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Cross-resistance in the 2′,2′-difluorodeoxycytidine (gemcitabine)-resistant human ovarian cancer cell line AG6000 to standard and investigational drugs

A.M. Bergman, G. Giaccone, C.J.A. van Moorsel, R. Mauritz, P. Noordhuis, H.M. Pinedo, G.J. Peters *

Department of Medical Oncology, University Hospital Vrije Universit., PO Box 7057, 1007 MB Amsterdam, The Netherlands

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Abstract

Gemcitabine (2′-2′-difluorodeoxycytidine; dFdC) is a deoxycytidine analogue which is effective against solid tumours, including lung cancer and ovarian cancer. dFdC requires phosphorylation by deoxycytidine kinase (dCK) for activation. In the human ovarian cancer cell line A2780 and its 30 000-fold dFdC-resistant variant AG6000 ($P < 0.001$), we investigated the cross-resistance profile to several drugs. AG6000, which has a complete dCK deficiency, was approximately 1000–10 000-fold resistant to other deoxynucleoside analogues such as 1-β-D-arabinofuranosyl cytosine, 2-chloro-deoxyadenosine, aza-deoxycytidine and 2′,2′-difluorodeoxyguanosine (dFdG) ($P < 0.001$). dFdG can be activated by dCK and deoxyguanosine kinase (dGK), but the latter enzyme was not altered in AG6000 cells. Thus dFdG resistance was only due to dCK deficiency. AG6000 was 1.6- and 46.7-fold resistant to 5-fluorouracil (5-FU) and ZD1694, respectively (the latter was significant; $P < 0.01$), which may be due to the 1.7-fold higher thymidylate synthase (TS) activity, but AG6000 cells were also 2.7-fold resistant to the lipophilic TS inhibitor AG337 ($P < 0.05$). Remarkably, AG6000 cells were 2.5-fold more sensitive to methotrexate (MTX) ($P < 0.01$) than A2780 cells, but 1.6-fold more resistant to trimetrexate (TMQ) ($P < 0.10$). However, no differences in reduced folate carrier activity, folylpolyglutamate synthetase (FPGS) activity and polyglutamation of MTX were found between the cell lines. AG6000 cells were approximately 2 to 7.5-fold more resistant to doxorubicin (DOX), daunorubicin (DAU), epirubicin and vincristine (VCR) (the latter was significant; $P < 0.02$) and approximately 4-fold more resistant to the microtubule inhibitors paclitaxel and docetaxel ($P < 0.001$). Fluorescent activated cell sorter (FACS) analysis revealed no P-glycoprotein (Pgp) or multidrug resistance-associated protein (MRP) expression, but less fluorescence of intercalated DAU in AG6000 cells. An approximately 2-fold resistance to the topoisomerase I and II inhibitors etoposide, CPT-11 and SN38 was found in AG6000 cells. *Topoisomerase I* and *IIα* RNA expression was decreased in AG6000 cells. AG6000 was 2.4, 2.4, 2.3 and 3.7-fold more resistant to EO9 ($P < 0.02$), mitomycin-C (MMC) ($P < 0.05$), cisplatin (CDDP) ($P < 0.10$) and maphosphamide (MAPH), respectively. DT-diaphorase (DTD), which activates EO9, was 2.2-fold lower in AG6000 cells. CDDP resistance might be related to a reduced retention of DNA adducts in AG6000. However, glutathione levels were equal in A2780 and AG6000 cells. A 24 h exposure to DOX, VCR and paclitaxel at equimolar and equitoxic concentrations, resulted in more double-strand breaks (1.5- to 2-fold) in A2780 than in AG6000 cells. MAPH at 1120 nM and 17 nM of EO9 did not cause DNA damage in either cell line. In conclusion, AG6000 is a cell line highly cross-resistant to a wide variety of drugs. This cross-resistance might be related to altered enzyme activities and/or increased DNA repair.

Keywords: Gemcitabine; Cross-resistance; DNA damage; Deoxyguanosine kinase activity (DGK); DT-diaphorase activity (DTD); Folylpolyglutamate synthetase (FPGS) activity; Methotrexate polyglutamination; Methotrexate uptake; Glutathione; Topoisomerase

1. Introduction

2′,2′-Difluorodeoxycytidine (dFdC, gemcitabine) is a deoxycytidine analogue which is active against non-small cell lung cancer and ovarian cancer, as predicted by *in vitro* and *in vivo* results [1–4]. Moreover, gemcitabine is very active in combination with drugs with other mechanisms of action, both in experimental models and in patients [5–8]. In the cell, gemcitabine is phosphorylated by deoxycytidine kinase (dCK) to its monophosphate and subsequentially to its triphosphate (dFdCTP), which can be incorporated into DNA and RNA [9,10].

* Corresponding author. Tel.: +31-20-444-2633; fax: +31-20-444-3844.

E-mail address: gj.peters@azvu.nl (G.J. Peters).

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After incorporation into DNA, one more deoxynucleotide can be incorporated whereafter the DNA polymerisation stops, resulting in single-strand DNA damage [10]. Moreover, the DNA exonuclease activity is unable to excise dFdCMP [10]. Gemcitabine can be inactivated by the action of deoxycytidine deaminase (dCDA) to dFdU [11]. Another pathway of inactivation is deamination of dFdCMP to dFdUMP, by the action of deoxycytidine monophosphate deaminase (dCMP deaminase), and subsequently to dFdU, which is excreted out of the cell [12]. More mechanisms of action have been described; dFdCDP has been identified as the metabolite which inhibits ribonucleotide reductase (RNR) resulting in a decrease in dCTP pools, leading to a decreased feedback inhibition of dCK and thus an increase in gemcitabine phosphorylation [13]. Possible inhibition of thymidylate synthase (TS) by the deaminated product dFdUMP might affect DNA synthesis resulting in DNA damage [14]. Both dCMP deaminase and cytidine triphosphate synthetase (CTP synthetase) are inhibited by dFdCTP, resulting in enhanced retention of gemcitabine nucleotides [15], a depletion of CTP pools and possibly enhanced incorporation into RNA [16].

Since gemcitabine has a complex mechanism of action with multiple cellular targets, resistance can be multifactorial. Several possible mechanisms of resistance to gemcitabine have been described [6]. Similar to 1-β-D-arabinofuranosylcytosine (ara-C), resistance to gemcitabine can include several other mechanisms besides dCK deficiency [6], such as an increased dCDA activity, an altered DNA polymerase, or altered membrane transport [17–19]. Since gemcitabine has more targets for its action then ara-C, resistance might be multifactorial, e.g. increased RNR and 5′-nucleotidase activities have been observed in gemcitabine-resistant cell lines [20,21].

Despite significant initial response rates for advanced ovarian carcinomas using platinum-based combination chemotherapy, many patients relapse as a result of the development of cross-resistance and $<15\%$ will be long-term survivors [22]. Overall, chemotherapy is relatively ineffective due to the emergence of a broad cross-resistance pattern that is either intrinsic to the tumour or acquired after chemotherapy [23]. Therefore, it is essential to know whether resistance to gemcitabine is associated with cross-resistance to other agents commonly used in diseases in which gemcitabine is active. These include anthracyclines, platinum-based drugs and topoisomerase inhibitors. For this purpose, we used AG6000 as a model, the first human cell line cultured to be resistant to gemcitabine. AG6000 is a 30 000-fold gemcitabine-resistant variant of the human ovarian cancer cell line A2780, which is the result of a total absence of dCK activity. Polymerase chain reaction (PCR)-amplified mRNA, using specific dCK primers, demonstrated that AG6000 expressed an aberrant amplicon [24].

The initial investigation of the cross-resistance profile of AG6000 revealed interesting aspects from a clinical point of view which can be used to circumvent the development of gemcitabine resistance such as the use of drug combinations. Here we describe the cross-resistance of AG6000 cells to a panel of drugs used in the therapy of ovarian cancer and to potentially interesting investigational drugs. The drugs were divided in four different groups: (1) deoxynucleoside analogues; (2) 5-fluorouracil (5-FU) and antifolates; (3) tubulin and topoisomerase inhibitors; and (4) miscellaneous drugs. Several parameters known to be important in the metabolism and resistance to the different drugs were studied.

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Flow Laboratories (Irvine, UK) and fetal calf serum (FCS) from Gibco (New York, NY, USA), trichloroacetic acid (TCA), L-glutamine and gentamycin from Merck (Darmstadt, Germany) and trypsin and sulphorhodamine B (SRB) from Sigma Chemical Co (St Louis, MO, USA). Gemcitabine, 2′,2′-difluorodeoxyuridine (dFdU) and 2′,2′-difluorodeoxyguanosine (dFdG) were kindly supplied by Eli Lilly Inc. (Indianapolis, USA). Cisplatin (CDDP) was purchased from Bristol-Myers Squibb (Weesp, The Netherlands). [8-^{3}H]-2 chloro-2′deoxyadenosine (CdA) and [3′, 5′, 7-^{3}H(N)]-methotrexate were from Moravek Biochemicals (Brea, Ca), α[^{32}P]-dCTP from Amersham (Bucks, UK) and [2,3-^{3}H]-glutamic acid from NEN-Dupont (Dreiech, Germany). All other chemicals were of analytical grade and commercially available.

2.2. Cell culture and chemosensitivity testing

The *in vitro* experiments were performed with A2780, a human ovarian cancer cell line [25] and its variant AG6000 which is highly resistant to gemcitabine [24]. Doubling times of the cell lines were 18.1±1.6 and 23.8±2.5 h (mean±standard error of the mean (SEM)), respectively. Cells were grown in monolayers in DMEM supplemented with 5% heat-inactivated FCS, and 250 ng/ml gentamycin, at 37°C at 5% CO_2.

The determination of the IC_{50} (the drug concentration causing 50% growth inhibition), was performed using the SRB assay [26,27]. On day 1, 100 μl of cell suspension was plated per well in 96-well plates (A2780 5000 cells/well and AG6000 18 000 cells/well), followed by 100 μl of the drugs on day 2. After exposure for 72 h,

allowing at least two doubling times, cells were precipitated with 50 μl ice-cold 50% (w/v) trichloroacetic acid (TCA) and fixed for 60 min. The SRB assay was then carried out as described in [27]. Growth inhibition curves were made by a calculation of the relative growth based on the optical densities of drug-treated and control wells of every SRB assay. The points were connected by straight lines and the IC_{50} values were determined from the interpolated graph [28]. Cell size of A2780 and AG6000 cells was microscopically checked on a regular basis during exposure to the various drugs, but the size of the cells did not change. In addition, comparison of chemosensitivity testing for some antimetabolites by cell counting, dimethylthiazolyl-2,5-diphenyltetrazolium bromide (MTT) and SRB assay in A2780 cells, revealed no differences in the IC_{50}s. Measurements of the protein content of the cells using a protein assay (see below) paralleled these results.

2.3. Fluorometric analysis of the DNA unwinding assay

To measure drug-induced DNA damage the fluorometric analysis of DNA unwinding (FADU) assay was used [5,29]. $3–5\times10^6$ A2780 and AG6000 cells were incubated for 24 h at 37°C with drugs in a 50% cell growth-inhibiting IC_{50} concentration at the 72 h exposure. Since the IC_{50} concentration of dFdC for AG6000 is very high, a concentration of 1000 nM dFdC was chosen. This enabled sufficient viable A2780 cells to be retained after exposure to be able to evaluate the DNA damage. In every assay, 50 μM of etoposide (VP16) was used as a positive control. After harvesting, cells were lysed in a 9 M urea solution and exposed to an alkaline environment (pH 12.8) for 60 min, allowing the DNA to unwind, which was terminated by a glucose solution. Double-stranded (ds)-DNA was stained by ethidium bromide and fluorescence was measured on a SPEX Fluoromax fluorescence spectrometer (Perkin-Elmer) (excitation, 520 nm; analyser, 590 nm). Percentage ds-DNA was calculated relative to no DNA unwinding and complete DNA unwinding.

2.4. DT-diaphorase assay

In 1×10^6 cells, lysate protein content was measured [30]. Bovine serum albumin (BSA) was added to a final concentration of 1% to act as a DT-diaphorase (DTD) activator [31]. Enzyme activity was measured as previously described [32,33] with 0.5–40 μg of cellular protein in the presence of 0.2 mM NADH and 40 μM 2,6-dichlorophenol-indophenol (DCPIP).

2.5. Deoxynucleoside kinase activity assay

Lysates of 3×10^6 cells were incubated with 0.33 mM [8-^{3}H]deoxyguanosine (134 Ci/mol) in a final volume of 80 μl for 60 min at 37°C. The reaction was terminated by heating and addition of excess deoxyguanosine, dGMP, guanine and xanthine [34,35]. For dCK activity, 230 μM [5-^{3}H] dCyd (40 Ci/mol) and 52 μM [8-^{3}H] CdA (192 Ci/mol) were used as substrates. Substrates and products were separated on PEI-cellulose thin-layers as previously described [34–36].

2.6. FACS analysis of Pgp and MRP activity

Detection of P-glycoprotein (Pgp) and multidrug resistance-associated protein (MRP) activity was performed as previously described [37]. 0.5×10^6 cells were incubated with 2 μM daunorubicin (DAU) or 200 ng/ml rhodamine 123 (RHO 123) for 60 min or for 10 min in 0.5 μM Calcein-acetomethyl ether (Calc-AM) in medium A at 37°C, with or without a modulator. Pgp was modulated by 2 μM SDZ PSC 833 (PSC; Pgp inhibitor). MRP was modulated by 200 μM genistein, only with DAU as a probe due to interactions with RHO 123 and Calc-AM. Both Pgp and MRP were modulated by 100 μM vincristine (VCR) [38]. DAU and RHO 123 retention was measured by incubating the cells in drug-free medium A with or without a modifier for 60 min after loading the cells with drugs for 60 min. The efflux was stopped by centrifuging the cells and adding ice-cold medium. Fluorescence was determined by FACS analysis (Becton Dickinson, San Jose, CA, USA).

2.7. Determination of methotrexate (MTX) polyglutamates

The formation of MTX polyglutamates was measured as previously described [39]. 1×10^7 cells were exposed to 100 μl [^{3}H]-MTX mix (1.91 Ci/mmol), and incubated for 24 h at 37°C. Cells were harvested, and precipitated by 10% TCA, neutralised with trioctylamine/1,1,2-trichlorofluoroethane (1/4, v/v) and analysed as previously described by high performance liquid chromatography (HPLC).

2.8. [^{3}H]-MTX transport

[^{3}H]-MTX transport was performed as previously described [40]. Uptake studies of [^{3}H]-MTX (0.2 Ci/mmol) by A2780 and AG6000 cells were carried out at 37°C at an extracellular concentration of 2 μM. At selected time intervals, uptake samples were analysed for radioactivity in Optifluor scintillation fluid (United Technologies Packard, Brussels, Belgium) using an Isocap/300 (Searle, Nuclear Chicago) scintillation counter.

2.9. Folyl polyglutamate synthetase (FPGS) assay

As previously described [41], the protein amount of 20×10^6 cells was determined and 200 μg protein was

added to the reaction mixture consisting of 250 μM MTX and 4 mM [^{3}H]-glutamate mixture ([2,3-^{3}H]-glutamic acid, 6.6 Ci/mmol, NET). After incubation for 3 h at 37°C, separation of product and substrate was performed on a Sep-Pack C_{18} cartridge pretreated with methanol and NaAc (pH 5.5). After loading a sample on the column, the excess of free [^{3}H]-glutamate was removed by NaAc (pH 5.5) and NaOH, while MTX-[^{3}H]-glutamate and unreacted MTX was eluted with methanol and counted for 1 min.

2.10. Glutathione (GSH) assay

The GSH assay was performed as previously described [42]. Briefly, 5×10^6 cells were incubated in the dark in a Tris buffer with 440 μM monobromobimane solution (pH 8.0) and terminated after 10 min by a aqueous 5-sulphosalicylic acid. Three hundred μl of the supernatant was mixed with an equal volume of 0.3 M 4-ethyl-morpholine prior to HPLC analysis on a Supelcosil LC18 column (Supelco, Bellefonte, PA, USA) at an excitation wavelength of 275 nm and an emission wavelength of 465 nm.

2.11. Northern blot for topoisomerase I and IIα mRNA expression

Northern blotting for *topoisomerase I* and *IIα* was performed as previously described [43]. Total RNA from 20×10^6 cells was denatured, run on a 1% multipurpose agarose gel, blotted onto a Qiabrane nylon membrane (Westburg, Leusden, The Netherlands) and cross-linked to the membrane. The probe, labelled with α[^{32}P]-dCTP using random primers and Klenow polymerase, was incubated with the membrane overnight; a photographic film was exposed and scanned on a GS-690 Bio-rad scanner (Hercules, CA, USA).

2.12. Statistical analysis

Differences in the IC_{50} values and DNA damage induced by drug exposure between the A2780 and AG6000 cells were evaluated using the Student *t*-test for unpaired data. The computer program Statistical Package for the Social Sciences (SPSS) (version 7.5, SPSS, Inc., Chicago, IL, USA) was used for the statistical analysis.

3. Results

3.1. Growth inhibition tests

The sensitivity of the A2780 and AG6000 cells to the various drugs, expressed as IC_{50} values, are listed in Table 1. The 30 000-fold gemcitabine-resistant AG6000 cells were cross-resistant to all deoxynucleoside analogues, although the extent varied. AG6000 cells were 1.6-fold resistant to 5-FU, 46.7- and 2.7-fold cross-resistant to ZD1694 (raltitrexed) and AG337, two thymidylate synthase inhibitors, respectively. AG6000 cells were 2.5-fold more sensitive to the dihydrofolate reductase (DHFR) inhibitor MTX, but 1.6-fold more resistant to trimetrexate (TMQ). The tubulin inhibitor VCR showed a 7.5-fold resistance and the taxanes paclitaxel and docetaxel a 4.4- and 4.6-fold resistance, respectively. The topoisomerase inhibitors displayed an approximately 2-fold cross-resistance. Of the miscellaneous drugs, a 2- to 3-fold cross-resistance of AG6000 cells was found for the anthracyclines and the quinone analogues mitomycin-C (MMC) and EO9. A 2- to 4-fold cross-resistance was found to CDDP and maphosphamide (MAPH), the active metabolite of the alkylating agent cyclophosphamide.

Table 1
IC_{50} values in the ovarian cancer cell lines A2780 and AG6000

Drug	A2780 (nM)	AG6000 (nM)	Fold-resistance
dFdC[e]	2.16±1.0	50 500±20 200	23 380[d]
dFdU[f]	2200±200	$>5\times10^6$	>2273[d]
dFdG	316.7±143.1	>50 000	>158[d]
ara-C[f]	14±4	135 000±44 000	9643[d]
CdA[f]	78±6	260 000±49 000	3333[d]
Aza-CdR[f]	5000±1600	$>5\times10^6$	>1000[d]
5-FU	8400±2200	13 600±2100	1.6[n.s.]
Methotrexate (MTX)	15.5±1.2	6.2±2.7	0.4[c]
Trimetrexate (TMQ)	170.0±32.0	267.0±27.0	1.6[n.s.]
ZD1694 (raltitrexed)	5.5±0.5	257.0±92.0	46.7[c]
AG337 (TS inhibitor)	3625±1116	9950±2090	2.7[a]
Vincristine (VCR)	0.2±0.0	1.5±1.0	7.5[b]
Paclitaxel	1.0±0.2	4.4±0.3	4.4[d]
Docetaxel	0.8±0.1	3.7±0.2	4.6[d]
CPT-11 (Camptothecin)	1280±493	2157±309	1.7[n.s.]
SN38	4.7±1.5	7.4±2.1	1.6[n.s.]
Etoposide (VP16)	140.8±29.3	270.0±81.7	1.9[n.s.]
Doxorubicin (DOX)	37.4±10.0	114.8±46.7	3.1[n.s.]
Daunorubicin (DAU)	6.7±1.4	13.5±8.1	2.0[n.s.]
Epirubicin	10.1±2.5	20.3±4.8	2.0[n.s.]
Mitomycin-C (MMC)	52.0±5.8	125.0±24.7	2.4[a]
EO9	17.5±1.6	42.8±8.9	2.4[b]
Cisplatin (CDDP)[e]	1960±770	4510±1010	2.3[n.s.]
Maphosphamide (MAPH)	1119±794	4188±1986	3.7[n.s.]

IC_{50} = concentration causing 50% growth inhibition.

Values are means±standard error of the mean (SEM) of three to eight experiments. Cells were exposed for 72 h. Previously published by [e]Bergman and associates [5]; [f]Ruiz van Haperen and associates [24].

For statistical analysis a Student *t*-test was used, revealing: [n.s.]non significant; [a]$P<0.05$; [b]$P<0.02$; [c]$P<0.01$; [d]$P<0.001$.

dFdC, 2′,2′-difluorodeoxycytidine; dFdU, 2′,2′-difluorodeoxyuridine; dFdG, 2′,2′-difluorodeoxyguanosine; ara-C, 1-β-D-arabinofuranosylcytosine; CdA [8-^{3}H]-2 chloro-2′deoxyadenosine (CdA).

3.2. Enzyme assays, [^{3}H]-MTX uptake, polyglutamate pools, glutathione levels, topoisomerase I and IIα mRNA expression

To investigate possible mechanisms for these cross-resistance patterns, we measured the activities of several enzymes known to be the target enzyme for drugs or responsible for the conversion of the drugs into active metabolites (Table 2). As described before, AG6000 cells are dCK-deficient. We also measured dCK with CdA, in contrast to dCyd, a specific substrate for dCK. With CdA, dCK activity was hardly detectable in AG6000 cells, underlining the complete dCK deficiency. Since dFdG can also be activated by the more specific dGK, we measured the dGK activity, but this was not altered in AG6000 cells, indicating that the cross-resistance of AG6000 cells to dFdG was possibly due to a lack of conversion by dCK.

Thymidylate synthase (TS), the target enzyme for 5-FU and the antifolates ZD1694 and AG337, was 1.7-fold increased in AG6000 cells. Since polyglutamates of ZD1694 are much more potent TS inhibitors, we reasoned that the enzyme catalysing polyglutamation might be changed, but activities of FPGS were similar in A2780 and AG6000 cells. In addition, [^{3}H]-MTX uptake and accumulation of the intracellular polyglutamate pools were comparable with a tendency to an increase of the higher polyglutamates in the AG6000 cells.

The bioreductive indoloquinone EO9 requires activation by DTD; the reduced sensitivity of AG6000 cells correlates with the 2.2-fold lower DTD activity in AG6000 cells compared with A2780 cells. The 2.3-fold lower sensitivity for CDDP of the AG6000 cells might be explained by the longer retention of DNA adducts in A2780 than in AG6000 cells. Differences in glutathione levels, which might detoxify drugs, were not found between the cell lines.

In AG6000 cells, a decreased *topoisomerase I* and *IIα* mRNA expression was found (Fig. 1). The decreased *topoisomerase I* mRNA expression might clarify the 1.7- and 1.6-fold cross-resistance to CPT-11 and SN38, respectively, and the decreased *topoisomerase IIα* mRNA expression the 2- to 3-fold cross-resistance to etoposide, DAU, doxorubicin (DOX) and epirubicin.

Table 2
Enzyme activities, [^{3}H]-MTX uptake, MTX polyglutamate accumulation, glutathione levels and DNA platination and retention of the human ovarian cell lines A2780 and AG6000

	A2780	AG6000
Enzyme		
dCK[a,c]	1.42±0.63	0.16±0.03
dCK[b]	0.30±0.03	0.02±0.00
dGK	1.39±0.17	1.17±0.27
DTD	28.9±2.5	13.2±1.2
TS	0.67±0.21	1.17±0.26
FPGS	0.50±0.01	0.40±0.14
[^{3}H]-MTX uptake	0.97±0.09	0.96±0.06
MTX polyglutamates		
Glutamate 1	1.92±0.18	1.92±0.13
Glutamate 2	1.43±0.19	1.52±0.26
Glutamate 3	0.73±0.11	1.13±0.20
Glutamate 4	0.20±0.01	0.32±0.05
Glutathione levels	1.77±0.27	1.81±0.11
Pt–DNA adduct retention[d]		
4 h	48.0±34.5	18.7±11.2
24 h	42.3±21.6	6.0±3.5

Enzyme activities in nmol/h/10^6 cells, dCK activity with dCyd[a] and CdA[b] as a substrate, [^{3}H]-MTX uptake in pmol/min/10^7 (2 μM for 3 min), MTX polyglutamates in pmol/10^6 cells, glutathione in nmol/10^6 cells and total Pt–DNA adduct levels (total Pt-GG and Pt-AG), determined by ^{32}P-postlabelling, in fmol platinum/μg DNA after 1 h exposure to equitoxic concentrations of cisplatin (3 μM for A2780 and 17 μM for AG6000) followed by a 4 and 24 h drug-free period. All values are means±SEM of at least three separate experiments. Previously published by [c]Ruiz van Haperen VWT and associates [24], [d]Van Moorsel and associates [46].

Pt, platinum; MTX, methotrexate; TS, thymidylate synthase; DTD, DT-diaphorase; DGK, deoxyguanosine kinase; FPGS, folylpolyglutamate synthetase; dCK, deoxycytidine kinase.

3.3. DNA damage

Since several of the drugs for which we observed cross-resistance, interact with DNA, we evaluated the possibility that the IC_{50}s observed were the result of differences in drug-induced DNA damage. For this purpose, the cells were exposed to equitoxic concentrations of several representative drugs. The differences

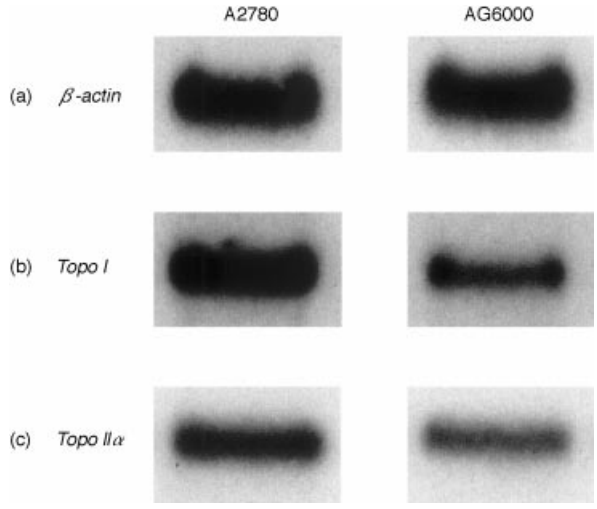

Fig. 1. Representative Northern blots of *β-actin* (a), *topoisomerase I* (b) and *topoisomerase IIα* (c) in A2780 and AG6000 cells. *Topoisomerase* mRNA expression was evaluated by comparison of the density with that of *β-actin*. The *topoisomerase I*/*β-actin* ratios were 2.55±0.04 and 0.58±0.06 in A2780 and AG6000 cells, respectively, and the *topoisomerase IIα*/*β-actin* ratios were 1.22±0.40 and 0.58±0.03 in A2780 and AG6000 cells, respectively. Values are means±SEM (standard error of the mean) of three experiments.

observed in DNA damage between A2780 and AG6000 cells are depicted in Fig. 2.

DNA damage shown as relative amounts of ds-DNA in A2780 and AG6000 cells revealed that exposure to dFdC at the IC_{50} concentration of A2780 cells resulted in significantly more DNA damage in AG6000 cells than in A27800 cells (Fig. 2a). However, at 1000 nM dFdC significantly more DNA damage was found in the A2780 cells compared with AG6000 cells. Exposure to the tubulin-inhibitors VCR and paclitaxel at concentrations of 0.2 and 1.0 nM, respectively, induced more DNA damage in the A2780 than in the AG6000 cells. At these concentrations, paclitaxel caused more DNA damage in both the A2780 and AG6000 cells than VCR. However, at 1.5 nM VCR and 3.3 nM paclitaxel no difference in DNA damage between A2780 and AG6000 cells was found. At 140 nM, the topoisomerase II inhibitor etoposide caused more DNA damage in A2780 than in AG6000 cells. However, at 270 nM no difference was found between A2780 and AG6000 cells, but at equitoxic concentrations etoposide caused more DNA damage in AG6000 than in A2780 cells. The topoisomerase I

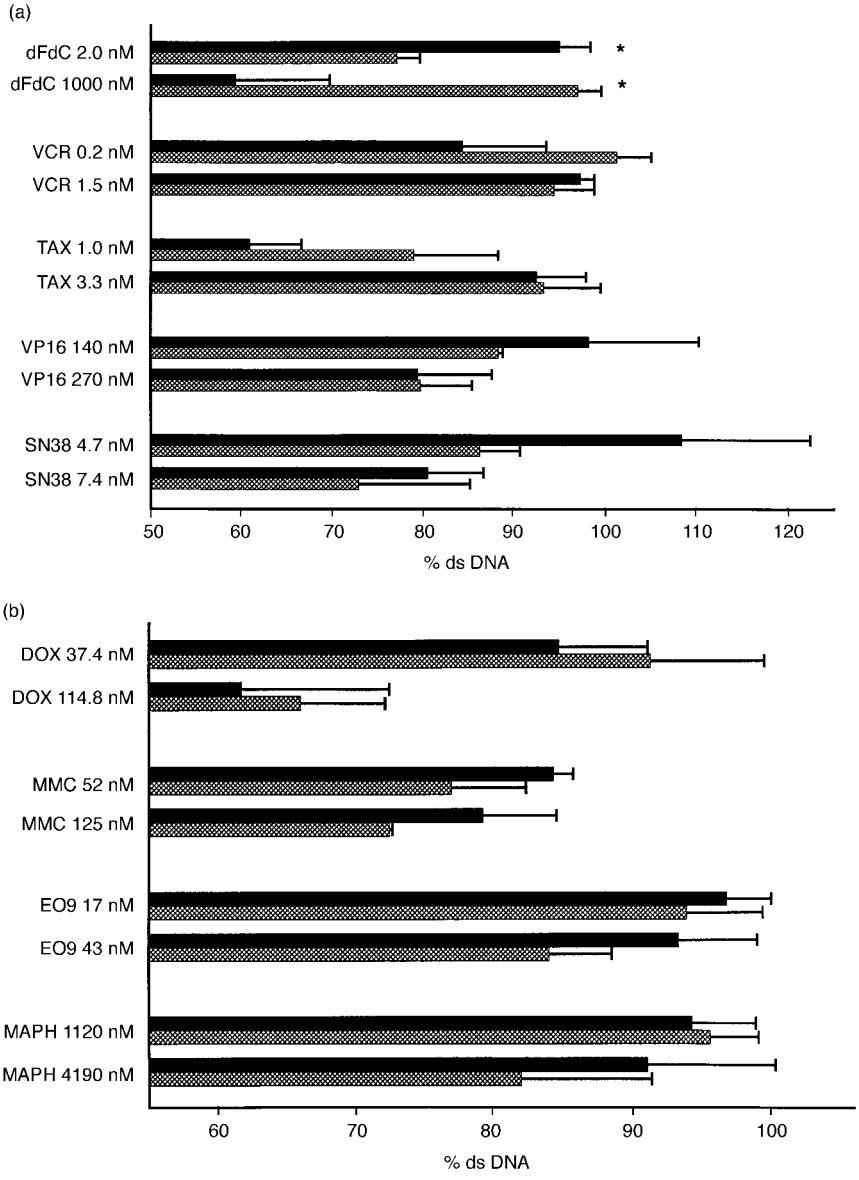

Fig. 2. Relative amount of double-stranded (ds)-DNA in A2780 ■ and AG6000 ▩ cells as a measure of the number of strand-breaks after incubation for 24 h with representative drugs; gemcitabine (dFdC), vincristine (VCR), etoposide (VP16), paclitaxel (TAX) and SN38 (a) doxorubicin (DOX), mitomycin-C (MMC), EO9 and maphosphamide (MAPH) (b). Cells were exposed to concentrations at IC_{50} values of both A2780 and AG6000 cells exposed for 72 h. The unwinding time in the fluorometric analysis of DNA unwinding (FADU) assay was 60 min for both cell lines. 1 h exposure to 50 μM etoposide (VP16) was always included as a positive control. Values are means±SEM of percentages of double-stranded (ds)-DNA in treated cells compared with that in untreated cells, which was set at 100% in three–six experiments. The actual extent of ds-DNA of cells not exposed to drugs were: A2780, 78.3±4.1% and AG6000, 70.1±2.2% of fluorescence of samples not exposed to an alkaline environment. *Significant differences between A2780 and AG6000 by Student *t*-test, $P = 0.03$.

inhibitor SN38 caused more DNA damage in AG6000 cells than in A2780 cells both at 4.7 and 7.4 nM.

Fig. 2(b) shows the relative amounts of ds-DNA in A2780 and AG6000 cells after exposure to representative drugs of the miscellaneous group. DOX caused more DNA damage in A2780 than in AG6000 cells at both 37.4 nM and at 114.8 nM. MMC induced more DNA damage in AG6000 than in A2780 cells at both 52 nM and 125 nM. At 17 nM, the bioreductive alkylating agent EO9 did not cause DNA damage in both the A2780 and AG6000 cells, but at 43 nM somewhat more DNA damage was found in AG6000 cells than in A2780 cells. MAPH did not cause DNA damage at 1120 nM, but at 4190 nM, DNA damage was found in both cell lines, with rather more observed in AG6000 than in A2780 cells.

3.4. FACS analysis of Pgp and MRP activity

In order to evaluate whether drug efflux might explain the cross-resistance to anthracyclines, we performed a FACS analysis of drug accumulation in A2780 and AG6000 cells (Table 3). In AG6000 cells accumulation of DAU, a substrate for Pgp and MRP, was 78.6% of that in A2780 cells, for RHO 123 a Pgp substrate, this was 37.2%, while for Calc-AM, a MRP substrate, this was 92.6%. It was also determined whether drug accumulation could be modulated by specific Pgp and MRP inhibitors. Fluorescence of intracellular DAU was not affected by the addition of the specific Pgp inhibitor PSC in both A2780 and AG6000 cells. In addition, the specific MRP inhibitor genistein did not substantially affect the accumulation of DAU in either cell line. The absence of Pgp expression was also indicated by the lack of an effect of PSC on RHO 123 accumulation. Furthermore, with the MRP modulator VCR, no effect was found on accumulation of the MRP probe Calc-AM.

Table 3
FACS analysis of drug accumulation and effect of Pgp and MRP modifiers on drug accumulation in the human ovarian cancer cell lines A2780 and AG6000

Drug	Uptake	Modifier	Effect modifier	
			A2780	AG6000
2 μM daunorubicin (DAU)	78.6	2 μM PSC	106.6	105.4
		200 μM gen.	83.7	90.5
200 ng/ml RHO 123	37.2	2 μM PSC	94.1	91.8
0.5 μM Calc-AM	92.6	200 μM VCR	101.8	95.3

Uptake, relative accumulation of the anthracyclines daunorubicin, rhodamine 123 (RHO 123) and Calcein-AM (Calc-AM) in AG6000 compared with A2780 cells. Accumulation in A2780 cells was set at 100%; effect modifier, values are average percentages of accumulation with modifier relative to drug alone.

Cells were exposed to 2 μM DAU or 200 ng/ml RHO 123 for 60 min or to 0.5 μM Calc-AM for 10 min with or without modifier.

PSC, Pgp inhibitor; gen, genistein, MRP inhibitor; VCR, vincristine, MRP inhibitor; MRP, multidrug resistance-associated protein.

4. Discussion

Knowledge on the mechanism of induction of resistance to gemcitabine, and possible cross-resistance patterns is limited. The gemcitabine-resistant cell line AG6000 was characterised by a cross-resistance to a wide variety of anti-cancer agents which were associated with various mechanisms. The most pronounced defect was the absence of dCK activity, which explains the resistance to dFdC and other deoxynucleoside analogues. However, dFdG can be phosphorylated by both dGK and dCK, but in AG6000 cells dCK deficiency appears to be responsible for the cross-resistance to dFdG, since dGK activity was not changed [44]. This is in contrast to another dCK-deficient cell line in which no cross-resistance to dFdG was found [45]. The large difference in DNA damage between A2780 and AG6000 caused by dFdC, is clearly the result of the absence of dFdC phosphorylation in AG6000 cells. However, differences in the induction of DNA damage may also be related to an increase in DNA repair, as found in the increased repair of DNA platina adducts in AG6000 cells [46].

Since pronounced differences were observed in the sensitivity to various antifolates, we determined the activities of RFC, TS, FPGS and MTX polyglutamation, but only TS was increased 1.7-fold. Both MTX and TMQ have DHFR as their target enzyme, but MTX requires active transport into the cell by RFC and subsequent polyglutamylation in order to be active and retained in the cell, while TMQ diffuses passively into the cell and does not require polyglutamylation [47,48]. The sensitivity of AG6000 to MTX cannot be explained by differences in RFC activity, FPGS activity or MTX polyglutamation. The TS activity was increased in AG6000, which might explain the cross-resistance to 5-FU and to the lipophilic TS inhibitor AG337 and partly that of ZD1694. In contrast to AG337, ZD1694 requires active transport by RFC into the cell. The 46.7-fold cross-resistance to ZD1694 can not be completely explained by the 2-fold increase in TS activity or differences in the RFC activity in AG6000.

AG6000 is cross-resistant to paclitaxel and docetaxel, which is possibly related to a difference in DNA damage between A2780 and AG6000 cells caused by paclitaxel. Other mechanisms of resistance include alterations in the expression levels of individual *β-tubulin* isotypes and increased expression of the *MDR1* gene [49,50]. However, no expression of the product of the *MDR1* gene, Pgp was found in AG6000 cells, although a decrease in transport of other compounds was found.

Camptothecin (CPT-11), a plant alkaloid, strongly inhibits mammalian topoisomerase I, but its metabolite SN38 is 1000-fold more potent *in vitro* [51,52]. Resistance to CPT-11 and SN38 may be attributed to a decrease of topoisomerase I protein and activity [51]. The decreased *topoisomerase I* mRNA expression fits with the moderate cross-resistance to CPT-11 and SN38. However, a decrease of *topoisomerase I* expression does not always relate to decreased topoisomerase I activity [43]. Decreased topoisomerase II activity has been identified as a frequent basis for resistance to the anthracyclines and the epipodophyllotoxin etoposide [53], which fits with the decreased *topoisomerase IIα* mRNA level seen in the AG6000 cells. This phenomenon was also found in an ara-C resistant baby hamster kidney cell line, which had a diminished topoisomerase II activity, and an increased IC_{50} for etoposide [54].

Among the most active anticancer agents are the anthracyclines DOX, DAU and epirubicin and the vinca alkaloid VCR. AG6000 cells were cross-resistant to all natural toxin-derived drugs tested. Although all of the above mentioned drugs are substrates for membrane-efflux pumps, no overexpression of Pgp or MRP was found in the AG6000 cells, which is in agreement with a previous study [24], but the cross-resistance to VCR and DOX can only partially be explained by differences in DNA damage between the A2780 and AG6000 cells. Despite the absence of a Pgp- or MRP1-associated membrane-efflux pump activity, accumulation of DAU in AG6000 cells was lower than in A2780 cells after a 1 h incubation, which might partially explain the cross-resistance of AG6000 cells to the anthracyclines. Differences in drug accumulation in the absence of a membrane-efflux pump activity might be related to differences in membrane permeability or the size of the cell surface. The cell surface of AG6000 cells is 80.7% of the cell surface of A2780 cells (cell diameter; A2780, 13.30 μm; AG6000, 11.95 μm), which might clarify the difference in DAU, but not in RHO 123 accumulation between the A2780 and AG6000 cells [24]. However, other mechanisms can play a role in anthracycline accumulation, such as the encapsulation of the drug in cytoplasmatic acidic vesicles followed by exocytosis [55,56]. Since RHO 123 accumulates in mitochondria, differences in mitochondrial number, membrane potential or active transporters are likely to influence the rate of accumulation of RHO 123 [57,58]. Calc-AM is transformed in the cell to an intensively fluorescent-free acid form by cleavage of the ester bonds by intracellular esterases [59]. Despite the difference in cell surface size, little difference was found in accumulation of Calc-AM between the cell lines, which might be related to an increased cleavage of the ester bonds in AG6000 cells.

The differences in DNA damage between A2780 and AG6000 cells exposed to EO9 and MAPH, an active analogue of cyclophosphamide, can not explain the cross-resistance to these drugs. The cross-resistance to MMC and EO9 could be related to the decreased DTD activity in AG6000 cells, which is in agreement with previous studies [33].

AG6000 cells were less sensitive to CDDP than A2780 cells. The possible mechanisms of CDDP resistance *in vitro* include enhanced platinum–DNA adduct repair capacity and elevated GSH. Decreased platinum–DNA adduct formation and increased repair of platinum–DNA adducts have been shown to be associated with CDDP resistance in several *in vitro* models [60]. Evidence for the formation of GSH–platinum complexes in cells exposed to cisplatin has been reported [61], and several investigators have observed increased glutathione levels in cells selected for CDDP resistance *in vitro* [62]. In a study in unrelated human ovarian cancer cell lines with different sensitivities to CDDP [63], multiple regression and correlation analysis revealed a strong correlation between cisplatin sensitivity and platinum (Pt)–DNA damage tolerance, but cellular GSH levels did not correlate. Moreover, a strong correlation was found between Pt–DNA damage tolerance and cross-resistance to DOX, paclitaxel, etoposide and MMC. It was suggested that failure in recognising and processing drug-induced damage (including Pt–DNA adducts) may result in a broad resistance phenotype [63]. Differences in retention of Pt–DNA adducts were found between A2780 and AG6000 cells [46]. Probably AG6000 cells have a increased Pt–DNA damage repair mechanism, which might be related to the decreased sensitivity to CDDP.

Cross-resistance might be due to gene amplification, which might result in, e.g. increased DNA repair. Gene amplification plays a role in oncogenesis and resistance to anticancer drugs [64]. Recombination is very likely an essential process in gene amplification [65]. Given that topoisomerases break and rejoin DNA as part of their various cellular functions, and that breakage and rejoining of DNA are steps necessary for genetic recombination, it is possible that topoisomerases may be involved in recombination and gene amplification [66]. Holm and colleagues reported that during mitosis, in yeast cells with a decreased topoisomerase II activity, separation of chromosomes is impaired and the chromosomes are damaged or broken as the spindle pulls the poorly separated chromosomes apart [67]. As cited above, topoisomerase downregulation was described in an ara-C-resistant baby hamster kidney cell line [54]. Since ara-C is structurally and functionally related to gemcitabine, a comparable mechanism might explain the cross-resistance to etoposide of AG6000 cells. Moreover, a decreased topoisomerase II activity, as a result of gemcitabine resistance, might explain recombinations and gene amplifications in AG6000 cells resulting in altered enzyme activities and cell functions, ultimately leading to a wide range of cross-resistance.

In conclusion, AG6000 is a cell line that is highly resistant to dFdC and cross-resistant to a wide variety of cytostatic agents. This resistance could partially be explained by differences in enzyme activities and the extent of DNA damage in A2780 and AG6000 cells.

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