Analysis of Glioma Cell Platinum Response by Metacomparison of Two-dimensional Chromatographic Proteome Profiles*

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Successful clinical development of cancer treatments is aided by the development of molecular markers that allow the identification of patients likely to respond. In the case of broadly cytotoxic drugs, such as the multinuclear series of platinum chemotherapeutic agents that we are evaluating for the treatment of glioma, one route to marker identification is proteomic profiling. We are using the two-dimensional chromatography system, the ProteomeLab PF2D, to compare proteomic profiles of glioma cells in culture before and after drug treatment. The existing software tools allowed the rapid identification of peaks increased by treatment of a given drug as compared with control untreated cells. To compare across these pairs, we developed new software, called the MetaComparison Tool (MCT). The MCT uses the chromatographic characteristics of peaks as identifiers, an approach that was validated by mass spectrometry of two independent isolations of a peak, from cells that were treated with two different platinum compounds. The MCT made it possible to rapidly query whether a given peak responded to more than one treatment and so allowed the identification of peaks that were specific to a given drug. As a result, this analysis greatly reduced the list of peaks whose isolation and downstream analysis by mass spectrometry is warranted, accelerating the search for protein markers of response. Molecular & Cellular Proteomics 5:35–42, 2006.

The successful clinical deployment of anticancer drugs relies on being able to identify patients whose tumors are likely to respond. In the case of drugs developed against tumor-specific molecules, detection of the relevant target is the natural approach. For more broadly cytotoxic therapies, including platinum chemotherapeutic agents, such a direct approach is not possible, and so markers of likely response must be identified by molecular comparisons. In this work we present an approach to comparing the response of glioma cells to novel platinum drugs at the protein level as a first step toward this goal. We show that proteome displays generated by two-dimensional liquid chromatography can be compared using new computer software, the MetaComparison Tool (MCT), and that this approach can rapidly lead to the identification of peaks worth further analysis as proteins whose expression is responsive to drug treatment.

The need for new clinical agents for the treatment of glioma has led us to investigate the potential of a newer class of platinum compounds, which are multinuclear, and whose clinical profile and mechanism of action are quite distinct from the established platinum compounds. The first member of this class, the trinuclear platinum BBR3464, is effective against cells with an inherent or acquired resistance to cisplatin, including glioma cells (1, 2), suggesting that there are important differences in their mode of action. BBR compounds, exemplified by BBR3464, show increased rates of cellular uptake and more rapid formation of DNA adducts than cisplatin and a different pattern of adduct structure (Fig. 1) (3–6) that is recognized by different proteins (7, 8). However, BBR3464 may not be an ideal subject for further study because it has encountered metabolism-related difficulties in clinical trials (9–11). Therefore, in the proteome profiling work presented here we have focused on BBR3610, which had the greatest potency on glioma cells in culture and in xenograft models.

The proteomic profiling platform chosen for this work is the ProteomeLab PF2D (Beckman Coulter, Inc., Fullerton, CA), which is based on recent advances in technology using chromatographic separation of proteins in liquid phase, from the work of Dr. David Lubman and colleagues (12, 13). The PF2D system separates proteins in two dimensions: according to pI in the first dimension using a chromatofocusing column and...
subsequently using nonporous reversed-phase HPLC in the second dimension. The use of liquid separations allows for the collection of hundreds of purified proteins in the liquid phase for rapid and easy further analysis, for example by peptide mass mapping using MALDI-TOF/TOF MS, which may allow identification of differentially expressed proteins. To avoid unnecessary analysis of uninteresting peaks, proteome profiles generated by the PF2D can be compared using the associated DeltaVue software analysis tool. DeltaVue allows easy pairwise comparisons of proteome profiles using a graphical interface and is used to mark and collect data describing differentially expressed peaks. There is currently no capability for the easy comparison of data from multiple proteome profiles, and yet most experiments contain more than two samples. In many comparison experiments, a control sample is used to first determine how molecules respond to each individual treatment in a series. This initial level of analysis as a comparison of each test sample with the control is readily achieved using DeltaVue and in this study allowed us to identify peaks that are increased in response to cisplatin, BBR3464, or BBR3610 as compared with untreated control. However, it is more difficult to rapidly assess whether a peak that responds to one of these drugs also responded to the others. Peaks that are associated specifically with exposure to one drug but not another are of particular interest for our efforts to isolate specific markers to the most potent family member. Here we present an approach and a software tool for the analysis of initial pairwise comparisons at a second or metalevel, allowing the rapid identification of peaks that show expression associated with one condition. These peaks are prime candidates for isolation and further analysis, and our approach has dramatically narrowed the number of peaks for which this additional analysis is necessary.

**EXPERIMENTAL PROCEDURES**

LNZ308 glioma cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum and maintained in a 7.5% CO2 humidified incubator. Cisplatin (Sigma) was prepared fresh in DMSO at 1000× working solution and diluted in medium before use. The BBR compounds were dissolved in water and stored at −80 °C until use. Cells were treated with IC50 concentrations of each drug (cisplatin, 2 µM; BBR3464, 0.1 µM; BBR3610, 80 nM) and collected at 6 or 18 h post-treatment.

For PF2D separation, samples were first lysed in an optimized lysis buffer (7.5 M urea, 2.5 M thiourea, 12.5% glycerol, 62.5 mM Tris-HCl (pH 7.8−8.2), 2.5% (w/v) n-octylglucoside, 6.25 mM Tris(carboxymethyl)phosphine hydrochloride, 1.2 µM protease inhibitor). Adherent cells were rinsed with PBS, scrapped off the dish into PBS, pelleted by centrifugation, and lysed with 2 ml of lysis buffer. The suspension was vortexed vigorously to ensure complete lysis. The lysates were cleared by centrifugation at 20,000 × g for 60 min at 4 °C, and the supernatant was recovered. The lysis buffer was then exchanged with start buffer (pH 8.5 ± 0.1; Beckman Coulter, Inc.) using a PD-10 column (Amersham Biosciences), and the first 3.5-ml fraction was collected. The sample was now ready for injection on the first dimension column.

Two milliliters of the samples were injected on the chromatofocusing column in the first dimension compartment of the PF2D instrument, and the separation method was initiated. This method, which operates at a constant flow rate of 0.2 ml/min, starts by washing the column with 100% start buffer pH 8.5 ± 0.1 for 45 min. Fractions were collected at 5-min intervals and were −1 ml. After the wash step, the method initiated a linear gradient to 100% eluent buffer pH 4 ± 0.1 (Beckman Coulter, Inc.) that takes −60 min to complete, and fractions were collected every 0.3 pH units, resulting in −17 fractions ranging in size from 0.6 to 1 ml. After the pH of the eluent is at pH 4.0, column was washed with 10 column volumes of high ionic strength wash to remove residual proteins, and fractions were collected by time as before, resulting in the collection of another three fractions of −1 ml. The column was then rinsed with 10 column volumes of distilled water before the next sample was injected.

The −30 fractions that were collected in the first dimension were then sequentially injected onto the second dimension reversed-phase HPLC column. This process is automated and occurs unattended because the fraction collector for the first dimension run doubles as an autoloader for the second dimension run. Injections of 200 µl were used, and it is important to note that there was ample first dimension fraction material left over for additional second dimension runs. No material was collected from the initial second dimension run because blind collection of material would rapidly result in a storage and sample management problem. Instead the data were analyzed, and then first dimension fractions were rerun in the second dimension as needed. The second dimension consists of a 30-min linear gradient of 5–100% B at 0.75 ml/min where A × 0.1% TFA in water and B is 0.08% TFA in acetonitrile. It was run at 50 °C, and the proteins were detected with UV light at 214 nm. At the end of each second dimension run, the column was equilibrated with an initial mobile phase for 10 column volumes.

Absorbance at 214 nm was measured in the second dimension, and the data were collected and analyzed in the ProteoVue/DeltaVue software packages. ProteoVue allows inspection of individual proteome maps, whereas DeltaVue allows comparison of two maps and tagging of peaks of interest (see “Results” for examples). To compensate for differences in protein loading between two samples, the sum of the area under all peaks in the first dimension was used to adjust the peak size values and so adjust the ratios that are observed. These data were automatically generated in the first dimension report by the HPLC System Gold software that runs the PF2D methods and

![The structure of platinum compounds](Image.png)
were derived from in-line 214 nm UV readings. Tagged peaks were prioritized on the basis of the ratio of their intensity between two samples that is an indication of the degree of differential expression as well as absolute size and separation from other peaks that are characteristics related to the ability to cleanly collect sufficient quantities for mass spectrometry analysis.

The DeltaVue data sets, with peaks picked, were then loaded into the MCT, evaluation versions of which can be obtained on line (www.daedalussoftware.com/website/products.asp). The MCT application was written using C#.NET, Microsoft Access, Crystal Reports 9, and Graphics Components software and custom code. MCT is equipped to deal with multiple Excel files in identical formats and uploads the data from those files through a click of a button into an Access database. This database then serves as the backbone to run all the queries and generate graphic-based outputs. A total of 172 peaks were selected for analysis in the MCT in this study. These peaks were chosen because they were within the range of the pH gradient, were seen to be up-regulated after drug treatment, were of at least 0.05 absorbance units in size, and were distinct from nearby peaks on the chromatogram meaning that they reached the base line on both sides and suggesting that their chromatographic separation would be achievable. Each peak was analyzed with MCT by designating fairly wide pI and retention time (RT) ranges. MCT analysis generates a number of candidate peaks that match the input criteria. These candidate peaks were further screened, and a peak was considered a “match” only if the following criteria were met: the peak had to have a size of at least 0.05 with at least a 2-fold change (=0.5 on the MCT output) and be within 0.05 retention time of the initial peak of interest. Mass spectrometry was performed at the Michigan Proteome Consortium core laboratory at the University of Michigan using an Applied Biosystems 4700 Proteomics Analyzer (TOF/TOF) mass spectrometer with high throughput option. Proteins were identified using the automated Applied Biosystems GPS Explorer MS/MS to summarize Mascot search of the NCBI nr database.

For Western analysis of triose-phosphate isomerase 1 (TIM), samples were either not treated (controls) or treated at IC_{50} concentrations of drug for 6 h. Samples were run through the first dimension on the PF2D system as described above. An aliquot of the first 10 fractions was immediately incubated with Laemmli SDS sample buffer and heated to 70 °C for 5 min. Samples were run on a 10% NuPage BisTris gel (Invitrogen), transferred to a PVDF membrane (Pall, East Hills, NY), and blocked with 5% BSA. Samples were incubated with anti-TIM antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:100 overnight at 4 °C. Membrane was washed with TBS with 0.1% Tween 20 times for 5 min each, incubated with the secondary antibody rabbit anti-goat conjugated with horseradish peroxidase (Pierce) (1:3000), developed with SuperSignal West Pico substrate (Pierce), and exposed to film.

RESULTS

In an attempt to identify protein markers of response to novel platinum chemotherapeutic agents in brain tumors, we treated cultured glioma cells with isotoxic doses of drug, displayed their proteins using a two-dimensional chromatography system, and analyzed these profiles using computer software. We studied LNZ308 glioma cells that were either left untreated or were treated for 18 h with cisplatin, BBR3464, or BBR3610 at a concentration that will cause a 90% reduction in colony formation in a clonogenic assay (IC_{90}; details of drug response of glioma cells to BBR compounds will be published elsewhere). Proteins were harvested and analyzed according to the PF2D protocols to generate profiles (see “Experimental Procedures”). Pairwise comparisons using DeltaVue software led to the identification of ~500 peaks that were altered by treatment with a single compound when compared with untreated controls. These comparisons were done lane by lane, and local alignment was optimized by eye using the DeltaVue lane adjustment capabilities. Examples of PF2D profiles displayed in DeltaVue are shown as chromatograms (Fig. 2). The traces on the left and colored red are from control samples, and those on the right and colored green are from drug-treated cells (Fig. 2, A and B). In the middle is a ‘bands on a gel’ representation of the difference with the stronger peaks determining the color. In Fig. 2, these are green representing the greater intensity of the peaks in the right-hand samples (note differences in scale of chromatograms), which are from cells treated with cisplatin (Fig. 2A) or BBR3610 (Fig. 2B).

The peak-picking feature of DeltaVue was used to tag peaks that appeared to be differentially expressed and display their characteristics in a spreadsheet (Fig. 2C). These included an arbitrary peak number assigned by the software, the lane, the pH range separately for the left- and right-hand peaks (as pI Min and pI Max) of the first dimension fraction, the RT of the peak in the second dimension, and the peak size (data). In the middle were calculated values of the ratio of the peaks (shown as modular [L/R] where L is left and R is right) expressing -fold difference and the difference in the retention time between the two peaks (ΔRT). Please note that the data in Fig. 2C are from two different DeltaVue files combined here for the ease of presentation.

Ideal markers of drug response are proteins that respond specifically to the drug of interest. Accordingly in our study the most interesting protein peaks are those that changed when cells were treated with the multinuclear platinum compounds and not cisplatin. In addition, if there were peaks that responded only to BBR3610, which is more effective in the treatment of glioma cells in culture or in xenografts than BBR3464, then these would be the highest priority for follow-up experiments. Therefore, the question of whether a peak identified in DeltaVue as changing in response to BBR3610 also changed in response to BBR3464 or cisplatin became important. Similarly peaks that responded to any of the other drugs would need to be evaluated for specificity. These considerations prompted the development of new software, called the MCT because it compares among existing pairwise comparisons generated by DeltaVue.

The MCT is capable of importing the peak data, which are exported from DeltaVue as a spreadsheet, one for each of the pairwise comparisons. It then displays peaks from any of these pairs that fall into a user-specified region of the two-dimensional display. The user sets these parameters as pI range and retention time range and can also set a lower limit for -fold difference between control and treated peaks, and the MCT displays all peaks within those parameters. For example, data sets representing control versus cisplatin and control versus BBR3610, each containing over 500 different
Identification of triose-phosphate isomerase by PF2D. LNZ308 glioma cells were mock-treated or treated with cisplatin or BBR3610, collected at 6 h, and run through the PF2D system. A and B show the chromatogram traces after DeltaVue analysis of control versus cisplatin (A) and control versus BBR3610 (B). The dotted line indicates a peak that is up-regulated after exposure to both drugs. The traces of drug-treated samples are shown in green, while the control sample trace is in red. C, the peak table generated by DeltaVue shows the characteristics of the peaks illustrated in A and B. In each case, the control sample has been designated as the left peak (red trace). D, MCT results displaying peaks with similar characteristics between different DeltaVue analyses. E, mass spectrometry analysis of the peaks highlighted in A and B indicate that both peaks are TIM. C.I., confidence interval; Pep., peptide; Instr., instrument; Calc., calculated; Obsrv., observed; Seq., sequence; L/R, left/right.
peaks, were loaded into the MCT. To compensate for differences in protein loading, the sum of the area under all peaks, which is automatically generated in the first dimension report by the HPLC System Gold software, was used to adjust the peak values prior to import into the MCT. Then the following parameters were set in the MCT: pI Min = 8.8; pI Max = 9; RT Min = 18; RT Max = 18.5, and minimum -fold difference = 2. This resulted in the identification of about a dozen peaks from the two data sets overall. Visual inspection of this small list of peaks rapidly identified two peaks as having similar pI and RT parameters and a similar response in that they were both up-regulated by drug treatment as indicated by the small graphics at the right of the output screen (Fig. 2D). The MCT provides this visual representation of the change observed for each peak by graphing its size in the left and right (i.e. control and treated) columns to aid visual screening for similar peaks.

Although in this example we only used two data sets, the MCT becomes much more useful when many more pairwise comparisons are considered at one time, a situation in which manual examination without prior windowing would be difficult (see below).

To determine whether the two peaks identified by use of the MCT were similar, they were first inspected again in DeltaVue (Fig. 2, A and B) and then physically isolated using the fraction and retention time information. The obtained protein fractions were then analyzed by mass spectrometry at the Michigan Proteome Consortium core at the University of Michigan using MALDI-TOF and MALDI-TOF/TOF. This analysis demonstrated that both peaks were triosephosphate isomerase 1 (Fig. 2E, peak 558 from Fig. 2A; Fig. 2F, peak 522 from Fig. 2B). Therefore the chromatographic data collected by DeltaVue and used in the MCT to compare peaks were sufficiently accurate to allow the isolation and identification of proteins.

Triose-phosphate isomerase is a common glycolytic enzyme that is unlikely to be a good marker for response to drugs. Nevertheless we chose to investigate it further to test whether the changes in its pattern in the PF2D profiles could be correlated with independent analysis. As this protein has been thoroughly studied, antibodies were readily available and allowed analysis by Western blot of fractions from the first dimension of the PF2D separation (Fig. 3). Interestingly this revealed that the treatment with platinum compounds did not alter the overall expression level of triose-phosphate isomerase but did change its profile in the fractions of the pH gradient of the first dimension of separation, giving the impression of appearing in the lane marked by an asterisk in Fig. 3, which corresponds to the lanes shown in Fig. 2. This is the equivalent of a spot shifting position on a 2-D gel and suggests that the protein is modified following treatment of cells with platinum chemotherapeutic agents. Analysis of triose-phosphate isomerase confirms that the peaks being analyzed in DeltaVue and with the MCT can represent identifiable proteins whose behavior can be confirmed using direct, independent detection of the protein.

The MCT was then used to define peaks that are increased by treatment of glioma cells with cisplatin, BBR3464, or BBR3610 and determine which of these was specific to each drug. In each case the proteome profile of drug-treated cells was first compared with untreated controls in DeltaVue, and a spreadsheet of ~500 responsive peaks was generated. All three of these data sets were loaded into the MCT, and a total of 172 peaks were selected for analysis in the MCT (Fig. 4). These peaks were chosen because they were seen to respond to one of the drugs, were of a good size (at least 0.05 absorbance units) suggesting sufficient material for isolation,
and were distinct from nearby peaks, meaning that they reached the base line on both sides, suggesting that their chromatographic separation would be achievable. For example, a set of peaks conforming to these criteria in the DeltaVue comparison of control and BBR3610-treated cells were taken, and the MCT was used to determine whether these peaks also existed in the other two pairwise comparisons. MCT parameters would be set around the characteristics of the peak that triggered the analysis with windows up to 1 pH unit and 1 min in retention time. A peak was considered a match only if the following criteria were met: the peak had to have a size of at least 0.008 with at least a 2-fold change (\( \times 0.5 \) on the MCT output) and be within 0.05 retention time of the initial peak of interest (Fig. 4A).

A summary of the complete MCT analysis in which care was taken not to count peaks twice if they responded to more than one drug shows that there were both common and unique peaks (Fig. 4B). The numbers of peaks that were observed in different compartments suggested that there might be more overlap between BBR3464 and BBR3610 than between either of these drugs and cisplatin, prompting us to calculate these numbers as percentages also. This analysis showed that of all the peaks that responded to a given drug, approximately the same proportion were specific to that drug in this analysis. Accordingly 45% of the 106 peaks that increased following treatment with BBR3610 were found not to respond to either cisplatin or BBR3464 (Fig. 4C). Similarly 47% of 45 peaks and 51% of 87 peaks responding to cisplatin or BBR3464, respectively, were unique to these conditions in the limits of this experiment. Overlap in the response between two drugs was...
calculated as the proportion of peaks that responded to both drugs out of all the peaks that responded to at least one of them: between 13.5 and 12% of peaks were common to BBR3464 or BBR3610 and cisplatin, respectively, but 28% of peaks were common to BBR3464 and BBR3610, suggesting that these two chemically similar drugs elicited more similar responses at the cellular level. Of course the peaks in this analysis were selected according to their chromatographic characteristics and so do not represent a randomized sample that could be used for meaningful statistical analysis of this phenomenon. However, the way the peaks were selected was not influenced by their performance in the MCT analysis, so the observed trends are suggestive of a greater commonality of responding peaks between BBR compounds. The peaks described in Fig. 4, particularly the 39 that are specific to BBR3610, are prime candidates for isolation and identification by mass spectrometry and then further analysis as markers of drug response.

In summary, we have developed a software tool that is capable of rapidly comparing the levels of peaks that are shown to be differentially expressed by PF2D profiling and pairwise comparison across many samples. This tool accelerated the analysis of complex proteomic information by allowing us to quickly focus on peaks with the desired profile across multiple experiments.

**DISCUSSION**

Successful development of effective therapies for glioma has been hampered by a lack of tools to stratify patients that respond. Although currently much effort is focused on targeting cancer-specific targets with exquisitely specific inhibitors, more broadly cytotoxic drugs, such as the BBR series platinum, still have a role to play. It is more difficult to develop markers of response for these compounds because they have broader mechanisms of action. We have chosen to attempt to develop such markers at the protein level and here present a refinement of a new two-dimensional chromatographic approach to proteome fractionation. Although comprehensive mass spectrometric analysis of proteomic profiles is possible, particularly with the two-dimensional chromatography approach (14, 15), it is not technically trivial to accomplish and is not yet available as an integrated solution. A more common approach for laboratories not primarily engaged in proteomics is to use mass spectrometry services offered by an expert core or collaborator to identify a relatively small number of proteins. In this situation, the ability to reduce the number of samples that are analyzed represents an important advantage both in terms of time and resources. We have used the ProteomeLab PF2D system to generate proteomic profiles and developed a new software, the MetaComparison Tool, to reduce the number of peaks that are worth analyzing to answer a specific question.

The approach made possible by the MCT depends on first identifying peaks whose expression changes in one sample as compared with another, a step accomplished in the Delta-Vue software. The identification of these peaks reduces the complexity of the analysis and focuses in on proteins whose response is likely to be biologically meaningful. The MCT further hones the list of peaks by providing a quick answer to the question of whether a peak that responds to one treatment would also respond to another. In this study, this reduced the list of peaks to be analyzed from 172 that were differentially expressed to 39 that were found to be specific to BBR3610, representing a reduction of 75%. This reduction in the number of peaks whose isolation is warranted also reflects a reduction in time and effort devoted to collecting peaks chromatographically because we do not routinely collect the second dimension output of the PF2D. Our approach is to collect data on the first run and after analysis is complete to return to the stored first dimension fraction, rerun the relevant ones, and collect fractions using the retention time data collected for each peak.

An important issue in performing comparisons at the levels of peaks as we do here, rather than proteins as would be the case with comprehensive mass spectrometry approaches, is the correspondence between a peak and a protein. There is no guarantee that a peak is a single protein or even represents one clear majority species. Our data on the TIM protein do suggest that a peak can lead to the unambiguous identification of a single protein but do not exclude that other peaks are composed of multiple species. Comparisons that focus at the level of peaks may, therefore, be weakened by false negatives. These would occur when a change in protein level was masked by a compensatory change in another protein that appeared in a similar part of the proteomic profile. It is less likely that an observed change in peak appearance is not related to a change in a protein. However, as our data for TIM indicate, this may not simply reflect a change in expression level but may also identify a modification that alters the chromatographic characteristics of the protein. Analysis of peaks is akin to comparing spot intensities on a 2-D gel and so is also likely to allow significant progress to be made toward identification of markers. Using the refinement afforded by the MCT approach accelerates this by focusing attention on peaks that show the expression characteristics of greatest interest.

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