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Tamoxifen Resistance?

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13. ABSTRACT (Maximum 200 Words) The development of resistance to tamoxifen is a pressing issue in breast cancer management, as this typically results in poor prognosis and an increased chance of patient relapse. Tamoxifen is metabolized by the cytochrome P450s, and these products may then be further metabolized to glucuronide conjugates, which can ultimately be eliminated from tumor cells by transport proteins including MRP1 and MRP3. We hypothesized that we could use specific enzyme inhibitors of the metabolizing and transport enzymes to increase tamoxifen concentrations in MCF7 breast cancer cells. The results demonstrated that several of the inhibitors could indeed reduce the amount and type of tamoxifen metabolites formed. In addition, one of the inhibitors increased the amount of tamoxifen retained in the cells. These compounds may be good candidates to design adjuvant therapies for breast cancer treatment.				
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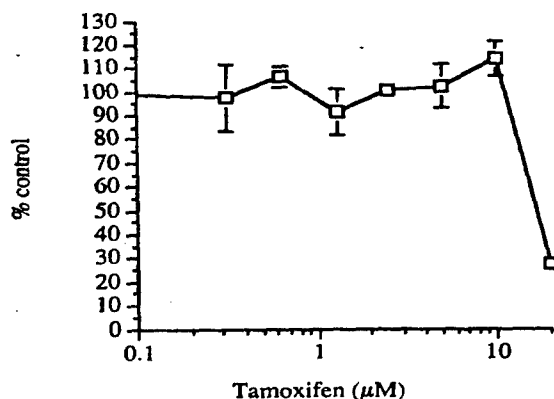
INTRODUCTION:

The question of whether breast tumors will acquire drug resistance to chemotherapeutic agents such as tamoxifen is a pressing one, as this will result in poor prognosis and increasing chance of patient relapse. Prolonged use of tamoxifen typically results in the loss of estrogen responsiveness in tumors, which can render tamoxifen useless as a treatment. The reasons for this loss of responsiveness are unclear, but since mechanisms such as down-regulation or mutations in the estrogen receptor are not found in the majority of tumors, alternative mechanisms must be investigated. Confounding estrogen non-responsiveness is a lowering of the tamoxifen concentration in the tumor. We proposed to examine the metabolism and transport of tamoxifen in MCF7 breast cancer cells, and determine whether specific enzyme inhibitors can increase the concentration of tamoxifen in these cells. The results of this study will help investigators and clinicians understand the underlying causes of tamoxifen resistance and in the future, may aid in the use of adjuvant therapies for treating breast cancer.

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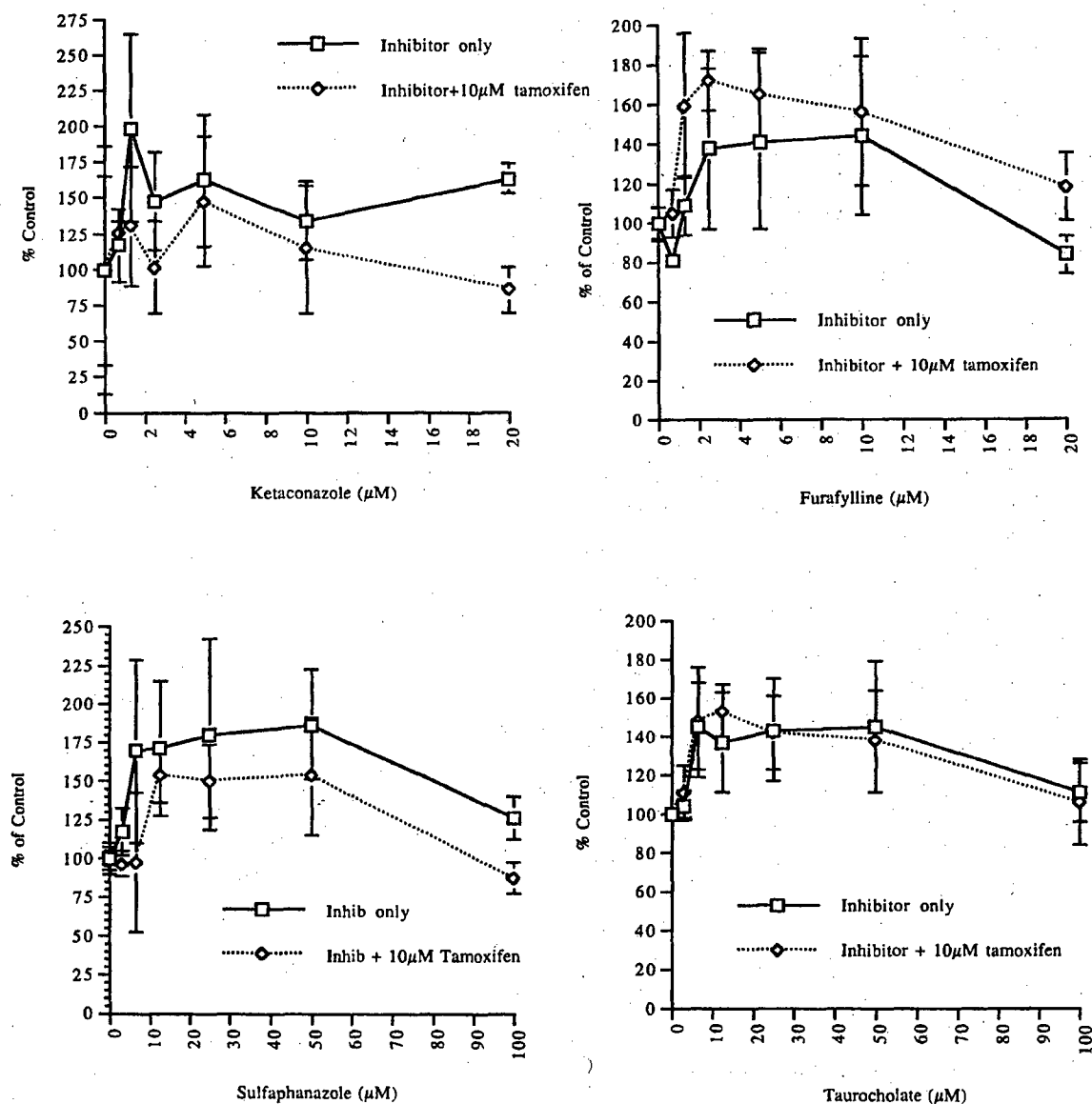
The goal of objective 1 was to determine whether inhibitors of the cytochrome P450s and the transporters MRP1 and MRP3 could increase the amount of tamoxifen remaining in MCF7 breast cancer cells. We employed two techniques to test this objective: cell viability assays and tamoxifen accumulation assays. For the cell viability assays, MCF7 breast cancer cells were incubated with tamoxifen at 10 μ M, which is the highest concentration tested that did not cause cell death compared to control cells (Figure 1) and increasing concentrations of the inhibitors for three days. All concentrations of the inhibitors were chosen based upon preliminary experiments to determine the highest amount of inhibitor that would not cause a significant increase in cell death. For all assays, cellular viability was assessed using 3-(4,5-dimethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) dye, which is converted into a brown colored dye only by active mitochondria (Cory, Owen et al. 1991). Initial experiments surprisingly demonstrated that using cell culture medium containing 10% dextran-charcoal stripped serum, which is done to remove hormones such as estradiol, did not impact cellular viability with tamoxifen or any of the inhibitors. Therefore, we decided to use only regular FBS supplemented medium for the remainder of the cellular viability assays.

Figure 1: Viability of MCF7 cells treated with tamoxifen



Incubation of MCF7 cells with 10 μ M tamoxifen and increasing concentrations of the inhibitors ketaconazole (CYP3A4) or sulfaphenazole (CYP2C9) (Baldwin, Bloomer et al. 1995) reduced cellular viability compared to cells incubated with the inhibitor alone (Figure 2). Incubation of taurocholate (MRP3) (Hirohashi, Suzuki et al. 2000) with tamoxifen did not alter cell viability while interestingly, incubation with furafylline (CYP1A2) (Kunze and Trager 1993) alone decreased viability slightly more than coincubation with 10 μ M tamoxifen. However, it should be noted that the variability in these assays was very high. We therefore decided to pursue tamoxifen accumulation assays.

Figure 2: Cell viability after incubation with inhibitors +/- 10 μ M tamoxifen



The second set of experiments we performed involved determining whether the inhibitors altered tamoxifen accumulation in MCF7 cells. Using the concentrations of inhibitors determined from the cell viability assays, 8×10^5 cells were plated in 6-well plates and allowed to attach overnight. In the morning, the medium was removed and fresh medium containing tamoxifen ($10 \mu\text{M}$) with or without the appropriate inhibitor, in both fetal bovine serum-supplemented medium or with dextran-charcoal stripped serum (to remove hormones such as estradiol), was added. Each assay was performed in triplicate.

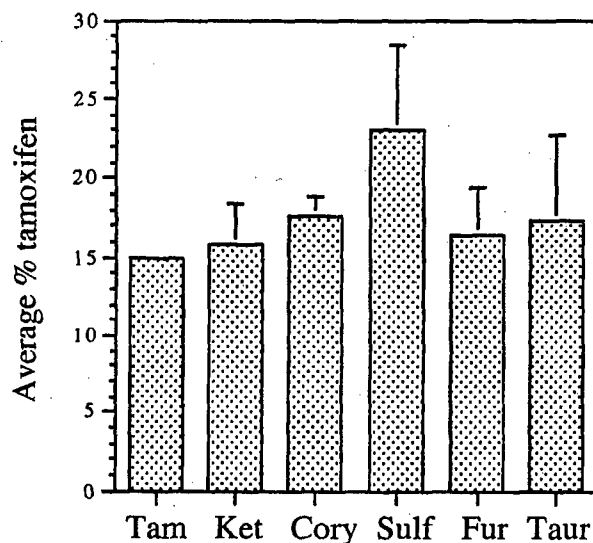
This set of assays was initially a bit problematic as the detection method we were attempting to use was not sensitive enough. We tried using HPLC with an ultraviolet wavelength detector to determine concentrations of tamoxifen and its metabolites (MacCallum, Cummings et al. 1997). Using stock solutions of $10 \mu\text{M}$, $1 \mu\text{M}$, and $0.1 \mu\text{M}$ tamoxifen, we could easily detect the lowest concentration of $0.1 \mu\text{M}$. However, $10 \mu\text{M}$ 4-hydroxytamoxifen gave us absorbance values of the same magnitude as $0.1 \mu\text{M}$ tamoxifen. We could barely detect $1 \mu\text{M}$ 4-hydroxytamoxifen as a stock solution in ethanol. When we incubated MCF7 cells in $10 \mu\text{M}$ tamoxifen for 0, 2, 4, 8, 24, or 48 hours we could detect tamoxifen accumulation in the cells, could detect what we believe to be desmethyl tamoxifen, but could not detect any other metabolites of tamoxifen. Thus, we switched to incubating the MCF7 cells with [^3H]tamoxifen and separating the metabolites by thin-layer chromatography (TLC) using a solid phase extraction technique (Kupfer and Dehal 1996) for both the cells and the cell culture medium (MacCallum, Cummings et al. 1997). Preliminary experiments with $10 \mu\text{M}$ [^3H]tamoxifen alone suggested that a 4-hour incubation would be the most appropriate time point.

We incubated the MCF7 cells with $10 \mu\text{M}$ [^3H]tamoxifen with or without $25 \mu\text{M}$ ketoconazole, $50 \mu\text{M}$ sulphaphenazole, $12.5 \mu\text{M}$ furafylline, $100 \mu\text{M}$ corynanthine (CYP 2D6) (Rane, Liu et al. 1995), $50 \mu\text{M}$ taurocholate, or $25 \mu\text{M}$ dihydrocapsaicin (CYP2E1) (Surh, Lee et al. 1995). At the end of the four hours, the medium was removed and placed into test tubes. The cells were then washed twice with 1 mL ice-cold phosphate buffered saline (PBS) to remove any unbound radioactivity, and the cells were then scraped up into 1 mL PBS, homogenized with 15 strokes of a Dounce-homogenizer and placed into test tubes. Solid phase extraction was performed to remove particulate matter and medium, the extracts were evaporated and resuspended in $50 \mu\text{L}$ ethanol. The suspension was spotted onto normal-phase TLC plates containing a pigment for UV detection. Plates were chromatographed in 80% chloroform/20% methanol/0.5% ammonium hydroxide and visualized under UV light to determine the location of the metabolites. Based upon previous studies, the first metabolite from the origin is tamoxifen N-oxide, the second is desmethyltamoxifen, the third is 4-hydroxytamoxifen, and the fourth is tamoxifen (Kupfer and Dehal 1996). We were able to compare the locations of both 4-hydroxytamoxifen and tamoxifen itself to authentic standards.

The average percentage of tamoxifen retained in the cells was approximately 14% of the total radioactivity. Sulphaphenazole increased the percentage to 23%, although due to variability, this was not statistically significant (Figure 3). Although none of the other inhibitors caused an increase in the amount of tamoxifen remaining in the cells, they did alter the amount and type of metabolites formed. Tamoxifen N-oxide is produced by the flavin monooxygenases while demethylation is catalyzed by CYP3A4 (Mani, Gelboin et al. 1993) (Jacolot, Simon et al. 1991) and CYP1A (Simon, Berthou et al. 1993) in humans, and by CYP1A, CYP2C, and CYP3A in

rats (Mani, Gelboin et al. 1993). 4-hydroxytamoxifen formation appears to be catalyzed predominately CYP2D6 (Crewe, Ellis et al. 1996), although indications are such that CYP3A4 and 2C9 play a role as well.

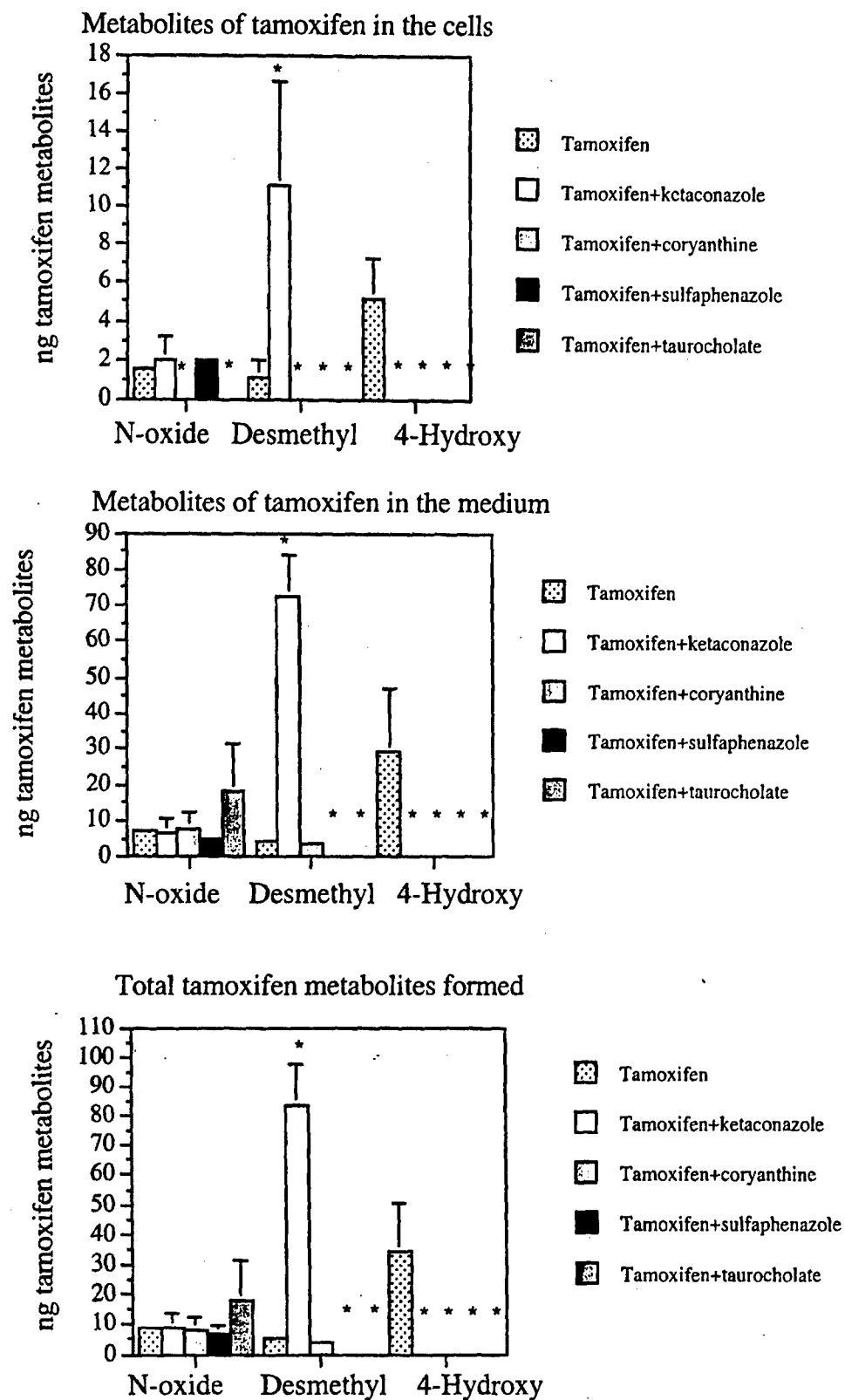
Figure 3. Percentage of tamoxifen retained in MCF7 cells



Although the primary route of tamoxifen metabolism is N-demethylation (Lim, Yuan et al. 1994), 4-hydroxytamoxifen is a much more potent estrogen antagonist (Jordan, Collins et al. 1977). However, 4-hydroxytamoxifen is also the metabolite that appears to contribute most to the formation of DNA adducts. We therefore hope that if we can decrease N-demethylation, we can reduce metabolism of tamoxifen.

Figure 4 shows the amount of N-oxide, desmethyl, and 4-hydroxytamoxifen in the cells, in the medium, and the total amount of metabolites formed. Both sulphaphenazole (CYP 2C9) and taurocholate (MRP3) significantly inhibited desmethyltamoxifen formation while ketaconazole (CYP3A4) significantly increased its formation. Therefore, because sulphaphenazole decreased desmethyltamoxifen formation while increasing the amount of tamoxifen retained in the cells, this may be a good compound to further examine in adjuvant therapy. Not shown in figure 4 is furafylline (CYP1A2), which inhibited the formation of all of the metabolites. Although the inhibition of desmethyltamoxifen is not surprising, the inhibition of 4-hydroxytamoxifen is, as its production is not supposed to be catalyzed by CYP1A. Even more interesting is the loss of tamoxifen N-oxide, which should be catalyzed by a different enzyme family altogether. Perhaps furafylline is not as specific of an inhibitor as currently believed. However, the fact that this compound can eliminate tamoxifen metabolism deserves further investigation. The other compound not depicted in figure 4 is dihydrocapsaicin, which inhibited all accumulation of tamoxifen in the cells. This obviously would not be a good compound to pursue for adjuvant therapy.

Figure 4. Metabolites of tamoxifen in MCF7 cells and in the cell culture medium



We did not complete objectives 2 or 3 of this study. Objective 2 was to prepare radiolabeled tamoxifen metabolites for use in objective 3, which was to examine the transport of the metabolites by MRP1 and MRP3. From the studies above, the inhibitor of MRP3, taurocholate, did not increase the retention of tamoxifen in the cells and therefore, MRP3 probably does not play a major role in tamoxifen elimination. In addition, during the principal investigator's move from Clemson University to the University of Texas at El Paso, the two transfected cells lines that overexpressed MRP1 and MRP3 were rendered useless. Although we are performing the transfections again, the cells are not ready to use at the current time. However, the results from this study suggest that at least two of the compounds, sulphaphenazole and furafylline, may have use as potential adjuvant therapies.

KEY RESEARCH ACCOMPLISHMENTS:

- Sulphaphenazole and taurocholate significantly inhibited the formation of desmethyltamoxifen
- Sulphaphenazole increased the amount of tamoxifen retained in MCF7 cells
- Furafylline completely inhibited tamoxifen metabolism, although this did not increase the retention of tamoxifen in MCF7 cells

REPORTABLE OUTCOMES:

- Part of the work was accomplished by two undergraduate students, Christian Kettlehut and Flor Lozano, a minority student. Both performed the assays as part of the requirements for the course Biol 4398-Special Problems in Biology.
- This research is being used as preliminary data to apply for an IDEA award through the Breast Cancer Research Program.

CONCLUSIONS:

Sulphaphenazole significantly inhibited desmethyltamoxifen formation while increasing the amount of tamoxifen retained in the cells. Therefore, this may be a good compound to further examine in adjuvant therapy. Furafylline inhibited the formation of all tamoxifen metabolites, which is surprising since it should be a CYP1A2-specific inhibitor. The fact that this compound can eliminate tamoxifen metabolism deserves further investigation. Both of these compounds were developed, tested, and even used in humans as drugs. Sulphaphenazole was developed as an antibiotic (one of the sulfa drugs). It was used to treat skin and venereal chancroids in developing countries, although the drug was found to be less effective than other commonly used antibiotics, including streptomycin, trimethoprim, and erythromycin (Kumar, Sharma et al. 1990), and there were issues with drug-drug interactions between it and tolbutamide, an antidiabetic drug (Komatsu, Ito et al. 2000). Furafylline, a trisubstituted xanthine was developed as an asthma medication (Segura, Garcia et al. 1986), although in early trials, patients consuming large amounts of caffeine-containing beverages decreased the elimination of both the caffeine and furafylline (Tarrus, Cami et al. 1987). As some of the pharmacokinetics of these two drugs are known, they might make ideal candidates to study tamoxifen adjuvant therapy.

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