Anti-Cancer Drugs 1996, 7, pp. 288–292

Phenylacetic acid halides inhibit estrogen receptor (ER)-positive MCF-7 cells, but not ER-negative human breast cancer cells or normal breast epithelial cells

DE Johnson,[1] J Ochieng[2] and SL Evans[1]

Departments of [1]Pharmacology and [2]Biochemistry, Meharry Medical College, Nashville, TN 37208, USA.
Tel: (+1) 615 327-6293; Fax: (+1) 615 327-6632.

Phenylacetic acid (PA) derivatives and conjugates have been reported to have antiproliferative and antitumor properties against various types of cancers. Based on these findings, recent *in vitro* experiments were devised to examine the antiproliferative properties of a series of *para* substituted (Br, Cl, F, H, NO_2 and OCH_3) PAs. The *in vitro* screening protocal involved the plating of MCF-7 cells in a 96-well plate assay. After 1 day, the cells were exposed to the PA derivatives for 2 days (log phase of MCF-7 growth curve). Cells growth was determined by the Alamar blue dye reagent. The optical density data was analyzed and IC_{50} concentration values determined. The results showed that PA halide derivatives caused a significant decrease in proliferation of the MCF-7 cells. The order of antiproliferative activity was BR > Cl ≥ F, with IC_{50} values (nM) of 10 ± 0.005, 100 ± 0.02 and 100 ± 0.04, respectively. The OCH_3, H and NO_2 compounds showed no significant antiproliferative activity. PA halide derivatives may have similar actions as tamoxifen because they show specificity for estrogen receptor-positive cells.

Key words: Alamar blue, antiestrogenic, antiproliferative, breast cancer, estrogen receptor-positive, substituted phenylacetic acids.

Introduction

Phenylacetate (PA), a metabolite of phenylalanine, is a naturally occurring plasma component that suppresses the growth of various tumor cells and it has also been shown to induce differentiation in cultured tumor cells. PA has shown profound anticancer activity in several *in vitro* cellular models. At concentrations of 2–6 mM, PA stimulated morphological differentiation of two human neuroblastoma cell lines IMR-32 and UKF-NB-3. These concentrations inhibited growth and DNA snythesis of cells in a dose-dependent manner without significant effect on cell viability.[1] Phenylacetate also has been shown to induce terminal differentiation of HL60 cells and has preclinical activity against prostate cancer and glioblastoma.[2] Gliomas, but not mature normal brain cells, are highly dependent on mevalonate (MVA) for production of sterols and isoprenoids, which are vital for cell growth. PA inhibits MVA-diphosphate decarboxylase, which is the key enzymatic step in the formation of MVA. Systemic treatment of rats bearing intracranial gliomas resulted in significant tumor suppression with no apparent toxicity to the host. The data indicated that PA had the ability to alter cell lipid metabolism by acting through the inhibition of protein prenylation and other mechanisms, which may offer a safe and effective novel treatment of malignant gliomas.[2,3] PA, demonstrated antitumor activity in experimental models and in man, prevented carcinogenesis by 5-aza-2′-deoxycyntidine (5-AzadC). Transient exposure of premalignant *ras*-transformed fibroblasts to 5-AzadC resulted in malignant conversion associated with increased *ras* expression and reduced collagen biosynthesis.[4] These profound changes were prevented by a simultaneous treatment with phenylacetate or phenylbutyrate. These data indicate that phenylacetate or phenylbutyrate could potentially benefit individuals predisposed to cancer development. These agents could also be used as adjuvant or combination treatment with other anticancer therapeutics to enhance their efficacy and minimize adverse side effects, and perhaps be used in maintenance therapy to prevent disease relapse.[4] Considering the increase in glutamine dependence of malignant cells, PA has been examined as a glutamine deprivation molecule because it binds and depletes glutamine, which is an important amino acid for lymphocyte metabolism. Treatment of hormone refractory human prostate carcinoma cell lines PC3 and DU145 with pharmacologically attainable non-toxic concentrations of PA resulted in

Phenylacetic acid halides were obtained commercially from Aldrich Chemical Company, Inc. This research was supported by NIH grant RR-03032.

Correspondence to DE Johnsson

selective growth arrest and reversal of malignancy (i.e. loss of invasiveness and tumorigenicity in athymic mice).[3] Interestingly, PA enhanced the antitumor effects of suramin, a drug known to be active in patients with advance disease. The data suggested that PA, used alone or in combination with other antitumor agents, may offer an effective and safe approach to the treatment of androgen-insensitive prostate carcinoma.

In the present study, we decided to examine whether some of the PA derivatives exhibited any anticancer activity against two breast cancer cell lines (MCF-7 and MDA-MB 231). We found that the PA halides showed significant antiproliferative activity against MCF-7 cells, while they showed no significant activity against estrogen receptor (ER)-negative or normal breast epithelial cells.

Materials and methods

Cell culture methods

The ER-positive MCF-7 human breast cancer cell line was obtained from American Type Culture Collection (Rockville, MD). MCF-7 cells were grown in T-75 tissue culture flasks as monolayer cultures in RPMI 1640 medium (phenol red) supplemented with 2 mM glutamine, penicillin (30 000 units/ml), insulin (2500 units/ml), 10% calf serum and fungizone (250 μg/ml). Cultures were grown at 37°C in a humid 5% CO_2 atmosphere and fed every 3 days. When cultures reached confluency (usually every 5–7 days), they were subcultured using a 1:2 splitting ratio every third day. Culture medium was changed every 3 days until the cells were confluent.

The ER-negative MDA-MB231 human breast cancer cell line was obtained from the American Type Culture Collection. The MCF-10 normal breast epithelial cell line was obtained from the Michigan Cancer Foundation (Detroit, MI). MDA-MB231 cells were grown under cell culture conditions which were similar to MCF-7 cells. MCF-10 cells were grown in T-75 tissue culture flasks as monolayer cultures in DMEM (phenol red 14.8 g/l), F-12 HAM BASE supplemented with glutamine (200 mM), amino acids (50 ×), Fungizone (5 ml), insulin (4 mg/ml), hydrocortisone (17 ml), EGCF (2 ml), sodium bicarbonate (2.2 g), penicillin/streptomycin (10 000 units/ml), cholera toxin (0.49 ml) and 10% bovine serum. The MCF-10 cell line was grown in a similar manner to MCF-7 cells, except they reached confluency in 7–9 days and were subcultured using a 1:3 splitting ratio every fifth day.

Cell proliferation studies

In each experiment the exponentially growing cells were trypsinized, counted and plated in multiwell plates at a density of 2×10^4 cells/well in 200 μl of media. After 2 days of incubation when the cells were in an exponential growth phase, the test compounds were added. The test compounds (Figure 1) were dissolved in dimethylsulfoxide and added to cell cultures following dilutions in culture medium. Control wells received the same amounts of vehicle alone. Exponentially growing viable cells were counted by a hemacytometer using the trypan blue exclusion method. On the third day of administration, the treated cells were measured for optical density (OD) at 570 and 600 nm using a microwell plate reader. The OD was determined by using Alamar blue dye reagent. Alamar blue measures the chemical environment surrounding cells in media. Dying cells undergo more extensive oxidation reactions which turns the media into a blue color. Viable cells undergo more extensive reduction reactions which tends to leave the media a light pink color. The microplate reader quantitates these color changes into numbers. The antiproliferative activity of the test compounds was calculated as a percentage of control as follows: antiproliferative activity = [viable cells (control) − viable cells (treated)]/[viable cells (control)] × 100.

Estradiol binding studies

ER binding competition studies were performed between [^{3}H]estradiol, estradiol, 4-hydoxytamoxifen, PAF, PACl and PABr. The procedure followed was that of Wakeling.[5]

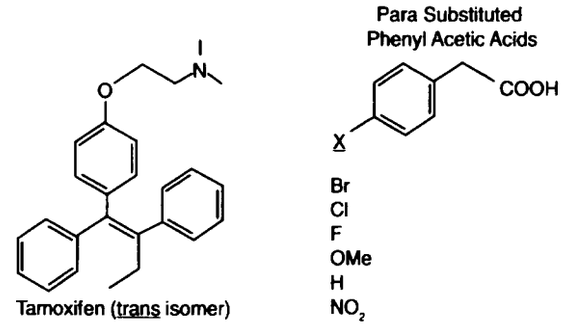

Figure 1. Structures of tamoxifen and *para* substituted PA halides.

Results

Antiproliferative activity

The experimental standard estradiol (100 nM) stimulated the growth of MCF-7 cells 50% above control. The antiestrogen standard tamoxifen (100 nM), PAF (100 nM), PACl (100 nM) and PABr (10 nM) significantly ($p < 0.005$) inhibited the growth of MCF-7 cells by 48, 49, 40 and 52%, respectively (Figure 2).

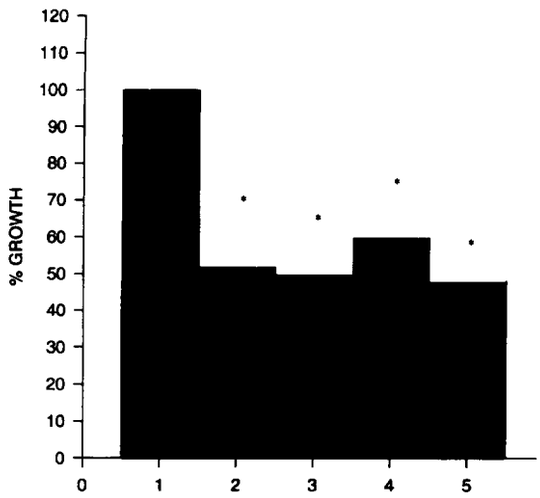

Figure 2. Tamoxifen (100 nM) and PA (F, Cl and Br) at 100 nM inhibit proliferation of MCF-7 cells. 1, Control; 2, tamoxifen; 3, PAF; 4, PACl; 5, PABr. *Statistically significant $p < 0.001$. Each value is the mean of quadruplet samples ± SEM.

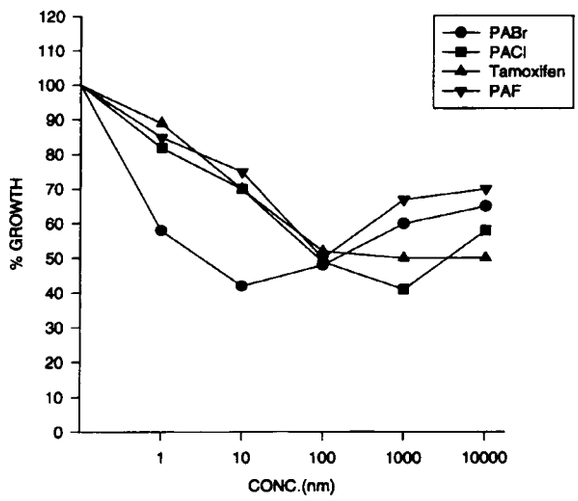

Figure 3. Effects of PA halides (F, Cl and Br) and tamoxifen on growth of MCF-7 cells. Each value is the mean of quadruplet samples ± SEM.

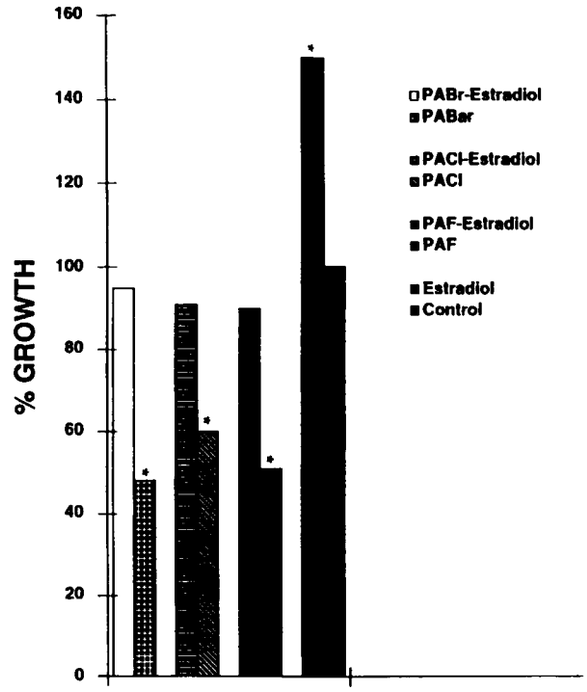

Figure 4. Inhibitory growth effects of PA halides (F, Cl and Br) at 100 nM against MCF-7 cells in the presence and absence of estradiol at 100 nM. Each value is the mean of quadruplet samples ± SEM. *Statistically significant.

Over a concentration range of 1–10 000 nM, tamoxifen and the PA halides inhibited the growth of MCF-7 cells in a dose-dependent manner (Figure 3). The antiproliferative effects of PA halides were found to be as potent as tamoxifen. PABr was found to be even more potent than tamoxifen. Estradiol (100 nM) significantly reversed ($p < 0.001$) the inhibition of MCF-7 cell proliferation caused by either tamoxifen (100 nM) or PA halides (100 nM) by the second day of administration (Figures 4 and 5). ER competition binding studies indicated that PAF, PACL and PABr were not competitive ligands to the ER with relative binding affinity (RBA) values of less than 1.5 for all PA halides, as compared with known ligands 4-hydroxytamoxifen (RBA = 500) and estradiol (RBA = 100).

Discussion

Antiestrogens, such as tamoxifen, are effective in controlling the growth of estrogen-dependent breast tumors in patients.[6] *In vitro*, specific antiestrogenic effects of tamoxifen on the growth of ER-positive tumors are usually observed at a range

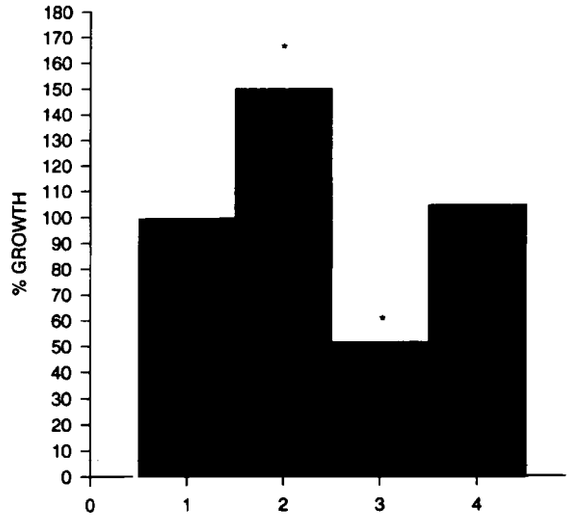

Figure 5. Inhibitory growth effects of tamoxifen on MCF-7 cells in the presence and absence of estradiol. Each value is the mean of quadruplet samples ± SEM. *Statistically significant, $p < 0.05$. 1, Control; 2, estradiol; 3, tamoxifen; 4, tamoxifen–estradiol.

Table 1. Influence of estradiol, tamoxifen, PA, P-OCH_3, PAF, PACl and $PANO_2$ on growth inhibition of MCF-7, MCF-10 and MDA-MB231 cell lines.

Treatment	Percent of control (viable cells)		
	MDA-MB231	MCF-10	MCF-7
Estradiol (0.1 μM)	109 ± 0.09	99 ± 0.06	151 ± 0.07
Tamoxifen (0.1 μM)	107 ± 0.08	99 ± 0.01	52 ± 0.08
PA (0.1 μM)	115 ± 0.09	103 ± 0.08	117 ± 0.08
P-OCH_3 (0.1 μM)	93 ± 0.08	125 ± 0.06	120 ± 0.09
PAF (0.1 μM)	92 ± 0.09	88 ± 0.05	51 ± 0.07
PACl (0.1 μM)	94 ± 0.08	103 ± 0.08	60 ± 0.08
PABr (0.1 μM)	95 ± 0.08	101 ± 0.05	48 ± 0.09
$PANO_2$ (0.1 μM)	92 ± 0.08	93 ± 0.07	88 ± 0.09

MCF-7-, MCF-10- and MDA-MB231-treated cells were counted on the third day of drug administration. Each value represents the mean of quadruplet samples ± SEM.

of 1000 nM or lower, whereas above this concentration the antiproliferative effects of tamoxifen on cell growth is believed to be due to cytotoxic action.[7,8] Therefore the PA halides were initially evaluated at concentrations of 1–1000 nM to examine their specific comparative antiestrogenic properties. Tamoxifen and estradiol were used as the standard antiestrogen and estrogen, respectively, in the evaluation of MCF-7 cell proliferation responsiveness. Estradiol (100 nM) stimulated and tamoxifen (100 nM) clearly inhibited the growth of MCF-7 cells by 50% of control. The PA halides were observed to be as potent, in the case of PABr more potent, as tamoxifen on the inhibition of MCF-7 cell proliferation. Since the antiproliferative activity of PA halides was observed on ER-positive human breast cancer cell lines, it could have been mediated through an ER competition mechanism. Another alternative explanation for the effects produced by PA halides against MCF-7 cells could be the binding of these compounds to extracellular receptors, such as epidermal growth factors or transforming growth factor-β, which prevents tyrosine phosphorylation and therefore blocking cellular growth modulating proteins.[9] In order to determine whether the antiproliferative activity of the PA halides was an ER-related mechanism, two experiments were performed. The first experiment involved studying the proliferative activity of MCF-7 cells by exposing them to tamoxifen as well as the PA halides in the presence and absence of estradiol. The dose-dependent inhibition of MCF-7 cells by tamoxifen as well as PA halides tends to indicate a similar potency profile against MCF-7 cells. The second experiment was designed to examine whether the PA halides were exerting their growth inhibitory effects on MCF-7 cells by competing with estradiol for the ER. In an attempt to rank the tested compounds in order of their relative binding affinities for the ER, increasing concentrations (4–4000 nM) for estradiol (E_2), 4-hydroxytamoxifen, PAF, PACl and PABr were allowed to compete with a fixed concentration of [^{3}H]estradiol (8 nM) for binding to ER of MCF-7 cell extract. The data showed that the PA halides were not competitive binders at the ER, as compared with known ER binders 4-hydroxytamoxifen and estradiol. Although the PA halides show a similar antiproliferative profile to tamoxifen against MCF-7 (ER-positive) cells, they are not exerting their effects by antagonism at the ER. In addition, the absence of a significant antiproliferative effect of tamoxifen and the PA halides against MDA-MB231 and MCF-10 lines seems to indicate a selective specificity of these agents towards ER-positive cancer cells. Thus, the results of the present study indicate that both potency and efficacy of PA halides against MCF-7 cells to be comparable to that of tamoxifen. Tamoxifen and PA halides did not show an increase in estrogenic activity over a range of 1–10 000 nM. Therefore, PA halides may be a good candidate for clinical trials on women with ER-positive tumors. It also may be of value in the primary or adjuvant treatment and/or prevention of breast cancer in patients who have tumors that are ER-positive. PA derivatives (PA and PA-OCH_3) showed enhanced proliferation of MCF-7 cells at a concentration of 100 nM.

Acknowledgments

The authors wish to thank Ms KiTani Parker, Ms Marilyn Saulsbury, Ms Monique Gibbs and Dr Daren LeBeau for assistance with the organization and presentation of information.

References

1. Cinatl J, Mainke M, Weissflog A, *et al. In vitro* differentiation of human neuroblastoma cells induced by sodium phenylacetate. *Cancer* Lett 1993; **70**: 15–24.
2. Samid D, Ram Z, Hudgins WR, *et al.* Selective activity of phenylacetate against malignant gliomas: resemblance to fetal brain damage in phenylketonuria. *Cancer Res* 1994; **54**: 891–5.
3. Ram Z, Samid D, Walbridge S, *et al.* Grwoth inhibition, tumor maturation, and extended survival in experimental brain tumors in rats treated with phenylacetate. *Cancer Res* 1994; **54**: 2923–7.
4. Call TG, Stenson MJ, Witzig TE. Effects of phenylacetate on cells from patients with B-chronic lymphocytic leukemia. *Leukocyte Lymphoma* 1994; **14**: 145–9.
5. Wakeling, AE. Antihormones and other steroid analogues. In: *Steroid hormones: a practical approach* 1987: 220–21.
6. Robinson SP, Goldstein D, Witt PL, Borden EC, Jorden, VC. Inhibition of hormone-dependent and independent breast cancer cell growth *in vivo* and *in vitro* with the antiestrogen toremifeme and recombinant human interferone-a2. *Breast Cancer Res Treat* 1990; **15**: 95–101.
7. Wilson AJ, Tehrani F, Baum M. Adjuvant tamoxifen therapy for early breast cancer: an experimental study with reference to oestrogen and progesterone receptors. *Br J Surg* 1982; **69**: 121–5.
8. Gottardis MM, Jordan VC. The antitumor actions of keoxifene and tamoxifen in the *N*-nitrosmethylurea-induced rat mammary carcinoma model. *Cancer Res* 1987; **47**: 4020.
9. Duax WL, Griffin JF, Rohrer DC, Weeks CM. Steroid agonists and antagonists: molecular conformation, receptor binding, and activity. In: Agarwal MK, ed. *Hormone antagonists.* New York: Walter de Gruyter 1982: 3–24.

(Received 12 September 1995; received in revised form 21 December 1995; accepted 10 January 1996)