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Modulation of multidrug resistance with dexniguldipine hydrochloride (B8509-035) in the CC531 rat colon carcinoma model

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Abstract The chemosensitizing potency of dexniguldipine hydrochloride (B8509-035) on epidoxorubicin was assessed in a multidrug-resistant (MDR) tumour model, the intrinsic MDR rat colon carcinoma CC531. In vitro in the sulphorhodamine B cell-viability assay the cytotoxicity of epidoxorubicin was increased approximately 15-fold by co-incubation with 50 ng/ml dexniguldipine. In vivo concentrations of dexniguldipine 5 h after a single oral dose of 30 mg/kg were 72 (\pm 19 SD) ng/ml in plasma and 925 (\pm 495 SD) ng/g in tumour tissue. Levels of the metabolite of dexniguldipine, M-1, which has the same chemosensitizing potential, were 26 (\pm 6 SD) ng/ml and 289 (\pm 127 SD) ng/g respectively. The efficacy of treatment with 6 mg/kg epidoxorubicin applied intravenously combined with 30 mg kg⁻¹ day⁻¹ dexniguldipine administered orally for 3 days prior to epidoxorubicin injection was evaluated on tumours grown under the renal capsule. Dexniguldipine alone did not show antitumour effects in vivo. Dexniguldipine modestly, but consistently, potentiated the tumour-growth-inhibiting effect of epidoxorubicin, reaching statistical significance in two out of four experiments. In conclusion, these experiments show that dexniguldipine has potency as an MDR reverter in vitro and in vivo in this solid MDR tumour model.

Key words Chemosensitizer · Dexniguldipine · Epidoxorubicin · Multidrug resistance · in vivo

Abbreviations MDR multidrug resistance · PBS phosphate-buffered saline

Introduction

Multidrug resistance (MDR) is an important mechanism in clinical drug resistance and expression of the *mdr* gene is found in a variety of tumours (Bellamy and Dalton 1994; Goldstein et al. 1989). In MDR a transmembrane efflux pump, P-glycoprotein, confers drug resistance on a group of chemically unrelated anticancer drugs by increasing the efflux. The P-glycoprotein pump can be blocked reversibly by so-called chemosensitizers, which are substrates for the protein themselves. As a result, higher intracellular levels of anticancer drugs are achieved and enhanced cell death occurs. Among the various compounds that can function as chemosensitizers are verapamil, cyclosporin A and its non-immunosuppressive analogue PSC 833, quinidine, tamoxifen, and many others (Bellamy and Dalton 1994). Previous studies have been carried out with verapamil, but levels necessary for modulation of MDR in vivo appeared too high, resulting in severe cardiovascular side-effects. In a clinical trial with verapamil, dose-limiting side-effects were hypotension and cardiac arrhythmias at levels of exposure anticipated to be inadequate for MDR reversal (Pennock et al. 1991). This has led to a search for related compounds that are devoid of the cardiovascular side-effects.

Stereoisomers of verapamil and related drugs vary in calcium-channel-blocking activity. For example, the (+)stereoisomer of verapamil is a 10-fold less potent calcium antagonist than the (-)isomer (Echizen et al. 1985), but has approximately the same chemosensitizing effectiveness (Plumb et al. 1990). Of the other calcium antagonists, the dihydropyridine drug

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niguldipine, was found to be a very effective chemosensitizer in MDR (Höllt et al. 1992). The (–) stereoisomer, dexniguldipine hydrochloride (B8509-035), displays a 45-fold lower affinity for calcium-channel-binding sites compared to the (+) isomer, while both have the same MDR-modulating potency (Höllt et al. 1992). In various preclinical models in vitro the chemosensitizing potency of dexniguldipine was either equal to or up to 50 times more effective than verapamil (Hill and Hosking 1994; Hofmann et al. 1991, 1992; Höllt et al. 1992; Reymann et al. 1993; Roller et al. 1993).

We tested the activity of dexniguldipine in the CC531 MDR tumour model. CC531 is a colon carcinoma, derived from and transplantable in the WAG/RIJ rat, that intrinsically expresses the multidrug-resistant phenotype. In a previous report the potency of cyclosporin A as a modulator of resistance to doxorubicin was shown in this model (Van de Vrie et al. 1993). Here we report on the chemosensitizing effect of dexniguldipine in vitro and in vivo and on levels of dexniguldipine and its active metabolite M-1 in plasma and tumours.

Materials and methods

Animals

Male rats of the inbred WAG/RIJ (RT1^a) strain were obtained from Harlan-CPB (Austerlitz, The Netherlands). Animals were bred under specific-pathogen-free conditions and fed standard rat chow (Hope Farms, Woerden, The Netherlands) and water *ad libitum*. In the experiments rats 12–18 weeks old, weighing 220–280 g, were used.

Tumour and cell line

CC531 is a colon carcinoma, which was induced chemically in the WAG/RIJ rat with 1,2-dimethylhydrazine. The tumour, a moderately differentiated adenocarcinoma, is weakly immunogenic and transplantable in WAG/RIJ rats (Marquet et al. 1984). In vitro the cell line grows as a monolayer. CC531 is an intrinsically multidrug-resistant tumour as it expresses the MDR phenotype. At the mRNA level, expression of *mdr-1a*, and not *mdr-1b*, has been detected by the polymerase chain reaction (De Greef et al. 1995). A low level of P-glycoprotein expression has been shown with the monoclonal antibody C-219 by Western blotting and by immunofluorescence techniques (De Greef et al. 1995; Van de Vrie et al. 1993). Intracellular accumulation of daunorubicin can be enhanced by chemosensitizers like verapamil and cyclosporin A (Gheuens et al. 1993; Van de Vrie et al. 1993). Cytotoxicity assays have shown the typical drug-resistance pattern of MDR and enhancement of cytotoxicity by chemosensitizers (Gheuens et al. 1993; Van de Vrie et al. 1993).

The cell line was grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, aspartic acid (0.1 mM), glutamic acid (0.3 mM), penicillin (111 IU/ml) and streptomycin (111 µg/ml), all obtained from Gibco (Paisley, UK), in a humidified atmosphere of 5% CO₂/95% air at 37°C. Regular screening for *Mycoplasma* infection was performed. Cells were isolated by trypsinization; viability, determined by trypan blue exclusion, was over 90% in all experiments.

Chemicals

Dexniguldipine hydrochloride (B8509-035), the metabolite M-1 (B8909-008) and B89003-001 (internal standard for dexniguldipine in the HPLC) were kindly provided by Byk Gulden, Konstanz, Germany; epidoxorubicin (Farmorubicin) was obtained from Farmitalia, Carlo Erba, Italy; sulphorhodamine B (SRB) was purchased from Sigma Chemicals, St. Louis, M., USA; deionized Milli-Q water was from Millipore, Etten Leur, The Netherlands; trichloroacetic acid from J.T. Baker, Deventer, The Netherlands; and dichloromethane/hexane/isobutyl alcohol (40:60:0.5) from Rathburn, Waltham, Scotland.

In vitro cytotoxicity assay

Chemosensitivity in vitro was determined with the sulphorhodamine B cell-viability assay, essentially carried out as described by Skehan et al. (1990). In brief, 2×10^3 trypsinized tumour cells/well in 200 µl complete medium were plated into 96-well flat-bottomed microtitre plates (Costar, Cambridge, Mass., USA). Tests were carried out in quadruplicate. The plates were incubated for 24 h at 37°C, 5% CO₂/95% air to allow the cells to adhere. Then the old medium was replaced by medium containing the test drug in graded concentrations; in the interaction studies epidoxorubicin together with a fixed concentration of dexniguldipine was added. On day 7 the incubation was terminated by washing the plates twice with phosphate-buffered saline (PBS). Subsequently the cells were fixed with 10% trichloroacetic acid in deionized Milli-Q water and placed for 1 h at 4°C. After five washings with tap water, the cells were stained for 15 min with 0.4% sulphorhodamine B dissolved in 1% acetic acid, and subsequently washed with 1% acetic acid to remove the unbound stain. The plates were air-dried and bound protein stain was dissolved in 150 µl 10 mM TRIS base. The absorbance was read at 540 nm using an automated microplate reader (Titertek, Flow Laboratories Ltd., Irvine, Scotland).

In vivo assay

Subcutaneously grown solid tumours of the CC531 cell line were used 20–30 days after implantation. In a subrenal capsule assay tumour pieces weighing 6–8 mg were implanted under the capsule of the kidneys. In the pharmacokinetic experiment, treatment with dexniguldipine was given 10 days after implantation. Rats were restrained from food 12 h prior to administration of the drug. A single dose of 30 mg/kg dexniguldipine was administered orally through a thin metal cannula. Five hours later rats were sacrificed, a blood sample was taken and the tumours were collected for analysis of dexniguldipine and M-1 levels.

In the pharmacodynamic experiment dexniguldipine treatment was started 24 h after implantation of the tumour. In contrast to the single dosing in the pharmacokinetic experiment, dexniguldipine was administered for 3 consecutive days orally at a dose of 30 mg kg⁻¹ day⁻¹. On day 4, 5 h after the last dexniguldipine dose, rats were injected intravenously with 6 mg/kg epidoxorubicin, or PBS in control rats. On day 10 animals were sacrificed, tumours were enucleated and weighed.

All experimental groups consisted of 6–8 rats.

Apparatus for dexniguldipine and M-1 measurement

Dexniguldipine was determined in plasma and tumour tissue with an automated reverse-phase isocratic high-performance liquid chromatography (HPLC) assay with UV detection at 230 nm. A model 710B WISP autosampler and a model M510 pump were

used (all Waters Assoc., Milford, Mass., USA). The detector was a UV2000 (Spectra Physics, San Jose, Calif, USA). The data were processed with a Shimadzu CR3A integrator (Shimadzu Corp., Kyoto, Japan). The column was a Shandon Hypersyl CPS, 3 μm 150 \times 4.6 mm (LC Services, Emmen, The Netherlands). The eluent consisted of a 5 mM phosphate buffer (pH 7.5) with 60% acetonitrile. The flow rate was 1.5 ml/min and the column temperature 40°C. Sample size was 100 μl for each analysis.

Sample preparation for dexniguldipine and M-1 measurement.

A volume of 150 μl plasma was collected, to which 50 μl 2000 ng/ml solution of internal standard (B89003-001) in methanol was added. Next, 800 μl deionized Milli-Q water was added and the sample was mixed on a whirl mixer for 15 s. For extraction of the test chemicals 7 ml dichloromethane/hexane/isobutyl alcohol (40:60:0.5) was added. The mixture was mixed for 30 min on a whirl mixer and subsequently centrifuged for 10 min at 4000 *g*. The organic layer was collected and evaporated to dryness at 50°C under vacuum. The residue was reconstituted in 150 μl eluent.

Tumour tissue was homogenized with a Turrax homogenizer (Boom, Meppel, The Netherlands) in 1 ml Milli-Q water. The homogenizer was flushed twice with 250 μl Milli-Q water. A 50- μl volume of a 2000-ng/ml internal standard solution in methanol and 7 ml dichloromethane/hexane/isobutyl alcohol (40:60:0.5) were added. Further handling of the tissue sample was as described for the plasma sample.

The recovery of dexniguldipine, M-1, and the internal standard was determined relative to direct injection of the individual dissolved compounds.

Statistics

Statistical analysis was carried out with SPSS/PC+, using the Mann-Whitney *U*/Wilcoxon rank-sum *W*-test. *P* < 0.05 was considered significant.

Ethical approval

The experimental protocols adhered to the rules laid down in *The Dutch Animal Experimentation Act* (1977) and the *Guidelines on the Protection of Experimental Animals* published by the Council of the E.C. (1986). Specific protocols were approved by the Committee on Animal Research of the Erasmus University, Rotterdam.

Results

In vitro chemosensitizing effect of dexniguldipine on epidoxorubicin

Dexniguldipine up to 1000 ng/ml had less than 10% growth-inhibiting effect on CC531 cells in vitro. The median inhibitory dose (ID₅₀) for dexniguldipine was approximately 5000 ng/ml. Epidoxorubicin showed a concentration-dependent growth-inhibiting effect with an ID₅₀ of 62 ng/ml. The chemosensitizer dexniguldipine at a concentration of 50 ng/ml enhanced cytotoxicity of epidoxorubicin approximately 15 times (Fig. 1). Higher concentrations of dexniguldipine were

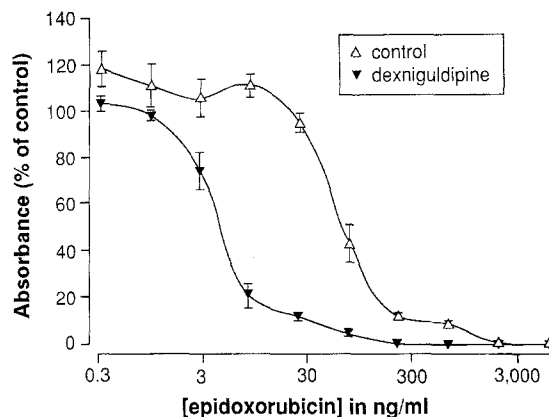


Fig. 1 Dose/response curves of the cell line CC531 to incubation with graded concentrations of epidoxorubicin in the absence (Δ) or presence (\blacktriangledown) of the chemosensitizer dexniguldipine (50 ng/ml) determined in the sulphorhodamine cell viability assay. Sulphorhodamine B absorbance is expressed as a percentage of the control absorbance on the y axis and represents the percentage cell viability.

equally effective (500 ng/ml) or too toxic for CC531 cells by themselves (5000 ng/ml) (data not shown).

Tissue concentrations of dexniguldipine

In this report we show some data on the levels of dexniguldipine and M-1 in plasma and in tumour tissue. In a separate report more elaborate studies on dexniguldipine and M-1 pharmacokinetics will be presented. (JHM Schellens, et al., manuscript in preparation). Dexniguldipine was readily absorbed after oral administration reaching levels in plasma after 5 h of 72 (\pm 19 SD) ng/ml. (see Fig. 2). Levels in tumour tissue were much higher: 925 (\pm 495 SD) ng/g. A similar pattern was observed for the metabolite M-1: in plasma 26 (\pm 6 SD) ng/ml and in tumour tissue 289 (\pm 127 SD) ng/g. The level of M-1 was approximately one-third of the level of dexniguldipine.

In vivo chemosensitizing effect of dexniguldipine on epidoxorubicin

Pilot experiments with epidoxorubicin alone showed that a dose of 6 mg/kg had a moderate but consistent growth-inhibiting effect on CC531 tumours grown in the subrenal capsule assay. Dexniguldipine treatment alone had no influence on growth of the tumour. In all experiments, tumours treated with the combination dexniguldipine and epidoxorubicin were the smallest and statistical significance was reached in two experiments. The results are shown in Table 1. Dexniguldipine had no additive effect in combination with a lower dose of 4 mg/kg epidoxorubicin (data not shown).

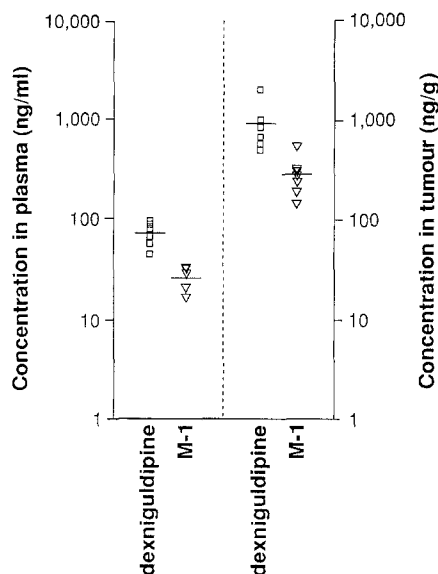


Fig. 2 Dexniguldipine (□) and M-1 (▽) levels in plasma (ng/ml) and tumour tissue (ng/g) determined by HPLC 5 h after a single oral dose of 30 mg/kg. Results from rats are represented individually on a logarithmic scale, with lines indicating the mean values. *Left* data from plasma; *right* data from tumour tissue

The experiments were not specifically designed for assessment of toxicity, but some effects on the body weight of rats were observed. Data on body weights were available from three experiments. All rats lost some weight during the experiment: control rats 1%–5%, dexniguldipine-treated rats 2%–6%, rats treated with epidoxorubicin alone 8%–12%, and rats treated with the combination dexniguldipine and epidoxorubicin 9%–13%. The total weight loss was significantly higher in the rats treated with epidoxorubicin compared to control groups. In one of the three experiments, rats treated with the combination dexniguldipine and epidoxorubicin had significantly more weight loss compared to epidoxorubicin-treated rats. In this particular experiment (number 3) no difference was observed in tumour weights between these groups.

Table 1 Results of experiments (1–4) on tumour growth inhibition *in vivo* with epidoxorubicin and the chemosensitizer dexniguldipine. Standard deviations are shown in parentheses

Treatment group	Tumour weight (mg)			
	1	2	3	4
Control	45.1 (± 15.6)	34.4 (± 7.8)	20.8 (± 6.5)	41.3 (± 9.2)
Dexniguldipine	38.6 (± 17.9)	35.1 (± 9.0)	16.4 (± 10.6)	40.6 (± 14.9)
Epidoxorubicin 6 mg/kg	23.2 (± 2.5)*	20.9 (± 3.2)**	12.3 (± 4.2)*	27.2 (± 5.1)*
Epidoxorubicin 6 mg/kg + dexniguldipine	18.5 (± 10.2)**	13.9 (± 4.7)**,**	9.9 (± 4.1)*	19.6 (± 5.2)**,**

*–***Statistically significant ($P < 0.05$) results: * epidoxorubicin or epidoxorubicin + dexniguldipine versus control; **epidoxorubicin or epidoxorubicin + dexniguldipine versus control and versus dexniguldipine; ***epidoxorubicin + dexniguldipine versus epidoxorubicin

Discussion

Dexniguldipine is a novel modulator of MDR that has low intrinsic calcium antagonist activity. *In vitro* studies have shown its efficacy as a chemosensitizer in various cytotoxicity tests and accumulation assays (Hill and Hosking 1994; Hofmann et al. 1991, 1992; Höllt et al. 1992; Reymann et al. 1993; Roller et al. 1993). Effective chemosensitizing was shown in experimental and in human tumour cell lines, and in induced as well as in intrinsic MDR. On a molar basis dexniguldipine was shown to be at least as effective as verapamil. Most studies report a superior drug-modulating effect of dexniguldipine over verapamil of 2.5- to 50-fold (Hill and Hosking 1994; Hofmann et al. 1992; Reymann et al. 1993; Roller et al. 1993).

This study expands these *in vitro* studies with *in vivo* data. After oral administration, dexniguldipine is readily absorbed and distributed into various tissues. Dexniguldipine has a very lipophilic nature and its volume of distribution is high (in animals 20–40 l/kg) (Zech and Herzog 1991). In the present study intratumoural levels of dexniguldipine were 925 ng/g tissue, which is more than ten times the plasma levels. Compared to the *in vitro* level of 50 ng/ml, which was effective in MDR modulation, these *in vivo* levels are high. The data on dexniguldipine levels were obtained after a single oral dosing. In the antitumour experiment dosing was tripled by administration on 3 consecutive days. Because of the lipophilic nature of the drug this will probably have resulted in even higher intratumoural levels. The wide variation in the results of the levels of the chemosensitizer is not readily explained. Wide interindividual variation in pharmacokinetics has been reported by others for dexniguldipine after oral administration, as well as for other dihydropyridine compounds (Ukena et al. 1995).

Part of the activity of dexniguldipine *in vivo* is mediated by the active metabolite M-1, which is shown to have the same MDR-modulating potency as dexniguldipine (Hofmann et al. 1992; Höllt et al. 1992). The pharmacokinetics of M-1 followed the results of

dexniguldipine closely. The M-1 level was approximately one-third of the level of dexniguldipine in plasma and in tumour tissue 5 h after administration. Recently, comparable levels of dexniguldipine and M-1 have been published from a phase I trial in patients (Ukena et al. 1995).

Dexniguldipine showed no direct antitumour activity against CC531 cells. Antiproliferative effects of dexniguldipine have been reported for some tumours, possibly tumours with a neuroendocrine differentiation depending on autocrine stimulating factors (Schüller et al. 1990, 1991). The chemosensitizing potency of dexniguldipine on the MDR CC531 tumour was observed in all *in vivo* experiments. Dexniguldipine had a significant potentiating effect on growth inhibition of CC531 tumours by epidoxorubicin in two out of four experiments, while in the other experiments the observed differences did not reach statistical significance. The results with dexniguldipine are comparable to those with earlier published experiments in the CC531 tumour model, which revealed the chemosensitizing effect of cyclosporin A in combination with doxorubicin (Van de Vrie et al. 1993).

Levels of epidoxorubicin in plasma and tumour have not been measured in these experiments. Therefore, we cannot rule out the possibility that altered pharmacokinetics of epidoxorubicin contribute to the chemosensitizing effect apart from direct modulation of MDR at the cellular level. The fact that combination treatment resulted in a small enhancement of toxicity in one experiment, as measured by body-weight loss, suggests that at least some systemic enhancement of epidoxorubicin activity may have occurred. This is in agreement with previous studies that showed enhancement of doxorubicin toxicity caused by combined treatment with cyclosporin A (Van de Vrie et al. 1994). Other investigators, however, have also furnished evidence for a direct effect of chemosensitizers on the tumour. Niwa et al. showed that PAK-200, like dexniguldipine a dihydropyridine analogue, enhanced the accumulation of doxorubicin in solid tumours *in vivo* (Niwa et al. 1992). The effect of PAK-200 on doxorubicin accumulation in the tumours was dependent on the level of P-glycoprotein expression: only the tumours with a clear expression of P-glycoprotein had a higher doxorubicin content in the presence of the chemosensitizer. Furthermore, in patients with refractory multiple myeloma, addition of cyclosporin A to the chemotherapeutic regimen vincristine, doxorubicin and dexamethasone resulted in enhancement of the response rate (Sonneveld et al. 1992). Additional studies showed that the effect was probably achieved by specific killing of the plasma cells expressing P-glycoprotein (Sonneveld et al. 1994). The studies clearly suggest drug modulation directly at the cellular level by a P-glycoprotein-dependent mechanism.

The results in our study are comparable to results obtained with dexniguldipine and doxorubicin in

a nude mouse xenograft model. Here partial reversal of resistance to doxorubicin was observed in solid tumours of the MDR1-overexpressing KB 8.5 cell line grown subcutaneously (Boss et al. 1994). Dexniguldipine has entered clinical studies now and promising results have been obtained in trials in acute myeloid leukemia and multiple myeloma (Scheulen et al. 1995; Thaler et al. 1994).

The present study confirms the chemosensitizing potency of dexniguldipine on MDR cells *in vitro*. It shows that *in vivo* relatively high levels of dexniguldipine in plasma and tumour tissue can easily be achieved by oral administration. *In vivo* this resulted in a strong trend towards a significant enhancement of the anti-tumour effect of epidoxorubicin in the solid MDR tumour CC531.

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