

Platinum Resistance Determined by Cell Culture Drug Resistance Testing (CCDRT) Predicts for Patient Survival in Ovarian Cancer

Larry M. Weisenthal, Constance M. Weisenthal, Mary E. Smith, Cindy G. Sanchez, and Robin F. Berglund

Weisenthal Cancer Group
15140 Transistor Lane
Huntington Beach, CA 92649

Phone: 714-894-0011; Fax: 714-893-3658; e-mail: mail@weisenthal.org

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Abstract

Background: We examined the relationship between long-term patient survival in ovarian cancer and the results of cell culture drug resistance testing (CCDRT). *Methods:* The in vitro activity of cisplatin and carboplatin was determined through the concurrent application of two different cell death endpoints (cell membrane dye exclusion/ DISC assay and mitochondrial metabolism/ MTT assay) following 96 hour culture of 3 dimensional microclusters of tumor cells. "Sensitive" / "Intermediate" / "Resistant" cut-offs were defined by calculating means and standard deviations of training set assays performed on a wide variety of human tumors (including non-ovarian tumors) and were reported prospectively. These cut-offs were also re-calculated retrospectively, based only on the datasets of ovarian cancer assays. *Results:* Specimens from previously-treated patients were significantly more resistant to platinum than were specimens from untreated patients, and this difference was most pronounced in the case of poorly-differentiated tumors. Well-differentiated tumors had significantly greater platinum resistance than poorly-differentiated tumors. In untreated patients (n = 115) resistance to cisplatin and (separately) to carboplatin correlated significantly with long-term survival, as reported prospectively. This relationship was strongest in

the case of poorly-differentiated tumors (hazards ratio "sensitive" versus "resistant" = 0.31, 95% confidence interval 0.039 - 0.62, for assay results reported prospectively and hazards ratio = 0.22, 95% C.I. 0.043 - 0.47, for cut-offs objectively calculated retrospectively, based on only the ovarian cancer dataset). There was no significant relationship between platinum resistance and patient survival in previously-treated patients (n = 327). *Conclusions:* Platinum resistance determined by CCDRT using cell death endpoints on tumor cell microclusters predicts for long term survival of untreated ovarian cancer patients with poorly-differentiated tumors. Furthermore, the CCDRT system described here is currently the most highly validated system for studying the circumvention of platinum resistance in human adenocarcinomas.

Introduction

There has been virtually no progress in the clinical science of drug selection for the treatment of ovarian cancer during the past 30 years. Prospective, randomized trials have failed to identify specific forms of first line therapy which result in superior patient survival when compared with the first line use of single alkylating agents (e.g. oral melphalan) which constituted first line therapy 4 decades ago. Thus, platinum combinations were found on meta-analysis¹ to be equivalent to single agent alkylators (hazard ratio = 0.93, C.I. 0.831 - 1.049, p = 0.246) and platinum/paclitaxel combinations were found to be equivalent to single agents cisplatin² and carboplatin^{3,4}. Improved methods of matching treatment to patient are clearly needed.

In 1978, it was reported in the NEJM that results obtained with a cell proliferation-based assay (the "human tumor clonogenic assay") correlated retrospectively with patient response to chemotherapy in ovarian cancer⁵. However, a subsequent prospective study was unable to demonstrate a significant response correlation and found no relationship between assay results and patient survival⁶.

Clinical correlations with cell culture drug resistance testing (CCDRT) with the DISC assay, based on the alternative concept of total tumor

cell kill (cell death), were first described 20 years ago⁷. There is, at present, a large and diverse literature documenting the biological relevance of this assay and a related family of cell death assays, in a wide array of hematologic and solid neoplasms, including ovarian cancer⁸.

Beginning in January, 1993, we prospectively reported results of cell death assays to clinicians and pathologists who referred fresh biopsy specimens of ovarian cancer to our clinical laboratory. We reported all of the relevant raw data, as well as the final classification of results (in terms the tumor being "sensitive," "resistant," or "intermediate" to a given drug), thus allowing for a true and verifiable prospective comparison between assay results and patient survival. Reported here are the relationships we found between in vitro resistance to cisplatin and carboplatin as compared with prior treatment status and long term overall patient survival.

Methods

Tumor Specimens and Patients

Fresh biopsies or fluid aspirates were obtained from patients with newly-diagnosed or previously-treated ovarian cancer. These specimens were submitted from a wide range of medical centers in the United States and occasionally abroad. They were submitted for cell culture drug resistance testing (CCDRT) for the purpose of identifying chemotherapy regimens with above and below average probabilities of providing clinical benefit on a non-investigational basis. Patients were informed prior to assay completion that they would be responsible for all costs associated with testing and that some insurance companies deny payment for testing on the grounds that these companies classified the tests as "investigational." The patients were given the opportunity to cancel the tests prior to completion to avoid financial responsibility. In practice, fewer than 2% of patients elected to cancel testing, most returned signed acceptances of financial responsibility, and more than 60% of all payment received came from third party insurance carriers.

Specimens were most typically submitted via the anatomic pathology laboratories of the submitting hospitals, but were occasionally submitted directly from the operating room. Solid tumor specimens (not exposed to fixatives or frozen) were to be placed in cold transport medium (CO₂-independent medium, Invitrogen / GIBCO, Grand Island, NY, supplemented with penicillin/ streptomycin, amphotericin B, insulin/selenium/ transferrin, and 10% low endotoxin, heat inactivated fetal bovine serum). Specimens were then to be placed in sturdy styrofoam shipping boxes, containing 350 gm blocks of "blue ice" frozen to minus 20 degrees Celsius. These were then shipped either via FedEx priority overnight delivery service or via local land courier. Fluid specimens were to be mixed well to suspend cell clusters and then poured into sterile 500 ml polypropylene transport bottles. Instructions were given to add 10 - 15 units of heparin sulfate per ml of fluid submitted. In practice, we do not know the extent to which all of the above instructions were followed. More than half of all specimens were submitted via FedEx and > 90% were received either the day of biopsy or the day following biopsy.

We received faxed copies of the official histopathology reports from the submitting hospitals for virtually all specimens received. These official pathology reports provided the tumor grading used in our study. Specimens described as "well differentiated" were entered as such in our database. Tumors described as "poorly differentiated" or "moderate to poorly differentiated" were entered as "poorly differentiated." Tumors described simply as "moderately differentiated" were entered as "moderately differentiated." There were no specimens from untreated patients labeled as "moderate to well differentiated." Tumors without any of these descriptors were not classified in our database as to degree of differentiation. Tumors were also described in the database as being "serous," "endometrioid," "clear cell," "mucinous," and/or "papillary" if and only if these descriptors appeared on the official histopathology report from the submitting institution. Tumors were not classified or reclassified as to differentiation or histology based upon our own assessments.

As described in the results, we analyzed the following subsets of data with respect to long-term patient survival: (1) all tumors. (2) all tumors minus tumors specifically identified on the official histopathology as being "well differentiated." (3) Only tumors specifically identified as being "poorly differentiated."

Isolation of three dimensional tumor cell clusters

Solid tumors were minced to pieces smaller than 1 mm (small enough to be aspirated into a standard disposable 10 ml pipette) with high quality curved surgical scissors. Transport medium was reserved, along with the supernatant from the tissue mince. Scissor-minced tumor pieces were digested with collagenase/DNAse in RPMI-1640 containing antibiotics and 10% fetal calf serum. Specimens were digested in 50 ml disposable polypropylene centrifuge tubes, assisted by gentle mixing with plastic-coated, magnetic stirring bars over a stirring plate. Specimens were thusly mixed until complete gross digestion had taken place -- typically about 2 - 3 hours for a 1 - 3 gram specimen. Cytospin slides were then prepared from all cell fractions (transport medium, supernatant from tissue mince, and enzyme digestate), and stained with Fast Green/Hematoxylin-Eosin, as described previously^{7,9}.

Fluid specimens were centrifuged in their entirety to collect all cells in the specimen. Cells were then resuspended in the above RPMI-1640-based medium and Cytospins were prepared as described above.

We wished to test three dimensional microclusters of cells and not single cell suspensions. Viable microclusters were preferentially enriched from medium containing a mixture of microclusters, single cells, normal cells, red blood cells, dead cells, and debris by means of repeated sedimentation at 1 x g, collecting and resuspending the sediment for repeated sedimentation steps. This process was monitored by preparing Cytospins of the resuspended cell clusters, until fractions containing 90% of the viable cells as tumor cell clusters were obtained. When it was not possible to achieve this ideal, we combined sedimentation fractions containing the

highest possible percentage of three dimensional cell clusters. We adjusted the concentration of cell clusters so that approximately 25% of the area of the Cytospin cellular "disk" ("spot") was comprised of reddish-pink (viable) tumor cell clusters, and 75% was comprised of empty space. This cell concentration was of critical importance, as overplating and underplating was capable of producing artifactual drug resistance and sensitivity. It was crucial to standardize assay conditions, as results were based on comparison with a universe of comparison assays, as described below.

At the beginning of the assays, we prepared "day zero" slides, depicting the condition of the non-drug-exposed cells at the beginning of the assays, and we also prepared "day four" slides of negative control (non-drug exposed cells at the end of the assay). Both day zero and day 4 slides were subjectively scored as to (1) percentage of total viable tumor cells which were in clusters (as opposed to being single cells); (2) average density of cell clusters, where "loose" clusters had clear spaces between the cells following Cytospin centrifugation, "medium" clusters did not contain clear spaces between cells but were flattened to a two dimensional appearance, and "tight" clusters maintained a three dimensional appearance following Cytospin centrifugation, and (3) median two dimensional area of the cell clusters, as measured with an ocular micrometer. In addition to cluster measurements, slides were subjectively scored to determine the ratio of viable tumor cells at the end of culture (96 hours or "day 4") relative to the number of viable cells at the beginning of culture (zero hours or "day 0"). All of the above measurements were included on the final report issued to the referring physicians, along with assay results and all raw data used to determine assay results. Also reported were the MTT formazan signal (negative controls minus positive controls) in the control culture, in Absorbency units read at 570 mu on a standard 96 well plate reader, as described below. Finally, a written description of the cytologic appearance of the tumor cells on the H&E-stained Cytospin slides was also provided with the report.

Cell Culture and Drugs

The cell cluster suspensions were mixed with 10% (volume/volume) drug solution or vehicle control (most typically 0.9% NaCl). Final volume of cell suspension/drug solution (or vehicle) plated for culture was 0.12 ml. Cell clusters were cultured in polypropylene round bottom, 96 well culture dishes in a humidified 37 degree incubator for 96 hours (maximum permissible time deviation was 2 hours, thus cell clusters were actually cultured for between 94 to 98 hours).

Clinical formulations of cisplatin (Platinol-AQ, Bristol) and carboplatin (Paraplatin, Bristol) were purchased from standard commercial suppliers. Stock solutions were prepared at ten times the desired concentrations (see below), aliquotted into single use, 0.5 ml conical polypropylene tubes, and frozen at -70 degrees Celsius prior to use.

Cells were cultured with the index concentration of each drug and also with 1/2 the index concentration, where the index concentration was defined as the concentration which produced the greatest scatter (standard deviation) in training set assays performed on prior specimens. In the case of cisplatin, the index concentration was 3.3 ug/ml. In the case of carboplatin, the index concentration was 23.5 ug/ml. Note that these index concentrations were determined by testing a broad range of human neoplasms, and not only ovarian cancer. Ovarian cancer specimens were, on average, significantly less resistant to cisplatin and carboplatin than was the average of the entire human tumor database. Negative controls consisted of 0.9% NaCl, the diluent used for both cisplatin and carboplatin. Positive controls consisted of 100 ug/ml of cisplatin plus 1 ug/ml of anguidine (obtained from the National Cancer Institute). Replicate 96 well plates were tested. One plate was assessed for cell death by means of the DISC assay; the second plate was assessed for cell death by means of the MTT assay.

DISC and MTT assays

On the fourth culture day, 0.010 ml of Alamar Blue dye solution (Trek Diagnostic Systems, Westlake, OH) was added to all culture wells in

the DISC assay 96 well culture dish. After 4 hours, Absorbancies at 570 mu and 600 mu were recorded on a standard microplate reader (Dynatech). Absorbancies at 600 were subtracted from Absorbancies at 570 and corresponding readings in the positive control (high concentration cisplatin/anguidine) wells were subtracted from the readings of each drug-exposed well. Each value so determined was divided by the corresponding values from the negative (vehicle) control wells (0.9% NaCl), also with positive control readings subtracted. The above result provided a crude (relatively insensitive) index of drug-induced cell death, which was, none-the-less, useful as an additional quality control to ensure that the microplate wells were correctly spun down on correctly-labeled DISC assay Cytospin slides.

DISC assay Cytospin slides were prepared as previously described ⁷, with the addition of acetaldehyde-fixed duck red blood cells ⁹, which, in the present assays, was used primarily as a quality control to gauge the uniformity of Cytospin cellular "disks" ("spots"). Post-culture slides were subjectively scored to gauge tumor cell death, most accurately scored by determining percent of control cell survival. The method of slide scoring is also of critical importance, and will be described in detail.

As originally described ⁹, a ratio of "living" (H&E-stained) tumor cells over duck red blood cells (DRBC) was determined by counting individual cells and keeping track of the cell counts with a standard hand tally counter, of the type used in manual complete blood counts. However, this method is not accurate for solid tumor microclusters. Therefore, the following method was used:

Slides are first inspected to determine which cells and clusters are tumor cells and which, if any, are normal cells, using standard cytopathologic criteria. Thereafter, DISC assay Cytospin slide "disks" are scored primarily at a magnification of 40X. The four negative control (0.9% NaCl vehicle) slides are scanned to determine mentally how slides appear in the complete absence of drug effect. The analogy is scoring Olympic diving or gymnastics, where the best possible score is a

"perfect" 10. Thus, the appearance of the negative control slides is calibrated mentally as a "perfect" 10, which is converted to 100, representing 100% of control cell survival. Then the positive control slides are scored. Typically (but not always), these slides show no viable cells, but many bluish-green dead cells and debris, and the appearance of these slides is mentally calibrated to represent a "belly flop," or zero percent of control cell survival. Thereafter, each slide is scored in turn. Slides appearing similar to the negative control slides would have percent cell survivals close to 100, while those similar to the positive control slides would have percent control cell survivals close to zero. A slide appearing half way between the negative and positive controls would have a value near 50, and so on. The slide scorer is permitted to use any degree of precision with which he/she is comfortable. The first author (LMW) who personally scored all of the assays reported in this paper, feels that he can distinguish between, for example, an "82" and an "87" and, thus, records his scores to the single digit. Other slide scorers have felt comfortable only with gradations of 5 (e.g. 80, 85, 90) or 10 (e.g. 70, 80, 90) and sometimes with even less precision than this (e.g. 0, 25, 50, 75, 100). This system of slide scoring may impress a reader with no experience at doing this as being impossibly subjective and imprecise, but it is actually very accurate and much more reproducible, as described below, than are, for example, cytopathologic scorings of immunocyto-chemical staining for estrogen receptors, Her2/neu or other antigens.

It is very important to note that it was not the purpose of this study to compare and contrast the DISC and MTT assays, in the form of a head to head competition. Indeed, there was only a single culture well (not a duplicate) for each drug concentration in both DISC and MTT assays. Both assays, taken together, provided a duplicate for each drug concentration tested. DISC results were not blinded as to MTT results and MTT results were at times discounted as being less reliable, because of obvious disagreement with the DISC assay, in cases where the DISC assay was deemed to be the more reliable. Likewise, when DISC and MTT results did not agree in the case of an individual drug or drug concentration,

the slide would be re-evaluated and the score revised, if necessary, in cases where the MTT assay alerted the slide scorer as to a possible error. In other cases, both DISC and MTT results were deemed to be credible and equally likely to be "correct," but remained in disagreement and were just left as such on the raw data portion of the final assay report provided to the referring physician. Such disparities were noted as such on the final report.

As described below, however, this was a true prospective study, and, once the assays were completed and faxed/mailed to the referring physicians, not a single DISC or MTT assay result (DISC assay slide score or MTT formazan signal measurement) was ever altered, either on the reports or in the archived database. The stringency of this database control is further described below.

Although the results reported to the referring physicians and reported here were not blinded between DISC and MTT, we did determine the reproducibility of the slide scoring by having two different people "blindly" score the DISC assay slides, without knowledge of the MTT results and without knowledge of the official slide scores (non-MTT-blinded) determined and reported by the first author (LMW). The non-blinded slide scorer (LMW) is a medical oncologist without formal cytopathology training who is self-taught by virtue of reviewing and scoring tens of thousands of slides from thousands of fresh human tumor specimens over a 23 year period. The first blinded slide scorer (MS) is a former clinical hematology technician who has worked with LMW carrying out fresh tumor drug resistance assays over a 10 year period. The correlation coefficients between both slide scorers for cisplatin assays in ovarian cancer were 0.94 (n=52) for the higher of the two tested concentrations and 0.85 (n=52) for the lower of the two tested concentrations. Even better agreement between the two slide scorers has been recently obtained when the scores of a second hematologic medical technologist with a stronger background in microscope slide evaluation were compared with those of LMW. The first author (LMW) is quite certain that the requisite degree of precision in slide scoring may be readily achieved

by cytopathologists and cytotechnologists, following training appropriate for the learning of other standard microscope slide evaluation skills of similar importance.

The MTT assay was plated, incubated with drugs, and cultured precisely as in the case of the DISC assay. MTT reagent (thiazolyl blue, Sigma) was prepared as follows: Firstly, 10 mg of succinic acid was dissolved in 10 ml H₂O to prepare a 1 mg/ml solution. Then 500 mg of MTT/thiazolyl blue was dissolved in 100 ml H₂O and 0.027 ml of 1 mg/ml succinic acid solution. The solution was decanted from any undissolved MTT crystals and aliquoted into single use, light protected vials for storage at -15 degrees. At 92 hours into the cell cultures (plus or minus 2 hours), 20 ul of MTT reagent (prepared with succinic acid, as described above) was added to each of the culture wells and cells were incubated for 4 hours. 110 ul of supernatant fluid was thereafter aspirated. 110 ul of greater than 4-week-aged, 0.4 N HCl-isopropyl alcohol was added to solubilize the purple MTT formazan crystals and plates were read at 570 mu. In cases where the coefficient of variation among the 4 negative control wells exceeded 10% or in cases where the formazan signal in the positive control wells exceeded 0.40 Absorbance units, plates were centrifuged, supernatants were withdrawn and transferred to new 96 well flat-bottomed polystyrene plates. Based on consideration of the results of the readings in the respective plates (in situ culture plates versus medium supernatant plates) a decision was made whether to average the two values or to use one versus the other (most typically they were averaged). Once reported, however, these MTT results thusly calculated were never changed in the database.

In rare cases, it was determined in advance that either the DISC or MTT assay would likely not yield useful results (e.g. in the case of an extremely mucinous specimen, where preparation of high quality DISC assay Cytospin slides would be problematic, or in a situation where only a minority of viable cells were consistent with tumor cells, in which case it would not be possible to separate drug effects on tumor versus non-tumor cells in the MTT assay). In these cases, both 96 well culture plates would be

processed as either duplicate DISC assays or duplicate MTT assays.

Technical features of the assays

In the 450 evaluable assays, the mean ratio of viable tumor cells at the conclusion of the cell cultures relative to the beginning of the cell cultures was 0.82 (standard deviation = 0.29, median = 0.85). Thus, drug effects were being measured on a population of presumably non-dividing or slowly-dividing cells, in which there was a net loss of viable tumor cells during culture. At the conclusion of the cell culture, a mean of 84% of all viable cells were judged to be tumor cells (standard deviation = 20, median = 90). This high percentage of tumor cells post culture is attributable to efficient enrichment for tumor cells with the cell isolation protocols, selectively rapid death of normal connective tissue cells during the four days in culture in the anchorage independent culture conditions, and selective loss of normal epithelial and inflammatory cells, when present. Based on 23 years experience with the DISC assay (in which culture conditions and drug exposure is identical to the those used by us in the MTT assay) the 4 day (96 hour) culture is optimum with respect to allowing for selective loss of normal cells, relative to tumor cells. This high percentage of tumor cells is also responsible for the overall high correlation coefficient between DISC and MTT assay results ($r = 0.85$ for both cisplatin and carboplatin, high concentration; $r = 0.81$ for cisplatin, low concentration; $r = 0.78$ for carboplatin, low concentration). The mean Absorbance in the MTT assays (negative controls minus positive controls) was 0.58 (standard deviation = 0.25, median = 0.56). It was important to avoid plating excessive numbers of cells in the cultures such that the MTT Absorbance (negative minus positive controls) exceeded 0.82. Cultures with MTT Absorbance signals greater than 0.82 tended to be associated with artifactual resistance to platinum (to be described in a future publication).

The median percent of total tumor cells in three dimensional clusters (as opposed to being discohesive cells) at the beginning of the cultures was 80 and the median at the conclusion of the

cultures was 85. The median cell cluster two dimensional area at the beginning of culture was 870 microns squared and at the conclusion of culture was 2300 microns squared. At the beginning of cell culture, 1% of all specimens were comprised entirely of discohesive single cells, and this remained 1% at the conclusion of culture. In specimens containing cell clusters, as scored at the beginning of the cell cultures, 17% of these contained predominately "loose" clusters (defined above), 78% predominately "medium-dense" clusters, and 4% predominately "tight" clusters. Corresponding percentage of assays having cell clusters at a given density at the conclusion of cell culture were 25% predominately "loose," 69% "medium-dense," and 6% "tight."

Assays were also rated according to technical quality, rating the following parameters: ratio of viable tumor cells on Day 4, relative to Day 0; internal agreement between MTT assay readings on negative control quadruplicates and positive control duplicates; "correct" dose-response relationships (higher drug concentrations producing greater degrees of cell death than lower drug concentrations; magnitude of MTT formazan signal; overall agreement between DISC and MTT assays; technical quality of DISC assay cytospin slides; number of drugs successfully tested, and occasionally other subjective factors. In general, an "excellent" assay required a Day 4/Day 0 viable tumor cell ratio of 0.75 or greater and no major problems with any of the other parameters; a "good" assay would require a Day 4/Day Zero ratio of 0.5 to 0.74 and no more than one, relatively minor, problem with one of the other parameters. A "fair" assay would require a Day 4/Day Zero ratio of 0.25 to 0.49 and problems with no more than two other parameters. A "poor" assay would have a Day 4/Day Zero ratio of less than 0.25 and/or problems with multiple other parameters. These technical quality ratings were also reported to the referring physicians along with the original assay results and were never changed thereafter. Technical quality is an important variable in the clinical and biological correlations with cell culture assays, to be reported in the future.

In the case of the present ovarian cancer dataset, 45% of assays were classified as being technically excellent, 33% good, 17% fair, and 4% poor. The high evaluability rate and preponderance of high quality assays, along with the usually generous quantity of viable tumor available, makes ovarian cancer an unusually attractive neoplasm from the standpoint of a target neoplasm in which to utilize cell culture drug resistance testing.

Database Structure and Integrity

It was our intention from the beginning of our operations to have the ability to analyze and report our data in the peer review medical literature in the form of prospective studies. Serious disadvantages for us included: (1) The fact that we are a commercial, for-profit laboratory, providing a clinical service which is deemed by many oncologists to be quite controversial^{8,10}. This fact raises the specter of conflict of interest, and we knew in advance that we must provide credible and verifiable documentation of the complete accuracy and integrity of the data to be reported. (2) The fact that we are an independent laboratory, not affiliated with a major medical center or formal research group, making the retrieval of clinical data for comparison with assay results highly problematic.

Therefore, the following procedures were followed:

1. As each specimen was physically received, it was immediately logged by hand in ink into a permanently-bound composition book, called the "accession book." Specimens were entered consecutively, with columns recording the date, patient name, laboratory case number, clinical diagnosis as reported either on the requisition accompanying the specimen (preferably) or with a diagnosis obtained by telephone, anatomic site of origin, hospital, city, state, and referring physician. This specimen log (which may be authenticated by many past and present employees) provides an auditable record of every specimen ever received by our laboratory.

2. In addition to the hand-entered specimen log, all pertinent data for analysis were entered into a

MicroSoft FoxPro (dBase-type) relational PC computer database. Pertinent data recorded included clinical diagnosis (occasionally revised prior to the report, based on the receipt of subsequent information), histologic diagnosis, including histologic subtype and tumor grade, if provided with the faxed copy of the official pathology report (always obtained prior to the reporting of the assay results), specimen site of origin, prior chemotherapy (Yes/No), drugs administered, date of last treatment, date of biopsy, date of specimen receipt, date of report, patient's name, birthdate, social security number, insurance company data, referring physicians (surgeon, pathologist, and oncologist all entered when this information was available), percentage of total viable cells which were consistent with tumor cells, ratio of viable tumor cells in the control cultures at the conclusion of the 96 hours of culture ("Day 4") as compared with viable tumor cells present at time zero ("Day 0"), magnitude of the MTT formazan signal (negative control minus positive control) in non-drug exposed cultures at the conclusion of the assay, percentage of total tumor cells which were in the form of three dimensional clusters on Days 0 and 4, average size of cell clusters Days 0 and 4, average density (as defined above) of cell clusters D0 and 4, drugs and drug concentrations tested, percent of control cell survival for each drug at each concentration, with each assay endpoint (DISC and MTT), results for DISC and MTT assays at each drug concentration averaged together, calculated correlation coefficient for DISC compared with MTT assay results, calculated (interpolated/extrapolated) "LC50" (drug concentration required to kill 50% of the tumor cells), overall technical quality of the assays (excellent, good, fair, poor, inevaluable, with each of these categories assigned according to predetermined criteria described above; n.b. assay results in the present paper are NOT subgrouped according to these categories of technical quality), and overall classification of result for each drug as far as being "sensitive," "intermediate," "resistant," or (extremely) resistant-"EDR" (based on comparisons with results from other assays, using criteria described below).

3. What is most important to emphasize is that EACH specimen received and logged by hand into the accession book, can be cross referenced with EACH record in the electronic database, to ensure the accuracy and completeness of the dataset to be analyzed. Furthermore, EACH of the assay-related parameters described above was PROSPECTIVELY reported to each of the (usually two or three, namely surgeon, pathologist, and/or oncologist) referring physicians on formal, written assay reports both faxed and then mailed at the conclusion of each assay. Thus, both faxed and mailed copies of complete reports, including not just "sensitive," "intermediate," and "resistant" results for each drug, but also all of the above raw data were typically sent to two or three different physicians associated with each specimen. Once the official reports were mailed (typically 6 to 30 days following specimen receipt), none of the data reported on the final assay reports were ever deleted from or altered in the electronic database. Thus, the integrity of the data and data analysis may be confirmed through an audit which would compare (1) consecutive handwritten entries in the bound accession book, (2) electronic database, along with hard copies of the official reports, archived in the individual chart permanently archived for each patient (which also contains all raw records from the microplate readers and hand entries of the DISC assay slide scoring, etc.), (3) the permanently-archived DISC assay Cytospin slides (nb: in the DISC assay, the entire content of each culture well is cytocentrifuged onto a permanently archived microscope slide, so that the post culture effects of each drug tested may be independently assessed and confirmed for each patient under consideration and at any future time), and (4) official reports submitted for each specimen to the referring physicians (e.g. a random sampling of cases could be selected for audit, referring physicians who received reports could be contacted by phone, and faxed copies of the original reports submitted to the physicians, which included all relevant raw data, as well as the "sensitive," "intermediate," "resistant" determinations could be compared with the data residing in the electronic database). Thus, we believe that the present study fulfills all reasonable requirements for an externally verifiable, true prospective study.

Determination of overall survival using on-line data from the Social Security Death Masterfile (Social Security Death Index/"SSDI").

Death dates were assigned to each patient using only data obtained through electronic searches of the SSDI, retrieved through the following World Wide Web site:

<http://www.ssdigenealogy.rootsweb.com>

The SSDI is a database maintained by the US Social Security administration and mirrored on a number of commercial internet sites, including the above site, which we have found to be generally the most "user friendly" (subjectively useful).

A number of studies have examined the accuracy of the SSDI for use in determining the death dates of medical patients. Past studies, using different "gold standards," have found the SSDI to be 99 - 100% specific and 86 - 88% sensitive in determining death dates¹¹. This degree of accuracy was specifically reported in a recently-published study which used our exact search algorithm (described below) on the above World Wide Web site¹¹. The above studies, however, examined deaths occurring in earlier time periods, and the current sensitivity may be substantially higher.

Searching the internet, we discovered a much larger and more recent study, covering a cohort of 6590 deaths in Department of Energy workers between 1994 and 1997¹². The first author of this study was interviewed by telephone by LMW to obtain details for the specific study cohort in which complete demographic information was available (i.e. social security number, name, and date of birth), as all of these parameters were available for patients in our own, presently-described study. In the telephone interview of October 28, 2002, the senior author (Dr. Mary Schubauer-Berigan of the National Institute of Occupational Safety and Health, Cincinnati, OH) provided the following statistics for sensitivity/specificity of the Social Security Death Master File in cases where the Social Security number, name, and birthday were available: 98.06% sensitivity (95% confidence interval 97.8

to 98.3) and specificity greater than 99. The "gold standard" for the sensitivity comparison was the National Death Index, which has, itself, been documented to be 97% sensitive^{13,14}.

We searched the SSDI, using the above web site, according to the following protocol. First, we entered the social security number. If this then resulted in a match for a patient with the same name and birthdate as the patient in question, this was considered to be a true date of death and this date was entered into our database. If the social security number did not result in a match, we then entered the patient's last name, and compared the resulting retrieval with first name and with date of birth. If we had a match by name and date of birth, but not by social security number, we then re-reviewed the chart to see if there was any indication that the social security number entered into the database was entered correctly. We cross-checked our financial (billing) database and billing records to determine if there was some mistake or omission. Ultimately, if we were able to match name, date of birth, and social security number obtained from a credible source with the name, date of birth, and social security number in the SSDI, then the date of death listed in the SSDI was entered into our database as the date of death of the patient. If we had a social security number, patient name, and patient date of birth and there were no SSDI matches searching by either social security number or last name and birth date, then the patient was (for purposes of data analysis) considered to be alive as of the date of the last SSDI update (listed on the website). To allow for adequate follow-up, the present data analysis was restricted to patients who had their biopsy (and assay) date a minimum of two years prior to the date of the last SSDI update in our data retrieval. In the case of the current paper, the date of the last SSDI update was June 30, 2002, and we therefore restricted the data analysis only to patients whose biopsies had been performed on or prior to June 30, 2000. The entire period covered by this data analysis included ovarian cancer specimens received between January 1, 1993 and June 30, 2000. The start date of January 1, 1993 was selected as the beginning date by which the above-described assay and reporting methods had been standardized.

It is important to note that patients for whom we did not have a social security number were censored in the data analysis, even in a case where we had a valid follow-up or death date from some other source of information. Among previously-untreated patients with reported results for cisplatin and/or carboplatin, there were 115 patients for whom we had names, birth dates, and social security numbers. Two additional patients did not have social security numbers, including one European patient whose specimen was sent from Europe and one 80 year old patient who never had a social security number. Only these two patients were censored. The European patient had an incomplete assay (MTT only; DISC technically unsuccessful). The 80 year old patient had an assay which showed clear cut sensitivity to platinum and was independently confirmed to be alive 5 years following biopsy, but was censored, none-the-less, to ensure consistency in data analysis. In the case of previously treated patients, there were 327 cases where we had all three types of required demographic information (name, birth date, social security number) and 6 additional cases which did not have a social security number. These latter 6 cases were, likewise, censored. Thus, of a total of 450 cases (117 untreated and 333 treated), there were 442 cases (98.2%) in which we had complete information and which comprised the database for the survival comparisons, and only 8 cases (1.8%) which required censorship for lack of a social security number. For untreated patients (the main subject of this paper), the corresponding numbers were 98.3% evaluable and 1.7% censored.

It is likewise important to note that all patients falling within a given assay category ("sensitive," "intermediate," "resistant") were included in the analysis, even if the patient was a very "early death," i.e. occurring within the first week from biopsy date. As in the case of the "censored" patients (for whom censoring actually weakened correlations), including "early deaths" likewise weakened correlations (e.g. as in the case of a previously-untreated patient with a highly "sensitive" assay, who died 7 days after the biopsy, before any chemotherapy was actually administered and before the assay was even completed (we did not learn of the patient death

until after the assay had been completed and reported. The inclusion of this "early death" obviously weakened the correlations between assay results and patient survival, but the patient was not censored to maintain consistency). It was the intent to be as neutrally-objective as possible in the data analysis, to prevent bias from skewing the results.

Methods for prospective comparisons between platinum resistance and patient survival

As noted above, prospective assay reports included both the overall assay results for each drug (in terms of "sensitive," "intermediate," "resistant," and "resistant-EDR," defined below), as well as the complete raw data (percent of control cell survival in DISC and MTT assays at both tested drug concentrations).

In categorizing a given result as "sensitive," "resistant," etc., the following methods were used. For each drug concentration, an overall database of "training set" assays was generated. This consisted of first hundreds and then thousands of fresh tumor assays, performed on a wide variety of human neoplasms, including adenocarcinomas of all types, squamous carcinomas, sarcomas, melanomas, neuroendocrine tumors, glioblastomas, and other, more rare types of neoplasms. For each drug concentration in each of the two assays, we calculated an overall assay mean and standard deviation. Fig. 1 shows how these data were used to define the ranges of results for "sensitive," "resistant," "intermediate," and "resistant-EDR" (where EDR is an acronym for extreme drug resistance, as defined previously by the first author¹⁵).

An "intermediate" result in each database would be a result falling within plus or minus 1/2 standard deviation from the mean. A "sensitive" result would be equal to or greater than 1/2 standard deviation below the mean (in terms of percent control cell survival). A "resistant" result would be equal to or greater than 1/2 standard deviation above the mean. "Resistant-EDR" would be equal to or greater than a full standard deviation above the mean.

Figure 1: % Control Tumor Cell Survival, Calculated as Deviations from Mean of Reference Database Assays

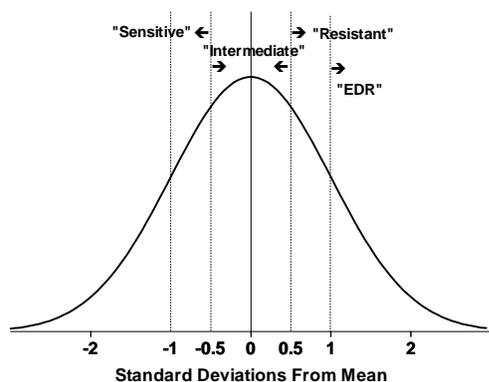


Figure 1. Method for objectively determining "sensitive," "intermediate," "resistant," and "resistant-EDR" cut-offs in cell culture drug resistance testing (CCDRT). For each dataset (e.g. DISC assay high concentration, MTT assay low concentration, etc.) an overall mean % control tumor cell survival (survival of tumor cells in drug exposed cultures, relative to survival of tumor cells in control cultures) is calculated, along with the standard deviation from the mean. Cut-off boundaries between "sensitive," "intermediate," etc. are objectively identified as shown in the schematic diagram.

In cases where the result in all 4 databases (DISC assay/high concentration, DISC assay/low concentration, MTT assay/high concentration, and MTT assay/low concentration) were concordant with respect to the categorization of the result as "S,I,R, or E," then this was the result reported. If there was disagreement between one or more categories, the result was generally rounded toward intermediate. For example, if a given result was "S,I,S,I" in the four respective databases, the result was reported as "I." As the purpose of performing the assays was to provide information to be considered in treatment planning, the first author (LMW) did apply what might be described as common sense subjectivity in classifying the final results to be reported. For example, if one of the 4 categories did not agree with the other three and there was some obvious reason to disbelieve the outlying result (e.g. poor slide preparation in the DISC assay; loss of formazan crystals while pipetting a mucous plug in the MTT assay, etc.) then the outlier was

ignored and the results based on the results which were technically more optimum. As another example, if two of the four results (particularly the results with the higher of the two tested concentrations) indicated clear cut resistance but the other two were near the borderline between "resistant" and "intermediate," then the final result (as reported) would usually be classified as "resistant."

It is important to recall that most cancer tests involving either anatomic pathology or radiology involve varying degrees of physician-dependent subjectivity. In these latter examples, as with our cell culture drug resistance tests, the consulting pathologist or radiologist is obliged to use his/her best judgement in conveying to the referring physician what is believed to be the most accurate interpretation of the tests which is possible, given all of the complex challenges inherent in performing the tests.

But it is again important to emphasize that all of these determinations were made prior to reporting the assay and prior to the time when any follow-up information regarding patient survival was obtained. It is additionally important to emphasize that, once reported, none of the results were ever changed or censored in the database used as the basis of the present analysis.

Methods for retrospective comparisons between platinum resistance and patient survival

After the entire dataset for this study had been accrued, we re-analyzed the data based on entirely objective, rigid cut-offs to define the various categories of drug resistance. For this analysis, we used the exact definitions of "S,I,R,E" described above, only these cut-offs were defined exclusively with the database subset of ovarian cancer assays being analyzed. For example, a computer program took the data subset of 115 assays performed on previously-untreated ovarian cancer assays and calculated an overall mean and standard deviation, and the categorized each of the 115 results as being "S,I,R,E" with respect to how each result fell into the "S,I,R,E" categories so defined. This might be termed "subset specific" "S,I,R,E" definitions, as opposed to "global" definitions (based on the entire database

of all assays from all types of tumors, not restricted to ovarian cancer and not restricted only to assays on specimens from previously-untreated patients). Likewise, when re-analyzing data for previously-untreated patients with poorly-differentiated tumors, the retrospective analysis was carried out by first retrieving (by computer program search) the assay results from only the 60 patients with tumors described specifically as being "poorly-differentiated," then using these results to define "S,I,R,E" cut-offs, and then classifying (by computer) each result (for DISC assay/high concentration, DISC/low, MTT/high, and MTT/low) as either S, I, R, or E, based on the specific cut-offs defined for the database of poorly-differentiated tumors.

For purposes of the present analysis, "Resistant-EDR" was lumped together with "Resistant" to comprise a single category of "Resistant" assays. The reason for this was that there were only about a half dozen cases of "Resistant-EDR" among the previously-untreated patients.

In these latter (retrospective) studies, S,I,R classifications were, as noted, determined for each of 4 individual databases (DISC high concentration database, DISC low concentration database, MTT high database, and MTT low database). Results were further categorized in the following manner: "Sensitive" required individual categorization of results in each of two databases (i.e. DISC high/MTT high; DISC high/DISC low; MTT high/MTT low; MTT low/DISC low) to fall in the "sensitive" range; "resistant" required results in each of the two databases to fall in the "resistant" range; "intermediate" included all other patterns of results (e.g., in this retrospective, but entirely objective categorization of results based on inclusion of results in two of the databases, the following results would all be categorized as "intermediate:" DISC high/resistant combined with MTT high/intermediate; DISC high/resistant combined with MTT high/sensitive; DISC high/intermediate combined with MTT high/intermediate, etc.).

In summary, both prospective and retrospective results will be shown. The prospective results are the (partially subjectively categorized) results

actually reported at the time of the original assay, while the retrospective results are the (completely objective, rigidly defined) results calculated using subset-specific databases at the conclusion of the study. Once again, however, it is important to note that the retrospective analysis was performed entirely on uncensored, unaltered raw data which was prospectively reported to the referring physicians at the time of the assays and that these data lend themselves easily to external audit.

Statistics

All statistical calculations and graph constructions were performed with Prism 2.01 software from Graph Pad Software, San Diego, CA, <http://www.graphpad.com>. Differences between Kaplan-Meier survival curves were tested by log rank test. Differences between means were tested by Student T test in cases where the standard deviations of the means of the two different groups were not significantly different and by Welch's alternate T test in the uncommon case where the standard deviations of the means were significantly different. All P values are 2-sided.

Clinical Results (other important, but purely technical results were described above in the Methods section)

Specimens received and evaluability rates

Between January 1, 1993 and June 30, 2000, we received 467 specimens which were diagnosed as either "ovarian cancer" or "probable ovarian cancer versus fallopian tube cancer" or "probable ovarian cancer versus primary peritoneal serous carcinoma" (or very similar words with same connotation). All of the above cases were included in the dataset which was analyzed. On the other hand, cases labeled "adenocarcinoma, possible ovarian," "ovarian versus pancreatic" (or similar non-gynecologic primary), or "peritoneal carcinoma (without the descriptor probable ovarian)," or "endometrial adenocarcinoma," or other non-ovarian or equivocal diagnoses were not included in this data analysis.

Of the above specimens received, 10 contained no viable tumor cells upon receipt or were subsequently officially reported out as

histologically or cytologically negative and no assays were plated. In 7 cases, assays were plated, but the assays failed for one of several reasons, such as cell death in culture, microbial contamination, or intractable mucin artifacts. Thus, 450 out of 467 cases provided evaluable cell culture drug resistance assays, for an overall assay evaluability rate of 96%. Note that this evaluability rate is based on every specimen received and is not based only on histologically or cytologically positive and/or viable specimens received. In the 96% of cases which were evaluable and in which results were reported back to the referring physicians, an average of 18 drugs were tested at two concentrations with both DISC and MTT endpoints (in addition to the 4 negative controls and 2 positive controls for each assay tested with both DISC and MTT endpoints).

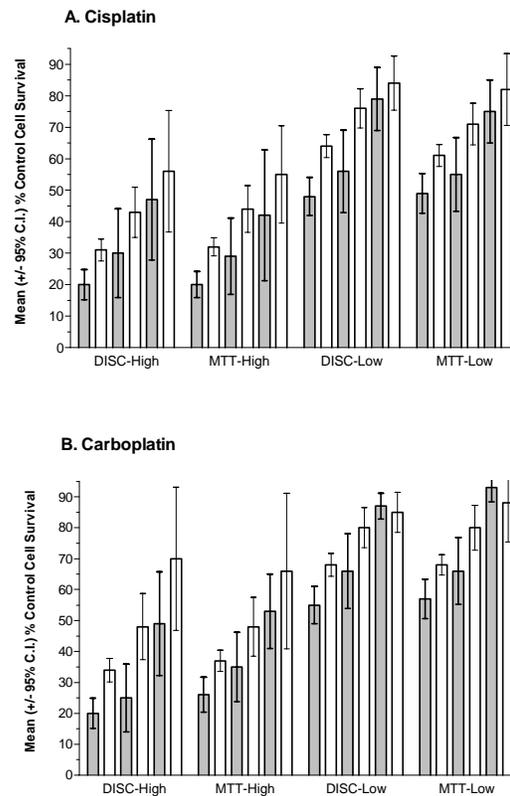
Platinum resistance as a function of prior treatment status and tumor histology

Figure 2 shows that specimens from previously treated patients were significantly more resistant to cisplatin and to carboplatin than were specimens from previously untreated patients. Considering the entire universe of all assays, not grouped according to the subsets shown in Figure 2, the 2-sided P value was <0.0001 in all four assay databases (DISC High concentration, DISC Low, MTT High, and MTT low) for the difference in activity between tumors from untreated versus treated patients for cisplatin and also for carboplatin.

Figure 2. Mean (+/- 95% confidence intervals) for CCDRT results on ovarian tumors, grouped by histopathologic differentiation and prior treatment status in each of the 4 assay datasets (DISC assay-high concentration, DISC assay-low concentration, MTT assay-high concentration, and MTT assay-low concentration). In each dataset, the shaded bars represent CCDRT results on specimens from previously-untreated patients, while open bars represent results on specimens from previously-treated patients. Each group of 6 bars is ordered according to tumor differentiation. The two left-most bars represent poorly-differentiated tumors. The two middle bars represent moderately-differentiated tumors. The two right-most bars represent well-differentiated tumors. All differences between untreated and treated for

poorly differentiated tumors are significant at two sided p-values ranging from <0.0001 to 0.0009. Most differences between untreated and treated for moderately differentiated tumors are significant at two sided p-values ranging from 0.0044 to 0.080. All differences between poorly-differentiated and well-differentiated for tumors from previously-untreated patients are significant at two sided p-values ranging from 0.0002 to 0.0058.

Figure 2: Platinum resistance as a function of prior treatment and tumor differentiation.



There was also a clear relationship between drug resistance and degree of differentiation, with the borderline (only 3 specimens; data not shown) and well differentiated tumors (for which the data are shown) being clearly more resistant to cisplatin and carboplatin than poorly-differentiated tumors, and with moderately-differentiated tumors falling in between. There were no significant differences between serous and endometroid tumors, and, in both cases, tumors from previously-treated patients were more resistant to cisplatin than tumors from previously untreated patients (2-sided P values ranging between 0.0007 and 0.0031 for serous tumors and between 0.0094

and 0.24 for endometrioid tumors). There were no significant relationships comparing untreated and treated tumors in the cases of clear cell (n=4 untreated and n=16 treated) and mucinous (n=7 untreated and n=8 treated) tumors, although the numbers of clear cell and mucinous tumors were obviously quite limited.

Patient survival as a function of tumor differentiation

Figure 3 shows that untreated patients with borderline and well differentiated tumors enjoyed a very favorable survival, despite the fact that these tumors were generally platinum resistant (see Figure 2). Also shown in Figure 3 is the survival of a subset of patients expected to have a very poor prognosis, namely patients previously-treated with at least two different chemotherapy regimens, who had received their most recent chemotherapy within three months prior to their assay date, and for whom the specimen submitted was either a pleural or pericardial effusion or ascites specimen. This group is included for the purpose of serving as a methods control relating to the use of the online Social Security Death Master File (Social Security Death Index, SSDI) and the search protocol described above in the Methods. We believe that these (and similar data not shown) provide further support for our use of the online SSDI to obtain clinically-relevant survival data.

Patient survival as a function of platinum resistance

A. Prospective evaluation

Table 1 shows that cisplatin resistance was predictive of survival in the entire dataset of previously-untreated patients. Figures 2 and 3 strongly imply that platinum resistance is not related to survival in patients with well differentiated tumors. Therefore, in the dataset of previously-untreated patients, we analyzed two subsets of data, firstly omitting only specimens which were specifically described as being well-differentiated tumors and secondly including only specimens which were specifically described as being poorly-differentiated tumors. There were insufficient numbers of patients with tumors described as being moderately-differentiated to include this as a separate data subset for survival analysis. Table 1 and Figure 4 show that omitting tumors specifically described as being well-differentiated increased the significance of the relationships between platinum resistance and patient survival. Including only tumors specifically described as being poorly differentiated further increased the significance of the relationship, despite the fact that the numbers of patients in the datasets were reduced, reducing the power of the datasets to detect differences (Table 1, Figure 5).

Figure 3: Patient survival as a function of tumor differentiation

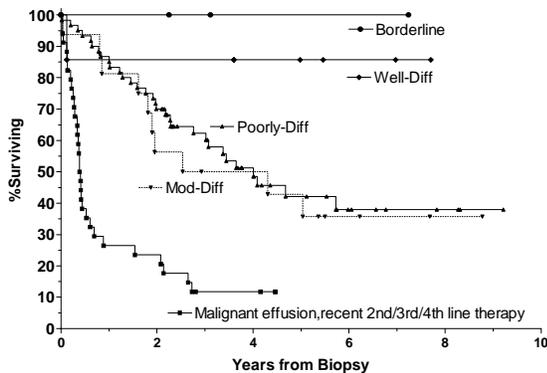


Figure 3. Kaplan-Meier survival curves of previously-untreated patients, grouped according to tumor differentiation (borderline, well-differentiated, moderately-differentiated, and poorly-differentiated). Also shown (as a methods control, as described in the text of the Results), is the survival of previously-treated patients who had been treated within the three months prior to CCDRT with either 2nd, 3rd, or 4th line chemotherapy and for whom the source of the specimen tested was a malignant effusion (peritoneal, pleural, or pericardial).

Table 1. Correlation between platinum resistance and overall patient survival (in days)

Basis of "S,I,R" Classification	n	Med.Surv."Resistant"	M.S."Intermediate"	M.S."Sensitive"	P2(S vs R)	P2(Trend)
Prospective Correlations						
UnRxd Patients,Cisplatin	115	680	1110	1491	.0370	.143
UnRxd Patients,Cispl,-Well Diff	108	662	1102	1491	.0108	.0383
UnRxd Patients,Cispl,Poorly-Diff	60	711	1008	1707	.0082	.0062
UnRxd Patients,Carboplatin	110	778	1231	1571	.175	.219
UnRxd Patients,Carbopl,-Well Diff	103	723	1102	1571	.0747	.0571
UnRxd Patients,Carbopl,Poorly-Diff	54	1082	1166	Not Reached	.0939	.0393
Rxd Patients,Cisplatin	336	447	421	700	.466	.484
Rxd Patients,Cisplatin,-Well Diff	326	404	421	700	.362	.362
Rxd Patients,Cisplatin,Poorly Diff	137	470	385	721	.160	.114
Rxd Patients,Carboplatin	286	470	443	541	.567	.549
Rxd Patients,Carbopl,-Well Diff	280	469	443	541	.554	.550
Rxd Patients,Carbopl,Poorly-Diff	125	658	439	870	.307	.241
Retrospective Correlations						
UnRxd Patients,Cisplatin	113	704	925	2775	.0057	.0048
UnRxd Patients,Cispl,-Well Diff	106	704	833	2058	.0023	.0018
UnRxd Patients,Cispl,Poorly-Diff	58	723	1707	Not Reached	.0013	.0035
UnRxd Patients,Carboplatin	107	833	1102	1971	.0302	.0179
UnRxd Patients,Carbopl,-Well Diff	101	723	925	1971	.0043	.0019
UnRxd Patients,Carbopl,Poorly-Diff	53	1008	1122	Not Reached	.0105	.0095
Rxd Patients,Cisplatin	311	409	498	592	.346	.305
Rxd Patients,Cisplatin,-Well Diff	302	367	503	622	.158	.143
Rxd Patients,Cisplatin,Poorly Diff	135	330	525	586	.140	.223
Rxd Patients,Carboplatin	278	509	415	541	.672	.602
Rxd Patients,Carbopl,-Well Diff	270	498	493	531	.838	.822
Rxd Patients,Carbopl,Poorly-Diff	123	395	493	701	.174	.155

The correlations between carboplatin resistance and patient survival were very similar to the correlations between cisplatin resistance and patient survival, although the carboplatin correlations were slightly less significant than the corresponding cisplatin correlations.

Using the same methods of analysis, there were no significant associations between platinum resistance and overall, long-term patient survival in patients who had been previously-treated (Table 1), more than 1/2 of whom had received at least two prior chemotherapy regimens). In analyzing smaller patient subsets, some significant correlations did emerge and will be described in a future report.

2. Retrospective evaluation

When cutoffs between "Sensitive," "Intermediate," and "Resistant" were objectively calculated (from the raw data reported prospectively to the referring clinicians) for the specific datasets being analyzed (as described in the Methods), the significance of the relationships between platinum resistance and patient survival increased substantially, as shown in Table 1 and Figure 6,7.

Figure 4: Prospective correlations between platinum resistance and patient survival, omitting well-differentiated tumors.

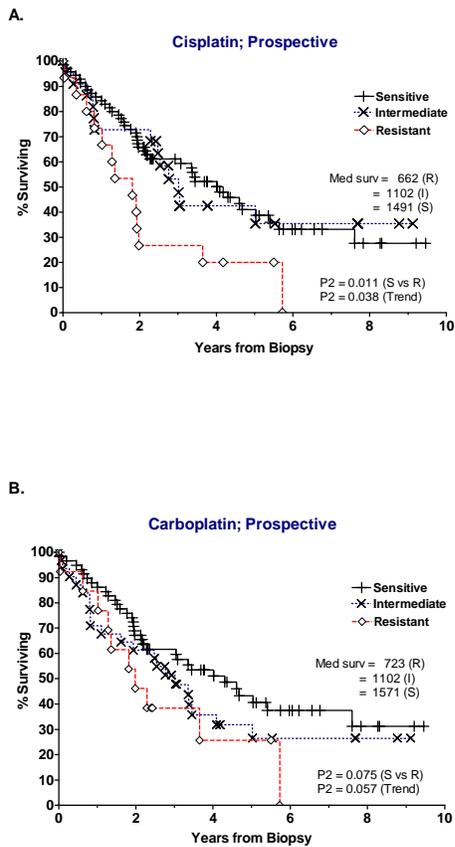


Figure 4. Kaplan-Meier survival curves from previously-untreated patients with tumors which were not specifically labeled as being either "well-differentiated" or "borderline." Patients are grouped according to results of CCDRT, as reported prospectively to at least two referring physicians (pathologist, surgeon, and/or oncologist).

As described in the Methods and shown in Fig. 1, we objectively classified tumors as being platinum sensitive, resistant, and intermediate by defining these categories specifically for each dataset based on calculated means and standard deviations for each dataset. Although correlations (and typically very strong correlations) emerged

between patient survival and platinum resistance with each dataset analyzed (e.g. DISC assay, high concentration database, considered alone; DISC assay, high concentration database considered along with DISC assay, low concentration database; DISC high/DISC low/MTT high/MTT low considered all together, etc.), the most consistently strong correlations emerged when the DISC assay, high concentration database was combined with the MTT assay, high concentration database. Tumors were considered to be "sensitive" to platinum if they were "sensitive" (one half standard deviation below the mean of percent control cell survivals of the dataset being analyzed) in both the DISC assay, high concentration database and MTT assay, high concentration database. Tumors were considered to be "resistant" if they were "resistant" (one half standard deviation above the mean of percent control cell survivals of the dataset being analyzed) in both the DISC assay, high concentration database and MTT assay, high concentration database. Tumors were considered to be "intermediate" with any other combination of DISC assay, high concentration and MTT assay, high concentration results.

Table 1 and Figures 6, 7 show highly significant correlations between the findings in the DISC and MTT assays, tested at a single (high) concentration, and overall, long-term patient survival. These strong correlations were found in (1) the entire universe of untreated patients, as well as in the subsets where (2) tumors described as being well-differentiated were omitted, and where (3) only tumors specifically described as being poorly-differentiated were included. These strong correlations were found both in the case of cisplatin and (independently) in the case of carboplatin. All hazard ratios ("sensitive" versus "resistant") were less than 0.5 and all were highly significant (data not shown).

Figure 5: Prospective correlations between platinum resistance and patient survival in poorly differentiated tumors.

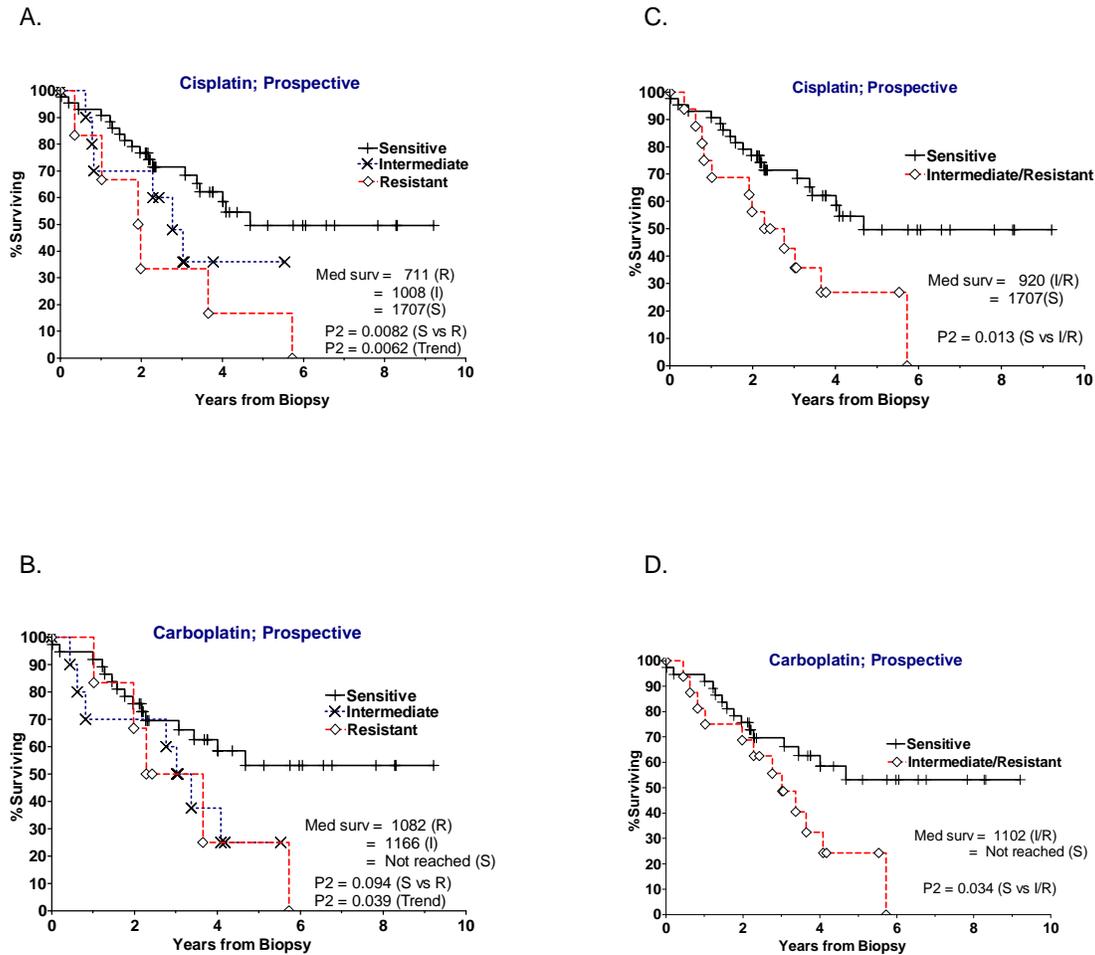


Figure 5. Kaplan-Meier survival curves from previously-untreated patients with tumors which were specifically labeled as being "poorly-differentiated" or "moderate to poorly-differentiated." Patients are grouped according to results of CCDRT, as reported prospectively to at least two referring physicians (pathologist, surgeon, and/or oncologist). Because of the relatively small number of tumors with "resistant" and "intermediate" results, these latter two categories are first shown separately in panels A and B, and then as lumped together in panels C and D.

As in the case of the prospective correlations, the patient survival relationship to cisplatin resistance determined retrospectively was most clearly evident in poorly-differentiated tumors (hazards ratio for cisplatin "sensitive" versus "resistant" = 0.31, 95% confidence interval 0.039 - 0.62, for results reported prospectively, and hazards ratio = 0.22, 95% C.I. 0.043 - 0.47, for cut-offs objectively calculated retrospectively, based on only the ovarian cancer dataset). In order to determine what effect, if any, a longer minimum follow-up

time would have on the results, we re-analyzed the data and restricted the analysis to patients with tumors tested a minimum of 5 years prior to the date of the last Social Security Death Master File update. Since the update we used was dated June 30, 2002, we restricted the analysis to the 66 patients with non-well differentiated tumors who were studied prior to June 30, 1997. For results reported prospectively, the hazard ratio was 0.38, 95% C.I. 0.11 - 0.64. For the objective, retrospective classifications of "sensitive"/ "resistant" cut-offs (as described previously) the hazard ratio was 0.35, 95% C.I. 0.11 - 0.68. Kaplan-

Meier survival curves on the patients with the minimum 5 year follow-up also showed highly significant differences on log rank test ($P_2 < 0.01$, analyzed both prospectively and retrospectively), data not shown.

Table 1 shows no significant relationship between platinum resistance and long-term survival for previously-treated patients, analyzed according to the same methods used for previously-untreated patients. Once again, this was a population of patients who had often received two or more different chemotherapy regimens prior to assay.

3. Parametric analysis of relationship between survival and platinum resistance

The median survival of all patients with tumor histologies not specifically classified as being well differentiated was 36 months. Figure 8 shows the mean percent of control cell survivals for assays on tumors from patients surviving (1) less than and (2) greater than 36 months. Once again, patients with shorter survivals had greater resistance to platinum. An additional, technical conclusion was that the higher of the two tested drug concentrations had greater power for revealing these differences. This is consistent with the finding (on the retrospective, but objective survival curve analysis) that the strongest relationships between assay results and patient survival were found when the high drug concentration results for DISC and MTT assays (considered together) were used as the basis for classifying tumors as sensitive, intermediate, and resistant.

As a methodologic consideration, we also infer from these data that fresh tumor assays of this type may be most accurate and useful when only one or two most optimal drug concentrations are used as the basis for classifying tumors as "sensitive" versus "resistant," rather than when this classification is based on a broad range of (strongly-predictive and non-predictive or only weakly predictive) drug concentrations to determine an "IC50" or "LC50" (nb: correlations based on interpolated/extrapolated LC50 values were always weaker than correlations based on results of testing at single, robust drug

Figure 6: Retrospective correlations between platinum resistance and patient survival, omitting well-differentiated tumors.

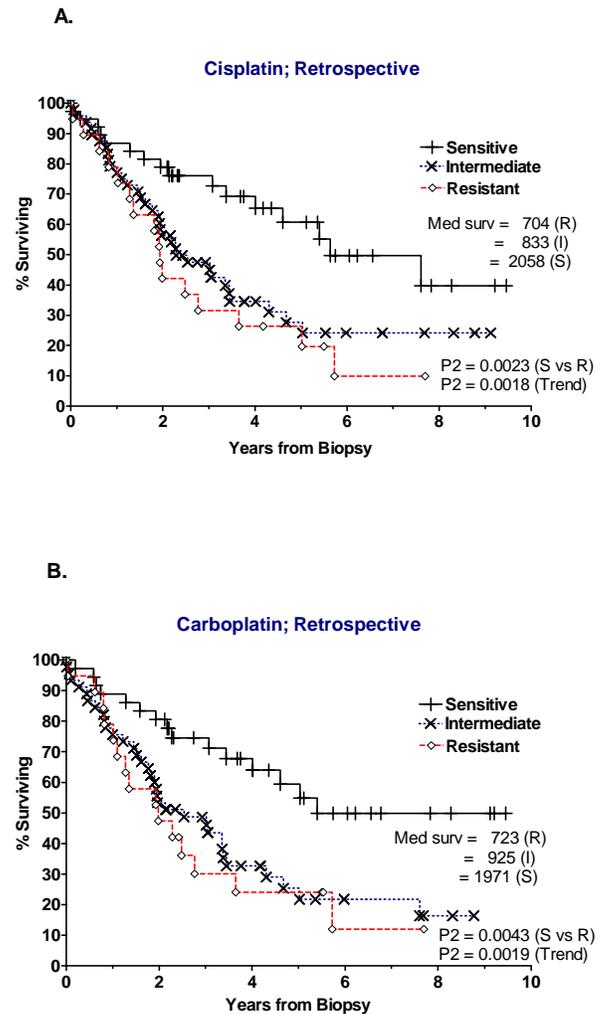


Figure 6. Kaplan-Meier survival curves from previously-untreated patients with tumors which were not specifically labeled as being either "well-differentiated" or "borderline." Patients are grouped according to results of CCDRT, as determined retrospectively (but objectively, as described in the text) from raw data reported prospectively to at least two referring physicians (pathologist, surgeon, and/or oncologist).

concentrations for DISC and MTT endpoints; however, it must be recalled that we tested only two drug concentrations, probably reducing the accuracy of LC50 determinations).

Discussion

There are two important conclusions from this study. Firstly, tumor cell resistance to cisplatin and carboplatin are clearly related to overall, long-term survival in previously-untreated ovarian cancer, as measured in short term cell culture assays in which 3 dimensional cell clusters are tested with cell death endpoints. Secondly, the cell culture drug resistance testing (CCDRT) system described here is now the most highly validated system for studying the circumvention of platinum resistance in human adenocarcinomas.

The present findings confirm and extend previous reports^{16,17} of a significant relationship between patient survival in ovarian cancer and the results of cell culture drug resistance testing (CCDRT) based on the concept of total cell kill (cell death, occurring in the total population of tumor cells). These previous reports, however, were retrospective studies using arbitrary cut-off boundaries, and they did not specifically examine platinum resistance. Previous studies have also showed consistently significant correlations between assay results and tumor response to chemotherapy⁸.

The associations between platinum resistance and overall, long-term survival were most clear in the group of patients with tumors described on the official histopathology reports as being poorly differentiated. Patients with well differentiated tumors had favorable survival despite having tumors which were largely platinum resistant. There were few patients with tumors specifically described as being moderately differentiated (n = 16) and there was not a clear relationship between platinum resistance and patient survival in this small group of patients (data not shown). These general findings are also consistent with the analysis of the overall platinum resistance in previously-untreated versus treated patients with poor, moderate, and well-differentiated tumors (Figure 2). Poorly-differentiated tumors were, on average, significantly less resistant to platinum than were well differentiated tumors and there were greater differences between platinum resistance in untreated versus previously-treated tumors in the case of the poorly differentiated

tumors. One possible interpretation of these data is that patient survival with well differentiated tumors is more related to biological aggressiveness and/or host factors and/or surgical skill, while patient survival with poorly differentiated tumors is closely related to platinum resistance (in an era where virtually all patients with poorly- differentiated tumors are treated with platinum-based therapy).

Figure 7: Retrospective correlations between platinum resistance and patient survival in poorly-differentiated tumors.

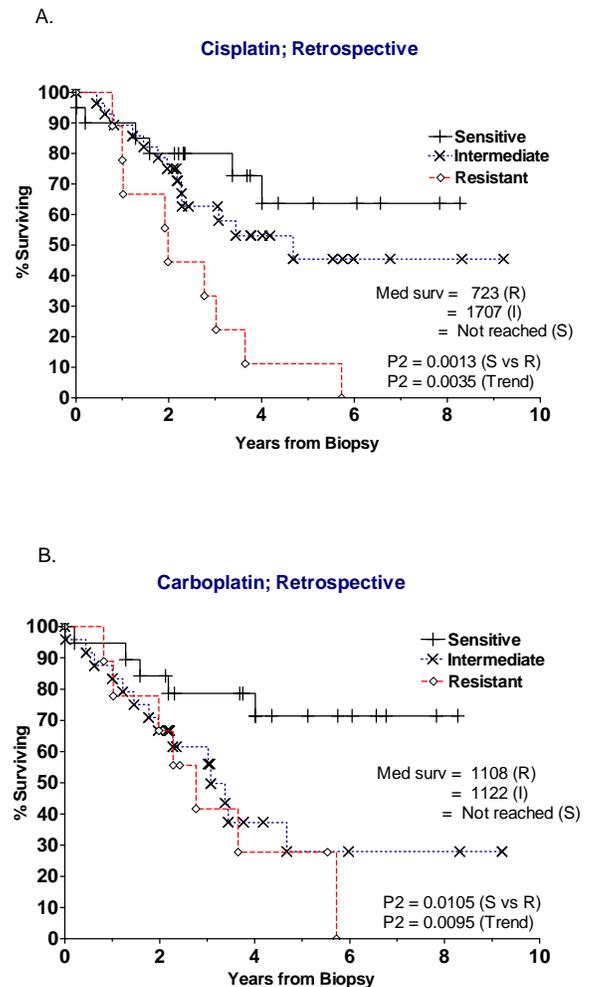


Figure 7. Kaplan-Meier survival curves from previously-untreated patients with tumors which were specifically labeled as being "poorly-differentiated" or "moderate to poorly-differentiated." Patients are grouped according to results of CCDRT, as determined retrospectively (but objectively, as described in the text) from raw data reported prospectively.

Figure 8: Platinum resistance as a function of duration of patient survival, omitting well-differentiated

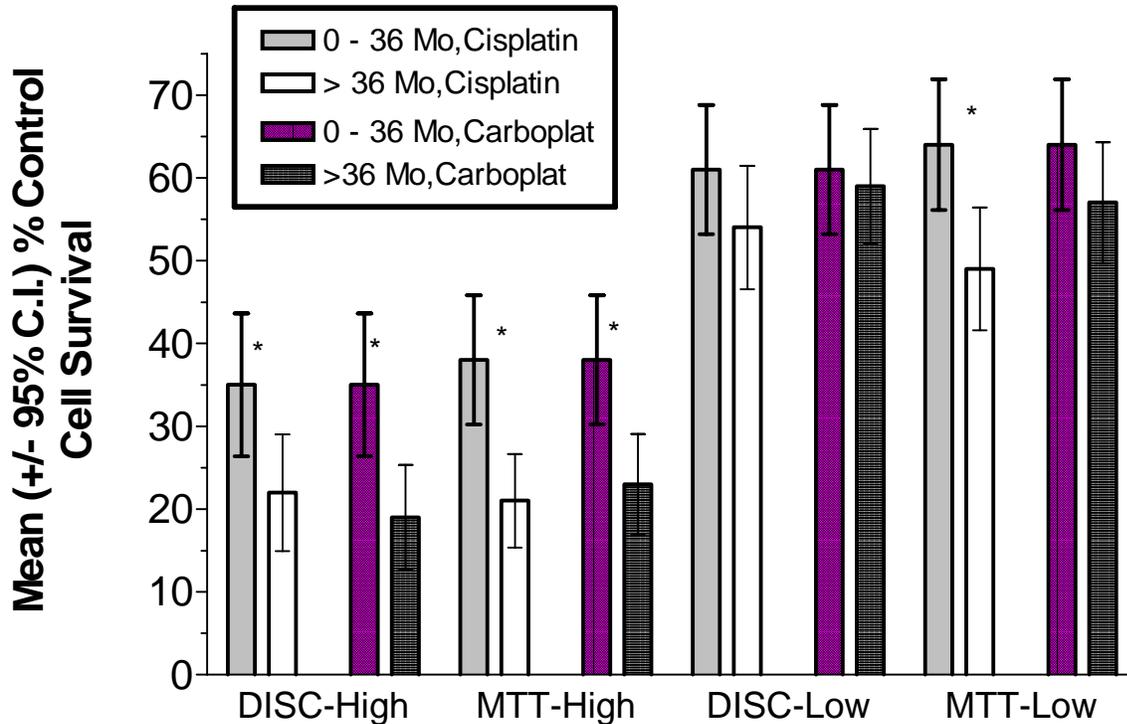


Figure 8. Mean (+/- 95% confidence intervals) for CCDRT results on ovarian tumors from previously-untreated patients with tumors which were not specifically labeled as being either "well-differentiated" or "borderline.", grouped by the duration of patient survival post-biopsy (surviving 0 - 36 months versus surviving greater than 36 months, which was the median survival determined by Kaplan-Meier analysis of all previously-untreated patients with non-well-differentiated tumors). Data are shown for each of the 4 assay datasets (DISC assay-high concentration, DISC assay-low concentration, MTT assay-high concentration, and MTT assay-low concentration), for both cisplatin and carboplatin. Differences between assays from patients surviving less than 36 months versus greater than 36 months were significant in the case of DISC and MTT assays, high concentration cisplatin at two sided p-values of 0.020 and 0.0005, respectively and for high concentration carboplatin at 0.0031 and 0.0028, respectively. Differences for low concentration carboplatin were not significant, while differences for low concentration cisplatin were significant only in the case of the MTT assay (P2 = 0.0064).

There was no significant relationship between platinum resistance and patient survival in the overall population of previously-treated patients,

more than half of whom had relapsed after both 1st and 2nd line (and often 3rd and 4th line) therapies, although some relationships did emerge

on very detailed subset analysis (these latter subset data are not shown and will be the topic of a future report).

It must be noted that tumors were referred to our laboratory specifically for the purpose of obtaining information which was to be considered in drug selection. We do not have information concerning with which agents patients were ultimately treated, nor do we have information on formal tumor staging, response to treatment, or time to first tumor progression. The only follow-up information we have is the overall survival of the patients, as determined from the public record social security death master file, using search methods previously validated in the epidemiology literature (as described in the Methods). As explained in the Methods section, the purpose of the study was to compare the relative survival of patients in relation to the findings in CCDRT. There is no reason to suspect that any errors in assessing date of death with our (consistently-applied and independently-validated) methods would be more prevalent or different in kind as a function of the results of CCDRT. Although we do not have information regarding the specific forms of chemotherapy administered post-assay, it is entirely reasonable to surmise that most, and most likely all, patients would have received some form of platinum-based therapy at some point in their illness.

We have provided detailed methods to allow other investigators to confirm and extend our findings. It must be noted that obtaining consistent, reliable results with CCDRT demands stringent standardization of assay conditions. Key features of our methods include (1) selective isolation and enrichment of three-dimensional microclusters^{18,19}, (2) anchorage-independent culture conditions in which our microclusters persisted as tumor cell spheroids and did not develop into monolayers, (3) standardization of plating densities to avoid artifacts of plating density-dependent drug resistance, (4) precise 96 hour duration of cell culture, which was determined to be optimum for the purposes of allowing selective attrition of normal connective tissue and inflammatory cells, while maintaining a reasonably high state of tumor cell viability, and (5) performing the testing on true fresh (non-

subcultured) tumor cells, under conditions where the bulk of the cells were either non-dividing or slowly dividing. It is also very important to stress that our endpoints were cell death endpoints and not cell proliferation endpoints (e.g. the latter being the case with thymidine-incorporation or so-called clonogenic endpoints and also being the case with assays performed on subcultured cell lines with high growth fractions). As a further note of caution, it should be noted that disappointing results were obtained by other investigators in non-small cell lung cancer when sub-cultured tumors (not true fresh tumors) were tested in monolayer cell culture²⁰.

In addition to the relationship between platinum resistance and patient survival, an equally important finding of the present study was the striking correlation between platinum resistance and prior treatment status. Tumors from previously-treated patients with poorly-differentiated and moderately-differentiated tumors were significantly more resistant to platinum than were tumors from untreated patients. The importance of this finding cannot be overemphasized. Firstly, it provides a relatively simple and immediate method for investigators who wish to emulate and confirm the present findings, without the need to accrue and follow large numbers of patients for many years. A relatively small dataset of assays on treated versus untreated patients should readily provide confirmation, given the magnitude of the differences reported here. Secondly, the data reported here establish the cell culture drug resistance testing (CCDRT) system presently described as being the most highly validated system yet reported for studying the circumvention of platinum resistance in human adenocarcinomas. For example, in the search for an effective "drug resistance reversing agent," one would test for agents which had the ability to shift the CCDRT results for platinum from the range characteristic of previously-treated patients to the range characteristic of untreated patients.

As a real-world example of the above, Table 2 shows the results of CCDRT performed on two patients, the first with pancreatic adenocarcinoma and the second with known, platinum resistant

Table 2: CCDRT results showing circumvention of platinum resistance. Results shown are percent control cell survivals for assays performed on specimens from two patients with ovarian and pancreatic cancers, respectively. Drugs were tested at an index concentration [x] and at one half the index concentration [x/2], as described in the Methods. The index concentration for gemcitabine and platinum tested in combination was 1/2 the index concentration when the drugs were tested individually, as single agents. In each case, the addition of gemcitabine markedly potentiated the otherwise poor activity of the platinum, and the patients subsequently achieved durable complete remissions to the platinum/gemcitabine combinations, as described in the Discussion.

Patient	Date	Drug	Concentration[x] ug/ml	%Cntrl DISC[x]	%Cntrl DISC[x/2]	%Cntrl MTT[x]	%Cntrl MTT[x/2]
Pancreatic	02-Feb-97	Carboplatin	23.5	61	89	72	120
		Cisplatin	3.3	63	80	51	98
		Gemcitabine	260	75	71	96	105
		Gemcit + Cispl	130/1.65	29	63	38	96
Ovarian	27-May-98	Cisplatin	3.3	85	95	58	89
		Carboplatin	23.5	87	98	68	94
		Gemcitabine	260	80	85	69	79
		Gemcit + Carbopl	130/11.75	12	55	22	48

ovarian cancer, each tested with platinum alone, gemcitabine alone, and platinum plus gemcitabine (tested at half the index concentrations utilized when platinum and gemcitabine were tested as single agents). Years before gemcitabine/ platinum became a widely used drug regimen, CCDRT identified this combination as the most active regimen in the patient with pancreatic cancer metastatic to kidney and spleen, a CA 19-9 level greater than 12,000, a 22 kg weight loss, and with parenteral-narcotic dependent pain, despite the poor activity in CCDRT of gemcitabine and cisplatin tested as single agents (Table 2).

This patient went on to achieve a complete remission with gemcitabine/cisplatin and remains alive with an excellent quality of life nearly 6 years later^{21,22}.

A second such patient was the ovarian cancer patient with clinical primary resistance to paclitaxel/carboplatin who then underwent tandem stem cell transplant/high dose chemotherapy regimens (at a cost of greater than

\$200,000) 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, also as shown in Table 2. 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 years²³, until a recent pericardial relapse (she has recently been re-started on assay-directed chemotherapy).

There is now a large, diverse, and consistent literature demonstrating that CCDRT with a variety of total cell kill (cell death) endpoints correlates with clinical response and patient survival, in both hematologic neoplasms and in solid tumors⁸. The most widely studied endpoints have been the two endpoints used in the present study (DISC and MTT), as well as the measurement of cellular ATP content (which rapidly disappears with cell death) and fluorescein diacetate metabolism and trapping (which is

dependent upon the presence of a functioning cell membrane). As long as the culture conditions are identical, all 4 of these endpoints have been shown to provide very similar results for most drugs, thus allowing studies with all 4 of these endpoints to be considered together for the purpose of meta-analysis⁸.

Most recently, it has become apparent that the current clinical trials paradigm in ovarian cancer is not achieving progress. For the past 30 years, the paradigm has been to perform empiric, prospective randomized trials comparing one form of empiric chemotherapy with another, putatively-improved form of empiric chemotherapy. Yet a critical analysis of the clinical trials literature shows that absolutely nothing relating to drug selection has been clearly proven during the past 30 years, save for the fact that cisplatin and carboplatin have similar therapeutic efficacy, although with different patterns of toxicity. It hasn't been clearly proven that initial treatment with platinum combinations provides superior outcomes compared to treatment with single agent alkylating agents¹, which have been used for at least 40 years, and it hasn't been proven that platinum + paclitaxel (considered to be current "standard" first line therapy for reasons which are not possible to justify, if the prospective, randomized clinical trials literature is to be used as the guide) are superior to single agent platinum²⁻⁴. Finally, it hasn't been proven that any of a half dozen or more forms of second and third line therapies are superior to the others, when drug selection is based on the empiric knowledge from prospective, randomized trials^{24,25}.

In spite of the general non-support for this field of inquiry over the past 20 years, cell death assays have been found to show strong and consistent correlations with both response and survival in ovarian cancer and other solid and hematologic neoplasms⁸, and controlled, but not randomized trials showed improved outcomes for assay-directed chemotherapy in both ovarian²⁶ and breast cancer²⁷. In contrast to the 96% evaluability rate described in the present study, only 43% of ovarian cancer specimens were evaluable with the "human tumor clonogenic assay"²⁸, in which a prospective trial was unable to document

significant response or survival correlations⁶. The presently-described methodology also allowed for the testing of a median of 18 drugs (plus negative and positive controls) at two different concentrations, tested with two different (but complementary) endpoints. The present results suggest that the best correlations may be obtained when a single (relatively high) drug concentration is tested with both a morphologic (DISC assay) and metabolic (MTT assay) endpoint. The substantial concordance between the results of these two cell death endpoints and also with the results of the ATP and fluorescein diacetate endpoints was referenced above.

There is currently an ongoing explosion of clinical trials with large numbers of agents which are likely to be only partially and/or less than universally effective. There is a serious need for improved methods to predict therapeutic outcomes with chemotherapy versus other forms of treatment and to better match treatment to patient. It is our hope that greater consideration will be given in the future to the many potential applications of cell death-based CCDRT in cancer research and treatment.

Acknowledgement: We would like to acknowledge the pioneering work of Dr. Hans Carstensen, a gynecologic oncologist at Uppsala University, in Uppsala, Sweden. To our knowledge, Dr. Carstensen was the first to carry out a clinical trial to test the clinical relevance of CCDRT using a cell death endpoint in ovarian cancer. Dr. Carstensen presented his preliminary results at the 3rd European Conference on Clinical Oncology in 1985, but died from a sudden cardiovascular event before he could complete his work. His studies were published only in abstract form, currently available only on the internet:

<http://weisenthal.org/carstensen.htm>

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