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Report

Growth inhibition of human melanoma tumor cells by the combination of sodium phenylacetate (NaPA) and substituted dextrans and one NaPA–dextran conjugate

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We have studied the cytostatic effects of sodium phenylacetate (NaPA) in association with several substituted dextrans on human tumor melanoma 1205LU cells. We show that NaPA alone inhibits the growth of these cells (IC_{50} = 3.9 mM) while a weak inhibitory effect appears at a concentration of 37 μM (10 μg/ml) for a dextran methyl carboxylate benzylamide (LS17-DMCB). The precursors of LS17-DMCB [T40 Dextran and carboxymethyl dextran (LS17-DMC)] did not affect the growth of 1205LU cells. To potentiate the inhibitory activity of NaPA at low concentrations (below 5.6 mM), we have tested NaPA and LS17-DMCB in physical mixture (association) or linked together covalently (this conjugate is termed 'LS17–NaPaC'). We have observed an increase of the 1205LU cell growth inhibition effect with NaPA in association (IC_{50} 1.8 mM). For a concentration of 5 mM of NaPA (free in the case of association or linked in the case of conjugate), the association with dextran derivative exhibits a 4.6-fold higher efficacy than with NaPA alone (9 versus 41% surviving fraction), while the conjugate is 1.3-fold smaller (52% growth inhibition). By performing isobologram analysis of the IC_{50} data, we have shown a synergistic effect for a particular molar ratio of NaPA and LS17-DMCB (NaPA:LS17-DMCB = 0.35). [© 2002 Lippincott Williams & Wilkins.]

Key words: Cytostatic agent, growth inhibition, human melanoma, sodium phenylacetate, substituted dextrans.

Introduction

Over the last few decades, the number of melanomas has increased in the Caucasian population. Melanoma is always malignant and can become rapidly metastatic. Dacarbazine (Deticene®) with a partial and transitory response rate of 15–20% has emerged as the chemotherapy agent of reference. A response rate of the order of 40% has been observed with polychemotherapy (cisplatin, dacarbazine and vinblastine) and cytokines (interferon and interleukin-2).[1]

Currently, new non-toxic molecules like sodium phenylacetate (NaPA), with fewer side effects, are in clinical trials.[2–5] NaPA is a phenylalanine metabolite found throughout the phylogenetic spectrum, ranging from bacteria to man. In humans and primates, NaPA is a natural plasma component capable of conjugating glutamine with subsequent renal excretion of phenylacetyl-glutamine. The formation of phenylacetyl-glutamine is used in the treatment of a number of inborn errors of the urea cycle enzymes that lead to the accumulation of toxic concentrations of ammonia in body fluids. Because of the objectionable smell of NaPA, the less-volatile component, sodium phenylbutyrate (*tri*Butyrate®), is often administered as a substitute. However, NaPA was proposed as a potential antitumor agent.[2] It acts as non-toxic cytostatic agent on several malignant cell lines including leukemia, breast, prostate and brain cancer cells.[6–9] Moreover, Adam *et al.*[10] demonstrated the antitumoral and antiproliferative effects of NaPA on malignant breast epithelial MCF-7*ras* cells and its lack of toxicity. The authors showed that NaPA enhances the activity of another cytostatic agent, tamoxifen (Novaldex citrate®). A dose-dependent growth inhibition of several ovarian carcinoma cell lines has also been reported by NaPA.[8] However, the mechanisms by which NaPA can affect cell growth are

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under active investigation. Several mechanisms have been proposed, such as depletion of circulating glutamine,[2,11] inhibition of protein isoprenylation[12,13] or hypomethylation of DNA.[14]

Substituted dextrans, termed dextran methyl carboxylate benzylamide (DMCBs), are soluble polysaccharides tailored by statistical grafting of suitable chemical groups on a polymeric backbone. These polysaccharides exhibit a range of biological properties and some synthetic analogs have been shown to be non-toxic malignant cell growth inhibitors.[15] We showed that the compound termed CMDB7 inhibited breast cancer cell proliferation *in vitro* and in nude mice.[16,17] This antiproliferative effect was observed on pretumoral and tumoral human mammary epithelial cells (HBL100, HH9 and MCF7). The results indicated that the chemical nature and the proportion of groups linked to glucosyl units of the dextran chains are required to promote cell growth inhibition.[18] In addition, we showed that CMDB7 prevents tumor angiogenesis by binding to angiogenic growth factors and leading to non-activation of their receptors.[17,19] In addition, previous works show that the mechanism of CMDB7 activity involves cytokines sequestering.[19–23] Recently, it was shown that CMDB7 inhibits the mitogenic effect of vascular endothelial growth factor on human umbilical vein endothelial cells via the formation of a stable complex with this growth factor.[24]

Although most efforts have been directed towards drug targeting, others important objectives may be achieved using drug carrier conjugates, including long-term efficiency and reduction of side effects. In this study, we evaluated the antiproliferative effect of the DMCB termed LS17-DMCB associated or conjugated with NaPA on the 1205LU human tumor cell line. We have termed 'association' for a physical mixture of the two components, and 'conjugate' for the covalent binding of the dextran derivative and phenylacetate. We investigated a possible synergistic effect of the two molecules both in association and conjugated.

Materials and methods

Materials

T40 Dextran (M_n = 28 800 g/mol, M_w = 40 400 g/mol, batch 228608) was supplied by Pharmacia (St Quentin en Yveline, France). All 'analytical grade' chemicals and solvents were purchased from Aldrich (St Quentin Fallavier, France), Acros (Noisy le Grand, France), Interchim (Montluçon, France) and Sigma (St Quentin Fallavier, France). NaPA was provided by Seratec (Paris, France) and used as neutral aqueous solution (pH 7) (20%, v/v).

The derivatized dextrans named LS17-DMC and LS17-DMCB were prepared as previously described.[25,26] In brief, the synthesis of LS17-DMC involved carboxymethylations of starting dextran performed with monochloroacetic acid in alkaline medium at 60°C for 1 h. Then, LS17-DMCB was obtained quantitatively by coupling benzylamine to carboxylic groups of carboxylated dextran using *N*-ethoxycarbomyl-2-ethoxy-1,2-dihydroquinoleine as coupling agent in water:ethanol (1:1, v/v) mixture at room temperature. The LS17–NaPaC conjugate was prepared by esterification of NaPA with free OH of the LS17-DMCB. This process involves the action of the pyridium salt of the LS17-DMCB with phenyl acetyl chloride in dimethylformamide at room temperature. Then the solution was purified with an ultrafiltration device using a cut-off membrane of 5000 Da (Filtron, St Quentin en Yveline, France). After freeze-drying, the product termed LS17–NaPaC (Figure 1) was obtained quantitatively in a yield of 70%. The methylcarboxylate content was determined on dried aliquots of dextran derivatives by acidimetric titration in water:acetone mixture (1:1;v/v) acidified with 2N nitric acid. The degree of substitution (DS) was determined from N and S elemental analysis performed by CNRS (Gif/Yvette, France). HPSEC chromatography analysis, ultraviolet spectrum (λ=250 nm) and acidimetric titration were used to determine the DS in sodium phenylacetate. Table 1 shows the characterization of these different components.

Cell line and cell culture

Human melanoma cells (1205LU; Wistar Institute, Philadelphia, PA) were selected from WM793, established from a primary lesion for their ability to induce metastasis in nude mice. After four sequential passages of WM793 cells *in vivo* in nude mice, variant cell lines 1205LU were obtained.[27] 1205LU cells constitutively express high levels of pleiotrophin mRNA and induce metastasis from s.c. tumors to the lungs of experimental animals.[28]

The cells were routinely grown at 37°C in a humidified atmosphere of 95% air and 5% CO_2 in tissue culture flasks (Costar, Dominique Dutscher, Issy Les Moulineaux, France). They were maintained in serum-free keratinocyte medium (Keratinocyte-

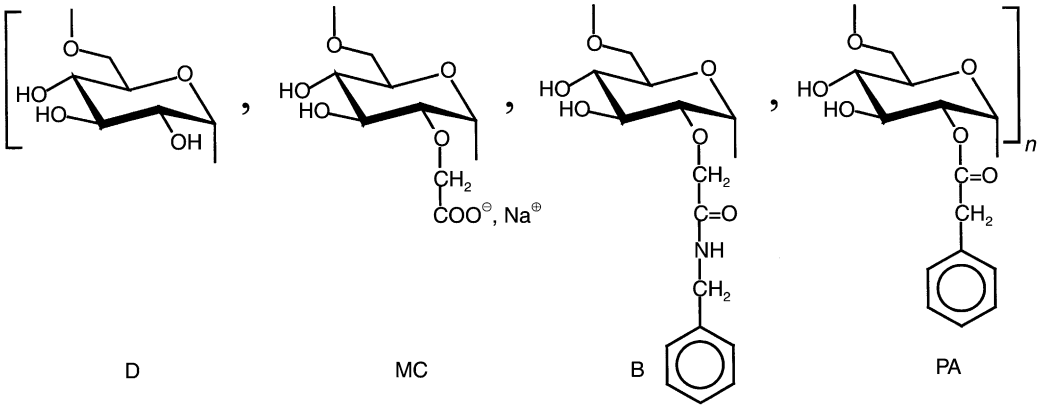

Figure 1. Structure of LS17–NaPAC. D= native dextran unit; MC= sodium methylcarboxylate dextran unit; B= benzylamide dextran unit and PA= phenylacetate dextran unit.

Table 1. Characterization of T40 Dextran and substituted dextrans

	DSa			
	D	MC	B	PA
T40 Dextran	3	0	0	0
LS17-DMC	2	1	0	0
LS17-DMCB	1.99	0.61	0.4	0
LS17–NaPaC	1.59	0.61	0.4	0.4

[a]The characterization is expressed in terms of DS of T40 Dextran (D), methylcarboxylate (MC), methylcarboxylate benzylamide (B) and phenylacetate (PA) groups for three available sites (OH groups) per glucosyl unit.

SFM; Life Technologies, Cergy Pontoise, France) supplemented with 20% Leibovitz with glutamaxI (L-15; Life Technologies) and 10% heat-inactivated fetal bovine serum (FBS; Eurobio, Les Ulis, France).

Assay for cell growth

Cells (2×10^5/well) were seeded onto 24-wells plate in 1 ml of Keratinocyte-SFM with 20% L15 containing 10% FBS and allowed to adhere overnight in a humidified 5% CO_2 atmosphere at 37°C. T40 Dextran, LS17-DMC, LS17-DMCB, NaPA, the association LS17-DMCB + NaPA and the conjugate LS17–NaPaC were formulated in Keratinocyte-SFM media with 20% L15 and 2% FBS. Each well was washed with Keratinocyte-SFM media with 20% L15 and the different compounds were added to the plate at different concentrations (three wells for each concentration). After 48, 72, 96 and 120 h of incubation, the cells were trypsinized and counted with a Coulter counter model ZM (Coultronics, Evry, France). The net growth of 1205LU cells was obtained by subtracting the cell number at the end of the experiment from the starting cell number (J_0 which is the number of cells just as added to each compound at different concentrations). This procedure was repeated 4 times. The percentage of growth inhibition was determined from the relation:

$$\%\,\text{Inhibition} = \left[1-\left(\frac{\text{net growth in the presence of compound}}{\text{net growth in control dishes}}\right)\right]\times 100$$

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay

The MTT microculture tetrazolium assay has been used to evaluate single and combined compound cytotoxicity *in vitro*. Single and combined drug cytotoxicity was assessed according to which follows protocol. MTT (Sigma) was dissolved in PBS with Ca^{2+} and Mg^{2+} (Sigma) at a concentration of 2 mg/ml. Exponentially growing tumor cells were harvested, counted and seeded at appropriate density into 96-well microtiter plates (10^4 cells/100 μl/well). Growth medium was used as a blank and controls (untreated cells) were included in the microtiter plate. Three wells were set up for each point. Tumor cells were continuously exposed to the drugs for 72 h. At the end of this treatment time, 100 μl of 2 mg/ml MTT was added to each well and the microtiter plates re-incubated at 37°C for 4 h in a 5% humidified and 95% CO_2 atmosphere. Then, the reduction of MTT in the presence of cellular dehydrogenases

yielded formazan crystals at the bottom of the plate. The formazan crystals were dissolved in 100 μl/well of dimethyl sulfoxide (Sigma). The plate was agitated for a minimum of 15 min to solubilize the crystals. The formazan absorbance was read on a microplate autoreader (Bio-Tek Instruments EL311; OSI, Paris, France) at 570 nm wavelength. Each value represents five experiments. The percentage of proliferation (P%) was determined from the relation:

$$P\% = \left(\frac{\text{DO product} - \text{DO J}_0}{\text{DO control} - \text{DO J}}\right) = 100 - I\%$$

J_0 is the number of cells just as added to each compound at different concentrations. I% is the percentage of inhibition observed for each compound.

Isobologram analysis

The *in vitro* cytotoxic effect by NaPA associated or conjugated with derivatized dextran was evaluated using isobologram analysis.[9] For each combination of the compound, we have constructed isobolograms for the isoeffect 50 (P%= 50% after 72 h of incubation time). An additive relationship between LS17-DMCB and NaPA would produce a straight line whose extremes are represented by the IC_{50} values calculated for each compound tested alone. Points to the right of this line indicate a sub-additive or antagonistic relationship, whereas points to the left of the line indicate a supra-additive or synergistic relationship. The IC_{50} values were determined for each compound alone (denominator) and then the concentration of compound combination corresponding to 50% inhibition (numerator) was determined. Results are expressed as the CI:

$$\text{CI} = \left(\frac{\text{concentration of A in combination}}{\text{concentration of A alone}}\right) + \left(\frac{\text{concentration of B in combination}}{\text{concentration of B alone}}\right)$$

Statistics

The difference between numerical results was considered significant if $p < 0.05$, as determined by the Student's t-test.

Results

Growth inhibition and cell viability

We first investigated whether the native T40 Dextran, LS17-DMC and LS17-DMCB can inhibit the 1205LU melanoma cell growth in the presence of increasing polymers concentrations ranging from 0.01. to about 10 mM, for incubations of 48 and 72 h. No cell growth inhibition effect of native T40 Dextran and LS17-DMC was observed at 48 and 72 h, irrespective of concentrations even over 8 mM (Figure 2A and B).

A cell growth inhibition of approximately 30% was observed at 2 mM of glucosyl units for 48 and 72 h of treatment with LS17-DMCB, which is a dextran

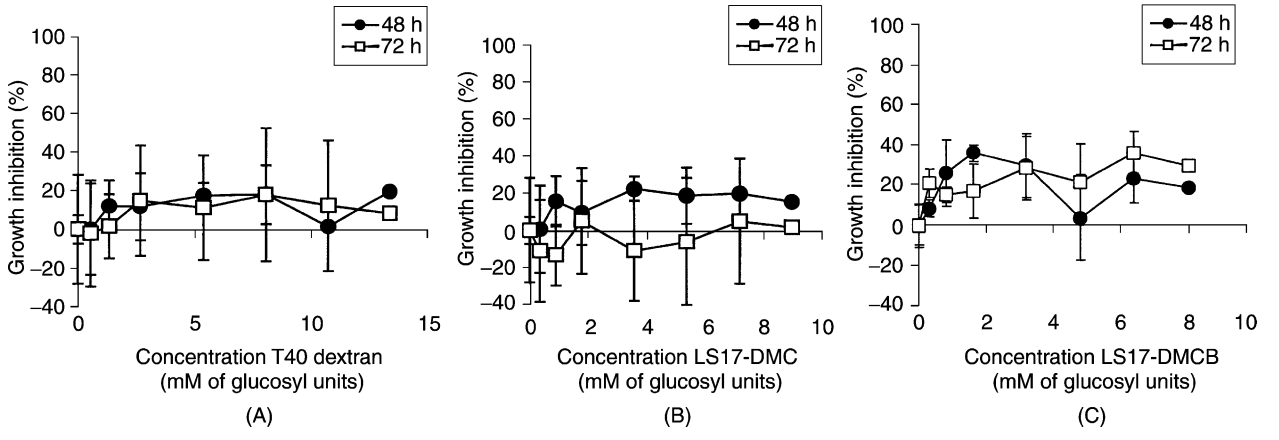

Figure 2. Inhibition of 1205LU cell growth treated for 48 and 72 h by native T40 Dextran, LS17-DMC and LS17-DMCB. Data are expressed as a percentage of cell number incubated with each polymer versus control incubated without polymer. Each point represents the mean of three experiments.

derivative bearing not only methylcarboxylate groups (DS=0.61) but also benzylamide groups (DS=0.4) (Figure 2C). Cell viability, as determined with the Trypan blue exclusion assay, was greater than 98% at concentrations of 73.6 mM (20 mg/ml) of each of the polymers. This result indicates that native T40 Dextran, LS17-DMC and LS17-DMCB are not cytotoxic (data not shown).

Likewise, we investigated the effect of NaPA alone, of the association of NaPA with LS17-DMCB and of the LS17–NaPaC conjugate on 1205LU cell growth. A dose-dependent inhibitory effect of NaPA on 1205LU cell growth was observed (Figure 3A) and this cell growth inhibition increases with time from 48 to 72 h. Cells treated with NaPA were perfectly viable (98% as compared to the untreated control cells). A significant inhibitory capacity of the association of NaPA with LS17-DMCB was found (Figure 3B). The dose–response plots reported in Figure 3(B) show an effect without difference between 48 and 72 h. It is noteworthy that this activity is observed at non-toxic concentrations (from 0.056 to 5.6 mM of NaPA in the association). By comparison to the results with NaPA alone, the effect is more important with the association after 48 h of exposure of the compound. At very small concentrations of NaPA (0.056 mM) when associated to LS17-DMCB, an inhibition of cell growth of the order of 10% was observed, while no significant inhibition appeared with the NaPA alone.

Finally, the conjugate LS17–NaPaC was investigated as shown in Figure 3(C). A time-dependent relationship was obtained between 48 and 96 h of incubation, while no time-dependent effect was detected over 96 h. Overall, the activity of the conjugate did not overcome that of the association of the two compounds.

Cytotoxic activity assays

Figure 4 shows the percentage of surviving 1205LU cells treated for 72 h in the presence of the different compounds at various concentrations. The IC_{50} doses (expressed in NaPA equivalent) were 3.9, 1.8 and 5.5 mM for NaPA, LS17-DMCB+NaPA association and LS17–NaPaC conjugate, respectively (Figure 4A–C). LS17-DMCB alone inhibited cell growth (Figure 4D).

At a concentration of 5 mM of NaPA (or NaPA equivalent), the surviving fraction is 41 and 9%, respectively, for NaPA alone and LS17-DMCB + NaPA association (Figure 4A and B). The efficacy in terms of surviving fraction is 4.6-fold higher with the association than with NaPA alone. Surprisingly, the conjugate is less efficient than NaPA alone: the surviving fraction is 52% at 5 mM of NaPA equivalent (Figure 4C). Under this condition, the conjugate is 1.3-fold smaller than the NaPA alone.

The IC_{50} doses (expressed in LS17-DMCB equivalent) were 20.3, 5.2 and 13.8 mM of glucosyl units for LS17-DMCB, LS17-DMCB+NaPA association and LS17–NaPaC conjugate, respectively. The LS17-DMCB+NaPA association had the lowest IC_{50}, and

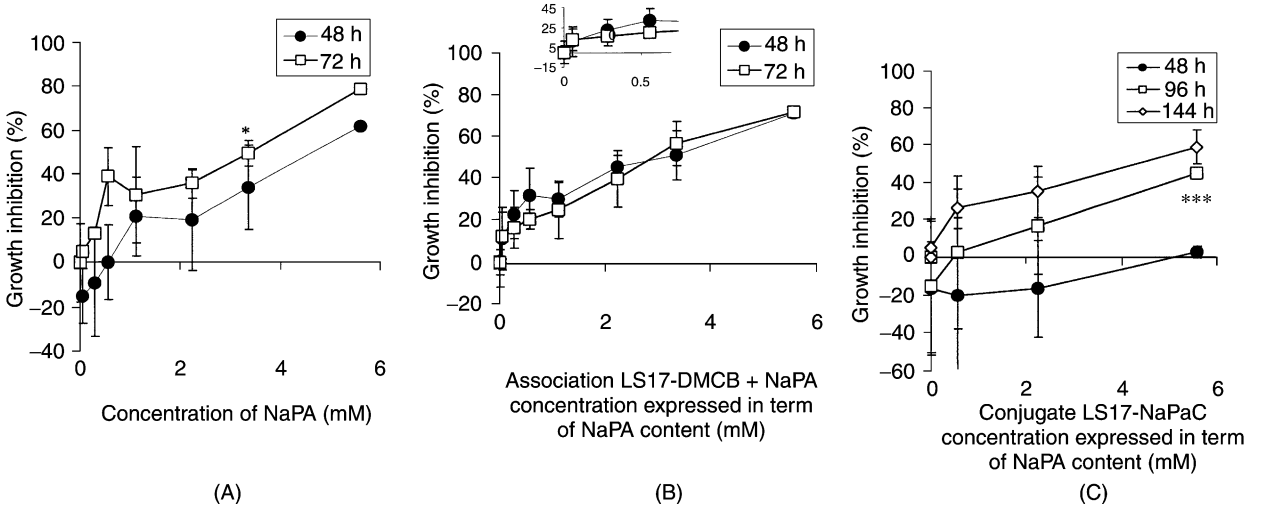

Figure 3. Inhibition of 1205LU cells growth by NaPA, associated or conjugated with LS17-DMCB. Concentration is expressed in terms of NaPA content. Data are expressed as a percentage of cell number incubated with each polymer versus control incubated without polymer. Each point represents the mean of three experiments ($^{*}p<0.05$, $^{***}p<0.004$).

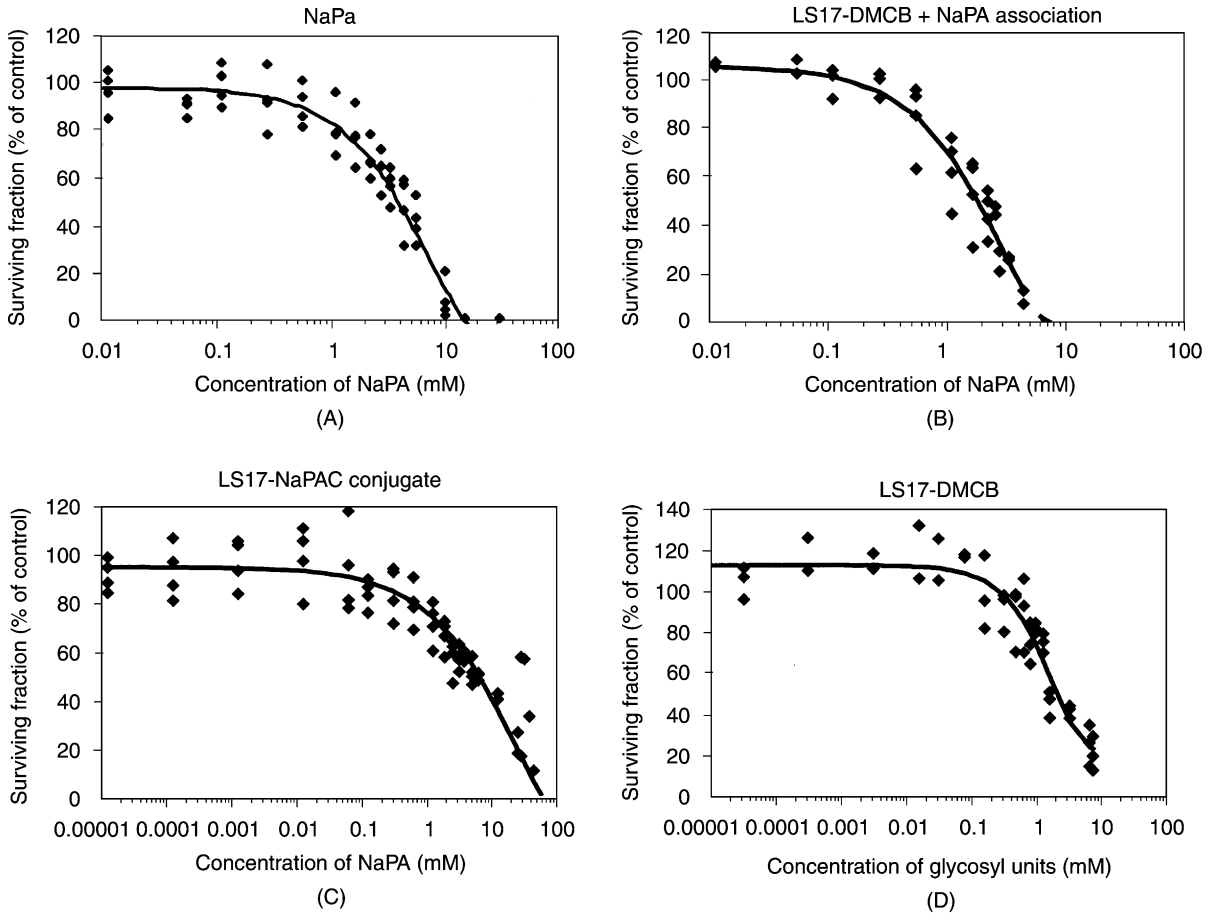

Figure 4. Percentage of viable 1205LU human melanoma cells treated for 72 h in the presence of increasing concentrations of NaPA, LS17-DMCB, association LS17-DMCB + NaPA and conjugate LS17–NaPaC. Each point represents the mean of five experiments.

was more efficient in inhibiting the growth of the 1205LU cells than NaPA alone, LS17-DMCB alone and the LS17–NaPaC conjugate.

Isobologram analysis

We examined the LS17-DMCB+NaPA association effect on 1205LU cells at different ratios after a treatment of 72 h by the MTT microculture tetrazolium assay. Table 2 shows the IC_{50} values (expressed as concentration of NaPA equivalent) and the index of combination (CI) for each ratio tested. At the 0.027 ratio, 50% inhibition of cell growth is obtained at a very low concentration of NaPA (1.11 ± 0.14 mM). The association of NaPA with LS17-DMCB is antagonistic ($CI > 1$) at all ratios except for the 0.35 ratio, which induces a synergistic effect on 1205LU cells ($CI < 1$). The isobologram

Table 2. IC_{50} values calculated from dose–response plots relative to each ratio of the association of NaPA and LS17-DMCB tested on tumor 1205LU cell line after 72 h [five independent experiments were performed (each concentration in triplicate)]

NaPA:LS17-DMCB ratio[a]	Concentration of NaPA (mM) required for 50% inhibition of 1205LU growth	CI[b]
0.027	1.00 ± 0.14	1.95 ± 0.27
0.135	3.03 ± 0.60	1.80 ± 0.40
0.35	1.84 ± 0.70	0.70 ± 0.26
0.54	3.70 ± 0.44	1.19 ± 0.11
1.36	4.11 ± 0.75	1.17 ± 0.17
5.4	5.09 ± 0.75	1.31 ± 0.20

[a]NaPA is expressed in mM. LS17-DMCB is expressed in mM of glucosyl units.
[b]Combination Index (*cf.* Materials and methods).

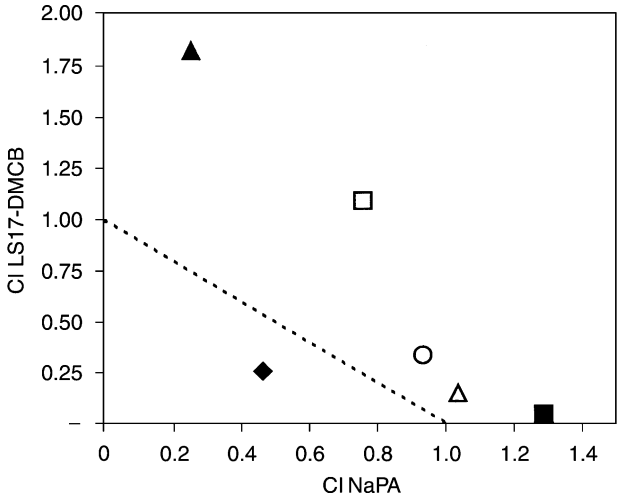

Figure 5. Isobologram for interaction of NaPA with LS17-DMCB at different ratios (5.4, solid squares; 1.36, open triangles; 0.54, circles; 0.35, diamonds; 0.135, open squares; 0.027, solid triangles). Each point represents the mean of five experiments.

presented in Figure 5 indicates the relative concentration of NaPA or LS17-DMCB required to inhibit 50% of tumor cells at the same incubation time (72 h). An additive relationship between NaPA and LS17-DMCB would produce a straight line as indicated by the dotted line whose extremes are represented by the IC_{50} values calculated for each compound tested alone. All points except one are located to the right of this line, indicating an antagonistic relationship. The single point to the left of the line (see Table 2: 0.35 ratio, CI=0.70) indicates a synergistic relationship, this effect being achieved with a relatively low concentration of NaPA (1.84 ± 0.70 mM).

Discussion

Our results show that sodium phenylacetate (NaPA) inhibits the growth of melanoma 1205LU cells at a concentration of 0.5 mM (79 μg/ml) after 72 h of treatment. This cytostatic effect is in agreement with previous studies showing that NaPA in the millimolar range has an antiproliferative activity against human melanoma cells *in vitro*[29] without any toxicity. A number of studies have reported a strong inhibitory activity of NaPA at low concentration (2.0–5.7 mM) causing 50% reduction in cell proliferation (IC_{50}). By comparing our results with those reported by Liu *et al.*[30] on human melanoma cells, we found a similar IC_{50} (3.9 mM). The mechanisms of NaPA antitumor activity are not completely understood, but a recent study showed that NaPA modifies the synthesis of growth factors secreted by MCF7 and MCF7*ras* tumor cells leading to cell proliferation inhibitions.[31]

On the other hand, our work evidences that the LS17-DMCB has a negative modulation effect on melanoma 1205LU cell growth. This effect confirms the results obtained with other tumoral cells.[15,18] A low but detectable inhibitory effect appears at a concentration of 37 μM (10 μg/ml) of LS17-DMCB. The absence of effect with the precursors, T40 Dextran and LS17-DMC, indicates that benzylamide groups linked to glucosyl units of dextran are required to promote cell growth inhibition. The chemical groups on dextran derivatives could be responsible of differences in uptake mechanisms of the compounds. Previous work has shown that such dextran derivatives displayed an *in vitro* growth-inhibitory activity on breast pretumoral and tumoral cells, depending on their composition.[16,18]

NaPA was found to potentiate the antitumor activity of suramin, a drug acting through different mechanisms.[6] Our study shown that the human melanoma cell phenotype was sensitive to NaPA. The effect of NaPA in association with different dextran derivatives has been performed using isobologram analysis, which represents a precise and widely used method to validate drug interactions. Our investigation demonstrates that the NaPA in association with LS17-DMCB potentiates the single drug effect. In our model, a synergistic effect has been detected for a combination of NaPA and LS17-DMCB, which corresponds to the composition of the LS17–NaPaC conjugate tested. Interestingly, an antagonistic relationship effect has generally been obtained for NaPA in association with LS17-DMCB, except for one ratio (NaPA:LS17-DMCB=0.35). Witzig *et al.*[32] had also shown a synergy or additivity with combinations of phenylacetate and conventional drugs in inducing apoptosis on both the MYS and 8226 human myeloma cell lines.

The LS17–NaPaC conjugate is 5.8-fold less efficient in inhibiting the 1205LU cell growth than the association of both compounds at 5 mM of PA. This result can reflect the lipophilicity of NaPA, which could affect its capability to enter into and cross the lipid bilayer of cell membranes.[7] It is possible that the free carboxyl group of NaPA (Figure 1) may be essential for some aspects of the antitumor activity.[33] Our synergistic effect can be also explained by the two different mechanisms of actions of these two molecules. Thus, polysaccharides like LS17-DMCB

prevent growth factors from reaching their receptor on the tumor cells.[17,19] On the other hand, NaPA was shown to inhibit signaling pathways of these growth factors.

Combination and conjugation of NaPA with a dextran derivative, LS17-DMCB, may provide a strategy to elicit a therapeutic effect without the need to maintain unrealistically high serum concentrations of drugs. Structure–function relationships of these compounds suggest that each derivative conjugated or associated acts on melanoma cells via different mechanisms. Further studies are now under investigation to clarify the mechanism of the capacity of sodium phenylacetate conjugated or associated to dextran derivatives to inhibit the growth of human melanoma cancer through *in vitro* and *in vivo* studies.

Acknowledgments

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