ks induces its activity, which is critical for lipid and ribosome biogenesis pathways and  
61 promotes translation via suppression of PDCD4 and activation of eIF4B [13,14]. In contrast,  
62 phosphorylation of 4E-BPs suppresses their ability to inhibit eIF4E, which promotes translation  
63 initiation [15]. Together, these effectors coordinately increase protein synthesis rates, a process  
64 whose dysregulation is a central driving mechanism in cancer [16,17]. Importantly, hyper-  
65 activating mutations in mTOR itself have been identified in many cancers and further indicates  
66 the importance of mTOR activity to tumorigenesis [18].

67

#### 68 *Evidence of mTOR activation in B-ALL and NHL*

69 Aberrant activation of mTOR is frequently associated with poorer prognosis and has been  
70 well described in B cell malignancies including B cell acute lymphoblastic leukemia (B-ALL) and  
71 non-Hodgkin's lymphoma (NHL). Given that mTOR is a convergence point for many distinct  
72 signaling pathways, there are many mechanisms by which it may become inappropriately  
73 activated (Figure 2). In B-ALL, the most common mode is through activation of upstream  
74 kinases. For example, the Philadelphia chromosome (Ph<sup>+</sup>), characterized by the BCR-ABL

75 translocation, induces robust activation of several parallel pathways leading to mTOR activation.  
76 Similarly, genomic profiling has recently identified a Ph-like subset of B-ALL, which exhibits a  
77 similar kinase activation signature to that of Ph<sup>+</sup> B-ALL. Notably, these mutations are strongly  
78 associated with poorer outcomes in both children and adults [19-22]. Empirical evidence has  
79 also shown a direct correlation between AKT and/or mTOR activation and poor prognosis in  
80 patients with pediatric and adult B-ALL [23-25].

81 Among NHL subtypes, activation of mTOR is consistently a reliable indicator of more  
82 aggressive disease and poorer prognosis [26-30]. Similar to B-ALL, activation of mTOR follows  
83 through direct mutations in key upstream pathways. In mantle cell lymphoma (MCL),  
84 amplification of *PIK3CA* (the gene encoding the catalytic subunit of PI3K) and/or PTEN loss (the  
85 negative regulator of PI3K activity) have been observed in a large fraction of primary tissue  
86 samples [31]. In diffuse large B cell lymphoma (DLBCL), activation may be similarly achieved  
87 via mutations in *PIK3CA* [32,33] or chronic B cell receptor activation [34]. In follicular lymphoma  
88 (FL), mTOR is aberrantly activated by way of PKC $\zeta$  or Syk kinases [35-38]. Collectively, these  
89 data highlight the impact of elevated mTOR activity on patient outcomes, and provide a solid  
90 rationale for the use of mTOR-targeted therapies in these B cell malignancies.

91

## 92 **Rapamycin and Rapalogs: partial mTORC1 inhibitors**

### 93 *Mechanism of action*

94 Upon entry into a cell, rapamycin binds to FKBP12 forming a complex that potently and  
95 selectively suppresses mTORC1 kinase activity by limiting substrate access to the active site  
96 [39,40]. Importantly, the rapamycin-FKBP12 complex cannot bind to mTORC2 [2,41], though in  
97 some cases prolonged exposure may limit the assembly of mTORC2 [42]. In this manner,  
98 rapamycin behaves as a highly potent and selective inhibitor of mTORC1 (Figure 3). However,  
99 poor solubility and pharmacokinetics spurred the development of rapamycin analogs (termed  
100 rapalogs) for oral dosing in cancer patients [43]. Most notable among these rapalogs are

101 temsirolimus (CCI-779, Wyeth Pharmaceuticals [44]), everolimus (RAD001, Novartis  
102 Pharmaceuticals [45]), and ridaforolimus (AP23573, Merck and ARIAD Pharmaceuticals [46]).

103

#### 104 *Rapamycin and Rapalogs in B-ALL*

105 Early testing with rapamycin unveiled potent anti-proliferative efficacy in several preclinical  
106 models of ALL. In an E $\mu$ -RET model of murine B-pre ALL, rapamycin as a single agent potently  
107 inhibited proliferation of leukemia cells both *in vitro* and *in vivo* [47,48]. Similar efficacy was later  
108 observed in models of Ph<sup>+</sup> B-ALL [49,50] as well as in Ph-like B-ALL driven by JAK pathway  
109 mutations or CRLF2 rearrangement [51]. Rapalogs also demonstrated marked preclinical  
110 efficacy in primary human ALL samples grown *in vitro* or in xenograft models [50-52]. Notably,  
111 rapamycin demonstrated single-agent cytotoxicity in primary pediatric ALL samples and  
112 sensitized cells to doxorubicin *in vitro* [52]. Both everolimus and temsirolimus have shown  
113 similar efficacy in xenograft models of adult and pediatric primary human ALL as single agents  
114 [53] and in combination with chemotherapy [54,55].

115 Clinically, rapamycin as a single agent exhibited no dose-limiting toxicities, but had  
116 lackluster efficacy compared to standard chemotherapeutic options (Table 1). In an early trial,  
117 rapamycin yielded stable disease in only three out of nine pediatric patients with relapsed ALL  
118 [56]. As a result, several trials have been launched to determine whether rapalogs can combine  
119 safely and effectively with standard chemotherapies. An early pilot trial combining rapamycin  
120 with glucocorticoids in relapsed ALL patients found that rapamycin effectively reduced the anti-  
121 apoptotic protein MCL-1 in various patients. This promising outcome suggested that rapamycin  
122 might sensitize ALL cells to apoptosis-inducing drugs. Indeed, in another study combining  
123 temsirolimus with intensive multi-drug re-induction therapy (dexamethasone, mitoxantrone,  
124 vincristine, and PEG-asparaginase) in relapsed childhood ALL yielded complete response in  
125 seven of sixteen patients, of which three had less than 0.01% minimal residual disease (MRD)  
126 by the end of treatment [57]. However, a separate trial evaluating everolimus combined with

127 intensive chemotherapy (hyper-CVAD) in relapsed B-ALL yielded complete remission rates that  
128 were similar to standard salvage chemotherapies (~35%) [58-60]. These trials highlight how the  
129 efficacy of rapalogs seem to be dependent on which chemotherapeutics are used, warranting  
130 further investigation.

131 A key question that remains to be answered is whether rapalogs combined with  
132 chemotherapy will demonstrate acceptable toxicity profiles. In the aforementioned trial  
133 combining temsirolimus with re-induction chemotherapy, the treatment was associated with  
134 unacceptable toxicities including severe infections that led to one death due to sepsis [57].  
135 However, a recent multi-center study testing the combination of everolimus with prednisone,  
136 vincristine, PEG-asparaginase and doxorubicin demonstrated that the combination was well  
137 tolerated in pediatric patients with first relapse [61]. Further trials are being performed, including  
138 an expansion of the aforementioned trial as well as one testing the safety of temsirolimus with  
139 less intensive re-induction (etoposide and cyclophosphamide; NCT01614197). Together, these  
140 results show that rapalogs have some potential in combination therapy, but an effective and  
141 tolerable regimen in B-ALL has yet to be identified. Moving forward, it will be important to  
142 identify which chemotherapeutics are best combined with rapalogs and whether modifications to  
143 the dose and/or schedule may alleviate dose-limiting toxicities.

144

#### 145 *Rapalogs in NHL*

146 Similar to B-ALL, preclinical testing of rapalogs in NHL revealed promising cytostatic effects  
147 both *in vitro* and *in vivo*, yet clinical responses were limited in most contexts. For example, in  
148 MCL, FL, and DLBCL, rapamycin potently suppressed the proliferation of cell lines and primary  
149 patient cells *in vitro* [62-66]. However, the clinical use of rapalogs has only made progress in  
150 MCL where responses to standard chemotherapies are limited (Table 1). In phase II trials of  
151 relapsed MCL, single agent administration of either temsirolimus or ridaforolimus yielded overall  
152 response rates (ORR) of 38% [67] and 33% [68] respectively. Notably, a subsequent phase II

153 trial using a 10-fold lower dose of temsirolimus revealed that similar responses could be  
154 obtained with lower toxicity [69]. Based on these results, a randomized phase III trial was  
155 conducted. Strikingly, the ORR and progression free survival were significantly higher in  
156 patients treated with temsirolimus compared to investigator's choice agent. These results  
157 ultimately led to approval for temsirolimus as a single agent therapy for relapsed/refractory MCL  
158 in Europe [70]. A subsequent phase II trial has also been completed combining temsirolimus  
159 with rituximab in relapsed/refractory MCL. Despite demonstrating higher response rates than  
160 single agent temsirolimus, the combination was also associated with higher toxicities including  
161 thrombocytopenia and neutropenia in a significant fraction of patients [71]. Rapalog  
162 monotherapy has also elicited responses in a subset of patients with other NHL subtypes. In a  
163 phase II trial of everolimus in relapsed lymphoma, the ORR in DLBCL was 30% (14/47) and  
164 38% (3/8) in FL [72]. Similar results were seen with temsirolimus where the ORR was 28% for  
165 DLBCL and 53% in FL [73]. While these studies highlight that rapalogs have some activity, the  
166 availability of better therapeutic options in both DLBCL and FL has limited the clinical progress  
167 of rapalogs in these diseases. Thus, across NHL subtypes it will be important to determine  
168 whether the addition of rapalogs to standard chemotherapy can provide additional benefit to  
169 patients, without increasing toxicities.

170

171 *Outlook:*

172 Overall, despite showing promising preclinical activity in hematologic malignancies, rapalogs  
173 have only gained regulatory approval for use in one disease setting (MCL) where standard  
174 chemotherapies have limited efficacy. A major issue is that rapalogs given as single agents tend  
175 to elicit primarily cytostatic responses in hematologic malignancies [62,63,66,74]. Clinically, the  
176 lack of inherent cytotoxicity is problematic since discontinuation of treatment may permit tumor  
177 cell regrowth [75-77]. While continued treatment may combat this issue, whether rapalogs at  
178 anti-leukemic doses will be safe for long-term use also remains to be seen. Clinical evidence of

179 several toxicities including thrombocytopenia, mucositis, and hyperlipidemia suggests that  
180 prolonged treatment will be difficult to manage [43]. Alternatively, combinations with  
181 chemotherapy are actively being investigated and may reposition rapalogs as an adjuvant to  
182 improve chemotherapeutic responses. On this note, it is important to point out that the cytostatic  
183 activity of rapalogs will likely limit its potential to combine with certain chemotherapies,  
184 necessitating the identification of cytotoxic drugs that will synergize with rapalogs productively  
185 while maintaining acceptable tolerability.

186 While rapalogs provided proof-of-concept for effective mTOR targeted anti-cancer therapies,  
187 they exhibit many unfavorable biochemical properties that may also limit their clinical potential.  
188 Most notably, failure to suppress mTORC2 kinase activity allows maintained survival signaling  
189 through AKT and other related kinases. This issue is exacerbated by the existence of a negative  
190 feedback loop downstream of mTORC1 (Figure 3). Selective inhibition of mTORC1 induces  
191 robust feedback activation of upstream PI3K/AKT and MAPK pathways allowing cancer cells to  
192 escape from the effects of rapamycin [57,78-82]. Additionally, rapalogs are known to  
193 incompletely inhibit the phosphorylation of a subset of mTORC1 substrates (Figure 3). Despite  
194 restricting access to the active-site, rapalog-induced suppression of 4E-BP1 phosphorylation is  
195 refractory to long-term treatment compared to phosphorylation of p70S6K [83]. The cause of  
196 this differential sensitivity has recently been attributed to distinct substrate sequences near the  
197 phosphorylation sites [84]. This incomplete suppression of mTORC1 may significantly impact  
198 the anti-cancer potential of rapalogs as sustained activation of eIF4E is known to promote  
199 oncogenesis [85]. Consequently, sustained 4E-BP phosphorylation may allow cancer cells to  
200 escape from rapamycin-induced cell cycle arrest [86]. Thus, more complete mTOR inhibition  
201 may be required to elicit more promising clinical responses.

202

203 **TOR-KIs: complete mTORC1/2 inhibitors**

204 The timely development of mTOR kinase inhibitors (TOR-KIs) directly addressed the  
205 biochemical disadvantages of rapalogs. By competing with ATP for binding to the mTOR active  
206 site, not only do TOR-KIs more completely block mTORC1 substrate phosphorylation (namely  
207 4E-BPs), but they also inhibit mTORC2 activity [87,88]. This results in reduced phosphorylation  
208 of AKT at Ser473 (Figure 3), dampening the feedback activation of PI3K/AKT that is known to  
209 limit rapalog efficacy [89-91]. It is important to note that by competing with ATP, TOR-KIs are  
210 capable of inhibiting several kinases at higher doses, including the structurally related protein,  
211 PI3K. Conversely, several compounds that are often used pre-clinically as PI3K inhibitors  
212 (wortmannin, LY294002) directly inhibit mTORC1 and mTORC2 at similar concentrations. Thus,  
213 it is important to fully understand the pharmacologic properties of ATP-competitive mTOR and  
214 PI3K inhibitors when interpreting their preclinical and clinical efficacy.

215 Several structurally distinct mTOR-selective inhibitors have been reported and tested in  
216 models of B cell malignancies. Most notable among them are PP242 [88], Torin1 [87], Ku-  
217 0063794 [92], AZD8055 [93], AZD2014 [93], MLN0128 (previously INK128 [94]), and CC-223  
218 [95]. In preclinical testing, these TOR-KIs proved superior to rapalogs in terms of cytostatic and  
219 cytotoxic potential. For example, in a mouse model of AKT-driven lymphangiogenesis, PP242  
220 strongly suppressed both 4E-BP1 phosphorylation and tumor growth compared to rapamycin  
221 [96]. These findings were also recapitulated *in vitro* using leukemia and DLBCL cell lines where  
222 TOR-KIs had a greatly improved biochemical effect on downstream 4E-BP phosphorylation [97-  
223 99].

224 Despite the broader biochemical impact of TOR-KIs over rapalogs, whether complete mTOR  
225 kinase inhibition is sufficient to elicit cytotoxic responses is yet to be established. Two reports of  
226 structurally distinct TOR-KIs in B-ALL demonstrated that mTOR kinase inhibition was sufficient  
227 to induce apoptosis in B-ALL cell lines compared to rapamycin [100,101]. However, in both  
228 studies, apoptosis was only observed at doses of TOR-KI that greatly exceed what was needed  
229 to fully suppress mTOR kinase activity as measured by western blot. At lower doses that still

230 fully suppress mTOR activity, our lab has found that both AZD8055 and MLN0128 maintain a  
231 primarily cytostatic response profile (that is greater than rapalogs) [98,102-104]. Notably, low  
232 doses of PP242 were sufficient to kill murine bone marrow cells immortalized by p190-BCR-ABL  
233 [99], suggesting that fully transformed B-ALL cells with additional oncogenic lesions may  
234 respond differently to mTOR inhibition. Thus, it remains unclear whether TOR-KIs will be  
235 effective in B-ALL or NHL as a single agents at doses that are highly selective for mTOR kinase  
236 activity.

237 Early clinical trials have suggested that while TOR-KIs are more effective than rapalogs at  
238 suppressing tumor growth, they may also be less tolerable [78]. A single agent tolerability test of  
239 AZD2014 showed dose-limiting toxicities that were similar to rapalogs including mucositis and  
240 fatigue [105]. Both CC-223 and MLN0128 also presented similar toxicities, but hyperglycemia  
241 also occurred and necessitated close monitoring of patient blood [106,107]. Several additional  
242 clinical trials are currently in progress to address the efficacy and tolerability of TOR-KIs and are  
243 summarized in **Table 2**. However, a key question is to investigate whether TOR-KIs will retain  
244 anti-cancer efficacy at lower doses that minimize these toxicities. While it is likely that lowering  
245 the dose of TOR-KIs may improve their tolerability, it will also impinge on their ability to fully  
246 suppress mTOR kinase activity. Moving forward, it may be important to determine whether  
247 these potentially suboptimal doses, which only partially inhibit mTOR, will be more effective than  
248 clinically tolerable doses of rapalogs, which potently inhibits phosphorylation of some, but not  
249 all, mTORC1 substrates.

250

### 251 **Emerging Combinations with mTOR Inhibitors:**

252 Recent research efforts have been dedicated to identifying promising combinations that can  
253 synergistically kill cancer cells. The rationales behind these emerging combinations can be  
254 loosely categorized into two broad groups. The first approach seeks to exploit known resistance  
255 mechanisms to mTOR inhibition; either by targeting feedback pathways or using apoptosis-

256 sensitizing agents. The second approach seeks to evaluate the potential of mTOR inhibitors as  
257 adjuvants to augment the effects of other agents targeting known oncogenic drivers. While both  
258 approaches have yielded several promising combinations, whether they can be translated to  
259 significant clinical responses with acceptable toxicity still remains to be determined.

260

261 *Combinations targeting resistance mechanisms*

262 Targeting parallel and downstream pathways

263 As with all targeted therapies, an understanding of how cells maintain survival in the  
264 presence of mTOR inhibitors has been crucial to the identification of promising combinations.  
265 Currently, there are several known acquired and *de novo* mechanisms of resistance to mTOR-  
266 targeted therapies. For example, in addition to feedback activation of PI3K/AKT, mTORC1  
267 inhibition may also activate the parallel MAPK/ERK pathway in B-ALL. In a similar fashion,  
268 PI3K/AKT/mTOR inhibition can also induce up-regulation of receptor tyrosine kinases (RTKs)  
269 leading to resistance in some solid tumors [108]. In agreement with these induced resistance  
270 mechanisms, the addition of MAPK inhibitors and RTK inhibitors have demonstrated  
271 significantly more efficacy in combination with both rapalogs and TOR-KIs in preclinical settings  
272 [80,109,110]. However, in other instances resistance to mTOR inhibition may be a result of  
273 sustained downstream effector activity, particularly cap-dependent translation. For example, our  
274 laboratory has noted resistance to TOR-KIs in DLBCL cell lines lacking expression of 4E-BPs  
275 [98] or over-expressing eIF4E [111]. Furthermore, recent evidence has indicated that PIM and  
276 MNK kinases can maintain cap-dependent translation downstream of mTORC1 inhibition [112].  
277 In these situations, targeting cap-dependent translation indirectly using combinations of PIM or  
278 MNK inhibitors with TOR-KIs has shown cytotoxic activity in AML cell lines [113,114] as well as  
279 in cutaneous T cell lymphoma cell lines *in vitro* [115]. Additional work is required to evaluate the  
280 potential of directly targeting the cap-dependent translation initiation machinery. It is likely that

281 other mechanisms of resistance will arise as our experience with mTOR inhibitors increases,  
282 and these may ultimately support the study of additional combinations.

283 While clinical data regarding the efficacy of these combinations in B cell malignancies has  
284 not reached maturity, similar combinations have been successfully deployed in non-hematologic  
285 malignancies. For example, inhibition of the upstream tyrosine kinase, HER2, significantly  
286 improved the efficacy of mTORC1/2 inhibition in patients with refractory breast cancer  
287 compared to single agent treatment [116]. Similarly, combinations of PI3K/AKT/mTOR and  
288 Ras/MAPK/ERK pathway inhibition yielded improved response rates in patients with advanced  
289 refractory solid tumors, but did so at the cost of significantly higher toxicities [117]. Collectively  
290 these studies highlight the potential of using mTOR inhibitors in combination with agents  
291 targeting known resistance pathways to mTOR inhibition or as an adjuvant therapy to augment  
292 the effects of other rational targeted therapies. However, it will be important to determine  
293 whether these combinations targeting multiple key survival pathways will remain selective for  
294 cancer cells as toxicity will be a major concern.

295

#### 296 Targeting apoptosis

297 Another straightforward approach to directly enhance the apoptotic potential of mTOR  
298 inhibition is to target the pro-survival BCL-2 family proteins. Apoptosis is regulated through  
299 dynamic and competitive binding interactions between anti-apoptotic proteins (e.g. BCL-2, BCL-  
300 XL, BCL-w, and MCL-1) and pro-apoptotic sensitizers (e.g. BAD, PUMA, and NOXA), activators  
301 (e.g. BIM and BID), and effectors (BAX and BAK) (Figure 4). While mTOR inhibition is known to  
302 suppress survival signaling through both mTORC1 (e.g. MCL-1 expression [96]) and AKT (e.g.  
303 inhibition of BAD and down-regulation of BIM [118,119]), TOR-KIs are insufficient to induce  
304 apoptosis through this pathway. Thus, a simple approach would be to use antagonists of the  
305 pro-survival proteins to disrupt their binding capacity, and subsequently lower the threshold for  
306 BIM to activate BAX/BAK-mediated MOMP and apoptosis [120].

307 ABT-737, and its orally bioavailable analog, ABT-263, represent the most potent and  
308 selective small molecule inhibitors of BCL-2 and BCL-X<sub>L</sub>. Both of these compounds  
309 demonstrated remarkable cytotoxic potential that was significantly enhanced when combined  
310 with mTOR inhibitors in DLBCL [121], FL [122], AML [123], and B-ALL [124]. However, due to  
311 on-target toxicity associated with BCL-X<sub>L</sub> inhibition [125], a more promising clinical candidate is  
312 ABT-199 [126]. ABT-199 is a selective inhibitor of BCL-2 and has elicited substantial clinical  
313 responses in patients with CLL as a single agent [127], leading to its designation as a  
314 breakthrough therapy for CLL patients with a 17p deletion (p53). Importantly, we and others  
315 have recently reported that ABT-199 synergizes with mTOR inhibition comparably to dual BCL-  
316 2/BCL-X<sub>L</sub> inhibitors [104,128], suggesting that the rationale established using first generation  
317 BCL-2 antagonists will hold true for ABT-199. However, a key concern is whether the addition of  
318 TOR-KIs to BCL-2 antagonists will enhance its toxicity towards non-cancer cells. In an effort to  
319 address this question, our lab has recently demonstrated that the combination does not  
320 synergize to kill peripheral blood mononuclear cells obtained from normal healthy donors [104].  
321 Further work must be done to ensure that these potent combinations will maintain favorable  
322 tolerability when administered to patients.

323

324

325 *mTOR inhibition as an adjuvant*326 Targeting oncogenic drivers

327 In contrast to targeting resistance mechanisms, others have found that combining  
328 oncogene-targeted therapies with mTOR inhibition also holds promise in B cell malignancies.  
329 For example, in Ph+ B-ALL driven by the BCR-ABL translocation, both rapamycin and PP242  
330 strongly synergized with imatinib to suppress leukemia growth [99]. Similarly, in  
331 myeloproliferative disorders characterized by JAK2 mutations, combinations of TOR-KIs or  
332 rapalogs with JAK2 inhibitors synergistically killed cells whereas single-agent treatments were

333 primarily cytostatic [129,130]. In the activated B cell like (ABC) subtype of DLBCL, which is  
334 driven by sustained activation of the B cell receptor (BCR) [34], inhibition of the downstream  
335 kinase, Bruton's tyrosine kinase (BTK), also synergized strongly with PI3K/AKT/mTOR inhibitors  
336 [131]. However, the limitations of this approach are also becoming apparent. In particular, the  
337 germinal center B cell-like (GCB) DLBCL subtype is unresponsive to combinations of BTK and  
338 mTOR inhibitors likely because BCR activation is not an oncogenic driver in this setting [132].  
339 More alarmingly, in some cases the addition of mTOR inhibitors may antagonize the effects of  
340 other agents either through suppression of proliferation or through induction of autophagy  
341 [133,134]. Studies like these serve as powerful reminders that a sound biological understanding  
342 supporting the use of these combinations must precede their clinical use.

343

#### 344 Targeting histone deacetylases (HDACs)

345 HDAC inhibitors are another promising class of drug that may benefit from the addition of  
346 mTOR inhibitors. In addition to modulating histone function and gene expression, HDACs also  
347 regulate the activity of non-histone proteins with relevance to B cell cancers (e.g. STAT, Hsp90,  
348 and FOXO) [135-138]. Importantly, mutations in genes regulating protein acetylation have been  
349 described in both B-ALL and NHL. For example, mutations in the CREBBP histone  
350 acetyltransferase (HAT) domain have been identified in a subset of patients with relapsed  
351 pediatric B-ALL where it may confer glucocorticoid resistance [139]. Similar mutations in HAT  
352 activity were identified as frequent mutations in both FL and DLBCL where their inactivation  
353 promotes aberrant up-regulation of BCL-6, a protein known to promote B cell malignancies  
354 [140-142]. Given the pervasive importance of protein acetylation, it is unsurprising that HDAC  
355 inhibitors have elicited promising responses in various leukemias and lymphomas. For example,  
356 in lymphomas with a t(14;18) translocation, HDAC inhibitors were shown to markedly reduce  
357 expression of BCL-2 leading to apoptosis [143]. In other contexts, HDAC inhibition can induce  
358 mitochondrial apoptosis via epigenetic regulation of other BCL-2 family proteins [144,145],

359 production of reactive oxygen species and ceramide [146], or activation of death receptors  
360 [147]. Potent anti-proliferative effects have also been described [145,148]. Importantly, recent  
361 evidence has suggested that the addition of mTOR inhibition may augment the effects of HDAC  
362 inhibitors. For example, our lab has recently identified synergy between HDAC inhibitors and  
363 TOR-KIs in B-ALL cell lines and primary patient samples [103]. Also, both temsirolimus and  
364 everolimus have demonstrated synergistic anti-proliferative and apoptotic effects when  
365 combined with the HDAC inhibitors in MCL [149,150]. In DLBCL, combining HDAC inhibitors  
366 with rapalogs or TOR-KIs also synergistically induced apoptosis [65,151]. While there is still  
367 debate as to the exact mechanism of synergy, it is clear that in a preclinical setting this  
368 combination has marked potential in B cell malignancies. However, in a phase I trial combining  
369 panobinostat and everolimus in relapsed/refractory lymphoma, the combination yielded ORRs  
370 similar to everolimus alone but with higher incidence of thrombocytopenia [152]. As this  
371 combination moves forward, it will be important to identify the exact mechanism of action so as  
372 to better predict which patients may benefit from these combinations. It may also be useful to  
373 explore compounds targeting selected subsets of cellular HDAC enzymes.

374

### 375 Targeting the proteasome

376 Another class of inhibitors that has shown promise in B cell malignancies are proteasome  
377 inhibitors [153]. Interestingly, even across several cancer subtypes these inhibitors have been  
378 most promising in B cell malignancies [154-159] as evidenced by FDA approval for bortezomib  
379 in both relapsed MCL and multiple myeloma [160]. By suppressing degradation of proteins,  
380 these inhibitors induce a plethora of cellular responses leading to anti-proliferative and pro-  
381 apoptotic effects [161,162]. Most notable among these effects are its ability to suppress NF- $\kappa$ B  
382 activity and modulate expression of BCL-2 family proteins [162-164], which provides the basis  
383 for single agent bortezomib efficacy in ABC-DLBCL [165,166]. However, in other B cell  
384 malignancies, single agent proteasome inhibition is not as effective [167-169]. While preclinical

385 data has suggested some synergy between rapalogs and bortezomib [150,170], whether  
386 combined proteasome and mTOR inhibition will have generalizable efficacy is still unclear. A  
387 major clinical concern with bortezomib is neurological toxicity [171,172], and while dose  
388 management may alleviate some risks, it is unclear what effects the addition of mTOR inhibitors  
389 may have on patient outcomes.

390

### 391 **Outlook**

392 While the initial discovery of mTOR inhibitors yielded a flood of promising and exciting  
393 preclinical data, the initial wave of rapamycin-based therapies have not elicited widespread and  
394 durable patient responses. Consequently, rapalogs have only achieved regulatory approval in  
395 one subtype. With the development of TOR-KIs that offered a distinct biochemical advantage  
396 over rapalogs, there was an expectation of much greater responses. While the clinical data are  
397 not yet mature, it is becoming more apparent that while TOR-KIs may indeed have higher  
398 efficacy, it comes with the cost of higher toxicities. Whether dose modifications or altered  
399 schedules can lower the toxicity while maintaining efficacy is still unknown, but is a critical  
400 question in determining the future of mTOR-targeted therapies. Given the modest performance  
401 of single-agent mTOR inhibitors, it is likely that identifying combinations, either with targeted  
402 agents or with chemotherapy, may be the key to unleashing the full potential of mTOR inhibition  
403 in cancer. While the preclinical data strongly support this claim, it is still unclear whether this  
404 approach will translate to improved clinical responses, and more importantly, whether it will do  
405 so with acceptable toxicities. Given the generally well-tolerated nature of rapalogs, it seems  
406 prudent to initiate these combination studies using rapalogs. It will also be important to  
407 emphasize the preclinical evaluation of cancer selectivity, specifically to address whether these  
408 combinations will synergize to kill normal cells. Thus, the field of mTOR targeted therapies has  
409 progressed rapidly over the past few decades, and as our knowledge of the biology increases,

410 so too will our capacity to augment and fine-tune these therapies to effect positive patient  
411 outcomes.

412

### 413 **Acknowledgements**

414 mTOR inhibitor studies from our laboratory have been supported by NIH grants CA158383 and  
415 HD081319 (to D.A.F). T.T.V. is supported by a Ruth L. Kirschstein National Research Service  
416 Award from the National Institutes of Health (F32-CA189629).

417

418

### 419 **Conflict of Interest Statement**

420 All authors have completed the Unified Competing Interest form at  
421 [http://www.icmje.org/coi\\_disclosure.pdf](http://www.icmje.org/coi_disclosure.pdf) (available on request from the corresponding author)  
422 and declare: no support from any organisation for the submitted work; no financial relationships  
423 with any organisations that might have an interest in the submitted work in the previous 3 years.  
424 D.A.F. reports a patent, mTOR modulators and uses thereof, licensed to Intellikine, Inc.

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**1067 Figure Legends****1068 Figure 1: mTOR signaling pathway**

1069 mTOR exists in two distinct complexes (mTORC1 and mTORC2) that are regulated separately  
1070 and have distinct substrates. Whereas mTORC2 is regulated downstream of PI3K, mTORC1 is  
1071 coordinately regulated by growth factor signals, nutrient availability (amino acids), and cellular  
1072 energy status (ATP levels). The outputs of their downstream effectors coordinate processes  
1073 required for cell growth including survival, inhibition of autophagy, protein translation and cell  
1074 cycle progression.

1075

**1076 Figure 2: Mechanisms of aberrant mTOR activation in B-cell malignancies**

1077 Different proteins are amplified or activated (shown in red) in B-cell malignancies that result in  
1078 increased mTOR activity. Where as the loss of the tumor suppressor PTEN (shown in blue)  
1079 promotes mTOR activation. These mutations negate the normal constraints on mTOR activity  
1080 to promote cancer cell proliferation.

1081

**1082 Figure 3: Different effects of rapamycin and TOR-KI on mTOR activity**

1083 Rapamycin (and all rapalogs) only partially inhibits mTOR activity. Rapamycin forms a complex  
1084 with FKB12 to inhibit TORC1 activation of S6K activity and only partially reduces effects on  
1085 4EBP. S6K normally negatively feeds back to inhibit the activation of PI3K. By suppressing  
1086 S6K, rapamycin negates the feedback inhibition resulting in increased PI3K, TORC2 and AKT  
1087 activation. Thus, survival signals from AKT highly active and 4EBP is partially active.  
1088 Conversely, TOR-KIs suppresses all mTOR survival outputs.

1089

**1090 Figure 4: Combination of targeting BCL-2 and mTOR**

1091 (A) The BCL-2 family of proteins consists of pro- and anti-apoptotic members that interact  
1092 antagonistically to determine cell fate. Survival signaling through AKT and mTOR increases the

1093 anti-apoptotic family members and decreases the pro-apoptotic members. (B) By inhibiting  
1094 mTOR activity the balance is shifted where anti-apoptotic family members are reduced and pro-  
1095 apoptotic members are increased. This reduces the capacity of anti-apoptotic proteins to  
1096 sequester the pro-apoptotic proteins. Addition of ABT-199, a BCL-2 inhibitor, further shifts this  
1097 balance to release the pro-apoptotic proteins and cause cancer killing.  
1098

1

1 **Targeting mTOR for the treatment of B cell malignancies**

2

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16 Running head: mTOR inhibitors in B cell malignancies

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18 Keywords: mTOR, rapamycin, rapalogs, TOR-KIs, leukemia, lymphoma

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20 Word count: 4,574

21 Figure count: 4

22 Table count: 2

## 23 | Summary Abstract

24 | Mechanistic target of rapamycin (mTOR) is a serine/threonine kinase that functions as a key  
25 | regulator of cell growth, division, and survival. Many hematologic malignancies exhibit elevated  
26 | or aberrant mTOR activation, supporting the launch of numerous clinical trials aimed at  
27 | evaluating the potential of single-agent mTOR-targeted therapies. While promising early clinical  
28 | data using allosteric mTOR inhibitors (rapamycin and its derivatives, rapalogs) have suggested  
29 | activity in a subset of hematologic malignancies, these agents have shown limited efficacy in  
30 | most contexts. Whether the efficacy of these partial mTOR inhibitors might be enhanced by  
31 | more complete target inhibition is being actively addressed with second generation ATP-  
32 | competitive mTOR kinase inhibitors (TOR-KIs), which have only recently entered clinical trials.  
33 | However, emerging preclinical data suggest that despite their biochemical advantage over  
34 | rapalogs, TOR-KIs may retain a primarily cytostatic response. Rather, combinations of mTOR  
35 | inhibition with other targeted therapies have demonstrated promising efficacy in several  
36 | preclinical models. This review investigates the current status of rapalogs and TOR-KIs in B cell  
37 | malignancies, with an emphasis on emerging preclinical evidence of synergistic combinations  
38 | involving mTOR inhibition.

## 39 | Introduction

### 40 | The mTOR Signaling Pathway

41 | *The mTOR Signaling Pathway*

42 | mTOR is a serine/threonine kinase that functions as a master regulator of cell growth,  
43 | proliferation, metabolism, and survival. mTOR is active in two distinct multi-protein complexes  
44 | (mTORC1 and mTORC2) that are characterized by the defining subunits RAPTOR and  
45 | RICTOR respectively [1,2]. Each complex is differentially regulated and has a distinct set of  
46 | substrates (Figure 1). Activation of mTORC2 activation is incompletely understood, but has  
47 | recently been shown to be directly dependent on the generation of regulated by the levels of  
48 | PI(3,4,5)P<sub>3</sub> produced by phosphoinositide 3-kinase (PI3K) [3]. Upon activation mTORC2

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49 ~~functions to amplify the activity of AKT, a key oncogene involved in cell survival and~~  
50 ~~metabolism [4,5] and is required for full activity of AKT [4], a key oncogene involved in cell~~  
51 ~~survival and metabolism [5].~~ On the other hand, mTORC1 ~~activation is coordinately regulated by~~  
52 ~~functions by integrating~~ growth factor signals (i.e. from the PI3K/AKT pathway), ~~and~~ nutrient  
53 ~~availability (amino acids), and cellular energy status (ATP levels). Under conditions of low~~  
54 ~~nutrients, amino acid sensors (such as SLC38A9 [6,7]) suppress mTORC1 activation. Similarly,~~  
55 ~~under conditions of low energy (low ATP), 5' AMP-activated protein kinase (AMPK) can also~~  
56 ~~suppress mTORC1 activation [8]. This multifaceted regulation to ensures~~ that the cell is at an  
57 appropriate bioenergetic state to support cell growth and division [9,10][6,7] (Figure 1).

58       Upon activation, mTORC1 promotes key biosynthetic pathways including translation,  
59 transcription, and lipogenesis, while suppressing apoptotic and autophagic processes  
60 [11,12][8,9]. The most well-characterized downstream targets of mTORC1 ~~include are~~ the p70  
61 ribosomal-S6 kinases (S6Ks), ~~which are critical for lipid and ribosome biogenesis pathways,~~ and  
62 eukaryotic initiation factor 4E (eIF4E) ~~binding proteins (4E-BPs),~~ ~~Phosphorylation of S6Ks~~  
63 ~~induces its activity, which is critical for lipid and ribosome biogenesis pathways and promotes~~  
64 ~~translation via suppression of PDCD4 and activation of eIF4B [13,14]. In contrast,~~  
65 ~~phosphorylation of 4E-BPs suppresses their ability to inhibit eIF4E, which promotes translation~~  
66 ~~initiation which promotes translation of cap-bound mRNA transcripts (Figure 1). Whereas~~  
67 ~~mTORC1 activates S6Ks directly, it activates eIF4E indirectly by suppressing the inhibitory~~  
68 ~~function of eIF4E-binding proteins (4E-BPs) [15][10].~~ Together, these effectors ~~promote~~  
69 ~~coordinately~~ increased protein synthesis ~~rates~~, a process whose dysregulation is a central  
70 driving mechanism in cancer [16,17][11,12]. Importantly, hyper-activating mutations in mTOR  
71 itself have been identified in many cancers ~~s and~~, further indicat~~esing~~ the importance of mTOR  
72 activity to tumorigenesis [18][13].

73

74 *Evidence of mTOR activation in B-ALL and NHL*

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75 Aberrant activation of mTOR is frequently associated with poorer prognosis and has been  
76 well described in B cell malignancies including B cell acute lymphoblastic leukemia (B-ALL) and  
77 non-Hodgkin's Lymphoma (NHL). Given that mTOR is a convergence point for many distinct  
78 signaling pathways, there are many mechanisms by which it may become inappropriately  
79 activated (Figure 2). In B-ALL, the most common mode is through activation of upstream  
80 kinases. For example, the Philadelphia chromosome (Ph<sup>+</sup>), characterized by the BCR-ABL  
81 translocation, induces robust activation of several parallel pathways leading to mTOR activation.  
82 Similarly, genomic profiling has recently identified a Ph-like subset of B-ALL, which exhibits a  
83 similar kinase activation signature to that of Ph<sup>+</sup> B-ALL. Notably, these mutations are strongly  
84 associated with poorer outcomes in both children and adults [19-22][14-17]. Empirical evidence  
85 has also shown a direct correlation between AKT and/or mTOR activation and poor prognosis in  
86 patients with pediatric and adult B-ALL [23-25][18-20].

87 Among NHL subtypes, activation of mTOR is consistently a reliable indicator of more  
88 aggressive disease and poorer prognosis [26-30][21-25]. Similar to B-ALL, activation of mTOR  
89 follows through direct mutations in key upstream pathways. In mantle cell lymphoma (MCL),  
90 amplification of *PIK3CA* (the gene encoding the catalytic subunit of PI3K) and/or PTEN loss (the  
91 negative regulator of PI3K activity) have been observed in a large fraction of primary tissue  
92 samples [31][26]. In diffuse large B cell lymphoma (DLBCL), activation may be similarly  
93 achieved via mutations in *PIK3CA* [32,33][27,28], or chronic B cell receptor activation [34][29].  
94 In follicular lymphoma (FL), mTOR is aberrantly activated by way of PKC $\zeta$  or Syk kinases [35-  
95 38][30-33]. Collectively, these data highlight the impact of elevated mTOR activity on patient  
96 outcomes, and provide a solid rationale for the use of mTOR-targeted therapies in these B cell  
97 malignancies.

98  
99 **Rapalogs****Rapamycin and Rapalogs: partial mTORC1 inhibitors**

100 *Mechanism of action*

101 | Upon entry into a cell, rapamycin binds to FKBP12, forming a complex that potently and  
102 | selectively suppresses mTORC1 kinase activity by limiting substrate access to the active site  
103 | [39,40][34,35]. Importantly, the rapamycin-FKBP12 complex cannot bind to mTORC2  
104 | [2,41][2,36], though in some cases, prolonged exposure may limit the assembly of mTORC2  
105 | [42][37]. In this manner, rapamycin behaves as a highly potent and selective inhibitor of  
106 | mTORC1 (Figure 3). However, poor solubility and pharmacokinetics spurred the development of  
107 | rapamycin analogs (termed rapalogs) for oral dosing in cancer patients [43][38]. Most notable  
108 | among these rapalogs are temsirolimus (CCI-779, Wyeth Pharmaceuticals [44][39]), everolimus  
109 | (RAD001, Novartis Pharmaceuticals [45][40]), and ridaforolimus (AP23573, Merck and ARIAD  
110 | Pharmaceuticals [46][41]).

111

#### 112 | *Rapamycin and Rapalogs in B-ALL*

113 | Early testing with rapamycin unveiled potent anti-proliferative efficacy in several preclinical  
114 | models of ALL. In an E $\mu$ -RET model of murine B-pre ALL, rapamycin as a single agent potently  
115 | inhibited proliferation of leukemia cells both *in vitro* and *in vivo* [47,48][42,43]. Similar efficacy  
116 | was later observed in models of Ph<sup>+</sup> B-ALL [49,50][44,45], as well as in Ph-like B-ALL, driven  
117 | by JAK pathway mutations or CRLF2 rearrangement [51][46]. Rapalogs also demonstrated  
118 | marked preclinical efficacy in primary human ALL samples grown *in vitro* or in xenograft models  
119 | [50-52]. Notably, rapamycin demonstrated single-agent cytotoxicity in primary pediatric ALL  
120 | samples, and sensitized cells to doxorubicin *in vitro* [52][47]. Both everolimus and temsirolimus  
121 | have shown similar efficacy in xenograft models of adult and pediatric primary human ALL as  
122 | single agents [53][48] and in combination with chemotherapy [54,55][49,50].

123 | Clinically, rapamycin as a single agent exhibited no dose-limiting toxicities, but had  
124 | lackluster efficacy compared to standard chemotherapeutic options (Table 1). In an early trial,  
125 | rapamycin yielded stable disease in only three out of nine pediatric patients with relapsed ALL  
126 | [56][54]. As a result, several trials have been launched to determine whether rapalogs can

127 combine safely and effectively with standard chemotherapies. An early pilot trial combining  
128 rapamycin with glucocorticoids in relapsed ALL patients found that rapamycin effectively  
129 reduced the anti-apoptotic protein MCL-1 in various patients. This promising outcome suggested  
130 that rapamycin might sensitize ALL cells to apoptosis-inducing drugs. Indeed, in another study  
131 combining temsirolimus with intensive multi-drug re-induction therapy (dexamethasone,  
132 mitoxantrone, vincristine, and PEG-asparaginase) in relapsed childhood ALL yielded complete  
133 response in seven of sixteen patients, of which three had less than 0.01% minimal residual  
134 disease (MRD) by the end of treatment [57][52]. However, a separate trial evaluating everolimus  
135 combined with intensive chemotherapy (hyper-CVAD) in relapsed B-ALL yielded complete  
136 remission rates that were similar to standard salvage chemotherapies (~35%) [58-60][53-55].  
137 These trials highlight how the efficacy of rapalogs seem to be dependent on which  
138 chemotherapeutics are used, warranting further investigation.

139 A key question that remains to be answered is whether rapalogs combined with  
140 chemotherapy will demonstrate acceptable toxicity profiles. In the aforementioned trial  
141 combining temsirolimus with re-induction chemotherapy, the treatment was associated with  
142 unacceptable toxicities including severe infections that led to one death due to sepsis [57][52].  
143 However, a recent multi-center study testing the combination of everolimus with prednisone,  
144 vincristine, PEG-asparaginase and doxorubicin demonstrated that the combination was well  
145 tolerated in pediatric patients with first relapse [61]. Further trials are being performed, including  
146 an expansion of the aforementioned trial as well as As a result, a trial one testing the safety of  
147 temsirolimus with less intensive re-induction with (etoposide and cyclophosphamide is currently  
148 underway; (NCT01614197). Additionally, a multi-center study is also testing the combination of  
149 everolimus with prednisone, vincristine, PEG-asparaginase and doxorubicin (NCT01523977).  
150 Together, these results show that rapalogs have some potential in combination therapy, but an  
151 effective and tolerable regimen in B-ALL has yet to be identified. Moving forward, it will be

152 important to identify which chemotherapeutics are best combined with rapalogs and whether  
153 modifications to the dose and/or schedule may alleviate dose-limiting toxicities.

154

#### 155 *Rapalogs in NHL*

156 Similar to B-ALL, preclinical testing of rapalogs in NHL revealed promising cytostatic effects  
157 both *in vitro* and *in vivo*, yet clinical responses were limited in most contexts. For example, in  
158 MCL, FL, and DLBCL, rapamycin potently suppressed the proliferation of cell lines and primary  
159 patient cells *in vitro* [62-66][56-60]. However, the clinical use of rapalogs has only made  
160 progress in MCL where ~~chemotherapeutic~~ responses to standard chemotherapies are limited  
161 (Table 1). In phase II trials of relapsed MCL, single agent administration of either temsirolimus  
162 or ridaforolimus yielded overall response rates (ORR) of 38% [67][64] and 33% [68][62]  
163 respectively. Notably, a subsequent phase II trial using a 10-fold lower dose of temsirolimus  
164 revealed that similar responses could be obtained with lower toxicity [69][63]. Based on these  
165 results, a randomized phase III trial was conducted. Strikingly, the ~~overall response rates~~ORR  
166 and progression free survival were significantly higher in patients treated with temsirolimus  
167 compared to investigator's choice agent. These results ultimately led to approval for  
168 temsirolimus as a single agent therapy for relapsed/refractory MCL in Europe [70][64]. A  
169 subsequent phase II trial has also been completed combining temsirolimus with rituximab in  
170 relapsed/refractory MCL. Despite demonstrating higher response rates than single agent  
171 temsirolimus, the combination was also associated with higher toxicities including  
172 thrombocytopenia and neutropenia in a significant fraction of patients [71][65]. Rapalog  
173 monotherapy has also elicited responses in a subset of patients with other NHL subtypes. In a  
174 phase II trial of everolimus in relapsed lymphoma, the ORR in DLBCL was 30% (14/47) and  
175 38% (3/8) in FL [72][66]. Similar results were seen with temsirolimus where the ORR was 28%  
176 for DLBCL and 53% in FL [73][67]. While these studies highlight that rapalogs have some  
177 activity, the availability of better therapeutic options in both DLBCL and FL has limited the

178 clinical progress of rapalogs in these diseases. Thus, across NHL subtypes it will be important  
179 to determine whether the addition of rapalogs to standard chemotherapy can provide additional  
180 benefit to patients, without increasing toxicities.

181

182 *Outlook:*

183 Overall, despite showing promising preclinical activity in hematologic malignancies, rapalogs  
184 have only gained regulatory approval for use in one disease setting (MCL) where standard  
185 chemotherapies have limited efficacy. A major issue is that rapalogs given as single agents tend  
186 to elicit primarily cytostatic responses in hematologic malignancies [62,63,66,74][56,57,60,68].  
187 Clinically, the lack of inherent cytotoxicity is problematic since discontinuation of treatment may  
188 permit tumor cell regrowth [75-77][69-74]. While continued treatment may combat this issue,  
189 whether rapalogs at anti-leukemic doses will be safe for long-term use also remains to be seen.  
190 Clinical evidence of several toxicities including thrombocytopenia, mucositis, and hyperlipidemia  
191 suggests that prolonged treatment will be difficult to manage [43][38]. Alternatively,  
192 combinations with chemotherapy are actively being investigated and may reposition rapalogs as  
193 an adjuvant to improve chemotherapeutic responses. On this note, it is important to point out  
194 that the cytostatic activity of rapalogs will likely limit its potential to combine with certain  
195 chemotherapies, necessitating the identification of cytotoxic drugs that will synergize with  
196 rapalogs productively while maintaining acceptable tolerability.

197 While rapalogs provided proof-of-concept for effective mTOR targeted anti-cancer therapies,  
198 they exhibit many unfavorable biochemical properties that may also limit their clinical potential.  
199 Most notably, failure to suppress mTORC2 kinase activity allows maintained survival signaling  
200 through AKT and other related kinases. This issue is exacerbated by the existence of a negative  
201 feedback loop downstream of mTORC1 (Figure 3). Selective inhibition of mTORC1 induces  
202 robust feedback activation of upstream PI3K/AKT and MAPK pathways, allowing cancer cells to  
203 escape from the effects of rapamycin [57,78-82][52,72-76]. Additionally, rapalogs are known to

204 incompletely inhibit the phosphorylation of a subset of mTORC1 substrates (Figure 3). Despite  
205 restricting access to the active-site, rapalog-induced suppression of 4E-BP1 phosphorylation is  
206 refractory to long-term treatment compared to phosphorylation of p70S6K [83][77]. The cause of  
207 this differential sensitivity has recently been attributed to distinct substrate sequences near the  
208 phosphorylation sites [84][78]. This incomplete suppression of mTORC1 may significantly  
209 impact the anti-cancer potential of rapalogs as sustained activation of eIF4E is known to  
210 promote oncogenesis [85][79]. Consequently, sustained 4E-BP phosphorylation may allow  
211 cancer cells to escape from rapamycin-induced cell cycle arrest [86][80]. Thus, more complete  
212 mTOR inhibition may be required to elicit more promising clinical responses.

213

#### 214 **TOR-KIs: complete mTORC1/2 inhibitors**

215 The timely development of mTOR kinase inhibitors (TOR-KIs) directly addressed the  
216 biochemical disadvantages of rapalogs. By competing with ATP for binding to the mTOR active  
217 site, not only do TOR-KIs more completely block mTORC1 substrate phosphorylation (namely  
218 4E-BPs), but they also inhibit mTORC2 activity [87,88][81,82]. This results in reduced  
219 phosphorylation of AKT at Ser473 (Figure 3), dampening the feedback activation of PI3K/AKT  
220 that is known to limit rapalog efficacy [89-91][83-85]. It is important to note that by competing  
221 with ATP, TOR-KIs are capable of inhibiting several kinases at higher doses, including the  
222 structurally related protein, PI3K. Conversely, several compounds that are often used pre-  
223 clinically as PI3K inhibitors (wortmannin, LY294002) directly inhibit mTORC1 and mTORC2 at  
224 similar concentrations. Thus, it is important to fully understand the pharmacologic properties of  
225 ATP-competitive mTOR and PI3K inhibitors when interpreting their preclinical and clinical  
226 efficacy.

227 Several structurally distinct mTOR-selective inhibitors have been reported and tested in  
228 models of B cell malignancies. Most notable among them are PP242 [88][82], Torin1 [87][84],  
229 Ku-0063794 [92][86], AZD8055 [93][87], AZD2014 [93][87], MLN0128 (previously INK128

230 | [94][88], and CC-223 [95][89]. In preclinical testing, these TOR-KIs proved superior to rapalogs  
231 | in terms of cytostatic and cytotoxic potential. For example, in a mouse model of AKT-driven  
232 | lymphangiogenesis, PP242 strongly suppressed both 4E-BP1 phosphorylation and tumor  
233 | growth compared to rapamycin [96][90]. These findings were also recapitulated *in vitro* using  
234 | leukemia and DLBCL cell lines where TOR-KIs had a greatly improved biochemical effect on  
235 | downstream 4E-BP phosphorylation [97-99][94-93].

236 | Despite the broader biochemical impact of TOR-KIs over rapalogs, whether complete mTOR  
237 | kinase inhibition is sufficient to elicit cytotoxic responses is yet to be established. Two reports of  
238 | structurally distinct TOR-KIs in B-ALL demonstrated that mTOR kinase inhibition was sufficient  
239 | to induce apoptosis in B-ALL cell lines compared to rapamycin [100,101][94,95]. However, in  
240 | both studies, apoptosis was only observed at doses of TOR-KI that greatly exceed what was  
241 | needed to fully suppress mTOR kinase activity as measured by western blot. At lower doses  
242 | that still fully suppress mTOR activity, our lab has found that both AZD8055 and MLN0128  
243 | maintain a primarily cytostatic response profile (that is greater than rapalogs) [98,102-  
244 | 104][92,96-98]. Notably, low doses of PP242 were sufficient to kill murine bone marrow cells  
245 | immortalized by p190-BCR-ABL [99][93], suggesting that fully transformed B-ALL cells with  
246 | additional oncogenic lesions may respond differently to mTOR inhibition. Thus, it remains  
247 | unclear whether TOR-KIs will be effective in B-ALL or NHL as single agents at doses that are  
248 | highly selective for mTOR kinase activity.

249 | Early clinical trials have suggested that while TOR-KIs are more effective than rapalogs at  
250 | suppressing tumor growth, they may also be less tolerable [78][72]. A single agent tolerability  
251 | test of AZD2014 showed dose-limiting toxicities that were similar to rapalogs including mucositis  
252 | and fatigue [105][99]. Both CC-223 and MLN0128, also presented similar toxicities, ~~but in~~  
253 | ~~addition to~~ hyperglycemia, ~~also occurred and~~ necessitated ~~teding~~ close monitoring of patient blood  
254 | [106,107][100,101]. Several additional clinical trials are currently in progress to address the  
255 | efficacy and tolerability of TOR-KIs and are summarized in **Table 2**. However, a key question is

256 to investigate whether TOR-KIs will retain anti-cancer efficacy at lower doses that minimize  
257 these toxicities. While it is likely that lowering the dose of TOR-KIs may improve their tolerability,  
258 it will also impinge on their ability to fully suppress mTOR kinase activity. Moving forward, it may  
259 be important to determine whether these potentially suboptimal doses, which only partially  
260 inhibit mTOR, will be more effective than clinically tolerable doses of rapalogs, which potentially  
261 inhibits phosphorylation of some, but not all, mTORC1 substrates.

262

### 263 **Emerging Combinations with mTOR Inhibitors:**

264 Recent research efforts have been dedicated to identifying promising combinations that can  
265 synergistically kill cancer cells. The rationales behind these emerging combinations can be  
266 loosely categorized into two broad groups. The first approach seeks to exploit known resistance  
267 mechanisms to mTOR inhibition; either by targeting feedback pathways or using apoptosis-  
268 sensitizing agents. The second approach seeks to evaluate the potential of mTOR inhibitors as  
269 adjuvants to augment the effects of other agents targeting known oncogenic drivers. While both  
270 approaches have yielded several promising combinations, whether they can be translated to  
271 significant clinical responses with acceptable toxicity still remains to be determined.

272

#### 273 *Combinations targeting resistance mechanisms*

##### 274 Targeting parallel and downstream pathways

275 As with all targeted therapies, an understanding of how cells maintain survival in the  
276 presence of mTOR inhibitors has been crucial to the identification of promising combinations.  
277 Currently, there are several known acquired and *de novo* mechanisms of resistance to mTOR-  
278 targeted therapies. For example, in addition to feedback activation of PI3K/AKT, mTORC1  
279 inhibition may also activate the parallel MAPK/ERK pathway in B-ALL (Figure 4). In a similar  
280 fashion, PI3K/AKT/mTOR inhibition can also induce up-regulation of receptor tyrosine kinases  
281 (RTKs) leading to resistance in some solid tumors [108][102]. In agreement with these induced

282 resistance mechanisms, the addition of MAPK inhibitors and RTK inhibitors have demonstrated  
283 significantly more efficacy in combination with both rapalogs and TOR-KIs in preclinical settings  
284 [\[80,109,110\]](#)[\[74,103,104\]](#). However, in other instances, resistance to mTOR inhibition may be a  
285 result of sustained downstream effector activity, particularly cap-dependent translation. For  
286 example, our laboratory has noted resistance to TOR-KIs in DLBCL cell lines lacking expression  
287 of 4E-BPs [\[98\]](#)[\[92\]](#) or, and over-expression of eIF4E [limits the efficacy of TOR-KIs](#) [\[111\]](#)[\[105\]](#).  
288 Furthermore, recent evidence has indicated that PIM and MNK kinases can maintain cap-  
289 dependent translation downstream of mTORC1 inhibition [\[112\]](#)[\[106\]](#) (Figure 4). In these  
290 situations, targeting cap-dependent translation indirectly using combinations of PIM or MNK  
291 inhibitors with TOR-KIs has shown cytotoxic activity in AML cell lines [\[113,114\]](#)[\[107,108\]](#) as well  
292 as in cutaneous T cell lymphoma cell lines *in vitro* [\[115\]](#)[\[109\]](#). Additional work is required to  
293 evaluate the potential of directly targeting the cap-dependent translation initiation machinery. It  
294 is likely that other mechanisms of resistance will arise as our experience with mTOR inhibitors  
295 increases, and these may ultimately support the study of additional combinations.

296 While clinical data regarding the efficacy of these combinations in B cell malignancies has  
297 not reached maturity, similar combinations have been successfully deployed in non-hematologic  
298 malignancies. For example, inhibition of the upstream tyrosine kinase, HER2, significantly  
299 improved the efficacy of mTORC1/2 inhibition in patients with refractory breast cancer  
300 compared to single agent treatment [\[116\]](#)[\[110\]](#). Similarly, combinations of PI3K/AKT/mTOR and  
301 Ras/MAPK/ERK pathway inhibition yielded improved response rates in patients with advanced  
302 refractory solid tumors, but did so at the cost of significantly higher toxicities [\[117\]](#)[\[111\]](#).  
303 Collectively these studies highlight the potential of using mTOR inhibitors in combination with  
304 agents targeting known resistance pathways to mTOR inhibition, or as an adjuvant therapy to  
305 augment the effects of other rational targeted therapies. However, it will be important to  
306 determine whether these combinations targeting multiple key survival pathways will remain  
307 selective for cancer cells as toxicity will be a major concern.

308

309 Targeting apoptosis

310 Another straightforward approach to directly enhancing the apoptotic potential of mTOR  
311 inhibition is to target the pro-survival BCL-2 family proteins. Apoptosis is regulated through  
312 dynamic and competitive binding interactions between anti-apoptotic proteins (e.g. BCL-2, BCL-  
313 XL, BCL-w, and MCL-1) and pro-apoptotic sensitizers (e.g. BAD, PUMA, and NOXA), activators  
314 (e.g. BIM and BID), and effectors (BAX and BAK) (Figure 45A). While mTOR inhibition is known  
315 to suppress survival signaling through both mTORC1 (e.g. MCL-1 expression [96][99]) and AKT  
316 (e.g. inhibition of BAD and down-regulation of BIM [118,119][442,443]), TOR-KIs are insufficient  
317 to induce apoptosis through this pathway. Thus, a simple approach would be to use antagonists  
318 of the pro-survival proteins to disrupt their binding capacity, and subsequently lower the  
319 threshold for BIM to activate BAX/BAK-mediated MOMP and apoptosis [120][444].

320 ABT-737, and its orally bioavailable analog, ABT-263, represent the most potent and  
321 selective small molecule inhibitors of BCL-2 and BCL-X<sub>L</sub>. Both of these compounds  
322 demonstrated remarkable cytotoxic potential that was significantly enhanced when combined  
323 with mTOR inhibitors in DLBCL [121][445], FL [122][446], AML [123][447], and B-ALL  
324 [124][448]. However, due to on-target toxicity associated with BCL-X<sub>L</sub> inhibition [125][449], a  
325 more promising clinical candidate is ABT-199 [126][420]. ABT-199 is a selective inhibitor of  
326 BCL-2, and has elicited substantial clinical responses in patients with CLL as a single agent  
327 [127][424], leading to its designation as a breakthrough therapy for CLL patients with a 17p  
328 deletion (p53). Importantly, we and others have recently reported that ABT-199 synergizes with  
329 mTOR inhibition comparably to dual BCL-2/BCL-X<sub>L</sub> inhibitors [104,128][98,422], suggesting that  
330 the rationale established using first generation BCL-2 antagonists will hold true for ABT-199.  
331 However, a key concern is whether the addition of TOR-KIs to BCL-2 antagonists will enhance  
332 its toxicity towards non-cancer cells. In an effort to address this question, our lab has recently  
333 demonstrated that the combination does not synergize to kill peripheral blood mononuclear cells

334 | obtained from normal healthy donors [104][98]. Further work must be done to ensure that these  
335 | potent combinations will maintain favorable tolerability when administered to patients.

336

337

338 | *mTOR inhibition as an adjuvant*

339 | Targeting oncogenic drivers

340 | In contrast to targeting resistance mechanisms, others have found that combining

341 | oncogene-targeted therapies with mTOR inhibition also holds promise in B cell malignancies.

342 | For example, in Ph+ B-ALL driven by the BCR-ABL translocation, both rapamycin and PP242

343 | strongly synergized with imatinib to suppress leukemia growth [99][93]. Similarly, in

344 | myeloproliferative disorders characterized by JAK2 mutations, combinations of TOR-KIs or

345 | rapalogs with JAK2 inhibitors synergistically killed cells whereas single-agent treatments were

346 | primarily cytostatic [129,130][123,124]. In the activated B cell like (ABC) subtype of DLBCL,

347 | which is driven by sustained activation of the B cell receptor (BCR) [34][29], inhibition of the

348 | downstream kinase, Bruton's tyrosine kinase (BTK), also synergized strongly with

349 | PI3K/AKT/mTOR inhibitors [131][125]. However, the limitations of this approach are also

350 | recently becoming apparent. In particular, the germinal center B cell-like (GCB) DLBCL subtype

351 | is unresponsive to combinations of BTK and mTOR inhibitors, likely because BCR activation is

352 | not an oncogenic driver in this setting [132][126]. More alarmingly, in some cases the addition of

353 | mTOR inhibitors may antagonize the effects of other agents, either through suppression of

354 | proliferation or through induction of autophagy [133,134][127,128]. Studies like these serve as

355 | powerful reminders that a sound biological understanding supporting the use of these

356 | combinations must precede their clinical use.

357

358 | Targeting histone deacetylases (HDACs)

359 HDAC inhibitors are another promising class of drug that may benefit from the addition of  
360 mTOR inhibitors. In addition to modulating histone function and gene expression, HDACs also  
361 regulate the activity of non-histone proteins with relevance to B cell cancers (e.g. STAT, Hsp90,  
362 and FOXO) [135-138][129-132]. Importantly, mutations in genes regulating protein acetylation  
363 have been described in both B-ALL and NHL. For example, mutations in the CREBBP histone  
364 acetyltransferase (HAT) domain have been identified in a subset of patients with relapsed  
365 pediatric B-ALL where it may confer glucocorticoid resistance [139][133]. Similar mutations in  
366 HAT activity were identified as frequent mutations in both FL and DLBCL where their  
367 inactivation promotes aberrant up-regulation of BCL-6, a protein known to promote B cell  
368 malignancies [140-142][134-136]. Given the pervasive importance of protein acetylation, it is  
369 unsurprising that HDAC inhibitors have elicited promising responses in various leukemias and  
370 lymphomas. For example, in lymphomas with a t(14;18) translocation, HDAC inhibitors were  
371 shown to markedly reduce expression of BCL-2 leading to apoptosis [143][137]. In other  
372 contexts, HDAC inhibition can induce mitochondrial apoptosis via epigenetic regulation of other  
373 BCL-2 family proteins [144,145][138,139], production of reactive oxygen species and ceramide  
374 [146][140], or activation of death receptors [147][144]. Potent anti-proliferative effects have also  
375 been described [145,148][139,142]. Importantly, recent evidence has suggested that the  
376 addition of mTOR inhibition may augment the effects of HDAC inhibitors. For example, our lab  
377 has recently identified synergy between HDAC inhibitors and TOR-KIs in B-ALL cell lines and  
378 primary patient samples [103][97]. Also, both temsirolimus and everolimus have demonstrated  
379 synergistic anti-proliferative and apoptotic effects when combined with the HDAC inhibitors in  
380 MCL [149,150][143,144]. In DLBCL, combining HDAC inhibitors with rapalogs or TOR-KIs also  
381 synergistically induced apoptosis [65,151][59,145]. While there is still debate as to the exact  
382 mechanism of synergy, it is clear that in a preclinical setting, this combination has marked  
383 potential in B cell malignancies. However, in a phase I trial combining panobinostat and  
384 everolimus in relapsed/refractory lymphoma, the combination yielded ORRs similar to

385 | everolimus alone, but with higher incidence of thrombocytopenia [152][146]. As this combination  
386 | moves forward, it will be important to identify the exact mechanism of action so as to better  
387 | predict which patients may benefit from these combinations. It may also be useful to explore  
388 | compounds targeting selected subsets of cellular HDAC enzymes.

389

### 390 | Targeting the proteasome

391 | Another class of inhibitors that has shown promise in B cell malignancies are proteasome  
392 | inhibitors [153][147]. Interestingly, even across several cancer subtypes, these inhibitors have  
393 | been most promising in B cell malignancies [154-159][148-153], as evidenced by the FDA  
394 | approval for bortezomib in both relapsed MCL and multiple myeloma [160][154]. By suppressing  
395 | degradation of proteins, these inhibitors induce a plethora of cellular responses leading to anti-  
396 | proliferative and pro-apoptotic effects [161,162][155,156]. Most notable among these effects are  
397 | its ability to suppress NF- $\kappa$ B activity and modulate expression of BCL-2 family proteins [162-  
398 | 164][156-158], which provides the basis for single agent bortezomib efficacy in ABC-DLBCL  
399 | [165,166][159,160]. However, in other B cell malignancies, single agent proteasome inhibition is  
400 | not as effective [167-169][161-163]. While preclinical data has suggested some synergy  
401 | between rapalogs and bortezomib [150,170][144,164], whether combined proteasome and  
402 | mTOR inhibition will have generalizable efficacy is still unclear. A major clinical concern with  
403 | bortezomib is neurological toxicity [171,172][165,166], and while dose management may  
404 | alleviate some risks, it is unclear what effects the addition of mTOR inhibitors may have on  
405 | patient outcomes.

406

### 407 | **Outlook**

408 | While the initial discovery of mTOR inhibitors yielded a flood of promising and exciting  
409 | preclinical data, the initial wave of rapamycin-based therapies have not elicited widespread and  
410 | durable patient responses. Consequently, rapalogs have only achieved regulatory approval in

411 one subtype. With the development of TOR-KIs that offered a distinct biochemical advantage  
412 over rapalogs, there was an expectation of much greater responses. While the clinical data are  
413 not yet mature, it is becoming more apparent that while TOR-KIs may indeed have higher  
414 efficacy, it comes with the cost of higher toxicities. Whether dose modifications or altered  
415 schedules can lower the toxicity while maintaining efficacy is still unknown, but is a critical  
416 question in determining the future of mTOR-targeted therapies. Given the modest performance  
417 of single-agent mTOR inhibitors, it is likely that identifying combinations, either with targeted  
418 agents or with chemotherapy, may be the key to unleashing the full potential of mTOR inhibition  
419 in cancer. While the preclinical data strongly support this claim, it is still unclear whether this  
420 approach will translate to improved clinical responses, and more importantly, whether it will do  
421 so with acceptable toxicities. Given the generally well-tolerated nature of rapalogs, it seems  
422 prudent to initiate these combination studies using rapalogs. It will also be important to  
423 emphasize the preclinical evaluation of cancer selectivity, specifically to address whether these  
424 combinations will synergize to kill normal cells. Thus, the field of mTOR targeted therapies has  
425 progressed rapidly over the past few decades, and as our knowledge of the biology increases,  
426 so too will our capacity to augment and fine-tune these therapies to effect positive patient  
427 outcomes.

428

#### 429 **Acknowledgements**

430 mTOR inhibitor studies from our laboratory have been supported by NIH grants CA158383 and  
431 HD081319 (to D.A.F). T.T.V. is supported by a Ruth L. Kirschstein National Research Service  
432 Award from the National Institutes of Health (F32-CA189629).

433

434

#### 435 **Conflict of Interest Statement**

436 [All authors have completed the Unified Competing Interest form at](#)

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437 | [http://www.icmje.org/coi\\_disclosure.pdf](http://www.icmje.org/coi_disclosure.pdf) (available on request from the corresponding author)  
438 | [and declare: no support from any organisation for the submitted work; no financial relationships](#)  
439 | [with any organisations that might have an interest in the submitted work in the previous 3 years.](#)  
440 | [D.A.F. reports a patent, mTOR modulators and uses thereof, licensed to Intellikine, Inc.](#)

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1081

1082

## 1083 Figure Legends

### 1084 Figure 1: mTOR signaling pathway

1085 mTOR exists in two distinct complexes (mTORC1 and mTORC2) that are regulated separately  
1086 and have distinct substrates. Whereas mTORC2 is regulated downstream of PI3K, mTORC1 is  
1087 coordinately regulated by growth factor signals, nutrient availability (amino acids), and cellular  
1088 energy status (ATP levels). The outputs of their downstream effectors coordinate processes  
1089 required for cell growth including survival, inhibition of autophagy, protein translation and cell  
1090 cycle progression.

1091

### 1092 Figure 2: Mechanisms of aberrant mTOR activation in B-cell malignancies

1093 Different proteins are amplified or activated (shown in red) in B-cell malignancies that result in  
1094 increased mTOR activity. Where as the loss of the tumor suppressor PTEN (shown in blue)  
1095 promotes mTOR activation. These mutations negate the normal constraints on mTOR activity  
1096 to promote cancer cell proliferation.

1097

### 1098 Figure 3: Different effects of rapamycin and TOR-KI on mTOR activity

1099 Rapamycin (and all rapalogs) only partially inhibits mTOR activity. Rapamycin forms a complex  
1100 with FKB12 to inhibits TORC1 activation of S6K activity and only partially reduces effects on  
1101 4EBP. S6K normally negatively feeds back to inhibit the activation of PI3K. By suppressing  
1102 S6K, rapamycin negates the feedback inhibition resulting in increased PI3K, TORC2 and AKT  
1103 activation. Thus, survival signals from AKT highly active and 4EBP is partially active.  
1104 Conversely, TOR-KIs suppresses all mTOR survival outputs.

1105

### 1106 Figure 4: Combination of targeting BCL-2 and mTOR

1107 (A) The BCL-2 family of proteins consists of pro- and anti-apoptotic members that interact  
1108 antagonistically to determine cell fate. Survival signaling through AKT and mTOR increases the

1109 anti-apoptotic family members and decreases the pro-apoptotic members. (B) By inhibiting  
1110 mTOR activity the balance is shifted where anti-apoptotic family members are reduced and pro-  
1111 apoptotic members are increased. This reduces the capacity of anti-apoptotic proteins to  
1112 sequester the pro-apoptotic proteins. Addition of ABT-199, a BCL-2 inhibitor, further shifts this  
1113 balance to release the pro-apoptotic proteins and cause cancer killing.  
1114

Table 1: Published trials of mTOR-targeted therapies in ALL and NHL

Study	Phase	Drug	Drug Class	Disease	Outcomes
Witzig et al. [67]	II	Temsirolimus	Rapalog	Relapsed MCL	13/34 ORR <sup>1</sup>
Ansell et al. [69]	II	Temsirolimus	Rapalog	Relapsed MCL	11/27 ORR
Hess et al. [70]	III	Temsirolimus	Rapalog	Relapsed/refractory MCL	12/54 ORR compared to 2/54 ORR for investigator's choice
Tobinai et al. [173]	I	Everolimus	Rapalog	Relapsed/refractory NHL	4/13 ORR
Smith et al. [73]	II	Temsirolimus	Rapalog	DLBCL	9/32 ORR
				FL	21/39 ORR
Witzig et al. [72]	II	Everolimus	Rapalog	DLBCL	14/47 ORR
				MCL	6/19 ORR
				FL	3/8 ORR
Rizzieri et al. [68]	II	Ridaforolimus	Rapalog	ALL	0/1 ORR
				MCL	3/9 ORR
Rheingold et al. [56]	I	Sirolimus	Rapalog	Relapsed/refractory ALL	3/9 ORR
Rheingold et al. [57]	I	Temsirolimus with intensive re-induction chemotherapy	Rapalog	Pediatric relapsed ALL	7/15 CR with 3/7 MRD < 0.01%. High toxicities.
Daver et al. [58]	I/II	Everolimus with hyper-CVAD	Rapalog	Relapsed/refractory ALL	8/24 ORR
Ansell et al. [71]	II	Temsirolimus with rituximab	Rapalog	Relapsed/refractory MCL	41/69
Oki et al. [152]	I	Everolimus with panobinostat	Rapalog	Relapsed/refractory NHL	10/30 ORR
Barnes et al. [174]	II	Everolimus with rituximab	Rapalog	DLBCL	9/24 ORR
Infante et al. [107]	I	MLN0128	TOR-KI	Multiple	Dose escalation
Basu et al. [105]	I	AZD2014	TOR-KI	Multiple	Dose escalation
Bendell et al. [106]	I	CC-223	TOR-KI	Multiple	Dose escalation

<sup>1</sup> Overall response rate

Table 2: Ongoing trials of mTOR targeted therapies/combinations in ALL and NHL

mTOR-targeted drug	Combination	Study	Phase	Disease
Everolimus		NCT00790036	III	DLBCL
Everolimus	Panobinostat	NCT00918333	I/II	NHL
Everolimus	Panobinostat	NCT00978432	II	DLBCL
Everolimus	Multiagent re-induction	NCT01523977	I	ALL
Temsirolimus	Etoposide and cyclophosphamide	NCT01614197	I	Relapsed ALL and NHL
Sirolimus	Hyper-CVAD	NCT01184885	I	Relapsed/refractory ALL
Temsirolimus	Rituximab and DHAP	NCT01653067	II	Relapsed/refractory DLBCL
Everolimus	Bortezomib	NCT00671112	I	Relapsed/refractory lymphoma
Temsirolimus	Rituximab and bendamustine	NCT01078142	I/II	Relapsed FL and MCL
Temsirolimus	Bortezomib	NCT01281917	II	Relapsed/Refractory NHL
Temsirolimus	Rituximab and cladribine	NCT00787969	I/II	Newly diagnosed MCL
Sirolimus	Multiagent chemotherapy	NCT01658007	I	Relapsed/refractory ALL and lymphoma
Everolimus	Rituximab	NCT01665768	II	Lymphoma
Temsirolimus	Vinblastine	NCT02343718	I	Recurrent/refractory lymphoma
Temsirolimus	Inotuzumab Ozogamicin	NCT01535989	I	Relapsed/refractory NHL
CC-115		NCT01353625	I	NHL and solid tumors
MLN0128		NCT02484430	II	Relapsed/refractory ALL
PQR309		NCT02249429	I	Relapsed/refractory lymphoma
CC-223		NCT01177397	I/II	NHL and solid tumors
CC-223	Rituximab	NCT02031419	I	DLBCL

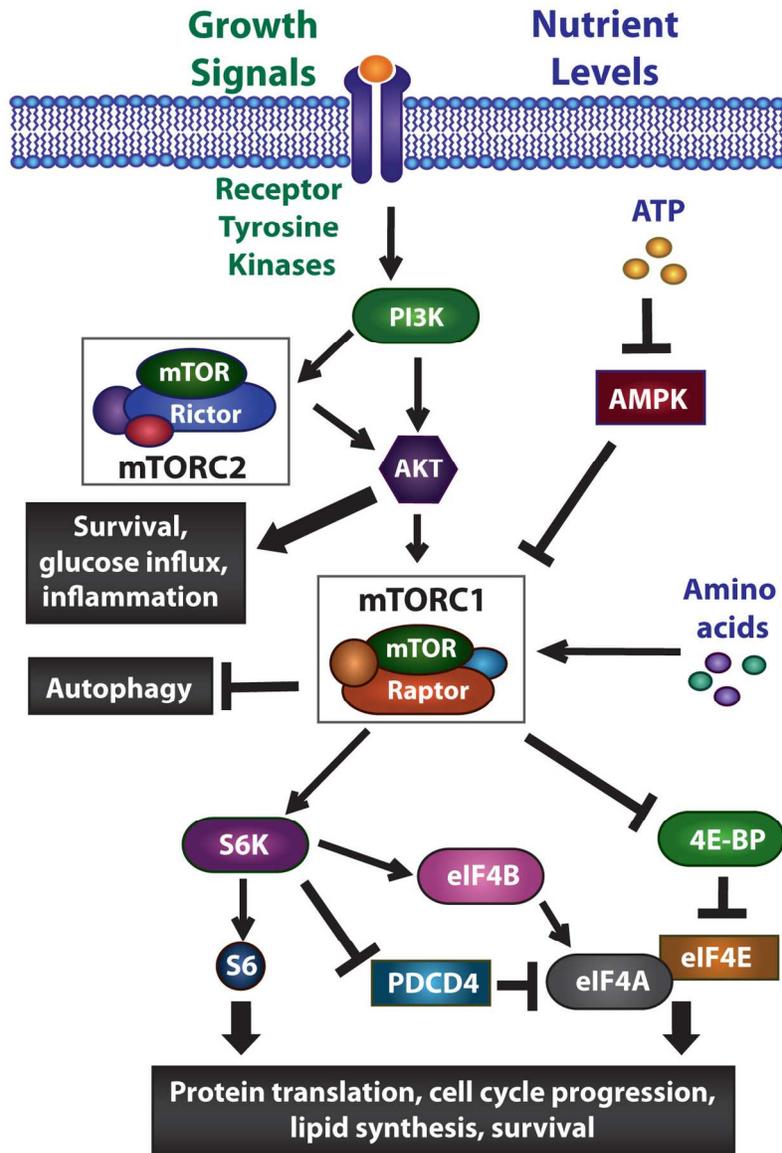


Figure 1  
122x171mm (300 x 300 DPI)

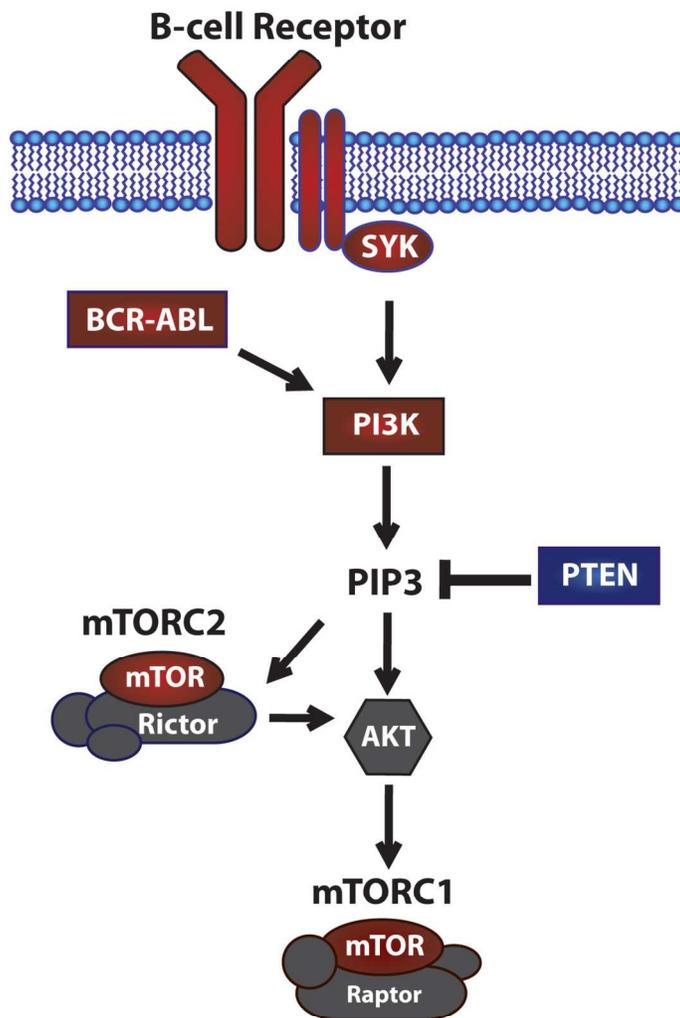


Figure 2  
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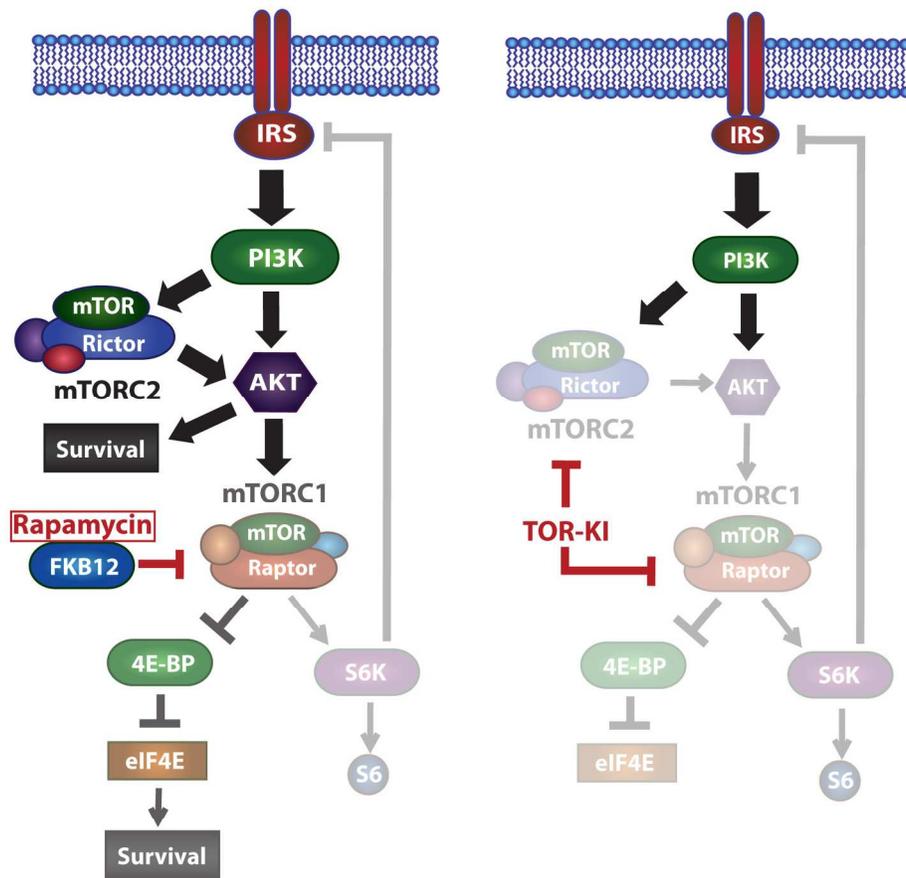


Figure 3  
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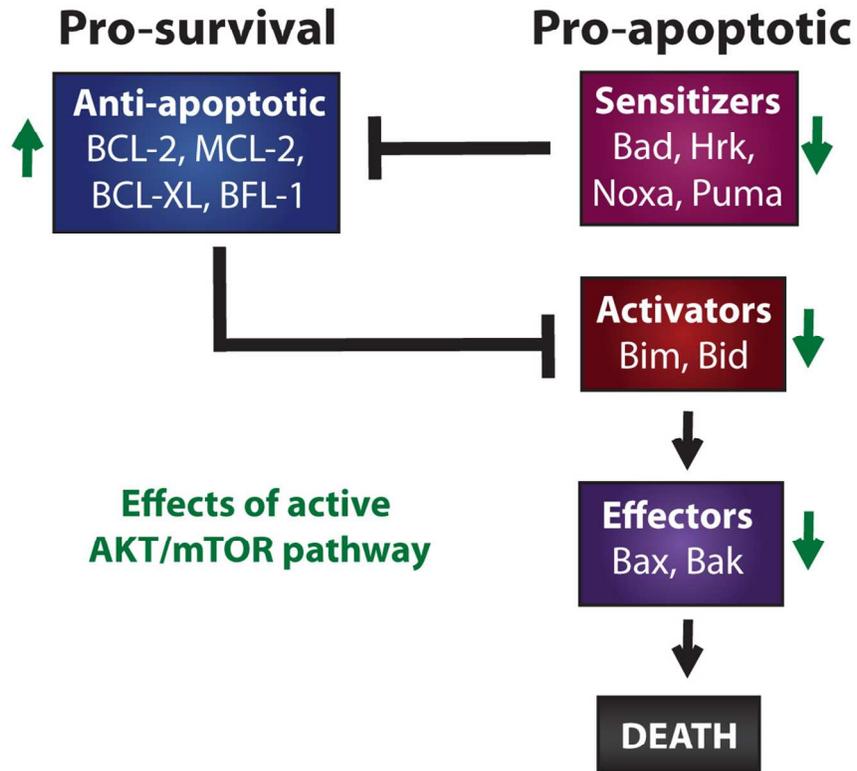


Figure 4A  
108x100mm (300 x 300 DPI)

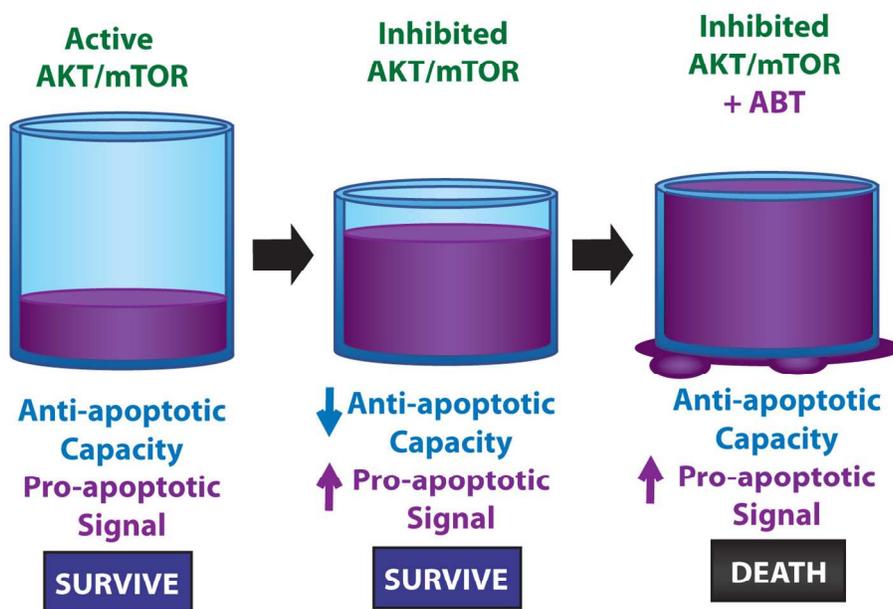


Figure 4B  
132x96mm (300 x 300 DPI)