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The Transsulfuration Pathway Significantly Contributes to Glutathione Biosynthesis in Human Mammary Epithelial Cells

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**THE TRANSSULFURATION PATHWAY SIGNIFICANTLY CONTRIBUTES TO
GLUTATHIONE BIOSYNTHESIS IN HUMAN MAMMARY EPITHELIAL CELLS**

**Thesis submitted to
the Graduate College of
Marshall University**

**In partial fulfillment of
the requirements for the degree of
Master of Science
in Chemistry**

by

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ABSTRACT

THE TRANSSULFURATION PATHWAY SIGNIFICANTLY CONTRIBUTES TO GLUTATHIONE BIOSYNTHESIS IN HUMAN MAMMARY EPITHELIAL CELLS

By Andrea Belalcázar

Cellular methylation and antioxidant metabolism are linked by the transsulfuration pathway, which converts the methionine cycle intermediate homocysteine to cysteine, a precursor for glutathione biosynthesis, principally in hepatic cells. In mammals, the transsulfuration pathway has been identified in liver, kidney, pancreas and brain. To determine whether the pathway exists in mammary cells human breast adenocarcinoma cells (MCF-7) and normal mammary epithelial cells (HMEC) were labeled with ^{35}S -methionine for 24 hours following pre-treatment with a vehicle control, the cysteine biosynthesis inhibitor propargylglycine (PPG) or the gammaglutamyl cysteine synthesis inhibitor buthionine sulfoximine (BSO). Cell lysates were prepared and reacted with glutathione-S-transferase (GST) and the fluorescent labeling compound monochlorobimane (mCBI) to cause glutathione (GSH) to form a fluorescent GSH-Bimane conjugate with mCBI. Thin layer chromatography (TLC) was used to separate conjugated glutathione (GSH-Bi) from the free mCBI fluor. Fluorescent images and autoradiography of the TLC plate were compared; incorporation of ^{35}S -methionine into glutathione-bimane spots identified through fluorescence (GSH-mCBI bands) indicated that functional transsulfuration occurs in mammary cells (there is no other known manner for the ^{35}S of methionine to incorporate into cysteine and subsequently into GSH). Given the role that glutathione plays as a major cellular antioxidant, we were interested in determining the extent to which transsulfuration contributes to glutathione production under conditions of oxidant stress; enzymatic determination of the impact of PPG pre treatment and 2hr 300uM hydrogen peroxide treatment of HMEC's on GSH levels indicated PPG reduced total GSH by one third, and blocked the ability of oxidatively stressed cells to upregulate GSH production. In summary, results of this study demonstrate the presence of the transsulfuration pathway in mammary epithelial cells and the importance of this pathway under oxidative stress conditions.

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TABLE OF CONTENTS

ABSTRACT.....	ii
ACKNOWLEDGMENTS	iii
TABLE OF CONTENTS.....	iv
LIST OF FIGURES	v
INTRODUCTION	1
MATERIALS AND METHODS.....	6
RESULTS	11
DISCUSSION.....	14
FIGURES.....	17
APPENDIX.....	38
REFERENCES.....	39

LIST OF FIGURES

Figure 1	The transsulfuration pathway linking the methionine cycle and the synthesis of glutathione. ...	17
Figure 2	PPG and BSO effects on the transsulfuration pathway.	18
Figure 3	Reaction scheme between monochlorobimane and glutathione to form GSH-mCB	19
Figure 4	Methionine cycle	20
Figure 5	Synthesis of Cysteine.....	21
Figure 6	Glutathione Synthesis Pathway	22
Figure 7	Role of glutathione as an antioxidant	23
Figure 8	Effect of H ₂ O ₂ in the methionine cycle	24
Figure 9	The two cell lines used for studies.....	25
Figure 10	Methods diagram for experimental protocol 2.	26
Figure 11	Diagram of oxidative stress treatment for experiment 3	27
Figure 12	Mass spec of glutathione-monochlorobimane (GSH-mCB) conjugate used as a control	28
Figure 13	Mass spec of MCF-7 lysate glutathione-monochlorobimane conjugate	29
Figure 14	A scanned TLC plate showing the selectivity of the monochlorobimane reaction with glutathione present in HMEC lysates.	30
Figure 15	A scanned TLC plate showing the selectivity of the monochlorobimane reaction with glutathione present in MCF-7 cell lysates.	31
Figure 16	Incorporation of ³⁵ S-methionine into glutathione (GSH-mCB bands) showed in the autoradiography demonstrates that functional transsulfuration occurs in HMEC.....	32
Figure 17	Incorporation of ³⁵ S-methionine into glutathione (GSH-mCB bands) showed in the autoradiography demonstrates that functional transsulfuration occurs in MCF-7.....	33
Figure 18	Mass spec of HMEC showing the glutathione-monochlorobimane conjugate peak.	34
Figure 19	Mass spec of HMEC showing that under the presence of BSO there is no presence of glutathione-monochlorobimane conjugate peak.	35
Figure 20	Mass spec of HMEC treated with PPG showing the peak at 498 for glutathione-monochlorobimane conjugate (GSH-mCB)	36
Figure 22	Impact of transsulfuration inhibition with PPG on cellular total glutathione levels in HMEC subjected to oxidative challenge (H ₂ O ₂).	37

INTRODUCTION

The transsulfuration pathway (see Figure 1) links two important processes for the human body, the regeneration of methionine (which occurs through the methionine cycle) to form S-adenosylmethionine, the principal cellular methyl donor, and the synthesis of glutathione, the principal cellular antioxidant. The transsulfuration pathway converts homocysteine, an intermediate of methionine cycle, to cysteine, a biosynthesis precursor of glutathione.¹ Since the transsulfuration pathway was first described 30 years ago by Reed and co-workers in liver,² it has also been identified in certain other tissues including the kidney,^{3,4} pancreas,^{5,6} intestine,⁷ lens,⁸ brain,^{9,10,11} and lymphoid cells.¹² Due the vital role of glutathione against oxidative stress,¹ we were interested in determining whether transsulfuration provides biosynthetic precursors for glutathione in other tissues. Breast cancer is a highly prevalent disease, and oxidant stress may contribute to its development.¹³ For this reason we were exploring the role of transsulfuration in mammary tissues. Our hypothesis was that human mammary epithelial cells (HEMC) and MCF-7 human breast adenocarcinoma cells would incorporate ³⁵S-methionine into glutathione via a metabolically active transsulfuration pathway. This would indicate that a link exists between the methionine cycle and the synthesis of glutathione in this tissue type. As part of our research strategy, two inhibitors were used, propargylglycine (PPG),¹⁴ an irreversible inhibitor of gamma-cystathionase, and buthionine sulfoximine (BSO), a synthetic amino acid that irreversibly inhibits gamma-glutamylcysteine synthetase (see Figure 2). Propargylglycine inhibition thus blocks the transsulfuration pathway, preventing the genesis of cysteine by blocking production of its precursor, cystathionine. Buthionine sulfoximine inhibition prevents the incorporation of cysteine from **any** source into glutathione biosynthesis.

To be able to identify glutathione, cell pellet lysates were reacted with monochlorobimane (mCBI) in the presence of added glutathione S transferase (GST). Monochlorobimane (mCBI) forms a stable fluorescent GSH-bimane adduct in a reaction catalyzed by GST (see Figure 3).¹⁵ This adduct can be detected fluorimetrically permitting us to identify the location of glutathione as a fluorescent spot on a TLC silica plate.¹⁶ This method has been tested before using liver tissue,^{15,16} cultured neural cells,^{17,18,19} and ocular tissues.²⁰

In the methionine cycle (see Figure 4), methionine is converted to S-adenosylmethionine (SAdMe) in a reaction catalyzed by methionine adenosyltransferase (MAT), a magnesium dependent enzyme. MAT catalyzes the only reaction that generates SAdMe.²¹ In this reaction methionine and adenosine triphosphate (ATP) combine to form SAdMe, releasing the triphosphosphate portion of ATP after ATP donates the adenosine group to methionine. The remaining triphosphosphate portion of ATP is hydrolyzed simultaneously to Pi and PPi by the intrinsic triphosphatase activity of MAT.²²

SAdMe, the principal methyl donor in the human body is converted to S-Adenosylhomocysteine (SAH) after its methyl group has been transferred to a large variety of acceptor molecules in reactions catalyzed by various methyltransferases.²³ These methyl groups participate in the methylation of DNA, RNA, proteins, membrane phospholipids and neurotransmitters.^{22,24} Such methylation plays an important role in processes such as epigenetic regulation (via DNA and histone methylation), protein stability, and phospholipid and neurotransmitter production.²⁵ Also, methylation by SAdMe is a critical step in the stabilization of many proteins, including myelin. SAdMe is also a key metabolite that regulates hepatocyte growth, death and differentiation.²³

In humans, homocysteine is derived usually from methionine through the transmethylation pathway from the hydrolysis of SAH via a reversible reaction catalyzed by adenosylhomocysteinase, releasing adenosine.²⁶ SAH is a potent competitive inhibitor of methylation reactions and prompt removal of adenosine and homocysteine is required to prevent accumulation of SAH.²³

Homocysteine has two major metabolic fates, the first is the transmethylation catalyzed by methionine synthase (MS) which regenerates methionine.¹ MS requires normal levels of folate and vitamin B₁₂, and serves to release N⁵-methyl tetrahydrofolate as tetrahydrofolate (THF), the active form of folic acid.¹ Methionine regenerates by retrieving the methyl radical from 5-methyltetrahydrofolate (5-MTHF) creating tetrahydrofolate (THF) which will then regenerate to 5-MTHF through the action of methylenetetrahydrofolate reductase (MTHFR). This process is called remethylation.^{27,28} THF supports a number of folate dependent one carbon transfer reactions. The efficiency of folate metabolism has an impact on the availability of SAdMe because it supplies the methyl group used to regenerate methionine from homocysteine.

Methionine can also be synthesized from homocysteine via a zinc dependent enzyme betaine homocysteine methyltransferase (BHMT), which is a betaine (a metabolite of choline) dependent reaction which also produces dimethylglycine (see Figure 4).²³ An alternative metabolite fate of homocysteine is the transsulfuration catalyzed by cystathionine β synthase (CBS) that converts homocysteine to cysteine via a cystathionine intermediate (see figure 5).¹

As a consequence of the biochemical reactions in which homocysteine is involved, a blockage in these pathways due to dietary deficiencies of any of the cofactors involved in methionine and homocysteine metabolism (folic acid, vitamin B12, vitamin B6 (pyridoxine)),²⁶ deficiency of cystathionine β -synthase (CBS), gamma-cystathionase, 5,10-methylenetetrahydrofolate reductase, methionine synthetase (MS) or from genetic variations, acquired pathology, toxicity, or nutritional inadequacy, can result in elevated levels of total homocysteine concentrations in plasma,^{26,27,28,29,30,31,32,33,34,35,36} hypermethioninemia,^{37,38} homocystinemia,^{31,37,39} cystathioninuria,^{31,32,38} cirrhosis,^{36,40,41,42,43,44} beta-mercaptolactate cysteine disulfideuria,³⁸ sulfite oxidase deficiency (SOD),³⁸ aging,^{45,46} chronic renal failure,⁴ cancer,^{46,47} anemia,⁴⁷ goiter,⁴⁸ impaired coagulation,^{49,50,51} and, hyperhomocysteinemia^{26,30,52,53,54} correlated with cardiovascular diseases,^{24,30,33,34,35,45,46,52,55,56,57,58,59,60,61} neural tube defect⁶² and neurodegenerative disorders^{10,63,64} as Parkinson disease,^{46,65} Alzheimer,^{46,66,67} Down syndrome,⁴⁶ and Autism.^{24,46,68,69,70,71,72}

Hormonal regulation has been discovered to be also a major factor in the metabolic control of folate, methyl groups, and homocysteine, thereby providing a potential link between the pathologies associated with these pathways and hormonal imbalance.⁷³ For example it has been demonstrated that CBS, one of the homocysteine-clearing enzymes, is downregulated by testosterone.⁷⁴

In mammals, cystathionine β -synthase (CBS) catalyzes the first step in the transsulfuration pathway (see Figure 5),²³ a pyridoxal 5'-phosphate (PLP)-dependent condensation of serine and homocysteine to cystathionine.^{75,76} PLP is one of the different forms of vitamin B6.⁷⁷ The second step of the transsulfuration pathway is the hydrolysis of cystathionine to cysteine, ammonia, and α -ketobutyrate catalyzed by the enzyme γ -cystathionase (cystathionine-gamma-lyase (CGL)).

CBS contains three functional domains. The middle domain contains the catalytic core, which is responsible for the pyridoxal phosphate-catalyzed reaction. The C-terminal domain contains a regulatory region that is responsible for allosteric activation of the enzyme by S-adenosylmethionine. The N-terminal domain contains heme,⁷⁶ and this domain regulates the enzyme in response to redox conditions.⁷⁸ Deficiency of CBS is the major cause of inherited homocysteinemia.³⁹

CBS is regulated by the SAME in the liver. When SAME is depleted, homocysteine is channeled to remethylation to regenerate SAME via MAT; whereas when SAME is abundant, homocysteine is channeled to the transsulfuration pathway via CBS.^{23,79}

Ischemia-reperfusion injury induces a systemic inflammatory response and causes oxidative stress producing reactive oxygen species.^{80,81} Recent studies have demonstrated that ischemia-reperfusion reduces the activity of CBS leading to homocysteine accumulation in the kidney, which in turn leads to increases in oxidative stress contributing to renal injury.^{82,83,84}

Low levels of cysteine result from a decrease in methionine levels, so cysteine synthesis can be sustained only if the dietary intake of methionine is adequate.²⁴ Biosynthesis of cysteine from methionine via the hepatic transsulfuration pathway is impaired in some cirrhotic patients.^{43,44}

Cysteine, which can be derived through diet, or synthesis from homocysteine via the transsulfuration pathway, is the limiting substrate for the biosynthesis of glutathione.⁹ Cysteine in the presence of glutamate, γ -glutamylcysteine synthase and ATP as source of energy, is converted into γ -glutamylcysteine; this product is converted to glutathione via the enzyme glutathione synthase in the presence of glycine, and ATP as source of energy (see Figure 6). In liver, approximately half of the cysteine in glutathione is derived from homocysteine via the transsulfuration pathway.^{1,2,9}

Glutathione (GSH) is a tripeptide that contains an unusual linkage between the amine group of cysteine and the carboxyl group of glutamate side chain. Glutathione plays an important role in cellular anti-oxidant defense and detoxification reactions.⁹ Deficits in glutathione have been implicated in aging and a host of diseases as reviewed by James et al.⁴⁶ Glutathione (GSH) is also an important intravascular scavenger that protects endothelial cells from atherosclerosis.⁸⁵

Glutathione also provides the major intracellular defense against mercury-induced neurotoxicity due the high affinity that mercury has for thiol groups.⁸⁶

Glutathione acts as an antioxidant when the thiol (sulfhydryl (-SH)) group of cysteine donates a reducing equivalent. After glutathione (GSH) acts as an antioxidant it becomes reactive and forms glutathione disulfide (GSSG) combining itself with another reactive glutathione.⁸⁷ Glutathione can be regenerated through reduction from the disulfide form via the enzyme glutathione reductase (see Figure 7).^{88,89,90}

Hydrogen peroxide is an oxidant specie that causes cellular oxidative stress. In the methionine cycle the presence of a peroxide will direct homocysteine to the transsulfuration pathway, activating the enzyme CBS and reducing the activity of MS (see Figure 8).¹

The transsulfuration pathway, which activity is reported in a small number of tissues, plays a clear role in the prevention of a variety of disorders which manifest themselves when the pathway is deficient. We are interested in whether transsulfuration occurs in breast tissue, to determine its possible role in protection versus chronic diseases of mammary tissue such as breast cancer. We will test our hypothesis using human mammary epithelial cells (HEMC) and MCF-7 human breast adenocarcinoma cells by measuring the incorporation of ³⁵S-methionine into glutathione in the presence and absence of transsulfuration and glutathione biosynthetic pathway inhibitors.

MATERIALS AND METHODS

Materials. Mammary epithelial basal medium (MEBM), HEPES buffer saline solution (HEPES-BSS), trypsin neutralizing solution (TNS), trypsin/EDTA (0.25 mg/ml), MEGM singlequots (supplements and growth factors), were from Lonza (Walkersville, MD); dulbecco's modified eagle's medium (DMEM) (With 4500 mg glucose/L, 110 mg sodium pyruvate/L and L-glutamine), dulbecco's modified eagle's medium (Without methionine, cystine and L-glutamine) (With 4500 mg glucose/L and NaHCO_3), insulin from bovine pancreas, D,L-propargylglycine, D,L-buthionine-(S,R)-sulfoximine (BSO), dymethyl sulfoxide (DMSO), glutathione s-transferase from equine liver (GST), L-methionine, L-glutathione (GSH), hydrogen peroxide 30%wt, were from Sigma (St. Louis, MO); fetal bovine serum (FBS) and penicillin streptomycin solution were from Hyclone (Logan, UT); L-[^{35}S] methionine was from Perkin Elmer (Waltham, MA); monochlorobimane (mCBI) was from Invitrogen (Carlsbad, CA); potassium chloride (KCl), potassium phosphate monobasic (KH_2PO_4), sodium phosphate dibasic (Na_2HPO_4), butanol, methanol, acetic acid and scintiverse (TM) BD cocktail were from Fisher (Chicago, IL); trypsin/EDTA 1X (0.25% Trypsin/2.21 mM EDTA in HBSS without sodium bicarbonate, calcium & magnesium, porcine parvovirus tested) was from Cellgro (Manassas, VA) and sodium chloride (NaCl) was from Fisher (Fair Lawn, NJ).

Cell lines. Human mammary epithelial cells (HMEC) were purchased from Lonza (Walkersville, MD). Human breast adenocarcinoma cells (MCF-7) were obtained as a gift from Dr. Michael Moore.

Experimental protocol 1:

The objectives of this experiment were to become familiarized with cell culture, follow reactions in cell culture, practice thin layer chromatography techniques, and to verify that the identity of the major fluorescent product present after reacting cell lysates with mCBI was the glutathione-bimane adduct by running mass spectroscopy.

Cell Culture. MCF-7 cells were cultured in T75 tissue culture flasks using water-Jacketed CO_2 incubators (Fisher Scientific, Thermo Forma Series II) at 37°C , 5% CO_2 and high levels of humidity. The standard medium for MCF-7 cells was Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS), 5 ml of 200mM L-glutamine, 5 mg of insulin and

5 ml of penicillin. The culture medium was changed every 3 days with 15 ml of standard medium.

To prepare experimental cells, 80% confluent T75 flasks were split and used to seed a T75 flask with 5E6 cells in 15 ml of standard medium and incubated for 24 hours.

Sample Collection. Cells were harvested after 24 hours of incubation; for this purpose the medium was removed, the cells were rinsed twice with PBS and then trypsinized covering with 4 ml of warm trypsin 0.25% solution (Cellgro). When the cells detached after 5 minutes of incubation at 37°C and 5% CO₂, they were collected in a 15 ml tube and trypsin was neutralized with 6 ml of a 10% FBS solution in PBS. The 15 ml tube was centrifuged at 4°C for 10 minutes and the supernatant was removed from on top of the cells. Then, the pellet was suspended with PBS. Cells were pelleted again and pellets resuspended in fresh PBS, then removed to an eppendorf tube, spun to pellet the cells, the PBS was removed and an equal amount of water added to it and frozen at -80°C. Cells were frozen and thawed 4 times with vortexing at each thaw. The supernatant with cytosolic contents was isolated by spinning the cells at 4°C and 15,000 rpm for 15 minutes in a Thermo Forma 3L GP 4500R Centrifuge and repeating the extraction and freeze-thaw 2 times more.

Fluorescent labeling of GSH with monochlorobimane (mCBI). Monochlorobimane (mCBI), in a reaction catalyzed by glutathione S-transferase (GST), forms a stable fluorescent GSH-bimane adduct that can be detected fluorimetrically and analyzed by thin layer chromatography (TLC). The reaction mixture to label GSH consisted of 100 µl of cell lysate, 2 µl of mCBI 20mM, 20 µl of 500 mM potassium phosphate (K₂PO₄) pH 6.5 and 2.5 µg of GST (20 µl of 25 µg GST dissolved in 100 µl of PBS) in a total reaction volume of 200 µl volume (water is used to equalize samples). Reactions were prepared on ice then initiated by incubation at 37°C for a period of 10 minutes. Reactions were stopped by freezing. A control reaction was run by substituting the cell lysate with 2 µl of 10mM GSH in the same reaction volume of 200 µl. 10 µl of each reaction mixture were spotted on 250 microns silica gel GF uniplates (Analtech, Newark, DE) and analyzed by TLC using a 3:1:1 mixture of 1-butanol:methanol:water. Two µl of 20mM mCBI were run in a separate lane as a control to indicate the migration of non reacted mCBI. The plates were removed from the TLC chamber when the mobile phase had covered approximately 90% of the height of the plate and allowed to air-dry ^[16]. Migrations of

fluorescent products were compared under UV light. The fluorescence silica spots were scraped from the plate into a tube under an UV lamp. Silica was extracted by adding 0.2% acetonitrile and extracts were sent for analysis by mass spectrometry.

The mass spectrometer used was the Finnigan LCQ quadrupole ion trap mass spectrometer. It is equipped with an atmospheric pressure ionization (API) source which can be operated in two modes: electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI). Both are soft ionization methods that can be utilized to produce either positive or negative ions. ESI is effective with most compounds, and is the default method of ionization. Polar compounds such as amines, peptides, and proteins are best ionized by ESI. Non-polar compounds such as hydrocarbons and steroids usually give better results with APCI.

Experimental protocol 2:

The objective of this experiment was to follow the incorporation of ^{35}S -methionine into the glutathione biosynthetic pathway, with and without the presence of specific inhibitors in MCF-7 cells and HMEC.

Cell Culture and Treatment.

MCF-7 cells were cultured as described under methods for experiment 1. To prepare experimental cells, 80% confluent T75 flasks were split and used to seed 6 T75 flasks with 5×10^6 cells in 15 ml of standard medium and incubated for 24 hours. After 24 hours of incubation, cells received the two inhibitors added to the medium. Two flasks were preincubated for 24 hours with 9 mM BSO, two flasks with 2.5mM PPG and as a control, two flasks with PBS vehicle. Then, to monitor the incorporation of radioactive methionine into glutathione the medium was replaced with 10 ml of radioactive labeling medium containing DMEM without methionine instead of standard DMEM, the inhibitors for each group, 0.4 mM methionine and ^{35}S methionine to give a 2.5 $\mu\text{Ci/ml}$ activity.

HMEC are a primary cell culture and require particular conditions to ensure that they do not become senescent due to irreversible contact-inhibition if their confluence too greatly exceeds 80%. For this reason, many preliminary experiments were conducted to gain a working knowledge of their growth parameters (data not shown). HMEC were cultured under the same

conditions of incubation as described under methods for experiment 1 but using as standard medium mammary epithelial basal medium (MEBM), supplemented with growth factors recommended by Lonza, the cell line distributor. These are supplied in aliquots at proprietary concentrations, and include 2 ml of bovine pituitary extract, 0.5 ml of epidermal growth factor, 0.5 ml of insulin, 0.5 ml of hydrocortisone and 0.5 ml of gentamicin sulfate / amphotericin-B. The culture medium was changed every 2 days with 15 ml of this standard medium. To prepare experimental cells, 80% confluent T75 flasks were split and used to seed 12 T75 flasks each with 2E5 cells in 15 ml of standard medium and were cultured changing the medium every 2 days. Seven days after seeding they were 50% confluent and experimental treatment began. Cells received the two inhibitors added to the medium. Four flasks were preincubated for 24 hours with 9 mM BSO, four flasks with 2.5mM PPG and as a control, four flasks with PBS. Then, to monitor the incorporation of radioactive methionine into glutathione, the medium was replaced in 2 flasks of each group with 10 ml of radioactive labeling medium containing the appropriate inhibitors and ^{35}S methionine to give a 2.5 $\mu\text{Ci/ml}$ activity.

Sample Collection. MCF-7 cells were harvested 24 hours later as described previously under experiment protocol 1. HMEC are trypsinized after 48 hours of incubation; for this purpose the medium was removed, the cells were rinsed twice with HEPES-BSS and then trypsinized covering with 37°C trypsin/EDTA solution from LONZA. When the cells were released after 6 minutes of incubation at 37°C and 5% CO_2 , they were collected in a 15 ml tube neutralizing the trypsin with TNS and doing a final rinse of the flask with HEPES-BSS to collect residual cells. The 15 ml tube was centrifuged at 4°C for 10 minutes and the supernatant was removed from on top of the cells. Then, the pellet was rinsed and re-suspended twice with PBS. The pellet was removed to an eppendorf tube, spun to pellet the cells, the PBS was removed and an equal amount of water added to it and frozen at -80°C. Cells were frozen and thaw 4 times. The supernatant with cytosolic contents was isolated by spinning the cells hard at 4°C and 15,000 rpm for 15 minutes and repeating the freeze-thaw 2 times more. Radioactive procedures were carried separately from non radioactive procedures to prevent radioactive cross contamination.

Detection of GSH. The mCBi conjugation reaction was run for this cell line as it was run for the MCF-7 but this time glutathione spots were fluorimetrically detected from the TLC plate using the BIO RAD universal hood imaging system. Radioactivity on the plates was detected through

phosphorimaging using the Typhoon 9200 variable mode imager, as well as by exposure to xray film.

To prove the presence of glutathione in the HMEC's that were not radioactively labeled, the fluorescence silica spots were scraped, silica extracted, and mass spectrometry conducted as described under protocol one.

Experimental protocol 3:

The purpose of this experiment was to determine the impact of oxidative stress on GSH production from transsulfuration pathway- and non transsulfuration pathway-derived cysteine as is shown in the flowchart in Figure 11.

Cell Culturing and treatment. HMEC cells were grown in T225 tissue culture flasks in 45 ml of standard medium, until they were 80% confluent. The culture medium was changed every 2 days.

Cells were split from the flasks and 24 T225 flasks were seeded at different densities, 8 flasks with 2.75E6 cells, 8 with 1.38E6 cells and 8 with 7E5 cells; yielding three separate replicates at different harvest times. Each of these was cultured by changing the medium every 2. Experiments began when cells were 50% confluent. For each group of 8 flasks, cells were treated for 24 hrs with either 2.5mM PPG or the vehicle control PBS. Then, the medium was replaced to 4 flasks for fresh medium with inhibitors and to the other 4 flasks with oxidative stress medium containing 300 μ M hydrogen peroxide (H_2O_2) and inhibitors during 2 hours.

Pellet Collection. The cells were harvested after the oxidative stress treatment as described previously for experimental protocol 2 under methods. After the final PBS rinse, the pellets were roughly divided into thirds, frozen after PBS removal and stored at -80°C.

Pellet analysis. One aliquot of each pellet was sent to Dr. Monica Valentovic's lab for determination of glutathione abundance, using their published methods.^{91,92,93,94,95}

RESULTS

Experiment 1:

5E6 MCF-7 cells were cultured in a T75 tissue culture flask with the standard medium specified under methods and incubated for 24 hours, harvested, the cell pellet isolated and lysed. A reaction with the cell lysate was run with monochlorobimane in the presence of GST to form fluorescent GSH-mCBI adducts. As a control, the reaction was run with glutathione and monochlorobimane in the absence of cell pellet. Ten μ l of reaction mixture were spotted on TLC silica plates and allowed them to dry. TLC was conducted using a 3:1:1 mixture of 1-butanol:methanol:water as the mobile phase. mCBI alone produced a single spot with fast migration (70 % of the height). This matched a similar spot found in all lanes, indicating that sufficient mCBI was present in each sample to react with all the available GSH. GSH-mCBI conjugate produced a fluorescent spot with slow migration (30% of the height). Fluorescent spots were detected using a UV-lamp and lines were draw around the spots, which then, were scraped and eluted from silica plates for subsequent mass spectrometry analysis.

Using a Finnigan LCQ quadrupole ion trap mass spectrometer, Dr. Frost helped us to determine the content of the fluorescent spots. As result, mass spectrometry shows a peak at 498 for the control sample (see Figure 12) and MCF-7 lysate sample (see Figure 13) verifying the presence of glutathione-mCBI conjugate in the spot we had identified by virtue of its comigration with the GSH-mCBI control reaction products.

Experiment 2:

MCF-7 and HMEC cell lines were used to follow the incorporation of ^{35}S -methionine into the glutathione biosynthetic pathway. Each cell line was pretreated with two inhibitors, 2.5 mM propargylglycine (PPG) and 9 mM buthionine sulfoximine (BSO), in standard medium for 24 hours; followed by replacement of the medium with radioactive labeling media, containing the same inhibitors and ^{35}S -methionine (2.5 $\mu\text{Ci/ml}$) for 24 hours and 48 hours, as described under methods.

Figure 14 and Figure 15 show the digital fluorescent images of the silica plates where HMEC and MCF-7 cells lysates reacted with monochlorobimane were analyzed by TLC. In these figures we can see that the fluorescent conjugated glutathione (GSH-mCB) was clearly separated from the free mCBi fluor (compare lanes 1-6 with lane 7). BSO blocks almost completely glutathione synthesis leading to very low GSH bimane levels, seen only as faint fluorescent spots (compare lanes 1 and 2 with lanes 3 and 4). Treatment with PPG inhibits the cysteine for GSH synthesis coming from the transsulfuration pathway, clearly a portion of, but not all glutathione present in the cells is derived from transsulfuration derived cysteine (compare lanes 1 and 2 with 5 and 6) leading to less fluorescent spots than controls, but more fluorescence than seen with BSO inhibition. Glutathione can also be derived from cysteine released during protein turnover or imported from the extracellular medium.

Position of the fluorescent spots from the TLC plates were marked under uv-light and TLC plates were scanned using a Typhoon 9200 variable mode imager, and autoradiography conducted with Xray film. Comparison of the positions of the fluorescent and radioactive spots indicate that the glutathione-bimane conjugate has incorporated ^{35}S -methionine as is shown in Figure 16 and Figure 17 in lanes 1 and 2. Incorporation of radioactivity into glutathione in BSO treated cells was inhibited by roughly 80%. Interestingly, incorporation of radioactivity into glutathione in PPG treated cells was also inhibited by roughly 80%. Since PPG inhibits the conversion of cystathionine to cysteine, this directly impacts the flux of sulphur from the methionine cycle. Incorporation of ^{35}S -methionine into glutathione (GSH-mCB bands) showed in the autoradiography demonstrates that functional transsulfuration occurs in mammary cells.

The HMEC cell line was treated with inhibitors and non radioactive cell extracts were prepared and processed with monochlorobimane. Extracts from GSH-mCB spots were analyzed by mass spectrometry. Mass spectrometry showed a peak at 498 when cells were treated with PBS (vehicle) (see Figure 18) and PPG (see Figure 20) demonstrating the presence of glutathione migrating to the spot we identified as glutathione-bimane on the fluorescent and autoradiography pictures of the TLC plates (see Figure 16). When HMECs were treated with BSO, the peak at 498 was not detectable (see Figure 19), indicating that BSO thoroughly blocks the glutathione synthesis. This is consistent with our fluorimetric findings (see Figure 16, fluorescent plate, comparing lanes 1 and 2 with lanes 3 and 4).

Experiment 3:

Our objective for this experiment was to determine the impact of oxidative stress on GSH biosynthesis from transsulfuration pathway and also it was designed to test the hypothesis that transsulfuration in mammary cells provides necessary cysteine for the production of glutathione under conditions of oxidant stress.

Therefore, HMEC were treated with a control or 300 μM H_2O_2 to induce oxidative stress responsive genes^[93], in the presence of inhibitors and vehicle for 2 hours. Cells were collected, lysates prepared, and GSH levels reported as mean \pm SEM were determined as described under experimental protocol 3. Results, depicted in Figure 21 indicate that 24 hours of PPG inhibition reduced the levels of glutathione to roughly 2/3 that of control cells, though this was not determined a significant difference. Cells treated with 300 μM H_2O_2 increased GSH levels relative to Basal controls by roughly 1/3, though this again was not determined a significant change. Propargylglycine inhibited cells, when treated with 300 μM H_2O_2 did not increase their GSH levels, as compared with the increase seen with uninhibited cells ($p < 0.02$, one way ANOVA).

DISCUSSION

My hypothesis was that the transsulfuration pathway provides cysteine for glutathione production in mammary cells. To approach testing this hypothesis I had a number of smaller learning objectives. First, I needed to become familiar with general tissue culture techniques, followed by mastering the more exacting conditions of primary cell culture. Cell tissue culture involves techniques that take time to learn and become familiarized with. A primary cell culture is more stringent than the culture of transformed cells. With a primary cell line, one has to be very careful with how confluent they can get and how often they are fed, to avoid triggering cellular senescence. A transformed cell line is easier to handle due to its ability to grow indifferently from frequency of media change and how much confluent they get. I had a great experience using MCF-7 cell line to become familiarized with the tissue culture technique.

My second learning objective was to learn how to conduct thin layer chromatography (TLC) analysis. TLC was needed as the glutathione we isolate from cells needs to be separated and identified from other cell constituents. Once we prepare cell lysates we react them with monochlorobimane. Reaction of glutathione instead of the cell lysates was used as a control. Then, we run the reaction in the presence of GST to form the fluorescent GSH-bimane conjugate, to identify the location of glutathione as a fluorescent spot following TLC. Also, to prove the glutathione presence in the fluorescent spot, the samples were analyzed by mass spectrometry obtaining a peak at 498 that represents the GSH-bimane conjugate.

My third learning objective was to master radioactivity techniques using ^{35}S -methionine to determine if the methionine cycle supplies sulphur for glutathione (GSH) synthesis by the transsulfuration pathway. As its name implies, the transsulfuration pathway supplies sulphur from the methionine cycle for glutathione synthesis. Thus to determine if this pathway is active in mammary cells, we treated the MCF-7 and the HMEC cell lines with ^{35}S -methionine. After the treatments were done and the cell lysates were reacted for each cell line, we compared the fluorescence pictures and the autoradiographs of the TLC silica plate. The positions and shapes of the fluorescent and radiographic spots on the plates were strikingly similar, indicating a co-migration of the fluorescent label with the ^{35}S from methionine. These data indicate that the ^{35}S from methionine did incorporate into the glutathione, demonstrating that a functional

transsulfuration pathway is present in human mammary epithelial cells. Glutathione can also be synthesized from cysteine released during protein turnover or imported from the extracellular medium but the only possible way that glutathione could incorporate ^{35}S in this experiment was by the transsulfuration pathway.

When the inhibitors PPG or BSO, were used, the levels of glutathione were reduced, appearing as less intense spots in the TLC plate due to less incorporation of ^{35}S methionine into glutathione (GSH-mCB bands). For both inhibitor treatments, the reduction of ^{35}S incorporation was around 80% as each inhibits the transfer of the ^{35}S from methionine to glutathione.

Fluorescent levels of GSH following treatment with PPG are higher than seen following treatment with BSO, indicating the key difference between these inhibitors. Propargylglycine blocks the formation of cysteine from cystathionine, effectively blocking only the transsulfuration pathway as a source of cysteine. Buthionine sulfoximine blocks the incorporation of cysteine from any source into the first step of glutathione biosynthesis. This results in BSO reducing total GSH levels more significantly (as is shown in Figure 19 where levels were below the ability of the mass spec to detect), while PPG only reduces the portion of GSH that derives its cysteine from transsulfuration.

When we tested whether oxidative stress would affect GSH biosynthesis using a treatment of H_2O_2 , we found the following response in mammary cells. Cells treated with $300\ \mu\text{M}$ H_2O_2 for two hours increased GSH synthesis by approximately ~30% as compared to untreated controls. In cells not treated with H_2O_2 , PPG pre treatment for 24 hours reduced GSH by over 30%. This result is similar to that which Reed and co-workers² obtained when they treated rat hepatocytes with 1 mM propargylglycine for 2h; this resulted in a 35% depletion of the glutathione pool compared to untreated controls. Interestingly, **PPG pretreatment blocked the ability of mammary cells to respond to H_2O_2** yielding a significant reduction in GSH in PPG pretreated, H_2O_2 treated cells versus H_2O_2 treated cells that had received no pre treatment (One way ANOVA, $p < 0.02$). These results demonstrate the importance of the transsulfuration pathway to HMECs under conditions of oxidant stress.

We feel that these experiments provide clear and conclusive evidence supporting our hypothesis, that the transsulfuration pathway provides cysteine for glutathione production in mammary cells.

Further, that this pathway is a significant source of cysteine under conditions of oxidant stress. These findings imply that transsulfuration may link oxidant stress to the critical methyl donor pool of S_AMe, providing a means by which oxidant stress might impact epigenetic regulation in mammary tissues. This in turn may provide a mechanism through which oxidant stress could lead to chronic diseases in mammary tissue such as breast cancer.

FIGURES

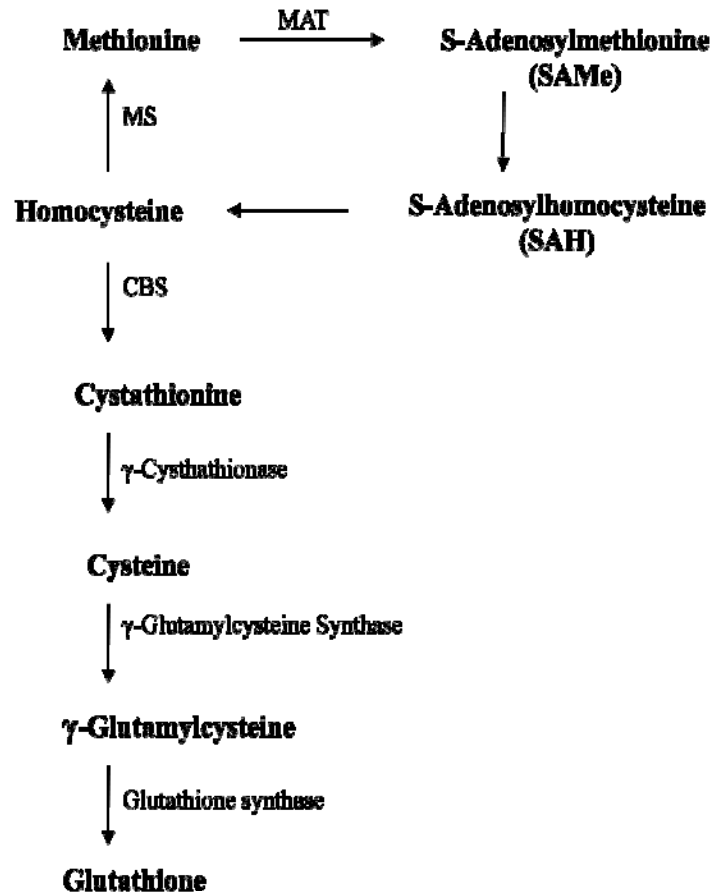


Figure 1 The transsulfuration pathway linking the methionine cycle and the synthesis of glutathione. A metabolic pathway that converts homocysteine to cysteine, through the intermediate cystathionine.

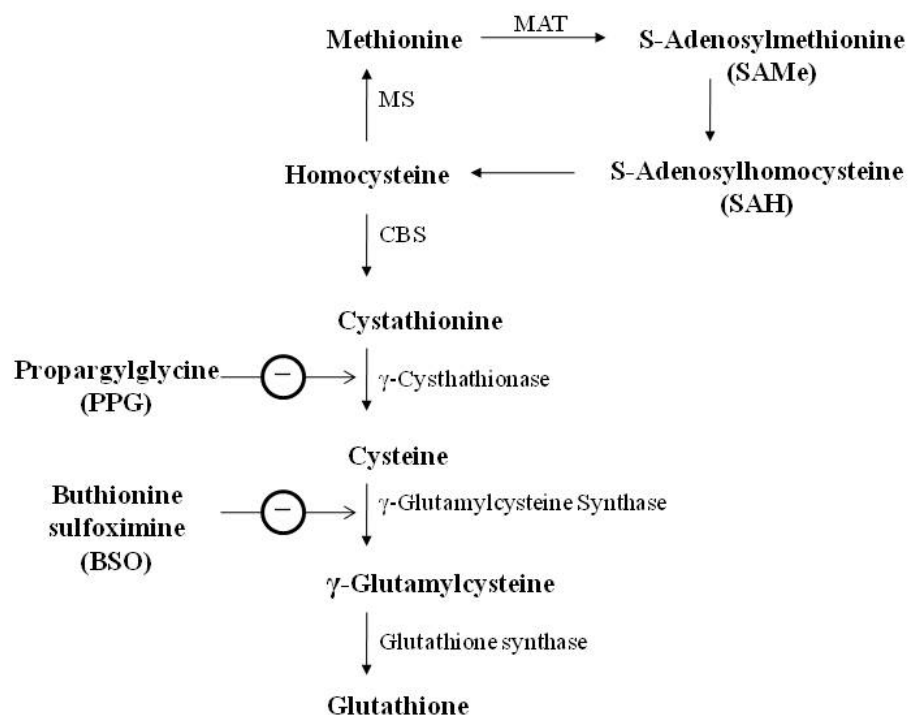


Figure 2 PPG and BSO effects on the transsulfuration pathway. Propargylglycine (PPG) is an irreversible inhibitor of gamma-cystathionase, and buthionine sulfoximine (BSO) is a synthetic amino acid that irreversibly inhibits gamma-glutamylcysteine synthetase. While both of them prevent the incorporation of cysteine derived from the transsulfuration pathway into GSH, PPG has no impact on how cysteine derived from the media may incorporate into GSH.

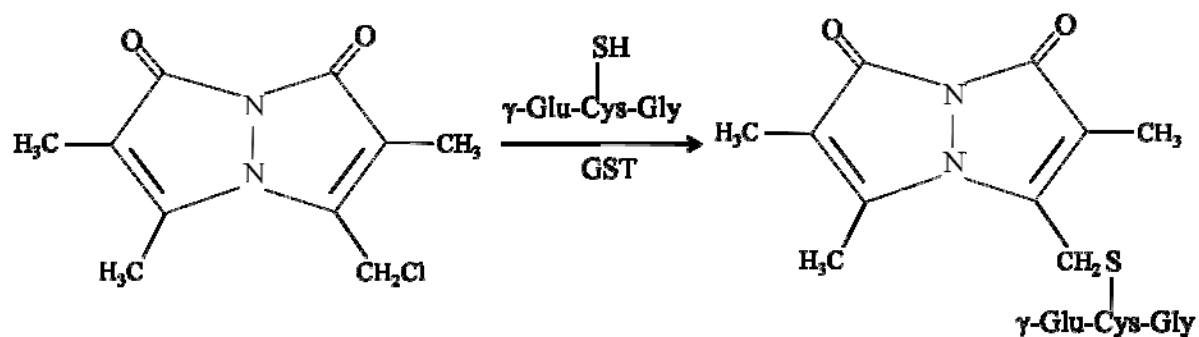


Figure 3 Reaction scheme between monochlorobimane and glutathione to form GSH-mCB. Monochlorobimane (mCBI) forms a stable fluorescent GSH-bimane adduct in a reaction catalyzed by glutathione S-transferase (GST). This adduct permits us to easily identify the position of GSH-bimane on TLC silica plates.

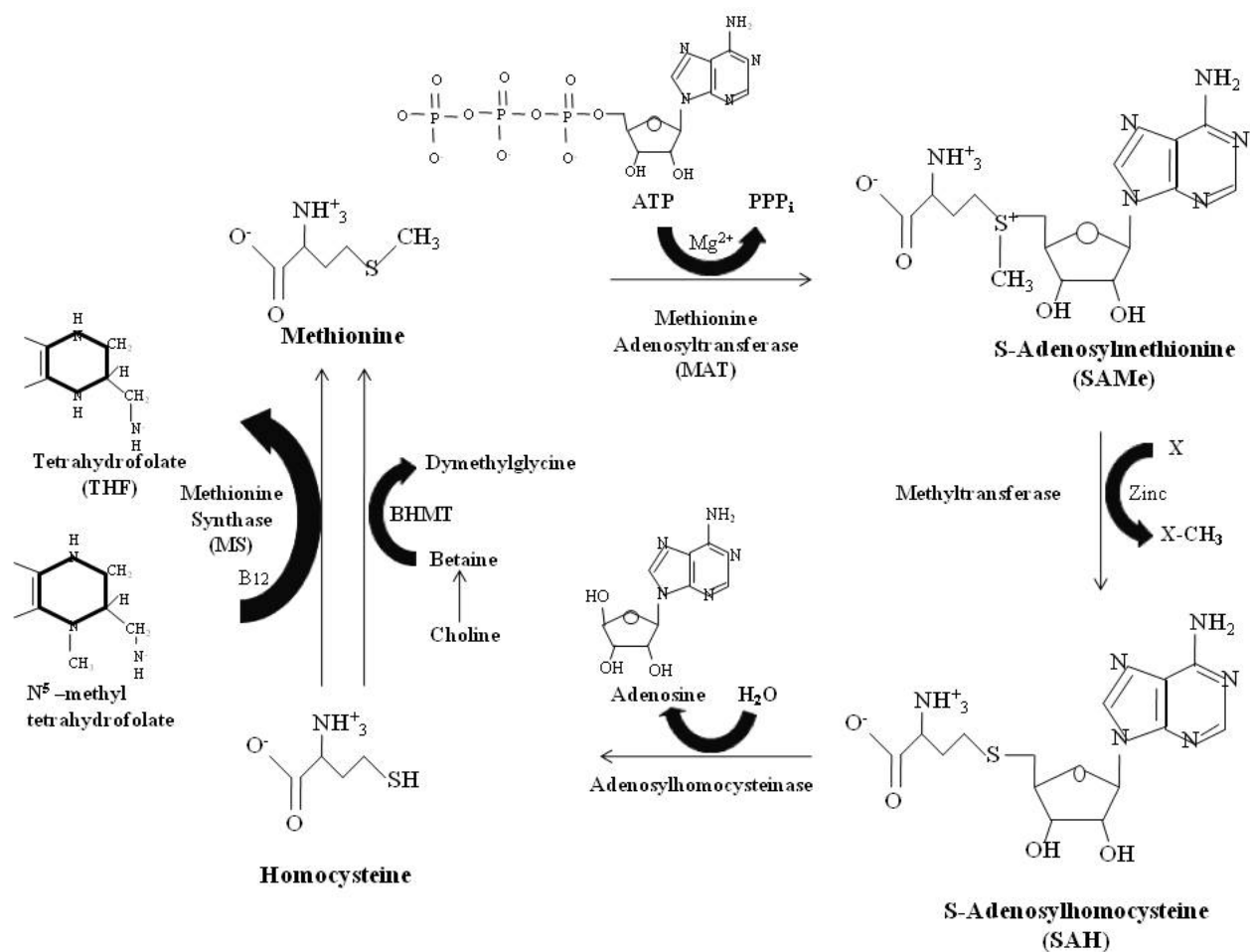


Figure 4 Methionine cycle. Methionine is converted to S-adenosylmethionine (SAME) by methionine adenosyltransferase (MAT). SAME is converted to S-adenosylhomocysteine (SAH) through donation of a methyl group by any methyltransferase. SAH is hydrolyzed to form homocysteine via adenosylhomocysteinase. Homocysteine can then regenerate methionine via methionine synthase or betaine homocysteine methyltransferase (BHMT), or follow the transsulfuration pathway via cystathionine- β -synthase (CBS) to produce cysteine (reaction not shown, see Figure 5).

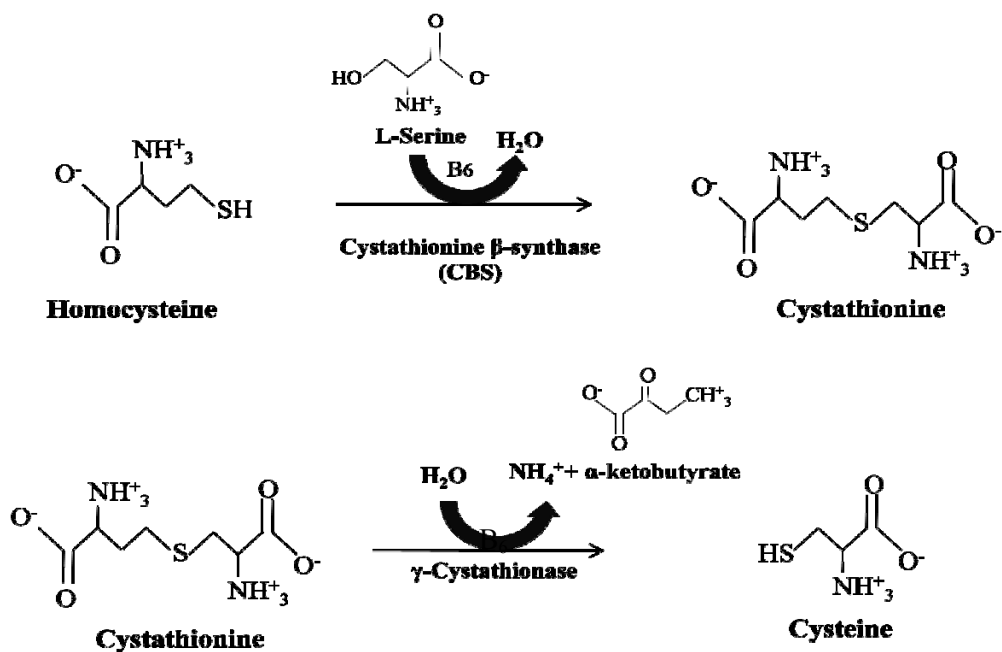


Figure 5 Synthesis of Cysteine. Cystathionine beta-synthase (CBS) catalyzes the first step in the transsulfuration pathway which converts homocysteine into cysteine in a two step reaction. In the first step, cystathionine is obtained from homocysteine by a condensation reaction with serine in the presence of CBS. The second step of the transsulfuration pathway is catalyzed by gamma cystathionase and it turns cystathionine into cysteine, ammonia and α -ketobutyrate.

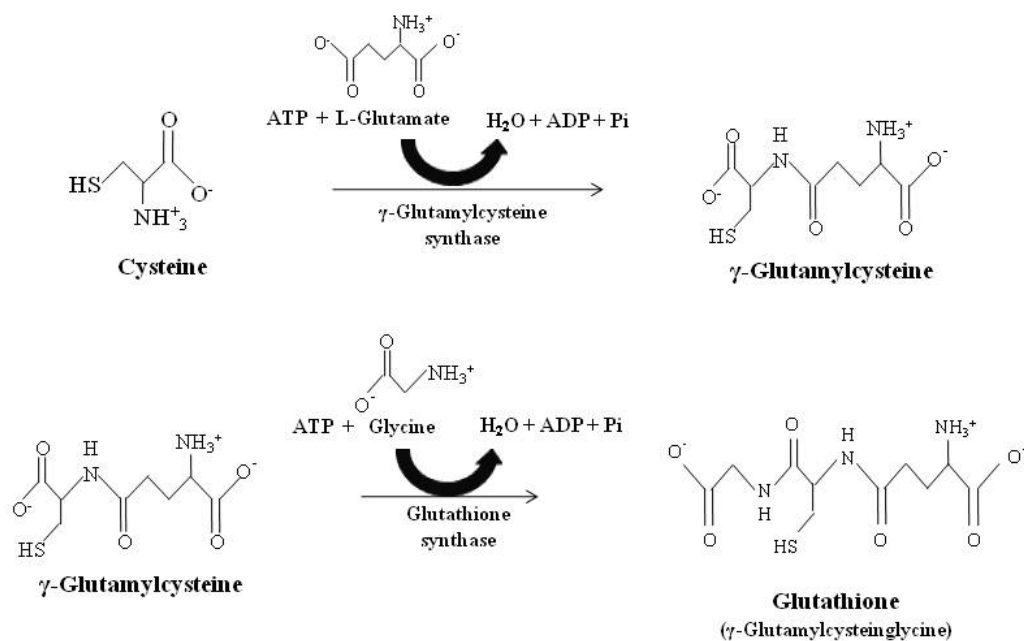


Figure 6 Glutathione Synthesis Pathway. Cysteine in the presence of glutamate, γ -glutamylcysteine synthase and ATP as source of energy, is converted into γ -glutamylcysteine; the latter is converted to glutathione via the enzyme glutathione synthase in the presence of glycine, and ATP as source of energy.

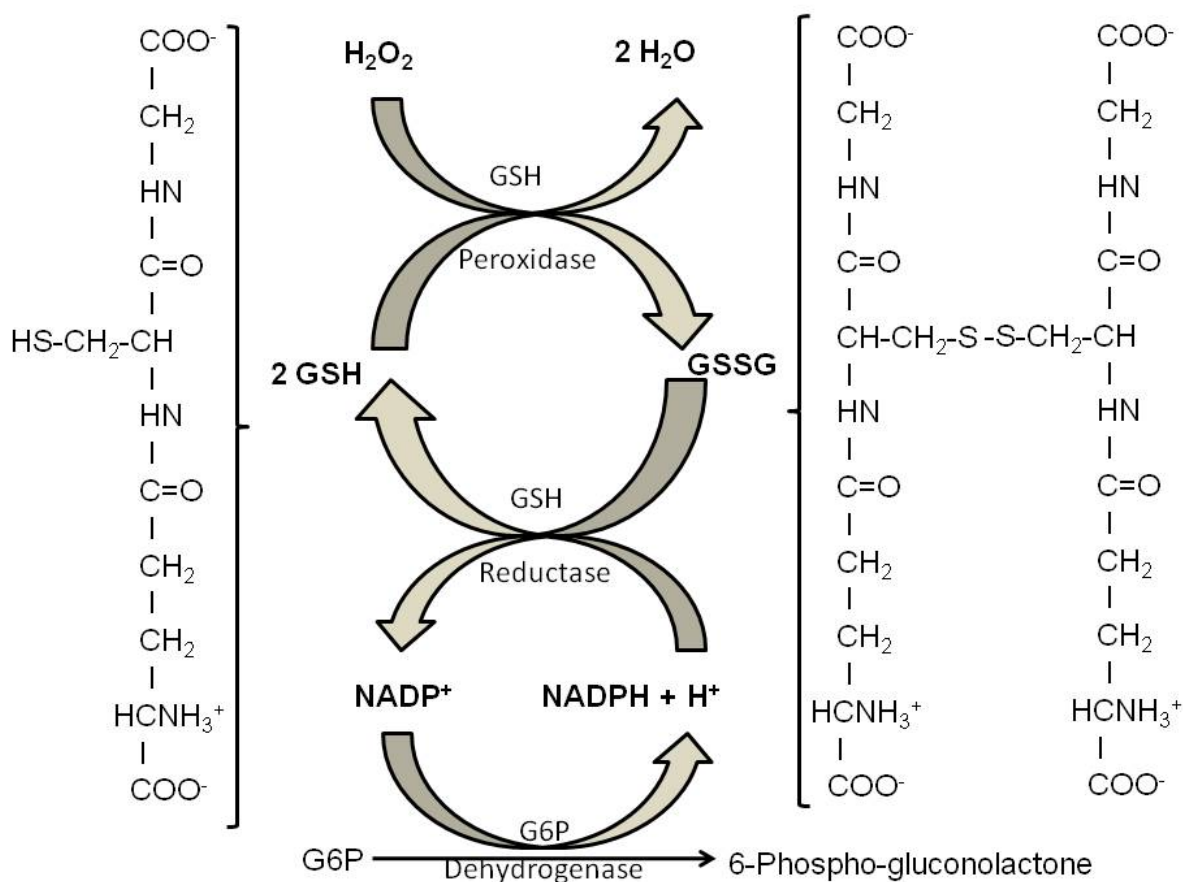


Figure 7 Role of glutathione as an antioxidant. In the presence of an oxidative stress agent, the thiol group of cysteine present in glutathione donates a reducing equivalent becoming reactive and forming glutathione disulfide (GSSG) by combining itself with another reactive glutathione. Then, glutathione can be synthesized again from the disulfide form via the enzyme glutathione reductase in the presence of NADPH produced in the glucose 6-phosphate dehydrogenase reaction.

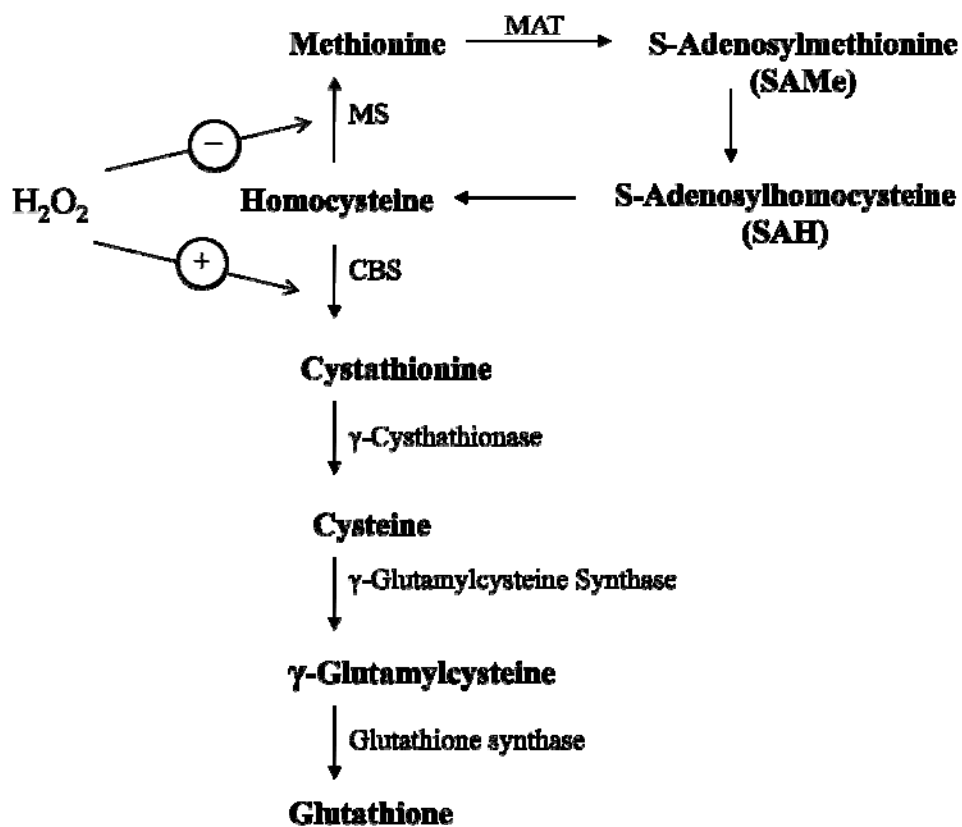
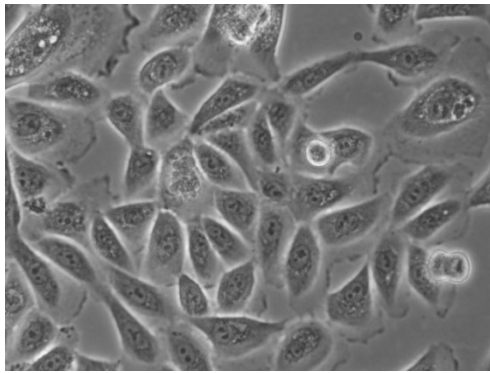
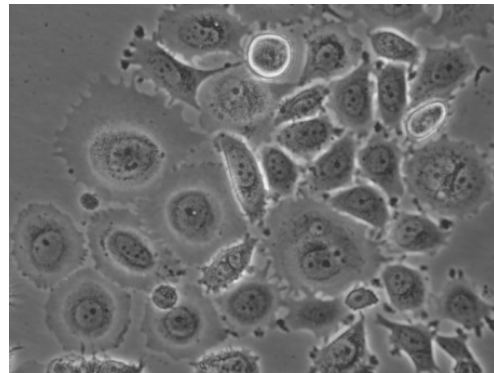


Figure 8 Effect of H_2O_2 in the methionine cycle. In the methionine cycle the presence of H_2O_2 will direct homocysteine to the transsulfuration pathway, activating the enzyme CBS and reducing the activity of MS.¹



HMEC



MCF-7

Figure 9 The two cell lines used for studies. Pictures were taken at 400x magnification. Human mammary epithelial cells (HMEC) were purchased from Lonza (Walkersville, MD). Human breast adenocarcinoma cells (MCF-7) were obtained as a gift from Dr. Michael Moore.

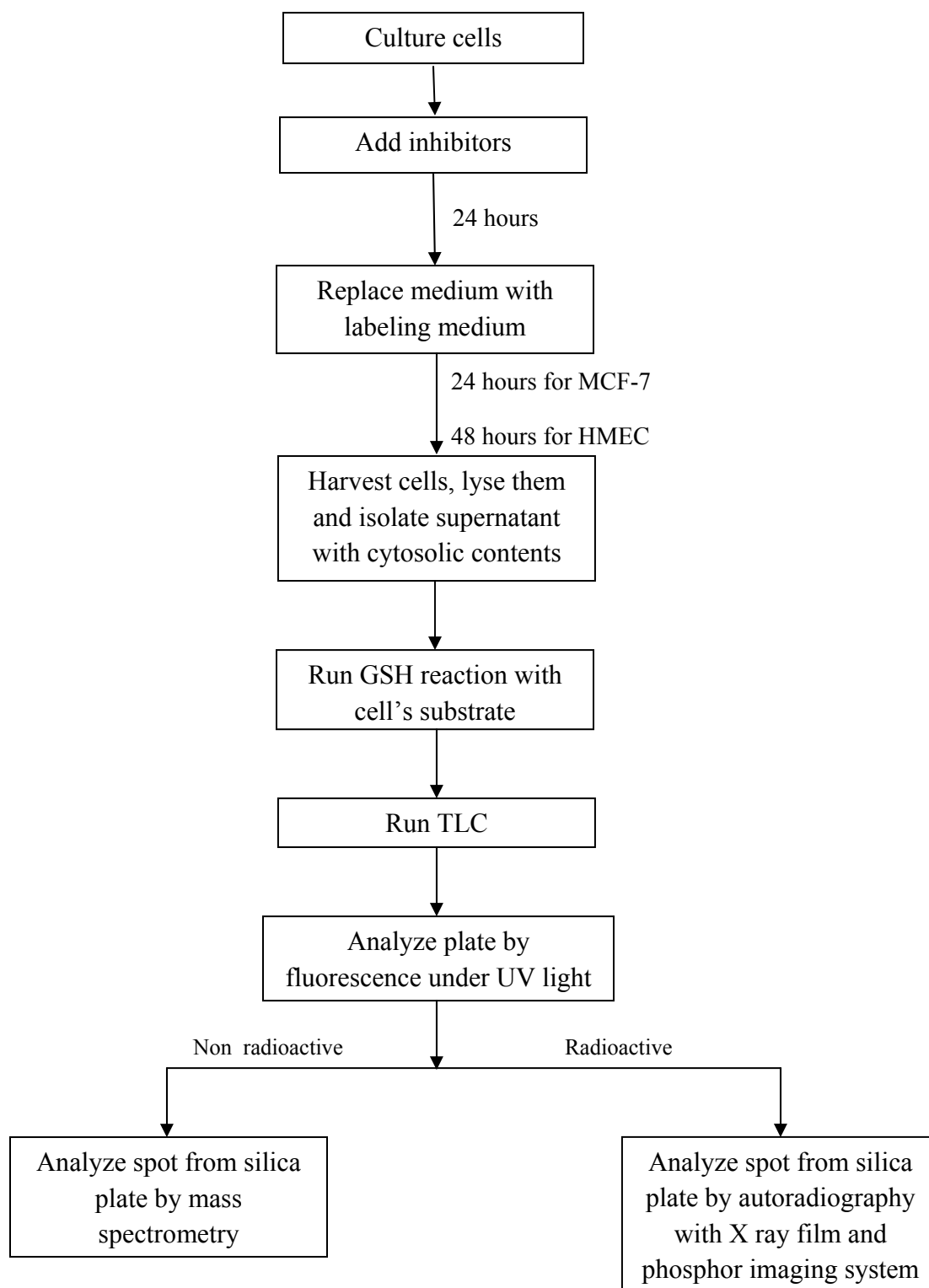


Figure 10 Methods diagram for experimental protocol 2.

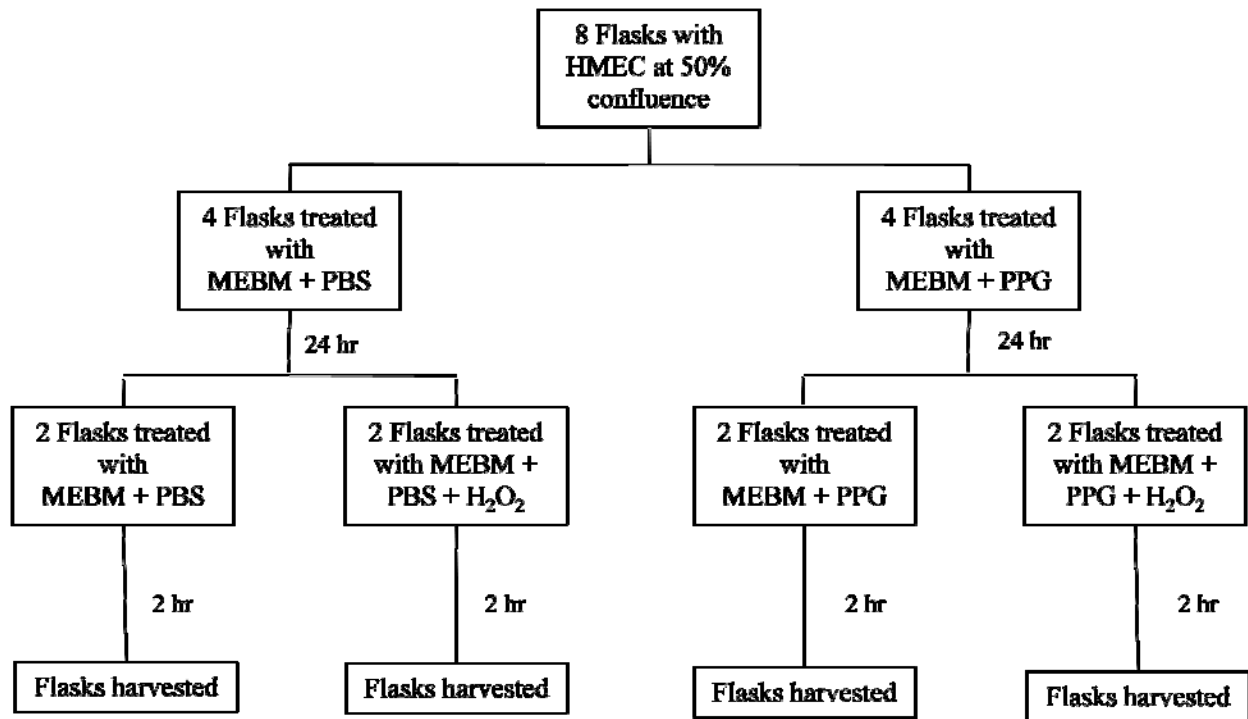


Figure 11 Diagram of oxidative stress treatment for experiment 3. Each replicate experiment was started when HMEC cells in the eight T75 flasks reached 50% confluence. Cells were pretreated with the inhibitor propargylglycine (PPG) or the PBS vehicle control. Twenty four hours later they were treated for 2 hours with 300 μ M H₂O₂ to generate an oxidant challenge followed by harvesting.

GSH_std_HPLC#100-105 RT: 1.64-1.72 AV: 6 NL: 3.26E7
T: + c ms [150.00-1000.00]

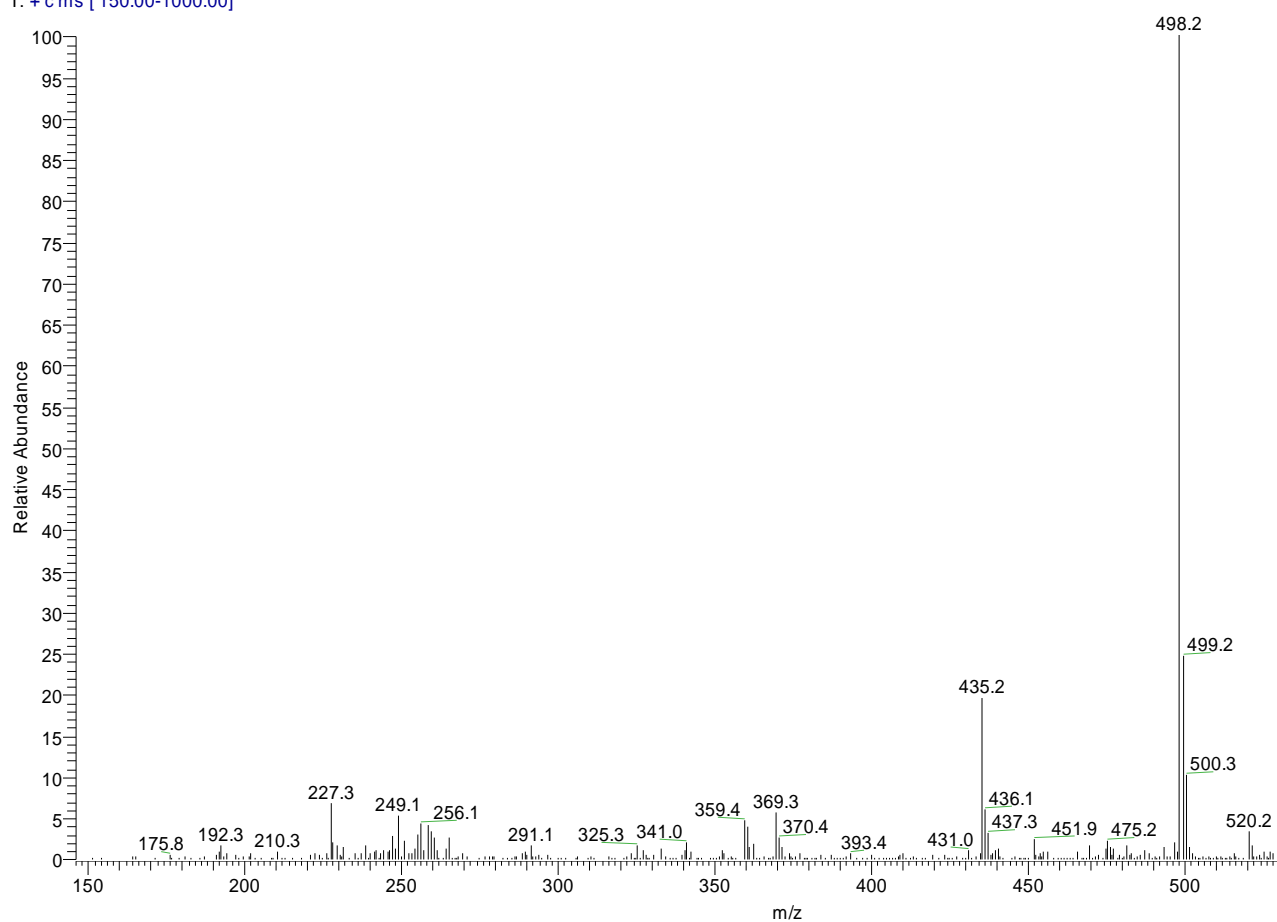


Figure 12 Mass spec of glutathione-monochlorobimane (GSH-mCB) conjugate used as a control.

GSH_sample6#100-104 RT: 1.74-1.80 AV: 5 NL: 7.41E6
T: + c ms [150.00-1000.00]

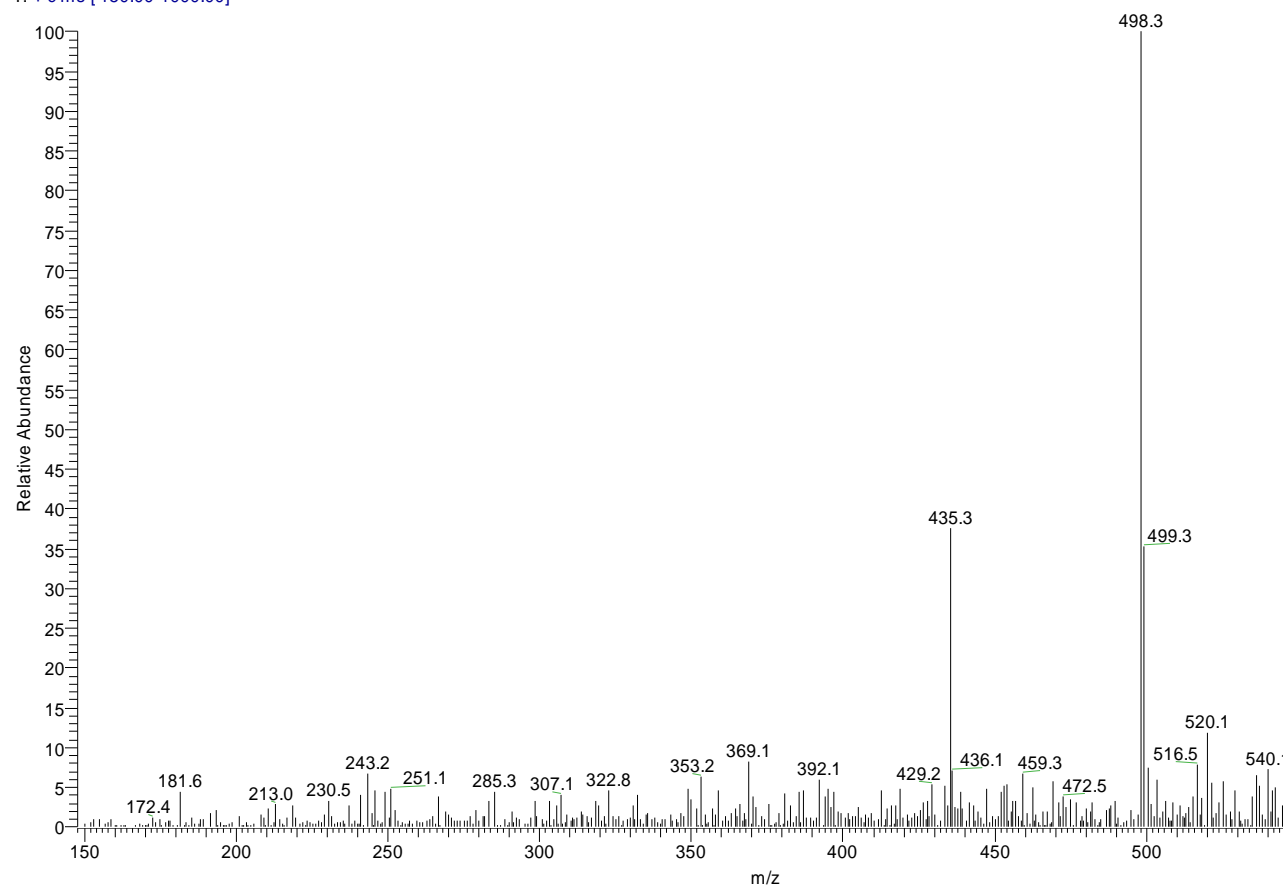


Figure 13 Mass spec of MCF-7 lysate glutathione-monochlorobimane conjugate.

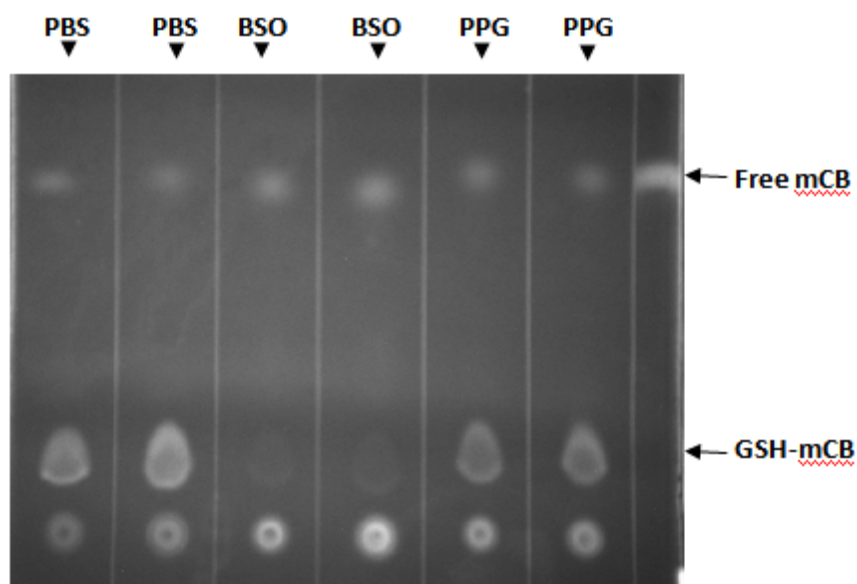


Figure 14 A scanned TLC plate showing the selectivity of the monochlorobimane reaction with glutathione present in HMEC lysates.

Cells treated with PBS (lanes 1 and 2) generated glutathione-bimane (GSHmCB) conjugates which produced an intense fluorescent spot. Cells treated with BSO (lanes 3 and 4) had little GSH-mCB conjugate, while cells treated with PPG (lanes 5 and 6) showed partial reduction of GSH levels, leading to less GSH-mCB conjugate. Lane 7 indicates the migration of free mCBi (present in the tops of all reactions). Inhibition of GSH levels by PPG (compare lanes 1 and 2 with lanes 5 and 6) is an indication that transsulfuration is taking place in these cells.

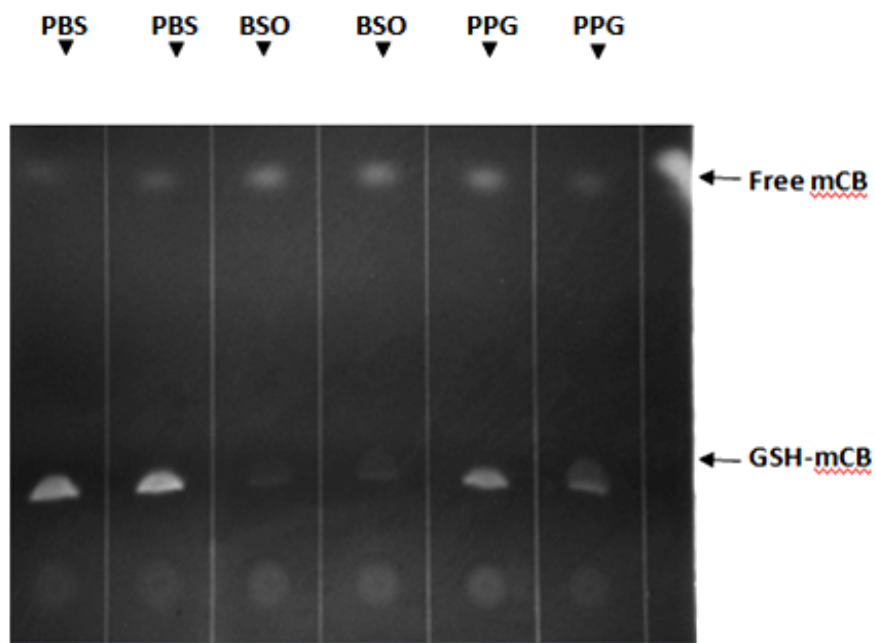


Figure 15 A scanned TLC plate showing the selectivity of the monochlorobimane reaction with glutathione present in MCF-7 cell lysates.

Cells treated with PBS (lanes 1 and 2) generated glutathione-bimane (GSHmCB) conjugates which produced an intense fluorescent spot. Cells treated with BSO (lanes 3 and 4) had little GSH-mCB conjugate, while cells treated with PPG (lanes 5 and 6) showed partial reduction of GSH levels, leading to less GSH-mCB conjugate. Lane 7 indicates the migration of free mCBi (present in the tops of all reactions). Inhibition of GSH levels by PPG (compare lanes 1 and 2 with lanes 5 and 6) is an indication that transsulfuration is taking place in these cells.

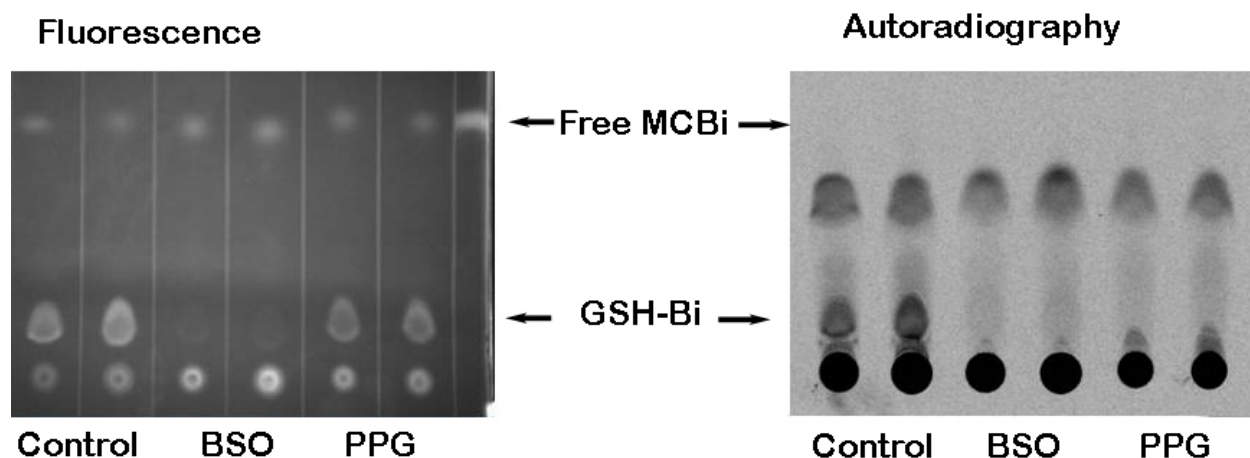


Figure 16 Incorporation of ^{35}S -methionine into glutathione (GSH-mCB bands) showed in the autoradiography demonstrates that functional transsulfuration occurs in HMEC.

HMEC cells treated with PBS (lanes 1 and 2) that generated glutathione-bimane (GSHmCB) conjugates producing an intense fluorescent spot also incorporated ^{35}S -methionine represented as a darker spot in the autoradiography. Cells treated with BSO (lanes 3 and 4) and with PPG (lanes 5 and 6) that had less GSH-mCB conjugate than controls, also show less incorporation of ^{35}S -methionine. Incorporation of ^{35}S -methionine in controls (lanes 1 and 2) demonstrates that functional transsulfuration occurs in mammary cells.

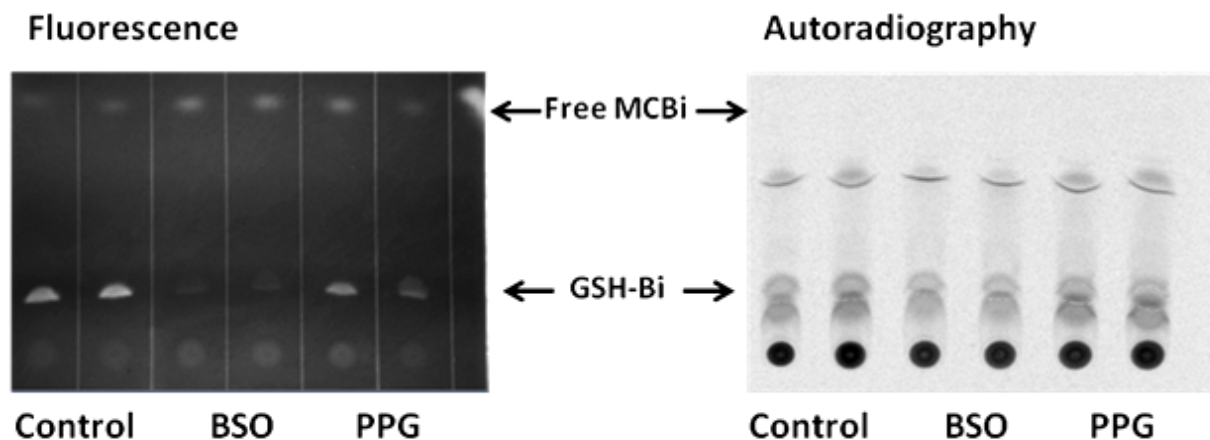


Figure 17 Incorporation of ^{35}S -methionine into glutathione (GSH-mCB bands) showed in the autoradiography demonstrates that functional transsulfuration occurs in MCF-7.

MCF-7 cells treated with PBS (lanes 1 and 2) that generated glutathione-bimane (GSHmCB) conjugates producing an intense fluorescent spot also incorporated ^{35}S -methionine represented as a darker spot in the autoradiography. Cells treated with BSO (lanes 3 and 4) and with PPG (lanes 5 and 6) that had less GSH-mCB conjugate than controls, also show less incorporation of ^{35}S -methionine. Incorporation of ^{35}S -methionine in controls (lanes 1 and 2) demonstrates that functional transsulfuration occurs in mammary cells.

Gsh_11#140-151 RT: 2.10-2.27 AV: 12 NL: 1.43E5
T: + c Full ms [100.00-800.00]

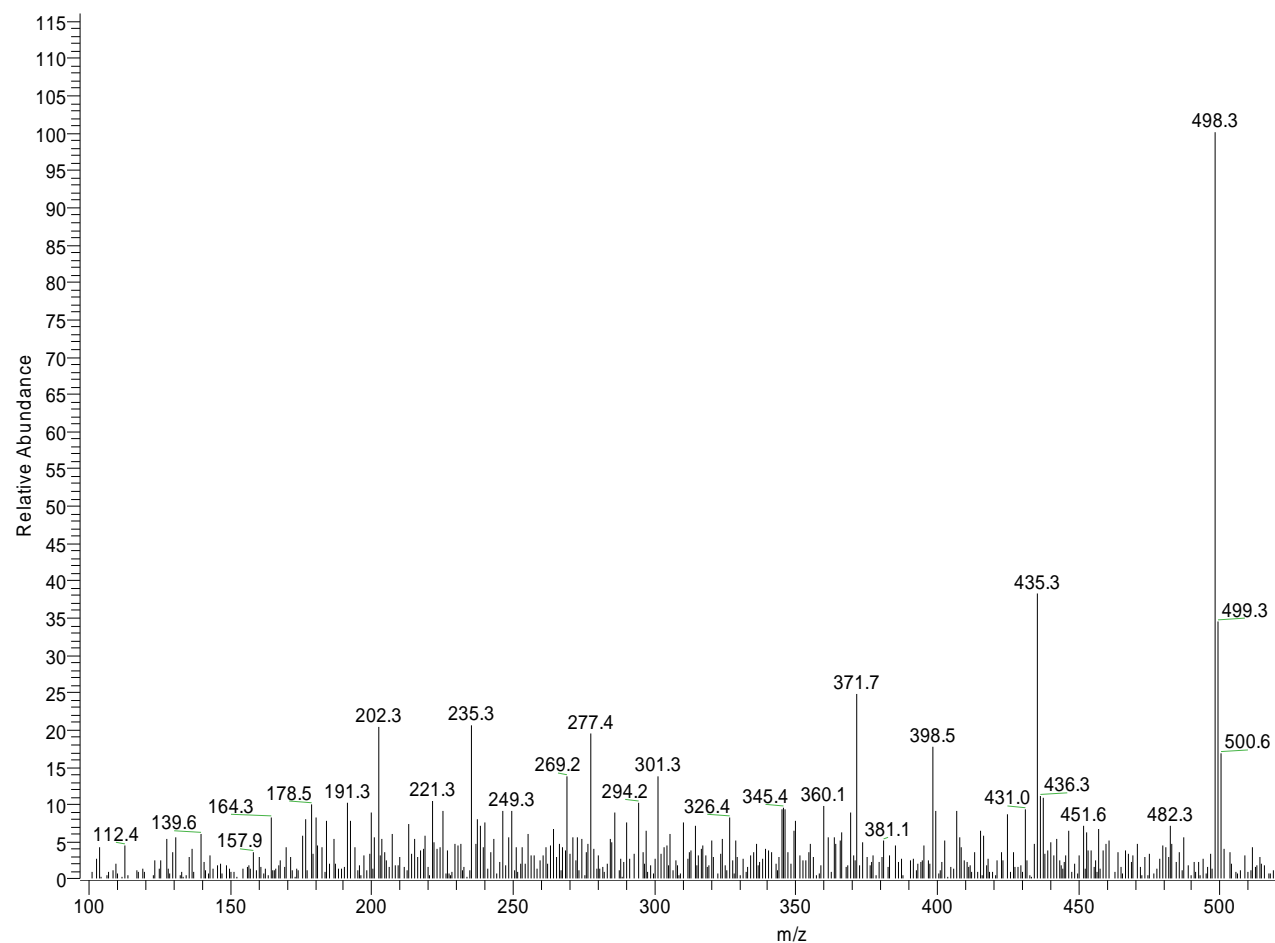


Figure 18 Mass spec of HMEC showing the glutathione-monochlorobimane conjugate peak.

Gsh_26#113-123 RT: 1.80-1.96 AV: 11 NL: 5.53E6
T: + c ms [100.00-800.00]

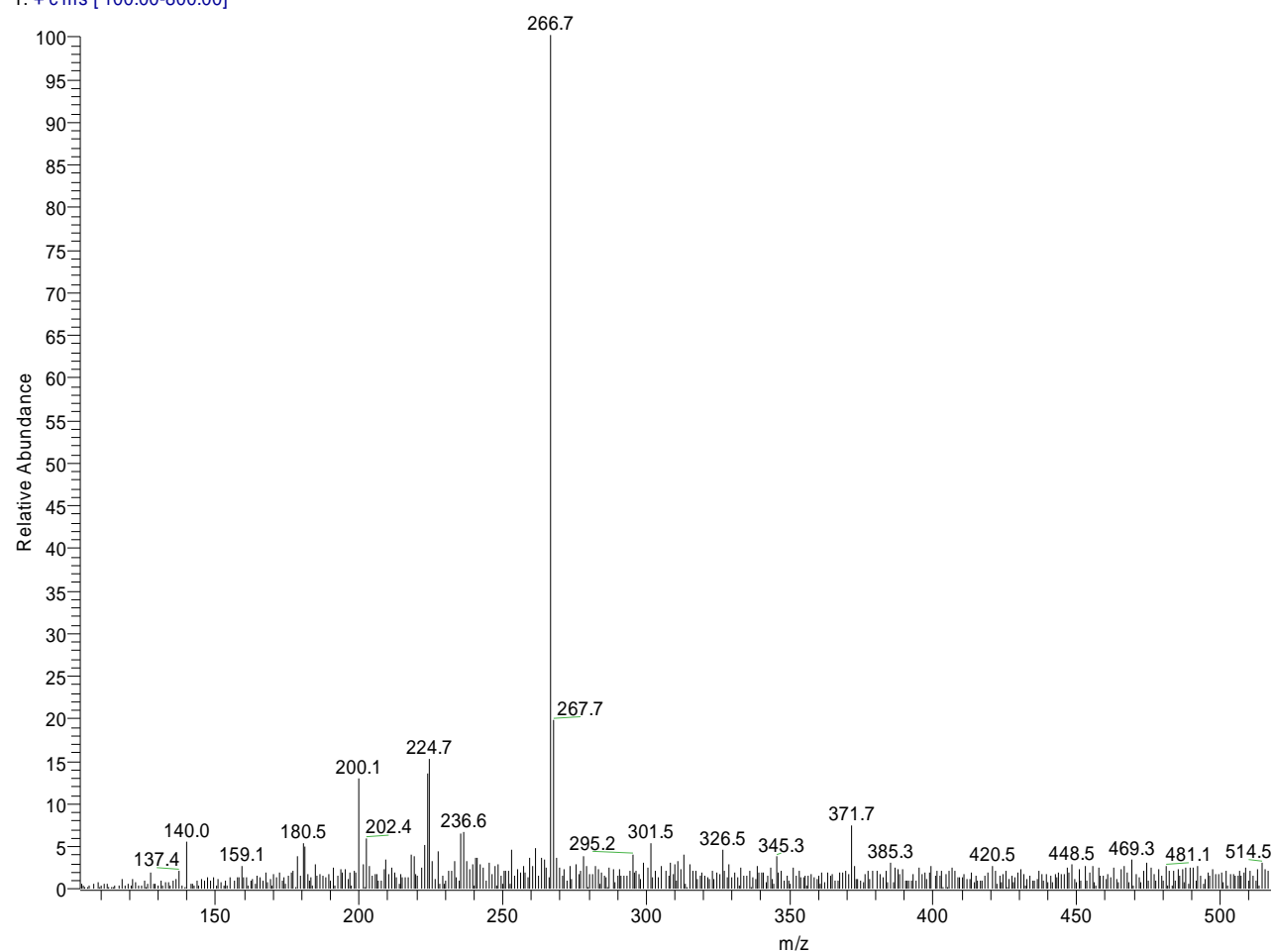


Figure 19 Mass spec of HMEC showing that under the presence of BSO there is no presence of glutathione-monochlorobimane conjugate peak.

Gsh_30#98-100 RT: 1.61-1.64 AV: 3 NL: 1.52E7
T: + c ms [80.00-800.00]

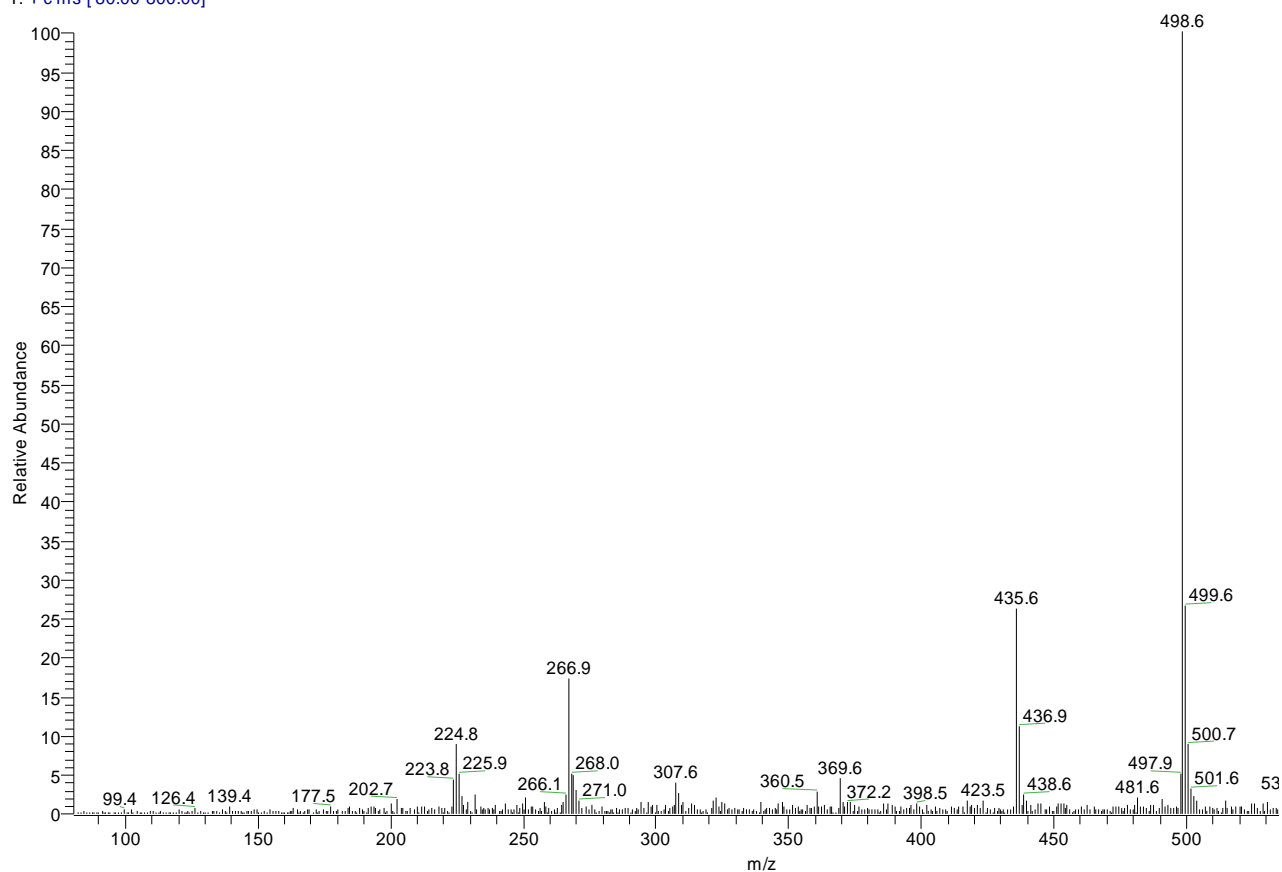


Figure 20 Mass spec of HMEC treated with PPG showing the peak at 498 for glutathione-monochlorobimane conjugate (GSH-mCB). Indicates that under the presence of PPG, glutathione can be still synthesized as shown in the fluorescent and autoradiography figure of the TLC plate.

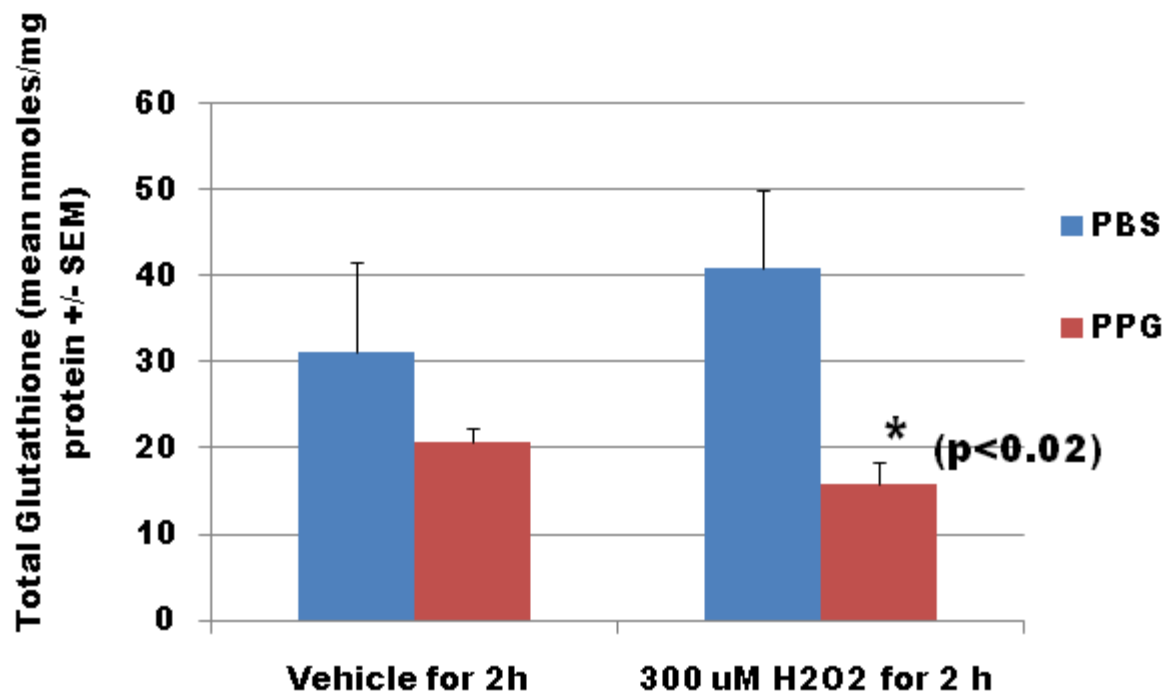


Figure 21 Impact of transsulfuration inhibition with PPG on cellular total glutathione levels in HMEC subjected to oxidative challenge (H₂O₂).

APPENDIX

List of abbreviations

ATP	Adenosine Triphosphate
BHMT	Betaine Homocysteine Methyltransferase
BSO	Buthionine Sulfoximine
CBS	Cystathionine β Synthase
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
FBS	Fetal Bovine Serum
GSH	Glutathione
GST	Glutathione-S-Transferase
HEPES-BSS	HEPES Buffered Saline Solution
MCF-7	Human breast adenocarcinoma cells
MEBM	Mammary Epithelial Basal Medium
MAT	Methionine Adenosyltransferase
MS	Methionine Synthase
mCBI	Monochlorobimane
HMEC	Human mammary epithelial cells
PBS	Phosphate Buffered Saline
PPG	Propargylglycine
SAH	S-Adenosylhomocysteine
SAMe	S-adenosylmethionine
THF	Tetrahydrofolate
TLC	Thin Layer Chromatography
TNS	Trypsin Neutralizing Solution

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