Establishment of in-vitro models of chemotherapy resistance

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Chemotherapy resistance is one of the most prevalent obstacles to the treatment of cancer, resulting in increased mortality and prolonged exposure to cytotoxic agents with no treatment benefit. One of the tools utilized in the study of mechanisms of chemotherapy resistance are established cell lines derived from human neoplasms. These cell lines can be challenged *in vitro* with controlled chemotherapy doses to produce chemotherapy-resistant variants. Analysis of these novel chemotherapy-resistant cell lines may then identify genetic and proteomic changes which are associated with the resistant phenotype. Two very important mediators of chemotherapy resistance (P-glycoprotein and multidrug resistance protein-1) were initially identified in chemotherapy-resistant cell lines. To make these in-vitro studies clinically relevant it is, however, necessary to duplicate as far as possible the treatment conditions used *in vivo*. Considerations should include clinically relevant drug concentrations, such as those derived from peak plasma values, and the type of treatment schedule to be employed. *Anti-Cancer Drugs* 18:749–754 © 2007 Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2007, **18**:749–754

Keywords: biomarkers, cell lines, chemotherapy, in-vitro models

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Received 10 November 2006 **Revised form accepted** 12 January 2007

Introduction

Tumour-derived cell lines are one of the most important tools for the study of cancer, from delineating the process of carcinogenesis through to testing new therapeutic agents. Cancer cell lines can also be used for the investigation of pathways and mechanisms by which tumours become resistant to therapy. This review first briefly outlines the use of cell lines derived from human neoplasms in the study of cancer. This is followed by an appraisal of the use of cell lines as models for investigating chemotherapy resistance, including an in-depth review of the methods for establishing clinically relevant surrogates.

The use of cell lines as models of chemotherapy resistance

The first documented use of a cancer cell line from a human neoplasm was in 1951, when George Gey [1] successfully maintained the serial culture of cells obtained from the epidermal cervical carcinoma of a 31-year-old cancer patient Henrietta Lacks. The resulting immortal cell line, subsequently named HeLa, continues to be used in cancer research to this day. In the 50 years following the establishment of the HeLa culture, many more immortal cell lines have been established from human neoplasms. The two main repositories of cell cultures, the European Collection of Cell Cultures (www.ecacc.org.uk) and the American Type Culture Collection (www.atcc.org) hold approximately 200 human carcinoma cell lines between them. The study of chemotherapy resistance *in vitro* utilizing cancer cell lines removes many of the problematical factors associated with an in-vivo study, and allows characterization of the mechanisms of resistance development and circumvention. Advantages of cell line models include good sample availability, an unlimited quantity of material, and greater control over the type, concentration and duration of treatment. In addition, experiments can be conducted upon cells, which could not be performed upon a patient. A preliminary in-vitro study can identify candidate markers of resistance to a particular chemotherapy agent, which can then be validated in a clinical study. Putative markers of chemotherapy resistance include molecules responsible for the detoxification of drugs or their removal from the cell, those involved in cellular pathways (such as apoptosis or DNA repair) which occur downstream of cytotoxic treatment or those linked to the specific mechanism of action of the individual drug. Of the insights into the nature of chemotherapy resistance resulting from in-vitro studies, the most important to date is probably the identification of cell membrane pumps. These drug efflux systems can actively transport chemotherapy agents out of the cell thus rendering them ineffective and leading to so-called multidrug resistance (MDR). MDR protein-1 (MRP1), coded by the *ABCC1* gene, was the first MRP to be identified using an in-vitro tumour cell line model [2] and is implicated in resistance to a wide range of drugs, including the vinca alkaloids (e.g. vinorelbine), antimetabolites [e.g. methotrexate (MTX)] and podophyllotoxins (e.g. etoposide). Overexpression of MRP1/*ABCC1* has been observed in drug-resistant cell lines derived from almost every kind cancer [3]. Although not initially discovered in chemotherapy-resistant cancer cell lines, the expression of P-glycoprotein

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(PGP/MDR1/*ABCB1*) is also important in chemotherapy resistance and is known to be extensively involved with in-vitro resistance to a wide variety of chemotherapy drugs, including the anthracycline antibiotics [4–7]. After the discovery of MRP1 and PGP it became clear that these were not the only such drug pumps to be found. In some MRP1 and PGP-negative cancer cell lines, or those which had been treated with inhibitors of these proteins, resistance to a wide variety of chemotherapy drugs was still observed. This led to the discovery of a family of MRP ABC transporter proteins as well as the breast cancer resistance protein (BCRP1/*ABCG2*) [6] and the lung resistance protein (LRP/*MVP*) [8], which is a major protein component of vaults. Proteins associated with MRD have been identified *in vitro*, and now chemoresistant cell lines are being put to further use in the search for new drugs and therapies, which can circumvent the MDR phenotype. Several potential drugs have been identified which act alone or synergistically with drugs whose toxicity is affected by MDR pumps to reverse this phenotype and enhance the toxic efficacy of these agents. MDR reversal agents (chemosensitizers), which have demonstrated an ability to reverse the MDR phenotype *in vitro*, include gemcitabine [9], fluoxetine [10], tipifarnib and bortezomib [11].

Application of in-vitro data to a clinical situation

Ultimately, proteins which are found to be associated with chemotherapy resistance in cancer cell line models must also be associated with resistance to chemotherapy in actual cancer patients if they are to be of any use in assessing prognosis or response to therapy, or as targets for new therapies. In the case of the MDR-associated pumps MRP1-9, PGP, LRP and BCRP, the correlation between the expression of these proteins and clinical prognosis/response to therapy have been extensively investigated. Recent examples include the identification of the expression of MRP or PGP proteins as predictors of poor prognosis or response in acute lymphoblastic leukaemia [12], early-stage breast cancer [13] and small cell lung cancer [14]. The expression of LRP has been identified as a predictor of poor clinical outcome in patients with advanced testicular germ-cell tumours [15] and BCRP expression has been associated with survival in patients with advanced nonsmall cell lung cancer (NSCLC) treated with platinum-based chemotherapy [16].

Methods of establishing novel chemotherapy-resistant cancer cell lines

Chemotherapy-resistant cell lines have been utilized to investigate the problem of drug resistance for over 20 years, but essentially the methods have remained the same [17,18]. By the continuous or intermittent exposure of a cell line to a given (often steadily increasing) concentration of the chosen chemotherapy agent, the expression of a drug-resistant phenotype is progressively produced. It is thought that establishment of this phenotype is brought about by one of two genetic events. Exposure (especially chronic exposure at low drug concentrations) may over time induce genetic events, which begin to confer a drug-resistant phenotype in those affected cells, which were not intrinsically resistant at the start. Alternatively, resistant cells can be selected from a culture on the strength of an intrinsic mutation conferring resistance in that cell or group of cells, thus establishing them as the dominant clone in the culture. These two types of resistance can be produced by altering the conditions of exposure of a cell line to the agent of choice.

Low-concentration/long-duration (continuous) treatment

One of the most well established methods of culturing drug-resistant cell lines is to utilize a low-concentration/long-duration approach. Cells are grown in standard media containing low (sublethal) concentrations of the chosen drug. At specified points, this concentration is increased in a stepwise manner. The exact methodologies vary but this approach can take anywhere between 6 and 16 months to establish a drug-resistant cell line (Table 1). Cell lines may be genetically unstable, and this must be considered and carefully controlled in long-term experimental treatments. Early examples of the continuous methodology include that of Twentyman *et al.* [17], who first established the highest concentration of doxorubicin (DOX) at which five lung cancer cell lines (NCI-H69, COR-L32, COR-L47, MOR, COR-L23) would grow. Cells were grown at this concentration (1.7×10^{-4} to 8.6×10^{-4} μmol/l) for 2–4 weeks, followed by a doubling of DOX concentration. If cell multiplication was observed, the cells were grown in this concentration of drug for a further 2–4 weeks and this cycle was repeated until a drug concentration was reached at which cell multiplication no longer occurred. The cells were maintained at this concentration indefinitely. Two years later, this methodology was applied by the same group for the establishment of five novel cisplatin (CDDP)-resistant lung cancer cell lines [19]. These cell lines grew in a maximum of 4 μmol/l CDDP and demonstrated a maximum 25.6-fold resistance to CDDP compared with the untreated parental cell lines. The continuous procedure has also been utilized to transform a renal cell carcinoma cell line (RCC8701) into several DOX-resistant sublines [20]. This process took 16 months and produced a variant with a near 80-fold increase in resistance to DOX compared with the parent line. Once established, the resistance of these sublines remained stable even after the withdrawal of DOX from the culture medium for several weeks. These cell lines demonstrated cross-resistance to epirubicin, CDDP, vinblastine and 5-fluorouracil (5FU), as well as changes in morphological characteristics and an increase in cell doubling time. Yoon *et al.* [21] were able to produce a novel

Table 1 Details of selected studies which have established novel chemotherapy-resistant cancer cell lines

Drug	Method used	Cell line	Cancer type	Duration (months)	Maximum resistance (fold)	Maximum drug concentration (μmol/l)	Maximum IC_{50} (μmol/l)	Reference
5-FU	C	SNU638	Gastric	12	2117	1538	5.57	[24]
CDDP	C	H460	NSCLC	3	20	6700	23.53	[21]
CDDP	C	NTUB1	Bladder	N/A	12	14	52.5	[22]
CDDP	P	TGW, GOTO	Neuroblastoma	12	2.1	66.6	N/A	[18]
CDDP	C	Sa-3	Oral	N/A	7.5	1.7	1.33	[26]
CDDP	C	H-1	Oral	N/A	10	0.16	2.14	[25]
CDDP	C	PC-7, PC-9, PC-14	NSCLC	3	3.1–22.9	4	2.14	[19]
CDDP	C	H69, N231	SCLC	3	8.4–25.6	1.33	0.41	[19]
DOX	C	RCC8701	Renal	16	80	1.37	6×10^{-3}	[20]
DOX	C	NCI-H69, COR-L32, COR-L47	SCLC	6–12	N/A	1.7×10^{-3}	N/A	[17]
		MOR, COR-L23	NSCLC					
DOX	C	HCT-15	Colorectal	12	7.8	0.34	N/A	[23]
DOX	P	NCI-H69	SCLC	7–13	37-fold	6.9×10^{-2}	N/A	[17]
		MOR, COR-L23	NSCLC					
MTX	P	MOLT-3	Leukaemia	5	5.3-fold	110	>100	[27]

The continuous (C) or pulsatile (P) method of establishment, the time required to produce a significantly resistant cell line (duration) and the maximum IC_{50} concentration achieved are indicated. The drug concentration used can be compared with the published RCAC PPC values: 4.25–76.7 μmol/l for 5-FU [33,34,47], 6.3–12.6 μmol/l for CDDP [31,32], 1.4–34.4 μmol/l for DOX [30,31] and 220 μmol/l for MTX [30].
CDDP, cisplatin; 5-FU, 5-fluorouracil; DOX, doxorubicin; MTX, methotrexate; NSCLC, nonsmall cell lung cancer; RCAC, relative clinically achievable concentration; PPC, peak plasma concentration; SCLC, small cell lung cancer.

CDDP-resistant NSCLC cell line (H460/CIS) by continuous exposure to this agent in only 3 months. The maximum concentration of CDDP used was 6.7 μmol/l and the new H460/CIS subline was 20 times more resistant to CDDP than the parent line (H460). H460/CIS was also cross-resistant to DOX (10.2-fold) and etoposide (6.7-fold). Hour *et al.* [22] utilized chronic CDDP treatment to produce three novel CDDP-resistant bladder transitional carcinoma cell lines. These three cell lines were 6-, 9- and 12-fold more resistant to CDDP than the parent line. Uchiyama-Kokubu and Watanabe [23] established three novel DOX-resistant HCT-15 colorectal cancer cell lines using a continuous approach without increasing the drug concentration in a stepwise manner. Instead, cells were continually cultured in 43.13, 86.25 or 345 nmol/l DOX for 1 year. The novel cell lines HCT-15/ADM1, ADM2 and ADM2-2 demonstrated 2.23-, 4.40- and 7.77-fold increase in resistance to DOX, respectively, when compared with the parental HCT-15 line. To maintain resistance, these cell lines had to be maintained in growth media supplemented with 43.13, 86.25 or 345 nmol/l DOX. Significant cross-resistance to MDR-related drugs (e.g. paclitaxel, etoposide mitoxantrone, epirubicin and vincristine) but not to non-MDR-related drugs (e.g. mitomycin C) was observed. In a further modification to the basic structure of this method, Chung *et al.* [24] continuously cultured a gastric carcinoma cell line SNU638 in gradually increasing concentrations of 5FU up to 1.54 mmol/l. After 1 year, two individual clones were selected and subcultured to produce two drug-resistant variants – SNU638-F1 and SNU638-F2. The two novel cell lines demonstrated 881- and 2117-fold resistance to 5FU, respectively, compared with the parental line. The SNU638-F1 cell line was cross-resistant to paclitaxel, and the SNU638-F2 cell line was cross-resistant to paclitaxel, DOX and CDDP. Most recently, Nakamura *et al.* [25] and Nakatani *et al.* [26] have established oral squamous cell carcinoma cell lines with significant levels of resistance to CDDP. The novel cell lines Sa-3R and H-1R were exposed to low-dose CDDP and demonstrated IC_{50} concentrations below the proposed clinically achievable concentration for this drug. The novel cell lines were 7.5- and 10-fold more resistant to CDDP than their respective parent cell lines.

Short-duration/high-concentration (pulsatile) treatment

The second method of producing a drug-resistant cell line is to utilize a higher concentration of drug for a shorter period of exposure (typically 3–24 h), to apply selective pressure for cell clones with intrinsic mutations, which already confer a degree of resistance to the chosen agent. This method of treatment has been termed 'pulsatile', as it often involves several cycles of high-concentration/short-duration treatment to select the desired phenotype (Table 1). Takemura *et al.* [27] utilized the pulsatile method to select MOLT-3 leukaemia cells, which demonstrated resistance to MTX. Cells were exposed to a high drug concentration for 24 h, washed and recultured in drug-free medium. Selection of resistant sublines required several repeated drug treatments interspersed with periods of drug-free growth and required occasional reexposure to maintain the resistant phenotype. After producing only limited results with a continuous regimen, Twentyman *et al.* [17] also adopted a pulsatile approach. Over a period of around 7 months, resistant variants of both COR-L23 and MOR were produced as well as two highly resistant variants of NCI-H69 (designated LX and LX4). These two lines showed 4- and 37-fold increases in resistance to DOX, respectively, when compared with the parental line. The resistance of the NCI-H69 variants was unstable

during the first 3 weeks of drug-free growth, but stabilized between weeks 3 and 9, with no loss of resistance if maintained in DOX-containing media. NCI-H69/LX and LX4 were cross-resistant to vincristine, mitoxantrone, epirubicin and etoposide, but not bleomycin. Iwasaki *et al.* [18] selected several stable resistant variants from the human neuroblastoma-derived TGW and GOTO cell lines using the pulsatile treatment method. CDDP-resistant TGW variants were produced by culturing cells with either 3 or 10 μmol/l CDDP for 1 week, followed by 3 weeks of drug-free growth, then three repetitions of this cycle. This produced two variants, TR1 and TR2, resistant to 10 and 3 μmol/l CDDP, respectively. The variant TR3 (resistant to 20 μmol/l CDDP) was produced by exposing TR1 cells to a single further treatment of 20 μmol/l CDDP. Three CDDP-resistant GOTO variants were produced: GR1, after three exposures to 3 μmol/l CDDP; GR2, after four exposures to 3 μmol/l CDDP; and GR3, after four exposures to 10 μmol/l CDDP. All variants displayed stable resistance to CDDP for over 6 months of continuous drug-free culture.

Continuous versus pulsatile treatment: mechanism of resistance

It appears that the type of drug exposure conditions used to produce a drug-resistant cell line, as well as the biochemical properties of the drug, may greatly influence the mechanism by which resistance develops. Takemura *et al.* [27] used both continuous and pulsatile treatment regimens in their quest to produce MTX-resistant MOLT-3 variants. MTX is a folate analogue and is preferentially transported into cells by the reduced folate carrier (RFC) system. It exerts its cytotoxic effects by inhibiting dihydrofolate reductase (DHFR), which is responsible for the synthesis of thymidylate and a key enzyme in DNA synthesis. Once inside the cell, MTX is converted into its active polyglutamated form by folylpolyglutamate synthetase (FPGS). Takemura *et al.* [27] identified upregulation of DHFR along with impaired RFC activity as a mechanism of resistance to MTX in those cells treated with a continuous MTX-exposure regimen. In contrast, cells treated with a pulsatile regimen displayed reduced activation of MTX via polyglutamination with no upregulation of DHFR or impaired RFC activity. Van der Laan *et al.* [28] also demonstrated decreased FPGS activity in a human head and neck squamous carcinoma cell line treated with a pulsatile MTX regimen. In cells treated with a continuous regimen, DHFR was increased 63-fold and FPGS activity was decreased, but to a lesser degree than in those cells treated with the pulsatile regimen [28]. Despite the fact that the mechanisms of resistance to MTX can be altered by the treatment conditions, there is currently no evidence suggesting that this is the case for other antineoplastic agents.

Continuous versus pulsatile treatment: clinical relevance

Another aspect of the method chosen for establishment of drug-resistant cell lines is how it relates to the clinical setting, i.e. does any method match the way in which chemotherapy is administered to a patient? Most chemotherapy treatments are administered over a period of several hours at cycles of every 21–28 days. In this respect, the pulsatile treatment regime potentially offers a more suitable approximation of this cyclic therapy protocol than the continuous treatment regime, in which cells are continuously exposed to the drug of choice for months at a time.

All chemotherapy drugs have half-lives of varying durations, along with different rates for metabolism and clearance from the body. For example, the biological half-life of DOX is 24–48 h, meaning that after this time period only half of the original dose of DOX is active. After administration to a patient, DOX is rapidly sequestered by plasma proteins and also metabolized by the liver, both of which render it inactive. Thus the exposure of a tumour to DOX would be limited both by drug concentration and by exposure duration. In contrast, CDDP has a biphasic pharmacokinetic profile with more than 90% of the drug undergoing detoxification via plasma proteins within a matter of a few hours but clearance takes approximately 30 days [29]. Low levels of some chemotherapy drugs such as CDDP may therefore reach the tumour for weeks to months. For these particular drugs, removal from the culture medium *in vitro* may be inappropriate and the continuous regime may give a more suitable approximation of *in vivo* conditions.

A point of criticism of cell line studies is how the concentrations of drug used in the experiments relate to those achievable in the clinical setting. A further complication is the discrepancy between the units of dose measurement utilized in the clinic (g/m^2) and the laboratory (g/l or molarity), making calculations of the correct dose to use in an in-vitro experiment difficult. This problem may, however, be overcome by the use of the peak plasma concentration (PPC) of the drug, derived from patients undergoing treatment. Moriuchi *et al.* [30] have quoted relative clinically achievable concentration values of 100 μg/ml (220 μmol/l) for MTX and 20 μg/ml (34.4 μmol/l) for DOX derived from PPC values. When these concentrations are correlated with those used in cell line studies (Table 1), the latter usually appear to fall well within the limits of those used in the clinic and are often far below the maximum tolerated concentration. Schöndorf *et al.* [31] exposed various ovarian cancer cell lines to CDDP and DOX after calculating a suitable dose based on PPC values derived after intravenous administration of a standard clinical dose of these drugs. Their calculations suggested a PPC

value of 3.8 μg/ml (12.6 μmol/l) for CDDP [31], which is supported by Bonetti *et al.* [32], who demonstrated PPC values for CDDP of 1.89 μg/ml (6.3 μmol/l) after the fourth cycle of treatment. These PPC values for CDDP are comparable to the dose used by Hour *et al.* (14 μmol/l) [22], but the range was exceeded in the studies by Iwasaki *et al.* (66.6 μmol/l) [18] and Yoon *et al.* (6700 μmol/l) [21] (Table 1). Employing a dose which is almost 1000 times higher than the PPC value does not reflect the clinical situation and may not present an accurate surrogate model. A PPC value of 0.5 μg/ml (1.4 μmol/l) was calculated for DOX [31], which is comparable to the dose used by Yu *et al.* (1.37 μmol/l) [20] and Uchiyama-Kokubu and Watanabe (0.34 μmol/l) [23] (Table 1). For 5-FU, the clinically achievable PPC values vary with the concentration given, but are within the range of 4.25–76.7 μmol/l [33,34], a dose range which was exceeded by Chung *et al.* (1538 μmol/l; Table 1) [24].

The use of PPC values as a way of determining suitable drug concentrations for use *in vitro* is not fool proof. As described above, the half-life of chemotherapy drugs varies and some drugs are rapidly sequestered by proteins within the plasma (e.g. DOX) or metabolized into noncytotoxic compounds by the liver, even before uptake by the tumour. All of these factors mean that the PPC value will vary depending upon sampling time after treatment. For cell line studies to accurately model clinical dynamics, more accurate (less empirical) clinical data such as the concentration of circulating drug actually reaching the tumour may be required. The most appropriate method of assessing chemosensitivity may vary according to the drug under study [35]; however, from the examples in Table 1 there does not appear to be a consensus regarding a clinically relevant range for the fold-change increase in the level of resistance that is achievable (or desirable) from in-vitro studies. For studies which have utilized a clinically relevant dose, based in the range of the PPC value, a low level change in resistance (in the region of a 2–10-fold as opposed to a 1000-fold increase) appears to be commonly achievable, which may also represent the clinical situation.

Cells as models: clinical relevance

To investigate whether the mechanisms of resistance produced *in vitro* are the same as those produced in the clinical setting, it is possible to compare pairs of cell lines established from the same patient before and after chemotherapy treatment. Kawai *et al.* [36] established and characterized two NSCLC cell lines from the pericardial effusions of a 20-year-old man before and after combination treatment with CDDP, ifosfamide and vindesine. Compared with the parent line (EBC-2), the posttreatment cell line EBC-2/R displayed higher IC_{50} values for CDDP (2.3-fold), ifosfamide (3.2-fold) and MTX (3.7-fold). Intracellular CDDP accumulation was decreased in the resistant cell line and overexpression of MRP3 was identified [36]. Reduced intracellular CDDP accumulation is also seen in some CDDP-resistant lung carcinoma cell lines produced *in vitro*, possibly due to decreased CDDP–DNA binding and increased efflux of unbound drug [37].

The real test of an in-vitro chemoresistant cell line model is the identification of a novel biomarker with demonstrable clinical value. The CDDP-resistant oral cancer cell line derived from H-1 cells, as detailed in Table 1 [25], was used to demonstrate that decreased caveolin-1 expression was associated with CDDP resistance both in the cell line model and clinical biopsy specimens [38]. In a landmark paper, Gyorrfy *et al.* [39] interrogated DOX-resistant breast cancer cell lines using expression microarray analysis and identified a 79-gene signature, which was associated with resistance. This gene panel predicted clinical survival in breast cancer patients undergoing DOX therapy.

Conclusion

Advances in clinical sample acquisition and preparation, and high-throughput analysis techniques such as microarray analysis, have moved the analysis of tumour samples to a stage where high-resolution profiles of gene expression are being developed, which may one day be able to reliably predict the response of individual patients to particular chemotherapy regimes [40–43]. Many authors, however, argue that current markers such as tumour grade and DNA ploidy are as good at predicting clinical outcome as microarray-based gene signatures, and that the profiles obtained from microarray studies of clinical samples are often nonspecific, prone to statistical bias and irreproducible across different platforms [44,45]. Although chemotherapy-resistant cancer cell lines have some disadvantages, they do provide model systems wherein all biological parameters can be controlled and assessed. The opportunity thus arises for undertaking large-scale expression profile screening of such cell lines to identify novel biomarkers of chemotherapy resistance [39,46,47]. These specific biomarkers could then be assessed in relation to response in clinical samples. To obtain reliable and clinically relevant data from such studies, it is essential that every effort is taken to approximate the cell line model as closely as possible to the clinical situation. This can be achieved by using appropriate drug concentrations, such as those derived from PPC values, and using treatment schedules which follow the same pattern, which is utilized for patients.

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