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MECHANISM OF ACTION OF DEXNIGULDIPINE-HCl (B8509–035), A NEW POTENT MODULATOR OF MULTIDRUG RESISTANCE

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Abstract—It has previously been shown that dexniguldipine-HCl (B8509–035) is a potent chemosensitizer in multidrug resistant cells [Hofmann *et al.*, *J Cancer Res Clin Oncol* **118**: 361–366, 1992]. It is shown here that dexniguldipine-HCl causes a dose-dependent reduction of the labeling of the P-glycoprotein by azidopine, indicating a competition of dexniguldipine-HCl with the photoaffinity label for the multidrug resistance gene 1 (MDR-1) product. Exposure to dexniguldipine-HCl results in a dose-dependent accumulation of rhodamine 123 in MDR-1 overexpressing cells. In the presence of 1 μM dexniguldipine-HCl, rhodamine 123 accumulated in multidrug resistant cells to similar levels as in the sensitive parental cell lines. At this concentration, dexniguldipine-HCl enhances the cytotoxicities of Adriamycin® and vincristine. The resistance modulating factors (RMF), i.e. IC_{50} drug/IC_{50} drug + modulator, were found to be proportional to the expression of MDR-1, ranging from 8 to 42 for Adriamycin and from 16 to 63 for vincristine. Transfection with the MDR-1 gene was found to be sufficient to sensitize cells to the modulation by dexniguldipine-HCl. The compound does not affect the expression of the MDR-1 gene. Dexniguldipine-HCl has no effect on a multidrug resistant phenotype caused by a mutation of topoisomerase II. It is concluded that dexniguldipine-HCl modulates multidrug resistance by direct interaction with the P-glycoprotein.

Key words: multidrug resistance; dexniguldipine-HCl; B8509–035; rhodamine 123

In preceding publications we and others described the new dihydropyridine derivative dexniguldipine-HCl‖ (B8509–035 or B859–35), which has proven to be extremely potent in overcoming resistance to Adriamycin® or Vinca-alkaloids [1–5]. Dexniguldipine-HCl is the *R*-enantiomer of niguldipine, an L-type Ca^{2+}-channel blocker [6]. Compared to niguldipine, the affinity of dexniguldipine-HCl to Ca^{2+}-channels is 40 times lower [6]. Consequently, side effects due to its Ca^{2+} antagonistic effects are low and according to ongoing clinical phase II trials the compound is well tolerated. As the clinical use of presently available MDR modulators is frequently limited by their toxic side effects, this apparently non-toxic but potent MDR modulator appeared especially interesting and warranted the elucidation of its mechanism of action. In addition to MDR reversal, the compound acts as a calmodulin antagonist [7], inhibits protein kinase C [8] and exerts an antitumor activity in its own right [7, 9].

A characterization of the mechanism by which dexniguldipine-HCl modulates MDR, however, appeared necessary, in view of the fact that in addition to an overexpression of P-glycoprotein, other mechanisms have been described which are correlated with MDR [10–14]. A combination of increased P-glycoprotein expression with other mechanisms involved in multidrug resistance has also been described [11–14]. This multifactorial resistance may be of particular importance for clinically occurring drug resistance [15]. Compounds which enhance vinblastine cytotoxicity in P-glycoprotein overexpressing cells by a P-glycoprotein-independent mechanism have also been described [16]. Furthermore, compounds able to overcome P-glycoprotein-mediated MDR may act at different targets. In addition to the direct interaction with the P-glycoprotein described for some MDR modulators, compounds affecting membrane lipid composition or fluidity [17, 18] or altering the expression or activity of protein kinase C [19–23] have been shown to reduce resistance in P-glycoprotein overexpressing cells as well.

In order to investigate whether the sensitizing potency of dexniguldipine-HCl is correlated to the

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‖ Abbreviations: dexniguldipine-HCl, B8509–035, (4*R*)-3-[4,4-diphenyl-1-piperidinyl-(propyl)]-5-methyl-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-pyridine-3,5-dicarboxylate-hydrochloride); MDR, multidrug resistance; MDR-1 gene, human multidrug resistance gene 1; HeLa-WT, HeLa wild type cells; HeLa-MDR-1, HeLa cells transfected with the human MDR-1 gene; RMF, resistance modulating factor = IC_{50} drug/IC_{50} drug + modulator.

expression of the P-glycoprotein we employed cells which had been transfected with the MDR-1 gene and in which drug resistance should be exclusively due to P-glycoprotein overexpression. The data are compared to results obtained from cells which had been selected for drug resistance by exposure to increasing antitumor drug concentrations and which express various levels of P-glycoprotein. Finally, cells which are multidrug resistant due to a mutated topoisomerase II were included.

MATERIALS AND METHODS

Drugs. Dexniguldipine-HCl (B8509–035) was from Byk-Gulden (Konstanz, Germany) and was dissolved in DMSO in glassware. The final concentration of DMSO did not exceed 0.1% and was not toxic. *R*-verapamil was obtained from RBI (Natick, U.S.A.). Adriamycin, vinblastine, vincristine, etoposide and rhodamine 123 were from Sigma Chemicals (Munich, Germany).

Tissue culture. CCRF-CEM (human lymphoblastoid cells), CCRF-VCR100, CCRF-VCR1000 and CCRF/VM-1 cells were grown as described [24]. CCRF-VCR100 were grown in the presence of 100 ng/mL, CCRF-VRC1000 cells in the presence of 1 μg/mL vincristine, except at the time of the experiments. CEM/VM-1 cells were kindly provided by Dr W. T. Beck, Memphis, U.S.A. and grown as described [25].

The multidrug resistant cell line HeLa-MDR-1 was obtained by transfection of human HeLa S3 (HeLa-WT) cervix carcinoma cells with an MDR-1 gene using the expression vector construct pSK1.MDR [26] kindly provided by Dr M. M. Gottesman, National Cancer Institute, Bethesda, MD, U.S.A. This vector contains the human MDR-1 cDNA harboring a Gly-Val mutation in codon 185 which confers an altered MDR pattern onto recipient cells. HeLa-MDR-1 cells were grown in the presence of 240 ng/mL colchicine, except at the time of the experiments. The medium for HeLa cells was the same as for CCRF cells.

The human KB cell lines KB-3–1, KB-8–5 and KB-Cl were obtained from Dr M. M. Gottesman, Bethesda, MD, U.S.A. and grown as described [27]. KB-8–5 cells were grown in 10 ng/mL, KB-C1 cells in 1 μg/mL colchicine.

Cell proliferation. Dose–response curves of drug sensitivities for CCRF cells were established by addition of drugs or drug combinations as indicated in the figures. Following incubation for 72 hr in the presence of the drugs, the cells were counted with an electronic counter (Coulter Electronics, Luton, U.K.). Cellular multiplication (M) was calculated by $M = (T_t - T_0)/(C_t - C_0) \times 100$, where C are untreated controls, T are drug treated cells, 0 and t equal the number of cells at time 0 and t (72 hr), respectively.

In all other cell lines inhibition of cell proliferation was detected by a modification of the MTT assay [28].

Detection of P-glycoprotein by Western blotting. Cells ($1–2 \times 10^8$) were sonified at 4° in 10 mM Tris–HCl, pH 7.4, 10 mM NaCl, 1.5 mM $MgCl_2$ and 0.1 mM phenylmethylsulfonylfluoride. After centrifugation at 300 *g* for 10 min, the resulting supernatant was centrifuged at 100,000 *g* at 4° for 20 min. The pellets were resuspended in PBS containing 0.1 mM phenylmethylsulfonylfluoride. Protein content was determined by a BCA-assay (Pierce, Rockford, IL, U.S.A.) using BSA as a standard. Membranes (50 μg) were loaded onto an 8.5% SDS–polyacrylamide gel. Proteins were transferred to a membrane (Immobilon-P, Millipore, Eschborn, Germany), using an electroblotting chamber from Hoefer Scientific Instruments, Inc. (San Francisco, CA, U.S.A.) in 25 mM Tris–HCl, pH 8.3, 192 mM glycine, 1% SDS in 20% (v/v) methanol at 300 mA for 16 hr. After three washing steps, the membranes were incubated with 1 μg/mL of the P-glycoprotein specific monoclonal antibody C494 (Signet Laboratories, Dedham, U.S.A.) at 4° for 14 hr followed by incubation with a horseradish peroxidase-conjugated anti-mouse IgG antibody (Dianova, Hamburg, Germany). After washing, the membranes were incubated in an ECL-detection system (Amersham, Little Chalfont, U.K.) and exposed to a Hyperfilm-ECL (Amersham).

Accumulation of rhodamine 123. The intracellular accumulation of rhodamine 123 was determined as described previously [2].

Determination of MDR-1 mRNA. For detection of the MDR-1 mRNA levels, total RNA was isolated using RNAzol (Biotexs Laboratories Inc., 5023 South Loop East, Houston, TX, U.S.A.). Synthesis of cDNA and amplification by polymerase chain reaction were performed as described previously [29]. The products of the PCR reaction were separated on 10% polyacrylamide gels and stained with ethidium bromide.

Photoaffinity labeling. Membranes from CCRF-CEM and CCRF-VCR1000 cells were prepared according to Hamada and Tsuruo [30]. Protein concentration was determined using a Bio-Rad protein assay kit. The experiments were performed under dim sodium light. Thirty microgrammes of protein was incubated with 0.7 μCi [^{3}H]azidopine (49 Ci/mmol, Amersham) in the presence or absence of dexniguldipine-HCl in a final volume of 50 μL phosphate buffer (40 mM, pH 7.4) at room temperature for 60 min. The incubation mixture was placed on ice and irradiated with a Philips TL 40 W/08 black–light lamp at a distance of 8 cm for 20 min. Samples were solubilized for 10 min at 56° in loading buffer (130 mM Tris–HCL, pH 7,4, 20% glycerol, 10% SDS) and separated on a 4–15% polyacrylamide gel (containing SDS). The region containing P-glycoprotein was cut from the gel after staining with Coomassie-blue. Radioactivity was eluted from the gel slices by incubation with 1 mL 30% H_2O_2/50 μL 25% NH_3 at 56° for 3–7 hr. Non-specific labeling was detected from the extent of [^{3}H]-azidopine incorporated into the 170 kDa region of membranes from the parental CCRF-CEM cell line.

RESULTS

The MDR-modulating effect of dexniguldipine-HCl is related to the expression of the P-glycoprotein

Dexniguldipine-HCl inhibits cellular proliferation with IC_{50} values between 3 and 6 μM. The expression

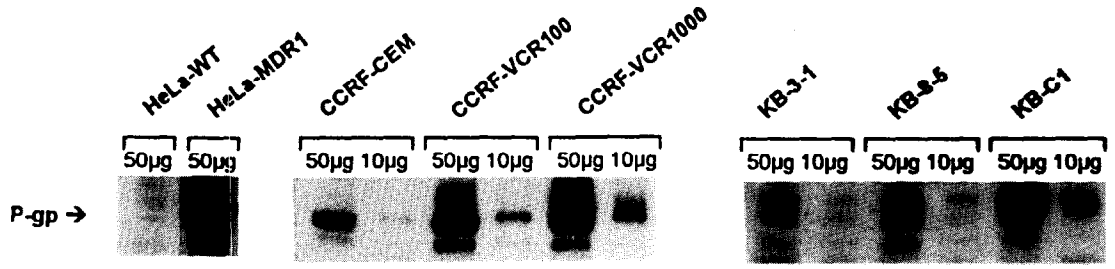

Fig. 1. Detection of P-glycoprotein by Western blotting. Membrane preparation of the indicated cell lines, gel electrophoresis and transfer to polyvinylidenedifluoride membranes were performed as described in Materials and Methods. The membranes were incubated with the P-glycoprotein specific monoclonal antibody C494. After incubation with a horseradish peroxidase-conjugated secondary antibody, the membranes were treated with an ECL-system and exposed to a Hyperfilm-ECL.

of P-glycoprotein in the various cell lines is shown in Fig. 1. Detection of MDR-1 mRNA by polymerase chain reaction and quantitation by HPLC revealed: KB-8–5 expressed approximately 9-fold and KB-C1 13-fold more than KB-3–1 cells. Compared to CCRF-CEM the extent in CCRF-VCR100 was approximately 5-fold and in CCRF-VCR1000 10-fold. In HeLa-MDR-1 the level was approximately 31 times higher than in HeLa-WT.

The modulation by dexniguldipine-HCl of the sensitivity of the various cell lines to Adriamycin, vinblastine or etoposide is demonstrated in Table 1. The effects of *R*-verapamil, an established inhibitor of the MDR-1 encoded pump, are also included.

The data in Fig. 1 and Table 1 clearly demonstrate that 1 μM dexniguldipine-HCl, corresponding approximately to the IC_{20} values for the particular cell lines, enhances the cytotoxicities of Adriamycin and vincristine in the MDR-1 transfected HeLa cells. In contrast the corresponding wild type cells exhibit only a minor increase in cytotoxicity to these agents in combination with dexniguldipine-HCl. In the other cell lines that had been selected for resistance by exposure to increasing drug concentrations, the resistance modulating factor (RMF), i.e. the ratio of the IC_{50} value for Adriamycin determined in the absence of the modulator over the IC_{50} value in the presence of the modulator, is proportional to the expression of P-glycoprotein (Table 1). Similar results are obtained with vincristine with the exception of the KB-C1 cells. These cells contain higher levels of MDR-1 mRNA and are more resistant to vincristine than the KB-8–5 line but the sensitizing effect of dexniguldipine-HCl for vincristine in these cells (expressed by the RMF) was found to be lower than in KB-8–5 cells. This may be due to the fact that in contrast to KB-8–5 cells, the MDR-1 gene in KB-C1 encodes a mutated protein with a glycine to valine substitution at position 185. Alternatively, an additional, dexniguldipine-sensitive mechanism for vincristine resistance may be operative in the KB-C1 cell line.

At equimolar concentrations, dexniguldipine-HCl is more potent than *R*-verapamil. In all cases resistance to etoposide, known to be a poor substrate for P-glycoprotein, was lower than resistance to Adriamycin or vincristine. Sensitivity to etoposide was only marginally affected by dexniguldipine-HCl or *R*-verapamil in MDR-1 overexpressing cells. Resistance to etoposide is a typical feature of cells with altered topoisomerase II. Cells which are Adriamycin resistant due to an altered topoisomerase II cannot be sensitized to Adriamycin by dexniguldipine (Fig. 2).

The modulating potency of dexniguldipine-HCl is related to its capacity to increase intracellular accumulation of rhodamine 123, *a substrate of the P-glycoprotein*

Rhodamine 123 is known to be accepted as a substrate by the P-glycoprotein [2]. In accordance with this assumption, rhodamine 123 levels are low in cells overexpressing P-glycoprotein (Fig. 3A, B). Treatment of cells with dexniguldipine-HCl at concentrations required to modulate drug resistance (Table 1) leads to a marked accumulation of rhodamine 123 in P-glycoprotein overexpressing cells, whereas rhodamine accumulation in sensitive cells with low P-glycoprotein levels is hardly affected by dexniguldipine-HCl (Fig. 3A, B). The accumulation of rhodamine 123 is dexniguldipine-HCl dose dependent. As shown in Fig. 3C, an elevation of rhodamine 123 is already detectable at a concentration of 0.1 μM dexniguldipine-HCl.

Dexniguldipine-HCl causes a dose-dependent inhibition in photoaffinity labeling of P-glycoprotein by azidopine

The dihydropyridine azidopine is a well-established photoaffinity label for P-glycoprotein. Figure 4 demonstrates that dexniguldipine-HCl causes a concentration-dependent reduction in azidopine labeling of the P-glycoprotein. The data indicate that dexniguldipine-HCl competes with azidopine for the same binding region at the P-glycoprotein.

Exposure to dexniguldipine-HCl does not affect the expression of the MDR-1 gene

A recent report described an increase in MDR-1 expression following exposure to various MDR

Table 1. Modulation of the cytotoxicities of Adriamycin, vincristine and etoposide by dexniguldipine or *R*-verapamil

	ADR IC_{50}	ADR + DN IC_{50}	ADR + DN RMF	ADR + R − V IC_{50}	ADR + R − V RMF	VINCR IC_{50}	VINCR + DN IC_{50}	VINCR + DN RMF	VINCR + R − V IC_{50}	VINCR + R − V RMF	ETOP IC_{50}	ETOP + DN IC_{50}	ETOP + DN RMF
HeLa-WT	0.014	0.014	1	ND	—	0.0018	0.001	1.8	0.0012	1.5	0.49	0.35	1.4
HeLa-MDR1	5	0.3	17	0.7	7	0.1	0.005	20	0.02	5	20	12.3	2
CCRF-CEM	0.0094	0.009	1	0.009	1	0.0004	0.0003	1	0.3	1	0.092	0.085	1
CCRF-VCR100	0.5	0.06	8.3	0.104	4.8	0.3	0.01	30	0.0343	8.7	1.45	1.25	1
CCRF-VCR1000	2.077	0.1	21	0.378	6	3.299	0.052	63	0.684	5	2.11	2.226	1
KB-3-1	0.2	0.2	1	0.2	1	0.003	0.003	1	0.003	1	2.5	2.5	1
KB-8-5	2.0	0.2	10	0.3	7	0.3	0.005	60	0.038	8	3.2	2.5	1
KB-C1	25.1	0.6	42	8.8	3	3.2	0.2	16	1.6	2	12.6	7.5	2

Cells were continuously incubated with the drugs or drug + dexniguldipine-HCl as indicated for 72 hr. The concentration of dexniguldipine-HCl or *R*-verapamil was always 1 μM. Inhibition of cell proliferation was detected by MTT-assay. The IC_{50}s were determined and are indicated in μg of the drug per mL medium. The RMF was calculated by IC_{50} drug/IC_{50} drug + 1 μM modulator. ADR = Adriamycin, VINCR = vincristine, ETOP = etoposide, DN = dexniguldipine − HCl, R − V = *R* − verapamil, ND = not determined.

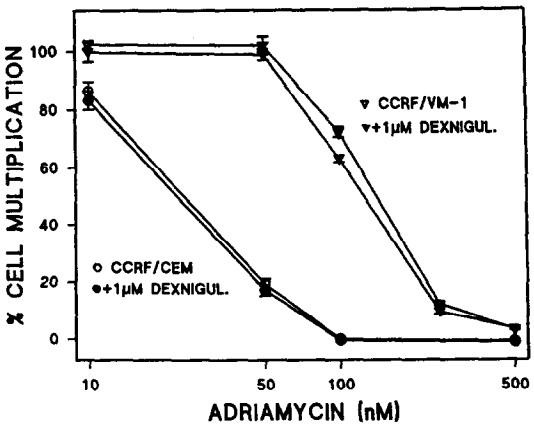

Fig. 2. Inhibition of cell proliferation after incubation of the human CCRF-CEM and the atypical multidrug resistant CEM/VM-1 cells with Adriamycin or a combination of Adriamycin/dexniguldipine-HCl for 72 hr. The mean of three independent experiments in which two samples were taken within each experiment (+/−SEM) is indicated.

modulators [31]. As such an effect would counteract the inhibition of the pumping activity of the P-glycoprotein, the usefulness of MDR modulators could be questioned if this phenomenon were to represent a general consequence of exposure to MDR modulators. Figure 5 demonstrates, however, that dexniguldipine-HCl is without any significant effect on MDR-1 expression during a 24 hr exposure to the drug.

DISCUSSION

The goal of these studies was to characterize the mechanism underlying the chemosensitizing activity of dexniguldipine-HCl. In view of the results described here it is concluded that dexniguldipine-HCl acts as an inhibitor of the P-glycoprotein, the product of the MDR-1 gene. This conclusion is based on the following data: dexniguldipine-HCl causes a dose-dependent reduction of the labeling of the P-glycoprotein by azidopine, indicating a competition of dexniguldipine-HCl with the photaffinity label for the MDR-1 product. The chemosensitizing effect of dexniguldipine-HCl is restricted to cells over-expressing the MDR-1 gene. In these cells dexniguldipine-HCl is capable of antagonizing drug resistance and causes a dose-dependent accumulation of rhodamine 123, an established substrate of the MDR-1 encoded P-glycoprotein. The modulating effect was found to be proportional to the extent of expression of the P-glycoprotein: i.e. the dexniguldipine-HCl-mediated shift in the IC_{50} values for Adriamycin and vincristine is proportional to the MDR-phenotype and reflects the contribution of the P-glycoprotein to the sensitivity of the cell lines to antitumor agents. Finally, resistance in cells which had been transfected with the MDR-1 gene can be reverted by dexniguldipine-HCl. As resistance in these cells should be exclusively due to P-glycoprotein overexpression, the data clearly indicate that P-

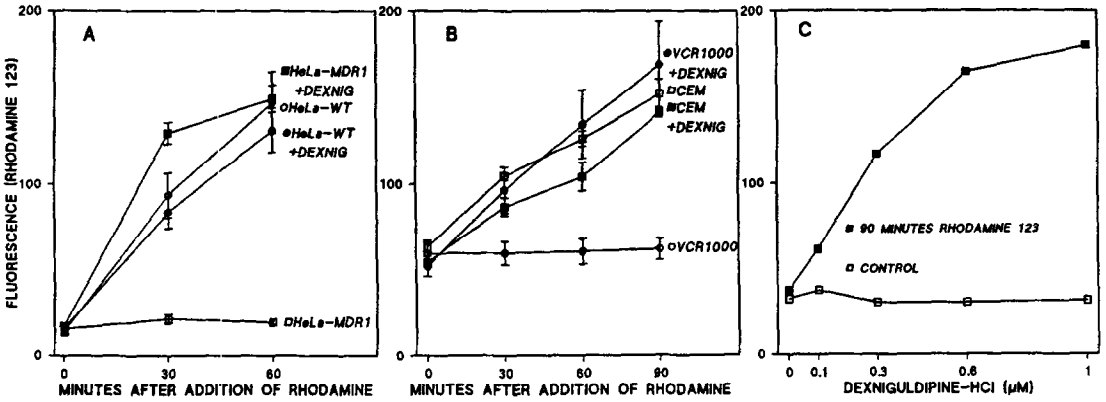

Fig. 3. Accumulation of rhodamine 123 (indicated as fluorescence) in (A) sensitive HeLa-WT, multidrug resistant HeLa-MDR1, (B) sensitive CCRF-CEM and multidrug resistant CCRF-VCR1000 cells after preincubation with 1 μM dexniguldipine-HCl for 1 hr. The mean (+/−SEM) of three independent experiments in which two samples were taken within each experiment is indicated. (C): dose-dependent accumulation of rhodamine 123 following treatment with dexniguldipine-HCl in CCRF-VCR1000 cells (control = no rhodamine 123). The mean of two independent experiments is indicated.

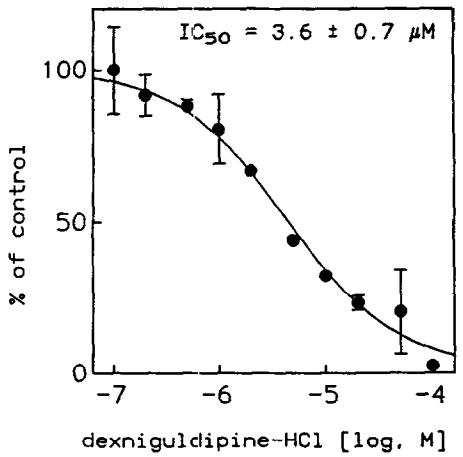

Fig. 4. Interaction of dexniguldipine-HCl with P-glycoprotein. Photoincorporation of [^{3}H]azidopine into P-glycoprotein enriched membranes of multidrug resistant CCRF-VCR1000 cells was measured in the absence or presence of the indicated concentrations of dexniguldipine-HCl. Conditions were as described in the Materials and Methods. The IC_{50} value is given as the mean of two independent experiments (+/−SD, N = 4). [^{3}H]Azidopine incorporated in the absence of dexniguldipine-HCl was normalized to 100% (control).

glycoprotein is the target of dexniguldipine-HCl. In accordance with this conclusion, the compound has no effect on an MDR phenotype caused by an altered topoisomerase II.

Our data on the sensitization by dexniguldipine-HCl to Adriamycin are in agreement with data published for doxo- and daunorubicin [3]. Our results also confirm recently published findings by Hill and Hosking [5] which appeared during the preparation of this manuscript. Hill and Hosking [5] found that dexniguldipine-HCl sensitizes to vinblastine only in the MDR-1 overexpressing VBL1000 cell line but not in the topoisomerase II mutant CEM/VM-1 and the parental sensitive cells. In the studies by Hill and Hosking [5] dexniguldipine-HCl was not found to modulate the toxicity of teniposide in VBL1000 cells. However, in our study dexniguldipine-HCl exerts a slight modulation of the growth inhibitory effect of etoposide in HeLa and KB-C1 cells. This may be due to cell type related differences and the use of etoposide instead of teniposide. It should be emphasized, however, that in all previous studies the cells employed had been selected by long-term exposure to increasing drug concentrations. Although these cells overexpress MDR-1 they may express a variety of additional alterations which makes it difficult to define the MDR-1 encoded P-glycoprotein as the only target of dexniguldipine-HCl. The use of MDR-1 transfected cells and of additional cell lines with varying degrees of MDR-1 expression permits a more clear-cut correlation between modulation by dexniguldipine and P-glycoprotein expression.

We employed *R*-verapamil as a reference compound for which the interaction with the P-glycoprotein is well known. In all cases dexniguldipine-HCl reflects the behavior of *R*-verapamil. On a molar basis, however, dexniguldipine proved to be more potent than verapamil in reversing drug resistance. This is in agreement with previously published findings [3, 5]. In contrast to the data reported here, Hill and Hosking [5] described a slight sensitization by verapamil to vinblastine in sensitive CEM cells. However, these authors employed 10 μM of racemic verapamil whereas in our studies 1 μM of *R*-verapamil was used. Thus, the difference may be explained by the higher growth inhibitory effect of the verapamil concentration employed by Hill and Hosking.

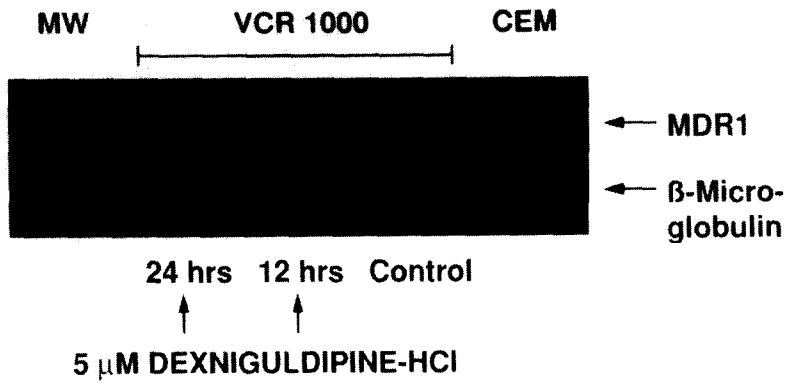

Fig. 5. Detection of MDR-1 mRNA levels by polymerase chain reaction. CCRF-CEM and CCRF-VCR1000 cells were incubated with dexniguldipine-HCl for the times indicated. RNA isolation and amplification were performed as described in Materials and Methods. β-Microglobulin was used to control the correct amount of RNA in the experiment.

In addition to its MDR-modulating potency, dexniguldipine-HCl also exerts an antiproliferative activity in its own right [7, 9]. Dexniguldipine-HCl has been shown to inhibit protein kinase C in cell-free extracts as well as in intact cells and acts as a calmodulin antagonist [7, 8]. These effects have been correlated to the antitumor activity of dexniguldipine-HCl. However, the MDR-modulating activity of the compound is expressed at concentrations which—for most cell systems—are significantly lower (10–100-fold) than those required to achieve an inhibition of cellular replication by dexniguldipine-HCl as a single agent. This is in agreement with the conclusion that the MDR-modulating activity of dexniguldipine-HCl is caused by a mechanism which differs from the effects responsible for its direct inhibition of tumor cell proliferation.

Ongoing clinical trials have revealed that orally administered dexniguldipine-HCl is well tolerated up to daily dosages of 2500 mg [32, 33]. Dexniguldipine-HCl does not affect the expression of the MDR-1 gene, an effect reported to occur following administration of other MDR modulators [31] one which might counteract the efficiency of compounds administered to overcome drug resistance. In view of the data available so far, dexniguldipine-HCl appears to be a promising candidate for a new clinically useful modulator of MDR.

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