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Aponecrotic, antiangiogenic and antiproliferative effects of a novel dextran derivative on breast cancer growth *in vitro* and *in vivo*

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1 1 Since the sodium phenylacetate (NaPa) was reported to enhance the inhibitory effect of carboxymethyl benzylamide dextran (CMDB) on the breast cancer growth, we performed the esterification of CMDB with NaPa to obtain a new drug carrying the characteristics of these two components. A new molecule, phenylacetate carboxymethyl benzylamide dextran, was named NaPaC.

2 We investigated *in vitro* and *in vivo* the effects of NaPaC on MCF-7ras cell growth as well as its apoptotic and antiangiogenic effects in comparison to NaPa and CMDB. In addition, we assessed *in vitro* the antiproliferative effects of these drugs on other breast cancer cells, including MDA-MB-231, MDA-MB-435 and MCF-7.

3 *In vitro*, NaPaC inhibited MCF-7ras cell proliferation by 40% at concentration lower than that of CMDB and NaPa (12 μM *vs* 73 μM and 10 mM). IC_{50}s were 6 and 28 μM for NaPaC and CMDB, respectively. The similar results were obtained for three other breast cancer cell lines. NaPaC reduced the DNA replication and induced cell recruitment in G_0/G_1 phase more efficiently than its components. Moreover, it induced a cell death at concentration 1000-fold lower than NaPa.

4 *In vivo*, CMDB (150 mg kg^{-1}) and NaPa (40 mg kg^{-1}) inhibited the MCF-7ras tumour growth by 37 and 57%, respectively, whereas NaPaC (15 mg kg^{-1}) decreased tumour growth by 66% without toxicity.

5 NaPa or CMDB reduced the microvessel number in tumour by 50% after 7 weeks of treatment. NaPaC had the same effect after only 2 weeks. After 7 weeks, it generated a large necrosis area without detectable microvessels. *In vitro*, NaPaC inhibited human endothelial cell proliferation more efficiently than CMDB or NaPa. NaPaC interacts with vascular endothelial growth factor as observed by affinity electrophoresis.

6 NaPaC acts like NaPa and CMDB but in more potent manner than components used separately. Its antiproliferative, aponecrotic and anti-angiogenic actions make it a good candidate for a new anti-cancer drug.

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Keywords: Sodium phenylacetate (NaPa); carboxymethyl benzylamide dextran (CMDB); phenylacetate carboxymethyl benzylamide dextran (NaPaC); antitumour activity; angiogenesis

Abbreviations: Ann-V, annexin-V; BrdU, bromodeoxyuridine; CMDB(s), carboxymethyl benzylamide dextran(s); ds, degree of substitution; EGF, epidermal growth factor; FCS, foetal calf serum; FGF-2 and FGF-4, fibroblast growth factor-2 and -4; HUV-EC-Cs, human umbilical vein endothelial cells; IGF-1, insulin-like growth factor-1; NaPa, sodium phenylacetate; NaPaC, phenylacetate carboxymethyl benzylamide dextran; PDGF, platelet-derived growth factor; PI, propidium iodide; s.c., subcutaneously; TGF-α and TGF-β, transforming growth factor-α and -β; VEGF, vascular endothelial growth factor

Introduction

We previously showed that carboxymethyl benzylamide dextran (CMDB) derivatives inhibit the breast cancer cell proliferation *in vitro* and *in vivo* (Bagheri-Yarmand *et al*., 1992; 1997; 1998a,b; 1999). The *in vitro* effect was associated with a decrease in the S-phase cell population and with an accumulation of cells in the G_0/G_1 phase of cell cycle (Bagheri-Yarmand *et al*., 1992; 1994). CMDB disrupts the mitogenic effect of growth factors by preventing their binding to specific receptors as reported for fibroblast growth factor-2 and -4 (FGF2, FGF4) (Bagheri-Yarmand *et al*., 1998a), platelet-derived growth factor-BB (PDGF-BB) and transforming growth factor-β (TGFβ) (Bagheri-Yarmand *et al*., 1998b). The inhibition of binding to growth factor receptors results from an alteration of the growth factor conformation induced by the CMDB binding to ligands (Bittoun *et al*., 1999). *In vivo*, CMDB treatment reduces the growth of MCF-7ras (Bagheri-Yarmand *et al*., 1998b) and FGF4-transfected HBL100 xenografts in nude mice (Bagheri-Yarmand *et al*., 1997; 1998a). Also, it decreases tumour angiogenesis (Bagheri-Yarmand *et al*., 1998b; 1999).

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Sodium phenylacetate (NaPa), a physiological metabolite of phenylalanine, is normally found in human plasma at micromolar concentrations. At higher concentrations, NaPa induces the cytostasis and reverses *in vitro* the malignant phenotype of different cancer cells (Samid *et al*., 1993; 1994; 1997; 2000; Adam *et al*., 1995). Furthermore, NaPa was described to modulate the synthesis and/or the release of some growth factors (Ferrandina *et al*., 1997; Thibout *et al*., 1998), and to increase, in a synergistic manner, the effect of some molecules affecting the intracellular signalling of growth factors (Samid *et al*., 1993; Prasanna *et al*., 1996). In addition, NaPa potentiates the antitumour activity of tamoxifene by increasing the apoptosis of breast cancer MCF-7ras xenografts in nude mice (Adam *et al*., 1997). Recently, we have observed that NaPa alone induced cell death associated with a new morphological characteristic of both apoptosis and necrosis (Di Benedetto *et al*., 2001) chimerically termed aponecrosis as proposed by Formigli *et al*. (2000). Finally, phase I and II clinical trials gave some evidence of activity in patients being struck down by malignant tumours (Thibault *et al*., 1994; Chang *et al*., 1999). In a recent study, we showed that NaPa enhanced, in a synergistic or additive manner, the inhibitory effect of CMDB on breast cancer cell growth *in vitro* and in nude mice when the CMDB/NaPa ratio of two molecules administrated in combination was four (Di Benedetto *et al*., 2001). To obtain a new drug with the same properties but easier to use as a future anti-cancer molecule than the combined treatment, we performed the esterification of CMDB by NaPa respecting the synergistic CMDB/NaPa ratio.

In the present study, we investigated the *in vitro* and *in vivo* effects of this new dextran derivative, phenylacetate carboxymethyl benzylamide dextran, named NaPaC, on breast cancer cell proliferation. In addition, we explored if this molecule displayed the apoptotic and anti-angiogenic effects of both components.

Methods

Compound preparation

NaPa was provided by Seratec (Paris, France). CMDB was synthesized by Sterilyo Laboratories (Levallois-Perret, France) with some modifications of the procedure described previously (Mauzac & Jozefonvicz, 1984; Chaubet *et al*., 1995). Briefly, CMDB was prepared in one step by simultaneous carboxymethylation and benzylamidation of dextran T40. New dextran derivative named NaPaC was synthesized by Sterilyo Laboratories performing a statistical esterification of CMDB with phenylacetic acid. After purification by ultrafiltration and lyophilization, the chemical composition or degree of substitution (d.s.) of CMDB and NaPaC was determined by acidimetric titration and elementary analysis of nitrogen. The composition of CMDB was: 0 d.s. for dextran, 0.67 d.s. for carboxymethyl and 0.39 d.s. for benzylamide. Concerning NaPaC, it displays the same d.s. as CMDB and a phenylacetate d.s. of 0.35. Thus, the ratio of CMDB and NaPa contained in this new molecule is closed to that found optimal in combined treatment (Di Benedetto *et al*., 2001). The calculated average molecular weights of NaPaC and CMDB mers were 314.2 and 264.1 g unit^{-1}, respectively. Their absolute molecular weights were calculated multiplying the average molecular weight by the number of units (247). This calculation leads to MW: 77,607 and 65,233 g mol^{-1}, for NaPaC and CMDB respectively. To compare the efficiencies of NaPaC and NaPa we have calculated a content of phenylacetate in NaPaC using the equation: molarity of NaPaC $\times$ 247 mers $\times$ 0.35 (d.s.). The esterification of CMDB did not change significantly its solubility in water.

Cell culture

The human breast cancer cell line MCF-7ras, established by transfection of MCF-7 cells with v-Ha-*ras* gene, was provided by Dr C. Sommers (Georgetown University, Washington DC, U.S.A.). Breast cancer MCF-7, MDA-MB-231, and MDA-MB-435 cells, human umbilical vein endothelial cells (HUV-EC-Cs) and BALBc/3T3 fibroblasts were purchased from American Tissue Culture Collection (Rockville, MD, U.S.A.). All cells were routinely grown in DMEM supplemented with 2 mM L-glutamine, 50 IU ml^{-1} penicillin-streptomycin and 10% foetal calf serum (FCS) (Life Technologies, Inc., Gaithersburg, MD, U.S.A.) at 37°C in a 5% CO_2 humidified atmosphere.

Growth inhibition experiments

MCF-7ras, MCF-7, MDA-MB-231, MDA-MB-435 and HUV-EC cell growth was assessed using the MTT-microculture tetrazolium assay (Mosmann, 1983). CMDB antiproliferative effect is FCS concentration-dependent with an optimum observed in the presence of 1 to 2% FCS (Bagheri-Yarmand *et al*., 1992). Briefly, the cells (4×10^3) were incubated in 2% FCS/DMEM for 24 h and then treated with NaPaC, CMDB or NaPa at different concentrations for 72 h. Then, the cells were washed with PBS and incubated with 0.1 ml of MTT (2 mg ml^{-1}). Complementary assay of cell cytotoxicity was performed by Trypan blue exclusion test.

Cell cycle analysis

MCF-7ras cells (5×10^4) were plated in DMEM supplemented with 2% FCS. After 24 h of culture, NaPaC, CMDB or NaPa were added for the next 72 h. Then, the cells were treated with bromodeoxyuridine (BrdU) (Pharmingen, San Diego, CA, U.S.A.) for 4 h. The incorporated BrdU was revealed using an anti-BrdU antibody conjugated with fluorescein isothiocyanate (Boerhinger Mannheim, Germany). Then, the cells were stained with propidium iodide (PI) (Boerhinger) for 10 min and analysed using a flow cytometer (Coulter Epics Laser, CA, U.S.A.).

Evaluation of cell death

To reveal a phosphatidylserine translocation specific to early apoptosis stage, the cells were stained with a FITC-labelled annexin-V (Ann-V) (Boerhinger). The ultimate stage of apoptosis or the first stage of necrosis was revealed by incorporation of PI, which enters into the cells when a cell membrane damage has occurred. After the treatments

with NaPaC, CMDB or NaPa, the cells were harvested, washed with annexin buffer (Boerhinger) and then stained with Ann-V followed by PI accordingly to the manufacturer's protocol. Then, the cells were analysed by flow cytometry. To visualize the apoptotic DNA fragmentation, we have used TumorTACS kit (R&D Systems, Abingdon, U.K.).

Preparation of conditioned media (CMs)

MCF-7ras cells (1×10^6) were treated or not with 24 μM NaPaC or 73 μM CMDB or 20 mM NaPa for 48 h in DMEM supplemented with 2% FCS and washed twice with PBS. To obtain conditioned media (CMs) containing only the growth factors produced by MCF-7ras, the cells were incubated with serum-free DMEM for 24 h at 37°C. These media (CMs) were then harvested and used directly. The quantity of cells submitted to every treatment was adjusted to obtain, at the end of treatment, always the same number of cells (5×10^6). Thus, the content of conditioned media was independent of direct growth inhibitory effects of drugs.

CM experiments

The mitogenic activity of CMs was determined using BALB/c3T3 fibroblasts (5×10^3 cells per well) grown until preconfluence in DMEM supplemented with 10% FCS, washed twice with PBS and growth-arrested by serum starvation for 24 h. Thus prepared, fibroblasts were incubated with different CMs (1 ml) in the presence or absence of 24 μM NaPaC, 73 μM CMDB or 20 mM NaPa for 48 h. The mitogenic activity of the CMs was estimated by counting the fibroblasts in a Coulter counter (Coultronics, Margency, France).

Xenografts in nude mice

MCF-7ras cells (5×10^6) were inoculated subcutaneously (s.c.) near the right mammary fat pad of 4-week-old athymic nude mice (nu/nu) (Harlan laboratory, Gannat, France). The animals ($n = 45$) were kept in a temperature-controlled room on a 12 : 12 light-dark schedule with food and water *ad libitum*. This protocol resulted in the development of single s.c. palpable tumours 4 weeks later in 90% of mice. Then, the animals were arbitrarily placed in control ($n = 10$) and three treated groups ($n = 10$). NaPaC (15 mg kg^{-1} = 0.18 μmol kg^{-1}), CMDB (150 mg kg^{-1} = 1.85 μmol kg^{-1}) or NaPa (40 mg kg^{-1} = 0.25 mmol kg^{-1}) was injected in 0.1 ml of 0.9% NaCl s.c. near the tumour, twice a week for 7 weeks. The control group received s.c. 0.1 ml of 0.9% NaCl. Tumour volumes were calculated as previously described (Bagheri-Yarmand *et al*., 1998b). In our experiment, we used the CMDB dose, which was reported efficient on MCF-7ras tumour growth inhibition (Bagheri-Yarmand *et al*., 1998b). Since in NaPaC, the CMDB/NaPa ratio is close to four, we have used for NaPa a dose of 40 mg kg^{-1}. Concerning NaPaC, we have tested a dose 10-fold lower than CMDB one because *in vitro* studies showed that the inhibition of MCF-7ras cell growth by CMDB can be achieved by NaPaC at a concentration 6–10 fold lower than CMDB one (Figure 1a).

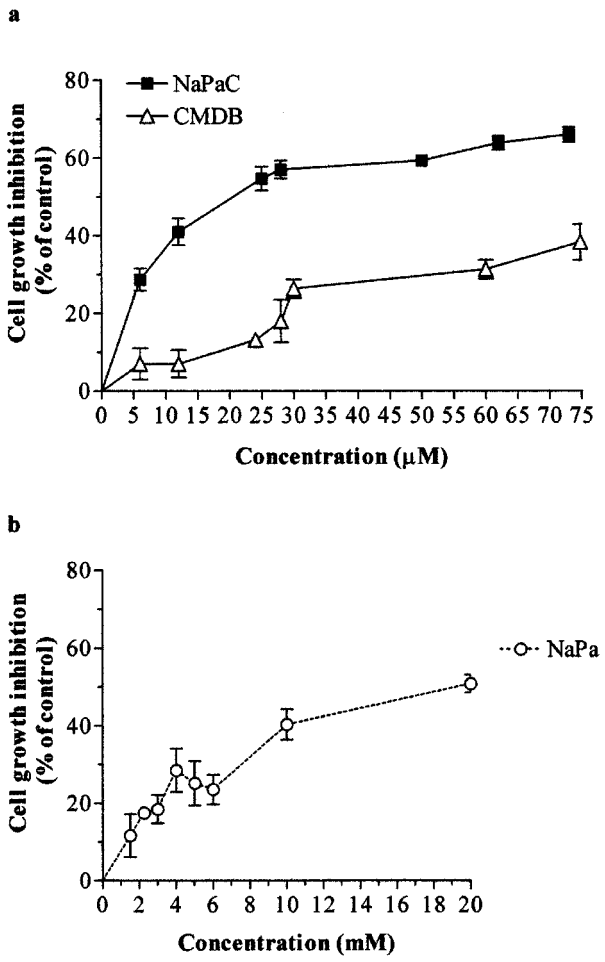

Figure 1 Effect of NaPaC and CMDB (a), and NaPa (b) on MCF-7ras cell proliferation. Cells were incubated for 72 h in the absence or presence of each drug at various concentrations. Cell growth was assessed using MTT-assay as described in Methods. Each point represents the mean (±s.d.) of three independent experiments.

Immunohistochemical analysis

Tumour specimens were fixed with a solution of paraformaldehyde (4%) and included into paraffin using standard procedure. Routinely, 5 μm-sections were stained in haematoxylin and eosin. For immunohistochemical studies the sections were deparaffinized and rehydrated. Endogenous peroxidase was inactivated with 3% H_2O_2 and washed in TBS (Tris 0.05 M, NaCl 1.5 M, pH 7.6) followed by preincubation in 10% normal goat serum for 1 h at room temperature. Endothelial cells were specifically labelled with GSL-1 isolectin B4 (Vector Laboratories, Burlingame, CA, U.S.A.). The GSL-1 lectin binds specifically to galactosyl residues and thus labels the vascular endothelium in mice (Alroy *et al*., 1987). The sections were incubated for 1 h with the 1 : 50 diluted GSL-1 isolectin at room temperature. The sections were then incubated with goat antibody against GSL-1 isolectin B_4 (1 : 400 dilution, Vector Laboratories) for 30 min, washed with TBS and incubated with biotinylated rabbit anti-goat immunoglobulins (1 : 400 dilution; Dako, Glostrup, Denmark) for 20 min in a moist chamber at room

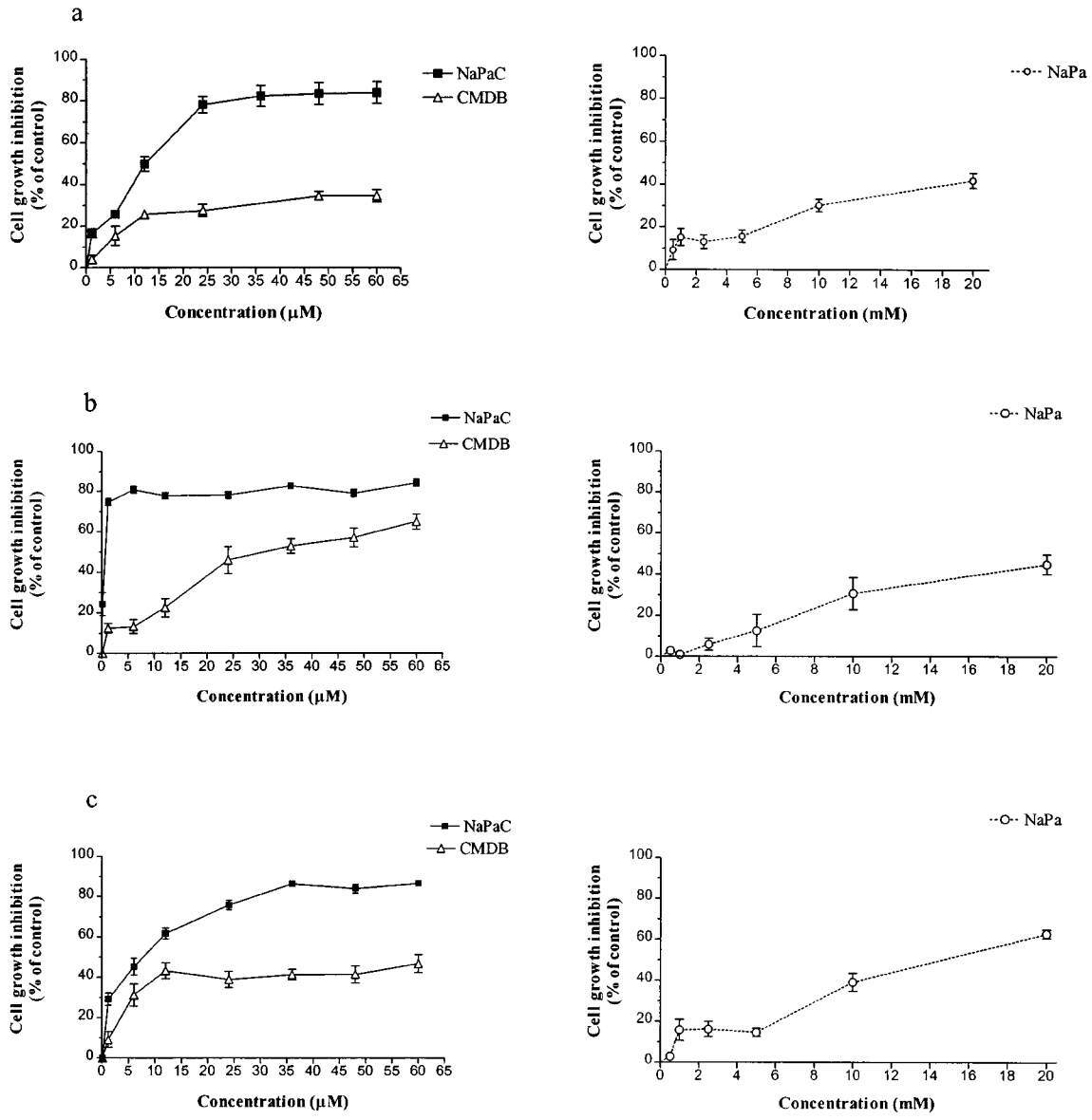

Figure 2 Effect of NaPaC and CMDB, and NaPa on MCF-7 (a), MDA-MB-231 (b), MDA-MB435 (c) cell proliferation. Cells were incubated for 72 h in the absence or presence of each drug at various concentrations. Cell growth was assessed using MTT-assay as described in Methods. Each point represents the mean (±s.d.) of three independent experiments.

temperature. After three washes with TBS, the samples were incubated with streptavidin-biotin peroxidase (LSAB kit; Dako) for 10 min using diaminobenzidine tetrahydrochloride as the chromogen. Finally, the slides were washed in water and counterstained with hematoxylin.

Microvessel quantification in tumour sections

Intratumour microvessel areas were determined using a point-counting grid over the endothelial cells and expressed as a fraction of the total point count of the point-counting grid (96 points in the grid corresponding to an area of 1.02 mm^2 on the picture) (Weibel, 1979). For each tumour, 10 randomly selected nonserial sections were studied. For each GSL-1 labelled section of control and treated tumours, 10 fields containing exclusively viable tumour cells, as indicated by the hematoxylin staining, were selected randomly for analysis. Using a Reichter-Jung (Polivar, Austria) microscope, each tumour was scanned at magnification ($\times 100$) to detect and select the areas with the most intense vascularization following the criteria defined by Weidner *et al.* (1991). For each section, two or three pictures were taken at a $\times 250$ magnification. Each count was expressed as the highest number of microvessels identified within any $\times 250$ field (1.02 mm^2). GSL-1 stained endothelial cell or group of cells, with or without lumen, clearly separated from other cells were considered as individual vessel. The coefficient of variation (s.d.) was used to assess the variability of counts divided by fields of the same tumour. Mean intratumour microvessel number values per area in the various tumours were compared using Student's *t*-test to identify significantly differences. For all statistical analyses, the level of significance was set at 0.05.

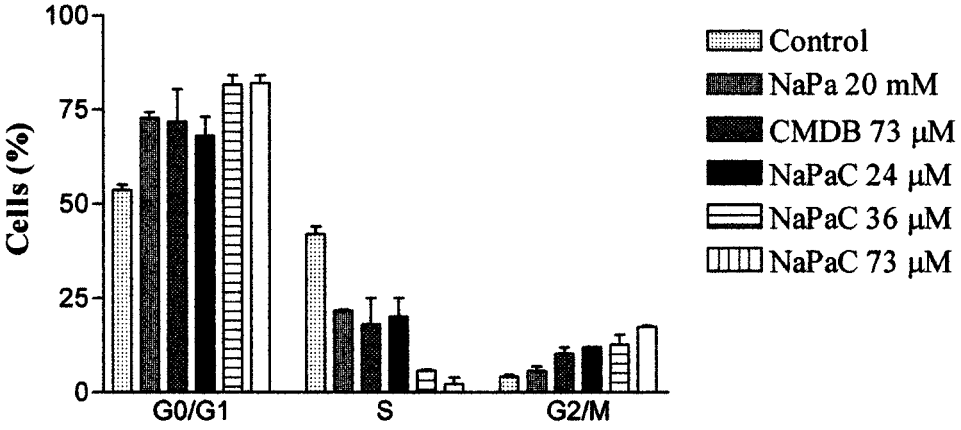

Figure 3 Effect of NaPaC (24–73 μM), CMDB (73 μM) and NaPa (20 mM) on the distribution of MCF-7ras cells in cell cycle phases. Each column represents the mean (±s.d.) of three independent experiments.

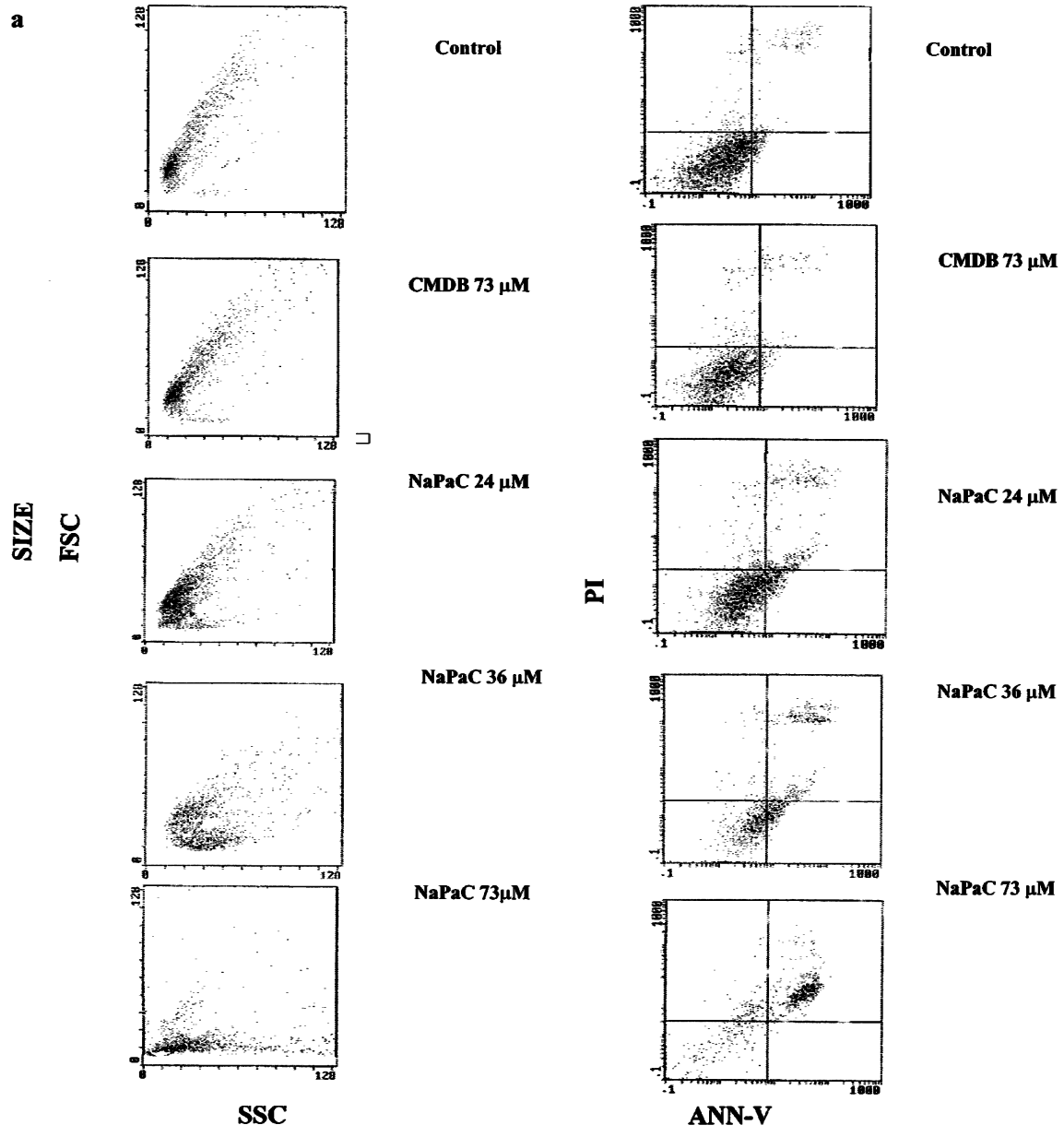

Figure 4 (*continued overleaf*)

Vascular endothelial growth factor (VEGF) stimulation experiment

HUV-EC-Cs (5×10^3 cells) were grown until 80% of confluence and made quiescent by serum starvation for 24 h. The culture medium was replaced with serum-free DMEM with or without VEGF 10 ng ml^{-1}, and NaPaC 24 μM, CMDB 73 μM or NaPa 20 mM were added. After 48 h treatment, the cells were counted using a Coulter counter (Coultronics, Margency, France).

Agarose gel electrophoresis

The NaPaC or NaPa effects on the electrophoretic mobility of ^{125}I-VEGF$_{165}$ (Amersham Pharmacia Biotech, Orsay, France) were analysed by non-denaturant agarose gel electrophoresis as described by Lee & Lander (1991). After a 1 h incubation of ^{125}I-VEGF$_{165}$ (10^5 c.p.m. = 3 ng) with NaPaC or NaPa at various concentrations at 4°C, the mixtures (10 μl) were analysed in agarose 1% gel at pH 7.0

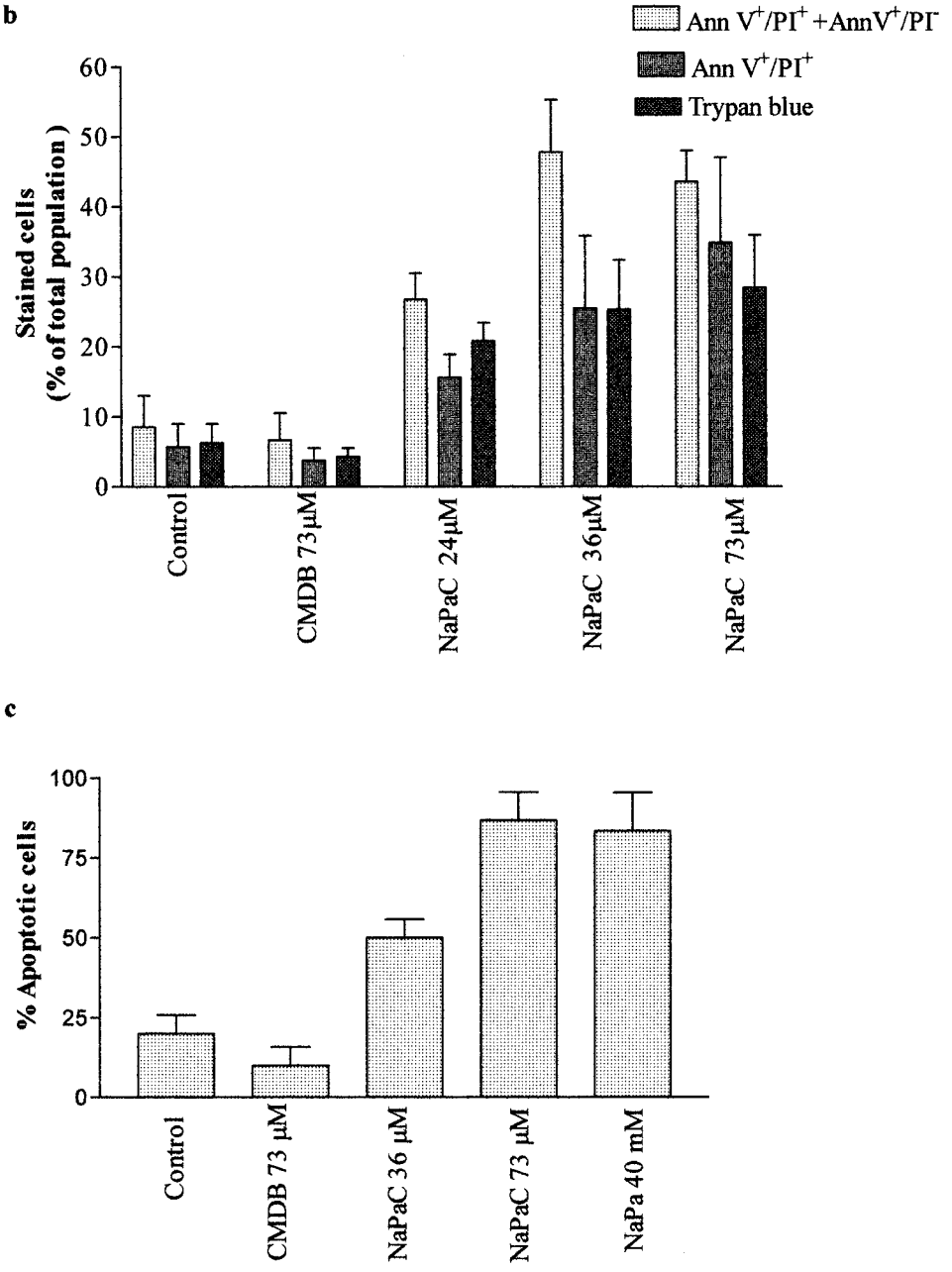

Figure 4 NaPaC induces the cell death of MCF-7ras cells. The cells were incubated for 72 h in the absence or presence of NaPaC at concentrations of 24, 36 and 73 μM or 73 μM CMDB (a,b). After staining with FITC-labelled annexin-V (Ann-V) and propidium iodide (PI), the cells were analysed by flow cytometer. (a). Size FSC (forward scatter)/SSC (side scatter) patterns (column 1) and Ann-V/PI patterns (column 2) for untreated (control) and treated cells. In column 2, the lower right region represents the AnnV$^+$/PI$^-$ positive cells and the one on the top right region contains the AnnV$^+$/PI$^+$ positive cells. (b) The columns represent the ratio of AnnV$^+$ + /PI$^+$ + AnnV$^+$/PI$^-$ or AnnV$^+$/PI$^+$ and Trypan blue positive cells *vs* total population and show the mean ($\pm$s.d.) of three independent experiments. Similar results were obtained by terminal deoxynucleotidyl transferase-mediated nick end labelling using TumorTACS kit (R&D system) (c). The data are presented as the percentage of TumorTACS-positive cells.

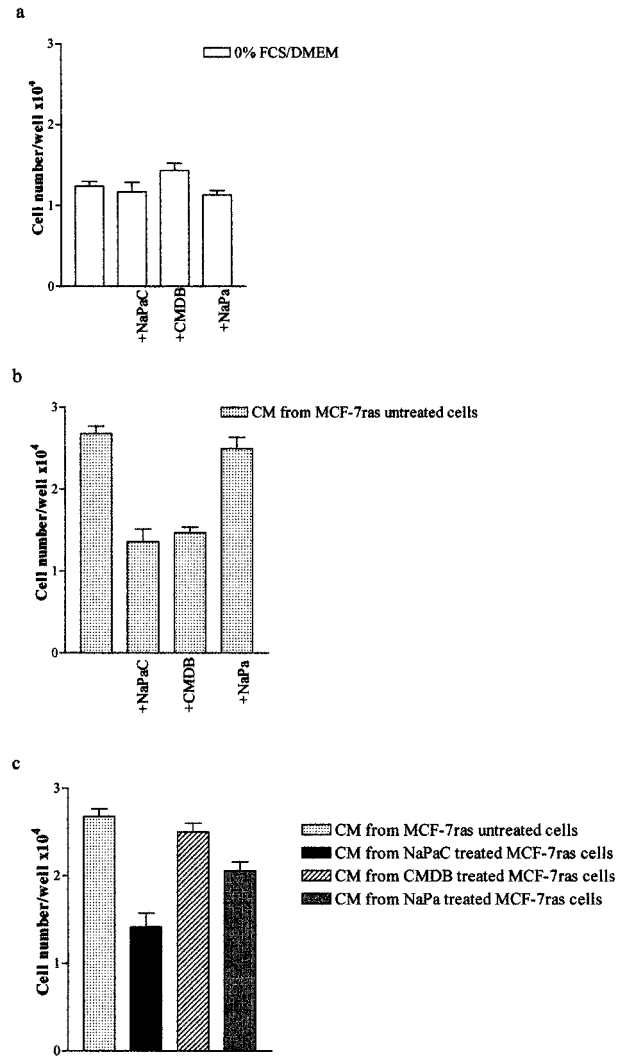

Figure 5 Effects of 24 μM NaPaC, 73 μM CMDB and 20 mM NaPa on the mitogenic activity of MCF-7ras conditioned medium (CM). Control represents the growth of fibroblasts in serum-free medium (a). The mitogenic activity of CMs from untreated MCF-7 ras cells supplemented or not with drugs (b) and CMs from MCF-7 ras treated cells (c) was evaluated on BALBc/3T3 fibroblast growth (see Methods). Each column represents the mean ($\pm$s.d.) of three independent experiments.

using running buffer containing 125 mM sodium acetate, 50 mM 3-(N-morpholino)-2-hydroxypropane-sulfonic acid, 6% sucrose and 0.5% (w v^{-1}) bromophenol. Typical electrophoresis was performed at 60–70 V for 2 h. Gels were dried and then exposed to Kodak X-Omat film (Amersham Pharmacia Biotech). If the negatively charged NaPaC or NaPa binds to essentially basic $VEGF_{165}$, an anodic shift in the migration of the tracer should be observed.

Statistical analysis

Multiple statistical comparisons were performed using ANOVA in a multivariable linear model. Some statistical comparisons were conducted using the Mann-Whitney *t*-test. $P<0.05$ was considered statistically significant.

Results

NaPaC is a more potent inhibitor than NaPa and CMDB components of breast cancer cell proliferation

After a 72 h incubation, NaPaC induced a dose-dependent inhibition of MCF-7ras cell growth with higher potencies than NaPa and CMDB (Figure 1). For example, to obtain 40% of cell growth inhibition, only 12 μM NaPaC is required whereas for the same inhibitory effect 73 μM CMDB (Figure 1a) or 10 mM NaPa is necessary (Figure 1b). Thus, the antiproliferative activity of NaPaC was significantly enhanced by 6.0 ($P=0.002$) and 1000 ($P=0.026$) as compared with CMDB and NaPa, respectively. If we consider that 12 μM NaPaC contained 1.04 mM of phenylacetate, the ratio between NaPaC and NaPa efficiencies was 11.54 ($P=0.002$). The concentrations inducing 50% of maximal inhibition (IC_{50}) were 6 and 28 μM for NaPaC and CMDB, respectively. Concerning NaPa, IC_{50} was previously determined equal to 20 mM for McF-7ras (Di Benedetto *et al*., 2001) and ovarian NSC 3039 carcinoma cells (Ferrandina *et al*., 1997). The comparison of tumour cell growth inhibition induced by NaPaC, CMDB and NaPa (at concentration which corresponds to NaPa content in NaPaC) shows that the hybrid molecule NaPaC is more efficient than each component used alone. It is noteworthy that NaPaC effect is at least the sum of the individual molecule effects as it was previously observed in the case of combined CMDB/NaPa treatment (Di Benedetto *et al*., 2001). For example, the 12 μM NaPaC induced 40% of cell growth inhibition while 12 μM CMDB and 1 mM NaPa (concentration corresponding to NaPa content in 12 μM NaPaC) generated 6 and 5% of inhibition, respectively. At higher concentration (30 μM), NaPaC inhibitory effect was 50% while those of 30 μM CMDB or 2.5 mM NaPa were only 20%.

Similar inhibitory effects of NaPaC were observed on other breast cancer cells, including MCF-7 (Figure 2a), MDA-MB-231 (Figure 2b) and MDA-MB-435 (Figure 2c) cells. The Trypan blue staining revealed that CMDB, up to 73 μM, and NaPa, up to 20 mM, induced the death of less than 5% of cell population (data not shown). NaPaC at the concentrations higher than 24 μM augmented a died cell population until 25% (Figure 4).

NaPaC has a more potent effect on cell cycle than CMDB and NaPa

NaPaC, starting from concentration 24 μM, inhibited the MCF-7ras cell DNA synthesis in a concentration-dependent manner as evidenced by a decrease in MCF-7ras cell number in the S-phase with a major recruitment of cells in the G_0/G_1 phase (Figure 3). NaPaC (24 μM) is as effective as 20 mM NaPa and 73 μM CMDB on these cell cycle phenomena. These results suggest that NaPaC is a more cytostatic molecule than its components.

NaPaC is more potent generator of cell death than NaPa

The Ann-V-positive/PI-negative (Ann-V^+/PI^-) population corresponds to cells in an early apoptotic phase and the Ann-V-positive/PI-positive (Ann-V^+/PI^+) one to cells in a late apoptosis phase and/or necrosis. The latter population is

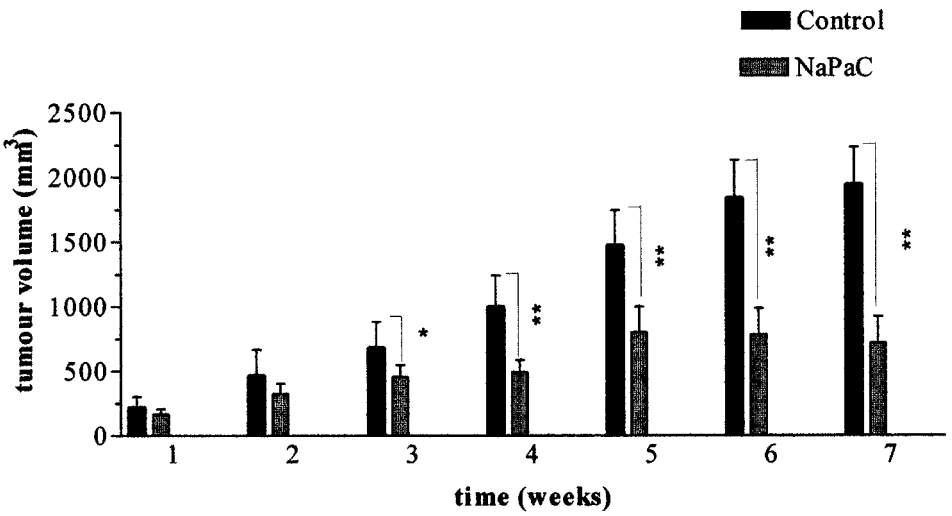

Figure 6 Inhibition of MCF-7ras xenograft growth by NaPaC. MCF-7ras cells were inoculated in nude mice as described in Methods. After 4 weeks, mice were treated with NaPaC (15 mg kg^{-1}), twice a week, for 7 weeks. Each column represents the mean of tumour volume (mm^3) ($\pm$s.d., $n=10$). *$P<0.05$ *vs* control; **$P<0.01$ *vs* control.

Table 1 Inhibition of MCF-7ras tumour growth by NaPaC and its components, CMDB and NaPa

Treatment	*Tumour volume at day 1* (mm^3)	*Tumour volume at day 50* (mm^3)	*Inhibition of tumour growth* (% of control)
Control	221 ± 90	2103 ± 328^a	–
NaPa 40 mg kg^{-1} (0.25 mmol kg^{-1})	157 ± 34	990 ± 192^b	53
CMBD 150 mg kg^{-1} (1.85 μmol kg^{-1})	199 ± 71	1326 ± 281^c	37
NaPac 15 mg kg^{-1} (0.18 μmol kg^{-1})	167 ± 40	717 ± 203^d	66

MCF-7ras cells were inoculated s.c in nude mice as described in Methods. After tumour uptake, the animals were treated for 7 weeks with NaPa ($n=10$), CMDB ($n=10$) and NaPaC ($n=10$). Tumour volume ($\pm$s.e.m.) for different experimental groups were compared to control. $P<0.05^{a}$ *vs* ^{b,c}; $P<0.01^{a}$ *vs* ^{d} and ^{c} *vs* ^{d}.

also revealed by Trypan blue staining. NaPaC induced cell death, in a concentration-dependent manner, starting at 24 μM (Figure 4a,b) which is a concentration 1666 fold lower than that found for NaPa (40 mM; Di Benedetto *et al.*, 2001). In the presence of 36 μM NaPaC, we observed 48% of Ann-V stained cells, including two populations: Ann-V^+/PI^+ (25%) and Ann-V^+/PI^- (13%) corresponding to cells in early stage of apoptosis (Figure 4b). In the presence of 73 μM the majority of Ann-V-stained cells progress in a later apoptosis and/or in an early stage of necrosis. In contrast, 73 μM CMDB did not induce cell death of MCF-7ras cells (Figure 4a,b). The staining of DNA fragments (Figure 4c) confirmed AnnV-PI analysis.

NaPaC acts as NaPa and CMDB on the mitogenic activity of MCF-7ras conditioned medium (CM)

Previous reports indicated that MCF-7ras cells secreted growth factors, mitogenic for BALBc/3T3 fibroblasts (Bagheri-Yarmand *et al.*, 1998a; Di Benedetto *et al.*, 2001). Here, we investigated whether NaPaC could influence the mitogenic activity of MCF-7ras cell conditioned medium (CM) in the same manner than its components CMDB and NaPa (Figure 5). The control experiments (Figure 5a) showed that NaPaC, CMDB or NaPa had no effect on fibroblast growth in DMEM-FCS 0%. MCF-7 ras-CM stimulated the BALBc/3T3 fibroblast proliferation by 2 fold after 48 h of treatment (Figure 5b) as compared to control medium (DMEM-FCS 0%; Figure 5a; $P=0.0002$). The addition of 24 μM NaPaC ($P=0.0018$) or 73 μM CMDB ($P=0.0004$) but not 20 mM NaPa to the CM abolished the stimulation of fibroblast growth (Figure 5b) as compared to control CM. The CM prepared from MCF-7ras cells pre-treated for 48 h (Figure 5c) with 24 μM NaPaC ($P=0.0023$) or 20 mM NaPa ($P=0.016$) but not with 73 μM CMDB diminished the CM efficacy on fibroblast growth as compared to CM from non-treated MCF-7ras cells. In this experimental design, NaPaC was more efficient than NaPa ($P=0.0011$). In addition, the CM prepared from NaPaC-pretreated MCF-7ras cells has no mitogenic effect on BALBc/3T3 fibroblast proliferation as

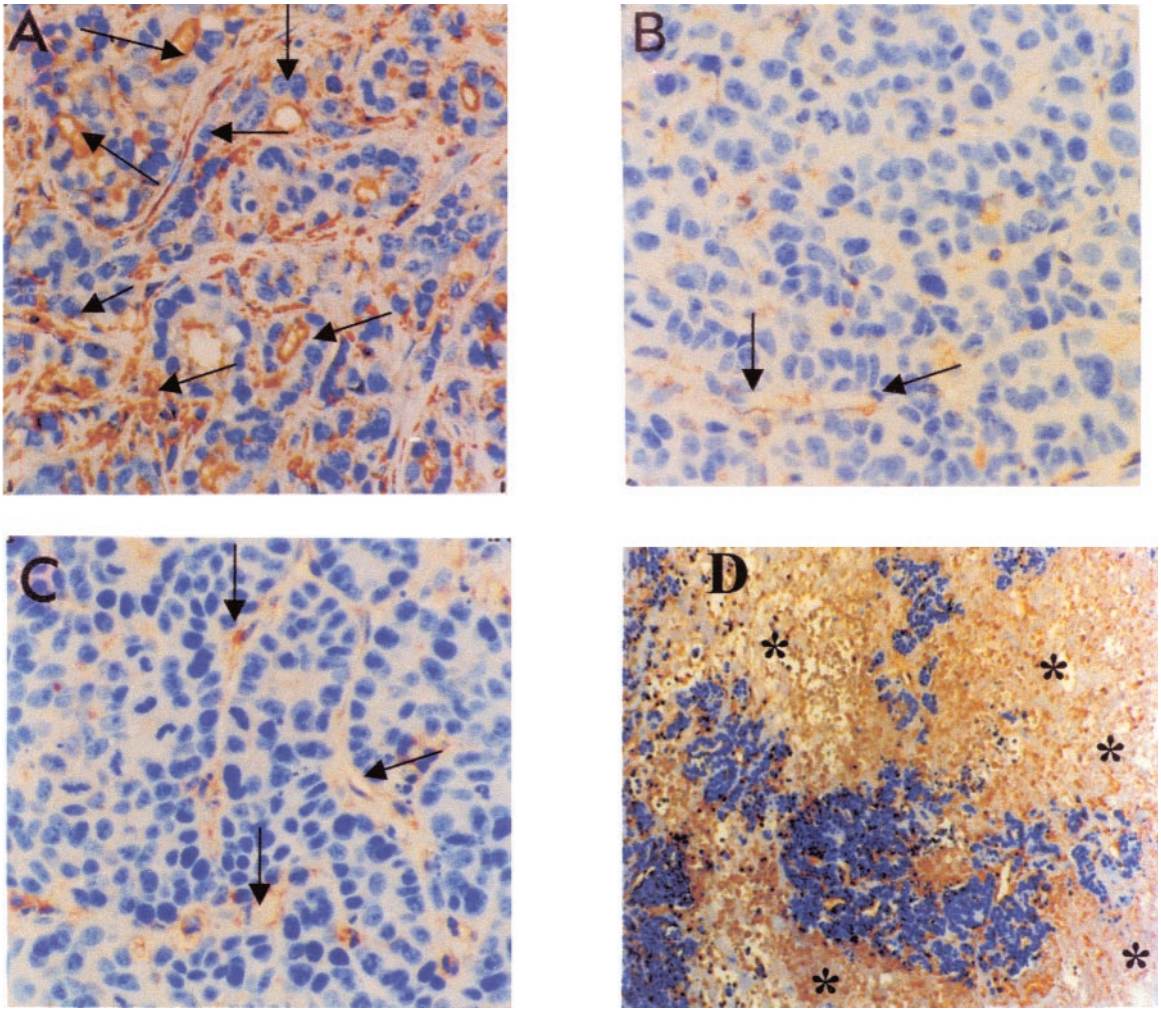

Figure 7 Inhibition of MCF-7ras tumour angiogenesis by NaPaC, CMDB and NaPa. Microvessel staining of tumours untreated (A) and treated with 150 mg kg^{-1} CMDB (B), 40 mg kg^{-1} NaPa (C) or 15 mg kg^{-1} NaPaC (D), twice a week, for 7 weeks, was performed using GSL-1 lectin. Necrotic areas in panel d are indicated with the asterisks. Magnification $\times 250$ was used for panels A, B and C, and magnification $\times 100$ was applied for panel D. The representative microvessels in panels A–C are marked with the arrows.

compared to control medium (DMEM-FCS 0%; Figure 5a). These results clearly demonstrate that NaPaC acts as NaPa and CMDB.

NaPaC has a higher antitumour activity on MCF-7ras tumours than NaPa or CMDB

After 7 weeks of treatment, NaPaC administrated at a dose of 15 mg kg^{-1}, twice a week, inhibited the MCF-7ras tumour growth by 66% ($P=0.007$) (Figure 6, Table 1). A similar tumour growth inhibition (53%) was observed after treatment with NaPa at 2.7-fold higher dose, 40 mg kg^{-1} (Table 1). It is noteworthy that the difference in antitumour activity of NaPaC and NaPa can be stronger when one considers that 15 mg kg^{-1} of NaPaC contains only 2.6 mg kg^{-1} of NaPa. Concerning CMDB, even at dose so high as 150 mg kg^{-1}, it inhibited the tumour growth only by 37%.

In all experimental conditions, no gross animal toxicity was observed, as the body weight of mice was not affected by treatment. No diarrhoea, infection, weakness or lethargy was stated. All of 40 studied mice were alive at the end of 7 weeks.

Anti-angiogenic effect of NaPaC on MCF-7ras tumours

A high number of microvessels stained with GSL-1 was detected in untreated tumours evidencing their angiogenic characteristic (Figures 7A and 8). CMDB (150 mg kg^{-1}) or NaPa (40 mg kg^{-1}) treatment for 7 weeks reduced the stained microvessel number by 50% as compared to control (untreated tumour) (Figure 7B,C, respectively, *vs* 7A; Figure 8). The similar inhibition of microvessel development was observed when CMDB and NaPa were administrated in combination (Figure 8a). In contrast, NaPaC treatment (15 mg kg^{-1}) for 7 weeks induced large necrotic areas (Figure 7D). In the remaining small non-necrotic areas of NaPaC-treated MCF-7ras tumours, no endothelial cells were detected (Figure 8a). To obtain non-necrotic NaPaC-treated tumours and to better evaluate the inhibitory effect of NaPaC on microvessel development, the same treatment of animals was limited to 2 and 4 weeks (Figure 8b). Indeed, in these

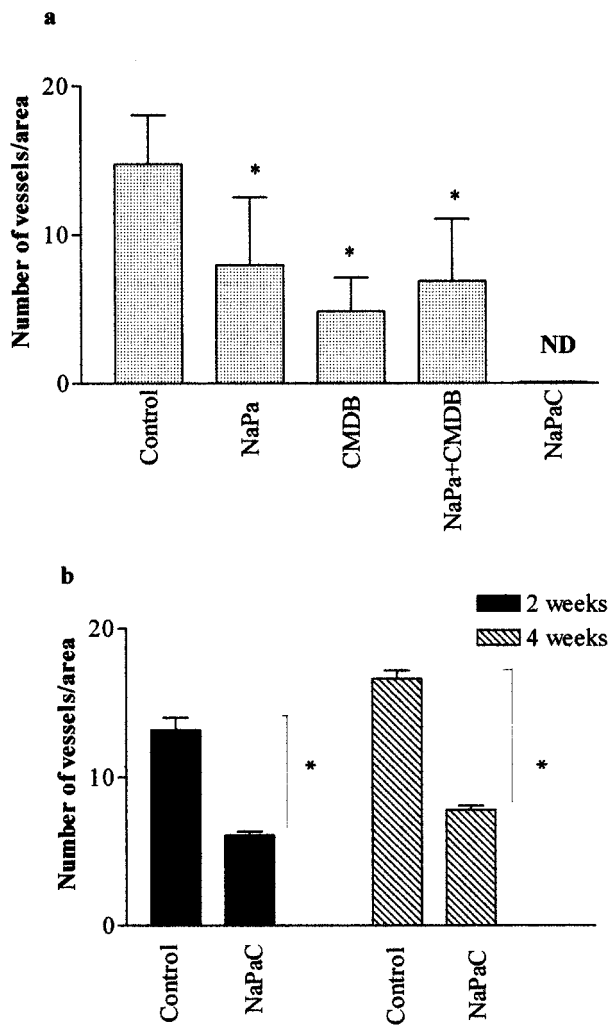

Figure 8 Quantification of angiogenesis inhibition in MCF-7ras xenografts after 7 week-treatment (a) or after 2 and 4 week-drug administration (b). Four weeks after tumour cell inoculation, mice were treated with NaPaC (15 mg kg^{-1}), CMDB (150 mg kg^{-1}), NaPa (40 mg kg^{-1}) or with combined NaPa and CMDB (40+150 mg kg^{-1}), twice a week. Microvessel staining and quantification were performed as described in Methods. Each column represents the mean of the vessel number per area (±s.d., $n=10$). *$P<0.05$ *vs* control. ND: non determined.

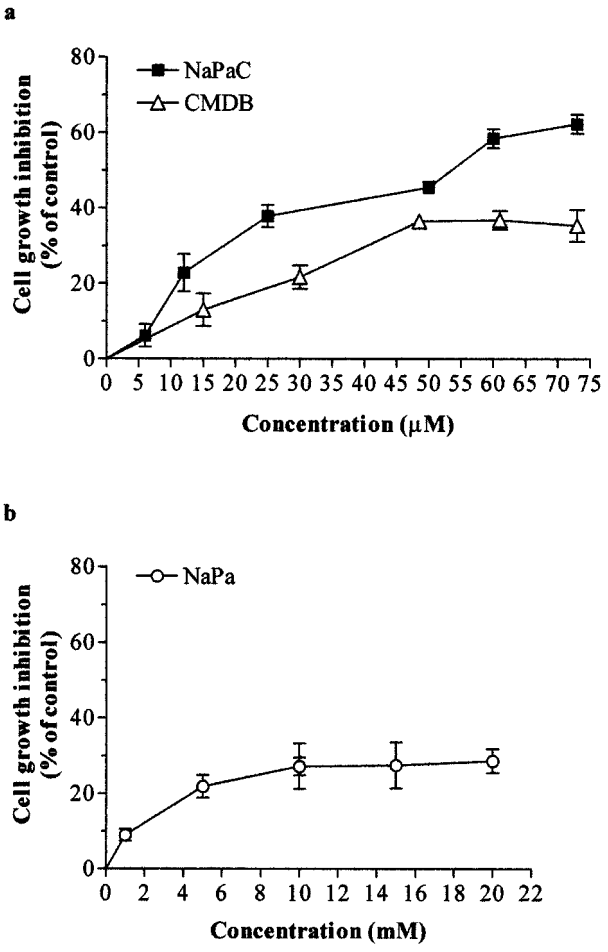

Figure 9 Effect of NaPaC and CMDB (a), and NaPa (b) on HUV-EC-C proliferation in DMEM-FCS 2%. The cells, treated for 72 h with drugs at various concentrations, were assessed using MTT-assay as described in Methods. Each point represents the mean (±s.d.) of three independent experiments.

experimental conditions, a decrease of 55% can be observed in microvessel number as early as after 2 weeks of treatment.

NaPaC is a potent inhibitor of endothelial cell proliferation

As NaPaC, CMDB and NaPa reduced the number of microvessels *in vivo*, we investigated their antiproliferative activity on human umbilical vascular endothelial cells (HUV-EC-Cs) in DMEM-FCS 2%. After 72 h exposure, the maximal inhibitory effect of NaPaC was 60% whereas those of CMDB, at the same concentration interval, and NaPa were only 27 and 23%, respectively (Figure 9). Thus, NaPaC is a twice more efficient inhibitor of HUV-EC-C proliferation than NaPa or CMDB. IC_{50}s for NaPaC and CMDB were similar (20 μM) whereas that for NaPa was 4 mM. The comparison of endothelial cell growth inhibition induced by NaPaC, CMDB and NaPa (at concentration corresponding to content of NaPa in NaPaC) shows that NaPaC effect is at least the sum of the individual molecule effects.

NaPaC, like CMDB, decreases the mitogenic activity of $VEGF_{165}$

We studied the effects of NaPaC, CMDB and NaPa on the mitogenic activity of the most specific growth factor for HUV-EC-Cs, Vascular Endothelial Growth Factor ($VEGF_{165}$). In the medium deprived of FCS, $VEGF_{165}$ stimulated the HUV-EC-C proliferation by 63% ($P=0.0073$ *vs* control) after 48 h of treatment (Figure 10). The addition of 24 μM NaPaC ($P=0.0179$ *vs* VEGF) or 73 μM CMDB ($P=0.045$ *vs* VEGF), but not 20 mM NaPa, abolished the $VEGF_{165}$-induced stimulation of HUV-EC-C growth. In control conditions (without FCS nor $VEGF_{165}$), NaPa, CMDB or NaPaC did not change the HUV-EC-C growth suggesting no toxicity or mitogenic effects of these three drugs.

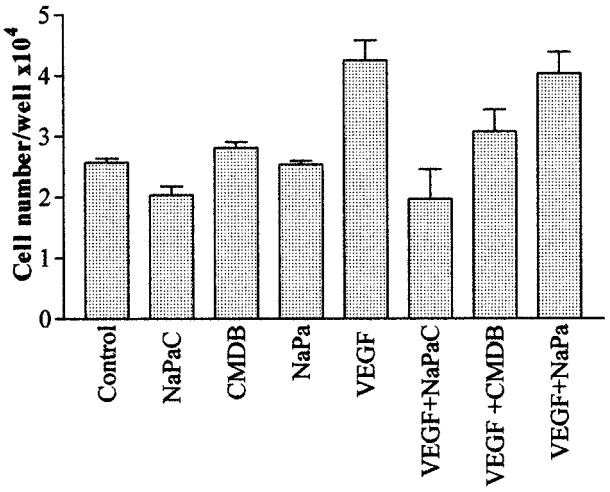

Figure 10 Effect of NaPaC, CMDB and NaPa on $VEGF_{165}$-stimulated HUV-EC-C proliferation. HUV-EC-Cs were incubated in serum-free medium supplemented or not with 10 ng ml^{-1} $VEGF_{165}$ in the absence or presence of 24 μM NaPaC, 73 μM CMDB or 20 mM NaPa for 48 h. Each column represents the mean ($\pm$s.d.) of three independent experiments.

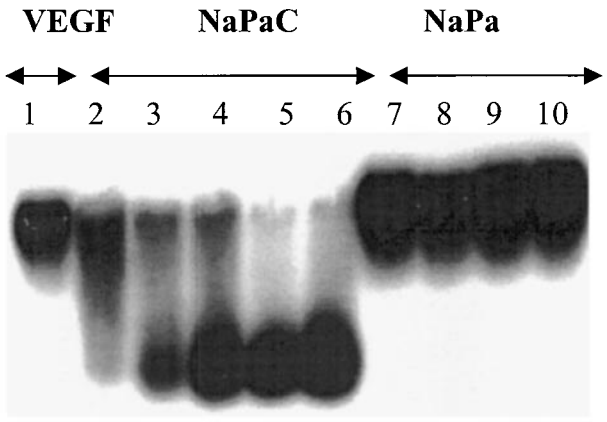

Figure 11 NaPaC directly interacts with $VEGF_{165}$. After a 1 h incubation of ^{125}I-$VEGF_{165}$ (10^5 c.p.m. = 3 ng) with NaPaC or NaPa at 4°C, the mixtures (10 μl) were electrophoretically analysed in non denaturant 1% agarose gel at pH 7.0. Lane 1 represents the migration of ^{125}I-$VEGF_{165}$ alone; lanes 2–6 correspond to 0.6, 1.8, 5.5, 15 or 48 μM NaPaC; lanes 7–10 represent the shifts after addition of 1.0, 5.0, 10 or 20 mM NaPa.

NaPaC forms a complex with $VEGF_{165}$

Clear evidence for direct interaction of NaPaC with $VEGF_{165}$ was obtained using an affinity-electrophoretic technique (ACE). As shown in Figure 11, lane 1, ^{125}I-$VEGF_{165}$, being weakly cationic in non-denaturing conditions, remains close to the loading well. The interaction with negatively charged NaPaC increases the migration of labelled growth factor evidencing the formation of ^{125}I-$VEGF_{165}$-NaPaC complex. Indeed, the shift toward the anode is visible in the presence of 0.6–48 μM NaPaC (Figure 11, lanes 2–6), but not in the presence of 1–20 mM NaPa (lanes 7–10). This result clearly demonstrates that NaPaC interacts directly with $VEGF_{165}$.

Discussion

In this study, we characterized the biological effects of a new molecule resulting from the esterification of CMDB with NaPa, named NaPaC. We showed that it displayed the antiproliferative, aponecrotic and anti-angiogenic effects observed for CMDB and NaPa on breast cancer cells *in vitro* and *in vivo* with potency higher than that of each component.

We have shown *in vitro* that NaPaC inhibited the growth of breast cancer MCF-7ras cells at a concentration lower than CMDB or NaPa. The comparison of IC_{50} for three drugs supplies the additional evidence for the highly enhanced efficiency of NaPaC as compared to CMDB and NaPa. Our data indicate that the hybrid molecule retains at least the additive effect of its two components observed previously (Di Benedetto *et al*., 2001). This effect is not only specific to MCF-7 ras cells as similar results were obtained for other breast cancer cell lines, including MCF-7, MDA-MB-231 and MDA-MB-435. The superiority of NaPaC *vs* CMDB and NaPa was also found in its effects on MCF-7 ras cell cycle. Indeed, NaPaC, more effectively than its components, decreased the cell number in the S-phase and blocked the cells in G_0/G_1-phase. This can be explained, at least in part, by the hypothesis that NaPaC operates involving the mechanisms of CMDB and NaPa actions at the same time. NaPa is believed to modulate the synthesis and/or release of growth factors (Ferrandina *et al*., 1997; Thibout *et al*., 1998) whereas CMDB interacts with growth factors changing their conformation (Bittoun *et al*., 1999) and thus, preventing their binding to specific receptors (Bagheri-Yarmand *et al*., 1998a,b). Our results of conditioned media experiments support the idea mentioned above. Thus, NaPaC was found to reduce the mitogenic effect of MCF7-ras cell conditioned medium after pre-treatment of cells with drug, like in the case of NaPa, as well as following the drug addition to conditioned culture medium, like observed for CMDB. However, we cannot discard the specific effect of NaPaC.

In this study, we observed that NaPaC induced a cell death, evidenced by the apparition of Ann-V-positive/PI-negative or PI-positive cells and staining of DNA fragmentation, on MCF-7ras cells. It seems that this NaPaC action can be due to the NaPa subunits since CMDB alone, even at higher concentration, was not observed to cause cell death. Interestingly, NaPaC was more efficient on cell death than NaPa reported aponecrotic only at high concentration, 40 mM (Di Benedetto *et al*., 2001). Indeed, 24 μM of NaPaC (efficient aponecrotic concentration) contain only 2.1 mmol of phenylacetate. Independently, it is possible that NaPaC could act also in specific manner.

Accordingly to our *in vitro* results, NaPaC inhibited *in vivo* MCF-7ras tumour growth more efficiently and at lower dose than CMDB or NaPa. This can be explained by the fact that NaPaC gathers the antiproliferative, aponecrotic and anti-angiogenic actions generally admitted to lead *in vivo* to concerted inhibition of tumour growth. The inhibition of the endothelium growth, causing the impaired delivery of nutrients and oxygen to tumour, leads to tumour cell death

(Folkman, 1995). Indeed, we observed *in vivo* that the inhibition of MCF-7ras tumour growth by NaPaC was concomitant with a poor microvessel density (as compared to control) at short-time treatment and with multifocal necrotic areas at long-term administration. Once more, NaPaC was more efficient than CMDB or NaPa. It decreased the microvessel density at dose lower than its components. Moreover, these large necrotic areas were undetected in tumours treated with CMDB or NaPa alone. In accord with our *in vivo* results on tumour neovascularization, NaPaC was observed *in vitro* to inhibit the growth of human endothelial cells more efficiently that CMDB or NaPa. The mechanisms involved in CMDB and NaPa actions on endothelial cell proliferation seem to be distinct. CMDB was reported to interact directly with $VEGF_{165}$ (Hamma-Kourbali *et al*., 2001), the most specific angiogenic factor (Plouet *et al*., 1989) and to inhibit the $VEGF_{165}$-induced HUVE-C-C growth. Here, we observed that NaPa did not interact with $VEGF_{165}$ molecule, as shown by affinity electrophoresis, and had not effect on $VEGF_{165}$-dependent cell growth. Up to date, the mechanism of NaPa action on HUVE-C-C growth is unknown. Concerning NaPaC, it inherited the CMDB ability to interact with angiogenic growth factor and blocked the $VEGF_{165}$-induced endothelial cell proliferation at lower concentration than CMDB. However, there is no argument to discard or not the involvement of the NaPa mechanism in NaPaC action on HUVE-C-C growth.

Altogether, our results clearly indicate that the increased efficiency of NaPaC on breast tumour growth inhibition, in comparison to CMDB and NaPa, is due to gathering activities specific to both components. Nevertheless, a possible specific NaPaC effect cannot be discarded. It could be that the enhanced effect of NaPaC on cell death is due not only to properties of NaPa but, at least in part, to new specific NaPaC mechanisms.

Finally, it is noteworthy that NaPa treatments at high concentrations can induce pathological effects (Thibault *et al*., 1994; Chang *et al*., 1999). The use of non-toxic new molecule, NaPaC, should limit side effects of NaPa and increase its therapeutic efficacy.

In conclusion, our results provide additional interesting clues in developing new anti-cancer drugs with specific triple activity: antiproliferative, aponecrotic and anti-angiogenic. The inhibition of angiogenesis is crucial for blocking tumour progression since tumour-associated high-density neovascularization is responsible for development of metastases.

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