Sodium Phenylacetate (NaPa) Improves the TAM Effect on Glioblastoma Experimental Tumors by Inducing Cell Growth Arrest and Apoptosis

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Abstract. Background: Multiform glioblastomas represent the most aggressive brain tumors. Here, the cooperative effects of sodium phenylacetate (NaPa) and/or tamoxifen (TAM) on CNS1 and 9L glioblastoma cell lines in vitro and in an experimental animal tumor model were investigated. Materials and Methods: The drug effects on cell cycle and apoptosis were investigated by flow cytometry. CNS1 cells were implanted subcutaneously in nude mice to form tumors which were then treated with NaPa, TAM or NaPa/TAM. Results: A significant inhibitory effect of NaPa on the two glioma cell lines (LD50 of 10 mM) was observed. 10–5 M of TAM inhibited approximately 35% of 9L cell growth, and 90% of CNS1 cell growth. When a combination of both drugs included 10–9 M of TAM, inhibition of about 50% of 9L cell growth and 75% of CNS1 cell growth occurred. The NaPa/TAM combined treatment increased the number of G0/G1 arrested cells and apoptotic cells as compared to treatments with NaPa or TAM alone. Inhibition of CNS1 tumor growth were observed after a two week treatment with NaPa (32 mg/kg/day) or TAM (6 mg/kg/day). Conclusion: These results showed a synergistic effect between these two drugs on tumor cell proliferation, caused by cell cycle arrest in the G0/G1 phase and by induction of apoptosis.

Keywords: Tamoxifen, sodium phenylacetate, glioma, apoptosis.

Malignant gliomas represent about 40% of all primary brain tumors in humans (1). They are considered incurable and even multimodal approaches, including surgery, radiation therapy and chemotherapy, prolong the survival of patients by only a few months (2). This resistance to therapy is due in part to glioma cell migration, phenotypic heterogeneity and a very poor immune response in the brain, as well as to difficulties in chemotherapeutic agents passing through the blood-brain barrier. Therefore, new approaches to therapy of these tumors should be evaluated, such as combined therapies.

Previous reports have shown an abnormal de novo synthesis of cholesterol from mevalonate (MVA) in malignant glioma cells as compared to their normal counterparts (3, 4). Intermediates of the MVA pathway include farnesyl and geranylgeranyl residues which are involved in the posttranslational modification of intracellular signaling proteins critical to tumor growth and maintenance of the malignant phenotype (5). Two enzymes control the MVA pathway: 3-hydroxy-3-methylglutaryl coenzymeA (HMG-CoA) reductase and MVA pyrophosphate (MVA-PP) decarboxylase which controls the MVA within cells. Sodium phenylacetate (NaPa), a physiological product of phenylalanine metabolism present in micromolar concentrations in human plasma, can specifically inhibit the MVA-PP decarboxylase (6, 8). This metabolite used in the treatment of hyperammonemic disorders (9) causes cytostasis and differentiation of glioma cells in vitro and in animal models (8, 10). Our previous results have shown a cytostatic inhibition of MCF-7ras cell growth by NaPa as a strong proapoptotic molecule both in vitro and in vivo in an experimental tumor model (11, 12).

Breast cancer is the second cancer morbidity cause in the USA after lung cancer and is the first cause of cancer death in Europe. About 50% of patients develop metastasis within
5 years of diagnosis. Two main types of treatment are used to treat breast cancer, hormone therapy and chemotherapy. Tamoxifen (TAM), a synthetic non-steroidal anti-estrogenic compound, is usually the first choice of hormone therapy for early and advanced breast carcinomas.

Tamoxifen (TAM) belongs to a class of drugs known as selective estrogen receptor (ER) modulators which function like estrogen in some tissues such as the uterus, but block actions of estrogen’s in others such as the breast. TAM inhibited the induction of rat mammary carcinoma by dimethylbenzanthracene (DMBA) and caused the regression of DMBA-induced tumors in a rat model; it seems that TAM exerted its anti-tumoral function by binding to the estrogen receptor. In addition to its inhibitory effect on the estrogen receptor, TAM may affect tumor growth by inhibiting angiogenesis. This activity may contribute to the therapeutic effect of TAM in estrogen receptor-negative tumors. The TAM/receptor complex seems to inhibit RNA synthesis in tumor cells and secretion of the growth factors TGF-β and EGF, mediated by estrogen (13). Furthermore, protein kinase C within tumor cells is inhibited by TAM. Such inhibition by high doses of TAM may be responsible for a reduction of malignant glioma proliferation (14, 15).

Our previous work has demonstrated strong tumor growth inhibition of MCF-7 ras mammary tumors in athymic mice by NaPa and TAM (12). Tumors treated by TAM alone showed strong growth inhibition until six weeks post-treatment, however subsequent progressive regrowth indicated an escape from anti-estrogen inhibition. Relapse was suppressed by simultaneous administration of the two drugs, suggesting that this combined therapy could treat breast cancer more efficiently.

A study was therefore designed to evaluate the potential of a combined therapy with NaPa and TAM on glioblastoma 9L and CNS1 cell lines. Based on the in vitro results, the in vivo experiments were carried out to evaluate potential clinical applications.

Materials and Methods

Cell lines. 9L is a rat gliosarcoma cell line and CNS1 is a rat glioblastoma cell line, which form tumors within one week when implanted subcutaneously in athymic mice. These two cell lines were generously given by Dr. J.Y. Delattre of the hospital Pitié-Salpêtrière, Paris.

Cell culture. Cells were grown in Dulbecco’s minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100,000 U/L penicillin and 100 mg/L streptomycin in a 5% CO₂ incubator at 37°C.

Chemicals. Tamoxifen was purchased from Sigma (St Quentin, France) solubilized and stored at −20°C. Sodium phenylacetate (NaPa) was purchased from Seratec (Courville, France) kept in solution and stored at +4°C. TAM and NaPa were simultaneously added to cell cultures as indicated in figure legends.

Proliferation assay. The cells were plated at a density of 20,000 cells per well in a 24-well plate. The next day, the drugs at different concentrations in the medium with 2% of serum were added to the cells. Four days later, the cells were trypsinised and counted with a Coulter counter (Coultronics, Margency, France). The assay was performed in triplicate. The values were given as mean±SD (standard deviation).

Quantification of apoptosis by flow cytometry. The CNS1 cells were treated with 5, 10 or 20 mM NaPa alone or with 0.01, 0.1 or 1 μM of TAM for 48 hours and then adherent and detached cells were harvested by 0.1% EDTA. DNA was stained with 50 μg/ml propidium iodide and analyzed with a FACScalibur (Becton Dickinson, Franklin Lakes, NJ, USA). The DNA histograms were interpreted using Cell Require and ModFit software (Verity Software House, Topsham, ME, USA).

Subcutaneous glioma tumor models. Four- to 6-week-old athymic nu/nu female mice (Harlan, France) were used for the experiments. The CNS1 cells (2x10⁶) in 100 μL of Hank’s balanced salt solution (HBSS) were injected into the right posterior flank of 20 mice (5 mice/group). At day 3 after tumor inoculation and once a day for 14 days, 5 mice/group were treated with a subcutaneous injection of tamoxifen (6 mg/kg/d; n=6), NaPa (32 mg/kg/d; n=6), or vehicle (n=6) at the periphery of the tumor. The mice were sacrificed by cervical elongation 27 days after tumor cell injection. Tumors were measured, excised and weighed. In situ apoptotic cell detection was performed using ApopTag Peroxidase Kit (Intergen, Burlington, USA). The apoptosis index was calculated by assessing the percentage of ApopTag-positive cells in five high-power microscopic fields (x400) for each specimen from different sections.

Results

NaPa and TAM effects on glioblastoma cell proliferation. The dose response experiment revealed that 10 mM of NaPa inhibited about 50% of cell proliferation and 20 mM of NaPa inhibited approximately 80-90% of cell proliferation, without any cell toxicity. Similar results were obtained with both cell lines (Figure 1A and 1B). On the other hand, concentrations up to 10⁻⁶ M TAM alone had no significant effect on cell growth. However, 10⁻⁵ M of TAM inhibited approximately 35% of 9L and 90% of CNS1 cell growth (Figure 1A and 1B).

Combined NaPa/TAM treatment of 9L and CNS1 glioblastoma cells. In order to determine if the combination of both drugs could further enhance the treatment efficiency, 10 mM of NaPa with increasing concentrations of TAM were added to the cells. In this case, 10⁻⁹ M of TAM was able to inhibit about 50% of 9L cell growth and 75% of CNS 1 cell growth. With higher concentrations (10⁻⁸, 10⁻⁷, 10⁻⁶ M of TAM) the efficacy of inhibition was not further increased. With 10⁻⁵ M of TAM, cell growth was dramatically inhibited: more than 95% for the 9L cells and
more than 90% for the CNS1 cells (Figure 2). The LD_{50} of both cell lines was dramatically reduced, from 10 μM to 0.001 μM (Table I) when NaPa and TAM were simultaneously used to treat the cells.

**NaPa and TAM effects on cell apoptosis.** Morphological changes of both cell lines were observed when the cells were treated with NaPa or NaPa/TAM. Cells became flatter and developed pseudopodia and filopodia (data not shown). The distribution of cells in the S phase (DNA content between 2n and 4n) was lower for the treated as compared to control cells (Table II). The proportion of apoptotic cells (DNA content less than 2n) increased from approximately 7% to 20% in the treated cells. The appearance of these apoptotic cells was associated with the antiproliferative effects of TAM, NaPa and the combined NaPa/TAM treatments (Table II). However, cytostatic effects were evident from the difference between the number of S-phase cells and G0/G1 arrested cells.

**Table I. LD_{50} of treated cell lines (9L and CNS1).**

<table>
<thead>
<tr>
<th></th>
<th>NaPa</th>
<th>TAM</th>
<th>NaPa + TAM</th>
</tr>
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<tbody>
<tr>
<td>9L</td>
<td>10 mM</td>
<td>&gt;10 μM</td>
<td>0.001 μM (TAM)</td>
</tr>
<tr>
<td>CNS1</td>
<td>10 mM</td>
<td>5 μM</td>
<td>&lt;0.001 μM (TAM)</td>
</tr>
</tbody>
</table>

Under this experimental condition, the LD_{50} of both cell lines was dramatically reduced, from 10 μM to 0.001 μM TAM by the combination treatment when the concentration of NaPa was 10 mM. These results indicated that this combined therapy could kill tumor cells more efficiently.

**NaPa, TAM and NaPa/TAM treatment of CNS1 subcutaneous tumors.** In order to extend our in vitro results, in vivo experiments were performed by treating subcutaneous CNS1...
Table II. Effects of NaPa and TAM on cell cycle and apoptosis (flow cytometry).

<table>
<thead>
<tr>
<th>CNS1</th>
<th>% of apoptosis</th>
<th>% of cells in S-phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.2%</td>
<td>57.0%</td>
</tr>
<tr>
<td>5 mM NaPa</td>
<td>7.0%</td>
<td>35.5%</td>
</tr>
<tr>
<td>10 mM NaPa</td>
<td>9.1%</td>
<td>29.4%</td>
</tr>
<tr>
<td>20 mM NaPa</td>
<td>18.7%</td>
<td>26.3%</td>
</tr>
<tr>
<td>0.01 µM TAM</td>
<td>6.1%</td>
<td>39.9%</td>
</tr>
<tr>
<td>0.10 µM TAM</td>
<td>8.4%</td>
<td>39.3%</td>
</tr>
<tr>
<td>1.00 µM TAM</td>
<td>7.9%</td>
<td>33.3%</td>
</tr>
<tr>
<td>10 mM NaPa + 0.01 µM TAM</td>
<td>21.4%</td>
<td>38.4%</td>
</tr>
<tr>
<td>10 mM NaPa + 0.10 µM TAM</td>
<td>19.3%</td>
<td>37.5%</td>
</tr>
<tr>
<td>10 mM NaPa + 1.00 µM TAM</td>
<td>23.8%</td>
<td>31.8%</td>
</tr>
</tbody>
</table>

Cells were plated per flask 1.0x10^6 in DMEM medium with 10% FCS. The next day, the drugs were added at different concentrations as indicated in medium with 2% of FCS. Cells were trypsinized, washed and assayed by flow cytometry 48 hours later.

Discussion

Tamoxifen has been used for more than two decades for the hormonal therapy of breast carcinomas that express the ER, consistent with the known function of tamoxifen as an ER agonist/antagonist. However it has been established that tamoxifen can also exert an antitumor effect in ER-negative breast cancer and other types of carcinoma. Several groups have reported that tamoxifen has antiglioblastoma activity in vitro and in vivo in animal models (16, 18). In clinical trials tamoxifen has been given to patients with recurrent malignant glioblastoma as a last treatment when all other therapies have failed (19, 20). Given the ease of administration and the low toxicity of tamoxifen it is an attractive proposition to improve its antitumor efficacy by its use as an adjuvant treatment with another molecule. Whether or not the cytostatic and proapoptotic effects of NaPa demonstrated by us and others (11, 12) can improve the antiglioblastoma effect of tamoxifen was explored.

To our knowledge, this is the first report on the treatment efficiency of a combined therapy with TAM and NaPa, an inhibitor of MVA-PP decarboxylase, on glioblastoma cells. These results indicated that the combination of NaPa and TAM allows the reduction of their concentrations used in this assay and gives a much stronger inhibition of tumor cell growth, showing a synergistic effect. Thus our results showed a synergistic effect between these two drugs on tumor cell proliferation, especially on the CNS1 cells, and apoptosis was observed following the drug treatments. In fact the NaPa/TAM combined treatment increased the number of G0/G1 arrested cells and apoptotic cells as compared to treatments with NaPa or TAM alone. The treated cells were viable as determined by the trypan blue exclusion test. This suggests that the doses used to observe the synergistic effects were not toxic, which was consistent
with the observation that the cells were undergoing apoptosis as opposed to cell necrosis. The determination of cell apoptotic index in untreated and treated tumors showed that the apoptotic index was significantly increased in the NaPa/TAM combined treatment as compared to single treatments with TAM or NaPa (p<0.001). Our results clearly showed a significant apoptotic effect of TAM, NaPa and combined NaPa/TAM treatments on glioblastoma tumors without significant and/or strong effect on tumor size. The results strongly suggested a qualitative more than a quantitative effect of combined therapy by inducing an apopto-necrotic effect, without exhibiting such a marked effect on the size of the treated tumor.

The comparison of the present results on glioblastoma cell lines with our previous work on breast cancer cell lines suggests that the synergistic effect of NaPa and TAM could be generalized to other types of tumors and molecules. We have previously shown that carboxy methyl benzylamide dextran (CMDB) inhibited the growth of the human breast cancer cell line MCF-7ras both in vitro and in vivo (21) and that CMDB strongly synergized the TAM effect on the MCF7 ras tumors (22). It seems that CMDB and TAM inhibited the tumor cell growth by disrupting the autocrine and paracrine effects of growth factors released from the tumor cells. It is possible that the lack of synergistic effect on the growth of the glioblastoma subcutaneous tumors as compared to the orthotopic mammary tumors could be due to differences in the cell micro-environment in terms of growth factors between the brain and the mammary gland. In this context, using the conditioned media from cell cultures, we have previously shown that NaPa modifies the synthesis of growth factors, such as TGF-beta, secreted by MCF-7 and MCF-7ras tumor cells, leading to cell proliferation inhibition (23, 24). Similarly, Hui et al. (18) have demonstrated that the antiglioma activity of TAM can be accentuated by inhibiting the nuclear factor NF-kB.

The mechanisms underlying the synergy between TAM and NaPa are not known. Nevertheless, because these molecules inhibit two different pathways, the synergy of proapoptotic tumor effects could result from a more complete blockade of survival factor synthesis and/or activities. The mechanisms of action of these two drugs appear to involve modulation of gene expression leading to changes in lipid metabolism with subsequent reversion to a more benign phenotype and cell death. However further studies should be addressed to investigate the mechanism of this synergistic action.

In agreement with previous work showing the synergistic activity of Lovastatin and NaPa on glioma cells (25), these data provide a potential strategy for enhancing the efficacy of glioblastoma TAM treatment with the proapoptotic NaPa molecule which displays little normal tissue toxicity as compared to chemotherapeutic molecules. Other treatment modalities, such as gene therapy (26, 27), could be considered as candidates for combined therapy to improve tumor treatment. In the future, this potential synergetic effect should be further evaluated in a clinical protocol for the treatment of glioblastoma.

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References


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