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Increased Chemosensitivity to Doxorubicin of Intrinsically Multidrug-resistant Human Colon Carcinoma Cells by Prolonged Exposure to Verapamil

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Resistance modifying agents (RMA) such as verapamil (VER) have proved effective in reversing multidrug resistance (MDR) in many *in vitro* experimental models, but clinical results with RMA have been disappointing. To clarify this apparent discrepancy we have evaluated the cytotoxic effects of doxorubicin (DOX) plus VER in four human colon carcinoma (HCOC) cell lines (LoVo, DLD-1, SW948, SW1116). These lines were selected on the basis of their levels of mdr1 mRNA being similar to those expressed by HCC obtained from non-drug-treated patients. In all cell lines the sensitising effect of VER on DOX cytotoxicity was schedule-dependent and maximal potentiation of DOX cytotoxicity was obtained by exposure to VER for a time ≥ the cells' population doubling time.
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SOME CANCERS, such as colon cancers, are intrinsically insensitive to most antitumour drugs, whilst others are initially chemosensitive but acquire resistance during chemotherapy [1]. Acquired resistance has been widely studied *in vitro* using cell lines in which resistance is selected by exposure to various antitumour drugs [2], many of which are characterised by a high level of expression of the mdr1 gene and its product the P-glycoprotein (P-gp) [3], associated with the multidrug resistance (MDR) phenotype [4]. Tsuruo *et al.* [5] demonstrated reversal of this MDR phenotype by co-administration of verapamil (VER) with vincristine using P388 murine cell lines. In numerous subsequent studies resistance modifying agents (RMA) have proved effective, to varying degrees, in reversing MDR, but results of clinical studies have proved generally disappointing [6–8]. This apparent discrepancy may be related to the use of cell lines *in vitro* which express P-gp levels much higher than those expressed by chemoresistant tumours taken from patients [4] and/or inadequate VER dose escalation *in vivo* because of associated cardiotoxicity sustained by VER plasma levels required for significant *in vitro* MDR reversal [6–8].

We have, therefore, investigated the effect of VER treatment on chemosensitivity to doxorubicin (DOX) in four human colon carcinoma (HCOC) cell lines (LoVo, DLD-1, SW948 and SW1116). These lines were selected on the basis of their level of mdr1 mRNA and P-gp expression which covers the range detected in primary non-drug-treated HCOC [9]. The aim of this study was to determine whether continuous exposure to RMA is effective in reversing the MDR phenotype in cell lines expressing low, yet clinically relevant levels of P-gp [9, 10]. Drug cytotoxicity was determined by clonogenic assay in liquid medium, as previously described [9], comparing drug-treated cultures with untreated controls. In all the four HCOC cell lines DOX cytotoxicity (expressed as IC_{50}) increased as the time of exposure to DOX increased, ranging from 282–343 ng/ml for 1-h exposure to 25–34 ng/ml for 24-h exposure ($P < 0.01$). Longer incubations (> 24 h) did not significantly increase drug cytotoxicity (not significant) (Table 1). Enhancement of DOX cytotoxicity by VER was a direct function of both the incubation time with DOX plus VER and VER concentration (Table 1). Short incubations (1 h) with DOX plus non-cytotoxic concentrations of VER (< 3 μg/ml) did not enhance DOX cytotoxicity (not significant). By increasing the time of cell exposure to DOX plus VER from 1 to 24 h, a significant potentiation ($P < 0.01$) of DOX cytotoxicity was observed in all four HCOC cell lines as compared to the cytotoxicity derived by treatment with DOX alone (Table 1). No further increase in DOX cytotoxicity resulted from simultaneous treatments with DOX plus VER > 24 h (not significant) (Table 2). The potentiation of DOX cytotoxicity was VER dose-dependent. After 24 h of simultaneous treatment the potentiation of DOX cytotoxicity ranged through 1.4–1.7 ($P < 0.05$), 1.8–2.6 ($P < 0.01$), 2.0–3.1 ($P < 0.01$) and 3.1–4.2 ($P < 0.01$) times using VER concentrations of 0.25 μg/ml, 0.5 μg/ml, 1 μg/ml and 3 μg/ml, respectively.

The continued presence of VER in the culture medium (up to 4 days) after simultaneous treatments < 8 h caused a significant enhancement of DOX cytotoxicity as compared to that of short (≤ 8 h) simultaneous treatments alone. Conversely, no potentiation was observed if VER was maintained in the culture medium after simultaneous treatments ≥ 16 h (Table 2).

Our data indicate that VER can increase sensitivity to DOX (a drug included in the MDR spectrum of resistance) in HCOC cell lines expressing mdr1 products levels comparable to those expressed by non-drug-treated primary HCOC and demonstrate that this phenomenon is strongly schedule-dependent. These findings may be explained in the light of previous reports which indicated that, in MDR cells, exposure to VER enhances DOX

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Table 1. DOX cytotoxicity (reported as ng/ml IC_{50}) after simultaneous cell exposure to DOX plus VER

Cell line	Time† (h)	VER (ng/ml)* 0‡	250	500	1000	3000
LoVo	1	282 (31.0)	ND	275 (48.0)	261 (41.0)	250 (33.0)
	24	25 (3.7)	17 (2.0)§	10 (0.7)‖	8 (0.4)‖	6 (0.7)‖
	72	23 (4.9)	15 (1.9)	9 (0.2)‖	8 (0.2)‖	7 (0.5)‖
DLD-1	1	343 (54.0)	ND	327 (27.0)	309 (36.0)	317 (19.0)
	24	34 (4.9)	20 (2.1)‖	13 (3.8)‖	13 (1.7)‖	9 (0.8)‖
	72	31 (4.3)	21 (1.7)§	13 (13.0)‖	10 (2.4)‖	8 (3.7)‖
SW948	1	303 (55.0)	ND	341 (68.0)	338 (52.0)	265 (43.0)
	24	28 (3.4)	19 (1.4)‖	14 (0.5)‖	12 (1.3)‖	8 (1.4)‖
	72	24 (3.0)	18 (1.1)§	13 (4.3)‖	10 (2.1)‖	10 (1.3)‖
SW1116	1	297 (31.0)	ND	289 (41.0)	275 (30.0)	264 (18.0)
	24	28 (3.4)	20 (1.9)§	16 (2.4)‖	14 (2.0)‖	9 (1.0)‖
	72	25 (1.9)	18 (2.5)‖	16 (0.8)‖	11 (1.4)‖	8 (1.1)‖

* VER concentrations used in the chemosensitivity test.
† Incubation times with DOX plus VER. ‡ Treatment with DOX alone.
Results were obtained from at least three independent experiments. S.D. values are in parentheses. ND, not done. For statistical analyses comparisons between treatments with and without VER were performed using one-way ANOVA. § $P < 0.05$; ‖ $P < 0.01$.

Table 2. Potentiation of DOX cytotoxicity by prolonged exposure to VER

Time (h)	Cell line: LoVo DOX + VER† (Simultaneous)	LoVo DOX + VER‡ (Prolonged)	DLD-1 DOX + VER (Simultaneous)	DLD-1 DOX + VER (Prolonged)	SW948 DOX + VER (Simultaneous)	SW948 DOX + VER (Prolonged)	SW1116 DOX + VER (Simultaneous)	SW1116 DOX + VER (Prolonged)
1	250 (33.0)	121 (28.0)‖	317 (19.0)	138 (18.0)‖	265 (43.0)	145 (20.0)‖	264 (21.0)	136 (18.0)‖
2	171 (20.0)	104 (7.0)‖	178 (12.0)	92 (13.0)‖	186 (15.0)	117 (13.0)‖	167 (27.0)	93 (13.0)‖
4	85 (6.0)	48 (2.9)‖	99 (5.2)	57 (3.4)‖	89 (4.2)	44 (7.3)‖	99 (13.0)	55 (4.7)‖
8	37 (3.1)	27 (4.3)§	46 (1.3)	28 (1.8)‖	45 (3.6)	28 (1.2)‖	65 (4.6)	41 (4.3)‖
16	23 (3.0)	18 (4.0)	21 (4.2)	17 (1.0)	24 (1.3)	17 (0.6)§	23 (1.8)	19 (1.2)
24	6 (0.7)	8 (0.6)	9 (0.8)	7 (1.1)	8 (1.4)	8 (1.3)	9 (1.0)	11 (2.1)

* Incubation times with DOX plus VER (3 μg/ml).
† Cytotoxicity of DOX (reported as ng/ml IC_{50}) after simultaneous cell exposure to DOX plus VER.
‡ IC_{50} DOX by prolonged (up to 4 days) exposure to VER (3 μg/ml) after simultaneous incubation with DOX plus VER. Data reported as ng/ml DOX. Data obtained from at least three experiments. For statistical analysis (one-way ANOVA test) the IC_{50} DOX obtained with the two modalities of treatment were compared. § $P < 0.05$, ‖ $P < 0.01$.

accumulation in the nucleus [11–13] and that DOX is rapidly removed from the nucleus and extruded from the cell when P-gp drug-transporting activity is restored [11]. The important suggestion which emerges from our study is that VER has to be administered in prolonged exposure to prevent DOX nuclear clearance and recovery of MDR [12]. In all four cell lines maximal DOX cytotoxic enhancement occurred when the simultaneous treatment was prolonged for a time ≥ the cells' population doubling time (24 h). This is in agreement with reports in the literature which demonstrated that DOX, although it cannot be considered a typical phase-specific drug, is maximally effective during the S-phase of the cell cycle [14].

Finally, prolonged cell exposure to VER after simultaneous treatments is advantageous only when combined incubations with DOX plus VER are shorter than cellular doubling time.

In vivo use of therapies including DOX and VER are limited by the concentrations of the drugs achievable in the serum without a prohibitive toxicity. It is, therefore, worth considering that DOX plus VER treatments, prolonged for a time corresponding to cells' doubling time, exerted significant cytotoxic effects at DOX concentrations (10–20 ng/ml) fully consistent with those achievable at serum levels in the patient during the postdistributive phase using a standard DOX administration (75 mg/m^2) [15] and VER concentrations (0.5–1 μg/ml) which can be maintained at serum level even for 4–5 days with acceptable toxic cardiovascular side-effects [ref. 16 and our unpublished results]. In our experimental model, the potentiation of DOX cytotoxicity might appear relatively small (about two to four times at VER concentrations of 0.5–3 μg/ml for 24 h of exposure). However, since the range of antineoplastic drug concentration in which human tumour cells are sensitive or resistant is probably small [17], slight enhancements of drug cytotoxicity might be useful in overcoming drug resistance of human tumour cells.

In conclusion, our data demonstrate that VER can modulate *in vitro* DOX cytotoxicity in HCOC cells expressing levels of mdr1 products comparable to those expressed by non-drug-treated primary HCOC and that drug cytotoxic enhancement is a schedule-dependent phenomenon.

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Feature Articles

Black (Air-cured) and Blond (Flue-cured) Tobacco and Cancer Risk VI : Lung Cancer

Ellen Benhamou and Simone Benhamou

FEW STUDIES have compared the risk of lung cancer incurred by smokers of black tobacco to the risk incurred by smokers of light tobacco. The small number of studies can be explained by the geographical distribution of black and light tobacco use all over the world: most studies on lung cancer and cigarette smoking have been conducted in countries where light tobacco has been mainly used, such as the U.S.A. or U.K., whereas black tobacco is common mainly in southern Europe and Latin America.

Between 1983 and 1989, four case–control studies addressing the differential role of light and black tobacco on lung cancer risk were performed in Cuba, France, Uruguay and Italy [1–4]. The main characteristics of these studies are summarised in Table 1. They were hospital-based studies and the controls were without tobacco-related diseases. All cases had a histologically verified diagnosis. The data were recorded from a face-to-face interview except in [3] in which 75% of cases and 60% of controls were interviewed through a next of kin. In all studies but [3], controls were matched to cases on sex and age. The total number of male manufactured or hand-rolled cigarette smokers ranges from 1253 in [3] to 3019 in [4].

Because of the small number of women enrolled in these studies, only the analysis on males will be presented here. Analyses were conducted on squamous cell type cancers in [3], on Kreyberg I type (squamous, small and large cell) in [2], and all histological types in [1, 4]. Table 2 shows the number of male

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