In vitro assay-assisted treatment selection for women with breast or ovarian cancer

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Abstract

The selection of chemotherapy for women with breast or ovarian carcinoma has been traditionally based on results from phase III comparative trials that define the most active drugs and drug combinations. This approach has led to a significant prolongation of the lives of these patients. Unfortunately, few patients with advanced stage IV disease are cured using the currently available regimens. In order to improve the selection process for individual patients, various types of in vitro tests that assess the activity of standard drugs on a patient's tumor have been developed over the past five decades. As with bacterial culture and sensitivity tests, significant predictive correlations between in vitro drug-response assays and cancer patient response and survival have been demonstrated. Medicare currently covers in vitro drug-resistance assays. This review discusses the historical development of in vitro drug-response assays and the clinical validation of various technologies currently available to assist the clinician in selecting the optimal therapy for each patient.

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Introduction

Treatment for patients with breast or ovarian cancer has traditionally been based on the selection of active agents and combinations identified by large phase III randomized clinical trials. Clinical trial-based identification of new chemotherapy agents with significant disease specific activity has been a cornerstone of modern oncology, providing statistical validation of their safety and activity. Perhaps of greatest importance, the impact of new treatment modalities on patient survival can be compared with previously proven treatment regimens. The clinical utility of agents developed through this process has led to significantly improved outcomes for women with advanced stage breast and ovarian cancer. Conventional chemotherapy agents with clinical activity in breast cancer currently include doxorubicin, Doxil, epirubicin, cyclophosphamide, 5-fluorouracil (5FU), capcitabine, methotrexate, gemcitabine, vinorelbine, paclitaxel, docitaxel, and cisplatin (Norton 1999, Esteva et al. 2001). Chemotherapy has been augmented by advances in hormonal therapy, including selective estrogen receptor antagonists and aromatase inhibitors. Of particular interest is the recent development of synergistic combinations of Herceptin with conventional chemotherapy agents, an approach that integrates rational and empirical treatment approaches. With respect to advanced stage epithelial ovarian cancer, while platinum-based regimens continue to be a standard of care for first-line treatment, many agents have been found to exhibit comparable second-line activity, including topotect, vinorelbine, etoposide, Doxil, cyclophosphamide, taxanes, 5FU, and Hexalen (McGuire et al. 1996, Alberts 1999, Markman & Bookman 2000). However, in spite of this armamentarium and newer pharmacological strategies, disease progression and patient death are still major problems that largely result from intrinsic and acquired drug resistance. Although current chemotherapy regimens produce clinical response rates for women with breast or ovarian cancer of 60% to 70%, 5-year survival rates for these women remain below 50%, and cures are rare (Greenlee et al. 2001).

In an attempt to improve treatment response and patient survival, investigators working over the past 50 years have developed in vitro drug-response assay systems to determine the potential activity of chemotherapy agents for a given patient prior to their administration. The central hypothesis underlying this approach is that the drug-response profile for each individual patient will differ based on their intrinsic genetic diversity and the development of subclones within tumors that have divergent phenotypes (Goldie & Coldman 1979, Iyer & Ratain 1998). In vitro assays that identify individual differences in tumor drug response make it possible to design patient-specific regimens targeted against each
patient’s tumor characteristics. By eliminating ineffective agents, the patient is spared toxic treatment without benefit, while the selection of agents active in vitro may increase the probability of response. Although several test systems have emerged that accurately predict patient response in a clinically useful time-frame, debate continues about the optimal application of these technologies (Fruehauf & Bosanquet 1993, Cortazar & Johnson 1999, Chu & DeVita 2001). Although this approach has obvious appeal, various technical hurdles have retarded the incorporation of these technologies into standard practice. This paper will briefly review the historical development of in vitro drug-response assays, and then focus on recent clinical validation studies that have led to more widespread use of these assays to assist in treatment selection.

Historical perspective

In vitro drug-response assays originated at the beginning of the last century when Louis Pasteur and Paul Ehrlich observed the effects of antimicrobial agents on cultured bacteria (Pasteur & Joubert 1877, Burger 1988). Their pioneering work subsequently led to Flemming’s development of penicillin, as well as to the general use of culture and sensitivity testing for antibiotics over the past century (Table 1). It was not until 1953, however, that the first publications appeared by Black & Spear (1953, 1954) describing the use of an in vitro assay using human tumor biopsy material to evaluate the activity of aminopterin, an early antimetabolite. Their assay evaluated the in vitro response of tumor tissue segments composed of both malignant and non-malignant components to aminopterin exposure. Black and Spear’s assay endpoint was cell viability determined by metabolic conversion in the mitochondria of a tetrazolium dye to a colored product that could be measured spectrophotometrically. These studies eventually led to the development of the methylthiazol diphenyl tetrazolium bromide dye conversion (MTT) assay employed by the National Cancer Institute to measure cellular proliferation. Radiolabeled thymidine had been used since 1953 to measure cellular proliferation. Radiolabeled thymidine had appeared by Black&Spear (1953,1954) describing the use of a radiolabeled thymidine incorporation in cell culture systems to evaluate the activity of aminopterin in transformed cells. Their identification of agar as a selective growth medium for the transformed cells within the tumor led to Salmon’s development of the agarose-based human tumor stem cell chemosensitivity assay in the early 1970s (Salmon et al. 1978). This ‘chemosensitivity’ assay was initially met with great enthusiasm, but technical problems prevented its widespread application. Technical pitfalls associated with this system included assay success rates less than 50%, turn around times of up to 3 weeks, and technical concerns related to the incomplete disaggregation of the tumor into pure single cell populations required to discern colony formation (Selby et al. 1983). During the late 1970s and early 1980s advances in scintillation counting technology made it relatively easy to accurately determine tritium-labeled thymidine incorporation in cell culture systems to measure cellular proliferation. Black and Spear’s seminal work was significant because it foreshadowed some of the technical hurdles that would confront subsequent investigators. First, their assay endpoint measured the metabolic activity of both cancer and normal cells, making it difficult to distinguish between drug effects on the normal versus cancer components. In addition, because they employed tumor segments, the contribution of the malignant component to the endpoint signal varied from patient to patient, making it difficult to standardize the system and compare results between patients. Finally, their findings suggested that the accuracy of their system to predict treatment response, the positive predictive value, was not as great as the negative predictive value to predict treatment failure. The overall predictive accuracy of their assay system was below 70%, a value too low for clinical application. On the other hand, the relative advantage of this early in vitro drug-response assay to better predict drug resistance rather than chemosensitivity has been borne out by many other investigators over the ensuing decades (Fruehauf & Bosanquet 1993).

A focused approach to obtain in vitro assay results specific for the malignant component of a tumor began with observations by Puck & Marcus (1955) that agarose-based culture systems preferentially supported the growth of transformed cells, while the non-malignant cellular components did not proliferate. Their identification of agar as a selective growth medium for the transformed cells within the tumor led to Hamburger and Salmon’s development of the agarose-based human tumor stem cell chemosensitivity assay in the early 1970s (Salmon et al. 1978). This ‘chemosensitivity’ assay was initially met with great enthusiasm, but technical problems prevented its widespread application. Technical pitfalls associated with this system included assay success rates less than 50%, turn around times of up to 3 weeks, and technical concerns related to the incomplete disaggregation of the tumor into pure single cell populations required to discern colony formation (Selby et al. 1983). During the late 1970s and early 1980s advances in scintillation counting technology made it relatively easy to accurately determine tritium-labeled thymidine incorporation in cell culture systems to measure cellular proliferation. Radiolabeled thymidine had

Table 1 Historical overview

<table>
<thead>
<tr>
<th>Year</th>
<th>Event</th>
</tr>
</thead>
<tbody>
<tr>
<td>1877</td>
<td>Pasteur and Joubert described the effects of ‘chemotherapy’ on cultured microbes (Pasteur &amp; Joubert 1877)</td>
</tr>
<tr>
<td>1928</td>
<td>Flemming’s discovery of penicillin ushers in modern chemosensitivity era (Burger 1988)</td>
</tr>
<tr>
<td>1953</td>
<td>Black and Spear report first in vitro assay for cancer specimens (Black &amp; Spear 1953)</td>
</tr>
<tr>
<td>1955</td>
<td>Marcus and Puck: agar based assay system selective for malignant cell growth (Puck &amp; Marcus 1955)</td>
</tr>
<tr>
<td>1978</td>
<td>Salmon and Hamburger report on second generation human tumor cloning assay (Salmon &amp; Hamburger 1978)</td>
</tr>
<tr>
<td>1984</td>
<td>Southwest Oncology Group: assay quality controls introduced (Clark &amp; Von Hoff 1984)</td>
</tr>
<tr>
<td>1990</td>
<td>Clinical trials indicate that assays predict response (Kern &amp; Weisenthal 1990)</td>
</tr>
<tr>
<td>1993</td>
<td>Clinical trials indicate that assay results are significantly associated with survival (Fruehauf &amp; Bosanquet 1993)</td>
</tr>
<tr>
<td>2000</td>
<td>Medicare covers in vitro drug resistance assays</td>
</tr>
<tr>
<td>2001</td>
<td>Assay directed therapy may improve outcomes (Kurbacher et al. 2001)</td>
</tr>
</tbody>
</table>
Table 2  Comparison of in vitro drug-response assay techniques

<table>
<thead>
<tr>
<th>Assay type</th>
<th>Culture conditions</th>
<th>Assay endpoint</th>
<th>Prediction</th>
<th>Cancer cell specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>Microtiter plate in defined media (no serum)</td>
<td>ATP content in cultured cells</td>
<td>Sensitivity</td>
<td>Moderate</td>
</tr>
<tr>
<td>FCPA</td>
<td>Microorgan culture in cellulose–collagen matrix</td>
<td>Cytosolic esterase formation of fluorescein</td>
<td>Sensitivity</td>
<td>Low</td>
</tr>
<tr>
<td>MTT</td>
<td>Microtiter plate in 15% serum–containing media</td>
<td>Succinate dehydrogenase formation of formazan</td>
<td>Sensitivity</td>
<td>Low</td>
</tr>
<tr>
<td>DISC</td>
<td>Cytophobic plates in 15% serum–containing media</td>
<td>Light microscopic reading of nonviable cells</td>
<td>Both</td>
<td>High</td>
</tr>
<tr>
<td>HTCA</td>
<td>Agar plates in 15% serum–containing media</td>
<td>Colony formation and counting</td>
<td>Sensitivity</td>
<td>High</td>
</tr>
<tr>
<td>³H-Uridine</td>
<td>Extracellular matrix strips in 1% serum media</td>
<td>³H-Uridine incorporation</td>
<td>Resistance</td>
<td>Moderate</td>
</tr>
<tr>
<td>EDR</td>
<td>Agarose suspension culture in 15% serum media</td>
<td>³H-Thymidine incorporation</td>
<td>Resistance</td>
<td>High</td>
</tr>
</tbody>
</table>

been employed since the 1960s to assess bacterial proliferation (Brock 1967). Tritiated thymidine uptake as an endpoint to determine cancer cell response to chemotherapy mirrored results of the clonogenic assay performed in soft agar (Johnson & Glaubiger 1983, Sondak et al. 1984). The merger of agarose-based cultures of cellular clumps with the tritiated thymidine incorporation endpoint resulted in a third generation technology capable of determining in vitro drug response in greater than 85% of the cases in as few as 5 days (Sondak et al. 1984). At the same time, alternative endpoints were developed, including measurement of mitochondrial function (e.g. MTT and adenosine triphosphate luminescence (ATP) assays), membrane integrity (e.g. fluorescent cyto-footprint, fluorescein diacetate, propidium iodine uptake, and differential staining cytotoxicity (DiSC) assays (Weisenthal & Lippman 1985, Bird et al. 1987, Bosanquet 1991, Meitner 1991, Wilbur et al. 1992, Kochli et al. 1994, Weisenthal 1994, Andreotti et al. 1995, Elledge et al. 1995, Bosanquet et al. 1999, Mason et al. 1999). Table 2 shows a comparison of these various assays, while Figs 1–6 provide cartoons of the assay schemas.

Assay technologies

As shown in Table 2, various assay endpoints have been developed to assess the effects of drugs on cancer cell growth and viability. The advantages or disadvantages of a given endpoint are related in part to their ease of use, reproducibility, precision, and success rate. Assay endpoints are generally related to measures of cell proliferation, metabolism, or survival. As depicted in Fig. 1, the human tumor cloning assay (HTCA) measures the capacity of single malignant cells to divide and form colonies in or on an agar-based matrix. After short-term drug exposure, single cells are plated in or on an agar matrix. Cells that have been killed or have undergone damage causing cell cycle arrest fail to form colonies. Colony counts after a 2- to 3-week period is the endpoint determined in the HTCA. Differential colony formation between untreated controls and treated cells measures drug activity. Agar, the growth substrate, mimics a suspension environment and suppresses the proliferation of non-transformed cells (Puck & Marcus 1955, Hamburger & Salmon 1977). Agar-based culture systems, such as used in the extreme drug resistance (EDR) assay (Fig. 2), or polypropylene plates employed in the DiSC (Fig. 3) and ATP assay (Fig. 4) systems, suppress cellular adherence to a growth surface. Fibroblasts, mesothelial cells and other stromal cells can proliferate in adherence-based culture systems, adding a non-cancer cell-specific growth signal or component to the endpoint. In vitro drug-response assay results are adversely affected by proliferation of non-malignant cells that add ‘noise’ to the cancer cell growth signal (Campling et al. 1991, Kitaoka et al. 1997). The growth signal of disaggregated cells obtained from tumor biopsies grown in non-adherent culture conditions is therefore relatively more cancer cell specific than growth in adherent culture systems. The use of low serum-containing media is another technique...
employed to suppress non-transformed cell proliferation. The use of agar or low serum-containing medium helps ensure that assay endpoints determined after several days of tissue culture measure cancer cell proliferation or metabolism with minimal contributions from normal cellular components present in each tumor biopsy specimen.

The EDR assay (Fig. 2) measures the effects of chemotherapy agents to inhibit proliferation of small tumor cell clumps suspended in a low density layer of agarose that overlays a solid layer of agarose (Sondak et al. 1984). Cell cultures are incubated with drugs for 4 days and pulsed with $^3$H-thymidine over the last 2 days of the 5-day culture. Radiolabeled thymidine is incorporated into the replicating DNA of dividing cells while non-proliferating cells and dead cells fail to incorporate the label. Cells are harvested onto glass fiber filters and lysed with deionized water. The filter traps the labeled macromolecular DNA, while unincorporated tritiated thymidine is washed through the filter. Filters and scintillation fluid are added to vials and radioactive decay is measured in a scintillation counter to determine the amount of DNA synthesis that took place in control and treated tumor cells. $^3$H-Uridine incorporation can also be used to evaluate in vitro drug effects on tumor cell proliferation (Elledge et al. 1995). Tumor cells are constantly exposed to the drug during the 5-day period employed in the EDR assay, resulting in in vitro drug exposures that are several-fold higher than those achieved clinically after intravenous bolus administration. As shown in Table 3, cisplatin exposure is 16.5-fold higher in the in vitro assay system than when administered intravenously as a bolus (Fruehauf & Bosanquet 1993). Similar exposures are employed in the ATP, DiSC, and MTT assays, whereas short-term drug exposures are used in the HTCA. Longer exposure times with higher areas under the curve (AUC) favor the identification of highly resistant tumor cells that can grow through AUC values in excess of those achieved clinically. Drugs found to be inactive under optimal conditions for activity in vitro are unlikely to be active under suboptimal conditions in vivo. On the other hand, assays that use short-term drug exposures may be more capable of identifying agents with greater sensitivity in vivo. However, as discussed below, this has not been borne out in clinical trials.
**Figure 4** *In vitro* assay schema: ATP assay. d, days; TCA, trichloroacetic acid; \( \lambda \), microliter.

**Table 3** *In vitro* versus *in vivo* drug exposures in the EDR assay*

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration (C) (µg/ml)</th>
<th>Exposure time (T) (h)</th>
<th>In vitro ((C \times T))</th>
<th>In vivo ((C \times T))</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin</td>
<td>0.1</td>
<td>96</td>
<td>8</td>
<td>1.4</td>
<td>5.8</td>
</tr>
<tr>
<td>5FU</td>
<td>3</td>
<td>96</td>
<td>288</td>
<td>20.0</td>
<td>14.4</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>1</td>
<td>96</td>
<td>33</td>
<td>2.0</td>
<td>16.5</td>
</tr>
<tr>
<td>BCNU</td>
<td>10</td>
<td>96</td>
<td>20</td>
<td>1.0</td>
<td>20.0</td>
</tr>
<tr>
<td>Melphalan</td>
<td>5</td>
<td>96</td>
<td>20</td>
<td>2.5</td>
<td>8.0</td>
</tr>
</tbody>
</table>


The EDR assay and other suspension assay systems measure the growth of tumor aggregates. Plating cells as small organoid clusters allows cells to grow in an environment that recapitulates the *in vivo* three-dimensional growth pattern. Clustered cells grow better compared with single cell suspensions, improving the assay success rate and shortening the time required for obtaining results. The EDR assay success rate is approximately 85% (Mehta *et al.* 2001) and takes 5 days. In contrast, the HTCA, which requires that tumors be disaggregated to the single cell level, has a 50% evaluability rate and takes 14 days or longer (Clark & Von Hoff 1984). It has also been reported that three-dimensional
Fruehauf: In vitro treatment selection

cultures demonstrate acquired drug resistance that may not always be detected when cells are grown in monolayers (Graham et al. 1994). The EDR assay has been found to be highly accurate for predicting drug resistance (≥97%) (Fruehauf & Bosanquet 1993, Chu & DeVita 2001).

Several assays measure the effects of cancer drugs on cellular metabolism. The ATP assay measures the concentration of intracellular ATP in the culture population (Andreotti et al. 1995, Sevin & Perras 1997) (Fig. 4). ATP is essential for cell viability and DNA replication. Metabolically active cells will produce more ATP, as will cells metabolizing glucose through the citric acid cycle linked to aerobic mitochondrial function. In vitro drug effects that inhibit glucose metabolism, mitochondrial function, or that cause cell death result in decreased intracellular ATP concentrations. ATP levels in cultured cells are measured by fluorochromes such as luciferin–luciferase that fluoresce after binding to ATP. Loss of cell viability after drug exposure results in decreased cellular ATP levels. The ATP assay utilizes 96-well round-bottomed polypropylene plates and serum-free media to suppress the proliferation of non-transformed cells. Andreotti et al. (1995) studied the growth advantage of the malignant component of 124 biopsies of various types of solid tumors using the ATP assay. Cytological analysis was performed to assess the ratio of malignant to non-malignant cells pre- and post-culture. They found that the mean proportion of malignant cells increased from 54% initially to 83% by the end of the 6- to 7-day assay period, with a significant expansion of the malignant population in 98% of the cases evaluated. Several clinical trials in Europe have demonstrated that the ATP assay’s predictive reliability favors drug resistance over chemosensitivity (Table 4) (Andreotti et al. 1995, Cree et al. 1996, Konecny et al. 2000).

The MTT assay detects mitochondrial succinate dehydrogenase (SDH) activity as a determinant of mitochondrial function and cell viability (Fig. 5). SDH is a component of the citric acid cycle, and generates FADH2 and fumarate from succinate and FAD. SDH activity resides on the mitochondrial inner membrane and requires a functioning electron transport system. SDH activity is therefore a measure of mitochondrial and cellular viability. SDH activity is measured by its capacity to convert 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltratrazolium bromide (MTT) to a blue crystallized compound that is dissolved in dimethyl sulfoxide at the end of the assay. The amount of crystal formation is determined by measuring the optical density of the tissue culture well using a spectrophotometer that measures absorbance at the wavelength absorbed by the blue solution. Tumors can be assessed in the MTT assay as organoids or small fragments grown on collagen gel sponges (Fig. 5), or as disaggregated cells grown in adherent cultures (Grever et al. 1992, Fruehauf et al. 1990, Furukawa et al. 2000, Ohie et al. 2000). While the MTT assay works well for cancer cell lines, it is not generally performed using culture conditions that preferentially favor the transformed component of the tumor. The MTT assay signal source stems from all functional mitochondria, and therefore does not distinguish between cancer cell and normal stromal cell response to in vitro drug exposures (Fruehauf & Bosanquet 1993).

The fluorescent cytoprint assay (FCPA) is also a metabolic function assay. The FCPA measures the enzyme activity of membrane cytosolic esterases (Fig. 6) (Rotman et al. 1988, Leone et al. 1991). Esterase activity is related to cell viability, and is assayed by monitoring hydrolysis of fluorescein-monooacetate to fluorescein. In the FCPA, tumors are disaggregated into small clumps, or ‘microorgans’, which are immobilized between sheets of cellulose-collagen that are subsequently placed onto a grid in tissue culture flasks containing medium. A baseline ‘fluorescent cytoprint’ is recorded by digital photography and cells are cultured for 24 h prior to drug exposures. After a subsequent 48-h drug exposure period, cells are rinsed and grown for an additional 48 h in drug-free medium. The second ‘cytoprint’ is obtained, and the difference in fluorescence between the two images is calculated. Computer capture of the images is utilized for an objective comparison. Each culture acts as its own control. While the ‘microorgan’ structure has the advantage of maintaining the in vivo tumor environment, this assay endpoint does not distinguish between the fluorescent signal produced by the malignant cells and the non-transformed component of each organoid. Because the malignant...
component is not enumerated, tumor heterogeneity makes it difficult to compare cases, and to discern tumor cell response to treatment from total cellular response. Tumors that have few cancer cells present a particularly troublesome challenge for the FCPA with respect to interpretation.

Clinical studies in breast and ovarian cancer correlating in vitro assay results with response and survival

The clinical utility of in vitro drug-response assays has been evaluated in clinical trials that evaluated the relationship between drug action on a given patient’s tumor in vitro and that patient’s clinical response to that drug. As shown in Table 4, recent reports have addressed the predictive accuracy of various in vitro drug-response technologies for 220 breast cancer cases and 284 ovarian cancer cases. The negative predictive accuracy (NPA) relates to the reliability of the assay to identify ineffective agents that will fail to produce a clinical response, while the positive predictive accuracy (PPA) is a measure of an assay’s reliability to identify agents that will cause clinical responses defined as a ≥50% reduction of measurable tumor size. The negative predictive value (NPV) for breast cancer cases ranged from 86% to 100%, while the positive predictive value (PPV) ranged from 47% to 91%. For ovarian cancer cases, the NPV ranged from 62% to 100%, while the PPV ranged from 58% to 91%. While these ranges are rather broad, it appears that the NPVs were generally higher than the PPVs, suggesting that these technologies were better at identifying ineffective agents. This has been a generally accepted axiom for several years based on the clear differences between in vitro models and in vivo pharmacodynamics (see below).

Table 4 Utility of in vitro assays to predict clinical response for patients with breast and ovarian carcinomas

<table>
<thead>
<tr>
<th>Reference</th>
<th>Assay type</th>
<th>Tumor</th>
<th>No.</th>
<th>NPA*</th>
<th>PPA†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kern (1990)</td>
<td>EDR</td>
<td>Breast</td>
<td>48</td>
<td>100</td>
<td>47</td>
</tr>
<tr>
<td>Blackman (1994)</td>
<td>FCFA</td>
<td>Breast</td>
<td>47</td>
<td>100</td>
<td>91</td>
</tr>
<tr>
<td>Elledge (1995)</td>
<td>3H-Uridine</td>
<td>Breast</td>
<td>25</td>
<td>94</td>
<td>71</td>
</tr>
<tr>
<td>Kochli (1994)</td>
<td>ATP</td>
<td>Breast</td>
<td>17</td>
<td>86</td>
<td>90</td>
</tr>
<tr>
<td>Xu (1998)</td>
<td>MTT</td>
<td>Breast</td>
<td>83</td>
<td>100</td>
<td>77</td>
</tr>
<tr>
<td>Alberts (1980)</td>
<td>HTCA</td>
<td>Ovarian</td>
<td>44</td>
<td>99</td>
<td>62</td>
</tr>
<tr>
<td>Kern (1990)</td>
<td>EDR</td>
<td>Ovarian</td>
<td>46</td>
<td>100</td>
<td>58</td>
</tr>
<tr>
<td>Taylor (1998)</td>
<td>MTT</td>
<td>Ovarian</td>
<td>37</td>
<td>80</td>
<td>61</td>
</tr>
<tr>
<td>Blackman (1994)</td>
<td>FCFA</td>
<td>Ovarian</td>
<td>72</td>
<td>96</td>
<td>71</td>
</tr>
<tr>
<td>Csinka (1997)</td>
<td>FCFA</td>
<td>Ovarian</td>
<td>47</td>
<td>100</td>
<td>56</td>
</tr>
<tr>
<td>Konecny (2000)</td>
<td>ATP</td>
<td>Ovarian</td>
<td>38</td>
<td>89</td>
<td>66</td>
</tr>
</tbody>
</table>

*NPA (%) to identify agents in vitro that will fail to cause a partial or complete tumor response when administered to the patient.
†PPA (%) to identify agents in vitro that will cause a partial or complete tumor response.

In vitro drug response and patient survival

While it is clear that in vitro drug-response assays effectively discriminate between clinically inactive and active agents, this does not necessarily translate to an accurate prediction of patient survival. Various clinical trials have identified agents capable of causing short-term responses without translating clinical response into a survival benefit. Clinical validation of in vitro drug-resistance assays requires that they predict poorer survival for patients treated with agents their tumors are resistant to in vitro, and improved survival for patients treated with agents their tumors are ‘sensitive’ to in vitro. Table 5 summarizes the results of three recently published studies that demonstrate significantly inferior survival for patients treated with agents found to be inactive in vitro.
Fruehauf: In vitro treatment selection

Table 5 Survival of patients who received assay sensitive versus resistant agents

<table>
<thead>
<tr>
<th>Reference</th>
<th>Tumor type</th>
<th>No.</th>
<th>Median PFS (months)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mehta et al. (2001)</td>
<td>Breast</td>
<td>96</td>
<td>100</td>
<td>48</td>
</tr>
<tr>
<td>Konecny et al. (2000)</td>
<td>Ovary</td>
<td>38</td>
<td>28.5</td>
<td>12.6</td>
</tr>
<tr>
<td>Holloway et al. (2001)</td>
<td>Ovary</td>
<td>79</td>
<td>24</td>
<td>6</td>
</tr>
</tbody>
</table>

PFS, progression free survival.

In the Mehta et al. (2001) study, newly diagnosed breast cancer patients were treated with either cyclophosphamide, methotrexate, 5-fluorouracil (CMF) or Adriamycin, cyclophosphamide (AC). Their tumors were studied in the EDR assay. Their therapy was blinded to assay results. For each patient’s tumor specimen, in vitro responses to individual drugs were scored as 0 for EDR when the percent cell inhibition (PCI) was > 1 s.d. below the population median, 1 for intermediate drug resistance when the PCI was between the median and 1 s.d. below the median, or 2 for low drug resistance when the PCI was above the median. EDR scores for 4-hydroxycyclophosphamide (4HC) and doxorubicin were summed for patients treated with AC. 4HC and 5FU scores were selected for summation for patients treated with CMF. Summed EDR scores ranged from 0 to 4. For example, a patient treated with AC and having a tumor with extreme resistance to 4HC and low resistance to doxorubicin was assigned an EDR score of 0 + 2 = 2.

If any one of the agents patients were treated with showed in vitro resistance at an intermediate or extreme level, patient progression-free survival was decreased by twofold. In multivariate analysis adjusted for stage and lymph node status, Mehta et al. (2001) found that patients who received agents resistant in vitro had a threefold increased relative risk of death. Figure 7A depicts the Kaplan–Meier survival curves for these cases, and demonstrates a clear relationship between the degrees of in vitro resistance seen in the assay and clinical outcome.

Konecny et al. (2000) and Holloway et al. (2001) both reported similar results for newly diagnosed stage III and IV ovarian cancer patients treated with platinum-based chemotherapy after initial cytoreductive surgery. The study of Konecny et al. (2000) employed the ATP assay, while Holloway et al. (2001) utilized the EDR assay. These two groups independently found that in vitro resistance to either cisplatin or carboplatin was an adverse factor, with significantly reduced progression-free survival seen in patients with tumors that were resistant to platinum in vitro. Konecny et al. (2000) found that median time to progression was 2.3-fold shorter for the platinum-resistant group, while Holloway et al. (2001) found a 4-fold difference. Figure 7B shows the Kaplan–Meier overall survival curves for the cases studied by Holloway et al. (2001). Patients who had tumors found to be extremely resistant to platinum compounds in vitro had an inferior survival time compared with those with low to intermediate drug resistance.

These recent studies are consistent with others that have evaluated survival differences between patients receiving in vitro-resistant versus in vitro-sensitive agents, and suggest that agents found to be inactive in vitro are unlikely to
produce clinical responses or improved survival (Fruehauf & Bosanquet 1993).

Assay-assisted therapy

Cortazar & Johnson (1999) recently summarized results for clinical trials that attempted to determine if patient survival could be improved by the administration of agents selected by \textit{in vitro} assays versus physician’s choice. They reviewed results for 1545 cases that were enrolled onto ‘\textit{in vitro} chemosensitivity’ trials. \textit{In vitro} assays of various types were actually performed in 72% of the cases. The primary technology evaluated was the human tumor cloning assay, which represented 52% of the cases studied, while the DiSC assay was performed in 25% of the cases, the MTT assay was performed in 15% of the cases, and the capillary cloning assay accounted for 8% of the cases. Drug-resistance assays were not included in their review. Of the 12 peer-reviewed reports analyzed, seven had response data: two for the clonogenic technique, four for the DiSC assay, and one for the capillary cloning assay. Cortazar & Johnson (1999) found that response rates for patients treated with the best \textit{in vitro} regimen was 27% versus 16% for empiric treatment. With respect to survival outcomes, survival was improved in two of five studies, and comparable in three others. They concluded that ‘patient response rates to \textit{in vitro} selected chemotherapy were at least as good as those achieved with empiric therapy’.

More recently, Kurbacher \textit{et al.} (2001) reported that ATP assay-directed therapy administered to women with platinum-resistant recurrent ovarian cancer versus platinum-sensitive recurrent ovarian cancer resulted in comparable response rates and survival times. With the advent of significantly increased numbers of agents to choose from for both breast and ovarian carcinoma, assay-assisted therapy is a rational approach that provides objectivity to the selection process. The study of Kurbacher \textit{et al.} (2001) provides an impetus for co-operative groups to revisit the issue of assay-directed therapy.

Another assay-directed study was published by Orr \textit{et al.} (1999), who evaluated EDR assay-assisted selection of initial chemotherapy for newly diagnosed ovarian carcinoma cases. After cytoreductive surgery, platinum-based therapy was combined with either paclitaxel or cyclophosphamide depending on which agent showed the lowest level of resistance in the assay. While no significant difference in survival was noted for the two groups, there was a significant cost saving of approximately $6000 per patient based on assay utilization. Taken together, the data summarized in Tables 4 and 5 on treatment response and survival, and the data on assay-directed therapy, suggest that assay-assisted therapy selection may improve survival as well as economic outcomes.

Drug resistance versus drug sensitivity: two different coins

During the 1980s, advances in laboratory technologies overcame many of the technical problems of the earlier systems. Advanced assay systems were developed that yielded answers for most cases in a less than a week, making broader clinical applications a reality. Significant progress was made to identify issues that prevented tumor growth \textit{in vitro}, and comparisons were made between the different endpoints. Results from clinical trials which determine the relative advantages and disadvantages of the different \textit{in vitro} systems were compared (Weisenthal 1985, 1991, 1994). Divergent assay endpoints were generally found to provide comparable predictive accuracy. One particularly notable finding that emerged from these comparisons was that, regardless of the assay endpoints employed, \textit{in vitro} drug-response assays were most reliable for accurately identifying drugs that were unlikely to be effective rather than for picking out drugs that would cause tumors to shrink. The NPA was generally in the order of 90% to 99%, while the PPA was generally in the order of only 50% to 70%.

That \textit{in vitro} drug-response tests should vary in their positive and negative predictive reliability is not too surprising when the complexity of drug delivery to the patient’s tumor \textit{in vivo} is considered. While \textit{in vitro} assay systems can dependably deliver active drug to the tumor cells in culture, the human side of the equation is far less certain. After intravenous administration, chemotherapy agents are subjected to significant individual differences in biotransformation and biodistribution. Biotransformation differs among patients in part as a function of their enzyme haplotype. Analysis of the impact of single nucleotide polymorphisms on drug activation and inactivation is an emerging area of pharmacogenetics that may lead to patient-specific drug dosing (Kim \textit{et al.} 2001). Recent data on single nucleotide polymorphism has also provided new insight into the genetic basis for why some patients rapidly inactivate drugs, while others suffer greater toxicity by virtue of their slower drug metabolism (Roses 2001). Individual differences in drug metabolism that might prevent an active form of the drug from reaching the tumor \textit{in vivo} cannot be modeled using the current \textit{in vitro} assay systems. Pharmacodynamic activity ultimately depends on biodistribution of active drug species to the tumor bed through the tumor’s blood supply. A great deal of evidence has emerged supporting the notion that tumors of a given type and grade may possess a wide range of microvessel densities. In fact, angiogenesis has become an important new prognostic factor, as well as a new target for cancer treatment (Chen \textit{et al.} 2001). Unfortunately, individual differences in tumor vascularity are not accounted for by current \textit{in vitro} drug-response assays, adversely impacting on their positive predictive capability. Current \textit{in vitro} assays lack the capability to account for these critical pharmacodynamic aspects of
drug delivery, making it difficult for them to accurately predict in vivo 'chemosensitivity.' On the other hand, while these pharmacodynamic factors mitigate against accurately predicting that a drug will work, they favor predictions of drug resistance. If the tumor sample is completely resistant after supraoptimal drug exposures in vitro, then suboptimal in vivo delivery resulting from poor tumor vascular supply and/or rapid drug inactivation, will most likely result in treatment failure.

While the holy grail may not be at hand for in vitro chemosensitivity, the ability to tailor or personalize treatment regimens based on in vitro testing is still a viable approach. In fact, in vitro tests for drug response provide a bridge between the current empirical approach to chemotherapy and the future era that will focus on treatment tailored by biochemical fingerprinting. STIS571, Herceptin, Rituximab, and tamoxifen are targeted treatments developed through rational screening programs based on an understanding of cancer cell biology and critical signaling pathways (Sledge 2001). However, even these therapies are not universally effective, and resistance emerges. One reason for the development of drug resistance is that the transformation process is almost universally multifactorial, yielding tumors composed of genetically and biochemically heterogeneous cells containing multiple aberrant pathways. Thus, drug resistance will remain a major obstacle until the black box of the cancer phenotype is fully dissected at a proteogenomic level (Mechelner et al. 1998, Houston et al. 1999).

The main advantage of in vitro drug-response assays is their determination of the net effect of drug action. Drugs either induce apoptosis or they do not. The measurement of a specific drug target may not account for all of the steps required for drug efficacy. For example, tamoxifen is expected to be effective as an initial treatment in patients with steroid receptor-positive breast carcinoma. However, co-expression of HER2 can diminish breast cancer sensitivity to tamoxifen (Houston et al. 1999). Thus, a myriad of pathways may be interacting simultaneously to impact on drug entry to a cell, drug movement to its site of action, and the effect of the drug to induce cell death. In vitro tests make it possible to determine if the process of drug action and cellular response is intact or not. The clinician can take advantage of these findings in two ways. First, because these assays can accurately identify ineffective drugs, such agents can be avoided. Secondly, while the accuracy of predicting that a drug will work is limited, agents to which the tumor demonstrates low resistance have been found to be more effective than those found to be extremely resistant in vitro. Integration of all pertinent clinical factors together with in vitro data on tumor response to agents defined to be effective for the patient’s tumor type may provide the best outcomes for patients with breast or ovarian cancer. Cancer drug development by the co-operative groups should be enhanced by assessing the value of in vitro-assisted chemotherapy selection technologies in combination with proteogenomic techniques that can stratify and assign patients to trials predicted to be of greatest benefit to the individual patient. Such studies may better define the optimal diagnostic criteria for treatment selection and accelerate the approval of new rationally designed agents that may be effective only in small subsets of patients that have developed tumors that bear the appropriate repertoire of drug targets. Integration of in vitro cellular technologies and proteogenomic diagnostics into clinical trial design should be a high priority for the co-operative groups. Assay-assisted treatment selection is now emerging as an important adjunct in the physician’s armamentarium.

References


Alberts DS 1999 Treatment of refractory and recurrent ovarian cancer, Seminars in Oncology 26 (Suppl 1) 8–14.


Endocrine-Related Cancer (2002) 9 171–182
Fruehauf: In vitro treatment selection

Puck TT & Marcus PI 1955 A rapid method for viable titration and clone production with Hela cells in tissue culture: the use of X-irradiated cells to supply conditioning factors. PNAS 41 432–437.
Weisenthal LM 1994 Clinical correlations for cell culture assays based on the concept of total tumor cell kill. Contributions in Gynecology and Obstetrics 19 82–90.