Targeting the ribosome to treat MYC-driven lymphoma

Combination therapy targeting ribosome biogenesis and mRNA translation synergistically extends survival in MYC-driven lymphoma.

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protein (RP); ribosomal DNA (rDNA); ribosomal RNA (rRNA); RNA polymerase I (Pol I); internal ribosome entry site (IRES); combination drug index (CDI); untranslated region (UTR); 5'terminal oligopyrimidine (TOP); quantitative real-time polymerase chain reaction (qRT-PCR); bromodomain and extra-terminal (BET); immunoprecipitation (IP); eIF4E binding protein 1 (4EBP1); mouse double minute 2 homolog (MDM2); Australia New Zealand Clinical Trials Registry Number (ACTRN)

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CONFLICTS OF INTEREST

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ABSTRACT

Ribosome biogenesis and protein synthesis are dysregulated in many cancers, with those driven by the proto-oncogene *c-Myc* characterized by elevated Pol I-mediated ribosomal rDNA transcription and mTORC1/eIF4E-driven mRNA translation. Here we demonstrate that coordinated targeting of rDNA transcription and PI3K/AKT/mTORC1-dependent ribosome biogenesis and protein synthesis provides a remarkable improvement in survival in MYC-driven B-lymphoma. Combining an inhibitor of rDNA transcription (CX-5461) with the mTORC1 inhibitor everolimus more than doubled survival of E μ -Myc lymphoma-bearing mice. The ability of each agent to trigger tumor cell death via independent pathways was central to their synergistic efficacy. CX-5461 induced nucleolar stress and p53 pathway activation, while everolimus induced expression of the pro-apoptotic protein BMF that was independent of p53 and reduced expression of RPL11 and RPL5. Thus targeting the network controlling the synthesis and function of ribosomes at multiple points provides a potential new strategy to treat MYC-driven malignancies.

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STATEMENT OF SIGNIFICANCE: Treatment options for the high proportion of cancers driven by MYC are limited. We demonstrate that combining pharmacological targeting of ribosome biogenesis and mTORC1-dependent translation provides a remarkable therapeutic benefit to E μ -Myc lymphoma-bearing mice. These results establish a rationale for targeting ribosome biogenesis and function to treat MYC-driven cancer.

INTRODUCTION

The abnormal expression and activity of the proto-oncogene *c-Myc*, a key regulator of cell growth, proliferation and survival, occurs in greater than 30% of cancers including a high proportion of hematologic malignancies and solid tumors such as ovarian, prostate, HER2/ER/PR-negative breast cancer and melanoma (1-6). The transcription factor c-MYC functions as a global controller of protein biosynthesis, with the regulation of components of the ribosome biogenesis machinery being one of the most consistent gene expression signatures associated with MYC activation (7, 8). MYC transcriptional targets are important for key steps in the ribosome biogenesis process including the synthesis of ribosomal RNAs (rRNA) and proteins (RPs) as well as the expression of specific translation initiation factors (e.g. eukaryotic initiation factor 4E (eIF4E)) (2, 9-11). In particular, the robust up-regulation of genes encoding all members of the RNA polymerase I (Pol I) complex, which is responsible for transcription of the 47S pre-rRNA encoding genes (rDNA), is a key component of MYC's gene expression signature (9, 10, 12, 13). Importantly, we recently demonstrated that rDNA transcription, a key rate-limiting step for ribosome biogenesis, is specifically targeted by the novel small molecule inhibitor CX-5461, which is currently in Phase I trial (14). CX-

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5461 exhibits potent and selective single-agent therapeutic efficacy against MYC-driven B-lymphoma cells *in vivo* (12). The efficacy of this agent was mediated through induction of a nucleolar stress response characterized by the activation of p53-mediated apoptosis following binding and sequestration of the ubiquitin ligase MDM2 by RPL11 and RPL5 (12) (reviewed in 13, 15-17).

Elevated rates of protein synthesis are also essential for MYC-driven lymphomagenesis with the up-regulation of mammalian target of rapamycin complex 1 (mTORC1)/eIF4E-dependent protein translation demonstrated to sensitize MYC-driven malignancies to mTOR inhibition (18, 19). The PI3K/AKT/mTOR and RAS pathways cooperate with MYC to control ribosome biogenesis and protein synthesis in normal and malignant cells (20, 21), with the PI3K/AKT/mTOR pathway in particular regulating ribosome biogenesis and function at multiple steps. PI3K/AKT/mTOR signaling controls rRNA synthesis at the levels of rDNA transcription and rRNA processing as well as RP synthesis by regulating translation initiation for 5' terminal oligopyrimidine (TOP) containing mRNAs (20, 22-24). PI3K/AKT/mTOR signaling modulates mRNA translation at both the initiation and elongation stages including the promotion of eIF4E-dependent 5'CAP-dependent translation initiation (20, 21, 23, 25, 26). We and others have previously demonstrated the vulnerability of MYC-driven B-lymphoma cells to a number of targeted therapeutic strategies that inhibit PI3K/AKT/mTOR signaling (18, 20, 27-31). However in all cases disease relapse occurred despite continuing single agent therapy.

It is becoming increasingly apparent that the maximum inhibition of entire signaling networks is a paradigm for improved anti-tumor response (32, 33). A

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common mechanism through which cancer cells acquire resistance is to bypass the signaling module that is targeted by a particular therapy. By targeting signaling and transcriptional networks at multiple nodes the development of resistance may be delayed and potentially even prevented (33). Despite the success of strategies targeting the biogenesis and function of the protein synthetic machinery at the levels of rDNA gene transcription (12, 14, 27), RP synthesis (30) and mTORC1-dependent mRNA translation (18) in isolation, it is clear that optimal treatment of MYC-driven malignancies will require the potency of therapeutic strategies that target the ribosome to be maximized. We hypothesized that multi-point inhibition of the ribosome network via coordinated targeting of the PI3K/AKT/mTOR signaling pathway and rDNA transcription would improve therapeutic outcomes in cancers driven by dysregulated MYC expression and activity. In this work we identify the combined inhibition of ribosome biogenesis and function with selected small molecule inhibitors targeting multiple points as a novel and highly potent strategy to treat MYC-driven B-cell lymphoma.

RESULTS

Inhibitors of PI3K/AKT/mTOR signaling suppress rDNA transcription independent of nucleolar-stress mediated p53 activation

Apoptosis and cell cycle arrest in response to the disruption of ribosome biogenesis triggered by knockdown of RPs or inhibition of rDNA transcription have been causally linked to induction of the nucleolar stress response (12, 14, 34, 35). Activation of the tumor suppressor protein p53, mediated via the binding of a 5S rRNA-RPL11-RPL5 complex to the E3-ubiquitin-ligase MDM2

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is a key feature of this stress response (9, 15-17, 36, 37). To evaluate whether PI3K/AKT/mTOR pathway inhibition induced apoptosis (29) via a nucleolar stress mechanism in response to disruption of ribosome biogenesis, we treated *Tp53*^{WT} Eμ-Myc B-lymphoma cells in culture with a range of PI3K/AKT/mTOR inhibitors (Fig.1; AKTi-1/2 (AKT), KU-63794 (pan-mTOR), BEZ-235 (PI3K/mTOR) and everolimus (mTORC1)). Measurement of the abundance of the 47S 5'external transcribed spacer sequence (ETS) was performed to evaluate the effect of inhibitors on rDNA transcription initiation rates that we have shown to reflect changes in newly synthesized 47S rRNA levels in Eμ-Myc B-lymphoma cells in response to CX-5461 treatment and inhibition of AKT and mTORC1 (12, 20). Importantly, measurement of 5'ETS levels allows evaluation of changes to the initiation of rRNA synthesis *in vivo* (12, 27).

Despite the robust suppression of rDNA transcription initiation rates (Fig.1A), the induction of Eμ-Myc B-lymphoma cell apoptosis achieved by multiple inhibitors of PI3K/AKT/mTOR pathway signaling (Fig.S1A) was not associated with p53 activation, as demonstrated by the absence of p53 protein accumulation (Fig.1B) or p53 target gene (*p21*, *puma*) up-regulation (Fig.S1B). This was despite potent inhibition of phosphorylation of key substrates of AKT (PRAS40) and mTORC1 (RPS6 and 4EBP1) signaling (Fig. S1C). In direct contrast to signaling inhibitors and consistent with previous results (12, 14), inhibition of rDNA transcription by the novel Pol I transcription inhibitor, CX-5461, was associated with a robust accumulation of p53 protein (Fig.1B) and increased p53 target gene expression (Fig.S1B). The effects of AKTi-1/2 and everolimus with respect to nucleolar morphology (assessed by localization of

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the nucleolar protein Fibrillarin) were compared to those of CX-5461. While CX-5461 resulted in the dispersal of Fibrillarin and the loss of multiple nucleoli, AKTi-1/2 and everolimus had no effect on nucleolar morphology of Eμ-Myc lymphoma cells (Fig.1C).

It is thought that the increased availability of free (i.e. non-ribosome incorporated) RPs, specifically RPL11 and RPL5, is essential for the nucleolar stress response upon disruption of ribosome biogenesis. Free RPL11 and RPL5 are able to bind in an inhibitory manner to the p53 ubiquitin ligase MDM2, resulting in p53 accumulation (16, 17, 36). However, while CX-5461 treatment robustly increased the binding of critical RPs (RPL11 and RPL5) to MDM2, as expected from previous studies (12), AKTi-1/2 treatment had no effect on RP binding to MDM2 compared to control (Fig.1D and S1D). To examine the ability of targeted inhibitors to modulate RP abundance, free RP populations were isolated from Eμ-Myc lymphoma cells treated with AKTi-1/2 or CX-5461. Interestingly assessment of free versus total RP populations revealed that AKTi-1/2 significantly decreased the abundance of free RPL11, as well as a modest decrease in free RPL5 (Fig.1E). In contrast, CX-5461 robustly increased the abundance of both free RPs (Fig.1E). Thus the reduced expression of RPL11 and RPL5, critical mediators of the nucleolar stress response, is likely to be responsible for the absence of nucleolar stress-dependent p53 pathway activation following inhibition of AKT/mTORC1 signaling, despite the robust inhibition of rDNA gene transcription. The reduced abundance of free RPs was not associated with changes in *Rpl11* or *Rpl5* mRNA levels (Fig.S1E) indicating that AKTi-1/2 treatment did not alter transcription of genes encoding the RPs. Instead decreased RPL11 and RPL5

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protein abundance was associated with altered mRNA translation profiles of Eμ-Myc B-lymphoma cells. Specifically there was a significant decrease (24.9 +/- 0.8 percent) in the ratio of the actively translating polysomes to the sub-polysomes (comprising the 40S, 60S and 80S monomer peaks) following AKTi-1/2 treatment (Fig.1F). Thus AKTi-1/2 treatment mediated changes in ribosomal activity in Eμ-Myc lymphoma cells resulting in decreased abundance of free RPs. The absence of any effects on either the transcription of RP-encoding genes (Fig.S1E; *Rpl11* and *Rpl5*) or the polysome profile following CX-5461 treatment (Fig.1F) suggests that the accumulation of free RPL11 and RPL5 induced by treatment with CX-5461 results from continued RP synthesis despite suppression of rDNA transcription and ribosome biogenesis.

PI3K/AKT/mTOR inhibitor- but not CX-5461-mediated B-lymphoma apoptosis is associated with induction of pro-apoptotic BMF protein.

In the absence of p53 pathway activation, the inhibitors of PI3K/AKT/mTOR signaling must induce Eμ-Myc B-lymphoma cell apoptosis via alternative mechanism(s). Inhibition of PI3K/AKT/mTOR signaling is reported to drive the transient up-regulation of the translation of specific mRNAs containing regulatory internal ribosome entry site (IRES) elements in the 5'untranslated region (UTR) (38), including the pro-apoptotic BH3-only protein *Bcl-2* modifying factor (BMF) (29, 39). In response to AKTi-1/2, KU-63794, BEZ-235 and everolimus treatment a robust increase in BMF protein abundance was detected in Eμ-Myc lymphoma cells (Fig.1G), while CX-5461 treatment had no effect. These findings implicate BMF in B-lymphoma cell apoptosis induced by our panel of PI3K/AKT/mTOR inhibitors, while CX-5461 induces p53-

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mediated-apoptosis independent of increased BMF expression. In support of this hypothesis, the absence of BMF protein expression (*Bmf*^{-/-} Eμ-*Myc* B-lymphoma cells – Fig.S1F) did not alter the sensitivity of B-lymphoma cells to CX-5461 induced apoptosis (Fig.S1G). In contrast, in comparison to B-lymphoma cells wild-type for *Bmf*, the absence of BMF protein significantly ablated the increase in apoptosis induced by AKTi-1/2 (Fig.S1H).

Combined inhibition of mTORC1 signaling and rDNA transcription cooperates to treat MYC-driven B-cell lymphoma *in vivo*

The ability of PI3K/AKT/mTOR inhibitors and CX-5461 to induce B-lymphoma cell apoptosis via independent pathways led us to hypothesize that coordinated targeting of ribosome biogenesis and protein synthesis would improve outcomes in MYC-driven B-lymphoma. To test this hypothesis *in vivo*, C57Bl/6 mice with transplanted Eμ-*Myc* B-lymphoma were treated with everolimus, the most clinically advanced PI3K/AKT/mTOR pathway inhibitor in our panel, in combination with CX-5461. In mice transplanted with two different *Tp53* wild-type (*Tp53*^{WT}) Eμ-*Myc* clones, co-treatment with everolimus and CX-5461 resulted in a robust survival benefit compared to either single agent therapy (Fig.2A-B). In both experiments the length of the survival extension provided by combination therapy was more than double that achieved with CX-5461 alone (Fig.2A: 50.0 +/- 5.6 (combination) vs. 20.7 +/- 1.9 (CX-5461) and Fig.2B: 36.2 +/- 4.2 (combination) vs. 15.1 +/- 1.9 (CX-5461) days survival beyond control group). Thus strong cooperation between everolimus and CX-5461 provides a major improvement for overall survival in this highly aggressive mouse model of MYC-driven cancer.

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Combined inhibition of mTORC1 signaling and rDNA transcription enhances apoptosis of MYC-driven lymphoma cells

To investigate the cellular mechanism(s) underlying this synergistic effect of inhibiting PI3K/AKT/mTOR signaling and rDNA transcription we analyzed the effects of combining everolimus or AKTi-1/2 with CX-5461 with respect to the induction of apoptosis in E μ -Myc lymphoma cells. Both everolimus (Fig.S2A) and AKTi-1/2 (Fig.S2B) synergized with CX-5461 to significantly increase apoptosis in cultured E μ -Myc lymphoma cells (Fig.S2C), suggesting that enhanced induction of apoptosis is a mechanism through which they cooperate to treat MYC-driven lymphoma *in vivo*. Similarly, combined treatment of E μ -Myc B-lymphoma-bearing mice with a single dose of everolimus plus CX-5461 for 6 hours resulted in a significant increase in cell death in the bone marrow (Fig.2C) and spleen (Fig.2D) compared to CX-5461 alone. The enhanced cell death with combination treatment correlated with increased reductions in the E μ -Myc tumour (GFP-positive) cell populations in the bone marrow (Fig.2E) and spleens (Fig.2F) compared to either single agent. Furthermore combination therapy (72 hours) in mice transplanted with a different *Tp53*^{WT} E μ -Myc B-lymphoma clone also resulted in significantly increased clearance of GFP-positive E μ -Myc tumor cells from the bone marrow (Fig.S2D) and a greater reduction of disease burden in the axillary lymph node (Fig.S2E) compared to CX-5461 treatment alone. Importantly, combination therapy did not adversely affect the wild-type B-cell population, with an increased proportion of GFP-negative plus B220-positive B-cells detected in the circulating blood of mice treated with CX-5461 and everolimus plus CX-5461 (Fig.S2F), consistent with the specificity of CX-5461 for tumour

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cells we demonstrated previously (12). Together these results demonstrate that AKT/mTORC1 inhibition and CX-5461 cooperate to enhance apoptosis of Eμ-Myc B-lymphoma cells in culture and *in vivo*, and this mechanism is likely to explain the significantly improved survival in lymphoma-bearing mice receiving combination therapy.

AKT/mTORC1 inhibitors combine with CX-5461 to inhibit ribosome biogenesis and induce p53 and BMF pro-apoptotic pathways

A functional p53-response is a key component of CX-5461-induced apoptosis (12), with Eμ-Myc B-lymphoma cells generated on a *Tp53*^{-/-} background (Fig.S3A) significantly less sensitive than those with a wild-type *Tp53* genotype to the induction of apoptosis by CX-5461 (Fig.S3B). Thus the ability of AKT/mTORC1 signaling blockade to reduce free RP abundance (Fig.1E) suggested that PI3K/AKT/mTOR pathway inhibitors might antagonize p53 activation and apoptosis induced by CX-5461 and potentially limit the cooperation between these two inhibitor classes. In contrast however, we found that the increase in p53 protein abundance induced by CX-5461 was not affected by combination with either everolimus (Fig.3A and S3C) or AKTi-1/2 (Fig.S3D), nor was CX-5461 induced disruption of nucleolar morphology (Fig.3B and S3E). Importantly p53 protein accumulation *in vivo* following acute treatment with CX-5461 for 6 (Fig.3C) and 72 hours (Fig.3D) was not prevented or reduced in B-lymphoma bearing mice co-treated with everolimus.

Due to PI3K/AKT/mTORC1 inhibition disrupting rDNA gene transcription via a different mechanism to CX-5461 (14, 20, 25), we hypothesized that the combination of both inhibitor classes would also be associated with enhanced

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effects on rRNA synthesis. Indeed, treatment of E μ -Myc B-lymphoma cells with everolimus (Fig.3E) or AKTi-1/2 (Fig.3F) in combination with CX-5461 further suppressed rDNA gene transcription initiation rates beyond that achieved with either single agent. Importantly, the combination of everolimus and CX-5461 *in vivo* (2 hours) further suppressed rDNA transcription initiation in the inguinal lymph nodes of mice with transplanted E μ -Myc B-lymphoma (Fig.3G). We hypothesize that this increased potency of the combination therapy for targeting rRNA synthesis underpins the maintenance of p53 induction by CX-5461 in the combination treatment setting. Furthermore, CX-5461 did not alter the increased expression of BMF protein induced by either everolimus (Fig.3H) or AKTi-1/2 (Fig.S3F). Taken together, this data supports a mechanism whereby the combination of PI3K/AKT/mTOR pathway inhibitors and CX-5461 potentiates the inhibition of rRNA synthesis, which is associated with enhanced E μ -Myc B-lymphoma apoptosis and the maintain induction of distinct apoptotic mechanisms (p53 and BMF).

Activation of both p53 and BMF apoptotic pathways are required for the therapeutic benefit of combining everolimus and CX-5461.

The requirement for functional p53 and BMF for cooperation between everolimus and CX-5461 to treat MYC-driven B-cell lymphoma was examined. Combination treatment did not alter the induction of apoptosis of cultured *Tp53* deficient E μ -Myc B-lymphoma cells beyond that achieved with everolimus as a single agent (Fig.S4A), indicating that functional p53 expression is required for enhanced induction of cell death in culture by these agents. However, combination treatment with everolimus and CX-5461 did

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provide a modest survival benefit beyond single agents in mice transplanted with *Tp53*^{-/-} Eμ-Myc B-lymphoma cells (Fig.4A). Thus, p53-independent mechanisms must contribute to the therapeutic response to CX-5461 *in vivo*, both as a single agent and in combination with everolimus. Importantly however, the survival extension in *Tp53*^{-/-} Eμ-Myc B-lymphoma provided by the combination therapy, beyond that of the single agents, was significantly less than that observed in mice transplanted with *Tp53*^{WT} Eμ-Myc B-lymphoma (Fig.2A-B, 4D and S4B). This confirms that the p53 pathway is critical for potent cooperation between these two agents in this model.

Similar results to *Tp53*^{-/-} Eμ-Myc B-lymphoma were observed in mice transplanted with *Bmf*^{-/-} Eμ-Myc B-lymphoma cells that either expressed wild-type p53 protein (Fig.4B) or did not express functional p53 due to a spontaneous mutation (*Tp53*^{Mutant}; Fig.4C and S4C-D). In both cases the survival extension provided by combination treatment beyond that of the best single agent (Fig.4B: CX-5461; Fig.4C: everolimus) was significantly less than the survival extension achieved by the combination in Eμ-Myc B-lymphoma with both wild-type p53 and wild-type BMF (Fig.4D and S4C). Functional p53 and BMF pathways were therefore required for optimal cooperation between everolimus and CX-5461 to treat MYC-driven B-cell lymphoma *in vivo* although p53- and BMF-independent mechanisms also exist. Overall however, while the absence of BMF alone reduced the potency of the combination treatment regimen with respect to survival extension, the absence of functional p53 had the most dramatic effect on the ability of everolimus and

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CX-5461 to provide a synergistic therapeutic benefit against aggressive B-cell lymphoma (Fig.4D and S4C).

DISCUSSION

We hypothesized that targeting the ribosome with combination therapy would exploit the intrinsic reliance of MYC-driven B-lymphoma cells on enhanced rates of ribosome biogenesis (12, 13, 15) and mTORC1/eIF4E-driven protein synthesis (18, 19, 24). We found that specific inhibition of rDNA transcription initiation with CX-5461 in combination with the inhibition of key mRNA translation regulatory molecules (AKT/mTORC1) markedly improved therapeutic potency against Eμ-Myc B-lymphoma by independently engaging the p53 and BMF pro-apoptotic pathways respectively. Consistent with previous findings CX-5461 potently induced nucleolar disruption and p53 activation in Eμ-Myc B-lymphoma cells (12, 14) that was demonstrated here to be associated with the increased abundance of free RPs available to interact with and inhibit MDM2. In contrast, PI3K/AKT/mTOR-inhibitor mediated suppression of rDNA transcription rates occurred in the absence of nucleolar disruption and p53 pathway activation and was instead associated with the up-regulation of BMF protein. The lack of p53 stabilization following selective inhibition of mTORC1 signaling by rapamycin has been previously reported (40, 41), however the mechanism underpinning the absence of a nucleolar stress response despite disruption of ribosome biogenesis remained undefined. Here we demonstrate that the absence of p53 activation following AKT/mTORC1 inhibition, despite the robust reduction of rDNA transcription rates, was associated with decreased proportions of ribosomes in

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translationally active polysomes, and reduced abundance of critical nucleolar-stress related RPs (RPL11 and RPL5). This is consistent with the well-documented roles of PI3K/AKT/mTOR pathway for the modulation of ribosome function and selective mRNA translation in addition to the regulation of rDNA transcription (20, 26, 27, 39).

The altered expression of RPL11 and RPL5 in response to treatment with both AKTi-1/2 (decreased) and CX-5461 (increased) is also consistent with a previous report suggesting the importance of an imbalance between rRNA and RP synthesis for the activation of p53 in response to the disruption of rDNA transcription (40). Specifically, the presence of free RPs in excess to rRNA, as a consequence of the selective inhibition of rDNA transcription, is intrinsic to the induction of p53 by CX-5461, while the concomitant decrease in the synthesis of RPs and rRNA following PI3K/AKT/mTOR inhibition prevents this response. Importantly, combined treatment of B-lymphoma cells with AKT/mTORC1 inhibitors and CX-5461 enhanced the suppression of rDNA transcription initiation rates and hence rRNA synthesis, facilitating p53 pathway activation despite the impact that PI3K/AKT/mTOR signaling blockade has on protein translation and critical RP abundance. The ability of PI3K/AKT/mTOR signaling inhibitors to disrupt rDNA transcription at multiple points (20) in addition to RP synthesis and mRNA translation is therefore likely to be key to their capacity to potentially cooperate with CX-5461 to kill MYC-driven cancer cells.

Our *in vivo* studies clearly showed that the activation of the p53 tumor suppressor pathway was integral for a strong therapeutic effect of combining

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CX-5461 with everolimus to treat MYC-driven B-lymphoma. BMF expression was also required for optimal combination therapy effects *in vivo*. However, it is clear that additional factors may also contribute to the potency of this treatment strategy, as some survival benefit was derived from combination therapy in *Tp53*^{-/-} and *Tp53*^{Mutant} Eμ-Myc clones, in direct contrast to that observed with *Tp53*^{-/-} B-lymphoma cells in culture. This is consistent with previous work demonstrating that the sensitivity of solid tumor cells to CX-5461 is independent of p53 mutational status and is not exclusively dependent on the induction of cell death (14). Additionally, the improved *in vivo* efficacy provided by the combination (everolimus plus CX-5461) was associated with increased tumor cell clearance from the bone marrow compartment. This is important as early disease relapse is thought to originate from tumor cells that remain within a protective bone marrow niche (42, 43). Our data therefore suggests potential bone marrow-specific effects of the combination therapy are associated with the improved response. Our previous studies revealed that everolimus-mediated survival advantage was associated with the induction of cellular senescence *in vivo* (28). Future experiments will therefore test the possibility that this response may contribute to microenvironment-specific effects or to improved immune-system mediated clearance of B-lymphoma cells in the response to single agent therapy with CX-5461.

The identification and establishment of therapeutic approaches for MYC-driven cancer is critical given the high proportion of tumors that exhibit deregulated MYC activity and the difficulty of directly targeting transcription factors (44). While there are many emerging efforts to target dysregulated

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MYC in cancer (reviewed in 45), current approaches are focused on the development of strategies that exploit the reliance of these malignancies on MYC-governed processes. For example, in addition to our approach of targeting the ribosome, inhibitors of bromodomain and extra-terminal (BET) proteins (e.g. JQ-1, iBET-151) that modulate RNA polymerase II-mediated transcription processes, including the expression of the *c-Myc* gene and downstream targets of MYC (46, 47). These inhibitors have shown efficacy in pre-clinical models of MYC-driven cancer and proceeded to clinical trial (47, 48). While these inhibitors do not mediate therapeutic responses exclusively via disruption of MYC-directed transcription programs, it will be of great interest to compare their efficacy with combination strategies targeting ribosome and protein synthesis in MYC-driven malignancies, thus allowing MYC to be targeted at yet another level in cancer cells.

We propose that clinical trials of this combination therapy should begin with a focus on hematological cancers. Pre-clinical studies demonstrated that hematological malignancies are particularly sensitive to CX-5461 (12, 14), with CX-5461 progressing to Phase I trials in patients with refractory blood cancers (ACTRN12613001061729). In addition, inhibitors of PI3K/AKT/mTOR signaling exhibit single agent efficacy in mouse models of lymphoma and multiple myeloma (18, 27-29) and have been approved for treatment of hematological malignancies, including the PI3K δ inhibitor idelalisib for B-cell lymphoma and chronic lymphocytic leukemia (CLL) and the mTORC1 inhibitor temsirolimus for mantle cell lymphoma (43, 49). Furthermore, this work has established the importance of *Tp53* and *Bmf* for potent cooperation between everolimus and CX-5461 to treat MYC-driven lymphoma *in vivo*.

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Hematological malignancies are associated with a relatively low frequency of *Tp53* deficiency (< 20 percent) in contrast to solid tumours, of which over 60 percent possess *Tp53* mutations (reviewed in 50). Additionally, BMF protein is widely expressed in hematological cells and has been shown to be important for the induction of tumour cell death malignancies such as acute lymphatic leukemia (ALL) and CLL upon treatment with glucocorticoids as well as histone deacetylase (HDAC) inhibitors (51-53). Taken together with our current study, these observations indicate that the key elements to underpin robust therapeutic responses to combination therapy with PI3K/AKT/mTOR inhibitors and CX-5461 are in place in *Tp53* wild type hematological cancers.

Despite some successes, many PI3K/AKT/mTOR pathway inhibitors have exhibited poor efficacy in the clinic despite robust pre-clinical efficacy (54). Importantly, our findings suggest that the efficacy of PI3K/AKT/mTOR inhibitors may be markedly improved by combination with selective inhibitors of rDNA transcription such as CX-5461 or one of the growing list of Pol I transcription inhibitors currently under development (37). Strikingly, this therapeutic regime is well tolerated by the animals and remained selective for tumor cells with no negative effect against the wild-type B cell population observed. Furthermore, another tantalizing possibility arising from this study is that targeting a housekeeping process such as ribosome biogenesis and targeting growth networks at multiple points will make it more difficult for tumour cells to bypass inhibition. This approach may therefore overcome at least some of the resistance mechanisms driven by tumor heterogeneity.

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In summary, inhibitors of AKT/mTOR signaling and rDNA transcription potentially cooperate to treat *Tp53^{WT}* and *Bmf^{WT}* Eμ-Myc B-cell lymphoma, enhancing the induction of apoptosis and more than doubling the survival of mice with transplanted B-lymphoma. This potent improvement in efficacy is characterized by increased inhibition of rDNA transcription and mTORC1/eIF4E-dependent protein synthesis leading to induction of both p53 and BMF-dependent apoptotic pathways. Importantly this work underscores the concept that MYC driven tumors are addicted to multiple regulatory steps associated with ribosome synthesis and protein synthesis. It establishes the coordinated inhibition of key players in the complex network controlling the ribosome as a novel and potent strategy for the treatment of cancers driven by dysregulated MYC activity.

METHODS

Detailed methods are provided in the supplementary material.

Cell culture and reagents

Eμ-Myc clonal B-lymphoma cell lines (clone #4242, #107, #3239, #3391, #14 and #31) were kindly provided by R. Johnstone, Peter MacCallum Cancer Centre, East Melbourne 3002, VIC, Australia between January 2010 and December 2014 and were cultured as described previously (12, 44). All Eμ-Myc clonal B-lymphoma cell lines utilized were generated from the lymphomas of transgenic Eμ-Myc mice and therefore have not been authenticated. AKTi-1/2 (124017) was from MERCK (Kenilworth, NJ, USA); KU-0063794 (KU-63794 - S1226) and everolimus (S1120) were from Selleckchem. NVP-BEZ-235 (BEZ-235) was provided by Novartis (Basel,

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Switzerland) and CX-5461 was provided by Cylene Pharmaceuticals and Senhwa Biosciences (San Diego, CA, USA). 10mM stocks of AKTi-1/2, KU-0063794 and NVP-BEZ-235 were prepared in DMSO. For use *in vitro* 1mM stocks of everolimus were prepared in 100% ethanol and 10mM stocks of CX-5461 in 50mM NaH₂PO₄. For use *in vivo* everolimus was given daily at 5mg/kg (oral gavage) in DMSO (5 percent) and 1 percent methylcellulose (95 percent) and CX-5461 every 3 days at 30 – 40mg/kg (oral gavage) in 25mM NaH₂PO₄ (pH 4.5).

Animal Experiments

All animal experiments were performed with approval from the Peter MacCallum Cancer Centre Animal Experimentation Ethics Committee (Project E462). 6 – 8 week old male C57Bl/6 mice (Walter and Eliza Hall Institute, Parkville, VIC, Australia) were intravenously injected with 2×10^5 E μ -Myc B-lymphoma cells and treated with pharmacological inhibitors from 10 days post-injection (survival analyses) or on day 13 or 14 post-injection (acute analysis). For survival analyses treatment of mice continued until an ethical end-point was reached (Supplementary Materials and Methods). White blood cells, axillary lymph node cells and bone marrow cells were analyzed for GFP and B220 (CD45R) expression or fixed with 95% ethanol and stained with PI for SubG₁ analysis (Supplementary Materials and Methods).

Protein and RNA analysis

Protein was extracted with SDS-lysis or Rac-lysis buffer (Supplementary Table 1), separated by SDS-PAGE, transferred to PDVF membranes, immunoblotted with primary and HRP-conjugated secondary antibodies

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(Supplementary Table 2) and protein visualized by enhanced chemiluminescence (Perkin Elmer NEL104001EA) and X-ray film (Fujifilm SuperRX). RNA was extracted with the Bioline Isolate RNA Kit (BIO 520-44) with $\alpha^{32}\text{P}$ -UTP riboprobe added to allow for RNA recovery correction. cDNA was synthesized with SuperScript III (Invitrogen 18080-044) from template RNA equivalent to either equal cell number or RNA concentration and analyzed by quantitative real-time polymerase chain reaction (qRT-PCR; Supplementary Table 3). For MDM2 immunoprecipitation (IP), protein was extracted with NP-40 lysis buffer (Supplementary Table 1), incubated with 3 μg MDM2 antibody cocktail and IP isolated with MagnaChIP beads (Millipore 16-661). For analysis of free RPs cells were incubated with 10 $\mu\text{g}/\mu\text{L}$ cycloheximide (Sigma Aldrich C-1988) for 15 minutes and protein extracted with hypotonic lysis buffer (Supplementary Table 1). Protein lysates equivalent to equal cell number were concentrated, fractionated through 1.8M sucrose. Post-spin supernatant and pellet fractions were isolated. For polysome profiling, cells were incubated with 10 $\mu\text{g}/\mu\text{L}$ cycloheximide and protein extracted with hypotonic lysis buffer. Protein lysates equivalent to equal cell number were centrifuged through 10-40% sucrose, separated and analyzed with ISCO TrisTM and UA-6 UV/VIS detector (Teledyne, Thousand Oaks, CA, USA). Sub-polysome:polysome ratios were determined by measuring the area under the curve as described previously (49).

Immunofluorescence

Cells were fixed (2% paraformaldehyde), cytospun onto Superfrost Plus[®] microscope slides and stained with α -fibrillarin (Abcam 5821) and 594-conjugated secondary antibody (Life Technologies A-11012). Slides were

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analyzed with the Olympus BX-61, images captured with SPOT Advanced software (4.6.4.3 Diagnostic Instruments) and analyzed with ImageJ software (1.47v, NIH, USA).

Flow Cytometry

For Annexin-V and propidium iodide (PI) analysis cells were stained with Annexin-V-APC (BD Pharmigen 550474) and 1µg/mL PI (Sigma Aldrich 81845) and analyzed with the BD LSR. For SubG₁ analysis cells were fixed with 95% ethanol, stained with 50µg/mL PI and analyzed with the BD Canto II. Flow cytometry data was analyzed with FCSExpress software (De Novo, Los Angeles, CA, USA).

Statistical tests

Student t-tests (two-tailed), two-way ANOVA, Mantel-Cox and Gehan-Breslow-Wilcoxin tests were performed with GraphPad Prism Software (Version 6, La Jolla, CA, USA). Combination drug index (CDI) was determined as described previously (55).

SUPPLEMENTARY MATERIAL

Supplementary material consists of supplementary materials and methods, tables (3) and figures (4).

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Targeting the ribosome to treat MYC-driven lymphoma

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FIGURES

Figure 1: PI3K/AKT/mTOR pathway inhibition suppresses rDNA transcription and induces BMF expression independent of p53 activation and nucleolar disruption.

(A) qRT-PCR and **(B)** western analysis of E μ -Myc B-lymphoma cells (clone #4242) treated as indicated for 3 hours. **(C)** Immunofluorescence analysis of E μ -Myc B-lymphoma cells (clone #4242 MSCV *Cherry Bcl-2*) treated as indicated for 3 hours. **(D)** Immunoprecipitation (IP) of MDM2 from protein lysates isolated from E μ -Myc B-lymphoma cells (clone #4242 MSCV *Cherry Bcl-2*) treated as indicated for 1 hour. **(E)** Western analysis of protein isolated from E μ -Myc B-lymphoma cells (clone #4242 MSCV *Cherry Bcl-2*) treated as indicated for 3 hours and fractionated through a sucrose cushion. Free RP protein abundance was normalized to input and fold change over vehicle was determined. **(F)** Polysome profiles of lysates isolated from E μ -Myc B-lymphoma cells (clone #4242 MSCV *Cherry Bcl-2*) treated as indicated for 3 hours. Sub-polysome:polysome ratio was determined by measuring the area under the curve. **(G)** Western analysis of E μ -Myc B-lymphoma cells (clone 4242 MSCV *Cherry Bcl-2*) treated as indicated for 24 hours. BMF protein abundance was normalized to α -Tubulin or β -Actin and fold change over vehicle was determined. Graphs represent mean $\pm$ SEM of n=3. Western

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blots, immunofluorescence figures and polysome profiles are representatives of 3 independent experiments. Scale bar is 5µm and * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Data was analyzed by student t-test.

Figure 2: Combined inhibition of mTORC1 signaling and rDNA transcription extends survival of mice with transplanted MYC-driven B-lymphoma.

Kaplan-Meier curve of wild-type C57Bl/6 mice transplanted with Eµ-Myc B-lymphoma cells (**A** – clone #4242 MSCV *Gfp*; **B** – clone #107 MSCV *Gfp*) treated with Everolimus (5mg/kg qd), CX-5461 (35mg/kg q3d) and combination. Proportion of SubG₁ cells present in the (**C**) bone marrow and (**D**) spleens of transplanted wild-type mice treated as indicated. Proportion of GFP-positive Eµ-Myc B-lymphoma cells (clone #4242 MSCV *Gfp*) cells present in the (**E**) bone marrow and (**F**) spleens of transplanted wild-type mice treated as indicated. Kaplan-Meier curves represent 9 or 10 mice per group. Graphs represent mean $\pm$ SEM of 3 mice per group (**C** – **F**). Data was analyzed by log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxin tests (**A** – **B**) and student t-test (**C** – **F**). * $p < 0.05$, ** $p < 0.01$.

Figure 3: Maintained activation of p53 and BMF pro-apoptotic pathways in response to combined inhibition of AKT/mTORC1 signaling and rDNA transcription.

(**A**) Western analysis of Eµ-Myc B-lymphoma cells (clone #4242) treated as indicated for 24 hours. (**B**) Immunofluorescence analysis of Eµ-Myc B-lymphoma cells (clone #4242 MSCV *Cherry Bcl-2*) treated as indicated for 3

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hours. **(C)** Western analysis of protein lysates isolated from the inguinal lymph nodes of mice with transplanted Eμ-Myc B-lymphoma cells (clone #4242 MSCV *Gfp*) treated as indicated for 6 hours. **(D)** Western analysis of protein lysates isolated from the bone marrow of mice with transplanted Eμ-Myc B-lymphoma cells (clone #107 MSCV *Gfp*) treated as indicated for 72 hours. qRT-PCR analysis of Eμ-Myc B-lymphoma cells (clone #4242) treated as indicated for **(E)** 24 and **(F)** 6 hours. **(G)** qRT-PCR analysis of inguinal lymph nodes isolated from mice with transplanted Eμ-Myc B-lymphoma cells (clone #4242 MSCV *Gfp*) treated as indicated for 2 hours. **(H)** Western analysis of Eμ-Myc B-lymphoma cells (clone 4242 MSCV *Cherry Bcl-2*) treated indicated for 24 hours. BMF protein abundance was normalized to β-Actin and fold change over vehicle was determined. Graphs represent mean +/- SEM of n=3 (F, H), n=4 (E) or 3 mice per group (G). Western blots are representative of (A, H) 3 independent experiments, (C) 3 mice or (D) 5 mice per group. Immunofluorescence figures are representative of 3 independent experiments and scale bar is 5μm. n.s, non-significant, *p<0.05, *** p<0.001. Data was analyzed by two-way ANOVA (E, F) and student t-test (G and H).

Figure 4: p53 and BMF mediates potent cooperation between Everolimus and CX-5461 for the treatment of MYC-driven B-lymphoma.

Kaplan-Meier curve of wild-type C57Bl/6 mice transplanted with Eμ-Myc B-lymphoma cells (**A** – clone #3239 (*Tp53*^{-/-}); **B** – clone #14 (*Bmf*^{-/-})) treated with Everolimus (5mg/kg qd), CX-5461 (35mg/kg q3d) and combination. **(C)** Kaplan-Meier curve of wild-type C57Bl/6 *Ptprca* mice transplanted with Eμ-Myc B-lymphoma cells (clone #31 (*Tp53*^{mutant}; *Bmf*^{-/-})) treated with Everolimus

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(5mg/kg qd), CX-5461 (30mg/kg q3d) and combination. 30mg/kg and 35mg/kg CX-5461 had no difference in therapeutic efficacy against transplanted E μ -Myc B-lymphoma (Fig.S4D). **(D)** Number of days of extension in survival provided by combined Everolimus + CX-5461 treatment compared to that of the best single agent. Curves represent 8 mice per group. Graph represents mean $\pm$ SEM of n= 8 – 10 mice. ** p<0.01 and *** p<0.001. Data was analyzed by student t-test.

Figure 2

(A)

(B)

(C)

(D)

(E)

(F)

Figure 4

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Combination therapy targeting ribosome biogenesis and mRNA translation synergistically extends survival in MYC-driven lymphoma

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