

Current Status of Cell Culture Drug Resistance Testing (CCDRT) May, 2002

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Abstract

Cell culture drug resistance testing (CCDRT) is purported to correlate with response to chemotherapy and/or with patient survival after chemotherapy. Advocates of CCDRT maintain that this information is of value in clinical drug selection, particularly in situations where there is a choice to be made between more than one acceptable drug regimen. Assays based on a cell proliferation or DNA synthesis endpoint were largely studied in the early to mid-1980s and are currently advocated chiefly for the identification of inactive drugs. Assays based on cell death as an endpoint were the subject of increasing study during the late 1980s and throughout the 90s. An extensive, diverse, and consistent literature documents the ability of cell death assays to identify forms of chemotherapy which are associated with both favorable and unfavorable prognoses. CCDRT should be much more widely utilized in clinical oncology practice and as an integral component of ongoing and future clinical trials.

Introduction

Cell culture drug resistance tests (CCDRT) are laboratory tests in which fresh biopsy specimens of human tumors are cultured in the presence and absence of anticancer drugs. At the conclusion of the cell culture, measurements are made to determine whether or not the drugs were effective in either killing the tumor cells or in preventing the growth of the tumor cells. Proponents of these tests maintain that this information correlates with drug effects in the patient and can therefore be used to assist the clinical oncologist in selecting the most appropriate drugs to be used in the treatment of individual patients. This paper will review the data relevant to this point of view.

To begin with, there has been an unfortunate proliferation of names/terms applying to this testing. It should be noted that the terms "chemosensitivity assay," "chemoresistance assay," "drug resistance assay," and "drug response assay" can be used interchangeably. Likewise, the terms "in vitro assay" and "ex vivo assay" can be used interchangeably in this context. Some authors have tried to draw a distinction between assays which are geared and/or used more for the identification of inactive drugs versus active drugs. These are, however, purely semantic distinctions. Depending on where cut-off lines are drawn, all assays will have differing specificities and sensitivities for identifying inactive drugs and active drugs. It is much more useful to describe the specificity and sensitivity of an assay than to arbitrarily label the assay to be either a "chemoresistance" or "chemosensitivity" assay. The generic term "cell culture drug resistance testing" (CCDRT) describes laboratory tests in which gradations of drug resistance are determined by measuring drug effects on short term cultures of viable cells.

Depending on the conditions of the assays, they will have greater and lesser specificities and sensitivities for identifying inactive drugs and active drugs.

One must begin by understanding that there is a clear divide between CCDRT based on *cell proliferation* as an endpoint and CCDRT based on *cell death* as an endpoint. Historically, the cell proliferation endpoint received great attention, as a result of studies by Salmon, Von Hoff, and others during the late 1970s and early 1980s [1,2]. These studies occurred during the heyday of the oncogene discovery period in cancer research, where oncogene products were frequently found to be associated with cell growth, and where cancer was most prominently considered to be a disease of disordered cell growth. In contrast, the concept of apoptosis (programmed cell death) had yet to become widely recognized. Also unrecognized were the concepts that cancer may be a disease of disordered apoptosis/cell death and that the mechanisms of action of most if not all available anticancer drugs may be mediated through apoptosis [3-5]. When problems with proliferation-based assays emerged [6,7], there was little enthusiasm for studying cell death as an alternative endpoint. These factors explain the abandonment of research into CCDRT by American universities and cancer centers by the mid-80s. However, clinical laboratories began to offer CCDRT as a service to patients in the USA by the late 1980s, and studies of CCDRT continued in Europe and Asia.

Chapter 1: Cell Proliferation Assays

During the last dozen years, the cell proliferation assay which has been most heavily promoted and provided as a service to patients in the USA is the radioactive thymidine incorporation assay originally described by Tanigawa and Kern [8]. In this assay, applied only to solid tumors and not to hematologic neo-

plasms, tumor cells suspended in soft agarose are cultured for 4 - 6 days in the continuous presence of antineoplastic drugs. At the end of the culture period, radioactive thymidine is introduced and differences in putative thymidine incorporation into DNA are compared between control and drug-treated cultures. Kern and Weisenthal analyzed the clinical correlation data and defined the concept of "extreme drug resistance," or EDR [9]. This was defined as an assay result which was one standard deviation more resistant than the median result for comparison, database assays. Patients treated with single agents showing EDR in the assay virtually never enjoyed a partial or complete response. Kern and Weisenthal also defined "low drug resistance" (LDR) as a result less resistant than the median and "intermediate drug resistance" (IDR) as a result more resistant than the median but less resistant than EDR (in other words, between the median and one standard deviation more resistant than the median).

The principles and clinical correlation data with the thymidine "EDR" assay were reviewed in this journal 10 years ago [10]. There have been only a few follow-up studies published since this time. One such study showed that EDR to one or more of the single agents used in a two drug combination is not apparently associated with a lower probability of response to the two drug combination in the setting of intraperitoneal chemotherapy of appendiceal and colon cancers [11]. It is, however, possible that response to the high drug concentrations achievable with intraperitoneal chemotherapy may be more closely associated with drug penetration to the tumor than to intrinsic drug resistance of the tumor cells. It was also shown that EDR to paclitaxel does not appear to be a prognostic factor in ovarian cancer patients or in patients with primary peritoneal carcinoma treated with paclitaxel plus platinum [12,13]. However, it was recently reported that EDR to platinum in

ovarian cancer may have prognostic implications (Fruehauf, J., et al Proc ASCO, v.20, Abs 2529, 2001). [Note added in proof]: It was also reported that previously-untreated breast cancer patients with tumors showing LDR (defined above) had superior times to progression and overall survivals than patients with tumors showing either IDR or EDR (Mehta, R.S., et al, Breast cancer survival and in vitro tumor response in the extreme drug resistance assay. Breast Cancer Res Treat 66:225-37, 2001).

Currently in the USA, the tritiated thymidine "EDR" assay is provided by two different national laboratories (Oncotech and Impath). Based on the publication validating the assay [9], it has a very high specificity (>98%) for the identification of inactive single agents, but a low sensitivity (<40%). In other words, a drug with assay-defined "EDR" is predicted to be almost certain to be inactive as a single agent (high specificity for identifying inactive drugs), but many drugs without "EDR" will also be inactive (low sensitivity for identifying inactive drugs).

A second form of cell proliferation assay currently provided as a service to patients (NuOncology Labs, Houston, TX) is the adhesive tumor cell culture system, based on comparing monolayer growth of cells over a proprietary "cell adhesive matrix" [14]. Positive clinical correlations were described with this system in 1987 [14], but confirmatory and follow-up studies have not been reported.

Chapter 2: Total Cell Kill/Cell Death Assays

As opposed to measuring cell proliferation, there is a closely-related family of assays based on the concept of *total cell kill*, or, in other words, *cell death* occurring in the entire population of tumor cells (as opposed to only in a small fraction of the tumor cells, such as

the proliferating fraction or clonogenic fraction) [15-18]. The concepts underlying cell death assays are relatively simple, even though the technical features and data interpretation can be very complex. There has been considerable work based on these assays reported during the past 15 years. This body of work is not currently well appreciated among clinical oncologists, and the remainder of this review will focus on the cell death assays.

The basic technology concepts are straightforward. A fresh specimen is obtained from a viable neoplasm. The specimen is most often a surgical specimen from a viable solid tumor. Less often, it is a malignant effusion, bone marrow, or peripheral blood specimen containing "tumor" cells (a word used to describe cells from either a solid or hematologic neoplasm). These cells are isolated and then cultured in the continuous presence or absence of drugs, most often for 3 to 7 days. At the end of the culture period, a measurement is made of cell injury, which correlates directly with cell death. There is evidence that the majority of available anticancer drugs may work through a mechanism of causing sufficient damage to trigger so-called programmed cell death, or apoptosis [3,4].

Although there are methods for specifically measuring apoptosis, per se, there are practical difficulties in applying these methods to mixed (and clumpy) populations of tumor cells and normal cells. Thus, more general measurements of cell death have been applied. These include: (1) delayed loss of cell membrane integrity (which has been found to be a useful surrogate for apoptosis), as measured by differential staining in the *DISC assay* method, which allows selective drug effects against tumor cells to be recognized in a mixed population of tumor and normal cells [10,19], (2) loss of mitochondrial Krebs cycle activity, as measured in the *MTT assay* [20],

(3) loss of cellular ATP, as measured in the *ATP assay* [21-23], and (4) loss of cell membrane esterase activity and cell membrane integrity, as measured by the *fluorescein diacetate assay* [24-26].

It is very important to realize that all of the above 4 endpoints can and do, in most cases, produce valid and reliable measurements of cell death, which correlate very well with each other on direct comparisons of the different methods [20,25-36]. This should not be surprising, any more than should the fact that auscultating heart sounds, observing spontaneous breathing, palpating a carotid pulse, measuring core body temperature, and recording an electroencephalogram or electrocardiogram are all good and reliable methods of determining patient death.

We have performed direct correlations between the DISC and MTT assays in approximately 6,000 fresh human tumor specimens, testing an average of 15 drugs per specimen at two different concentrations. Thus, we have approximately 180,000 direct comparisons between DISC (membrane integrity) and MTT (mitochondrial Krebs cycle activity) endpoints in fresh human tumor specimens. The overall correlation coefficient between these endpoints in specimens containing > 60% tumor cells is 0.85 (These data do not include assays on 5FU, which, for biological reasons, may be tested in the MTT assay but not the DISC assay. These data also do not include assays for paclitaxel and docetaxel, which, for different biological reasons, may be tested in the DISC assay but not the MTT assay).

The above studies, demonstrating the comparability of results with the 4 different cell death endpoints, are important for the following reason. For perfectly understandable reasons, clinical studies correlating assay results with clinical outcome are very difficult to per-

form. The literature in this field may be characterized as including a great many small studies, but no big studies. Additionally, different investigators have favored different cell death endpoints, depending on the laboratory and clinical situation.

For example, the DISC assay is extremely labor intensive, and requires expertise in recognizing and counting tumor cells using a microscope, but it may be applied to specimens containing a heterogeneous mixture of tumor cells and normal cells. MTT, ATP, and FDA endpoints use semi-automated instrument readouts, but can only be applied to specimens which are relatively homogeneous for tumor cells. In addition, there are a number of additional reasons why one type of cell death endpoint may be advantageous in a given tumor specimen and why laboratories may apply several different cell death endpoints in the testing of a single specimen.

It should be noted that, historically, the DISC assay studies of the early 1980s provided the prototype for later studies of the other cell death endpoints. When the MTT endpoint was first introduced in the late 1980s, the first published studies compared the MTT results to the DISC results, with culture conditions and drug exposures being otherwise identical [20,27,29,31]. Many laboratories have preferred the MTT endpoint (and later the ATP and FDA endpoints), because of the difficulty in manually scoring the DISC assay microscope slides. But what is important is that each of the above cell death endpoints do give essentially the same results (except in the case of isolated drugs, such as taxanes and 5FU). *Thus, it is entirely reasonable and proper to consider as a whole the clinical validation data which has been published using the above 4 endpoints.*

The second point to understand is that cell death assays are not intended to be scale

models of chemotherapy in the patient. The DISC assay was designed to address the major practical problems with the popular clonogenic assays of the late-70s/early-80s. Chief among these problems were (1) low evaluability rates and (2) uncertainty of what was being measured in individual assays (true tumor cell colonies, arising from clonogenic cell growth versus artifactual colonies arising from cell aggregation). Unlike the case with the clonogenic assays, there was no attempt to model in vivo pharmacokinetics (i.e. no attempt to utilize clinically-achievable drug concentrations or to determine something analogous to an anti-bacterial minimal inhibitory concentration). Instead, the assay conditions were rigorously fixed, with respect to culture media and drug exposure time (the latter being, most typically, 96 hours). Drugs were first tested in training set assays to determine the drug concentration which gave the widest scatter of results (mathematically defined as the greatest standard deviation). The hypothesis to be tested with clinical correlations was a very simple one - that above-average drug effects in the assays would correlate with above-average drug effects in the patient, as measured by both response rates and patient survival.

Chapter 3: Correlations between cell death assay results and chemotherapy response

The hypothesis to be tested with clinical correlations was a very simple one - that above-average drug effects in the assays would correlate with above-average drug effects in the patient, as measured by both response rates and patient survival.

The tables and figures described below show that the above hypothesis has been confirmed to be true in every single study of these assays ever carried out.

Table 1. Correlations between assay results and clinical response

Summary of Data Correlating Clinical Response with Cell Death Assay Results

Author	Dx	N	TP	TN	FP	FN	Ref.
Leone	Adrenal	1	0	1	0	0	[37]
Beksac	ALL	3	2	1	0	0	[38]
Bosanquet	ALL	17	14	2	1	0	[39]
Hongo	ALL	25	16	3	5	1	[40]
Hongo	ALL	133	90	18	20	5	[41]
Kaspers	ALL	58	40	6	0	12	[42]
Larsson	ALL	4	1	2	1	0	[43]
Lathan	ALL	4	3	1	0	0	[44]
Weisenthal	ALL	29	18	5	2	4	[45]
Weisenthal	ALL	2	2	0	0	0	[19]
Kirkpatrick	ALL/CLL	55	38	7	10	0	[31]
Bosanquet	AML	4	0	1	2	1	[39]
Langkjer	AML	11	6	5	0	0	[46]
Larsson	AML	21	11	8	2	0	[43]
Staib	AML	83	74	9	0	0	[47]
Santini	AML	27	6	13	0	8	[48]
Sargent	AML	21	10	9	2	0	[49]
Stute	AML	33	11	8	4	10	[50]
Larsson	AML/ALL/NHL	73	45	16	9	3	[51]
Beksac	ANLL	12	7	3	2	0	[38]
Hongo	ANLL	14	9	1	2	2	[40]
Hongo	ANLL	14	9	2	1	2	[41]
Lathan	ANLL	17	11	4	1	1	[44]
Santini	ANLL	27	12	12	2	1	[52]
Tidefelt	ANLL	34	20	11	2	1	[53]
Leone	Bladder	2	0	2	0	0	[37]
Blackman	Breast	47	31	13	3	0	[54]
Cree	Breast	29	17	5	4	2	[55]
Koechli	Breast	17	9	6	1	1	[56]
Xu	Breast	83	56	10	17	0	[57]
Weisenthal	Breast	4	2	2	0	0	[19]
Bosanquet	CLL	80	12	48	18	2	[39]
Bosanquet	CLL	34	26	6	2	0	[58]
Larsson	CLL	1	1	0	0	0	[43]
Silber	CLL	15	11	3	0	1	[59]
Weisenthal	CLL	15	9	4	1	1	[45]
Weisenthal	CLL	3	2	1	0	0	[19]
Bosanquet	CLL/ALL/NHL	226	102	76	41	7	[60]
Weisenthal	CML (blastic)	9	2	6	1	0	[45]
Furukawa	Colon	16	3	13	0	0	[62]
Imai	*Colon	37	5	29	2	1	[63]
Weisenthal	Colon	1	0	1	0	0	[19]
Weisenthal	Esophagus	1	0	1	0	0	[19]
Furukawa	Gastric	22	3	16	3	0	[62]

Table 1, Page 1 of 2

[Table 1 \(this page\)](#) and [Table 1 continuation \(next page\)](#) show the raw published data from which the results were taken, with literature references. [Figure 1](#) shows the results of each individual study, arrayed in order of increasing response rates in the total patient population studied. In every single case, without exception, assay "sensitive" patients were more likely to respond than the total patient population as a whole and assay "resistant" patients were much less likely to respond than the patient population as a whole. In every case, patients treated with assay "resistant" drugs were less likely to respond than patients treated with assay "sensitive" drugs. This should not be a surprising finding.

Table 1. Correlations between assay results and clinical response (page 2; continued from page 1)

Author	Dx	N	TP	TN	FP	FN	Ref.
Leone	GI (Msc.)	5	0	4	1	0	[37]
Asanuma	GI (Msc.)	23	1	17	5	0	[61]
Furukawa	GI (Msc.)	22	1	18	3	0	[64]
Weisenthal	Pancreas	2	0	2	0	0	[19]
Meitner	Head & Neck	18	13	1	2	2	[65]
Leone	Melanoma	3	3	0	0	0	[37]
Weisenthal	Melanoma	1	0	1	0	0	[19]
Bosanquet	Myeloma	7	0	7	0	0	[39]
Weisenthal	Myeloma	6	2	2	1	1	[45]
Weisenthal	Myeloma	5	0	5	0	0	[19]
Larsson	Myelofibrosis	1	1	0	0	0	[43]
Beksac	NHL	1	1	0	0	0	[38]
Bosanquet	NHL	10	3	3	3	1	[39]
Leone	NHL	3	2	0	1	0	[37]
Nygron	NHL	50	27	10	11	2	[36]
Weisenthal	NHL	10	6	3	1	0	[45]
Weisenthal	NHL	3	0	3	0	0	[19]
Leone	NSCLC	8	5	2	1	0	[37]
Smit	NSCLC	6	0	6	0	0	[66]
Weisenthal	NSCLC	8	0	8	0	0	[19]
Wiibur	NSCLC	25	7	11	5	2	[67]
Meitner	Gynecologic	4	0	3	1	0	[65]
Blackman	Ovary	72	31	27	13	1	[54]
Carstensen	Ovary	22	6	12	4	0	[68]
Ng	Ovary	20	3	14	3	0	[69]
Konecny	Ovary	38	18	8	10	1	[70]
Ohie	Ovary	15	7	6	1	1	[71]
Sargent	Ovary	25	9	9	6	1	[72]
Sevin	Ovary	31	23	6	0	2	[73]
Sevin	Ovary	65	45	12	3	5	[74]
Taylor	Ovary	37	11	17	6	3	[75]
Weisenthal	Ovary	3	1	2	0	0	[19]
Leone	Renal Cell	2	1	1	0	0	[37]
Leone	Sarcoma	2	1	1	0	0	[37]
Beksac	SCLC	1	1	0	0	0	[38]
Smit	SCLC	8	2	4	0	2	[66]
Weisenthal	SCLC	10	0	7	3	0	[19]
Leone	Teratoma	1	0	1	0	0	[37]
Leone	Testicular	3	2	1	0	0	[37]
Leone	Thymoma	1	1	0	0	0	[37]
Leone	Unk Primary	3	1	2	0	0	[37]
Weisenthal	Unk Primary	1	1	0	0	0	[19]

N = 1945 TP = 983 TN = 626 FP = 245 FN = 90

Specificity = .92 (Specificity for drug resistance)

Sensitivity = .72 (Sensitivity for drug resistance)

Table 1, Page 2 of 2

Figure 1

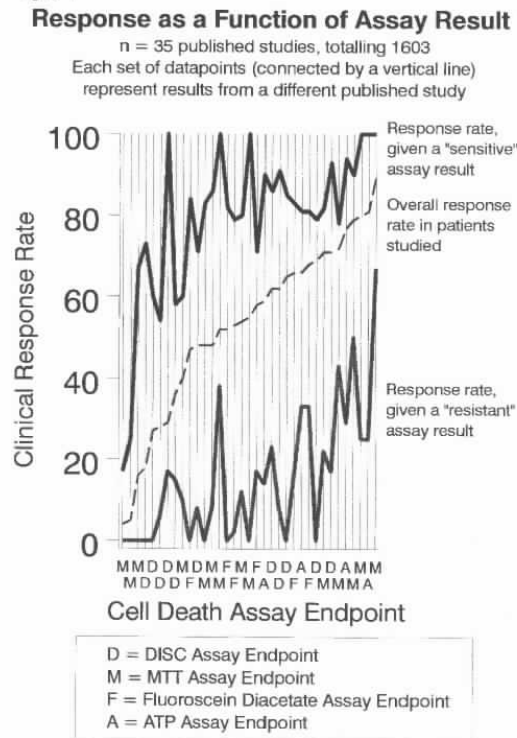
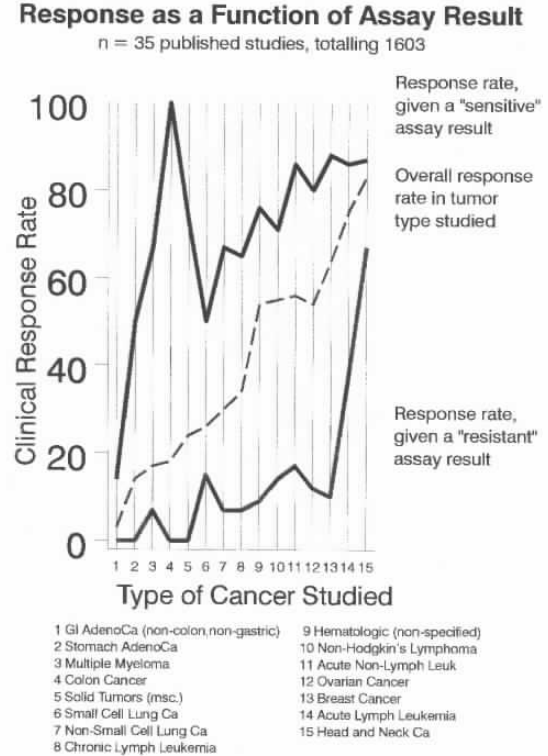


Figure 2 shows the correlations between assay results and treatment, broken down as to clinical/histopathologic-diagnosis.

Fig. 2



These are also arrayed in order of increasing overall response rates of the patient populations under study. In each case, assay "sensitive" patients were more likely to respond than the overall patient population and assay "resistant" patients were less likely to respond. In every case, patients treated with assay "sensitive" drugs were more likely to respond than patients treated with assay "resistant" drugs." The only "near exception" to this point was in the case of head and neck cancer, in which results were available only from a single study, in only a handful of patients. It may further be concluded from Figure 2 that cell death assays are broadly applicable to a broad range of neoplasms. This does not prove, for example, that the assays are clinically valid for a given rare tumor, such as esthesioneuroblastoma, but there is no reason to

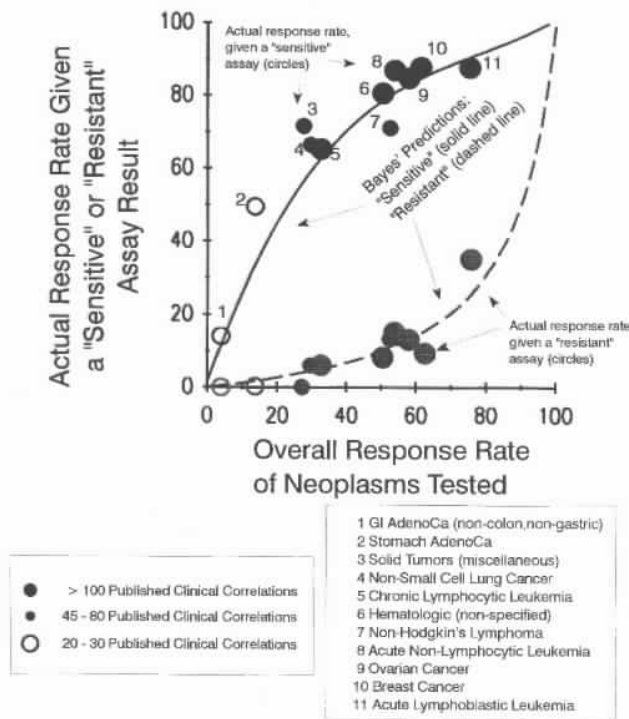
expect that the cell death assays should not be valid in any given type of neoplasm.

Chapter 4: Assay Results in the Context of Bayes' Theorem

The absolute predictive accuracy of the tests varies according to the overall response rate in the patient population being studied, in accordance with Bayesian principles [76].

Figure 3 is of greatest importance, and is well worth considering. If one understands this figure, one goes a long way to understanding how the results of these assays should be used in patient management.

Fig. 3
Assay Results and Bayes' Theorem
 Relationship between assay result and probability of response as a function of overall response rate



The solid and dashed lines in this figure show the theoretical expectations for the cell death

assays, based on Bayes' Theorem, applied to assays with an overall specificity for drug resistance of 0.92 and an overall sensitivity of 0.72, which represent the overall findings from the studies included in the meta-analysis. The circles show the actual response rates of patients with different types of neoplasms, given that either "sensitive" or "resistant" results were obtained. It may be seen that, in every case, the actual performance of the assays in each type of tumor precisely matched predictions made from Bayes' Theorem, projected from the overall assay sensitivity and specificity.

The findings in **Figure 3** show conclusively that the cell death assays are broadly applicable to a wide range of human neoplasms, ranging from low response rate tumors, such as pancreatic cancer and cholangiocarcinoma (group 1, the non-colon, non-stomach GI adenocarcinomas) to acute lymphoblastic leukemia (group 11), and including breast cancer and ovarian cancer.

Of equal importance, this figure shows how the assay may be best applied to patient management decisions. It is obvious that, in high response rate neoplasms, there will be many "false negative" predictions. No one should ever use these assays to deny chemotherapy to such patients, if chemotherapy is otherwise indicated, any more than one should deny antibiotics in an infection with an in vitro drug resistant bacterium. In cases where there is one particular drug regimen which has been shown to produce a very high cure rate and this regimen is widely accepted as being superior to all other regimens (e.g. testicular cancer, where dose-intense cisplatin/etoposide/bleomycin has been shown to produce the highest cure rate), it would be most unwise to forgo this regimen solely on the basis of today's available cell culture assays.

On the other hand, *the assays could be appropriately used to identify patients with above and below-average clinical prognoses if treated with given drugs*. In cases where more than one acceptable regimen exists, the physician could select the regimen containing the most favorable drugs and avoid the regimen containing the most unfavorable drugs. This would apply to clinical decisions at all points along the curve. Thus, the absolute probability of response with assay "sensitive" and "resistant" drugs varies according to the overall prior response probability in the patient population studies, but, at all points, assay "resistant" patients have a below average probability of response and assay "sensitive" patients have an above average probability of response and treatment with assay "sensitive" drug(s) is more likely to be associated with a favorable outcome than treatment with assay "resistant" drugs.

Chapter 5: Specific Diseases - Hematologic Neoplasms

The preceding was an overview of the forest of the literature supporting the hypothesis that above-average drug effects in cell death assays correlate with above-average clinical efficacy in the patient, and below-average drug effects in the assays correlate with below-average clinical efficacy in the patient. These (remarkably consistent) data supported the correlation between in vitro and clinical drug effects for a wide range of neoplasms.

We will now consider several individual "trees," or disease types, which have received the greatest amount of study. The diseases considered are (1) lymphatic neoplasms (CLL, ALL, and non-Hodgkin's lymphoma), (2) acute non-lymphocytic leukemia, (3) stomach and colorectal cancer, (4) ovarian cancer, and (5) breast cancer.

(Studies in hematologic neoplasms will be described below, and studies in GI neoplasms, ovarian cancer, and breast cancer will be described in the following Chapter 6)

Lymphatic Neoplasms and ANLL

Considering first only correlations between assay results and clinical response (defined as a complete response in the case of acute leukemia and as a partial or complete response for CLL and NHL), [Table 1 \(page 1\)](#) and [Table 1 \(page 2\)](#) show the following correlations:

Acute Lymphoblastic Leukemia: n = 275 published correlations between assay results and response. Overall response rate for patients studied = 76%. Response rates for patients treated with drugs with good activity in the assays = 87%. Response rates for patients treated with drugs with poor activity in the assays = 37%.

CLL: n = 157. Overall response rate = 43%. Response rate with good assay activity drugs = 74%. Response rate with poor assay activity drugs = 6%.

NHL: n = 77. Overall response rate = 55%. Response rate with good assay activity drugs = 71%. Response rate with poor assay activity drugs = 14%.

ANLL: n = 318. Overall response rate = 67%. Response rate with good assay activity drugs = 90%. Response rate with poor assay activity drugs = 23%.

There is a long, extensive, and consistent body of evidence supporting the clinical relevance of cell death assays in human hematologic neoplasms. It is very important to consider this evidence as a whole. One must remember that we are evaluating a laboratory technology and not a therapy. The issue to be

considered is the claim that the cell death measured in the assays correlates with tumor cell death measured in the patient. If one considers the CLL and ALL data as a whole, and then also considers the more limited but also consistent data in non-Hodgkin's lymphoma, a very powerful case is made to support the clinical relevance of this testing in human lymphatic neoplasms. If one then goes on to consider the ANLL data in the context of the lymphatic neoplasm data, a powerful case is made to support the clinical relevance of this testing in hematologic neoplasms in general.

The body of literature supporting cell death assays in lymphatic neoplasms dates to studies in CLL published by Schrek in the 1960s [77-80]. Schrek measured the in vitro cell death effects of drugs, heat, and radiation on CLL cells by means of phase contrast microscopy (undoubtedly measuring what we would today recognize as apoptosis and undoubtedly being precisely congruent with the DISC assay). Radiation effects were correlated with clinical outcome [77,80]. Schrek was, non-incidentally, the investigator who first described the identification of viable cells by means of dye exclusion [81].

In the late 1970s, Durkin compared in vitro drug effects in NHL and CLL by means of trypan blue dye exclusion with clinical drug effects and reported good correlations in a small study [82]. Independently, the *DISC assay* was developed as an improved variation of the trypan blue test, in which suspension cultures of cells were first exposed to trypan blue, spun down onto Cytospin slides, and then counterstained with either Hematoxylin/Eosin or Wright/Giemsa (to identify the non-trypan blue-stained cells with respect to whether these surviving cells were tumor cells or normal cells). With further improvement (substitution of fast green stain for trypan blue and the addition of acetaldehyde-fixed duck erythrocytes as an internal standard to aid in

scoring the Cytospin slides), clinical correlations in CLL and other neoplasms were first reported in abstract form and at meetings in the US and Europe in 1981 [83,84].

The first full journal publication of clinical correlations with the DISC assay occurred in 1983 and 1984, which included studies of the activity of glucocorticoids and standard cytotoxic agents correlated with prior therapy and with clinical outcome in ALL and CLL [15,19,85]. This was followed, in 1986, with a study showing the clinical relevance of the DISC assay in CLL, ALL, and NHL using several clinical endpoints: (1) correlations with known disease-specific activity profiles, (2) individual patient correlations with clinical response, (3) greater resistance of specimens from previously-treated patients versus previously-untreated patients, and (4) a shift to significantly greater drug resistance in metachronous assays in the presence of intervening chemotherapy, but no shift in the absence of intervening chemotherapy [45]. It should be noted that these findings were subsequently independently confirmed by other investigators in more comprehensive studies [26,31,36,39,40,43,51,59,86-91]. Additionally, studies in pediatric ALL reported that resistance to dexamethasone in the DISC assay predicted for poor survival [92,93]. These findings were also independently confirmed (see below).

By the late 80s, a number of other investigators had begun to look at the DISC assay and related cell death assays. These began with a head to head comparison of the DISC assay with the MTT assay in established cell lines by the NCI lung cancer group [20,27]. These studies established the comparability of these endpoints in homogeneous cell populations.

A group at the Free University of Amsterdam carried out a head to head comparison of the DISC endpoint with the MTT endpoint in

acute lymphoblastic leukemia [29,30]. This group showed that the endpoints were comparable in specimens in which the percentage of leukemia cells (relative to normal cells in the specimen) was greater than 80 [29,30,94]. This group found the MTT endpoint to be much less labor intensive. They used the same general conditions originally described for the DISC assay (including a 96 hour continuous drug exposure, followed by comparisons between drug exposed and control cultures with the cell death endpoint). These Dutch authors went on to publish an extensive, elegant, and ongoing series of rigorous studies which have established that the assay results correlate with and predict for both response and survival in ALL, and that the assay results are, in fact, the only factor which independently predicts for survival in pediatric ALL [87,88,90,95-103]. They have also extended this work to ANLL [89,104,105]. Taken in the context of the entire literature, these studies in pediatric ALL provide complementary support for the validity of complementary studies in CLL (described below).

Other investigators also showed strong correlations between cell death assay results and clinical outcome (response and/or survival) in pediatric ALL [40,41,91-93,106], adult ALL and ANLL [25,31,44,46,47,49,52,53,99,107-113], CLL [58,59,114-116], and adult NHL [19,36,45,51]. These studies included further confirmation of the comparability between DISC and MTT endpoints in assays on clinical specimens and also introduced the fluorescein diacetate cell death endpoint, which, like the DISC endpoint, measures cell membrane integrity and which correlates very well with the DISC endpoint in homogeneous cell populations [26,36].

In 1991, Bosanquet published in *Lancet* a relatively large number of correlations between clinical response and DISC assay results, chiefly in CLL [39]. He showed, fur-

thermore, highly significant correlations between assay results and patient survival. This paper also confirmed the relevance of the "EDR" (extreme drug resistance) endpoint, which is defined as an assay result more than one standard deviation more resistant than the median of comparison assays. Bosanquet later described a paradoxical shift toward increased methylprednisolone sensitivity in previously-treated CLL and used the DISC assay to identify high dose methylprednisolone as an effective treatment for otherwise refractory CLL [117,118].

These studies with the DISC and MTT assays are supported by studies with the fluorescein diacetate (FDA) endpoint. Fluorescein diacetate is a lipid soluble material which readily penetrates cell membranes. Viable cells contains a membrane esterase which cleaves the dye to non-lipid soluble fluorescein, which is concentrated in cells containing a functionally-intact membrane. Thus, the assay is conceptually similar to the DISC assay, which measures the ability of cells with functionally-intact membranes to exclude non-lipid soluble dyes. Delayed loss of this membrane integrity is a marker of apoptotic cell death [119].

Investigators at Uppsala University in Sweden began work in the 1980s by comparing the DISC and FDA assays and establishing their comparability [26,36,51]. They proceeded to publish a series of studies showing (1) strong correlations with assay results and treatment outcomes in NHL and ANLL [25,26,43,107,108,120], (2) confirming the specificity of the EDR endpoint in predicting for clinical non-response [108], and (3) confirming and extending earlier reports of the capability of the cell death endpoint to identify the general disease-specific activity patterns of a diverse spectrum of drugs [86,121].

Within the past several years, additional studies have provided strong support for the clinical relevance of the information provided by cell death assays in hematologic neoplasms.

Bosanquet and colleagues reported a study in 243 CLL patients [116]. "Standard" first-line chemotherapy in the USA is fludarabine, but there are acceptable alternatives, such as single agent chlorambucil, cyclophosphamide, cladribine, and combinations such as "CVP" (cyclophosphamide/vincristine/ prednisone). In Bosanquet's study, fludarabine-sensitive patients treated with fludarabine had a 69% response rate (80% for untreated patients; 64% for previously-treated patients), while fludarabine-resistant patients had a 7% response rate (25% for previously-untreated; 0% for previously-treated). 66 patients received fludarabine within a year of the performance of the DISC assay. 15 of these were test-resistant. Not a single one of these 15 patients resistant to fludarabine but treated with fludarabine survived 17 months, and their median survival was 7.9 months. In contrast, the fludarabine-sensitive patients treated with fludarabine had an 80% chance of surviving beyond 17 months, a 41.7 month median survival, and a 25% chance of surviving beyond 6 years. Patients with DISC assay resistance to fludarabine, but treated with other regimens than fludarabine had a median survival of 16.3 months and 10% survived beyond 4 years. The relative risk of death for patients with DISC assay fludarabine resistance treated with fludarabine versus treated with a non-fludarabine regimen was 2.9. On multivariate analysis, fludarabine test resistance was a more important determinate of survival in patients treated with fludarabine than was any other clinical characteristic, including sex, Binet stage, prior chemotherapy, and patient age. In a separate analysis, DISC assay-directed therapy of CLL was calculated to be cost effective [122].

Other investigators, as noted, have reported that assay results are important predictors of patient survival in *pediatric* acute lymphoblastic and non-lymphoblastic leukemia [103,123-126].

Similar studies have been reported for *adult* acute non-lymphocytic leukemia [47,53,111,127]. Three different groups have published strong correlations between CCDRT results and survival in ANLL. Correlations between DISC assay results and patient survival in ANLL were first published by a Swedish group in 1989 [53]. These results were recently confirmed and extended by a group at the University of Cologne [47,111], in follow-up to their earlier report of strong correlations between DISC assay results and clinical remission of adult acute non-lymphocytic leukemia a decade earlier [44]. In their recent follow-up studies, the DISC assay results "precisely" predicted clinical outcome, and identified a group of patients with a 100% early death rate, when treated with conventional induction therapy [47]. These studies are very analogous to Bosanquet's work identifying a group of CLL patients in whom conventional treatment is uniformly fatal.

The German group followed up with a presentation at the American Society of Hematology (ASH) meetings in December, 1999, in which multivariate analysis showed DISC assay results to be the strongest factor predicting for clinical outcome in adult ANLL [111]. Most recently, a Danish group reported studies correlating MTT assay results with both overall and relapse-free survival in 85 adult ANLL patients [127]. Assay results remained significantly correlated with survival on multivariate analysis. This work on ANLL is precisely analogous and complementary to the studies by the Dutch (Amsterdam) group in pediatric ALL, discussed above.

The only "negative" study ever published concerning total cell kill (cell death) assays in hematologic neoplasms was an otherwise "positive" study in adult acute non-lymphocytic leukemia, in which strong correlations between anthracycline activity and survival were shown, but poor correlations between cytarabine activity and survival were seen [113], in contradistinction to several other studies in which assay results with cytarabine were found to be strongly correlated with patient survival [47,53,104,111]. The "negative" study was the only one to use the ATP endpoint, which is disadvantageous in hematologic neoplasms, as normal cells, red blood cells, and platelets all produce an appreciable "contaminating" ATP signal, in contradistinction to the other cell death endpoints, which are less affected by such artifacts. The authors of the "negative" cytarabine study acknowledged that they did not determine the percentage of leukemic blast cells at the conclusion of the cell culture and noted the advantages of the DISC assay in being able to discriminate neoplastic from normal cells.

Thus we have, in hematologic neoplasms, a 35 year history of highly positive studies, published by investigators in the USA, the United Kingdom, the Netherlands, Germany, Sweden, Canada, Italy, and Japan all showing consistent, strong correlations between the results of cell death assays and clinical outcomes. In summary, there is a strong scientific rationale for these tests and that the clinical relevance of the information provided by the tests has been documented in a collectively large and diverse literature in hematologic neoplasms.

Chapter 6: Specific Disease-Solid Tumors

General Considerations

Studies in solid tumors are technically different than studies in hematologic neoplasms because solid tumor most commonly are present as three-dimensional aggregates of cohesive cells, while hematologic neoplasms are almost exclusively discohesive. Studies by Teicher and Kerbel in murine tumors showed that in vitro drug activity correlated with in vivo drug activity when tumors were tested in vitro as three dimensional clusters, but not when they were tested in two dimensional monolayers [128]. There is now an extensive literature on what has been labeled "multicellular resistance" [129-131].

All published clinical correlations with true fresh tumor assays with cell death endpoints have tested the tumor cells largely in the form of three dimensional clusters. The only exception to this statement is the non-small cell lung cancer study of the NCI-Navy medical oncology group, in which subcultured cells (not true fresh tumors) were tested in monolayer culture [132]. This latter study not surprisingly showed poor correlations; all of the other cited studies, which used true fresh (non-subcultured) tumor cells tested largely as three dimensional cell clusters (and not in monolayer culture), showed good correlations.

The solid tumors which have received the greatest degree of study are gastrointestinal adenocarcinomas (colon and gastric adenocarcinoma), breast cancer, and ovarian cancer. There have been relatively few clinical correlations published in the cases of melanoma, soft tissue sarcoma, glioblastoma, and squamous cell carcinomas in general.

GI Neoplasms

In the case of gastrointestinal neoplasms, there have been 129 published correlations between assay results and clinical response [Table 1 \(page 1\)](#) and [Table 1 \(page 2\)](#) . Overall, patients treated with drugs having good activity in the assays had a 48% response rate, while those treated with drugs having poor activity in the assays had a response rate of less than 1%, in a population of patients who overall had a 11% response rate. Also reported in many additional patients were positive associations between assay results and patient survival [64,133,134].

Colon and stomach cancer have been studied mainly with the MTT endpoint, most prominently by Kubota and colleagues at Keio University in Tokyo [64,133,135-140], but also by other Japanese investigators [63,134,141-144]. The Keio group has published studies with the MTT assay in which tumors were cultured both in suspension and also as macroclusters (0.5 mm tissue fragments) using an "in vivo-like" culture technology developed by Hoffman [145]. These studies showed correlations between assay results and both response and survival [64,133,135-139]. These studies support the relevance of the MTT endpoint for fluoropyrimidine-based chemotherapy [133]. As MTT measures mitochondrial function, the response and survival correlations raise the interesting possibility that fluoropyrimidine activity in colon cancer may be directed at (and mediated through) autonomously-replicating mitochondria. What is most important here, however, is that the MTT endpoint has been shown to identify colon and gastric cancer patients who will have relatively favorable versus relatively unfavorable outcomes when treated with fluoropyrimidine-based therapy. This is very important for the reason that the cell death endpoint had previously been most problematic for

fluoropyrimidines, of the currently FDA-approved cytotoxic drugs.

Taken in the broad context of the entire literature, these studies provide important confirmation of the broad (with respect to both drugs and tumor types) applicability of cell death assays. The issue of whether the MTT assay or measurements of thymidylate synthetase [146] is more accurate in gauging probability of response to specific types of fluoropyrimidine-based therapy awaits future head to head comparisons.

Ovarian Cancer

In the case of ovarian cancer, there have been 328 published correlations between assay results and clinical response [Table 1 \(page 2\)](#) . Overall, patients treated with drugs having good activity in the assays had a 77% response rate, while those treated with drugs having poor activity in the assays had a response rate of 11%, in a population of patients who overall had a 51% response rate. Also reported were highly positive associations between assay results and patient survival [147,163].

Kurbacher and colleagues treated 25 patients with ovarian cancer with ATP-assay-directed chemotherapy and compared outcomes with 30 non-randomized but clinically well-matched controls [148]. In the control group, there was a response rate of 37% (2 complete responders), with median progression-free survival of 20 weeks and median overall survival of 69 weeks. In the assay-directed group, there was a response rate of 64% (8 complete responders), with a median progression-free survival of 50 weeks ($P=0.003$) and a median overall survival of 97 weeks ($P=0.145$). Assay directed therapy also produced a greater benefit with respect to both response rate and progression-free survival in the subgroup of patients with platinum-

resistant disease. A current multi-institutional, international trial is currently in progress to determine if assay-directed therapy is superior to empiric therapy.

Breast Cancer

In the case of breast cancer, there are a total of 179 published correlations between assay results and patient treatment [Table 1 \(page 1\)](#). Patients treated with assay "sensitive" drugs had an 82% response rate. Patients treated with assay "resistant" drugs had a 7.7% response rate. The overall response rate for the patients in the studies was 66%.

Xu and colleagues treated 73 breast cancer patients on the basis of MTT-assay directed chemotherapy, and compared outcomes with 73 patients treated with "physician's choice" chemotherapy [57]. This was also a non-randomized study, but the patients receiving assay-directed therapy actually had less favorable prognostic factors, such as having significantly more sites of disease (the author informed me in a personal communication that the patients at her medical center with unfavorable disease were more often referred for biopsy and assay-directed therapy, while patients with more favorable disease were more likely to receive standard empiric chemotherapy). The response rate of the assay-directed group was 77%, while the response rate for the empiric therapy group was 44%. In a small group of 10 patients who received assays but in which no active drugs were identified, empiric therapy was given with no responses (0% response rate). One year survivorship for the two groups was 74% for assay-directed therapy and 67% for empiric therapy. Three year survivorships were 25% and 19%, respectively. Five year survivorships were 20.5% and 12.3%, respectively.

The above study showed a clear response advantage to assay-directed therapy and a trend

for a survival advantage, despite less favorable prognostic factors for the group receiving assay-directed therapy. The lack of statistical significance for survival is no doubt owing to the small numbers of patients enrolled in the study. Putting things into perspective, the adjuvant Cancer and Acute Leukemia Group B study comparing doxorubicin/cyclophosphamide with and without Taxol required 2,000 patients to show an absolute 2% difference in survival. And yet triple drug therapy has now become the standard of care in this setting. It also required a meta-analysis of studies totalling close to 50,000 patients to establish a small survival advantage for adjuvant chemotherapy of postmenopausal patients.

Chapter 7: Editorial Conclusions

The title of this review is current status of cell culture drug resistance testing. This review focused on a description of the technologies and a review of the clinical correlation data, because there are many misconceptions and much ignorance about both technology and data.

Several years ago, Cortazar and Johnson reviewed clinical trials of therapy ostensibly based on the results of cell culture assays [149]. However, the Cortazar/Johnson review is not relevant to the technologies discussed here or to any technologies offered through clinical laboratories in the United States as a service to patients at any time within the past ten years. A critical discussion of this review is available on the Internet (http://weisenthal.org/cort_rev.htm).

One must recall the extraordinary difficulty in proving the efficacy of chemotherapy in general and of specific drug regimens in particular in studies of non-assay-directed chemotherapy. Only with extremely large studies (and sometimes only with meta-analyses of

extremely large studies) has it been possible to document that chemotherapy of any type produces survival advantages compared to no chemotherapy at all, in many clinical situations. The quite impossible challenge of documenting the clinical standard of "efficacy" (as opposed to the heretofore traditional laboratory standard of "accuracy") with these non-proprietary, public domain technologies was, in fact, pointed out by Dr. Maurie Markman (a noted critic of Human Tumor Assays) who correctly wrote that "even if it were possible to establish the efficacy of [the assays] in a particular situation, this would do nothing at all to establish the efficacy of [the] assays in any other situation" [135].

The challenge of "validating" a single test for a single treatment in a single disease is challenging enough (e.g. estrogen receptor in breast cancer, which has still, after 30 years, only been shown to correlate with clinical outcome and has yet to be shown to improve clinical outcome). Now consider the challenge of "validating" a test for 40 different drugs which can be used in tens of thousands of combinations in hundreds of diseases. If documented clinical "efficacy" is the standard to be demanded of non-proprietary laboratory tests, then clinicians should abandon all tests currently used in their practices. It will be interesting to see which standard is applied in the future to other laboratory tests associated with the prediction of drug resistance, such as tests based on mechanisms of drug resistance (e.g. expression of thymidylate synthetase [146,150,151]) and Her2/neu expression [152-154].

While evaluating the data discussed here, please consider that it has taken 20 years to amass this body of evidence in an environment of continued hostility and non-support by the academic oncology community toward work in this area and consider also the little which has been achieved in the area of em-

piric methods of drug selection, despite billions of dollars spent on empiric clinical trials enthusiastically supported by this same academic oncology community.

If one [critically evaluates](#) the clinical trials data in ovarian cancer, for example, one finds that there is no advantage for platinum-based combination chemotherapy over single agent alkylator therapy and no advantage for platinum + paclitaxel over single agent cisplatin or carboplatin [155-157]. But this did not prevent platinum combination therapy from becoming "standard of care" before the introduction of paclitaxel and it did not prevent platinum/paclitaxel from becoming standard of care over single agent carboplatin or cisplatin. In point of fact, the only thing clearly established after 30 years of clinical trials is that carboplatin and cisplatin are therapeutically equivalent, albeit with different toxicity profiles. And there are absolutely no data to support any of the half dozen or so available drug choices for second and third line therapy over any other choice. So what is the "risk" in using currently available assays to help guide these choices?

Only when these assays are widely performed and used and routinely included as an integral part of clinical trials will these already promising technologies be improved and only then will their role in patient management become better defined. But this is true for all complex laboratory technologies (a good example being immunohistochemical staining for batteries of cell antigens).

Absent this testing, on what basis are drugs chosen today for use in the many clinical settings in which a single "best" empiric regimen has not been well-defined? An objective reviewer would admit that many oncology practices would base choice of drug regimen, at least in part, on the profit "spread" between the wholesale cost of the drug(s) and the re-

imbursement which the third party payers provide. This is a conflict of interest as well as a cost-ineffective method for selecting therapy; yet it is a method which the oncology and insurance communities support every single day in their treatment and coverage decisions. It is the loss of this "freedom to choose" and the overzealous dedication to a weak clinical trials paradigm (identification of the "best" treatment to give to the average patient) which is largely behind the reluctance to introduce these technologies as an important component of current clinical trials and as a part of the process of clinical drug selection in situations where clear empiric "best regimens" have not been well defined through prior clinical trials.

The private sector laboratories offering CCDRT as a patient service ([Table 2](#)) have been able to make considerable progress in improving the assay technologies and in building databases which improve the interpretation of "raw" assay results. But this progress has only been possible because insurers and often patients have been willing to pay for the tests and because clinicians have wanted to have the information provided by the tests. The progress would have been much faster (and doubtless even more substantial) had the academic oncology community not done everything it could to oppose this work at every step of the way.

By raising the bar of acceptance to levels unprecedented for a laboratory test, in essence a tariff has been erected to protect the paradigm of the "best" empiric treatment for the average patient, as identified in appallingly non-productive clinical trials. This tariff also serves to protect the paradigm of drug selection with consideration of the spread between wholesale cost and reimbursement.

Finally, the tariff discourages discovery of new, effective drug regimens through the use

of CCDRT to guide drug selection. Take, for example, the gemcitabine/cisplatin combination. Years before gemcitabine/cisplatin became a widely used drug regimen, CCDRT identified this as the most active regimen in a patient with pancreatic cancer metastatic to kidney, omentum, and liver, despite the poor activity of gemcitabine and cisplatin tested as single agents. This patient went on to achieve a complete remission with gemcitabine/cisplatin and remains alive with an excellent quality of life 5 1/2 years later [158,159]. A second such patient was an ovarian cancer patient with primary resistance to paclitaxel/carboplatin who then underwent tandem stem cell transplant/high dose chemotherapy regimens (at a cost of more than \$250,000) without ever achieving a response. At a time when she had bulky, non-cytoreducible abdominal and pleural disease, CCDRT confirmed resistance to single agent cisplatin, carboplatin, and gemcitabine, but good activity for the gemcitabine/carboplatin combination. She subsequently received gemcitabine/carboplatin as an outpatient, achieved a durable complete response, and returned to work full time as an oncology nurse, where she remained well, for four years [160], until a recent relapse (she has recently been restarted on assay-directed chemotherapy). Indeed, early anecdotal results of this type occurring in diseases in which there was no existing clinical trials literature accelerated clinical trials of this regimen in diseases in which assay-directed responses had been observed [161].

With more widespread use of these assays in clinical oncology, it is very likely that the activity of new drugs and new regimens would be identified at a much earlier time than with the current system relying exclusively on usually-empiric, Phase II trials [162].

Why is it so necessary to protect the patient from the information provided by a perfectly

rational laboratory test, supported by a wealth of entirely consistent, if understandably incomplete data? If used to assist in the selection of a regimen chosen from a series of otherwise reasonable alternatives, then patients will never be harmed and best available evidence strongly indicates that they will often be helped.

Think of all the objections to this testing. Now try to design all of the clinical trials which would be needed to meet all of these objections and think of how much money these would require and who is going to provide this money and how many years the studies would take and how many patients will continue to receive ineffective or suboptimum treatment in the interim. The body of information will never be sufficiently large and complete and definitive to encompass even a reasonable fraction of the situations where the information provided by the tests would be helpful. Now ask the questions: What is the potential risk? What are the potential benefits? What is the probability that these tests really do provide information which can improve the drug selection process in individual clinical situations? What is the potential cost? How does the benefit/risk ratio balance out? What is the (financial) cost as a percentage of total costs relating to management of patients on chemotherapy (including the costs of radiographic and laboratory studies performed only to determine if a given form of treatment is working or not)? What are the long term costs if drug selection always remains an empiric, one-size-fits-all, trial and error process? What would be the impact on improving existing technologies (through the attraction of more laboratory and clinical investigators into the field) and developing new technologies should these assays become more widely used?

If one wishes to see an example of an entirely rational technology advance, in a human dis-

ease crying out for precisely such a technology advance, supported by an entirely consistent (if understandably incomplete) body of data, where the advance continues to be held hostage to a high bar of extraordinarily difficult clinical trials which the critics have been entirely unwilling to support, in an area (laboratory testing) for which such trials would be entirely unprecedented, one need look no further.

Table 2 shows a partial listing of laboratories from which CCDRT as a clinical service is currently available. For specific information concerning the practical and technical aspects of these services, and for cost and reimbursement issues, the director of each laboratory should be contacted.

Table 2. Laboratories Providing Cell Culture Drug Resistance Testing (CCDRT) as a Service to Patients and Clinicians

Laboratory / Internet address

Anticancer, Inc. www.anticancer.com
Bath Cancer Research Unit (U.K.)
www.bath.ac.uk/departments/postmed/bcr/home.htm

Carl-von-Hess Hospital (Germany)
www.klinik-hammelburg.de/index.htm

Clinical Pharmacology/Oncology Uppsala University (Sweden) e-mail:
PeterNygren@medsci.uu.se

Human Tumor Cloning Laboratory
www.ahsc.arizona.edu/~htclweb/ (Note: Web check 7/30/2002 -> link for U of Arizona Human Tumor Cloning Laboratory no longer active)

Impath, Inc. www.impath.com

Table 2 (continued).

Oncotech, Inc. www.oncotech.com

Precision Therapeutics, Inc. www.percisiontherapeutics.com

Rational Therapeutics, Inc. www.rational-t.com

Weisenthal Cancer Group
www.weisenthal.org , e-mail:
mail@weisenthal.org

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