Biochemical Pharmacology, Vol. 37, No. 15, pp. 3038–3041, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

Reduced cytotoxicity of tetracyclines to a multi-drug resistant human cell line

(*Received* 8 *October* 1987; *accepted* 15 *February* 1988)

A major mechanism of resistance to anthracyclines (identified in tumor cells selected *in vitro* and *in vivo*) is a reduced level of intracellular drug, due in part to energy-dependent drug extrusion from resistant cells [1, 2]. Cells resistant to anthracyclines are cross-resistant to a variety of cytotoxic compounds such as vinca alkaloids, m-AMSA, VP-16 and mitoxantrone. It appears that resistant cells are able to recognize and extrude different chemical structures; although several compounds can antagonize drug efflux, no competition has been demonstrated among the various cross-resistant compounds themselves [3].

Although the extrusion mechanism has not been fully elucidated, a striking alteration observed in many multiresistant cells of different origin is an increased expression of a high molecular weight membrane glycoprotein (p170) [4]. Recently it has been shown that p170 presents sequence homologies with an *E. coli* membrane protein required for the extracellular transport of haemolysin [5].

Thus, it appears that p170 has been conserved during evolution, and that it can represent an important mechanism of transport and/or detoxification for both prokaryotes and eukaryotes.

In this report we present evidence that a multiresistant human colon adenocarcinoma cell line, LoVo/DX* [6] is less susceptible than its sensitive parent line, LoVo, to the cytotoxic effects of the antibiotics tetracycline and demethylchlorotetracycline (demeclocycline). As evaluated with cDNA probe, a 10-fold amplification of the *mdr* gene, responsible for p170 expression, was evidenced on LoVo/DX (Ballinari *et al.*, in preparation). Partial restoration of sensitivity to tetracyclines in the resistant cell line was observed by combination of the antibiotics with verapamil (V) a calcium channel blocker able to revert *in vitro* resistance to doxorubicin (DX) and other cross-resistant compounds in multiresistant cells [7]. Tetracyclines block ribosome function both on prokaryotes and eukaryotes, their selective antibiotic effect being due to an active uptake system present only on bacteria [8]. A well-documented mechanism of resistance to tetracyclines in bacteria is an amplified, energy-requiring efflux system [8, 9]. The reversion of the cytotoxic effect of tetracyclines, observed with verapamil suggests that the multidrug resistance mechanism in mammalian cells may be somewhat similar to that reported in bacteria.

Materials and methods

Drugs

DX and tetracycline were from Farmitalia C. Erba (Milan, Italy); demethylchlorotetracycline was a gift from G. Lancini, Dow Merrel (Milan, Italy). They were dissolved in water at the concentrations of 1.72 mM (DX) and 4.5 mM (tetracyclines) and adjusted to the final concentrations with the growth medium. Verapamil was a pharmaceutical preparation (Isoptin, Knoll AG, Liestal, Switzerland).

Cell lines and culture conditions

LoVo and LoVo/DX were maintained at 37° in humidified atmosphere of 5% CO_2. The growth medium was Ham's F12 medium supplemented with 10% fetal calf serum, 1% of a 200 mM glutamine solution, 100 U/ml penicillin, 100 μg/ml streptomycin and 1% of a BME vitamin solution 100X. Cells were passaged twice weekly; DX was added to LoVo/DX medium at the final concentration of 0.172 μM at every passage.

Cytotoxicity assay

(a) *Continuous exposure with cell count assay.* Exponentially growing cells were harvested with 0.25% trypsin, adjusted to the concentration of 5×10^4 cells/ml and distributed in plastic dishes (Falcon, 36 mm ∅, 2 ml/dish) containing graded drug concentrations. After 72 hr incubation, cells were harvested with trypsin, resuspended in 2 ml growth medium and the cell number was measured with a model .ZBI Coulter Counter. Cytotoxicity is expressed as the percentage of surviving cells in treated samples vs untreated controls.

(b) *Short-term exposure with colony growth assay.* Exponentially growing cells were harvested with 0.25% trypsin and adjusted to the concentration of 300 cells/ml. The cell suspension was seeded in plastic dishes (Falcon, 36 mm ∅, 2 ml/dish) 48 hr before treatment; cells were exposed to the drugs by replacing the growth medium with drug-containing medium. Exposure was 4 hr; the medium was then withdrawn, the cells were rinsed once with saline and fresh growth medium was added. Colonies were counted under an inverted microscope after 7–10 days of incubation; cytotoxicity is expressed as the percentage of colonies in treated samples vs untreated controls.

Results and discussion

Cytotoxic activity of tetracyclines. The cytotoxic activity of tetracycline and demeclocycline on LoVo and LoVo/DX after 72 hr continuous treatment is reported in Fig. 1. The drug concentrations which turn out to be cytotoxic on the sensitive line, LoVo, (4–20 μM) do not differ substantially from those reported to affect the growth of most gram-positive bacteria (2–10 μM) [10], although a direct comparison cannot be made, due to the different test systems employed and the much faster division rate in bacteria. On the contrary, higher doses of tetracycline and demeclocycline have to be employed to inhibit proliferation of LoVo/DX (20–100 μM). The resistance index (R.I.), determined as the ratio between the ID_{50} (drug concentration inhibiting cell proliferation by 50%) on LoVo/DX vs LoVo is low when compared with the R.I. of DX on the same cell lines (3–6 vs 30) [6]. However, it is evident that the two antibiotics are less cytotoxic to the DX-resistant cell line than to the sensitive counterpart. This reduced sensitivity cannot be due to a difference in the cell cycle, since flow cytometric analysis has demonstrated that the growth fraction and the duration of the cell cycle are the same in both cell lines (Broggini *et al.*, submitted). We also tested the cytotoxic activity of the compounds after short-term exposure (4 hr) as inhibition of colony growth (Fig. 2). Although the cytotoxic doses are higher than in the 72-hr test, a reduced effect of tetracyclines on LoVo/DX is evident.

Effect of verapamil on tetracyclines cytotoxicity. V, like other calcium channel blockers, calmodulin inhibitors and a variety of different compounds [7, 11, 12], is an effective agent in reverting *in vitro* resistance to anthracyclines and other cross-resistant compounds.

* Abbreviations used: DX, doxorubicin; ID_{50}, dose inhibiting by 50% colony growth; R.I., resistance index; V, verapamil; SD, standard deviation.

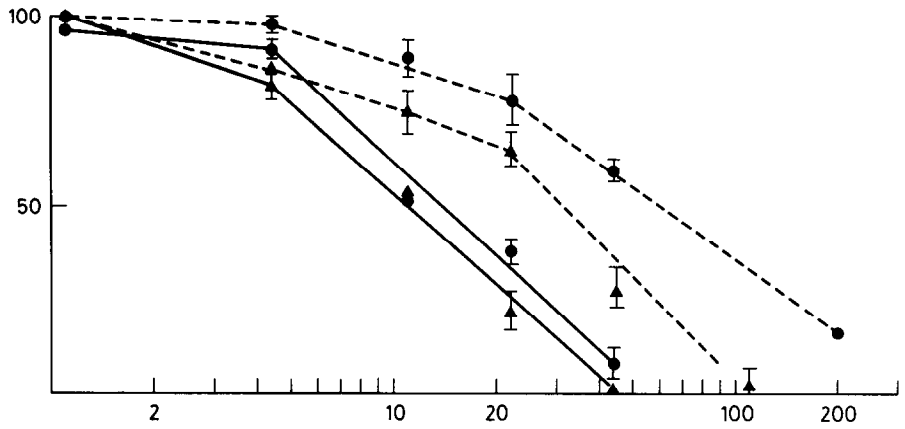

Fig. 1. Effect of tetracycline (●) and demeclocycline (▲) on the survival of LoVo (continuous line) and LoVo/DX (dotted line) after 72 hr treatment (average of three experiments). Abscissa, drug concentration (μM); ordinate, % survival.

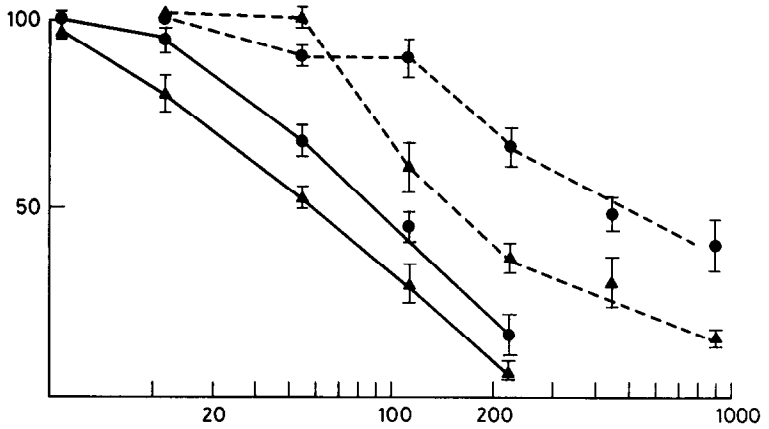

Fig. 2. Effect of tetracycline (●) and demeclocycline (▲) on the survival of LoVo (continuous line) and LoVo/DX (dotted line) after 4 hr treatment (average of three experiments). Abscissa, drug concentration (μM); ordinate, % survival.

V blocks the drug extrusion mechanism in resistant cells, with a consequent increase in intracellular drug accumulation. The effect is believed not to be related with its activity on calcium channels, but to interaction with membrane proteins, in particular with p170 [11]. A recent report showed that V is effective in reverting chloroquine resistance in *Plasmodia* [13]: on this parasite too, V increases the intracellular concentration on chloroquine in resistant strains. When tested on LoVo/DX in combination with tetracyclines, V was able to increase the antibiotics cytotoxicity on the resistant subline (Table 1), thus confirming that the reduced effect of tetracyclines on LoVo/DX is, at least in part, due to a reduced intracellular concentration. We were able to observe the effect of V only when the test was carried out with the 72-hr continuous treatment; only an additive effect between V and tetracyclines was detected with the 4-hr treatment assay (Table 2). When combination V + DX was tested, both assays revealed the synergistic activity of the drugs on LoVo/DX (Tables 1 and 2).

The different antibiotics concentrations that were used in the two assays suggest a possible explanation for this discrepancy: in the 4-hr assay, the tetracyclines concentration was 112 μM, a concentration far exceeding that of V (22 μM). In these conditions, a competitive effect of V on the protein(s) responsible for drug extrusion might be missed. A more favourable test condition is obtained in the 72 hr where the tetracyclines concentration is 22 μM and even more when DX activity is tested.

In conclusions, the data reported show that a multiresistant cell line, LoVo/DX, is more resistant than the sensitive parent line, LoVo, to the growth inhibiting effect of tetracyclines.

Moreover, in the long-term exposure experiment, a reverting effect of V on tetracycline resistance can be demonstrated. This effect is not observed in the 4-hr treatment assay, most probably because of the unfavourable molar ratio antibiotic/revertant agent.

Tetracyclines are thus another class of substances that can be added to the variety of compounds recognized

Table 1. Cytotoxic effect of tetracycline, demeclocycline and DX on LoVo and LoVo/DX cells in the absence and in the presence of verapamil (treatment time 72 hr)

	LoVo	LoVo/DX
	Survival (% of controls)*	Survival (% of controls)*
V (22 μM)	61 ± 7†	41 ± 2†
Tetracycline (22 μM)	38.5 ± 2.1	83 ± 2.1
Tetracycline (22 μM) + V (22 μM)	40 ± 4.1	22 ± 7
Demeclocycline (22 μM)	24.5 ± 4.1	68 ± 5.6
Demeclocycline (22 μM) + V (22 μM)	4 ± 2.1	9.5 ± 0.7
DX (0.009 μM)	73 ± 12	—
DX (0.009 μM) + V (22 μM)	67.5 ± 0.7	—
DX (0.69 μM)	—	92.6 ± 9.1
DX (0.69 μM) + V (22 μM)	—	6.5 ± 0.7

* Survival is based on cell number expressed as percentage of the untreated controls.
† Mean ± SD from triplicate experiments.

Table 2. Cytotoxic effect of tetracycline, demeclocycline and DX on LoVo and LoVo/DX cells in the absence and in the presence of verapamil (treatment time 4 hr)

	LoVo	LoVo/DX
Treatment	Survival (% of controls)*	Survival (% of controls)*
V (22 μM)	91.5 ± 15†	73.3 ± 11†
Tetracycline (112 μM)	64.4 ± 2.1	87 ± 6.2
Tetracycline (112 μM) + V (22 μM)	54 ± 1.4	68 ± 25
Demeclocycline (112 μM)	32 ± 10	65 ± 12.7
Demeclocycline (112 μM) + V (22 μM)	27 ± 14	55 ± 18
DX (0.072 μM)	83 ± 19	—
DX (0.072 μM) + V (22 μM)	74 ± 25	—
DX (3.4 μM)	—	58 ± 9
DX (3.4 μM) + V (22 μM)	—	2.3 ± 0.7

* Survival is based on colony counts expressed as percentage of the untreated controls.
† Mean ± SD from triplicate experiments.

by multiresistant cells: moreover, the similarities in the resistance mechanism to tetracyclines in bacteria and to anthracyclines and other drugs in mammalian cells, coupled with sequence homologies among p170 and bacterial transport proteins suggest a common evolutionary origin for this phenomenon.

Acknowledgements—The authors wish to thank Mr. A. Dadda for skillful technical assistance, and Ms. G. Viscardi for typing the manuscript.

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Biochemical Pharmacology, Vol. 37, No. 15, pp. 3041–3043, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

Organ distribution of neuropathy target esterase in man*

(*Received* 28 *October* 1987; *accepted* 15 *February* 1988)

Some organophosphorous esters (OP)† induce a delayed polyneuropathy (OPIDP) in man and other susceptible species [1, 2]. The molecular target for the initiation of OPIDP is a protein called Neuropathy Target Esterase (NTE) [3]. The progressive phosphorylation of NTE in the nervous system soon after dosing represents the initial reaction [4–6] which is followed by a molecular rearrangement of the phosphorylated protein called "aging". This non-enzymatic cleavage of an alkyl group, generates a charged monosubstituted phosphoric acid residue on the protein [7–9]. It has also been shown in the hen, the animal of choice for OPIDP studies, that the threshold for initiation of this toxic response is 70–75% inhibition of axonal NTE [10, 11] and that a progressive deficit of retrograde axonal transport heralds the clinical expression of OPIDP [12].

Some of these biochemical aspects of OPIDP have been validated so far in man [13]. Here we report the distribution and some biochemical characteristics of NTE from several tissues in man as compared with those in the hen. For comparison the distribution of acetylcholinesterase (AChE) was also measured.

Experimental

Chemicals. Di-isopropyl phosphorofluoridate (DFP) and phenylmethylsulphonyl fluoride (PMSF) were from Fluka AG Chem. Fabrik (Buchs, Switzerland). Diethyl *p*-nitrophenyl phosphate (paraoxon), which was purified according to Johnson [14], acetylthiocholine iodide and DTNB (5,5′-dithiobis-2-nitrobenzoic acid) were from Sigma Chem. Co. (St Louis, MO). A purified preparation of *N,N′*-di-isopropylphosphorodiamidic fluoride (Mipafox) and phenylsulphonyl fluoride (PSF) were a gift of Dr M. K. Johnson, MRC Laboratories (Carshalton, U.K.). Phenyl valerate (PV) was synthetized and purified according to Johnson [14].

Buffers. Tris buffers for NTE studies were as follows: 50 mM Tris/0.2 mM EDTA was adjusted (at 23°) to pH 8.0 with HCl or to pH 5.2 with 50 mM citric acid/0.2 mM EDTA; in the text these buffers will be identified by pH. For AChE assay, 100 mM phosphate buffer pH 7.4 at 23° was used.

Tissue preparation. Samples of human tissues were obtained from post-mortem examinations performed within 36 hr after death, during which time NTE and AChE activities are known to be stable [15]. The analyzed organs are listed in Table 1. Organs with evidence of gross pathology were discarded. Tissue samples were washed in cold Tris pH 8.0, dried and stored at −20° until assayed. Tissues were homogenized (10% w/v) in the same buffer with a Polytron homogenizer and then further diluted in Tris pH 8.0 or Phosphate buffer pH 7.4 for NTE and AChE assays, respectively.

Identification and determination of NTE activity. NTE is a PV-hydrolyzing esterase in the nervous tissue which is dissected from other esterases by using selective inhibitors [14]. Pre-incubation of tissue homogenates with paraoxon at pH 8.0, 37°, for 20 min, inhibits most of the PV esterase activity. If mipafox is also added in the incubation medium, more activity is inhibited. This additional decrease represents NTE activity. To define NTE activities in all tissues, we derived inhibition curves with paraoxon (0.1–1000 μM) of all PV esterases ("A" activity) in order to dissect activity resistant to paraoxon inhibition; PV esterases insensitive to paraoxon (40 μM) ("B" activity) were further titrated with mipafox (0.1–500 μM). The residual activity resistant to paraoxon and mipafox is called "C" activity. The reported NTE activities (B–C) were calculated according to Johnson [14] as follows: PV activity insensitive to paraoxon (40 μM) minus PV activity insensitive to both paraoxon (40 μM) and mipafox (50 μM).

NTE I_{50}s for mipafox, DFP and PMSF (pH 8.0, 37°, 20 min) were calculated as previously described [16].

Determination of AChE activity. AChE was measured in the organs where NTE activity was found, according to Ellman *et al.* [17].

Aging of phosphorylated NTE. Aging of DFP-inhibited NTE from human brain, liver and kidney, and hen brain was studied according to the procedure described by Clothier and Johnson [8]. Sample P, where paraoxon was substituted by PSF (250 μM), was prepared by incubating the 10% homogenate with PSF for 25 min at 37°, pH 8.0. The reaction was stopped by cooling at 0°, the mixture was centrifuged at 30,000 *g* for 20 min at 4°, the supernatant discarded and the pellet resuspended in Tris pH 5.2. The same procedure was adopted to prepare sample M where mipafox (200 μM) was added 5 min before cooling. The difference between the ability of P and M to hydrolyse PV represented NTE activity. P and M were incubated with DFP (20 μM at 37°, pH 5.2 for 2 min) and incubation was stopped by dilution (25-fold) with Tris pH 5.2, at 37°. Immediately and also 5 min after the dilution, aliquots of diluted samples were added to a KF (for reactivation) or KCl (for controls) solution (both 200 mM final concentration) at 37°. Reactions were stopped 10 min thereafter by cooling to 0°. After centrifugation at 30,000 *g* for 60 min at 4°, pellets were resuspended in Tris pH 8.0 and the substrate was added to measure NTE activity, as usual. Uninhibited samples from hen brain and human kidney were not significantly affected by the whole procedure. The recovery of NTE activity was somehow lower for human brain and liver (Table 2) and we don't know if this was due to partial solubility of NTE. Nevertheless for all organs the recovery of NTE activity was similar for KCl and KF-treated samples.

* Part of these results was communicated to the Second International Meeting on Cholinesterases, Fundamental and applied aspects, Bled, Yugoslavia, 17–21 September 1983.

† Abbreviations used: AChE, acetylcholinesterase; DFP, di-isopropyl phosphorofluoridate; NTE, Neuropathy Target Esterase; OP, organophosphorus esters; OPIDP, organophosphate-induced delayed polyneuropathy; PMSF, phenylmethylsulphonyl fluoride; PSF, phenylsulphonyl fluoride; PV, phenyl valerate.