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A Nanoemulsion Formulation of Tamoxifen Increases Its Efficacy in a Breast Cancer Cell Line

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Abstract: This paper reports on the preparation of a water-soluble nanoemulsion of the highly lipid-soluble drug tamoxifen (TAM). In addition, relative to a suspension of TAM, the nanoemulsion preparation demonstrated a greater ζ potential (increased negative charge) which has previously been associated with increasing drug/membrane permeability. This study also reports that relative to suspensions of TAM with particle sizes greater than 6000 nm, nanoemulsions of TAM, having mean particle sizes of 47 nm, inhibited cell proliferation 20-fold greater and increased cell apoptosis 4-fold greater in the HTB-20 breast cancer cell line. Thus, this work suggests that a nanoemulsion compared to a suspension preparation of TAM increases its anticancer properties relative to breast cancer.

Keywords: Nano-emulsion; tamoxifen; breast cancer; cell proliferation; apoptosis

Introduction

Breast cancer is a malignant growth that begins in the tissues of the breast which over the course of a lifetime results in one in eight women being diagnosed with one of several types of breast cancer. For example, ductal carcinoma, which begins in the cells lining the ducts that bring milk to the nipple, accounts for more than 75% of breast cancers. Another breast cancer type, lobular carcinoma, begins in the milk-secreting glands of the breast but is otherwise similar in its behavior to ductal carcinoma. Alternatively, other varieties of breast cancer can arise from the skin, fat, connective tissues, and other cells present in the breast. Breast cancer is the most common nonskin cancer in women and the second most common cause of cancer-related deaths in U.S. women. An estimated 215 000 new breast cancer diagnoses and 40 000 breast cancer deaths occurred in 2004. Greater than 570 000 deaths were expected in the United States in 2005, with currently one in four deaths in the United States due to cancer[1] with an estimate of more than 1 000 000 new cases and 370 000 deaths annually worldwide.[2]

Tamoxifen (TAM), a widely used lipid-soluble drug considered as first choice in chemotherapy and prevention of estrogen receptor-positive breast cancer,[3] inhibits proliferation and induces apoptosis of breast cancer cells by estrogen receptor-dependent modulation of gene expression.[4] TAM is a nonsteroidal agent with potent antiestrogenic effects in animal and in vitro models.[5] The drug's pharmacologic properties are related to its ability to compete with estrogen for estrogen receptors in breast tissue and to inhibit the stimulatory effect of estrogen for tumor growth. However,

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patient response to this chemotherapeutic drug is often associated with significant problems in efficacy, bioavailability, and toxicity. The long list of adverse effects of TAM includes but are not limited to causing cancer of the uterus, strokes, blood clots in the lungs, abnormal vaginal bleeding, pain or pressure in the pelvis, leg swelling or tenderness, chest pain, shortness of breath, tingling or numbness of the face, arms, or legs, sudden confusion, difficulty speaking or understanding, sudden difficulty seeing in one or both eyes, dizziness, loss of balance or coordination or sudden severe headache, and increased risk of developing cataracts. Despite these numerous adverse effects, TAM continues to be used to (a) treat breast cancer that has spread to other parts of the body in men and women, (b) treat early breast cancer in women who have already been treated with surgery, radiation, and/or chemotherapy, (c) reduce the risk of developing a more serious type of breast cancer in women who have had ductal carcinoma in situ (DCIS) and who have been treated with surgery and radiation, and (d) reduce the risk of breast cancer in women who are at high risk for the disease due to their age, personal medical history, and family medical history.[4–9] Thus, delivery systems such as but not limited to nanoemulsions that increase the water solubility of lipid-soluble drugs may be particularly advantageous.

In an attempt to overcome some of these adverse effects and increase its efficacy, a nanoemulsion system has been developed for delivery of TAM. Nanoemulsions are a class of stable emulsions formed by a monolayer of phospholipids composed of surfactant and vegetable oil suspended in water with a particle diameter usually less than 100 nm.[10–12] The stability of nanoemulsions make them extraordinary, and they are often referred to as "Approaching Thermodynamic Stability".[13] It has been suggested that emulsion systems offer an appealing substitute for the formulation of poorly soluble drugs such as paclitaxel and amiodarone.[14] Compared to typical suspension preparations which can be thousands of nanometers in size, nanoemulsion delivery systems with particle sizes in the hundred nanometer range or less have been shown to increase bioavailability and efficacy of a number of compounds such as anti-inflammatory agents,[15,16] insulin,[17–19] tetanus toxoid,[20] and Dicumarol.[21]

Nanoemulsion delivery systems are fast becoming fundamental approaches for innovative strategies in the prevention and treatment of cancer. Nanoemulsion delivery systems which are particularly germane to this communication can (1) convert fat-soluble to water-soluble compounds, thereby allowing delivery into a polar versus nonpolar matrices potentially reducing toxicity and (2) reduce particle sizes of existing drugs that are usually 1000's of nm in size to less than 100 nm size, possibly for more in-depth and longer penetration. During the production process, we are suggesting that the surface/volume ratios of the nanoemulsions containing the drug are increased raising the bioavailability and efficacy of pharmaceuticals, as reported previously.[14–20] Support for increased efficacy of nanoemulsion delivery substances has also recently been reported from our laboratory, which demonstrated that nanoemulsions produced by the high sheer forces during microfluidization and containing an antioxidant synergy formulation (ASF), dramatically reduced tumor size in a neuroblastoma-bearing xenograft mouse model compared to a suspension formulation of ASF.[22]

Materials and Methods

1. Micro- and Nanoemulsion Preparations of TAM. TAM was administered in a range of 0.4–0.8 mg/kg/animal. For cell culture-based experiments, 0.37 mg/mL of TAM

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was used to prepare a 10^{-3} M stock solution, and 100 mL of 3×10^{-5} M was used for all cell culture treatments. A suspension containing 260 mg of TAM was prepared by first dissolving it in ethanol and adding it to soybean oil and Polysorbate 80 in a ratio of 1 and 4% for tissue culture and mouse injections (see the Supporting Information), respectively. The mixture was then homogenized (Polytron model PT 10/35, Brinkmann Instruments, Westbury, NY) in either HPLC-grade water and/or cell culture medium for 60 s at 25 °C. The nanoemulsion formulation of TAM generated from the suspension was prepared using the microfluidizer processor model M-110 EH (Microfluidics Corp., Newton, MA). Microfluidizer processors provide high pressure and a resultant high shear rate by accelerating the product through microchannels to a high velocity for size reduction to the nanoscale range. The fluid is split in two and is then pushed through microchannels with typical dimensions on the order of 75 μm at high velocities (in the range of 50–300 m/s). As the fluid exits the microchannels it forms jets which collide with jets from opposing microchannels. In the channels, the fluid experiences high shear (up to 10×7 L/s), which is orders of magnitude greater than other conventional high sheer force/stress technologies. Jet collisions result in mixing in the submicron level. Therefore, high shear and impact are responsible for particle size reduction and mixing of multiphase fluids in the microfluidizer technology.

The viscosities of the nanoemulsion formulations were measured using a Ubbelohde viscometer (VWR International, Boston, MA) at 25 °C. For measurement of the mean droplet size and polydispersity index (width of the particle size distribution), a dynamic laser light scattering Malvern Nano-S instrument (Malvern Instruments, Inc., Southborough, MA) capable of measuring particle sizes between 0.6 and 6000 nm was used (Figures S1 and S4, Supporting Information).

2. ζ Potential. The measurement of ζ potential is based on the following principle: colloidal particles of TAM dispersed in soybean oil (density 0.917 g/mL) and Polysorbate 80 (density 1.064 g/Ll) solutions are electrically charged due to their ionic characteristics and dipolar attributes. Each particle dispersed in the solution using the Malvern Zetasizer nano series Zen 3600 (Malvern Instruments Ltd., Enigma Business Park, Grovewood Road, Malvern, Worcestershire WR14 1XZ, U.K.) is surrounded by oppositely charged ions called the fixed layer. Outside the fixed layer, there are varying compositions of ions of opposite polarities, forming a cloudlike area. This area is called the diffuse double layer, and the whole area is electrically neutral. When a voltage is applied to the solution in which the particles are dispersed, particles are attracted to the electrode of the opposite polarity, accompanied by the fixed layer and part of the diffuse double layer, or internal side of the "sliding surface. This system uses dispersion technology software (DTS v4.20), which changes to the ζ potential measuring mode and, the sample for which ζ potential is measured, is taken in disposable capillary cuvettes (DTS1060) equipped with electrodes. Each determination is done in triplicate Figures S1 and S4, Supporting Information).

3. Cell Culture Line, Growth Conditions, and Reagents. The human culture breast tumor line HTB-20 was obtained from the American Type Culture Collection (ATCC, Manassas, VA). HTB-20, a ductal breast carcinoma from a 60 year old Caucasian female, was propagated in an ATTC complete growth medium of 90% Hybri-care medium (Modified Dulbecco's medium) and 10% fetal bovine serum (FBS) formulated from Dulbecco's modified Eagle's medium with 4 mM of L-glutamine. In addition, the cell culture media composition was adjusted to contain 1.5 g/L of sodium bicarbonate, 4.5 g/L of glucose, and 1 mM sodium pyruvate, supplemented with 1.2 mM oxaloacetic acid, 0.01 mg/ml of bovine insulin, and 10% (v/v) NCTC 135 medium. All cells were cultured at 37 °C in 5% CO_2. Reagents such as ammonium phosphate (monobasic), ammonium hydroxide, triethylamine, methanol, acetonitrile (HPLC grade), ethyl acetate, NaOH−glycine, hexane, isopropyl alcohol, phosphoric acid, hydrochloric acid, TAM, propranonol, and 3-methylxantine (internal standard, I.S.) were purchased from Sigma (St. Louis, MO). Water was obtained from a Millipore Milli-Q plus water purification system (Bedford, MA).

4. Cell Proliferation Assay. Treated cells were harvested by trypsinization every 2–3 days and were counted using a Beckman Coulter Z1 Series particle counter (Beckman Coulter, Inc., Fullerton, CA). Cell proliferation was expressed as percentage of the untreated or control cells ± SE.

5. Apoptosis Assay. Cells were plated in duplicate on glass coverslips on small 35 mm plates to about 30% confluency. The cells were incubated at 37 °C and 5% CO_2 in a completely supplemented plus 10% FBS-containing media. Three days after reaching 60−70% confluency, the cells were treated with equal concentrations but different preparations of TAM (empty nanoemulsion, suspension of TAM and nanoemulsion of TAM). The cultures were incubated with the appropriate drug preparations for 72 h at 37 °C, 5% CO_2, and in vitro DAPI and VYBRANT assays (from Upstate Biotechnology, Lake Placid, NY) for apoptosis detection were performed. Determination of the percentage apoptotic cells and the percentage of necrotic cells was made using the following formula: percent apoptotic cells = (number of apoptotic cells with and without loss of membrane integrity/total cells counted) × 100.

Statistical Methods

Values in duplicate of pooled data were analyzed, and significant differences for all parameters measured were determined by student's "t-test" with $p < 0.05$ established for statistically significant differences.

Results

1. Nanoemulsion Preparation, ζ Potential, and Particle Size. The highly lipid soluble suspension of TAM became water soluble upon preparation of the nanoemulsion of TAM.

For these studies, the particle size Z-average of the TAM suspension was greater than 6000 nm, the upper limit of detection for the Malvern particle size analyzer, and subsequently reduced to 47 nm after microfluidization (Figure 1).

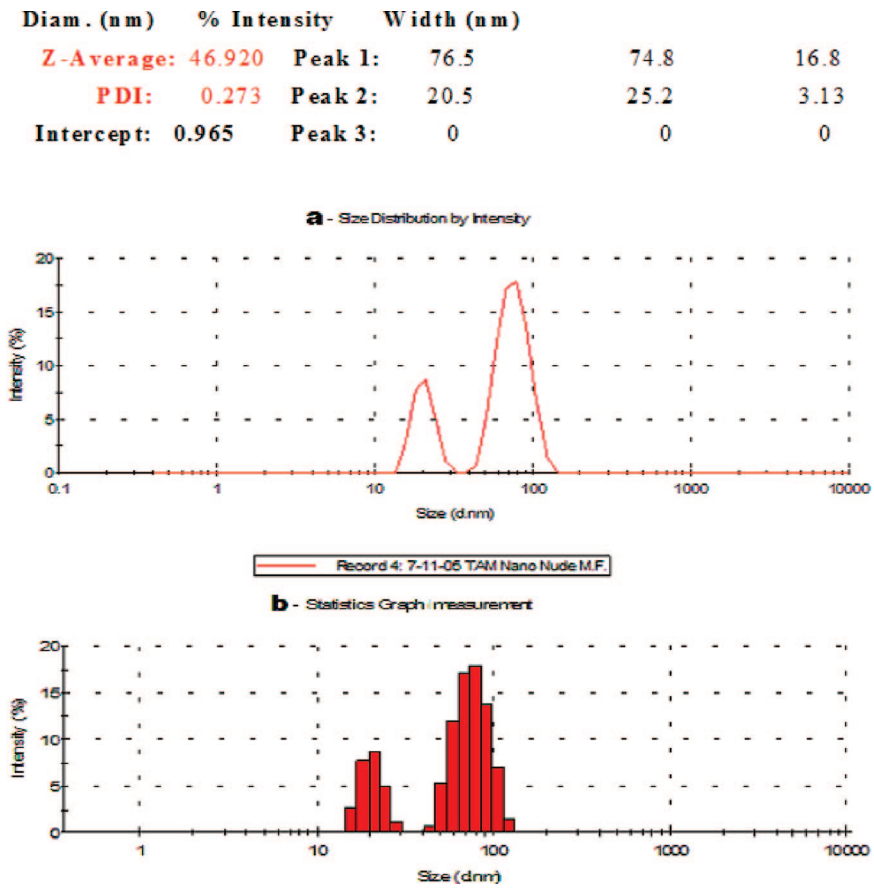

Figure 1. Dynamic laser light scattering particle size analysis of Nano Tam showing (a) the Z-average size distribution of the particle and (b) the statistics graph measurement by model distribution. As shown in parts a and b, microfluidization results in a dramatic decrease in particle size (a) and also demonstrates the heterogeneity of particle size even within what appears to be a homogeneous distribution (b).

Table 1. Composition, Physical, and Chemical Properties of Tamoxifen and Formulations[a]

formulation	composition	PDI	size (nm)	ζ potential (mV)	average mean
empty nano	SO + P80	0.284	145	0.304	0.304
microemulsion	SO + P80 + TAM	0.366	2800	−0.517	−68.00
nanoemulsion	SO + P80 + TAM	0.273	46	−4.570	−81.90

[a] SO = soybean oil, P_{80} = polysorbate 80, TAM = tamoxifen.

As noted in Table 1, the interaction of the drug with the process of emulsification dramatically increased ζ potential up to 11-fold and the average mean 223-fold, while in addition, the nanoemulsions further increased the ζ potential 9-fold and the average mean 1.2-fold relative to the suspension of TAM (Figures S1 and S4, Supporting Information).

2. HTB-20 Cell Proliferation Assay. As described in Figure 2 and Table S1 (Supporting Information), relative to the empty nanoemulsion preparation, the suspension of TAM and the nanoemulsion of TAM reduced cell proliferation by 66% ($p < 0.040$) and 97% ($p < 0.025$), respectively.

3. HTB-20 Apoptosis Assay. Chromatin condensation, nuclear shrinkage, and formation of apoptotic bodies are easily observed under fluorescence microscopy using the Vybrant assay showing the high morphologic features typical of apoptosis such as the formation of apoptotic bodies containing chromatin fragments (Figure 3a) and DAPI assay showing the appropriate staining of nuclei with DNA-specific fluorochromes (Figure 3b). An early observation concerning apoptosis from the Vybrant assay was that cells entered apoptosis from non mitotic parts of the cell cycle and showed dramatic and characteristic changes in nuclear shape and organization (Figure 3a). In addition to changes in nuclear morphology, loss of DNA integrity also characterizes apoptosis (Figure 3b).

Compared to the nanocontrol, the % increase in apoptosis for the nano- and microsuspension preparations of TAM was approximately 40- and 10-fold greater, respectively (Figure 4 and Table S2, Supporting Information). The increase in % apoptosis for the nanoemulsion of TAM was 4-fold greater compared to the suspension of TAM.

4. Xenograft. To determine the in vivo efficacy, mice were inoculated subcutaneously with viable HTB-20 cancer cells and then treated once daily by IM for almost 3 months with the drug TAM and their formulation (0.4−0.8 mg kg-1 day-1) after growth of the cancer. From the five groups of mice xenograft study and because of the metastasis tumor, we evaluated the results in mortality rate. The death rate of the negative control group was evidently higher than that of any other groups ($P < 0.05$), of course, because experimental animals were not treated. More severe pathology was

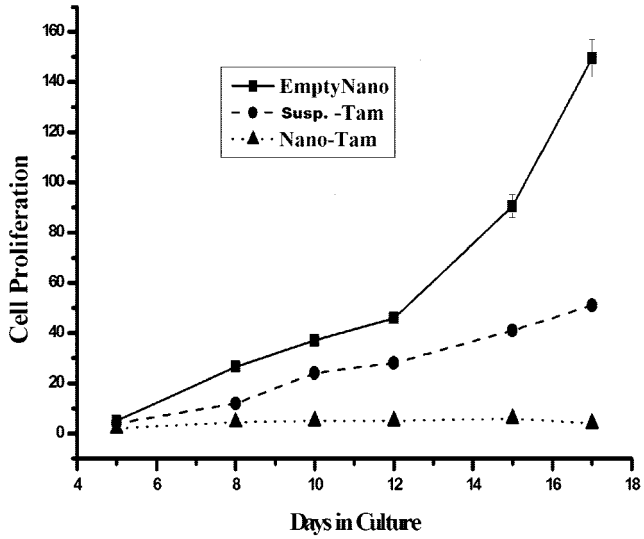

Figure 2. Results of cell proliferation assay showing that relative to the empty nanoemulsion control, the suspension and nanoemulsion preparations of TAM reduced cell proliferation 66% and 97%, respectively. Error bars represent the standard error of the mean for experiments done in duplicate over the 18-day period. At every time point measured, relative to the empty nanoemulsions, both the micro- ($p < 0.04$) and nanoemulsion preparations of TAM ($p < 0.025$) significantly reduced cell proliferation, $p < 0.02$. Relative to the suspension preparation of TAM, the nanoemulsion preparations of TAM significantly reduced cell proliferation, $p < 0.01$.

developed in those mice. However, incidence and mortality were almost the same among animals with aggressive tumors treated with a topical application of TAM, while mortality from cancer decreased for animals treated with our topical application of nano-TAM and TAM suspension successively. About 93% of mice treated with our nanoformulations of the drug TAM recovered.

Discussion

The mechanism(s) of action of TAM, similar to other anticancer agents, involves interfering with cell cycle progression[23,24] or generating DNA strand breaks, consequently affecting cell division and/or DNA synthesis.[25,26] The use of conventional delivery vehicles for chemotherapeutic agents such as a TAM suspension in the treatment of cancer have several disadvantages. First, the cells may develop resistance to the chemotherapeutic agent because not all cells receive an initially lethal dose due to nonuniform distribution. Such resistance results either in the requirement for higher dosages of the drug and/or the renewed spread of the cancer.[27,28] Alternatively, cellular resistances may result from biochemical metabolism of the anticancer agent or a functional resistance whereby the cell remains unaffected in the presence of the agent.[27,29]

It is well-established that conventional administration vehicles may also result in chemotherapeutic agents being toxic to the patient causing more pain and discomfort than the disease itself. Therefore, there is a practical upper limit to the amount of anticancer agent that a patient can receive. However, when a chemotherapeutic agent is delivered by a vehicle with enhanced cellular permeability, the dosage of any single drug may be lowered. This is especially beneficial to the patient since using lower levels of chemotherapeutic agents is generally safer and associated with fewer adverse side effects for the patient. The reports that nanosuspensions are advantageous because they can deliver a wide range of dosages would support this notion.[30,31]

The increased water solubility of our nanoemulsion preparations of TAM would also be expected to reduce adverse side effects.[9] Additionally, when anticancer agents are delivered under conditions of enhanced membrane permeability, cancer cells are less likely to generate resistance because a greater percentage of the cancer cell will be killed upon initial exposure. It is well-known that human solid tumors, as in breast cancer, remain resistant to most therapeutic agents, even when used in various drug combinations by inducing resistance to apoptosis.[32] This may also be due to the fact that extremely high pressure in the interstitial matrix of the solid tumor retards the passage of chemotherapy molecules across the vessel wall and into the interstitial matrix.[33] Although in our studies there were not sufficient animals at the appropriate time points to measure pharmacokinetics, the nanoemulsion of TAM, because of its

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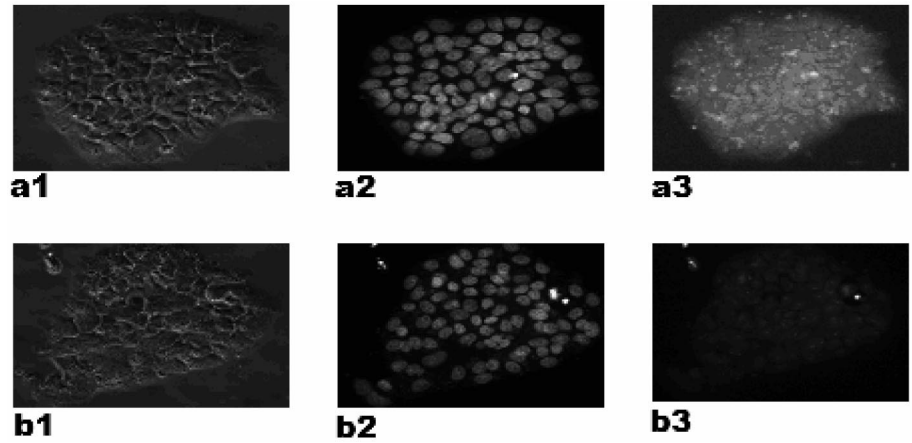

Figure 3. (a and b) Chromatin condensation, nuclear shrinkage, and formation of apoptotic bodies are easily observed under fluorescence microscopy using Vybrant assay showing the high morphologic features typical of apoptosis such as the formation of apoptotic bodies containing chromatin fragments and DAPI showing the appropriate staining of nuclei with DNA-specific fluorochromes. Visualization of the compacted stage of the chromatin and the nuclear bind DNA morphology, respectively, by Vybrant (a1–a3) and 4,6-diamidino-2-phenylindole (DAPI) (b1–b3) staining in HTB-20 breast cancer cells after 24 h nano-TAM treatments to detect apoptosis. Parts a1 and b1 represent normal visualization of treated cells, parts a2 and b2 show distinctive apoptotic cells, respectively, after vibrant and DAPI, and parts 3a and 3b represent the different pattern of staining between vibrant a3 showing the formed protein/DNA complexes and DAPI b3 showing only visualization of the nuclei.

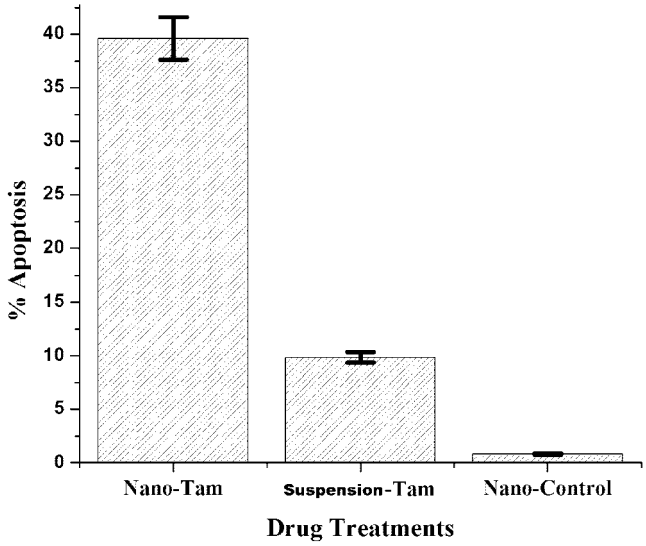

Figure 4. Effect of tamoxifen alone, a nanoemulsion preparation of tamoxifen, and empty nanosphere on HTB-20 breast cancer cell line after 48 h exposure at a concentration of 3×10^{-5} M each. Compared to the nanocontrol, the percent increase in apoptosis for the nano and suspension preparations of TAM was approximately 40- ($p < 0.01$) and 5-fold ($p < 0.01$) greater, respectively. The increase in percent apoptosis for the nanoemulsion of TAM was 4-fold greater compared to the suspension of TAM ($p < 0.01$.

reduced particle size, may have increased bioavailability upon intramuscular injection by quickly entering the blood stream and being released in the vicinity of the tumor cells for a longer time as recently reported.[34,35] The decline in mortality can be attributed to this increase in bioavailability and the release process of the drug. There are other studies including our own[22] to suggest that particle size can affect the efficacy of TAM, i.e., the smaller the particle size, the greater the surface to volume ratio and presumably the greater the efficacy.[36,37]

There is also recent evidence to suggest that there may exist an association between greater ζ potential (negative charge of the particle) and the degree of penetration through biological membranes.[38] For example, nanoemulsions, which in this report are dispersions of oil in water, are more stabilized by a negative ζ potential which prevents droplet coalescence upon random collisions of particles resulting in repulsion forces which can stabilize the preparation.[38] Our finding of a greater ζ potential for the nanoemulsion of TAM (Table 1) compared to the nanocontrol and the suspension of TAM would appear to support these observations. Also, it has been reported that anionic nanoparticles (NP) at higher concentrations significantly affect the blood−brain barrier process.[38] This report also indicated that the advantages of a NP system include but are not limited to (a) masking the role that

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the blood−brain barrier plays in limiting permeability of a therapeutic drug molecule, (b) having the fastest rate of drug absorption,[39] and (c) having slower drug release in the brain, thereby decreasing peripheral toxicity.[40] The bioavailability of a nanoemulsion, by any route of administration, ultimately depends on the dissolution of the drug and the temporal rate of apoptosis. The finding that the anticancer response in this study using the nanoemulsion of TAM in cell culture occurred in less than 24 h of treatment would suggest a relatively fast rate of cellular uptake of the drug.

The purpose of the fluorescent Vibrant and DAPI assays are to simultaneously measure apoptotic cells, necrotic cells, normal (nonapoptotic/non-necrotic) cells, and apoptotic cells that have undergone secondary necrosis. 4′,6-Diamidino-2-phenylindole (DAPI) is known to form fluorescent complexes with natural double-stranded DNA, showing fluorescence specificity for adenine−thymine (AT), adenine−uracil (AU), and inosine−cytosine (IC) clusters. Because of this property, DAPI is a useful tool in various cytochemical investigations such as apoptosis.[41,42] The observation that when DAPI binds to DNA its fluorescence is strongly enhanced has been interpreted to mean that a highly energetic and intercalative type of interaction exists, allowing us to visualize nuclei[43] (Figure 3b), while the Vybrant assay provides us with a rapid and convenient method, based upon strict fluorescence detection, of the compacted state of the chromatin in apoptotic cells. An early observation concerning apoptosis was that cells treated with the nanoemulsion of TAM entered into more apoptosis from nonmitotic parts of the cell cycle and showed dramatic and characteristic changes in nuclear shape and organization (Figure 3a). In addition to changes in nuclear morphology, loss of DNA integrity also characterized apoptosis (Figure 3b). It may be appropriate to speculate that the characteristic change in nuclear morphology is the most accurate indicator of the involvement of apoptosis in the death of a cell, even though programmed cell death is a normal phenomenon that occurs most of the time in the turnover of adult tissue.[44]

Using these two assays, compared to the nanocontrol, the percent increase in apoptosis for the nano and suspension preparations of TAM was approximately 40- and 10-fold greater, respectively (Figure 4). The increase in percent apoptosis for the nanoemulsion of TAM was 4-fold greater compared to the suspension of TAM. The finding of increased apoptosis, particularly with the nanoemulsions of TAM, is especially important in light of the reports that increased programmed cell death may be a significant mechanism(s) of action for anticancer drugs.[45–47]

In addition, as seen in Figure 2, relative to the empty nanoemulsion preparation, the suspension of TAM and the nanoemulsion of TAM reduced cell proliferation by 66% and 97%, respectively. The ability of an anticancer drug to decrease cell proliferation is an obvious necessary characteristic for efficacy.[48–50] We are proposing that one of the mechanisms of action of the nanoemulsion of TAM is to effectively compete more with estrogen for ER binding, leading to greater inhibition of cell cycle progression and G1 arrest of cancer cells.

In conclusion, our new nanoemulsion formulations of TAM may have the potential to reduce the incidence and severity of breast cancer due to their permeability enhancement (increased ζ potential), increased water solubility. and greater bioavailability attributed to decreased particle size.

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Supporting Information Available: Description of xenograft model, cream formulation, and preparation of xenograft animals and treatment groups. This material is available free of charge via the Internet at http://pubs.acs.org.

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