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Our research is directed at determining the specific metabolic pathways and the individual reactions affected by estrogen and TAM in both ER+ and ER- cells through the use of novel NMR methodology and quantitative analysis. In this report period, we have identified the primary metabolic pathways that are operative in ER+ cells. This was accomplished using a comprehensive metabolic model and NMR data from cell extracts. We have also performed preliminary studies necessary for the design and development of HFBR experiments. Investigations to characterize TAM and estrogen rescue response in ER+ cells were performed first in 96-well plates to determine simple dose response. Microcarrier culture, flask, and roller bottle studies were performed in order to demonstrate the ability and further develop methodology used to quantify breast cancer cell metabolism under varied treatment conditions. Metabolic fluxes were calculated for TAM-treated cells using NMR data from cell extracts and our metabolic model.

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INTRODUCTION

Breast cancer, the most prevalent cancer among women, has proven to be a devastating disease. The pain of being diagnosed with the disease is compounded by the lack of a definitive treatment. Currently, the most widely used post-operative hormonal therapeutic is the antiestrogen tamoxifen (TAM). TAM acts at least in part as a competitive inhibitor of estrogen, a hormone which signals cell growth in breast cells. Although effective in postmenopausal patients with estrogen receptor-positive (ER+) tumors, its effectiveness in premenopausal patients and in patients with estrogen receptor-negative (ER-) tumors is substantially lower. Additionally, TAM resistance and cancer recurrence strikes almost all patients with advanced breast cancer undergoing TAM therapy[1]. The serious limitations of this current treatment highlight the need for a greater understanding of both estrogen and TAM action.

A consequence of anti-cancer therapy is an alteration of carbon flows through metabolic pathways. Changes in metabolic fluxes allow cells to provide cellular components to accommodate increased growth. In human breast cancer cells, increased growth and consequent metabolic changes are initiated by estrogen binding to the estrogen receptor. Conversely, TAM is a cytostatic drug which appears to compete with endogenous estrogen for binding to the ER, preventing communication of the growth signal. The effect of this broken communication on metabolism is presently not known. The proposed research uses novel methodology to identify the specific metabolic pathways and the individual metabolic reactions affected by estrogen and TAM in both ER+ and ER- cells. The metabolic effects of these agents, manifested as changes in metabolic fluxes, will be quantified with the aid of *in vivo* NMR techniques. This will enable a description of the relationship between primary metabolism and growth of cells in response to estrogen and TAM treatment to be developed. This new information will help guide the development of new therapies and new therapeutic agents for arresting the metabolism of breast cancer cells and tumors. The specific purposes stated in the original proposal were as follows:

- *Identify the primary metabolic pathways that are operative in ER+ and ER- cells.* This will be achieved by *in vivo* ^{13}C NMR tracer studies and extracellular measurements of ER+ and ER- cells grown in a hollow fiber bioreactor (HFBR).
- *Quantify the primary and secondary metabolic fluxes in breast cancer cells in the presence and absence of TAM and estrogen, including those effects which may be reversed by increased concentrations of estrogen ("estrogen rescue"), and those effects of TAM that are not related to ER binding.* Flux and label balance equations will be developed for carbon pathways. Using both extracellular assays and *in vivo* NMR measurements of cells grown in an HFBR, these equations will be solved to determine metabolic fluxes in response to estrogen and TAM treatment. Fluxes through branchpoints, indeterminable by extracellular measurements alone, can be quantified using this methodology.
- *Examine modes of estrogen and tamoxifen action by investigating the relationship between fatty acid synthesis and the pentose phosphate pathway, and other pathways requiring $NADP^+$/NADPH.* We will examine the effects of impaired fatty acid synthesis (resulting from the addition of the fatty acid synthase inhibitor, cerulenin) on other metabolic pathways in the presence of estrogen and tamoxifen.

In this report period, we have made significant progress toward the first two goals. We have performed preliminary studies necessary for the design and development of HFBR experiments. We have identified the primary metabolic pathways that are operative in ER+ cells using a refined metabolic model and NMR data from cell extracts. Additional experiments to determine the metabolic requirements of ER+ cells have been performed in microcarrier culture. Investigations to characterize TAM and estrogen rescue response in ER+ cells were performed first in 96-well plates to determine simple dose-response relationships. Microcarrier culture, flask, and roller bottle studies were performed in order to develop and demonstrate the ability to quantify breast cancer cell metabolism under varied treatment conditions. Metabolic fluxes were calculated for TAM-treated cells using NMR data from cell extracts and our metabolic model.

BODY

A. Methods and Procedures

1. General Methods

Cell Cultures. Human breast cancer cell lines T47D and MCF7 were obtained from American Type Culture Collection and maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 5% fetal bovine serum (FBS) at 37°C in a humidified atmosphere with 5% CO_2. Confluent Falcon tissue culture flasks were subcultured weekly following trypsinization with 0.05% trypsin in saline /ethylaminediaminetetraacetic acid (EDTA). Cell counts were obtained using a Coulter Multisizer or Neubauer hemacytometer.

Glucose, Lactate, and Amino Acid Measurements. Glucose and lactate concentrations were determined using a Yellow Springs Instrument Co. 2700 Select Biochemistry Analyzer. Amino acid concentrations were determined using an Applied Biosystems 130A high pressure liquid chromatography system (HPLC) and 420A derivatizer equipped with a 920 data module.

Roller Bottle Culture. Cells were grown in 850 cm^2 Falcon or Corning roller bottles. After seeding at 15,000 - 20,000 cells/cm^2, the bottles were rotated at 1/3 RPM for twenty-four hours and at 1 RPM thereafter[2]. The headspace of the bottles was purged with 5% CO_2 in air.

2. TAM Dose Response in 96-well Plates

Cells were seeded at 5,000-6,000 cells/well (15,625-18,750 cells/cm^2) in 100 µl of media. Twenty-four hours after seeding (day 0), cells were administered various TAM concentrations or a vehicle control (0.1% absolute ethanol). Cells were again fed TAM-containing media on day 3, and cell number determinations were made on day 6. Cell numbers were determined using a crystal violet assay described previously[3]. Dose-response (percent final cell number in response to a given concentration of TAM) was reported as percent of control. For estrogen rescue experiments, cells were fed varied estrogen(E2)/tamoxifen-containing media on day 6, and cell determinations were made on day 9.

3. TAM Effects in Microcarrier Cultures

Cells were seeded in spinner flasks containing 0.67 g/L Cytodex 3 microcarriers (Pharmacia) and 50,000-60,000 cells/ml (15,625-18,750 cells/cm^2). Cultures were initiated in 2/5 of the final culture volume and stirred for 2 minutes every half hour for the first five hours. After this time, cultures were brought up to a final volume of 500 ml and stirred constantly at 60 RPM. Culture media was replaced

with TAM or vehicle control-containing media 24 hours after initiation (day 0) and on day 3. Samples were removed daily for determination of cell number. Three samples were removed for each time point. Released nuclei stained with crystal violet were counted on a hemacytometer[4]. Media samples were also removed regularly for glucose, lactate and amino acid measurement. Cell pellets were prepared for cell cycle analysis using flow cytometry. Flow cytometry measurements of propidium iodide-stained nuclei were obtained using a Coulter EPICS-XL flow cytometer..

4. Cell Extraction

Cells were removed from either bottles or flasks by trypsinization and then pelletted by centrifugation at 1200 RPM for 5 minutes. Cell pellets were treated with 1ml 5% perchloric acid (PCA) per 250µl of cell volume. Cell solids were removed by centrifugation and the supernatent neutralized with 250µl of 2M K_2CO_3. Precipitated potassium perchlorate was removed and the extract dried by lyophilization[5]. Extracts were stored at -4°C and rehydrated in 500 µl of D_2O just prior to NMR analysis.

Using extraction to characterize the intracellular isotope distribution and subsequently determine intracellular flux has been questioned because of concerns that extraction affects the chemical make-up of the isotope-containing metabolites. However, preliminary measurements indicate that the major metabolites observable in breast cancer cells by ^{13}C-NMR are the amino acids glutamate and aspartate, both of which are unaffected by acid extraction.

There are many advantages to the extraction NMR method that make it a powerful preparatory step to *in vivo* NMR experiments. High concentrations of intracellular aspartate and glutamate can be attained that produce very informative spectra on a high powered 500 MHz spectrometer. In these spectra, isotopomers, or multiply labeled species, can be discerned. A measured isotopomer distribution can be used to increase the accuracy of the calculated fluxes. In addition, numerous flasks or bottles can be grown simultaneously prior to extraction. Running different treatments and controls concurrently allows efficient comparison of treatments and verification of metabolic consequences.

5. Metabolic Response to TAM Treatment Using 1-^{13}C NMR and Cell Extraction

Eight Corning T150 culture flasks were seeded with T47D cells at a density of 18,000 cells/cm^2. This initial time point was designated day zero. Samples of the culture media were taken every 12 hours to determine the concentrations of glucose, lactate, and the amino acids. On day one, media containing 3µM TAM was added to four of the flasks. The other four flasks received 0.1% (v/v) ethanol as vehicle control. At the same time, the media added to all four flasks contained 1-^{13}C glucose. On day three the experiment was ended. Four of the flasks (two of each) were trypsinized and the cell pellet resuspended in 5 ml conditioned media. Conditioned media was used to limit transport of unlabeled metabolites into the cells. A small portion of this resuspension (0.5 ml) was used for cell density determination on a Coulter Counter. The rest of the cells were re-centrifuged and used for extraction. Sampling was maintained beyond the end of the experiment for the other four flasks to increase the accuracy of the fitted external fluxes.

6. NMR Analysis

^{13}C spectra were acquired on a 500MHz Bruker DRX spectrometer with a 5 mm broad-band probe. A DEPT-45 pulse sequence was used with a 2 second interscan delay. Spectra were acquired overnight, resulting in the signal averaging of approximately 20,000 scans. Concentrations of labeled

species were determined by calibration to the α- and β-1-^{13}C glucose peaks and corrected for DEPT enhancement.

7. Development of a Biochemical Pathway Model and Mathematical Description

Measured external metabolite fluxes and fractional enrichments are inputted into a mathematical description of biochemical pathways model to determine the unknown internal fluxes. In batch growth (flask and roller bottle culture), external fluxes, $f_{i,ex}$ (μmol/cell·hr), are calculated from linear time-course concentration profiles using equation 1[6]:

$$f_{i,ex} = \frac{1}{N} \cdot \frac{dC_i}{dt} = \frac{b_i}{N} \tag{1}$$

where C_i is the concentration of metabolite i, N is number of cells and b_i is the slope of the linear profile. Currently the metabolically important substrates that are measured are glucose, lactate, glutamate, glutamine, aspartate, serine, and alanine. Fractional enrichments are determined by integrating decoupled carbon spectra to find the concentration of label, $L_{i,y}$, on atom y in metabolite i. The total metabolite concentration in the extract, C_i, was measured by HPLC or enzyme assay and was combined with the concentration of label to calculate the absolute fractional enrichment, $M_{i,y}$:

$$M_{i,y} = \frac{L_{i,y}}{C_i} \tag{2}$$

Conversion of fractional enrichments and external flux data into internal fluxes requires the development of a biochemical pathway model. There are too many pathways within the cell to all be included in the model. Pathways retained in the model have either significant flows of carbon or considerable effects on the isotope distribution. The current model is shown in Figure 1 and contains the following pathways: glycolysis, the TCA cycle, the pentose phosphate pathway, glutaminolysis, malate transport, tricarboxylate transport, malic enzyme, and pyruvate carboxylase.

Fluxes are calculated using metabolite and isotope mass balances. Because metabolic steady state is assumed[7], the intracellular accumulation is negligible, and metabolite balances simply equate the sum of the fluxes producing a metabolite to the sum of the fluxes consuming it. All of the balances derived from Figure 1 are given in Table 1:

Table 1 Metabolite balances derived from Figure 1

$f_1 = f_2 + f_3$	$f_{12} + f_{24} = f_{14}$
$f_2 + 2/3\ f_3 = f_4$	$f_{14} = f_{13} + f_{16}$
$f_4 = 2\ f_5$	$f_{15} + f_{16} + f_{22} = f_{17} + f_{18} + f_{21} + f_{25}$
$f_5 + 1/3\ f_3 = f_6$	$f_{17} + f_{18} + f_{19} = f_9 + f_{14}$
$f_6 + f_7 + f_{20} = f_{10} + f_{11} + f_{12} + f_{19}$	$f_9 + f_{13} = f_{20}$

Isotope balances equate the sum of the fluxes of isotope into a single *atom* of a metabolite to the sum of the fluxes out.

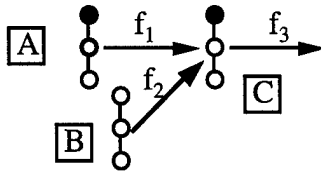

Figure 2. Example of an isotope balance. Each circle represents carbon atoms in one of three example molecules: A, B and C. The darkened circles are partially enriched by ^{13}C. The three carbon atoms of each molecule are numbered 1 - 3 starting at the top. This particular set of reactions represents the recombination of two pathways.

The isotope balance around the first carbon of metabolite C in Figure 2 is written as:

$$f_1 \cdot A1 + f_2 \cdot B1 = f_3 \cdot C1 \quad (3)$$

Where A1, B1, and C1 are the fractional enrichments of the first carbons of metabolite A, B, and C, respectively. The product of $f_i \cdot C$ thus has units of µmol/cell·hr. Two more balances can be written for the example, one for the second carbon and one for the third. All of the label balances in Figure 1 are listed in Table 2:

Table 2. Label balances derived from model 1.

Gp1 (f2 + f3) = G1 f1	C5 (f13 + f16) = M4 f14	Cc1 f13 = C1 f13
Fp1 f4 = Gp1 f2	C6 (f13 + f16) = M1 f14	Cc2 f13 = C2 f13
Fbp1 (1/2) f5 = Fp1 f4	K1 (f17 + f18 + f21 + f25) = C5 f16	Cc3 f13 = C3 f13
GA3 f6 = Fbp1 (1/2) f5	K2 (f17 + f18 + f21 + f25) = C4 f16	Cc4 f13 = C4 f13
PG3 f6 = GA3 f6	K3 (f17 + f18 + f21 + f25) = C3 f16	Cc5 f13 = C5 f13
PE3 f6 = PG3 f6	K4 (f17 + f18 + f21 + f25) = C2 f16	Cc6 f13 = C6 f13
P1 (f19 + f10 + f11 + f12) = Mc4 f20	K5 (f17 + f18 + f21 + f25) = C1 f16	Ac1 f13 = Cc1 f13
P2 (f19 + f10 + f11 + f12) = Mc3 f20	M1 (f14 + f9) = K5 f17 + K2 f18 + P1 f19	Ac2 f13 = Cc2 f13
P3 (f19 + f10 + f11 + f12) = Mc2 f20 + PE3 f6	M2 (f14 + f9) = K4 f17 + K3 f18 + P2 f19	L1 f11 = P1 f11
A1 f14 = P2 f12	M3 (f14 + f9) = K3 f17 + K4 f18 + P3 f19	L2 f11 = P2 f11
A2 f14 = P3 f12	M4 (f14 + f9) = K2 f17 + K5 f18	L3 f11 = P3 f11
C1 (f13 + f16) = A1 f14	Mc1 f20 = M1 f9 + Cc6 f13	A1 f10 = P1 f10
C2 (f13 + f16) = A2 f14	Mc2 f20 = M2 f9 + Cc3 f13	A2 f10 = P2 f10
C3 (f13 + f16) = M2 f14	Mc3 f20 = M3 f9 + Cc4 f13	A3 f10 = P3 f10
C4 (f13 + f16) = M3 f14	Mc4 f20 = M4 f9 + Cc5 f13	

Because very few of the enrichment fractions in table 2 are measured, it is necessary to reduce the number of equations so that there is an equal number of equations to the number of measured fractional enrichments. In equations 4 - 8, fractional enrichments are in boldface.

$$\mathbf{P2}\beta_p = \left[\left(\frac{f_9}{\beta_m}\right)+\left(\frac{f_{14}}{\beta_m}\right)\left(\frac{f_{13}}{\beta_c}\right)\right][f_{17}\mathbf{K3}+f_{18}\mathbf{K4}+f_{19}\mathbf{P3}] \tag{4}$$

$$\mathbf{P3}\beta_p = \left[\left(\frac{f_9}{\beta_m}\right)+\left(\frac{f_{14}}{\beta_m}\right)\left(\frac{f_{13}}{\beta_c}\right)\right][f_{18}\mathbf{K3}+f_{17}\mathbf{K4}+f_{19}\mathbf{P2}]+\frac{f_2}{\beta_g}\mathbf{G1} \tag{5}$$

$$\mathbf{K2}\beta_k = \left(\frac{f_{14}}{\beta_m}\right)\left(\frac{f_{16}}{\beta_c}\right)[f_{17}\mathbf{K3}+f_{18}\mathbf{K4}+f_{19}\mathbf{P3}] \tag{6}$$

$$\mathbf{K3}\beta_k = \left(\frac{f_{14}}{\beta_m}\right)\left(\frac{f_{16}}{\beta_c}\right)[f_{18}\mathbf{K3}+f_{17}\mathbf{K4}+f_{19}\mathbf{P2}] \tag{7}$$

$$\mathbf{K4}\beta_k = \left(\frac{f_{16}}{\beta_c}\right)[f_{12}\mathbf{P3}] \tag{8}$$

Where:

$$\beta_g = f_1 = f_2 + f_3 \tag{9}$$

$$\beta_c = f_{14} = f_{13} + f_{16} \tag{10}$$

$$\beta_m = f_{17} + f_{18} + f_{19} = f_9 + f_{14} \tag{11}$$

$$\beta_p = f_6 + f_7 + f_{20} = f_{10} + f_{11} + f_{12} + f_{19} \tag{12}$$

$$\beta_k = f_{15} + f_{16} + f_{22} = f_{17} + f_{18} + f_{21} + f_{25} \tag{13}$$

The method of least squares is used to find the set of unknown fluxes by minimization of the sum-of-squares, F, which is given by:

$$F = \sum_{i=1}^{n} \frac{[y_i - y_i(x)]^2}{\sigma_i^{\,2}} \tag{14}$$

Where y_i is any measured value, either fractional enrichment or external flux, and $y_i(x)$ is the adjusted value of that variable constrained by the metabolite and isotope balances. There are n measurements and x represents the adjusted unknown fluxes. The standard deviation of each measurement, σ_i, is included to weigh the sum in favor of more accurate measurements.

Errors in the values of the internal fluxes were calculated by simulation around the errors of the input measurements[8]. This is done by first generating a statistically probable set of input data inside a Gaussian distribution of the measurements. A new set of internal fluxes was calculated from this new set of input "data." This process is repeated M times, after which the error of each internal flux is given by:

$$E_i = \sqrt{\sum_{j=1}^{M}\left(f_{ij} - \hat{f}_i\right)^2 \Big/ M} \tag{15}$$

Where $\hat{\underline{f}}$ is the set of best-fit fluxes, determined from the initial values of the measurements. These errors of the internal fluxes have three uses: they establish the accuracy of the results, they discriminate plausible from implausible pathway models, and they establish the statistical significance between different test conditions.

B. Results and Discussion

1. TAM dose response in 96-well plates

Response of T47D cells to TAM was investigated in three different serum concentrations (10, 5, and 2%). It was determined that serum concentration does not greatly affect T47D response to TAM except at a very high concentration (10 μM). Ten μM TAM more adversely affected cells in 2% serum than in 5% or 10% serum. At this point, it was decided to perform subsequent experiments in 5% serum to reduce the number of variables investigated. Response of MCF7 cells to TAM was investigated in 5% serum. The inhibitory concentration of TAM was found to be between 1μM and 5 μM for both MCF7 and T47D cells. Estrogen rescue was performed in MCF7 cells. MCF7 cells treated with 5 μM for 6 days were rescued with E2/TAM ratios of 1:1000, 1:100, 1:10. Under all rescue conditions, the cell growth as determined by final cell number was approximately four-fold higher than in the unrescued case.

2. TAM effects in microcarrier cultures

Based on the results of the 96-well plate studies, T47D cells were subjected to 1 μM TAM. The cell number on day 6 for TAM-treated flask was approximately 70% of the vehicle control flask. The growth curves are shown in Figure 3. Profiles of glucose and lactate concentrations as a function of time were determined. These profiles were then adjusted to remove the discontinuities due to media replacement, as shown in Figures 4 and 5. These profiles were then fit to a standard quadratic equation. The derivative of the fit was used to calculate uptake and production rates of glucose and lactate, respectively. The results are shown in Figure 6 and 7. The ratio of glucose uptake to lactate production was determined and is shown in Figure 8. Cell cycle phase was determined on day 6 and is shown in Figure 9.

The above experiment indicated that certain metabolic effects of TAM can be quantified using microcarrier cultures. Glucose uptake and lactate production on a per cell basis were significantly lower for TAM-treated cultures than for control cultures. TAM treatment also resulted in a lower ratio of glucose uptake to lactate production compared to control cultures. Cell cycle analysis was used to investigate the nature of TAM action. The results indicate that TAM was acting as a cytostatic agent, causing cell cycle arrest in G0/G1.

This experiment also demonstrated the need for more accurate nuclei counts. The large errors in some data points for glucose uptake and lactate production were due to error propagation of nuclei counts. The sampling procedure was then modified to ensure more uniform samples. Note that the error of the ratio of glucose consumption to lactate production is unaffected by nuclei count errors, as it is independent of nuclei number.

3. Metabolic response of TAM treatment using 1-^{13}C NMR and cell extraction

The time profile for glucose consumption and lactate production are shown in Figure 10. A cubic equation was fit through the concentration points. The external flux rate, on a per flask basis, is

the slope of the cubic fit at day 2.87, which is the time when the extraction was performed. Similar profiles and fluxes were generated for serine, alanine, glutamine, and glutamate. The number of cells in the control and TAM flasks were $6.33 \cdot 10^6$ cells/flask and $6.06 \cdot 10^6$ cells/flask, respectively. Specific consumption rates were determined by dividing the external flask consumption rates (μmole/flask/hour) by the cell density of the flasks (cells/flask). The absolute fractional enrichments were found for 2- and 3-lactate and 2-, 3-, and 4-glutamate. All of the measured external fluxes and fractional enrichments are summarized in Table 3.

Table 3. Summary of the measurements for the 1-^{13}C NMR / TAM response experiment

	Control	TAM
glucose consumption (μmole/hour/10^9 cell)	243.8	257.1
lactate production (μmole/hour/10^9 cell)	245.6	258.9
glutamine consumption (μmole/hour/10^9 cell)	67.5	44.7
alanine production (μmole/hour/10^9 cell)	26.8	23.2
serine consumption (μmole/hour/10^9 cell)	19.6	12.1
glutamate production (μmole/hour/10^9 cell)	0.8	3.6
lactate 2 fractional enrichment	0.013	0.012
lactate 3 fractional enrichment	0.4	0.39
glutamate 2 fractional enrichment	0.066	0.14
glutamate 3 fractional enrichment	0.061	0.1
glutamate 4 fractional enrichment	0.1	0.3

Figure 11 shows fluxes determined by inputting the measurements into the pathway model. All of the fluxes were adjusted with the What's Best optimization add-in to Microsoft Excel in order to minimize the sum-of-squares. Many model formulations were tried until one was found that gave physiologically possible results. The different fluxes that were included or removed from the different models were pyruvate carboxylase (f_{19}), fatty acid catabolism (f_{24}), glutamate exchange with proteins ($f_{21,22}$), the malate shunt (f_9), and channeling in the TCA cycle ($f_{17,18}$). It is possible to see from this result that there are obvious differences in the internal fluxes between TAM-treated and control cells. These fluxes represent an intermediate result. Improved measurement and model assessment techniques are being developed to produce more accurate results and quantify more fluxes.

C. Recommendations

The Statement of Work in the original proposal is included below. Recommendations in relation to the Statement of Work are given in Italics.

Task 1: Months 1-2: Serially wean MCF7 ER+, MCF7 ER-, and T47D ER+ cells to 2% serum. *Concerns were raised about the use of low levels of serum. MCF7 and T47D cell lines are being maintained in 5% serum. Another ER- cell line, MDA-MB-231, is currently being used in place of ER- MCF7, but we are seeking to obtain ER- MCF7s.*

Task 2: Months 2-5: Implement more precise analytical control and monitoring capabilities of existing hollow fiber bioreactor (HFBR) support apparatus. *A more robust temperature control algorithm*

implementing cascade control has been created. This controller is capable of maintaining the desired set point (37 °C) plus or minus 0.1°C.

Task 3: Months 5-6: Treat HFBR with attachment factors and test cell growth. *T47D cells were grown in an HFBR for 3 months at which point the cells where removed by trypsinization. Both NMR and culturing techniques indicated that there was little viability in the 5g cell pellet. A modified HFBR will be needed to attain a higher viable cell density.*

Task 4: Months 7-8: Perform growth studies to determine cerulenin concentration to be used in HFBR experiments. *This task will be performed in months 17-18. We found it necessary to perform growth studies to determine appropriate TAM and estrogen concentrations first.*

In order to prepare for Tasks 6 and 10, we performed the preliminary experiments in 96-well plates, microcarrier culture, flasks, and roller bottles as described above.

Task 5: Month 9: Inject treated HFBR with ER+ MCF7 cells and allow them to grow to high density. *See Task 3. T47D cells were used for these preliminary studies because of their more rapid growth in HFBR's.*

Task 6: Months 10-14: Execute serial step changes in medium composition, such as TAM concentration and ^{13}C-labeled metabolites, while continually measuring internal and external metabolites.

Task 7: Month 15-16: Perform cell extract experiments to confirm spectral assignment of metabolites. *As was described in Materials and Methods, the extraction technique proved to be more beneficial than expected. The ability to detect isotopic distribution by ^{13}C-NMR measurements of extracts prompted us to perform this task as a precursor to more complex in vivo experiments in tasks 6 and 10.*

Task 8: Months 17-18: Perform data analysis. Check for inconsistencies with existing metabolic model. *It was possible to perform this task with ^{13}C-NMR extract measurements. As described in the results section, many models have been tested and a simulation method has been developed to discern between models.*

Task 9: Month 19: Inject treated HFBR with ER+ T47D cells and allow to grow to high density.

Task 10: Months 20-28: Repeat tasks 6-8 but for the T47D cell line.

Task 11: Month 28: Inject treated HFBR with ER- MCF7 cells and allow to grow to high density.

Task 12: Months 20-28: Repeat tasks 6-8 but for the ER- cell line.

CONCLUSIONS

In summary, we have demonstrated the ability to perform several important tasks critical to the success of this research. Specifically, these accomplishments are: 1) quantification of external metabolic fluxes in ER+ human breast cancer cells in response to TAM treatment based on extracellular measurements, 2) calculation of internal and external metabolic fluxes using cell extract NMR data and a comprehensive metabolic model, 3) use of these internal and external metabolic fluxes to quantify the effects of TAM treatment, and 4) cultivation of human breast cancer cells in a HFBR. We will use data from microcarrier, flask, and roller bottle studies to make comparisons with HFBR data and as simpler tests of drug treatment conditions. This level of quantification of TAM effects has not been previously demonstrated in the field of breast cancer research.

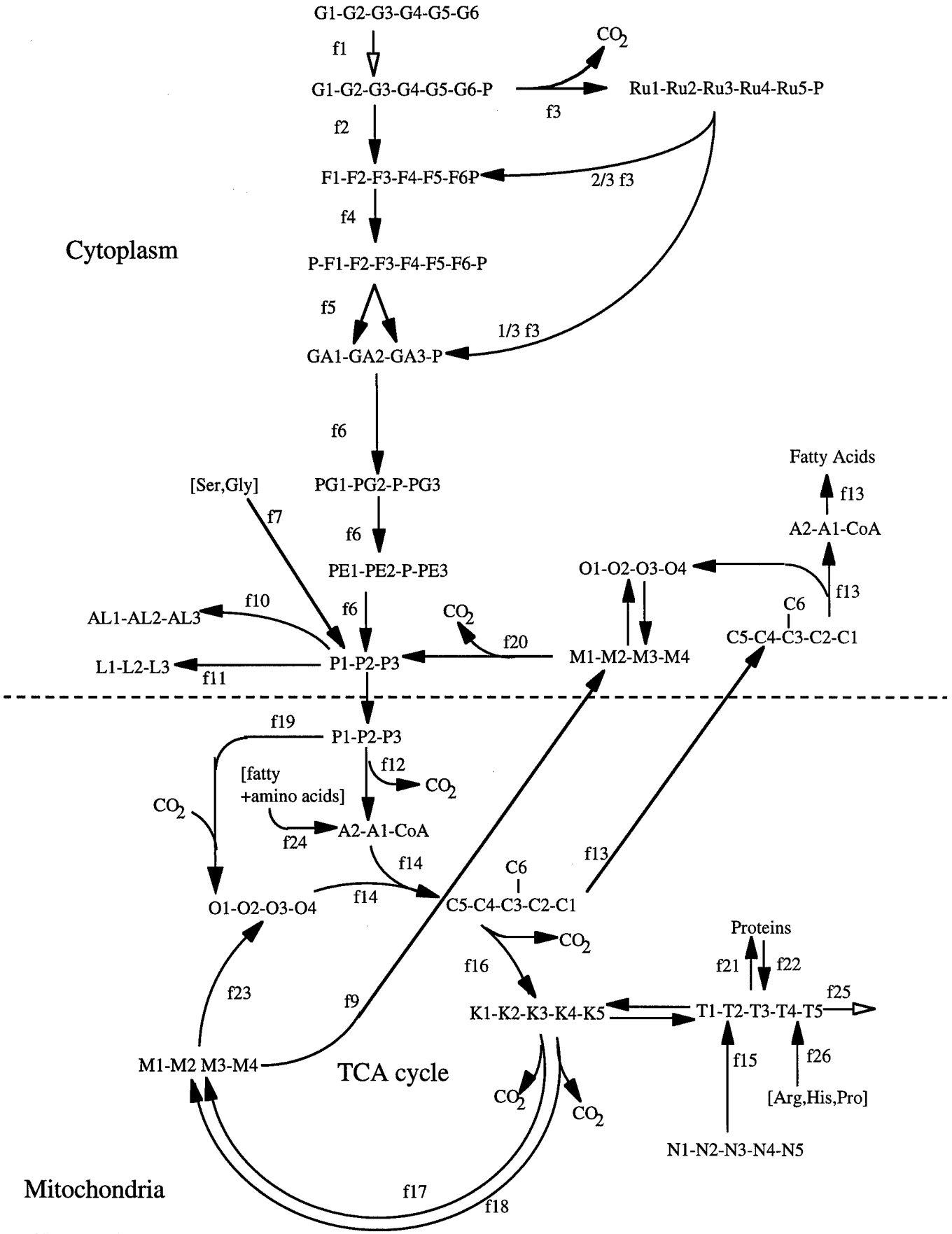

Figure1. Current metabolic pathway model. Abbreviations are: G-glucose, Ru-ribose-5-phosphate, F-fructose, GA-glyceraldehyde-3-phosphate, PG-phosphoglycerate, PE-phophenolpyruvate, P-pyruvate, AL-alanine, L-lactate, M-malate, O-oxaloacetate, C-citrate, A-acetyl-CoA, K-a-ketogltarate, T-glutamate, and N-glutamine.

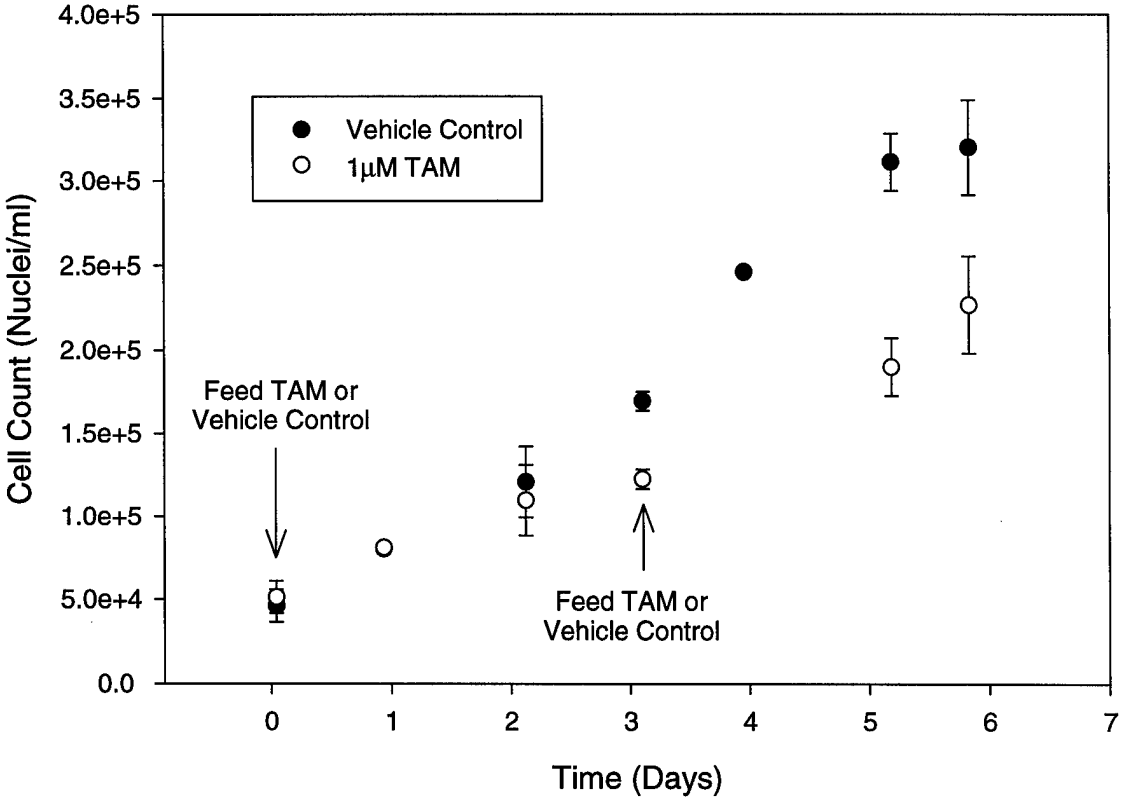

Figure 3. Growth curve for T47D microcarrier cultures. Cell number was determined as nuclei/ml. Cultures were fed 1 μM TAM or vehicle control on day 0 and day 3. Error bars represent the standard deviation of triplicate measurements.

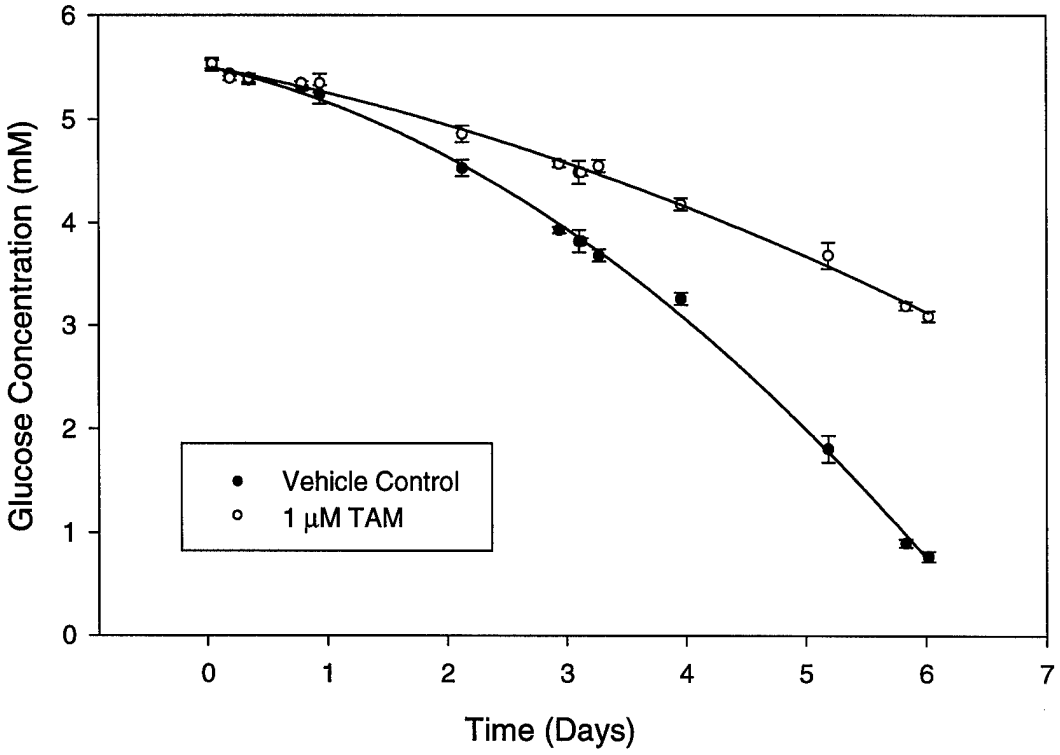

Figure 4. Glucose concentrations v. time for T47D microcarrier cultures. Cultures were fed 1μM TAM or vehicle control on days 0 and 3. Concentrations have been adjusted for media replacement. Data are fit to a quadratic equation. Error bars represent the standard deviation of triplicate measurements.

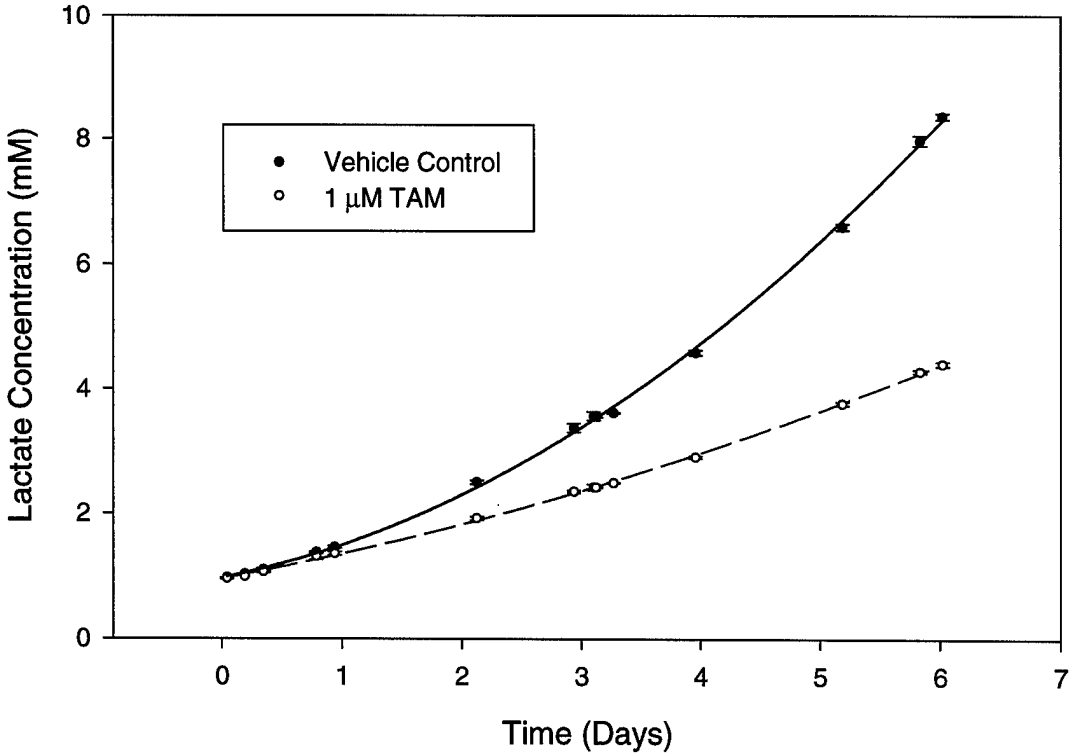

Figure 5. Lactate concentrations v. time for T47D microcarrier cultures. Cultures were fed 1μM TAM or vehicle control on days 0 and 3. Concentrations have been adjusted for media replacement. Data are fit to a quadratic equation. Error bars represent the standard deviation of triplicate lactate measurements.

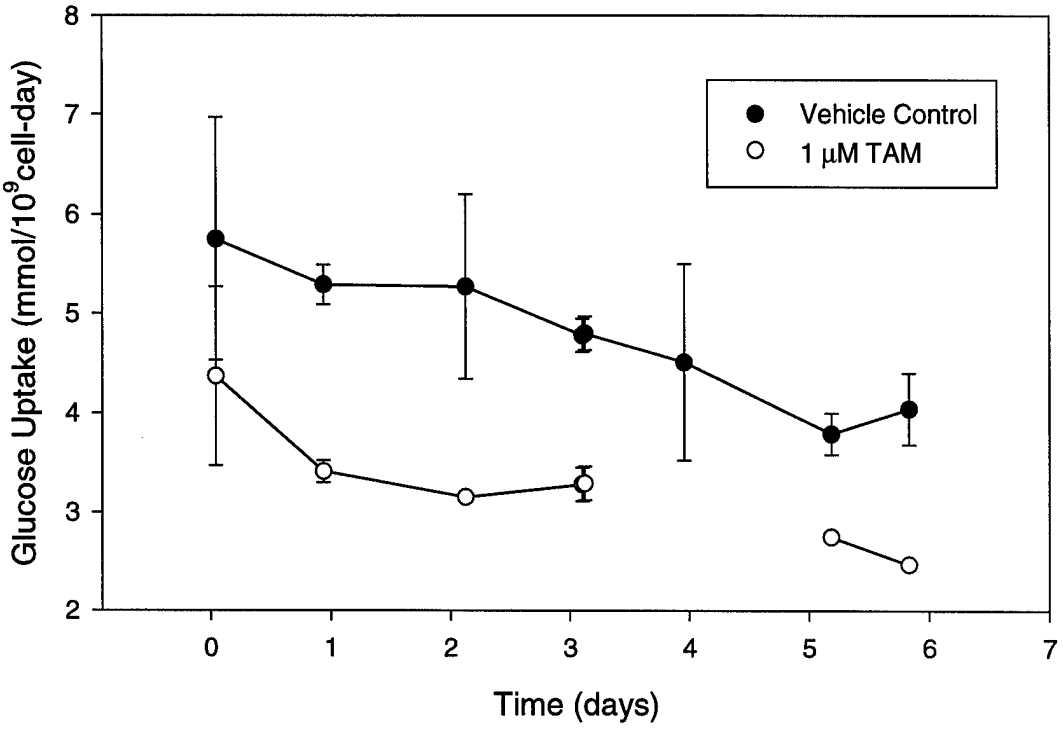

Figure 6. Glucose uptake rates v. time for T47D microcarrier cultures. Error bars represent propagated error from triplicate glucose measurements and nuclei counts.

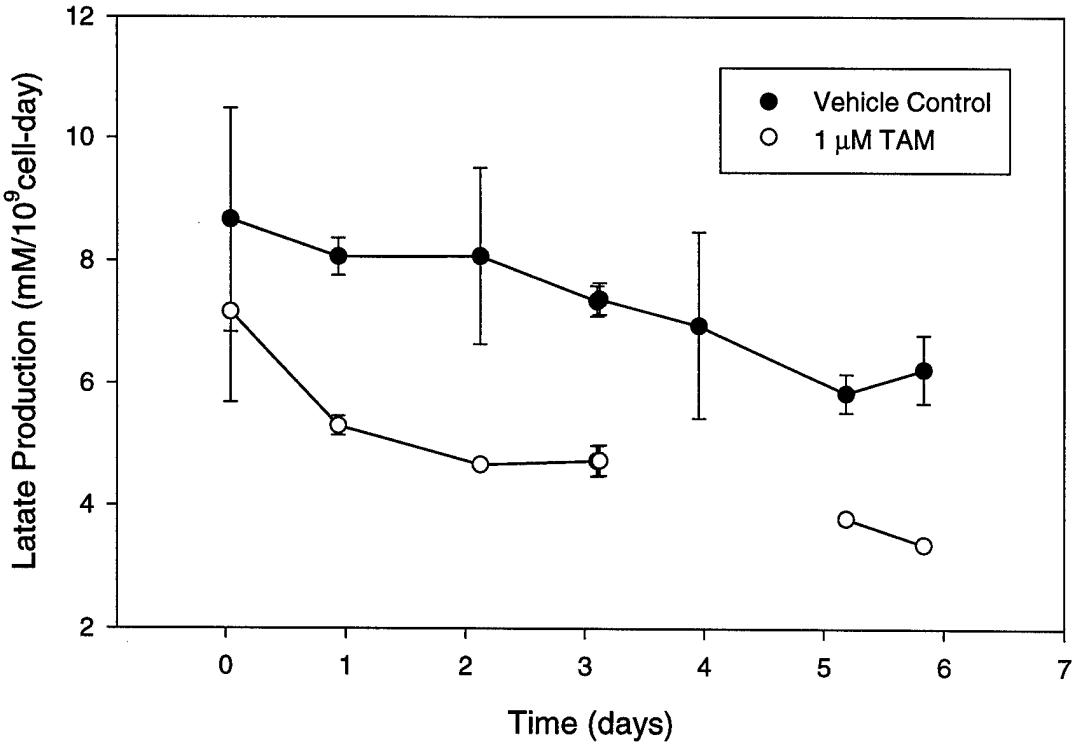

Figure 7. Lactate production rates v. time for T47D microcarrier cultures. Error bars represent propagated error from triplicate lactate measurements and nuclei counts.

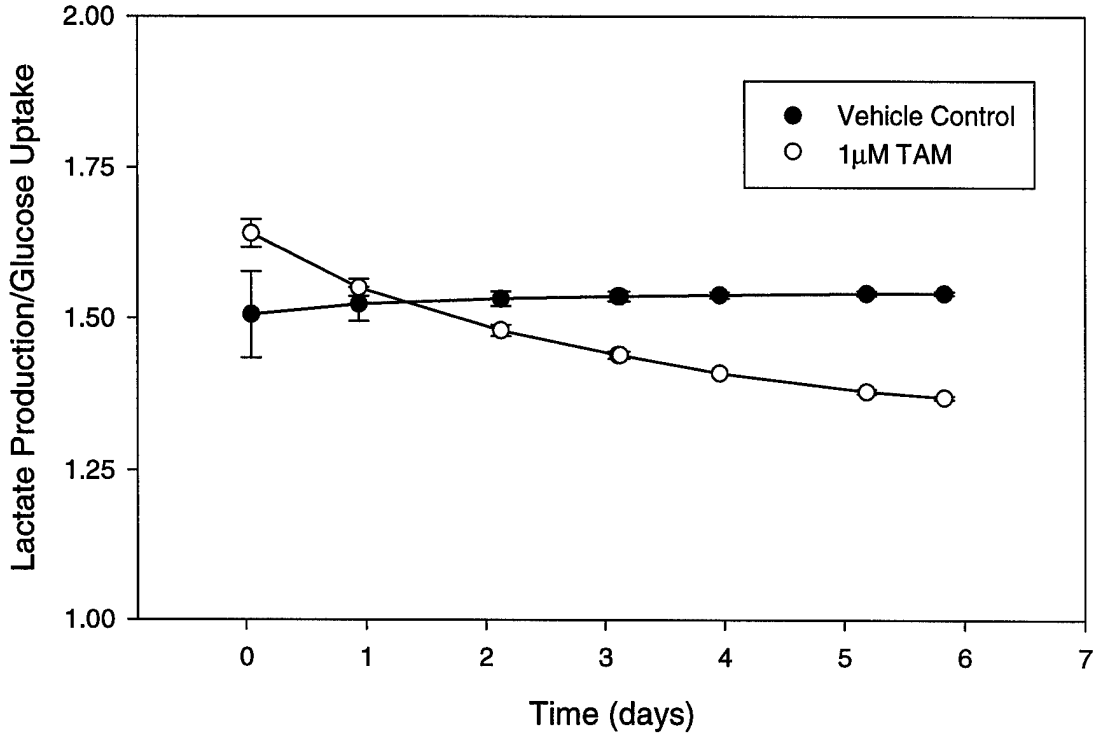

Figure 8. Ratio of lactate production to glucose uptake for T47D microcarrier cultures.

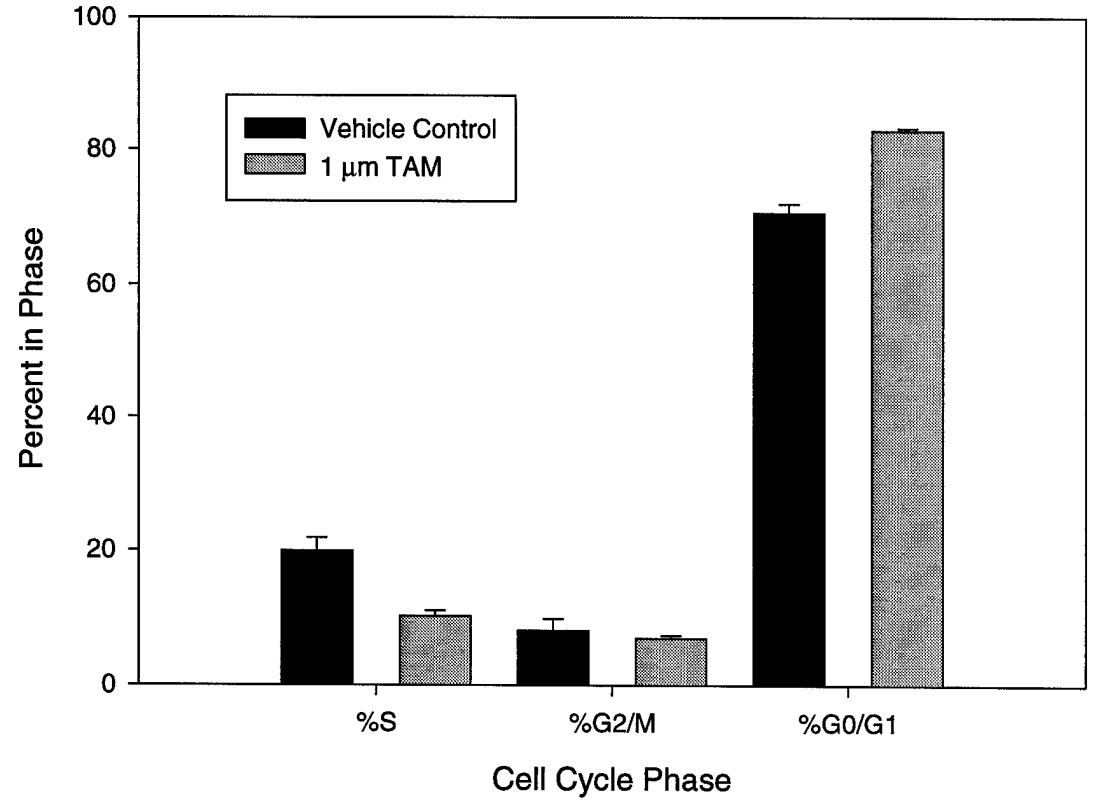

Figure 9. Cell cycle analysis of T47D microcarrier cultures on day 6.

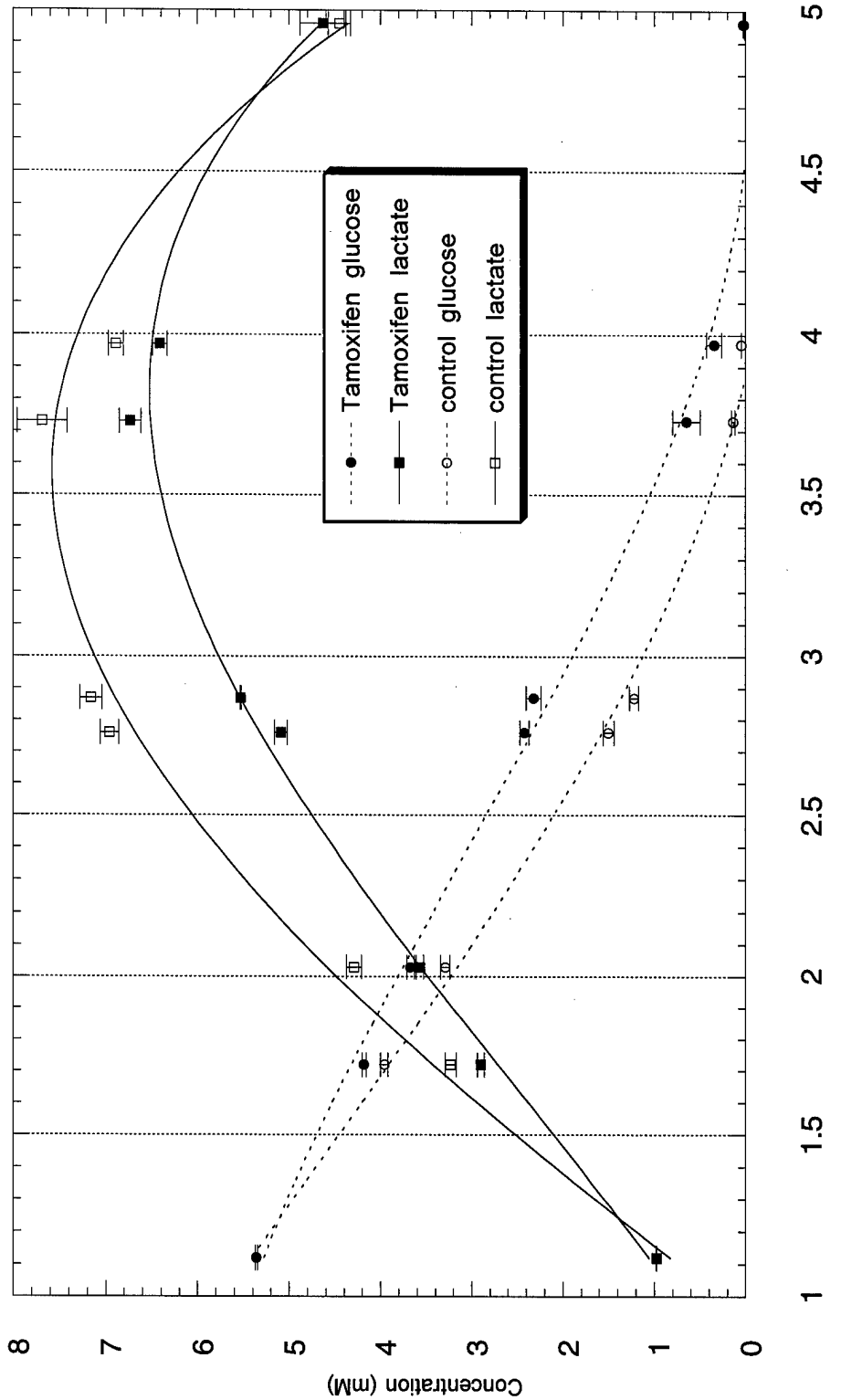

Figure 10. Glucose and lactate consumption and production response to Tamoxifen treatment. The fitted line is a cubic polynomial.

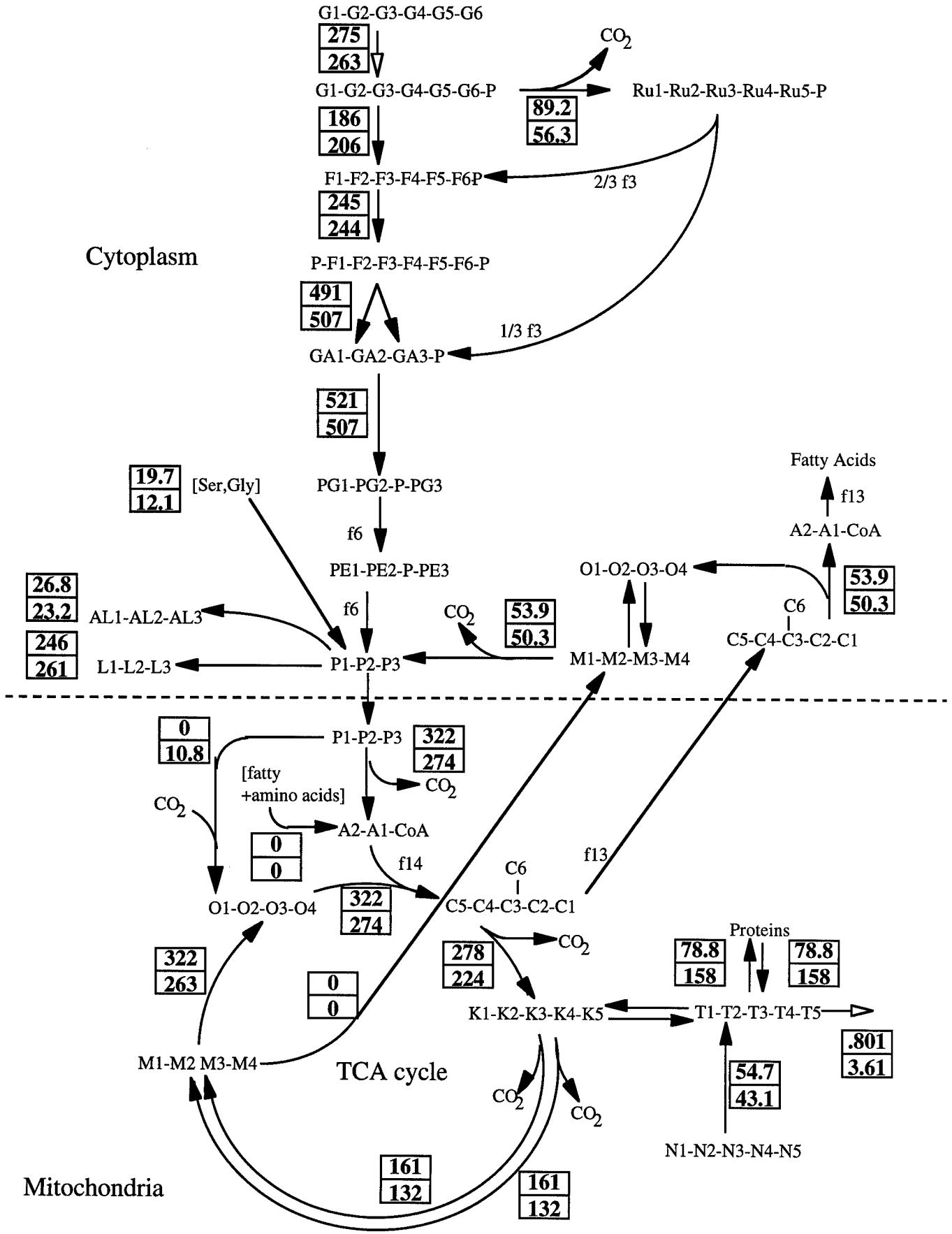

Figure11. Metabolic resonse of T47D cells to 3 mM TAM, determined in flask culture by extraction and ^{13}C-NMR. The top number is the flux in the control and the bottom the TAM response.

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