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14. ABSTRACT

The L-methioninase-annexin V fusion protein (FP) was produced from recombinant E. coli in good purity and relatively good yield. As indicated by measuring the dissociation constant (Kd), purified FP binds strongly to human endothelial cells, MCF-7 breast cancer cells, and MDA-MB-231 breast cancer cells grown in vitro. Hydrogen peroxide was found not to be needed to induce exposure of PS on the endothelial cells. The following Kd values were determined: 1.7 nM for endothelial cells, 1.9 nM for MCF-7 cells, and 2.2 nM for MDA-MB-231 cells. The amount of FP bound on the three cell lines in vitro was found to decline steadily over 3 days, but there was still some FP bound at day 3. The rate of change of FP bound, normalized by the number of viable cells present, was the lowest for the endothelial cells. In tests of this enzyme/prodrug system in vitro, significant killing of endothelial and MDA-MB-231 cells was found at a SeMet concentration of 10 μM; for MCF-7 cells there was significant killing of cells at 100 μM SeMet. These results provide strong support for the idea that this enzyme/prodrug system will lead to damage of the tumor vasculature.

15. SUBJECT TERMS
Enzyme prodrug and methionine-depletion therapy for breast cancer, tumor vasculature targeting

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INTRODUCTION

The focus of this project is on the development of new ways to treat breast cancer with minimal or no side effects. The project aims to develop a novel enzyme prodrug and methionine-depletion combination cancer therapy in which the enzyme L-methioninase is targeted by the human protein annexin V to the breast tumor vasculature, using selenomethionine as the prodrug. Annexin V is known to bind with high affinity to phosphatidyl serine (PS) in phospholipids bilayers. PS has recently been shown to be expressed on the external surface of endothelial cells that line the blood vessels in tumors but is not expressed on the external surface of the vascular endothelium in normal organs. The enzyme L-methioninase catalyzes the conversion of methionine to methanethiol, α-ketobutyrate, and ammonia. It also catalyzes the conversion of selenomethionine to toxic methylselenol, α-ketobutyrate, and ammonia. Methylselenol has been shown to be cytotoxic to various cancer cells. To accomplish the specific aims of this project, the L-methioninase-annexin V fusion protein (FP) will be produced and purified, the strength of binding of the FP to human endothelial cells and two breast cancer cell lines *in vitro* will be determined, the cytotoxicity *in vitro* of the FP in combination with the selenomethionine prodrug will be determined for endothelial cells and two breast cancer cell lines, and the FP will be tested in nude mice with tumor xenografts (two breast cancer cell lines) for its effect as an enzyme prodrug by itself and also in combination with methionine-depletion therapy.

BODY

The research accomplishments for the first year of this project are summarized as follows:

Task 1 – Production of the recombinant L-methioninase-annexin V fusion protein to be tested

The FP was produced by recombinant expression in *E. coli* using a vector previously developed in the PI's laboratory. The FP was purified using procedures previously developed in the PI's laboratory (Zang et al. 2006), which consist of the following main steps in sequence: (1) cell lysis by sonication, (2) immobilized metal affinity chromatography (IMAC), (3) cleavage of the protein with HRV 3C protease to remove the $(His)_6$ tag, (4) IMAC with collection of the FP in the flow-through, and (5) freeze drying. To reduce the amount of FP lost in the flow-through of the first IMAC, the imidazole concentration in the wash buffer was reduced from 40 mM in the original protocol to 30 mM. Also, compared to the original protocol, the dialysis time after both chromatography steps was lowered from overnight to 3 hours, to accelerate the purification process to 2 days instead of 3, and to reduce the loss of activity occurring during the dialysis. The complete procedures for the expression and purification of the FP are given in Appendix I.

For a 1 liter batch of cell culture, the yield of purified protein was 21 mg. An SDS-PAGE gel of the FP is shown in Figure 1, lane 3; the purity of the FP was estimated to be >99% using densitometry software analysis. The recovery of L-methioninase activity from the starting broth was 26%, and the specific activity of the purified protein was 1.0 U/mg protein. The specific activity was originally thought to be 10 U/mg protein, but it was discovered that a factor of 10 error was made previously in the standard curve for the enzyme assay. The activity was

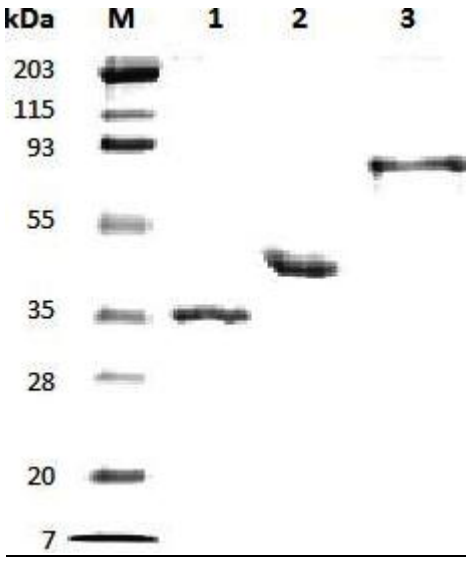

Figure 1. SDS-PAGE analysis with Coomassie blue staining of three of the purified proteins. *Lane 1* annexin V, *lane 2* L-methioninase, *lane 3* methioninase-annexin V, *M* marker proteins with molecular masses indicated on the left in kiloDaltons.

also calculated using the extinction coefficient for the azine derivative produced and the recorded absorbance; this activity was within 3% of the specific activity measured using the new standard curve. To investigate this further, the L-methioninase gene was sequenced and compared with the L-methioninase gene sequence in the gene database. It was found that a mismatched base pair gave a point mutation and caused the change of the amino acid Gly 377 of the FP into an Arg. We are now working to correct this mutation using site-directed mutagenesis.

Task 2 – Test of the function *in vitro* of annexin V for the L-methioninase-annexin V fusion protein

The ability of the FP to bind to human endothelial cells and breast cancer cells with PS exposed on the cell surface was evaluated by equilibrium binding experiments in which increasing concentrations of biotin-labeled FP were used. For this labeling, a kit obtained from KPL was used; the procedure used is given in Appendix II. In initial experiments with endothelial cells, hydrogen peroxide was used at a low concentration (1 mM) to induce exposure of PS. In later experiments, the H_2O_2 was omitted with little change in the results; therefore, the data reported here is with no H_2O_2 added. No H_2O_2 was added in the experiments with the breast cancer lines, since it has been reported that cancer cells express PS when grown *in vitro* (Sugimura et al. 1994; Utsugi et al. 1991). The procedures for growing all the cell lines are given in Appendix III.

A typical equilibrium binding result is shown in Figure 2 for endothelial cells. The non-specific binding, obtained in the absence of Ca^{2+}, is subtracted from the total binding to obtain the specific binding. The dissociation constant (K_d) for each cell line tested was obtained from the specific binding data using GraphPad Prism 5 software to give the following results: 1.7 ± 0.6 nM for endothelial cells, 1.9 ± 1.2 nM for MCF-7 breast cancer cells, and 2.2 ± 1.2 nM for

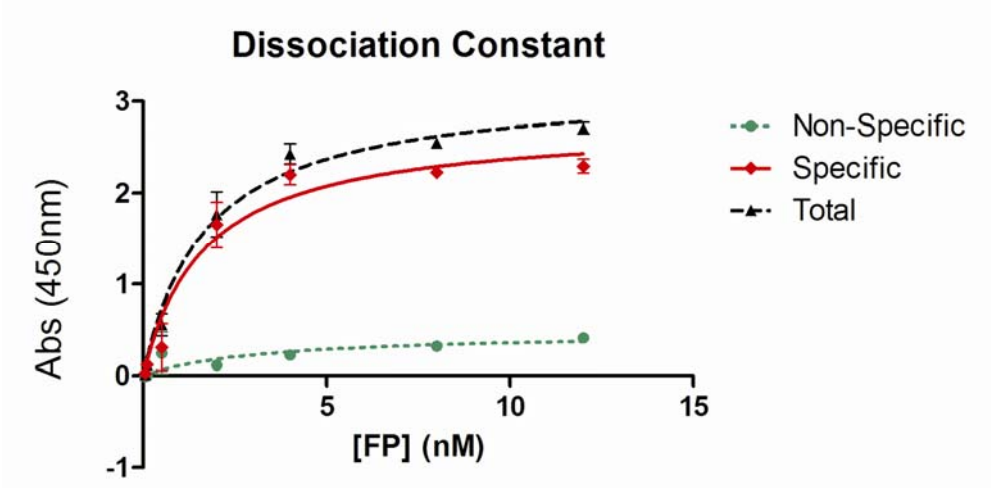

Figure 2: Determination of FP binding strength to exposed PS on endothelial cells. FP was biotinylated and streptavidin-HRP was used to quantify the binding. Total binding was obtained by having 2 mM of Ca^{2+} in the binding buffer. Non-specific binding was obtained by removing the Ca^{2+} for the binding buffer and replacing it with 5 mM of EDTA to chelate Ca^{2+}. Specific binding was obtained by subtracting the non-specific binding from the total binding. Bars indicate the standard error of the mean (SEM), n=3.

MDA-MB-231 breast cancer cells. These results indicate that the binding of the FP to these cells is relatively strong. Literature values of annexin V binding alone to endothelial cells have been reported from 2.7—15.5 nM (van Heerde et al. 1994a; van Heerde et al. 1994b). Thus, the K_d values that we have measured are lower than those reported in the literature for annexin V alone, indicating the binding is stronger; this is probably because the FP exists as a tetramer, giving four annexin V molecules able to bind per FP molecule. The procedure for measuring the equilibrium binding of the fusion protein to endothelial cells and breast cancer cells is given in Appendix IV.

To assess how long the FP remains bound to the surface of the endothelial cells, a modified binding assay was used. Cells on 24-well plates were first incubated for 2 h at 37˚C in a saturating concentration of biotinylated FP (50 nM) in complete growth medium with 2 mM Ca^{2+}. Increased amounts of L-methionine were added to offset the methionine depletion effect (500 µM for endothelial cells and MDA-MB-231 cells, and 2000 µM for MCF-7 cells). Then, the cells were fixed either at day 0, 1, 2 or 3 with 0.25 % glutaraldehyde in binding buffer. Excess aldehyde groups were quenched by incubation in a 50 mM NH_4Cl in binding buffer. The binding of FP was then quantified using the same procedure used in the equilibrium FP binding assay. The stability of FP binding for 3 days was normalized by the viability of the cells as

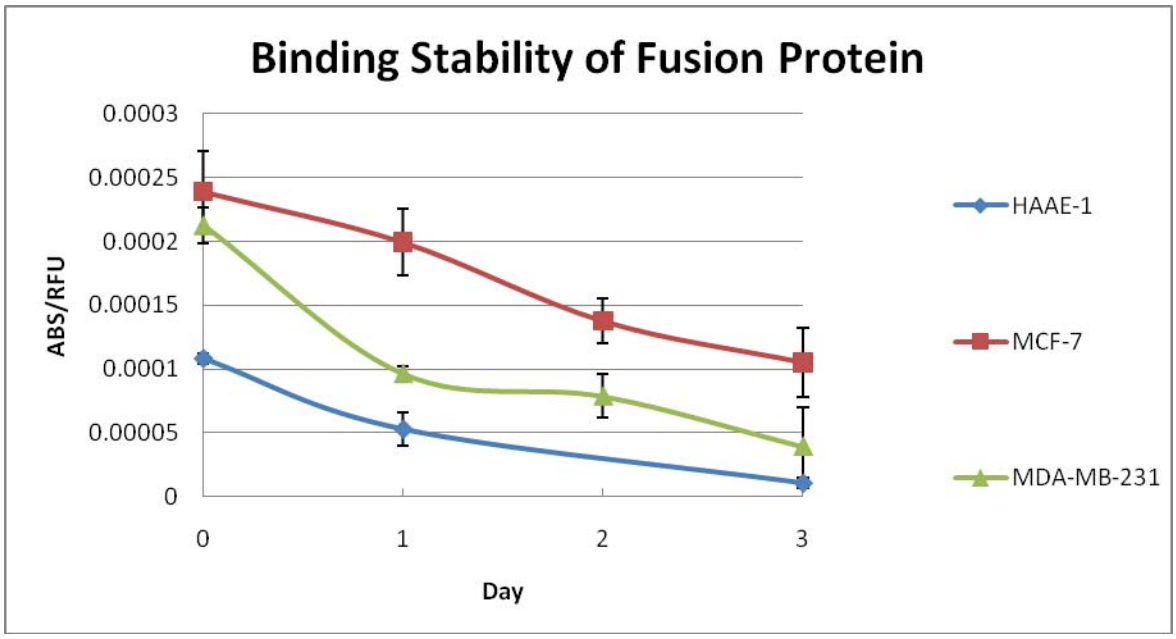

Figure 3: Fusion protein binding stability. The Alamar Blue assay was performed each day, followed by the binding assay to determine the duration of binding of annexin V to the exposed PS on the surface of each cell line. FP was found to stay bound to the cell lines for at least 3 days, the period of the experiment. ABS is the absorbance at 450 nm that is measured in the binding assay. RFU is relative fluorescence units, measured by the Alamar Blue assay for cell viability. Bars indicate the standard error of the mean (SEM), n=3.

shown in Figure 3 for the three cell lines. Cell viability, as measured by the Alamar Blue assay, was found to be linearly proportional to the number of cells (data not shown). The data in Figure 3 indicate a steady decline in FP bound over 3 days, but there is still some FP bound at day 3. For each cell line, the slope by linear regression of the absorbance/RFU versus time data was obtained, with results as follows: 3.07×10^{-5} per day for the endothelial cells (HAAE-1), 4.63×10^{-5} per day for the MCF-7 cells, and 5.38×10^{-5} per day for the MDA-MB-231 cells. Thus, the rate of change of absorbance/RFU was the lowest for the endothelial cells.

Task 3 – Test of the anticancer activity *in vitro* of the L-methioninase-annexin V fusion protein in combination with selenomethionine prodrug on endothelial cells and breast tumor cells

The ability of the enzyme/prodrug system to eliminate human endothelial cells and breast cancer cells was evaluated using cells grown *in vitro* and plated on 24-well plates. A saturating concentration of fusion protein was added, followed by concentrations of SeMet ranging from 0 – 500 μM. Hydrogen peroxide was not used in the tests with endothelial cells because of the previous finding in the binding studies (see Task 2) that deletion of hydrogen peroxide did not affect the binding results. For each cell line, the methionine concentration in the medium was set at a level that would not lead to a significant decrease in cell viability because of methionine

depletion with FP present. Each of the cell lines in question metabolized the Alamar Blue to produce a fluorescence that was measured to quantify total cell viability. For each day, the fluorescence data from the Alamar Blue assay was expressed as a percentage of the fluorescence for the cells with no FP and 0 μM SeMet (control). Cells that were treated with different SeMet concentrations but no FP were compared to the control, whereas cells that had the FP were compared to cells with the same SeMet concentration but no FP. The procedures used are given in Appendix V.

The results for endothelial cells in medium that contained 500 μM L-methionine are shown in Figure 4. Significant cell killing was found at days 2 and 3 for 10 and 100 μM SeMet (at day 2, $p < 0.05$; at day 3, $p < 0.05$ for 10 μM SeMet and $p < 0.01$ for 100 μM SeMet). For MCF-7 breast cancer cells containing 2000 μM L-methionine, there was significant cell killing at day 2 with 500 μM SeMet and at day 3 with 100 and 500 μM SeMet (Figure 5, $p < 0.01$). At day 3 with no FP and 500 μM SeMet, cell viability was significantly lower ($p < 0.05$), indicating that SeMet by itself is somewhat toxic to cells. Figure 6 shows the results for MDA-MB-231 breast cancer cells with 500 μM L-methionine. With no SeMet present, there was a significant decrease in cell viability when the FP was added, indicating that the L-methionine level needs to be higher. However, the addition of 10 μM SeMet caused a further drop in cell viability, and addition of 500 μM SeMet caused almost complete cell killing. It is planned to repeat this test for MDA-MB-231 cells with 750 μM L-methionine.

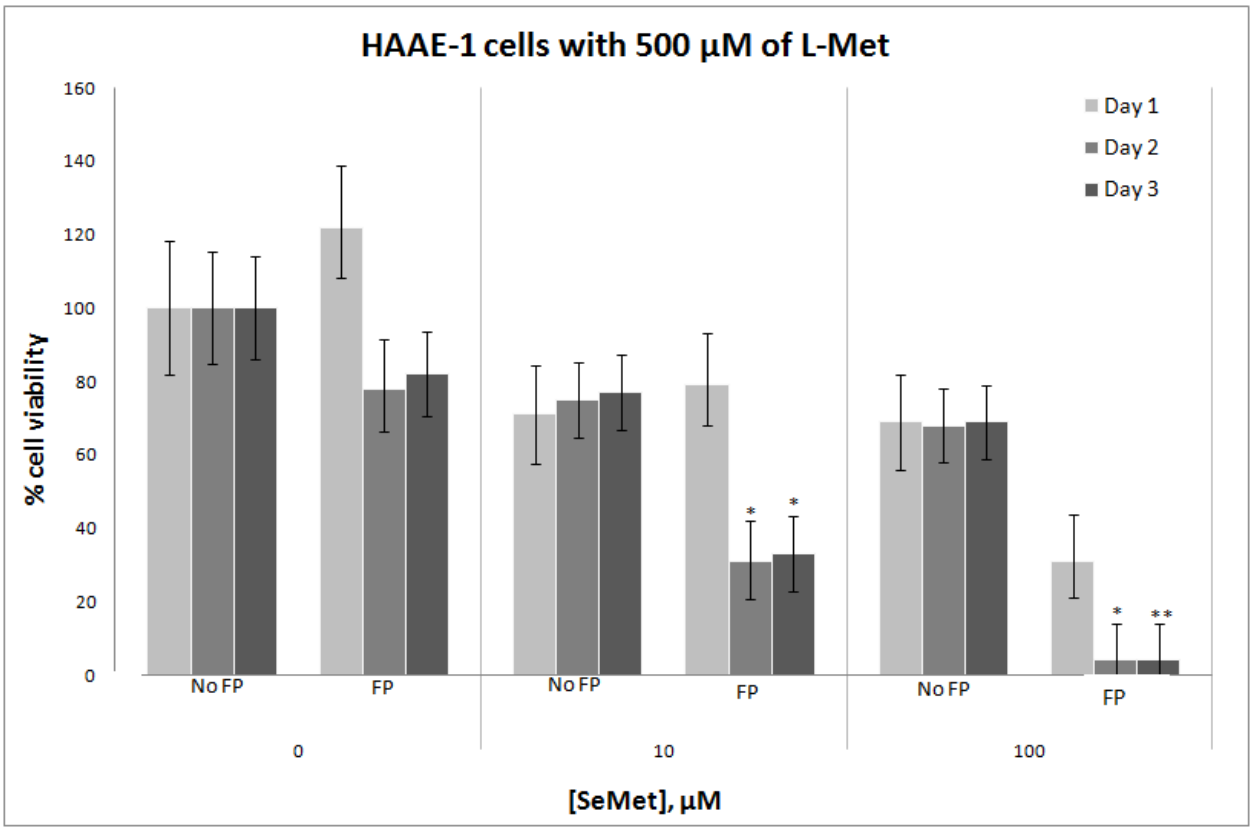

Figure 4: Effect of SeMet conversion to methylselenol on HAAE-1 endothelial cells. Cells were grown in medium adjusted to 500 µM of L-methionine. Cell viability was assessed using Alamar Blue assay and normalized to the control (i.e. no FP and no SeMet). A two-tailed T-test was performed for statistical analysis. Cells treated with different SeMet concentrations but with no FP were compared to the control and statistical significance was represented with (#). Cells treated with different SeMet concentrations and FP were compared to cells that were treated with the same SeMet concentration but no FP, and statistical significance was represented with (*). * $p < 0.05$; ** $p < 0.01$. Bars indicate the standard error of the mean (SEM), n=3.

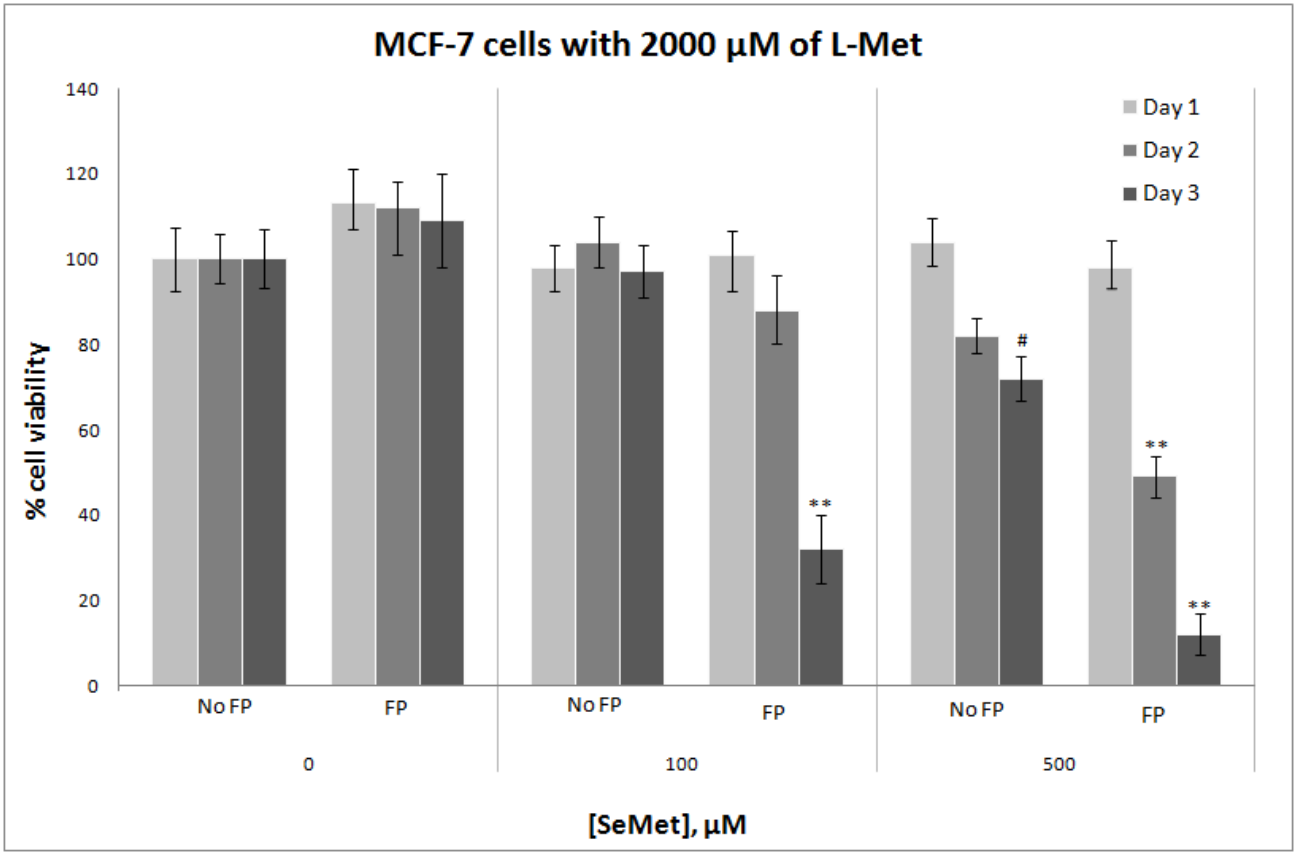

Figure 5: Effect of SeMet conversion to methylselenol on MCF-7 breast cancer cells. Cells were grown in medium adjusted to 2000 µM of L-methionine. Cell viability was assessed using Alamar Blue assay and normalized to the control (i.e. no FP and no SeMet). A two-tailed T-test was performed for statistical analysis. Cells treated with different SeMet concentrations but with no FP were compared to the control and statistical significance was represented with (#). Cells treated with different SeMet concentrations and FP were compared to cells that were treated with the same SeMet concentration but no FP and statistical significance was represented with (*). # $p < 0.05$. * $p < 0.05$; ** $p < 0.01$. Bars indicate the standard error of the mean (SEM), n=3.

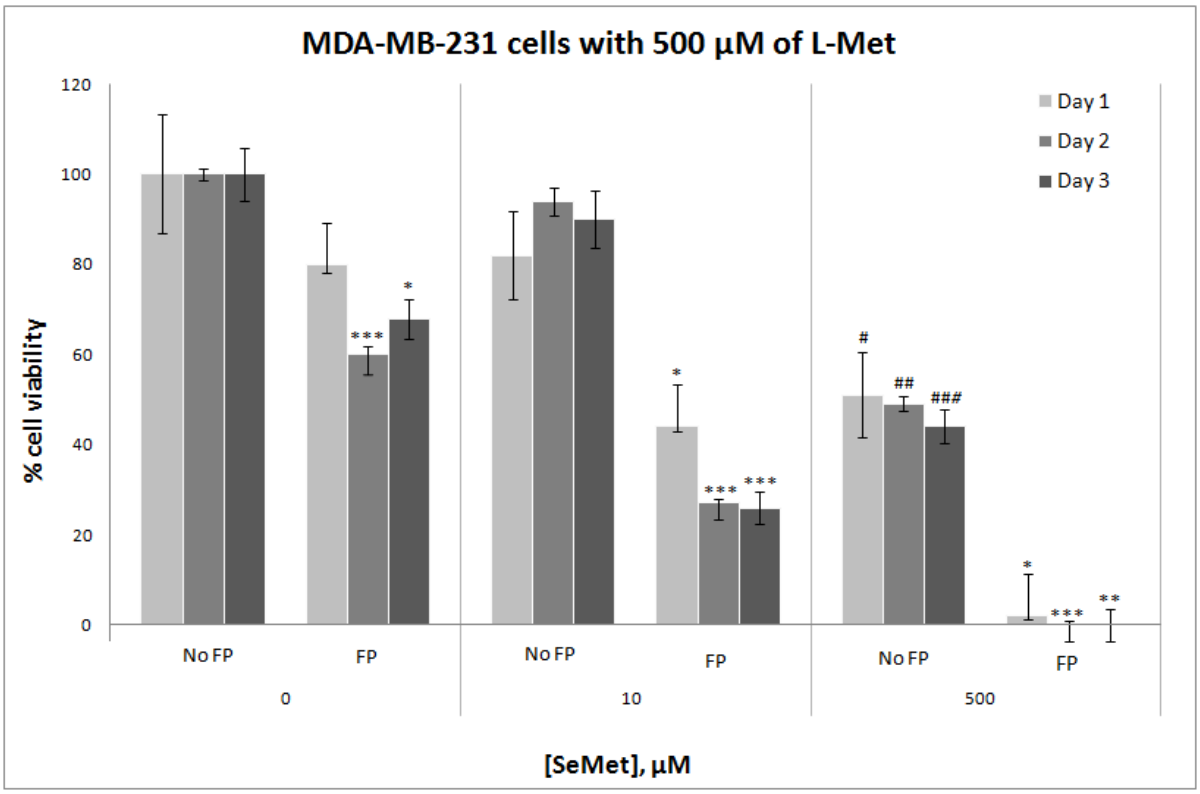

Figure 6: Effect of SeMet conversion to methylselenol on MDA-MB-231 breast cancer cells. Cells were grown in medium containing 500 µM of L-methionine. Cell viability was assessed using Alamar Blue assay and normalized to the control (i.e. no FP and no SeMet). A two-tailed T-test was performed for statistical analysis. Cells treated with different SeMet concentrations but with no FP were compared to the control and statistical significance was represented with (#). Cells treated with different SeMet concentrations and FP were compared to cells that were treated with the same SeMet concentration but no FP and statistical significance was represented with (*). #, * $p < 0.05$; ##, ** $p < 0.01$; ###, *** $p < 0.001$. Bars indicate the standard error of the mean (SEM), n=3.

KEY RESEARCH ACCOMPLISHMENTS

- Purified recombinant FP was produced in good purity (>99%) and relatively good yield (21 mg/liter of starting culture broth).
- As indicated by measuring the dissociation constant (K_d), purified FP binds strongly to human endothelial cells, MCF-7 breast cancer cells, and MDA-MB-231 breast cancer cells grown *in vitro*. Hydrogen peroxide was found to not be needed to induce exposure of PS on the endothelial cells. The following K_d values were determined: 1.7 nM for endothelial cells, 1.9 nM for MCF-7 cells, and 2.2 nM for MDA-MB-231 cells.
- The amount of FP bound on the three cell lines *in vitro* was found to decline steadily over 3 days, but there was still some FP bound at day 3. The rate of change of FP bound, normalized by the number of viable cells present, was the lowest for the endothelial cells.

- In tests of this enzyme/prodrug system *in vitro*, significant killing of the cells was found at a SeMet concentration of 10 μM for endothelial cells and MDA-MB-231 breast cancer cells; for MCF-7 breast cancer cells there was significant killing at 100 μM SeMet.

REPORTABLE OUTCOMES

Harrison, R.G., Van Rite, B.D., Lazrak, Y.A., Pagnon, M., and McFetridge, P.S., "Targeting of Solid Tumor Vasculature with Enzyme/Prodrug and Methionine-Depletion Therapy," poster presentation, Biomedical Engineering Society Annual Meeting, Pittsburgh, October, 2009.

Harrison, R.G., "Enzyme Prodrug Cancer Therapy Selectively Targeted to Tumor Cells or Tumor Vasculature and Methods of Production Thereof," U.S. Provisional Patent Application, March, 2009.

Lazrak, Y.A. (speaker), Neves, L.F.F., McFetridge, P.S., and Harrison, R.G., "Novel Enzyme-Prodrug Therapy for Cancer," oral presentation, Biomedical Engineering Society Annual Meeting, St. Louis, October, 2008.

Harrison, R.G. (speaker), Lazrak, Y.A., Neves, L.F.F., McFetridge, P.S., and Tfayli, A., "Novel Enzyme-Prodrug Therapy for Cancer," oral presentation, American Institute of Chemical Engineers Annual Meeting, Philadelphia, November, 2008.

CONCLUSIONS

Recombinant FP was produced in good purity and yield, and it has been shown to bind strongly to PS exposed on human endothelial cells and MCF-7 and MDA-MB-231 breast cancer cells (dissociation constants ranging from 1.7 to 2.2 nM). The non-specific binding, obtained in the absence of Ca^{2+}, was subtracted from the total binding to obtain the specific binding. In a binding experiment over 3 days for each of the three cell lines, it was found that there was a steady decline in FP bound over this period, but there was still some FP bound at day 3. The rate of change in binding was lowest for the endothelial cells. Tests with the enzyme prodrug over a period of 3 days showed significant cell killing at 10 μM SeMet for endothelial cells and MDA-MB-231 cells. Significant cell killing was obtained at 100 μM SeMet for MCF-7 cells. These results provide strong support for one of the basic ideas for the project, which is that the conversion of the SeMet to methylselenol at the surface of the tumor vasculature will lead to damage of the tumor vasculature; this damage will lead to clotting of the tumor vasculature, thus cutting off the oxygen supply of the cancer cells.

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APPENDIX I

Fusion Protein Expression

1. Culture 5 µl of *E. coli* BL21 (DE3) harboring pET- 30 Ek/LIC with the fusion gene L-methioninase-annexin V in 10 ml of LB medium containing 35 µg/ml kanamycin overnight at 37^{o}C with shaking at 200 rpm.
2. Add the 10 ml of cell culture to 1 L of LB medium with 35 µg/ml kanamycin, and incubate at 37^{o}C with shaking at 200 rpm. Take 2 mL of medium, before adding the bacteria, to use it as a blank. This cell culture was grown to mid-log phase (OD_{600} = 0.5)
3. Add isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM and incubate at 30^{o}C with shaking at 180 rpm for 5 h to induce protein expression.
4. Harvest the cells by centrifugation for 10 min at 1000 x *g*, at 4ºC.

Fusion Protein Purification

It is important to take samples at each step of the purification in order to measure the protein concentration and enzymatic activity as well as to perform SDS-PAGE. Chromatography steps were performed using a HisTrap column at a superficial velocity of 240 cm/hr.

1. Resuspend the cell pellet in 40 ml of sonication buffer which is composed of:
    a. 0.05 mM N- *p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)
    b. 1 mM phenylmethylsulfonyl fluoride (PMSF)
    c. 1% HPLC ethanol
    d. 0.02 mM pyridoxal phosphate,
    e. 0.01% β-mercaptoethanol
    f. 0.02 M sodium phosphate at pH 7.4.
2. Lyse the cells by sonication at 4^{o}C for 30 sec at 4.5 watts and then allow it to cool for 30 sec on ice. Repeat this cycle 4 times for a total sonication time of 2.5 min.
3. Centrifuge the lysate obtained at 12,000 x *g* for 30 min, to remove the cell debris, and take the supernatant.
4. Add 30 mM of imidazole and 500 mM of NaCl to the lysate to reduce non-specific protein binding.
5. Wash a 5 ml HisTrap column with immobilized Ni^{2+}, HisTrap, with wash buffer until the pen reaches the baseline. The wash buffer is composed of:
    a. 20 mM sodium phosphate,
    b. 30 mM imidazole,
    c. 500 mM NaCl,
    d. 0.02 mM pyridoxal phosphate at pH 7.4.
6. Feed the lysate to the HisTrap column.
7. Wash the column with the wash buffer to remove unwanted proteins, until the pen reaches the baseline.
8. Elute the protein using elution buffer which is composed of:
    a. 20 mM sodium phosphate,
    b. 500 mM imidazole,
    c. 500 mM NaCl,
    d. 0.02 mM pyridoxal phosphate at pH 7.4.

9. Dialyze the eluted protein for 3 h against “dialysis buffer 1”, which contains 20 mM sodium phosphate and 0.02 mM pyridoxal phosphate at pH 7.4, to remove NaCl and imidazole from the protein solution and make it suitable for N-terminal His-tag cleavage.
10. Measure the concentration of protein using the Bradford assay.
11. Cleave the N-terminal His-tag by adding HRV-3C protease (10 Units/mg of protein) and recommended 10X buffer (1.5 M NaCl, 0.5 M Tris-HCl, pH 7.5), incubate for 16 h at 4^{o}C with gentle shaking (volume added is 10% of the original volume).
12. Add imidazole (30 mM) and NaCl (500 mM) to the cleaved protein solution.
13. Wash the HisTrap column with wash buffer until the pen reaches the baseline.
14. Feed the solution to the HisTrap column.
15. Collect pure protein that comes out with the flow-through.
16. Regenerate the HisTrap column by feeding it these solutions in the following order:
    a. 25 ml of 1 M KCl
    b. 25 ml of 1 M NaOH
    c. 25 ml of dH_2O
    d. 25 ml of 1 M ethanol HPLC grade
17. Dialyze the purified protein for 3 h against “dialysis buffer 2”, which contains 20 mM sodium phosphate buffer at pH 7.4, 0.02 mM pyridoxal phosphate and 0.1 M NaCl.
18. Sterilize the dialyzed protein by filtering it trough a 0.2 µm cellulose acetate filter.

APPENDIX II

Procedure for the Labeling of the L-Methioninase-annexin with Biotin

The concentration of the protein to be biotinylated needs to be between 0.2 to 5 mg/ml.

1. Dissolve 1.0 mg biotin in 50 µL anhydrous DMF immediately prior to use.
2. Using a 60-fold excess of biotin for conjugation, add the appropriate volume of 20 mg/ml SureLINK Chromophore Biotin to the protein solution.

$$\text{Volume (µL) of 20 mg/mL biotin for conjugation reaction} = \frac{1000 \times (\text{protein amount in mg}) \times (\text{biotin molar excess ratio})}{(\text{protein molecular weight in kDa}) \times (25\ \text{nmol/µL})}$$

3. Incubate at room temperature for 2 hours with gentle agitation.
4. Remove the unconjugated chromophore biotin by extensive dialysis using a 10 kDa membrane in a 1X modification buffer. Two 3 hour dialyses and an overnight dialysis were performed, with dialysis buffer change after each.
5. Determine the degree of biotin incorporation was determined using the following protocol.

Determining the Degree of Biotin Incorporation

1. Transfer the labeled protein sample into a cuvette and measure the absorbance 354 nm using a UV spectrophotometer. Absorption values should fall within the linear range of the spectrophotometer, typically 0.05 – 2.00.
2. Calculate the molar concentration of biotin. [Biotin] (M) = $A_{354\ nm}$ / 29,000.
3. Calculate the molar substitution ratio (MSR), MSR = [Biotin] / [Protein]. Protein concentration was determined using Bio-Rad's Bradford assay.

APPENDIX III

Procedures for Growing the Cell Lines

Human HAAE-1 aorta endothelial cells were grown in F-12K medium with 2 mM L-glutamine and 1.5 g/L sodium bicarbonate and supplemented with 10% fetal bovine serum (FBS), 0.03 mg/ml endothelial cell growth supplement, and 0.1 mg/ml heparin. MCF-7 human breast cancer cells were maintained as monolayer cultures in Eagle's Minimum Essential medium containing 1 mM sodium pyruvate and 1.5 g/L sodium bicarbonate, and supplemented with 10% FBS, 2 mM L-glutamine, and 0.01 mg/ml bovine insulin. MDA-MB-231 human breast cancer cells were grown in Leibovitz's L-15 medium supplemented with 10% FBS and 2 mM L-glutamine. Penicillin (50,000 U) and streptomycin (50,000 μg) were also added to each medium. HAAE-1 and MCF-7 cells were grown at 37 ^{o}C in a 5% CO_2 atmosphere, while MDA-MB-231 cells were grown without CO_2 at 37 ^{o}C.

APPENDIX IV

Assay for the Binding of L-Methioninase-annexin V on Externally Positioned PS on the Surface of Endothelial and Cancer Cells

1. Coat 24-well plates with gelatin (for endothelial cells only).
2. Seed $5x10^4$ cells in each well, and grow them until they reach 70% confluence
3. Remove the existing medium.
4. Fix the cells by adding 100 µl/well of binding buffer (PBS with 2 mM Ca^{2+}) containing 0.25% glutaraldehyde. Incubate 5 minutes at room temperature. Remove after incubation period.
5. Quench excess aldehyde groups by adding 100 µl/well of 50 mM NH_4Cl, diluted in binding buffer for 5 minutes at room temperature. Remove after incubation period.
6. Perform serial dilutions of biotinylated fusion protein in binding buffer containing 0.5% BSA, with concentrations of 12, 8, 4, 2, 0.5, 0.1, and 0.05 nM. For the blank, delete the FP addition.
7. Add 300 µl of each dilution to one triplet of wells, and incubate for 2 hours at 37ºC, 5% CO_2.
8. Wash with 300 µl of the binding buffer + BSA.
9. Add 300 µl of streptavidin-HRP (2 µg/ml) solution and incubate for 1 hour at room temperature.
10. Wash with 300 µl of the binding buffer.
11. Add 300 µl of the chromogenic substrate O-phenylenediamine (OPD) to each well.
    a. The OPD solution is made with phosphate-citrate buffer (1 capsule in 100 ml dH_2O).
    b. Immediately prior to use, add 40 µl of 30% H_2O_2 to the 100 ml citrate buffer.
    c. Prepare the desired volume of OPD at a concentration of 0.4 mg/ml.
12. Incubate for 30 minutes at room temperature in the dark.
13. Transfer 100 µl of the supernatant to 96-well plates.
14. Measure absorbance at 450 nm. Use a blank that was subjected to the same procedure but with no FP added.
15. To determine the non-specific binding, perform the same procedure with no Ca^{2+} and 5 mM EDTA in the binding buffer.

APPENDIX V

Procedures for Determination of the Cytotoxicity of the Fusion Protein in Combination with the Selenomethionine Prodrug

The medium used in this assay is the complete growth medium of the cells being tested, adjusted to have 2 mM Ca^{2+} and 0.02 mM pyridoxal phosphate. Starting with step 3, additional methionine is added to the medium, in order not to cause a decrease in cell viability because of methionine depletion.

1. Coat 24-well plates with gelatin (for endothelial cells only).
2. Seed $5x10^4$ cells in each well, and grow them until they reach 70% confluence.
3. Remove medium and
   a. For set 1, add 300 µl of medium containing 100 nM methioninase-annexin V fusion protein (FP).
   b. For set 2, add 300 µl of medium (no FP).
4. Incubate for 2 h at 37ºC, 5% CO_2 (except do not use CO_2 for MDA-MB-231 cells).
5. Wash with medium.
6. Add 300 µl of medium containing different concentrations of selenomethionine (SeMet). Use triplicates for each concentration.
7. Perform the Alamar Blue assay.
   a. Add 10% (30 µl) of Alamar Blue (the blank should have medium with no cells).
   b. Incubate for 4 hours at 37ºC, 5% CO_2.
   c. Transfer 250 µl to an opaque 96-well plate.
   d. Read fluorescence at 590 nm using excitation at 530 nm.
8. Remove the remaining Alamar Blue solution from the wells, and add 300 µl of medium to each well with the desired SeMet concentration.
9. Repeat steps 6 and 7 on day 1, 2 and 3 (change the medium just before adding the Alamar Blue).

Perform the enzymatic activity and protein concentration tests to make sure that there are no significant changes in the concentration and activity of the FP.