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Deprivation of estrogen, called EndocrineTherapy (ET), is commonly used to treat women with estrogen receptor (ER) positive breast cancer. Resistance to ET occurs in many women after about 18 months of treatment. Upregulation of growth factor pathways mediated by the 21 kD RasGTPase protein may contribute to resistance to ET. A novel Ras antagonist, farnesylthiosalicylate (FTS) causes Ras downreguation with concomitant abrogation of growth factor pathways. We tested the ability of FTS, which was complexed to a cyclodextrin moiety for solubility, to reduce the growth of ER positive breast cancer cells that were resistant to ET. FTS prevented growth of ER positive cells by increasing apoptosis and reducing proliferation. Surprisingly the FTS enhanced apoptosis in breast cancer cells was synergistic with the effects of estradiol. Accompanying loss of cell growth was a significant reduction in the response to estrogen. Of major interest was the unexpected observation that FTS blocked the signaling molecule, mTOR via a novel mechanism, the dissociation of RAPTOR from mTOR. In vivo studies have demonstrated a reduction in tumor size with FTS at doses that caused no demonstrable toxicity. We suggest that FTS should enter preclinical trials against ER positive breast cancer.

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Introduction :

One-third of human breast cancers depend upon estrogen for growth and regress upon exposure to antiestrogens or inhibitors of estrogen biosynthesis (e.g. aromatase inhibitors). In advanced breast cancer, initial responses to the antiestrogen tamoxifen last for 12-18 months on average but tumors nearly uniformly begin to regrow later . Secondary therapies with aromatase inhibitors often cause additional tumor regressions but are again followed by relapse . Extensive recent work has focused upon the mechanisms underlying relapse during tamoxifen or aromatase inhibitor therapy. These studies demonstrate upregulation of growth factor pathways involving the MAP kinase and PI-3-kinase signaling cascades. Based upon this concept, a number of investigative groups have suggested that growth factor inhibitors might serve as ideal agents to prolong responses to hormonal therapy in breast cancer or to control progression into an estrogen independent state.

Binding of a number of specific growth factor ligands to their cognate receptors activates a pathway involving Ras and leads to activation of MAP kinase and PI-3-kinase. Because of the key role of Ras, this signaling molecule has been a prime target for drug development. Major efforts have been directed toward development of farnesyltransferase inhibitors (FTIs). Since Ras must be farnesylated to be anchored in the plasma membrane, the FTIs prevent Ras from localizing in the plasma membrane and result in accelerated degradation in the cytoplasm. While the FTIs exert anti-tumor effects, a body of recent work suggests that mechanisms other than Ras depletion explain the efficacy of these agents. Current speculation is that the FTIs may block Rho B as their primary mechanism of action.

Another anti-Ras strategy is to block the binding of Ras to its membrane acceptor sites. Ras must be farnesylated and bound to GTP as a pre-requisite for forming a high affinity complex with membrane acceptor proteins and for its activity in activating the MAP kinase pathway. The group of Kloog et al. have developed a compound capable of dissociating GTP-Ras from its membrane binding sites. This agent, called farnesylthiosalicylic acid (FTS), binds specifically to galectin 1 and displaces GTP-Ras from it. As a consequence, GTP-Ras loses its anchor to galectin 1 in the plasma memberane and rapidly traverses the raft like structures as well as the non-caveolar regions of the plasma membrane. Lacking an anchor in the

membrane, Ras re-enters the cytoplasm where it is degraded and inactivated over a period of several hours. Through this mechanism, FTS interrupts the ability of Ras to signal in the plasma membrane.

While activating mutations of Ras are uncommon in human breast cancer, the MAP kinase pathway plays a major role in mediating the proliferative effects of estradiol. In addition, the MAP kinase and PI-3-kinase pathways are frequently up-regulated in response to estrogen deprivation therapy and may play a role in development of hormonal resistance. Accordingly, several clinical trials are examining the effect of drugs designed to abrogate Ras effects to prolong the beneficial actions of tamoxifen and the aromatase inhibitors.

Several investigators have demonstrated that FTS blocks the activation of MAP kinase and causes inhibition of the growth of tumors containing activating mutations of Ras (i.e. pancreatic cancer and malignant melanomas). However, no previous studies have examined the effect of FTS on breast cancer because Ras is only infrequently mutated in this neoplasm. However, we reasoned that the frequent up-regulation of the MAP kinase pathway through Ras, which occurs in response to estrogen deprivation therapy, might uncover a role for FTS in this cancer. The present studies examined the effects of FTS on growth of estrogen dependent breast cancer cells in vitro **(Specific Aim 1)** and in xenografts in vivo **(Specific Aim 2)**.

Methods and Procedures: Cell culture methods utilize MCF-7 cells grown under standard conditons are described in the original grant application. Xenografts of MCF-7 cells grown in nude mice use previously published techniques. Briefly, cells are grown up in tissue culture and then implanted into the flanks of castrate nude mice. Estradiol is administered by silastic implant which delivers the required dose of estradiol (i.e. estradiol clamp method). The measurements of tumor size utilize a caliper accoridng to previously publsihed techniques. The western blot studies using phosphospecifc anitbodies are standard in our laboratory and have been extensively publsihed.

Results:

Specific Aim 1: Wild type MCF-7 cells responded to estradiol with maximal stimulation at a dose of 10^{-11}M which increased cell number approximatley three fold (Figure 1A). Increasing concentrations of FTS

suppressed E2 dependent growth in a dose responsive fashion with initial inhibition at 50μM and maximal effects at 75 μM FTS. As a reflection of hypersensitivity to estradiol (as previously extensively described, the LTED cells (Figure 1B) responded maximally to two log lower concentrations of estradiol than did wild type cells (i.e. 10^{-13}M vs 10^{-11}M respectively). In contrast to its effects in wild type cells, FTS suppressed LTED growth completely at a dose of 25 μM with continued suppression at 50 μM. Unexpectedly, we observed lesser inhibition of growth at 75 μM FTS in LTED cells.

FTS is a relatively hydrophobic lipid analogue. It was initally thought that it would not be absorbed by patients in an oral formulation. We reasoned that cyclodextrins (CDs) which have been used previously to solubilize hydrophobic drugs, might be a practical means to develop a practical formulation of FTS for ultimate use in humans. Accordingly, FTS was complexed with CD and compared to free FTS and to the DMSO vehicle (for free FTS) and to buffer vehicle (for CD-FTS) in our in vitro system. FTS and FTS-CD exhibited almost identical MCF-7 cell growth inhibition profiles (Figure 2A). CD alone or buffer vehicle had no significant effect on cell growth of MCF-7 and LTED cells (Figures 2B and 2C) under conditions where FTS-CD significantly reduced cell growth. Even though LTED cells have higher MAPK activity than MCF-7 cells, the degree of inhibition was the same between the two cell lines (Figure 2C). We have also observed similar results in tamoxifen resistant breast cancer cell lines (data not shown).

Ras-mediated growth factor pathways are required to maintain cellular proliferation and are important regulators of the apoptotic response. Reduction of cell number can reflect either an inhibition of proliferation, an enhancement of apoptosis, or a combination of these two effects. Accordingly, we systematically examined the effects of FTS specifically on apoptosis and then on BRD-U incorportion as a marker for proliferation. FTS enhanced apoptosis in wild type and LTED cells starting at a dose of 60 μM and maximally stimulating at a dose of 100 μM FTS-CD to levels 3000-4000 fold higher (Figures 3A and 3B). Proliferation was also reduced to very low amounts in both cell lines by FTS-CD, but not by CD or buffer alone (Figures 4A and 4B). The disruption of both proliferation and apoptosis indicates a global disruption of signaling pathways in breast cancer cells.

Effect of FTS on serum-induced activation of MAP kinase, PI3 kinase and mTOR in MCF-7 and LTED cells. To dissect out the mechanisms whereby FTS inhibits the proliferation of breast cancer cells, we first examined its effect on the MAP kinase pathway under serum containing conditions (serum is a source of endogenous growth factors). We utilized an antibody to detect phospho-ERK 1 and 2, a commonly used indirect assay to assess MAP kinase activation. Surprisingly, FTS did not inhibit the phosphorylation of ERK1/2 MAP kinase at doses causing 50% inhibition of cell growth (i.e. 50 μM). Only higher doses of FTS (i.e. 75 μM) blocked this kinase (Fig. 5A). To obtain more direct evidence of MAP kinase effects, we then utilized a MAP kinase activity assay. ERK1/2 activity was measured in vitro by monitoring phosphorylation of Elk-1. Pre-incubation of LTED cells with FTS at concentrations of 25-75 μM for 3 h did not reduce the levels of Elk-1 phosphorylation. However, reduction in the level of phospho-Elk-1 was seen in the cells that were exposed to 100 μM of FTS for 24 h (Fig. 5B). Used as a positive control, U0126, a known MEK inhibitor, completely blocked ERK1/2 activity for the same 3 h treatment. These results suggest that inhibition of MAP kinase activation is not the predominant mechanism for FTS-induced inhibition of proliferation of breast cancer cells.

We next turned our attention to the PI3 kinase pathway. We first examined the effect of FTS on activation of PI3 kinase by monitoring changes in phosphorylation of Akt (Ser^{473}). FTS, at 50 μM, caused only 30% inhibition of Akt phosphorylation in LTED cells. The inhibition was only slightly increased with higher concentrations of FTS. The inhibitory effects of FTS on Akt phosphorylation were smaller in MCF-7 than in LTED cells (Fig. 5C). In contrast to its effects on Akt, FTS dramatically inhibited phosphorylations of p70 S6K (Thr^{389}) and PHAS-I (Ser^{65}) in LTED cells (Fig. 5D-E). The inhibitory effects of FTS on phosphorylations of these two proteins appeared to be smaller in MCF-7 cells where basal levels of phosphorylated proteins were lower than in LTED cells. These studies demonstrating blockade of the downstream effectors of mTOR (i.e. p70 S6K and PHAS-1) with only minimal blockade of the upstream mediator, Akt, provided our first evidence that FTS blocks mTOR signaling.

Effect of FTS on EGF and IGF-1 induced activation of MAP kinase, PI3 kinase, and mTOR. Serum stimulation represents the combined effect of multiple growth factors but does not allow dissection of the

effects of individual factors. For this reason, we examined the separate effects EGF and IGF-1, two growth factors known to be involved in the stimulation of breast cancer cells. Rapamycin was used to identify effects mediated by mTOR. LY 294002, was used to block both PI3 kinase and mTOR. MCF-7 and LTED cells were serum-starved for twenty-four hours, then incubated for one hour with FTS and three hours with LY 294002 or rapamycin before challenge with EGF (1 μg/ml). As the peak increases in phosphorylated ERK1/2, Akt, p70S6K and PHAS-I occurred at variable times, we chose to examine the effects of EGF after 90 minutes of incubation (all levels remained elevated at this time point, data not shown). As responses to EGF and inhibitors were similar in MCF-7 and LTED cells, only the data from LTED cells are presented.

With respect to the MAP kinase pathway, EGF-stimulated ERK1/2 phosphorylation was not blocked by FTS, even at a concentration of 100 μM. As expected, neither LY 294002 nor rapamycin blocked the activation of ERK 1/2 (Fig. 6), consistent with the known activities of these drugs to inhibit PI3 kinase and mTOR signaling but not MAP kinase.

We then examined the effects of FTS on the PI3 kinase pathway, first by examining Akt activation and then by evaluating the more downstream mediator, mTOR. FTS was without effect on EGF stimulated Akt phosphorylation whereas LY 294002, as expected, completely blocked this phosphorylation. Then we examined the effect of FTS on mTOR by evaluating EGF-induced phosphorylation of p70 S6 kinase and PHAS-I. Phosphorylation of p70 S6K at Thr^{389} was abolished by FTS and by the specific mTOR inhibitor, rapamycin (Fig. 6). Similarly, FTS significantly reduced the levels of Ser^{65} phosphorylated PHAS-I. The immunoblot with the antibody against total PHAS-I showed that PHAS-I in the cells treated with FTS, LY 294002, and rapamycin migrated more rapidly when subjected to SDS-PAGE. Phosphorylation of Ser^{65} and Thr^{70} in PHAS-I reduces the rate of mobility of the protein in SDS-PAGE, so that changes in the mobility reflect changes in phosphorylation state. Thus, the gel shifts confirmed that the levels of phosphorylated PHAS-I were reduced by these compounds. The results shown in Fig. 6 indicate that FTS, like rapamycin, blocks EGF-induced phosphorylation of p70 S6K and PHAS-I without affecting Akt.

Similar to EGF, IGF-1 activated the MAP kinase, PI3K/Akt, and mTOR pathways in both MCF-7 and LTED cells. Effects of shorter treatment with FTS on IGF-1-induced phosphorylation of signal transduction molecules were examined in LTED cells and were compared with those of LY 294002. We first evaluated the MAP kinase pathway and then PI3K and mTOR mediated events. With respect to MAP kinase, neither FTS nor LY 294002 inhibited ERK1/2 phosphorylation stimulated by IGF-1 (Fig. 7). Paradoxically LY 294002 enhanced IGF-1 induced ERK phosphorylation at 10 min and 30 min. This could result from enhanced interaction between IRS-1 and Grb2 as reported in intestinal epithelial cells. As for Akt, FTS did not inhibit IGF-1 induced phosphorylation of Akt but, as expected, LY 294002 caused profound inhibition. Thus, FTS does not appear to inhibit IGF-1 activation of PI3 kinase as reflected by activation of its downstream target, Akt. In marked contrast, FTS did effectively inhibit the Thr^{389} phosphorylation of p70 S6K and the Ser^{65} phosphorylation of PHAS-I. The inhibitory effect of FTS on these two molecules was apparent within 10 minutes and increased with time (Fig. 7). The finding that FTS inhibits phosphorylation of both p70 S6K and PHAS-I, but not the phosphorylation of Akt provides additional evidence of an inhibitory effect on mTOR activity.

Lack of effect of FTS on Thr^{229} phosphorylation of p70 S6K. To further verify that inhibition of FTS on p70 S6K and PHAS-1 is not through inhibition of PI3 kinase, we examined the effect of FTS on phosphorylation of p70 S6K at Thr^{229}, a site known to be phosphorylated by phosphoinositide-dependent kinase 1 (PDK1). If the inhibition of p70 S6K by FTS occurs downstream of PDK 1 and Akt, phosphorylation of p70 S6K at Thr^{229} should not be affected by this compound.

FTS had little, if any, effect on Thr^{229} phosphorylation in LTED cells treated with EGF (Fig. 8A) or IGF-1 (Fig. 8B). In contrast, LY 294002 blocked growth factor-induced Thr^{229} phosphorylation in a dose- and time-dependent manner (Fig. 8A, B). Fig. 8C shows that the inhibitory effect of FTS (100 μM) on Thr^{389} phosphorylation in serum containing medium occurred at 2 h and maximized at 24 h. Thr^{229} phosphorylation, in contrast, was only slightly reduced. These findings provide additional evidence that the inhibitory effect of FTS on p70 S6K occurs at a point downstream of PI3 kinase/PDK1/Akt.

Role of mTOR in breast cell growth. If inhibition of mTOR accounts for the effects of FTS on cell proliferation, then rapamycin and FTS should inhibit the proliferation of breast cancer cells to a similar extent. To investigate this possibility, growth rates of two breast cancer cell lines in the presence and absence of rapamycin were examined. Rapamycin markedly reduced the numbers of both MCF-7 and LTED cells. The IC_{50} of rapamycin was approximately 0.2 nM. In a marked contrast, benign MCF-10A cells were completely resistant to rapamycin (Fig. 9A), which suggests that MCF-10A cells are less dependent on the mTOR pathway for growth. This is supported by the result of Western analysis shown in Fig. 9B that MCF-10A cells expressed very low levels of p70 S6K, and phosphorylation of this kinase was undetectable in these cells. These data indicate that the rapamycin-sensitive mTOR pathway is an important mediator of proliferation for MCF-7 and LTED breast cancer cells but not for the benign MCF-10A cells. Further these data demonstrate the specificity of FTS for cancer cells and eliminate the possibility that this compound might be non-specifically toxic.

Specific Aim 2: In the xenograft model, we initrally compared the administration of vehicle alone, cyclodextrin (CD) alone and CD-complexed to FTS. CD-FTS caused a statistically significant reduction of tumor weight when measured at the end of the two month experiment. (Figure 10). After discussion with consulting pharmacologists, we learned that FTS, as it is highly lipid soluble, would be absorbed rapidly by the stomach without being complexed to CD. In preliminary data, a collaborting laboratory measured the absorption of FTS into the blood of mice when not complexed to CD. Forty percent absorption was detected (data not shown). For this reason, we conducted the additional experiements using FTS dissolved in sesame oil. The experiments involved 10 animals each which were castrated and implanted with wild type MCF-7 cells on each flank. In order for tumors to be established, the tumors were allowed to grow in response to 85 pg/ml estradiol without FTS. After that time, animals were given vehicle, 10, 30 and 90 mg/kg FTS in sesame oil by gavage. After two weeks, it became apparent that all animals were losing up to 20% body weight and all developed severe diarrhea (data not shown). We attributed this to the laxative effects of sesame oil and changed the formulation to corn oil and reduced the volume of administration of oil by 50%. With this regimen, body weight rapidly returned back to baseline. The 10 and 30 mg/kg doses did not cause tumor regression whereas the 90

mg/kg dose caused a substantial reduction (data not shown). We are now repeating this experiment using corn oil exclusively.

Proposed Plan of Research :

The experiemnts using the LTED cells have not as yet been completed. Since we had a lapse in personel during the transition from Dr. McPherson to Dr. Santen as prinicipal investigator, we have the funding to complete the experiments in the LTED tumors that were originally proposed. A no cost extension has been requested to complete these studies.

Key Research Accomplishments:

1. FTS blocks the growth of MCF-7 cells in culture
2. FTS was shown to be a potent inhibitor of mTOR by mechanisms that have not been previously described and its effect of MAP kinase was confirmed
3. FTS blocks the growth of breast tumors in vivo in xenografts
4. FTS synergizes with estradiol to increase apoptosis in long term estrogen deprived breast cancer cells.

Reportable outcomes:

1. Lynch, A.R., Neal, L.R., Santen, R.J. and McPherson, R.A. Farnesyl-thiosalicylic acid (FTS): Inhibition of proliferation and enhancement of apoptosis of hormone dependent breast cancer cells. (in preparation)
2. Lloyd P. McMahon, Wei Yue, Richard J. Santen and John C. Lawrence, Jr Farnesylthiosalicylic Acid Inhibits Mammalian Target of Rapamycin (mTOR) Activity Both in Cells and *in Vitro* by Promoting Dissociation of the mTOR-Raptor Complex.
3. Yue W, Wang J-P, Li Y, Fan P, Santen RJ. Farnesylthisalicylic acid blocks mammalian target of rapamycin signaling in breast cancer cells. International Journal of Cancer 2005, in press

Conclusions : A variety of recent studies have examined the effects of estrogen deprivation therapy on up-regulation of growth factor pathways in breast cancer cells. Our data have demonstrated a marked up-

regulation of activated MAP kinase and of the downstream PI-3-kinase effectors, AKT, p70S6 kinase, and 4E-BP-1 in MCF-7 cells deprived of estradiol long term. The group of Dowsett et al confirmed these findings regarding MAP kinase and also demonstrated an increase in HER-2 activation. Other investigators also found a marked up-regulation of the MAP kinase pathway in cells teated with tamoxifen long term. These investigators also demonstrated that blockade of MAP kinase and of EGF-receptor pathways in cells subjected to these two forms of estrogen deprivation therapy caused a reduction in cell growth. Based upon these findings, we considered that FTS might provide an effective means of blocking Ras mediated growth factor signaling in breast cancer cells. Our results show significant effects of FTS to inhibit cell proliferation and to enhance apoptotic cell death. Based upon these findings, FTS appears to be a candidate drug for testing in patients after we have completed our xenograft studies.

Our data demonstrated the FTS is active in vitro both in cells that have been subjected to estrogen deprivation therapy and in wild type cells. These results are best explained by the fact that estradiol stimulates proliferation in breast cancer cells through the stimulation of growth factor pathways. It would appear then that FTS blocks estradiol stimulated growth by interrupting these pathways. Our data have shown that FTS blocks MAP kinase activation in both wild type and in LTED cells and the growth of long term tamoxifen exposed cells. We have also shown that FTS partially blocks AKT activation and more effectively inhibits the activation of p70S6 kinase and 4E-BP-1.

The observation that FTS-CD suppresses E2 dependent growth but does not shift hypersensitivity may be significant. We have previously demonstrated that inhibition of MAP kinase with the MEK inhibitor, PD 98059, reverted LTED cells back to the level of estradiol sensitivity observed in wild type cells. This observation suggests that FTS may exert effects in addition to those mediated by MAP kinase. This possibility is also supported by the marked suppression of breast cancer cell growth observed. We now have obtained data indicating that FTS is a direct inhibitor of mTOR. In several model systems, mTOR functions as a meditor of cell proliferation. It is possible, therefore, that a major action of FTS is to block mTOR. If correct, FTS would exert effects both on the MAP kinase and the mTOR pathways. Our recent studies demonstrated upregulation of mTOR in LTED cells. Accordingly, FTS may serve as a unique agent to block more than one signaling pathway

involved in breast cancer growth. An additional advantage of FTS is its ability to stimulate apoptosis. Although its mechanism for enhancing apoptosis is not known, recent data suggest that blockade of mTOR can stimulate apoptosis through activation of ASK-1 (apoptosis stimulating kinase 1).

Both FTS and the farnesyltransferase inhibitors were designed to block Ras activity and to inhibit Ras induced growth in cancer cells. At first consideration, one might consider FTS and the farnesyltransferase inhibitors to be agents in the same class and to potentially exert similar actions. However, the farnesyltransferase inhibitors are now considered to act through mechanisms other than Ras. On the other hand, FTS exerts unexpected effects to markedly enhance apoptosis. In addtion, recent data demonstrate that FTS also blocks mTOR. Taken together, these data strongly support the concept that FTS and the farnesyltransferase inhibitors are not in the same class and probably will work very differently when admistered to patients.

The dose of FTS used in vitro approaches concentrations that can exert detergent effects. This observation would raise the possibility of non-specific toxic effects of FTS. To address this issue, we examined the effects of a geranyl analogue of FTS (S-geranylthiosalicyylic acid or GTS) with similar detergent properties to FTS. GTS did not inhibit mTOR whereas FTS did not (data not shown). This provides evidence of the lack of non-specific detergent effects of FTS. Finally, a wide range of doses of FTS have been used in vivo in models of pancreatic cancer and malignant melanoma . Even a dose of 90 mg/kg causes no weight loss when given in corn oil and no evidence of toxicity in nude mice bearing these tumors. Full toxicolgy studies are now ongoing in two animal species in preparation for application for an IND to do Phase I studies in patients.

In summary, we have shown that FTS and its complexed form, FTS-CD exert strong anti-proliferative effects on both wild type and LTED breast cancer cells. An additional action of FTS is to markedly enhance apoptosis. This agent appears to be effective both in breast cancer cells subjected to estrogen deprivation therapy and in wild type cells. On this basis, FTS might be active as intial treatment of hormone dependent breast cancer in addition to actions on tumors adapting with upregulation of MAP kinae pathways in response to estrogen deprivation therapy. Further in vivo studies are now required before consideration of use of FTS in Phase I studies in women with breast cancer.

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FIGURE LEGENDS

Figure 1: FTS suppresses E2 dependent breast cancer cell growth. MCF-7 (**A**) and LTED (**B**) cells were grown under conditions where they demonstrate an E2 response as described in the methods. Increasing concentrations of FTS suppressed this E2 dependence. Representative of two experiments, mean and standard deviation of 4 samples are shown.

Figure 2: FTS and FTS-CD have similar growth inhibition profiles. **A.** MCF-7 cells were grown in the presence of FTS dissolved in DMSO, the equivalent volume of DMSO, FTS-CD dissolved in PBS or the equivalent amount of CD in PBS. Cells were treated for 5 days as dsescribed in the methods section. Representative of 2 experiments, mean and standard deviation of 4 samples are shown. **B.** and **C.** FTS-CD inhibits growth of both MCF-7 (**B**) and LTED (**C**) cells. Cells were assayed according to the techniques described in the methods section. Responses to either PBS, CD in PBS or FTS-CD in PBS added. Representative of 6 experiments, mean and standard deviation of 4 samples are shown.

Figure 3: FTS-CD increases apoptosis. MCF-7 (**A**) and LTED (**B**) cells were incubated with buffer (equivalent to 100 μM FTS-CD), CD (equivalent to 60 or 100 μM FTS-CD) or 60 or 100 μM FTS-CD for five days as described in Methods. Apoptosis was measured by DNA nick site quantitative ELISA. Representative of two experiments. Mean and standard deviation of two samples are shown.

Figure 4: FTS-CD reduces proliferation. MCF-7 (**A**) and LTED (**B**) cells were incubated with buffer, CD or FTS-CD for five days as described in Figure 3 legend. Proliferation was measured by Brd-U incorporation. Representative of two experiments. Mean and standard deviation of two samples are shown.

Figure 5: Effect of FTS on serum-stimulated activation of MAP kinase, PI3 kinase, and mTOR. Sub-confluent MCF-7 and LTED cells grown in 60 mm dishes were treated with FTS in their culture media for 24 h. Cells were then harvested and cell lysate prepared. Phosphorylation of kinases or effector was detected by Western analysis using specific antibodies and quantitated by densitometry scanning. ERK1/2 MAP kinase activity was measured as described in Materials and Methods. (A) Phosphorylation of ERK1/2 MAP kinase; (B) Activity of ERK1/2 MAP kinase; (C) Phosphorylation of Akt at Ser^{473}; (D) Phoshporylation of p70 S6 kinase at Thr^{389}; and (E) Phosphorylation of PHAS-I at Ser^{65}.

Figure 6: Effect of FTS on EGF induced activation of MAP kinase, PI3 kinase, and mTOR in LTED cells. Sub-confluent LTED cells grown in 60 mm dishes were serum starved for 24 h, pretreated for 1 h with FTS, 3 h with LY 294002 (LY), or rapamycin (Rapa) at indicated concentrations before addition of EGF (1 μg/ml, 1 h). Cells were then harvested and cell lysate prepared. Phosphorylated and total kinases were detected by Western analysis using specific antibodies.

Figure 7: Effect of FTS on IGF-1 induced activation of MAP kinase, PI3 kinase, and mTOR in LTED cells. Sub-confluent LTED cells grown in 60 mm dishes were serum starved for 24 h, pretreated with FTS (100 μM) or LY 294002 (LY, 20 μM) for 10, 30 or 60 min before addition of IGF-1 (20 ng/ml for 10 min). Cells were then harvested and cell lysate prepared. Phosphorylated and total kinases were detected by Western analysis using specific antibodies.

Figure 8: Effect of FTS on serum- and growth factor-induced phosphorylation of p70 S6 kinase at Thr^{229} in LTED cells. (A) Comparison of FTS and LY 294002 (LY) on Thr^{229} phosphorylation of p70 S6K induced by EGF (same treatment as described in Fig. 6); (B) Comparison of FTS and LY 294002 on Thr^{229} phosphorylation of p70 S6K induced by IGF-1 (same treatment as described in Fig. 7); (C) Time course of FTS (100 μM) on Thr^{389} and Thr^{229} phosphorylation of p70 S6K in LTED cells cultured in serum containing IMEM.

Figure 9: Effect of rapamycin on growth of MCF-7, LTED, and MCF-10A cells. (A) Sixty thousand cells were plated into each well of 6-well plates in their culture media. Two days later, the cells were treated in triplicate wells for five days with rapamycin at indicated concentrations. The results (mean ± S.E.) are expressed as percentage of the vehicle control. (B) LTED and MCF-10A cells grown in 60 mm dishes were treated with rapamycin at 2 nM for LTED cells and 20 nM for MCF-10A cells fro 24 hours. Cells were then harvested and cell lysate prepared. Levels of phosphorylated and total p70 S6 kinase were detected by Western analysis using specific antibodies.

Figure 10: In vivo effects of FTS-CD on cell growth. LTED cells were implanted into castrate nude mice to form xenografts. Siliastic implants delivering estradiol at amounts sufficient to provide plasma levels of estradiol of 5 pg/ml were implanted. One group (n=9) received buffer alone (PBS), the second (n=9) cyclodextrin (CD) alone, and the third (n=7) FTS-CD complex (40 mg/kg). The Student *t*-test was used to compare the effects of FTS-CD to buffer or CD alone.

FIGURES

Figure 1A

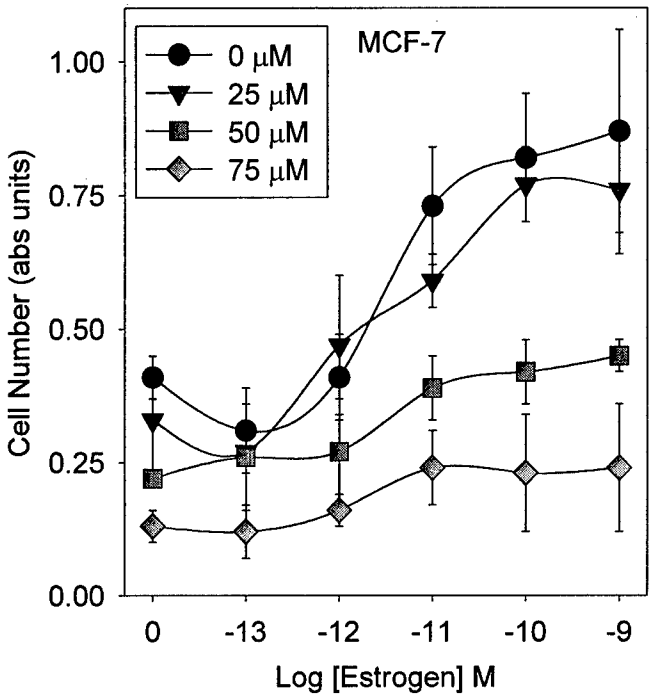


Figure 1B

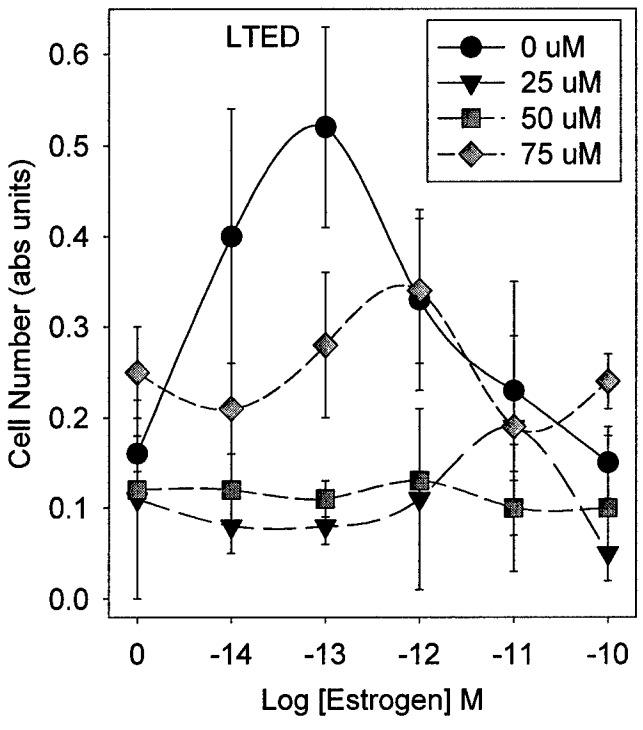

LTED
0 uM
25 uM
50 uM
75 uM
Cell Number (abs units)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
-14
-13
-12
-11
-10
Log [Estrogen] M

Figure 2 A

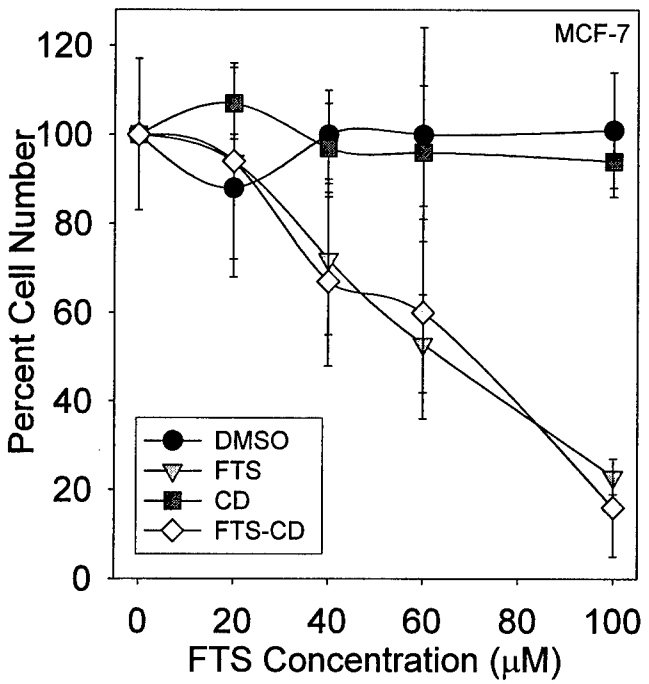

MCF-7
Percent Cell Number
120
100
80
60
40
20
0
DMSO
FTS
CD
FTS-CD
0
20
40
60
80
100
FTS Concentration (μM)

Figure 2B

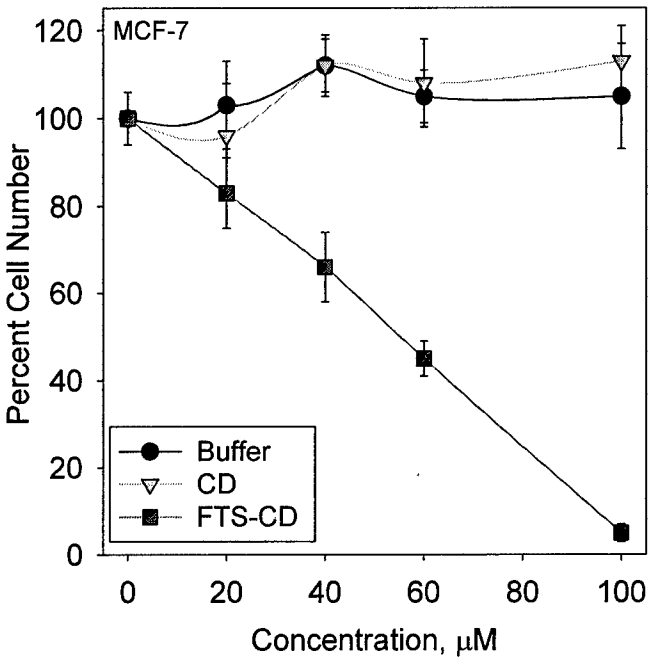

MCF-7
120
100
80
60
40
20
0
Percent Cell Number
Buffer
CD
FTS-CD
0
20
40
60
80
100
Concentration, μM

Figure 2C

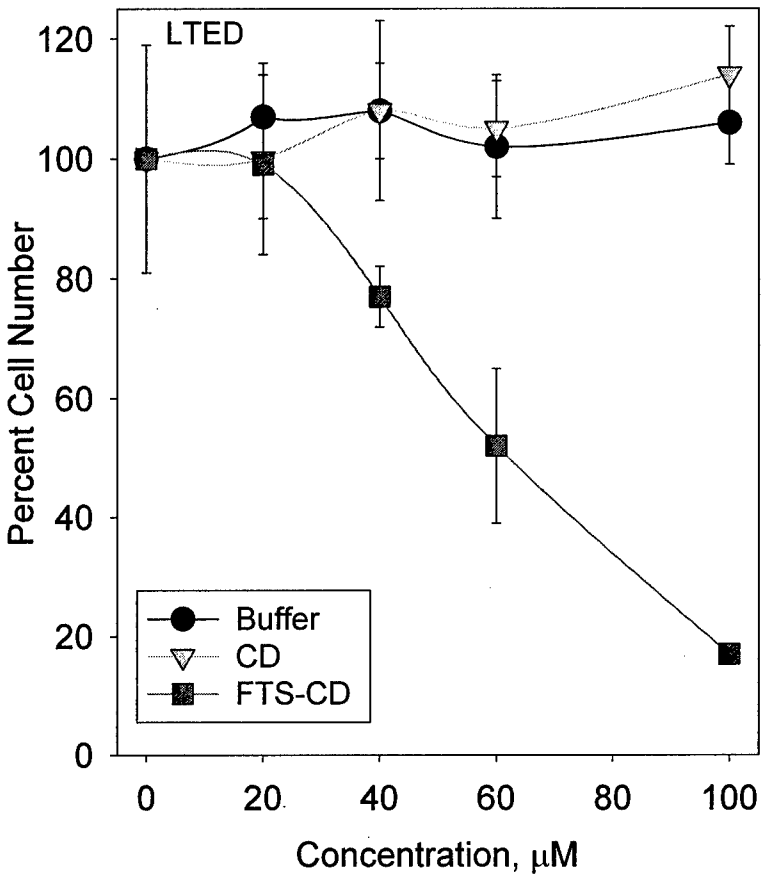

LTED
120
100
80
60
40
20
0
Percent Cell Number
Buffer
CD
FTS-CD
0
20
40
60
80
100
Concentration, μM

Figure 3A

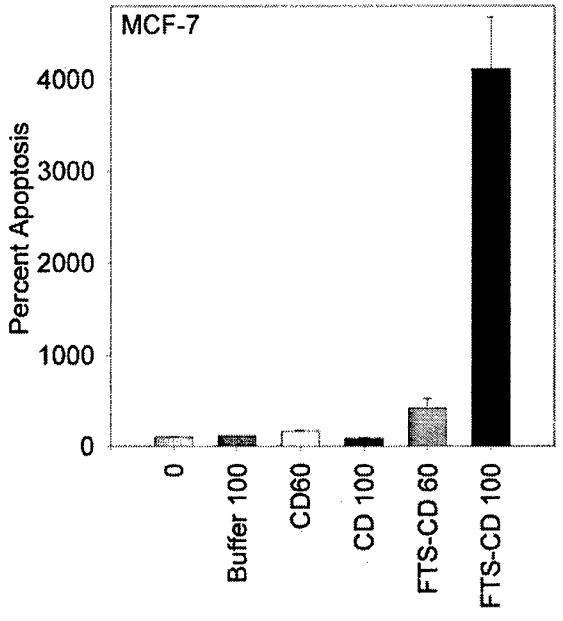

MCF-7
Percent Apoptosis
4000
3000
2000
1000
0
0
Buffer 100
CD60
CD 100
FTS-CD 60
FTS-CD 100

Figure 3B

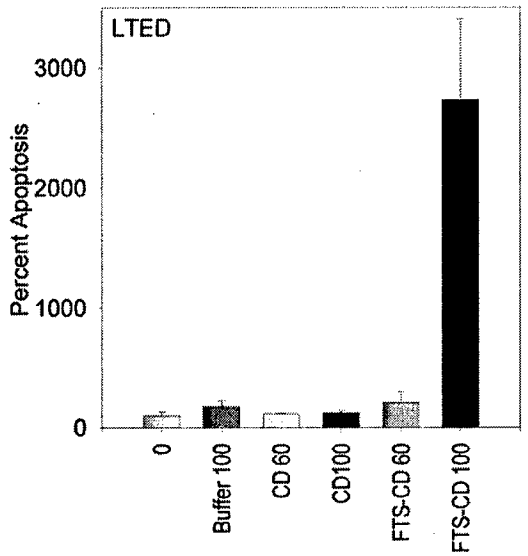

LTED
Percent Apoptosis
3000
2000
1000
0
0
Buffer 100
CD 60
CD100
FTS-CD 60
FTS-CD 100

Figure 4A

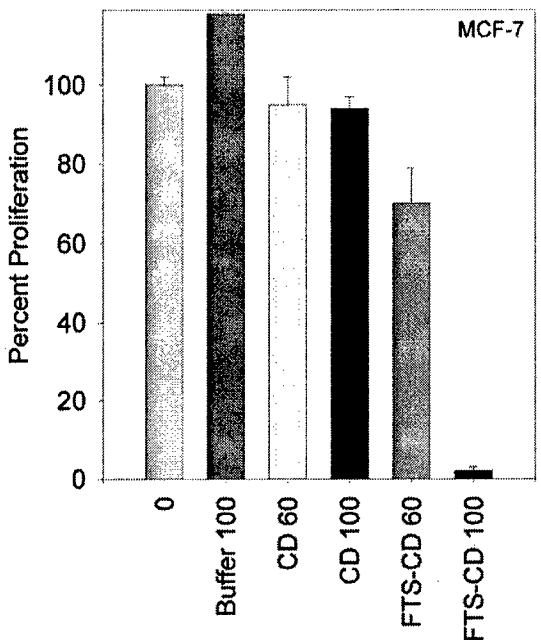

MCF-7
Percent Proliferation
100
80
60
40
20
0
0
Buffer 100
CD 60
CD 100
FTS-CD 60
FTS-CD 100

Figure 4B

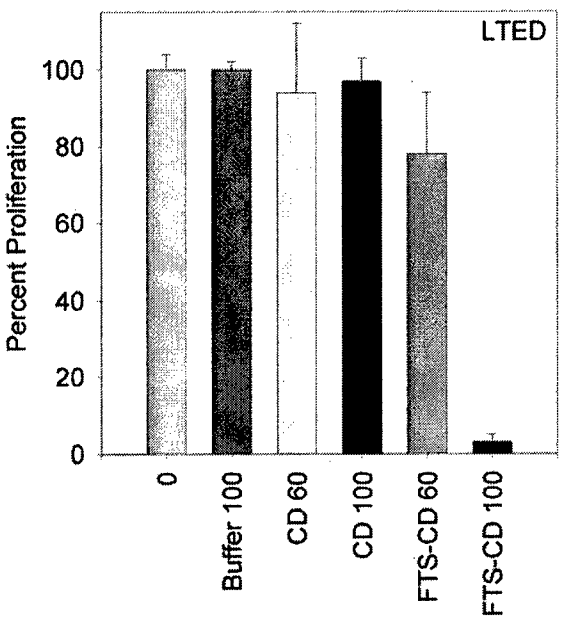

LTED
Percent Proliferation
100
80
60
40
20
0
0
Buffer 100
CD 60
CD 100
FTS-CD 60
FTS-CD 100

Figure 5

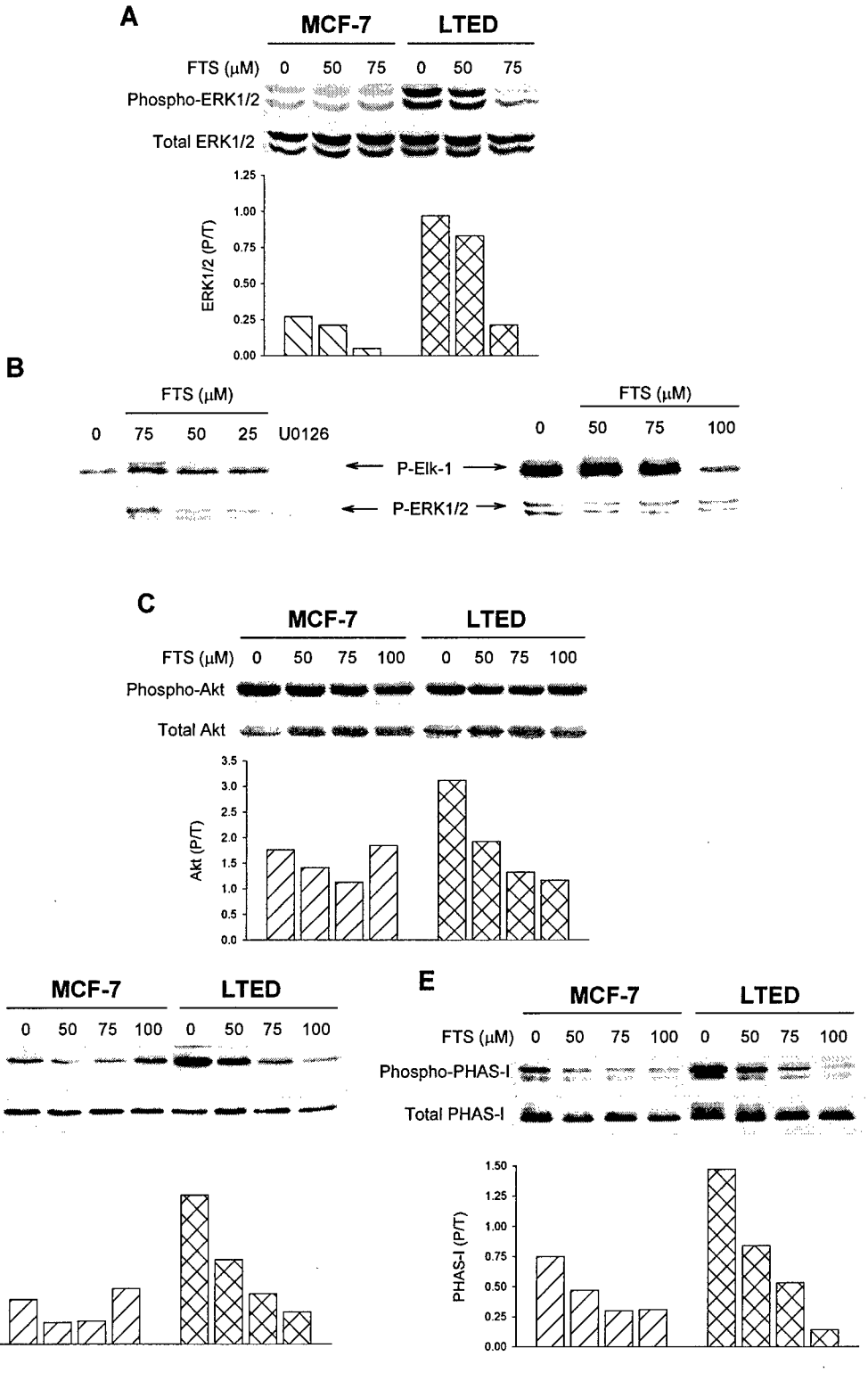

A
MCF-7
LTED
FTS (μM) 0 50 75 0 50 75
Phospho-ERK1/2
Total ERK1/2
ERK1/2 (P/T)
B
FTS (μM)
0 75 50 25 U0126
FTS (μM)
0 50 75 100
P-Elk-1
P-ERK1/2
C
MCF-7
LTED
FTS (μM) 0 50 75 100 0 50 75 100
Phospho-Akt
Total Akt
Akt (P/T)
D
MCF-7
LTED
FTS (μM) 0 50 75 100 0 50 75 100
Phospho-p70S6K
Total p70S6K
p70S6K (P/T)
E
MCF-7
LTED
FTS (μM) 0 50 75 100 0 50 75 100
Phospho-PHAS-I
Total PHAS-I
PHAS-I (P/T)

Figure 6

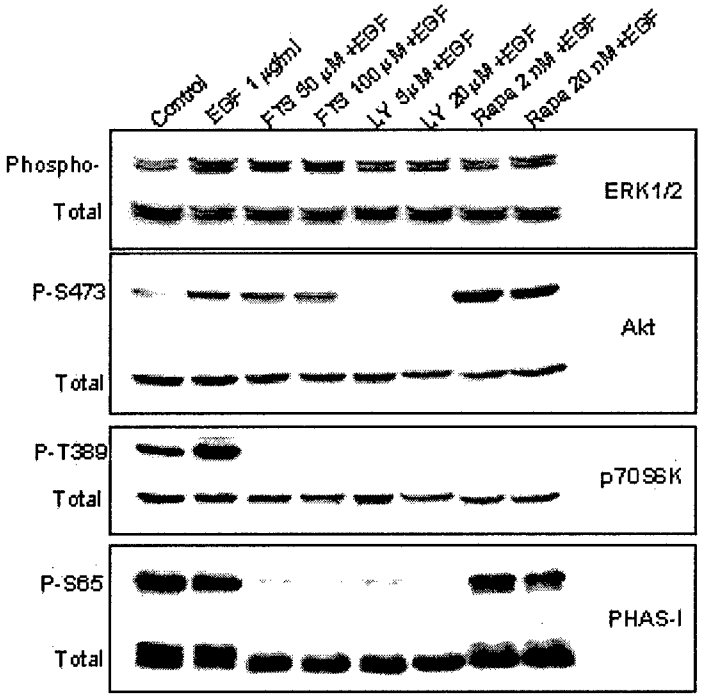

Control
EGF 1 μg/ml
FTS 50 μM +EGF
FTS 100 μM +EGF
LY 5μM +EGF
LY 20 μM +EGF
Rapa 2 nM +EGF
Rapa 20 nM +EGF
Phospho-
Total
ERK1/2
P-S473
Total
Akt
P-T389
Total
p70S6K
P-S65
Total
PHAS-I

Figure 7

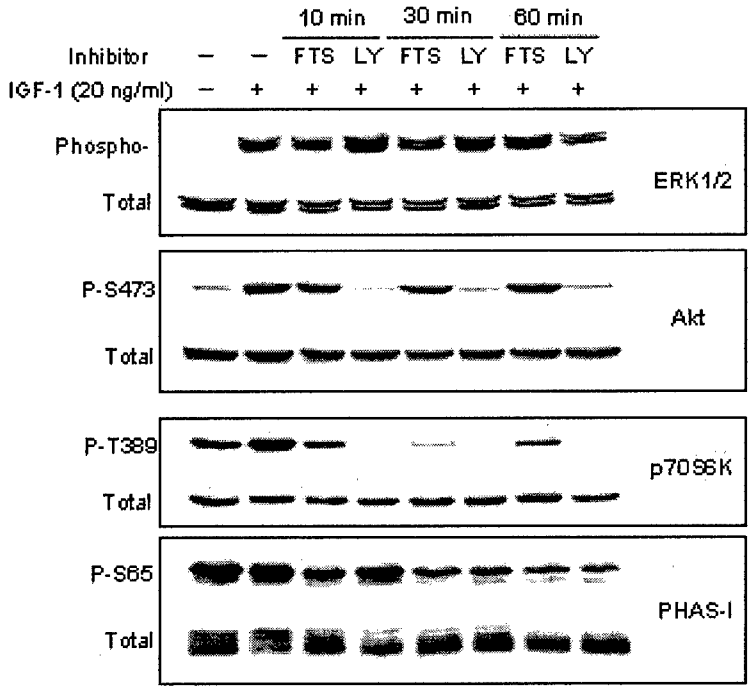

10 min
30 min
60 min
Inhibitor – – FTS LY FTS LY FTS LY
IGF-1 (20 ng/ml) – + + + + + + +
Phospho-
Total
ERK1/2
P-S473
Total
Akt
P-T389
Total
p70S6K
P-S65
Total
PHAS-I

Figure 8

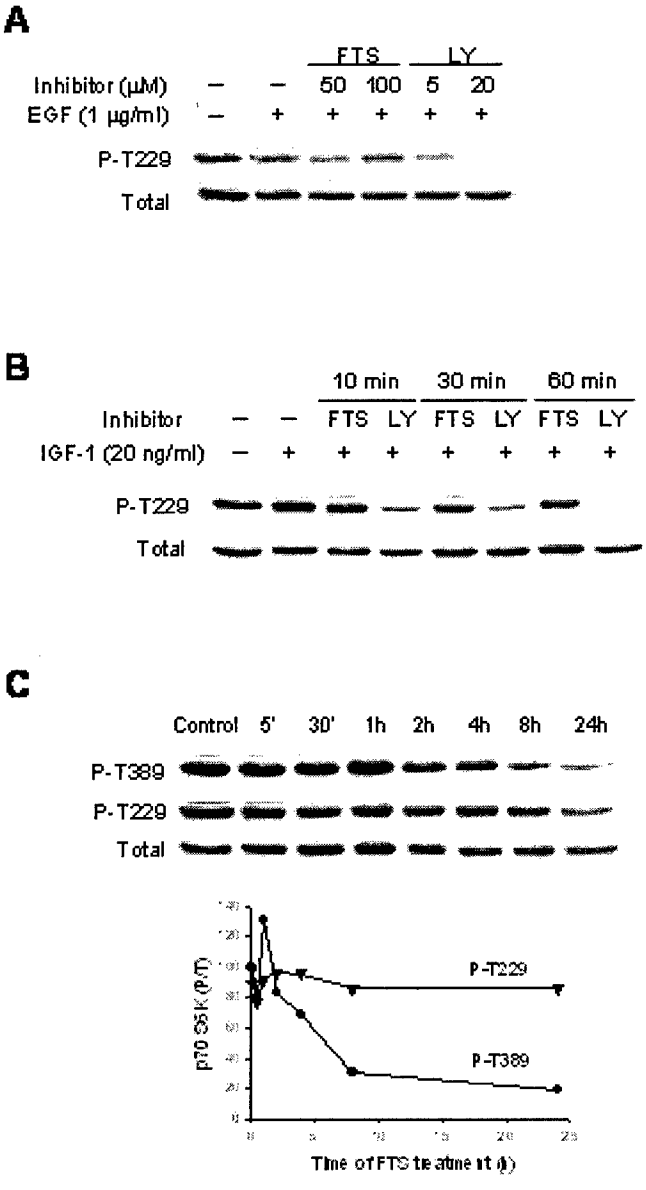

A
FTS
LY
Inhibitor (μM) – – 50 100 5 20
EGF (1 μg/ml) – + + + + +
P-T229
Total
B
10 min
30 min
60 min
Inhibitor – – FTS LY FTS LY FTS LY
IGF-1 (20 ng/ml) – + + + + + + +
P-T229
Total
C
Control 5' 30' 1h 2h 4h 8h 24h
P-T389
P-T229
Total
p70 S6K (P/T)
P-T229
P-T389
Time of FTS treatment (h)

Figure 9

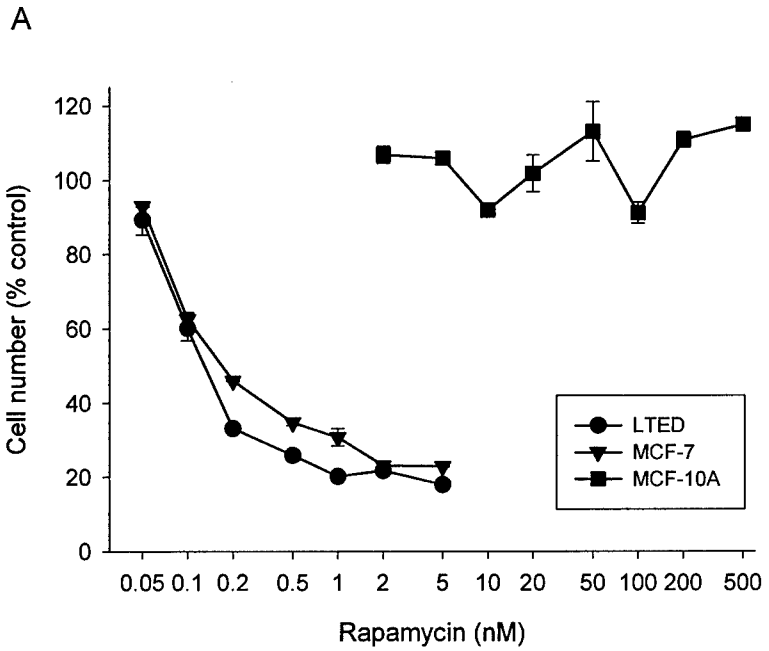
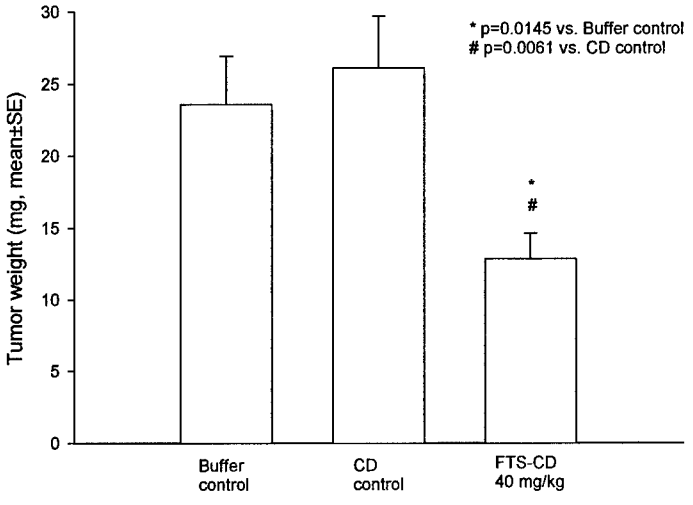


Figure 10


* p=0.0145 vs. Buffer control
# p=0.0061 vs. CD control
Tumor weight (mg, mean±SE)
30
25
20
15
10
5
0
*
#
Buffer control
CD control
FTS-CD 40 mg/kg

Appendices:

Manuscript in preparation: Lynch, A.R., Neal, L.R., Santen, R.J. and McPherson, R.A. Farnesyl-thiosalicylic acid (FTS): Inhibition of proliferation and enhancement of apoptosis of hormone dependent breast cancer cells.

Manuscriptspublished: Lloyd P. McMahon, Wei Yue, Richard J. Santen and John C. Lawrence, Jr Farnesylthiosalicylic Acid Inhibits Mammalian Target of Rapamycin (mTOR) Activity Both in Cells and *in Vitro* by Promoting Dissociation of the mTOR-Raptor Complex. Molecular Endocrinology 19:175-183, 2005

Yue W, Wang J-P, Li Y, Fan P, Santen RJ Farnesylthiosalicylic acid blocks mammalian target of rapamycin signaling in breast cancer cells. International Journal of Cancer 2005, in press

Farnesyl-thiosalicylic acid (FTS): Inhibition of Proliferation and Enhancement of Apoptosis of Hormone Dependent Breast Cancer Cells

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ABSTRACT

Estradiol exerts major actions on cell proliferation through Ras and the MAP kinase signaling pathway. Ras is a prime target for antineoplastic drug development. We examined the effects of farnesyl-thiosalicylic acid (FTS), an inhibitor of Ras binding to its membrane acceptor site galectin 1, on breast cancer growth in vitro. FTS and its solubilized conjoiner FTS/cyclodextrin markedly inhibited growth. We then dissected out its separate effects on proliferation and apoptosis. FTS inhibited proliferation but also markedly enhanced apoptosis. These data suggest that FTS is a promising agent to be developed for treatment of hormone dependent breast cancer.

Key Words: Ras, farnesyl-thiosalicylic acid, FTS, breast cancer, apoptosis

INTRODUCTION

One-third of human breast cancers depend upon estrogen for growth and regress upon exposure to antiestrogens or inhibitors of estrogen biosynthesis (e.g. aromatase inhibitors) [1]. Initial responses to the antiestrogen tamoxifen last for 12-18 months on average but tumors nearly uniformly begin to regrow later [1-2]. Secondary therapies with aromatase inhibitors often cause additional tumor regressions but are again followed by relapse [1]. Extensive recent work has focused upon the mechanisms underlying relapse during tamoxifen or aromatase inhibitor therapy [3-5]. These studies demonstrate upregulation of growth factor pathways involving the MAP kinase signaling cascade [5-9]. Based upon this concept, a number of investigative groups have suggested that growth factor inhibitors might serve as ideal agents to prolong responses to hormonal therapy or to control progression into an estrogen independent state [5,9-12].

Binding of a number of specific growth factor ligands to their cognate receptors activates a pathway involving Ras and leads to activation of MAP kinase [13]. Because of the key role of Ras, this signaling molecule has been a prime target for drug development [14-15]. With respect to human breast cancer, the MAP kinase pathway plays a major role in mediating the proliferative effects of estradiol [16]. In addition, the MAP kinase pathway is frequently up-regulated in response to estrogen deprivation therapy and may play a role in development of hormonal resistance [17-19]. Accordingly, several clinical trials are examining the effect

of drugs designed to abrogate Ras effects to prolong the beneficial actions of tamoxifen and the aromatase inhibitors [20].

Blockade of Ras may be accomplished by preventing its translocation to the cell membrane or by competing for binding sites to its acceptor protein in the membrane. Farnesylation of Ras is an important mechanism necessary for its membrane translocation. Inhibition of farnesylation is thus a major target for drug development. While effective in inhibiting the growth of several tumors, the farnesyl transferase inhibitors unexpectedly do not exert their anti- tumor effects through blockade of Ras [21]. Other, as yet unidentified targets such as Rho-B are now thought to mediate the anti-proliferative effects of farnesyl transferase inhibitor [22]. Another strategy is to block the binding of Ras to its membrane acceptor site, the protein galectin 1 [23-32]. Ras must be bound to GTP as a pre-requisite for forming a high affinity complex with galectin 1 and for its activity in activating the MAP kinase pathway. Thus another anti-tumor strategy is to competitively inhibit the binding of GTP-Ras to galectin 1.

The group of Kloog et al. have developed a compound capable of dissociating GTP-Ras from galectin 1 [23-32]. This agent, called farnesyl-thiosalicylic acid (FTS), binds specifically to galectin 1 and displaces GTP-Ras from it. As a consequence, GTP-Ras loses its anchor to galectin 1 in the plasma memberane and rapidly traverses the raft like structures as well as the non-caveolar regions of the plasma membrane. Lacking an anchor in the membrane, Ras re-enters the cytoplasm where it is degraded and inactivated over a period of several hours. Through this mechanism, FTS interrupts the ability of Ras to signal in the plasma membrane and prevents the activation of MAP kinase.

Several investigators have demonstrated that FTS blocks the activation of MAP kinase and causes inhibition of the growth of tumors containing activating mutations of Ras (i.e. pancreatic cancer and malignant melanomas) [23-32]. However, no previous studies have examined the effect of FTS on breast cancer because Ras is only infrequently mutated in this neoplasm. However, we reasoned that the frequent up-regulation of the MAP kinase pathway through Ras, which occurs in response to estrogen deprivation therapy, might uncover a role for FTS in this cancer.

The present study examined the effects of FTS on growth of estrogen dependent breast cancer cells in vitro. Since this agent may need to be complexed to solubilizing agents to be orally absorbed, we have examined both free FTS and FTS compexed with cyclodextrin to make it more water soluble. Herein we report that the farnesyl analogue, FTS, blocks the growth of breast cancer cells in tissue culture but unexpectedly also stimulates cell death. Based upon these observations, we believe that this agent provides a promising drug for further study in women with hormone dependent breast cancer, either concomitantly with estrogen deprivation therapy or following this strategy.

METHODS

Materials: FTS and cyclodextrin (CD) were donated by Dr. C. Wayne Bardin of the Thyreos Corporation. The FTS-CD complex was prepared according to instructions from Thyreos. The "pure" antiestrogen, ICI 182,780, was donated by Astra-Zeneca, United Kingdom. The Cell Death Detection ELISA and Cell Proliferation ELISA kits were from Roche; the Neutral Red was from Aldrich; the IMEM was from Biosource; and Fetal Bovine Serum (FBS) from Gibco. Dextran T70 was from Pharmacia and Charcoal (NoritA) from Sigma.

Cell Culture: All culture methods have been previously described in detail. Briefly, we utilized wild type E2-dependent, ER positive MCF-7 breast cancer cells which are called "wild type". When deprived of E2 over several months, MCF-7 cells develop hypersensitivity to the proliferative and pro-apoptotic effects of estradiol and display upregulated MAPK activity [17-19, 33]. These cells, described previously in several publications, are termed LTED as an acronym for **L**ong **T**erm **E**stradiol **D**eprived. [17-19,33].

Cell number was assayed by a modified neutral red method. [34] We have correlated this method with direct cell counts using a Coulter counter and shown excelent agreement (unpublished data). To assay E2 dependent growth, MCF-7 cells were seeded into 96 well plates and 24 hours later, the medium was changed to IMEM DCC (Improved Modified Eagles Medium Dextran Coated Charcoal stripped) . Five days later, E2 was added in fresh IMEM/DCC. After 3 additional days, fresh E2 in IMEM/DCC was added and cell number measured 2 days later. To assess responses to E2, LTED cells were seeded into 96 well plates. Twenty-four hours later, the media were changed to IMEM with glutamine added. Seven days later, E2 was added in the

doses indicated in the figures. In addition, we routinely added 10^{-9} M ICI 182,780 in fresh IMEM to counteract residual estradiol leached from the plastic in the culture dishes. Three days later, fresh E2 was added in IMEM/ICI and 2 days later cell number was measured.

To assay the effects of FTS-CD on cell number, proliferation and apoptosis, cells were seeded into 96 well plates and the next day drug was added in fresh medium. Three days later, fresh drug in medium was added and cell number, apoptosis and proliferation was measured 2 days later.

RESULTS

Wild type MCF-7 cells responded to estradiol with maximal stimulation at a dose of 10^{-11}M which increased cell number approximatley three fold (Figure 1A). Increasing concentrations of FTS suppressed E2 dependent growth in a dose responsive fashion with initial inhibition at 50μM and maximal effects at 75 μM FTS. As a reflection of hypersensitivity to estradiol, the LTED cells (Figure 1B) responded maximally to two log lower concentrations of estradiol than did wild type cells (i.e. 10^{-13}M vs 10^{-11}M respectively). In contrast to its effects in wild type cells, FTS suppressed LTED growth completely at a dose of 25 μM with continued suppression at 50 μM. Unexpectedly, we observed lesser inhibition of growth at 75 μM FTS in LTED cells.

FTS is a relatively hydrophobic lipid analogue and may not be absorbed by patients in an oral formulation. We reasoned that Cyclodextrins (CDs) which have been used previously to solubilize hydrophobic drugs, might be a practical means to develop a practical formulation of FTS for ultimate use in humans[35-36]. Accordingly, FTS was complexed with CD and compared to free FTS and to the DMSO vehicle (for free FTS) and to buffer vehicle (for CD-FTS) in our in vitro system. FTS and FTS-CD exhibited almost identical MCF-7 cell growth inhibition profiles (Figure 2A). CD alone or buffer vehicle had no significant effect on cell growth of MCF-7 and LTED cells (Figures 2B and 2C) under conditions where FTS-CD significantly reduced cell growth. Even though LTED cells have higher MAPK activity than MCF-7 cells [18], the degree of inhibition was the same between the two cell lines (Figure 2C). We have also observed similar results in tamoxifen resistant breast cancer cell lines (data not shown).

Ras-mediated growth factor pathways are required to maintain cellular proliferation and are important regulators of the apoptotic response [36-37]. Reduction of cell number can reflect either an inhibition of proliferation, an enhancement of apoptosis, or a combination of these two effects. Accordingly, we systematically examined the effects of FTS specifically on apoptosis and then on BRD-U incorportion as a marker for proliferation. FTS enhanced apoptosis in wild type and LTED cells starting at a dose of 60 μM and maximally stimulating at a dose of 100 μM FTS-CD to levels 3000-4000 fold higher (Figures 3A and 3B). Proliferation was also reduced to very low amounts in both cell lines by FTS-CD, but not by CD or buffer alone (Figures 4A and 4B). The disruption of both proliferation and apoptosis indicates a global disruption of signaling pathways in breast cancer cells.

DISCUSSION

A variety of recent studies have examined the effects of estrogen deprivation therapy on up-regulation of growth factor pathways in breast cancer cells [17-19,33,38-40]. Our group and that of Dowsett et al demonstrated a marked up-regulation of activated MAP kinase in MCF-7 cells deprived of estradiol long term [17-19,40]. Other investigators also found a marked up-regulation of the MAP kinase pathway in cells teated with tamoxifen long term [9]. These investigators also demonstrated that blockade of MAP kinase and of EGF-receptor pathways in cells subjected to these two forms of estrogen deprivation therapy caused a reduction in cell growth [9]. Based upon these findings, we considered that FTS might provide an effective means of blocking Ras mediated growth factor signaling in breast cancer cells. Our results show significant effects of FTS to inhibit cell proliferation and to enhance apoptotic cell death. Based upon these findings, FTS appears to be a candidate drug for testing in vivo in xenograft breast cancer models and then in patients.

Our data demonstrated the FTS is active both in cells that have been subjected to estrogen deprivation therapy and in wild type cells. These results are best explained by the fact that estradiol stimulates proliferation in breast cancer cells through the stimulation of growth factor pathways involving MAP kinase. It would appear then that FTS blocks estradiol stimulated growth by interrupting this pathway. Althogh not presented in

this manuscript, our data have shown that FTS blocks MAP kinase activation in both wild type and in LTED cells and the growth of long term tamoxifen exposed cells.

Agents such as testosterone are not well absorbed orally and can be rendered orally effective by complexing to cyclodextrin. Other drugs have also been complexed to cyclodextrin to enhance solubility and absorption [41-42]. Anticipating the future use of FTS in patients, we examined whether complexing with cyclodextrin would alter the in vitro efficacy of FTS. We demonstrated that the FTS-CD complex clearly prevents growth of cellular models of E2 dependent (MCF-7) and E2 hypersensitive (LTED) breast cancers at the same doses as with free FTS. CD-FTS also induces apoptosis up to several thousand fold and reduces proliferation to very low levels.

The observation that FTS-CD suppresses E2 dependent growth but does not shift hypersensitivity may be significant. We have previously demonstrated that inhibition of MAP kinase with the MEK inhibitor, PD 98059, reverted LTED cells back to the level of estradiol sensitivity observed in wild type cells [43]. This observation suggests that FTS may exert effects in addtion to those mediated by MAP kinase. This possibility is also supported by the marked suppression of breast cancer cell growth observed. We now have obtained data indicating that FTS is a direct inhibitor of mTOR [44-45]. In several model systems, mTOR functions as a meditor of cell proliferation. It is possible, therefore that a major action of FTS is to block mTOR. If correct, FTS would exert effects both on the MAP kinase and the mTOR pathways. Our recent studies demonstrated upregulation of mTOR in LTED cells [45]. Accordingly, FTS may serve as a unique agent to block more than one signaling pathway involved in breast cancer growth. An additional advantage of FTS is its ability to stimulate apoptosis. Although its mechanism for enhancing apoptosis is not known, recent data suggest that blockade of mTOR can stimulate apoptosis thorugh activation of ASK-1 (apoptosis stimulating kinase 1) [46-47].

Both FTS and the farnesyl transferase inhibitors were designed to block Ras activity and to inhibit Ras induced growth in cancer cells. At first consideration, one might consider FTS and the farnesyltransferase inhibitors to be agents in the same class and to potentially exert similar actions. However, the farnesyltransferase inhibitors are now considered to act through mechanisms other than Ras. On the other hand,

FTS exerts unexpected effects to markedly enhance apoptosis. In addtion, recent data demonstrate that FTS also blocks mTOR [44]. Taken together, these data strongly support the concept that FTS and the farnesyltransferase inhibitors are not in the same class and probably will work very differently when admistered to patients.

In summary, we have shown that FTS and its complexed form, FTS-CD exert strong antiproliferative effects on both wild type and LTED breast cancer cells. An additional action of FTS is to markedly enhance apoptosis. This agent appears to be effective both in breast cancer cells subjected to estrogen deprivation therapy and in wild type cells. On this basis, FTS might be active as intial treatment of hormone dependent breast cancer in addition to actions on tumors adapting with upregulation of MAP kinae pathways in response to estrogen deprivation therapy. Further in vivo studies are now required before consideration of use of FTS in Phase I studies in women with breast cancer.

ACKNOWLEDGMENTS

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FIGURE LEGENDS

Figure 1: FTS suppresses E2 dependent breast cancer cell growth. MCF-7 (**A**) and LTED (**B**) cells were grown under conditions where they demonstrate an E2 response as described in the methods. Increasing concentrations of FTS suppressed this E2 dependence. Representative of 2 experiments, mean and standard deviation of 4 samples are shown.

Figure 2: FTS and FTS-CD have similar growth inhibition profiles. **A.** MCF-7 cells were grown in the presence of FTS dissolved in DMSO, the equivalent volume of DMSO, FTS-CD dissolved in PBS or the equivalent amount of CD in PBS. Cells were treated for 5 days as dsescribed in the methods section. Representative of 2 experiments, mean and standard deviation of 4 samples are shown. **B.** and **C.** FTS-CD inhibits growth of both MCF-7 (**B**) and LTED (**C**) cells. Cells were assayed according to the techniques described in the methods section. Responses to either PBS, CD in PBS or FTS-CD in PBS added. Representative of 6 experiments, mean and standard deviation of 4 samples are shown.

Figure 3: FTS-CD increases apoptosis. MCF-7 (**A**) and LTED (**B**) cells were incubated with buffer (equivalent to 100 μM FTS-CD), CD (equivalent to 60 or 100 μM FTS-CD) or 60 or 100 μM FTS-CD for five days as described in Methods. Apoptosis was measured by DNA nick site quantitative by number ELISA. Representative of two experiments. Mean and standard deviation of two samples are shown.

Figure 4: FTS-CD reduces proliferation. MCF-7 (**A**) and LTED (**B**) cells were incubated with buffer, CD or FTS-CD for five days as described in Figure 3 legend. Proliferation was measured by Brd-U incorporation. Representative of two experiments. Mean and standard deviation of two samples are shown.

FIGURES

Figure 1A

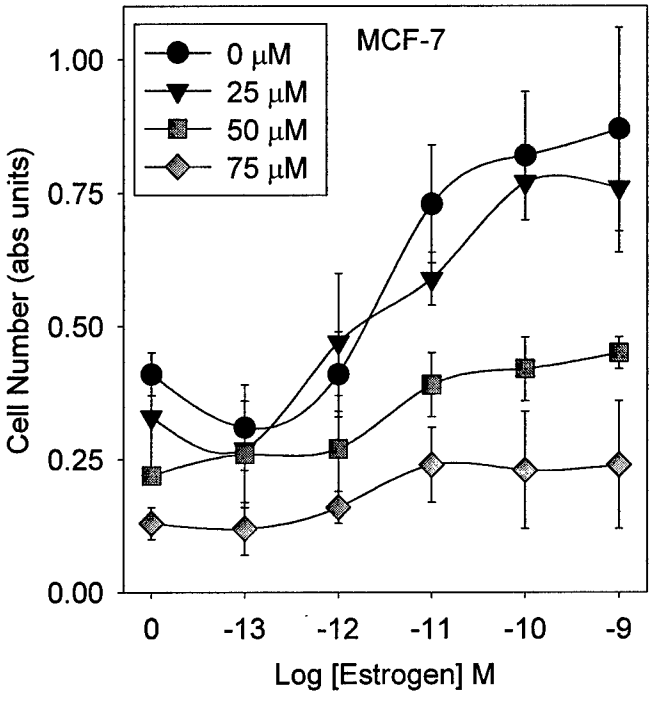


Figure 1B

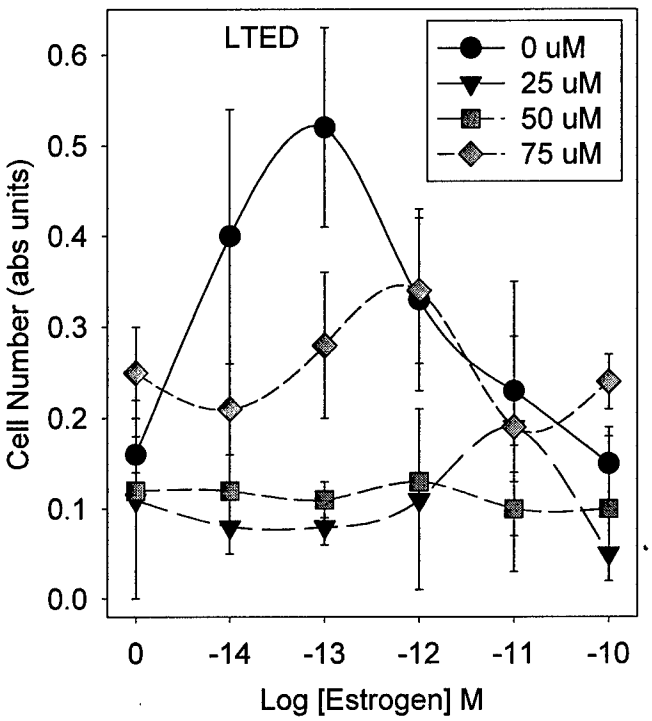

LTED
0 uM
25 uM
50 uM
75 uM
Cell Number (abs units)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
-14
-13
-12
-11
-10
Log [Estrogen] M

Figure 2 A

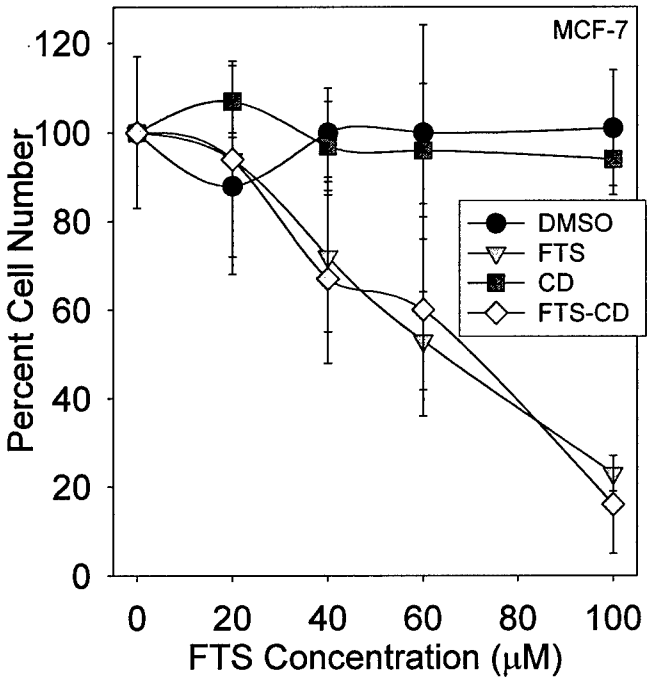

MCF-7
120
100
80
60
40
20
0
Percent Cell Number
0
20
40
60
80
100
FTS Concentration (μM)
DMSO
FTS
CD
FTS-CD

Figure 2B

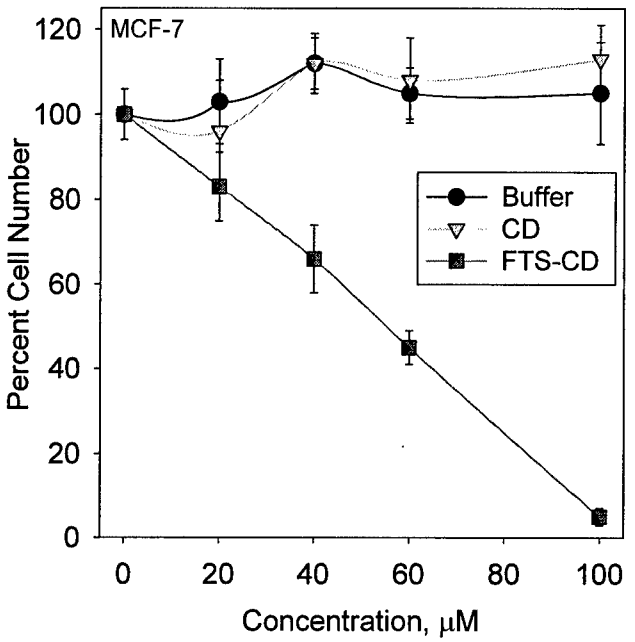

MCF-7
120
100
80
60
40
20
0
Percent Cell Number
0
20
40
60
80
100
Concentration, μM
Buffer
CD
FTS-CD

Figure 2C

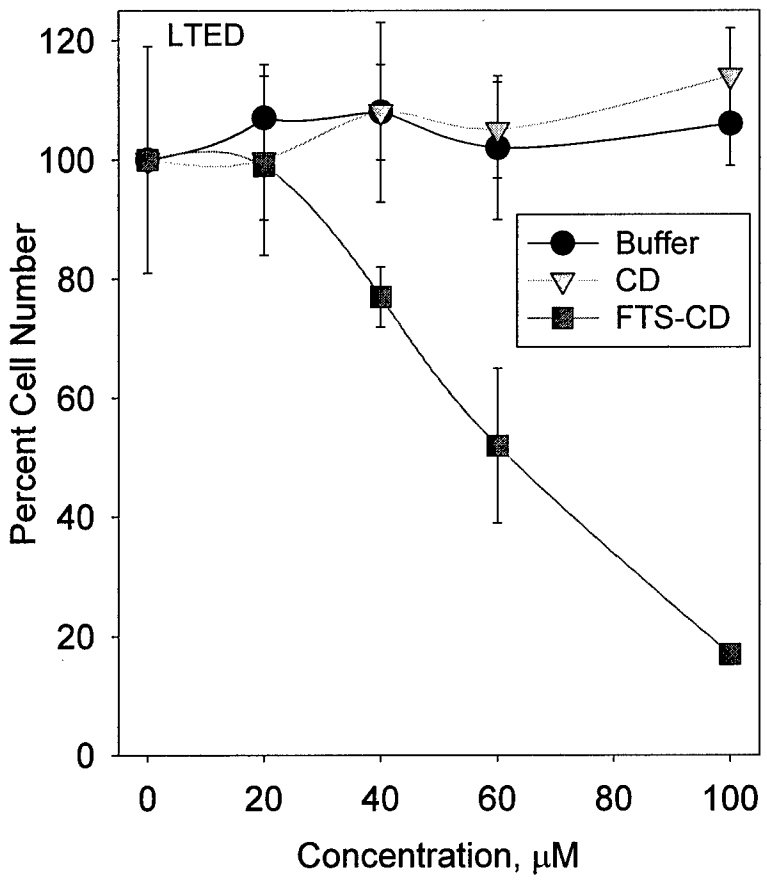

LTED
120
100
80
60
40
20
0
Percent Cell Number
0
20
40
60
80
100
Concentration, μM
Buffer
CD
FTS-CD

Figure 3A

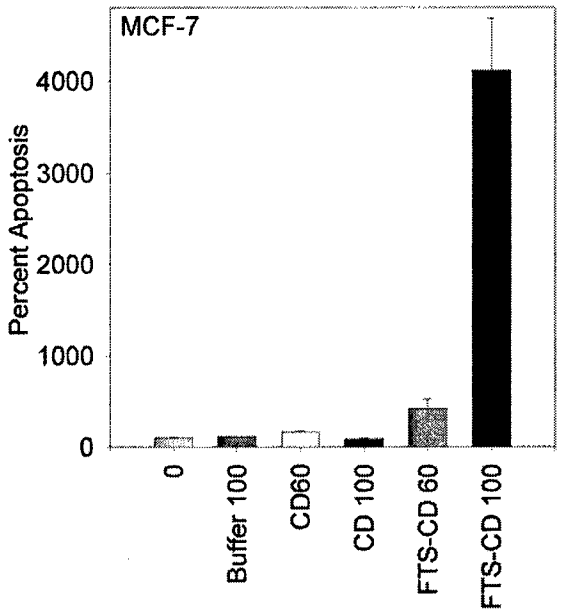

MCF-7
Percent Apoptosis
4000
3000
2000
1000
0
0
Buffer 100
CD60
CD 100
FTS-CD 60
FTS-CD 100

Figure 3B

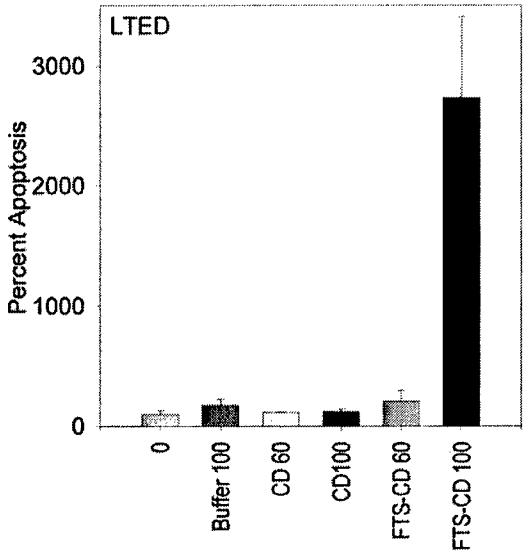

LTED
Percent Apoptosis
3000
2000
1000
0
0
Buffer 100
CD 60
CD100
FTS-CD 60
FTS-CD 100

Figure 4A

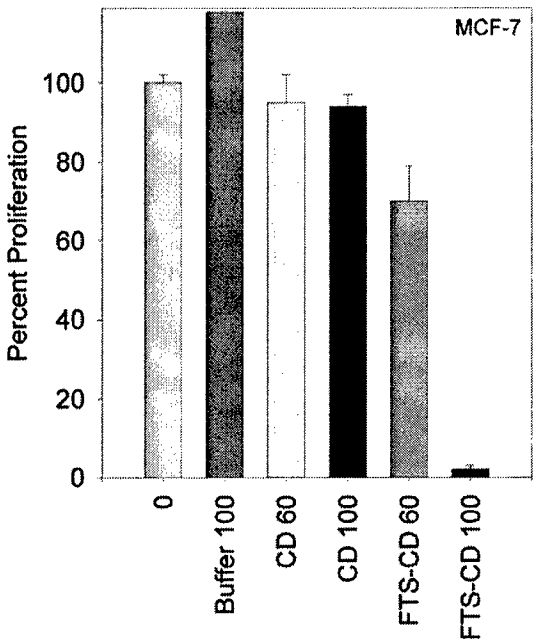
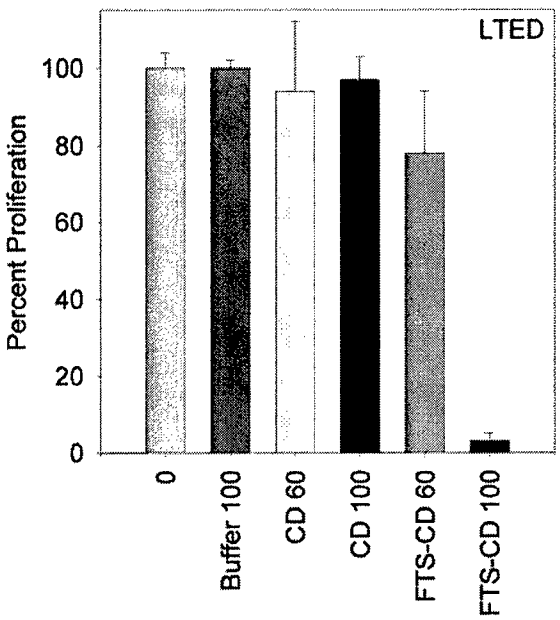

MCF-7
Percent Proliferation
100
80
60
40
20
0
0
Buffer 100
CD 60
CD 100
FTS-CD 60
FTS-CD 100

Figure 4B


LTED
Percent Proliferation
100
80
60
40
20
0
0
Buffer 100
CD 60
CD 100
FTS-CD 60
FTS-CD 100

Farnesylthiosalicylic acid inhibits mTOR activity both in cells and *in vitro* by promoting dissociation of the mTOR- raptor complex.

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The mammalian target of rapamycin, mTOR, functions with raptor and mLST8 in a signaling complex that controls rates of cell growth and proliferation. Recent results indicate that an inhibitor of the Ras signaling pathway, farnesylthiosalicylic acid (FTS), decreased phosphorylation of the mTOR effectors, PHAS-I and S6K1 in breast cancer cells. Here we show that incubating 293T cells with FTS produced a stable change in mTOR activity that could be measured in immune complex kinase assays using purified PHAS-I as substrate. Similarly, FTS decreased the PHAS-I kinase activity of mTOR when added to cell extracts or to immune complexes containing mTOR. Incubating either cells or extracts with FTS also decreased the amount of raptor that coimmunoprecipitated with mTOR, while having relatively little effect on the amount of mLST8 that coimmunoprecipitated. The concentration effect curves of FTS for inhibition of mTOR activity and for dissociation of the raptor-mTOR complex were almost identical. Caffeine, wortmannin, LY294002, and rapamycin-FKBP12, also markedly inhibited mTOR activity *in vitro*, but unlike FTS, none of the other mTOR inhibitors appreciably changed the amount of raptor associated with mTOR. Thus, our findings indicate that FTS represents a new type of mTOR inhibitor, which acts by dissociating the functional mTOR-raptor signaling complex.

INTRODUCTION

The mammalian target of rapamycin, mTOR, is a Ser/Thr protein kinase involved in the control of cell growth and proliferation (1,2). One of the best characterized substrates of mTOR is PHAS-I (a.k.a. 4E-BP1) (3-6). PHAS-I binds to eIF4E and represses cap-dependent translation by preventing eIF4E from binding to eIF4G (7,8). When phosphorylated by mTOR, PHAS-I dissociates from eIF4E, allowing eIF4E to engage eIF4G, thus increasing the formation of the eIF4F complex needed for the proper positioning of the 40S ribosomal subunit and for efficient scanning of the 5'-UTR. In cells mTOR is found in mTORC1, a complex also containing raptor and mLST8 (5,9-11). Raptor (a.k.a. mKOG1) is a newly discovered Mr = 150,000 protein, which possesses a unique NH_2 terminal region followed by three HEAT motifs and seven WD-40 domains (5,9,11). mLST8 (a.k.a. Gβl) is homologous to members of the family of β subunits of heterotrimeric G proteins, and it consists almost entirely of 7 WD-40 repeats (10,11). The roles of the two mTOR-associated proteins are still not fully defined, but both appear necessary for optimal mTOR function, since depleting cells of either raptor or mLST8 with siRNA decreases mTOR activity (5,9,10). Raptor binds directly to PHAS-I and mutations in PHAS-I that decrease raptor binding also inhibit phosphorylation of PHAS-I by mTOR *in vitro* (12,13). It has been proposed that raptor functions in TORC1 as a substrate-binding subunit which presents PHAS-I to mTOR for phosphorylation (5).

Rapamycin is the prototypic inhibitor of mTOR function (14). Determining the sensitivity to rapamycin has been an invaluable approach for identifying processes in cells controlled by mTOR. In addition to its experimental use, rapamycin and/or the related drug, CCI-779, are used clinically to inhibit host rejection of transplanted organs, the occlusion of coronary arteries following angioplasty, and the growth of tumor cells (15-17). Rapamycin action is complicated in that in order to bind mTOR with high affinity, the drug must first form a complex with the peptidylprolyl isomerase, FKBP12 (14). Rapamycin-FKBP12 binds upstream of the kinase domain in a region of mTOR referred to as the FRB. Binding of the complex markedly attenuates, but does not fully inhibit, mTOR activity *in vitro* (4,6). The incomplete inhibition raises the possibility that there are rapamycin-insensitive functions of mTOR in cells. Thus, agents that interfere with mTOR by mechanisms different from that of rapamycin may prove to be useful experimental and/or clinical tools.

Results in the accompanying report demonstrate that farnesylthiosalicylic acid (FTS) inhibits phosphorylation of the mTOR effectors, PHAS-I and S6K1, in response to estrogen stimulation of breast cancer cells (18). FTS is best known for its effects on the Ras signaling pathway, which it inhibits by disrupting the association of Ras with the plasma membrane, a localization essential for both the action and stability of Ras (19,20). In this report we present evidence that FTS inhibits mTOR activity through a novel mechanism involving dissociation of raptor from TORC1.

RESULTS

To investigate the effects of FTS on mTOR function in 293T cells, we monitored changes in the phosphorylation of PHAS-I, a well characterized target of mTOR (1,8). Phosphorylation of Ser64 and Thr69 in PHAS-I causes a dramatic decrease in the mobility of the protein in SDS-PAGE (8), so that changes in the mobility provide an index of changes in phosphorylation state. Incubating cells with increasing concentrations of FTS decreased the phosphorylation of PHAS-I, as evidenced by a decrease in the electrophoretic mobility (Fig. 1A). To determine whether FTS also promoted dephosphorylation of Thr36 and Thr45, the preferred sites for phosphorylation by mTOR (21,22), an immunoblot was prepared with PThr36/45 antibodies (Fig. 1A). Increasing FTS markedly decreased the reactivity of PHAS-I with the phosphospecific antibodies. While the change in the intensity of the immunoblot does not provide an exact measure of the change in phosphorylation, as the antibodies react with PHAS-I phosphorylated in either Thr36 or Thr45 (23), it is clear that the drug promotes the dephosphorylation of these sites.

To investigate further the inhibitory effects of FTS on mTOR signaling, we determined the effect of the drug on the association of mTOR, raptor, and mLST8. AU1-mTOR and HA-tagged forms of raptor and mLST8 were overexpressed in 293T cells, which were then incubated with increasing concentrations of FTS before AU1-mTOR was immunoprecipitated with anti-AU1 antibodies. Immunoblots were prepared with anti-HA antibodies to assess the relative amounts of HA-raptor and HA-mLST8 that coimmunoprecipitated with AU1-mTOR (Fig. 1A). Both HA-tagged proteins were readily detectable in immune complexes from cells incubated in the absence of FTS, indicating that mTOR, raptor, and mLST8 form a complex in 293T cells. FTS did not

change the amount AU1-mTOR that immunoprecipitated; however, increasing concentrations of FTS produced a progressive decrease in the amount of HA-raptor that coimmunoprecipitated (Fig. 1A). When results from three experiments were analyzed, the half maximal effect on raptor dissociation from mTOR was observed at approximately 30 μM FTS (Fig. 2B). FTS did not appear to change the amount of HA-mLST8 associated with AU1-mTOR (Fig. 1A and 2B).

Results obtained with overexpressed proteins are not necessarily representative of responses of endogenous proteins. Therefore, experiments were conducted to investigate the effect FTS on the endogenous TORC1 in nontransfected cells. 293T cells were incubated with increasing concentrations of FTS before mTOR was immunoprecipitated with the mTOR antibody, mTAb1 (Fig. 1B). Immunoblots were then prepared with antibodies to mTOR, mLST8, and raptor. FTS markedly decreased the amount of raptor that coimmunoprecipitated with mTOR. Thus, FTS had comparable effects on the association of endogenous and overexpressed mTOR and raptor proteins. FTS also decreased the amount of mLST8 that coimmunoprecipitated with mTOR, but this effect was much less pronounced than the effect of the drug on the recovery of raptor (Figs. 1A, 1B, and 2).

Incubating cells with FTS produced a stable decrease in mTOR activity that persisted even when mTOR was immunoprecipitated. Fig. 2A presents results of immune complex kinases assays with AU1-mTOR from extracts of 293T cells that had been incubated with increasing concentrations of FTS. The dose response curves for FTS-mediated inhibition of AU1-mTOR activity (Fig. 2A) and dissociation of AU1-mTOR and HA-raptor (Fig. 2B) were very similar, with half maximal effects occurring between 20-30 μM. These results indicate that FTS inhibits mTOR in cells by promoting dissociation of raptor from mTORC1.

We also investigated the effects of incubating cells with increasing concentrations of S-geranylthiosalicylate (GTS) on mTOR activity and the association of AU1-mTOR and HA-raptor (Fig. 2). GTS is identical to FTS except that it contains the 10-carbon geranyl group instead of the 15-carbon farnesyl group. GTS is much less effective than FTS in down-regulating the Ras signaling pathway (24), and it serves as a control for nonspecific detergent-like actions that occur with high concentrations of isoprenoid derivatives.

Incubating cells with increasing concentrations of GTS slightly decreased mTOR activity (Fig. 2A); however, GTS was clearly less effective than FTS. GTS had relatively little effect on the association of AU1-mTOR with either HA-raptor or HA-mLST8 (Fig. 2B). Incubating cells with 200 μM sodium salicylate was also without effect on either mTOR activity or the association of mTOR and raptor.[2]

The findings with FTS in intact cells would be consistent with either an action of FTS on TORC1 or an action on a signaling pathway controlling the association of mTOR and raptor. Since the integrity of most signaling pathways is disrupted when cells are homogenized, we investigated the effects of FTS in extracts of cells in which AU1-mTOR, HA-raptor, and HA-mLST9 had been overexpressed. Incubating extracts with increasing concentrations of FTS progressively decreased the PHAS-I kinase activity of AU1-mTOR, assessed both by ^{32}P incorporation from [γ-^{32}P]ATP and by immunoblotting with PThr36/45 antibodies (Fig. 3A). Approximately four times lower concentrations of FTS were needed to inhibit mTOR activity *in vitro* than in intact cells. Presumably factors related to protein binding and membrane permeability account for the difference in concentrations of FTS needed in cells and extracts. Incubating extracts with increasing concentrations of FTS also decreased the amount of HA-raptor that coimmunoprecipitated with AU1-mTOR. The dose response curves of kinase inhibition (Fig. 4A) and dissociation of AU1-mTOR and HA-raptor (Fig. 4B) were almost identical, indicating that loss of raptor from mTORC1 accounted for the inhibition of mTOR activity by FTS under these *in vitro* conditions. Again, the effects of FTS did not depend on overexpression of the mTORC1 components. Incubating extracts of nontransfected cells with FTS decreased the amount of endogenous raptor that coimmunoprecipitated with mTOR (Fig. 3B). The effects occurred at the same concentrations that promoted dissociation of the complex between the transfected proteins (Fig. 3A). The inhibitory effects of FTS on mTOR activity were apparent either when FTS was added directly to immune complexes just before the protein kinase assay, or when FTS was added to extracts prior to the immunoprecipitation of mTOR.[2] Thus, if FTS action depends on factors other than the known components of mTORC1, such factors must coimmunoprecipitate with the complex. FTS also modestly decreased the amounts

of both transfected and endogenous mLST8 proteins that coimmunoprecipitated with mTOR proteins (Fig. 3A and 3B), but the effects were observed only at the highest concentrations of FTS investigated (Fig. 4B).

As a portion of mTORC1 may be associated with membranous structures, we determined whether the effect of FTS depended on the presence of membranes. A fraction containing soluble mTORC1 was generated by centrifuging detergent-free extracts of HEK293 cells for 40 min at 200,000 x *g*. Adding 100 μM FTS to the supernatant fraction completely dissociated the complex between mTOR and raptor.[2]

Incubating extracts of transfected cells with increasing concentrations of GTS also led to progressive decreases both in AU1-mTOR activity (Fig. 4A) and in the association of AU1-mTOR and HA-raptor (Fig. 4B). These effects of GTS occurred at approximately ten times higher concentrations than those of FTS. Thus, a degree of selectivity is conferred by the isoprenyl component of the drug.

We next compared the effects of FTS to those of rapamycin and several other inhibitors of mTOR that have been described previously (6,25,26). Incubating extracts from transfected cells with FTS decreased both mTOR activity (Fig. 5A and 5B) and the association of AU1-mTOR and HA-raptor (Fig. 5A and 5C). In contrast, incubating these complexes with concentrations of caffeine, rapamycin-FKBP12, LY294002 or wortmannin that decreased mTOR activity by more than 80% had little, if any, effect on decreasing the association of AU1-mTOR and HA-raptor.

DISCUSSION

The results of this study provide direct evidence that FTS inhibits mTOR activity. The finding that the inhibition of mTOR activity by increasing concentrations of FTS correlated closely with the dissociation of the mTOR-raptor complex, both in cells (Fig. 2) and *in vitro* (Fig. 3), supports the conclusion that FTS acts by promoting dissociation of raptor from mTORC1.

The peptidomimetic farnesyltransferase inhibitor, L-744,832, has also been shown to inhibit mTOR signaling (27,28). By analogy to the Ras signaling pathway, it is logical to suspect that FTS and farnesyltransferase inhibitors might act at the same target in the mTOR signaling pathway. Both farnesyltransferase inhibitors and FTS disrupt the plasma membrane localization of Ras, one by blocking in the

isoprenylation of Ras necessary for its membrane localization, the other by displacing Ras from its membrane binding sites. Farnesyltransferases prenylate the Cys found in a COOH terminal motif, sometimes referred to as the *CAAX* box (where *C* is Cys, *A* is an aliphatic amino acid, and *X* is any amino acid) (29). The COOH terminal sequence in mTOR is CysProPheTrp, which has some features of a *CAAX* box. However, based on studies with model peptides, the Phe in the mTOR sequence represents a strong negative determinant (29). Indeed, peptides with Phe in this position served as the basis for the design of L-744,832, and other potent competitive inhibitors of farnesyltransferase.

While none of the proteins in mTORC1 are known to be prenylated, there are potential targets for FTS upstream in the mTOR signaling pathway. One example is the farnesylated GTP-binding protein Rheb (Ras homologue enriched in brain) (30). Rheb is activated in response to growth factors that inhibit the TSC1/TSC2, which functions as the Rheb GTP'ase activating protein (GAP) (31-33). Although the mechanism is still unclear, activation of Rheb increases mTOR signaling. Mutating the Cys in the *CAAX* box of Rheb abolished the ability of overexpressed Rheb to increase S6K activity (33,34). Thus, Rheb is a potential target for farnesyltransferase inhibitors, and it is feasible that an action of FTS to displace Rheb from intracellular binding sites contributes to the inhibitory effects of FTS on mTOR signaling in intact cells. However, Rheb does not appear to coimmunoprecipitate with mTOR (32). Thus, it is not clear that Rheb was involved in the inhibitory effects of FTS on mTOR activity and the association of mTOR and raptor *in vitro*. Interestingly, the inhibition of mTOR signaling by L-744,832 in cells seems to occur too rapidly (within 1.5 h) to be explained by inhibition of protein farnesylation (28,35). Moreover, incubating 293T cells for 18 h with 60 μM L-744,832 did not promote dissociation of endogenous or overexpressed mTORC1.[2] Additional studies will be required to determine the actual sites of action of both FTS and L-744,832.

Consistent with its action to inhibit Ras signaling, FTS blocks the activation of MAP kinase (20), and it inhibits the proliferation of several types of tumor cells, both *in vitro* and *in vivo* (18,36-39). In view of the number of different proteins that are farnesylated, the actions of FTS would be expected to involve more than inhibition of Ras signaling. As evidence of the complexity of FTS action, results presented in the accompanying report demonstrate inhibition by FTS of the effect of estrogen on stimulating the proliferation of

breast cancer cells correlate much better with dephosphorylation of PHAS-I and S6K-1, two downstream elements of the mTOR signaling pathway, than with the inhibition of MAPK (18).

Inhibition of mTOR with rapamycin has been shown to inhibit translation of capped mRNAs (40) and messages having a TOP (tract of pyrimidines) motif adjacent to the cap site (41). There are also reasons to suspect that the inhibition of mTORC1, with the decrease in PHAS-I phosphorylation and the resulting decrease in the availability of eIF4E for translation, contributes to the antiproliferative effect of FTS. Increasing eIF4E may result not only in an increase in cap-dependent translation, but also in an increase in cell proliferation. eIF4E levels are elevated in most breast cancer cells (44). Overexpressing eIF4E increased the rate of growth and caused an aberrant morphology of HeLa cells (42). Stable overexpression of eIF4E in 3T3 fibroblasts not only increased the rate of proliferation but actually caused malignant transformation, as evidenced by anchorage independent growth and formation of tumors when implanted in nude mice (43).

It has been suggested that eIF4E stimulates proliferation by preferentially increasing translation of proteins that facilitate mitogenesis (45). The 5'-UTRs of mRNAs encoding many oncogenes, growth factors and signal transduction proteins are predicted to contain regions of relatively stable secondary structure (46). These structured regions have been shown to interfere with binding and/or scanning by the 40S ribosomal subunit (47). Translation of such messages appears to be more dependent on eIF4E availability than translation of mRNAs having unstructured 5'UTRs, a characteristic of many messages encoding house-keeping proteins. The dependency on eIF4E is believed to be explained by the requirement of eIF4E for formation of eIF4F, which melts secondary structure in the 5'-UTR via the helicase activity of the eIF4A subunit (45). As predicted from this mechanism, overexpressing PHAS-I, which decreases eIF4E availability, caused reversion of cells overexpressing eIF4E (48). Interestingly, overexpressing a constitutively active PHAS-I protein was recently shown to decreased the proliferation of MCF7 breast cancer cells (49).

By inhibiting mTOR activity and decreasing PHAS-I phosphorylation, FTS should decrease the contribution of eIF4E to proliferative responses. Other mTOR-dependent processes that are independent of changes in eIF4E availability are surely involved in the control of cell proliferation. While not investigated in

the present study, it can be predicted that FTS will be found to inhibit those processes requiring the raptor-mTOR interaction.

MATERIALS AND METHODS

Antibodies—Antibodies recognizing endogenous mTOR (mTAb1 and mTAb2) (50), PHAS-I (51), and raptor (12) were generated by immunizing rabbits with peptides having sequences corresponding to regions in the respective proteins. The phosphospecific antibodies, P-Thr36/45 and P-Thr69, that recognize phosphorylated sites in PHAS-I were generated as described previously (23). P-Thr36/45 antibodies bind to PHAS-I phosphorylated in either Thr36 or Thr45, as the sequences surrounding these sites are almost identical (52). Ascites fluid containing monoclonal antibody to the AU1 epitope tag was from Berkley Antibody Company. 9E10, which recognizes the myc epitope tag, and 12CA5, which recognizes the HA epitope tag, were purified from hybridoma culture medium by the University of Virginia Lymphocyte Culture Center.

To generate antibodies to mLST8, a synthetic peptide (CVETGEIKREYGGHQK) having a sequence identical to positions 298-313 of human mLST8 was coupled to keyhole limpet hemocyanin, and the conjugate was used to immunize rabbits as described previously (53). Antibodies were purified using a column containing affinity resin prepared by coupling the peptide to Sulfolink beads (Pierce).

cDNA Constructs--The pcDNA3$^{AU1\text{-}mTOR}$ (3), pcDNA3$^{3HA\text{-}Raptor}$ (12), and pCMV-Tag 3A$^{PHAS\text{-}I}$ (54) constructs for overexpressing AU1-mTOR, HA-Raptor, and myc-PHAS-I were described previously (3,12,54). pcDNA3$^{3HA\text{-}mLST8}$ encodes mLST8 having an NH_2 terminal triple HA epitope tag (HA- mLST8). To generate pcDNA3$^{3HA\text{-}mLST8}$ a 5' *Eco*RI site and a 3'*Not*I site were introduced into mLST8 cDNA by PCR using I.M.A.G.E. clone 3910883 as template, and GAGTCGAATTCATGAACACCTCCCCAGGC and CGACTGCGGCCGCTCGAGCTAGCCCAGCACACTGTC as forward and reverse primers, respectively. After digesting the product with *Eco*RI and *Not*I, the mLST8 cDNA was inserted in pcDNA3$^{3HA\text{-}Raptor}$ in place of raptor insert, which had been removed with *Eco*RI and *Not*I. The coding region of the resulting pcDNA3^{3HAmLST8} was sequenced and found to be free of errors.

Overexpression of AU1- mTOR, HA- raptor, HA- mLST8 and Myc-PHAS-I--293T cells were cultured for 24 h in growth medium composed of 10 % (v/v) fetal bovine serum in Dulbecco's modified Eagle medium (DMEM) (6). AU1-mTOR, HA-raptor, and HA-mLST8 were coexpressed by transfecting 293T cells (100 mm diameter dish) with 4 μg each of pcDNA3$^{AU1\text{-}mTOR}$, pcDNA3$^{3HA\text{-}Raptor}$, and pcDNA3$^{3HA\text{-}mLST8}$ by using TransIT-LT2 (Mirus Corp., Madison, WI) as described previously (3). Other cells were transfected with pcDNA3 vector alone. Where indicated, cells were transfected with pCMV-Tag 3A$^{PHAS\text{-}I}$ to coexpress Myc-PHAS-I. Cells were used in experiments 18-20 hours after transfection.

Immune complex assay of mTOR activity--AU1-mTOR was immunoprecipitated by using anti-AU1 antibody bound to protein G-agarose beads as described previously (6). Endogenous mTOR was immunoprecipitated in the same manner, except that mTAb1was used instead of anti-AU1 antibody. To measure kinase activity, exhaustively washed immune complexes were suspended in 20 μl of Buffer A (50 mM NaCl, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 0.5 mM microcystin LR, 10 mM Na-HEPES, and 50 mM β-glycerophosphate, pH 7.4.). The kinase reactions were initiated by adding 20 μl of Buffer A supplemented with 2 mM [γ-^{32}P]ATP, 20 mM $MnCl_2$ and 40 μg/ml of [His^6]PHAS-I. In experiments in which the effects of wortmannin were investigated, DTT was omitted from the reactions, and [His^6]PHAS-I that had been reduced and alkylated with N-ethylmaleimide was used as substrate. Reactions were terminated after 10 min at 30° by adding SDS sample buffer. Samples were subjected to SDS-PAGE and the relative amounts of ^{32}P incorporated into [His^6]PHAS-I were determined.

Electrophoretic analyses-- SDS-PAGE was performed using the method of Laemmli (55). Immunoblots were prepared after electrophoretically transferring protein to Immobilon (Millipore) membranes. The relative amounts of ^{32}P incorporated into [His^6]PHAS-I were determined by phosphorimaging. Signal intensities of bands in immunoblots were determined by scanning laser densitometry.

Other materials--FTS and S-geranylthiosalicylic acid (GTS) were kindly provided by from Dr. Yoel Kloog (Tel-Aviv University, Tel-Aviv, Israel) and Wayne Bardin (Thyreos, New York, NY). Rapamycin and LY294002 were from Calbiochem-Novabiochem International. Caffeine was from Sigma Chemical Co.

Glutathione S transferase (GST)-FKBP12 (56) and [His^6]PHAS-I (57) were expressed in bacteria and purified as described previously (56,57). [γ-^{32}P]ATP was from NEN Life Science Products.

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FOOTNOTES

1. The abbreviations used are as follows: DMEM, Dulbecco's Modified Eagle's Medium; DTT, dithiothreitol; eIF, eukaryotic initiation factor; FTS, S-farnesylthiosalicylic acid; GST, glutathione-S-transferase; GTS, S-geranylthiosalicylic acid; HA, hemagglutinin; mTAb, mTOR antibody; mTOR, mammalian target of rapamycin; 5'-UTR, 5' untranslated region

2. McMahon, L. P., Choi, K. M., and Lawrence, J. C., Jr., unpublished observations.

FIGURE LEGENDS

Fig. 1 FTS inhibits PHAS-I phosphorylation and promotes dissociation of the mTOR-raptor complex in 293T cells. A, 293 T cells were transfected with pcDNA3$^{AU1\text{-}mTOR}$, pcDNA3$^{3HA\text{-}Raptor}$, pcDNA3$^{3HA\text{-}mLST8}$, and pCMV-Tag3A$^{PHAS\text{-}I}$. After 18 h the cells were incubated for 45 min with increasing concentrations of FTS before extracts were prepared. A sample of each extract was subjected to SDS-PAGE and immunoblotted to detect PHAS-I or PThr36/45. AU1-mTOR was also immunoprecipitated from each extract, and immune complexes were subjected to SDS-PAGE and immunoblotted with mTAb2, to detect mTOR, and with anti-HA antibodies, to detect HA-mLST8 and HA-raptor. B, nontransfected 293T cells were incubated with increasing concentrations of FTS for 1 h before extracts were prepared. mTOR was immunoprecipitated with mTAb1, and samples were subjected to SDS-PAGE. Immunoblots of mLST8, mTOR, and raptor are presented.

Fig. 2 Effects of incubating cells with increasing concentrations of FTS and GTS on mTOR activity and mTOR association with raptor and mLST8. AU1-mTOR, HA-raptor, and HA-mLST8 were overexpressed in 293T cells. The cells were then incubated for 45 min with increasing concentrations of FTS (●,▲,■) or GTS (○, Δ, □) before extracts were prepared. Immunoprecipitations were then conducted with anti AU1 antibodies. A, mTOR kinase activity (●,○) was determined by measuring ^{32}P incorporation into [His^{6}]PHAS-I in immune complex kinases assays performed with [γ-^{32}P]ATP. B, the relative amounts of HA-raptor (▲, Δ) and HA-mLST8 (■, □) that coimmunoprecipitated with AU1-mTOR were determined after immunoblotting with anti-HA antibodies. The results (mean values $\pm$ S.E. from 3 experiments) are expressed as percentages of the mTOR activity (A) or coimmunoprecipitating proteins (B) from samples incubated without FTS or GTS, and have been corrected for the amounts of AU1-mTOR immunoprecipitated.

Fig. 3. FTS promotes raptor dissociation and inhibits mTOR activity in cell extracts. A, 293T cells were transfected with pcDNA3 alone (Vec.) or with a combination of pcDNA3$^{AU1\text{-}mTOR}$, pcDNA3$^{3HA\text{-}Raptor}$, and pcDNA3$^{3HA\text{-}mLST8}$. Extracts of the cells were incubated with increasing concentrations of FTS for 30 min before

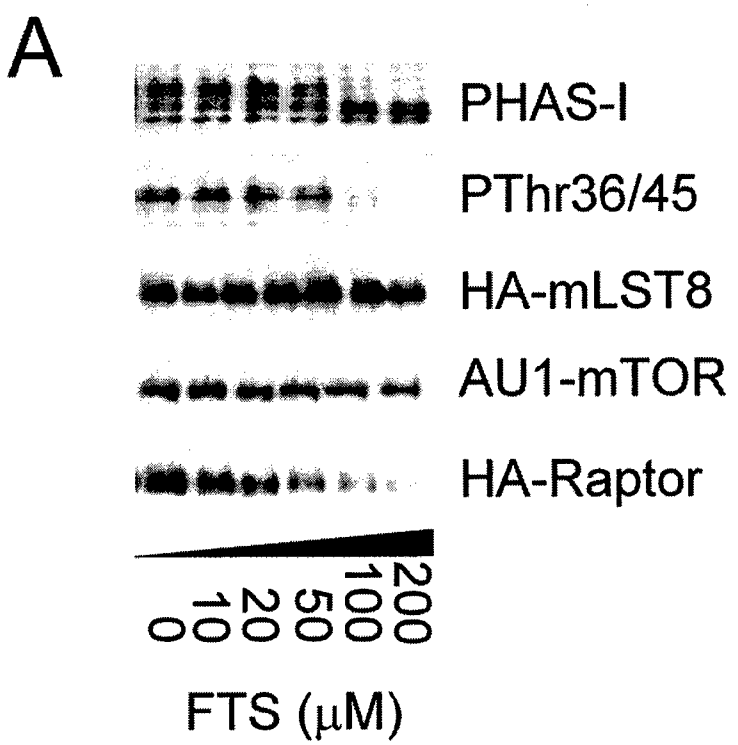
AU1-mTOR was immunoprecipated. Samples of the immune complexes were incubated with [γ-^{32}P]ATP and recombinant [His^{6}]PHAS-I, then subjected to SDS-PAGE. A phosphorimage of a dried gel was obtained to detect ^{32}P-PHAS-I, and an immunoblot was prepared with PThr36/45 antibodies. Other samples of the immune complexes were subjected to SDS-PAGE and immunoblots were prepared with antibodies to the HA epitope or to mTOR. B, extracts of nontransfected 293T cells were incubated with increasing concentrations of FTS before mTOR was immunoprecipated with mTAb1. A control immunoprecipitation was conducted using nonimmune IgG (NI). Immune complexes were subjected to SDS-PAGE and immunoblots were prepared with antibodies to mLST8, mTOR, and raptor.

Fig. 4. Relative effects of increasing concentrations of FTS and GTS on mTOR activity and the association of mTOR and raptor. Samples of extracts from 293T cells overexpressing AU1-mTOR, HA-raptor, and HA-mLST8 were incubated for 1 h with increasing concentrations of FTS (●,▲,■) or GTS (○, Δ, □) before immunoprecipitations were conducted with anti-AU1 antibodies. A, mTOR kinase activity (●,○) was determined by measuring ^{32}P incorporation into [His^{6}]PHAS-I in immune complex kinases assays performed with [γ-^{32}P]ATP. B, the relative amounts of HA-raptor (▲, Δ) and HA-mLST8 (■, □) that coimmunoprecipitated with AU1-mTOR were determined after immunoblotting with anti-HA antibodies. The results (mean values $\pm$ S.E. from 5 experiments) are expressed as percentages of the mTOR activity (A) or coimmunoprecipitating proteins (B) from samples incubated without FTS or GTS, and have been corrected for the amounts of AU1-mTOR immunoprecipitated.

Fig. 5. Effect of mTOR inhibitors on the association of mTOR and raptor A, 293T cells were transfected with pcDNA3 alone (Vec.) or with a combination of pcDNA3$^{AU1\text{-}mTOR}$, pcDNA3$^{3HA\text{-}Raptor}$, and pcDNA3$^{3HA\text{-}mLST8}$. Before AU1-mTOR was immunoprecipitated cell extracts were incubated for 30 min without or with the following: caffeine (1 mM), FTS (50 μM), LY294002 (10 μM), rapamycin (1 μM) plus FKBP12 (10 μM), and 1 μM wortmannin. After washing, AU1-mTOR immune complexes were subjected to SDS-PAGE, and

immunoblotted with anti-HA and anti-AU1 antibodies to determine the amounts of HA-raptor and HA-mLST8 associated with AU1-mTOR. To assess mTOR activity the samples of the washed immune complexes were incubated for 10 min at 30° with the same concentrations of inhibitors in solutions containing 10 mM $MnCl_2$, 1 mM [γ-^{32}P]ATP, and 50 μg/ml [His^6]PHAS-I. A, a phosphorimage of ^{32}P-PHAS-I from the kinase assay and immunoblots of HA-mLST8, AU1-mTOR and HA-raptor are shown. B, the relative amounts of ^{32}P incorporated into PHAS-I were determined by phosphorimaging. C, the amounts of HA-raptor and HA-mLST8 that coimmunoprecipitated with AU1-mTOR were determined from immunoblots. In B and C, the results were corrected for the amounts of AU1-mTOR immunoprecipitated and are expressed as percentages of the respective controls. Means $\pm$ 1/2 the range from 2 experiments are presented.

Figure 1


A
PHAS-I
PThr36/45
HA-mLST8
AU1-mTOR
HA-Raptor
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FTS (μM)
B
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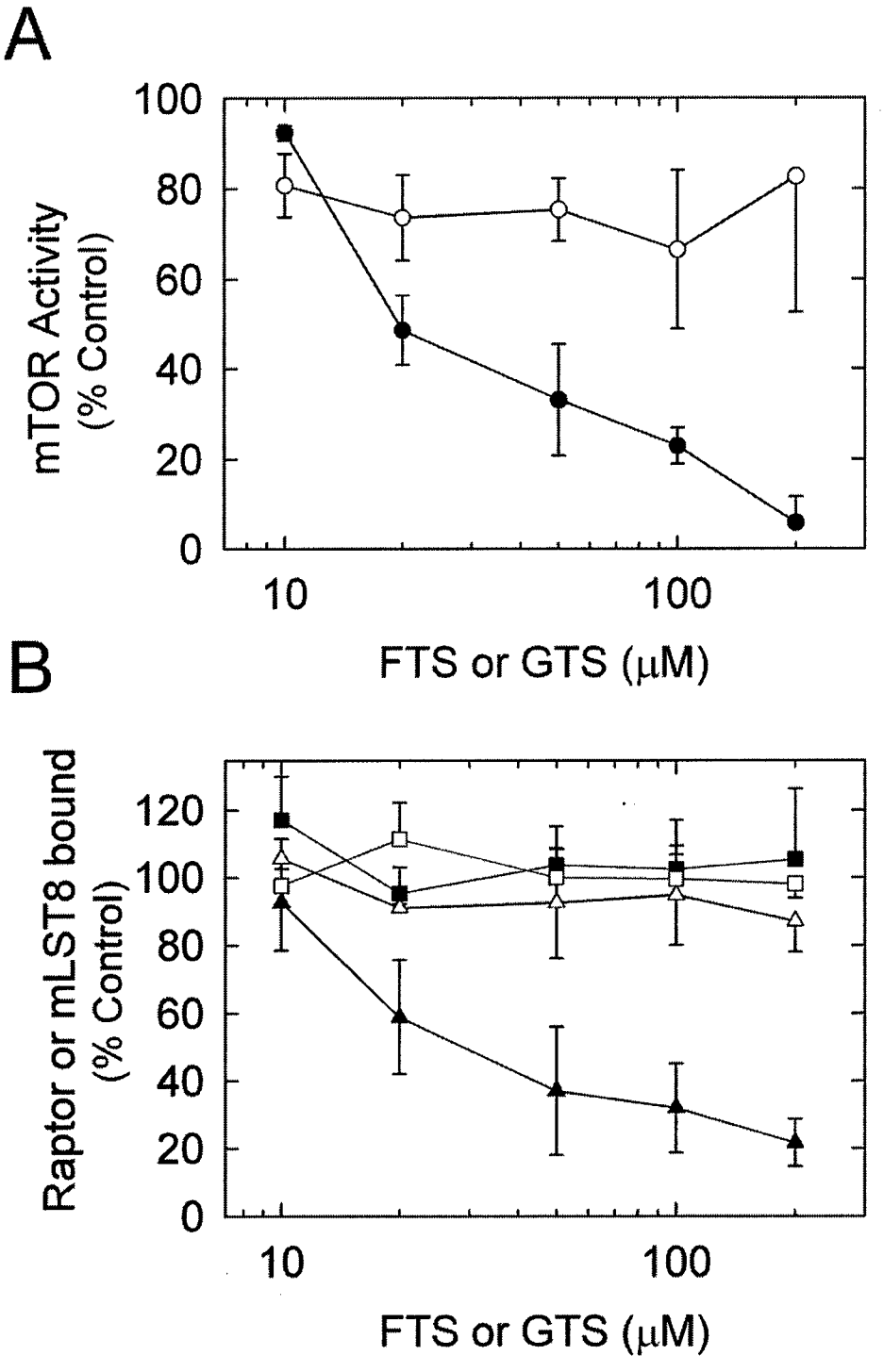
Figure 2


A
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80
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40
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mTOR Activity
(% Control)
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FTS or GTS (μM)
B
120
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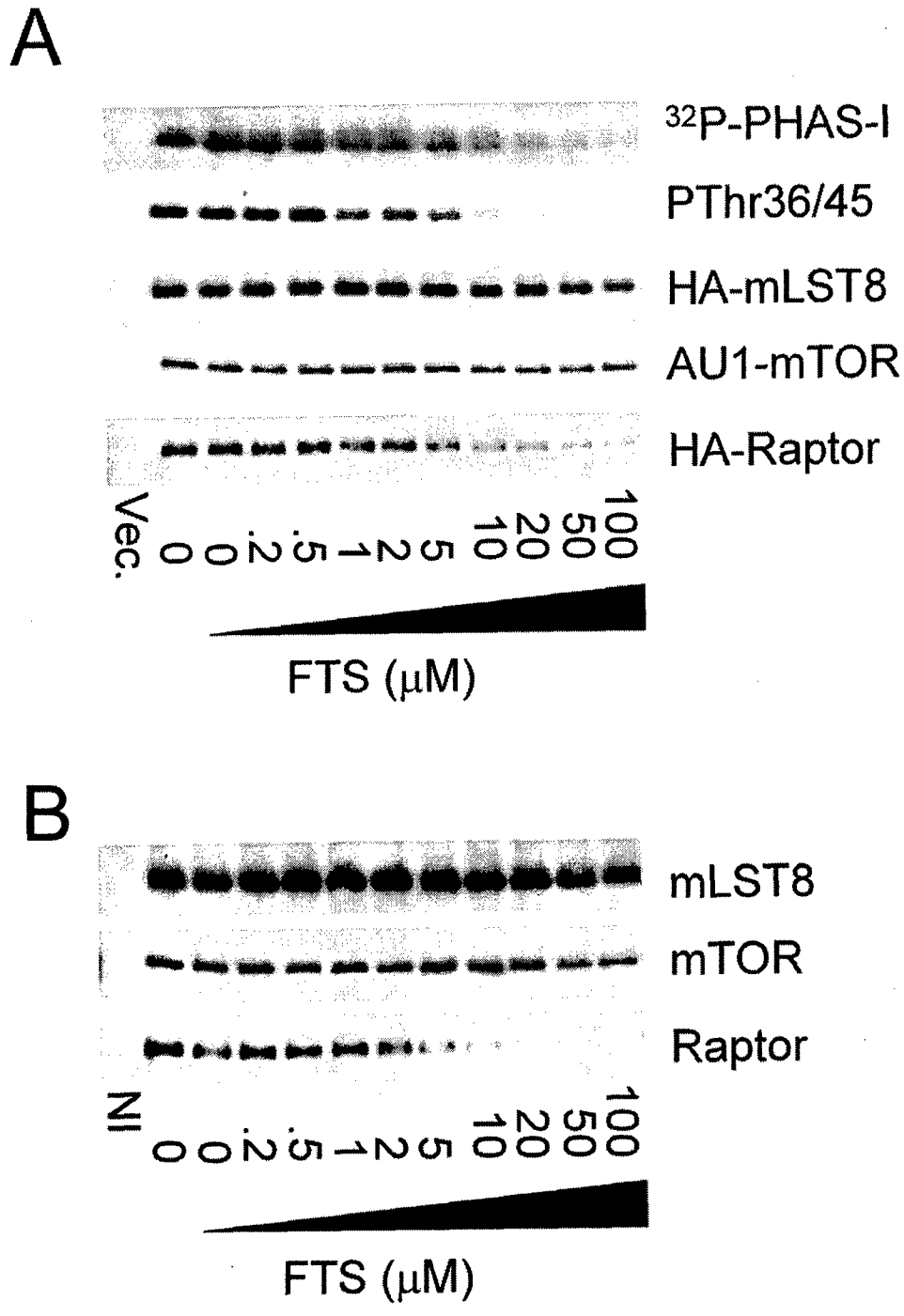
40
20
0
Raptor or mLST8 bound
(% Control)
10
100
FTS or GTS (μM)

Figure 3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32P-PHAS-I
PThr36/45
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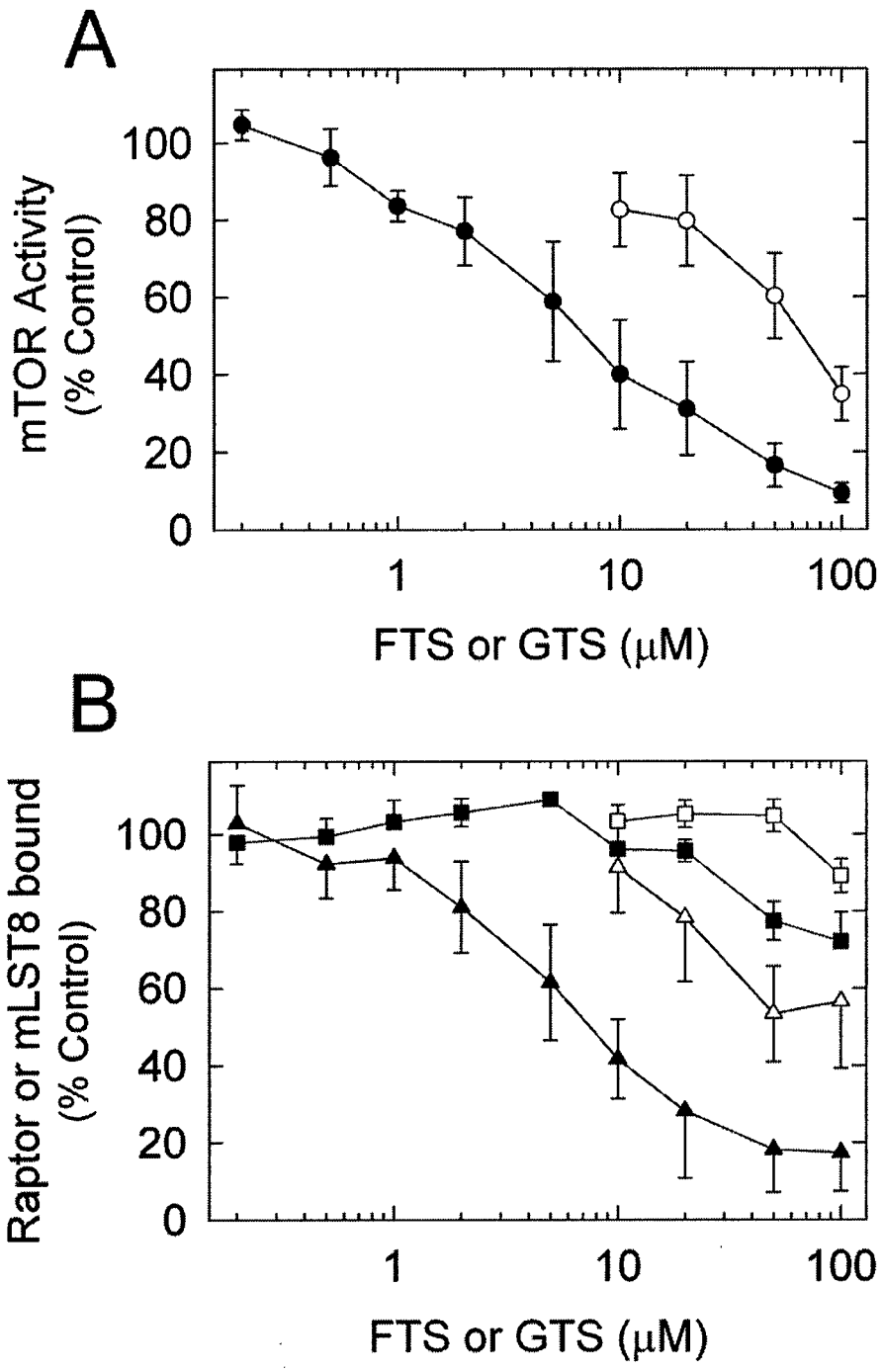
Figure 4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mTOR Activity
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B
Raptor or mLST8 bound
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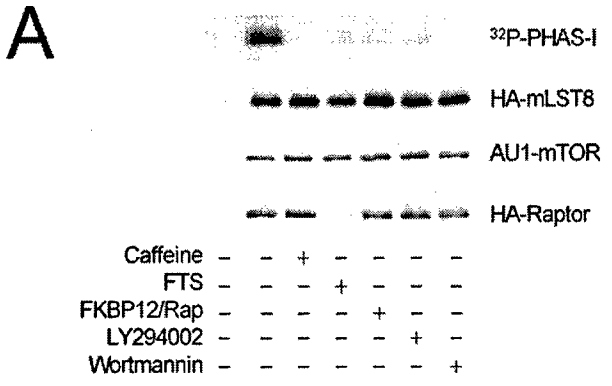
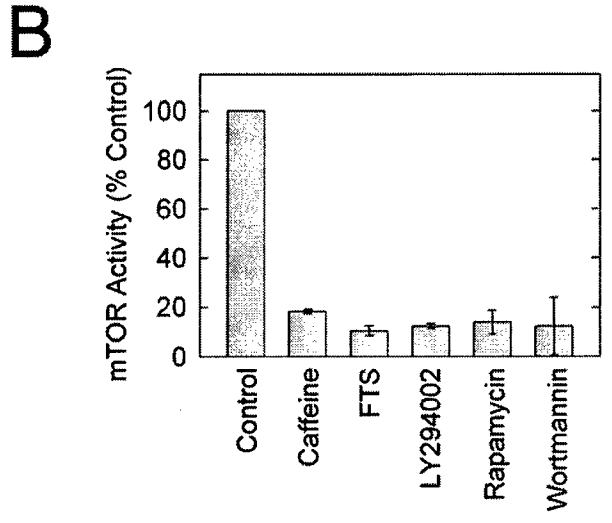
Figure 5


A
32P-PHAS-I
HA-mLST8
AU1-mTOR
HA-Raptor
Caffeine – – + – – – –
FTS – – – + – – –
FKBP12/Rap – – – – + – –
LY294002 – – – – – + –
Wortmannin – – – – – – +


B
mTOR Activity (% Control)
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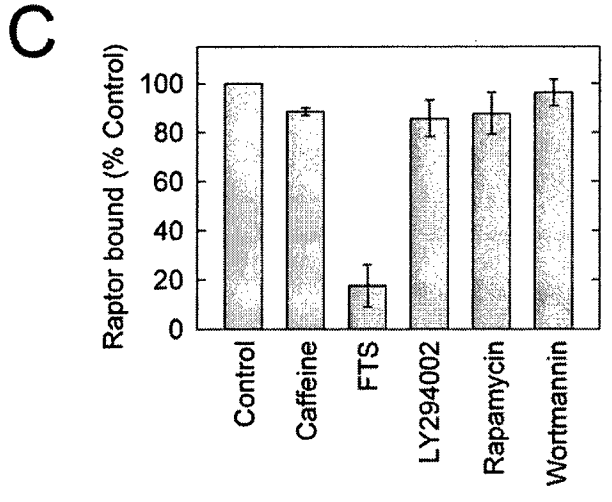
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LY294002
Rapamycin
Wortmannin

Farnesylthiosalicylic Acid Blocks Mammalian Target of Rapamycin Signaling in Breast Cancer Cells[1]

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Abbreviated title: mTOR signaling in breast cancer cells

Key words: FTS, breast cancer, mTOR, p70 S6 kinase, PHAS-I, PI3 kinase, MAP kinase

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4. The abbreviations used are: FTS, farnesylthiosalicylic acid; LTED, long term estrogen deprivation; E_2, estradiol; ERK, extracellular signal-regulated kinase; mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol-3 kinase; p70 S6K, p70 S6 kinase; PHAS-I, eukaryotic initiation factor 4E binding protein 1.

ABSTRACT

Estradiol (E_2) stimulates proliferation of hormone-dependent breast cancer and exerts downstream effects on growth factors and their receptors. Key among the pathways mediating growth factor action is the MAP kinase signaling cascade and the PI-3 kinase pathway with its downstream effector mTOR. We postulated that farnesylthiosalicylic acid (FTS), a novel anti-Ras drug, could effectively inhibit hormone-dependent breast cancer since Ras activates both the MAP kinase and the PI3 kinase pathways. Wild type MCF-7 cells and a long-term estrogen deprived subline (LTED) were used to examine the effect of FTS on cell growth and on several biochemical parameters. FTS inhibited growth of both cell lines by reducing proliferation and inducing apoptosis. These effects correlated best with blockade of phosphorylation of PHAS-I and p70 S6 kinase, two downstream effectors of mTOR. We observed only minimal inhibition of Akt, an effector upstream of mTOR. Taken together, these findings demonstrate a novel effect of FTS to inhibit mTOR signaling and also suggest that mTOR has a key role in breast cancer cell proliferation. Unexpectedly, only minimal inhibition of MAP kinase occurred in response to FTS at concentrations that markedly reduced cell growth. These later data provide support for the concept that FTS exerts its effects predominantly by blocking mTOR and to a lesser effect by inhibition of MAP kinase in breast cancer cells.

INTRODUCTION

Clinical and biochemical data provide evidence that one-third of human breast cancers are hormone dependent. The predominant mitogen for these tumors is estrogen, which exerts much of its effect through activation of growth factor signaling pathways. Specifically, estrogen induces activation of growth factor receptors[1] as well as increasing the level of the ligands themselves, such as IGF-1 and TGFα[2, 3]. Up-regulation of growth factor signaling pathways is associated with failure of endocrine therapy and targeting of the growth factor signaling pathway has become an emerging therapeutic strategy for treatment of breast cancer[4].

Interaction of growth factors with their receptors activates multiple downstream kinase pathways crucial for cell proliferation and survival. The MAP kinase and PI3 kinase cascades represent two of the most important[5-11]. The MAP kinase pathway exerts downstream effects on Elk-1, a transcription factor related to cell proliferation. The PI-3 kinase pathway activates several downstream signaling molecules including Akt and its more distal effector, mTOR (the mammalian target of rapamycin).

An increasing body of evidence suggests that cell growth and proliferation are tightly controlled by mTOR. This molecule is a member of phosphoinositide 3-kinase-related kinase family and functions as a serine/threonine protein kinase. Evidence of the key role of mTOR in proliferation is the observation that mTOR inhibitors result in delayed cell cycle progression, G1-phase arrest, and reduction of cell number[12-14].

The regulation of mTOR activity is complex and involves both activation by growth factors and regulation by availability of nutrient and energy. When growth factors bind to their cognate receptors, a signaling cascade involving PI3 kinase and Akt is

initiated. Akt in turn causes phosphorylation of tuberous sclerosis complex 2 (TSC2) and activation of mTOR[15]. Enhanced nutrient availability also increases mTOR but through unknown mechanisms. Activation of mTOR results in the phosphorylation of two downstream effectors: p70 S6 Kinase and PHAS-I (4E-BP-1). p70 S6 kinase in turn activates the major ribosomal protein S6. PHAS-I binds to eIF4E, the mRNA cap-binding protein and prevents the interaction of eIF4E with eIF4G, thus inhibiting cap-dependent translation. When phosphorylated in a mTOR-dependent manner in response to growth factor stimulation, PHAS-I dissociates from eIF4E, resulting in an increase in cap-dependent translation[16, 17].

It was reported recently that both the p70 S6K and PHAS-I/eIF4E pathways are required for mTOR-dependent cell cycle progression[18]. Amplification of the p70 S6K gene is found in approximately 10% of all primary breast cancer cases and is associated with poor prognosis[19, 20]. It has been reported that eIF4E levels are elevated in breast cancer[21]. This elevation increases the relative risk for cancer recurrence and cancer-related death[22]. Results from these studies indicate that the mTOR pathway may play an important role in breast cancer.

A common feature of signal transduction pathways is that they can be activated by several growth factors. The redundancy of signal transduction networks enables tumor cells to survive the blockade of a single pathway and to re-grow. Therefore, simultaneous inhibition of multiple pathways or blocking signal transduction at a nodal point where multiple pathways converge might be needed to provide an effective strategy for treatment of hormone-dependent breast cancer.

Farnesylthiosalicylic acid (FTS), a recently developed compound, could potentially serve as an agent capable of inhibiting several pathways. A series of studies demonstrates that this compound inhibits binding of GTP-Ras to a plasma membrane acceptor protein, galectin 1, leading to an increase in Ras degradation[23, 24]. FTS has been shown to exhibit substantial anti-tumor effects in neoplasms with activating mutations of Ras, such as pancreatic cancer and melanoma. The anti-tumor effect of FTS in these tumors correlates with inhibition of MAP kinase activation[25-28]. Our recent report suggests an additional potent effect of FTS to inhibit mTOR activity through a unique ability to dissociate mTOR from its binding partner, RAPTOR[29].

Because of its dual effect on MAP kinase and mTOR, we reasoned that FTS might be a useful inhibitor for hormone-dependent breast cancer. In the present study, we examined the effects of FTS on cell proliferation and apoptosis in wild type and the LTED variant of MCF-7 breast cancer cells. We also studied its effect on the major downstream growth factor pathways involving MAP kinase, PI3 kinase, and mTOR. Surprisingly, FTS exhibited relatively weak blocking effects on MAP kinase and Akt activation, while markedly inhibiting the phosphorylation of PHAS-I and p70 S6 kinase, two effectors of mTOR. These results, taken together with the lack of potent inhibition of Akt activation, provide further evidence that FTS is a potent inhibitor of mTOR signaling. The results of these studies indicate that the mTOR pathway is a key component of cell proliferation in models of hormone dependent breast cancer and that FTS is a promising agent to interrupt mTOR signaling in breast cancer.

MATERIALS AND METHODS

Materials. Farnesylthiosalicylic acid (FTS) was a gift from Drs. Yoel Kloog (Tel-Aviv University, Tel-Aviv, Israel) and Wayne Bardin (Thyreos, New York, NY). Estradiol was from Steraloids (Wilton, NH). Fulvestrant was kindly provided by Dr. Alan Wakeling (AstraZeneca Pharmaceuticals, Cheshire, UK). LY 294002 and rapamycin were purchased from Sigma (St. Louis, MO). U0126 was obtained from Promega (Madison, WI). EGF and IGF-1 were obtained from Collaborative Biomedical Products (Bedford, MA). Sources of antibodies for Western analysis are as follows: phospho-MAPK monoclonal antibodies (Sigma), total MAPK (Zymed Laboratories, Inc., South San Francisco, CA), Ser^{473}-phospho-Akt, total Akt, Thr^{389}-phospho-p70 S6K, total p70 S6K, Ser^{65}-phospho-PHAS-I and total PHAS-I (Cell Signaling Technology, Beverly, MA), Thr^{229}-phospho-p70 S6K (R&D Systems, Inc., Minneapolis, MN), and cyclin D1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Secondary antibodies conjugated with horseradish peroxidase were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Cell culture medium, Improved Minimum Essential Medium, Eagle's (IMEM) was from Biosource International, Inc. (Camrillo, CA). Fetal bovine serum, Dulbecco's modified Eagle medium/F12 (DMEM/F12), glutamine, and trypsin were from Invitrogen (Carlsbad, CA). Most commonly used chemicals were obtained from Sigma.

Cell culture. Wild type MCF-7 cells (kindly provided by Dr. R. Bruggemeier, Ohio State University, Columbus, OH) were grown in IMEM containing 5% fetal bovine serum (FBS). Estrogen hypersensitive MCF-7 subline was generated from MCF-7 cells by long term culture under estrogen-deprived conditions and are called LTED cells (**L**ong **T**erm **E**stradiol **D**eprivation)[30]. These cells represent a model of breast cancer cells treated with a hormonal therapy and undergoing adaptation in response to this treatment.

LTED cells were routinely grown in phenol red free IMEM containing 5% charcoal-dextran-stripped FBS (DCC-FBS). MCF-10A cells obtained from American Type Culture Collection (Manassas, VA) were cultured in DMEM/F12 medium supplemented with insulin (10 μg/ml), hydrocortisone (500 ng/ml), cholera toxin (100 ng/ml), EGF (20 ng/ml), and 5% horse serum. Other breast cancer cell lines, SK-Br-3, MDA-MB-231, and T47D cells were kept in DMEM medium with 10% (for SK-Br-3) or 5% FBS.

Growth assay. Cells were plated in 6-well plates at a density of 60,000 cells/well in their culture media. Two days later the cells were treated as described in figure legends for five days with medium change on day three. The final concentration of vehicle (ethanol or DMSO) was 0.1%. At the end of treatment, cells were rinsed twice with saline. Nuclei were prepared by sequential addition of 1 ml HEPES-$MgCl_2$ solution (0.01 M HEPES and 1.5 mM $MgCl_2$) and 0.1 ml ZAP solution (0.13 M ethylhexadecyldimethylammonium bromide in 3% glacial acetic acid (v/v)), and counted using a Coulter counter.

LTED cells are hypersensitive to the mitogenic effect of estradiol[30]. They proliferate in response to residual estrogen that remains in the serum after charcoal stripping or leaches out of plastic culture dishes[31, 32]. To block the effect of residual estrogen, we routinely include 10^{-9} M of the pure antiestrogen, fulvestrant (formerly called ICI 182,780) in LETD cultures to assess proliferative effect of exogenous estradiol.

Thymidine incorporation assay. Cells (10^5) were plated into each well of 24-well plates. One day later, the medium was replaced with phenol red-free IMEM with 5% DCC-FBS and the cells were cultured in this medium for 24 hours. The cells were

incubated in the same medium with FTS for 24 h, and then incubated with [^{3}H]thymidine (1 μCi/well, Perkin Elmer, Boston, MA) for 2 h at 37°C. In the experiments involving estradiol stimulation, this steroid was incubated with the cells for 18 h in the presence or absence of FTS. Cells were washed twice with phosphophate-buffered saline (PBS). Proteins and DNA were precipitated with 10% trichloroacetic acid. An aliquot of 0.5 ml of 0.1 N NaOH was added to each well to solubilize DNA. The amount of [^{3}H]thymidine incorporated into cellular DNA was measured in a scintillation counter. The result was normalized by protein content, which was measured by the method of Lowry et al[33].

Apoptosis assay. The effect of FTS on apoptosis was evaluated using a Cell Death Detection ELISA^{PLUS} kit (Roche Molecular Biochemicals, Indianapolis, IN) designed to detect fragmented DNA. Eighty thousand cells were plated into each well of 12-well plates. Two days later, fresh culture medium containing FTS was applied. The cells were treated for 72 h. Preparation of cell lysate and ELISA assay was carried out following the manufacture's instruction. Parallel plates with identical treatment were prepared for cell counting. The result was expressed as optical density at 405 nm per 10,000 cells. Prior studies validated the use of this assay for apoptosis by confirming the presence of apoptosis with annexin V staining and by time lapse photography[34].

Immunoblotting. Cells grown in 60 mm dishes were washed with ice cold PBS. To each dish were added 0.5 ml lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate, 1% Triton X 100, 1 mM β-glycerophosphate, 1 μg/ml leupeptin and aprotinin, 1 mM PMSF). After a 5-min incubation on ice, the lysates were pulse-sonicated and centrifuged at 14,000 rpm for 10 min. Cell lysates were stored at -80°C until analysis. Samples (50 μg

total protein) were subjected to SDS-PAGE using 10% polyacrylamide gels before proteins were transferred to nitrocellulose membranes. The membranes were probed with primary antibodies in 5% BSA dissolved in Tris-buffered saline with 0.05% Tween 20. Secondary antibodies conjugated to horseradish peroxidase (1:2,000) were then applied. After reacting with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL), targeted protein bands were visualized by exposing the membrane to X-ray film. Bands of specific proteins were scanned and relative optical densities of bands were determined using a Molecular Dynamics scanner and ImageQuant program.

Measurement of ERK1/2 MAP kinase activity. ERK1/2 MAP kinase activity was measured using the kit from the Cell Signaling Technology following the manufacturer's instruction. Briefly, LTED cells pretreated with inhibitors were collected in lysis buffer. An aliquot of cell lysate containing 750 μg protein was immunoprecipitated with anti-phospho-ERK1/2 antibody. Kinase activity was measured by incubating immobilized ERK1/2 with Elk-1 (3 μg) at 30°C for 30 min and phospho-Elk-1 was detected by Western blot analysis.

RESULTS

FTS inhibits serum and estradiol-stimulated growth of MCF-7 and LTED breast cancer cells but not benign breast cells. The effects of FTS on serum mediated growth of MCF-7 and LTED breast cancer cells were compared in the experiments presented in Fig. 1A. FTS reduced the number of cells in a dose-dependent fashion in the two cancer cell lines but the benign MCF-10A cells were inhibited only minimally at 75 μM (Fig. 1A). We examined the effect of FTS on four additional breast cancer cell lines.

FTS dose-dependently inhibited growth of T47D, BT20, and MDA-MB-231 cells whereas SK-Br-3 cells were not inhibited at all at concentrations less than 75 μM (Fig. 1B). Taken together, these observations suggest that FTS-induced growth inhibition of breast cancer cells is not due to non-specific toxicity.

Five-day treatment with E_2 (10^{-10} M) increased cell number by 8-fold compared with the vehicle control in both MCF-7 and LTED cells and FTS reduced cell number with a half maximum effect occurring at a concentration of approximately 50 μM (Fig. 2).

Effect of FTS on DNA synthesis and apoptosis. The decrease in cell number produced by FTS could result from inhibition of cellular proliferation, induction of apoptosis, or both. To discriminate among these possibilities, we separately explored the effect of FTS on DNA synthesis as a marker of cell proliferation and on apoptosis. FTS (75 μM) significantly reduced the amount of [^{3}H]thymidine incorporated into DNA, in MCF-7 (Fig. 3A) and LTED cells (Fig. 3B) both under basal conditions and in response to estradiol.

Decreased thymidine incorporation in FTS treated cells reflected a reduction in the number of cells entering the S phase. To confirm that FTS interferes with cell cycle progression, expression of cyclin D1 in these cells was examined by Western analysis. In MCF-7 cells, FTS (48 h treatment) reduced the levels of cyclin D1 starting at 25 μM and the effect was more significant with higher concentrations. In LTED cells, reduction in cyclin D1 expression was less substantial and only seen with 75 μM FTS (Fig. 3C).

Apoptosis was assessed by measuring fragmented DNA by a specific ELISA assay. Concentrations of FTS of 50 μM or lower did not induce apoptosis in either cell line whereas a dramatic increase in apoptosis occurred at a concentration of 75 μM (Fig.

4). LTED cells were much more sensitive than MCF-7 cells to the apoptotic effect of FTS. The results indicate that induction of apoptosis contributes to the reduction in cell number of MCF-7 and LTED cells produced by higher concentrations of FTS.

Effect of FTS on serum-induced activation of MAP kinase, PI3 kinase and mTOR in MCF-7 and LTED cells. To dissect out the mechanisms whereby FTS inhibits the proliferation of breast cancer cells, we first examined its effect on the MAP kinase pathway under serum containing conditions (serum is a source of endogenous growth factors). We utilized an antibody to detect phospho-ERK 1 and 2, a commonly used indirect assay to assess MAP kinase activation. Surprisingly, FTS did not inhibit the phosphorylation of ERK1/2 MAP kinase at doses causing 50% inhibition of cell growth (i.e. 50 μM). Only higher doses of FTS (i.e. 75 μM) blocked this kinase (Fig. 5A). To obtain more direct evidence of MAP kinase effects, we then utilized a MAP kinase activity assay. ERK1/2 activity was measured in vitro by monitoring phosphorylation of Elk-1. Pre-incubation of LTED cells with FTS at concentrations of 25-75 μM for 3 h did not reduce the levels of Elk-1 phosphorylation. However, reduction in the level of phospho-Elk-1 was seen in the cells that were exposed to 100 μM of FTS for 24 h (Fig. 5B). Used as a positive control, U0126, a known MEK inhibitor, completely blocked ERK1/2 activity for the same 3 h treatment. These results suggest that inhibition of MAP kinase activation is not the predominant mechanism for FTS-induced inhibition of proliferation of breast cancer cells.

We next turned our attention to the PI3 kinase pathway. We first examined the effect of FTS on activation of PI3 kinase by monitoring changes in phosphorylation of Akt (Ser^{473}). FTS, at 50 μM, caused only 30% inhibition of Akt phosphorylation in

LTED cells. The inhibition was only slightly increased with higher concentrations of FTS. The inhibitory effects of FTS on Akt phosphorylation were smaller in MCF-7 than in LTED cells (Fig. 5C). In contrast to its effects on Akt, FTS dramatically inhibited phosphorylations of p70 S6K (Thr^{389}) and PHAS-I (Ser^{65}) in LTED cells (Fig. 5D-E). The inhibitory effects of FTS on phosphorylations of these two proteins appeared to be smaller in MCF-7 cells where basal levels of phosphorylated proteins were lower than in LTED cells. These studies demonstrating blockade of the downstream effectors of mTOR (i.e. p70 S6K and PHAS-1) with only minimal blockade of the upstream mediator, Akt, provided our first evidence that FTS blocks mTOR signaling.

Effect of FTS on EGF and IGF-1 induced activation of MAP kinase, PI3 kinase, and mTOR. Serum stimulation represents the combined effect of multiple growth factors but does not allow dissection of the effects of individual factors. For this reason, we examined the separate effects EGF and IGF-1, two growth factors known to be involved in the stimulation of breast cancer cells. Rapamycin was used to identify effects mediated by mTOR. LY 294002, was used to block both PI3 kinase and mTOR[35]. MCF-7 and LTED cells were serum-starved for twenty-four hours, then incubated for one hour with FTS and three hours with LY 294002 or rapamycin before challenge with EGF (1 μg/ml). As the peak increases in phosphorylated ERK1/2, Akt, p70S6K and PHAS-I occurred at variable times, we chose to examine the effects of EGF after 90 minutes of incubation (all levels remained elevated at this time point, see Supplementary Figure 2). As responses to EGF and inhibitors were similar in MCF-7 and LTED cells, only the data from LTED cells are presented.

With respect to the MAP kinase pathway, EGF-stimulated ERK1/2 phosphorylation was not blocked by FTS, even at a concentration of 100 μM. As expected, neither LY 294002 nor rapamycin blocked the activation of ERK 1/2 (Fig. 6), consistent with the known activities of these drugs to inhibit PI3 kinase and mTOR signaling but not MAP kinase.

We then examined the effects of FTS on the PI3 kinase pathway, first by examining Akt activation and then by evaluating the more downstream mediator, mTOR. FTS was without effect on EGF stimulated Akt phosphorylation whereas LY 294002, as expected, completely blocked this phosphorylation. Then we examined the effect of FTS on mTOR by evaluating EGF-induced phosphorylation of p70 S6 kinase and PHAS-I. Phosphorylation of p70 S6K at Thr^{389} was abolished by FTS and by the specific mTOR inhibitor, rapamycin (Fig. 6). Similarly, FTS significantly reduced the levels of Ser^{65} phosphorylated PHAS-I. The immunoblot with the antibody against total PHAS-I showed that PHAS-I in the cells treated with FTS, LY 294002, and rapamycin migrated more rapidly when subjected to SDS-PAGE. Phosphorylation of Ser^{65} and Thr^{70} in PHAS-I reduces the rate of mobility of the protein in SDS-PAGE[36], so that changes in the mobility reflect changes in phosphorylation state. Thus, the gel shifts confirmed that the levels of phosphorylated PHAS-I were reduced by these compounds. The results shown in Fig. 6 indicate that FTS, like rapamycin, blocks EGF-induced phosphorylation of p70 S6K and PHAS-I without affecting Akt.

Similar to EGF, IGF-1 activated the MAP kinase, PI3K/Akt, and mTOR pathways in both MCF-7 and LTED cells. Effects of shorter treatment with FTS on IGF-1-induced phosphorylation of signal transduction molecules were examined in LTED cells and were

compared with those of LY 294002. We first evaluated the MAP kinase pathway and then PI3K and mTOR mediated events. With respect to MAP kinase, neither FTS nor LY 294002 inhibited ERK1/2 phosphorylation stimulated by IGF-1 (Fig. 7). Paradoxically LY 294002 enhanced IGF-1 induced ERK phosphorylation at 10 min and 30 min. This could result from enhanced interaction between IRS-1 and Grb2 as reported in intestinal epithelial cells[37]. As for Akt, FTS did not inhibit IGF-1 induced phosphorylation of Akt but, as expected, LY 294002 caused profound inhibition. Thus, FTS does not appear to inhibit IGF-1 activation of PI3 kinase as reflected by activation of its downstream target, Akt. In marked contrast, FTS did effectively inhibit the Thr^{389} phosphorylation of p70 S6K and the Ser^{65} phosphorylation of PHAS-I. The inhibitory effect of FTS on these two molecules was apparent within 10 minutes and increased with time (Fig. 7). The finding that FTS inhibits phosphorylation of both p70 S6K and PHAS-I, but not the phosphorylation of Akt provides additional evidence of an inhibitory effect on mTOR activity.

Lack of effect of FTS on Thr^{229} phosphorylation of p70 S6K. To further verify that inhibition of FTS on p70 S6K and PHAS-1 is not through inhibition of PI3 kinase, we examined the effect of FTS on phosphorylation of p70 S6K at Thr^{229}, a site known to be phosphorylated by phosphoinositide-dependent kinase 1 (PDK1)[38,39]. If the inhibition of p70 S6K by FTS occurs downstream of PDK 1 and Akt, phosphorylation of p70 S6K at Thr^{229} should not be affected by this compound.

FTS had little, if any, effect on Thr^{229} phosphorylation in LTED cells treated with EGF (Fig. 8A) or IGF-1 (Fig. 8B). In contrast, LY 294002 blocked growth factor-induced Thr^{229} phosphorylation in a dose- and time-dependent manner (Fig. 8A, B). Fig. 8C

shows that the inhibitory effect of FTS (100 μM) on Thr^{389} phosphorylation in serum containing medium occurred at 2 h and maximized at 24 h. Thr^{229} phosphorylation, in contrast, was only slightly reduced. These findings provide additional evidence that the inhibitory effect of FTS on p70 S6K occurs at a point downstream of PI3 kinase/PDK1/Akt.

Role of mTOR in breast cell growth. If inhibition of mTOR accounts for the effects of FTS on cell proliferation, then rapamycin and FTS should inhibit the proliferation of breast cancer cells to a similar extent. To investigate this possibility, growth rates of two breast cancer cell lines in the presence and absence of rapamycin were examined. Rapamycin markedly reduced the numbers of both MCF-7 and LTED cells. The IC_{50} of rapamycin was approximately 0.2 nM. In a marked contrast, benign MCF-10A cells were completely resistant to rapamycin (Fig. 9A), which suggests that MCF-10A cells are less dependent on the mTOR pathway for growth. This is supported by the result of Western analysis shown in Fig. 9B that MCF-10A cells expressed very low levels of p70 S6K, and phosphorylation of this kinase was undetectable in these cells. These data indicate that the rapamycin-sensitive mTOR pathway is an important mediator of proliferation for MCF-7 and LTED breast cancer cells but not for the benign MCF-10A cells. Further these data demonstrate the specificity of FTS for cancer cells and eliminate the possibility that this compound might be non-specifically toxic.

DISCUSSION

The present study demonstrated that FTS inhibits activation of the mTOR signaling targets, PHAS-I and p70 S6 kinase and results in a reduction of cell

proliferation and enhancement of apoptosis. These conclusions are based upon the concordance of several findings obtained under a variety of experimental conditions. As evidence of mTOR blockade, FTS inhibited the phosphorylation of PHAS-I and p70 S6 kinase, but not that of Akt under most conditions examined. In addition, FTS inhibited cell proliferation to the same extent as did the specific mTOR inhibitor, rapamycin. Moreover, in vitro studies previously published by our group, provided direct molecular evidence that FTS blocks mTOR by an action different from any other mTOR inhibitor. We had shown that FTS causes the dissociation of mTOR from its partner protein RAPTOR[29] whereas the other mTOR inhibitors, rapamycin, caffeine and LY 294002 do not.

FTS has previously been shown to inhibit the in vitro and in vivo growth of cancer cells containing activating Ras mutations[25, 26, 28, 40, 41]. Inhibition of ERK1/2 MAP kinase activity was thought the primary mechanism of the anti-tumor effect of FTS because the MAP kinase pathway is one of the major downstream effectors of Ras. We expected that the same mechanisms might be applicable to explain the inhibitory effect of FTS in breast cancer cells. However, growth inhibition by FTS correlated much better with blockade of phosphorylation of p70 S6K and PHAS-I, two downstream effectors of mTOR than with the inhibition of phosphorylation of ERK1/2 or Akt (Fig. 5). At 50 μM, the concentration that FTS reduced cell number by 50%, the phosphorylations of p70 S6K and PHAS-I was significantly inhibited whereas the phosphorylations of ERK1/2 and Akt were unaffected.

Differential sensitivity of MAPK and the mTOR targets to FTS appeared more striking in the experiments where EGF or IGF-1 were used to activate growth factor

pathways than in experiments in which serum was used. Both EGF and IGF-1 are known to activate the MAP kinase and PI3 kinase pathways, but via different mechanisms. In our studies, FTS uniformly blocked the phosphorylations of p70 S6K and PHAS-I induced by theses two growth factors but did not inhibit the phosphorylations of ERK1/2 and Akt.

The differential effects of FTS on MAP kinase and mTOR were also reflected by the time course of action. FTS-induced inhibition of p70 S6K and PHAS-I phosphorylation occurred very rapidly (10-60 minutes) whereas inhibition of MAP kinase activation was only seen when cells were incubated with higher concentration of FTS for hours (24 hours as shown in Fig. 5). This delay in activity is consistent with the time course of dislodgement of Ras proteins from the plasma membrane and the resulting MAP kinase inhibition in the cells with activated Ras[24, 25, 41]. Kloog et al have shown that FTS first displaces GTP-Ras from its anchor in the plasma membrane and then Ras returns to the cytoplasm where it is degraded over several hours. Clearly, our results suggest that FTS blocks MAP kinase and mTOR via different mechanisms.

Comparison of the effects of rapamycin with those of FTS provided evidence of the site of action of FTS. Theoretically, inhibition of mTOR signaling by FTS could result from direct suppression of mTOR activity or from indirect inhibition of the elements upstream of mTOR such as PI3 kinase. Rapamycin is known to target mTOR specifically without exerting upstream effects. Consequently, demonstration that FTS acts in a fashion similar to rapamycin provides evidence of the site of action of FTS at the level of mTOR. Rapamycin and FTS both inhibited p70 S6K and PHAS-I

phosphorylation with little effect on Akt. These differential effects of FTS are most consistent with an inhibitory action on mTOR.

Additional evidence of direct mTOR effects resulted from studies of p70 S6 phophorylation sites. We compared the effect of FTS and LY 294002 on two different phosphorylation sites of p70 S6K. Phosphorylations of Thr^{389} in the regulatory domain and of Thr^{229} in the activation loop are crucial for p70 S6K to be active. mTOR phosphorylates Thr^{389} directly in vitro[42, 43], and phosphorylation of this site is exquisitely sensitive to inhibition by rapamycin and LY 294002. Phosphorylation of Thr^{229} is catalyzed by PDK1, a downstream kinase in the PI3 kinase pathway[39]. LY 294002 showed strong inhibition of phosphorylation of both sites. In contrast, FTS strongly inhibited phosphorylation of Thr^{389} but not Thr^{229}. These results provide additional evidence that FTS acts downstream of PI3K/PDK1/Akt to block mTOR signal transduction.

Inducing apoptosis is another mechanism whereby FTS inhibits growth of MCF-7 and LTED cells. Although the precise molecular mechanism of FTS induced apoptosis in breast cancer cells remains unclear, the linkage between apoptosis and translational control has been described in other cell types. During apoptosis, protein synthesis is inhibited and several translational factors are cleaved in a caspase-dependent manner[44, 45]. Decreasing protein synthesis with rapamycin increases the susceptibility of Ras-transformed fibroblasts to cytostatic drug-induced apoptosis[46]. Activation of the tumor suppressor protein p53 in murine erythroleukemia cells causes a rapid decrease in the overall protein synthesis, which is associated with dephosphorylation of PHAS-I and inhibition of p70 S6 kinase[47]. Our data also showed a correlation between apoptosis and

reduction in the levels of phosphorylated PHAS-I and p70 S6K induced by FTS in both MCF-7 and LTED cells. In LTED cells (Fig. 5), the level of phosphorylated PHAS-I was much higher than in MCF-7 cells indicating that more eIF4E molecules were available for cap-dependent translation in these cells. This may partially explain why LTED cells are more vulnerable to the apoptotic effect of FTS. A recent study demonstrated that mTOR blockade induced sustained activation of the JNK cascade through stimulation of its upstream apoptosis signal-regulating kinase-1 (ASK1), which led to apoptosis[48]. This response depended on expression of PHAS-I. Inducing p53 expression might be another mechanism of the apoptotic effect of FTS[27].

In summary, our studies demonstrate that the mTOR pathway plays an essential role in mediation of proliferation and survival of hormone-dependent breast cancer cells. Blockade of the mTOR pathway results in significant inhibition of malignant cells but not benign breast cells. Results from the current studies and from our published paper[29] uncover a new mechanism whereby FTS inhibits growth of breast cancer cells. Because of its dual blockade of mTOR and Ras, we believe that FTS has the potential to become a useful drug to treat hormone-dependent breast cancer and prolong the beneficial duration of hormonal therapy in breast cancer patients.

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FIGURE LEGENDS

Fig. 1. (A) Effect of FTS on growth of MCF-7, LTED, and MCF-10A cells. (B) Effect of FTS on growth of SK-Br-3, MDA-MB-231, BT20, and T47D breast cancer cells. Sixty thousand cells were plated into each well of 6-well plates. Two days later, the cells were treated in triplicate for five days with FTS at indicated concentrations. The results (mean ± S.E.) are expressed as percentage of the vehicle control.

Fig. 2. Effect of FTS on E_2 stimulated growth of MCF-7 and LTED cells. Thirty thousand cells were plated into each well of 6-well plates. Two days later, the cells were re-fed with IMEM containing 5% DCC-FBS. After another two days, cells were treated in triplicate with estradiol (10^{-10} M) plus various concentrations of FTS for five days. In LTED cells, fulvestrant (10^{-9} M) was included in all wells to block the effect of residual estrogen in the culture. The results (mean ± S.E.) are expressed as percentage of cell number from the wells containing only E_2.

Fig. 3. Effect of FTS on DNA synthesis in MCF-7 (A) and LTED cells (B). One hundred thousand cells were plated into each well of 24-well plates. Two days later, the cells were re-fed with IMEM containing 5% DCC-FBS. After another two days, cells were treated in quadruplicate with the compounds indicated for 18 hours. [^{3}H] thymidine (1 μCi/well) was added during the last 2 h of incubation. [^{3}H] thymidine incorporated into DNA was normalized by protein content. (C) Effect of FTS on cyclin D1 expression by Western analysis.

Fig. 4. Induction of apoptosis in MCF-7 and LTED cells by FTS. Eighty thousand MCF-7 and LTED cells were plated into each well of 12-well plates in their culture media. Two days later, the cells were treated with FTS for 3 days. Apoptosis was measured using Cell Death Detection Kit from Roche Molecular Biochemicals. Parallel plates subjected to identical treatment were prepared for cell counting. The results were expressed as the values of optical density at 405 nm per 10,000 cells.

Fig. 5. Effect of FTS on serum-stimulated activation of MAP kinase, PI3 kinase, and mTOR. Sub-confluent MCF-7 and LTED cells grown in 60 mm dishes were treated with FTS in their culture media for 24 h. Cells were then harvested and cell lysate prepared. Phosphorylation of kinases or effector was detected by Western analysis using specific antibodies and quantitated by densitometry scanning. ERK1/2 MAP kinase activity was measured as described in Materials and Methods. (A) Phosphorylation of ERK1/2 MAP

kinase; (B) Activity of ERK1/2 MAP kinase; (C) Phosphorylation of Akt at Ser^{473}; (D) Phoshporylation of p70 S6 kinase at Thr^{389}; and (E) Phosphorylation of PHAS-I at Ser^{65}.

Fig. 6. Effect of FTS on EGF induced activation of MAP kinase, PI3 kinase, and mTOR in LTED cells. Sub-confluent LTED cells grown in 60 mm dishes were serum starved for 24 h, pretreated for 1 h with FTS, 3 h with LY 294002 (LY), or rapamycin (Rapa) at indicated concentrations before addition of EGF (1 μg/ml, 1 h). Cells were then harvested and cell lysate prepared. Phosphorylated and total kinases were detected by Western analysis using specific antibodies.

Fig. 7. Effect of FTS on IGF-1 induced activation of MAP kinase, PI3 kinase, and mTOR in LTED cells. Sub-confluent LTED cells grown in 60 mm dishes were serum starved for 24 h, pretreated with FTS (100 μM) or LY 294002 (LY, 20 μM) for 10, 30 or 60 min before addition of IGF-1 (20 ng/ml for 10 min). Cells were then harvested and cell lysate prepared. Phosphorylated and total kinases were detected by Western analysis using specific antibodies.

Fig. 8. Effect of FTS on serum- and growth factor-induced phosphorylation of p70 S6 kinase at Thr^{229} in LTED cells. (A) Comparison of FTS and LY 294002 (LY) on Thr^{229} phosphorylation of p70 S6K induced by EGF (same treatment as described in Fig. 6); (B) Comparison of FTS and LY 294002 on Thr^{229} phosphorylation of p70 S6K induced by IGF-1 (same treatment as described in Fig. 7); (C) Time course of FTS (100 μM) on

Thr^{389} and Thr^{229} phosphorylation of p70 S6K in LTED cells cultured in serum containing IMEM.

Fig. 9. Effect of rapamycin on growth of MCF-7, LTED, and MCF-10A cells. (A) Sixty thousand cells were plated into each well of 6-well plates in their culture media. Two days later, the cells were treated in triplicate wells for five days with rapamycin at indicated concentrations. The results (mean ± S.E.) are expressed as percentage of the vehicle control. (B) LTED and MCF-10A cells grown in 60 mm dishes were treated with rapamycin at 2 nM for LTED cells and 20 nM for MCF-10A cells fro 24 hours. Cells were then harvested and cell lysate prepared. Levels of phosphorylated and total p70 S6 kinase were detected by Western analysis using specific antibodies.

Fig. 1

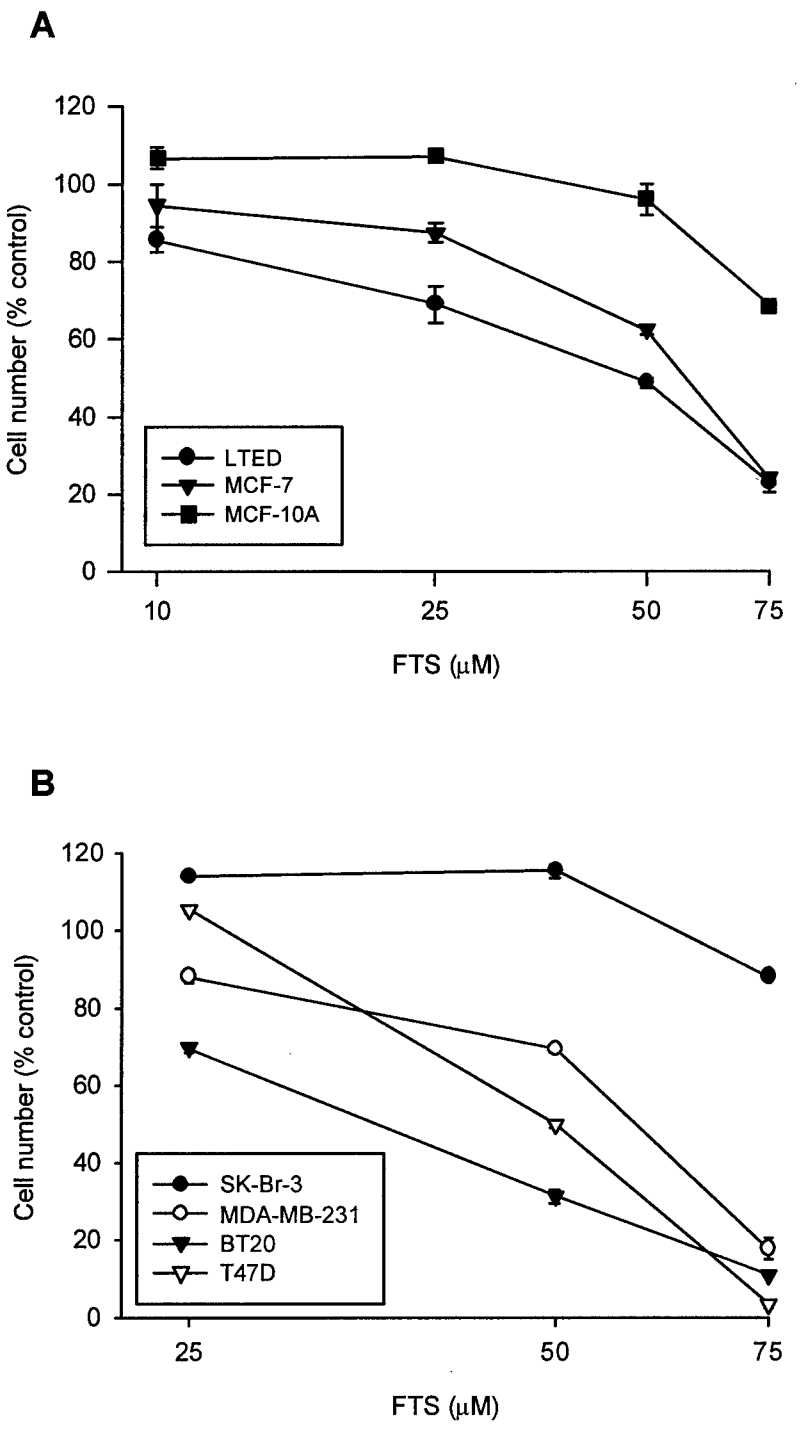

A
120
100
80
60
40
20
0
Cell number (% control)
LTED
MCF-7
MCF-10A
10
25
50
75
FTS (μM)
B
120
100
80
60
40
20
0
Cell number (% control)
SK-Br-3
MDA-MB-231
BT20
T47D
25
50
75
FTS (μM)

Fig. 2

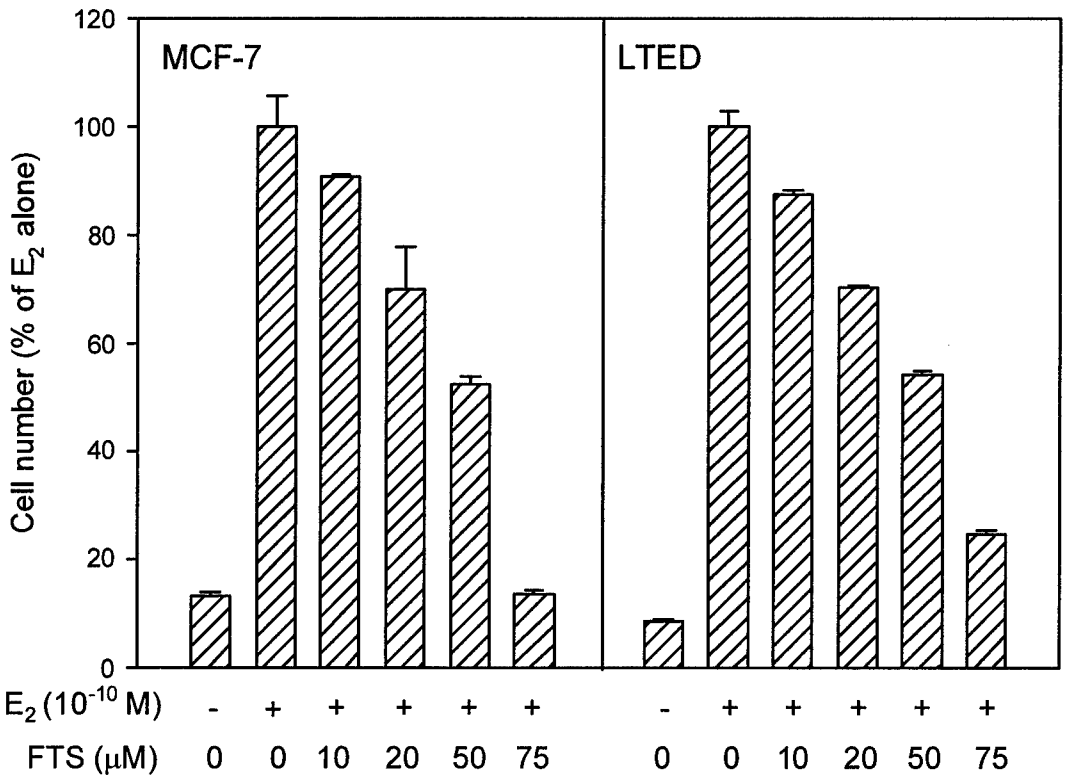

MCF-7
LTED
Cell number (% of E_2 alone)
120
100
80
60
40
20
0
E_2 (10^{-10} M) - + + + + + - + + + + +
FTS (μM) 0 0 10 20 50 75 0 0 10 20 50 75

Fig. 3

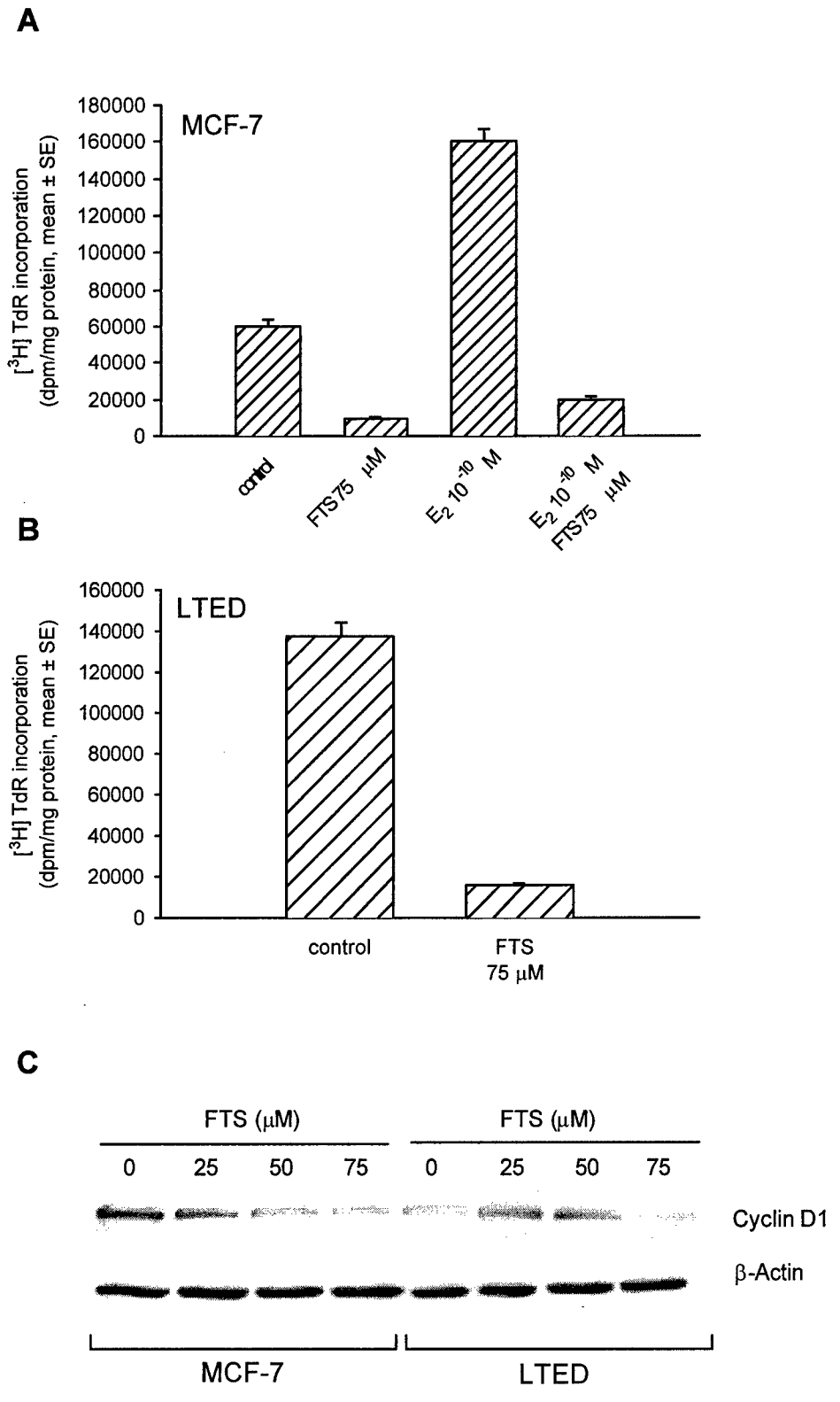
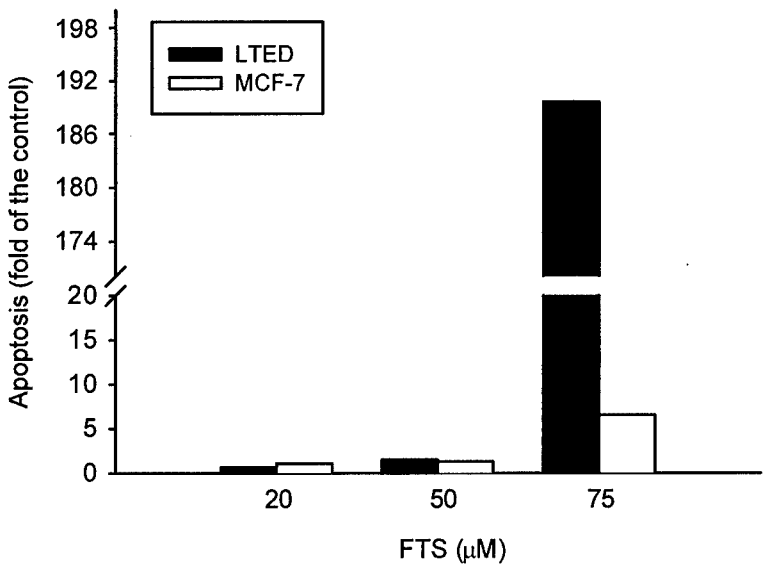

A
MCF-7
[3H] TdR incorporation (dpm/mg protein, mean ± SE)
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
control
FTS75 μM
E2 10-10 M
E2 10-10 M FTS75 μM
B
LTED
[3H] TdR incorporation (dpm/mg protein, mean ± SE)
160000
140000
120000
100000
80000
60000
40000
20000
0
control
FTS 75 μM
C
FTS (μM)
FTS (μM)
0 25 50 75 0 25 50 75
Cyclin D1
β-Actin
MCF-7
LTED

Fig. 4


LTED
MCF-7
Apoptosis (fold of the control)
198
192
186
180
174
20
15
10
5
0
20
50
75
FTS (μM)

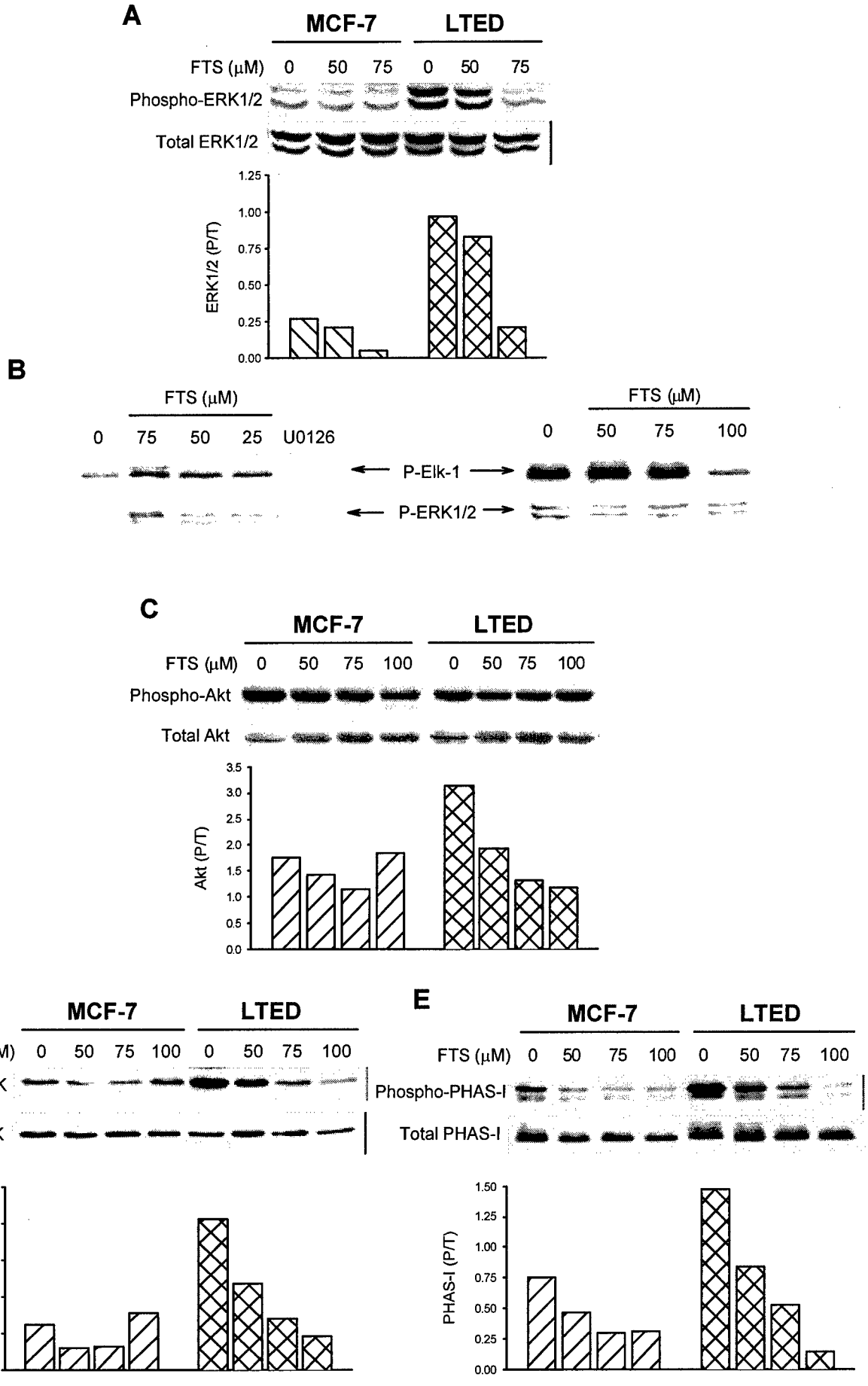

A
MCF-7
LTED
FTS (μM) 0 50 75 0 50 75
Phospho-ERK1/2
Total ERK1/2
ERK1/2 (P/T)
1.25
1.00
0.75
0.50
0.25
0.00
B
FTS (μM)
0 75 50 25 U0126
FTS (μM)
0 50 75 100
P-Elk-1
P-ERK1/2
C
MCF-7
LTED
FTS (μM) 0 50 75 100 0 50 75 100
Phospho-Akt
Total Akt
Akt (P/T)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
D
MCF-7
LTED
FTS (μM) 0 50 75 100 0 50 75 100
Phospho-p70S6K
Total p70S6K
p70S6K (P/T)
2.5
2.0
1.5
1.0
0.5
0.0
E
MCF-7
LTED
FTS (μM) 0 50 75 100 0 50 75 100
Phospho-PHAS-I
Total PHAS-I
PHAS-I (P/T)
1.50
1.25
1.00
0.75
0.50
0.25
0.00

Fig. 6

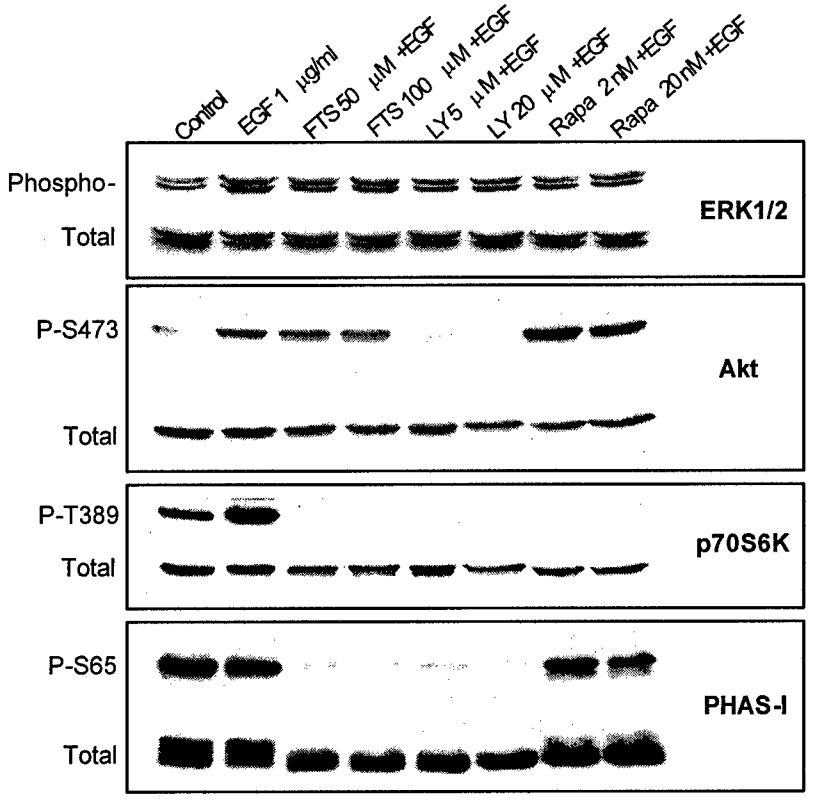

Control
EGF 1 μg/ml
FTS 50 μM +EGF
FTS 100 μM +EGF
LY 5 μM +EGF
LY 20 μM +EGF
Rapa 2nM+EGF
Rapa 20nM+EGF
Phospho-
Total
ERK1/2
P-S473
Total
Akt
P-T389
Total
p70S6K
P-S65
Total
PHAS-I

Fig. 7

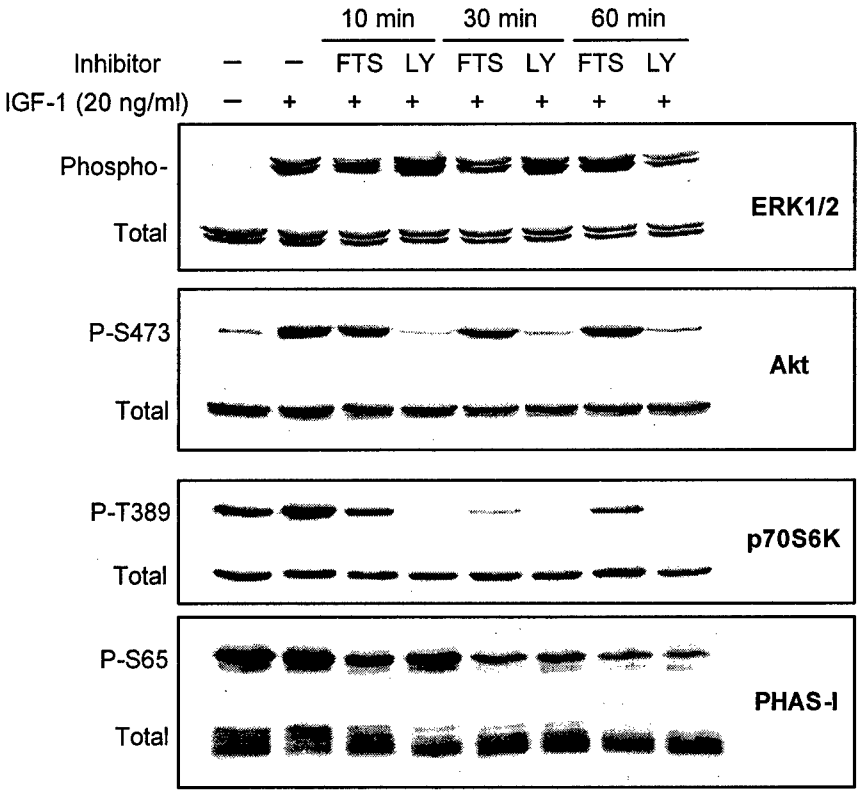

10 min
30 min
60 min
Inhibitor
–
–
FTS
LY
FTS
LY
FTS
LY
IGF-1 (20 ng/ml)
–
+
+
+
+
+
+
+
Phospho-
Total
ERK1/2
P-S473
Total
Akt
P-T389
Total
p70S6K
P-S65
Total
PHAS-I

Fig. 8

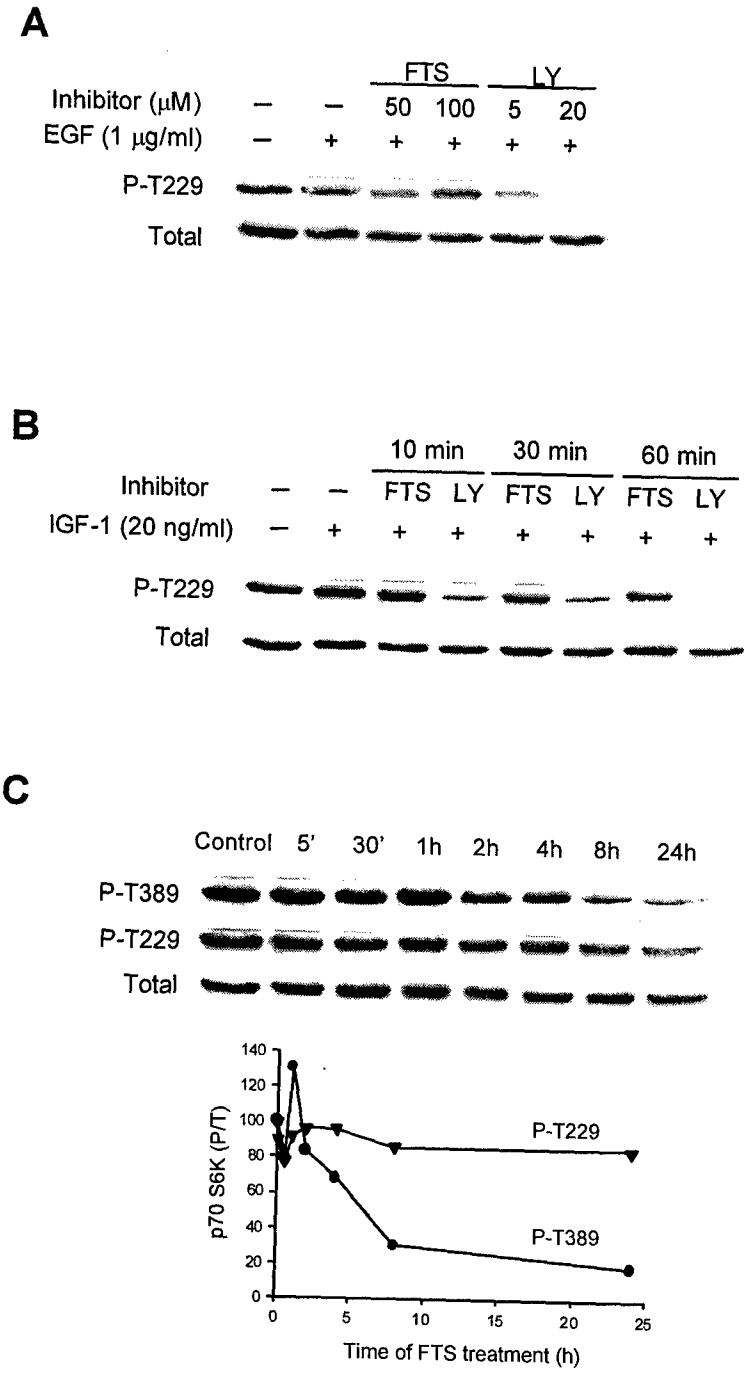
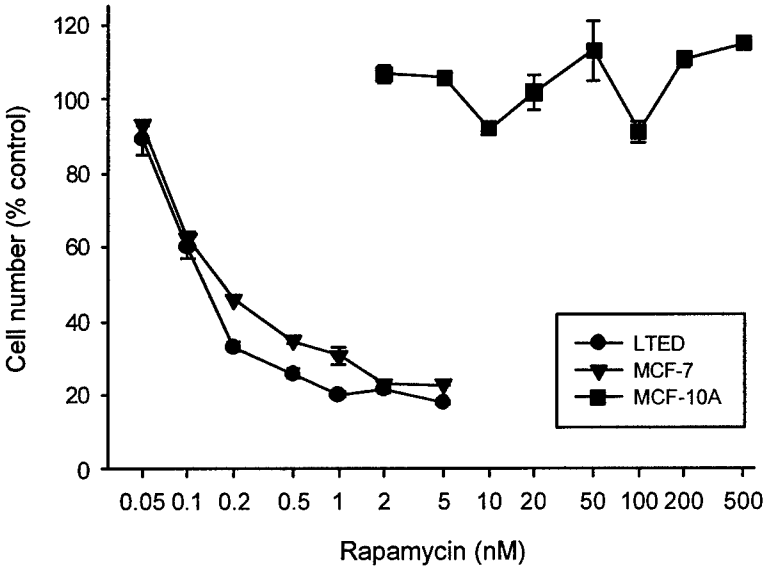
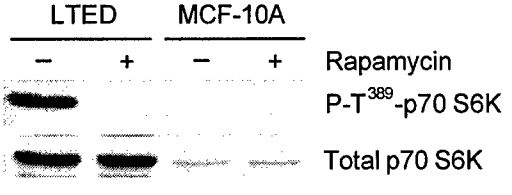

A
FTS
LY
Inhibitor (μM)
– – 50 100 5 20
EGF (1 μg/ml)
– + + + + +
P-T229
Total
B
10 min
30 min
60 min
Inhibitor
– – FTS LY FTS LY FTS LY
IGF-1 (20 ng/ml)
– + + + + + + +
P-T229
Total
C
Control 5' 30' 1h 2h 4h 8h 24h
P-T389
P-T229
Total
p70 S6K (P/T)
140
120
100
80
60
40
20
0
0 5 10 15 20 25
Time of FTS treatment (h)
P-T229
P-T389

Fig. 9

A


Cell number (% control)
120
100
80
60
40
20
0
0.05 0.1 0.2 0.5 1 2 5 10 20 50 100 200 500
Rapamycin (nM)
LTED
MCF-7
MCF-10A

B


LTED
MCF-10A
–
+
–
+
Rapamycin
P-T389-p70 S6K
Total p70 S6K