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# Inhibition of Cell Proliferation by Potential Peroxisome Proliferator-activated Receptor (PPAR) Gamma Agonists and Antagonists

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**Abstract.** *This study was initiated to determine if potential PPAR gamma antagonists could block the inhibition of cell proliferation caused by 4-phenylbutyrate. The action of 4-phenylbutyrate differed from other PPAR gamma ligands examined in that it induces histone acetylation. Proliferation of DS19 mouse erythroleukemia cells was inhibited by PPAR gamma agonists (4-phenylbutyrate, rosiglitazone, ciglitazone and GW1929) and by potential PPAR gamma antagonists: BADGE (Biphenol A diglycidyl ether), GW9662, PD068235 and diclofenac. Combined incubations tended to exhibit additive inhibitory effects. Potential PPAR gamma agonists and antagonists inhibited the incorporation of thymidine into DNA of human prostate (PC3), colon (Caco-2) and breast (T47D) cancer cells but also affected NIH3T3 cells that have little or no expression of PPAR gamma. Lipid accumulation in T47D cells was seen after incubation with 4-phenylbutyrate and both potential PPAR gamma agonists and antagonists. The extent to which the effects of 4-phenylbutyrate on cell proliferation are mediated through PPAR gamma or induction of histone acetylation remains an open question. We conclude that potential PPAR gamma antagonists may fail to reverse the growth inhibitory effect of PPAR gamma ligands and may themselves act as growth inhibitory agents.*

4-phenylbutyrate is a compound that is in clinical trials as a cancer chemotherapeutic agent. Among the actions that have been reported for 4-phenylbutyrate are inhibition of histone deacetylase (1-5) and action as a peroxisome proliferator-activated receptor (PPAR) gamma ligand (6). In an attempt to distinguish the importance of these actions, we studied the combined effects of phenylbutyrate and compounds, that have been reported to act as PPAR gamma antagonists. The compounds studied were bisphenol A diglycidyl ether (BADGE) (7,8), PD068235 (9), GW9662 (10,11) and diclofenac (12-14). Growth inhibitory effects of phenylbutyrate and some commonly studied PPAR gamma agonists were not blocked by the putative PPAR gamma antagonists. In the course of these studies, it became apparent that the compounds reported to act as PPAR gamma antagonists can have growth inhibitory effects. The objective of this report was to document that cell proliferation can be inhibited by a variety of compounds that have been reported to act as either PPAR gamma agonists or antagonists and to suggest that some of these effects may be independent of action on PPAR gamma.

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*Key Words:* 4-phenylbutyrate, histone acetylation, PPAR gamma agonists and antagonists.

## Materials and Methods

*Cells and determination of cell proliferation.* DS19 mouse erythroleukemia cells, NIH3T3 mouse fibroblasts, T47D human breast cancer cells, PC-3 human prostate cancer cells and Caco-2 human colon cancer cells were incubated at 37˚C in RPMI 1640 medium with 5% fetal calf serum and 25 mM N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) buffer.

Proliferation of DS19 cells was measured by plating the cells at an initial density of $10^5$ cells per ml and counting cell density at 24-hour intervals for 72 hours, using a hemocytometer. The incorporation of [$^3$H]thymidine into DNA was measured after incubating cells for 2 hours with 2 microcuries [$^3$H]thymidine as previously described [5],

*Reagents.* 4-phenylbutyric acid was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). 5-phenylpenta-2,4-dienoic acid (CG1255) was provided by CircaGen Pharmaceutical (Phoenix, MD, USA). These compounds were studied as their sodium salts. PD068235 was donated by Pfizer Inc. (Groton, CT, USA) through Mr. Donnie W. Owens, Sr. Ciglitazone was obtained from BIOMOL Research Laboratories Inc. (Plymouth Meeting, PA, USA). Sigma-Aldrich (St. Louis, MO, USA) was the supplier for pertussis toxin, GW9662 and diclofenac. Rosiglitazone maleate and GW1929 were purchased from Alexis Biochemicals (San Diego, CA, USA).

Table I. *Increased acetylation of H4 histone in DS19 cells was induced by 4-phenylbutyrate (PBA) but not by PD068235 (PD), docosahexaenoic acid (DHA) or ciglitazone (CIG).*

| | Percentage of total H4 histone | | | | |
|---|---|---|---|---|---|
| Incubation | H4-0 | H4-1 | H4-2 | H4-3 | H4-4 |
| Control (3) | 70.2±1.8 | 24.6±2.0 | 1.0±1.2 | 0.8±0.6 | 2.9±0.8 |
| 2 mM PBA (2) | 39.8±3.0* | 41.0±5.9 | 13.8±0.1* | 3.1±1.9 | 2.4±1.2 |
| 30 μM PD (4) | 64.5±5.1 | 27.4±7.2 | 0.1±0.1 | 1.6±0.6 | 5.5±3.2 |
| 50 μM DHA (2) | 69.3±7.2 | 19.3±12.2 | 0.1±0.1 | 5.1±3.7 | 6.3±1.3 |
| 100 μM DHA (2) | 70.6±2.3 | 23.6±2.3 | 0.2±0.1 | 0.8±0.1 | 4.8±0.0 |
| 15 μM CIG (2) | 69.6±1.8 | 26.1±2.4 | 0.1±0.1 | 1.5±1.1 | 2.6±2.6 |
| 30 μM CIG (1) | 75.0 | 21.7 | 0.0 | 0.4 | 3.0 |

DS19 cells were incubated 2 hours before isolation of histones, electrophoresis and densitometry. The data for H4 histones with 0-4 acetyl groups are expressed as a percentage of the total H4 histone. The data represent means ± S.D for the number of determinations given in parentheses. Data were evaluated by Dunnett's test.
*$p<0.01$ relative to control.

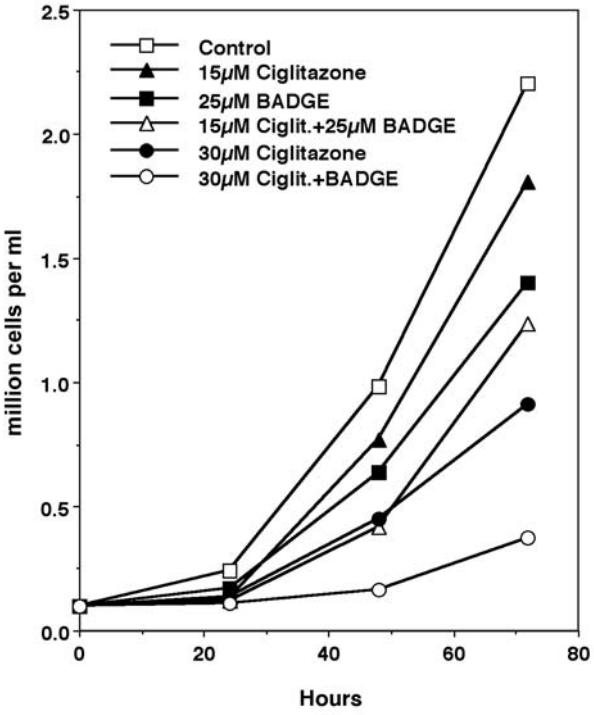


Figure 1. *Inhibition of proliferation of DS19 cells by ciglitazone and 25 μM BADGE. The data represent means of duplicate flasks.*

*Histone isolation and electrophoresis.* The isolation of histones and electrophoresis on urea-acetic acid polyacrylamide gels was performed as previously described (2). The relative levels of acetylated H4 histones were quantitated by densitometry of Coomassie-blue-stained gels.

*Evaluation of lipid accumulation.* Cells plated on UV-irradiated coverslips were fixed with 4% paraformaldehyde, washed with PBS and examined with a Nikon Eclipse TE300 fluorescence microscope.

*Statistical evaluation.* Statistical significance of the results was determined by a two-tailed Student's *t*-test using the Instat program. A probability of less than 5% was considered significant.

## Results

Before examining effects on cell proliferation, we investigated the potential effects of PPAR gamma ligands on histone acetylation. The results presented in Table I confirm the increased acetylation of H4 histone, induced by incubation of DS19 cells with 2mM 4-phenylbutyrate, as revealed by a decrease in unacetylated H4 histone and an increase in diacetylated H4 histone. There was no increase in histone acetylation after incubation with 15 or 30 μM ciglitazone, 30 μM PD068235, 50 μM or 100 μM docosahexaenoate. In subsequent studies it was observed that 20 μM GW9662 and 80 μM farnesol did not affect histone acetylation as single agents and did not prevent the hyperacetylation of H4 histone induced by incubation of DS19 cells with 2 mM 4-phenylbutyrate.

Although histone acetylation was not affected by incubation with 15 or 30 μM ciglitazone, this PPAR gamma ligand was an effective inhibitor of the proliferation of DS19 cells (Figure 1). BADGE as a single agent was growth inhibitory and there was an additive inhibitory effect on cell proliferation with a combination of 30 μM ciglitazone and 25 μM BADGE. In other studies, we have seen additive inhibitory effects of 50 μM BADGE in combination with either 2 mM 4-phenylbutyrate or 0.4 mM 5-phenylpenta-2,4-dienoate (CG1255). The inhibition of DS19 cell proliferation by incubation with GW9662 is illustrated in Figure 2. There was little, if any, reversal of the inhibitory action of 2 mM 4-phenylbutyrate when coincubated with 5 μM GW9662.

Studies on the effects of potential PPAR gamma agonists and antagonists on thymidine incorporation into DNA were performed initially with human cancer cell types that have been reported to express PPAR gamma: breast, colon and prostate (Table II). The potential agonists and antagonists were studied as single agents and in combinations. All of the potential PPAR gamma agonists studied (4-phenylbutyrate, CG1255, ciglitazone, rosiglitazone and GW1929) were observed to inhibit thymidine incorporation into DNA after incubation with the cells for 3 days. Inhibitory effects were

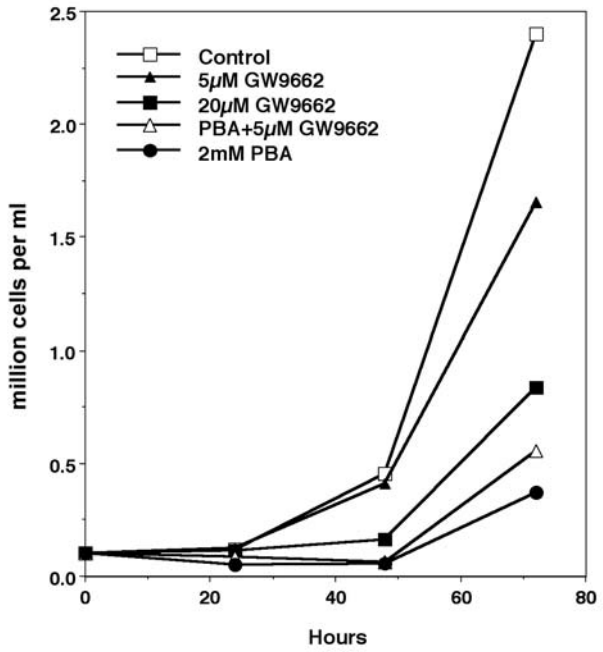


Figure 2. *Inhibition of proliferation of DS19 cells by GW9662 and 2 mM 4-phenylbutyrate. The data represent means of duplicate flasks.*

Table II. *Inhibition of thymidine incorporation in human cancer cell lines by incubation with compounds that can act as PPAR gamma agonists or antagonists.*

| Incubation | Incorporation (% control) | | |
|---|---|---|---|
| | T47D | Caco-2 | PC-3 |
| Control | 100 | 100 | 100 |
| *Potential PPAR γ agonists* | | | |
| 1 mM PBA | 59 | 42 | 75 |
| 2 mM PBA | 29 | 28 | 18 |
| 0.5 mM CG1255 | 22 | 20 | 14 |
| 1 mM CG1255 | 2 | 16 | 4 |
| 30 μM ciglitazone | 50 | 50 | 18 |
| 60 μM ciglitazone | 1 | 39 | 3 |
| 10 μM rosiglitazone | 49 | N.D. | 47 |
| 5 μM GW1929 | 66 | 68 | 70 |
| 10 μM GW1929 | 66 | 40 | 47 |
| 20 μM GW1929 | 54 | 40 | 31 |
| *Potential PPAR γ antagonists* | | | |
| 25 μM BADGE | 65 | 63 | 84 |
| 50 μM BADGE | 46 | 52 | 59 |
| 30 μM PD68235 | 69 | 42 | 43 |
| 5 μM GW9662 | 71 | 52 | 49 |
| 10 μM GW9662 | 65 | 65 | 85 |
| 20 μM GW9662 | 44 | 26 | 22 |
| *Combinations* | | | |
| 1 mM PBA + 25 μM BADGE | 29 | 15 | 25 |
| 1 mM PBA + 30 μM PD68235 | 22 | 32 | 17 |
| 2 mM PBA + 50 μM BADGE | 26 | 22 | 8 |
| 2 mM PBA + 5 μM GW9662 | 25 | N.D. | 17 |
| 2 mM PBA + 10 μM GW9662 | 10 | N.D. | 22 |
| 2 mM PBA + 20 μM GW9662 | N.D. | 5 | 2 |
| 0.5 mM CG1255 + 25 μM BADGE | 8 | 15 | 5 |
| 1 mM CG1255 + 50 μM BADGE | 1 | 11 | 2 |
| 30 μM ciglitazone + 25 μM BADGE | N.D. | 30 | 16 |
| 30 μM ciglitazone + 30 μM PD68235 | N.D. | 35 | 6 |
| 30 μM ciglitazone + 10 μM GW9662 | 54 | N.D. | 10 |
| 30 μM ciglitazone + 20 μM GW9662 | N.D. | 8 | 1 |
| 10 μM rosiglitazone + 5 μM GW9662 | 42 | N.D. | 37 |
| 10 μM GW1929 + 5 μM GW9662 | 46 | 35 | N.D. |

Cells were incubated for 3 days before addition of 2 microcuries [$^3$H]thymidine and incubated for a further 2 hours. The data are expressed as a percentage of the incorporation into DNA in controls and are means for at least 4 wells. N.D. = not determined.

also seen with the potential PPAR gamma antagonists that were examined (BADGE, PD068235 and GW9662). When combinations of the potential PPAR gamma agonists and antagonists were studied there was generally an additive rather than an antagonistic effect on thymidine incorporation. In subsequent studies with T47D cells we observed that preincubation with 10 μM GW9662 or 10 μM PD068235 for 24 hours did not reverse the inhibitory effect of 2 mM 4-phenylbutyrate or 30 μM ciglitazone when the compounds were coincubated for a further 72 hours.

Evidence has been presented that thiazolidinedione drugs can exert effects that are independent of PPAR gamma and can be blocked by pertussis toxin (15). To test for such a mechanism in T47D cells, we performed incubations with pertussis toxin (PTX) at 0.5 μg per ml. As a single agent, PTX treatment caused a small inhibition of thymidine incorporation into DNA and it did not block the inhibitory effects of 20 μM rosiglitazone, 2 mM 4-phenylbutyrate or 20 μM GW9662 (Table III).

The data in Table IV summarize a study with diclofenac which in some systems has been reported to antagonize the action of PPAR gamma agonists (12-14). At a concentration of 30 μM, diclofenac caused significant inhibition of the incorporation of thymidine into DNA in NIH3T3 cells and T47D cells. In combined incubations, the inhibitory effect of 30 μM rosiglitazone was not blocked by 15 μM or 30 μM diclofenac. The NIH3T3 fibroblast cell line lacks expression of PPAR gamma (16). In view of the inhibitory effect of the PPAR gamma agonist, rosiglitazone, on NIH3T3 cells recorded in Table IV, we extended this study to other PPAR gamma ligands (Table V). The inhibitory effect of rosiglitazone on thymidine incorporation in NIH3T3 cells was confirmed and inhibitory effects were also seen with ciglitazone, 4-phenylbutyrate, GW9662 and PD068235, suggesting that these agents were acting independently of PPAR gamma.

Table III. *Effect of co-incubation with pertussis toxin (PTX) on the inhibition of thymidine incorporation in T47D cells by compounds that can serve as PPAR gamma agonists or antagonists.*

| Incubation | Control |
|---|---|
| Control | 100 |
| PTX, 0.5 μg/ml | 73* |
| 20 μM rosiglitazone | 43* |
| 20 μM rosiglitazone + PTX, 0.5 μg/ml | 39* |
| 2 mM 4-phenylbutyrate | 22* |
| 2 mM 4-phenylbutyrate + PTX, 0.5 μg/ml | 12* |
| 20 μM GW9662 | 39* |
| 20 μM GW9662 + PTX, 0.5 μg/ml | 32* |

T47D cells were incubated for 3 days before addition of 2 microcuries [$^3$H]thymidine and then incubated for a further 2 hours. The data are expressed as a percentage of the incorporation into DNA in control cells. The data represent means of 4 determinations.
*$p<0.05$ relative to control values.

Table V. *Inhibition of thymidine incorporation in NIH3T3 cells by compounds that can serve as PPAR gamma agonists or antagonists.*

| Incubation | % Control |
|---|---|
| Control | 100 |
| 20μM rosiglitazone | 33* |
| 30μM rosiglitazone | 18* |
| 30μM ciglitazone | 8* |
| 2mM 4-phenylbutyrate | 3* |
| 20μM GW9662 | 13* |
| 30μM GW9662 | 4* |
| 30μM PD068235 | 4* |

NIH3T3 cells were incubated for 3 days before addition of 2 microcuries [$^3$H]thymidine and then incubated for a further 2 hours. The data are expressed as a percentage of the incorporation into DNA in control cells. The data represent means of 4 determinations.
*$p<0.05$ relative to control values.

Table IV. *Combined effects of diclofenac and rosiglitazone on thymidine incorporation into DNA.*

| Incubation | NIH3T3 | Caco-2 | PC-3 | T47D |
|---|---|---|---|---|
| Control | 100 | 100 | 100 | 100 |
| 15 μM diclofenac | 67* | 101 | 139 | 98 |
| 30 μM diclofenac | 48* | 82 | 73 | 52* |
| 30 μM rosiglitazone | 14* | 44* | 36* | 47* |
| 15 μM diclofenac + 30 μM Ros. | 7* | 30* | 25* | 22* |
| 30 μM diclofenac + 30 μM Ros. | 4* | 22* | 15* | 7* |

Cells were incubated for 3 days before addition of 2 microcuries [$^3$H]thymidine and incubated for a further 2 hours. The data are expressed as a percentage of the incorporation into DNA in control cells. The data represent means of 4 determinations.
*$p<0.05$ relative to control values.

4-phenylbutyrate serves as a differentiating agent for some cancer cell types. We examined the action of 4-phenylbutyrate on lipid accumulation in T47D cells to determine if there is any specificity in the action of PPAR gamma ligands on the induction of lipid accumulation. The data in Table VI indicate that lipid accumulation in T47D cells can be induced by incubation with 4-phenylbutyrate, CG1255 and PD068235 and that the action of 4-phenylbutyrate or CG1255 was not blocked by PD068235. Similarly the data in Table VII indicate that the induction of lipid accumulation in T47D cells by 4-phenylbutyrate was not blocked by co-incubation with GW9662 and that the effect of ciglitazone was not blocked by coincubation with PD068235.

## Discussion

Docosahexaenoic acid has been observed to suppress the activity of peroxisome proliferator-activated receptors in HCT116 colon cancer cells (17) , while docosahexaenoic acid and butyrate had additive effects on growth reduction in ras-transformed mouse colonocytes (18). This prompted us to determine if docosahexaenoic acid might influence histone acetylation in a similar manner to sodium n-butyrate and 4-phenylbutyrate. At concentrations in the range used by other investigators, 50 μM and 100 μM, docosahexaenoate did not influence histone acetylation, suggesting that it is not an effective inhibitor of histone deacetylase like sodium n-butyrate and 4-phenylbutyrate. The action of 4-phenylbutyrate as an inducer of histone acetylation was found to distinguish it from the PPAR gamma antagonist, ciglitazone and the PPAR gamma antagonist, PD068235.

Nakamuta *et al.* (19) reported that BADGE served as a PPAR gamma agonist for the murine macrophage-like cell line, RAW 264.7. This is in contrast to the antagonist action reported for BADGE by Wright *et al.* (7) and Zander *et al.*

Table VI. *Lipid accumulation in T47D cells after incubation with 4-phenylbutyrate (PBA), 5-phenyl-2,4-pentadienoate (CG1255) and PD068235.*

| Incubation for 3 days | Lipid accumulation | Probability |
|---|---|---|
| Control | 1.3±0.4 | |
| 1 mM 4-phenylbutyrate | 3.8±0.4 | <0.01 |
| 0.5 mM CG1255 | 3.6±0.4 | <0.01 |
| 6 μM PD068235 | 2.0±0.7 | N.S. |
| 12 μM PD068235 | 2.8±0.4 | <0.01 |
| 30 μM PD068235 | 3.2±0.4 | <0.01 |
| 1 mM PBA + 6 μM PD068235 | 4.0±0.0 | <0.01 |
| 1 mM PBA + 30 μM PD068235 | 4.0±0.0 | <0.01 |
| 0.5 mM CG1255 + 6 μM PD068235 | 2.9±0.6 | <0.01 |
| 0.5 mM CG1255 + 30 μM PD068235 | 3.3±0.3 | <0.01 |

Cells were fixed with paraformaldehyde and staining with Nile Red. Lipid accumulation was rated on a 1-4 scale by 3 observers. The data are expressed as the mean ± S.D for 4 wells. Data were evaluated by Dunnett's test. N.S. = not significant.

Table VII. *Lipid accumulation in T47D cells after incubation with 4-phenylbutyrate, ciglitazone, GW9662 and PD068235.*

| Incubation for 3 days | Lipid accumulation | Probability |
|---|---|---|
| Control | 1.1±0.1 | |
| 1 mM 4-phenylbutyrate | 3.7±0.4 | <0.01 |
| 2 mM 4-phenylbutyrate | 3.3±0.3 | <0.01 |
| 15 μM ciglitazone | 2.8±0.4 | <0.01 |
| 30 μM ciglitazone | 2.3±0.1 | <0.01 |
| 10 μM GW9662 | 1.8±0.2 | <0.05 |
| 2 mM PBA +10 μM GW9662 | 3.9±0.1 | <0.01 |
| 15 μM PD068235 | 3.8±0.2 | <0.01 |
| 15 μM ciglitazone + 15 μM PD068235 | 4.0±00 | <0.01 |

Cells were fixed with paraformaldehyde and staining with Nile Red. Lipid accumulation was rated on a 1-4 scale by 3 observers. The data are expressed as the mean ± S.D for 4 wells. Data were evaluated by Dunnett's test. N.S. = not significant.

(8). PPAR gamma agonist activity was reported for BADGE in ECV304 cells by Bishop-Bailey *et al.* (20). Fehlberg *et al.* (21) presented evidence that BADGE induces apoptosis -independently of PPAR gamma, in caspase-dependent and independent manners. Fehlberg *et al.* suggested that BADGE could represent a promising substance for improving the antitumoral activity of TRAIL (22). Our data suggest that tumor growth inhibitory properties may be exerted by other potential PPAR gamma antagonists, including PD068235 and GW9662, although their actions may not necessarily be exerted through PPAR gamma.

Diclofenac can antagonize the action of PPAR gamma agonists, but it can also act as a partial agonist (12). Diclofenac has been reported to decrease the induction of ppEnk mRNA by 4-phenylbutyrate in rat PC12 cells (23). In DU-145 prostate cancer cells, diclofenac was found to counter the growth inhibitory effect of rosiglitazone (12) but, in our studies with human colon, mammary and prostate cancer cells, diclofenac exhibited an additive inhibitory action with rosiglitazone on thymidine incorporation into DNA. Inhibition of proliferation and differentiation of neural stem cells have been observed with diclofenac (24).

NIH3T3 cells have very little PPAR gamma expression and show very little response to the PPAR gamma agonist, pioglitazone, unlike cells that are transfected with PPAR gamma (16). However, in our studies on thymidine incorporation into DNA, NIH3T3 cells were responsive to compounds that are potential PPAR gamma agonists or antagonists, suggesting that the compounds are acting through mechanisms that are not mediated by PPAR gamma.

Induction of cell differentiation by 4-phenylbutyrate has been reported in several cell types (25-28). The induction of lipid accumulation in T47D breast cancer cells is a measure of cell differentiation, but it does not distinguish the action of inhibitors of histone deacetylase and PPAR gamma ligands. It is possible that both these mechanisms can induce lipid accumulation in breast cancer cells. It is also possible that changes in gene expression mediated by phenylbutyrate (2,29,30,31) involve induction of histone acetylation and/or PPAR gamma-related regulation of gene transcription. In some systems, there is evidence that 4-phenylbutyrate can inhibit protein isoprenylation and that the growth inhibitory action of 4-phenylbutyrate can be partially blocked by farnesol (32). However, in the systems that we examined, the effects of 4-phenylbutyrate were not blocked by 40 or 80 μM farnesol (unpublished observations).

A number of publications have presented evidence that PPAR ligands can exert effects that appear to be independent of PPARs (33-35). Our observations suggest that this may hold true for both potential agonists and antagonists of PPAR gamma.

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