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Selective ATP-competitive Inhibitors of TOR Suppress Rapamycin Insensitive Function of TORC2 in S. *cerevisiae*

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Abstract

The target of rapamycin (TOR) is a critical regulator of growth, survival and energy metabolism. The allosteric TORC1 inhibitor rapamycin has been used extensively to elucidate the TOR related signal pathway but is limited by its inability to inhibit TORC2. We used an unbiased cell proliferation assay of a kinase inhibitor library to discover QLIX- 55 as a potent inhibitor of S. *cerevisiae* growth. The functional target of QL-IX-55 is the ATP-binding site of TOR2 as evidenced by the discovery of resistant alleles of TOR2 through rational design and unbiased selection strategies. QL-IX-55 is capable of potently inhibiting both TOR complex 1 and 2 (TORC1 and TORC2) as demonstrated by biochemical IP kinase assays (IC50: <50 nM) and cellular assays for inhibition of substrate YPK1 phosphorylation. In contrast to Rapamycin, QL-IX-55 is capable of inhibiting TORC2-dependent transcription which suggests that this compound will be a powerful probe to dissect the Tor2/TORC2 related signaling pathway in yeast.

The resurgence of pathogenic yeast infections has stimulated interest in developing new classes of antifungal agents.(1) Currently approved antifungals target a limited number of biological pathways that are unique to fungi in order to obtain agents that are well tolerated in humans. Protein kinases have become an intensely pursued enzyme class particularly for the treatment of cancer where 12 compounds have received approval and approximately 120 compounds are in different stages of clinical testing.(2) The kinome of S. *cerevisiae* encodes approximately 130 protein kinases of which at least 15 have been shown to be essential based upon genetic deletion.[3] The analog-sensitive (AS) approach to generating mutant kinases that can be uniquely and potently inhibited by small molecules such as 1-napthylmethyl-PP1 (1-NM-PP1) has provided proof that pharmacological inhibition of

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kinases such as Cdc28, Pho85, Hog1 and others can lead to cytostatic or cytotoxic effects in S. *cerevisiae*.(4–6) Recently a small molecule TORC1 inhibitor, CID3528206, which is believed to act by a mechanism similar to rapamycin was discovered using a multiplex flow cytometry assay.(7) We sought to address whether the large pharmacopeia of small molecule ATP-competitive inhibitors that have been developed to target human kinases could be leveraged to discover inhibitors of homologous or non-homologous yeast kinases that are efficacious in cellular assays. Should potent inhibitors of *S. cerevisiae* kinases be discovered they will likely need to be optimized to evade the efficient efflux mechanisms of yeast, to possess selectivity relative to human kinases and to have the ability to overcome resistance that is likely to result from emergence of point mutations in target kinases

To discover efficacious *in vivo* inhibitors of yeast kinases, we performed a medium-throughput growth assay of a 1000 compound kinase library containing most of the known ATP-competitive pharmacophores and a large number of clinical stage kinase inhibitors. As our first question was how many of these compounds would recognize yeast kinases and lead to growth inhibition, we decided to circumvent the drug penetration and efflux problem by using a 'drug-sensitized' yeast strain. We used the RDY98 yeast strain which contains deletion is ERG6, an essential enzyme in ergosterol biosynthesis and in PDR1 and PDR3, two ABC-multidrug transporters.(8,9)(Supplementary Figure S1) This strain has previously been shown to greatly facilitate uptake of a variety of different drugs in yeast. Screening at a concentration of 10 μM in liquid phase cultures, we discovered that a number of analogs of Torin1, an inhibitor that we had previously developed as the first ATP-competitive inhibitor of mTOR, were strongly growth inhibitory.(10) Follow-up titrations of the most active compound resulted in the identification of QL-IX-55 as the most potent Torin1 analog which inhibited growth to 50% of the DMSO control at a concentration of 163 nM (Figure 1a and Supplementary Figure S2). QL-IX-55 is not capable of inhibiting growth of wild-type yeast at concentrations below 10 μM demonstrating that the drug-sensitizing mutations are required for its cellular efficacy (Data not shown). Interestingly, other reported potent inhibitors of human mTOR that were present in our library such as, AZD8055, KU63794, WYE354 and PP242 were not growth inhibitory up to a concentration of 1 μM (Supplementary Table S1).(11–14)

We next sought to identify what molecular target(s) might be responsible for the growth inhibitory effects of QL-IX-55. Because QL-IX-55 is a structural analog of Torin1, we naturally speculated that QL-IX-55 might also target TOR in yeast. Yeast TOR was originally identified as the pharmacological target of the natural product rapamycin through genetic resistance screens and affinity chromatography approaches.(15) Yeast TOR exhibits a high degree of sequence and domain conservation relative to human TOR and rapamycin, which binds to the FRB-domain, is known to be a potent allosteric inhibitor of the kinase activity of both human and yeast enzymes. The kinase domains of S. *cerevisiae* TOR and human mTOR bear over 60 % sequence identity in the catalytic domain and there are substitutions of approximately 10 amino acids that are predicted to form the ATP-binding pocket (Supplementary Figure S3). However unlike in mammalian cells, which only encode a single *TOR* gene, in yeast there are two *TOR* genes, *TOR1* and *TOR2*.(16,17) The TOR1 and TOR2 proteins form two functionally and structurally distinct complexes termed TORC1 and TORC2. Yeast TORC1 consists of Kog1, Tco89, Lst8 and either TOR1 or TOR2. In contrast, TORC2 contains only TOR2 in complex with Avo1, Avo2, Avo3, Bit61, and Lst8.(18) To determine whether QL-IX-55 inhibits TORC1 and/or TORC2, we performed kinaseassays with both complexes individually immunoprecipitated from yeast cells expressing tagged Kog1 (TORC1) or Avo3 (TORC2).(18) QL-IX-55 potently inhibited both complexes with an IC_{50} of approximately 50 nM (Figure 1B). Torin1 which exhibits a less potent GI_{50} of 450 nM, is also a less potent inhibitor of TORC1 (IC_{50} = 500 nM) and of TORC2 (IC_{50} = 250–500 nM). A close structural analog, QL-VIII-56, which was inactive as

a growth inhibitor below concentrations of 10 μM, also lost its ability to inhibit TORC1 and TORC2. To elucidate the mechanism of the inhibition, we performed an ATP competition assay which demonstrated that QL-IX-55 is an ATP competitive inhibitor of TOR2/TORC2. This mode of inhibition is fundamentally distinct from rapamycin which is non-ATP competitive and binds to the FRB-domain located N-terminal to the kinase domain. (Figure 1C). Since there are no readily available methods to screen for selectivity comprehensively against all yeast kinases, we instead profiled QL-IX-55 (1μM) against a panel of 440 human kinases using the DiscoveRx KinomeScan™ technology (Supplementary Table S2). This analysis demonstrated that QL-IX-55 maintained the ability to bind to human mTOR and structurally related PI3K but is otherwise a remarkably selective kinase inhibitor, comparable to some of the most highly selective ATP-competitive inhibitors currently known.

To determine whether inhibition of TOR1 and/or TOR2 kinase activity was functionally related to the potent growth inhibitory activity of QL-IX-55, we performed both molecular model-guided mutagenesis and an unbiased drug selection screen. A molecular model for QL-IX-55 in complex with yeast TOR2 was constructed based upon the most closely related crystal structure (PI3Kγ, PDB:3DBS). This model predicted the existence of a hydrogen bond between the phenol of Y2166 located deep in the ATP-binding site with the aminopyrimidine motif of the inhibitor (Figure 2A). To evaluate this hypothetical interaction, we created a Y2166F mutation in *TOR2* and constructed a yeast strain bearing this mutation integrated into the native locus using homologous recombination. We also performed an unbiased selection experiment where yeast cells were grown in rich medium containing 300 nM QL-IX-55 for three days. Single clones were isolated by streaking the selected cultures onto YPD agar plates also containing 300 nM QL-IX-55. Targeted sequencing of *TOR1* and *TOR2* from genomic DNA isolated from resistant colonies revealed no mutations in *TOR1* and a recurrent I2290S/K2293I mutation in *TOR2*. Interestingly, these mutations map closely to the ATP-binding site and I2290S is located four amino acids N-terminal to the highly conserved DFG motif which marks the start of the activation loop and often undergoes dramatic rearrangements upon inhibitor binding. As other undetected mutations may also be present in these resistant colonies, we re-engineered strains with I2290S, K2293I, I2290S/K2293I and I2290S/Y2166F *TOR2* alleles and examined their ability to grow in the presence of QL-IX-55. In liquid-phase cultures, the I2290S mutation resulted in a 5-fold increase in GI_{50}, the Y2166F mutation induced a 4-fold increase in GI_{50}, the K2293I mutation had no effect and the double I2290S/Y2166F double mutation had an additive effect which resulted in a 9-fold elevation in GI_{50} relative to wild-type (Figure 2B and Supplementary Table S3). A similar resistance profile was observed when the assays were repeated using colony dilution and halo assays (Figure 2C,D). To determine whether growth resistance correlated with a loss in ability of QL-IX-55 to inhibit the kinase activity of mutant TOR2, we performed immunoprecipitation assays from I2290S/Y2166F *TOR2* yeast. The IC_{50} for inhibition of I2290S/Y2166F TOR2 was elevated approximately 20-fold relative to wild-type TOR2, which is consistent with TOR being a functionally relevant target for the growth inhibitory phenotype (Figure 2E,F).

We next sought to use our novel inhibitor to investigate the physiological consequences of ATP-competitive inhibition of TOR kinase activity, in comparison to allosteric inhibition using rapamycin. In yeast, TORC1 but not TORC2 is sensitive to rapamycin.(17) By looking at gene expression, we discovered that QL-IX-55 was capable of modulating transcriptional outputs of both TORC1 and TORC2. For example, similar to rapamycin, QL-IX-55 decreased expression of RPL30 and increased expression of CIT2 and GAP1, as demonstrated by Northern blot analysis (Figure 3A).(19) However, unlike rapamycin, QL-IX-55 also modulated transcriptional outputs specific for TORC2, including increased expression of YLR194C, CMK2 and DIA1 (Figure 3A).(20) We also observed that QL-

IX-55, but not rapamycin, rapidly inhibited TORC2-dependent phosphorylation of Ypk1, a recently identified substrate of TORC2 (Figure 3B), middle panel, compare lanes 3 and 5). We next investigated the effects of QL-IX-55 on actin morphology, where inhibition of either TORC1 or TORC2 is known to result in depolarization of actin patch structures.(21) Here we observed that both rapamycin and QL-IX-55 perturbed actin morphology with similar kinetics, where fewer than 20% of cells displayed normal polarization following 60 minutes of drug treatment (Figure 3C).

The TOR (Target of Rapamycin) signaling network couples cell growth to intracellular and environmental cues in all eukaryotic organisms examined to date (22). This network was discovered through the action of rapamycin, an immunosuppressant and antifungal compound that acts as a specific allosteric inhibitor of the TOR kinase, a member of the PI3-like kinase family of serine/threonine protein kinases (15). TOR associates with a number of other proteins to form two distinct protein complexes, termed TORC1 and TORC2 (or mTORC1 and mTORC2 in mammalian cells), where TORC1 is uniquely inhibited by rapamycin. These complexes control diverse downstream processes, including protein synthesis and actin cytoskeletal organization, and thus collaboratively regulate both temporal and spatial aspects of cell growth. Much of what has been learned about TOR signaling has come from studies using budding yeast, *S. cerevisiae*, and this remains an important model system for exploring the architecture and function of TOR. In particular, rapamycin has been invaluable as a specific small molecule inhibitor of TOR activity and has revealed a role for TOR signaling in a wide array of activities, including protein synthesis, ribosome biogenesis, autophagy, nutrient-regulated gene expression, as well as protein trafficking (22). Because rapamycin only targets TORC1, however, our understanding of the scope of processes controlled by TORC2 has been limited by comparison. To a large extent this problem has now been circumvented in mammalian cells by the development of a cadre of ATP-competitive inhibitors specific for mTOR, which have been shown to inhibit the activity of both mTORC1 and mTORC2 (23). Unfortunately these compounds have proven to be mostly ineffective against yeast, either because of problems with drug uptake and/or retention, subtle differences in the structure of the ATP binding pocket between yeast TOR and mTOR, or both.

By screening a diverse library of 1000 different kinase inhibitors for inhibitors of yeast growth, we have discovered a compound QL-IX-55, a structural analog of Torin1, which functions as an ATP-competitive inhibitor of yeast TOR and is capable of potently inhibiting the kinase activity and modulating the outputs of both TORC1 and TORC2. As QL-IX-55 inhibits both TORC1 and TORC2, it most likely targets both TOR1 and TOR2. QL-IX-55 resistance mutations were obtained only in *TOR2* because only TOR2 is in both TORC1 and TORC2 and mutations in *TOR1* would not affect the sensitivity of TORC2. The ability of QL-IX-55 to potently inhibit yeast growth depends on its ability to inhibit TOR *in vivo*. However, our currently best *TOR2* resistance allele I2290S/Y2166F confers approximately 20-fold resistance using a TOR2 *in vitro* kinase assay but only 9-fold resistance to growth inhibition. This apparent discrepancy is likely due to QL-IX-55 also targeting TOR1 and possibly other yeast enzymes.

Just as rapamycin has proven to be invaluable for mapping TORC1-dependent outputs, QL-IX-55 will serve as a useful means to map both TORC1 and TORC2-dependent outputs in yeast. Our study also demonstrates that ATP-competitive inhibition of TOR is a pharmacologically valid means of obtaining anti-fungal activity. QL-IX-55 possesses two major deficits that must be overcome to improve its use as a pharmacological tool and potential as a therapeutic lead compound. First, the ability of QL-IX-55 to accumulate in yeast needs to be improved to obtain activity against wild-type and pathogenic yeast. Second, although QL-IX-55 is approximately a 50-fold weaker inhibitor of human TORC1

and a 10-fold more potent inhibitor of yeast TORC1 relative to Torin1, further optimization of its yeast versus human selectivity will be needed. Our screen also demonstrated that highly potent ATP-competitive human mTOR inhibitors such as AZD8055, KU63794, WYE354 and PP242 are not growth inhibitory up to a concentration of 1 μM which again demonstrates that achieving selectivity between yeast and human mTOR can be accomplished. Finally our screen demonstrated that surprisingly few of the currently known human kinase inhibitors including highly promiscuous compounds such as dasatinib, sunitinib and sorafenib possess any significant antifungal activity even at a high concentration of 10 μM. This proves credence to the notion that achieving selectivity for fungal versus human kinases is an achievable goal but will require dedicated medicinal chemistry efforts potentially with new chemical templates.

Methods

Drugs, yeast strains and antibodies were described in detail in the supplemental information.

Drug induced mutagenesis and mutant regeneration

Yeast cells of RDY98 were grown in YPD liquid medium to mid-log phase and then diluted to A_{600}=0.05 using fresh medium containing 200 nM of QL-IX-55 for 2 days incubation. A couple of cell divisions later, 200 ul of cultures were spread on YPD agar plate containing 300 nM of XL-IX-55 for 3 days until colonies appear. 72 Colonies were picked and streaked on YPD agar plate containing 300 nM of QL-IX-55. TOR1 and TOR2 genes were amplified and sequenced. Homologous recombination was used for generation of yeast mutants[1]. LiAc method was applied for yeast transformation,

In vitro Torc1/2 biochemical Assay

TORC1 and TORC2 were purified by Protein A Sepharose crosslinked with mouse monoclonal HA antibody from exponentially growing *KOG1-HA* and *AVO3-HA* cells, respectively. Inhibitors were tested 8-fold serial dilutions from 100 μM to 10 nM. Kinase reactions were performed in kinase buffer in a final volume of 30 μl containing 20 μg of PHAS-I (4EBP1). Kinase reactions were started with the addition of 80 μM ATP and 5 μCi [gamma-^{32}P] ATP. 23 μL of the supernatants were transfer to Filter P18 (Whatman). The incorporation of ^{32}P was measured by Liquid-Scintillation Analyzer. IC_{50} values were determined by fitting the data to a sigmoid dose-response-curve using the BioDataFit 1.02 software package.

Other methods are described in detail in the Supplemental Experimental Procedures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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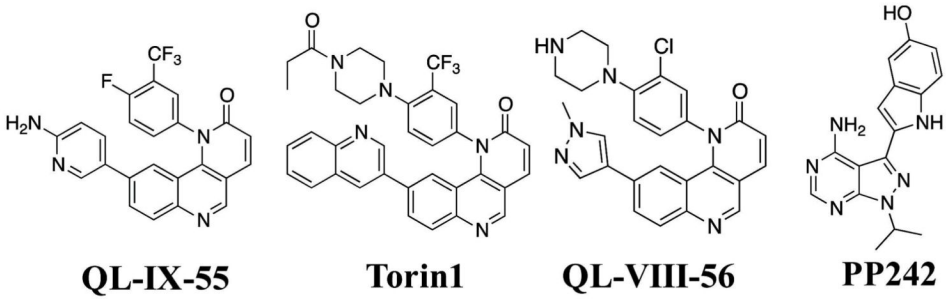
A



B

Drugs	GI_{50}(nM)	Human mTORC1 IC_{50}(nM)	Yeast TORC1 IC_{50}(nM)	Yeast TORC2 IC_{50}(nM)
QL-IX-55	163	50	50	10-50
Torin1	450	1	500	250-500
QL-VIII-56	>5000	590	>5000	>5000
PP242	>5000	25	>100000	50000-100000

C

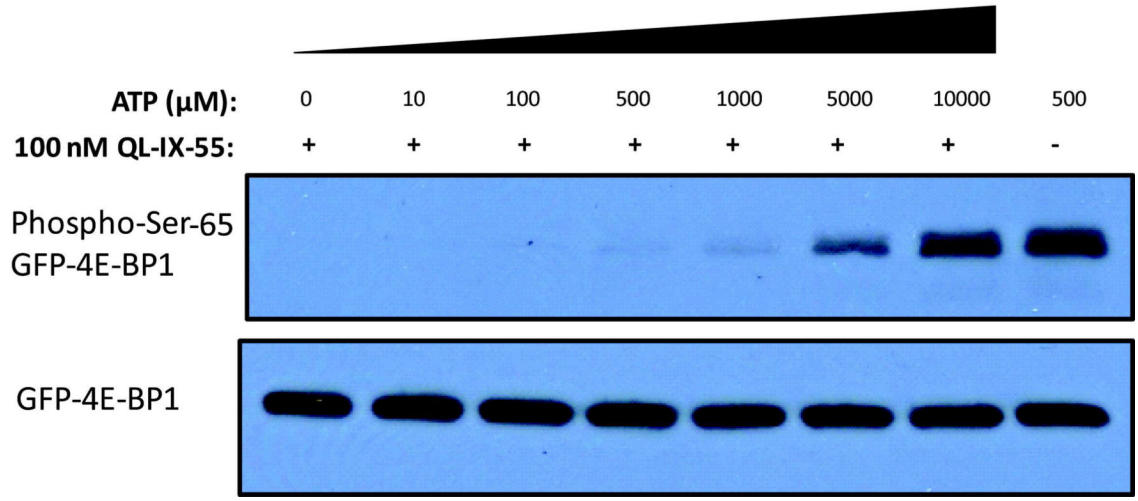


Figure 1. Discovery and characterization of QL-IX-55 as a TORC1/2 inhibitor

(A) Chemical structures of QL-IX-55, Torin 1, QL-VIII-56, and PP242.

(B) QL-IX-55 inhibits TORC1 and TORC2.

(C) QL-IX-55 is an ATP competitive inhibitor.

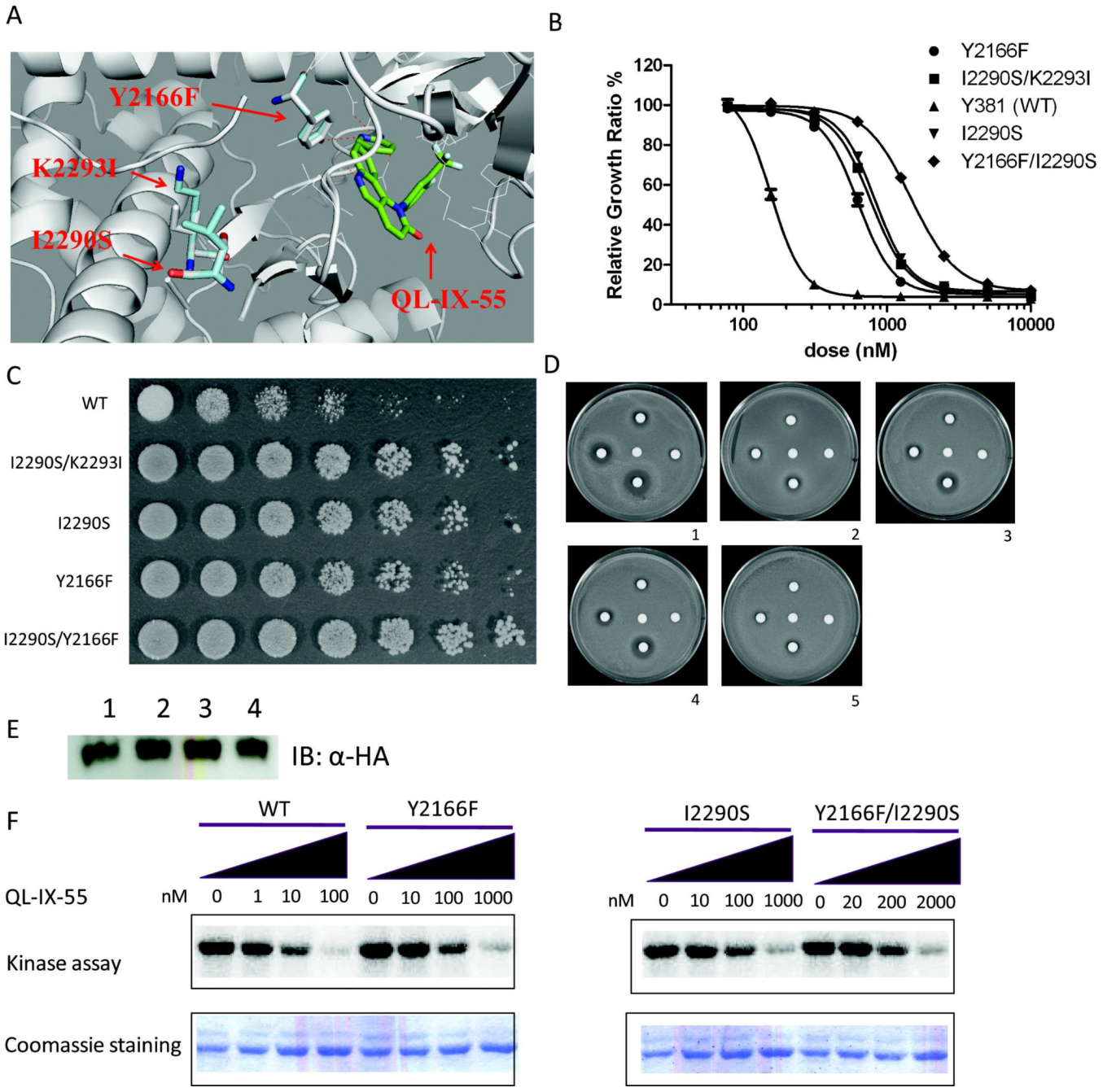


Figure 2. QL-IX-55 inhibition of TOR2

(A) Docking QL-IX-55 to a mTOR homology model.

(B,C,D) Growth inhibition assay, Clonogenic assay and Halo assay of QL-IX-55 on TOR2 wide/mutant strains

(E) Detection of HA-tagged Tor2 (HA-TOR2) by immunoblotting with Anti-HA antibody.

(F) In vitro TOR2 kinase assay

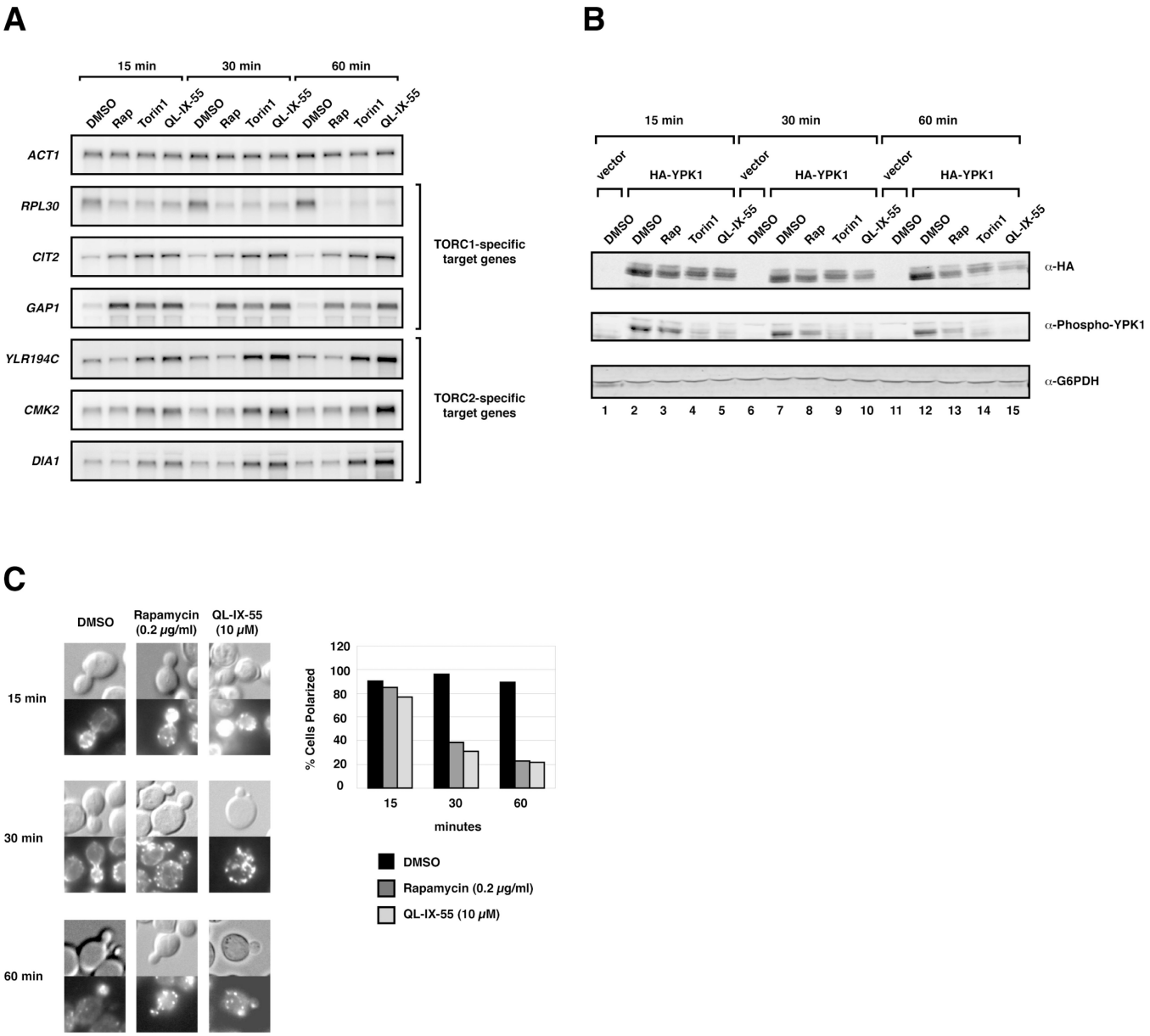


Figure 3. QL-IX-55 inhibition of both TORC1 and TORC2 signaling

(A) Northern blot analysis of total mRNA level

(B) TORC2-dependent phosphorylation of YPK1 inhibition.

(C) Actin cytoskeletal organization perturbation