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TITLE: Use of Mitochondria-Specific Dye MKT-077 as a Radiosensitizer to Preoperatively Treat Locally Advanced Breast Cancer

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14. ABSTRACT The major goal of this project is to determine if the rhodacyanine analog dye, MKT-077, can be used to inhibit breast cancer cell oxygen metabolism and raise tumor oxygen levels, thereby radiosensitizing the tumor. In the second year, we had to switch breast cancer cell lines from the human MDA-MB 231 line to the rat R3230Ac mammary adenocarcinoma line, because we were unable to grow xenografts from the human cells. We have now used the R3230Ac cells in the in vitro experiments to determine drug uptake and subsequent MKT-077-induced metabolic inhibition, as outlined in Tasks 1 and 2. As noted in the Year 1 report, we are determining MKT-077 drug uptake and metabolic inhibition using cell suspensions. This approach has been successful, and we have been able to show that the cells rapidly take up the drug in a dose-dependent manner, whether the cells have been raised on air or under hypoxic conditions. We have also shown that MKT-077 can inhibit cellular oxygen metabolism by up to 70% at a dose of 6 µg/ml in R3230Ac cells grown on 2.5% O ₂ . Our modeling has shown that the magnitude and time course of the inhibition are both concentration-dependent. In addition, we have begun the in vivo work in Fischer 344 rats. Infusion of 7.5 mg/kg MKT-077 resulted in a small rise in PO ₂ of about 2 mm Hg 10 minutes after the end of the infusion. We are currently completing the in vitro experiments and plan to expand the in vivo work by determining the effects of other drug doses and measuring PO ₂ histograms.					
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INTRODUCTION

The driving hypothesis behind this project is that the rhodacyanine dye analog MKT-077 can be used to inhibit breast cancer cell respiration, leading to increased oxygen levels within the tumor and a resultant increase in tumor radiation response. This central hypothesis is built upon the following rationale. First, the response of tumors to radiation therapy is dependent upon oxygen levels,⁴ and tumors with low oxygen levels (i.e., hypoxic tumors) are radioresistant.⁵ Second, over 60% of human breast tumors are severely hypoxic.¹³⁻¹⁵ Third, of the two methods to increase oxygen levels, inhibition of tumor cell oxygen consumption is theoretically a better method than increasing the oxygen supply.^{8, 9} Fourth, MKT-077 has been shown to inhibit mitochondrial respiration in some cancer cell types.⁶ Therefore, we expect to be able to decrease oxygen consumption and increase the oxygen levels in breast carcinoma by infusing MKT-077. If oxygen can be increased, then infusion of MKT-077 before radiotherapy should increase radiation response and permit lower levels of radiation to be used for preoperative treatment of locally advanced breast cancer (LABC). Lower radiation doses would result in less collateral damage to normal tissue and better cosmetic outcome. In addition, it might be possible to shorten treatment schedules, which would be a benefit to both patients and physicians. Finally, the use of the radiosensitizer could result in better locoregional control in LABC patients. This could increase the number of these women eligible for breast conserving treatment and lead to their improved survival. The use of MKT-077 in combination with radiotherapy should also be useful in patients with early stage breast cancer, who are receiving radiation as part of breast conserving treatment^{10, 16} or locoregional post-mastectomy radiotherapy.^{10, 12}

BODY

As noted in our original Statement of Work, we planned to complete Task 2 and begin Task 3 in Year 2 of the grant. Since we encountered some difficulties in Year 1 (see last year's report), we had to alter the methods used in Tasks 1 and 2. We have continued work on Tasks 1 and 2, and they are still ongoing. Meanwhile, we have begun work on Task 3. As reported last year, we were having difficulty growing tumors in the nude rat model, and we have now switched to the R3230Ac tumor growing in Fischer 344 rats. Experiments addressing Task 3 have begun in this new model. Details of our progress on each specific task are given below.

Task 1. To determine the transport parameters and cellular uptake of MKT-077 in a model of human breast cancer parenchyma at different drug concentrations and oxygen levels (Months 1-7):

Model: We will use an in vitro 3-D model of breast tumor parenchyma, the multicellular layer (MCL), grown from human MDA-MB 231 breast carcinoma cells.

Methods: MCLs will be exposed to media bubbled with different amounts of oxygen. We will add different concentrations of MKT-077 to one side of the MCL and take samples of the media from the other side at different times. The level of MKT-077 in the samples will be determined spectrophotometrically, and the time-concentration data will be fitted to a mathematical model to determine important transport parameters, e.g., the MKT-077 diffusion coefficient and the MKT-077 cellular uptake rate.

Objective:

1) Determine the effect of oxygen level on MKT-077 transport parameters and cellular uptake rate of MKT-077 as a function of drug dose in breast cancer MCLs.

Progress on Task 1: We originally planned to determine uptake of MKT-077 by MDA-MB 231 cells using the MCL model of tumor parenchyma. As noted in last year's report, we had difficulty growing the MCLs, and we decided to use a different method to determine MKT-077 uptake rate. This

new technique involves growing the breast cancer cells in tissue culture, harvesting the cells, and placing the suspension in a specially built, water-jacketed, plexiglas chamber. Then, a known concentration of MKT-077 is added to the chamber. At specific times for the next two hours, an aliquot is removed, and the cells are counted. Immediately thereafter, the aliquot is centrifuged and the subsequent pellet is washed. Ethanol is then added to the cells to lyse them and free the MKT-077. The concentration of MKT-077 in the ethanol is measured using a spectrophotometer, and the amount of MKT-077 taken up by the cells is expressed as ng MKT-077/100,000 cells. Because we are now using rat R3230Ac cells in our in vivo experiments, we have performed these uptake experiments in that cell line, rather than in the MDA-MB 231 line.

To date, we have performed experiments determining MKT-077 uptake at different concentrations in R3230Ac cells grown on air or on 2.5% O₂ in a hypoxia chamber. Results from several experiments, in which cells were grown on air and were then exposed to 2, 4, or 6 µg/ml MKT-077 at time zero, are shown in Figure 1. The cells take up the drug relatively quickly, and the uptake rate is linear out to two hours. The fast uptake rate is important, since the concentration of drug exposed to the tumor will diminish after infusion into the rat's circulation.¹¹ In these particular examples, the uptake rates are noted next to the lines, and they range from 0.08 to 0.44 ng MKT-077/(100,000 cells min) in a dose-dependent fashion. So far, we have performed 20 uptake experiments on R3230Ac cells grown on air and four experiments on cells grown on 2.5% O₂ at MKT-077 concentrations near 2, 4, or 6 µg/ml. The results are summarized in Figure 2. There is a positive correlation between uptake rate and MKT-077 concentration for cells grown on air over the range we have studied ($r = 0.823$; $p < 0.001$; $n = 20$). Early results with hypoxic cells show that the uptake rates are intermingled with those for the cells grown on air, however, it is too early to tell if growth under hypoxic conditions affects drug uptake. These experiments are ongoing and should be completed in the next six months. In that time, we plan to complete the measurements with cells grown on 2.5% O₂ and may test the effects of growth at 1% O₂ as well. We also plan to investigate how the oxygen availability affects the drug uptake, which is mitochondria-dependent.

Task 2. To determine the effect of MKT-077 on tumor metabolism in a model of human breast cancer parenchyma at different drug concentrations and oxygen levels (Months 8-18):

Model: We will use an in vitro 3-D model of breast tumor parenchyma, the multicellular layer (MCL), grown from human MDA-MB 231 breast carcinoma cells.

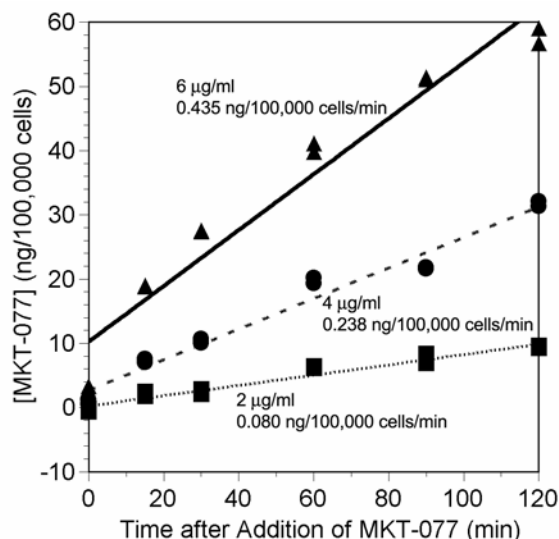


Figure 1. [MKT-077] of R3230Ac cells in suspension after addition of 3 different concentrations of MKT-077 (2, 4, and 6 µg/ml) to the medium. Slopes of lines (uptake rate) are shown in figure for each MKT-077 concentration.

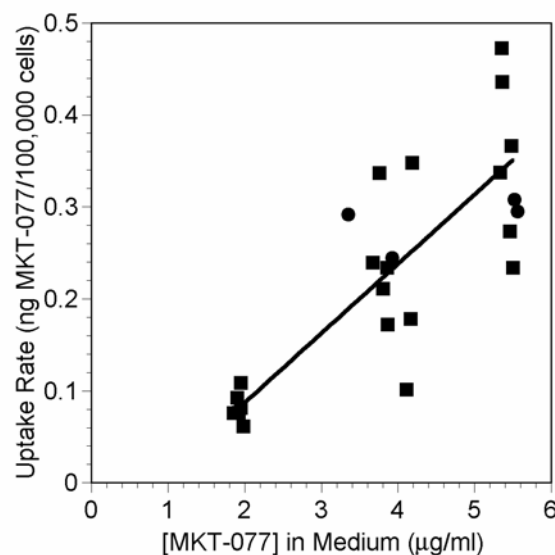


Figure 2. Uptake rate of MKT-077 by R3230Ac cells grown on air (■) or 2.5% O₂ (●) as a function of [MKT-077] in the medium. Uptake rate = $0.0754[\text{MKT-077}] - 0.064$; $r = 0.823$; $p < 0.001$; $n = 20$ (air only).

Methods: We will use oxygen microelectrodes to measure oxygen tension (PO_2) across an MCL exposed to different concentrations of MKT-077. PO_2 profiles will be measured while the MCL is exposed to different levels of oxygen in the media. The PO_2 data will be fitted to a mathematical diffusion model to determine the oxygen consumption rate of the MCL.

Objectives:

- 1) Determine if exposure of MCLs to different doses of MKT-077 causes a decrease in oxygen consumption and a concomitant increase in intratumoral oxygen tension (PO_2). We predict that higher doses of MKT-077 will cause a greater decrease in oxygen consumption.
- 2) Determine if the MKT-077-induced decrease in oxygen consumption is a function of local oxygen level.

Progress on Task 2: Again, we originally planned to determine the effect of MKT-077 on the metabolism of MDA-MB 231 cells by using the MCL model of tumor parenchyma. As noted in the Year 1 report, we abandoned the MDA-MB 231 MCL model during Year 1 and decided to measure drug-induced oxygen consumption changes in the cancer cells using a different technique. In addition, early in Year 2 we switched to the R3230Ac cell line (see the “Progress on Task 1” section).

The current technique involves growing the R3230Ac cells to 90% confluency in air in a standard incubator or in 2.5% O_2 in a hypoxia chamber. The cells are then trypsinized, counted, and resuspended in fresh tissue culture media to a concentration of 1×10^6 cells/ml. Five and one-quarter ml of the suspension are placed in the inner chamber of a specialized tonometer that is heated to $37^\circ C$ by circulating warm water. A Clark-type oxygen electrode (InO2 Dissolved Oxygen System, Innovative Instruments, Inc., Tampa, FL) is positioned in the suspension within the inner chamber. The suspension is exposed to air and allowed to equilibrate by gently stirring the suspension with a small magnetic stir bar. The chamber is then sealed, and oxygen tension (PO_2) is recorded continuously. After at least 20 minutes, concentrated MKT-077 is added to the inner chamber to bring the drug concentration to the desired level. The recording is continued for another two hours or until the PO_2 drops below 20 mm Hg. In preliminary experiments, we determined that consumption sometimes changed when the oxygen dropped this low in undisturbed cells. Examples of experiments performed on cells grown on air or

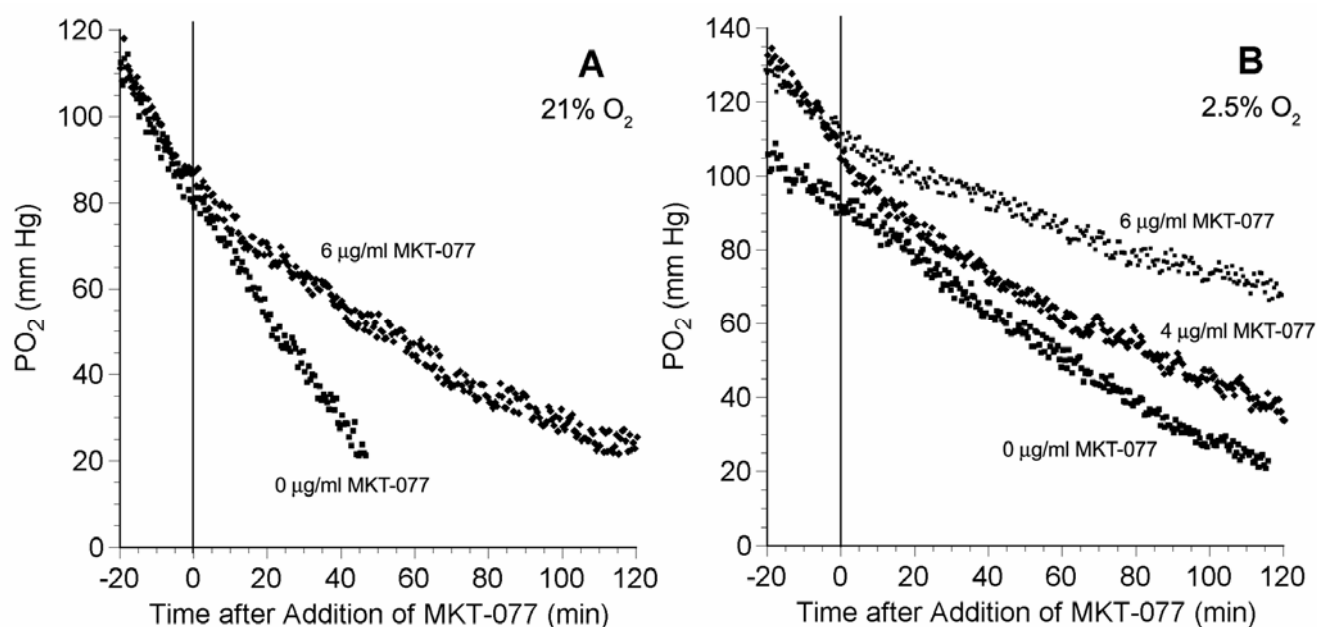


Figure 3. PO_2 measured in R3230Ac cell suspension before and after addition of different concentrations of MKT-077 (0, 2, or 6 $\mu g/ml$) to the media at $t=0$ minutes. A. R3230Ac cells grown on 21% O_2 (air). B. R3230Ac cells grown on 2.5% O_2 .

2.5% O₂ at different MKT-077 doses are shown in Figure 3. The time has been rescaled, so that saline or MKT-077 was added at time zero. Whether the cells were grown on air (Figure 3A) or on 2.5% O₂ (Figure 3B), addition of saline (0 µg/ml), did not change the slope of the line describing PO₂ as a function of time, indicating no change in O₂ consumption. Addition of MKT-077 clearly changed the course of the PO₂ decrease, and showed that the O₂ consumption rate was decreased by the drug. Qualitatively, the higher the dose of MKT-077, the larger the decrease in consumption appeared (Figure 3B).

As noted in last year's report, we have developed a mathematical model to quantify these changes in PO₂. We first assume that the oxygen consumption rate, q [ml O₂/(10⁵ cells min)], changes monoexponentially from q_1 to q_2 , following addition of MKT-077 at time t^* :

$$q(t) = q_1, t < t^*$$

$$q(t) = q_2 - (q_2 - q_1)e^{-\left(\frac{t-t^*}{tc}\right)}, t \geq t^*$$

where q_1 = basal O₂ consumption rate [ml O₂/(10⁵ cells min)], q_2 = O₂ consumption rate at steady state after MKT-077 addition [ml O₂/(10⁵ cells min)], and tc = time constant of change in q (min). This function can be substituted into the mass balance equation for this system: $k(dP/dt) = -Cq(t)$, where k is the oxygen solubility of the suspension (ml O₂/ml suspension/mm Hg), P is the PO₂ (mm Hg), t is time (min), C is the concentration of cells (1x10⁶ cells/ml suspension), and $q(t)$ is the oxygen consumption rate as a function of time given by the above equations. By integrating this function, PO₂ can be expressed as a function of time and four unknown parameters (P_0 , q_1 , q_2 , and tc):

$$P(t) = P_0 - \left(\frac{C}{k}\right)q_1t, t < t^*$$

$$P(t) = P_0 - \left(\frac{C}{k}\right)q_2t + \left(\frac{C}{k}\right)(q_2 - q_1)\left[t^* + tc\left(1 - e^{-\left(\frac{t-t^*}{tc}\right)}\right)\right], t \geq t^*$$

where P_0 = initial PO₂ at time $t=0$ (mm Hg). Using MatLab software (MathWorks, Inc., Natick, MA), we have written a program to fit the experimental PO₂ data to this model. An example of a fit of the model to experimental data is shown in Figure 4A, while the postulated change in q is shown in Figure 4B. The model clearly fitted the data well, and the results show that q changed rapidly following addition of 6 µg/ml MKT-077. The model did not yield unique parameter values for the control experiments (0 µg/ml, Figure 3), and those data could be better fitted by a straight line, i.e., q did not change over time, $q_1=q_2$.

After optimizing the system over the past year, we have completed the experiments on cells grown at 2.5% O₂ in a hypoxia chamber. The results are summarized in Figure 5. MKT-077 decreases oxygen consumption in a dose-dependent fashion, and seems to reach a plateau at ~60-70% inhibition (Figure 5A). Interestingly, the time course of the change in oxygen consumption is also time dependent (Figure 5B). A dose of MKT-077 as low as 2 µg/ml decreases oxygen consumption by about 40% (Figure 5A), but the consumption change is slow and it does not decrease completely until 1-2 hours after addition of the drug (Figure 5B). The difference in the time course of consumption change (Figure 5B) may be directly related to the concentration-dependent difference in MKT-077 uptake rate (Figure 2), but this needs to be studied in greater detail.

We are currently performing experiments on R3230Ac cells grown on air (21% O₂) in a standard incubator, and we have completed eight experiments to date. Although we cannot yet fully comment on all of the effects of raising cells on hypoxia compared to normoxia in these experiments, we do know that growing the R3230Ac cells on 2.5% O₂ causes a 17% decrease in their basal oxygen consumption

rate, from $3.91 \pm 0.72 \times 10^{-6}$ (n=8) to $3.15 \pm 0.68 \times 10^{-6}$ (n=27) ml O₂/(10⁵ cells min) (mean $\pm$ SD, p=0.011).

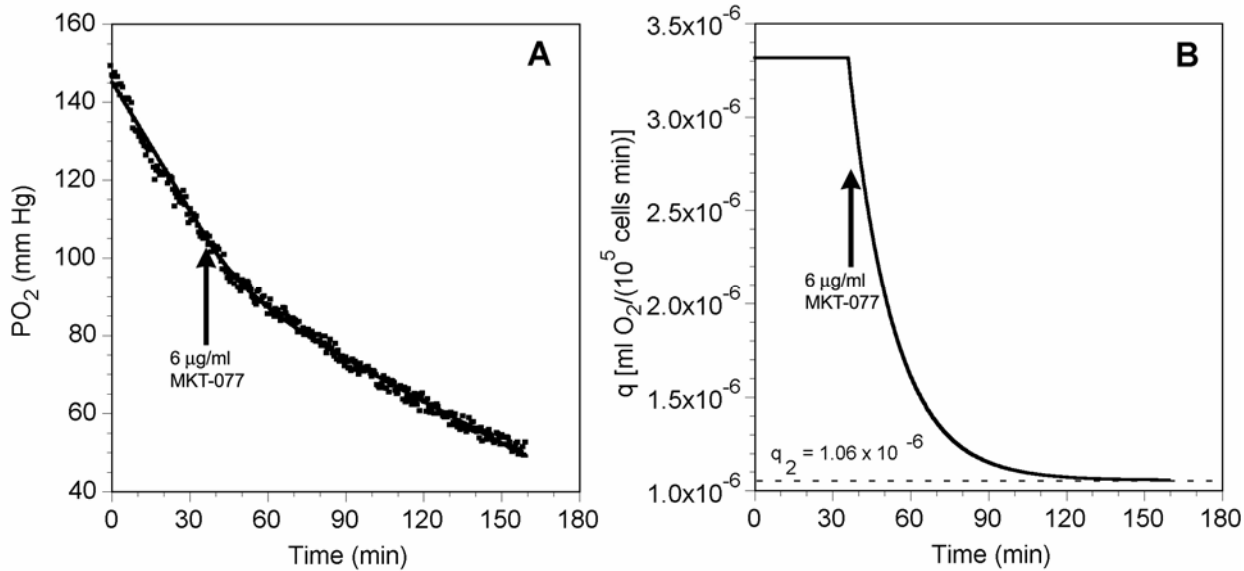


Figure 4. A. PO₂ measured in a R3230Ac cell suspension grown on 2.5% O₂ after addition of MKT-077 to the media at t=t*=36 minutes. The final MKT-077 concentration was 6 μg/ml. The solid line is a least-squares fit, assuming that oxygen consumption decreases monoexponentially after addition of MKT-077. B. The monoexponential drop in consumption, q, as determined by the fit: $q = q_2 - (q_2 - q_1)e^{-(t-t^*)/t_c}$. Fit parameters: P₀ = 149.5 mm Hg, q₁ = 3.32x10⁻⁶ ml O₂/(10⁵ cells min), q₂ = 1.06x10⁻⁶ ml O₂/(10⁵ cells min), and t_c = 17.1 min (95% confidence interval: 15.1-19.0 min).

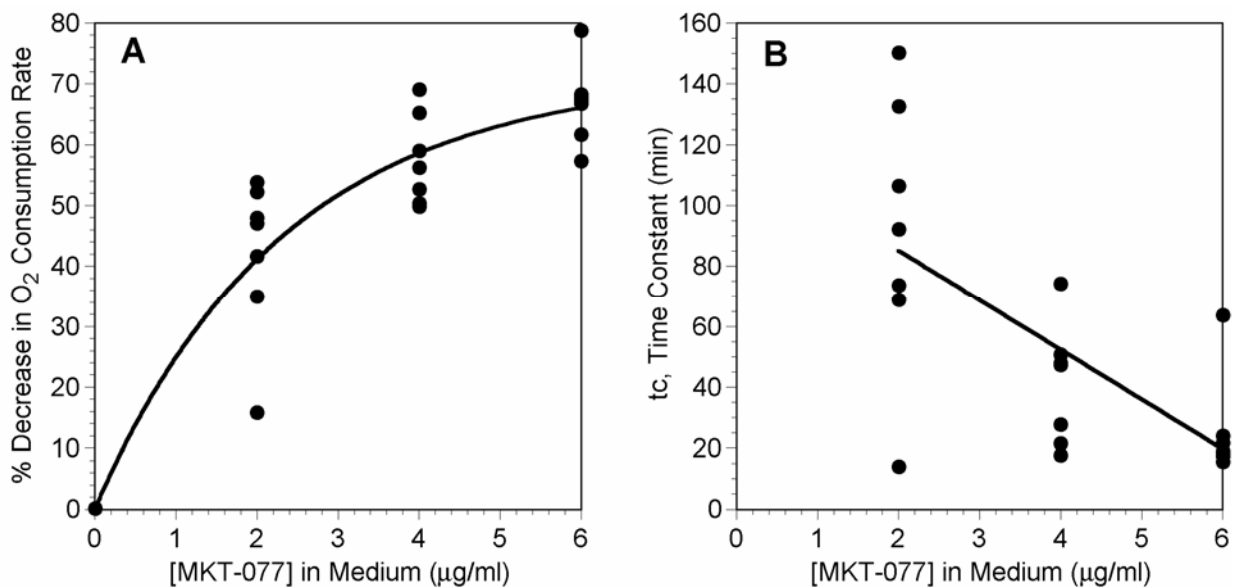


Figure 5. A. Decrease in O₂ consumption determined from the model after R3230Ac cells grown on 2.5% O₂ are exposed to different concentrations of MKT-077. The curve is a least-squares fit of the data to the function: % decrease in q = 71.5(1 - e^{-[MKT-077]/2.34}), r² = 0.909, n = 27. B. Time constant (t_c) of oxygen consumption decrease as a function of [MKT-077] in media: t_c = -16.38[MKT-077] + 117.8, r² = 0.458, r = -0.677, p < 0.001, n = 21.

In summary, we are behind the proposed schedule for these experiments, but we should complete them soon. As was the case for the experiments in Task 1, we would like to expand these to test the effects of more severe hypoxia on the magnitude and time course of the MKT-077-induced change in oxygen consumption. In addition, we may explore the effect of a transient exposure of MKT-077 on

oxygen consumption. In the current experiments, MKT-077 is in the media during the entire experiment, but a brief “peak” exposure may be more representative of the situation in vivo.

Task 3. To determine if MKT-077 infusion can increase tumor PO_2 in orthotopic xenografts grown from human breast cancer cells without altering tumor blood flow (Months 19-33):

Model: We will grow MDA-MB 231 human breast carcinomas orthotopically as xenografts in the mammary fat pads of female nude, athymic rats. We estimate requiring 120 rats for this study.

Methods: We will use oxygen microelectrodes to measure PO_2 at a single site in the xenograft following infusion of different doses of MKT-077. Tumor blood flow will be measured simultaneously at multiple sites using laser Doppler flowmetry to see if MKT-077 affects tumor perfusion. In a second set of experiments, we will measure PO_2 histograms in the xenografts either 2 or 24 hours after infusion of MKT-077 and determine the median PO_2 and other characteristics of the overall distribution.

Objectives:

- 1) Determine if MKT-077 infusion will transiently increase PO_2 in orthotopic breast tumors without altering local blood flow in a dose-dependent manner (Months 19-23). These are important measurements, since changes in perfusion can greatly affect tumor PO_2 .
- 2) Determine if MKT-077 infusion will increase the median PO_2 in orthotopic breast tumors in a dose- and time-dependent manner (Months 24-33).

Progress on Task 3: As noted above, these experiments were scheduled to begin in the middle of the last funding period (Year 2). Actually, we began preliminary in vivo studies during Year 1. Unexpectedly, we had great difficulty getting MDA-MB 231 xenografts to grow in the mammary gland of female nude rats. In the last report, we stated that we achieved no tumor growth following injection of MDA-MB 231 cell suspensions or spheroids into the mammary glands of Hsd:RH-rnu/rnu nude rats. We planned to implant MDA-MB 231 cells enmeshed in Matrigel[®], which has been found to enhance growth of MDA-MB 231 tumors in nude mice.^{1, 7} If that failed, our final fallback model was the

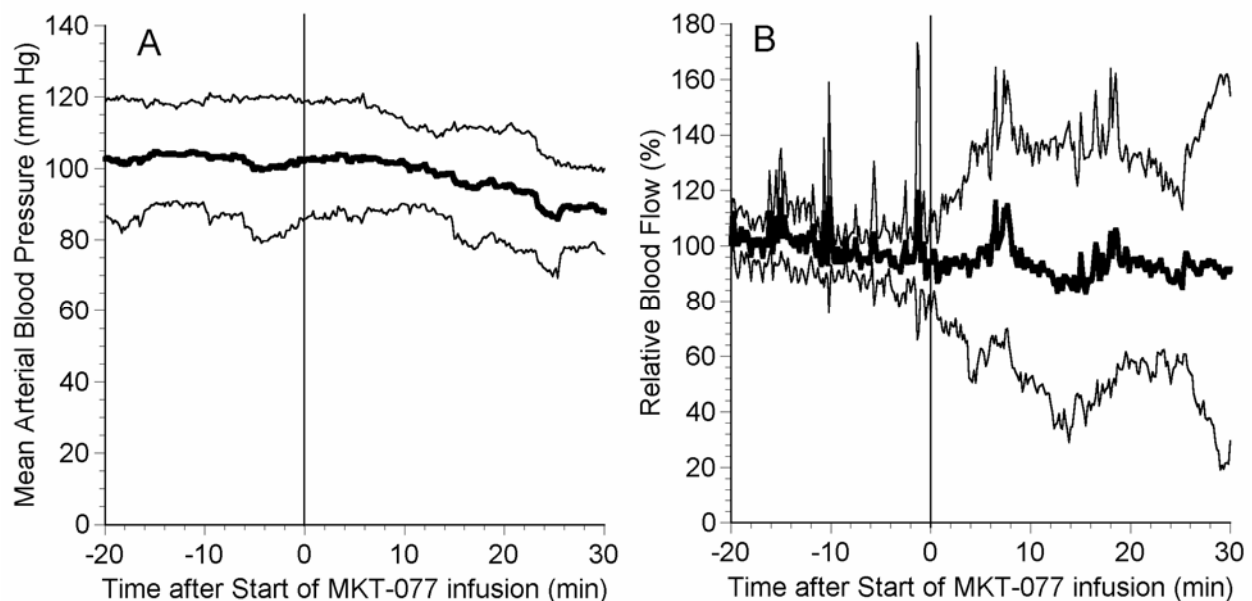


Figure 6. The effect of IV infusion of 7.5 mg/kg MKT-077 on mean arterial blood pressure (A) and tumor laser Doppler blood flow (B) in 5 Fischer 344 rats bearing R3230Ac tumors in the mammary gland. A 2.5 mg/ml solution of MKT-077 in saline was infused at a rate of 0.5 ml/(kg min) for ~6-7 minutes, beginning at t=0 (0-7 minutes). Mean values are shown as bold lines and standard deviations are shown as thinner lines.

orthotopic R3230Ac rat mammary adenocarcinoma growing in the mammary gland of the Fischer 344 rat. We did indeed follow this plan early in Year 2 of the grant, but we were unable to elicit any tumor growth from the MDA-MB 231 cells, even when they were implanted with Matrigel®. We implanted MDA-MB 231 cells at different concentrations in different volumes of Matrigel® in six nude rats, and no tumors grew. Given this inability to grow the human tumors in vivo, we had no choice but to switch to the R3230Ac model of rat mammary adenocarcinoma. We have had experience growing this tumor in Fischer 344 rats before.^{2,3}

It took several months to establish the R3230Ac tumors in the rats, since these tumors must be grown from passaged donor tumor pieces, rather than from cell suspensions. Over the last four months, we have performed nine control experiments (saline infusion) and five successful experiments at a dose of 7.5 mg/kg MKT-077. A 2.5 mg/ml solution of drug in saline was infused intravenously at a rate of 1.25 mg/(kg min) [0.5 ml/(kg min)] for ~6-7 minutes. This dose of MKT-077 slightly decreased mean arterial blood pressure 5-10 minutes after the end of the infusion (Figure 6A). There was no significant change in tumor blood flow following MKT-077 infusion, although blood flow did tend to increase or decrease, as shown by the large standard deviations after infusion (Figure 6B). Despite the small blood pressure drop (Figure 6A), tumor PO₂ increased in four of the five rats shortly after the MKT-077 infusion (Figure 7A). The mean change in PO₂ was about 2 mm Hg and was sustained for 30 minutes (Figure 7B). Control infusions of saline at the same rate had no significant effect on blood pressure, tumor blood flow, or tumor PO₂ (data not shown). Since MKT-077 did not increase blood flow and actually slightly decreased blood pressure, the most likely explanation for the PO₂ increase is that the drug was already beginning to decrease oxygen consumption in the tumor. This interpretation is consistent with the modeling of in vitro oxygen consumption, which showed an immediate monoexponential decrease in consumption rate (Figure 4).

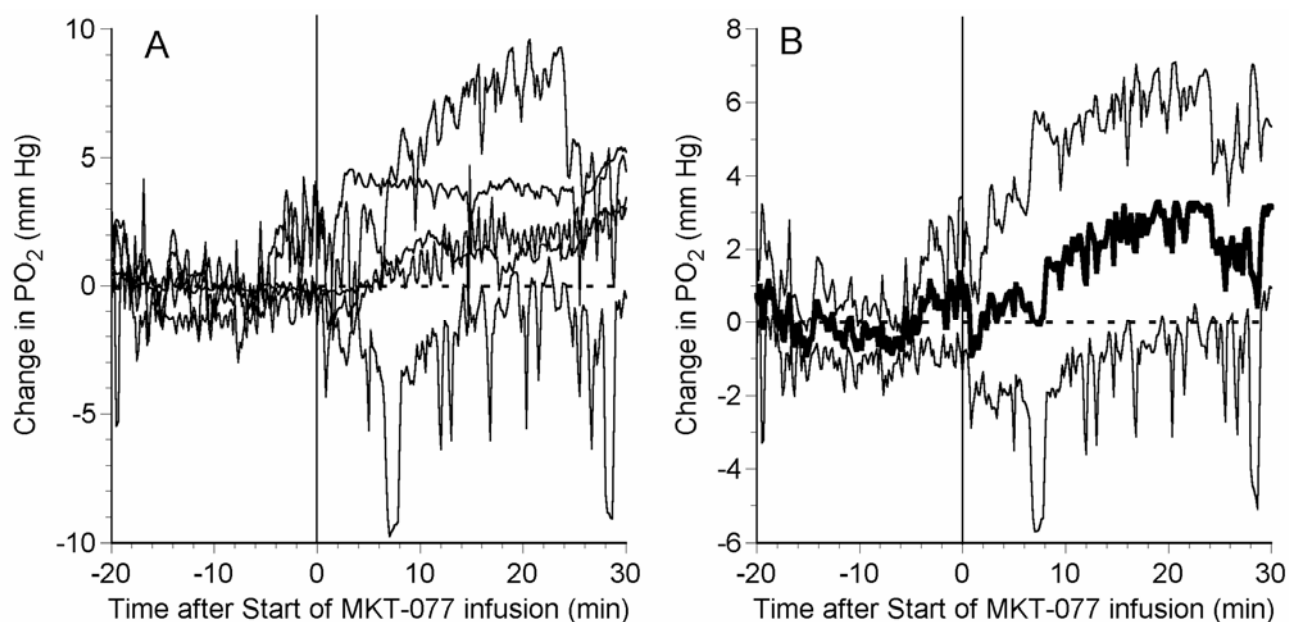


Figure 7. The effect of IV infusion of 7.5 mg/kg MKT-077 on tumor PO₂ in 5 Fischer 344 rats bearing R3230Ac tumors in the mammary gland. A. Change in PO₂ in individual tumors. B. Mean change (bold line) in PO₂ ± SD (thinner lines). A 2.5 mg/ml solution of MKT-077 in saline was infused at a rate of 0.5 ml/(kg min) for ~6-7 minutes, beginning at t=0 (0-7 minutes).

We are planning to add more experiments to the 7.5 mg/kg group and also infuse at 5 and 10 mg/kg. The pressure drop in the 7.5 mg/kg group was unexpected, since we were able to infuse 10 mg/kg MKT-077 into nude rats without any effects on blood pressure (see Year 1 report). We have some concerns about the general health of our Fischer 344 rats, and the pressure drop may be related to

this issue. We are in the process of changing our vendor source for these rats. We are also planning to decrease the MKT-077 concentration in the infusion solution and increase the infusion time. This may also minimize the pressure change. Following transient measurements at the different doses, we will measure PO₂ histograms to determine if the MKT-077-induced changes in PO₂ are sufficient to shift the PO₂ histogram and decrease the hypoxic fraction. These histograms would be recorded at different times after the MKT-077 infusion. Based on the data, we are currently planning to measure histograms several hours after drug infusion and 24 hours after drug infusion.

In summary, we are making significant progress on this task, and should complete these experiments during the upcoming year.

Task 4. To determine if MKT-077 infusion before single-dose radiation therapy can delay the growth of orthotopic human breast cancer xenografts better than either radiation or drug treatment alone (Months 34-36).

Model: We will grow MDA-MB 231 human breast carcinomas orthotopically as xenografts in the mammary fat pads of nude, athymic rats. We estimate requiring 45 rats for this study.

Methods: We will irradiate established MDA-MB 231 tumors that have either received MKT-077 or the saline vehicle. Parallel groups will be sham irradiated. Tumor volume will be measured 3 times per week until the tumors have reached 5 times the initial volume or for a maximum of 60 days.

Objective:

- 1) Determine if MKT-077 infusion before irradiation will result in significant tumor growth delay compared to the other three groups: saline with sham irradiation, MKT 077 with sham irradiation, and saline with irradiation.

Progress on Task 4: In the original Statement of Work, these experiments are not scheduled to be performed until the end of Year 3 of the grant. We believe this is still a reasonable goal and would plan to perform these at the end of the grant year. These studies would only be performed if we can find a dose of MKT-077 and a dosing schedule that is effective at decreasing the hypoxic fraction in the R3230Ac tumors.

KEY RESEARCH ACCOMPLISHMENTS

- We have shown that MKT-077 is readily and rapidly taken up by the R3230Ac rat breast cancer cells in vitro, in a dose-dependent fashion. Early data shows that cells raised under hypoxic data can take up the drug as well.
- We have been able to quantify the time course of changes in oxygen consumption following addition of MKT-077 to a cell suspension using a new mathematical model. To our knowledge, this is the first time that such a model has been used to quantify these parameters for in vitro oxygen consumption measurements.
- We have shown that MKT-077 effectively inhibits oxygen consumption in the R3230Ac cells in a dose-dependent manner, up to a maximum of ~70% inhibition. Not only is the magnitude of the inhibition dose-dependent, but the time course of the effect is dose-dependent as well.
- We have also shown that doses as low as 2 µg/ml MKT-077 are capable of rapidly inhibiting oxygen consumption in the R3230Ac cells, although complete inhibition may take more than an hour. This is consistent with the in vivo data showing small oxygen changes in the tumor soon after MKT-077 infusion.
- We have shown that intravenous infusion of 7.5 mg/kg MKT-077 could modestly increase PO₂ in an R3230Ac mammary adenocarcinoma growing orthotopically in a Fischer 344 rat.

REPORTABLE OUTCOMES

Due to the difficulties noted above, we have not yet published any results on the inhibition of cellular respiration by MKT-077 in vitro. We hope to complete this portion of the work soon and publish a manuscript based on the uptake data and consumption inhibition work, particularly the modeling aspect. The in vivo portion of the work is in its early phases, but initial results indicate that MKT-077 may be able to increase tumor PO₂ in vivo.

CONCLUSION

In this second year of the grant, we have demonstrated that MKT-077 is rapidly taken up by the rat breast cancer cell line, R3230Ac. We have also shown that the drug is capable of inhibiting oxygen consumption in vitro in this cell line in a dose-dependent manner, whether the cells are raised under normoxic or hypoxic conditions. The time course of the inhibition of oxygen consumption was also found to be dose-dependent. Finally, we have also demonstrated that the drug can modestly increase tumor PO₂ in vivo when infused at a dose of 7.5 mg/kg.

The ability of MKT-077 to inhibit oxygen consumption in vitro is very impressive. Two µg/ml of MKT-077 was able to decrease oxygen consumption in R3230Ac cells by about 40% after one to two hours of exposure. Higher concentrations inhibited the consumption much more quickly and to a greater extent. Secomb and coworkers demonstrated theoretically that inhibition of consumption by 30% was sufficient to eradicate hypoxia in a model tumor.^{8, 9} Thus, even relatively low doses of MKT-077 are able to inhibit R3230Ac metabolism to an extent that is physiologically relevant. These in vitro results suggest that MKT-077 might be a very effective in vivo inhibitor, especially if the drug is allowed to act for several hours.

Our in vivo results during Year 2 were also very promising. We showed that infusion of 7.5 mg/kg MKT-077 can modestly increase in vivo tumor PO₂ shortly after infusion, which is consistent with the rapid inhibition of tumor cell oxygen consumption demonstrated in vitro. Although the mean increase was only 2 mm Hg, the in vitro data suggest that a higher dose of MKT-077 might result in a larger increase in PO₂. This hypothesis will be tested in the upcoming year. As noted above, the in vitro data also suggest that the in vivo PO₂ increase might be larger several hours after the drug infusion. The PO₂ histogram measurements proposed in Task 3 should answer this question, because PO₂ histograms will be measured shortly (2-3 hours) and long (24 hours) after drug administration. It will be important to determine when the oxygen change is maximal following drug infusion. This will guide the radiation study in Task 4 and would suggest a protocol for testing in humans should MKT-077 prove to be a successful radiosensitizer in the rat.

In the third year, we will continue to use the modified in vitro techniques to measure MKT-077 uptake under physiologically relevant conditions, and we will continue to study the kinetics of MKT-077-induced changes in oxygen consumption. In addition, we plan to expand the in vivo work to measure the transient effects of infusion of 10 mg/kg MKT-077. Most importantly, we will measure PO₂ histograms to test if MKT-077 can actually be used to raise oxygen levels throughout these orthotopic tumors. If this is possible, we hope to perform a radiation study to determine if this drug can indeed act as a radiosensitizer.

REFERENCES

1. Bandyopadhyay A, Lopez-Casillas F, Malik SN, et al. Antitumor activity of a recombinant soluble betaglycan in human breast cancer xenograft. *Cancer Res.* 2002;62:4690-5.
2. Braun RD, Lanzen JL, Dewhirst MW. Fourier analysis of fluctuations of oxygen tension and blood flow in R3230Ac tumors and muscle in rats. *Am J Physiol Heart Circ Physiol.* 1999;277:H551-68.

3. Braun RD, Lanzen JL, Snyder SA, Dewhirst MW. Comparison of tumor and normal tissue oxygen tension measurements using OxyLite or microelectrodes in rodents. *Am J Physiol Heart Circ Physiol*. 2001;280:H2533-44.
4. Gray LJ, Conger AD, Ebert M, Hornsey S, Scott OCA. The concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy. *Br J Radiol*. 1953;26:638-48.
5. Harrison LB, Chadha M, Hill RJ, Hu K, Shasha D. Impact of tumor hypoxia and anemia on radiation therapy outcomes. *Oncologist*. 2002;7:492-508.
6. Modica-Napolitano JS, Koya K, Weisberg E, Brunelli BT, Li Y, Chen LB. Selective damage to carcinoma mitochondria by the rhodacyanine MKT-077. *Cancer Res*. 1996;56:544-50.
7. Noel A, De Pauw-Gillet MC, Purnell G, Nusgens B, Lapiere CM, Foidart JM. Enhancement of tumorigenicity of human breast adenocarcinoma cells in nude mice by matrigel and fibroblasts. *Br J Cancer*. 1993;68:909-15.
8. Secomb TW, Hsu R, Braun RD, Ross JR, Gross JF, Dewhirst MW. Theoretical simulation of oxygen transport to tumors by three-dimensional networks of microvessels. *Adv Exp Med Biol*. 1998;454:629-34.
9. Secomb TW, Hsu R, Ong ET, Gross JF, Dewhirst MW. Analysis of the effects of oxygen supply and demand on hypoxic fraction in tumors. *Acta Oncol*. 1995;34:313-6.
10. Taghian AG, Powell SN. The role of radiation therapy for primary breast cancer. *Surg Clin North Am*. 1999;79:1091-115.
11. Tatsuta N, Suzuki N, Mochizuki T, et al. Pharmacokinetic analysis and antitumor efficacy of MKT-077, a novel antitumor agent. *Cancer Chemother Pharmacol*. 1999;43:295-301.
12. Truong PT, Olivotto IA, Whelan TJ, Levine M. Clinical practice guidelines for the care and treatment of breast cancer: 16. Locoregional post-mastectomy radiotherapy. *CMAJ*. 2004;170:1263-73.
13. Vaupel P, Briest S, Hockel M. Hypoxia in breast cancer: pathogenesis, characterization and biological/therapeutic implications. *Wien Med Wochenschr*. 2002;152:334-42.
14. Vaupel P, Hockel M. Blood supply, oxygenation status and metabolic micromilieu of breast cancers: characterization and therapeutic relevance. *Int J Oncol*. 2000;17:869-79.
15. Vaupel P, Mayer A, Briest S, Hockel M. Oxygenation gain factor: a novel parameter characterizing the association between hemoglobin level and the oxygenation status of breast cancers. *Cancer Res*. 2003;63:7634-7.
16. Woerdeman LA, Hage JJ, Thio EA, Zoetmulder FA, Th Rutgers EJ. Breast-Conserving therapy in patients with a relatively large (T2 or T3) breast cancer: long-term local control and cosmetic outcome of a feasibility study. *Plast Reconstr Surg*. 2004;113:1607-16.

APPENDICES

None.