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Increased rate of glutathione synthesis from cystine in drug-resistant MCF-7 cells

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Abstract

The rate of glutathione synthesis was determined in drug-sensitive and -resistant MCF-7 cells by monitoring the rate of label uptake from [3,3′-$^{13}C_2$]-cystine using NMR spectroscopy and mass spectrometry. Compared with the wild-type human mammary adenocarcinoma cell line (MCF-7wt), the isotope incorporation rate was increased 1.6-, 2.4-, and 5.3-fold in the etoposide-resistant MCF-7 cell line (MCF-7vp), doxorubicin-resistant MCF-7 cell line (MCF-7adr), and 4-hydroperoxycyclophosphamide-resistant MCF-7 cell line (MCF-7hc), respectively. The increase in glutathione metabolism in the MCF-7hc line correlated with steady-state levels as determined by biochemical assay. In contrast, both the MCF-7vp and MCF-7adr lines showed increased metabolic synthesis of glutathione but displayed lower steady-state levels compared with the MCF-7wt line. The increased synthetic rates of all resistant lines reflected, in part, contributions from the increased activities of both γ-glutamyltranspeptidase and γ-glutamylcysteine synthetase. These results emphasize the importance of monitoring glutathione metabolic rates, rather than steady-state levels of enzymes or substrates, for assessing drug resistance.

Keywords: Glutathione; Biosynthesis; Drug resistance; Breast cancer; Stable isotope; Metabolism

1. Introduction

The tripeptide glutathione and its associated enzymes have been implicated in a number of mechanisms by which cancer cells resist the cytotoxic effects of therapy [1–3]. Many studies have shown a strong correlation between drug resistance and increased levels of glutathione; however, a significant number of studies failed to detect this relationship [4–6]. As a result, the value of using glutathione levels in a cancer cell or tissue as a predictor of therapy response is certainly in question. Since drug response is a function of both drug concentration and its time of exposure, the ability of a cell to maintain its glutathione concentration to meet this challenge, i.e. its glutathione rate of synthesis, may be a better determinant of cellular resistance.

Normally, glutathione is measured in cell lysates or in intact cells using spectrophotometric or fluorescent probes. These assays are accurate and easy to perform and constitute the bulk of glutathione measurements in cultured cells and biopsy samples. However, each of these methods yields a static picture of steady-state glutathione levels in cells. Glutathione synthetic rates are measured less frequently, probably due to the complexity of the experiment as compared with those measuring steady-state levels. Usually rate measurements require introduction of a radioactive tracer followed by isolation, identification, and quantitation of the radiolabeled metabolites. When these studies are done, however, they have illustrated the value of using dynamic information on glutathione metabolism in relation to resistance [7,8].

We have developed methods to monitor glutathione metabolism in cell extracts [9] and non-invasively in perfused cancer cells [10]. Both of these methods utilized NMR spectroscopy to monitor the rate of incorporation of the isotope label from [3,3′-$^{13}C_2$]-cystine in the growth medium into the cysteinyl residue of glutathione. The present study reports the use of both NMR and mass spectrometry to measure the rate of glutathione synthesis

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; MCF-7adr, doxorubicin-resistant MCF-7 cell line; MCF-7hc, 4-hydroperoxycyclophosphamide-resistant MCF-7 cell line; MCF-7vp, etoposide-resistant MCF-7 cell line; MCF-7wt, wild-type human mammary adenocarcinoma cell line; MDR, multidrug resistance; MRP, multidrug resistance-associated protein.

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in drug-sensitive and -resistant breast cancer (MCF-7) cells. Mass spectrometry offers sensitivity advantages over NMR and has found increasing use in mapping glutathione metabolism [11].

From the analysis of extracts, the rate of glutathione synthesis was found to be increased in three drug-resistant cell lines (MCF-7vp, MCF-7adr, and MCF-7hc) compared with the parental drug-sensitive cell line (MCF-7wt). The relative rates of glutathione synthesis did not correlate with steady-state levels of this tripeptide. The synthetic rates in all resistant cell lines reflected increased activities of both γ-glutamylcysteine synthetase and γ-glutamyltranspeptidase. Both enzymes contributed, in part, to the overall synthesis. These results indicate that prediction of drug sensitivity based on steady-state levels would be in error and that metabolic rates may be a more reliable indicator.

2. Materials and methods

2.1. Materials

[3-^{13}C]-L-Cysteine was purchased from Cambridge Isotopes Inc. [3,3′-$^{13}C_2$]-cystine was prepared by stirring a slightly basic solution of [3-^{13}C]-L-cysteine in an open container for 72 hr. Cystine- and methionine-free DMEM was purchased from Sigma. This medium was supplemented with 200 μM methionine and 200 μM [3,3′-$^{13}C_2$]-cystine. Solid-phase extraction cartridges (C_{18}) were obtained from Burdick & Jackson Inc.

Monobromobimane was purchased from Calbiochem. L-γ-Glutamyl-7-amino-4-methylcoumarin and glycylglycine were purchased from Sigma.

2.2. Cell culture

The parental MCF-7wt human mammary adenocarcinoma cell line was obtained from the American Type Culture Collection and from Dr. A. Miron, Duke University Medical Center. MCF-7adr and 4-hydroperoxycyclophosphamide (MCF-7hc) were obtained from Teicher of the Dana-Farber Cancer Institute [12–15]. MCF-7wt and MCF-7vp lines were obtained from Dr. Charles Morrow at Bowman-Gray Medical Center, Wake Forest University.

Cells were cultured in monolayer in DMEM supplemented with 10% FBS. We supplemented low glucose DMEM (1 g/L) with an additional 1 g/L of glucose due to the high glycolytic rate of the MCF-7adr cell line.

2.3. Isotope labeling and cell extraction

Glutathione synthesis in cells growing in monolayer was determined by a slight modification of the methods detailed earlier [9]. At 24 and 4 hr prior to feeding the cells isotope-labeled medium, the cells were fed with fresh, unlabeled medium. This ensured that the cells were well supplied with all nutrients when fresh, isotope-labeled medium was introduced. In all experiments, cells were in the log-phase of growth. Immediately before the addition of labeled medium, cells were washed with Hanks' Balanced Salt Solution to remove traces of unlabeled medium. DMEM/10% FBS containing [3,3′-$^{13}C_2$]-cystine was added, and the cells were incubated for periods between 1 and 24 hr. Cells were harvested by trypsinization, treated with monobromobimane, and extracted with perchloric acid. The extract was adjusted to pH 3 with KOH, and the supernatant was applied to a solid-phase extraction cartridge. The cartridge was washed with 0.2% acetic acid to remove unwanted metabolites, and the glutathione–bimane adduct was eluted from the cartridge using 30% methanol in water. This solution can be used directly for mass spectrometry. For NMR spectroscopy, the methanol was removed under a stream of nitrogen, and the samples were lyophilized.

2.4. NMR spectroscopy

NMR spectroscopy was performed at 11.75 T on a Varian Unity 500 NMR spectrometer at the Duke University Medical Center. The conditions used have been outlined previously [9]. Lyophilized cell extracts for NMR spectroscopy were taken up in 0.65 mL of D_2O for analysis.

2.5. Mass spectrometry

Cell extracts were analyzed by electrospray ionization mass spectrometry on a PE Sciex API 150 EX mass spectrometer in the Department of Chemistry at Duke University. Extracts were diluted into 50% water, 50% acetonitrile containing 0.1% trifluoroacetic acid. The samples were injected into the spectrometer at a constant flow rate of 10 μL/min using a Harvard syringe pump. The peak intensities at *m/z* 498, 499, and 500 atomic mass units (amu) were quantitated. Each sample was analyzed three times, and the average intensities for each of the peaks were used in calculating the isotopic enrichment.

2.6. Reduced glutathione assay

Glutathione was assayed in cell extracts by HPLC analysis of the monobromobimane derivatives as described previously [16]. All HPLC assays were performed using a Zorbax RX C_{18} column (Hewlett-Packard) on a Waters Millennium system equipped with a model 474 fluorescence detector.

2.7. Isotope incorporation rate

The fractional enrichment of glutathione was determined from NMR or mass spectrometry data. Using these enrichments and the total glutathione content determined by HPLC, the incorporation rate was measured by our published methods [9]. In all cell lines, the fractional

Table 1
Synthetic rates of MCF-7 cells[a]

Cell line	[Glutathione] (fmol per cell)	Synthesis rate (fmol/min per cell)	Turnover time (hr)
MCF-7wt	28.8 ± 5.6 (1.00)	0.0179 ± 0.0059 (1.00)	26.8
MCF-7adr	18.4 ± 4.4** (0.64)	0.0427 ± 0.0131** (2.39)	7.2
MCF-7hc	64.8 ± 12.4** (2.25)	0.0952 ± 0.0221** (5.31)	11.3
MCF-7vp	22.1 ± 3.6* (0.77)	0.0283 ± 0.0041** (1.58)	13.0

[a] Values are the means of at least four determination ± one standard deviation. The numbers in parentheses refer to values relative to that of the MCF-7wt line.
* $P < 0.05$; ** $P < 0.01$.

enrichment of glutathione obtained from extracts exposed for 2 hr to labeled medium was used in the calculation.

2.8. Enzyme activities

The activity of γ-glutamyltranspeptidase was determined in cell lysates by a fluorescence assay, using L-γ-glutamyl-7-amino-4-methylcoumarin and glycylglycine [17]. The high sensitivity of this assay, compared with the more commonly used spectrophotometric assay [18], was needed for measuring transpeptidase activity, especially in the MCF-7wt line. To recover maximal activity of this enzyme, cells growing in monolayer were washed with PBS and harvested by scraping. The activity of γ-glutamylcysteine synthetase in cell lysates was determined by monitoring the rate of formation of γ-glutamylcysteine by HPLC [19]. For homogenates from each cell line, a linear increase ($R^2 > 0.99$) in the amount of γ-glutamylcysteine was observed during four measurements between 0 and 90 min. Unlike the transpeptidase, synthetase activity was identical in cells harvested by trypsinization or scraping.

Protein assays were performed using an assay kit (Sigma) which is a modified version of the biuret and phenol procedure of Ohnishi and Barr [20].

3. Results

3.1. Glutathione content

The glutathione content in three of the cell lines used in this study has been determined previously in this laboratory by an HPLC assay of the bimane conjugate [16]. However, since glutathione levels can vary over time, new assays of the MCF-7wt, MCF-7hc, and MCF-7adr lines were performed and yielded results (Table 1) similar to those found previously. The MCF-7hc line exhibited a greater than 2-fold higher level of glutathione relative to the parental MCF-7wt cell line. The MCF-7adr and MCF-7vp lines showed lower levels of glutathione.

3.2. Glutathione synthesis

To measure the rate of glutathione synthesis, an isotope label was introduced in a biosynthetic precursor, and the incorporation rate of this label into glutathione was monitored. The enrichment rate measured was the net result of a number of transport and synthetic processes (Fig. 1). Rather than using radiolabeled tracers to follow metabolism, our laboratory has developed methods utilizing stable

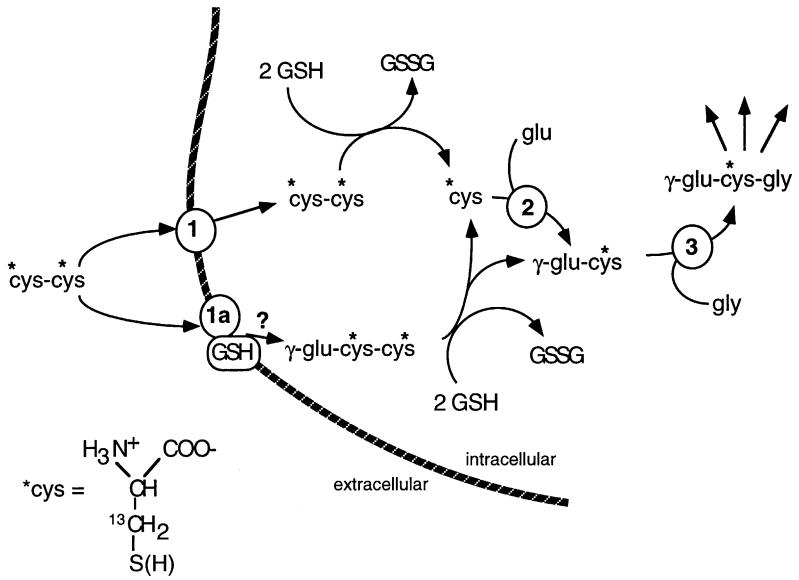

Fig. 1. Incorporation of isotope label from cystine into glutathione. The asterisk denotes the position of the ^{13}C isotope. Transport of cystine into the cell may occur *via* processes such as x_c^- (**1**), or possibly by γ-glutamyltranspeptidase (**1a**) with glutathione contributing the γ-glutamyl group (see text). Glutathione may be synthesized in steps catalyzed by γ-glutamylcysteine synthetase (**2**) and glutathione synthetase (**3**).

isotopes. Previously, we had employed NMR spectroscopy to monitor glutathione metabolism in intact cells [10] and extracts [9]. This paper extends these studies to a comparison of enrichment rates in drug-resistant cells and includes the use of mass spectrometry to further improve the sensitivity of the method.

Each cell line growing in monolayer was treated with medium containing [3,3′-$^{13}C_2$]-cystine. Cells were extracted at various time points, and the ^{13}C-enrichments were determined by ^{1}H NMR spectroscopy or mass spectrometry. The ^{1}H NMR spectra for each cell line, after a 2 hr exposure to [3,3′-$^{13}C_2$]-cystine-containing medium, are shown in Fig. 2. The amount of ^{13}C-enrichment is indicated at the left side of each spectrum and reflects the percentage of the total glutathione pool synthesized during the first 2 hr. For the MCF-7wt and MCF-7vp cell lines, less than 20% of the total pool was enriched in 2 hr, whereas both the MCF-7 hc and MCF-7adr lines showed higher enrichment levels.

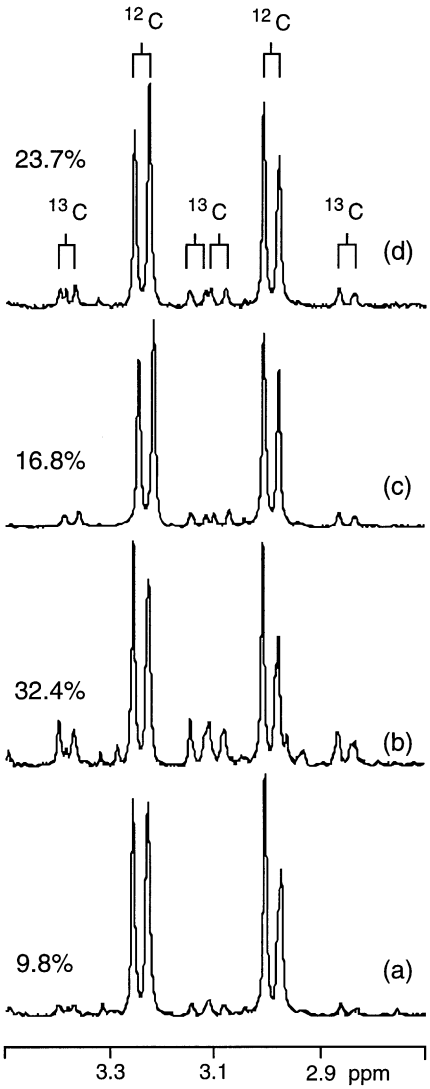

Fig. 2. A portion of the ^{1}H NMR spectrum showing the resonances of the β-cysteinyl protons of glutathione–bimane isolated from extracts of: (a) MCF-7wt; (b) MCF-7adr; (c) MCF-7vp, and (d) MCF-7hc cells. Each extract was obtained after a 2 hr exposure to medium containing 200 μM [3,3′-$^{13}C_2$]-cystine. The resonances due to protons attached to either the ^{12}C or ^{13}C isotope of carbon are indicated. Integration of the area beneath the resonances for ^{13}C-attached protons is divided by the total area beneath the resonances for both ^{12}C- and ^{13}C-attached protons to yield the percentage enrichment shown to the left of each spectrum.

The mass spectrum of the glutathione–bimane conjugate isolated from an extract of MCF-7vp cells is shown in Fig. 3a. The relative intensities of the peaks at 498, 499, and 500 amu (Fig. 3b) reflects an isotopic enrichment of 16%. Several samples were analyzed by both NMR and mass spectrometry. The inset shows a corresponding relationship between the enrichment determined by mass spectrometry and that found by NMR spectroscopy, indicating that both methods yield equivalent results.

For each cell line, replicate measurements of the percentage of label incorporation were performed for periods ranging from 1 to 8 hr (Fig. 4). Initially (at $t = 0$), the enrichment was due to the natural abundance of ^{13}C of 1.1%. Using this as the initial point, these data demonstrate fairly linear increases in label incorporation for at least 2 hr for the MCF-7adr line and at least 4 hr for the other cell lines. For this reason the isotopic enrichments determined in extracts after a 2 hr exposure to labeled medium were used to calculate the rate of synthesis as outlined in Section 2. The rates calculated are given in Table 1.

For the MCF-7hc line, the 5.3-fold increased rate of synthesis relative to the MCF-7wt line correlated with the relative 2.3-fold increase in steady-state levels of glutathione. The results for the MCF-7vp and MCF-7adr lines show important differences. The steady-state levels of glutathione in both these drug-resistant lines were lower than in the MCF-7wt line, but the rate of isotopic enrichment was increased (Table 1). These data indicate that each resistant cell line has a higher rate of turnover than the parental MCF-7wt line. For the MCF-7adr and MCF-7vp lines, the higher turnover rates reflect the combination of lower intracellular glutathione concentrations and rapid synthesis from cystine. This is shown in the far right column of Table 1. The turnover time was calculated by dividing the total glutathione content per cell by the turnover. This provides a theoretical time in which the entire glutathione pool can be resynthesized in a particular cell.

3.3. Enzyme activities

To determine what enzyme(s) might contribute to the overall turnover, the activities of γ-glutamyltranspeptidase and γ-glutamylcysteine synthetase were determined in each of the cell lines. Both enzymes have been implicated in regulating key steps in glutathione biosynthesis. The results are shown in Table 2. The activities of both of these enzymes appear to be higher in all of the drug-resistant cell lines. The relative activities of γ-glutamylcysteine synthetase more closely reflect the relative synthetic rates in each of the cell lines. However, this cytosolic enzyme is

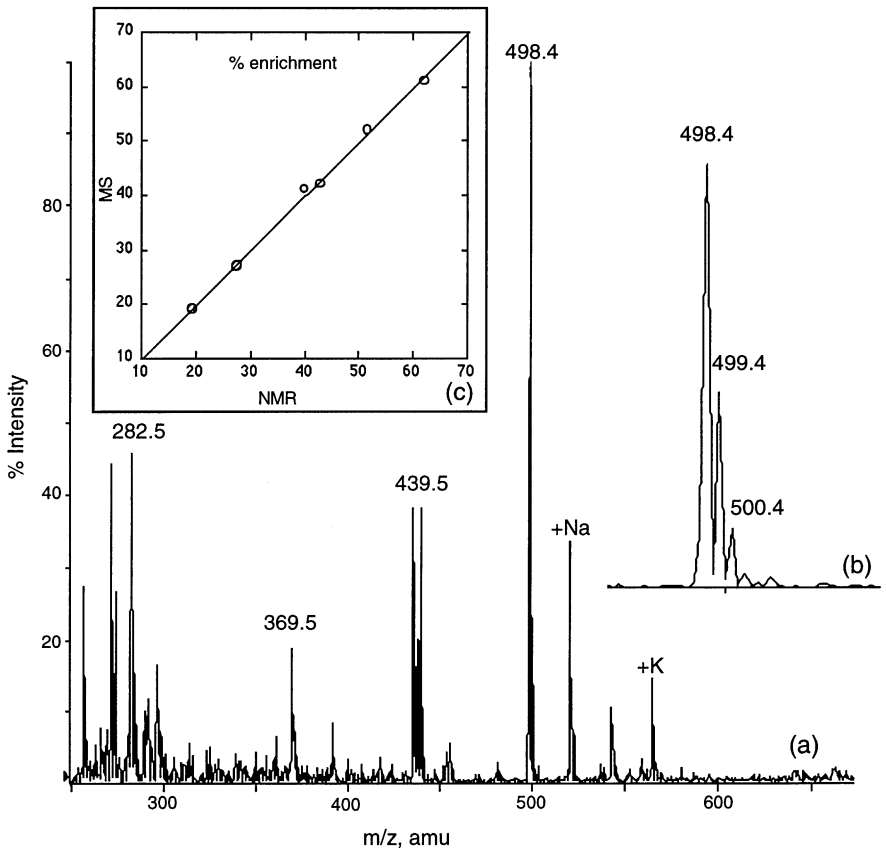

Fig. 3. (a) A portion of the mass spectrum of glutathione–bimane. (b) Expansion of the region near 498 amu. The isotope enrichment is calculated from the ratio of peak intensities at *m/z* 500, 499, and 498 amu. (c) Correspondence of enrichment calculated from NMR and mass spectrometry shows agreement to within 1%.

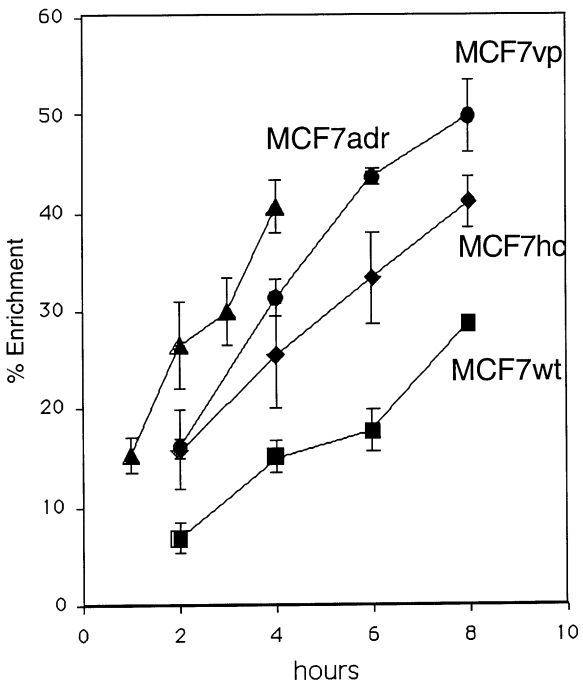

Fig. 4. Isotopic enrichment of glutathione as a function of time for the four cell lines. Data shown were obtained from both mass spectrometry and NMR spectroscopy and are the average of at least three determinations for each time point ± one standard deviation.

Table 2
Enzyme activities of MCF-7 cells[a]

Cell line	γ-Glutamyltranspeptidase (pmol/mg min)	γ-Glutamylcysteine synthetase (pmol/mg min)
MCF-7wt	21.1 ± 19.5 (1.00)	371 ± 120 (1.00)
MCF-7adr	31.0 ± 9.91* (1.47)	542 ± 105** (1.46)
MCF-7hc	44.2 ± 12.7** (2.09)	900 ± 251** (2.42)
MCF-7vp	152 ± 66.4** (7.20)	569 ± 75** (1.53)

[a] Values are the means of at least four determination ± one standard deviation. The numbers in parentheses refer to values relative to that of the MCF-7wt line.
* $P < 0.057$; ** $P < 0.01$.

dependent upon delivery of cyst(e)ine, which, in turn, is dependent upon transport processes including that of the membrane-bound γ-glutamyltranspeptidase. The activity of this transpeptidase was elevated in both the MCF-7hc and MCF-7vp lines. The elevation in activity in the MCF-7adr line bordered on statistical significance.

4. Discussion

It is unlikely that the resistance of the cancer cell to a single agent is the result of alterations in one process.

Therefore, it is also unlikely that a single measurement of a metabolite, protein, or gene will describe how a cell will respond to this agent much less a number of agents that differ in their mechanisms of action. Elevation of glutathione or its associated enzymes is thought to be involved with resistance to alkylating agents such as cyclophosphamide [6,21]. However, glutathione appears to play an important role in apoptosis [22], DNA repair [23], and gene expression [24] and, therefore, may be a key to determining whether a cell responds to or resists therapy. Since the effectiveness of a drug is a function of both its concentration and its time of exposure, the ability of a cell to maintain its glutathione concentration to meet this challenge as a function of time is important to drug response. This study presents evidence that such measurements may be a better indicator of therapy response.

The MCF-7adr, MCF-7vp, and MCF-7hc sublines were chosen because they are known to possess different resistance pathways due to the varied mechanisms of action of the drugs doxorubicin, etoposide, and 4-hydroperoxycyclophosphamide. Since cyclophosphamide is a prodrug requiring activation by hepatic mixed-function oxidases, the chemically activated form, 4-hydroperoxycyclophosphamide, is used *in vitro*. This MCF-7hc line was reported to be 9-fold resistant to 4-hydroperoxycyclophosphamide [14], which is consistent with the finding in our laboratory [25]. This cell line consistently showed increased glutathione levels relative to the MCF-7wt line in this (Table 1) and previous studies [10,16,26]. It also has increased levels of a class III aldehyde dehydrogenase implicated in resistance to cyclophosphamide-type drugs [27], but glutathione or its associated enzymes likely contribute to resistance in this cell line [26].

For the other sublines, the contribution of glutathione to overall resistance is less clear. Doxorubicin is thought to act by damaging DNA *via* free radical formation or a topoisomerase II-mediated process [28]. Resistance in the MCF-7adr cell line appears to be mediated, in part, by the presence of the MDR *P*-glycoprotein [12]; however, glutathione also appears to play a role [29]. It has been reported that a commonly used MCF-7adr line may be unrelated to the MCF-7 line and should be designated NCI/ADR-RES [30]. To avoid confusion in this discussion, and its relation to previous results, we will continue to use the designation MCF-7adr for our cell line but with the origin in question. Whatever the origin of this line, of greatest interest to us is its relatively low glutathione content observed in this work (Table 1) and in previous studies by others [31].

Etoposide is thought to act by targeting topoisomerase II, and resistance in this MCF-7vp line has been attributed to altered inhibition of this enzyme, as well as higher levels of the MRP [32]. Further studies demonstrated that depletion of glutathione sensitized these cells to treatment [33]. Our assay (Table 1) and previous work [33] demonstrate a slightly decreased glutathione level in this line relative to the MCF-7wt line.

The data from this and other studies show that the steady-state glutathione levels measured in the MCF-7adr and MCF-7vp cell lines do not correlate with resistance. The hypothesis driving this study is that a dynamic measure of glutathione metabolism would be a better predictor of drug response.

To determine how well a cell can maintain its glutathione pool, an isotope tracer was introduced to monitor metabolism. In the past, many studies have relied on radioactive tracers to obtain this information. More recently, the use of stable isotopes combined with mass spectrometry [11] or NMR spectroscopy [34–38] has offered an attractive alternative.

Quantitation of isotope incorporation by NMR spectroscopy (Fig. 2) or mass spectrometry yielded identical data (Fig. 3). The mass spectrometry method offers significant sensitivity advantages. For example a typical 1 hr NMR analysis requires 0.1 μmol of glutathione–bimane, whereas mass spectrometry requires about 10 pmol. The NMR method, however, is often less sensitive to sample preparation variables, such as the presence of salts or other interfering metabolites. The analytical method chosen may be dependent upon the amount of tissue available or access to the appropriate instrumentation. Both of these methods can be used with most tissue samples.

It should be noted that the rates reported herein reflect the rate of isotopic enrichment of glutathione from cystine and are not the actual rates of glutathione synthesis. To calculate the glutathione synthetic rate, the isotope incorporation into the immediate glutathione precursor pool must be known [11]. If the precursor pool is rapidly and completely enriched with isotope from cystine, the glutathione enrichment rate may equal the synthetic rate.

The enrichments obtained by both of these analytical methods were used to calculate the synthetic rates given in Table 1. Without assumptions on transport or equilibria, the enrichment rate reflects the transport and utilization of cystine. Cystine may be transported into the cell by a number of processes. Transport of cystine *via* mechanisms such as the x_c^- pathway (pathway **1**, Fig. 1; [39]) has been established. More controversial [40] is the possible role of γ-glutamyltranspeptidase-mediated transport of cystine as γ-glutamylcystine in which the γ-glutamyl group is derived from glutathione (pathway **2**, Fig. 1). It is apparent that in some cell lines cystine transport may be mediated, at least in part, by γ-glutamyltranspeptidase [41–43]. Whether cystine is actually transported in these cell lines as γ-glutamylcystine was not determined. However, if this pathway is present, incorporation of the label into glutathione may occur without the involvement of γ-glutamylcysteine synthetase. In normal tissue, this enzyme is considered the rate-limiting enzyme in glutathione biosynthesis and regulates intracellular levels through a feedback inhibition mechanism [44]. Label incorporation,

therefore, is the result of processes that are involved in the transport or biosynthesis of the substrates and a number of synthetic and regulatory pathways. This type of information is difficult to obtain by measuring static levels of substrates, transport proteins, or synthetic enzymes.

From the data in Table 1, it can be seen that the rate of synthesis in the MCF-7hc line correlates with a greater than 2-fold increase in steady-state levels of glutathione. This increased rate of synthesis resulting in higher levels of glutathione is consistent with earlier studies that linked overexpression of γ-glutamylcysteine synthetase with increased glutathione levels in melphalan-resistant cells [45,46]. The 2-fold increased activity of this enzyme (Table 2) supports the hypothesis that its overexpression influences glutathione levels in the cell. Quantitatively, the increase in activity of either γ-glutamylcysteine synthetase or γ-glutamyltranspeptidase alone for the MCF-7hc line (Table 2) is not responsible for the relative 5-fold increase in the rate of synthesis (Table 1). Most likely, the rate of synthesis is a reflection of several pathways with contributions from both γ-glutamyltranspeptidase and γ-glutamylcysteine synthetase and also is dependent upon substrate availability.

In contrast to the MCF-7hc line, the other two resistant lines showed relatively lower steady-state levels of glutathione but higher synthetic rates (Table 1). This higher rate of synthesis was correlated with increased activities of γ-glutamylcysteine synthetase and γ-glutamyltranspeptidase. For the MCF-7adr line, an earlier study noted the same lower glutathione content and measured a 1.6-fold increase in the activity of γ-glutamylcysteine synthetase [31]. That study also noted a more rapid depletion of glutathione in the MCF-7adr line than in the MCF-7wt line when cells were treated with the γ-glutamylcysteine synthetase inhibitor buthionine sulfoximine [31]. Based on these results, the authors hypothesized a more rapid glutathione synthetic and utilization rate in the resistant cell line. Our results confirm their hypothesis.

As already mentioned, the MCF-7adr cell line shows high expression of the MDR *P*-glycoprotein [12] but also increased glutathione *S*-transferase activity [47]. Unlike MRP, the MDR system is not associated with transport of glutathione or its conjugates. Earlier studies from this laboratory, however, noted rapid efflux of glutathione–bimane conjugates from the MCF-7adr line [16], suggesting that a glutathione-mediated efflux process is present in this cell line. Therefore, the presence of both the glutathione *S*-tranferases and *P*-glycoprotein or other efflux processes in the MCF-7adr cell line may account for the increased utilization of glutathione resulting in lower steady-state levels. This hypothesis was also advanced by Batist *et al.* [31].

Similarly, increased utilization may also be the reason for the relatively low levels of glutathione in the MCF-7vp line, which is known to express the MRP efflux pathway [32]. Coordinated overexpression of MRP and γ-glutamylcysteine synthetase is often observed [48]. This cell line also expresses unusually high levels of γ-glutamyltranspeptidase activity, perhaps reflecting increased reliance on this pathway resulting in increased glutathione utilization and lower steady-state levels.

Based on these data, we hypothesize that glutathione levels in the cell are not controlled by the levels of synthetic enzymes but by the pathways that utilize glutathione. It may be difficult to predict, therefore, the drug response of a given cell based solely on glutathione levels. Whether the rate of synthesis itself or the turnover time is a better prognostic factor in predicting response requires further testing. Likely the turnover time or a similar factor that combines steady-state glutathione content with the rate of synthesis would best predict drug response in light of the use of concentration vs. time curves in computing effective drug exposures. We must emphasize that these studies offer only preliminary data in support of our hypothesis. Many more cell lines must be tested in order to judge the value of this type of analysis in predicting therapy response.

Measuring glutathione enrichment reflects the concentration and delivery of substrates and activities of a number of enzymes, all of which may be altered in drug-resistant cancers. Since resistance is unlikely to be the result in alteration of only one gene product [49], prognostic factors must be found that reflect the contribution of multiple components. Even determining the levels of overexpressed enzymes may not be an accurate measure of increased activity if substrates are not readily available in the cell. Conversely, the effect of metabolic inhibitors would be hard to assess when measuring enzyme expression alone. For this reason, measuring flux rates for key metabolites may offer a better prognostic marker for therapy outcome.

Acknowledgments

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