Acta Oncologica Vol. 33, No. 7, pp. 787–791, 1994

REVERSAL OF MULTIDRUG RESISTANCE BY NEW DIHYDROPYRIDINES WITH LOW CALCIUM ANTAGONIST ACTIVITY

LAURA CAPOLONGO, NADIA AMBOLDI, DARIO BALLINARI, PAOLO COZZI, GIULIA MELEGARO, MARINA RIPAMONTI, FABRIZIO VAGHI and MARIA GRANDI

The clinical use of Ca^{++} antagonist agents as modulators of multidrug resistance is limited by their strong vasodilator activity. This study reports data obtained by testing a series of new 1,4 dihydropyridine derivatives (DHPs) for their in vitro resistance modulating activity and their Ca^{++} antagonist effect. All the tested DHPs are active to increase doxorubicin activity with dose modifying factor values ranging between 2 and 47 on P388/DX cells and 12 and 36 on LoVo/DX cells. Their resistance modulating action is exerted through an increase of DX intracellular level. The Ca^{++} antagonist activity of DHPs, evaluated as capacity to inhibit the KCl-induced contractions in isolated Guinea pig ileum strips, is not related to their resistance modulating activity. This finding makes it possible to select, for further in vivo evaluations, compounds IX, X and XI, which have strong ability to overcome multidrug resistance and low Ca^{++} antagonist effect.

One approach to overcome multidrug resistance (mdr) in tumor cells is the use of drugs such as anthracyclines or vinca alkaloids combined with resistance modulating agents (rmas) (1, 2). These compounds, which possess different pharmacological activities, generally exert their modulating effects through inhibition of drug efflux from mdr cells mediated by high levels of a membrane glycoprotein (p170). The rmas include Ca^{++} channel blockers such as verapamil (VRP) and dihydropyridine derivatives (DHPs) (3–5); calmodulin inhibitors (6, 7); cyclosporines (8) and others. Among the Ca^{++} channel blockers, compounds which have strong ability to overcome mdr but low Ca^{++} antagonist activity could be of value in cancer chemotherapy since cardiovascular side-effects would be avoided (9). For this purpose, a series of new DHPs have been evaluated in parallel for their ability to increase doxorubicin (DX) activity in mdr cells and to inhibit KCl-induced contraction in isolated Guinea pig ileum strips.

Received 1 October 1993.
Accepted 9 April 1994.
From Pharmacia-Farmitalia Carlo Erba, Research Center R.&D./Business Area Oncology—Oncology Lab., Nerviano, Italy (all authors).
Correspondence to: Dr Laura Capolongo, Pharmacia-Farmitalia Carlo Erba, Research Center R.&D./Business Area Oncology—Oncology Lab., Via Giovanni XXIII No. 23, I-20014 Nerviano (MI), Italy.
Presented at 18th International Congress of Chemotherapy, Stockholm, Sweden, June 27–July 2, 1993.

Material and Methods

Drugs. The DHP compounds were synthesized at the Pharmacia-Farmitalia Carlo Erba R&D Laboratories (10). Elemental analyses of the compounds were performed on a Carlo Erba 1106 Instrument and C, H and N results were within $\pm$ 0.4% of theoretical values. ^{1}H NMR spectra were performed on a Varian VXR-200 instrument and spectral data were consistent with the reported formulas. Their chemical structures are shown in Fig. 1. Compounds I, II, IV, and VI were dissolved in HCl 0.1N; compounds III, V, VIII, IX, X, XI and nifedipine in ethanol, and compound VIII in water. Verapamil (VRP) (Knoll AG, Knoll Farmaceutici, Italy) and doxorubicin (DX) (Farmitalia Carlo Erba) were dissolved in water. All the solutions were prepared immediately before use.

Cells and culture conditions. The wild-type and the DX-resistant murine leukemia cell lines, P388 and P388/DX (11), were maintained in RPMI 1640 (Gibco, Grand

© Scandinavian University Press 1994. ISSN 0284-186X

DHPs	Phenyl substitution	R_1	R_2	R_3	R_4	R_5
I	3	Et	OEt	Me	Me	H
II	3	Me	OEt	Me	Me	H
III	2	Et	OEt	Me	Me	H
IV	3	Et	OEt	Et	Et	H
V	3	Et	OEt	Me	Me	$(CH_2)_2$-N⟨O⟩
VI	3	Me	OEt	Me	CH_2-O-Me	H
VII	3	Me	OEt	Me	CH_2-O-$(CH_2)_2$-NH_2	H
VIII	3	Me	Me	Me	Me	H
IX	3	Et	OEt	Me	Me	Me
X	4	Et	OEt	Me	Me	H
XI	4	Et	OEt	Me	Me	Me

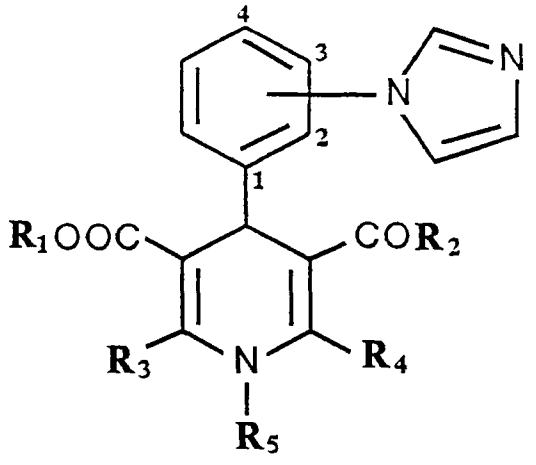

Fig. 1. Chemical structure of the tested DHPs.

Island Biological Co, Grand Island, NY) supplemented with 10% foetal calf serum (Flow laboratories, U.K.), 1% L-glutamine 200 mM (Gibco) and 2% β-mercapto-ethanol solution (1 mM). The wild-type and the DX-resistant human colon adenocarcinoma cell lines, LoVo and LoVo/DX (12), were maintained in Ham's F12 (Gibco) supplemented with 10% foetal calf serum, 1% vitamins (vitamins BME solution 100x, Gibco) and 1% L-glutamine 200 mM.

Cytotoxicity assay. P388 and P388/DX cells (50 000 cells/ml; 1 ml/well) were treated with graded concentrations of DX in the absence or in the presence of DHPs or VRP at 37°C. After 48 h incubation, the number of cells was determined with a Coulter counter (Kontron mod. ZM). The concentration of DX inhibiting cell growth by 50% (IC_{50}) was determined from concentration-response curves. LoVo and LoVo/DX cells (200 cells/ml; 2 ml/well) were seeded 48 h before treatment. The cells were treated with graded concentrations of DX in the absence or presence of DHPs or VRP for 4 h. Medium was replaced and colonies were counted after 8 days using an optical microscope. The concentration of DX inhibiting colony formation by 50% (IC_{50}) was determined. Results are reported as dose modifying factor (DMF):

$$DMF = \frac{IC_{50}\ DX}{IC_{50}\ DX + rmas}$$

Treatment with rmas was carried out at concentrations which caused $< 20\%$ inhibition of cell growth (see Table 1).

Ca^{++} antagonist activity. The Guinea pig terminal ileum was immediately removed, washed and mounted in a 20 ml organ bath containing Tyrode's solutions (composition in mM: NaCl 136.8, KCl 2.68, $CaCl_2$ 1.8, NaH_2PO_4 0.41, $NaHCO_3$ 11.9, $MgCl_2$ 1.03, glucose 5.55) gassed with 95% O_2 and 5% CO_2 and thermoregulated at 37°C. The tissue was loaded with 1 g and contractions were recorded via a Basile isometric transducer on a Watanabe Mark V recorder. Contractions in response to 60 mM KCl were obtained at 15 min intervals in the absence and presence of increasing concentrations of the compounds. The antagonist potency was expressed as IC_{50} values (the concentration of antagonist which inhibits KCl response by 50%) by means of a computerized regression analysis.

Intracellular content. LoVo and LoVo/DX cells were seeded into 6 well plates (2.5×10^5/ml; 2 ml/wells) in complete growth medium. After 24 h incubation cells were treated with DHPs, VRP and nifedipine at the doses reported in Results and after 5 min with DX 2.5 μg/ml. After 4 h incubation, cells were washed twice with saline, detached with trypsin-EDTA (0.05%–0.02% respectively) and centrifuged 10 min (2 000 rpm). The number of cells was evaluated with a Coulter counter (Kontron, Mod. ZM) and the pellet was processed for evaluating the content of DX.

Kinetics of uptake and efflux. LoVo and LoVo/DX cells were seeded into 6 well plates (2.5×10^5/ml; 2 ml/wells) in complete growth medium. After 24 h incubation cells were treated with 20 μg/ml of DHPs or VRP and after 5 min with DX 2.5 μg.ml. At different times, cells were collected and processed to assess the DX content. For evaluating the effect on the efflux, the medium was replaced with drug-free medium with or without the modulating agent; after 1 h the cells were processed as described.

Drug extraction and HPLC analysis. To the cell pellets 250 μl of HCl (0.6 N) : EtoH 1:1 were added and samples vigorously shaken for 20 min at 4°C. Then samples were spun at 7 000 rpm for 10 min and 20 μl of the supernatants injected into HPLC. HPLC analysis was performed using the following instruments and conditions:

Column: C18 μBondapak (Waters)
Precolumn: C18 Guardpak (Waters)
Pump: SP 8810 (Spectra-Physics)—1.5 ml/min
Mobile phase: KH_2PO_4 (0.05 M)—CH_3CN 70:30, pH 3
Detectors: Spectrofluorimeter 650/40 (Perkin-Elmer)
Excitation wavelength 478 nm
Emission wavelength 593 nm

Table 1

Potentiation of DX activity on P388/DX and LoVo/DX cells by DHPs, nifedipine and verapamil

Compounds	P388/DX				LoVo/DX			
	Conc.[1] ng/ml	Growth[2] ±S.E. %	IC50[3] ±S.E. ng/ml	DMF[4]	Conc.[1] ng/ml	Growth[2] ±S.E. %	IC50[3] ±S.E. ng/ml	DMF[4]
DX alone	—	—	518 ± 39	—	—	—	5357 ± 602	—
I	5000	87 ± 5	17 ± 2	30	20000	100 ± 7	320 ± 130	17
II	5000	89 ± 6	30 ± 10	17	50000	100 ± 5	210 ± 40	26
III	n.d.	n.d.	n.d.	n.d.	20000	88 ± 15	190 ± 27	28
IV	5000	81 ± 6	18 ± 2	29	10000	85 ± 6	155 ± 3	35
V	10000	86 ± 6	15 ± 1	35	50000	88 ± 4	150 ± 25	36
VI	5000	93 ± 2	22 ± 5	24	20000	85 ± 5	210 ± 85	26
VIII	10000	86 ± 0	57 ± 23	9	100000	107 ± 4	440 ± 96	12
VIII	2500	91 ± 0	291 ± 49	2	n.d.	n.d.	n.d.	n.d.
IX	5000	82 ± 4	18 ± 2	29	20000	91 ± 9	200 ± 10	27
X	5000	92 ± 1	11 ± 2	47	20000	100 ± 2	195 ± 30	27
XI	5000	92 ± 4	24 ± 4	22	20000	91 ± 3	213 ± 13	25
Nifed.	2500	91 ± 6	423 ± 23	1	10000	103 ± 4	6100 ± 1103	1
Verap.	2500	92 ± 6	25 ± 11	21	50000	85 ± 3	183 ± 53	29
					20000	89 ± 1	318 ± 33	17

[1] Concentrations of DHPs, nifedipine or verapamil.
[2] Percentage of survival ± S.E. after treatment with DHPs, nifedipine or verapamil at the reported concentrations.
[3] IC50 ng/ml: concentrations inhibiting 50% of cell growth or colony formation ± S.E. after treatment with DX alone or DX plus DHPs, nifedipine or verapamil.
[4] DMF: Dose modifying factor: IC50 DX alone/IC50 DX plus DHPs, nifedipine or verapamil.

Autosampler: 232 (Gilson)
Integrator: SP 4270 (Spectra-Physics)
Results are reported as ng of DX/10^6 cells.

In the experimental conditions the % recovery of DX is 99% and the intracellular accumulation in LoVo/DX cells is linear in the range of 0.312–5.0 μg/ml ($r^2 = 0.922$).

Results

Potentiation of DX activity on mdr cell lines and Ca^{++} antagonist effect of DHPs. The structures of the DHPs employed are shown in Fig. 1. Table 1 reports the cytotoxic activity of DX on the mdr cell lines P388/DX and LoVo/DX in the absence and in the presence of concentrations of the tested compounds which caused < 20% inhibition of cell growth. Results are expressed as IC_{50} (DX concentration inhibiting cell growth or colony formation by 50%) and DMF (dose modifying factor i.e. the ratio between the IC_{50} of DX alone and the IC_{50} of DX in combination with rmas). All DHPs were active in increasing DX cytotoxic activity with DMF values ranging between 2 and 47 on P388/DX cells and between 12 and 36 on LoVo/DX cells. Table 2 shows the Ca^{++} antagonist activity of the DHPs expressed as IC_{50} (dose inhibiting KCl-induced contractions by 50%). As can be seen, the Ca^{++} antagonist effect is not related to the resistance modulating activity. Nifedipine and VRP were employed as reference compounds. The DMF of nifedipine was 1 on both cell lines, that of VRP 21 on P388/DX and 29 on LoVo/DX cells (Table 1). Both compounds show high Ca^{++} antagonist activity ($IC_{50} = 2$ ng/ml for nifedipine and 14 ng/ml for VRP) (Table 2). According to these results, compounds IX, X and XI, which are active in modulating resistance and present low Ca^{++} antagonist

Table 2

Ca^{++} antagonist effect of DHPs, nifedipine and verapamil

Compounds	Ca^{++} antagonist[1] effect IC_{50} = ng/ml
I	25 ± 5
II	103 ± 14
III	186 ± 11
IV	415 ± 26
V	457 ± 41
VI	782 ± 91
VII	1614 ± 311
VIII	> 3514
IX	3604 ± 68
X	9096 ± 790
XI	40950
Nifedipine	2 ± 0.13
Verapamil	14 ± 1

[1] concentration inhibiting 60 mM KCl contraction by 50% in Guinea pig ileum strips ± S.E.

Table 3

DX intracellular levels into LoVo/DX cells with graded concentrations of DHPs, verapamil and nifedipine

Conc.[1] μg/ml	DX intracellular content (ng/10^6 cells ± SE)[2]				
	DX + compound IX	DX + compound X	DX + compound XI	DX + verapamil	DX + nifedipine
0	12.32 ± 2.02	12.32 ± 2.02	12.32 ± 2.02	12.32 ± 2.02	12.32 ± 2.02
2.5	14.97 ± 0.03	52.65 ± 3.82	124.54 ± 0.81	45.56 ± 0.81	n.d.
5	115.63 ± 7.42	98.88 ± 2.39	169.57 ± 3.87	60.12 ± 3.73	n.d.
10	174.89 ± 6.06	154.43 ± 5.79	221.65 ± 1.84	106.00 ± 13.34	n.d.
20	254.92 ± 1.01	230.75 ± 10.73	283.12 ± 2.87	163.43 ± 4.96	12.90 ± 1.25

[1] DHPs, verapamil and nifedipine concentrations.
[2] Intracellular content of DX into LoVo/DX cells after treatment with 2.5 μg/ml DX alone or in association with the compounds.

activity meet the positivity criteria set in our study, and they have therefore been selected for further studies. The methyl group in position R5 (compounds IX and XI) and the imidazolyl moiety in position 4 of the phenyl ring (compounds X and XI) (Fig. 1) have already been reported to decrease the Ca^{++} antagonist effect (13, 14).

Doxorubicin intracellular level into LoVo/DX cells. Table 3 presents the intracellular levels of DX in LoVo/DX cells after 4 h of treatment with 2.5 μg/ml of DX in the absence and in the presence of graded concentrations of compounds IX, X, XI and VRP (from 2.5 to 20 μg/ml) and of 20 μg/ml of nifedipine. A dose related increase of DX intracellular levels was observed with the tested DHPs and with VRP. The combined treatment of DX plus 20 μg/ml of these compounds caused an increase of DX intracellular level of at least 13-fold in respect to the treatment with DX alone. No effect on DX content was observed in cells treated with nifedipine, even at 20 μg/ml. Fig. 2 presents the kinetics of DX accumulation and efflux in LoVo/DX cells at different times after treatment with 2.5 μg/ml DX alone or plus 20 μg/ml of compounds IX, X, XI and VRP. DX levels were similar at all tested times with the three DHPs and slightly lower with VRP. DX efflux 1 h after withdrawal of the drug from the culture medium was fast when the rmas were absent (dotted line) and significantly slower when the rmas were present (continuous lines) in the medium. DHPs, verapamil and nifedipine were not significantly effective in potentiating the cytotoxicity and the intracellular accumulation of DX for the parent lines P388 and LoVo (data not shown).

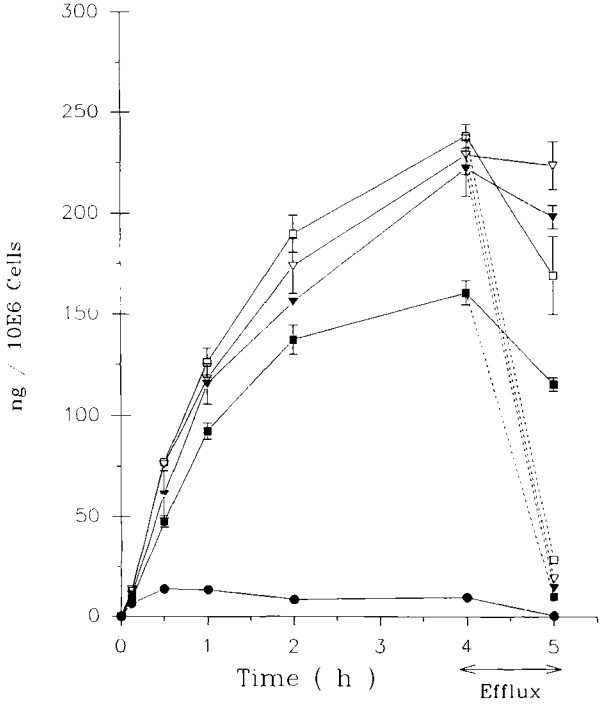

Fig. 2. Kinetics of DX accumulation and efflux in LoVo/DX cells at different times after treatment with 2.5 μg/ml DX alone or plus 20 μg/ml of compounds IX, X, XI and VRP. Bars: ± standard error of the mean. ● DX alone; ■ DX plus VRP; ▼ DX plus compound IX; ▽ DX plus compound X; □ DX plus compound XI.

Discussion

The present paper reports results obtained during a program aimed at identifying DHPs able to modulate mdr and devoid of Ca^{++} antagonist activity. As already reported for other agents of the same pharmacological class, the Ca^{++} antagonist effect of the tested DHPs is not related with their resistance modulating activity (4, 7). This finding made it possible to select compounds IX, X and XI which have shown strong ability to overcome mdr and, like other DHPs (3, 4, 15, 16), a significantly lower Ca^{++} antagonist effect in respect to nifedipine and VRP. The increase of DX activity after treatment in combination with DHPs is exerted, as for VRP, through an increase of DX intracellular level. This effect is presumably due to an interaction with P170 as reported for VRP and other

DHPs (15, 17, 18). Nifedipine, which failed to increase DX intracellular level, is not active to improve DX cytotoxic effect. As reported in the results, the DX intracellular level in LoVo/DX cells is DHPs dose-dependent and strictly related to the presence of DHPs in cell medium.

This observation suggests the need to reach and maintain high plasma levels in animals and in patients. Compounds IX, X and XI, showing similar modulating activity but significantly lower (at least 250-fold) Ca^{++} antagonist effect vs VRP, could meet this requirement and be used as active chemosensitizer agents without severe side-effects related to Ca^{++} antagonist effect.

REFERENCES

1. Gottesman MM. How cancer cells evade chemotherapy: sixteenth Richard and Hinda Rosenthal Foundation Award Lecture. Cancer Res 1993; 53: 747–54.
2. Stewart DJ, Evans WK. Non-chemotherapeutic agents that potentiate chemotherapy efficacy. Cancer Treat Rev 1989; 16: 1–40.
3. Kiue A, Sano T, Suzuki K, et al. Activities of newly synthesized dihydropyridines in overcoming of vincristine resistance, calcium antagonism, and inhibition of photoaffinity labeling of P-glycoprotein in rodents. Cancer Res 1990; 50: 310–7.
4. Yoshinari T, Iwasawa Y, Miura K, et al. Reversal of multidrug resistance by new dihydropyridines with lower calcium antagonistic activity. Cancer Chemother Pharmacol 1989; 24: 367–70.
5. Watanabe Y, Takano H, Kiue A, Kohno K, Kuwano M. Potentiation of etoposide and vincristine by two synthetic 1,4-dihydropyridine derivatives in multidrug-resistant and atypical multidrug-resistant human cancer cells. Anticancer Drug Des 1991; 6: 47–57.
6. Tsuruo T, Iida H, Tsukagoshi S, Sakurai Y. Potentiation of vincristine and adriamycin effects in human hemopoietic tumor cell lines by calcium antagonists and calmodulin inhibitors. Cancer Res 1983; 43: 2267–72.
7. Tsuruo T, Iida H, Tsukagoshi S., Sakurai Y. Increased accumulation of vincristine and adriamycin in drug-resistant P388 tumor cells following incubation with calcium antagonists and calmodulin inhibitors. Cancer Res 1982; 42: 4730–3.
8. Keller RP, Altermatt HJ, Nooter K, et al. SDZ PSC 833, a non-immunosuppressive cyclosporine: its potency in overcoming P-glycoprotein-mediated multidrug resistance of murine leukemia. Int J Cancer 1992; 50: 593–7.
9. Cairo MS, Siegel S, Anas N, Sender L. Clinical trial of continuous infusion verapamil, bolus vinblastine, and continuous infusion VP-16 in drug-resistant pediatric tumors. Cancer Res 1989; 49: 1063 6.
10. Cozzi P, Meninchincheri M, Capolongo L, Mongelli N. European Patent Appl. 0533504 A1 1992.
11. Johnson RK, Chitnis MP, Embrey WM, Gregory EB. In vivo characteristics of resistance and cross-resistance of an adriamycin-resistant subline of P388 leukemia. Cancer Treat Rep 1978; 62: 1535–47.
12. Grandi M, Geroni C, Giuliani FC. Isolation and characterization of a human colon adenocarcinoma cell line resistant to doxorubicin. Short communication. Br J Cancer 1986; 54: 515–8.
13. Triggle DJ, Langs DA, Janis RA. Calcium channel ligands: structure-function relationship of the 1,4 dihydropyridines. Med Res Rev 1989; 9: 123–80.
14. Cozzi P, Carganico G, Fusar D, et al. Imidazol-1-yl and pyridin-3-yl derivatives of 4-phenyl-1,4-dihydropyridines combining Ca^{++} antagonism and thromboxane A_2 synthase inhibition. J Med Chem 1993; 36: 2964–72.
15. Niwa K, Yamada K, Furukawa T, et al. Effect of a dihydropyridine analogue, 2-[benzyl (phenyl)amino]ethyl 1,4-dihydro-2,6-dimethyl-5-(5,5-dimethyl-2-oxo-1,3, 2-dioxaphosphorinan-2-yl)-1-(2-morpholino-ethyl)-4-(3-nitrophenyl)-3-pyridinecarboxylate on reversing in vivo resistance of tumor cells to adriamycin. Cancer Res 1992; 52: 3655–60.
16. Shinoda H, Inaba M, Tsuruo T. In vivo circumvention of vincristine resistance in mice with P388 leukemia using a novel compound, AHC-52. Cancer Res 1989; 49: 1722–6.
17. Kiue A, Sano T, Naito A, et al. Reversal by two dihydropyridine compounds of resistance to multiple anticancer agents in mouse P388 leukemia in vivo and in vitro. Jpn J Cancer Res 1990; 1: 1057 64.
18. Nogae I, Kohno K, Kikuchi J, et al. Analysis of structural features of dihydropyridine analogs needed to reverse multidrug resistance and to inhibit photoaffinity labeling of P-glycoprotein. Biochem Pharmac 1989; 38: 519–27.