Tumor Growth Inhibition, Apoptosis, and Bcl-2 Down-Regulation of MCF-7ras Tumors by Sodium Phenylacetate and Tamoxifen Combination

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Abstract
We demonstrated previously the antitumoral and antiproliferative effects of sodium phenylacetate (NaPA) on malignant breast epithelial MCF-7ras cells and its lack of toxicity. The present in vivo protocols were as follows: (1) a control group; (2) a NaPA-receiving group (450 mg/kg) through s.c. osmotic pumps (ALZA Corp.) for 2 weeks, followed by 2 weeks without treatment; and (3) a tamoxifen (TAM)-receiving group (20 mg/kg two times per week). The second group was further divided as follows: (a) a group receiving same doses of NaPA; (b) a TAM-receiving group; and (c) a group receiving both NaPA and TAM. Although tumors treated by TAM alone (group 3) showed progressive regrowth after 6 weeks, indicating an escape from antiestrogen inhibition, the TAM-administered group, following 2 weeks of NaPA pretreatment (group 2b), showed significant tumor regression of about 40% after 8 weeks. This effect was amplified to over 60% (P < 0.001) by simultaneous administration of the two drugs (group 2c). The last group displayed about 30% apoptotic-like nuclei, together with lower proliferation index, and less tumor vascularization, as compared to less than 5% terminal deoxytransferase-mediated dUTP-X nick end labeling-positive nuclei, highly vascularized tumors, in the TAM-treated group. Furthermore, in situ administration of 4-OH-tamoxifen induced a Bcl-2 up-regulation in MCF-7ras cells, which was completely abolished by NaPA pretreatment. The combination of NaPA and OHT induced significant cell differentiation with cell cycle accumulation in the G0-G1 phase.

Introduction
MCF-7 cells are known to express functional ERs1 (1—4) and to form tumors in nude mice stimulated with estrogens (5). By contrast, MCF-7 cells transfected with Ha-ras, although expressing ERs and responding to E2 stimulation, can form tumors in nude mice in the absence of E2 supplementation (6). On the other hand, TAM and its active metabolite OHT, although able to inhibit MCF-7 tumors, may in certain conditions induce estrogen-like morphological changes in cultured MCF-7 cells (7) and an invasiveness of an artificial basement membrane (8, 9). The same is evident when ras-transfected MCF-7 cells are used (10).

Having shown previously that NaPA may induce apoptosis and Bcl-2 down-regulation in MCF-7ras cells, we designed a study to explore the effects of TAM and OHT with or without pretreatment with NaPA on tumor growth, apoptosis, differentiation, and Bcl-2 regulation.

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5 The abbreviations used are: ER, estrogen receptor; E2, estradiol; OHT, 4-hydroxytamoxifen; TAM, tamoxifen; NaPA, sodium phenylacetate; TUNEL, terminal deoxytransferase-mediated dUTP-X nick end labeling; SF, steroid free.
(Boehringer Mannheim). Each sample was performed in triplicate. For in vitro apoptotic death assay, cells cultured on Lab-Tek chamber slides (Nunc, Inc., Naperville, IL) were treated with NaPA and/or OHT as indicated. Adherent cells were stained with the DNA-specific fluorochrome diamino-2-phenylindole (Boehringer Mannheim Biochemica) in a 1 μg/ml methanol solution. Cell counts were performed within 20 min of staining on a Zeiss Axiophot epifluorescence microscope. Experiments were performed in triplicate with at least 150 cells scored at each point. Fragmented or condensed nuclei were scored as apoptotic. Intact or mitotic nuclei were scored as normal.

**Direct Immunofluorescence Coupled to Flow Cytometry.** MCF-7ras cells (5 × 10⁶) detached from stock cultures under different treatments, were incubated with FITC-conjugated anti-Bcl-2 monoclonal antibody (F-7053) and with a FITC-conjugated negative control reagent (Mo IgG1/FITC; X-927) from DAKO. Fluorescence intensity was measured with a FACSscan III (Becton Dickinson, Mountain View, CA). Cell cycle assays were done using propidium iodide, following the manufacturer’s directions.

**Statistical Analysis.** In the figures, the data are presented as the mean values for a 95% confidence interval. Multiple statistical comparisons were performed using ANOVA in a multivariate linear model. Other statistical comparisons were completed using Student’s unpaired t test; P < 0.05 was considered statistically significant.

### Results

Studies were carried out in vivo and in vitro, comparing the effects of NaPA with estrogen and antiestrogens on the proliferation and apoptosis induction of human breast cancer cells, MCF-7ras.

**Effect of NaPA Pretreatment on TAM-treated MCF-7ras Tumor Xenografts.** Tumors treated by TAM showed regrowth, indicating an escape from drug antihormonal effect after 7 weeks of treatment (Fig. 1A). The NaPA-treated group displayed growth inhibition without regression of tumors. In the TAM-treated group following 2 weeks of continuous NaPA infusion, tumors displayed a 40% volume regression as compared to the NaPA-treated group. This effect was amplified to over 60% (P < 0.001) by simultaneous administration of the two drugs following the same NaPA pretreatment.

**Apoptosis Induction by TAM and NaPA on MCF-7ras Xenografts.** The TUNEL assay showed that after 2 months of TAM treatment, the NaPA-pretreated MCF-7ras tumors displayed about 30% apoptotic-like nuclei as compared to less than 5% in the relapsing group (Table 1). The NaPA-treated group displayed also about 30% of apoptotic-like nuclei (Table 1).

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**Fig. 1.** The NaPA pretreatment prevents OHT-induced tumor regrowth and MCF-7ras cell proliferation in vitro. Tumors were measured at weekly intervals beginning 3 weeks after 6 × 10⁶ cells/animal inoculation. The treatment (NaPA and TAM) was started 2 weeks after the appearance of single fat pad tumors. After 2 weeks of continuous s.c. infusion and 2 weeks with no treatment, the NaPA-treated group was further divided into a NaPA-treated group, a TAM-treated group, and a group receiving both NaPA and TAM (A): MCF-7ras cells were maintained for 3 weeks in E₂-free DMEM and then pretreated with E₂ (B) or with 5 mM NaPA (C) 24 h before exposure for 4 days to NaPA and/or OHT (B) and E₂ and/or OHT (C). Results are expressed as relative percentages to controls (vehicle-treated only) and represented as mean value for four experiments; bars, 95% confidence interval.
The number of positive cells for each monoclonal antibody, or of TUNEL (+) apoptotic nuclei, was estimated in six high-power fields containing 60–80 cells/field at x400. Results were described as follows: +++, >90%; +++, 50–80%; +++, 30–50%; +, 5–20% of cells stained; −, no staining in a few cells (<1%). Stromal fibroblasts: +++, dense; +, moderate; +, sparse. Number of vessels was measured on Mason’s trichromic stained sections: +, few (3–4); +++, intense (7–10); ++++, (>10) at ×100; and +/+−, very rare (1–2 tumor).

Effect of Estrogen (E2) Pretreatment on the MCF-7ras Growth Response to NaPA and OHT. In SF media, the E2 treatment of MCF-7ras cells caused a growth stimulation of about 230% as compared to untreated cells (Fig. 1B). The addition of NaPA showed a 80% growth inhibition. Administration of 10−9 M OHT on MCF-7ras grown in SF media stimulated cell proliferation about 30% after 4 days of treatment (Fig. 1B). This stimulatory effect was blocked by the addition of NaPA (Fig. 1B).

Effect of NaPA Pretreatment on the MCF-7ras Cell Growth Responses to E2 and OHT. Pretreatment with 5 mm of NaPA of MCF-7ras cells cultured in SF media blocked the agonistic effect of OHT and inhibited about 35% of the stimulatory effect of E2 (Fig. 1C).

Effect of NaPA Pretreatment on the Bcl-2 Up-Regulation Induced by OHT Treatment. Administration of NaPA showed a time-dependent down-regulation of Bcl-2 as compared to controls (Fig. 2G, profiles 2 and 1 compared to 3). Thus, whereas after 24 h of NaPA treatment cells were displaying a heterogeneous population of Bcl-2 expressions (Fig. 2G, profile 2), after 4 days of treatment, they showed a homogeneously reduced Bcl-2 expression (Fig. 2G, profile 1).

These findings were correlated with some morphological patterns as follows: (a) few cells began to show apoptotic-like changes with slight irregular perinuclear condensed chromatin (Fig. 2B) as compared to untreated cells (Fig. 2A); (b) cells displayed a pseudotrabecular morphology where both processes of proliferation and apoptosis were present (Fig. 2C); and (c) cells were disposed in a well-differentiated pseudo-alveolar structure (Fig. 2D).

By contrast, MCF-7ras cell proliferation exerted by OHT in SF media was associated with an up-regulation of the Bcl-2 oncoprotein expression, as compared to the untreated cells (Fig. 2G, profiles 6 compared to 3, respectively), and cells did not display apoptotic-like nuclei (Fig. 2E). When OHT was added to the NaPA-pretreated cells, about 35% of cells with apoptotic-like nuclei were observed (Fig. 2F). This was associated with the block of OHT-induced Bcl-2 up-regulation (Fig. 2G, profile 5 compared to 6, respectively).

Differentiation Effect of the Simultaneous Administration of NaPA and OHT. We tested to see if the combination of these two drugs would have any increased efficiency on cell growth inhibition in standard media, which contained steroids. Results showed that 48 h of 5 mm NaPA treatment exerted an inhibitory effect on the S phase of the cell cycle: 20% of cells entered into G2 as compared to 35% for the untreated cells (Fig. 3F, cycle 3 compared to 4, respectively).

The effect of OHT (10−9 M) was not as significant after the same time period (Fig. 3F, cycle 2). On the contrary, administration of both drugs exerted an enhanced growth inhibition even after this short time of treatment (Fig. 3F, cycle 1).

Furthermore, after 10 days after a single NaPA and OHT simultaneous administration, MCF-7ras cell cycles displayed G0–G1 arrested cells (Fig. 3G, cycle 1). This block was accompanied by the appearance of well-differentiated cells disposed on the entire peripheral zone of the cell clusters (Fig. 3E and detailed in C) as compared to clusters of NaPA-treated cells with occasionally differentiated cells but with apoptotic bodies presenting cells as well (Fig. 3D). These features were very different when compared to the untreated cells that displayed a higher cellular density (Fig. 3A), a changed pH media (acidic) with appearance of dead cells suggesting nutrient deprivation and catabolite accumulation. This observation was correlated with the appearance of altered DNA strands characteristic of dead cells (Fig. 3G, cycle 4). On the contrary, both treatments, by OHT or by NaPA, induced cell growth inhibition, where lower cell density-displaying cells (Fig. 3, B and D) were correlated with G1 phase cell accumulation (Fig. 3G, cycles 2 and 3, respectively). Furthermore, MCF-7ras cells treated by a combination of NaPA and OHT displayed higher intracytoplasmic lipid synthesis and accumulation (Fig. 4D) as compared to the untreated cells (Fig. 4A). This differentiation was observed also in the NaPA-treated cells (Fig. 4B). However, lipid droplet accumulation was a pattern given exclusively by the NaPA treatment, because they were absent in the OHT-treated cells (Fig. 4, C compared to B).

Discussion

Our study shows that the growth of experimental MCF-7 tumors in nude mice is affected by TAM stimulation after long-term antiestrogen treatment (14). MCF-7ras cell xenografts in nude mice are known to be estrogen independent, although the expression of ERs may in a way mimic the clinical situation, where tumors escape from the antiestrogenic effect of TAM is seen after some duration of treatment (15, 16).

MCF-7ras cells cultured in DMEM supplemented with charcoal-stripped FCS and treated with OHT (10−9−10−7 M) showed a dose-dependent cell growth stimulation from 25 to 50% as compared to controls (data not shown). In contrast, no stimulation was seen for cells after a 24-h NaPA pretreatment (5 mm). The estrogen-like effects of the TAM-active metabolite or of the 17β-estrogen were effectively blocked when they were added after a NaPA pretreatment. Furthermore, direct immunofluorescence coupled with flow cytometry analysis of the variation of Bcl-2 expression in MCF-7ras cells showed a direct correlation between the growth-stimulatory effect of OHT and the Bcl-2 up-regulation. This agonistic effect, in SF media, on cell proliferation or and Bcl-2 synthesis was blocked by NaPA treatment. Furthermore, simultaneous administration of NaPA and OHT displayed a significant synergistic inhibitory effect on the growth proliferation of the same cell line in standard media.

About 50% of ER-positive breast tumors respond to TAM treatment. Several explanations for the nonresponsiveness of these tumors have been suggested. Estrogens initiate gene transcription by binding to the hormone-binding domain of ER and inducing the activity of two activating factors, AF1 and AF2 (17). TAM inhibits ER activity at AF2 but stimulates it at AF1. Thus, if a tumor becomes resistant to the inhibitory effect of TAM on AF2, it might stimulate cell growth via AF1. Growth stimulation by TAM may be synergistic with growth factors acting via AF1. This can activate Ras followed by activation of the protein kinase Raf. The loss of control by steroids of the cross-talk between steroids and growth factors via the ER appears to be the major mechanism for the control of breast tumor growth (17).

It was shown that one of the mechanisms by which NaPA exerts its differentiation effect is by interfering with isoprenylation of GAP-GTP-dependent proteins like Ras (18). Thus, it may be possible to...
Fig. 2. Induction of programmed cell death and Bcl-2 down-regulation by NaPA. After E2 depletion, cells were seeded at 10^4 cells/well in 24-well plates, left for overnight attachment, and then treated with NaPA. Morphological changes characteristic of programmed cell death, including chromatin condensation and nuclear fragmentation in MCF-7ras cells by 4',6-diamidino-2-phenylindole staining are shown: controls (A), after exposure to NaPA (5 mM) for 24 h (B), 48 h (C), and 4 days (D); 4 days of OHT treatment (10^{-8} M) (E); or OHT after a 24-h NaPA pretreatment (F). Cells are stained with 4',6-diamidino-2-phenylindole. G. differential expression of Bcl-2 expression of MCF-7ras by cytometry immunostaining. Immunofluorescent cycles correspond to: a NaPA treatment during 4 days (1), 24 h (2), versus controls (3), 24 h NaPA pretreatment followed by 4 days of OHT treatment (5), or 4 days after single OHT addition (6). Black profiles 4 and 7 correspond to cells incubated with irrelevant isotype antibodies. Values indicate relative cell counts (vertical lines) and log fluorescence intensity (horizontal lines).
Fig. 3. Effect of NaPA and OHT on morphology of MCF-7ras cells (phase-contrast microscopy) and cell cycle (flow cytometry). Untreated cells cultivated in: A, 10% FCS containing DMEM; B, 10 days after a single addition of OHT; C, a combination of NaPA and OHT (with detail in D); or D, NaPA. Flow cytometry of 1 × 10⁶ MCF-7ras cells after 48 h treatment (F) or 10 days (G). Cell cycles are: 1, NaPA and OHT; 2, OHT; 3, NaPA (J); versus 4, no treatment. The same cells presented here in phase-contrast microscopy A–E were assayed by flow cytometry in G. A, B, and D, ×100; F and G, ×250.

delay the onset of loss of cross-talk using such agents, for example by pharmacological inhibition of tyrosine kinase growth factor pathways with NaPA.

Recent studies indicate that signals which ultimately cause cellular proliferation must interplay with mechanisms that control cell death. The bcl-2 proto-oncogene plays an important role in this process because the expression of the protein prevents programmed cell death induced by a variety of stimuli and stress factors including growth
factor depletion (19), c-myc expression in the absence of growth factors (20), p53 (21), and most chemotherapeutic agents (22). In addition, Bcl-2 synthesis in the ER-positive MCF-7 human breast cancer cell line is dependent on E2 (23), and very recently, it was shown that E2 promotes increased survival of MCF-7 cells exposed to different chemotherapeutic drugs and develops drug resistance through Bcl-2 protein induction (24). The up-regulation of the Bcl-2 synthesis by OHT observed by our flow cytometry coupled with direct immunofluorescent studies was coherent with the absence of apoptotic cells in vitro as well as the proportion of the apoptotic-like nuclei observed in tumor biopsies. These results strongly suggest that Bcl-2 may have a role in TAM-induced resistance in MCF-7ras cells.

Taken together, these results suggest that: (a) adjuvant treatment by NaPA may prevent tumor escape, which usually develops after a long-term treatment with TAM; and (b) the differentiating effect of NaPA can be enhanced by simultaneous administration of TAM. The differentiation effect of NaPA may lead to the “normalization” of the interactions between AF1 and AF2, thus allowing TAM and OHT to act only as inhibiting factors. In any case, the synergy shown between the two nontoxic agents (25) holds promise as to a possible clinical effect of their combination.

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