1. The Gastrointestinal Tumour Study Group. Further evidence of effective adjuvant combined radiation and chemotherapy following curative resection of pancreatic cancer. *Cancer* 1987, **59**, 2006–2010.
2. Neoptolemos JP. Adjuvant radio-chemotherapy following resection of pancreatic adeno-carcinoma. *Preliminary Results in Digestion* 1992, **3**, 127.
3. Moertel CG, Childs DS Jr, Reitemeier RJ, *et al.* Combined 5-fluorouracil and supervoltage radiation therapy of locally unresectable gastrointestinal cancer. *Lancet* ii, 865–867.
4. The Gastrointestinal Tumour Study Group. Therapy of locally unresectable pancreatic carcinoma: a randomized comparison of high dose (6000 rads) radiation alone, moderate dose radiation (4000 rads + 5-fluorouracil) and high dose radiation + 5-fluorouracil. *Cancer* 1981, **48**, 1705–1710.
5. Kahn PJ, Skornick Y, Inbar M, *et al.* Surgical palliation combined with synchronous therapy in pancreatic carcinoma. *Eur J Surg Oncol* 1990, **16**, 7–11.
6. Watanapa P, Williamson RCN. Surgical palliation for pancreatic cancer: developments during the past two decades. *Br J Surg* 1992, **79**, 8–20.
7. O'Connell MJ. Current status of chemotherapy for advanced pancreatic and gastric cancer. *J Clin Oncol* 1985, **3**, 1032.
8. Cullinan SA, Moertel CG, Fleming TR, *et al.* A comparison of three chemotherapeutic regimens in the treatment of advanced pancreatic and gastric carcinoma. *JAMA* 1985, **253**, 2061.
9. Topham C, Glees J, Rawson NSB. Randomised trial of epirubicin alone versus 5-fluorouracil, epirubicin and mitomycin C in locally advanced and metastatic carcinoma of the pancreas. *Br J Cancer* 1991, **64**, 179–181.
10. Bakkevold KE, Pettersen A, Arnesjo B, *et al.* Tamoxifen therapy in unresectable adenocarcinoma of the pancreas and the papilla of Vater. *Br J Surg* 1990, **77**, 725–730.
11. Aigner KR, Muller H, Bassermann R. Intra-arterial chemotherapy with MMC, CDDP and 5-FU for non-resectable pancreatic cancer—a phase II study. *Reg Cancer Treat* 1990, **3**, 1–6.

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Papers

Multiple Drug Resistance in the Human Ovarian Carcinoma Cell Line OAW42-A

Alice Redmond, Elizabeth Moran and Martin Clynes

A new multidrug-resistant variant (OAW42-A) of a human ovarian carcinoma line has been selected by exposure to increasing concentrations of doxorubicin. The variant is resistant to doxorubicin, vincristine (but surprisingly not to colchicine), etoposide, tenoposide and also to cisplatin (a drug not usually involved in classical multidrug resistance), but not to 5-fluorouracil. Overexpression of P-glycoprotein in the resistant line was demonstrated by immunofluorescence and western blotting. Direct evidence for P-glycoprotein as a determinant of resistance was provided by transfection with a specific antisense oligonucleotide. Reversal was incomplete and this, along with the pattern of cross-resistance observed, suggests that additional mechanisms of resistance may also be involved. Substantial clonal variation in resistance exists within the cell line.
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INTRODUCTION

OVARIAN CARCINOMA in humans often responds well initially to combination chemotherapy, but unfortunately the cancer often reappears in a form which is resistant to a wide range of drugs.

Multiple drug resistance (MDR) in human cancer and in cancer cells *in vitro* has thus far been principally associated with overexpression of a membrane efflux glycoprotein (P-glycoprotein, P-170), [1–3], alterations in glutathione-related enzyme levels, [4, 5], or reduced or altered expression of a topoisomerase II enzyme [6]. Other mechanisms of MDR, for example, non-P-glycoprotein efflux-related proteins [7, 8], and alterations in cellular calcium levels [9] exist. Studies on tissue from ovarian carcinoma patients suggest that P-glycoprotein overexpresion and possibly also reduction in topoisomerase II activity may relate to acquired and intrinsic drug resistance in ovarian carcinomas *in vitro* [11–13].

Bradley *et al.* [14] selected a range of MDR variants of the human ovarian carcinoma cell line SKOV3 by growth in increasing concentrations of vinca alkaloids. All but one of 16 variants selected overexpressed P-glycoprotein. At lower resistance levels, *mdr*-1 mRNA and protein were overexpressed, whereas at intermediate levels *mdr*-1 gene amplification was also observed. In one of the most resistant variants, P-glycoprotein levels were much higher (relative to other variants) than would be expected from mRNA levels and gene copy number, indicating possible control at the post-translational level (e.g. increase in half-life of the protein). Different glycosylated forms of P-glycoprotein are known to exist [15] but the relevance of this form of post-translational modification to stability and half-life of P-glycoprotein is unknown. Bernard *et al.* [16] have also isolated by exposure to vincristine a variant of a human ovarian carcinoma line which overexpresses P-glycoprotein. Lau *et al.* [17] have described a variant of the human ovarian carcinoma cell line ES-2, selected by exposure to cyanomorpholino doxoru-

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bicin, which has low-level resistance to the selective agent and also to doxorubicin, etoposide, bleomycin, cisplatin and carmustine; and a 2-fold resistance to ionising radiation. Chemoresistance was attributed at least in part to an increase in cellular glutathione levels and in glutathione-related enzymes (including glutathione *S*-transferase-π) and to increased repair of chemically-induced DNA cross-linkages.

This paper describes the selection and partial characterisation of a new MDR variant of the human ovarian carcinoma line OAW42.

MATERIALS AND METHODS

Cells

OAW42 is a cell line derived from human cystadenocarcinoma of the ovary obtained from the ECACC (8507310) (European collection of animal cell cultures, PHLS, Porton Down, Salisbury, U.K.). OAW42 was cultured in DMEM/HAM's F12, at 37°C in medium supplemented with 5% fetal calf serum (FCS) and 20 mmol/l Hepes (Sigma H-9139).

Toxicity assays

Toxicity was assessed by the acid phosphatase method [18] as described in the legend to Table 1.

Adaptation of MDR variants

To generate the resistant variant, doxorubicin was added (at a concentration permitting approximately 5% survival) until the cells appeared healthy and attaining high numbers; the drug concentration was then doubled. This process was continued until, after 15 months, the cells were growing in 0.85 μg/ml doxorubicin.

Cloning efficiency assay

Cells in exponential phase of growth were plated at 100 cells/500 μl per well in a 24-well plate (Costar 3524). Doxorubicin at varying concentrations was added 24 h later. The plates were incubated for 10 days at 5% CO_2 at 37°C, then stained for exactly 10 min with 0.25% crystal violet and dried thoroughly. Colonies ≥ 25 μm in diameter were counted.

Table 1. Cross resistance patterns of OAW42-A (0.95 μg/ml)

IC_{50} nmol/l	OAW42	OAW42-A	Fold-resistance OAW42 *
Doxorubicin	26.36 ± 1.7	1812 ± 82.9	68.7
Etoposide	33.1 ± 4.5	187 ± 21.2	5.7
Tenoposide	82.9 ± 9.9	233 ± 17.41	2.9
Vincristine	1.2 ± 0.09	3.5 ± 0.3	2.8
Cisplatin	583 ± 12.9	5349 ± 283.25	9.2
5-Fluorouracil	6917 ± 619	7095 ± 84.55	1.02
Colchicine	31.7 ± 3.9	33 ± 2.54	1.04

Toxicity is expressed as the 50% inhibitory concentration (in units of 10^{-6} × mmol/l, i.e. nanomolar). *IC_{50} OAW42-A/IC_{50} OAW42 for each drug.

Cells were seeded at 10^6 cells per 75 cm^2 flask, 2 days prior to assay, and fed on the following day. For the toxicity assay, 2 × 10^3 cells were plated in 100 μl medium per well of 96-well plates and 24 h later, drug, at 2 × concentration was added. The cells were incubated for 5 days, and cell number was then assessed using the acid phosphatase method [18].

Immunofluorescence and western blotting

The methods (including design of appropriate controls) used with C219 monoclonal antibodies have been described in detail elsewhere [19].

Antisense oligonucleotides

The sequences of the oligomers used in this study were: d5' (GTC CCC TTC AAG ATC CAT)3' antisense oligomer; d5' (ATG GAT CTT GAA GGG GAC)3' sense oligomer. These sequences represent the first 18 bases of the human *mdr*1 coding sequences. Details of oligonucleotide treatment of cells are given in the legend to Fig. 1.

RESULTS

Cross resistance patterns of OAW42-A at 0.95 μg/ml doxorubicin selective concentration

OAW42-A was found to be cross resistant to the classical MDR drugs, doxorubicin, etoposide, tenoposide and vincristine, but not to 5-fluorouracil or colchicine (Table 1). OAW42-A cross resistance patterns are unusual in that the highest level of resistance (apart from the selective agent doxorubicin) is to cisplatin. Doxorubicin resistance in OAW42-A was found to be unchanged after 3 months in the absence of the drug, signifying a genetically stable population.

Clonal variation

Cloning assays indicated the presence of different populations in OAW42-A. There is 90% survival up to 0.75 μg/ml doxorubicin; between 0.75 μg/ml and 1.0 μg/ml doxorubicin, survival reduces to 50%, then plateaus out to 35% at 1.5 μg/ml doxorubicin, 26% at 2 μg/ml and 8% at 3 μg/ml.

Immunochemical detection of P-170

Approximately 60% of the population was positive for C219 immunofluorescence in OAW42-A, indicating the presence of a heterogenous population. No fluorescence was noted in the parental cell line OAW42.

Analysis for P-170 glycoprotein by western blotting

Immunoreactive P-170 was evident in OAW42-A, but not in the sensitive cell line. The level of C219 staining on western blot was, however, lower than in other MDR lines known to express high levels of P-170, such as CHrC5.

Transfection of antisense and sense oligonucleotides

No change in doxorubicin resistance was seen after culture with sense oligomers for OAW42-A but increased doxorubicin sensitivity occurred after treatment with antisense oligomers (Fig. 1). Enhanced reversal of resistance was observed when, in addition to pretreatment, the oligonucleotide was included in the assay medium during the doxorubicin toxicity assay for OAW42-A.

DISCUSSION

The adapted line OAW42-A is resistant (in comparison to the parental line OAW42), to doxorubicin (69×), etoposide (6×), tenoposide (3×), vincristine (3×) and also to cisplates (9×). It is not resistant to 5-fluorouracil nor (surprisingly, in view of the vincristine resistance) to colchicine. Cross-resistance to doxorubicin, vincristine and epipodophylotoxins is consistent with P-glycoprotein involvement in resistance, but the relatively high level of resistance to the selecting agent, doxorubicin, as well as the quite high level of resistance to cisplatin (not usually associated with 'classical' MDR) suggests that other mechanisms may be operative in this line. Resistance to doxorubicin was a

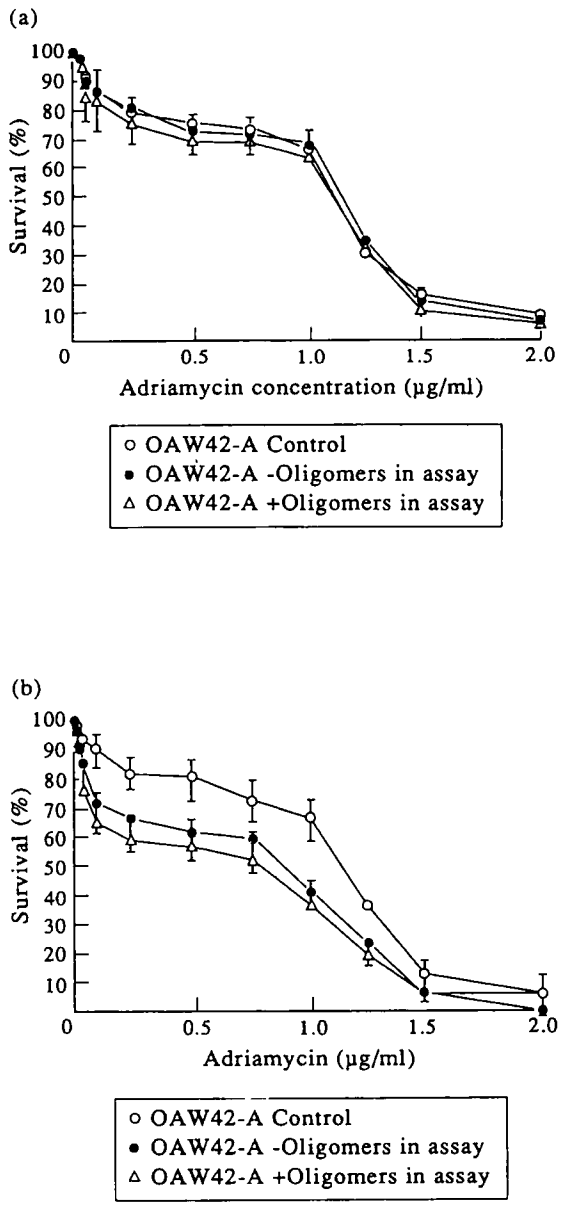

Fig. 1. Cells were set up in 25 cm² flasks at a density of 4 × 10⁶ cells per flask. All culture and assay medium was prepared with 10% heat inactivated FCS, (heat treated at 65°C for 15 min), to eliminate serum nucleases. Oligomers were added at a dose of 80 μg per flask on the first day, and 40 μg per flask on the second and third days. Growth medium volume per flask was 3.0 ml. After pretreatment with oligonucleotide the cells were plated at a density of 2 × 10³ cells/100 μl in each well of a 96-well plate. Doxorubicin at varying concentrations was added 24 h after plating the cells. A 4-day incubation period was used to test for doxorubicin sensitivity. Five assays were set up for each cell line: a control doxorubicin toxicity profile; antisense and sense pretreatment without oligomer (− oligo) present in the toxicity assay; and antisense and sense with oligomer (+ oligo) also present in the toxicity assay. Oligomers were prepared at a concentration of 40 μg per 3 ml and 2 μl of this solution added per 100 μl on each of the 4 days of the toxicity assay. The doxorubicin concentrations may be converted to molar concentrations according to the equation — 1 μg/ml = 1.72 × 10⁻³ mmol/l. (a) Effect of sense oligomers in OAW42-A. (b) Effect of antisense oligomers on OAW42-A.

genetically stable trait, since it was maintained after 3 months and 10 passages in the absence of drug.

Immunofluorescence and western blotting show that OAW-42-A cells overexpress P-glycoprotein (recognised by C219 monoclonal antibody). Transfection with an antisense oligonucleotide specific for the first 15 bases of the human *mdr*-1 gene results in partial reversal of resistance. This proves conclusively that overexpression of P-glycoprotein is not merely associated with, but actually has a causal role in resistance in this cell line. Control experiments involving transfection with the corresponding sense sequence had no effect on resistance, confirming that the effect observed with the antisense sequence was specific. The fact that reversal by the antisense treatment was only partial suggests that P-glycoprotein overexpression is not the only mechanism of resistance in this cell line, although use of stable antisense constructs would be desirable to elucidate this point, since factors related to cellular concentration of oligonucleotide and the half-life of P-glycoprotein in the cells [20] could play a role in the incomplete nature of the reversal. Verapamil, a calcium channel blocking agent, known to block P-glycoprotein-mediated drug efflux [1] also causes only partial (3-fold) reversal of doxorubicin toxicity in OAW42-A (and reversal in the parental line is 1.9-fold) (A. Redmond and M. Clynes, unpublished), giving further support to the existence of additional resistance mechanisms in this cell line.

Experiments involving clonal growth of OAW42-A suggest that some subpopulations are more resistant and some less resistant than would be expected from the selective concentration of drug. It is hypothesised that the less drug-resistant population may survive as a result of metabolic co-operation with more resistant cells which are more actively extruding drugs. Under conditions of clonal growth, such co-operation could not occur, and sensitive cells could not form colonies. If such clonal variation occurs also *in vivo*, it could have significant implications for the effectiveness of chemotherapy.

The OAW42-A line described here should provide a useful addition to the battery of lines available to investigate mechanisms of MDR and improved treatment in human ovarian carcinoma.

1. Ford J, Hait W. Pharmacology of drugs that alter multidrug resistance. *Cancer Pharmacol Rev* 1990, **42**, 155–199.
2. Bradley G, Juranka P, Ling V. Mechanism of multidrug resistance. *Biochem Biophys Acta* 1988, **948**, 87–128.
3. Clynes M. Cellular models for multiple drug resistance in cancer. *In Vitro Cell Dev Biol* 1992, in press.
4. Wang Y, Teicher B, Shea T, *et al.* Cross-resistance and GST-π levels among four human melanoma lines selected for akylating agent resistance. *Cancer Res* 1989, **49**, 6185–6191.
5. Tidefelt U, Elmhorn-Rosenborg A, Paul C, Hao X, Mannervik B, Eriksson L. Expression of glutathione transferase π as a predictor for treatment results at different stages of acute nonlymphoblastic leukemia. *Cancer Res* 1992, **52**, 3281–3285.
6. Giaccone G, Gazdar A, Beck H, Zunino, Capranico G. Multidrug sensitivity phenotype of human lung cancer cells associated with Topoisomerase II expression. *Cancer Res* 1992, **52**, 1666–1674.
7. Marquardt D, Center M. Involvement of vacuolar H^+-adenosine triphosphatase activity in multidrug resistance in HL60 cells. *J Natl Cancer Inst* 1991, **83**, 1098–1102.
8. Coley HM, Workman P, Twentyman P. Retention of activity by selected anthracyclines in a multidrug resistant human large cell lung carcinoma line without P-glycoprotein hyper-expression. *Br J Cancer* 1991, **63**, 351–357.
9. Nygren P, Larsson R, Gruber A, Peterson C, Bergh J. Doxorubicin selected multidrug-resistant small cell lung cancer cell lines characterised by elevated cytoplasmic Ca^{2+} and resistance modulation by

verapamil in the absence of P-glycoprotein overexpression. *Br J Cancer* 1991, **64**, 1011–1018.
10. Bourhis J, Goldstein L, Riou G, Pastan I, Gottesman M, Benard J. Expression of a human multidrug resistance gene in ovarian carcinomas. *Cancer Res* 1989, **49**, 5062–5065.
11. Van der Zee A, Hollema H, de Jong S, *et al.* P-glycoprotein expression and DNA topoisomerase I and II activity in benign tumours of the ovary, before and after platinum/cyclophosphamide chemotherapy. *Cancer Res* 1991, **51**, 5915–5920.
12. Roninson IB. The role of *mdr*-1 (P-glycoprotein) gene in multidrug resistance *in vitro* and *in vivo*. *Biochem Pharmacol* 1992, **43**, 95–102.
13. Holzmayer T, Hilsenbeck S, Von Hoff D, Roninson I. Clinical Correlates of *mdr*-1 (P-glycoprotein) gene overexpression in ovarian and small cell lung carcinomas. *J Natl Cancer Inst* 1992, in press.
14. Bradley G, Naik M, Ling V. P-glycoprotein expression in multidrug-resistant human ovarian carcinoma cell lines. *Cancer Res* 1989, **49**, 2790–2796.
15. Ichikawa M, Yoshimura A, Furukawa T, Sumizawa T, Nakazima Y, Akiyama S. Glycosylation of P-glycoprotein in a multi-drug-resistant KB cell line, and in the human tissues. *Biochim Biophys Acta* 1991, **1073**, 309–315.
16. Benard J, Da Silva J, Teyssier J, Riou G. Overexpression of *mdr*-1 gene with no DNA amplification in a multiple-drug-resistant human ovarian carcinoma cell line. *Int J Cancer* 1989, **43**, 471–477.
17. Lau D, Lewis A, Ehsan M, Sikic B. Multifactorial mechanisms associated with broad cross-resistance of ovarian carcinoma cells selected by cyanomorpholino doxorubicin. *Cancer Res* 1991, **51**, 5181–5187.
18. Martin A, Clynes M. Acid phosphatase: endpoint for *in vitro* toxicity tests. *In Vitro Cell Dev Biol* 1991, **27A**, 183–185.
19. Clynes M, Redmond A, Moran E, Gilvarry U. Multiple drug-resistance in a human non-small cell lung carcinoma cell line DLKP. *Cytotechnology* 1992, **10**, 75–89.
20. Muller C, Ling V. P-glycoprotein stability is affected by serum deprivation and high cell density in multidrug resistant cells. *Proc Am Assoc Cancer Res* 1992, **33**, 452.

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Treatment Results, Survival and Prognostic Factors in 109 Inflammatory Breast Cancers: Univariate and Multivariate Analysis

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Between 1978 and 1987, 109 patients without metastatic disease were treated by induction chemotherapy for inflammatory breast cancer (IBC) or "neglected" locally advanced breast cancer (LABC): 62 patients had a clinical history of rapidly growing tumours (doubling time ≤ 4 months) and inflammatory signs; conversely, the 47 neglected patients had local inflammation with a longer history of LABC. 103 patients were fully evaluable. All patients received the same induction chemotherapy with doxorubicin, vincristine, cyclophosphamide and 5-fluorouracil. After six cycles, locoregional treatment was by radiotherapy if a complete or nearly complete response had been obtained, and total mastectomy, with pre or postoperative radiotherapy, in other cases. The chemotherapy after local treatment comprised of six cycles for LABC and 12 cycles for IBC (six without doxorubicin). With a median follow-up of 120 months, the median overall survival (OS) time was 70 months as against 45 months for disease-free survival (DFS). No difference was observed for OS and DFS between LABC and IBC. The regional recurrence rate was 24% (15% for radiotherapy alone). 20 factors of potential prognostic significance were evaluated by univariate and multivariate analysis. For DFS and OS, univariate analysis suggested a worse prognostic significance for "peau d'orange" appearance of the skin, clinical evidence of node involvement and poor response to chemotherapy after three cycles, on mammographic criteria. The cumulative dose of doxorubicin after three cycles seemed to have a significant effect on OS ($P < 0.03$) but was too closely correlated with age to draw definite conclusions. In the multivariate analysis, "peau d'orange", menopausal staus and clinical node involvement predicted DFS. "Peau d'orange" and clinical node involvement also predicted OS. Our results indicate that IBC and LABC do not behave differently when treated with our procedure.
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INTRODUCTION

INFLAMMATORY BREAST cancer (IBC) is an uncommon cancer constituting only 1–4% of breast cancer cases in western countries [1]; most of our knowledge comes from retrospective and prospective (single-armed) studies, devoid of control groups. IBC is characterised by a high rate of locoregional and mainly metastatic failures, which occur rapidly. With well-managed locoregional treatment (surgery and/or radiotherapy) alone the overall survival (OS) may not exceed 5% at 5 years [1, 2]. Therefore, IBC has been considered to be a systemic disease, i.e. with micrometastasis at the time of diagnosis, requiring systemic treatment with combination chemotherapy. This effectively improves OS to within the range of 30–50% at 5 years [2–5].