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13. ABSTRACT (Maximum 200 Words) The aim of this project is preclinical testing of a novel Ras antagonist, farnesylthiosalicylate (FTS) in complex with a cyclodextrin (CD) carrier. Ras is involved in estrogen and growth factor signaling in breast cancer cells. Inhibition of Ras should prevent growth of breast cancer cells. We have tested FTS-CD against several breast cancer cell lines in vitro and found that: <ul style="list-style-type: none"> - FTS-CD is effective against estrogen receptor positive breast cancer in vitro. - FTS-CD is effective against Tamoxifen resistant cells in vitro. - FTS-CD interferes with estrogen signaling. - FTS-CD induces profound changes in cell signaling, manifested as increase in apoptosis and decrease in proliferation. 				
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Introduction

A standard treatment of receptor positive, node negative breast cancer is Endocrine Therapy. Endocrine Therapy involves starving the tumor of the Estrogen it requires for growth. A serious problem with Endocrine Therapy is tumor re-growth in low Estrogen levels. These adapted tumors typically require chemotherapy and there is a resultant high death rate. Because Endocrine Therapy is relatively benign compared to chemotherapy, ideally it should be possible to either re-induce a response to Endocrine Therapy or prolong the regression of tumor in response to lowered Estrogen.

Our data and that of others suggests that breast cancer tumors which have adapted to low Estrogen have activated MAPK. MAPK is dependent on Ras for activity. We have selected an anti-Ras compound which abrogates MAPK activity, FarnesylThioSalicylate (FTS). FTS inactivates Ras and has been used successfully in several animal models of Ras dependent tumors without observable side effects, but not breast cancer. This project will examine the effect of abrogating the MAPK pathway via inhibition of Ras with FTS on models of adaptive breast cancer in vitro and in vivo.

Statement of Work (verbatim)

Task 1: Test FTS and FTS-CD in vitro.

- a) Titrate FTS and FTS-CD on LTED cells. 6 months. Milestone is defining exact conditions of growth inhibition and confirming MAPK inhibition.
- b) Establish growth of Tam-R cells. 6 months. Milestone will be getting cells to grow.
- c) Test FTS and FTS-CD on Tam-R cells. 6 months. Milestone is defining exact conditions of growth inhibition and confirming MAPK inhibition.

Task 2: Test FTS and FTS-CD in vivo.

- a) Establish growth of all cell lines in nude mice (Strain Crl:CD-1 nu/nu). 4 months. Milestone will be confirming all cells grow as well as training in correct animal welfare procedures. Number of mice 8 for each cell line, total 24.
- b) Test FTS and FTS-CD on MCF-7 and LTED models in nude mice. 6 months. Milestone will be testing whether Endocrine Resistance reversed. Number of mice 192.
- c) Test FTS and FTS-CD on Tam-R model in nude mice. 6 months. Milestone will be testing whether Endocrine Resistance reversed. Number of mice 192.

Body

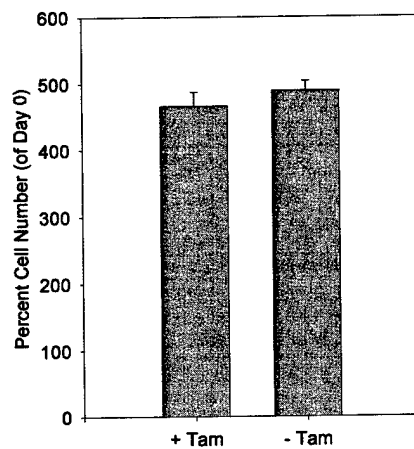
We are very happy with the progress of the project to date. We hired a technician mid 2002 but that person had to withdraw because of sudden illness. After re-advertising, Ms Mandy Lynch began work in October of 2002. She has proven to be an excellent choice. We are preparing a manuscript based on our work (attached) and are on schedule.

Task1:

a) FTS (farnesylthiosalicylate) and FTS-CD (cyclodextrin) have similar inhibition profiles on breast cancer cells, indicating that conjugation with CD does not affect activity in vitro. This has important clinical implications as the complexed form is water soluble, relatively stable and bio-available. FTS-CD induces a mild reduction in proliferation and a dramatic increase in apoptosis. This data is presented in the accompanying manuscript draft. We have found that MAPK activity responds to FTS-CD in a confounding way. The activity of MAPK depends on cell density and when it is measured after FTS-CD addition. We are analyzing the molecular mechanisms further as they may be critical in understanding drug activity.

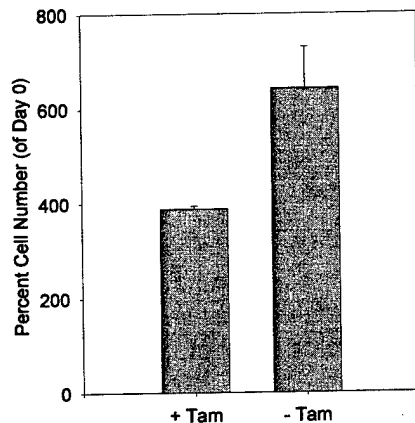
b) We have established growth of Tam-R (TR-1) cells and confirmed resistance to Tamoxifen.

TR-1 Cells, 3000 Cells/200 μ l, 2/26/03
With or Without 1×10^{-6} M Tamoxifen Treatment
3 day, 3 day Additions in IMEM Medium (5% Serum)



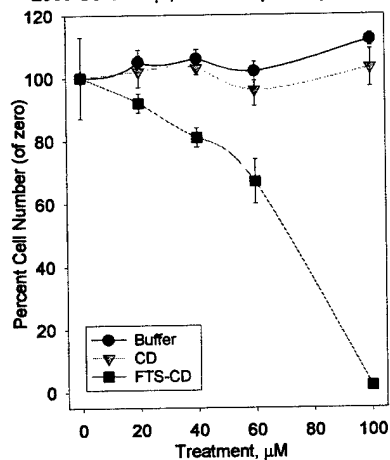
We have also confirmed control breast cancer ("P" cells) cells are sensitive to Tamoxifen.

P Cells, 1500 Cells/200 μ l, 2/26/03
With or Without 1×10^{-6} M Tamoxifen Treatment
3 day, 3 day Additions in IMEM Medium (5% Serum)



c) FTS-CD induces a large decrease in Tam-R cell growth. This is exciting because it may mean FTS-CD could be used to treat women who have developed Tamoxifen resistance. A medical student, Lindsey Neal, will be joining us for the summer of 2003 to study if Tamoxifen and FTS-CD synergize and to look at the mechanisms of FTS-CD on Tam-R cells versus control Tamoxifen sensitive cells.

TR-1 Cells in IMEM Medium, 5% Serum
2/21/03; Buffer, CD, or FTS-CD
2500 Cells/200 μ l; n=4: 3 Day, 2 Day Additions



Task 2:

a) Both Ms Lynch and I are now fully trained in mouse surgery and care. We have just started the tumor growth experiments and it appears that MCF-7 cells establish well as a xenograft but that LTED cells require Matrigel to establish in vivo. We are now comparing tumor growth in the flank to that in the mammary fat pad.

b) We will begin this stage when 2a) is fully complete.

c) We will begin this stage when 2a) is fully complete.

Key Research Accomplishments

- FTS-CD is effective against estrogen receptor positive breast cancer in vitro.
- FTS-CD is effective against Tamoxifen resistant cells in vitro.
- FTS-CD interferes with estrogen signaling.
- FTS-CD induces profound changes in cell signaling, manifested as increase in apoptosis and decrease in proliferation.

Reportable Outcomes

- Manuscript prepared for submission to Breast Cancer Research and Treatment.
- Summer studentship application for Ms Lindsey Neal submitted to Endocrine Society.

Conclusions

We remain cautiously optimistic about the prospects of FTS-CD. It has proven effective in vitro and in vivo against other cancers. We have shown effectiveness against breast cancer in vitro. The real proof will be our in vivo studies and, ultimately, in humans.

So what? The good news is the overall survival rate for women with breast cancer has improved over the last couple of decades. The bad news is the survival rate for women with advanced breast cancer has not changed significantly. If FTS-CD makes it to the clinic we hope that it will save lives.

Running Title: Ras Antagonist Complex

ArticleType: Standard Laboratory Research Article

Title: A RAS ANTAGONIST COMPLEX: CANDIDATE THERAPY FOR ESTROGEN RECEPTOR POSITIVE BREAST CANCER.

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Key Words: Breast Cancer, Endocrine Therapy, Estrogen Receptor, Experimental Therapeutics, Ras.

ABSTRACT

Deprivation of estrogen, called Endocrine Therapy (ET), is commonly used to treat women with estrogen receptor (ER) positive breast cancer. Resistance to ET occurs in a majority of women after about 18 months treatment. Upregulation of growth factor pathways mediated by the 21 kDa Ras GTPase protein may contribute to resistance to ET. A novel Ras antagonist, farnesylthiosalicylate (FTS), causes Ras downregulation with concomitant abrogation of growth factor pathways. We tested the ability of a FTS complex to reduce the growth of ER positive breast cancer cells that were resistant to ET. The FTS complex prevented growth of ER positive breast cancer cells by increasing apoptosis and reducing proliferation. Accompanying loss of cell growth was a significant reduction in the growth response to estrogen of these cells. The loss of estrogen response may have been due to a degradation of ER on FTS complex treatment. FTS complex might be causing a loss of cell growth in part by increasing ubiquitin mediated degradation of the ER in ER positive breast cancer cells. We are investigating this response further and suggest that the FTS complex should enter preclinical trials against ER positive breast cancer.

INTRODUCTION

For women between the ages of 40 and 60, breast cancer is the leading cause of cancer death. One third of these tumors are dependent on steroid hormone, Estrogen (E2), for growth and regress upon administration of anti-Estrogens or the inhibitors of the E2 synthesis enzyme, Aromatase, or by medical or surgical ovarian ablation, called Endocrine Therapy. Of these, 30 to 50% will regrow in all patients, in postmenopausal patients at least 50% will regrow with death within 5 years, estimates vary from study to study. Increased levels of E2 Receptor (ER) and Progesterone Receptor predicts women likely to have hormone dependent tumors. Hormonal therapies, as opposed to cytotoxic chemotherapy, are tolerated well without major side effects or toxicity. Experience over the past 30 years suggests that hormonal therapy is superior to chemotherapy in inducing objective tumor regression in women with receptor positive tumors.

Exactly how E2 stimulates breast tumor proliferation is unknown. However, a large body of recent data suggests that E2 partly mediates its effects on breast cancer through stimulation of growth factor pathways. E2 stimulates the synthesis of specific growth factors such as TGF alpha and results in activation of MAPK, a major mediator of the proliferative effects of growth factors. Inhibition of MAPK or of other steps in the growth factor signaling pathway causes inhibition of breast cancer cellular growth in vitro. Breast cancer cells bearing constitutively active growth factor pathway components such as EGF receptor, Ras, Raf, or MAPK itself do not require E2 for growth. Many breast tumors over express some component of the pathway and advanced tumors have increased active MAPK. Blockade of these pathways reduces the rate of cell proliferation and may also cause cell death. The 21 kDa Ras protein is a prominent transducer of signals from growth factors to MAPK.

Activating mutations of Ras can transform benign cells into a malignant phenotype and a large number of human tumors are associated with Ras mutation or overexpression. Ras proteins are post-translationally modified at their carboxy terminal regions with the C-15 prenyl group, farnesyl. Prenylation assists in plasma membrane anchoring. After synthesis, each form of Ras is prenylated in the cytoplasm via one of three different enzymes; farnesyl transferase (FTase) and GeranylGeranyl transferases I and II (GGTase) (20, 21).

One potential strategy for blocking Ras activity is to prevent farnesylation. Since Ras must be farnesylated and thus bound to the "membrane acceptor" to be fully active, a strategy is to block the enzymes mediating addition of the isoprenyl groups, C-15 farnesyl and C-20 geranylgeranyl. FTase inhibitors (FTI's) were developed as inhibitors (usually farnesyl analogues) of FTase (14, 15) that prevent activation of Ras by stopping the addition of farnesyl groups on Ras.

An alternative strategy is to prevent Ras from binding to the plasma membrane. Farnesylthiosalicylate (FTS) binds to the plasma membrane anchor for Ras, dislodging Ras into the cytoplasm where it is degraded. FTS and FTI's are very different. FTS removes activated Ras from a specific binding site on the membrane (21, 25), reducing MAPK activity by about 80%. FTI's prevent Ras from getting to the membrane, but do allow some Ras that is normally farnesylated to be geranylgeranylated (26). FTI's can cause resistance in vitro (23), FTS does not after six months exposure (27). FTI's have been associated with toxicities in xenografts (22), FTS has not shown any toxicity after testing in several different models.

We have tested FTS here in in vitro models of early and late stage ER positive breast cancer. MCF-7 cells are E2-dependent, ER positive breast cancer cells that have wild type p53, as do most early stage ER positive breast cancers. When MCF-7 cells are successively passaged in E2 stripped media, they become capable of growing in very low amounts of E2 and have upregulated MAPK activity. These hypersensitive ER positive cells are called Long Term Estrogen Deprived (LTED) and have many characteristics of advanced breast cancers that recur after Endocrine Therapy. We have found that a complexed form of FTS prevents growth of early and late stage ER positive breast cancer cells and appears to work at least in part by suppressing ER signaling.

METHODS

Materials: FTS and cyclodextrin (CD) were donated by Thyreos Corporation, New Jersey. FTS-CD complex was prepared according to instructions from Thyreos and CD alone and PBS buffer controls were prepared exactly the same way. ICI 182,780 was generously donated by Astra-Zeneca, United Kingdom. E2 was obtained from Steraloids (Newport, Rhode Island). Cell Death Detection ELISA and Cell Proliferation ELISA were from Roche. Neutral Red was from Aldrich. IMEM was from Biosource and Fetal Bovine Serum (FBS) from Gibco. DextranT70 was from Pharmacia and Charcoal (NoritA) from Sigma. The ER α antibody from Cell Signaling and beta-actin antibody from Sigma.

Cell Culture: To remove E2 and related metabolites from serum, FBS was treated with Dextran and charcoal. Dextran Charcoal Coated stripped serum (DCC) was prepared using a method modified from Horwitz and McGuire , JBC 253: 2223-2228 1978. Heat inactivated FBS (500 ml) was added to 5 g of washed charcoal and 167 mg of DextranT70. The mixture was stirred at 4C overnight and the Dextran/charcoal pelleted out. This was repeated with fresh Dextran/charcoal two more times and in the final step, the FBS was spun twice at 33,000 rpm in an ultracentrifuge and then filtered through a 0.1 µm filter. The resulting Dextran/charcoal coated stripped serum is referred to as DCC.

MCF-7 cells were cultured in IMEM, glutamine and 5% FBS, LTED cells were cultured in phenol red free IMEM, glutamine and 5% DCC. Genesis of the LTED cells is described by Santner et al. ****. Cell number was assayed by a modified neutral red method (McPherson, 2003). To assay E2 dependent growth, MCF-7 cells were seeded into 96 well plates, the next day the medium was changed to IMEM DCC and 5 days later E2 in fresh IMEM/DCC was added. Three days later fresh E2 in IMEM/DCC was added and cell number measured 2 days later. For measuring LTED E2 response, LTED cells were plated into 96 well plates, the next day, media was changed to IMEM with just glutamine added. Seven days later, E2 plus 10^{-9} M ICI182,780 in fresh IMEM was added, three days after that 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 measured 2 days later.

Cell Extract Preparation and Western Blotting: Cells were seeded at 2 million cells per 10 cm diameter dish. The next day CD or FTS-CD was added and three days later the cells were harvested into RIPA buffer. Cell extracts were normalized and loaded onto 10% SDS-PAGE gels, transferred to PVDF membrane and probed with the indicated antibodies.

RESULTS

Breast cancers that are susceptible to Endocrine Therapy are usually ER positive and wild type p53. MCF-7 cells are a wild type p53, ER positive, E2 dependent cell line and are a generally accepted model for early stage ER positive breast cancer that is treated by Endocrine Therapy. LTED cells were generated from MCF-7 cells by depriving them of E2 over several months. LTED cells have many of the characteristics of breast cancers that regrow in the low E2 serum levels after Endocrine Therapy. Upregulation of growth factor and ER pathways occurs during regrowth after Endocrine Therapy therefore we tested whether a growth factor pathway inhibitor suppressed the growth of breast cancer cells in vitro. We assayed the effect of FTS, a Ras inhibitor, as the 21 kDa Ras protein is at the nexus of several important growth factor pathways, as well as being implicated in plasma membrane based ER signaling. FTS suppressed the E2 dependent growth of MCF-7 cells (Figure 1A) with the greatest effects at 25 and 75 µM. LTED cells display the characteristic maximal hypersensitive growth at two log lower E2 concentration than MCF-7 cells (Figure 1B), consistent with the original observations of Santner et al.. FTS started suppressing LTED growth at 25 µM and there was a paradoxical slight stimulation of growth at 75 µM FTS. There was little growth of either cell line in the absence of E2. These data support previous observations that E2 dependent ER signaling can pass through Ras.

We next compared free FTS to FTS complexed with CD. Complexing FTS with CD is required to solubilize the hydrophobic FTS molecule and would be essential for any possible future clinical use of FTS. FTS and FTS-CD were very similar in inhibiting cell growth of MCF-7 cells (Figure 2A). CD alone or Buffer vehicle had little effect on cell growth of MCF-7 and LTED cells (Figures 2B and 2C) under conditions where FTS-CD significantly abrogated cell growth. Even though LTED cells have higher MAPK activity than MCF-7 cells, the degree of inhibition was the same.

Ras activity is required for cellular proliferation. Ras underexpression or overexpression can also induce apoptosis under the right conditions. Apoptosis was induced by between several hundred (not shown) and several thousand fold (Figures 3A and 3B) by 100 µM FTS-CD, which is known to decrease the amount of cellular Ras. 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), consistent with a reduction in an essential component of proliferative pathways.

Downregulation of the ER is known to block E2 responsiveness, induce apoptosis and decrease proliferation of breast cells. ER protein levels were downregulated after three days of FTS-CD treatment (Figure 5). Our data supports a hypothesis that a major mechanism of FTS-CD on breast cancer cells is abrogation of E2 signaling, possibly by downregulation of the ER protein.

DISCUSSION

The concept that E2 signals through plasma membrane based growth factor pathways is relatively new. If correct, it implies that growth factor antagonists could also be used to treat illnesses that once were the main province of anti-estrogen regimens. Endocrine Therapy for ER positive breast cancer is a well established treatment method and better tolerated than chemotherapy. Upregulation of growth factor or ER pathways can occur in tumors that regrow after initially responding to Endocrine Therapy. We hypothesized that a novel Ras antagonist complex would suppress the growth of advanced breast cancers and have presented supporting evidence for that hypothesis here.

The soluble FTS-CD complex clearly prevents growth of cellular models of E2 dependent (MCF-7) and E2 hypersensitive (LTED) breast cancers. The compound induces apoptosis up to several thousand fold and reduces proliferation to very low levels. The LTED cells have upregulated MAPK activity compared to parental MCF-7. Addition of a specific MAPK inhibitor to LTED cells changes the E2 concentration that stimulates maximal cell growth. That is, they become less sensitive to E2 but growth is not suppressed. The FTS-CD suppresses growth but does not markedly shift E2 sensitivity. If the main effect of FTS-CD were mediated by MAPK, then the LTED cells with higher MAPK activity should have altered sensitivity to FTS-CD. The LTED and MCF-7 cells have the same sensitivity to FTS-CD so we conclude that a major cellular effect of FTS-CD is not through MAPK, but is on ER mediated pathways that are required for cell growth. Although FTS-CD does change MAPK activity substantially in these cells (Lynch, McPherson, unpub.).

The observation that FTS-CD suppresses E2 dependent growth but does not shift hypersensitivity is important. Suppression of ER mediated growth suggested that ER signaling was being negated, rather than modified as seen with a specific MAPK inhibitor. Decreased levels of ER protein suggest the suppression of the E2 response might be through ER degradation. ER protein is degraded by proteasomal mechanisms and proteasomal inhibitors are very effective inhibitors of breast cancer cell growth in vitro (McPherson and Santen, unpub.). We are examining further the role of proteasomes in regulating breast cancer cell growth.

An exception to our hypothesis is the apparent mild shift in hypersensitivity of LTED cells at 75 μ M FTS-CD. This observation implies that maximal inhibition of Ras activity in LTED cells, which have hyperactivated MAPK, might partially restore E2 sensitivity. Because we do not know why MAPK activity is upregulated in LTED cells, this anomalous observation is more difficult to rationalize. However LTED cells do have more ER than parental MCF-7 cells, and it is possible that there is a different response to degradative pathways. We are investigating this phenomenon further.

Proteasomal degradation pathways that coordinately regulate cell signaling are essential to normal function of a cell. Proteasomal inhibitors are in clinical development as anti-cancer agents. Conversely, hyperactivation of proteasomal degradation pathways can also kill a cell. We have observed degradation of ER protein in response to a Ras antagonist. The proteasomal pathway can be activated by oncogenic hyper activated Ras under certain conditions (J Biol Chem 2001 Aug 3;276(31):29531-7 Oncogenic ras represses transforming growth factor-beta /Smad signaling by degrading tumor suppressor Smad4. Saha D, Datta PK, Beauchamp RD) J Biol Chem 2000 Jul 28;275(30):22916-24 Oncogenic Ras-mediated cell growth arrest and apoptosis are associated with increased ubiquitin-dependent cyclin D1 degradation. Shao J, Sheng H, DuBois RN, Beauchamp RD). Under other conditions, hyperactivated Ras can inhibit the proteasomal degradation of myc protein (Mol Cell 1999 Feb;3(2):169-79 Ras enhances Myc protein stability by inhibiting the proteasome. Sears R, Leone G, DeGregori J, Nevins JR.). Our observation that inhibition of Ras activates degradation pathways is consistent with a role for Ras as an inhibitor of proteasomal pathways in ER positive breast cancer cells. We are currently investigating proteasomal mediated pathways in more detail and conducting further preclinical studies on FTS-CD as an agent against breast cancer.

ACKNOWLEDGMENTS

This work was supported by Department of Defence Breast Cancer Clinical Bridge Grant Number DAMD17-02-1-0609 and the University of Virginia NCI Cancer Center Support Grant P30-44579.

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 according to the methods. 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 methods with 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 μ M FTS-CD), CD (equivalent to 60 or 100 μ M FTS-CD) or 60 or 100 μ M FTS-CD for five days as described in Methods. Apoptosis was measured by DNA nick 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 BrDu incorporation. Representative of two experiments. Mean and standard deviation of two samples are shown.

Fig 5: FTS-CD causes degradation of the ER. MCF-7 (left four lanes) and LTED (right four lanes) cells were incubated with 60 or 100 μ M FTS-CD (F-CD) or CD equivalent for three days. The cells were harvested and proteins separated by SDS-PAGE, transferred to PVDF membrane and probed for ER (top) and Actin (bottom). Representative of two experiments.

FIGURES

Figure 1A

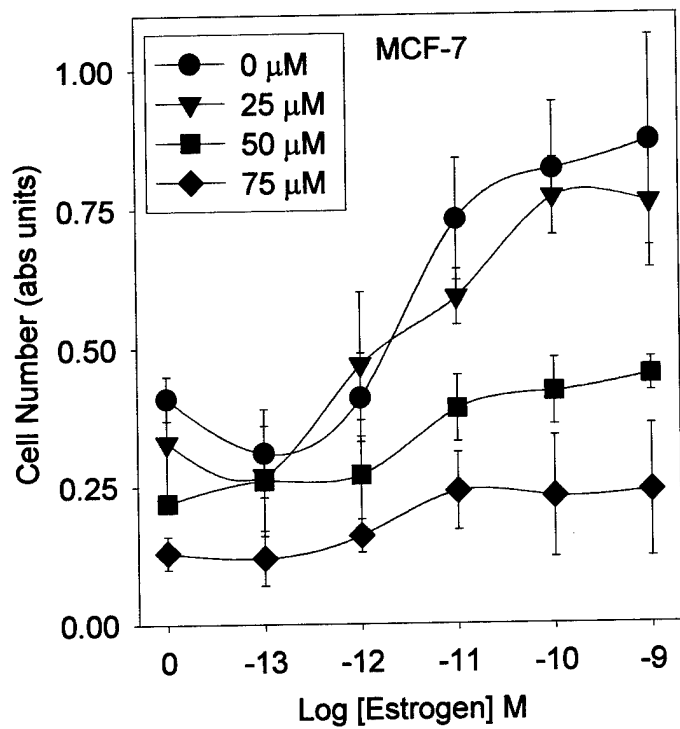


Figure 1B

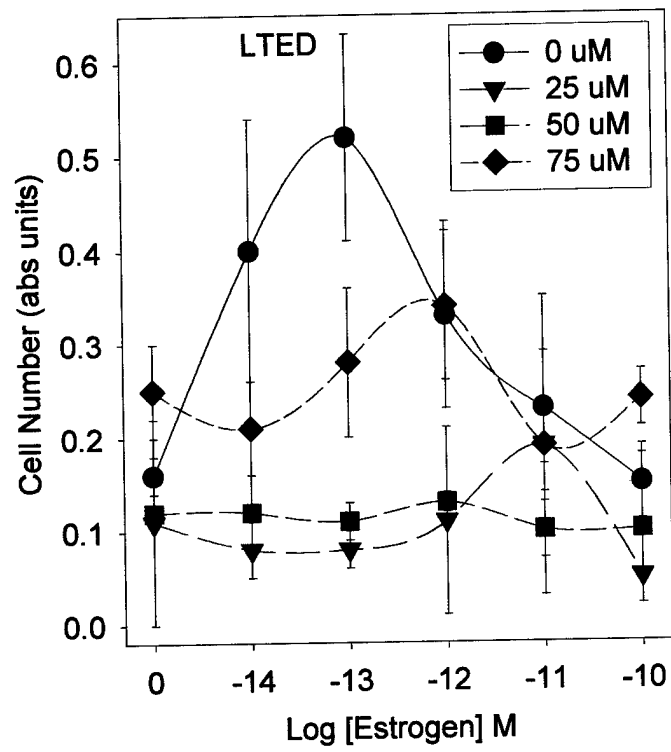


Figure 2 A

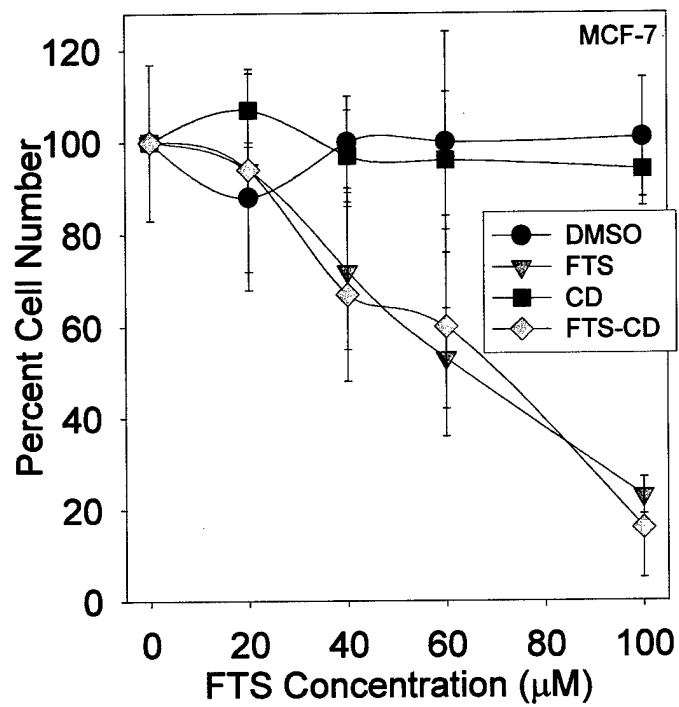


Figure 2B

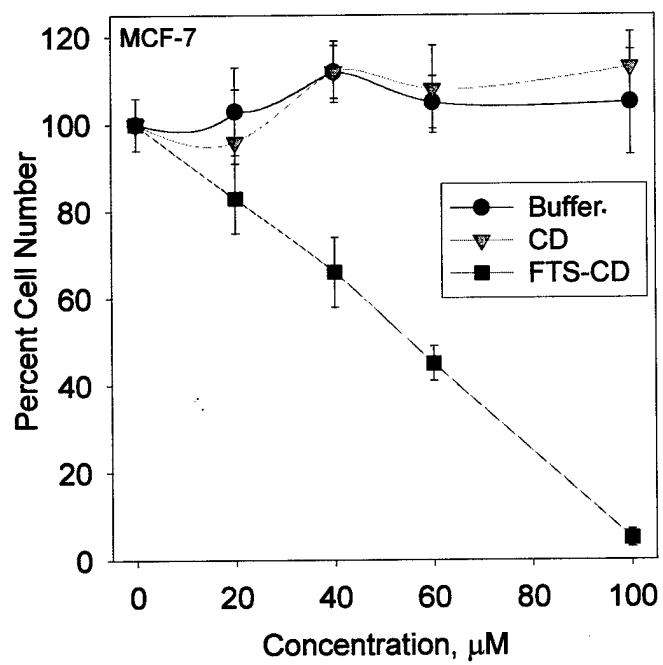


Figure 2C

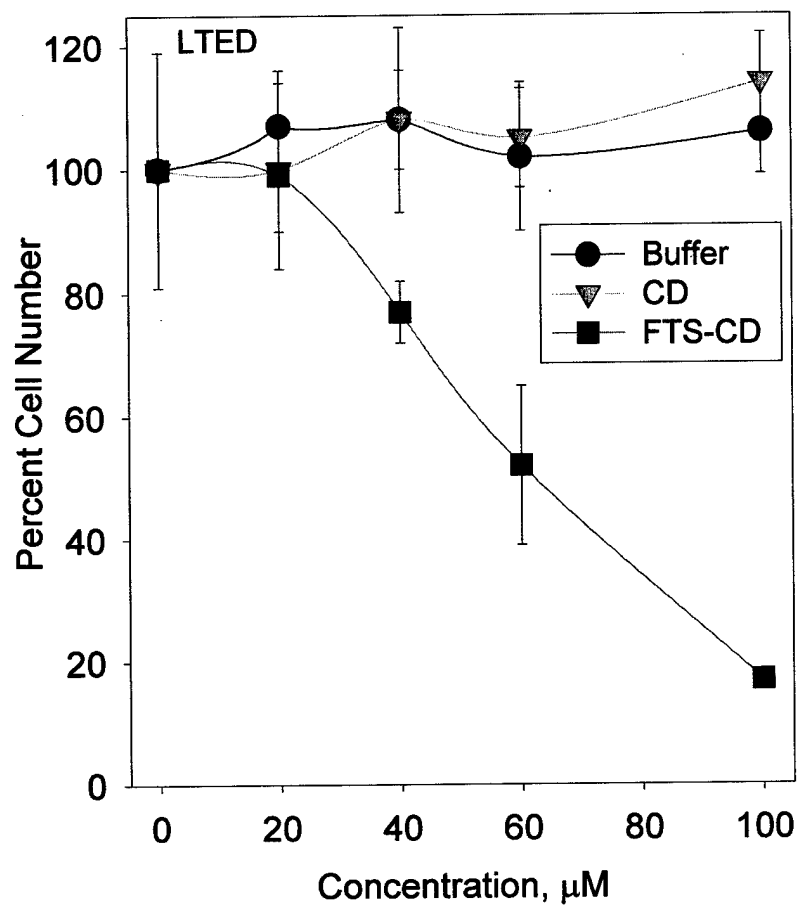


Figure 3A

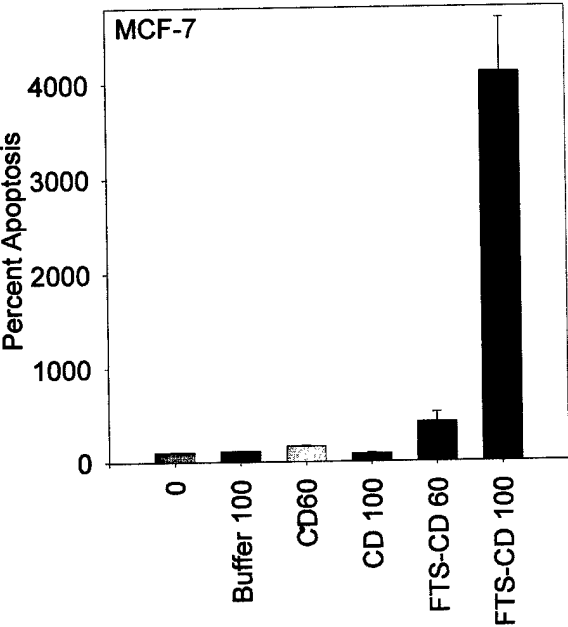


Figure 3B

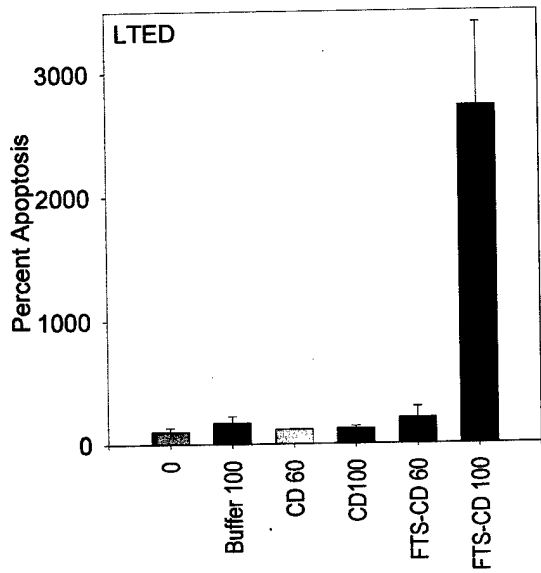


Figure 4A

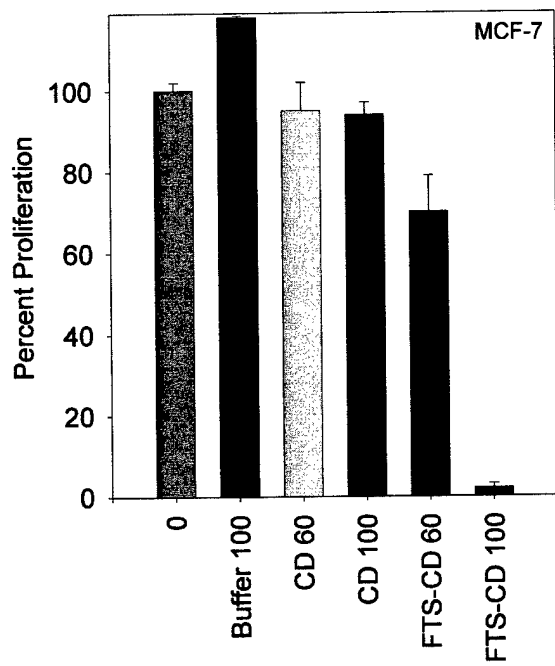


Figure 4B

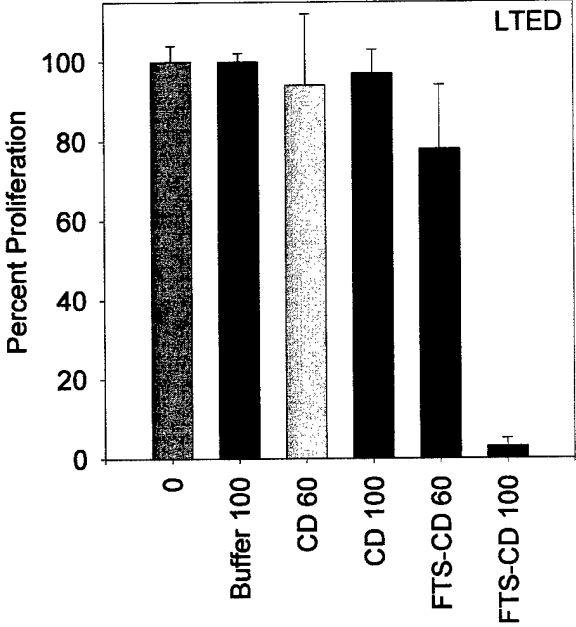


Figure 5

