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Remarkable inhibition of mTOR signaling by the combination of rapamycin and 1,4-phenylenebis(methylene)selenocyanate in human prostate cancer cells

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Abstract

Preclinical studies and clinical analyses have implicated the mammalian target of rapamycin (mTOR) pathway in the progression of prostate cancer, suggesting mTOR as a potential target for new therapies. mTOR, a serine/threonine kinase, belongs to two distinct signaling complexes: mTORC1 and mTORC2. We previously showed that the synthetic organoselenium compound, *p*-XSC, effectively inhibits viability and critical signaling molecules (e.g., androgen receptor, Akt) in androgen responsive (AR) and androgen independent (AI) human prostate cancer cells. Based on its inhibition of Akt, we hypothesized that *p*-XSC modulates mTORC2, an upstream regulator of the kinase. We further hypothesized that combining *p*-XSC with rapamycin, an mTORC1 inhibitor, would be an effective combinatory strategy for the inhibition of prostate cancer. The effects of *p*-XSC and rapamycin, alone or in combination, on viability and mTOR signaling were examined in AR LNCaP prostate cancer cells and AI C4-2 and DU145 cells. Phosphorylation of downstream targets of mTORC1 and mTORC2 was analyzed by immunoblotting. The interaction of mTORC1- and mTORC2-specific proteins with mTOR was probed through immunoprecipitation and immunoblotting. *p*-XSC inhibited phosphorylation of mTORC2 downstream targets, Akt and PCKα, and decreased the levels of rictor, an mTORC2-specific protein, co-immunoprecipitated with mTOR in C4-2 cells. The combination of *p*-XSC and rapamycin more effectively inhibited viability and mTOR signaling in C4-2, LNCaP, and DU145 cells than either agent individually.

Keywords

Prostate cancer; selenium; mTOR; combination therapy

INTRODUCTION

Despite advances in androgen deprivation therapy, prostate cancer recurrence and the development of androgen independence remain major challenges to prostate cancer survivorship. Hormone refractory prostate cancer is difficult to treat even though resistant

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cells express androgen receptors and rely on those receptors for growth and progression. Thus, an urgent need exists for the development of effective treatments against androgen independent (AI) prostate cancer by perhaps targeting androgen receptors or other cell proliferative targets. Specific mechanisms that account for the transition of androgen responsive (AR) tumors to AI tumors are not well understood but cell survival strategies that rely on signaling between androgen receptors and the PI3K/Akt/mTOR pathway may be critical for maintaining growth of prostate cancer cells. A study by Wu et al (1) suggest that repression of mTOR signaling results in a compensatory increase in androgen receptor function and thus promotes prostate cancer cell survival during deprivation therapy. Thus, agents that can modulate this signal pathway may be useful against AI prostate disease.

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that is an important transducer for cell growth and survival due to its involvement in protein synthesis, cell cycle progression, ribosome biogenesis and nutrient uptake (2). Additionally, mTOR signaling may be a key regulator of cancer cell growth as dysregulation of this pathway has been observed in a number of cancer types including prostate (3). Clinical analyses have shown increased expression and constitutive activity of components of the mTOR pathway in prostatic intraepithelial neoplasia (PIN) and prostate tumors compared with those of normal tissue (4-6). mTOR is a component of two compositionally and functionally distinct signaling complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 is rapamycin-sensitive and includes the mTOR catalytic subunit as well as proteins raptor and mLST-8/GβL. mTORC1 regulates cap-dependent translation initiation through phosphorylation of downstream targets 40S ribosomal protein S6 kinase (p70S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) (3). mTORC2 is composed of mTOR, rictor, mSin1, mLST-8/GβL, and PRR5 proteins. This complex activates Akt by phosphorylating serine 473 (7) and also plays a role in cytoskeletal organization through phosphorylation of PKCα (8).

Rapamycin selectively inhibits mTORC1 kinase activity and thus may induce autophagy to enable cells to recycle nutrients by breaking down expendable substrates. Clinically, Rapamycin is commonly used to prevent kidney graft rejection and as a component of arterial stents (9). Based on the preclinical effectiveness of mTOR inhibitors against a variety of cancer types, rapamycin-based mTOR inhibitors are being tested alone or in combination in over 150 ongoing trials for the treatment of a broad spectrum of malignancies including AI prostate cancer (10). However, some clinical trials that have used rapamycin as a monotherapy, including a phase I/II trial for the treatment of AI prostate cancer have reported disappointing results (11). Preclinical studies show that rapamycin in combination with other chemotherapeutic agents or signal pathway inhibitors has enhanced efficacy against prostate cancer (12-15). These studies re-enforce the clinical finding that combination regimens show significant tumor-responses than single agent therapies.

Preclinical studies in several laboratories including our own provide evidence supporting the potential use of organoselenium for treatment of late stage prostate cancer (16). Various selenium-containing compounds have been shown to modulate key targets mechanistically linked to prostate cancer progression and the development of AI disease (16-19). We previously showed that the synthetic organoselenium compound, 1,4-phenylenebis(methylene)selenocyanate (*p*-XSC), can modulate the androgen receptor and Akt signaling cascades in human prostate cancer cells (20).

The current study expands our understanding of the molecular targets of *p*-XSC by demonstrating its direct regulatory effects on components of the mTOR signaling pathway. Our previous findings determined that *p*-XSC inhibits Akt expression which suggested that inhibition of prostate cancer by *p*-XSC may be mediated by the mTORC2 signaling

pathway. We hypothesize that *p*-XSC in combination with rapamycin has a significant advantage over each agent alone and provides a complementary composite for the treatment of prostate cancer through inhibition of multiple critical cell signaling pathways. Coupled with rapamycin, various forms of organoselenium enhance or manifest a synergistic effect against prostate cancer when combined with other chemopreventive or chemotherapeutic agents (21-24). The current study demonstrates that *p*-XSC preferentially inhibits mTORC2 signaling in AI human prostate cancer cells. In addition, *p*-XSC in combination with rapamycin is superior to each agent alone in inhibiting prostate cancer cell growth. Further examination of the effects of combining *p*-XSC and rapamycin in prostate cancer cells shows that the combination inhibits downstream targets of both mTOR signaling complexes, likely contributing to their synergistic effects on cell viability primarily during androgen independent growth.

MATERIALS AND METHODS

Reagents and cell lines

Rapamycin was purchased from ALEXIS Biochemicals-Enzo Life Sciences (Plymouth Meeting, PA). *p*-XSC was synthesized as described previously (25). Androgen independent (AI) LNCaP C4-2 cells were obtained from Dr. Warren D.W. Heston, The Lerner Research Institute, The Cleveland Clinic Foundation, Ohio. Androgen responsive (AR) LNCaP cells and AI DU145 cells were obtained from the American Type Culture Collection (Manassas, VA).

Cell culture and treatments

C4-2 cells were maintained in RPMI-1640 medium supplemented with 10% Fetal Bovine Serum (FBS). LNCaP cells were maintained under the same conditions but with heat inactivated FBS. DU145 cells were cultured in Eagles minimum essential medium with Earle's balanced salt solution and 10% FBS. All cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and were routinely passaged when they were 70-80% confluent. Cells were plated in 10 cm dishes (1 million cells/plate) or 96-well plates (5,000 cells/well) depending on the assay, grown for 24 or 48 hr and then treated with *p*-XSC, rapamycin, or a combination of *p*-XSC and rapamycin at doses described below. The vehicle for both *p*-XSC and rapamycin was DMSO.

Detection of protein expression and phosphorylation by immunoblotting

Immunoblotting was performed as previously described to determine changes in downstream targets of mTOR (26). Briefly, cells were grown in 10 cm dishes for 48 hr and treated with *p*-XSC (5 and 10 μM), rapamycin (10 nM), or a combination of *p*-XSC and rapamycin (2.5 or 5 μM *p*-XSC with 1 or 10 nM rapamycin) for 90 min, harvested by scraping and washed with PBS containing phosphatase inhibitors (New England Biolabs, Ipswich, MA). The doses of *p*-XSC chosen were previously reported by us to inhibit Akt phosphorylation at 90 minute post incubation (20). The doses of rapamycin used in the combination studies (1 and 10 nM) were considerably lower than the doses of *p*-XSC as it is a potent and specific inhibitor of mTORC1 signaling and therefore individually inhibits mTORC1 target phosphorylation at such concentrations without notable effects on mTORC2 targets. Protein extraction was carried out using cell lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin) with freshly added 1 mM phenylmethylsulfonyl fluoride (PMSF). Equal amounts of protein (50 μg) were separated by electrophoresis on either 10 or 12% SDS-polyacrylamide gels and transferred to PVDF membranes. The following primary antibodies were used at dilutions ranging from 1:500 to 1:1000 for immunoblotting: Akt, phospho-Akt (Ser473), p70S6K, phospho-

p70S6K (Thr 389), RPS6, phospho-RPS6 (Ser 235/236), rictor, raptor, and mTOR from Cell Signaling Technology (Beverly, MA), and PKCα and phospho-PKCα (Ser 657) from Millipore (Billerica, MA). HRP-conjugated anti-mouse and anti-rabbit secondary antibodies (Cell Signaling, Beverly, MA) were used at a final dilution of 1:3000. Antigen-antibody reactions were developed using ECL reagents from Amersham (Piscataway, NJ) and density was analyzed using VisionWorks™ software (UVP, Inc. Upland, CA). All immunoblotting experiments were repeated three times. The results are presented as representative blots from single experiments.

Analysis of protein complex components by immunoprecipitation

In order to investigate the interactions of mTOR with its mTORC1 and mTORC2 binding partners, raptor and rictor, respectively, we immunoprecipitated mTOR from C4-2 cell lysates. C4-2 cells were grown in 10 cm for 48 hr and then treated with *p*-XSC (5 and 10 μM), rapamycin (10 nM), or a combination of *p*-XSC and rapamycin (5 μM *p*-XSC with 10 nM rapamycin) for 15, 30, 60, 90 min and 6 hr. After treatment, cells were washed with cold PBS and 500 μl of cell lysis buffer was added to each plate. The cells were incubated on ice for 5 min, harvested by scraping, transferred to microfuge tubes, and sonicated on ice by three 10-sec bursts using a Sonic Dismembrator Model 100 (Fisher Scientific, Pittsburgh, PA). Samples were then centrifuged (12,000 × g) at 4°C for 10 min and the supernatant fractions were transferred to fresh tubes. Equal amounts of protein (200 μg) from each sample were incubated with an anti-mTOR primary antibody (Cell Signaling, Beverly, MA) at a final dilution of 1:100 and rocked overnight at 4°C. Next, 20 μl of a Protein A agarose bead slurry (Invitrogen, Carlsbad, CA) was added and samples were rocked at 4°C for additional 3 hr. The protein-antibody-bead complexes were then collected by centrifugation (5,000 × g for 5 minutes) and washed with cell lysis buffer. Samples were resuspended in 25 μl of 3X SDS-PAGE sample buffer, separated by electrophoresis on 7.5% SDS-polyacrylamide gels, and transferred to PVDF membranes for immunoblotting analysis.

MTT assay for cell viability

C4-2, LNCaP, and DU145 cells were plated (5000 cells/well) in triplicate in 96 well format. After 24 hr, cells were treated with a range of doses of *p*-XSC (0.625, 1.25, 2.5, 5, 10, and 20 μM), rapamycin (0.1, 1, 10, 100, and 1000 nM), or a combination of the two agents including each dose of *p*-XSC with each dose of rapamycin. After 48 hr, MTT assays were performed as previously described to determine cell viability (27). Analyses were repeated three times and data averaged. Results are expressed as percent viability compared to the vehicle-only control.

Median effect analysis for combined effects

Synergistic effects of *p*-XSC and rapamycin were determined using a constant molar ratio of *p*-XSC to rapamycin (5000:1). Maintaining this ratio, we assessed the viability of C4-2 using a dose range of each agent as follows: 2.5, 5, 10, 20, and 40 μM *p*-XSC with 0.5, 1, 2, 4, and 8 nM rapamycin, respectively. The molar ratio of 5000:1 was chosen initially because a strong enhancement of inhibition was seen when C4-2 cells were treated for 48 hr with a combination of 5 μM *p*-XSC and 1 nM rapamycin. Also, the dose of *p*-XSC is within a physiological attainable concentration that is a relatively nontoxic level for this organoselenium compound and for rapamycin, one that is clinically achievable (11, 28). C4-2 cells were grown in triplicate in 96 well plates (5000 cells/well) for 24 hr, treated with the aforementioned combinations of *p*-XSC and rapamycin for 48 hr, and evaluated by MTT assay for cell viability.

The interaction between the combined agents was evaluated for synergism using the median effect and combination index (CI) equations described by Chou and Talalay (29-31). The efficacies of a given agent on cell viability are described by the median effect equation:

$$f_a/f_u=(D/D_m)^m$$

where f_a and f_u represent the affected and unaffected fractions respectively, by a given dose (D). D_m is the dose that elicits the median effect and m is the coefficient of the sigmoidocity of the dose effect curve. We generated dose-effect curves and median effect plots [log(D) vs. $\log(f_a/f_u)$] for *p*-XSC, rapamycin, and the combination of the two at the constant molar ratio of 5000:1. The parameters D_m and m were determined from the median effect plots. We assessed the fractional effects associated with each agent individually and in combination over a range of concentrations.

The nature of the interactive effects of *p*-XSC and rapamycin were evaluated by calculating the CI defined as:

$$CI=(D)_1/(D_x)_1+(D)_2/(D_x)_2+\alpha(D)_1(D)_2/(D_x)_1(Dx)_2$$

where $\alpha = 0$ and $\alpha = 1$ for p-XSC and rapamycin indicating whether the compounds are mutually exclusive and non-exclusive, respectively. $(D_x)_1$ and $(D_x)_2$ are the doses of *p*-XSC and rapamycin alone required to achieve a given effect level (f_a). $(D)_1$ and $(D)_2$ are the doses of *p*-XSC and rapamycin in combination that achieve the same f_a. The value of CI reflects synergism when the calculated value is < 1, antagonism when it is > 1 and additivity when it is = 1. CI values were calculated for the combination of *p*-XSC and rapamycin over a range of effect levels (0.2 to 0.9) indicating strong to moderate synergy between the test compounds.

Statistical analysis

Histogram results are expressed as mean ± standard error. Statistical significance was evaluated using either one or two-factor analysis of variance (ANOVA). Differences were considered significant at $p < 0.05$.

RESULTS

Time-course effects of *p*-XSC on mTOR complex proteins

To determine whether *p*-XSC interferes with mTOR complex formation, we immunoprecipitated mTOR from C4-2 cells treated with 5 and 10 μM *p*-XSC and probed to identify whether raptor and rictor co-immunoprecipitated. Immunoprecipitation was conducted after 15, 30, 60, and 90 min of treatment as well as after 6 hr of treatment to evaluate how early *p*-XSC mediated changes occur. No change in rictor or raptor binding to mTOR was observed at 15 (data not shown) or 30 min (Figure 1A) post-treatment with *p*-XSC. *p*-XSC decreased the amount of mTORC2 binding protein, rictor, that coimmunoprecipitated with mTOR as early as 60 min after treatment (Figure 1A). This inhibition lasted for at least 90 min and began to return to control levels at 6 hr; rictor levels in cells after 6 hr of treatment with 10 μM *p*-XSC were still less than those in the control samples (Figure 1A). At 6 hr of treatment, *p*-XSC caused a dose-dependent decrease in the amount of immunoprecipitated Ser 2481 phospho-mTOR, an autophosphorylated form of mTOR suggested to be a biomarker for intact mTORC2 and an indicator of mTOR catalytic activity (Figure 1B) (32,33).

The effects of *p*-XSC on mTOR target proteins

We assessed *p*-XSC modulation of mTOR by immunoblotting the levels of phospho-p70S6K (Ser 235/236), phospho-Akt (Ser 473), and phospho-PKCα (Ser 657) in C4-2 cell lysates that had been treated with *p*-XSC for 90 min. p70S6K is a downstream target of mTORC1, whereas Akt and PKCα are downstream targets of mTORC2. *p*-XSC, at 5 and 10 μM decreased phospho-Akt (Ser 473) (as we had also seen previously, 20) and phospho (Ser 657)- PKCα (Figure 1C). *p*-XSC had little to no effect on the phosphorylation of mTORC1 target p70S6K (Figure 1C). These results indicate that p-XSC affects the ratio of phosphorylated to non-phosphorylated proteins as no change in total p70S6K, Akt, or PKCα was observed with treatment.

The effects of *p*-XSC and rapamycin on prostate cancer cell viability

We initially examined the effects of mixtures of *p*-XSC and rapamycin on viability in the AI cell line, C4-2, and its AR parental cell line, LNCaP, by combining four doses of *p*-XSC (0.625, 1.25, 2.5, and 5 μM) with six doses of rapamycin (0.1, 1, 10, 100, 1000, and 10,000 nM). Data acquired at 48 hr of treatment showed that the combinations of *p*-XSC and rapamycin in the C4-2 cells, especially those with low-dose rapamycin (0.1, 1, and 10 nM), had significantly ($p < 0.05$) superior efficacy to inhibit growth compared with each agent individually (Figure 2A). For example, the combination of 0.1 nM rapamycin with 1.25, 2.5, and 5 μM *p*-XSC resulted in an 18.3, 12.3, and 26.8 percent increased inhibition, respectively. The addition of 1 nM rapamycin to 0.625, 1.25, 2.5, and 5 μM *p*-XSC resulted in 23, 32.2, 27.4, and 37.9 percent increased inhibition of C4-2 cell viability, respectively. The 10 nM dose of rapamycin enhanced inhibition mediated by 0.625, 1.25, 2.5, and 5 μM *p*-XSC by 19.7, 22.4, 30.1, and 37.6 percent, respectively. In LNCaP cells, treatment with the same mixtures of *p*-XSC and rapamycin showed similar trends (Figure 2B). However, the combination was less effective than that observed in C4-2 cells; significant ($p < 0.05$) reductions in viability were only achieved for the combination of 2.5 μM *p*-XSC with 1 μM rapamycin or when 5 μM *p*-XSC was combined with 1 nM or higher dose of rapamycin. Figure 2C shows that while the mixture of 5 μM *p*-XSC and 1 nM rapamycin was significantly more effective at inhibiting both C4-2 and LNCaP cell viability than either agent individually, DU145 cells did not appear to be sensitive to the same combination.

The C4-2 cells also achieved the most dramatic decreases in IC_{50} when *p*-XSC was combined with rapamycin. Table 1 compiles the calculated IC_{50} values that occurred when each agent at a range of doses was combined with a single dose of the other agent (represented graphically by each row and column in Figure 2A). For example, when 1 nM rapamycin was added to *p*-XSC, the IC_{50} decreased more than 2-fold. The addition of 5 μM *p*-XSC lowered the IC_{50} of rapamycin from the micromolar range to sub-nanomolar range.

Determination of the Combination Index (CI) for *p*-XSC and rapamycin

The combinatory efficacy of *p*-XSC and rapamycin was evaluated further in C4-2 cells, as these cells were the most sensitive of the prostate cancer cells examined. The above experiments clearly show that the combination of *p*-XSC and rapamycin have inhibitory effects on C4-2 cell viability after 48 hr which are significantly greater than with use of each agent individually. Our approach to evaluate synergistic or additive effects for the combination of *p*-XSC and rapamycin involved the method of Chou and Talalay (29-31). We chose a constant molar ratio of *p*-XSC to rapamycin (5000:1) and treated C4-2 cells with a range of doses at that ratio for viability. D_m values for *p*-XSC alone and in combination with rapamycin were determined to be 7.13 and 5.06 μM, respectively, while those for rapamycin, alone and in combination with *p*-XSC were 107.17 and 0.001 μM, respectively. The individual median effect plots for *p*-XSC and rapamycin were not parallel and therefore exclusivity could not be determined with certainty. Thus, we calculated CIs assuming that

both compounds exhibited mutual and non-mutual exclusivity (α = 0 and 1, respectively). All CI values were similar when calculated using both values for α except for the two lowest effect levels (0.2 and 0.3) (See Table 2). At effect levels ranging from 0.2 to 0.9, the CI values indicated varying degrees of synergy, with the degree of synergy increasing as the fraction affected increased (Table 2).

The effects of combining *p*-XSC and rapamycin on mTOR target proteins

We investigated whether combining *p*-XSC and rapamycin at a set ratio (2.5 or 5 μM *p*-XSC and 1 or 10 nM rapamycin) for 90 min decreased phosphorylation of their early mTOR molecular targets in C4-2 cells. Treated cells were analyzed by immunoblotting and phosphorylations of downstream targets of the mTOR pathway were determined using combinations of *p*-XSC and rapamycin. Results showed that the combination had greater efficacy to inhibit phosphorylation of the mTORC2 downstream targets, Akt and PKCα, than either agent alone at the same dose (Figure 3A). By contrast, treatment with 10 nM rapamycin alone was sufficient to eradicate phosphorylation of the mTORC1 downstream target, RPS6, and therefore we were unable to conclude whether *p*-XSC caused enhancement of the activity of rapamycin at this dose and time point. As a result of this finding, the dose of rapamycin was decreased to 1 nM in combination with *p*-XSC. This combination was sufficient to inhibit phosphorylation of PKCα at Ser 657, an mTORC2 specific site (8). Treatment with 1 nM rapamycin alone still decreased phospho-RPS6 (Ser 235/236), however, the combination of *p*-XSC and rapamycin was considerable more efficacious in decreasing phosphorylation. Results similar to these were observed in C4-2 cells treated with 2.5 μM *p*-XSC and 1 nM rapamycin, reinforcing the concept that combination of these agents are effective at low and clinically achievable doses. Immunoblotting analysis also showed that the responses of the LNCaP and DU145 cells paralleled those of the C4-2 cells in that the combination of *p*-XSC (5 μM) with rapamycin (1 or 10 nM) inhibited phosphorylation of both the mTORC1 downstream target, RPS6, and the mTORC2 target, Akt (Figure 3B).

DISCUSSION

This study determined that the synthetic organoselenium compound, *p*-XSC, inhibits mTORC2 signaling in human AI prostate cancer cells. A previous report showed that high doses (0.125–2 mM) of inorganic sodium selenate inhibit IGF-1-stimulated phosphorylation of Akt and mTOR in HT-29 human colon cancer cells (34). This study suggested that selenate inhibits mTORC1 by both an Akt-dependent and an Akt-independent mechanism. In other studies, 24 hr treatment of prostate cancer cells with an extract of selenium-enriched broccoli, which accumulates two active anticancer agents: sulforaphane and Se-methylselenocysteine, reduced phosphorylation of mTOR (35). Our data show that *p*-XSC exhibits specificity and selectivity in that the mTORC2 is preferentially targeted. *p*-XSC inhibited phosphorylation of Akt and PKCα both downstream targets of mTORC2 whereas it had no effect on phosphorylation of downstream targets of mTORC1. In addition, *p*-XSC decreased the levels of mTORC2 protein, rictor, associated with mTOR but had no effect on the levels of raptor, the protein bound to mTORC1. The present study was specifically designed to examine early changes (t = 90 min) mediated by *p*-XSC. Subsequent studies will evaluate whether extended exposures to *p*-XSC in AI and AR prostate cancer cells would affect changes in mTORC1 signaling.

Our findings markedly expand our current understanding of *p*-XSC-mediated inhibition of prostate cancer cell growth (20,26,27). Inhibition of mTORC2 by *p*-XSC, subsequently results in inhibition of Akt as well as its other downstream targets and thus greatly expands the impact that organoselenium compounds have on growth of androgen independent prostate cancer cells (Figure 4). A recent study in PTEN null mice showed that mTORC2

was required for the development of prostate cancer caused by the loss of *Pten* but was non-essential in normal prostate, highlighting mTORC2 as a significant target for prostate cancer treatment (36) especially apropos to hormone refractory disease. This further supports the potential of *p*-XSC for the management of advanced prostate cancer, which is frequently characterized by deletion of *Pten* (37,38) and significant "crosstalk" between androgen receptors and mTOR signaling to evoke a survival advantage of prostate cancer cells during androgen deprivation and under conditions of low nutrient intake (1).

A major highlight of our study is the finding that mixtures of *p*-XSC and rapamycin are more effective than either agent alone at low dose to inhibit prostate cancer cell viability. After 48 hr of treatment, C4-2 cell viability was significantly inhibited. The most promising combinations were those comprised of *p*-XSC with low-dose rapamycin (0.1, 1, and 10 nM). The efficacy of these compounds was markedly increased by 38% when *p*-XSC and rapamycin were mixed rather than used as single agent therapies thus providing dose ranges which are physiologically achievable and potentially clinically safe. The physiological level of rapamycin achieved in patients is on the order of 1 nM, although higher trough levels (up to 20 nM) have been reached in some clinical studies with marginal side effects (11,28,39). When added to rapamycin, *p*-XSC decreased its IC_{50} from the micromolar range to the low-nanomolar range (Table 1). In a similar fashion, addition of 1 nM rapamycin to *p*-XSC decreases its IC_{50} more than two-fold (Table 1). It is of vital importance to clinical management of prostate cancer to be able to achieve inhibition of growth and proliferation of both AR and AI prostate cancer cells using doses of these agents that are sustainable in humans.

An ideal objective in creating a strategy for combination therapy is to achieve synergy between drugs. In this study, we used median effect analysis described by Chou and Talalay (29) to determine the combination index (CI) for *p*-XSC and rapamycin at a constant molar ratio of 5000:1. The median effect plots generated for *p*-XSC and rapamycin alone and in combination had correlation coefficients greater than 0.9, confirming the applicability of this means of analysis for our study. We could not determine the exclusivity of the mechanisms of action of *p*-XSC and rapamycin because the slopes of their median effect plots were not parallel. Our analyses clearly indicate that the combination of *p*-XSC with rapamycin at a constant molar ratio of 5000:1 does exhibit synergistic inhibition of C4-2 cell viability. The calculated CIs indicate a moderate but highly significant degree of synergism that can be achieved by combining *p*-XSC with rapamycin. The strength of synergy of this combination increased as the effect level increased, reaching a maximal degree (CI = 0.684) at 90% inhibition.

Our data also show that treatment of C4-2 cells with combinations of *p*-XSC and rapamycin achieve early target inhibition (at least by 90 min) at concentrations that have little or no effect when administered individually. Mixtures of 2.5 or 5 μM *p*-XSC with 1 nM rapamycin significantly inhibit phosphorylation of downstream targets of both mTORC1 and mTORC2, likely contributing to their detrimental effects on cell viability. This study is of clinical significance as it identifies a combination regimen that fits the medical paradigm of a highly effective two-agent therapy. Future studies are needed to provide exact mechanisms by which rapamycin sensitizes AI prostate cancer cells to the actions of *p*-XSC.

The combination of *p*-XSC and rapamycin was also effective in AR LNCaP cells as well as in prostate cells with mutated p53 and absence of androgen receptors, namely, DU-145 cells. Although less responsive to *p*-XSC alone, AI DU-145 cells respond well to the combination of *p*-XSC with rapamycin. RPS6 phosphorylation was dramatically inhibited in DU-145 cells treated with mixtures of *p*-XSC and rapamycin compared to cells treated with the individual agents. Mixtures of *p*-XSC and rapamycin at doses higher than 5 μM and 1 nM,

respectively, significantly inhibited DU-145 cell viability to extents greater than either agent alone (data not shown). Interestingly, the combination was effective in DU145 cells despite this line being known to have less of a dependence on the PI3K pathway than the other lines used. *p*-XSC has been shown to inhibit multiple targets and it may be unknown mechanisms of action of this compound and its synergy with rapamycin that drive inhibition in the DU145 line.

Often multiple survival pathways are activated in cancer cells. The use of combinations of agents that inhibit different pathways is an attractive alternative to treatments with a single chemotherapeutic agent. Our studies show that *p*-XSC and rapamycin inhibit prostate cancer cell viability more effectively than either agent alone, in part, by targeting two distinct arms of the mTOR signaling cascade (Figure 4). Other studies have shown that inhibition of mTORC1 can activate Akt (40). Rapamycin treatment, while suppressing mTORC1 signaling, may be regulating Akt signaling in prostate cancer cells. Thus simultaneous treatment of prostate cancer cells with *p*-XSC, which specifically inhibits mTORC2 and subsequently Akt, along with rapamycin, may be acting to compensate for feedback activation of this regulatory cell survival pathway.

The ability of *p*-XSC to inhibit mTORC2 signaling, a potentially critical target in cancer treatment, is a significant finding in support of its use as a chemotherapeutic tool against advanced prostate cancer. These data together with the finding that rapamycin can enhance the anti-cancer effects of *p*-XSC in C4-2 cells, as well as LNCaP and DU145 cells, provide a rationale for continuing exploration of this and other organoselenium derivatives, alone or in combination, as means for improving patient survivorship especially for those with hormone refractory prostate cancer. Further characterization of the anti-cancer mechanisms of *p*-XSC in prostate cancer cells will be useful for understanding and subsequently improving cancer treatments using targeted approaches with organoselenium. Investigations of the effects of *p*-XSC and rapamycin individually and in combination on tumorigenesis in animal models of prostate cancer are a necessary next step in the evaluation of the potential applicability of these agents to a clinical setting. Lastly, our studies provide a framework for future investigations to treat hormone refractory disease using other organoselenium compounds in combination with analogs of rapamycin or other signal pathway inhibitors.

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ABBREVIATIONS USED

AI	androgen independent
AR	androgen responsive
CI	combination index
DMSO	dimethylsulfoxide
FBS	fetal bovine serum
mTOR	mammalian target of rapamycin
mTORC1	mTOR complex 1
mTORC2	mTOR complex 2

MTT	(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide)
PBS	phosphate buffered saline
***p*-XSC**	1,4-phenylenebis(methylene) selenocyanate

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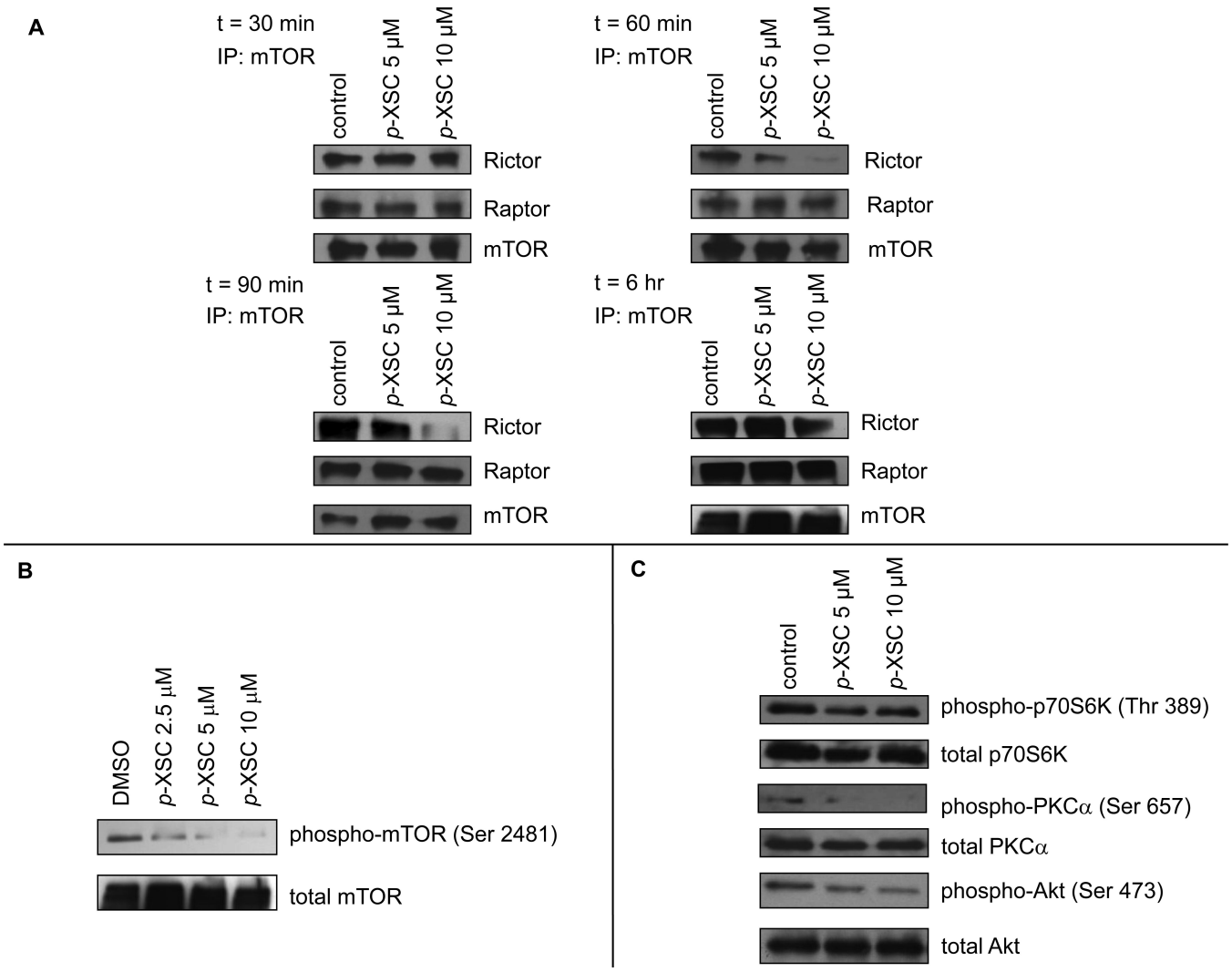

Figure 1.

The effects of *p*-XSC on mTOR pathway molecules. **A.** Levels of mTOR binding proteins raptor and rictor co-immunoprecipitated with mTOR from C4-2 cells treated with *p*-XSC for 30, 60, and 90 min and 6 hr. **B.** Immunoblot analysis of mTOR phosphorylation in C4-2 cells treated with *p*-XSC for 6 hr. **C.** Immunoblot analysis of phosphorylated downstream targets of mTOR in C4-2 cells treated with *p*-XSC for 90 min.

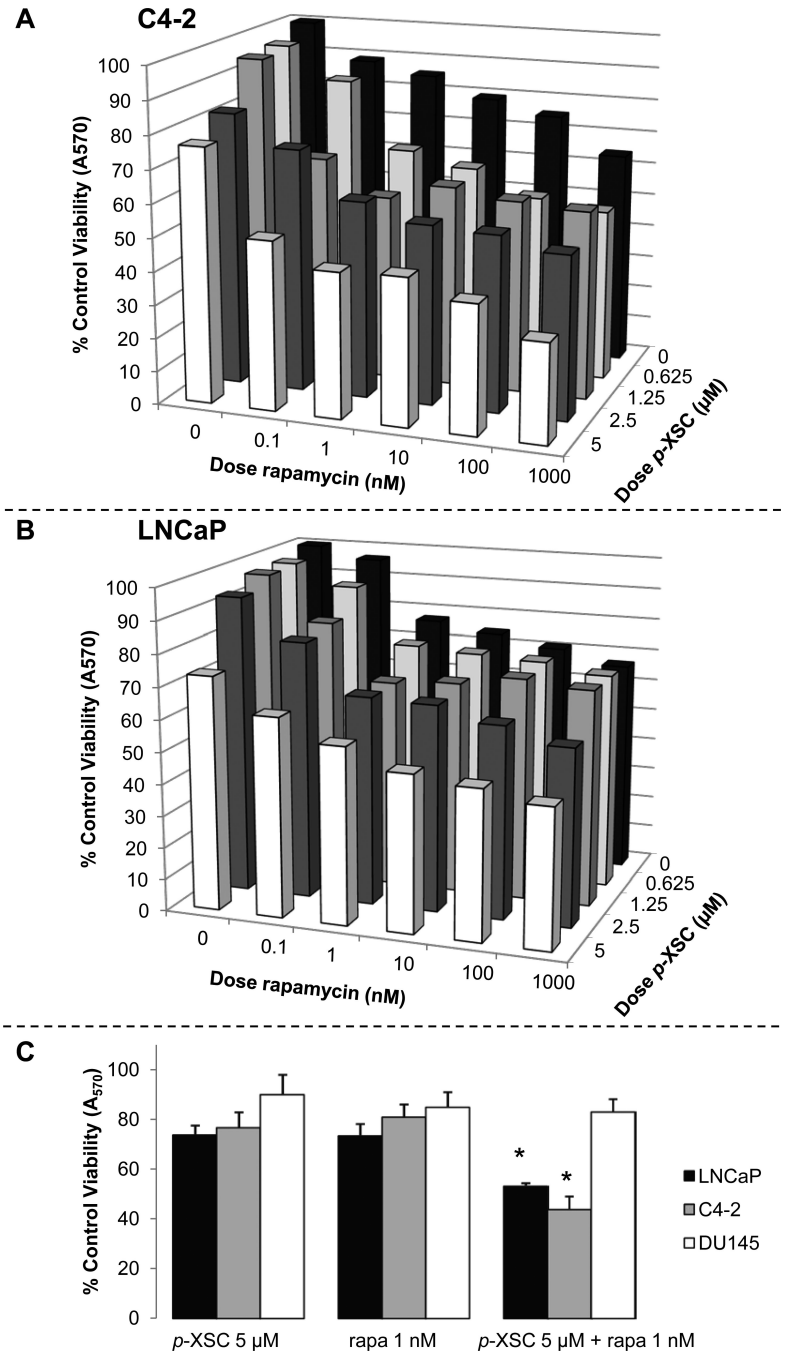

Figure 2.

The effects of selected combinations of *p*-XSC and rapamycin on cell viability and IC_{50} values (t = 48 hr). **A**. C4-2 and **B**. LNCaP cells were treated for 48 hr with a range of doses of rapamycin (x-axis; 0.1 - 1000 nM) and *p*-XSC (z-axis; 0.625 – 5 μM) alone and in combination and assayed for viability using the MTT method. **C**. Comparison of the effects of 5 μM *p*-XSC and 1 nM rapamycin (rapa), alone and in combination, on LNCaP, C4-2, and DU145 cell viability. Cells were treated for 48 hr with 5 μM *p*-XSC and 1 nM rapa, alone and in combination, and assayed for viability using the MTT method ($p < 0.05$).

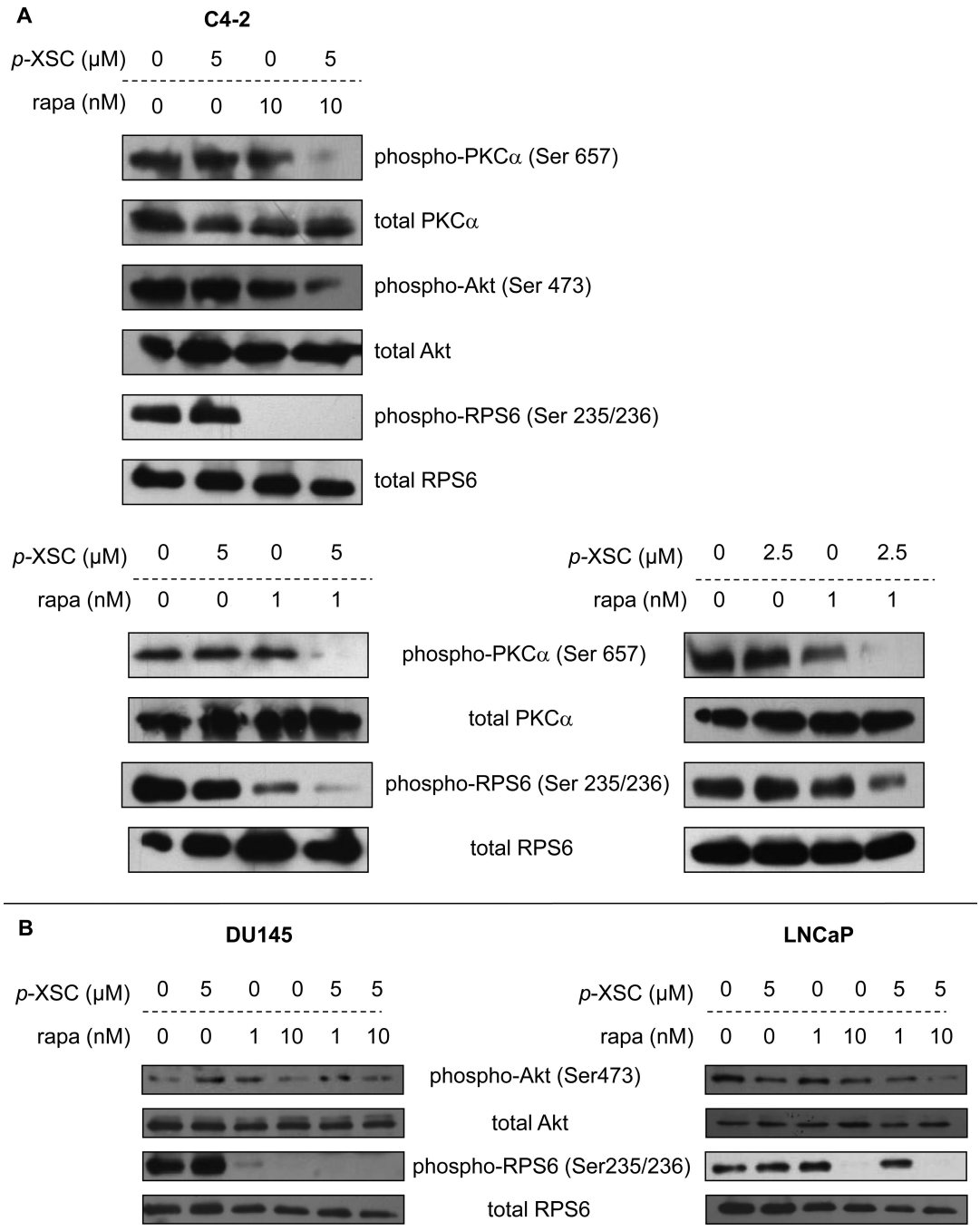

Figure 3.

The effects of combining *p*-XSC and rapamycin on mTOR pathway molecules. Immunoblot analysis of phosphorylated downstream targets of mTOR in **A**. C4-2 cells treated with 5 μM *p*-XSC and 10 nM or 1 nM rapamycin (rapa) or 2.5 μM *p*-XSC and 1 nM rapa. **B**. DU145 and LNCaP cells treated with 5 μM *p*-XSC and 1 or 10 nM rapa.

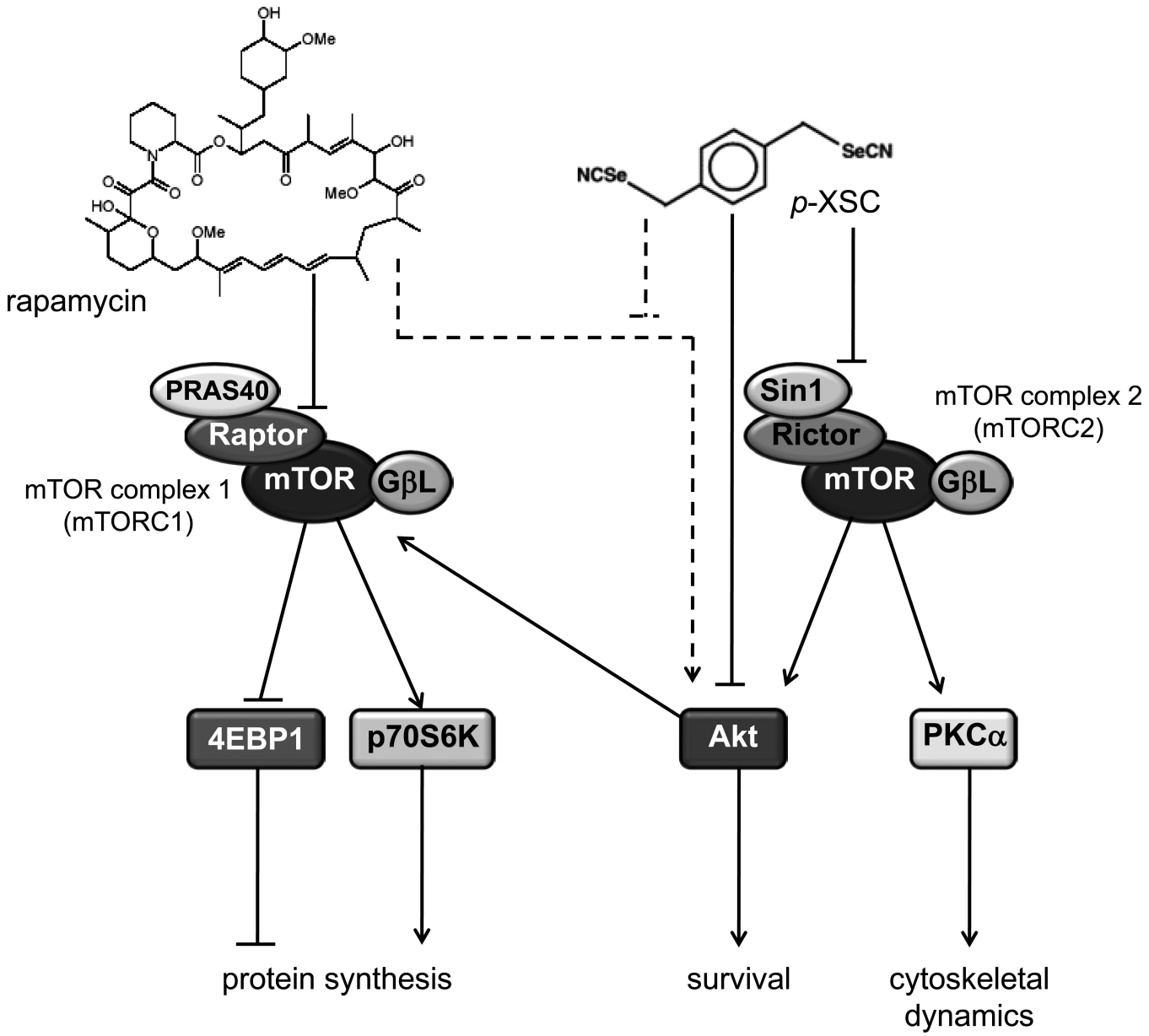

Figure 4.

Proposed scheme for the inhibition of both arms of the mTOR pathway by *p*-XSC and rapamycin in C4-2 cells. Rapamycin inhibits mTORC1 signaling while *p*-XSC inhibits mTORC2 signaling. *p*-XSC treatment may offset feedback activation of Akt by rapamycin (represented by broken lines).

Table 1

IC_{50} values for *p*-XSC in combination with varying doses of rapamycin (0.1 - 1000 nM) and IC_{50} values for rapamycin in combination with single doses of *p*-XSC (0.625 - 5 μM) in C4-2 cells treated for 48 hr. MTT assays were used to generate the dose curves from which the IC_{50} values were calculated.

[Rapa] (nM)	IC_{50} *p*-XSC (μM)	[*p*-XSC] (μM)	IC_{50} Rapa (nM)
0	7.2	0	49000
0.1	5.9	0.625	1400
1	3.5	1.25	570
10	3.3	2.5	560
100	2	5	0.2
1000	1.3		

Table 2

Calculated values for the combination index (CI) as a function of fractional inhibition (F_a) of C4-2 cell viability for a mixture of *p*-XSC and rapamycin (molar ratio 5000:1) after 48 hr of treatment.

Fractional Inhibition (F_a)	Combination Index (CI)	Description of combined effect
0.2	0.808	Moderate Synergism
0.3	0.724	Moderate Synergism
0.4	0.715	Moderate Synergism
0.5	0.710	Moderate Synergism
0.6	0.705	Moderate Synergism
0.7	0.700	Synergism
0.8	0.694	Synergism
0.9	0.684	Synergism