

# Sodium phenylacetate enhances the inhibitory effect of dextran derivative on breast cancer cell growth in vitro and in nude mice

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**Summary** Sodium phenylacetate (NaPa) and carboxymethyl benzylamide dextran derivative (CMDB<sub>LS4</sub>) are able to inhibit growth of breast tumour cells. In this study, we explored whether the combination of NaPa and CMDB<sub>LS4</sub> may enhance their respective inhibitory effects on the MCF-7ras cell growth in vitro and in vivo. NaPa inhibited MCF-7ras cell proliferation by reducing the DNA replication concomitantly with a recruitment of cells in G0/G1 phase and by inducing apoptosis in a dose- and time-dependent manner. The addition of CMDB<sub>LS4</sub> potentiated the NaPa antiproliferative effect in the manner dependent on the ratio of CMDB<sub>LS4</sub> and NaPa concentrations. In nude mice, CMDB<sub>LS4</sub> (150 mg kg<sup>-1</sup>) or NaPa (40 mg kg<sup>-1</sup>) administrated twice a week, for 7 weeks inhibited MCF-7ras xenograft growth by 40% and 60%, respectively. The treatment by both, CMDB<sub>LS4</sub> and NaPa, decreased tumour growth by 83% without any toxicity. To better understand the mechanism of NaPa and CMDB<sub>LS4</sub> action we assessed their effect on mitogenic activity of MCF-7ras conditioned medium (CM) on BALBC/3T3 fibroblasts. CMDB<sub>LS4</sub> added to the CM, inhibited its mitogenic activity whereas NaPa had an anti-mitogenic effect when CM was prepared from MCF-7ras cells pretreated with NaPa. Thus, the antiproliferative effects of NaPa and CMDB<sub>LS4</sub> involve 2 different mechanisms explaining, at least in part, the possible synergism between them. Overall, this study points to the potential use of a combination of dextran derivatives with NaPa to inhibit the breast tumour growth. © 2001 Cancer Research Campaign <http://www.bjcancer.com>

**Keywords:** sodium phenylacetate (NaPa); carboxymethyl benzylamide dextran derivative (CMDB); synergism; MCF-7ras; breast cancer

We recently showed that carboxymethyl benzylamide dextran derivative (in particular CMDB7) inhibits the breast cancer cell proliferation in vitro and in nude mice (Bagheri-Yarmand et al, 1992, 1997, 1998a, b, 1999). This effect in vitro is associated with a decrease in the S-phase cell population and with an accumulation of cells in G1 phase of cell cycle (Bagheri-Yarmand et al, 1992). CMDB7 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 and Transforming Growth Factor-β1 (PDGF-BB, TGFβ Bagheri-Yarmand et al, 1998b). In vivo, CMDB7 treatment reduces the growth of MCF-7ras (Bagheri-Yarmand et al, 1998b) and FGF4-transfected HBL100 xenografts and decreases the tumour angiogenesis (Bagheri-Yarmand et al, 1998a).

Sodium phenylacetate (NaPa), a physiological metabolite of phenylalanine, is normally found in human plasma at micromolar concentrations. At higher concentrations, NaPa was reported to induce the cytostasis and the reversion of malignant phenotype of different cancer cells in vitro (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 synergistic manner, the effect of some molecules affecting the

growth factor pathways (Samid et al, 1993; Prasanna et al, 1996). For example, NaPa potentiated the antitumour activity of tamoxifen by increasing apoptosis induction in breast cancer xenografts in nude mice. Finally, NaPa had been used in phase I and II clinical trials on patients with malignant tumours (Thibault et al, 1994; Chang et al, 1999).

In this study, we investigated in vitro and in vivo the efficacy of combined treatment with NaPa and an industrial dextran derivative LS4 (Sterilyo Laboratories) whose composition is similar to CMDB7 one, on breast cancer cell growth. We have used the MCF-7ras cell line obtained by transfection of MCF-7 cells, isolated from pleural metastasis of breast adenocarcinoma, with v-Ha-ras oncogene. The MCF-7ras cells secrete high quantities of TGFα, TGFβ, epithelial growth factor (EGF) and insulin growth factor (IGF) (Albini et al, 1986). This cell line represents an oestrogen-independent cellular model corresponding to some malignant breast tumours (Spandidos and Agnantis, 1984) and it no requires the oestrogen supplementation to induce a high incidence of tumours in nude mice (Sommers et al, 1990). The analysis of CMDB<sub>LS4</sub>-NaPa combination effect was performed by the isobole method. Then, we explored the mechanism of action of CMDB<sub>LS4</sub>-NaPa combination.

## MATERIALS AND METHODS

### Compound preparation

NaPa was provided by Seratec (Paris, France). Industrial water-soluble dextran derivative CMDB named CMDB<sub>LS4</sub> was synthesized by Sterilyo Laboratories (Levallois-Perret, France)

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with some modifications of the procedure described by Mauzac and Jozefonvicz (1984) and by Chaubet et al (1995). In brief, CMDB<sub>LS4</sub> was prepared by simultaneous carboxymethylation and benzylamidation of dextran T40 in one step. After purification by ultrafiltration and lyophilization, the chemical composition (or degree of substitution, ds) of CMDB<sub>LS4</sub> was determined by acidimetric titration and elementary analysis of nitrogen. The dextran, carboxymethyl (CM) and benzylamide (B) was 0, 0.67 and 0.33, respectively. The average molecular weight, calculated considering CMDB subunits was  $PM_{T40} + ds_{CM} \times PM_{CM} + ds_B \times PM_B = 264.1 \text{ g mol}^{-1}$ .

### Cell culture

The human breast cancer MCF-7ras cells, derived from the pleural effusion MCF-7 cells transfected with v-Ha-ras, were kindly provided by Dr C Sommers (Georgetown University, Washington, DC, USA). BALB/c3T3 fibroblasts were purchased from American Tissue Culture Collection (Rockville, MA, USA). All cells were routinely grown in DMEM supplemented with L-glutamine 2 mM, penicillin 50 IU ml<sup>-1</sup>, streptomycin 50 µg ml<sup>-1</sup>, and fetal calf serum 10% (FCS) (Life Technologies, Inc, Gaithersburg, MD, USA) at 37°C in a 5% CO<sub>2</sub> humidified atmosphere.

### Growth inhibition experiments

MCF-7ras cell growth was assessed using the MTT-microculture tetrazolium assay (Mosmann, 1983). As previously described, CMDB7 antiproliferative effect is FCS concentration-dependent with an optimum observed in the presence of 1 to 2% of FCS (Bagheri-Yarmand et al, 1992). Briefly, the cells ( $4 \times 10^3$ ) were incubated in 2% FCS/DMEM for 24 h and then treated with NaPa or/and CMDB<sub>LS4</sub> at different concentrations for 72 h. Then, the cells were washed with PBS and incubated with 0.1 ml of MTT 2 mg ml<sup>-1</sup>. Complementary assay of cell cytotoxicity was performed by trypan blue exclusion test.

For analysis of DNA synthesis, the bromodeoxyuridine (BrdU) (Boehringer Kit, Mannheim, Germany) labelling was used. After incubation of MCF-7ras cells with NaPa at concentrations 1–40 mM, the BrdU 10 µM was added for 6 h and its uptake was measured by monoclonal antibody from mouse–mouse hybrid cells conjugated with peroxidase (1/100). After 3 washes with PBS, the substrate reaction with tetramethyl-benzidine was stopped with 25 µl of H<sub>2</sub>SO<sub>4</sub>, then the absorbance was determined at 405 nm wavelength using a microplate reader (Biorad, Marnes-La-Coquette, France).

The drug interaction between NaPa and CMDB<sub>LS4</sub> was evaluated by comparison of the dose–response curves of MTT assay for single agents and combined treatment. The characteristics of the combined treatment were then analysed by the isobole method (Berenbaum, 1981) analysing the equation  $Ac/Ae + Bc/Be = D$ , where Ac and Bc correspond to concentrations of drugs A and B used in the combined treatment, and Ae and Be are the concentrations of drugs able to produce the same magnitude of effect if used individually. If D (combination index) < 1, the effect of combination is synergistic, when D = 1 its effect is additive and in the case of D > 1 the combined action is antagonistic. Each experiment was performed in triplicate and the statistical significance (P) of the difference between D obtained and D = 1 was calculated by the Student's *t*-test.

### Preparation of conditioned medium (CM)

MCF-7ras cells ( $1 \times 10^6$ ) were treated with or without NaPa 20 mM for 48 h in DMEM supplemented with FCS 2% and washed twice with PBS. To obtain conditioned medium (CM) containing only the growth factors produced by MCF-7ras, the cells were incubated with serum-free DMEM for 24 h at 37°C. This medium (CM) was then harvested and used directly.

### CM experiments

BALB/c3T3 fibroblasts ( $5 \times 10^4$ ) were grown until preconfluence in DMEM supplemented with FCS 10%, washed twice with PBS and growth-arrested by serum starvation for 24 h. Then, 0.75 ml of the CM from MCF-7ras cells, treated or not with NaPa, was added to 0.25 ml of DMEM containing or not the CMDB<sub>LS4</sub> or NaPa. After 48 h, the proliferative effects of the CM was measured by counting the fibroblasts using a Coulter counter (Coultronics, Margency, France).

### Cell cycle analysis

MCF-7ras cells ( $5 \times 10^4 \text{ ml}^{-1}$ ) were plated in DMEM supplemented with FCS 2%. After 24 h of culture, NaPa was added for 72 h. Then, the cells were treated with BrdU (Pharmingen, San Diego, CA, USA) for 4 h. The incorporated BrdU was revealed using an anti-BrdU conjugated with fluorescein isothiocyanate (Boehringer Mannheim, Germany). Then, the cells were stained with propidium iodide (PI) (Boehringer) for 10 min and were analysed using a flow cytometer (Coulter Epics Laser, CA, USA).

### Evaluation of cell death

Trypan blue and propidium iodide (PI) enter into the cells during the ultimate stage of apoptosis or predominantly the first stage of necrosis when damage of the cell membrane has occurred. After the different treatment, the cells were harvested and the cell viability was determined by trypan blue exclusion. Other aliquot of the same sample cells were centrifuged and washed with annexin buffer (Boehringer). To reveal a phosphatidylserine translocation specific to apoptosis stage, the cells were treated with a FITC-labelled annexin V (Boehringer) followed by PI (Boehringer) and finally the cells were analysed using a flow cytometer.

### Xenografts in nude mice

MCF-7ras cells ( $5 \times 10^6$ ) were inoculated s.c. near the right mammary pad of 3-week-old athymic nude mice (nu/nu) (Harlan laboratory, Gannat, France). The animals ( $n = 40$ ) were kept in a temperature-controlled room on a 12h:12h light–dark schedule with food and water ad libitum. This protocol resulted in the development of single s.c. palpable tumours 4 weeks after cell inoculation in 90% of the mice. Then, the animals were arbitrarily placed in control ( $n = 10$ ) and treated groups ( $n = 10$ ). NaPa (40 mg kg<sup>-1</sup>), CMDB<sub>LS4</sub> (150 mg kg<sup>-1</sup>) or CMDB<sub>LS4</sub>/NaPa combination (150/40 mg kg<sup>-1</sup>, respectively) were injected s.c. in 0.1 ml of NaCl 0.9% twice a week for 7 weeks. The control group received s.c. 0.1 ml of NaCl 0.9%. A tumour volume was calculated as previously described (Bagheri-Yarmand et al, 1998a).

## Statistical analysis

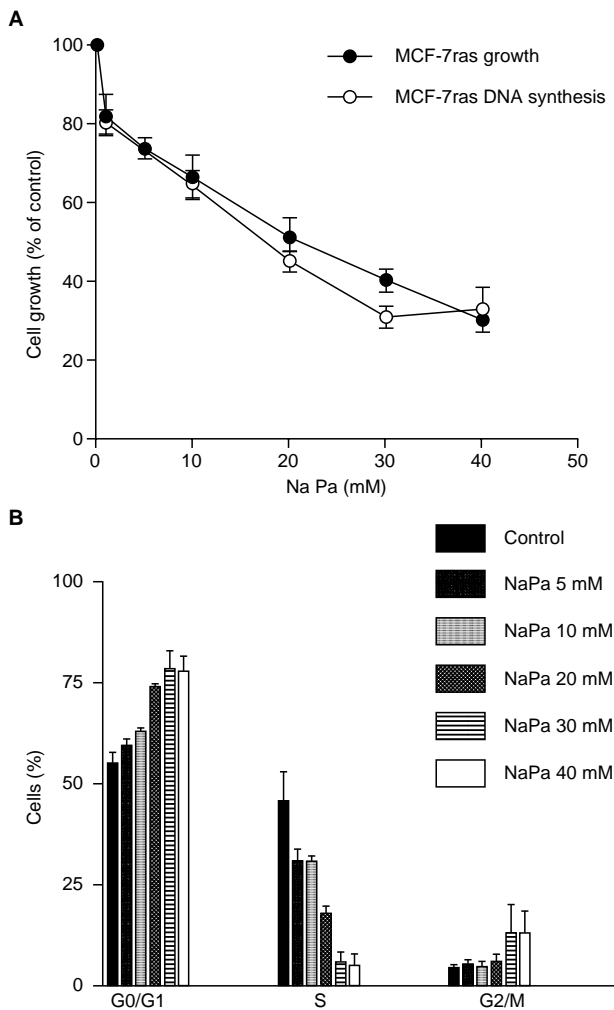
Multiple statistical comparisons were performed using ANOVA in a multivariable linear model. Some statistical comparisons were conducted using the Mann-Whitney *t*-test. *P* < 0.05 was considered statistically significant.

## RESULTS

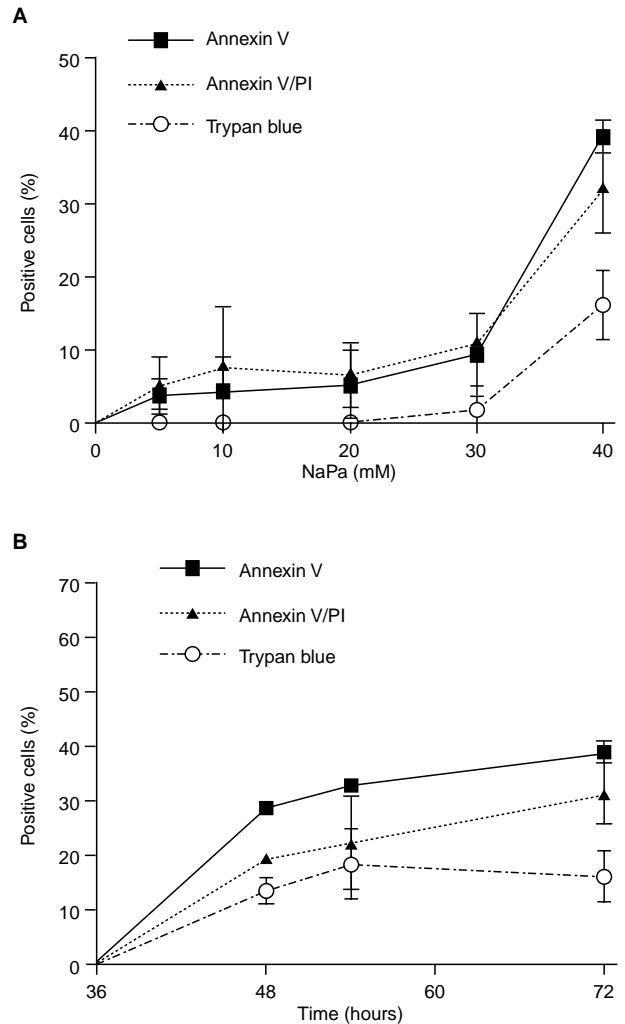
### Inhibition of MCF-7ras cell growth by NaPa

After 72 h exposure, NaPa induced a dose-dependent inhibition ( $IC_{50} = 20$  mM) of MCF-7ras cell growth (Figure 1A). This decline was associated with a similar inhibition of DNA synthesis (Figure 1A) as well as with a decrease in MCF-7ras cell number in S-phase and a major recruitment of cells in the G0/G1 phase (Figure 1B).

NaPa induced the cell death but only at high concentration (40 mM) (Figure 2A). At concentrations ranged from 5 to 30 mM NaPa did not induce apoptotic, necrotic or toxic effects as showed by the negative results of trypan blue exclusion test and annexin V



**Figure 1** Effect of NaPa on MCF-7ras proliferation (A) and cell cycle distribution (B). (A) Cells treated with NaPa for 72 h were assessed using MTT-assay as described in 'Materials and Methods'. Points represent the mean ( $\pm$  SD) of 3 independent experiments. (B) Distribution of cells treated with or without NaPa 5–40 mM in different phases of cell cycle. Each column represents a mean ( $\pm$  SD) of 3 independent experiments



**Figure 2** Apoptosis of MCF-7ras induced by NaPa. (A) Apoptosis of MCF-7ras cells treated with NaPa at various concentrations for 72 h. (B) Apoptosis of MCF-7ras cells treated with NaPa 40 mM for various time. Each point represents the mean ( $\pm$  SD) of 3 independent experiments

labelling. In the presence of NaPa 40 mM, we observed 40% of cells double labelled with annexin V/PI. Moreover, this result was confirmed using trypan blue exclusion test. The apoptotic effect of NaPa 40 mM on MCF-7ras cells was time-dependent (Figures 2B and 3). No apoptotic or necrotic effects were observed after 24 h of treatment (data not shown). After 48 h of treatment, 2 separate annexin V positive cell populations were observed. The first one represented 10% of total cells and contained annexin V single stained cells being in an early apoptotic phase. The other one included 18% of annexin V/PI double stained cells or trypan blue-stained cells progressing in a later apoptosis or in the early stage of necrosis. After 72 h of treatment, only annexin V/PI double-stained cell population (40%) was found.

### Improvement of MCF-7 ras cell growth inhibition by CMDB<sub>LS4</sub>-NaPa combination

As shown in Figure 4, the antiproliferative effect of CMDB<sub>LS4</sub> on MCF-7ras cells was potentiated in the presence of NaPa 5 mM. Indeed, CMDB<sub>LS4</sub> 18 mM alone was not able to inhibit the MCF-7ras cell growth by more than 38% ( $IC_{50}$  not reached), whereas in

**Table 1** Growth of MCF-7ras cells treated with different CMDB<sub>LS4</sub>/NaPa combined ratio

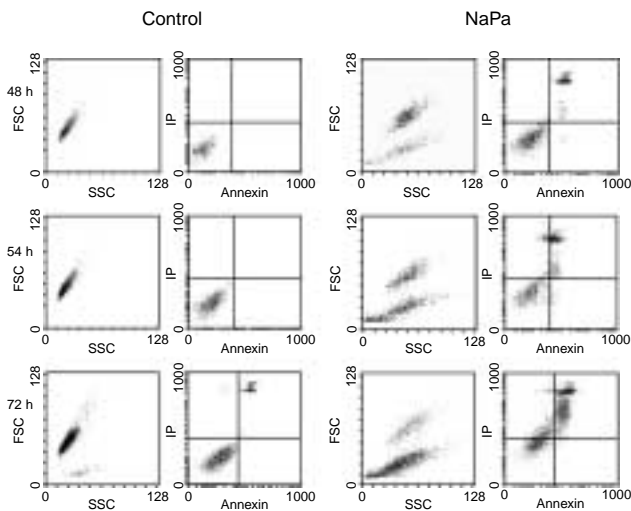
CMDB <sub>LS4</sub> /NaPa ratio	0.01		0.1		1		2		5		8	
Concentrations (mM)	C	N	C	N	C	N	C	N	C	N	C	N
	0.05	5	0.5	5	5	5	10	5	18.5	3.7	18	2.25
CMDB <sub>LS4</sub>	87.5 ± 0.8		82 ± 2		73 ± 1.7		69 ± 1		65 ± 2		65 ± 2	
NaPa	74 ± 1.7		74 ± 1.7		74 ± 1.7		74 ± 1.7		78 ± 2		78 ± 0.8	
CMDB <sub>LS4</sub> +NaPa	75 ± 2.8		70 ± 0.5		57 ± 1.8*		51 ± 0.8*		44 ± 2.2*		44 ± 2*	

MCF-7ras cells were treated with various ratios of CMDB<sub>LS4</sub>/NaPa (0.01, 0.1, 1, 2, 5, 8). Cell growth was determined by MTT assay (see 'Materials and Methods'). Each value represents the mean of 3 independent experiments. C = Concentration of CMDB<sub>LS4</sub> (mM); N = Concentration of NaPa (mM); \*P < 0.05 versus CMDB<sub>LS4</sub> or NaPa alone.

**Table 2** Effects of NaPa and CMDB<sub>LS4</sub> combination with a ratio = 5 on MCF-7ras cell proliferation

<sup>a</sup> CMDB <sub>LS4</sub> (A <sub>c</sub> ; mM)	NaPa (B <sub>c</sub> ; mM)	% I	CMDB <sub>LS4</sub> (A <sub>e</sub> ; mM)	NaPa (B <sub>e</sub> ; mM)	D
48 h					
3.7	0.75	33	18.5	17	0.44 <sup>b</sup>
7.4	1.5	45.8	>18.5	30	0.45 <sup>b</sup>
14.8	3	48.1	>18.5	32.1	0.89
18.5	4	50	>18.5	34	1.12
72 h					
3.7	0.75	51.4	>18.5	20.6	0.23 <sup>b</sup>
7.4	1.5	54.8	>18.5	23	0.46 <sup>b</sup>
14.8	3	53.3	>18.5	24	0.93
18.5	4	60.2	>18.5	29	1.13

<sup>a</sup> A<sub>c</sub> and B<sub>c</sub>, concentrations of agents A and B used in the combination treatment; A<sub>e</sub> and B<sub>e</sub>, concentrations of agents A and B able to produce the same magnitude of effect if used individually. D combination index (see Materials and Methods). <sup>b</sup> P < 0.05. % of inhibition determined by MTT assay: [1-(absorbance of cells in medium containing agents/absorbance of cells in control culture medium)] × 100.



**Figure 3** Flow cytometric analysis of annexin V/PI labelled MCF-7ras cells treated or not with NaPa. MCF-7 ras cells were treated with NaPa 40 mM for 48, 54 and 72 h. Cells were washed with annexin buffer and directly analysed after addition of annexin V-FITC and PI. FSC (forward scatter)/SSC (side scatter) pattern are shown in column 1 for control and 3 for NaPa treatment and annexin V/PI staining profile are shown in column 2 (control) and 4 (NaPa)

the presence of NaPa 5 mM, CMDB<sub>LS4</sub> was more potent and reduced the cell growth with an IC<sub>50</sub> of 12 mM.

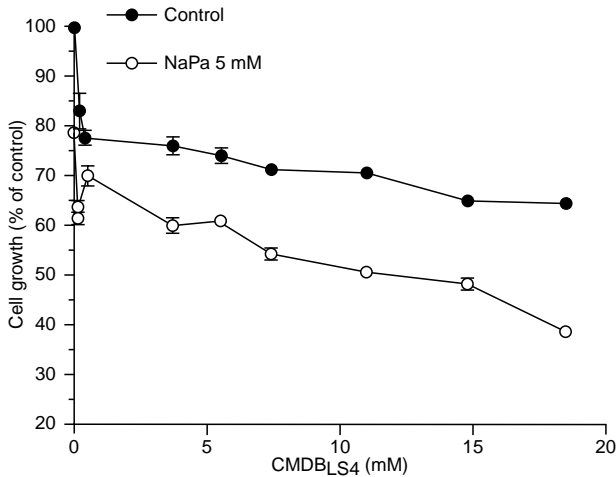
In order to better characterize the inhibition of MCF-7ras cell growth by CMDB<sub>LS4</sub>-NaPa combination, we evaluated this

inhibitory effect for various CMDB<sub>LS4</sub> and NaPa concentrations and for their different ratios (CMDB<sub>LS4</sub>/NaPa) in a range from 0.01 to 8 (Table 1). The inhibition of MCF-7ras cell growth induced by CMDB<sub>LS4</sub>-NaPa combination was compared to their single respective effect. Only for CMDB<sub>LS4</sub>/NaPa ratios > 1 the inhibitory effect was potentiated.

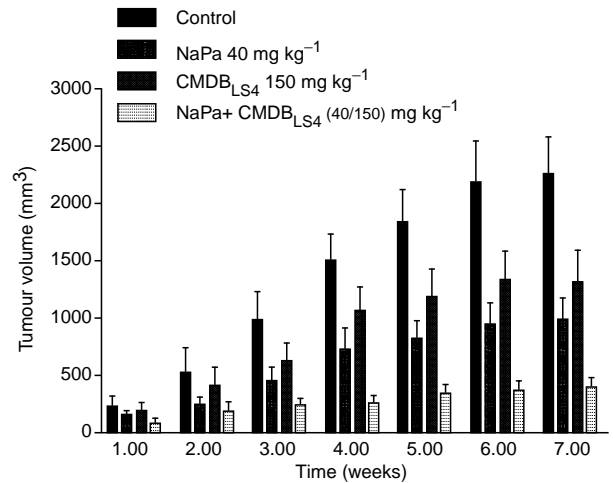
For CMDB<sub>LS4</sub>/NaPa ratio = 5, an additive inhibition (40–50%) was observed after 48 h and 72 h when higher drug concentrations were used (Table 2). A synergistic inhibitory effect was detected for lower concentrations of CMDB<sub>LS4</sub> and NaPa. In all cases of combined treatment, an increase in MCF-7ras cell growth inhibition was time-dependent. When used in combination, NaPa and CMDB<sub>LS4</sub> are efficient at much lower concentrations than when applied separately to induce the same inhibitory effect. The best combination seems to be 3.7 mM CMDB<sub>LS4</sub>/0.75 mM NaPa. The different combined treatments were not cytotoxic as determined by a trypan blue exclusion test (data not shown).

### Effect of NaPa and CMDB<sub>LS4</sub> on MCF-7 ras CM mitogenic activity on fibroblast proliferation

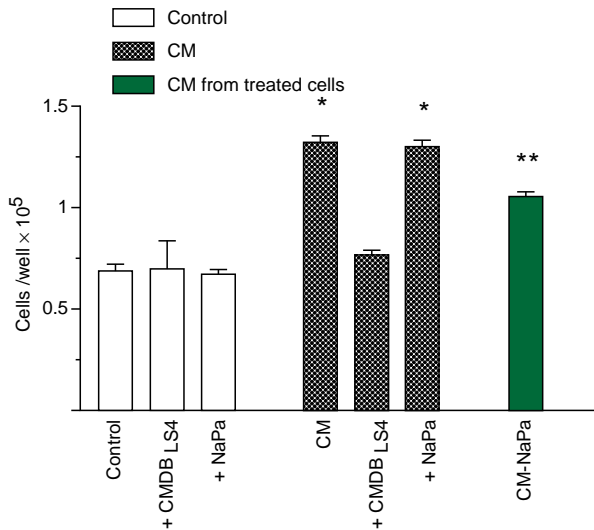
Previous reports indicated that MCF-7ras cells secreted the growth factors mitogenic for BALBc/3T3 fibroblasts (Bagheri-Yarmand et al, 1998a). We investigated whether CMDB<sub>LS4</sub> or NaPa could influence the mitogenic activity of MCF-7ras cell conditioned medium (CM) (Figure 5). CM stimulated BALBc/3T3 fibroblast proliferation 2-fold after 48 h of treatment. The addition of CMDB<sub>LS4</sub> 18.5 mM abolished the MCF-7ras CM stimulation of



**Figure 4** Antiproliferative effect of CMDB<sub>LS4</sub> in the presence of NaPa on MCF-7ras cell proliferation. The cells were treated with NaPa 5 mM and increasing concentrations of CMDB<sub>LS4</sub> for 72 h. Each point represents the mean ( $\pm$  SD) of 3 independent experiments



**Figure 6** In vivo growth inhibition of MCF-7ras carcinoma by NaPa, CMDB<sub>LS4</sub> and CMDB<sub>LS4</sub>-NaPa-combination (CMDB<sub>LS4</sub>/NaPa ratio = 3.75). MCF-7ras cells were inoculated in nude mice as described in 'Materials and Methods'. After 4 weeks, mice were treated with NaPa (40 mg kg<sup>-1</sup>), CMDB<sub>LS4</sub> (150 mg kg<sup>-1</sup>) and CMDB<sub>LS4</sub> combined with NaPa (150 mg kg<sup>-1</sup> and 40 mg kg<sup>-1</sup> respectively) twice a week for 7 weeks. Each chart represents a mean tumour volume (mm<sup>3</sup>) ( $\pm$  SD; *n* = 10)



**Figure 5** The effects of CMDB<sub>LS4</sub> and NaPa on the mitogenic activity of MCF-7ras conditioned medium (CM). CM from MCF-7ras cells treated (CM-NaPa) or not (CM) with NaPa 20 mM was tested for its effect on BALBc/3T3 fibroblasts growth. Control represents the fibroblasts treated with serum-free medium (see 'Materials and Methods'). Each column represents the mean ( $\pm$  SD) of 3 independent experiments. \**P* < 0.05 versus control, \*\**P* < 0.05 versus control and CM

fibroblasts growth. In contrast, NaPa 20 mM addition did not affect this CM. However, the pre-treatment of MCF-7ras cells with NaPa 20 mM for 48 h made a CM less effective on fibroblast proliferation.

**Antitumour activity of CMDB<sub>LS4</sub>-NaPa combination on MCF-7ras cell xenografted into nude mice**

We focused our attention on the in vivo effect of CMDB<sub>LS4</sub>-NaPa combination in a ratio of CMDB<sub>LS4</sub>/NaPa > 1 (Figure 6). NaPa (40 mg kg<sup>-1</sup> twice a week) inhibited by 60% (*P* < 0.05) the growth of MCF-7ras tumours after 7 weeks of treatment. No apparent toxicity was reported during mice treatment as their weight

remained stable between day 0: 28.4 g  $\pm$  1 and day 7: 30  $\pm$  1. CMDB<sub>LS4</sub> (150 mg kg<sup>-1</sup> twice a week) inhibited the tumour growth by 40% (*P* < 0.05) after 7 weeks of treatment without toxicity. When NaPa and CMDB<sub>LS4</sub> were administered at the same dose but in combination, the tumour growth was inhibited by 83% (*P*  $\leq$  0.001) after 7 weeks and no mice toxicity (mice weight at day 0: 28.9 g  $\pm$  0.6, day 7: 33.0  $\pm$  0.01) was noticed.

**DISCUSSION**

Our study demonstrates, for the first time to our knowledge, that NaPa enhances dextran derivative, CMDB<sub>LS4</sub>, antiproliferative effect on breast cancer MCF-7ras cells in vitro and in vivo. Indeed, NaPa or CMDB<sub>LS4</sub>, delivered alone for 7 weeks, inhibited MCF-7ras tumour growth by 60% and 40%, respectively, while the CMDB<sub>LS4</sub>-NaPa combination decreased MCF-7ras tumour growth by 83% without any toxicity. The effectiveness of the NaPa and CMDB<sub>LS4</sub> combination could be explained by their distinct mechanisms of action. MCF-7ras breast cancer cells secrete an important amount of mitogenic growth factors such as TGF $\beta$  and PDGF (Bronzert et al, 1987; Dickson et al, 1987; Knabbe et al, 1987). The mitogenic effects of these growth factors could be reduced by inhibition of their synthesis or/and their action on target cells. In our study, we showed that a treatment of cells with NaPa decreased the mitogenic activity of MCF-7ras conditioned medium on BALBc/3T3. This could be explained by a modulation of the synthesis and the release of growth factors like TGF $\beta$  in MCF-7ras breast cancer cells as reported previously by our laboratory (Thibout et al, 1998). Concerning CMDB<sub>LS4</sub>, we have demonstrated here that when added to CM it inhibited CM mitogenic effect on BALBc/3T3 fibroblasts. This finding argues for CMDB<sub>LS4</sub> interactions with growth factors contained in CM. Indeed, previous studies showed that dextran derivative interacted with heparin-binding growth factors like TGF $\beta$ , PDGFBB or FGF-2 and inhibited their mitogenic effect (Bagheri-Yarmand et al, 1998a, b). All our and others' observations suggest that NaPa

and CMDB<sub>LS4</sub> act on distinct targets involved in the tumour development. NaPa alters the mitogenic growth factor production and rendered the tumour cells quiescent in the G1 phase while CMDB<sub>LS4</sub> interacts with MCF-7ras growth factors and inhibits their mitogenic activities.

The additive and synergistic effect in vitro and in vivo was observed when the concentrations of CMDB<sub>LS4</sub> were 1 to 8-fold higher than NaPa concentration. If the CMDB<sub>LS4</sub>/NaPa ratio in vitro is lower than 0.1, the concentrations of CMDB<sub>LS4</sub> are probably too low to potentiate the NaPa inhibitory effect although they are efficient when CMDB<sub>LS4</sub> is used alone. When the ratio of CMDB<sub>LS4</sub> and NaPa concentrations is higher than 5, the inhibitory effect was unchanged suggesting that maximal efficiency of treatment is achieved at ratio 5. Within this same ratio, the combined antiproliferative effect was synergistic when NaPa and CMDB<sub>LS4</sub> concentrations were low and it became additive in the case of higher concentrations. This complexity of interaction between 2 drugs may be due to the fact that at different concentrations the drugs could induce various effects. For example, we showed that NaPa at concentration 5 mM, begun to induce G1-S transition arrest, whereas at higher concentration (40 mM), it generated the apoptosis after 48 h of treatment of 10% of cell as revealed by a single annexin V labelling and after 72 h, the destruction of cell membrane integrity probably due to necrosis or/and later apoptosis as showed by annexin V/PI labelling. The difficulty to distinguish the apoptotic cells from the necrotic ones may be, in part, explained by the fact that some drugs at high doses can induce new morphological features characteristic of both apoptosis and necrosis chimerically termed aponecrosis as proposed by Formigli et al (2000). MCF-7ras cell apoptosis induced by NaPa was previously described and associated with a reduction of the antiapoptotic *Bcl2* gene expression (Adam et al, 1995).

It is noteworthy that NaPa at high concentrations can induce pathological effects (Thibault et al, 1994; Chang et al, 1999) that may involve an induction of cell death as we observed in vitro. The use of combined treatments, reported in this paper, should reduce the dose of NaPa without decreasing a therapeutic efficacy and consequently should limit side effects. Indeed, we observed here an important MCF-7ras xenograft growth cytostatic inhibition (83%) using much lower concentrations of NaPa which are not toxic. Moreover, NaPa does not induce a multi-drug resistance (Adam et al, 1995).

In conclusion, our results clearly indicate the complementary action of NaPa and CMDB<sub>LS4</sub> on the autocrine or/and paracrine loops of growth factors to induce, at low doses, a tumour cell cytostatic effect. An important perspective of this study is based on the improvement of the antiproliferative properties of dextran derivatives depending on active subunit substitutions (Bagheri-Yarmand et al, 1992). Currently, we are investigating the antiproliferative effect of a carboxymethylbenzylamide dextran derivative substituted with active phenylacetate subunits.

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