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Induction of Apoptosis in Human Prostate Cancer Cells by Resveratrol

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INDUCTION OF APOPTOSIS IN HUMAN PROSTATE CANCER CELLS BY RESVERATROL

by

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ABSTRACT

THE EFFECT OF RESVERATROL ON APOPTOSIS IN HORMONE SENSITIVE PROSTATE CANCER CELLS

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Recently attention has been brought to *trans*-resveratrol's (TR) anticancer activity, as determined through a number of cultured cancer cell models. This activity was attributed to TR behaving as an estrogen, and the orientation of TR's hydroxyl groups. Based on this work it was of interest to determine whether TR would also be toxic in prostate cancer cells; if toxic, did TR induce necrosis or apoptosis in the cells; was it toxic through hormone mediated pathways; and were TR's hydroxyl groups responsible for its biological activity. To this end, cellular viability was assessed in two different prostate cancer cell lines, LNCaP and DU 145, in the presence of TR. The onset of apoptosis in LNCaP cells treated with TR was also measured through three distinct techniques, which measured apoptotic morphology, phosphatidylserine (PS) externalization and caspase activity, three events that occur in cells undergoing apoptosis. The measurement of apoptotic morphology and PS externalization, distinguished between apoptotic and necrotic cells. An immuno blot was also used to determine which of two possible caspases were being activated. Trimethoxy-TR (TmTR), a methylated TR analogue, was synthesized to determine whether TR's hydroxyl groups were important to its biological activity. It was found through the cell viability assays that, although TR was toxic to LNCaP cells, it was not toxic only through hormone mediated pathways. It was also found that TR induced apoptosis in

LNCaP cells in all three assays used, and that apoptosis was taking place through caspase-3. Finally it was determined that TR's hydroxyl groups were important to its biological activity.

"Stranger, if you passing meet me and desire to speak to me, why should you not speak to me? / And why should I not speak to you?"

Walt Whitman, *Leaves of Grass*

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My wife, Athena has had to work in retail (almost as bad as selling her soul to the devil) to support my academic ways. Athena along with my two sons, Kevin and Sebastian, had to put up with my absences, where I spent more time with the list of colleagues that follow in this acknowledgment, than I did at home. Yet, Athena, Kevin and Sebastian have always managed to make me feel like I was never gone for long, and have never questioned my pursuit of higher education.

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INTRODUCTION

3, 4', 5-Trihydroxy-*trans*-stilbene or *trans*-resveratrol (TR) has been the focus of much research in the last two years. When research for this thesis was started, there were only three publications exploring the relationship between TR and apoptosis. As of May 2000, there were 11 more papers exploring this connection in cell models ranging from neuronal to adipose tissue. The interest in TR and apoptosis in prostate cancer stemmed from previous work performed by Dr. Williams and Dr. Elliott (1), that looked at the effect phytoestrogens (genistein, diadzein, apigenin, biochanin and TR) had on cell viability of LNCaP cells, a cultured cell model for prostate cancer. The results from this study suggested that TR behaved differently from the other phytoestrogens. TR seemed to stimulate cell growth in LNCaP cells while the other phytoestrogens caused the cells to remain static, they did not grow or die (Figure 1). It has since been shown that the study lacked proper controls, thus the conclusion drawn from it was incorrect. But the work that followed gave rise to this thesis, some very interesting results, and more questions, as any good project worth pursuing should. Two hypotheses on the mechanisms of action, which are not mutually exclusive, were proposed to explain the effects observed in Figure 1. The first hypothesis suggested that TR was interfering with proteins in LNCaP cells responsible for the regulation of apoptosis, thus allowing the cells to grow. The second hypothesis suggested that TR was acting through a hormone receptor, stimulating cellular growth.

The model for this thesis was the *Journal of Biological Chemistry*

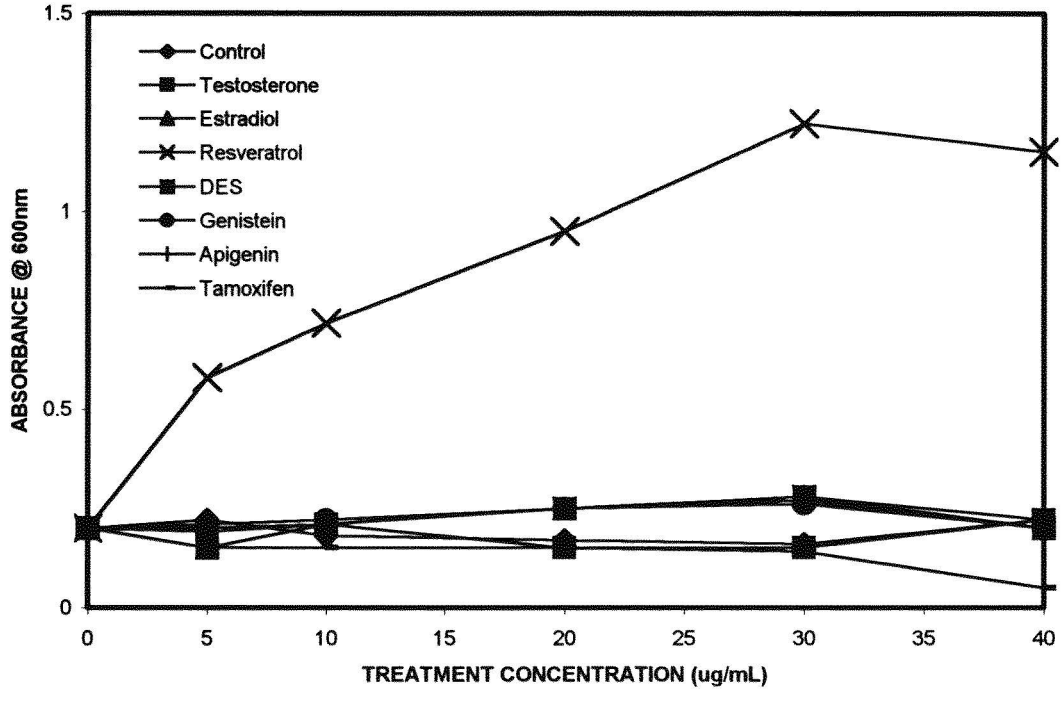

FIG. 1. **Early work: LNCaP cell viability assay in the presence of hormones and phytoestrogens, including TR.** Absorbance by TR treated LNCaP cells increased in a concentration dependent manner, suggesting the cells were growing in the presence of TR. Absorbance by cells in the seven other conditions neither increased, nor decreased, suggesting that the cells were neither growing nor dying in these treatments. It has since been determined that TR interacts with the assay used for this measurement, increasing absorbance.

In order to address the first hypothesis, apoptosis first needed to be characterized in LNCaP cells in the presence of known apoptosis inducers. Any observed effects could then be compared to the effect TR had on the cells. To address the second hypothesis, TR's biological effects needed to be assessed in a cell line that lacked hormone receptors or remained unresponsive to hormones. LNCaP cells are responsive to hormones, becoming static when hormones are removed from their growth media (2), and serve as a model for early, hormone-responsive prostate cancer. DU 145 cells, a second cell model for prostate cancer, were used in studies parallel to those done with LNCaP cells to address the second hypothesis. DU 145 cells are unresponsive to hormones and serve as a model for late stage, hormone-unresponsive prostate cancer. TR was focused on in this research because of the study that suggested TR induced LNCaP cells to grow (Figure 1) in contrast to the other phytoestrogens tested, which seemed to have little or no effect on LNCaP cells. Another reason for focusing on TR was that it was structurally homologous to two drugs currently used to treat cancer in hormone-sensitive tissue, diethylstilbestrol (DES) and tamoxifen (Figure 2). Four objectives were established for this thesis to address these observations 1) determine if TR was toxic to LNCaP cells and quantify the effect; 2) determine whether the effects measured in LNCaP cells were through hormone mediated pathways; 3) examine LNCaP cells treated with TR for the onset of apoptosis; 4) determine whether TR's hydroxyl groups were significant to its biological activity through use of a synthetic analog. The studies presented in this thesis involve three broad topics, cancer of the

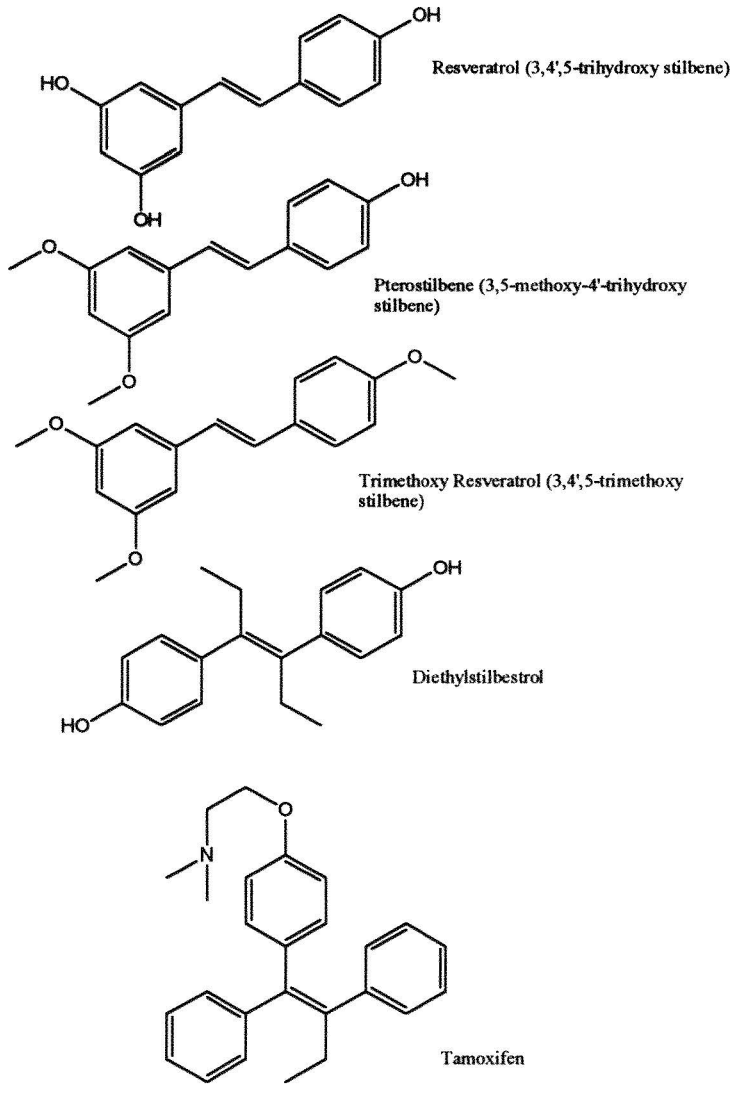

FIG. 2. **Structures of several natural and synthetic stilbenes**. Resveratrol and pterostilbene are natural occurring stilbenes produced by plants to stave off fungal infections. Trimethoxy stilbene is a synthetic stilbene produced for comparative assays with TR. DES and tamoxifen are both synthetic stilbenes used in hormone treatment of prostate and breast cancers.

prostate, phytoestrogens, and apoptosis, which have only been mentioned briefly, so introductions to each follow. In the end, this study showed that TR is toxic to LNCaP cells and that TR does not elicit its effect exclusively through hormone mediated pathways by comparing TR's effect on LNCaP cells to that in DU 145 cells. Further more this study determined that LNCaP cells treated with TR undergo apoptosis. Finally this study demonstrates that TR's hydroxyl groups are important to its effect on LNCaP cells, through comparative studies with its methylated analogue trimethoxy-TR (TmTR).

BACKGROUND ON PROSTATE CANCER

According to the 16th edition of Taber's Cyclopedic Medical Dictionary, the prostate "is a gland that surrounds the neck of the bladder and the urethra in the male. It is partly muscular and partly glandular, with ducts opening into the prostatic portion of the urethral lobes" (3). Prostate size, development and maintenance are controlled by testosterone, and it reaches its stationary size (~ $24cm^3$, 20g) when men are about 20 (4). It secretes a fluid mixture into the semen as it passes from the seminal vesicle into the prostatic end of the ampulla (4). The fluid makes the semen slightly alkaline, which may be important to fertilization, as vaginal secretions are slightly acidic, 3.5 to 4.0, and sperm becomes optimally motile in the pH range of 6.0 to 6.5. The prostate also produces prostate specific antigen (PSA), an enzyme that lyses postejaculatory semen (5). Under pathological conditions the prostate produces higher levels of PSA, which leaks into the serum. Monitoring PSA levels in the serum is currently one of the few techniques available for the early detection of prostate cancer (5).

The American Cancer Society predicts that 180,400 men will develop prostate cancer and 31,900 will die from the disease this year, in the United States alone (6). Prostate cancer is the second leading cause of cancer deaths in this country, after lung cancer, yet its rate of incidence has changed very little since1973. Even so, there will be more men, predominantly over the age of 65 (6), who will develop prostate cancer as the country's population and life expectancy increase. Prostate cancer has a high incidence in men over the age of 65 and is associated with the decrease in production of testosterone by the testes, which occurs in men around the age of 40 (4), yet the exact cause of prostate cancer is still not known. Unlike other cancers such as lung and liver cancer, which can be linked to smoking and drinking, respectively, there is no obvious mechanism by which prostate cancer seems to be triggered. The only thing in common that links patients with prostate cancer is age and gender.

Dr. Donald S. Coffey, a leading researcher in the field of oncology at Johns Hopkins University of Medicine, was the keynote speaker at the 20th Annual Seminar of Cancer Researchers in Virginia. Dr. Coffey addressed the lack of understanding on what induces cancer in hormone-sensitive prostate tissue in men and breast tissue in women (7). He made the observation that the only animals to develop these forms of cancer were dogs and humans (8) and stated, "If you have prostate and breasts, you have cancer." He attributed this to the fact that these are the only animals that eat treated and processed foods containing environmental estrogens such as polychlorinated biphenols, o, p'-dichlorodiphenyltrichloroethane, 4-nonylphenol, *p*-hydroxybenzoic acid, ethyl-, propyl- and butylparaben. He was suggesting that long term exposure to some of these environmental estrogens could lead to cancer later on

in life. Recently it was shown that male mice exposed to low doses of estrogens *in utero* had prostate glands that were 30% larger than in untreated mice when they reached adulthood (9). Prostate enlargement is one of the pathological conditions associated with prostate cancer (10).

There is epidemiological evidence relating diet to low incidence in prostate cancer, comparing the incidence in western to eastern countries. These studies showed that second- and third- generation Japanese immigrants had an incidence of prostate cancer similar to white American males (11), while the incidence in black American males is 50-60 times higher than males in Shanghai, China (12). Furthermore, there is a trend showing a high incidence of prostate cancer in countries with high dietary fat consumption such as the United States, and a low incidence in countries with low dietary fat consumption such as Japan and China (11). In addition to diets low in fat, it was found that people in China and Japan consumed diets containing high levels of phytoestrogens (13,14), a family of compounds demonstrated to have anticancer properties. Thus, the lower incidence of prostate cancer in Chinese men could be due to diets higher in vegetable content, lower in meat and processed food and the presence of compounds, such as phytoestrogens. These studies support Coffey's hypothesis.

Dr. Garnick described the four development stages of prostate cancer, A-D, in a clinical review on prostate cancer (10). Stage A of the disease can be divided into two categories, A1 and A2. The disease in patients with stage A1 is unsuspected through history and examination; but the point of origin for the disease is usually localized on the gland and cells are well differentiated. In stage A2 the clinical

presentation are similar to A1 patients, except the disease is more diffuse on the gland and may already have metastasized to pelvic lymph nodes. In most cases it is hard to distinguish stage A prostate cancer from benign prostate hypertrophy, which presents a problem to the clinician in determining a proper course of treatment for patients at this stage of the disease. Stage B includes cellular aggregation limited to the prostate gland, and hardening of the prostate, but the disease is still limited to the gland. In patients with stage C, the disease is manifested through acute onset of objective urinary symptoms and regions of diffuse hardening and cellular aggregation throughout the prostate, but there is still no evidence of metastasis. In stage D the disease is metastatic, and can be divided into four categories, D0- D3. In D0 the disease is limited to the prostate with elevated levels of acid phosphatase, in D1 the disease has metastasized to the pelvic lymph nodes, D2 it has metastasized beyond the pelvic lymph nodes to bone tissue, and in D3 the disease has become refractory to hormone therapy.

Treatment for prostate cancer in stages A1, B and C includes radical prostatectomy, which decreases the levels of testosterone in circulation, or radiation therapy, which kills the neoplastic cells (10). Since testosterone promotes the growth of metastastatic prostate cells, treatment of stages A2, if metastatic, or D, where the cancer is no longer localized, involves androgen ablation. This therapy consists of bilateral orchiectomy of the prostate and/or hormone treatment with estrogens (e.g. DES) or luteinizing hormone releasing hormone analogues (10,15). This treatment decreases the levels of testosterone to those of castration, but this only prolongs an inevitable death. Most patients on hormone therapy relapse when the cancer becomes

hormone-unresponsive (10,15-17).

Once prostate cancer has metastasized, the prospects of recovery for the patient are very poor. The best chance a patient has is early detection while the prostate cancer is contained within the prostate, before there is any chance of metastasis. Unfortunately, the two screening methods currently used, measuring PSA levels in the blood serum and transrectal ultrasonography, cannot distinguish reliably between early stages of prostate cancer and benign prostatic hyperplasia (10,16). A better solution for prostate cancer is prevention. If compounds are found in the products that are part of a daily diet, with the capability to eliminate the cells that develop into prostate cancer, in a selective manner, then prostate cancer could be prevented. Prostate cancer is a slow-developing adenocarcinoma (18), thus there is time for elimination of the pre-cancerous cells, before the disease develops and gets out of control. The search for the "silver bullet" is on, but prevention through diet is a feasible proposition, based on epidemiological studies comparing incidences between the East and West, and Dr. Coffey's hypothesis.

CELL MODELS USED TO STUDY PROSTATE CANCER

There are several cell models for prostate cancer that are well characterized in the literature and commercially available through American Type Culture Collection (ATCC). The two prostate cancer cultured cell models for this study were LNCaP and DU 145. LNCaP cells are characterized as a model for early, hormone-responsive, prostate cancer; and DU 145 cells are characterized as a model for late stage, hormone-unresponsive, prostate cancer.

LNCaP cells were isolated in 1977 from the superclavicular lymph node of a 50 year old, white, male patient diagnosed with prostate cancer (18,19). Horoszewicz et al. (18,19) did the initial work with this cell line and characterized it as having low anchoring potential to the plastic surfaces of the culture vessels, capable of growing at high densities in culture, and as exhibiting a much slower rate of growth than most "typical" cell cultures with a cell population doubling time of 72 hours. They confirmed that the cell line originated from human prostate cancer tissue through morphological analysis of the cells, preservation of functional differentiation, and maintenance of malignant properties in athymic nude mice (18). They also described organ-specific glycoproteins, such as human prostatic acid phosphatase (18). Several authors have also characterized LNCaP cells as producing PSA, in an androgen responsive manner (5,17). In addition, LNCaP cells remain responsive to physiological concentrations of androgen in their media (17,18). LNCaP cells undergo growth arrest if androgen concentrations in their media are high (17), and initiate apoptosis if they are treated in charcoal-stripped media (a treatment which removes survival factors from the media) (2). LNCaP cells serve as a cultured cell model for hormone responsive prostate cancer because they remain androgen responsive.

DU 145 cells were isolated from a central nervous system lesion of a 69 year-old white male, with prostate cancer, during a craniotomy (20). Mickey et al. (20) were the first to work with and characterize this cell line, showing that the cell line originated from human prostate cancer tissue, through morphologic analysis of the cells. The authors also showed that the cell line is not hormone-responsive by showing that the cells grew equally well in fetal bovine serum and bull serum. This has been

confirmed by others that have shown DU 145 cells grow in the absence of serum in their growth media (17), which may be due to the fact that DU 145 cells do not express estrogen or androgen receptors (21,22). DU 145 cells serve as a cultured cell model for hormone refractive prostate cancer because they are characterized as androgen insensitive.

PHYTOESTROGENS: RESVERATROL

Phytoestrogens are a family of organic compounds produced by plants that are of clinical interest because they interact with the human estrogen receptor (ER) (23). This specific biological activity leads to the name phytoestrogen: *phyto-* meaning it is of plant origin, and *–estrogen* suggests interaction with the estrogen receptor or eliciting an estrogen-like response. A lot of work has been done looking at the effects these compounds have on breast cancer tissue *in vitro*, because breast cancer, like prostate cancer, develops in a hormone dependent manner. It has been only in the past four to five years that the scientific community has began to look at the effects these compounds have on male prostate tissue.

Phytoestrogens can be grouped into four classes: flavones, isoflavones, lignans and stilbenes based on the chemical structure of the parent compound. Figures 2 and 3 show examples of several stilbenes, flavones and isoflavones in the phytoestrogen family. Epidemiological studies have linked phytoestrogens to low incidence of certain diseases, such as prostate cancer in China and Japan (14,24) and cardiovascular disease in France (14,25). The low incidence in cardiovascular disease in France was dubbed the "the French Paradox" in the mid 1990's (25), because the low incidence

was in spite of a French diet high in fat. The scientific community has been studying the biological effects of phytoestrogens in the hopes of understanding the mechanism by which they seem to convey these health benefits. Oxidative stress due to the presence of free radicals has been associated with some of the diseases phytoestrogens seem to decrease (14). All of these compounds contain hydroxyl groups. This functional group has the ability to chemically react with free radicals. Free radicals are

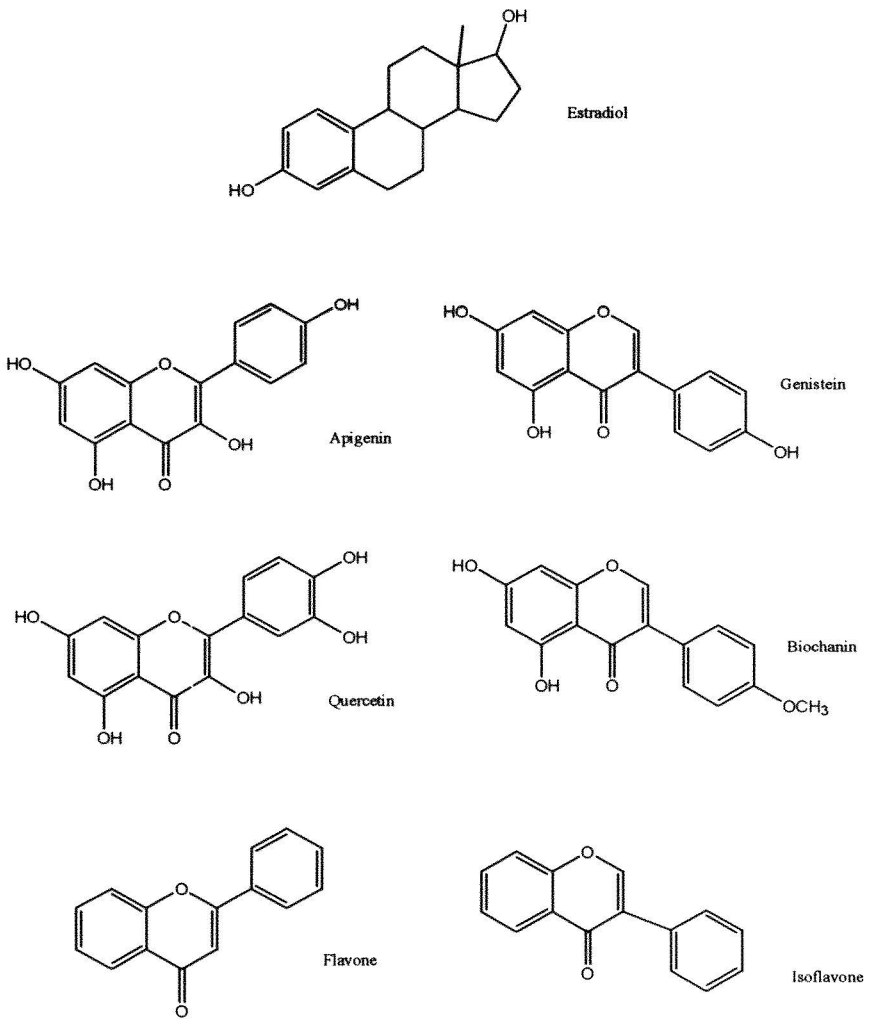

FIG. 3. **Structures of estradiol, flavone, isoflavone and four phytoestrogens**. Apigenin and quercetin are both structural analogues of flavone, thus are in the flavone family of phytoestrogens. Likewise, genistein and biochanin are isoflavone analogues, and are in the isoflavone family of phytoestrogens. This figure shows the similarity between the structures of phytoestrogens and estradiol, which leads to the suggestion that phytoestrogens could act like estrogen in biological systems through structure activity relationship studies.

reactive species capable of chemically modifying macromolecules such as proteins and DNA causing these structures to lose function. Compounds that have the ability to react with and neutralize free radicals are called antioxidants. One plausible mechanism of action for these organic compounds is that they behave as antioxidants within a cell. Since many oxidation reactions occurring in the cell occur in the mitochondria, this may be one location within the cell where these compounds may be most active, as has been suggested in some studies (14,26,27).

TR is a naturally occurring compound found in a variety of plants of the spermatophyte family (26). TR has also been found in wine grapes, *Vitis vinifera* (28) and in the weed, *Polygonum cuspidatum* (29,30), where it is produced as an antifungal agent (a phytoalexin). Both *Vitis* and *Polygonum* have been used in medicinal practice in India (31), Japan and China (29,30). In India, TR was part of *darakchasava*, an Indian herbal preparation prescribed as a cardiotonic (31). In Japan and China, *Polygonum* was used in *Ko-jo-kon*, a Japanese folk medicine used to treat fungal disease, liver and heart disease (27). TR is also carried over into wine from the grape during the wine making process. Red wines carry higher concentrations, ranging from 0.1 μg/L to 6.9 μg/L (0.4 – 30 nM)(26), than white wines, which have concentrations of less than 0.1 μg/L in most samples. Wine grapes produce TR in response to mold growth on the grapes, such as *Botrytis* infection, as part of their defense mechanism. *Botrytis* grows preferably under humid and cool conditions, and not in regions of low humidity and high temperature (32). Thus the concentration of TR in wine depend on the region of the world in which the wine is produced and on whether the grapes were challenged by a *Botrytis* infection (33). Interestingly the muscadine grape, *Vitis*

rotundifolia, the only wine grape native to the United States, is highly resistant to mold growth (34). This resistance is attributed to consistently high levels of TR in the grape. Wines produced from muscadine grapes also contain high levels of TR (34). TR concentrations range from 0.74 – 1.97 μg/L (3.2-8.6 nM) in wines produced from dark-skinned muscadine berries to 0.35-0.97 μg/L TR (1.5-4.2 nM) in wines produced from bronze-skinned berries. Other products that contain TR are peanuts (0.032- 8.0 mg/g of peanuts) (35,36) and rhubarb (37).

At present there are at least 200 articles in peer-reviewed journals describing TR's biological activity. Fremont and Soleas et al. (26,27) have published in-depth review articles on TR's biological activity. These include antioxidant activity (38), inhibition of platelet aggregation (39-42), anticancer activity (43-46), protein-tyrosine kinase inhibitor (47), estrogenic activity (23), inducing the expression of Fas ligand in HL60 cells (45), and activation of p53 in mouse fibroblast cells (42). A likely mechanism by which TR may elicit a protective effect, based on its chemical structure (Figure 2), is through antioxidant activity. TR's antioxidant activity has been demonstrated in a number of systems. Williams and Rutledge (48) measured TR's antioxidant activity using a Randox total antioxidant status (TAS) system, which measures a compounds antioxidant activity (status) through its ability to inhibit a chemical redox reactions *in vitro*, and compared the values obtained to those of the established antioxidant trolox, estradiol (the female hormone) and several phytoestrogens. Some of these values are presented in Table I. The values obtained suggest a trend of increasing antioxidant activity with increasing number of hydroxyl groups. This is clearly demonstrated among the stilbenes where piecatenol

TABLE I

Total anti-oxidant status (TAS) values for representative hormones, flavones, isoflavones and stilbenes

TAS values determined for several phytoestrogens and estradiol, through measurement with a Randox system (48). The assay compared the antioxidant ability of a compound to trolox, an established antioxidant, given the arbitrary value of one. Values greater than one suggest greater antioxidant activity. The values presented suggest a correlation between the number of functional groups present and anti-oxidant capability.

Compound	TAS Value
Estradiol	2.70
Quercetin (flavonoid)	2.90
Apigenin (flavonoid)	2.34
Genistein (isoflavonoid)	2.09
Diadzein (isoflavonoid)	0.058
Biochanin (isoflavonoid)	1.91
Piceatannol (tetrahydroxy stilbene)	3.35
Resveratrol (trihydroxy stilbene)	2.26
Triacetyl Resveratrol (triacetyl stilbene)	-1.02

(tetrahydroxy stilbene) > TR (trihydroxy stilbene) > tri-acetyl resveratrol (triacetyl analogue of TR, whose hydroxyls are acetylated and hidden from redox chemistry). TR's anitoxidant activity within cellular systems, such as reducing copper-catalyzed oxidation of low-density lipoproteins, which plays a role in atherosclerosis, has also been established (27). Finally, TR also inhibits cellular toxicity due to oxidation of membrane lipids, an event associated with Alzheimer's disease (38).

The interest in TR arises from the fact that it is a naturally occurring compound present in products consumed on a daily basis which has varied, selective biological effects in certain types of cancer cell lines (44). Further more TR has little or no effect on normal, healthy cells, such as peripheral blood lymphocyte (45) and fibroblasts (1), at concentrations used in most studies (< 32 μM). But it still remains to be demonstrated that TR is getting into the body from consumed food at concentrations relevant to the studies done here and elsewhere. Bertelli et al. (49) demonstrated that resveratrol administered orally to male Wistar rats was absorbed into the plasma, and attained its highest concentration one hour after administration. The plasma concentrations measured after a one time dose of dilute wine spiked with TR (86 μg/Kg) was 88.0 nM. The concentration of TR in the plasma of rats that were administered TR daily (43 μg/Kg) for 15 days was 33.0 nM. In the same study Bertelli et al. also determined the concentration of TR required to inhibition platelet aggregation to be 5.30 nM. This is a pharmacological effect associated with decreasing cardiovascular disease. This concentration is well within those measured in the plasma of rats, after prolonged oral consumption of TR (49). This concentration of TR agrees with those found in certain types of wine (26).

However, the concentrations measured in the plasma of the rats were at least 1,000 times lower than those used in studies measuring the biological activity of TR in human, cultured, cell models for cancer. And the bioavailability of TR in humans has not been determined yet. Until similar studies are done with humans, it will not be known if the concentrations of TR present in the diet are high enough to treat or prevent cancer.

APOPTOSIS

Apoptosis is the name for cell suicide, also called cell-mediated death or programmed-cell death. It is likely that most, if not all, cells of the body have the biochemical tools to undergo apoptosis (50). Apoptosis plays an important role in maintaining the cell population of an organism in homeostasis, ensuring that cells die at a rate parallel to cell division (50,51). Apoptosis is also important in the immune system of mammals, where cells of the immune system that express self-recognition are destroyed through apoptosis (52). The observation that cells in the body die naturally through apoptosis is important because without this mechanism it is hard to explain the mechanism by which cell density is maintained in the body without a net gain of cells. This dilemma is poignant in systems where there are large numbers of cells undergoing rapid division such as cells of the immune and hemopoietic systems. Loss of the ability to undergo apoptosis contributes to the establishment of certain diseases, such as cancer (50,53), where cells divide more rapidly than they die, eventually spreading beyond the organ boundaries. Apoptosis has also been suggested to have a role in Alzheimer's disease (54).

The other way for cells to die is through necrosis. Necrosis occurs when the cell loses membrane integrity, releasing its content into its surroundings, while in some cases the nucleus remains intact (55-57). Cell contents include proteases, free radicals and secondary messengers, all of which can interact in different ways with surrounding cells, leading to inflammation (58,59), which causes more damage to the surrounding tissue than the initial insult responsible for the onset of the necrosis.

Apoptosis, in contrast, is an orchestrated death in which events occur in a chronological order and can be triggered through paracrine signals (cell to cell signals), autocrine signals (cell signals itself) or by external stressors such as ultraviolet radiation, xenobiotics, or viruses (50,56,60). Apoptotic signal cascades can originate from trans-membrane receptors (60), the mitochondria (56) and/or from the nucleus (61), depending on the region being signaled or stressed and on what the signal or stress is. Signals from each of these origins initiate protein localization and activation through protein-protein interactions, propagating the signal through a cascade (50,60). The general order of events during caspase-dependent apoptosis includes: 1) caspase activation, 2) phosphatidylserine (PS) externalization, which binds and signals phagocytic cells (62), and chromatin condensation, 3) nuclear fragmentation and cell shrinkage, and 4) cellular fragmentation into apoptotic bodies (50,63). Throughout the entire process the cell maintains membrane integrity (50), preventing the content of the cell from being released to induce an inflammatory response. The process is terminated when the cell breaks up into apoptotic bodies, which *in vivo* are phagocytized by macrophages (58) and *in vitro* undergo secondary necrosis.

The pathways leading up apoptosis may overlap or apoptosis may be initiated through a single pathway (64). In either case the pathways usually lead to the activation of caspases. Caspases are a family of proteases containing the amino acid cysteine in their active site, which cleave other proteins at specific tetra-peptide sequences following an aspartic acid residue (65,66). Caspases are divided into two groups, initiator and/or effector based on whether their main function is to activate, through proteolysis of another caspases (initiators), or to be cleaved and activated by initiator caspases, to then cleave other essential proteins throughout the cell (effectors) (65). Each caspase has affinity for a specific tetra-peptide sequence, but as can be seen in Table II, some caspases cleave the same sequence, with different degrees of affinity (66). Although caspases seem to be very important to apoptosis, apoptosis may not always occur through caspase-mediated dependent pathways. It has recently been shown (67) that when the expression of the antiapoptotic chaperon protein, heat shock protein 70, is inhibited in cultured breast cancer cells, the cells die in a caspase independent manner, but still exhibit classical apoptotic morphology.

While cell surface receptors involved in apoptotic signaling are numerous they initiate apoptosis through similar biochemical pathways. The TNF receptor family (the family includes Fas, CAR1, nerve growth factor, and DR5) is a system described and well understood in the literature, which is found in most cells (60). Fas (CD95/Apo-1) is linked to intracellular proteins, which form a complex called the death domain. The ligand for Fas receptors binds three receptors at once, causing the death domains of these receptors to be brought together (60). This, in turn will, recruit adaptor proteins, the Fas-associated death domain, which in turn recruit and activate,

Table II

Tetra-peptide sequences for various caspases and their categories

Table shows the preferred tetra-peptide sequence by each of the caspases (66) and the role of each caspase (65) in apoptosis. The peptide sequence for each caspase is presented using the amino acid's one letter symbol: D aspartic acid, E glutamic acid, I isoleucine, L leucine, M methionine, Q glutamine, V valine, and Y tyrosine.

Enzyme	Preferred small substrate
Caspase-1	YEVDx > YVADx
Caspase-2 (initiator/effector)	YDVADx
Caspase-3 (effector)	DMQDx > DEVDx
Caspase-4	LEVDx > YEVDx
Caspase-5	Unknown
Caspase-6 (effector)	VEIDX
Caspase-7 (effector)	DEVDx > DMQDx
Caspase-8 (initiator)	IETDx
Caspase-9 (initiator)	Unknown
Caspase-10 (initiator)	IEADx

initiator caspases such as caspase-8. Initiator caspases will cleave effector caspases such as caspase-3 (65), which cleave proteins important to cell survival. This will in turn activate some proteins including PKC, gelsolin and PAK2 (65,68) or cause others to loose function such as I^{CAD}/DFF-45, and Bcl-2 (65). Yet in other proteins it is not clear what the outcome is due to processing by effector caspases. One example of this is PARP, whose proteolytic processing is important to apoptosis, but it is not clear if it becomes activated or loses function when processed (69). These actions lead to the break down of the cell's structure, cause a change in cellular and nuclear morphology, and eventually generate the formation of apoptotic bodies. It is interesting that the Fas receptor is not expressed in LNCaP cells (70). This is one example in which prostate cancer cells have lost a way of responding to apoptosis-inducing signals, which may have led to cancer.

One extracellular surface receptor especially important in prostate tissue is transforming growth factor-β (TGF-β1), a cytokine capable of inducing apoptosis in several cell lines (50), and important to the regulation of prostate epithelial cell's growth cycle (71). Production of TGF-β by prostate tissue occurs in response to changing concentrations of androgen (71), and is implicated in the growth arrest of prostate tissue in the absence of androgens (17,72). Castration induces the production of TGF-β, which in turn induces apoptosis in prostate tissue, due to the loss of androgen (72,73). Interestingly, LNCaP cells only express one of the two TGF-β receptors needed for the cells to respond to the ligand (71). It is not clear if this mutation was present prior to its removal from the patient or if it developed later, in culture.

In addition to paracrine and autocrine pro-apoptotic signals, endogenous molecules, such as ceramide and ATP, can trigger apoptosis (56,59) along with external stresses such as UV radiation (56). These signals can lead to mitochondria mediated apoptosis, which is regulated by members of the Bcl-2 protein family (53,74), which includes Bcl-2 and Bcl-x as antiapoptotic proteins and Bax and Bid as pro-apoptotic proteins (53,75). Mitochondria-mediated apoptosis is inhibited when Bcl-2 forms a heterodimer with Bax (53) or when Bcl-2 is expressed at high enough levels to form homodimers (74). If the levels of Bax are higher than those of Bcl-2, Bax forms homodimers, and the cell undergoes mitochondria-mediated apoptosis (53). It has been proposed that Bax homodimers can form pores in the outer membrane of the mitochondria (56), making the membrane semi-permeable, allowing cytochrome c to be released from the mitochondria's intermembrane space into the cytosol (56).

Cytochrome c is an integral part of the electron transport chain, so its release from the mitochondria will disrupt cellular respiration. Disruption of the electron transport chain will cause a drop in ATP production and consequently, the amount of ATP available to the cell (75). This step is important to deciding whether the cell will undergo necrosis or apoptosis. If the levels of cytochrome c are depleted from the mitochondria the cell undergoes necrosis, through the generation of reactive oxygen species (oxygen free radicals) and the depletion of ATP (56). If the levels of cytochrome c are not depleted when cytochrome c is released from the mitochondria, ATP production can continue, cytochrome c takes part in the formation of the apoptosome, and the cell undergoes apoptosis (56). The apoptosome is a complex that consists of three proteins, Apaf-1, cytochrome c, and pro-caspase-9 (*pro-* states the

enzyme is inactive). The formation of the apoptosome is ATP dependent (75). When the complex is formed, pro-caspase-9 is processed to caspase-9, an initiator caspase, which will then cleave and activate caspase-8, another initiator caspase, along with caspases-3 and -7, both effector caspases (56).

The expression of Bcl-2 in prostate epithelial cells has been one of the things associated with the neoplastic transformation of prostate tissue. Normal (non-transformed) prostate epithelial cells do not express Bcl-2, where as LNCaP cells increase expression of the protein in the presence of androgen (76). The presence of Bcl-2 in LNCaP cells confers on them resistance to apoptosis triggered through the mitochondria (77).

The protein primarily associated with nuclear mediated apoptosis is p53 (78). p53 is a nuclear protein, with the ability to induce the cell to enter growth arrest or undergo apoptosis, in response to DNA damage (78). If the cell can repair the DNA damage the cell will continue cell division. If the cell cannot repair the DNA damage, p53 induces the cell to undergo apoptosis. One pathway by which p53 mediates apoptosis seems to be through a down regulation of Bcl-2. The absence of p53 from cells has also been associated with the neoplastic transformation of the cell. Normal cells express detectable levels of p53, while transformed cells do not express p53 (78), suggesting a decrease in expression of p53 by transformed cells. LNCaP cells heterogeneously express p53 (79).

Cancer is a disease that results from multiple changes in the genome (78). LNCaP cells have undergone several changes in the genome by which they avoid apoptosis induced through both autocrine or paracrine pathways, such as the lack of

Fas and TGF-β1 receptor, over expression of Bcl-2 and decreased expression of p53. One of roles of apoptosis is to eliminate damaged cells from the population (50). However, transformed cells do not respond to apoptosis, allowing them to remain in the population and continue dividing, eventually leading to cancer. Since natural signals cannot induce apoptosis in cells that have undergone neoplastic transformation, other means need to be devised to eliminate transformed cells.

STRUCTURE ACTIVITY RELATIONSHIP BETWEEN RESVERATROL AND ITS HYDROXYL GROUPS

The last objective of this research was to determine whether the hydroxyl groups were important to TR's biological activity in LNCaP cells. To achieve this, the trimethoxy analog (Figure 2) was synthesized and used to treat the cells. Trimethoxy resveratrol (TmTR) masks TR's hydroxyl groups. This makes TR more lipophilic, and more likely to get into the cell. Lipophilicity and availability of TR is a question that has not been answered satisfactorily, to date. If TmTR elicited comparable effects to TR in LNCaP cells it would suggest two things. The first, TR was probably acting within the cell, instead of through surface receptors. The second, the hydroxyl groups are not biologically important to effects being measured. Hydroxyl groups would be important to antioxidant activity, so TmTR would eliminate TR's antioxidant capability. TmTR will be synthesized through a substitution reaction of iodomethane onto TR through a Williamson-ether synthesis (Figure 4).

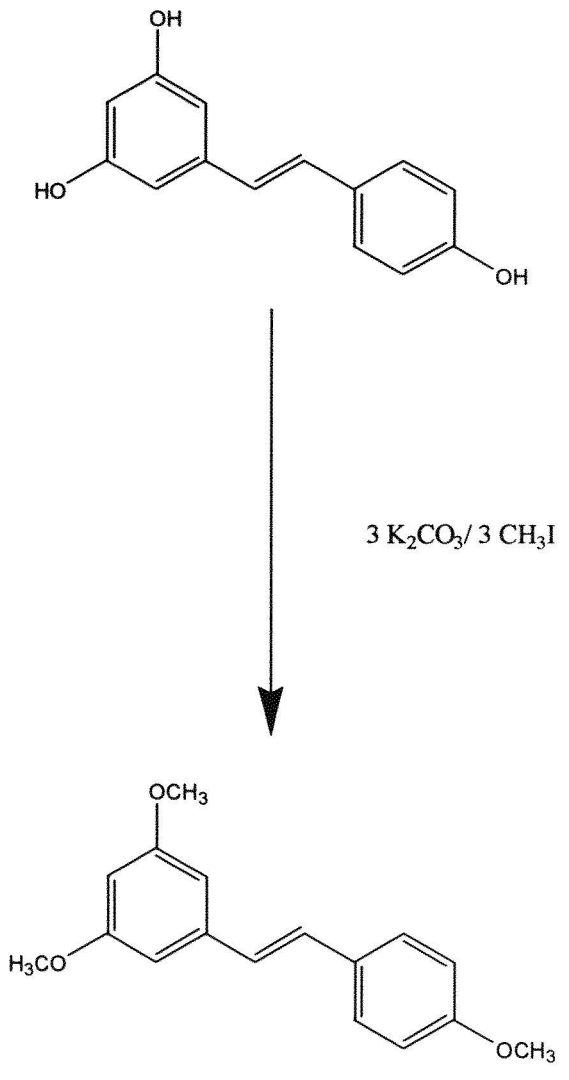

FIG. 4. **Williamson-ether synthesis of trimethoxy resveratrol**. The reaction is carried out with resveratrol in acetone in the presence of potassium carbonate and methyl iodide (see materials and method section). The reaction mechanism is through an S_N2 reaction: the base removes the protons from the hydroxyl groups on resveratrol, forming anions; the anions attack the methyl iodide, the iodide leaves and the methoxy groups are formed.

MATERIALS AND METHODS

CELL CULTURE AND TREATMENT CONDITIONS

LNCaP cells were purchased from American Type Culture Collection. They were grown in RPMI 1640 supplemented with 25 mM HEPES buffer, 2mM L-glutamine, 8 μg/mL insulin, 10% heat inactivated fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin (media and supplements from GIBCO BRL). In order to subculture the cells, 1.5 mL 0.05% Trypsin (GIBCO BRL) was added to each culture flask. The cells were then transferred to centrifuge tubes with growth media, containing serum, which inhibits trypsin, and centrifuged at 1,850 x g (Beckman CPR centrifuge). Cells were sub-cultured every four days (doubling time) and seeded at a density of 2×10^4 cells·cm^{-2} in T-25 or -75 cm^2 tissue culture flasks with canted neck and filtered seal (Falcon and Corning).

DU145 cells were purchased from American Type Culture Collection. In the present experiments the DU 145 cells were grown in Minimum Essential Media (MEM), supplemented with phosphate buffer, 2mM L-glutamine, 10% heat inactivated fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin (media and supplements from GIBCO BRL). DU 145 cells were sub-cultured using the same procedure used with LNCaP cells.

Treatments were carried out in Chemically Defined Chinese Hamster Ovary (CD CHO) media (GIBCO BRL) a proprietary formula lacking peptide components coming from plant, animal or synthetic. CD CHO media was used to show that the effects measured in the presence of TR are due to TR directly and not to TR

interacting with something in the serum or to a latent effect of some unknown present in the serum. CD CHO was supplemented with 8 mM L-glutamine, 100 mM hypoxanthine and 1.6 mM thymidine. LNCaP cells are an adherent cell line, so prior to treatment the growth media was removed from the treatment vessel, and the cells were rinsed with PBS. Stock solutions of 1 mM TR (Pharmascience) and 5 mM DES (Aldrich Chemical Co., Milwaukee, WI) were prepared in 95% ethanol and stored at -20°C. Cells were treated with 50 μM DES and 1000, 100 and 10 μM TR in viability assay but only 50 μM DES and 100 μM TR in apoptosis assays. DES was selected as a positive control based on a paper that demonstrated it induced apoptosis in LNCaP cells (21). The concentration of 50 μM was the highest concentration of DES used in this paper's cell viability assay (21), and was selected for the present studies to ensure a measurable levels of apoptosis. The final concentration of ethanol, the carrier vehicle, was 1% in all assays.

Once the effects TR and DES were shown in the absence of serum, in CD CHO media, the cells were treated in the appropriate growth media with TR, DES and TmTR (1, 10, 50 and 100 μM). All three compounds were prepared from 100 mM stocks in DMSO kept at -20°C.

MEASUREMENT OF CELL VIABILITY IN PRESENCE OF TREATMENTS

To determine the concentrations of DES, TR and TmTR to be used in this study both time and dose response experiments were done measuring cellular viability through cellular respiration. These experiments were done using Promega's CellTiter AQueous assay with MTS tetrazolium which measures mitochondrial respiration

through the NADH or NADPH dependent reduction of the tetrazolium salt to a water soluble, formazan salt, which is a colored product that absorbs at 490 nm. Assays were carried out in 96-well plates seeded with 5000 cells/well seeded in growth media. Experiments that treated in CD CHO media, the cells were incubated for 48 hours after which the media was switched to CD CHO media containing DES (50 μM) or TR (10, 100, and 1000 μM). Cells were treated for 72 hours and assayed with MTS solution at designated time points (6, 12, 24, and 48). Cell viability was determined as the ratio of absorbency of treated cells to control cells.

DETERMINATION OF APOPTOSIS IN LNCaP CELLS

The classical physiological and biochemical markers of an apoptotic cell are, caspase activation, loss of cell membrane asymmetry, DNA fragmentation, blebbing of the cell membrane and cell fragmentation into apoptotic bodies. Each of these events can be quantified in the laboratory through different techniques. Apoptosis was quantified in LNCaP cells through morphological changes by staining and observation under the microscope and biochemical techniques were used to measure loss of cell membrane asymmetry and caspase activity.

Assessment Of Apoptotic Morphology In LNCaP Cells

Cell morphology was used to characterize and quantify apoptosis in TR and DES treated LNCaP cells. Treated LNCaP cells were trypsinized and 40 μL aliquots were applied to a glass slid using a Cytospin III centrifuge (Shandon Southern; Sewickley, PA). They were then centrifuged at 7.06 x g for seven minutes. The slides

were stained with modified Wright-Giemsa stain (Diff-Quick; Baxter Scientific, Miami, FL), rinsed, dried and assessed under the microscope (1000x magnification with emersion oil) for apoptotic and necrotic morphology. Criteria for apoptotic morphology in LNCaP cells included 1) dense nuclear staining 2) cell shrinkage and 3) membrane blebbing, while maintaining membrane integrity. Criteria for necrotic morphology in LNCaP cells was 1) separation of cytosol from nucleus 2) loss of chromatin from nucleus and/or 3) obvious abrasions in the nuclear and/or plasma membrane. Quantification was determined as a percent of the population by taking the ratio of counted cells showing apoptotic or necrotic morphology to the total number of cells counted per slide. At least 200 cells/slide were counted.

Assessment of Externalization of Phosphatidylserine in LNCaP cells

Annexin V-FITC with ethidium homodimer (EthD-1) binding was the second method used to quantify apoptosis in LNCaP cells. Phosphatidylserine (PS) normally found on the inner leaflet of the cell membrane is externalized during apoptosis while the cell maintains membrane integrity excluding most dyes (80), including EthD-1. In contrast necrotic cells lose membrane integrity allowing dyes to enter the cell. The presence of PS can be measured by it's Ca^{2+} dependent association with Annexin V (81). This 35-36 kD protein has a Kd of 5×10^{-10} M for PS (82). Annexin V- FITC (Alexis Corp.; San Diego, CA) a conjugate of annexin V with fluorescein isothiocyanate (FITC) emits green light (530 nm) when it binds PS (81). The binding of Annexin V-FITC alone cannot be used to distinguish an apoptotic cell from a necrotic cell as annexin V will bind PS inside or outside of the cell indiscriminately.

EthD-1 (Molecular Probe, Eugene, OR) binds dsDNA in the cell if its membrane has been permeabilized or the cell is necrotic, emitting orange-red light (617 nm). This dual dye technique distinguishes between apoptotic and necrotic cells, as necrotic cells will be EthD-1 and annexin-V FTIC positive, while apoptotic cells will be annexin V-FITC positive, EthD-1 negative. Dual dye labeling was performed in LNCaP cells treated up to 72 hours as follows. Cells were trypsinized, washed in PBS and 2 x 10^5 LNCaP cells were suspended in 195 μL binding buffer (10 mM Hepes, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) with 5 μL annexin V-FITC. This was incubated for 10 minutes at room temperature. The cells were subsequently washed in PBS, resuspended in 40 μL binding buffer with 3 μM EthD-1 and observed microscopically on a Zeiss Axioscope fluorescence microscope. Data was quantified by calculating the ratio of apoptotic and necrotic cells counted to the total number of cells counted per slide. At least 200 cells/slide were counted.

Measurement of Caspase-3 like Enzyme Activity

Caspase activity was measured through the use of the acetylated tetrapeptide Ac-aspartic acid-glutamic acid-valine-aspartic acid-7-amino-4-trifluoromethylcoumarin (Ac-DEVD-AFC) an analog of the substrate for caspase-3 (CPP-32). This sequence is cross-reactive for other effector caspases such as caspase-7 (66). For detection the tetrapeptide is conjugated with AFC, an aromatic amine fluorophore, which is excited at 400 nm and fluoresces at 505 nm. Treated cells were lysed in ICE buffer (100 μL of 10 mM potassium phosphate, 1 mM EDTA buffer containing 0.5% Triton X-100) supplemented with 2 mM phenylmethylsulfonyl

fluoride, 10 μg/mL pepstatin, 10 mM dithiothreitol for 15 minutes. These cells were spun at 10,000x g for 10 minutes at 4°C and the supernatant kept. 50 μL of supernatant were diluted with 10 mM dithiothreitol, 50 μM DEVD-AFC and 390 μL of ICE buffer and incubated for 45 minutes at 37°C. The supernatant was diluted with 1.5 mL ICE buffer and fluorescence measured with excitation at 400 nm and emission at 505 nm on a LS50B spectrofluorometer (Perkin-Elmer). Results were reported as activity of caspase in pmol of substrate cleaved/ minute/mg of protein present in cell lysate (pmol/min/mg). Measured fluorescence was converted to picomoles of substrate cleaved through use of a standard curve prepared from the fluorescence measured using 7-amino-4-trifluoromethylcoumarin (AFC.) This was standardized with the mass of protein (mg) present in the cell lysate measured using the Bradford method.

IMMUNO BLOTTING DETERMINATION OF CASPASE ACTIVATION

The tetra-peptide used (DEVD) to determine caspase activity in TR and DES treated LNCaP cells was selective for caspase-3, but also cleaved by caspase-7 with a lower affinity (66). Western blot was used in order to determine if there was more than one effector caspase being activated in LNCaP cells treated with TR.

Cells were lysed using the same buffer used in the caspase assay (80 μL). Sample buffer, 20 μL, (7 mL 4x Tris-Cl/SDS, pH 6.8, 3.6 mL glycerol, 1 g SDS, 0.93 g DTT, 1.2 mg Bromophenol Blue, final volume 10 mL H_20) was added to the cell lysate and incubated for ten minutes at 100°C in a heating block (17600 Dri-Bath, Thermolyne). The samples were run on a 10% discontinuous sodium dodecyl sulfate

(SDS) polyacrylamide slab gel. The discontinuous gel consists of a stacking gel (the upper potion of the gel) and a separating gel (the lower portion of the gel).

The separating gel was prepared by mixing 7 mL of 30% acrylamide, 1 mL 10% SDS, 5 mL of 4X Tris (pH 8.8) and brought to a final volume of 21 mL with deionized water. Polymerization of the acrylamide was initiated by adding 50 µL of ammonium persulfate (0.1 g/mL) and 50 µL *N, N, N', N'*- tetramethylenediamine (TEMED) to the final volume. The stacking gel was prepared using the same concentrations of acrylamide and SDS used in the separating gel, but the 4X Tris had a pH of 6.8 instead of a pH of 8.8.

The separating gel was added between the glass plates and covered with a layer of water-saturated butanol and allowed to polymerize for 30 minutes. Once polymerization occurred, the water-saturated butanol was rinsed off with deionized water, and a ten well comb was placed between the glass plates. The stacking gel was added and allowed to polymerize for 15 minutes. Once polymerization was complete the comb was removed and the glass plates with the gel were placed in the gel apparatus (Bio-Rad Mini-PROTEIN II Cell, Bio-Rad Hercules, CA). 20 µL of lysate from each of the treatments and 10 µL of a protein-stacking ladder were each added to a lane. The gel was run for approximately four hours at 15 Amps (E-C 500, E-C Apparatus Corporation, St. Petersburg FL). Gels were prepared and run in pairs for each immuno blot analysis. Once the gel was complete (the bromophenol blue reached the lower portion of the gel), it was ready for blotting.

Blotting consists of transferring the protein loaded in the gel into a blotting membrane (Sequi-Bot PVDF Membrane, 0.2 µm, 10x15 cm, Bio-Rad). Prior to

blotting the membrane was cut to gel size and, along with the filter paper (extra-thick, Bio-Rad), soaked in transfer buffer (48 mM Tris base, 39 mM glycine, 3.75 mL of 10% SDS, 200 mL methanol, adjusted to a final volume 1L in water) for 30 minutes at 4°C. Manufacturer's directions were followed in setting up the blotting apparatus (Trans-Blot® SD Semi-Dry transfer cell, Bio-Rad). After setting up the blotting apparatus it was run at 25 volts for 45 minutes. If the immunodetection assay was not going to be performed immediately, it was placed in the blotting buffer (25 mM Tris, pH 7.5, 0.15 M NaCl, 0.05% Tween 20) with 0.05% sodium azide (Sigma Co.) at 4°C until the assay could be performed.

To perform the immunodetection assay, the filter needed to be washed with blocking buffer (blotting buffer with 2% non-fat dry milk). Antibodies were used that recognized caspase-3 (polyclonal rabbit anti-caspase-3, PharMingen) and caspase-7 (affinity-purified rabbit anti-human/mouse caspase-7, R&D Systems). Each was added 1:1000 (15 μL) to 15 mL blocking buffer with the membrane containing the protein. These were rocked gently overnight at 20°C. The blocking buffer was removed and the filters were washed three times with wash buffer (1 mM Tris, 10 mM NaCl, and 0.1 mL/L 2% Tween, pH 7.5), followed by the addition of 20 mL blocking buffer and 20 μL secondary antibody (anti-rabbit IgG, peroxidase-linked species-specific whole antibody, Amersham Pharmacia Biotech) and placed on shaker. After one hour the blocking buffer and antibody were removed and the membrane was washed three times with washing buffer. The proteins were visualized using Amersham's ECL secondary antibody detection system (followed manufacturers suggestion in use of product).

SYNTHESIS OF 3-, 4'-, 5- TRIMETHOXYSTILBENE

A solution of 200 mg (0.9 mM) TR in 10 mL acetone was treated with 300 □L methyl iodide (4.80 mM) and 500 mg potassium carbonate (3.6 mM). The solution was stirred with a magnetic stir bar and refluxed for 24 hours and then stirred at room temperature for an additional 72 hours. The course of the reaction was followed with thin layer chromatography (TLC) using a developing solvent consisting of 25 mL methyl chloride and 3 mL methanol. There were three spots present on the TLC plate after 24 hours, which may have been monomethoxy-, dimethoxy- and trimethoxystilbene. Only one spot was present after 96 hours. The acetone and excess methyl iodide were removed with a stream of N_2 gas. The remaining solid was resuspended in approximately 50 mL of ether and vacuum filtered to remove the potassium carbonate. The ether was removed with N_2 gas, leaving a yellow oil. The oil was resuspended in 50 mL methanol to which 15 mL water was added. This was refrigerated (20°C). Off-white crystals formed overnight in the methanol/water mixture. The crystals were filtered off through a filter crucible with vacuum filtration. The filtered crystals were placed in a desiccator. The methanol/water filtrate was placed in the refrigerated overnight to obtain a second crop of crystals. The purity of the crystals was determined through melting point (m.p.), high performance liquid chromatography (HPLC), TLC, and submitted for elemental analysis (Desert Analytical, Tucson, AZ). HPLC analysis was done using an isocratic 50/50, 0.1% trifluoroacetic acid (TFA): acetonitrile solvent system run through a C18 reverse phase column with guard column, at a flow rate of 0.8 mL/minute, monitoring absorbance at

306 nm. The structure of both crops was determined with infrared (IR) and nuclear magnetic resonance (NMR) spectroscopy. The crystals were prepared in deuterated DMSO for ^{1}H-NMR analysis (Varian UnitPlus, 400 MHz), and potassium bromide pellets for infrared (IR) analysis (Nicolet 510P FT-IR Spectrometer).

RESULTS

MEASUREMENT OF CELL VIABILITY

The effects of three compounds TR, DES and TmTR, on two different cell lines, LNCaP and DU 145, were assessed through MTS cell viability assays. DU 145 cells were used to determine if cell viability changes measured in TR treated LNCaP cells were exerted through hormone mediated pathways. TmTR was used to asses the importance of TR's hydroxyl groups to changes in the viability of TR treated LNCaP cells. The effects the compounds had on the cells will be described as being concentration-dependent and/or time-dependent. Concentration-dependent effects depend on whether there is an increase or decrease in cell viability as the treatment concentration changes within a specified time. In time-dependent effects cell viability increased or decreased with time at a specific treatment concentration.

The effects of TR and DES on LNCaP cells were initially assessed in CD CHO media to ensure that there would not be a loss of activity due to absorbance of the compounds by serum in the growth media, to be sure that any measured effects were due directly to TR and DES and not to any interactions with components in the serum of the growth media, and to allow for treatment in media with consistent composition. Under these conditions, TR was shown to be toxic to LNCaP cells in a time dependent manner over 48 hours and in a dose dependent manner (10-1000 μM) (Figure 5). DES was used as a putative apoptotic agent based on the literature (21). DES (50μM) demonstrated a time-dependent toxic effect on the cells over 48 hours (Figure 5).

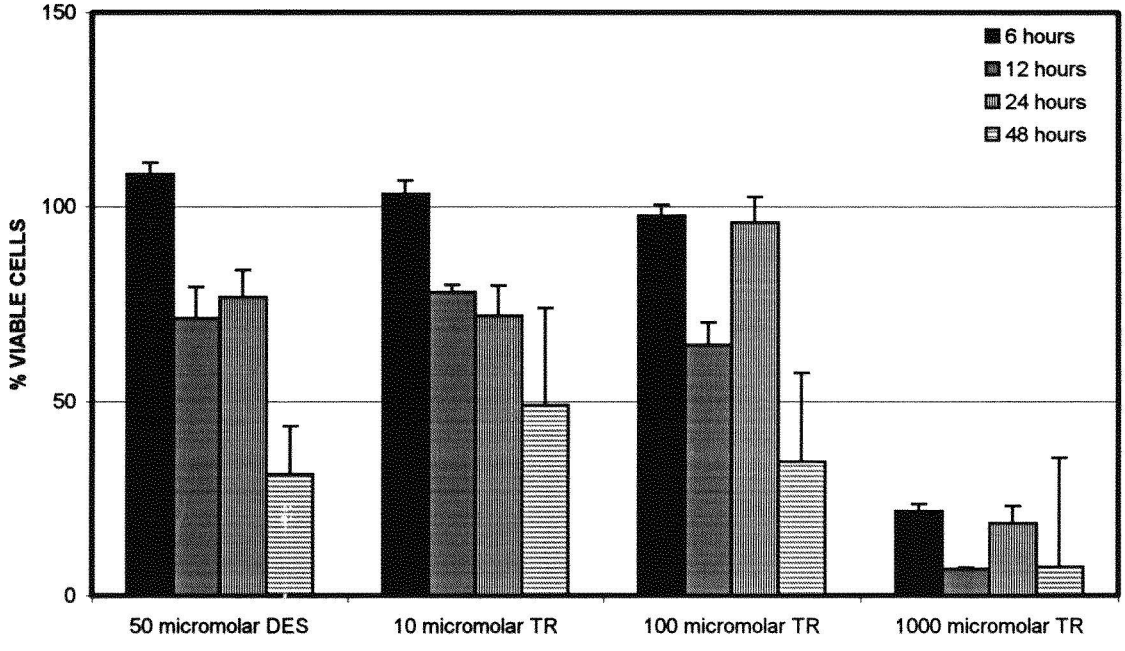

FIG. 5. **Viability assay of LNCaP cells treated with DES and TR in chemically defined Chinese Hamster Ovarian (CD CHO) media**. TR has a time and dose dependent toxic effect on the cells while DES, at the concentration selected from the literature as a positive control for apoptosis, has a time dependent toxic effect on the cells.

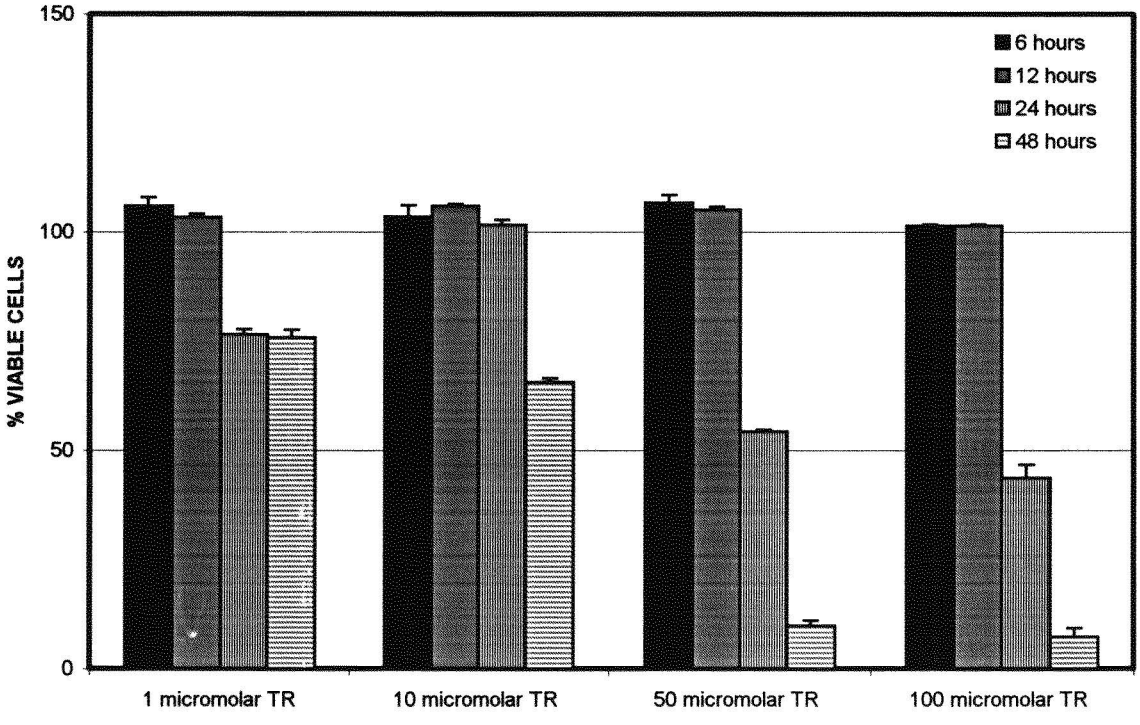

FIG. 6. **Viability assay of LNCaP cells treated with TR in RPMI 1640 growth media.** TR has a time and dose dependent toxic effect on the cells in the growth media similar to the one observed in CD CHO media.

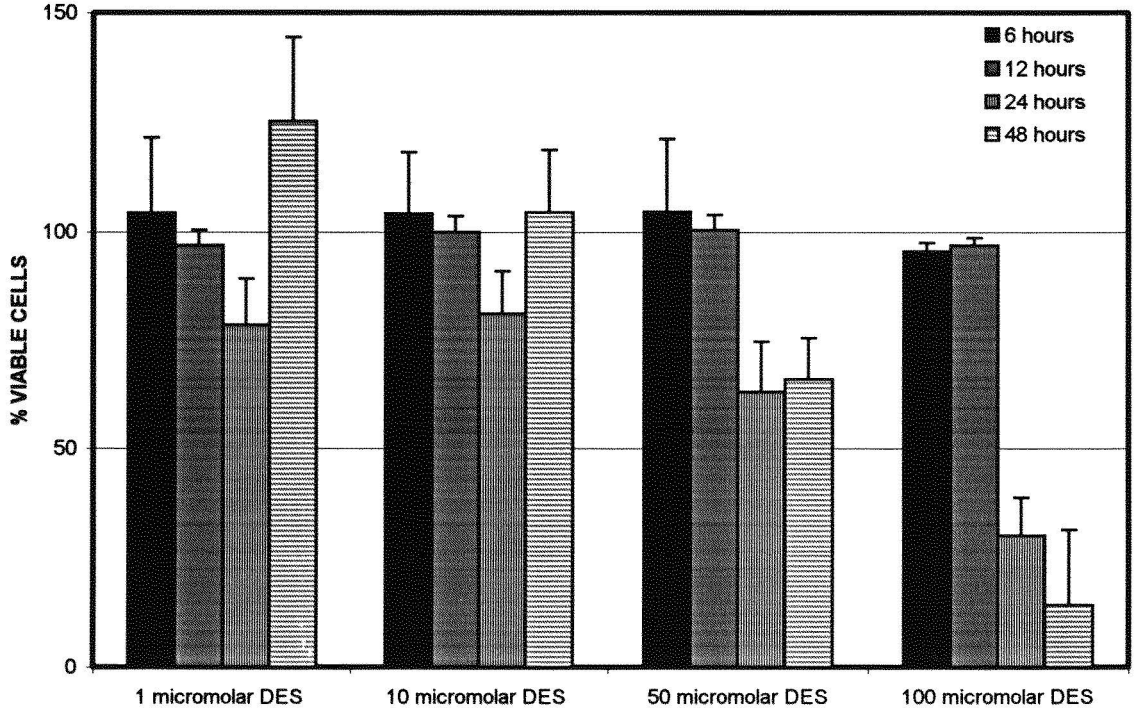

FIG. 7. **Viability assay of LNCaP cells treated with DES in RPMI 1640 growth media.** DES has a time and dose dependent toxic effect on the cells in the growth media.

Once it was ascertained that TR and DES were both toxic to LNCaP cells in CD CHO media, LNCaP cells were treated in their growth media, RPMI 1640. Under these conditions, TR demonstrated a dose dependent effect in the range used to treat the cells (1-100 μM), in a time dependent manner (Figures 6 and 7). TmTR was also toxic to LNCaP cells in a dose and time dependent manner but the concentration response curve shifted to the right, suggesting TmTR was less toxic than TR (Figure 8). TmTR, 100 μM, decreased cell viability by 48% in 48 hours, while 100 μM TR decreased cell viability by 80% in 48 hours.

DU 145 cells, which lack estrogen and androgen receptors (21,22), were treated with TR, DES and TmTR in order to determine if the effects measured in LNCaP cells were due to the compounds behaving through hormone mediated pathways. DU 145 cells were treated in their growth media, MEM. TR and DES affected DU 145 in a biphasic manner. At 1 and 10 μM TR and DES cell growth in a time dependent manner, over 48 hours (Figures 9 and 10). In contrast, at 50 and 100 μM TR decreased cell viability by 25% and 35% respectively by 6 hours of treatment and 63% by 48 hours. TmTR caused DU 145 cells to grow in a time dependent manner, which was independent of concentration (Figure 11).

From the cellular viability assays it can be summarized that TR, DES and TmTR are toxic to LNCaP cells in a time and dose dependent manner. TR and DES have a biphasic effect in DU 145 cells, causing an increase in viability at lower concentrations and a decrease in viability at higher concentrations. In contrast, TmTR increases viability of DU 145 cells at all concentrations used for treatment in a time dependent manner.

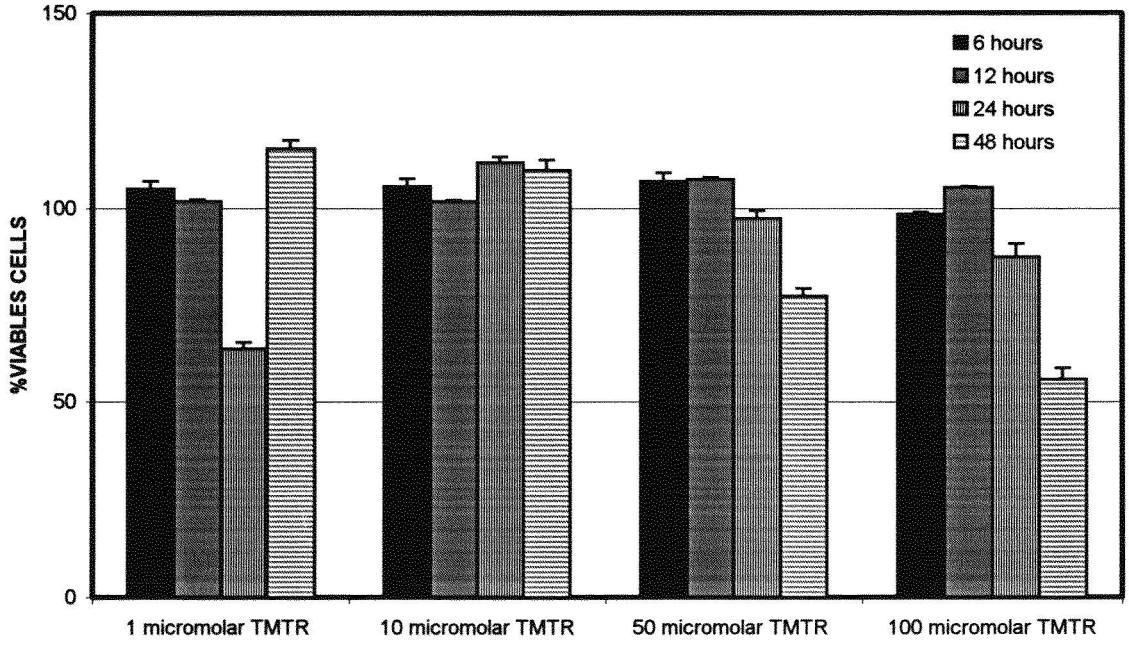

FIG. 8. **Viability assay of LNCaP cells treated with TmTR in RPMI 1640 growth media**. TmTR has a time and dose dependent toxic effect on the cells in the growth media. But it can be seen comparing to Figure 6, that at 48 hours TmTR (100 μM) is less toxic to LNCaP cells than TR (100 μM).

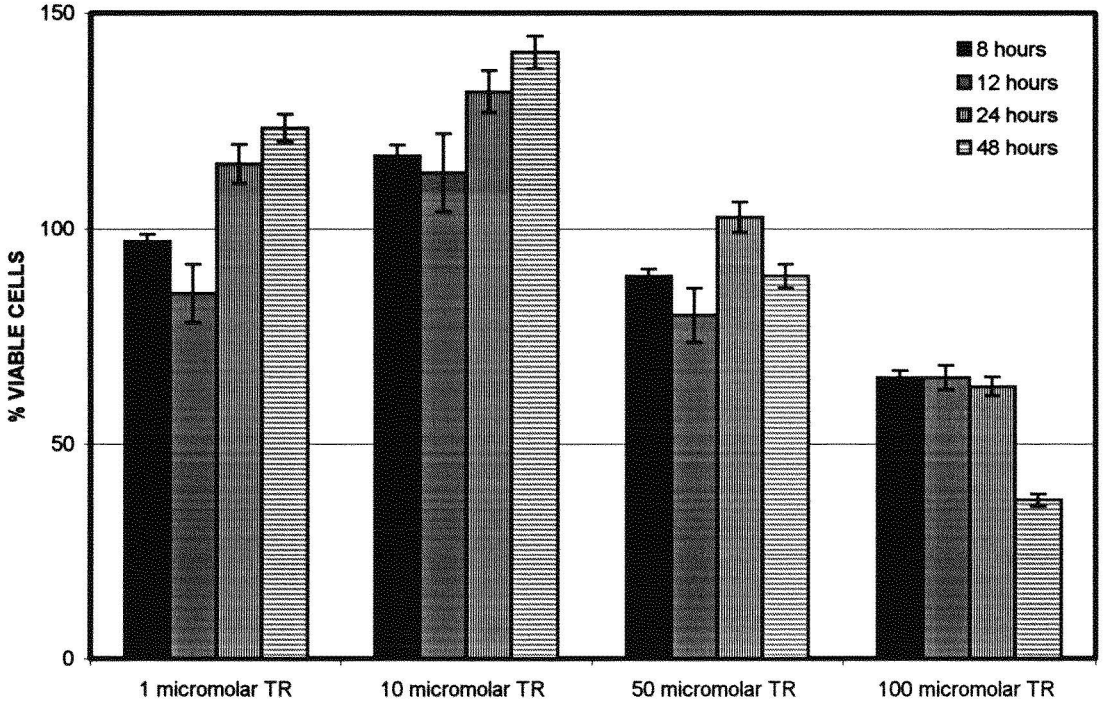

FIG. 9. **Viability assay of DU 145 cells treated with TR in MEM growth media**. TR had a biphasic effect on DU 145 cells. At the lower treatment concentrations (1 and 10 μM), TR increased cellular viability in a time dependent manner, while at the higher concentrations (50 and 100 μM) TR decreased cellular viability.

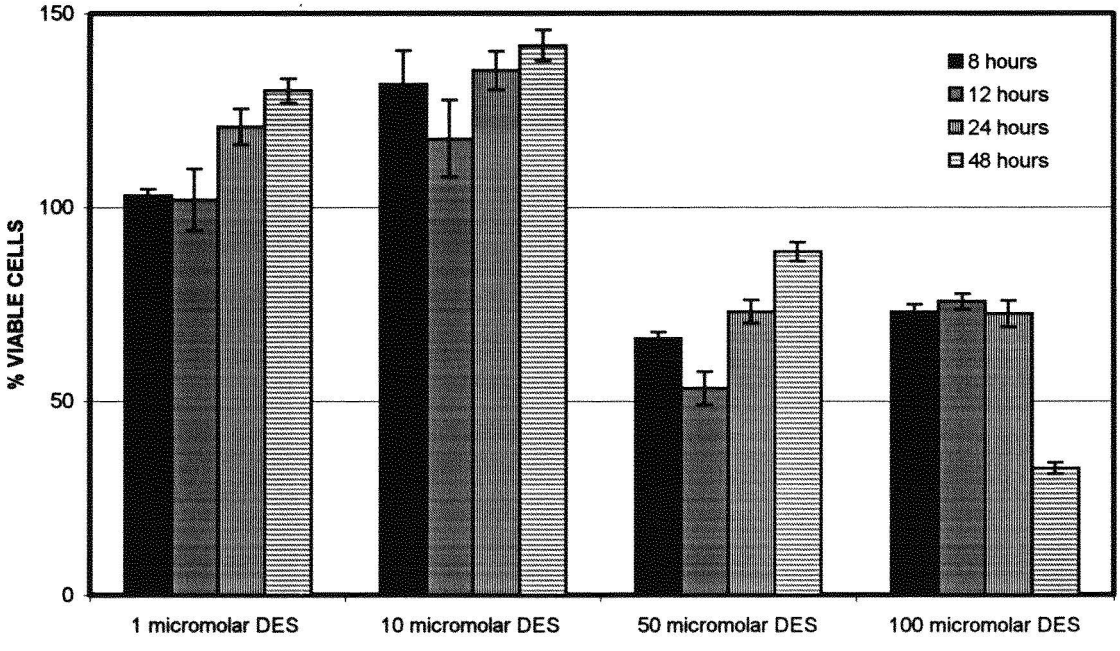

FIG. 10. **Viability assay of DU 145 cells treated with DES in MEM growth media**. DES had a biphasic effect on DU 145 cells, similar to TR. At lower treatment concentrations (1 and 10 μM), DES increased cellular viability in a time dependent manner, but at higher concentrations (50 and 100 μM) it decreased cellular viability.

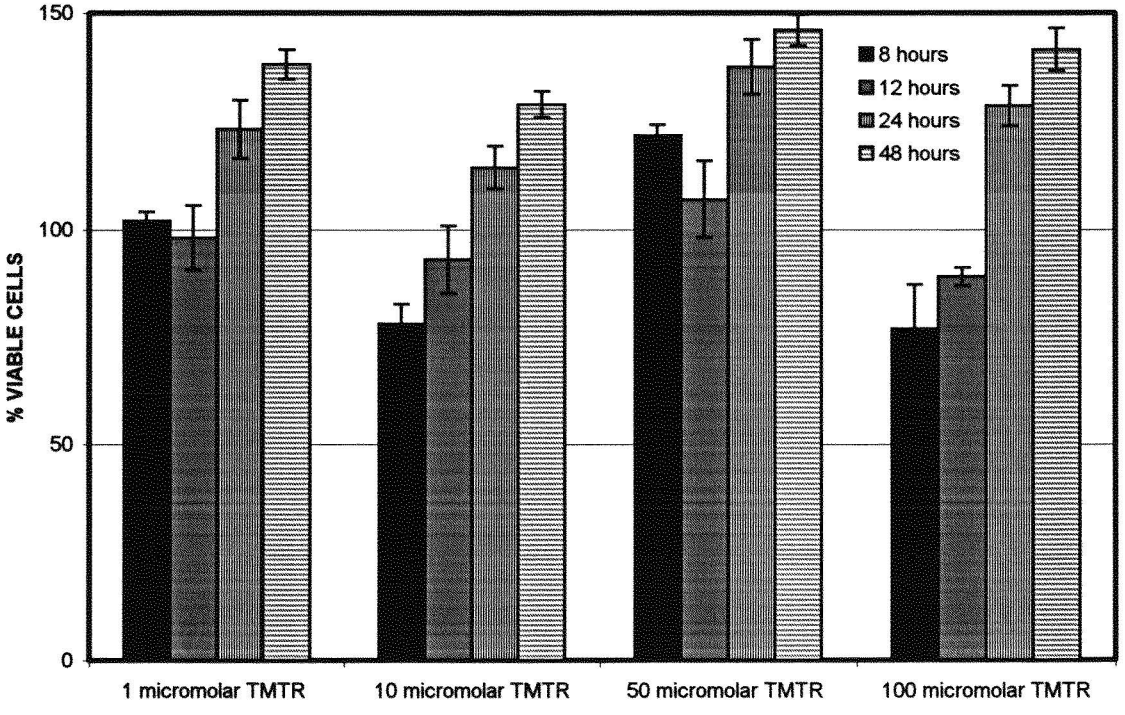

FIG. 11. **Viability assay of DU 145 cells treated with TR in MEM growth media**. TmTR increases cellular viability in a time dependent manner, at all treatment concentrations (1-100 μM). This is in contrast to TR and DES (Figures 9 and 10), which had a biphasic effect on this cell line.

DETERMINATION OF APOPTOSIS IN LNCaP CELLS

Four different events were measured to determine whether apoptosis was taking place in TR treated cells. These included: 1) onset of apoptotic morphology, 2) phosphatidylserine (PS) externalization, 3) caspase activation, and 4) immuno blot analysis using caspase-3 and caspase-7 specific antibodies that detect procaspase (34-35 kD) and the processed fragment (17 kD) from the activated caspases. This chronology looks at events that occur late in a cell undergoing apoptosis first, and then at events to occur relatively early during apoptosis. If a cell exhibits apoptotic morphology it should externalize PS. If the cell has externalized PS there should be activated caspases. All treatments in this section were carried out in CD CHO media.

Apoptotic morphology was scored in LNCaP cells based on the following criteria: 1) dense staining of cells, 2) cell shrinkage, and 3) the absence of cellular debris such as chromatin. Apoptotic morphology was distinguished from necrotic morphology, which included: 1) nucleus present without the cytosol, 2) chromatin spilling from the nucleus, 3) cells with disrupted cytosol, and 4) cellular debris surrounding the cell in question (Figure 12). Quantification of cells exhibiting apoptotic morphology is seen in Figure 13. It can be seen that DES and TR treated cells did not differ from control cells in the number of cells displaying apoptotic morphology at 24 hours. By 48 hours 16% of TR treated cells displayed apoptotic morphology in contrast to 3% in DES.

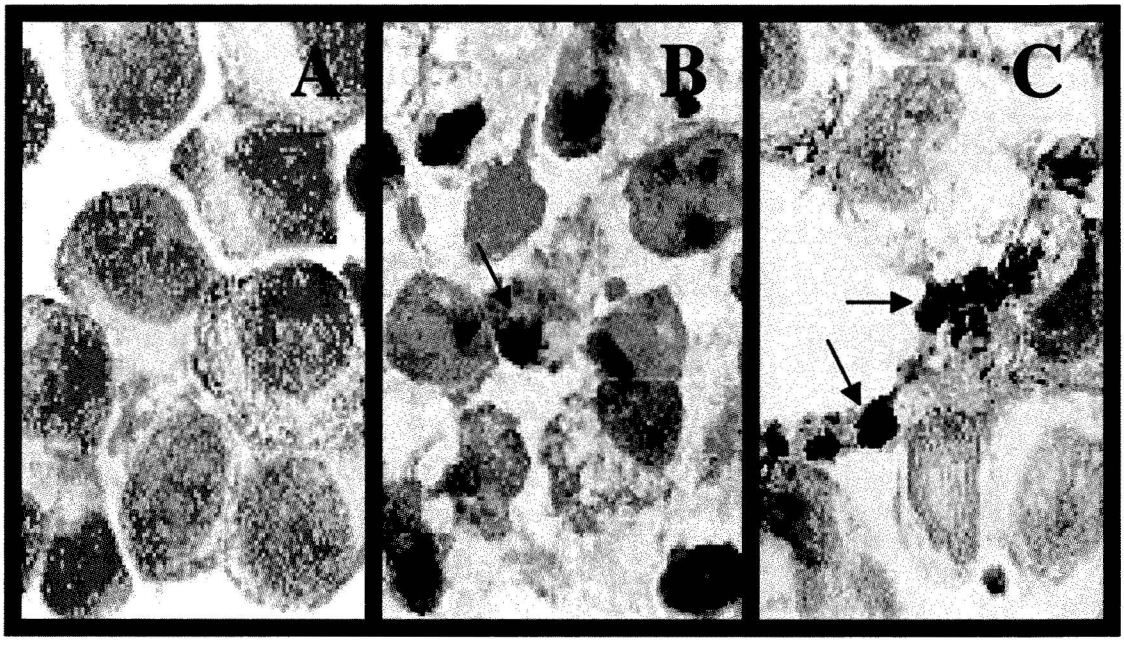

FIG. 12. **Microscopic, morphology assessment of untreated and treated LNCaP cells.** *A*, asymptomatic LNCaP cells, display a large, symmetric nucleus (light purple), surrounded by membrane bound, cytosol (light blue). *B*, arrow points at a necrotic cell, spilling out its chromatin and without a surrounding cytosol. *C*, arrows point at apoptotic cells, cells have shrunk, are densely stained (dark purple), and maintain membrane integrity, as they do not have cellular debris surrounding them.

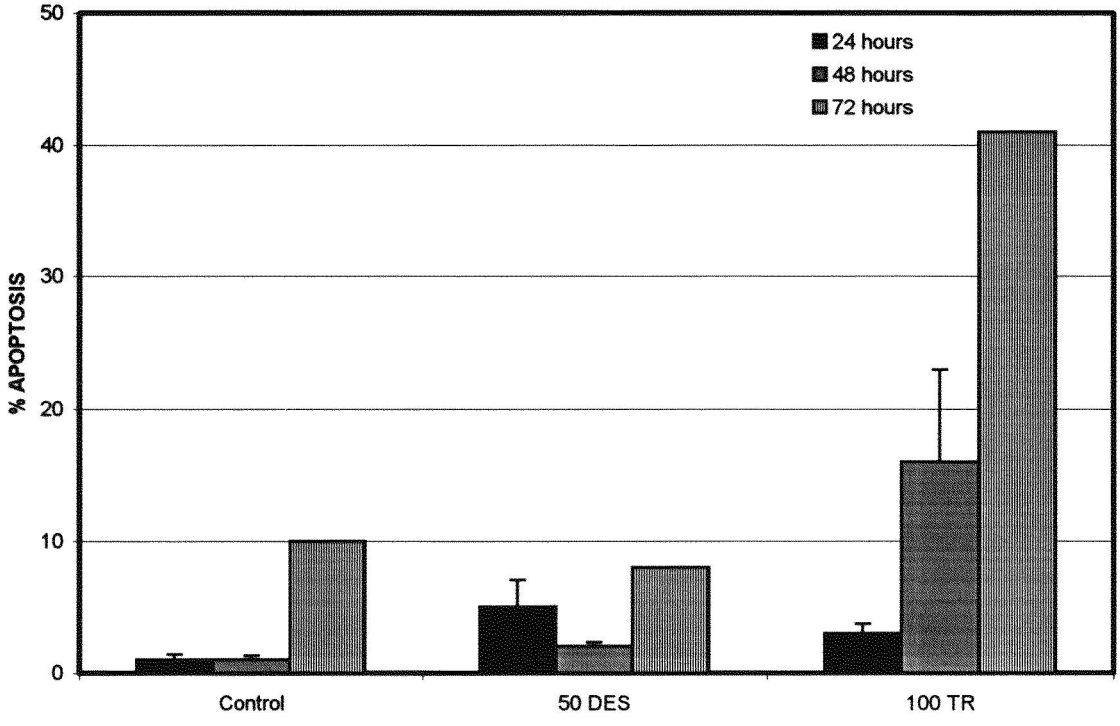

FIG. 13. **Effects of TR and DES on morphology of LNCaP cells**. DES (50 μM) has little effect on morphology of LNCaP cells over 72 hours, as the number of DES treated cells exhibiting apoptotic morphology are similar to those present under control conditions. In contrast, TR (100 μM) induces an increase in the number of cells with apoptotic morphology by 48 and 72 hours.

By 72 hours 40% of TR treated cells exhibited apoptotic morphology in contrast to only 8% in DES treated cells. It is important to note that DES was reported as inducing apoptosis in this cell line (21), yet by 48 hours, at 50 μM DES, the number of cells exhibiting apoptotic morphology were similar to those measured in the control, and five times lower than those in cells treated with TR.

Once the onset of apoptotic morphology was determined in the presence of TR, it was of interest to measure externalizing PS in treated cells. Externalizing PS is an event described as occurring some time after the activation of effector caspases and the expression of apoptotic morphology by the cells (81). In apoptotic cells, externalizing PS occurs while the cell maintains membrane integrity. This was distinguished from necrotic cells, which have lost membrane integrity, with the use of two dyes, annexin V-FITC, which binds PS, and membrane impermeable ethidium homodimer (EthD-1), which binds DNA. Cells that were annexin V-FITC positive, EthD-1 negative were counted as apoptotic cells, indicating binding is occurring on the exterior of the cell. Cells that took up both dyes have lost membrane integrity, and were not counted as apoptotic (Figure 14). Using this technique, a trend similar to the one observed in quantifying apoptotic morphology was measured (Figure 15). There was little difference among the three conditions, control, 50 μM DES, and 100 μM TR, in the first 24 hours. By 48 hours, 28% of TR treated cells had externalized PS, while the event was not detected in DES treated cells. Once again the effects measured in DES treated cells are in contrast with observations by others (21).

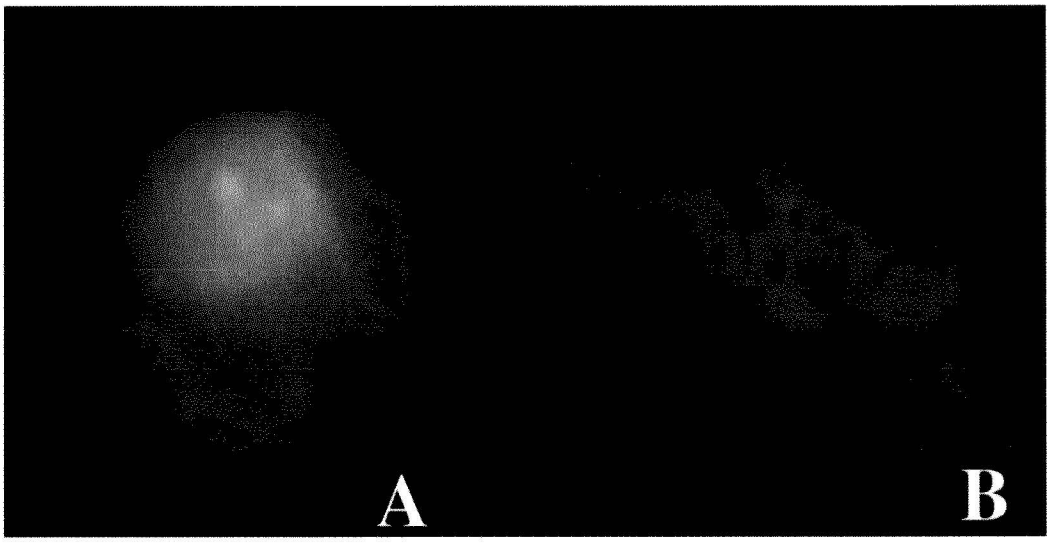

FIG. 14. **Microscopic, phosphatidylserine externalization assessment of untreated and treated LNCaP cells.** *A*, necrotic LNCaP cell, displays fluorescence of two dyes binding, annexin-V FITC (green) binding PS inside the cell, and ethidium homodimer (orange) binding DNA in the nucleus, indicating loss of membrane integrity. *B*, apoptotic cell, displays only annexin-V FITC binding. The absence of ethidium homodimer binding in the nucleus indicates maintenance of membrane integrity.

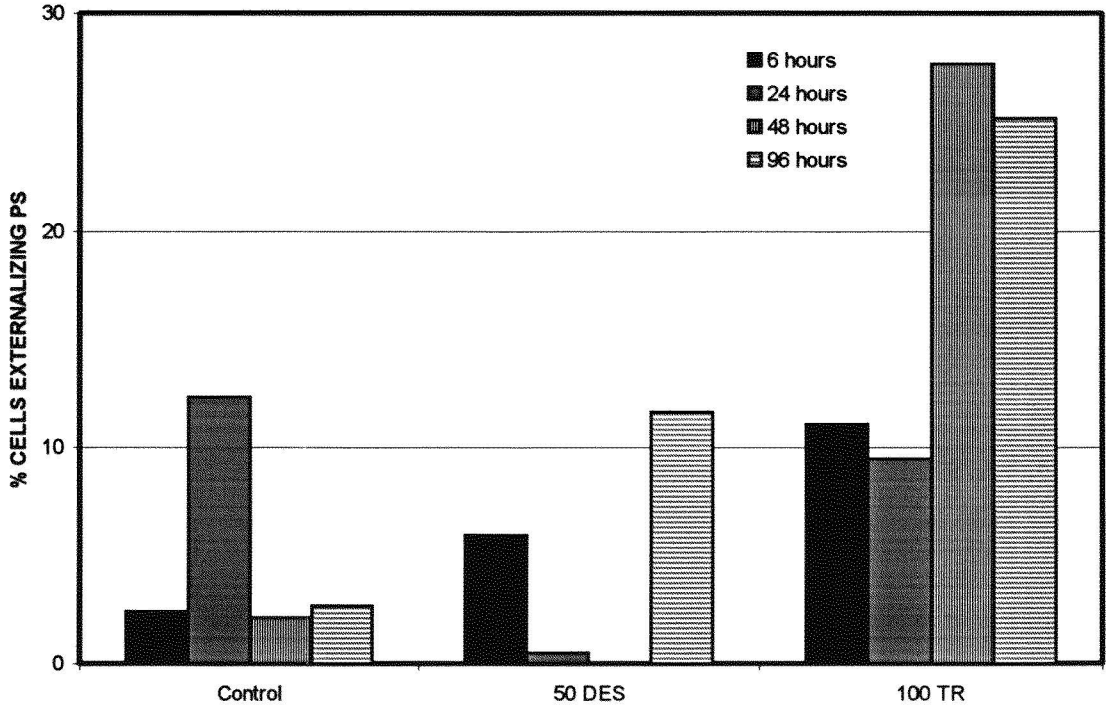

FIG. 15. **Effects of TR and DES on phosphatidylserine externalization by LNCaP cells**. There is little difference between DES and TR treated cells for initial 24 hours. By 48 hours 28% of cells treated with TR (100 μM) are externalizing PS. In contrast, only 12% of DES treated cells are externalizing PS. These levels are similar to those seen in control.

Having determined that morphology and PS externalization were occurring in TR-treated cells, it was of interest to look at another very specific marker for apoptosis, the activation of caspases. Caspase activity in the TR and DES treated cells was measured using an acetylated, tetra-peptide sequence specific substrate for caspase-3 and caspase-7, DEVD, which was conjugate to the flurophore 7-amino-4-trifluoromethylcoumarin. Caspase activity was reported as pmol of substrate cleaved/minutes of incubation/mg protein. Caspase activity in TR and DES treated cells was similar in the first six hours of treatment (Figure 16). But by 12 hours caspase activity in TR treated cells (941 pmol/min/mg) is twice as high as in control cells and nine times that of DES treated cells. Caspase activity in TR treated cells plateaued by 24 hours (1,991 pmol/min/mg), but changed little in DES treated cells (359 pmol/mg/min).

Apoptosis was clearly absent from DES treated LNCaP cells in all three assays used to measure apoptosis, even though it was shown to be toxic to LNCaP cells in the cell viability assays. The absence of apoptosis in the presence of a compound shown to be toxic, suggests that necrosis is taking place in DES treated cells. In contrast, apoptosis was clearly taking place in the presence of TR in all three assays. These analyzed for relatively early (caspase) and late (morphology) apoptotic events. Apoptosis in LNCaP cells is caspase-dependent. However the caspase activity assay did not distinguish between the two caspases, if not both, capable of cleaving the substrate used (DEVD) was being activated.

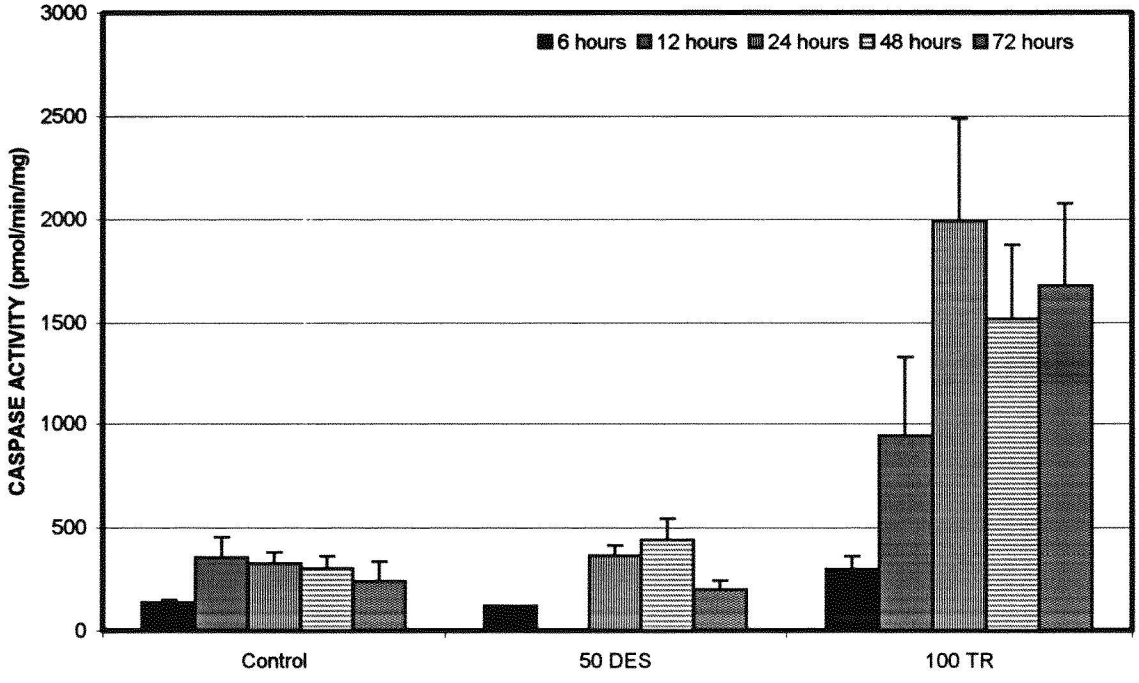

FIG. 16. **Caspase activity in TR and DES treated LNCaP cells**. There is little difference between DES (50 μM) and TR (100 μM) treated cells for initial 6 hours of treatment. By 12 hours caspses activity is three times as high in TR treated cells as in control cells and nine times as high as in DES treated cells. Caspase activity plateaus by 24 hours, yet DES treated and control cells have similar, relatively low, caspase activity.

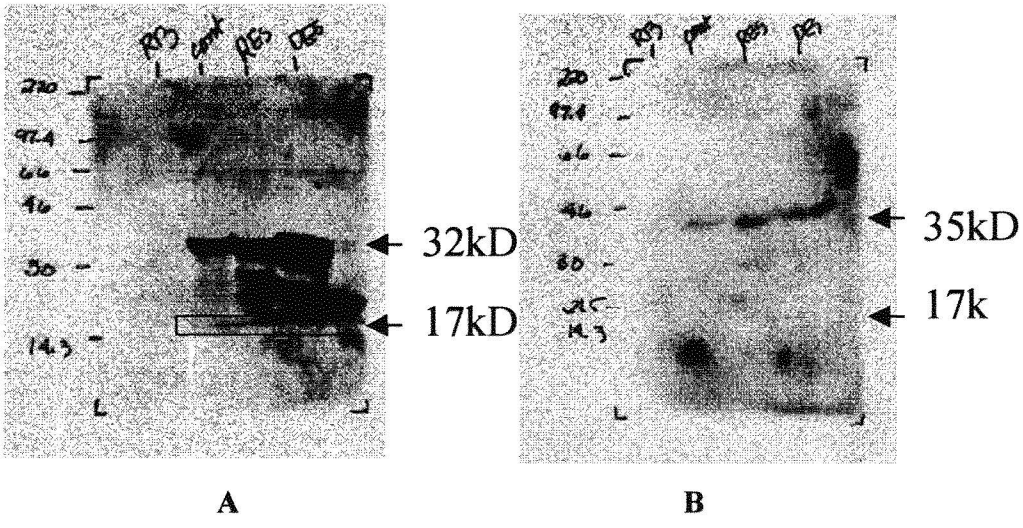

FIG. 17. **Immuno blot determining whether caspase-3 and caspase-7 are present and/or processed in TR and DES treated LNCaP cells**. *A*, Caspase-3 is present (band at 32 kD) in LNCaP cells, and the processed fragment (band at 17 kD) is present, indicating that caspase-3 is activated under all three conditions. *B*, Caspase-7 is present in LNCaP cells, but not processed, as may be seen by the absence of the processed fragment (band at 17 kD).But only the processed fragment for caspase-3 (17 kD) was present, indicating that caspase-3 was the only caspase being activated in the presence of TR.

To answer this an immuno bolt was carried out to measure the presence and activation of caspase-3 and caspase-7, both of which have been shown to be present in LNCaP cells (83). Figure 17 shows that procaspase-3 (32 kD band), panel A, and procaspase-7 (35 kD band), panel B, were both present in the cell line. But only caspase-3 was processed to the active form, as can be seen by the processed fragment at 17 kD (Figure 17,panel A). This, along with the caspase activity assay, indicate that caspase-3, but not caspase-7, is being activated in the presence of TR.

ANALYSIS OF 3-, 4'-, 5-TRIMETHOXYSTILBENE

3-, 4'-, 5- *trans*-trimethoxystilbene synthesis had a 70% yield and was analyzed for purity by measuring its melting point (m.p.), R*f* on thin layer chromatography (TLC), retention time on high performance liquid chromatography (HPLC), and determining it's elemental carbon and hydrogen composition. The melting point range of the product was to be 50.5 - 51.0 °C. This is a significant decrease of the melting point of TR, 253-255 °C (84). The decrease in melting point suggests a loss of hydrogen bonding capability, which would accompany the methylation of TR, and the narrow mp range suggests a pure product. The R*f*, determined as the ratio of distance traveled by compound:distance traveled by solvent front, was found to be 0.7 for TmTR and 0.3 for TR. The difference between these values indicates a loss of polarity in TmTR, as less polar compounds will have higher R*f* values on silica gel TLC plates. This again suggests a loss of the functionality of the hydroxyl groups in TR. TmTR's purity was further confirmed by HPLC analysis of the product, in which only one peak was present in a 90 minute run. TmTR had a retention time of 69.75 minutes, while TR had a retention time of 5.28 minutes. Thus, TmTR is very non-polar relative to TR.

The last method used to confirm the purity of the product obtained in the synthesis of TmTR was carbon and hydrogen percent composition of the product. The product was submitted for elemental analysis and found to be (%): C, 75.22, and H, 6.78. The theoretical calculation for $C_{17}H_{18}O_3$ was (%): C, 75.5 H, 6.70. The relative percent error in the carbon measurement was 0.03% and –0.012% for hydrogen. This is a clear indication that the product is TmTR and that it is pure.

The structure of TmTR was confirmed through spectral analysis using infrared (IR) and nuclear magnetic resonance (NMR) spectroscopy. The regions of interest in the IR analyses were 3000, 2948, 2836, 1590, 1150, 967, 839, 825 cm^{-1}. The product lacked the hydroxyl (OH) absorbance band at 3300 cm^{-1}, present in the spectra of TR. The ^1H NMR chromatogram had the following pattern (ppm/splitting/integration): 3.786/singlet/9H, 6.387/triplet/1H, 6.742/doublet/2H, 6.945/doublet/2H, 7.02/doublet/1H, 7.22/doublet/1H, 7.532/doublet/2H. This assignment is in agreement with the structure of TmTR.

DISCUSSION OF RESULTS

The studies described in this thesis have demonstrated that the structural homologues, DES and TR, were toxic to LNCaP cells, but induced death through different mechanisms. TR induced apoptosis in LNCaP cells, while DES did not. It was also shown that apoptosis in TR treated cells occurred through caspase-3, not caspase-7. It should not be a surprise that TR is toxic to eukaryotic cells, since plants produce TR to kill fungus. What is interesting is that TR selectively kills human, cultured, cancer cells, yet has little or no effect on normal cells (1,45). Further more, TR, at relatively high concentrations, kills these cells through apoptosis, which is a specific and heavily regulated mechanism. There are numerous mechanisms by which TR could induce apoptosis in LNCaP cells. TR has been shown to be a tyrosine kinase inhibitor (47). Tyrosine kinases are important to regulation of the cell cycle. If TR inhibited tyrosine kinase's function it could cause cells that are chronically stimulated to divide to enter cell cycle arrest, which would lead to apoptosis. In one study, it was found that TR induced cell cycle arrest, leading to apoptosis in LNCaP cells (46). This pathway may explain TR selective toxicity in cancer cells, as cancer cells divide at a higher rate than normal cells, and thus would be more susceptible to interruptions in cellular division. TR has also been shown to be an inhibitor of cyclooxygenase and hyperoxidase (44), topoisomerase and DNA polymerase (85). It is very likely that at the concentrations used for treatment in the studies described here, TR is acting through multiple pathways.

Cellular redox state has been suggested to be important in the regulation of apoptosis in murine W 7.2 lymphocyte cells, and the hormone sensitive MCF-7 breast cancer cell line (51). There is a significant amount literature sighting TR as an antioxidant (26,27). This is the mechanism by which TR was proposed to inhibit apoptosis in PC12 cells (38). TR has been shown to be a good antioxidant in studies using a total antioxidant status assay (Table I) (48). TR's antioxidant capability has been attributed to the number of hydroxyl groups it contains, and the presence of the hydroxyl groups on the phenyl rings (Figure 2), which form very stable free radicals. Thus TR can react with free radicals, neutralizing them, and become a stable free radical. If the levels of TR are high enough so that the levels of TR radicals are higher than the cellular antioxidant mechanisms, then TR may begin to induce oxidative stress in the cell leading to apoptosis. The importance of the hydroxyl groups to TR's biological activity was demonstrated with TmTR. The hydroxyl groups of TR were shown to be important to its effect on LNCaP and DU 145 cells at 50 and 100 μM in the comparative viability assays with TmTR (Figures 6, 8, 9 and 11). In TmTR the hydroxyl groups are not available for interaction with active sites of proteins such kinases, free radical scavenging, or interaction with the estrogen receptor three major biological activities for TR. LNCaP cells treated with 100 μM TmTR decreased 48%in viability by 48 hours (Figure 7). In contrast, TR treated LNCaP cells decreased in viability by 80% under the same conditions (Figure 6). This would suggest that the hydroxyl groups are important to the biological activity of TR. Further more, TmTR increased DU 145 cell viability at all treatment concentrations (1-100 μM, Figure 9) while TR decreased cellular viability at 50 and 100 μM.

TR's toxic effect has been shown not to occur exclusively through hormone-mediated pathways. TR has been demonstrated to be toxic to the hormone sensitive prostate cancer cell line, LNCaP. It is equally toxic in DU 145 cells, which do not respond to hormones. This suggests that TR is toxic in a hormone independent manner. This is not a surprise, since the mechanism of action by estrogens used to treat patients with prostate cancer, is indirect, acting through the pituitary (86). Estrogens, such as DES, act, *in vivo*, through a feedback mechanism on the pituitary, suppressing secretion of gonadotropin, thus decreasing the levels of testosterone produced by the Leydig cells of the testes (86). This suppresses the growth of cancer cells that grow in an androgen dependent manner.

LNCaP cells are not a homogeneous cell population. The chromosome counts vary from 84 to 87 within the LNCaP population (18), in contrast normal somatic cells contain 46 chromosomes. LNCaP cells also heterogeneously express the nuclear, pro-apoptotic protein p53 (79). TR may be inducing apoptosis in LNCaP cells that are more susceptible to the mechanism(s) by which TR elicits its biological effect, maybe in LNCaP cells that express p53. TR has been demonstrated to activate p53 and p53 dependent protein transcription in various murine, cancer cell models (42). As apoptosis progresses in the susceptible cells, they shrink, bleb and form apoptotic bodies (50). *In vivo*, these apoptotic bodies are removed rapidly through phagocytosis (50). *In vitro* there are no macrophages to clear the cells, so they eventually undergo necrosis. Necrosis initiated through this pathway is termed secondary necrosis, because necrosis was not induced directly by the treatment conditions. When cells die through secondary necrosis they loose membrane integrity, releasing

compartmentalized matter onto surrounding cells. This matter includes, chromatin, proteases and secondary messengers, such as ATP. Some of these components may trigger neighboring cells to undergo apoptosis or necrosis. Extracellular ATP triggers apoptosis in dendritic cells through purinoreceptors (59) and activate caspases-1, -3, and -8 (87). This would increase the levels of secondary necrosis within the population, amplifying the number of cells undergoing apoptosis. This would agree with the time dependent increase in caspase-3 activation, but is not supported by the morphology assay. When TR treated cells were evaluated for PS externalization, using annexin V-FITC and ethidium homodimer, 14% of the cells were scored positive for necrosis at 6 hours, and 33% at 24 hours (data not presented). These high levels of necrotic cells, in the presence of high levels of apoptotic cells, support some degree of secondary necrosis in these cells.

It was a surprise that DES did not induce apoptosis in LNCaP cells. Loss of cell viability in DES treated LNCaP cells in the absence of apoptosis suggests that cell death may have been through necrosis. There was little difference in the number of cells exhibiting necrotic morphology between TR or DES treatments, in the morphology assay (data not shown) but necrosis has previously been observed in LNCaP cells treated with DES (1), and 33% of the DES treated cells exhibited necrotic morphology by 96 hours in the annexin V-FITC/ethidium homodimer assay (data not shown). A plausible explanation for why apoptosis was not observed in the presence of DES, when DES had previously been demonstrated to induce apoptosis in LNCaP cells (21). The concentration selected from the literature was the highest concentration assessed in the referenced study. At this concentration DES may stress

the cell beyond apoptosis, leading to necrosis. This model of action has been demonstrated in the mitochondria (56). In this model, if the mitochondria is stressed to the point where there is osmotic disequilibrium, the mitochondria's outer membrane swells and ruptures, causing the cell to die through necrosis. If the mitochondria is stressed so that it does not swell and can concomitantly form pores which allow the release of cytochrome c, the cell undergoes apoptosis. Hydrogen peroxide has been demonstrated to elicit this type of effect in cells, where at low treatment concentrations cells undergo apoptosis, and at high concentrations, the cells undergo necrosis (50). DES has the chemical structure of an antioxidant, which may allow it to target the mitochondria, where it may elicit similar effects to hydrogen peroxide in the cells.

The induction of apoptosis by TR poses an interesting question because there is a significant lag time between caspase activation, which occurs by 12 hours (Figure 14) and the presence of apoptotic morphology in the cells, which appears by 48 hours (Figure 11). A reasonable explanation for this observation is difficult to formulate based on the existing body of literature. Even though it is accepted that apoptosis occurs through specific sequences of events, it is does not seem likely that empirical time values could be assigned to each of the specific events occurring during apoptosis. Even though it may be impossible to determine this for all systems, it would be nice to have some time reference, perhaps for the time that transpires between Fas ligands binding, to DNA fragmentation and exhibition of apoptotic morphology. But the duration of apoptosis is going to vary from cell line to cell line and may depend on the number of anti-apoptotic mechanisms (e.g., Bcl-2) a cell type has in place, which

need to be overcome before each of the events during apoptosis can transpire. This would be more pronounced in cancer cells, in which mutations have occurred causing them to be more resistant to apoptosis. Timing will also depend on the type of apoptotic signal given and its target sights. Parvathenani et al. (81) demonstrated that caspase-3 activity was significant by 2 hours, and DNA fragmentation and morphology changes occurred by 4 hours in cyclohexamide treated neutrophils, through a calcium-calmodulin-dependent protein kinase mediated pathway. But neutrophiles are terminally defferentiated, normal cells, destined to die through apoptosis. LNCaP cells, unlike neutrophiles, are cancer cells, which have become resistant to the normal apoptotic signals such as TGF-β and FasL (17,71,88,89), and TR may be interacting with several targets at once, at the concentrations used to treat the cells (100 μM). But, the glucocorticoid, dexamethasone, has been shown to induce apoptosis in murine W7.2 lymphocyte cells through the glucocorticoid receptor, after only 12 hours of treatment (90). W7. 2 cells seem to share this pathway to apoptosis with prostate cancer in rats. Briehl et al. (51) proposed that activation of apoptosis through this pathway causes a decrease in the expression of proteins important to the cells antioxidant defense. This allows for oxidative damage to occur through out the cell, leading to the onset of apoptosis. This is interesting because: 1) it involves the cellular oxidative status, in which TR may play a roll, and 2) the time that transpires between the treatment of W7.2 cells with dexamethasone and the onset of apoptosis are close to the times observed in TR treated LNCaP cells.

CONCLUSION

The implicit question in this thesis is whether TR's presence in foods could prevent prostate cancer through dietary exposure. This is particularly appropriate for prostate cancer, since it is a slow developing cancer (91). But the concentrations of TR found to induce apoptosis in prostate cancer cells are 50-100 times higher than concentrations normally found in food products. Thus, via diet alone, TR may not be effective therapeutically, even though it has been shown to selectively kill cancer cells (45). The levels needed in blood serum to treat cancer may be attained through supplements. But before this is even considered the mechanism by which TR elicits its effects should be further elucidated, and TR's toxicity should be ruled out in normal cells and organ systems in the body. The scientific community has this obligation to the general public. This warning is not to be taken lightly as TR is a structural analogue of DES (Figure 2), and DES was administered to women in the early '50s to help pregnant women carry to term (92,93), but latter DES was found to induce vaginal cancer in those women. It has yet to be shown whether TR can induce the neoplastic transformation of a cell, and this may not be a relevant question if TR were used to treat late stages of prostate cancer. But if TR is to be considered to prevent cancer, this is a very relevant question. TR has the chemical characteristics of an adduct-forming agent. It has aromatic rings that can be oxidized to the very reactive epoxide-reactive intermediates, through monooxygenase pathways in the cell. These could react to form adducts with bases in DNA. TR is also a planar compound, which would allow it to intercalate between bases in the DNA, disrupting regulation and

transcription process in the nucleus. These events have been shown to lead to neoplastic transformation, through multiple hits in the genome. Because of these points it should be reiterated that consumption of TR at high doses, for prolonged periods of time, may not be advisable until it is clear whether TR will have negative effects in cells other than cancer cells.

Here it has been demonstrated that TR is toxic to LNCaP and DU 145 cells, and that its measured effect is not exclusively through a hormone mediated pathway. It has also been demonstrated that TR induces apoptosis in LNCaP cells through a pathway leading to the activation of caspase-3. Finally, it was demonstrated that TR's hydroxyl groups are important to its mechanism of action.

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Employment

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Papers, Presentations and Awards

2000 **Virginia Academy of Science 78th Annual Meeting.**
–"Phytoestrogens and Apoptosis in Human Cancer Cells" **G. Z. Morris (3rd Place Award)**, M. S. Elliott, R. L. Williams, Old Dominion University and S. Beebe, Eastern Virginia Medical School.

2000 **Twentieth Annual Seminar of Cancer Research in Virginia.**
–"Effects of Two Stilbenes on Apoptosis in Androgen-Sensitive Prostate Cancer Cells" **G. Z. Morris** (presenter), M. S. Elliott, R. L. Williams, Old Dominion University and S.Beebe, Eastern Virginia Medical School.
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1999 **Eastern Virginia Medical School Research Day.**
–"Different Effects of Two Stilbenes on Apoptosis in Androgen-Sensitive Prostate Cancer Cells, LNCaP" (poster presentation), **G. Z. Morris**, M. S. Elliott, R. L. Williams, Old Dominion University and S. Beebe, Eastern Virginia Medical School.

1999 **Virginia Academy of Science 77th Annual Meeting.**
–"Naturally Occurring Phytoestrogens and Apoptosis in Human Cancer Cells" **G.Z. Morris** (presenter), R. L. Williams, M. S. Elliott, Old Dominion University and S. Beebe, Eastern Virginia Medical School.

1999 **American Association for Biochemistry and Molecular Biology.**
–" Resveratrol: a naturally occurring phytoestrogen as a tool to study apoptosis" (poster presentation), **G. Z. Morris (minority travel award)**, R. L. Williams, R. C. Morris, M. S. Elliott, Old Dominion University and S. Beebe, Eastern Virginia Medical School.
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