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The antitumor efficacy of cytotoxic drugs is potentiated by treatment with PNU 145156E, a growth-factor-complexing molecule

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Abstract PNU 145156E (formerly FCE 26644) is a noncytotoxic molecule whose antitumor activity is exerted through the formation of a reversible complex with growth/angiogenic factors, thus inhibiting their induction of angiogenesis. We studied in vitro and in vivo the activity of PNU145156E in combination with the four cytotoxic drugs doxorubicin, cyclophosphamide, methoxymorpholinyldoxorubicin (MMDX, FCE 23762, PNU152243), and 9-aminocamptothecin against M5076 murine reticulosarcoma. In vitro, PNU 145156E did not modify the cytotoxicity of the four drugs or the cell-cycle block induced by doxorubicin. In vivo, at the optimal dose of each compound, the antitumor activity was significantly increased in all combinations, with no associated increase in general toxicity being observed. In healthy mice treated with cyclophosphamide or doxorubicin the association with PNU 145156E did not enhance the myelotoxic effect induced by the two cytotoxics. These results indicate that two drugs affecting solid tumor growth through two different mechanisms – growth factor blockage and cell proliferation – can be combined, resulting in increased antitumor efficacy with no additive toxicity.

Key words Growth factors · Angiogenesis · Suramin

Abbreviations *DX* Doxorubicin · *9-AC* 9-Aminocamptothecin · *CTX* Cyclophosphamide · *4-HC* 4-Hydroperoxycyclophosphamide · *MMDX* (FCE 23762, PNU 152243; methoxymorpholinyldoxorubicin) · *DMSO* Dimethylsulfoxide · *bFGF* Basic fibroblast growth factor

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Introduction

Blood vessel formation occurs when angiogenic factors [16] produced by tumor cells stimulate quiescent endothelial cells to proliferate, destroy the basal membrane, migrate, adhere, and proliferate to form new capillaries. The search for compounds capable of modulating angiogenesis-related pathologies, with a special focus on tumor angiogenesis, is very active and has given rise to several classes of compounds that act at different levels in the process of new vessel formation [6, 11]. Although no information pertaining to a definitive clinical response is presently available with regard to the activity of the angiogenesis inhibitors undergoing clinical trials, experimental evidence indicates that modulation of blood vessel growth alone may be insufficient for the efficient control of tumor progression. It is thus conceivable that combined therapy with nontoxic angiostatic drugs and cytotoxic antitumor drugs might represent a new effective clinical regimen [6, 14]. One successful approach to the inhibition of angiogenesis is the blockage of angiogenic factors and inhibition of their binding to the receptors; tumor growth suppression has been obtained with antibodies neutralizing vascular endothelial growth factor (VEGF) [1, 8] or basic fibroblast growth factor (bFGF) [7] as well as with molecules complexing bFGF, such as pentosan sulfate [17] and suramin [9].

PNU145156E, 7,7' -(carbonyl-bis[imino-*N*-methyl-4, 2 pyrrole carbonyl-imino {*N*-methyl-4, 2-pyrrole} carbonylimino])-bis-(1,3-naphthalene)disulfonic acid, is a noncytotoxic molecule that is active in inhibiting the binding of bFGF, platelet-derived growth factor (PDGFβ), interleukin-1 (IL-1), and VEGF to their receptors [2] (M.Ciomei, personal communication). It also inhibits receptor activation and cell motility induced by

hepatocyte growth factor (HGF) [3] and insulin growth factor-1 (IGF-1)-dependent proliferation of human lung cancer cells [5]. The formation of a reversible complex of PNU 145156E with bFGF with a dissociation constant in the range of 10^{-6}–10^{-7} has been documented [19]. The compound blocks neovascularization in chicken chorioallantoic membrane and, when given i.v. or i.p., inhibits angiogenesis induced by bFGF-containing collagen sponges and the growth of a series of solid tumor models [2, 12]; it is currently undergoing, phase I clinical trial.

This paper presents results obtained in tests of the effect of combined treatment with PNU145156E and the four cytotoxic agents doxorubicin (DX), cyclophosphamide (CTX), methoxymorpholinyl-DX (MMDX, FCE 23762, PNU 152243), and 9-aminocamptothecin (9-AC). These drugs were selected as representatives of compounds that are active in inhibiting cell proliferation via the following different modes of action: DX is a DNA-intercalating agent and a topoisomerase II inhibitor, MMDX is a DX derivative that is active against DX-resistant tumors [10], CTX is an alkylating agent, and 9-AC is a topoisomerase I inhibitor [4]. Both MMDX and 9-AC are Phase1-2 clinical trials. The combinations were assayed in vitro on M5076 murine reticulosarcoma cells and in vivo on the same tumor cells implanted i.m.; their effect on the cell cycle was evaluated on M5076 cells in combination with DX, and their myelotoxicity was evaluated in healthy mice in combination with DX and CTX.

Materials and methods

Drugs

PNU 145156E (FCE 26644), 9-AC, MMDX (FCE 23762; PNU 152243), and DX were synthesized in the Chemical Laboratories of Pharmacia-Upjohn (Milan, Italy). Cyclophosphamide (CTX) was a pharmaceutical preparation (Endoxan-Asta), and 4-hydroperoxycyclophosphamide (4-HC), the in-vitro-active form of CTX, was obtained from Sigma Chemical Co. (St.Louis, Mo.).

PNU 145156E, DX, CTX, and 4-HC were weighed and dissolved in distilled water immediately prior to use. DX and MMDX concentrations were verified spectrophotometrically at $\lambda = 496$ nm, ($E1\% = 200$ in water) and $\lambda = 495$ nm, ($E1\% = 173$ in water), respectively. 9-AC was formulated for in vivo in studies as a colloidal dispersion (CD) at 1 mg/vial containing dimyristoylphosphatidylcholine (56 mg/vial), dimyristoylphosphatidylglycerol (24 mg/vial), and mannitol (100 mg/vial) and was reconstituted in 20% dextrose/0.9% sodium chloride. 9AC was dissolved in DMSO for in vitro studies.

Cells

M5076 murine reticulosarcoma cells [13] were grown as a suspension in a solution of RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (Biological Industries), 1% 200m*M* glutamine (Gibco), and 1% 1 m*M* β-mercaptoethanol (Sigma).

In vitro activity

M5076 cells were seeded at 50 000 cells/ml and treated for 72 h with graded concentrations of the tested compounds alone or in association with PNU 145156E (50 μg/ml). At the end of treatment the in vitro activity was determined by counting of surviving cells with a Coulter Counter (Kontron Mod. ZM). Results are reported as percentages of surviving cells in treated versus control cultures. The experiments were repeated at least twice.

Assessment of cell-cycle distribution

Cells were treated for 72 h, harvested, and fixed in 70% ethanol. Ethanol-fixed cells were stained for DNA analysis with propidium iodide (PI) at 50 μg/ml following treatment with RNAse at 10 μg/ml. Flow-cytometry analysis was performed using a Facstar plus, tuning of the excitation laser at 488 nm, and measurement of PI fluorescence above 620 nm. The percentages of cells in the G_1, S, and G_2-M phases of the cell cycle were calculated using a software program developed at the Mario Negri Institute [15].

Animals

First-generation hybrid C57BL/6 × DBA/2 (B6D2F1) female mice ages 6–8 weeks and supplied by Charles River (Calco, Como, Italy) were used. Animal health was monitored every 4–6 weeks by serological testing. The animals were free of infectious pathogens, including mouse hepatitis virus, Sendai virus, and *Mycoplasma pulmonis*. Animals were housed in plastic cages under temperature- and humidity-controlled conditions. Food and water were available ad libitum, and a 12-h light/dark schedule was maintained. The body weight at the start of the experiments ranged between 20 and 23 g. The care of animals was undertaken in accordance with international guidelines [18].

Tumor model

The murine M5076 reticulosarcoma tumor [13] was maintained by serial i.m. passage and was transplanted i.m. (5×10^5 cells/mouse) into compatible C57BL/6 mice.

Antitumor activity

Primary tumor growth was assessed twice weekly. The two diameters were measured by digital calipers (MAX-CAL, Cole-Palmer International, USA). Output data were elaborated using a software program developed in house. Tumor weight was estimated according to the formula $d^2 \times D/2$, where d and D are the smaller and larger diameters, respectively.

The AUC (area under the curve) of the tumor growth was calculated using the trapezoidal method and refers to the measurement of tumor growth performed 1 week after the last treatment. The percentage of inhibition (% AUC) was calculated using the following formula:

$$100 = \frac{\text{AUC tumor growth treated mice}}{\text{AUC tumor growth control mice}} \times 100.$$

Mice were monitored according to guidelines and were killed when the tumor weight exceeded 10% of their body weight. Tumor growth delay (TGD) is the time in days required to reach 1 g of tumor weight in treated animals as compared with control animals. The number of long-term survivors (LTS) refers to mice surviving for >120 days after tumor implantation. Groups of nine or ten mice were used.

Toxicity

For PNU 145156E the toxicology evaluation was performed in terms of spleen or liver enlargement and renal discoloration, and for cytotoxic drugs it was carried out in terms of body weight loss and spleen and liver reduction.

Hematology

Mice were treated with PNU 145156E, DX, and CTX alone and in combination; blood samples were drawn from all mice on day 13, since this was the average calculated for the DX and CTX nadir. Blood was collected from the orbital plexus through a glass Pasteur pipette and was distributed into tubes containing 8% ethylenediaminetetraacetic acid (EDTA) for hematology determinations. Samples were subjected to routine hematology analysis using a Technicon H1E (Bayer Diagnostic).

Statistical analysis

Evaluation of statistical significance was performed using Student's test for cytotoxic activity and Dunnett's test for hematology results. The potentiation of the tumor weight reduction achieved with combined therapy in comparison with the reduction exerted by single effects of the drugs was evaluated using the Tukey test. The criterion for statistical significance was the 0.01 level.

Results

Under the experimental conditions employed for in vitro growth-inhibition studies, that is, 72 h of treatment and counting of cells, PNU 145156E at concentrations of 50 and 100 μg/ml caused only a 10–15% and a 50% reduction in cell numbers versus control values, respectively; this effect was promptly reverted after seeding of cells in drug-free medium (data not shown). Combination studies were performed at a fixed PNU 145156E concentration of 50 μg/ml and at graded concentrations of DX (5–10–20–40 ng/ml), 4-HC (6.25–100–400 ng/ml), 9-AC (2.5–10–40 ng/ml), and MMDX (2.4–10–40 ng/ml).

The results reported in Fig. 1 indicate that the cytotoxicity of DX (Fig. 1A), 4HC (Fig. 1B), 9-AC (Fig. 1C), and MMDX (Fig. 1D) remained unchanged following their combination with PNU 145156E. The combination of PNU 145156E at 50 μg/ml with DX (5–10–20 ng/ml) on M5076 cells after 72 h treatment was also evaluated in terms of its effect on cell-cycle perturbation. The results reported in Fig. 2 indicate that DX alone induced a dose-dependent reduction in the percentage of cells in the S phase, paralleled with an increase in the percentage of cells in the G_2-M phase. This effect was not modified when DX was applied in combination with PNU 145156E, which alone does not affect cell-cycle distribution.

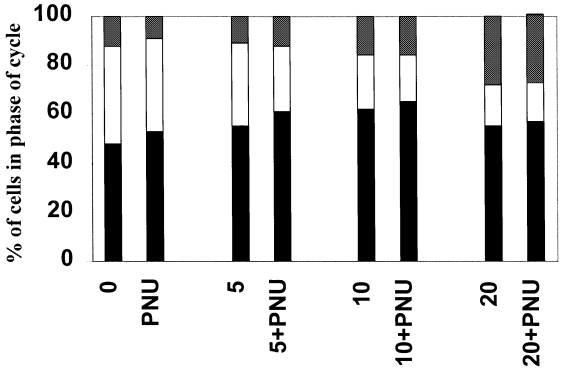

Fig. 2 Cell-cycle distribution of M5076 cells exposed to PNU 145156E at 50 μg/ml and to graded concentrations of DX (*X-axis* DX concentration in ng/ml, *Y-axis* % cell cycle, *white bars* S phase, *black bars*, G_1 phase, *gray bars* G_2-M phase.

Fig. 1A–D Growth inhibition on M5076 cells after 72 h of exposure to PNU 145156E at 50 μg/ml and to graded concentrations of ***A*** DX, ***B*** 4-HC, ***C*** 9-AC, and ***D*** MMDX (*X-axis* Cytoxics concentrations in ng/ml *Y-axis* % cell growth, *white bars* cytotoxics alone, *gray bars* PNU145156E alone, *black bars* cytotoxics plus PNU145156E)

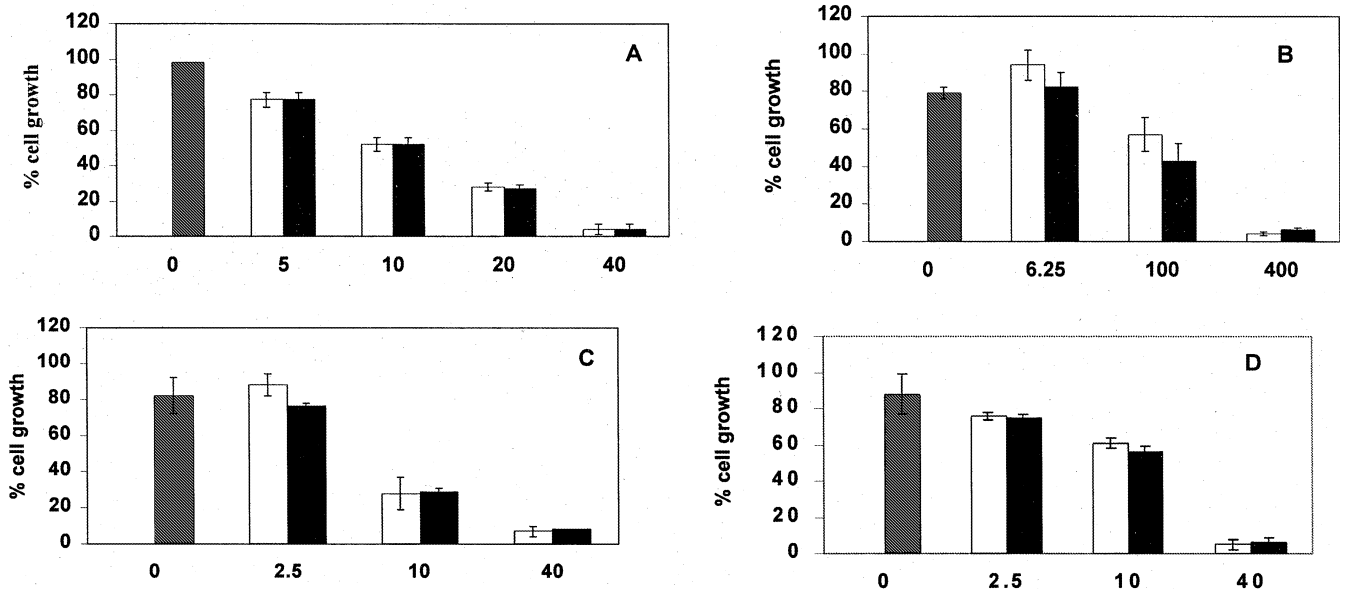

Table 1 Efficacy of PNU 145156E and DX combined treatment

Treatment group[a]	Tumor weight (g)[b]	A.U.C. % inhib.[c]	TGD[d]	Toxic/total mice
DX 4.5 mg/kg	2.34 ± 0.9	77	8	0/10
DX 6 mg/kg	1.3 ± 0.7	93	9	0/10
PNU145156E 100 mg/kg	1.45 ± 0.7	88	9	0/10
PNU 145156E + DX 4.5 mg/kg	0.07 ± 0.005*	100	18	0/10
PNU 145156E + DX 6 mg/kg	0.01 ± 0.001*	100	23	0/10

* $P < 0.01$
[a] PNU 145156E was given i.p. at 2 h prior to i.v. DX administration; treatment was performed on days 1,4,7, and 11
[b] Tumor weight measured at day 29
[c] Inhibition of solid tumor growth
[d] Difference in the number of days required to reach 1 g relative to controls (14 days)

Table 2 Efficacy of PNU 145156E and CTX combined treatment

Treatment group[a]	Tumor weight (g)[b]	A.U.C. % inhib.[c]	TGD[d]	LTS[e]	Toxic/total mice
CTX 90 mg/kg	0.01 ± 0.001	100	25	0/10	0/10
PNU 145156E 100 mg/kg	2.63 ± 0.8	85	10	0/10	0/10
PNU 145156E + CTX	0 ± 0*	100	90	5/11*	0/11

* $P < 0.01$
[a] PNU145156E was given i.p. at 2 h prior to i.v. CTX administration; treatment was performed on days 1,4,7, and 11
[b] Tumor weight measured at day 46
[c] Inhibition of solid tumor growth
[d] Difference in the number of days required to reach 1 g relative to controls (13 days)
[e] Mice surviving at day 120 from after tumor implantation

Antitumor efficacy in combination with cytotoxic drugs

All experiments involved the i.p. administration of PNU145156E at a dose of 100 mg/kg (the most effective dose) at 2 h prior to i.v. administration of the cytotoxics at their maximal doses and one or more lower doses; mice were treated on days 1, 4, 7, and 11 following tumor implantation. This schedule was selected as the optimal one from a previous scheduling study performed on the same tumor model. The parameters of efficacy were tumor weight, % AUC inhibition at 1 week after the last treatment, tumor growth delay (TGD), and the presence of long-term survivors (LTS).

Antitumor efficacy in combination with DX

DX given individually at 4.5 and 6 mg/kg as a single agent after PNU 145156E was associated with a % AUC inhibition of 77%, 93%, and 88% and a TGD of 8, 9, and 9 days, respectively; the combined treatment resulted in a 100% AUC inhibition of tumor growth and an increase in TGD (18–23 days; Table 1). On the combination of PNU145156E with both DX doses, a significant reduction in tumor weight was observed.

Antitumor efficacy in combination with CTX

CTX was highly active when given alone at 90 mg/kg as expressed by a % AUC inhibition of 100% and a TGD of 25 days; combined treatment with PNU 151484E was associated with a % AUC inhibition of 85% and a TGD of 10 days (Table 2). The combination resulted in a significant reduction in tumor weight and an increase in TGD (> 90 days) associated with 5/11 LTS.

Antitumor efficacy in combination with MMDX

Treatment with MMDX at 0.05 mg/kg and that with PNU 145156E was associated with a % AUC inhibition of 90% and a TGD of 12 and 16 days, respectively (Table 3). The combination resulted in a significant reduction in tumor weight and an increase in TGD (28 days).

Antitumor efficacy in combination with 9-AC

At the three tested doses of 2.53, and 3.5 mg/kg, treatment with 9-AC was associated with a % AUC inhibition of 45%, 48% and 68% and a TGD of 5, 6, and 8 days, respectively; treatment with PNU 145156E was associated with a % AUC inhibition of 75% and a TGD of 9 days (Table 4). The combination resulted in a significantly enhanced reduction in tumor weight and an increase in TGD (13–15 days).

Hematology in mice treated with DX or CTX

Healthy mice were treated i.p. with PNU145156E at 100 mg/kg and, 2 h later, with CTX at 90 mg/kg or DX at 6

Table 3 Efficacy of PNU 145156E and MMDX combined treatment

Treatment group[a]	Tumor weight (g)[b]	A.U.C. % inhib.[c]	TGD[d]	Toxic/total mice
MMDX 0.05 mg/kg	2.86 ± 0.8	90	12	0/9
PNU 145156E 100 mg/kg	1.4 ± 0.7	90	16	0/9
PNU 145156E + MMDX	0 ± 0*	100	28	0/9

* $P < 0.01$
[a] PNU 145156E was given i.p. at 2 h prior to i.v. MMDX administration ; treatment was performed on days 1,4,7, and 11
[b] Tumor weight measured at day 48
[c] Inhibition of solid tumor growth
[d] Difference in the number of days required to reach 1 g relative to controls (14 days)

Table 4 Efficacy of PNU 145156E and 9AC combined treatment

Treatment group[a]	Tumor weight (g)[b]	A.U.C. % inhib.[c]	TGD[d]	Toxic / total mice
9 AC 2.5 mg/kg	2.4 ± 0.9	45	5	0/10
9 AC 3 mg/kg	2.13 ± 0.8	48	6	0/10
9 AC 3.5 mg/kg	1.58 ± 0.6	68	8	1/10
PNU 145156E 100 mg/kg	1.4 ± 0.7	75	9	0/10
PNU 145156E + 9AC 2.5 mg/kg	0.2 ± 0.01*	96	13	0/10
PNU 145156E + 9AC 3 mg/kg	0.14 ± 0.07*	100	14	1/9
PNU 145156E + 9AC 3.5 mg/kg	0.05 ± 0.001*	100	15	1/9

* $P < 0.01$
[a] PNU145156E was given i.p. at 2 h prior to i.v. 9AC administration; treatment was performed on days 1,4,7, and 11
[b] Tumor weight measured at day 23
[c] Inhibition of solid tumor growth
[d] Difference in the number of days required to reach 1 g relative to controls (14 days)

mg/kg, and blood samples were drawn on day 13. Day 13 was chosen for blood sampling since it is the average day calculated for the DX and CTX nadir; no decrease in blood cells was observed for PNU 145156E in any toxicology study.

CTX given alone and in combination induced a similar marked decrease in leukocytes (80% and 84%, respectively), which was mainly sustained by reductions in lymphocytes, though neutrophils were also clearly affected, showing a decrease of 78% and 73%, respectively.

DX given alone and in combination induced a similar decrease in leuocytes (63% and 68%, respectively); however, although both neutrophils and lymphocytes decreased to a similar extent (59% and 64%, respectively) after treatment with DX alone, the reduction obtained wih the combination was primarily due to decreases in lymphocytes, whereas neutrophils did not appear to be affected.

Finally, slight decreases in platelets (about 35% with CTX and about 20% with DX) were seen only following treatment with the combinations, whereas the higher values observed after treatment with each of the cytotoxic drugs alone could have been due to a rebound phenomenon following the nadir, which probably occured earlier than on day 13.

Discussion

This paper presents data obtained in tests of the in vitro and in vivo activity of PNU 145156E, a noncytotoxic growth-factor-complexing molecule, in combination with the four cytotoxic drugs DX, CTX, MMDX, and 9-AC. The model selected was the M5076 murine reticulosarcoma, while was responsive to all drugs examined.

The rationale underlying these experiments was that greater inhibition of solid tumor growth could be obtained via action on two different steps in tumor progression, that is, inhibition of growth/angiogenic factors and inhibition of cell proliferation.

The combined treatment of PNU145156E with the four tested drugs was associated in all combinations with increased antitumor activity and increased delay in tumor growth; the potentiation of antitumor efficacy observed in vivo was not related to any interference of PNU 145156E with the antiproliferative effect of the cytotoxic drugs as evidenced by results showing that in vitro the cytotoxic activity of the four tested drugs was unchanged in the presence of PNU145156E at 50 μg/ml; also, in terms of its effect on the cell cycle, the combination with DX was found not to modify the cell-cycle distribution induced by DX alone.

It is noteworthy that no obvious increase in general toxicity was ever observed for the combinations as evaluated in terms of early deaths or gross pathological findings at necropsy. This finding was confirmed in experiments on healthy mice treated with PNU145156E and DX or CTX, showing that the association did not enhance myelotoxic effects of the two cytotoxic drugs.

All of these results indicate that PNU 145156E inhibits tumor progression via a mechanism different from that of cytotoxic drugs and that the potentiation of

activity observed is due to distinct, nonoverlapping interference with tumor growth. These results support the utilization of PNU 145156E in therapy in combination with cytotoxic drugs.

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