SHORT COMMUNICATION

The polyoxyethylene castor oil Cremophor EL modifies multidrug resistance

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In vitro the presence of P-glycoprotein is associated with the possibility to reverse multidrug resistance (MDR) with compounds with different structural features (Bradley et al., 1988). Although many such compounds have been described, only a few can be expected to be active at clinically achievable concentrations: quinidine (Tsuruo et al., 1984), amiodarone (Chauffert et al., 1987), bepridil (Schuurhuis et al., 1987) and cyclosporin A (Slater et al., 1986; Twentyman, 1988) are examples. During our investigations on the in vitro effects of resistance modifiers on daunorubicin and vincristine accumulation in freshly obtained human tumour cells (Schuurhuis et al., 1989b) we found that Cremophor EL, which is a polyethoxylated castor oil used as a solubiliser, e.g. of vitamins and of the immunosuppressant drug cyclosporin A, had effects on drug accumulation similar to other resistance modifiers used. In order to study whether this effect of Cremophor EL was related to MDR, we investigated the effects of Cremophor EL on anthracycline accumulation and anthracycline and vincristine cytotoxicity in MDR and sensitive model cell lines and we compared the results with those obtained using cyclosporin A and verapamil. Both human squamous lung cancer cells (SW-1573, Keizer et al., 1989; Broxterman et al., 1989) and human myeloma cells (8226, Dalton et al., 1986, 1989b) were used. The latter ones are of special interest because of the increased in vivo sensitivity of myeloma to a regimen containing vincristine, doxorubicin and dexamethasone (the VAD regimen) when verapamil is used as a modulator (Dalton et al., 1989a). Both types of MDR cells overexpress P-glycoprotein (Dalton et al., 1989b; Kuiper et al., 1990).

Cells were cultured in Dulbecco’s modified essential medium supplemented with 10% fetal bovine serum (Gibco, Paisley, Scotland). The human multiple myeloma 8226Dox4 and 8226Dox40 cells were cultured in the presence of 40 and 400 nM doxorubicin (Adriablastina, Farmitalia, Milan, Italy) respectively. SW-1573/2R160 cells were derived from SW-1573/2R50 cells (Keizer et al., 1989; Broxterman et al., 1989) by continuous exposure to 160 nM doxorubicin. Experiments were performed on cells cultured for 1–2 weeks without doxorubicin. Cells were allowed to adhere (SW-1573 cells) or equilibrate in suspension (8226 cells) in six-well tissue culture plates (Costar, Cambridge, MA, USA). Then they were incubated under 5% CO2 with doxorubicin or vincristine sulfate (Sigma Chemical Co., St Louis, MO, USA) with or without resistance modifiers for at least three cell doubling times. Cell doubling times were 22 h (SW-1573), 45 h (SW-1573/2R160), 36 h (8226D5), 42 h (8226Dox4) and 44 h (8226Dox40). Thereafter the cells were counted as described by Schuurhuis et al. (1987) using a Sigmex microcell counter model CC-110. With high concentrations of Cremophor EL (≥ 132 μg ml⁻¹) or when fresh human plasma was used, the cells were co-incubated with doxorubicin and Cremophor EL for 2 h, post-incubated for 2 h with Cremophor EL only and further incubated in fresh medium as described above. Resistance modifiers used were verapamil.HCl (Sigma), cyclosporin A (Sandoz AG, Basel, Switzerland), cyclosporin A in Cremophor EL (Sandimmune, Sandoz, AG) and Cremophor EL (Sandoz AG). Cyclosporin A in Cremophor EL was used because in this form (Sandimmune) cyclosporin A is administered clinically. The choice of the concentration of the modifiers used in the cytotoxicity experiments in this study is based on other in vitro studies: 1–2 μM cyclosporin A and 4 μM verapamil usually are effective in modulating MDR (Durie & Dalton, 1988; further reviewed in Twentyman, 1988 and Kaye, 1988). The choice of Cremophor EL concentrations is based on the amounts present in the cyclosporin A solutions which are administered in the clinic (as Sandimmune), e.g. a final dilution of 2 μM cyclosporin A contains 33 μg ml⁻¹ Cremophor EL.

Cellular accumulation and efflux experiments were performed essentially as described earlier (Schuurhuis et al., 1987). Some 0.1–0.3 × 10⁶ cells were incubated for 2 h at 37°C in 550 μl Dulbecco’s medium, pH 7.4, lacking NaHCO3, but containing 20 mM HEPES and 10% fetal bovine serum, to which 14C-doxorubicin (Amersham Laboratories, Amersham, UK) or 14C-daunorubicin (Amersham) was added with or without resistance modifiers. The final concentration of doxorubicin and daunorubicin was made 0.5 μM by adding unlabelled doxorubicin and daunorubicin (Specia, Paris, France). After two washes with ice-cold phosphate-buffered saline, the cells were transferred to liquid scintillation fluid. No corrections were made for direct binding of anthracyclines to the cells since binding was the same whether or not modifiers were present and was too low to affect the conclusions (5–20% at maximum). For efflux experiments sensitive cells were incubated with 0.5 μM doxorubicin or daunorubicin. Resistant cells were incubated with 2.5 μM daunorubicin (SW-1573/2R160) or 1 μM doxorubicin (8226Dox4); this resulted in about the same intracellular drug amounts as in the sensitive cells in these experiments after 2 h of incubation. After washing with ice-cold Dulbecco’s medium, the cells were resuspended in fresh ice-cold medium and incubated for 1 h at 37°C. After washing the cell-associated radioactivity was determined.

In Table 1 it is shown that Cremophor EL (132 μg ml⁻¹) partly reversed doxorubicin resistance in SW-1573/2R160 cells (the dose modifying factor, DMF, was 6.3; resistance index = 77) while only a small effect was observed on the parent cell line. With concentrations higher than 132 μg ml⁻¹ higher dose modifying factors were found (> 10). At a concentration of 35 μg ml⁻¹ Cremophor EL had a small although significant effect in SW-1573/2R160 cells (DMF = 1.9, see Table 1). Two μM pure cyclosporin A had a DMF of 8.3 ± 1.5 (mean ± s.d. in three experiments, P < 0.01), while 2 μM cyclosporin A (Sandimmune), which

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contains 33 μg ml⁻¹ Cremophor EL, had a DMF of 16.8 (Table I). These results show that both compounds as such are able to sensitize SW-1573/2R160 cells to doxorubicin and that the effects are additive when cyclosporin A is given as Sandimmune.

Also in the human myeloma cell line 8226Dox4 with a moderately high doxorubicin resistance index (45, see Table I) Cremophor EL (33 μg ml⁻¹) had only a small effect (DMF of 2.6, see Table I). Like in SW-1573/2R160 cells, with higher concentrations of Cremophor EL the effects on doxorubicin cytotoxicity became more pronounced (Table I). On the other hand, in 8226Dox4 cells with a low doxorubicin resistance index (7.9, see Table I) 33 μg ml⁻¹ Cremophor EL largely reversed doxorubicin resistance (Table I and Figure I). One μM cyclosporin A in Cremophor EL (16.5 μg ml⁻¹) or verapamil (4 or 16 μM) had no greater effect than Cremophor EL (33 μg ml⁻¹) alone (Table I). Figure 1 shows the dose–response relationship for Cremophor EL on doxorubicin cytotoxicity: even at concentrations of 4.1 and 8.2 μg ml⁻¹ (which correspond to dilutions of 1:25,600 and 1:128,000 respectively), significant effects were observed. These data show that in cells with low levels of MDR reversal of resistance with cyclosporin A may have been achieved at least partly due to the carrier (Cremophor EL) effects alone. This may have important implications for the design and interpretation of clinical trials with cyclosporin A as reversing agent.

Interestingly, our results indicate that cells with intermediate to high levels of resistance, like 8226Dox4 and SW-1573/2R160 cells, may not be good models to predict the possible clinical usefulness of resistance modifiers in α−glycoprotein-containing tumours. This may be due to the fact that drug efflux from cells with low amounts of α−glycoprotein can be blocked more efficiently.

Drug accumulation experiments confirmed the findings reported above. Cremophor EL significantly stimulated doxorubicin and daunorubicin accumulation in 8226Dox4 and SW-1573/2R160 cells, respectively, but not in the sensitive cells (Table II). In SW-1573/2R160 cells daunorubicin was used instead of doxorubicin, since drug accumulation differences between sensitive and resistant cells and importantly, effects of modifiers on drug accumulation in resistant cells, were much more pronounced for daunorubicin than for doxorubicin in these cells. Cremophor EL (132 μg ml⁻¹) stimulates anthracycline accumulation at least partly by in-

### Table I

| Cell line       | IC₅₀ (nM) (control) | CEL (33 μg ml⁻¹) | CEL (132 μg ml⁻¹) | Cycl. A (8 μM) | Vₑ (16 μM) | Vₑ (16 μM) |
|-----------------|---------------------|-------------------|-------------------|----------------|-------------|-------------|
| SW-1573         | 22 ± 3              | 1.6 ± 0.4         | 1.8 ± 0.6         | 1.4 ± 0.2      | 1.5 ± 0.1   |             |
| SW-1573/2R160   | 1700 ± 300          | 1.9 ± 0.1         | 6.3 ± 0.1         | 16.8 ± 6.1     | 5.4 ± 0.5   | 11.0 ± 1.4  |
| 8226S           | 12 ± 2              | 1.2 ± 0.1         | 1.11 ± 0.3        | 1.1 ± 0.1      | 1.2 ± 0.2   | 4.9 ± 0.5   |
| 8226Dox4        | 95 ± 12             | 5.4 ± 1.7         | 7.1 ± 1.2         | 4.6 ± 0.2      | 3.4 ± 0.3   | 4.9 ± 0.5   |
| 8226Dox40       | 540 ± 110           | 2.6 ± 1.3         | 13.3 ± 2.8        | 5.6 ± 2.2      | 8.4 ± 2.7   |             |

Values are means ± s.d. from 2–5 independent experiments. *Values within brackets: IC₅₀ doxorubicin cell line/IC₅₀ parent cell line. **Significantly different from 1 (P < 0.05, Student's t test). ***Significantly different from 1 (P < 0.05). 

### Table II

| Cell line       | anthracycline accumulation (pmol per 10⁶ cells) | Cel (132 μg ml⁻¹) | Cycl. A (8 μM) | Vₑ (16 μM) | Anhe retention | REFₕ |
|-----------------|-----------------------------------------------|-------------------|----------------|-------------|----------------|-----|
| SW-1573         | 346 ± 52                                       | 0.99 ± 0.08⁴      | 1.05 ± 0.12     | 1.18 ± 0.11⁴| 63 ± 12⁴       | 1.12 ± 0.17⁴|
| SW-1573/2R160   | 56 ± 6 (2.6⁴)                                  | 2.05 ± 0.39⁴      | 4.39 ± 0.88⁴    | 3.09 ± 0.4⁵  | 34 ± 8         | 1.37 ± 0.0⁹⁵|
| 8226S           | 147 ± 17                                      | 0.94 ± 0.19       | 1.05 ± 0.18     | 0.99 ± 0.11  | 72 ± 5         | 0.98 ± 0.04⁵|
| 8226Dox4        | 107 ± 20 (1.4⁴)                               | 1.24 ± 0.11⁴      | 1.34 ± 0.16⁴    | 1.30 ± 0.1⁹  | 67 ± 9         | 1.08 ± 0.0⁵⁴|
| 8226Dox40       | 84 ± 1 (1.8⁴)                                 | 1.27 ± 0.17⁴      | 1.54 ± 0.26⁴    | 1.30 ± 0.1⁸  |               |     |

Abbreviations as in Table I. *Drug accumulation (2 h at 37°C) was carried out with 0.5 μM daunorubicin for SW-1573 cells and with 0.5 μM doxorubicin for 8226S cells. AEF, accumulation enhancement factor = drug accumulation with modifier/drug accumulation without modifier. #Anthracycline retention was measured after 1 h of drug efflux; shown are means (± s.d.) of initial amounts. Vₑ, retention with CEL (132 μg ml⁻¹)/drug retention without CEL. 

Values are means ± s.d. from 2–6 independent experiments each performed in triplicate. Values between brackets: drug accumulation in sensitive cells/drug accumulation in resistant cells. Significantly different from 1 (P < 0.05, Student's t test). Significantly different from 1 (P < 0.05).
increasing its retention in the MDR cells (Table II), as seems to
be the case for cyclosporin A (Nooter et al., 1989). No
significant effects of Cremophor EL on anthracycline reten-
tion were seen in the parent cells. The effects of the modifiers
on drug cytotoxicity in MDR cells seems to be due for an
important part to a change in intracellular drug distribution
instead of to stimulation of drug accumulation as will be
discussed later. In addition, stimulation of anthracycline
accumulation by modifiers occurs in a dose-dependent way
and therefore low concentrations of modifiers stimulate
anthracycline accumulation only slightly. In order to show
clearly that the resistance modifiers used stimulate drug
accumulation in our MDR cells we have chosen higher con-
centrations of modifiers for accumulation and retention
experiments than for cytotoxicity experiments.

We have also determined the effect of Cremophor EL on
vincristine cytotoxicity in 8226Dox4 cells since vincristine is
included in clinical protocols for myeloma patients. Figure 2
shows that Cremophor EL is active in reversing vincristine
resistance with dose modifying factors of 2.2, 3.2, 8.4 and
28.7 for the concentrations of 8.2, 16.5, 33 and 132 µg ml⁻¹,
respectively. Since the resistance index was 15, this means a
more than complete reversal of resistance at 132 µg ml⁻¹.
Interestingly, the sensitive cells were affected too, although
to a limited extent (DMF: 2.2, see Figure 2). Two µM
verapamil, a concentration which is only achievable clinically
with serious side-effects (Benson et al., 1985; Ozols et al.,
1987) was less effective than Cremophor EL at a concentra-
tion of 33 µg ml⁻¹ (see Figure 2).

One major determinant of the efficacy of a drug in the
clinic can be its ability to bind to proteins (Koch-Weser &
Sellers, 1976). We have shown previously that an increase in
the protein concentration significantly decreased the potency
of resistance modifiers such as verapamil, bepridil, diltiazem
and Ro 11-2933/001 to stimulate anthracycline accumula-
tion in MDR cells (Broxterman et al., 1987). Table III shows
that Cremophor EL at concentrations of 33 and 132 µg ml⁻¹
largely retains its ability to reverse doxorubicin resistance in
8226Dox4 cells at a high protein concentration (compare
Tables I and III). At this protein concentration the dose
modifying factors of verapamil, even at a concentration of
16 µM, are somewhat lower than for Cremophor EL (Table III).
In addition, when 8226Dox4 cells were incubated in fresh
human plasma for 2 h with doxorubicin and Cremophor EL
(132 µg ml⁻¹), followed by a 2 h post-incubation in plasma with
Cremophor EL only, the effect was about the same as in control experiments using 10% fetal bovine serum in the same incubation protocol (DMF of 2.5–3.5). These results indicate that proteins probably do not strongly interfere with the capacity of Cremophor EL to modulate MDR.

Despite the many studies addressing the mechanism of
action of resistance modifiers, the answers offered are not yet
satisfactory. Resistance modifiers seem to act at least partly
by binding to P-glycoprotein (Safa et al., 1986; Cornwell et
al., 1986; Foxwell et al., 1989) and competing for drug efflux
via P-glycoprotein (Bradley et al., 1988), thereby increasing
drug accumulation in the cell. As an alternative some resist-
ance modifiers may act via their detergent effect on mem-

![Figure 2](image)

**Figure 2** Effect of Cremophor EL on vincristine cytotoxicity in
8226Dox4 and 8226S cells. Cells were incubated with vincristine
in the presence or absence of Cremophor EL (CEL) or verapamil.
Each point represents mean ± s.e. of 2–4 independent experi-
ments. 8226Dox4: ○—○, control; □—□, CEL (8.2 µg ml⁻¹); △—△,
CEL (16.5 µg ml⁻¹); ▽—▽, verapamil (0.5 µM): Δ—Δ, CEL
(132 µg ml⁻¹); ■—■, verapamil (1 µM); ▼—▼, verapamil (2 µM).
8226S: ●—●, control; ♦—♦, CEL (132 µg ml⁻¹).

branes as reported for Tween 80 (Carlsen et al., 1976). We
have shown previously that the action of resistance modifiers
may be due largely to their effects on the intracellular drug
localisation instead of on drug accumulation: in cells with
high levels of MDR doxorubicin is present mainly in the
cytoplasm in the absence of resistance modifiers. However,
in the presence of resistance modifiers doxorubicin is mainly
in the nucleus, as is the situation in drug-sensitive cells (Willi-
ingham et al., 1986; Schuurhuis et al., 1989a; Broxterman
et al., 1990). Cremophor EL also was able to produce a similar
change in drug localisation (from mainly cytoplasmic to
mainly nuclear) in SW-1573/2R160 cells as determined with
fluorescence microscopy (results not shown). These observa-
tions offer an explanation for the finding that modifiers such
as cyclosporin A are able to reverse drug resistance to a large
extent without strongly affecting drug accumulation (Slater
et al., 1986; Schuurhuis et al., 1989a; this study).

Since protein kinase C (PKC) activity has been associated
with MDR and its reversal (Aquino et al., 1988; Fine et al.,
1988; O'Brien et al., 1989; Ferguson & Cheng, 1987), it is of
interest that Cremophor EL, like other resistance modifiers
such as verapamil, tamoxifen, cyclosporin A and phenothia-
azines (Mori et al., 1980; O'Brien et al., 1985; Walker et al.,
1989; Schatzman et al., 1981), strongly inhibits PKC activity at concentrations comparable to those used in
this study (Zhao et al., 1989).

In conclusion, our findings demonstrate that Cremophor
EL is a potent modifier of MDR in human myeloma cells at
protein concentrations which closely mimic the in vitro situa-
tion. Clinical studies in myeloma with Cremophor EL as a
resistance modifier thus seem warranted. Further, Cremophor

**Table III** Reversal of doxorubicin resistance in 8226 MDR cells in protein-rich* medium

| Cell line | IC₅₀ (nm) | CEL (33 µg ml⁻¹) | CEL (132 µg ml⁻¹) | Vₚ 4 µM | Vₚ 16 µM |
|-----------|----------|-----------------|------------------|--------|--------|
| 8226S     | 26.3 ± 5.9| —               | 1.0 ± 0.1        | —      | 1.2 ± 0.3 |
| 8226Dox4  | 250 ± 71  | 5.5 ± 0.7*      | 6.2 ± 1.0*       | 2.0 ± 0.7 | 3.5 ± 0.7* |

Abbreviations as in Table I. *The growth medium contained 4% bovine serum albumin (Sigma) in addition to 10% fetal calf serum. **DMF, dose modifying factor: IC₅₀ minus resistance modifier/IC₅₀ plus resistance modifier. *Values are means ± s.d. from 2–3 experiments. **Significantly different from 1 (P < 0.05, Student's t test). ～Significantly different from 1 (P < 0.02).
EL may turn out to be useful in the treatment of other P-glycoprotein-containing tumours in addition to myeloma since we have found that in vitro the compound was active on other P-glycoprotein-containing human MDR cancer cells like squamous lung cancer cells (this paper) and ovarian cancer cells (submitted) as well as intrinsically resistant P-glycoprotein-containing human colon cancer cells (submitted).

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