Farnesylthiosalicylic acid: inhibition of proliferation and enhancement of apoptosis of hormone-dependent breast cancer cells

Richard J Santen[a], Amanda R Lynch[a], Lindsey R Neal[a], Robert A McPherson[a,*] and Wei Yue[a]

Farnesyltransferase inhibitors (FTIs) are being developed to block Ras-mediated actions, but current data suggest that the FTIs act through other non-Ras pathways. A new agent, farnesylthiosalicylic acid (FTS), blocks the binding of Ras to membrane acceptor sites and causes a marked reduction in Ras levels. Accordingly, FTS could be a useful new agent for the treatment of hormone-dependent breast cancer. We examined the dose–response effects of FTS on the growth of MCF-7 breast cancer cells *in vitro* and *in vivo*. Further, we dissected out its specific effects on cell proliferation and apoptosis by measuring BrdU incorporation into DNA and by using an ELISA assay to quantitate the magnitude of apoptosis. FTS and its solubilized conjoiner FTS–cyclodextrin markedly inhibited cell growth in MCF-7 breast cancer cells in culture and in xenografts. This agent exerted dual effects to reduce cell proliferation as assessed by BrdU incorporation and to enhance apoptosis as quantitated by ELISA assay. These data suggest that FTS is a promising agent to be developed for treatment of hormone-dependent breast cancer. *Anti-Cancer Drugs* 17:33–40 © 2006 Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2006, **17**:33–40

Keywords: apoptosis, breast cancer, cyclodextrin, farnesylthiosalicylic acid, Ras

[a]Division of Endocrinology, University of Virginia Health Sciences Center, Charlottesville, Virginia, USA.

Correspondence to R.J. Santen, University of Virginia Health Sciences Center, PO Box 801416, Charlottesville, VA 22908, USA.
Tel: +1 434 924 2961; fax: +1 434 924 1284;
e-mail: rjs5y@virginia.edu

Sponsorship: This work was supported by Department of Defence Breast Cancer Clinical Bridge grant DAMD17-02-1-0609 and the University of Virginia NCI Cancer Center support grant P30-44579.

Received 1 September 2005 **Accepted** 17 September 2005

Introduction

One-third of human breast cancers depend upon estrogen for growth and regress upon exposure to anti-estrogens or inhibitors of estrogen biosynthesis (e.g. aromatase inhibitors) [1]. In advanced breast cancer, initial responses to the anti-estrogen 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 mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K) signaling cascades [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 in breast cancer 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 MAPK and PI3K [12,13]. Because of the key role of Ras, this signaling molecule has been a prime target for drug development [14,15]. 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 [16]. Current speculation is that the FTIs may block Rho B as their primary mechanism of action [16].

Another anti-Ras strategy is to block the binding of Ras to its membrane acceptor sites [17–27]. 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 MAPK pathway. Kloog *et al.* have developed a compound capable of dissociating GTP-Ras from its membrane binding sites [17–27]. 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 membrane and rapidly

*Current address: School of Animal Studies, University of Queensland, Queensland, Australia

0959-4973

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 MAPK pathway plays a major role in mediating the proliferative effects of estradiol [28]. In addition, the MAPK and PI3K pathways are frequently upregulated in response to estrogen deprivation therapy, and may play a role in the development of hormonal resistance [29–32]. 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 [13].

Several investigators have demonstrated that FTS blocks the activation of MAPK and causes inhibition of the growth of tumors containing activating mutations of Ras (i.e. pancreatic cancer and malignant melanomas) [17–27]. 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 upregulation of the MAPK 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* and in xenografts *in vivo*. Since this agent may need to be complexed to solubilizing agents to be orally absorbed, we have examined both free FTS and FTS complexed with cyclodextrin (CD) to make it more water soluble. Herein, we report that the farnesyl analog, FTS, blocks the growth of breast cancer cells in tissue culture and *in vivo*. As an additional, but unexpected, therapeutic advantage, FTS 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.

Materials and methods

FTS and CD were donated by Dr C. Wayne Bardin (Concordia Pharmaceuticals, Bridgewater, New Jersey). The FTS–CD complex was prepared according to instructions from Thyreos. The 'pure' anti-estrogen, ICI 182,780, was donated by Astra-Zeneca (Cheshire, UK). Cell Death Detection ELISA and Cell Proliferation ELISA kits were from Roche (Indianapolis, Indiana), Neutral Red was from Aldrich, improved modified Eagles medium (IMEM) was from Biosource (Camarillo, California, USA), and FBS was from Gibco (Grand Island, New York, USA). Dextran T70 was from Pharmacia (Piscataway, New Jersey) and Charcoal (NoritA) from Sigma (St Louis, Missouri, USA).

Cell culture

All culture methods have been previously described in detail [29–32]. Briefly, we utilized estrogen receptor-positive MCF-7 breast cancer cells which are called 'wild-type'. When deprived of estradiol over several months, MCF-7 cells develop hypersensitivity to the proliferative and pro-apoptotic effects of estradiol, and display upregulated MAPK activity [29–32]. These cells, described previously in several publications, are termed LTED as an acronym for long-term estradiol deprived [29–32].

Cell number was assayed by the Neutral Red method [33]. We have correlated this method with direct cell counts using a Coulter counter and shown excellent agreement (unpublished data). For all experiments, cells were initially seeded, and allowed to attach and grow for 5 days before estradiol or vehicle was added. Specifically, wild-type MCF-7 cells were initially seeded into 96-well plates in IMEM containing 5% FCS. After a sufficient period of time for the cells to become attached (i.e. 24 h), the medium was changed to that containing 5% charcoal stripped serum (dextran-coated charcoal) in IMEM. The cells were accommodated to this medium for a 5-day period. At this point, the estradiol stimulation component of the studies was initiated. Estradiol and FTS–CD or its vehicle were added in fresh IMEM/5% DCC with a media change after 3 days. Cell number was measured 2 days later (i.e. after 5 days total in estradiol-containing media).

The protocol for LTED cells differed slightly. LTED cells were seeded into 96-well plates and allowed to attach. Twenty-four hours later, the media were changed to IMEM with glutamine added and the cells allowed to grow for 7 days without addition of estradiol. At this time point, the estradiol stimulation protocol was initiated by adding estradiol and FTS–CD or its vehicle at the doses indicated in the figures. In addition, we routinely added 10^{-9} mol/l ICI 182,780 in fresh IMEM to counteract residual estradiol leached from the plastic in the culture dishes. As with the wild-type MCF-7 cells, the media were changed and cells counted 2 days later (i.e. after 5 days total in estradiol-containing media).

It should be noted that our unpublished data indicated that pre-treatment of culture dishes with ethanol removes traces of estrogenic substances which can stimulate the hypersensitive LTED cells. As a practical method to circumvent this problem, small amounts of the pure anti-estrogen ICI 182,780 are added at the time of estradiol. This method has been in use in our laboratory for several years [29–32].

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 were measured 2 days later.

Xenografts of long-term estradiol-deprived cells involved castration of nude mice, insertion of silastic implants to clamp plasma estradiol levels at 5 pg/ml and implantation of cells at two sites on the animal. This technique has been described in detail previously [34]. FTS–CD, CD or PBS were administrated daily by i.p. injection for 8 weeks.

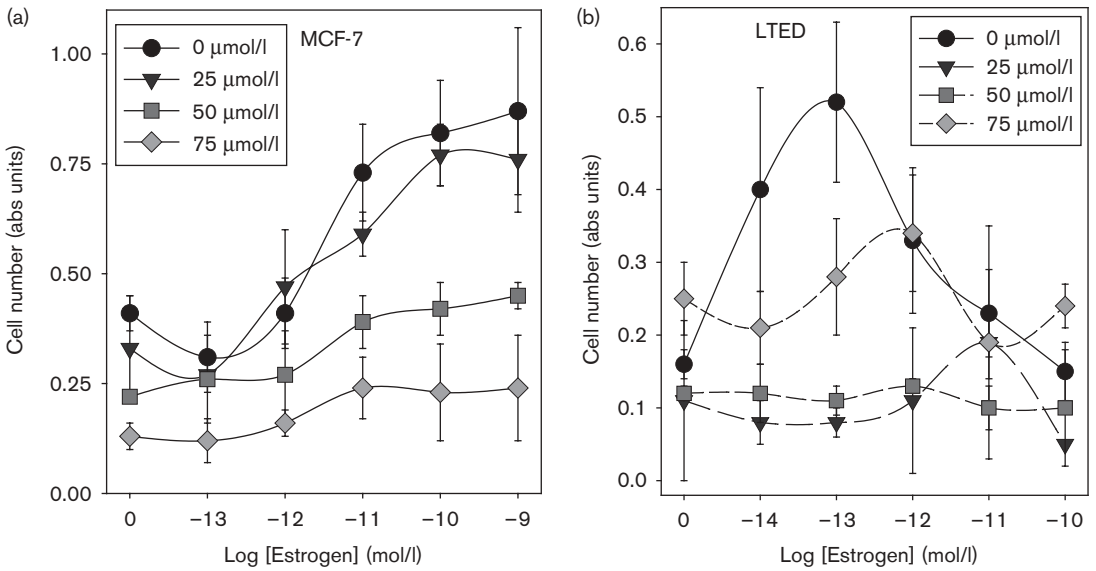
Results

Wild-type MCF-7 cells responded to estradiol with maximal stimulation appearing to plateau at a dose of approximately 10^{-11} mol/l which increased cell number nearly 3-fold (Fig. 1a). Increasing concentrations of FTS suppressed estradiol-dependent growth in a dose-responsive fashion with initial inhibition at 50 µmol/l and maximal effects at 75 µmol/l FTS. As a reflection of hypersensitivity to estradiol (as previously extensively described [29–32]), the LTED cells (Fig. 1b) responded maximally to 2 log lower concentrations of estradiol than did wild-type cells (i.e. 10^{-13} versus 10^{-11} mol/l, respectively). In contrast to its effects in wild-type cells, FTS suppressed LTED growth completely at a dose of 25 µmol/l with continued suppression at 50 µmol/l. Unexpectedly, we observed lesser inhibition of growth at 75 µmol/l FTS in LTED cells.

FTS is a relatively hydrophobic lipid analog and may not be absorbed by patients in an oral formulation. We reasoned that 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]. 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 (Fig. 2a). CD alone or buffer vehicle had no significant effect on cell growth of MCF-7 and LTED cells (Fig. 2b and c) under conditions where FTS–CD significantly reduced cell growth. Even though LTED cells have higher MAPK activity than MCF-7 cells [30], the degree of inhibition was approximately the same between the two cell lines (Fig. 2b and c). We have also observed similar results in tamoxifen-resistant breast cancer cell lines (data not shown).

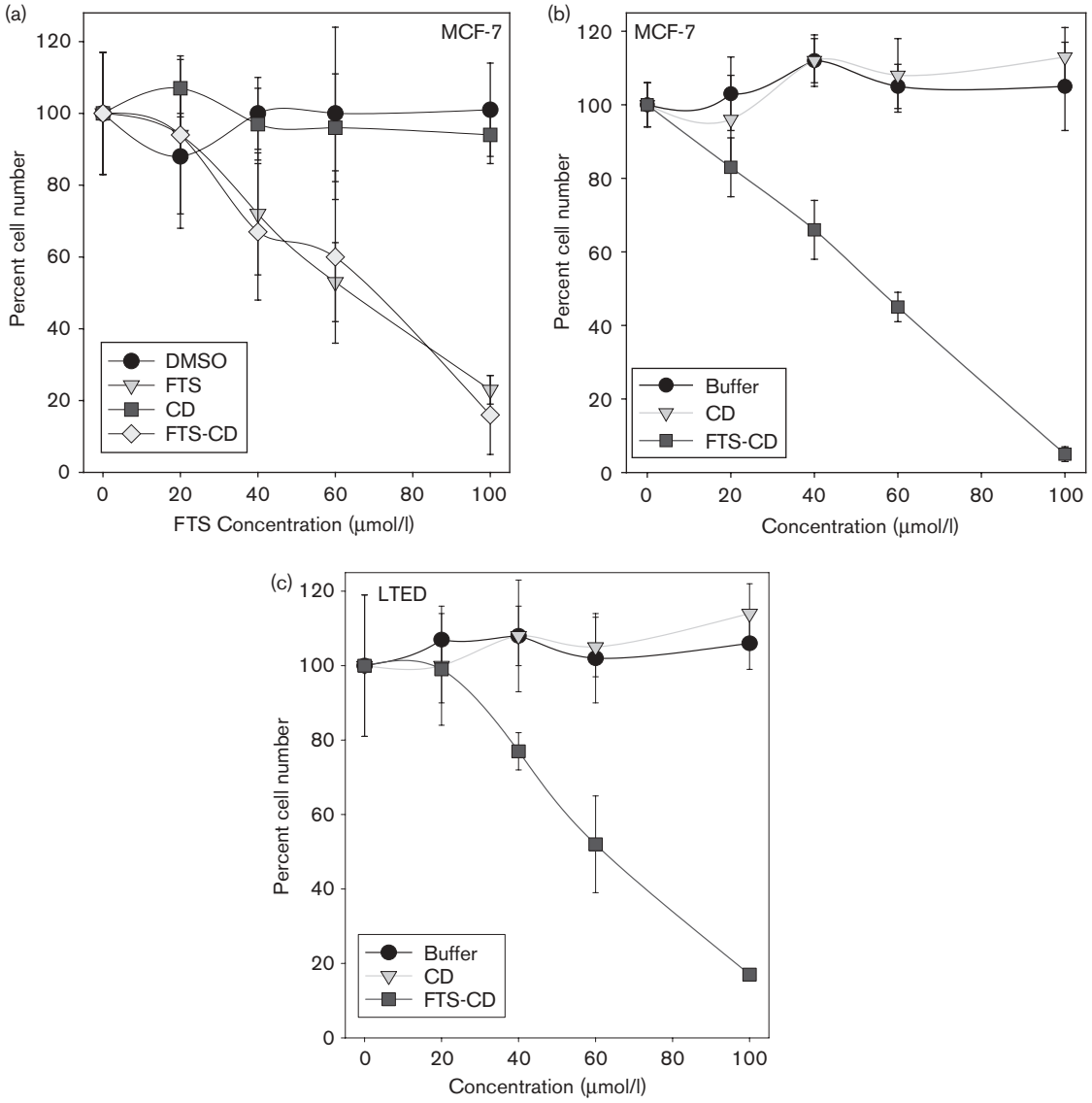
Ras-mediated growth factor pathways are required to maintain cellular proliferation and are important regulators of the apoptotic response [36,37]. Reduction of cell number can reflect either an inhibition of proliferation, an enhancement of apoptosis or a combination of these two effects. Accordingly, we systematically examined the effects of FTS specifically on apoptosis and then on

Fig. 1



FTS suppresses estradiol-dependent breast cancer cell growth. MCF-7 (a) and LTED (b) cells were grown under conditions where they demonstrate an estradiol response as described in Materials and methods. Increasing concentrations of FTS suppressed this estradiol dependence. Representative of two experiments, means and SDs of four samples are shown. The term (abs units) refers to the absorption units of optical density of the Neutral Red dye used to assess cell number.

Fig. 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 described in Materials and methods. Representative of two experiments, means and SDs of four 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 Materials and methods. Responses to either PBS, CD in PBS or FTS–CD in PBS added. Representative of six experiments, means and SDs of four samples are shown.

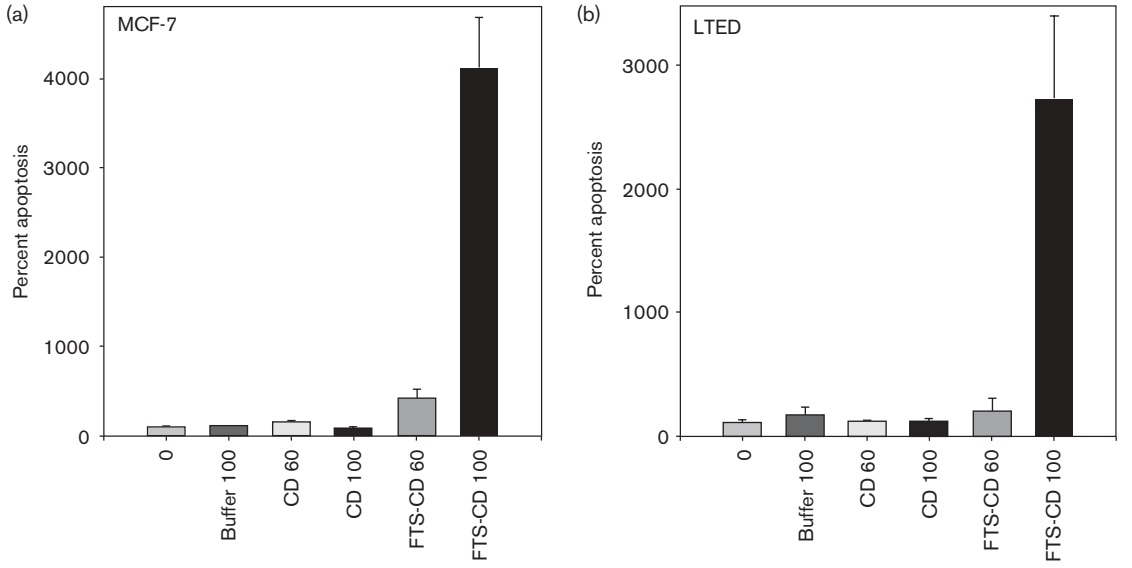
BrdU incorporation as a marker for proliferation. FTS enhanced apoptosis in wild-type and LTED cells starting at a dose of 60 μmol/l and maximally stimulating at a dose of 100 μmol/l FTS–CD to levels 3000- to 4000-fold higher (Fig. 3a and b). Proliferation was also reduced to very low amounts in both cell lines by FTS–CD, but not by CD or buffer alone (Fig. 4a and b). The disruption of both proliferation and apoptosis indicates a global disruption of signaling pathways in breast cancer cells.

In the xenograft model, we compared the administration of vehicle alone, CD alone and CD-complexed with FTS. CD-FTS caused a statistically significant reduction of tumor weight when measured at the end of the 2-month experiment (Fig. 5).

Discussion

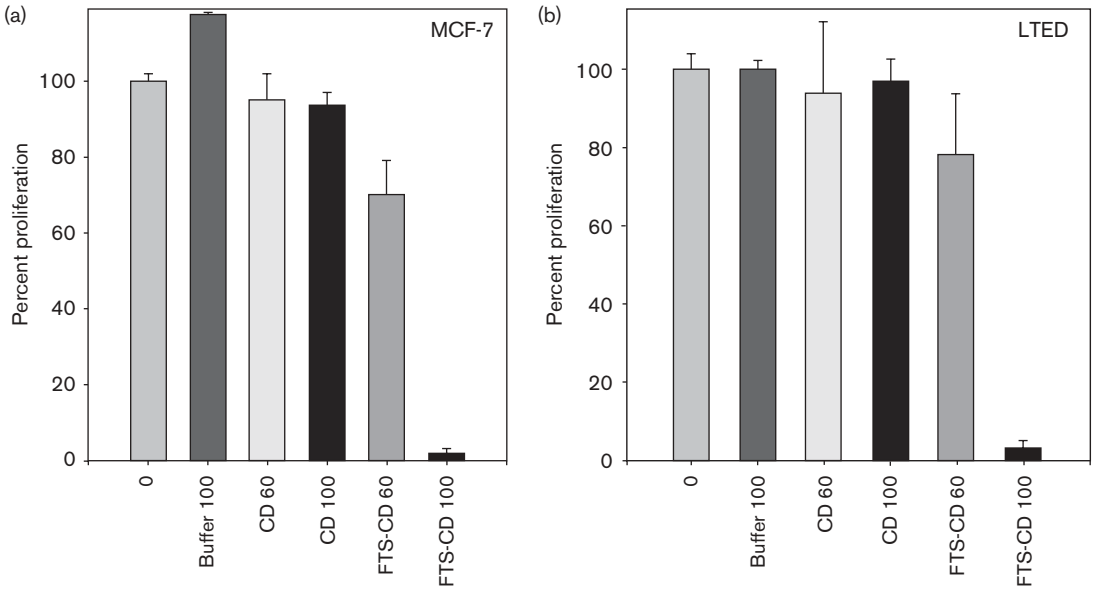
A variety of recent studies have examined the effects of estrogen deprivation therapy on upregulation of growth factor pathways in breast cancer cells [29–32,38–40]. Our group demonstrated a marked upregulation of activated MAPK and of the downstream PI3K effectors, AKT, p70S6 kinase and 4E-BP-1 in MCF-7 cells deprived of estradiol long term [29–32,41,42]. Dowsett *et al.*

Fig. 3



FTS–CD increases apoptosis. MCF-7 (a) and LTED (b) cells were incubated with buffer (equivalent to 100 μmol/l FTS–CD), CD (equivalent to 60 or 100 μmol/l FTS–CD) or 60 or 100 μmol/l FTS–CD for 5 days as described in Materials and methods. Apoptosis was measured by DNA nick site quantitative ELISA. Representative of two experiments, means and SDs of two samples are shown.

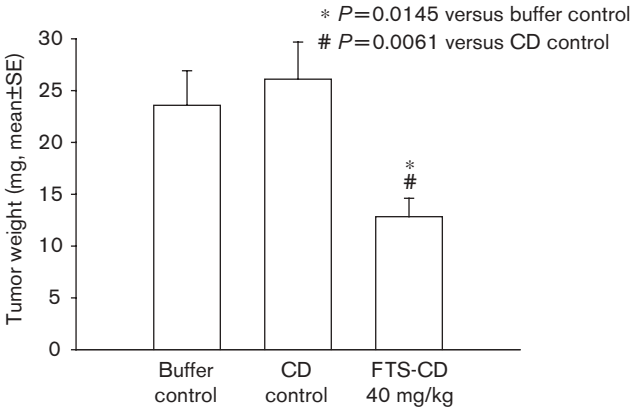
Fig. 4



FTS–CD reduces proliferation. MCF-7 (a) and LTED (b) cells were incubated with buffer, CD or FTS–CD for 5 days as described in Fig. 3. Proliferation was measured by BrdU incorporation. Representative of two experiments, means and SDs of two samples are shown.

confirmed these findings regarding MAPK and also demonstrated an increase in HER-2 activation [40]. Other investigators also found a marked up-regulation of the MAPK pathway in cells treated with tamoxifen long term [9]. These investigators also demonstrated that blockade of MAPK and of epidermal growth factor 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

Fig. 5



In-vivo effects of FTS–CD on cell growth. LTED cells were implanted into castrated nude mice to form xenografts. Silastic 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 ($n=9$) CD alone and the third ($n=7$) FTS–CD complex (40 mg/kg). Student's *t*-test was used to compare the effects of FTS–CD to buffer or CD alone.

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 or through permissive effects that require the growth factor pathways to be active. It would appear then that FTS blocks estradiol-stimulated growth by interrupting these pathways. Although not presented in this paper, our data have shown that FTS blocks MAPK 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 [42,43].

Agents such as testosterone are not well absorbed orally and can be rendered orally effective by complexing to CD. Other drugs have also been complexed to CD to enhance solubility and absorption [44,45]. Anticipating the future use of FTS in patients, we examined whether complexing with CD would alter the in-vitro efficacy of FTS. We demonstrated that the FTS–CD complex clearly prevents growth of cellular models of estradiol-dependent (MCF-7) and estradiol-hypersensitive (LTED) breast cancers at the same doses as with free FTS. FTS–CD also induces apoptosis up to several thousand-fold and reduces proliferation to very low levels.

The observation that FTS–CD suppresses estradiol-dependent growth, but does not shift the dose–response curve to the right as evidence of reduced sensitivity to estradiol, may be significant (Fig. 1a and b). Our prior data indicated that MAPK upregulation in long-term deprived MCF-7 cells causes increased sensitivity to estradiol [29–32]. We have previously demonstrated that inhibition of MAPK with the MEK inhibitor, PD 98059, reverted LTED cells back to the level of estradiol sensitivity observed in wild-type cells [41]. This observation suggests that FTS may exert effects in addition to those mediated by MAPK. 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 mammalian target of rapamycin (mTOR) [42,43]. In several model systems, mTOR functions as a mediator 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 MAPK and the mTOR pathways. Our recent studies demonstrated upregulation of mTOR in LTED cells [29]. 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 apoptosis stimulating kinase (ASK)-1 [46,47].

Both FTS and the FTIs were designed to block Ras activity and to inhibit Ras-induced growth in cancer cells. At first consideration, one might consider FTS and the FTIs to be agents in the same class, and to potentially exert similar actions. However, the FTIs are now considered to act through mechanisms other than Ras [16]. FTS exerts unexpected effects to markedly enhance apoptosis however, in addition, recent data demonstrate that FTS also blocks mTOR [42,43]. Taken together, these data strongly support the concept that FTS and the FTIs are not in the same class, and probably will work very differently when administered 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 analog of FTS [*S*-geranylthiosalicylic acid (GTS)] with similar detergent properties to FTS [43]. GTS did not inhibit mTOR, whereas FTS did [43]. 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 [19–21]. Even a dose of 100 mg/kg causes no

weight loss and no evidence of toxicity in nude mice bearing these tumors. Full toxicology studies are now ongoing in two animal species in preparation for application for an Investigational New Drug Application to perform 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 initial treatment of hormone-dependent breast cancer by blocking proliferation and increasing the rate of apoptosis. However, we postulate that FTS may exhibit enhanced activity in patients previously treated with aromatase inhibitors which could result in upregulation of the MAPK pathways. Further in-vivo studies are now required before consideration of use of FTS in phase I studies in women with breast cancer.

References

1 Santen RJ, Manni A, Harvey H, Redmond C. Endocrine treatment of breast cancer in women. *Endocr Rev* 1990; **11**:221–265.
2 Early Breast Cancer Collaborative Group. Tamoxifen for early breast cancer: an overview of the randomized trials. *Lancet* 1998; **351**:1451–1467.
3 Santen RJ. Long term tamoxifen therapy: can an antagonist become an agonist? *J Clin Endocrinol Metab* 1997; **81**:2027–2029.
4 Horwitz KB. When tamoxifen turns bad. *Endocrinology* 1995; **136**:821–823.
5 Ali A, Coombes RC. Endocrine-responsive breast cancer and strategies for combating resistance. *Nat Rev Cancer* 2002; **2**:101–112.
6 Katzenellenbogen BS, Montano MM, Ekena K, Herman ME, McInerney EM. Antiestrogens: mechanisms of action and resistance in breast cancer. *Breast Cancer Res Treat* 1997; **44**:22–38.
7 Schiff R, Massarweh S, Shou J, Osborne CK. Breast cancer endocrine resistance: how growth factor signaling and estrogen receptor coregulators modulate response. *Clin Cancer Res* 2003; **9 (1 pt 2)**:447S–4454S.
8 King RJ, Darbre PD. Interaction of growth factors during progression towards steroid independence in T-47-D human breast cancer cells. *J Cell Biochem* 1990; **43**:199–211.
9 Nicholson RI, Hutcheson IR, Knowlden JM, Jones HE, Harper ME, Jordan N, *et al.* Non-endocrine pathways and endocrine resistance: observations with anti-oestrogens and signal transduction inhibitors in combination. *Clin Cancer Res* 2004; **10**:346s–354s.
10 Nicholson RI, Hutcheson IR, Harper ME, Knowlden JM, Barrow D, McClelland RA. *et al.* Modulation of epidermal growth factor receptor in endocrine-resistant, oestrogen receptor-positive breast cancer. *Endocr Relat Cancer* 2001; **8**:175–182.
11 Knowlden JM, Hutcheson IR, Jones HE, Madde T, Gee JM, Harper ME, *et al.* Elevated levels of epidermal growth factor receptor/c-erbB2 heterodimers mediate an autocrine growth regulatory pathway in tamoxifen-resistant MCF-7 cells. *Endocrinology* 2003; **144**:1032–1044.
12 Schiff R, Massarweh SA, Shou J, Bharwani L, Mohsin SH, Osborne CK. Cross-talk between estrogen receptor and growth factor pathways as a molecular target for overcoming endocrine resistance. *Clin Cancer Res* 2004; **10**:t6S.
13 Johnston SR, Head J, Pancholi S, Detre S, Martin LA, Smith IE, *et al.* Integration of signal transduction inhibitors with endocrine therapy: an approach to overcoming hormone resistance in breast cancer. *Clin Cancer Res* 2003; **9**:t32S.
14 Joneson T, Bar-Sagi D. Ras effectors and their role in mitogenesis and oncogenesis. *J Mol Med* 1997; **75**:587–593.
15 Dinsmore CJ, Bell IM. Inhibitors of farnesyltransferase and geranylgeranyltransferase-I for antitumor therapy: substrate based design, conformational constraint and biological activity. *Curr Top Med Chem* 2003; **3**:1075–1093.
16 Prendergast GC. Farnesyltransferase inhibitors: antineoplastic mechanism and clinical prospects. *Curr Opin Cell Biol* 2000; **12**:166–173.
17 Elad-Sfadia G, Haklai R, Ballan E, Gabius HJ, Kloog Y. Galectin-1 augments Ras activation and diverts Ras signals to Raf-1 at the expense of phosphoinositide 3-kinase. *J Biol Chem* 2002; **277**:37169–37175.
18 Marom M, Haklai R, Ben-Baruch G, Marciano D, Egozi Y, Kloog Y. Selective inhibition of Ras-dependent cell growth by farnesylthiosalicylic acid. *J Biol Chem* 1995; **270**:22268–22270.
19 Egozi Y, Weisz B, Gana-Weisz M, Ben-Baruch G, Kloog Y. Growth inhibition of ras-dependent tumors in nude mice by a potent ras-dislodging antagonist. *Int J Cancer* 1999; **80**:911–918.
20 Weisz B, Giehl K, Gana-Weisz M, Egoz Y, Ben Baruch, Marciano G, *et al.* A new functional Ras antagonist inhibits human pancreatic tumor growth in nude mice. *Oncogene* 1999; **18**:2579–2588.
21 Jansen B, Schlagbauer-Wadl H, Kahr H, Heere-Ress E, Mayer BX, *et al.* Novel Ras antagonist blocks human melanoma growth. *Proc Natl Acad Sci USA* 1999; **96**:14019–14024.
22 Halaschek-Wiener, Wacheck JV, Schlagbauer-Wadl H, Wolff K, Kloog Y, Jansen B. A novel Ras antagonist regulates both oncogenic Ras and the tumor suppressor p53 in colon cancer cells. *Mol Med* 2000; **6**: 693–704.
23 Gana-Weisz M, Haklai R, Marciano D, Egozi Y, Ben Baruch G, Kloog Y. The Ras antagonist *S*-farnesylthiosalicylic acid induces inhibition of MAPK activation. *Biochem Biophys Res Commun* 1997; **239**: 900–904.
24 Gana-Weisz M, Halaschek-Wiener J, Jansen B, Elad G, Haklai R, Kloog Y. The Ras inhibitor *S-trans*,*trans*-farnesylthiosalicylic acid chemosensitizes human tumor cells without causing resistance. *Clin Cancer Res* 2002; **8**:555–565.
25 Rotblat B, Niv H, Andre S, Kaltner H, Gabius HJ, Kloog Y. Galectin-1(L11A) predicted from a computed galectin-1 farnesyl-binding pocket selectively inhibits Ras-GTP. *Cancer Res* 2004; **64**:3112–3118.
26 Aharonson Z, Gana-Weisz M, Varsano T, Haklai R, Marciano D, Kloog Y. Stringent structural requirements for anti-Ras activity of *S*-prenyl analogues. *Biochim Biophys Acta* 1998; **1406**:40–50.
27 Elad G, Paz A, Haklai R, Marciano D, Cox A, Kloog Y. Targeting of K-Ras 4B by *S-trans*,*trans*-farnesyl thiosalicylic acid. *Biochim Biophys Acta* 1999; **1452**:228–242.
28 Pearson G, Robinson F, Gibson TB, Xu BE, Karandikar M, Berman K, *et al.* Mitogen activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr Rev* 2001; **22**:153–183.
29 Yue W, Wang JP, Conaway M, Li Y, Santen RJ. Adaptive hypersensitivity following long-term estrogen deprivation: involvement of multiple signal pathways. *J Steroid Biochem Mol Biol* 2003; **86**:265–274.
30 Jeng MH, Yue W, Eischeid A, Wang JP, Santen RJ. Role of MAPK in the enhanced cell proliferation of long term estrogen deprived human breast cancer cells. *Breast Cancer Res Treat* 2000; **62**:167–175.
31 Santen RJ, Song RX, McPherson R, Kumar R, Adam L, Jeng MH, *et al.* The role of mitogen-activated protein (MAP) kinase in breast cancer. *J Steroid Biochem Mol Biol* 2002; **80**:239–256.
32 Masamura S, Santner SJ, Heitjan DF, Santen RJ. Estrogen deprivation causes estradiol hypersensitivity in human cancer cells. *J Clin Endocrinol Metab* 1995; **80**:2918–2925.
33 Fiennes AG, Walton J, Winterbourne D, McGlashan D, Hermon-Taylor J. Quantitative correlation of neutral red dye uptake with cell number in human cancer cell cultures. *Cell Biol Int Rep* 1987; **11**:373–378.
34 Shim WS, Conaway M, Masamura S, Yue W, Wang JP, Santen RJ. Estradiol hypersensitivity and MAPK expression in long term deprived human breast cancer cells *in vivo*. *Endocrinology* 2000; **141**:396–405.
35 Pitha J, Gerloczy A, Olivi A. Parenteral hydroperoxypropyl cyclodextrins: intravenous and intracerebral administration of lipophiles. *J Pharm Sci* 1994; **83**:833–837.
36 Sebolt-Leopold JS. MEK inhibitors: a therapeutic approach to targeting the Ras-MAPK pathway in tumors. *Curr Pharm Des* 2004; **10**:1907–1914.
37 Cox AD, Der CJ. The dark side of Ras: regulation of apoptosis. *Oncogene* 2003; **22**:8999–9006.
38 Daly RJ, Darbre PD. Cellular and molecular events in loss of estrogen sensitivity in ZR-75-1 and T-47-D human breast cancer cells. *Cancer Res* 1990; **50**:5868–5875.
39 Brunner N, Boulay V, Fojo A, Freter CE, Lippman ME, Clarke R. Acquisition of hormone-independent growth in MCF-7 cells is accompanied by increased expression of estrogen-regulated genes but without detectable DNA amplifications. *Cancer Res* 1993; **53**:283–290.
40 Martin LA, Farmer I, Johnston SR, Ali S, Marshall C, Dowsett M. Enhanced estrogen receptor (ER) alpha, ERBB2, and MAPK signal transduction

pathways operate during the adaptation of MCF-7 cells to long term estrogen deprivation. *J Biol Chem* 2003; **278**:30458–30468.
41 Yue W, Wang JP, Conaway M, Masamura S, Li Y, Santen RJ. Activation of the MAPK pathway enhances sensitivity of MCF-7 breast cancer cells to the mitogenic effect of estradiol. *Endocrinology* 2002; **143**:3221–3229.
42 Yue W, Wang JP, Li Y, Fan P, Santen RJ. Farnesylthiosalicylic acid blocks mammalian target of rapamycin signaling in breast cancer cells. *Int J Cancer* 2005; **117**:746–754.
43 McMahon LP, Yue W, Santen RJ, Lawrence Jr. JC. Farnesylthiosalicylic acid inhibits mTOR activity both in cells and *in vitro* by promoting dissociation of the mTOR–raptor complex. *Mol Endocrinol* 2005; **19**:175–183.
44 Pitha J, Irie T, Sklar PB, Nye JS. Drug solubilizers to aid pharmacologists: amorphous cyclodextrin derivatives. *Life Sci* 1988; **43**:493–502.
45 Stella VJ, Rajewsk RA. Cyclodextrins: their future in drug formulation and delivery. *Pharm Res* 1997; **14**:556–557.
46 Huang S, Shu L, Dilling MB, Easton J, Harwood FC, Ichijo H, *et al.* Sustained activation of the JNK cascade and rapamycin-induced apoptosis are suppressed by $p53/p21^{Cip1}$. *Mol Cell* 2003; **11**:1491–1501.
47 Matsuura H, Nishitoh H, Takeda K, Matsuzawa A, Amagasa T, Ito M, *et al.* Phosphorylation-dependent scaffolding role of JSAP1/JIP3 in the ASK1–JNK signaling pathway. A new mode of regulation of the MAPK cascade. *J Biol Chem* 2002; **77**:40703–40709.