

INCREASE OF DAUNORUBICIN AND VINCRIStINE ACCUMULATION IN MULTIDRUG RESISTANT HUMAN OVARIAN CARCINOMA CELLS BY A MONOCLONAL ANTIBODY REACTING WITH P-GLYCOPROTEIN*

HENRICUS J. BROXTERMAN, CATHARINA M. KUIPER, GERRIT J. SCHUURHUIS,
TAKASHI TSURUO,† HERBERT M. PINEDO and JAN LANKELMA

Department of Oncology, Free University Hospital, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands, and † Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo, Japan

(Received 23 June 1987; accepted 29 December 1987)

Abstract—An overexpression of plasma membrane 170–180 kDa P-glycoproteins is consistently found in multidrug-resistant (MDR) cell lines. In this study MRK-16, a monoclonal antibody (mAb) reacting with P-glycoprotein is used to study the putative functional role of this protein in vincristine (VCR) and daunorubicin (DNR) cellular accumulation in the MDR human ovarian carcinoma cell line 2780^{AD}. We established that this cell line is highly cross-resistant to vincristine and daunomycin, related to a greatly reduced drug accumulation. Verapamil (Vp) (8 μ M) caused a 3.6-fold increase in DNR as well as VCR accumulation. Exposition of 2780^{AD} cells to MRK-16 led to an increase of 30% in cellular accumulation of VCR, both in normal growth medium as well as in medium without added glucose and with sodium azide, which largely depleted cellular ATP levels. No increase in DNR accumulation was found under these conditions. However, in the presence of 8 μ M Vp, MRK-16 increased not only VCR but also DNR accumulation with about 30%. The relative increase of DNR accumulation was constant in a concentration range of 0.2–4 μ M DNR. These data indicate that mAbs against P-glycoprotein might potentiate the action of calcium antagonists like Vp to increase cellular anthracycline accumulation.

The development of drug resistance is a major cause of the failure of chemotherapeutic treatment of human malignancies. The problem is the more urgent, because of the general occurrence of clones of tumor cells which are resistant to multiple drugs. This drastically reduces the number of potentially active drugs to treat the individual patient [1].

The phenomenon of multidrug resistance (MDR)‡ has been studied intensively *in vitro* using cell lines made resistant to colchicine, anthracyclines or vinca alkaloids, which display cross-resistance to unrelated drugs [2–4]. Although MDR is a complex phenotype, it appears that a decreased cellular accumulation caused by increased efflux of drugs is a common feature of these cell lines, accounting for at least part of the resistance. From a number of independent lines of evidence it has emerged that a genetic defect resulting in an overexpression of a 170–180 kDa glycoprotein, called P-glycoprotein is a consistent finding in MDR cells [5–7]. Moreover, by gene transfection experiments it was possible to confer multidrug resistance to otherwise drug-sensitive cells [8].

There is, however, still considerable controversy

in the literature as to what extent a common event is responsible for resistance to anthracyclines and to vinca alkaloids in MDR cells. This is related to the findings that calcium antagonists like Vp and calmodulin inhibitors like trifluoperazine, which block the enhanced efflux of drugs from MDR cells, are found only partially to reverse resistance in MDR cell lines even at very high concentrations [9, 10]. Generally, a greater effect of resistance modifiers on VCR compared to anthracycline cytotoxicity is found in P388 mouse leukemia [10, 11], K562 human myelogenous leukemia [10] and CEM human leukemic lymphoblasts [12]. However, for a human sarcoma MDR cell line the reverse has been found, i.e. Vp only enhanced the effect of drugs that interact with DNA, like anthracyclines and dactinomycin again questioning the correlation between Vp effects and drug efflux modulation [13].

One way of clarifying the role of a P-glycoprotein related enhanced drug efflux mechanism as a common factor in resistance to vinca alkaloids as well as anthracyclines is to study whether and in what manner mAbs reacting to P-glycoprotein can interfere with the cellular accumulation and cytotoxicity of both classes of drugs. A mAb (MRK-16) that recognizes a cell-surface exposed epitope of P-glycoprotein was developed and was shown to increase VCR but not adriamycin toxicity in MDR K562 human leukemia cells [14]. In this study we describe effects of MRK-16 on both VCR and DNR accumulation in 2780^{AD} human ovarian carcinoma MDR cells.

* This work was supported by the Netherlands Cancer Foundation (KWF grant IKA-VU 85-05).

‡ Abbreviations used: MDR, multidrug resistant; VCR, vincristine; DNR, daunorubicin; mAb, monoclonal antibody; Vp, verapamil; EDTA, ethylenediaminetetraacetate disodium salt; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; DMF, dose-modifying factor.

MATERIALS AND METHODS

Tumor cells and chemicals. The source and maintenance conditions of A2780 and 2780^{AD} human ovarian carcinoma cell lines have been described before [15, 16]. Verapamil . HCl and vincristine-sulfate were from Sigma Chemical Corp. (St. Louis, MO). Daunorubicin . HCl was from Specia (Paris, France). Ro 11-2933/001 (*N*-[3,4-dimethoxyphenethyl] - 2 - [2-naphthyl] - *N* - methyl - *m* - dithiane - 2 - propylamine hydrochloride) was a gift from Hoffman-La Roche (Mijdrecht, The Netherlands). [¹⁴C] daunorubicin (sp. act. 45 Ci/mol) and [³H] vincristine sulfate (sp. act. 4.8 Ci/mmol) were obtained from Amersham (Amersham, U.K.). The radiochemical purity of both was checked and found to be >98%. MRK-16 was purified by precipitation with ammoniumsulphate and DEAE-Sephacel chromatography and stored at -70° [14].

Cellular accumulation of drugs in vitro. Cells from exponentially growing cultures were suspended with trypsin/EDTA (0.0025% and 0.25% w/v respectively) or EDTA alone in Ca²⁺ and Mg²⁺-free Hank's balanced salt solution (pH = 7.4). Both resuspension methods gave essentially similar results. Cells were resuspended in densities of about 1 × 10⁶/ml in Dulbecco's modified essential medium without bicarbonate, but with 20 mM HEPES (Serva, Heidelberg, F.R.G.) (pH = 7.4) and 10% heat-inactivated fetal calf serum (Flow Laboratories, Irvine, U.K.) (medium A). Medium A without glucose is referred to as medium B, while medium B + 10 mM sodiumazide (Baker Chemicals, Deventer, The Netherlands) is called Medium C. Cellular ATP levels after incubation in media A, B and C were determined by an HPLC method as described [17]. Cells (3–4 × 10⁵) were incubated with radiolabeled drugs in polypropylene vials in 440 µl medium, washed and radioactivity was determined as described before [16]. In experiments with MRK-16 (whole antibody; 15 µg protein/sample), the cells were preincubated with all reagents for 30 min at 4°. As a control, samples were incubated without mAb or in some experiments with mAb against human HLA-A-B-C (MAS 5326, Sera-Lab). The latter had no effects on drug accumulation compared to controls without mAb. A paired *t*-test was used to evaluate the results.

Growth-inhibition assays in vitro. IC₅₀ Values, i.e. drug concentrations inhibiting cell growth by 50% compared to drug-free controls, were determined in microtiter plates as described [16]. For short-time incubations with drugs, the drug containing medium was aspirated after 3 hr and fresh growth medium was added to allow cells to grow for 72 hr, i.e. about three doubling times.

RESULTS

Reversal of resistance by calcium antagonists

Resistance factors (IC₅₀ of resistant cells divided by IC₅₀ of sensitive cells) for DNR and VCR in 2780^{AD} cells, which were developed from the parent cell line A2780 by selection with doxorubicin were 240 and 610 respectively (continuous drug exposure). The dose modifying factors (DMF) of Vp and Ro

Table 1. Reversal of drug resistance in 2780^{AD} cells

Treatment	DNR 72 hr	DMF*	
		DNR 3 hr	VCR 72 hr
2 µM Vp	4.4 ± 2.0	3.1 ± 0.7	3.0 ± 1.3
4 µM Vp	8.5 ± 0.7	4.3 ± 2.1	7.1 ± 2.8
8 µM Vp	17.8 ± 6.0	17.2 ± 6.6	21.0 ± 2.8
1 µM Ro 11-2933	14.8 ± 4.1	—	19.0 ± 5.7

* Values are expressed as means ± SD of at least 3 experiments. DMF is dose-modifying factor, which is IC₅₀ without calcium antagonist divided by IC₅₀ with calcium antagonist.

11-2933/001 are shown in Table 1. It appears that in this cell line the effects of calcium antagonists on DNR and VCR cytotoxicity are very similar. Vp itself at 8 µM caused a significant growth-inhibition to 62 ± 14% (mean ± SD of 12 experiments) after 72 hr exposure, but not after 3 hr (95 ± 6%, N = 4). However, we also determined for DNR, that the DMF of Vp is not dependent on time of drug exposure. In A2780 the calcium antagonists did not significantly increase DNR or VCR toxicity (not shown).

Accumulation defects in 2780^{AD}

It has been determined before that 2780^{AD} expresses increased amounts of P-glycoprotein [14, 18] as well as a large decrease in DNR accumulation compared to the parent sensitive line [16]. We now determined VCR accumulation in 2780^{AD} and A2780 cells. Figure 1 shows that A2780 accumulates about 20 pmol VCR/10⁶ cells (at 2 µM VCR) while 2780^{AD} maintained a low intracellular VCR level, which in fact did not rise above the direct binding measured at 0°. For these reasons we feel confident that 2780^{AD} is a good model MDR cell line to study the effects of a mAb to P-glycoprotein on anthracycline as well as vinca alkaloid accumulation and cytotoxicity.

Effect of MRK-16 on DNR and VCR accumulation

The effects of MRK-16 were studied under different conditions with respect to energy supply to the cells, because it has been shown for a number of cell lines that the accumulation in and efflux of drugs from MDR cells is energy-dependent. For the media used in this study, only in medium C did cellular energy depletion occur, which was checked by measurement of cellular ATP levels (Fig. 2). However, cell viability (exclusion of trypan blue) was not reduced during 60 min incubation.

We found that in medium A, which is comparable to normal growth medium, MRK-16 increased VCR but not DNR accumulation in 2780^{AD} cells (Table 2). This confirms the initial observations on K562/AD, human leukemia cells [14]. Similar results were found in mediums B and C, although the percentage of stimulation of VCR uptake tended to be lower in medium B. No effects on DNR accumulation were seen in any medium. However, when MRK-16 was combined with Vp, which itself causes an increase in DNR or VCR accumulation with a factor 3.6 (see Ref. 16, Fig. 3, for DNR and 3.6 ± 0.6, mean ± SD

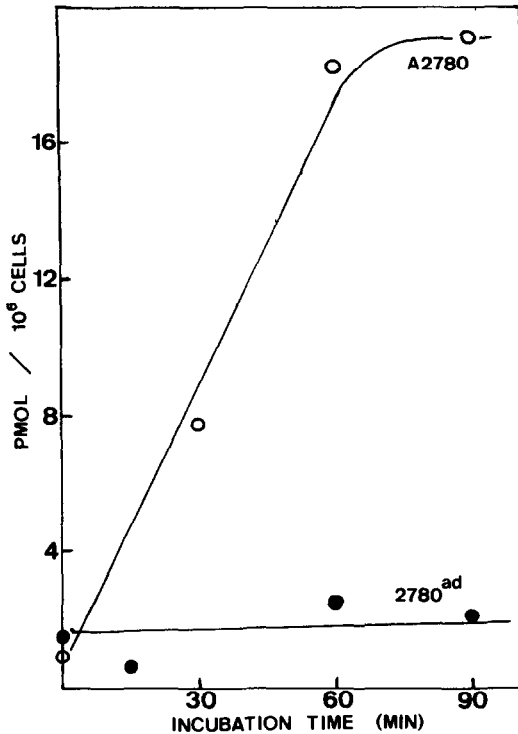


Fig. 1. [^3H] vincristine accumulation in A2780 (○) and 2780^{AD} (●) cells. Cells were suspended in medium A and exposed to 2 μM VCR. Mean of two experiments. SD < 10%.

of 4 exp. for VCR), net cellular uptake of DNR was stimulated by MRK-16. The largest effect was found in medium B and was similar to enhancement of VCR accumulation by MRK-16. A small, but significant increase in DNR accumulation was also seen in medium A and C. The accumulation of VCR was stimulated by MRK-16 with rather similar percentages, whether Vp was present or not. Therefore the stimulation in absolute amounts of VCR is much greater in the presence of Vp than in its absence (about 1 pmol and 0.2 pmol/ 10^6 cells respectively). In none of the conditions used here MRK-16 affected drug accumulation in A2780 cells.

The MRK-16 effect on DNR accumulation was also studied in medium B + 8 μM Vp at different

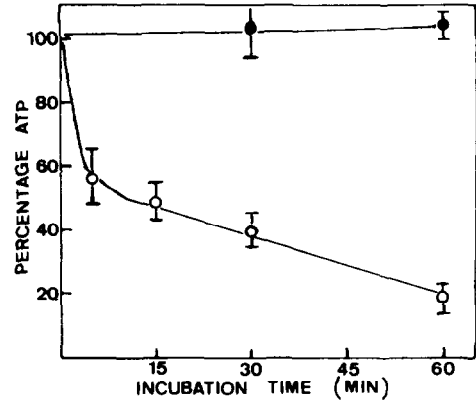


Fig. 2. Effect of incubation of 2780^{AD} cells in medium B (●) and medium C (○) on ATP levels. Mean \pm SD of 4 experiments.

DNR concentrations. Within the range of DNR concentrations studied (0.2 μM –4 μM DNR), the percentage of uptake stimulation was the same as in Table 2 (not shown). This means that at 0.2 μM DNR MRK-16 brought about a net stimulation of about 10 pmol/ 10^6 cells, but at 4 μM DNR of about 150 pmol/ 10^6 cells at steady-state levels. Thus no saturation of MRK-16 effect as to amount of extra DNR net uptake was apparent.

DISCUSSION

Multidrug cross-resistance to chemotherapeutic agents of different, structurally unrelated classes is tentatively explained by the existence of a common drug efflux mechanism [4]. Also, the overexpression of membrane-glycoproteins (P-glycoprotein) has been implicated in the enhanced drug efflux [19, 20]. Further, the reversal of drug resistance by membrane-active agents like Vp has been shown to be related to inhibition of the enhanced drug efflux from resistant cells [10]. The question whether Vp acts by a common mechanism on vinca-alkaloid and anthracycline transport to reverse resistance has not been clarified. In fact, verapamil induced resensitization of vinca-alkaloid resistance is usually greater than reversal of anthracycline resistance [10, 12], leading to the conclusion that Vp does not effectively reverse MDR phenotype of human leukemic blasts [12]. In

Table 2. Effect of MRK-16 on drug accumulation in 2780^{AD} cells

Drug	Medium A		Medium B		Medium C	
	–Vp	+Vp†	–Vp	+Vp	–Vp	+Vp
DNR	99.3 \pm 5.7*‡	115.5 \pm 1.7§	98.0 \pm 7.5‡	130.0 \pm 6.9§	96.3 \pm 3.3‡	111.2 \pm 7.2‡
VCR	130.0 \pm 14.0	131.2 \pm 5.4	115.5 \pm 4.9	138.3 \pm 16.6§	149.4 \pm 11.7§	129.4 \pm 5.3§

* Values are expressed as percentage of drug accumulation without MRK-16, mean \pm SD of at least 4 experiments. Extracellular DNR concentration was 1 μM . Absolute amounts of cellular drug accumulation are stimulated by 8 μM Vp with 300–500%.

† +Vp means: +8 μM Verapamil.

‡ Not statistically different from controls without MRK-16.

§ $P < 0.01$ compared to controls without MRK-16.

|| $P < 0.02$ compared to controls without MRK-16.

contrast it has been found that Vp does not reverse VCR resistance in a human sarcoma cell line [13]. We now used a human ovarian carcinoma MDR cell line and found that Vp effects on DNR as well as on VCR toxicity were quantitatively similar. Both DNR and VCR accumulation are very low compared to the sensitive parent cells. Further, we showed before that Vp effects on DNR accumulation are linearly correlated with its effects on DNR cytotoxicity (DMF) in this cell line [16]. Also in that study it was found that 8 μ M Vp caused a 3.6 times increase in DNR accumulation, which is the same as found in this study for VCR. Thus, in 2780^{AD} both vinca alkaloid as well as anthracycline resistance seem to be of MDR-type. Overexpression of P-glycoprotein as detected with MRK-16 has also been shown for 2780^{AD} [14]. We now have shown that with MRK-16 it is possible to increase DNR accumulation in 2780^{AD} cells. The magnitude of MRK-16 effects on VCR as well as DNR accumulation remains rather modest, since the maximal effect seems to be a 30% stimulation of net uptake over baseline level, independent of the conditions used. Since the absolute increase in DNR accumulation (in pmol/10⁶) was not saturated, the maximal percentage of drug increase may not be due to saturation of any intrinsic drug transport involved, but rather to the inability of MRK-16 to completely block drug efflux. In addition, the results indicate that the MRK-16 effects on P-glycoprotein function are to a large extent independent of energy state of the cells. Energy depletion on itself was not a sufficient nor necessary condition to bring about enhancement of DNR net uptake by MRK-16. However, the presence of Vp was required for this effect to become apparent.

One possible explanation for this effect is that Vp binding to P-glycoprotein as recently reported [20] by competition [9] may decrease the number of binding sites available for DNR binding, thereby limiting the capacity for DNR extrusion. Also it could be that Vp alters some aspect of P-glycoprotein function making it more prone to modulation by mAb. However, since no greater effects are seen on VCR accumulation whether Vp is present or not, the latter possibility does not seem to be likely. A third possibility is that MRK-16 increases intracellular Vp concentration, which by action at other sites than the plasmamembrane might increase DNR accumulation [21]. It remains to be tested how this effect of Vp is mediated.

In conclusion, the present study shows that *in vitro* a mAb to P-glycoprotein may increase the effect of calcium channel blockers on anthracycline uptake in MDR cells, but clearly not on normal not P-glycoprotein expressing cells. This may make Vp or other calcium channel blockers more selective in reversing drug resistance. Whether this finding may have any relevance for the modulation of clinical anthracycline resistance remains to be determined.

REFERENCES

- DeVita VT Jr, Principles of chemotherapy. In: *Principles and Practice of Oncology* (Eds. DeVita VT Jr, Hellman S and Rosenberg SA), pp. 132–155. J. B. Lippincott, Philadelphia, 1982.
- Biedler JL and Riehm H, Cellular resistance to actinomycin D in chinese hamster cells *in vitro*: cross-resistance, radioautographic and cytogenetic studies. *Cancer Res* 30: 1174–1184, 1970.
- Inaba M and Johnson RK, Decreased retention of actinomycin D as the basis for cross-resistance in anthracycline-resistant sublines of P388 leukemia. *Cancer Res* 37: 4629–4634, 1977.
- Skovsgaard T, Carrier-mediated transport of daunorubicin, adriamycin and rubidazole in Ehrlich ascites tumour cells. *Biochem Pharmacol* 27: 1221–1227, 1978.
- Peterson RHF and Biedler JF, Plasma membrane proteins and glycoproteins from chinese hamster cells sensitive and resistant to actinomycin D. *J Supramol Struct* 9: 289–298, 1978.
- Kartner N, Riordan JR and Ling V, Cell surface P-glycoprotein associated with multidrug resistance in mammalian cell lines. *Science* 221: 1285–1288, 1983.
- Van der Bliek AM, Van der Velde-Koerts T, Ling V and Borst P, Overexpression and amplification of fine genes in a multidrug-resistant chinese hamster ovary cell line. *Mol Cell Biol* 6: 1671–1678, 1986.
- Gros P, Neriai YB, Croop JM and Housman DE, Isolation and expression of a complementary DNA that confers multidrug resistance. *Nature* 323: 728–731, 1986.
- Kessel D, Circumvention of resistance to anthracyclines by calcium antagonists and other membrane-perturbing agents. *Cancer Surveys* 5: 109–127, 1986.
- Tsuruo T, Iida H, Nojiri M, Tsukagoshi S and Sakurai Y, Circumvention of vincristine and adriamycin resistance *in vitro* and *in vivo* by calcium influx blockers. *Cancer Res* 43: 2905–2910, 1983.
- Ganapathi R, Grabowski D and Schmidt H, Factors governing the modulation of vinca-alkaloid resistance in doxorubicin-resistant cells by the calmodulin inhibitor trifluoperazine. *Biochem Pharmacol* 35: 673–678, 1986.
- Beck WT, Cirtain MC, Look T and Ashmun RA, Reversal of vinca alkaloid multiple drug resistance in human leukemic cells by Verapamil. *Cancer Res* 46: 778–784, 1986.
- Harker WG, Bauer D, Etiz BB, Newman RA and Sikic BI, Verapamil-mediated sensitization of doxorubicin-selected pleiotropic resistance in human sarcoma cells: selectivity for drugs which produce DNA scission. *Cancer Res* 46: 2369–2373, 1986.
- Hamada H and Tsuruo T, Functional role for the 170- to 180-kDa glycoprotein specific to drug-resistant tumor cells as revealed by monoclonal antibodies. *Proc Natl Acad Sci USA* 83: 7785–7789, 1986.
- Rogan AM, Hamilton TC, Young RC, Klecker RW and Ozols RF, Reversal of adriamycin resistance by verapamil in human ovarian cancer. *Science* 224: 994–996, 1984.
- Broxterman HJ, Kuiper CM, Schuurhuis GJ, Van der Hoeven JJM, Pinedo HM and Lankelma J, Daunomycin accumulation in resistant tumor cells as a screening model for resistance modifying drugs: role of protein binding. *Cancer Lett* 35: 87–95, 1987.
- Leyva A, Appel H and Pinedo HM, Purine modulation of thymidine activity in L1210 leukemia cells *in vitro*. *Leukemia Res* 6: 483–490, 1982.
- Scheper RJ, Bulte JWM, Quak JJ, Brakkee JG, Broxterman HJ, Kuiper CM and Lankelma J, Production of a monoclonal antibody (JSB-1) with high affinity for multidrug resistant tumor cells. In: *Proc Ann Meeting American Assoc Cancer Res* 28: 360, 1987.
- Gerlach JH, Endicott JA, Juranka PF, Henderson G, Sarangi F, Deuchars KL and Ling V, Homology between P-glycoprotein and a bacterial haemolysin transport protein suggests a model for multidrug resistance. *Nature* 324: 485–489, 1986.

20. Cornwell MM, Pastan I and Gottesman MM, Certain calcium channel blockers bind specifically to multidrug resistant human KB carcinoma membrane vesicles and inhibit drug binding to P-glycoprotein. *J Biol Chem* **262**: 2166–2170, 1987.
21. Schuurhuis GJ, Broxterman HJ, Pinedo HM and Lankelma J, Accumulation defects can quantitatively account for doxorubicin resistance in 2 multi-drug resistant cell lines. In: *Proc Ann Meeting American Assoc Cancer Res* **28**: 298, 1987.