Reversal of Multidrug Resistance in Breast Cancer

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MDR can be reversed in vitro and we will test this hypothesis in a Phase I study of Cyclosporine A and quinine as MDR reversers of Vinblastine resistance. Together these studies will address the major goal of circumventing drug resistance in breast cancer. When the data of the MDR1 gene expression in breast cancer specimens from this proposal are available, clinical trials incorporating the modulators of MDR, Cyclosporine and quinine, will be designed for breast cancer as well. An alteration in drug efflux potentially may have an impact on response to chemotherapy and may result in improved survival for breast cancer patients. During the period between March 15, 1995 and March 14, 1996, we have outfitted our laboratory with staff, equipment, supplies and reagents, have been performing control experiments and have been pursuing activation of the various clinical trials to support this project. We now have some preliminary data on the expression of MDR1 and MRP (Multidrug resistant associated protein) in breast cancer specimens and normal adjacent breast tissue. Further follow-up of these patients is needed to determine the clinical significance of expression of these drug resistance genes.

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### Reversal of Multidrug Resistance in Breast Cancer

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#### ABSTRACT (Maximum 200)

Our data show that MDR1 gene expression is important in breast cancer resistance. The role of the MDR1 gene in breast cancer treatment will be further defined by sequentially determining MDR1 gene expression pre and post treatment with doxorubicin in the context of three prospective clinical trials. In addition, this study will allow a correlation of MDR1 gene expression and clinical outcome. To determine what level of MDR1 gene expression is clinically significant, various molecular methods of determining MDR1 gene expression, including immunohistochemistry and quantitative reverse transcription followed by polymerase chain reaction, will be evaluated.

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INTRODUCTION

Drug resistance is a major obstacle in the treatment of cancer. The multidrug resistance gene (MDR1) encodes an energy dependent drug efflux pump, P170, that confers cellular resistance to multiple therapeutic agents such as anthracyclines, vinca alkaloids, epipodophyllotoxins, taxol, and actinomycin-D. MDR1 gene expression is tumor specific in both de novo resistant tumors and those that acquire drug resistance following chemotherapy. The central role of P-170 in this multidrug resistance (MDR) phenotype suggests that modulation of either MDR1 gene expression or the function of P-170 may provide an effective means of clinically reversing drug resistance.

Our data show that MDR1 gene expression is important in breast cancer resistance. The role of the MDR1 gene in breast cancer treatment will be further defined by sequentially determining MDR1 gene expression pre- and post-treatment with doxorubicin in the context of prospective clinical trials. In addition, these studies will allow a correlation of MDR1 gene expression and clinical outcome. To determine what level of MDR1 gene expression is clinically significant, various molecular methods of determining MDR1 gene expression, including immunohistochemistry and quantitative reverse transcription followed by polymerase chain reaction, will be evaluated. We have also had the opportunity to analyze our samples for expression of the MRP gene (Multidrug Resistant Related Protein). The MRP gene is a new member of the ATP-binding cassette transporter superfamily. It is overexpressed in a variety of cell lines which exhibit acquired drug resistance, yet do not express P-glycoprotein. In addition, recent work has shown that this gene confers a multidrug resistant phenotype when transfected into sensitive cells. Currently, little is known of the mechanism by which MRP confers drug resistance. Furthermore, the clinical relevance of MRP is unknown.

MDR can be reversed in vitro and recent data from the in vivo transgenic mouse model suggests that combining MDR modulators such as cyclosporine and quinine, may have an advantage over either alone. We will test this hypothesis in a Phase I study of an analogue of cyclosporine A; PSC 833, and quinine as MDR reversers of vinblastine resistance. Together these studies will address the major goal of circumventing drug resistance in breast cancer. When the data of the MDR1 gene expression in breast cancer specimens from this proposal are available, clinical trials incorporating the modulators of MDR, cyclosporine and quinine, will be designed for breast cancer as well. An alteration in drug efflux potentially may have an impact on response to chemotherapy and may result in improved survival for breast cancer patients.

BODY

The aim of this project is to test the hypothesis that drug resistance in breast cancer is mediated by the MDR1 gene. Moreover, once MDR1 gene expression has been established in breast cancer and correlated with response and resistance to chemotherapy, such data may be used to predict drug resistance and design clinical trials to overcome such resistance using pharmacologic agents proven to reverse MDR in vitro and in vivo.

To accomplish the tasks outlined in the initial proposal, we have accomplished the following from March 15, 1995 to March 14, 1996:

1. Personnel
   a) Scientific Technician - Rajashekar K. Reddy, MS.
   b) Post-Doctoral Associate - Dwayne Dexter, Ph.D.
   c) Research Fellow - Jack Leighton, M.D.

2. Space and Facilities

   With the assistance of the personnel above, our 500 sq. ft. laboratory space has been fully equipped to perform the molecular experiments described in our original proposal.
3. **Reagents and Supplies**

With the assistance of the laboratory staff above, we have now fully equipped the laboratory with the necessary reagents and supplies. We have established reproducible, quality controlled experiments with regard to cell culture, RNA isolation, hybridization with our various cDNA probes and reverse transcription-PCR (RT-PCR) using our competitive template for the MDR gene as an internal control. In addition, the laboratory staff has been evaluated for proper handling of tissue specimens and RNA isolation by doing experiments of tumor bank specimens looking for MDR1 gene expression.

4. **Methods**

a) RNA isolations competitive RT-PCR, slot blot methodology as per previous reports.

b) Immunohistochemistry

Five μM cyrosections were prepared using standard techniques. Cyrosections were used because two of the antibodies (MRK16, UIC2) in this study cannot be used on paraffin-block sections since this procedure destroys the apparent epitope for these antibodies. Cyrosections were air-dried overnight then fixed with either acetone at 4°C for 10 minutes (C219, JSB1, QCRL1, MRPm6) or normal buffered formalin (NBF) at room temperature for 10 minutes (MRK16, UIC2). Acetone slides were air-dried for 1 hour and NBF slides were rinsed with copious amounts of water and air-dried for 2-3 hours. Slides were stored at -20°C until processing.

Slides were processed in an identical manner. Isotype non-specific mouse antibodies were used as a negative control. Cytospins of KB 3-1 and KB 8-5 were used as controls for staining. The staining protocol is outlined below. Except for the first 2 steps, the staining procedure is identical to that outlined by the manufacturer of the staining kit (DAKO).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>[µg/ml]</th>
<th>Isotype Control</th>
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<tbody>
<tr>
<td>MDR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C219</td>
<td>10</td>
<td>IgG2a</td>
</tr>
<tr>
<td>JSB1</td>
<td>10</td>
<td>IgG1</td>
</tr>
<tr>
<td>MRK16</td>
<td>5</td>
<td>IgG2a</td>
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<tr>
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<td>IgG1</td>
</tr>
<tr>
<td>MRPm6</td>
<td>1:20</td>
<td>IgG1</td>
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Antibodies were diluted in antibody dilution buffer (DAKO) as per manufacturer instructions. Slides were allowed to come to room temperature and the section outlined with a wax pen. Sample were rehydrated with PBS for 5 minutes, then blocked with 0.1% avidin followed by 0.01% biotin for 10 minutes each. Slides were rinsed with 0.2% Tween/PBS, followed by PBS between blocking steps. After blocking with avidin/biotin, the slides were blocked with 3% non-fat dry milk/PBS (NFM) for 10 minutes. Excess buffer was removed and the samples covered with the appropriate antibody. Samples were covered with diluted antibodies such that the entire sample was immersed in antibody solution (approximately 10-20 µl). The primary antibody was allowed to incubate overnight at 4°C in a humidified chamber, then the slides were rinsed as before. The secondary antibody was added and the slide incubated at room temperature for 15 minutes. The slides were rinsed as before and strepavidin-peroxidase complex was added. After a 15 minute incubation, the slide was rinsed as before and chromagen-substrate was added (DAB). After a 5 minute incubation, the slide was...
rinsed with ddH2O, counterstained with hematoxylin and a coverslip mounted. The stained slide was scored by 2 independent observers blinded to PCR and slot blot results.

5. Results

Thus far through the Fox Chase Cancer Center Tumor Bank, we have obtained a total of 109 total breast tissue specimens. Of these, 60 are tumor samples, 49 of which have accompanying normal adjacent tissue. 82 of these 109 specimens have been analyzed by QRT-PCR. 47 of the 109 have been analyzed by immunohistochemistry and 31 of 109 have been analyzed by slot blot. The majority of these tumor samples obtained were from patients with primary breast cancer, most of which were stage II. These samples were analyzed by QRT-PCR, slot blot and immunohistochemistry for expression of MDR 1 and MRP as demonstrated below:

a) MDR 1 and MRP expression QRT-PCR
Figure 1. MDR1 and MRP expression in human breast cancer. Expression levels were determined by QRT-PCR. In most instances, normal adjacent tissue was obtained with the tumor sample. □ = tumor. □□ = normal adj. tissue.

b) Slot Blot
Slot blot data is shown in Table 1. For MDR1, 93% of the samples examined by slot blot had detectable levels of transcript, while 100% of the samples were positive for MRP. As we reported earlier, the sensitivity of the slot blot does not appear to be as great as PCR since some of the samples that were reported as negative by slot blot were positive by PCR and immunohistochemistry (F64, F81). There appears to be no concordance between the two techniques for either gene (Pearson's correlation, p>0.05), in fact slot blot probably overrepresents the level of expression for both genes. Slot blot data show that most MDR1 positive samples have 2-3 times the amount of KB 8-5 signal and 3-7 times that of MRP. Conversely, QRT-PCR data show that the samples have 15-100 times less signal than KB 8-5 for both genes.

c) Immunohistochemistry
The immunohistochemistry (IHC) data is presented in Table 1. The staining was scored on a 1+, 2+, 3+ scale for intensity of staining, combined with a percentage of positive cells score for each intensity level. For instance, a sample with 80% of the positive cells being 1+ and 10% being 2+ would receive a score of 80/20 (80*1/10*2). Previous reports have used variations of both scoring systems, but have not combined them. This type of system eliminates arbitrary cut off values for percentage of cells stained and allows some relative level of expression to be applied to the 1+ system. The usefulness of this scoring system allows a clear differentiation between the level of staining seen in KB 3-1 versus KB 8-5. The basis for the staining in KB 3-1 is unclear, however, in the case of
C219, this antibody has been shown to cross-react with MDR2, myosin and recognize other ABC-like proteins (Childs et al, 1994; Thiebaut et al 1989). In addition, this staining may be background staining for these specific monoclonals in this particular staining scheme; however, only one isotype-matched control, IgG2a, had any detectable staining on the acetone fixed slides.

Overall, C219 and JSB1 gave the best staining reactions, while MRK16 and UIC2 were weak to very weak in most samples. The discrepancy in staining is not known at this time. However, it should be noted that C219 and JSB1 tend to stain the cytoplasm and occasionally the plasma membrane, if strong reactivity is present, while MRK16 and UIC2 are strictly localized to the outer plasma membrane. Thus, the epitopes for MRK16 and UIC2 may be more sensitive to sample processing than the other antibodies, resulting in a low signal.

Like the QRT-PCR and slot blot, IHC results indicated that MDR1 and MRP were expressed in both tumor and NAT. The reactivity of both proteins was confined to the epithelial cells of ducts and lobules, in situ and invasive tumor cells, vascular endothelial cells, and perhaps myoepithelial cells. No staining, other than background staining, was observed in the stroma, and in several cases lymphatic invasion was also negative for MDR1. In several cases, in situ and invasive components were present in sufficient quantity to make judgements concerning the expression of both genes relative to each component. While only a few samples exhibited such a characteristics, it was noted that the in situ component tended to have greater staining than the invasive component for MDR1, but not MRP.

Overall, C219 and JSB1 data correlated with each other, while MRK16 and UIC2 data tended to correlate with each other. We are currently in the process of analyzing the concordance between the different antibodies and slot blot and QRT-PCR.
### Table 1. Quantitative values of MDR1 and MRP expression obtained by slot blot, QRT-PCR, and immunohistochemistry. Slot blot and QRT-PCR values are normalized against KB 8-5 expression of both genes (30 U). Immunohistochemistry was scored on a combined scale of 1+ and % positive cells.

#### 6. Discussion

One of the major goals of this project is to determine the correlative significance of MDR1 gene expression with treatment outcome. In order to achieve this goal, the measurement of MDR1 gene expression must be reliable and consistent. There are several techniques that are currently employed to determine MDR1 gene expression levels. At issue is the reproducibility, sensitivity, specificity and quantitative nature of these methods and the correlation between them. A major focus of this study is to determine the concordance between the most common techniques (immunohistochemistry, RNA slot blot hybridization, and RT-PCR) used to measure MDR1 expression.
A considerable amount of time has been devoted to establishing appropriate procedures for each technique and evaluating the lab's consistency in performing these techniques. The sensitive nature of RNA isolation and RT-PCR require that strict protocols are followed. Thus, each technique has been scrutinized and potential pitfalls that may affect yield or introduce unwanted variability have been addressed.

General comparisons of the various techniques will establish the relative reliability of each technique and the concordance between them. In Figure 1, all the breast samples that express MDR1 do so at levels less than KB-8-5. The sensitivity of slot blot hybridization does not appear to be as great as PCR. We have found the limit of resolution for RT-PCR to be no less than a two-fold difference in expression for both MDR1 and MRP. A recent study by Brophy, et. al. (1994), demonstrated that slot blot hybridization had a high false positive rate while PCR was extremely sensitive and specific supporting that MDR1 expression levels be determined utilizing RT-PCR and immunohistochemistry.

The finding of expression of both MDR and MRP in normal adjacent tissue using RT-PCR was unexpected since most previous studies have not reported expression of MDR in normal breast tissue. Wishart et al, however, has noted expression in stromal cells of breast cancer but not of normal breast. Our planned immunohistochemical studies should assist in sorting out which specific cells are PGP positive. If indeed adjacent stromal tissue stains for PGP this might suggest up regulation of MDR1 in cells at risk for tumorigenesis. It might also indicate that since MDR is a transmembrane protein, cell-cell interaction might be important for function, however, investigation of these possibilities are not within the scope of this proposal. Recent work has demonstrated that insulin like growth factor II (IGF II) is primarily stromal in origin and possibly functions as a paracrine growth promotor in breast cancer suggesting that stromal epithelial interactions may be important in breast cancer.

We are still evaluating immunohistochemical results for MDR1 and MRP expression.

7. Clinical Trials

The clinical trials described in this project are at various levels of accrual, approval and development as outlined below:

a) Philadelphia Bone Marrow Consortium - PBT-3 (IRB94041). Phase II Trial of High Dose Chemotherapy with Cyclophosphamide, Thiotepa and Carboplatin and Peripheral Blood Stem Cell Infusion in Women with Inoperable Locally Advanced and Inflammatory Breast Cancer who achieved partial response to Induction Chemotherapy this protocol has been approved by the IRBs of the four member institutions including Fox Chase Cancer Center (FCCC), University of Pennsylvania, Hahnemann University and Temple University. This study was activated 6/94 and thus far, 16 patients have been enrolled and 28 specimens have been collected. From 7 patients we received only one sample thus far, from another 6 we have received two serial specimens, and from 3 patients we have received three serial samples.

b) Eastern Cooperative Oncology Group (ECOG) Registration Study of Induction with Adriamycin in Inoperable Locally Advanced and Inflammatory Breast Cancer to Evaluate for Multidrug Resistance. Since the Breast Biology Committee and the Breast Committee's Annual Report, this concept has been approved by the ECOG Breast Core Committee July 7, 1994, and a draft of the schema and eligibility were included in the appendix of the previous report. Currently, we are still awaiting ECOG Laboratory Committee approval. Both this study and PBT-3 will permit us to obtain sequential breast tumor samples before and after treatment with Adriamycin to support us in accomplishing the aims of this grant proposal i.e. to determine the clinical significance of MDR1 gene expression in breast cancer and to correlate expression with response and
accomplishing the aims of this grant proposal i.e. to determine the clinical significance of MDR1 gene expression in breast cancer and to correlate expression with response and resistance to treatment with MDR substrate. The ECOG activation process has been delayed due to a relocation of the Operations office.

c) Phase II Study of R-Verapamil (Dexverapamil) in Advanced Breast Cancer. This study has received FCCC IRB approval and consent forms have been approved by the DOD. R-Verapamil will likely be replaced by an alternative MDR modulator since it has not been shown to have significant clinical activity. The protocol has been written, and it has been submitted for appropriate review. The most recent draft of this protocol (E1195), a Phase II study of PSC 833 to modulate MDR mediated resistance, was enclosed in the previous and should be activated shortly.

d) Phase I study of Cyclosporine and Quinine to Reverse MDR in Refractory Malignancy treated with Vinblastine. This study has been approved by the FCCC IRB and consent forms have already been approved by the DOD. Because Cyclosporine A (CSA) initially planned to be used in this trial has been reformulated to enhance its immunosuppressive activity and has subsequently lost its potency in mediating reversal of MDR, we are in the process of discussing with Sandoz and the NCI the possibility of substituting CSA with its analogue PSC 833 which is a more potent MDR inhibitor. When this is accomplished this study will be open for accrual.

CONCLUSION

Drug resistance is a major obstacle in the treatment of malignancies. Although MDR1 mediated drug resistance has been well characterized in preclinical models, its role in clinical drug resistance is not as well characterized and requires further investigation. That is the aim of the studies proposed here. The ability to identify tumors with increased MDR1 gene expression has several potential applications, for example; the prediction of the response to chemotherapy or the design of studies of the reversal of resistance with agents that inhibit MDR1-mediated drug efflux. Prospective studies as described above are necessary to establish the role of MDR1 gene expression in clinical resistance. The initial goal of such trials is to demonstrate the ability to reverse MDR1 mediated drug resistance in appropriate advanced refractory malignancies. Ultimately, it will be important to incorporate these reversal strategies in the treatment of early stage disease at which time the tumor burden is smaller and fewer mechanisms of resistance may be present.

Well designed phase I and II prospective clinical trials using reversing agents in conjunction with chemotherapy in malignancies that express the MDR1 gene are necessary prior to routine use of agents such as verapamil and quinidine which carry innate toxicities. Epithelial tumors such as colon and renal cell carcinoma express the MDR1 gene and are clinically resistant to most cytotoxic agents, many of which are not substrates of P-170. In this situation, MDR may be one of a complex array of drug resistance mechanisms. Breast cancer would be a more appropriate human tumor model since it is a tumor for which many active chemotherapeutic agents are handled by MDR. In such a setting an alteration in drug efflux may indeed have an impact on response and possibly improve survival for breast cancer patients. The transgenic mouse model may be used to assess novel MDR reversing agents, non-toxic analogues of known reversing agents and combinations of various MDR modifiers to be subsequently investigated in Phase I studies. Over the period of March 15, 1995 to March 16, 1996, we have successfully outfitted our laboratory with staff, equipment, supplies and reagents to perform the necessary control experiments of MDR1 gene expression assays as described in the body of this report. We now have some preliminary data on the expression of MDR1 and MRP in breast cancer specimens and normal adjacent breast tissue. We will now follow these patients prospectively to determine the clinical significance of such expression as it relates to response and/or resistance to cytotoxins which are substrates for the proteins encoded by these genes.
REFERENCES


