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Reversal of multidrug resistance in Friend leukemia cells by dexniguldipine-HCl*

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Abstract. Dexniguldipine-HCl (DNIG) – a prospective clinical modulator of p170-glycoprotein (pgp170)-mediated multidrug resistance (MDR) – was evaluated in a drug-accumulation assay in MDR murine leukemia cell strain F4-6RADR expressing pgp170. The compound elevated low accumulation of either doxorubicin (DOX), daunorubicin (DNR), or mitoxantrone (MITO) in resistant F4-6RADR cells to the very levels observed in drug-sensitive F4-6 precursor cells. In parallel with the increase in DNR content (F4-6RADR, solvent: 303 ± 27 pmol/mg protein; DNIG (3.3 μmol/l): 1,067 ± 174 pmol/mg protein; F4-6P, solvent: 948 ± 110 pmol/mg protein; n = 8–9, SEM), the amount of DNR tightly bound to the acid precipitate pellet obtained from F4-6RADR (i.e., protein, DNA, RNA) increased 3.9-times to the levels observed in sensitive F4-6 cells. The main pyridine metabolite of DNIG displayed similar activity. Concentration-response analysis revealed that DNIG and R,S-verapamil (VER) induced 100% reversal of the DNR accumulation shortage associated with the MDR phenotype but DNIG was 8 times more potent than VER (50% inhibitory concentration (IC_{50}), 0.73 vs 5.4 μmol/l). In keeping with the accumulation assay, DNIG was about 10 times more potent than VER in sensitizing F4-6RADR cells to the cytostatic and cytotoxic effects of DNR in proliferation assays. In conclusion, DNIG is a potent in vitro modulator, improving (a) the accumulation of anthracycline-like cytostatics, (b) drug access to cellular binding sites, and (c) the cytostatic action of DNR in F4-6RADR leukemia cells of the MDR phenotype.

Introduction

Dexniguldipine-HCl (DNIG) is currently being evaluated in phase I-II clinical trial [14, 32] as a modulator of P-glycoprotein 170 (pgp170)-mediated multidrug resistance (MDR), one of the major obstacles in cancer chemotherapy [3, 30]. DNIG is reported to be a compound of low systemic toxicity [14, 32]. In particular, it is markedly less active than racemic verapamil (VER) regarding calcium-channel antagonistic and cardiovascular effects in clinical pharmacology [14, 18]. Reversal of in vitro MDR, however, appears to be induced by DNIG at concentrations 10 times *lower* than those required for VER in proliferation assays using MDR variant HeLa cells [11]. Rhodamine-uptake assays have indicated that DNIG might interfere with the pgp170-mediated drug extrusion system [11, 12]. We measured the effects of DNIG on mouse leukemia cell strain F4-6RADR expressing pgp170 and maintaining low anthracycline levels by drug extrusion as opposed to the drug-sensitive parental cell line F4-6. Concentration-response data for DNIG were analyzed in comparison with VER and additional compounds, including the major pyridine degradation product of DNIG in human metabolism. DNIG-mediated elevation of the cellular daunorubicin (DNR) content of F4-6RADR cells was related to sensitization for DNR-induced cytostasis.

Materials and methods

Cells. Friend erythroleukemia cell line F4-6 [8] and the MDR subline F4-6RADR were propagated in suspension cultures in Eagle's minimal essential medium (MEM alpha, Gibco, Karlsruhe, Germany) supplemented with 10% (v/v) fetal calf serum. MDR had originally been induced in a subclone of the parent line (selecting agent, doxorubicin (DOX); factor of resistance, 51-fold) by Dr. G. Steinheider (Department of Toxicology, University of Hamburg, Germany). The MDR-1 phenotype of F4-6RADR[1] has been verified [5, 7, 26] using immunocytochemistry (monoclonal antibody C219, MDR1 polyclonal antibody rabbit

* Dexniguldipine-HCl is the proposed INN for compound B859-35, the R-enantiomer of niguldipine. Segments of this work have been reported in the abstract form

Abbreviations: DOX, doxorubicin; CSA, cyclosporin A; DMSO, dimethylsulfoxide; DNIG, dexniguldipine-HCl; DNR, daunorubicin; MDR, multidrug resistance; MITO, mitoxantrone; pgp170, permease glycoprotein 170; VER, R,S-verapamil

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[1] Cell strain F4-6RADR has been referred to as strain F4-6R in previous communications [7, 26]

IgG; Oncogene Science, Manhasset, N.Y., USA), Northern blotting (for a description of the method, see [4]; data not shown), and polymerase chain reaction for pgp170 mRNA [5]. As compared with its parent line F4-6, this cell line displays a typical pattern of cross-resistance [7] to drugs such as DNR (35-fold), mitoxantrone (MITO, 16-fold), vincristine (19-fold), etoposide (17-fold), and amsacrine (11-fold).

Transport assays. Cells were collected from flasks at the late log phase of proliferation and were resuspended (5×10^6 cells/ml) in MEM alpha without fetal calf serum. Aliquots were used in parallel to determine the intracellular fluid space and the intracellular accumulation of the transport substrates using a silicone-oil filtration method [31]. Accuracy was improved by using both ^{3}H- and ^{14}C-labeled tracer substances. One radiolabeled marker [polyethylene glycol, (mol. wt., 4,000 Da) or inulin (mol. wt., 5,000 Da)] was used to determine the amount of extracellular fluid remaining after silicone-oil filtration in each individual test vial. As the second marker, radiolabeled DOX, DNR, or MITO was used in the transport assays; cell volume was measured with ^3H_2O. Continous incubation conditions in propagation medium (see above, pH 7.4 at 37° C) were used and experiments were terminated by centrifugation of 200 µl of the cell suspension at 15,000 *g* for 4 min through a layer of silicone oil into trichloroacetic acid (TCA; 10%, w/v; 200 µl). The radioactive label of cytostatic drugs and extracellular marker was measured in the TCA phase. The amounts of drug measured herein were termed "cytosolic" and were related to the cytosolic fluid space (the ^3H_2O space minus the [^{14}C]-PEG space; volume data obtained under parallel conditions of incubation). The precipitated pellet of cellular matter (DNA, RNA, proteins) was washed thoroughly with 10% TCA, and the radioactivity was measured after alkaline resuspension (2 N NaOH, 400 µl, 56° C, 30 min) and neutralization (2 N HCl). The cytostatic drug found in this fraction was referred to as "bound" and was related to cell protein as determined using a commercial dye (Bio-Rad, Munich, Germany) and bovine globulin as a standard [2]. The sum of cytosolic and bound drug per milligram of protein was termed "total uptake." Results were corrected for adherent incubation fluid (extracellular space marker).

Anthracycline extrusion was measured after preincubation with DNIG for 10 min and subsequent loading of 5.5×10^7 cells/ml with DOX (6 µmol/l) for 20 min. Following 100-fold dilution with anthracycline-free medium, DOX efflux from the cells to the medium was assayed after removal of the cells by silicone-oil filtration. This uptake assay has been validated for physiological transport substrates and with regard to anthracycline equilibration time ($\leq$30 min [20, 21]). Drug recovery exceeded 90% of the DOX, DNR, or MITO added. In accordance with other reports [15], cellular metabolism of DOX or DNR was negligible within the experimental period as checked by high-performance liquid chromatographic (HPLC) analysis (results not shown).

Proliferation assays. Cells were seeded at 10^6 cells/15 ml in propagation medium (see above) without drugs (controls) or in the presence of DNR and/or modulators as indicated. Proliferation of cell cultures was determined after 48 h by counting of vital cells using the trypan-blue dye-exclusion method. Experiments were done with 5- or 6-fold replicates as indicated. The antiproliferative effects of the drugs were related to the growth of controls, and their cytotoxic effects were related to the 10^6 cells initially seeded. Before testing, the viability of cells based on trypan-blue dye exclusion was >90%.

Chemicals. Silicone oils of types AR-20 and AR-200 were obtained from Wacker Chemie (Munich, Germany) and DOX and DNR, from Farmitalia (Freiburg, Germany). MITO was a gift from Lederle Pharmaceuticals (Wolfratshausen, Germany). [14-^{14}C]-doxorubicin hydrochloride (2.04 GBq/mmol, temporarily not commercially available) and [^{14}C(U)]-polyethylene glycol (mol. wt., 4,000 Da) were supplied by Amersham (Buckinghamshire, England). [^{3}H(G)]-DNR (118.4 GBq/mmol), ^3H_2O (37 MBq/g), [carboxyl-^{14}C]-inulin (425 MBq/mmol), and [^{3}H(G)]-inulin (18.5 GBq/mmol) were obtained from Dupont-NEN (Dreieich, Germany). Radiolabeled MITO (5,8-bis-([1-^{14}C]-((2-hydroxyethyl)-amino)-ethyl)-amino))-1,4-dihydroxy-9,10-anthracenedione. 2HCl (4.18 GBq/mmol) was provided by Dr. J. Blanz (Department of Organic Chemistry, University of Tübingen, Germany). DNIG (4R)-3-(3-(4,4-diphenyl-1-piperinidyl)-propyl)-5-methyl-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-pyridine-3,5-dicarboxylatehydrochloride and its main pyridine degradation product in human metabolism, 3-(3-(4,4-diphenyl-1-piperinidyl)-propyl)-5-methyl-2,6-dimethyl-4-(3-nitrophenyl)-pyridine-3,5-dicarboxylate, were supplied by Byk Gulden Pharmaceuticals (Konstanz, Germany). These compounds were dissolved in dimethylsulfoxide (final concentration in medium, 1%). Solutions were handled in glassware due to the high lipophilicity and known adherence of DNIG to plastic materials; DNIG was handled with presaturated pipet tips only. Other compounds were obtained from the usual sources at the highest purity available.

Statistical analysis. Data were analyzed using *t*-test evaluation or analysis of variance according to the recommendations of Tallarida and Murray [27]. The graded dose-response method [27] was used for calculation of potencies for modulators of MDR in the drug-uptake assay. We defined 100% efficacy as the complete reversal of reduced levels of the total DNR content of resistant F4-6RADR cells as compared with the drug-sensitive parental cell line F4-6, tested in parallel without modulators.

Results

Cellular anthracycline content

Resistant F4-6RADR cells maintained low DOX contents by means of a drug-extrusion system (Table 1). Despite the lower DOX loading levels obtained in these cells as compared with the sensitive F4-6 cells, initial anthracycline efflux occurred markedly faster from resistant F4-6RADR cells. This extrusion process in F4-6RADR cells was impaired by DNIG. Under conditions of steady state for intracellular cytostatic drug levels (observed by 30 min incubation; data not shown), DNIG increased the contents of either DOX, DNR, or MITO in F4-6RADR cells to the higher levels measured in the drug-sensitive strain F4-6 (Fig. 1). The concentration-dependent reversal of the DNR accumulation shortage (Fig. 1B) was half-maximal at a DNIG concentration of 0.73 µmol/l (95% confidence interval, 0.48–1.1 µmol/l); the complete reversal of accumula-

Table 1. DOX extrusion from F4-6 and F4-6RADR cells

	DOX (pmol DOX/10^6 cells)			
	F4-6 cells		F4-6RADR cells	
	Control	DNIG	Control	DNIG
DOX loading[a]:				
	18.3 ±1.5	18.1 ±2.9	11.0 ±1.0	17.5 ±.4
DOX release[b]:				
2 min	2.55 ±.04	2.13 ±.40	5.31 ±.17	3.12 ±.03
10 min	3.33 ±.01	2.96 ±.09 (NS)	6.95 ±.19	5.33 ±.11 (*)

[a] A total of 5.5×10^7 cells/ml were loaded in medium containing DOX (6 µmol/l) for 20 min. DNIG 3.3 µmol/l was added 10 min prior to DOX
[b] DOX release from cells into the medium was measured after 100-fold (v/v) dilution of the cell suspension with anthracycline-free medium. Data represent mean values ± SEM ($n = 3$). According to 2-way analysis of variance (*, $P < 0.05$ vs control; NS, not significant), F4-6RADR cells extruded DOX faster than did F4-6 cells and the effects of DNIG were significant in F4-6RADR cells only

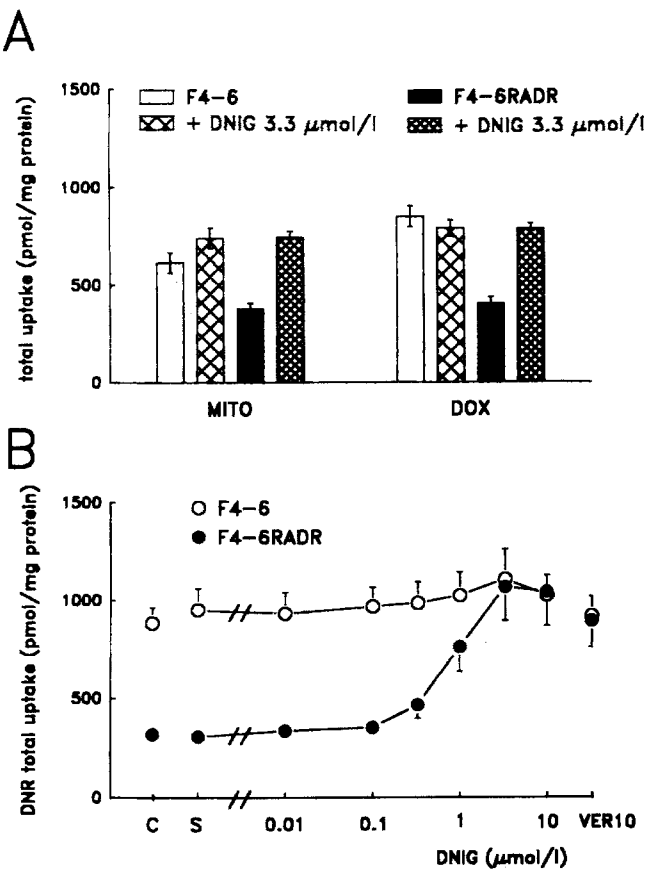

Fig. 1 A, B. Cytostatic drug content in sensitive and resistant cells. **A** DNIG increased the content of MITO or DOX in resistant F4-6RADR cells ($P < 0.001$) without prominently affecting the drug content of sensitive cells ($P > 0.1$). Data represent mean values ± SEM ($n = 6$; unpaired t-test). **B** Concentration-dependent increase induced by DNIG in the DNR content of F4-6RADR cells but not of F4-6 cells. *C*, drug free control; *S*, solvent (DMSO 1%, v/v); *VER10*, R,S-verapamil (10 μmol/l). Data represent mean values ($n = 8$–9); the SEM is included in symbols where not shown

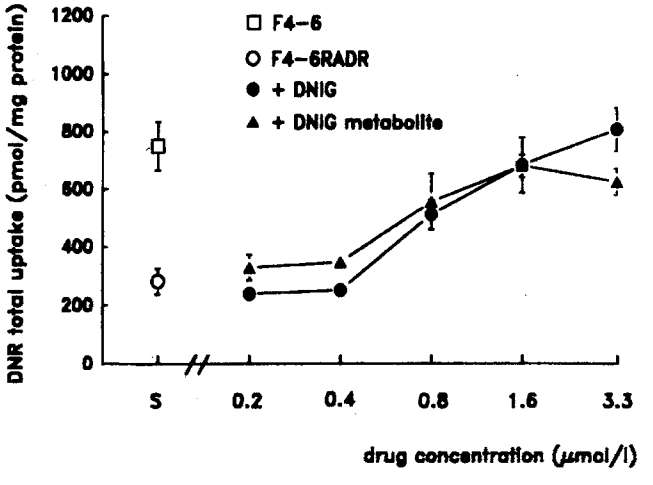

Fig. 2. Comparative assay of the elevating effects of DNIG and its main metabolite on the DNR content of F4-6RADR cells. *S*, DMSO 1% (v/v). Data represent mean values ($n = 4$–6); the SEM is included in symbols where not shown

Table 2. Relative potencies of modulators in the DNR-uptake assay

	100% efficacy in reversing DNR accumulation defects in F4-6RADR cells (μmol/l)	IC_{50} (μmol/l)
Dexniguldipine-HCl (DNIG)	3.3	0.73
R,S-verapamil (VER)	10	5.4
R-nitrendipine	100	51
Cyproheptadine	100	35.6
Cyclosporin A[a]	10	0.5–1
Cremophor EL[a]	330	100

Concentration-response data were obtained in the DNR-accumulation assay in F4-6RADR cells (see Materials and methods). IC_{50} values were graphically estimated from the concentration-response curve. Data were obtained by testing the respective compounds by stepwise increases in concentrations by a factor of 3 in comparison to the drug content in sensitive F4-6 precursor cells in the absence of modulators (based on $n = 6$–9)

[a] These substances increased the DNR content of both F4-6 and F4-6RADR cells [23]; the other compounds listed had no effect on the (high) DNR content of F4-6RADR cells

tion deficits for DNR in F4-6RADR (i.e., 100% efficacy) was found at 3.3 μmol/l. Other experimental MDR modulators evaluated in the DNR-accumulation assay (Table 2) revealed 100% efficacy in reversing DNR accumulation deficits in F4-6RADR (for VER, see Fig. 1B) but differed markedly in potency. DNIG was about as potent as cyclosporin A (CSA) and was 8 times more potent than VER. In clinical studies, about 25% of the applied DNIG was present in the plasma in the form of a pyridine metabolite under conditions of steady state (personal communication, Dr. R. Boer, Byk Gulden Pharmaceuticals, Konstanz, Germany). As assessed in a direct comparison over the steep part of the concentration-response curve for DNIG (0.2–3.3 μmol/l), this DNIG metabolite revealed full efficacy and almost equivalent potency (Fig. 2).

In concentration-dependent responses occurring in parallel, DNIG elevated the cytosolic DNR content (Fig. 3A) and increased the amount of DNR bound to acid-precipitable cellular matter of resistant F4-6RADR cells (DNA, RNA, protein) after thorough washing of the pellet (Fig. 3B). DNIG had no effect on (high) drug accumulation or binding in F4-6 cells (for DNR, see Fig. 3A, B; DOX and MITO not shown). To explore the working hypothesis that improved DNR binding to F4-6RADR would occur secondary to improved anthracycline supply in the cytosol, we challenged cells with varying concentrations of DNR in the absence of DNIG. Resistant F4-6RADR cells followed the same linear function relating DNR binding to DNR supply as did drug-sensitive F4-6 cells when the cytosolic DNR content was modulated by supplying 0.01–10 μmol DNR/l in the incubation medium. As shown in Fig. 3C, DNR binding in F4-6RADR cells responded linearly to cytosolic DNR, regardless of whether this was accomplished due to DNIG-induced effects or with increased extracellular drug concentrations alone. The data indicate that limited intracellular availability of cytosolic DNR is a sufficient cause for poor DNR binding in F4-6RADR cells.

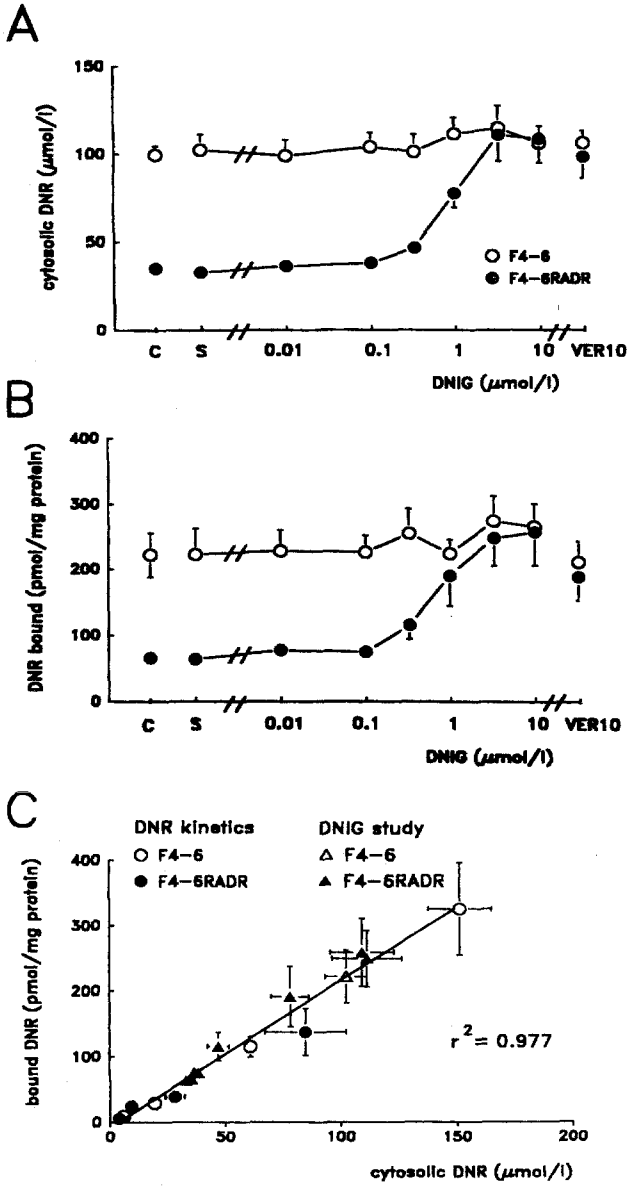

Fig. 3 A–C. Cytosolic and bound fractions of DNR (see Materials and methods). **A, B** Parallel elevation of cytosolic and bound fractions of DNR in response to increasing concentrations of DNIG. *C*, drug-free control; *S*, solvent (DMSO 1%, v/v); *VER10*, R,S-verapamil (10 µmol/l). Data represent mean values (n = 8–9); the SEM is included in symbols where not shown. **C** DNR binding was plotted vs cytosolic DNR content employing various concentrations of DNR in the incubation medium (*DNR kinetics*; 0.01–10 µmol/l, range of 0.1–3.3 µmol/l shown for clarity, n = 9 each) and compiling data from **A** and **B** for the effects of DNIG at 1 µmol DNR/l in the incubation medium (*DNIG study*; F4-6RADR, F4-6 controls, additional data on F4-6 ommitted for clarity). Analysis of 1st-order regression (least-squares method, unweighted means) revealed a common linear function for F4-6 and F4-6RADR cells in the absence of DNIG (r^2 = 0.997) and a good common linear curve fit including F4-6RADR cells showing elevated DNR contents in response to DNIG. Data represent mean values; bars indicate the SEM

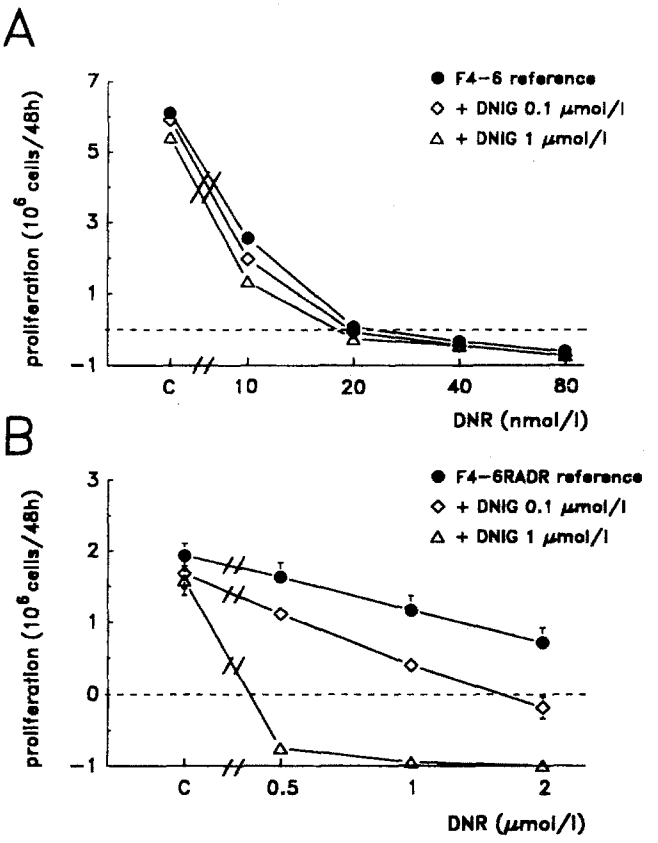

Fig. 4 A, B. Proliferation of **A** sensitive and **B** resistant cells as affected by exposure to DNR alone (*reference*) and in the presence of DNIG. **C**, Drug-free controls or DNIG alone; *dashed line*, zero growth of cells seeded (10^6 cells/15 ml) after 48 h; *negative values* indicate decreased cell counts. In resistant cells (**B**) but not in sensitive ones (**A**), the addition of DNIG had significant effects ($P < 0.05$, unpaired t-test) at any given concentration of DNR. At concentrations of DNIG up to 1 µmol/l, DNIG alone induced no significant growth inhibition (see Results). Data represent mean values (n = 6); the SEM is included in symbols where not shown

Proliferation assays

DNIG sensitized resistant F4-6RADR cells to the cytostatic effects of DNR (Fig. 4B) but had little effect on concentration-dependent cytostatic effects in F4-6 cells (Fig. 4A). At concentrations of up to 1 µmol/l, DNIG revealed slight intrinsic antiproliferative action [9, 24, 29] in F4-6 and F4-6RADR cells, regardless of the status of resistance (cf. Fig. 4 A, B). At 3.3 µmol DNIG/l, cell proliferation was reduced to 38% of control values in F4-6 cells (n = 6; SEM, <10%) or to 5% of control levels in F4-6RADR cells. DNIG at 10 µmol/l was 100% cytotoxic in both cell lines. In correspondence with the rank order of potencies in the DNR-uptake assay (cf. Table 2), at least 10-fold higher concentrations were required for VER to induce effects comparable with those produced by DNIG: DNR at 2 µmol/l + VER at 1 µmol/l resulted in 53% proliferation as compared with drug-free control values (n = 6; SEM, >5% vs DNIG at 0.1 µmol/l: no proliferation and 17% cell death; Fig. 4B). DNR at 2 µmol/l + 10 µmol VER/l caused a 43% loss of viable cells as compared with the initial cell-culture values (DNIG at 1 µmol/l: 100% cell death; Fig. 4).

Discussion

At present, DNIG is one of those compounds [17] emerging from the diverse array of in vitro modulators of pgp170-MDR [1, 30] into clinical trials. DNIG reverses resistance to DOX or etoposide and interacts with rhodamine 123 uptake in vitro [9, 11, 12]. The present work directly relates the DNIG-induced inhibition of the anthracycline-efflux function in an MDR leukemia cell line with the restoration of cytostatic drug access to cells and with their sensitization to anthracycline-induced cytostasis. DNIG elevated the drug content of F4-6RADR cells for DOX, DNR, or MITO and completely reversed the drug accumulation deficits associated with MDR in these cells as compared with the drug-sensitive F4-6 cells but did not affect drug content in the latter.

Although it is now generally accepted that pgp170-MDR is associated with the exclusion of cytostatic drugs from intracellular target sites [1, 30], particulars of the putative pump mechanism remain a matter of debate [1, 3]. Hence, prevailing information on modulator pharmacodynamics must also be considered incomplete [1]. Besides acting as a dihydropyridine calcium-channel blocker of minor clinical efficacy [14], DNIG reportedly interacts with protein kinase C activity, the Ca^{2+}/calmodulin system, and phorbol ester-induced *fos* expression [6, 29]. On the basis of our data, the effect of DNIG as an inhibitor of pgp170 function could be described to some extent on a quantitive basis (Fig. 3). DNIG elevated both cytosolic and bound fractions of DNR in resistant cells in parallel concentration-dependent responses. Likewise, DNR binding could be improved by elevating DNR concentrations in the incubation medium in the absence of DNIG (Fig. 3C). Interestingly, the DNR binding in resistant F4-6RADR cells followed the same kinetic function as that in sensitive F4-6 cells, depending on the DNR supply in the cytosol (Fig. 3C). It may be concluded that DNIG improved DNR access to cellular drug-binding sites by restoring the DNR supply to the cytosol and thus reestablished sensitivity to DNR in F4-6RADR cells. Binding sites and subcellular compartments for DNR [10, 16] as well as putative intracellular DNR redistribution [25] will be subjected to further study.

The DNR-uptake assay proved suitable for evaluating the relative potencies (Table 2) of MDR modulators providing results comparable with those obtained by other methods used for VER [11, 28] or CSA [3, 13]. CSA revealed non-specific effects in the uptake assay, i.e., this drug increased the DNR content of sensitive cells as well [23]; without compensation for these effects, CSA was estimated to be about equipotent to DNIG on a molecular-weight basis. DNIG compared favorably with VER in that the 50% inhibitory concentration (IC_{50}) for DNIG (0.73 μmol/l) was close to those concentrations that appear to be tolerated in the clinical setting (DNIG at >0.7 μmol/l, dose escalation ongoing; personal communication, Dr. F. Rathgeb, Byk Gulden Pharmaceuticals, Konstanz, Germany). The dose-limiting toxicity of VER at concentrations insufficient for MDR modulation, reflected in our data by an IC_{50} value of 5.4 μmol/l in the uptake assay versus the maximally tolerated plasma levels of <1–2 μmol/l, has been well documented [18, 30]. The main DNIG metabolite in human metabolism, a pyridine compound accounting for 25% of the DNIG load under conditions of steady state, was equipotent and equieffective as compared with its parent compound and might thus contribute to the efficacy of a prospective DNIG medication. The antihistaminic cyproheptadine [19], nitrendipine, or cremophor EL (solubilizer in clinical preparations of, e.g., CSA or taxol) were not effective modulators at clinically relevant concentrations.

DNIG sensitized resistant F4-6RADR cells but not F4-6 parent cells to the antiproliferative action of DNR. Proliferation assays using DNIG or VER confirmed the difference in potencies established in the uptake assay. The data illustrate the gradual sensitization of resistant cells to DNR in the presence of DNIG. Less DNIG was required to sensitize F4-6RADR cells to DNR in the proliferation assay as compared with the accumulation studies. This discrepancy might be explained by differences in the experimental intervals (48 vs 1 h) or in the cell-to-medium ratios (cell density, 10^6/ml; proliferation assays, 0.07; uptake assay, 5.5), which are relevant in assays in which highly lipophilic compounds such as DNIG are extracted from the incubation medium by leukemia cells in large quantities (our unpublished results).

A good correlation was shown in the pgp170-MDR cell line F4-6RADR between DNIG-induced sensitization to DNR and improved drug access to intracellular compartments via interference with the anthracycline-export function. This drug-accumulation assay may be a suitable functional test for the direct exploration of pgp170 transport features and may prove to be a useful tool for the analysis of concentration-dependent responses to modulators of pgp170-MDR.

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