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Adding a Reverser (Verapamil) to Combined Chemotherapy Overrides Resistance in Small Cell Lung Cancer Xenografts

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Small cell lung carcinomas (SCLC) are characterised by chemosensitivity to diverse antitumoral compounds. However, responses are transitory and relapses are commonly observed. We examined the ability of verapamil, a reverser of P-glycoprotein (Pgp)-related resistance, to improve the efficacy of CyCAV combined chemotherapy (Cy, cyclophosphamide (CPA); C, cisplatin (CDDP); A, doxorubicin (ADM);V, etoposide (VP16)), as currently administered to SCLC patients at Institut Gustave-Roussy, France, and adapted to the treatment of nude mice implanted with these tumours. Although Pgp encoded by the *MDR1* (multidrug resistance) gene is not the only mechanism for multidrug resistance (MDR), and not all drugs included in this regimen are recognised by Pgp, we anticipated a therapeutic benefit. Four different SCLC lines, expressing the *MDR1* gene and recently grafted into nude mice, were used. SCLC-75, SCLC-6 and SCLC-41 originated from untreated patients, and SCLC-74T was derived from a patient treated with a combination of ADM, CPA and VP16. SCLC-41T and SCLC-6T tumours were used after having undergone, respectively, five and nine cycles of *in vivo* passage and CyCAV treatment of the tumour-bearing nude mice, to reinforce their chemoresistance. The efficacy of the CyCAV regimen, associated with or without verapamil (given 24 h before CyCAV on days 1–5), was tested on the growth of these SCLC. Verapamil (25 mg/kg) improved the antitumour effect of CyCAV in mice bearing SCLC-6T, SCLC-41T and SCLC-75 tumours, although toxicity was observed. Verapamil modestly delayed the plasma clearance of ADM. Two daily injections of 10 mg/kg of verapamil, administered at a 3 h interval, proved to be effective, whereas the same total dose administered as a bolus was not. These results indicate that the association of some reversers of MDR, including drugs possibly interacting with Pgp, might potentiate SCLC combined chemotherapy.

Key words: small cell lung cancer, multidrug resistance, combined chemotherapy, reversion, xenograft, pharmacokinetic parameters
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INTRODUCTION

SMALL CELL lung carcinoma (SCLC) is recognised as a distinct entity among the various forms of lung cancer, because its biological behaviour, clinical presentation and response to therapy are quite different from those of other histological types, as reviewed by Minna and associates [1] and Gazdar [2]. SCLC is one of the most chemosensitive solid tumours, and systemic therapy plays a critical role in its management [3–7]. Current investigations include the assessment of different drug combinations, in an attempt to use cytotoxic agents more effectively. At our hospital (Institut Gustave-Roussy, France), the antitumour effect of the CyCAV regimen—cyclophosphamide (CPA), cisplatin (CDDP), doxorubicin (ADM) and etoposide (VP16)—given to patients with SCLC has been demonstrated [4–7]. Administration of the same regimen to mice bearing the same type of tumour has confirmed the clinical results [8] thereby validating the experimental model used. The efficacy of each drug alone has also been analysed [9]. Although apparently complete responses to initial treatment can be obtained in a large proportion of patients with limited disease, long-term survival is only achieved in a minority of patients. Most responders relapse

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after 1–2 years, and recurrent tumours are often resistant to further therapy. The main obstacle encountered in the treatment of SCLC is the prevention of recurrences once an initial response has been achieved.

The development of resistance has been ascribed to tumour site repopulation by resistant cells selected by antitumour drugs. Such a phenomenon is thought to be reflected in the known heterogeneity of the tumour [10]. The rapid development of resistance to multiple chemotherapeutic agents is a common clinical problem in the treatment of this cancer. Such resistance may exist prior to the initiation of therapy or may be acquired during treatment. The most intensively investigated mechanism of multidrug resistance (MDR) is the expression of the 170 kDa multidrug transporter P-glycoprotein (Pgp) encoded by the *MDR1* gene [11]. Varying results of studies on Pgp expression in SCLC have been published [12–16]. In our own series of 17 SCLC, five overexpressed the *MDR1*-mRNA transcript, as evaluated by simple Northern blot analysis [8, 17]. *MDR1* overexpression correlated with Pgp levels, assessed using the JSB1 antibody [8].

Recent pharmacological studies in experimental tumour models have sought to circumvent the MDR phenotype, and have aimed at applying their findings to treatment strategies for non-responsive patients [18–20]. A wide range of compounds, including calcium channel blockers and calmodulin antagonists, have been shown to modulate MDR, and have been used to restore the sensitivity of resistant cells [21–23]. Some of these substances, for example, verapamil and cyclosporin A, have been shown to reverse MDR to varying degrees when administered together with the appropriate cytotoxic agents [23–28]. Horton and associates [29] showed that verapamil modulated the accumulation of the cytotoxic agent, vincristine.

The present study was undertaken to investigate whether verapamil could improve the response of resistant SCLC xenograft tumours to CyCAV combined chemotherapy, postulating that verapamil could reverse MDR and at least partially counteract the ineffectiveness of CyCAV attributable to Pgp.

MATERIALS AND METHODS

Xenografts

Tumour specimens, obtained from patients by biopsy or during surgery and histologically diagnosed as SCLC, were xenografted by subcutaneous (s.c.) implantation of tumour fragments into the interscapular area of 18–20 g, 8–9 week-old female athymic Swiss mice (IFFA-Credo©, L'Arbresles, France), maintained in specific pathogen-free conditions. Human tumours growing in nude mice were serially transplanted from mouse to mouse and tumour lines were thus established [30]. The xenografts retained their human karyotype and the characteristics of each tumour are summarised in Table 1. Xenografted SCLC-74T and SCLC-75 tumours were used between the fourth and eighth passage. SCLC-6T and SCLC-41T tumours had previously been subjected to, respectively, nine and five cycles of transplantation and CyCAV treatment of the tumour-bearing mice, in order to increase their chemoresistance [17]. These latter tumours were used at the 15th (SCLC-41T) and 34th passages (SCLC-6T).

Chemotherapy and evaluation of therapeutic effect

When the tumours reached a mean diameter of 6–8 mm, the xenografts were randomised into groups of between five and seven animals each and treatment was started. Each drug was given to the mice at an optimal dose, determined during preliminary experiments: ADM (Laboratoires Roger Bellon, France) 6 mg/kg day 1; CDDP (Laboratoires Eli Lilly, France) 3 mg/kg day 2; VP16 (Laboratoires Sandoz, France) 8 mg/kg days 1–3; CPA (Laboratoires Lucien Colombes, France) 50 mg/kg days 3–5. Verapamil (Laboratoires Biosedra, Malakoff, France) 20 or 25 mg/kg was given 24 h prior to CyCAV and was then administered concurrently with the CyCAV compounds. All agents were injected intraperitoneally (i.p.) in a volume of 0.2 ml/20 g body weight diluted in a 5% glucose solution. During the treatment period, 5% glucose solution, 0.3–0.5 ml per mouse per day, was injected in order to maintain sufficient hydration. Control groups received only the vehicle.

Tumour growth was monitored by measuring two perpendicular diameters with a caliper every 2 days. Tumour volume was calculated as a function of an ellipsoid volume, using the formula $V = a^2 \times b/2$, where a is the tumour width and b is its length in mm. This is considered a valid estimation of volume [31]. The tumour sizes were standardised in the different groups by using the relative tumour volume (*RTV*) calculated by the formula $RTV = V_x/V_0$, where V_x is the tumour volume at any given time and V_0 is the tumour volume at the onset of treatment. The effect of the drugs was expressed as the relative tumour growth, which is a *T* (treated group)/*C*(control group) ratio (mean of the individual *RTV* of the treated group divided by mean *RTV* of the tumours in the control group) × 100. These mean ratios and their corresponding standard deviations (S.D.) were calculated. The statistical evaluation of the differences between the control and treated groups was calculated using the non-parametric Wilcoxon test, on days 9–14 after the onset of treatment.

Treatment protocol and pharmacokinetics (pK) study

Two groups of 20 nude mice were injected with ADM 10 mg/kg, intravenous (i.v.) bolus, with or without verapamil, 25 mg/kg given by i.p. route. Blood samples were taken from four mice at 1, 3, 6, 15 and 30 min after ADM injection. Plasma was recovered after immediate centrifugation and stored frozen until analysis.

ADM extraction procedure

ADM was extracted as described previously [32]. Briefly, 100 µl of daunorubicin (DNR) at 2 µg/ml in water were added to each sample as an internal standard. Each plasma sample (250 µl) was mixed with 5 ml of choroform/methanol (4/1 v/v) and (0.5 ml) of borate buffer (0.1 M, pH = 10) shaken vigorously for 1 min and centrifuged at 0°C for 10 min at 2500 rpm. The organic layer was concentrated until dry. The residue was resuspended in 300 µl of chromatographic solvent: 0.1% ammonium formate buffer/acetonitrile (68/32 v/v), as described by Robert [33]. Quantification of peaks was conducted by reference to standard curves of blank sample spikes with increasing ADM concentrations.

HPLC technique

ADM was a gift of Dakota. The HPLC conditions were those described by Robert [33]. We used a Waters associates Liquid Chromatography system with a micro-Bondapak C18 column and precolumn. The solvent was used isokinetically at a flow rate of 1.8 ml/min. Drugs were detected by spectrofluorimetry (Kontron); the excitation and emission wavelengths were 480 nm and 592 nm, respectively.

RESULTS

Four tumour lines derived from human SCLC were used in this study. As previously published [8] and summarised in

Table 1. Characteristics of four human small cell lung cancers xenografted into nude mice

Tumour	Sex/Age (yr)	Location	Histological type	Previous treatment	MDR1 expression*
SCLC-6T	M/44	Metastasis	INT	CyCAV†	++
SCLC-41T	M/55	Metastasis	OAT	CyCAV†	+
SCLC-74T‡	M/69	Metastasis	INT	CAV§	++
SCLC-75	M/47	Primary	INT	None	++++

*Previously published in [30]. *MDR1*-mRNA expression was rated visually (0, no visible transcript; +, trace level; and + to ++++, increasing levels detected in Northern blot analysis); †The mice were treated during passage; ‡Progression after therapeutic failure; §The patient had been treated. INT, intermediate type; OAT, oat cell. For composition and schedules, see Materials and Methods.

Table 1, these lines overexpressed the *MDR1*-encoded Pgp, detected either at the protein level with specific antibodies (JSB1) or at the RNA-transcript level, with a decreasing gradient of expression from SCLC-75 (very high), to SCLC-6T (high), and SCLC-74T and SCLC-41T (detectable). SCLC-6T and SCLC-75 tumour cells expressed Pgp spontaneously, prior to any treatment. The *MDR1*-mRNA transcript was detected in SCLC-41T tumours after the first cycle of CyCAV treatment had been given to the tumour-bearing nude mice. SCLC-74T tumours, derived from the tumour of a treated patient, expressed the *MDR1*-mRNA transcript and Pgp [8, 17].

The findings reported here concern the effects of CyCAV alone or associated with verapamil on SCLC-xenograft tumour lines. The results of one experiment per tumour line are reported in Table 2. Therapeutic responses were usually clearly detectable as early as days 3–7, depending on the tumour line studied. The five mice bearing tumour line SCLC-6T and treated simultaneously with 25 mg/kg of verapamil and CyCAV showed a mean tumour growth inhibition of 70%, as early as day 3 after the onset of treatment, while only a partial response was noted in two of the five mice treated with CyCAV alone (22% mean growth inhibition), noticeable on day 7. The relative tumour growth measured in these two groups is reported in Figure 1a. On days 9 and 11, the P value was < 0.02.

All SCLC-41T tumour-bearing mice that received the 25 mg/kg dose of verapamil and CyCAV therapy responded (70% mean tumour growth inhibition) (Figure 1b), while only one of the five mice treated with CyCAV alone responded. The peak SCLC-41T response to CyCAV plus verapamil occurred on day 13 after the onset of treatment ($P < 0.02$). Surprisingly, a transient increase of tumour volumes was observed in the first days after onset of CyCAV treatment. This effect, also observed early after treatment of SCLC-75T (Figure 1c) might be due to an increase of intratumoral vascularisation and oedema, in relation to tumour cell death.

All SCLC-75 tumours completely disappeared 9 days after treatment with 25 mg/kg of verapamil plus CyCAV (Figure 1c), after an initial increase of tumour growth. However, three of the five mice died on day 10. CyCAV alone strongly inhibited tumour growth (62%) as early as day 10, but no complete regression was achieved. A daily dose of 20 mg/kg of verapamil was well tolerated, but had little effect on the progression of SCLC-75 tumours.

Table 2. Efficacy of combined chemotherapy (CyCAV) associated with or without verapamil on SCLC xenografts

Tumours	Treatment*	Per cent responders†	Number of deaths at day 10‡	Mean per cent of growth inhibition
SCLC-6T	CyCAV	40	0/5	22 ± 4
	CyCAV + verapamil 25 mg/kg	100	0/5	70 ± 15
				$P < 0.02$§
SCLC-41T	CyCAV	20	1/5	13 ± 6
	CyCAV + verapamil 25 mg/kg	100	0/5	70 ± 0
				$P < 0.02$
SCLC-74T	CyCAV	60	0/6	49 ± 7
	CyCAV + verapamil 20 mg/kg	60	1/6	54 ± 5
	CyCAV + 2 × verapamil 10 mg/kg	100	0/7	74 ± 10
				$P < 0.05$
SCLC-75	CyCAV	75	1/5	62 ± 8
	CyCAV + verapamil 25 mg/kg	100	3/5	100
	CyCAV + verapamil 20 mg/kg	100	1/5	82 ± 5

*For CyCAV composition and schedule, see Materials and Methods; †Per cent responders corresponds to the number of mice that showed a response (≥ 50% inhibition) out of the total number of mice. Inhibition was quantified between days 10 and 20; ‡Number of dead mice, 10 days after the onset of treatment; §P values were calculated using the non-parametric Wilcoxon test.

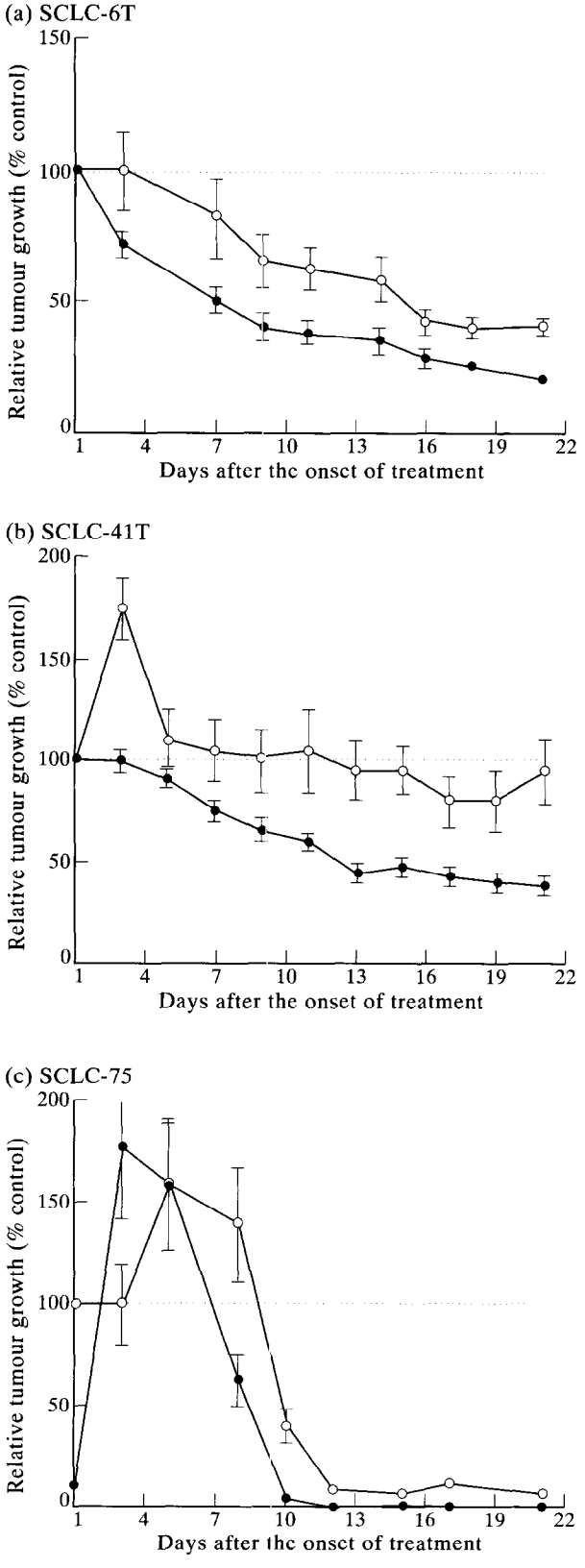

Figure 1. Relative tumour growth of SCLC xenografts treated with verapamil (25 mg/kg) and CyCAV (solid symbols) (for composition and schedule, see Materials and Methods) or treated with CyCAV alone (open symbols). The mean tumour growth in the control group is represented by the horizontal line at 100%. When tumours reached a diameter of 6–8 mm, the treatment cycle started on day 1 and finished on day 6. (a) SCLC-6T tumours (five mice/group); (b) SCLC-41T tumours (five mice/group); (c) SCLC-75 tumours (five mice per group). The treatment cycle was started on day 1 and finished on day 6.

The antitumoral enhancing effect of verapamil also depended upon the administration schedule (Figure 2). A total dosage of 20 mg/kg, administered as two 10 mg/kg doses, was given to SCLC-74T tumour-bearing mice according to the following protocol: one injection 3 h before the second dose was given together with CyCAV (based on the recommendations of the pharmaceutical company (Knoll, Ludwighaffen, Germany)). This schedule induced a response on day 7 in all the treated mice (Figure 2), with a mean of 74% tumour growth inhibition, compared with only four responders of the six (54% tumour growth inhibition, $P < 0.05$) tumour-bearing mice treated with CyCAV alone, or compared with four responders of the six (49% tumour growth inhibition, $P < 0.05$) tumour-bearing mice treated with CyCAV and a single verapamil dose of 20 mg/kg. The peak responses for these regimens occurred between days 10 and 11.

Pk results

The results of ADM concentration were analysed with Micropharm Software by extended least square regression using a Gauss Newton algorithm. The two curves are shown in Figure 3. We noted a delay of plasma ADM clearance when ADM was injected simultaneously with verapamil. The Pk parameters are detailed in Table 3.

DISCUSSION

An implicit hypothesis in many pharmacological trials on drug activity and resistance is that the degree of cytotoxicity is dependent upon stable and predictable biochemical pathways within tumour cells. Furthermore, it is presumed that intrinsic sensitivity and resistance are predominantly determined by variations in the expression of genes responsible for drug resistance, which arise as a result of spontaneous mutation (or chromosome rearrangements) in tumour cell populations [34]. In our laboratory, preliminary assays of chemotherapy intensification were performed using the SCLC-6T xenograft model. In comparison with the reference protocol, CDDP doses were doubled, ADM was increased 1.3-fold, VP16 was tripled, and CPA was quadrupled. No improvement of any antitumoral effect was obtained and toxicity was more acute (30% died during the first 10 days of treatment). This lack of benefit of chemotherapy intensification might be attributable to the MDR mechanisms at work in this type of tumour. Therefore, improvement of chemotherapy must be sought by other approaches.

This process of MDR is often accompanied by overexpression of the *MDR1* gene, which encodes a 170 kDa membrane pump protein, P-glycoprotein (Pgp). This overexpression was previously detected in our SCLC xenografts [8]. Although *MDR1* gene overexpression in SCLC remains a subject of controversy [12–15], we have found *MDR1*-mRNA to be overexpressed in 5 of the 17 SCLC tumour lines that we tested with the widely used Northern blot technique [17]. However, Pgp-mediated drug efflux cannot completely explain MDR. Another mechanism involved in drug resistance is the intracellular formation of drug-filled vesicles. It has been suggested that, in addition to Pgp-mediated drug efflux, these vesicles contribute to the reduction of the intracellular drug concentration by drug capture and exocytosis [35]. Glutathione-*S*-transferases (GST) are also implicated in the protection of cells against cytotoxic and carcinogenic chemicals, and the acidic class GST3 protein was, in general, the most abundant GST form in all the human tumours studied, including lung, colon, bladder and breast carcinomas [36, 37]. Although several reports have stressed the role of topoisomerases [38] and new molecular determinants such as MRP (multidrug-related protein), discovered in MDR-

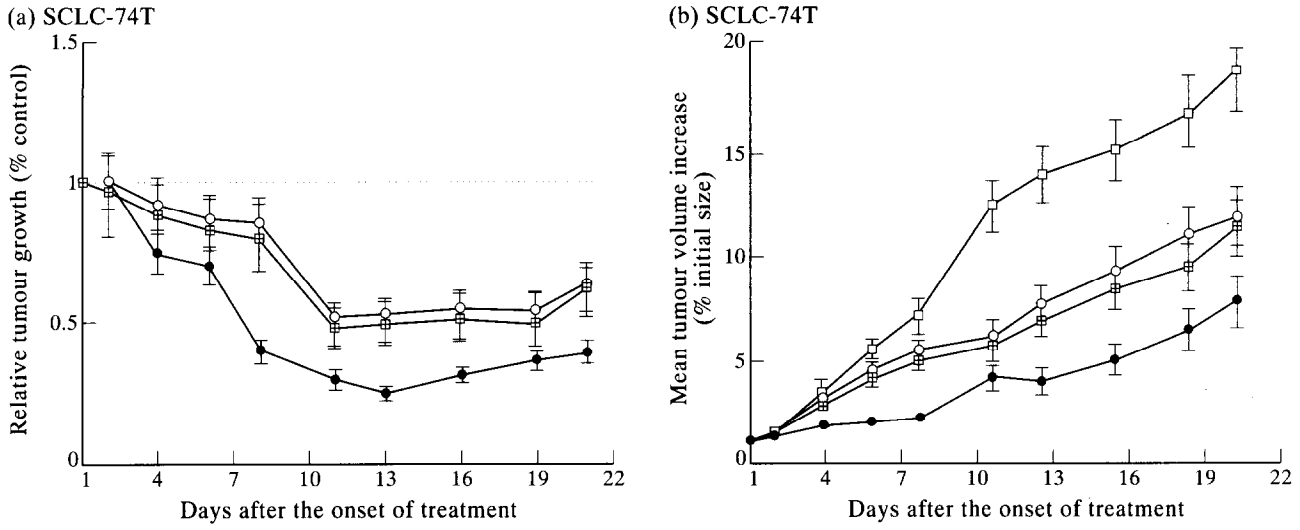

Figure 2. The modulating effect of verapamil (20 mg/kg) administered in two 10 mg/kg doses, one injection 3 h before the second, given simultaneously with CyCAV (seven mice) (solid circles) compared to CyCAV alone (six mice) (open circles) and CyCAV plus one verapamil bolus (six mice) (hatched squares) on the SCLC-74T xenografts. (a) The mean tumour growth in the control group is represented by the horizontal line at 100%. The treatment cycle was started on day 1 and finished on day 6. (b) The mean tumour volumes of all groups, including those of the control (open squares) are shown.

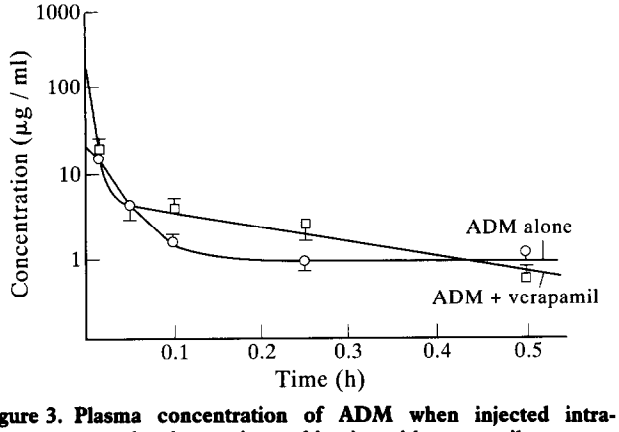

Figure 3. Plasma concentration of ADM when injected intravenously, alone or in combination with verapamil.

Table 3. Pk parameters

Pk parameters	ADM without VER	ADM with VER
Cmax (1') (μg/ml)	16.6	21.7
AUC (μg/ml h)	0.89	1.27
Cl (l/h)	0.335	0.235
$T^{1/2}\alpha$ (h)	0.015	0.004
V_d(l)	165.2	122.2

AUC, area under curve; Cl, clearance; $T^{\frac{1}{2}}$, half-life; V_d, volume of distribution; VER, verapamil.

SCLC cell lines [16], we focused our study on the *MDR1*-dependent mechanism of resistance, because of the clinical possibilities of its reversion in the near future.

A broad spectrum of compounds, including many that do not possess any direct antitumour activity, have been shown to potentiate the cytotoxic effects of some antineoplastic agents [39]. Verapamil, a calcium antagonist widely used in cardiology, has been shown, in preclinical studies, to be a potent reverser of multidrug resistance [18, 22, 23, 29, 40–45], and was used here as a reverser prototype. Since the demonstration by Tsuruo and associates [21] of a reversal of the MDR phenotype in P388 leukaemia cells by the co-administration of verapamil and vinblastine, there have been numerous reports of synergism between verapamil and vinca alkaloids [29, 41]. The clinical use of verapamil as a reverser is controversial because, to date, it has not been successful. It is possible that verapamil concentrations obtained in the patients' tumours may have been too low [28], since the plasma verapamil concentration must reach 5–6 μM to be active as a reverser [42–44]. Therefore, in our assays, we administered the maximal tolerated verapamil dose, postulating that the critical plasma concentration threshold of verapamil would be reached. Alternatively, in many clinical trials, verapamil has been used in the treatment of tumours not characterised in terms of Pgp expression, whereas in our SCLC xenografts, *MDR1*-mRNA expression had been determined previously [8].

In this study, we examined the effects of verapamil in combination with a complex polychemotherapeutic regimen, including CPA, CDDP, ADM and VP16, at doses comparable to those administered clinically, on the growth of four SCLC xenografts. These four transplantable tumours exhibited different levels of sensitivity to CyCAV, apparently not associated with Pgp expression. The SCLC-75 tumour line clearly illustrated this point with its generated tumours expressing high Pgp levels, but responding to the CyCAV treatment, although complete regression was never achieved after an initial response and tumours progressed. High dosage verapamil combined with CyCAV gave complete responses, but was highly toxic. SCLC-6T and SCLC-74T, previously subjected to chemotherapy, in mouse and man, respectively, responded to CyCAV but to a lesser degree. Association with a reverser improved the therapeutic response, but did not lead to complete regression. SCLC-41T tumours (treated in mice) did not respond at all to CyCAV. Addition of verapamil to the protocol led to a constant inhibition of tumour growth. It is possible that the resistance mechanism(s) of these three tumours (SCLC-41T, SCLC-74T and SCLC-6T)

is more complex than that of SCLC-75, involving several escape routes [16, 36, 38] which cannot be bypassed by MDR reversers, as in the case of the previously untreated SCLC-75.

The pharmacokinetics of our drug combination may be affected by co-administration of verapamil. To approach this question simply, we measured the plasma concentration of ADM injected i.v. alone or in combination with verapamil. We observed that (1) the area under curve (AUC) was lower when ADM was given alone than when ADM was given with verapamil; (2) the total clearance was higher (0.335 l/h versus 0.235 l/h); and (3) the total elimination (metabolic and urinary) was more important, revealing that, probably, in the presence of a reverser, such as verapamil, the ADM metabolism in the liver and body elimination were decreased.

The results presented here indicate that it is possible to improve the therapeutic benefit of CyCAV in resistant tumours by association with a reverser, in this case, verapamil. Does verapamil act by reversing MDR or not? We have no arguments that can firmly establish such a relationship. Interactions between verapamil and antitumoral drugs, that were described by Scheithauer and associates [44] and Morss and associates [42], could also been involved in our model. Nevertheless, this finding demonstrates the potential of verapamil to improve SCLC response to CyCAV.

It has previously been shown that defective intracellular drug uptake is correlated with the level of drug resistance [46], and a similar relationship exists between the enhancement of drug efficacy by modulating agents and the dosage used. Therefore, if the reverser agent increases intracellular drug uptake, decreased drug resistance should be observed, as we did. A similar dose–response effect of modulating agents on cytotoxicity has been described by Bellamy and associates [40]. To the best of our knowledge, the reversion of MDR can only occur with two of the drugs in the CyCAV regimen, ADM and VP16, which interact with the Pgp encoded by the *MDR1* gene; CPA and CDDP do not interfere. Although these latter two drugs are not recognised by Pgp, Chaudhary and associates [47] have shown that MDR could be induced by exposure to them. In addition, some reversers are able to improve CDDP efficacy, by mechanisms that have not yet been elucidated [48].

As reported previously, the verapamil dosage is critical for its efficacy [42–44]. A daily dose of 20 mg/kg was used to treat xenografts SCLC-74T and SCLC-75 and had no effect. However, the same dosage increased the efficacy of CyCAV against SCLC-74T tumours when it was administered in two 10 mg/kg doses, given at an arbitrary interval of 3 h. This latter protocol led to a good therapeutic response and reduced the toxicity to the mice. These results suggest that verapamil efficacy can be improved by injecting it both before and in combination with cytotoxic agents. Cass and associates [41] reported that combining verapamil with vincristine intensified the latter's toxicity in human leukaemia cell lines.

The present approach of using a reverser in association with combined chemotherapy should advance our therapeutic success at four levels: (1) circumvention of drug resistance in tumour cells; (2) restriction of the heterogeneity of tumour cell responses to drugs; (3) restoration of the efficacy of various antitumour agents that are transported outside the cells by blocking the efflux mechanism; and (4) the possibility of lowering doses of antitumour agents. However, the toxicity of the CyCAV and verapamil combination remains the main obstacle to its application in patients. New compounds have to be designed for clinical use, and many are being developed for this purpose [26–28, 48].

In conclusion, this study confirms that CyCAV efficacy is enhanced by co-administration with verapamil, perhaps by overriding escape mechanisms conferring resistance to small cell lung cancers. The administration of fractionated verapamil doses was able to increase the efficacy of CyCAV, while reducing the toxicity of the drug combination.

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