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PPARα signaling mediates the synergistic cytotoxicity of clioquinol and docosahexaenoic acid in human cancer cells

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ABSTRACT

This study investigated the involvement of PPARγ and PPARα signaling in the synergistic anticancer activity of clioquinol (5-chloro-7-iodo-8-hydroxyquinoline) and docosahexaenoic acid (DHA) in human cancer cells. The synergistic cytotoxicity of DHA and clioquinol was demonstrated in nine human cancer cell lines representing different tissues of origin. A2780, a well-established ovarian cancer model system, was chosen for further characterization because of its sensitivity to DHA and clioquinol. Both PPARα and PPARγ were expressed in A2780 cells when analyzed with western blotting and reporter gene technique. Treatment of the cells with clofibrate (a PPARα agonist) and clioquinol for three days mimicked the synergy of DHA and clioquinol, whereas this synergy could not be seen with the use of troglitazone (a PPARγ agonist) and clioquinol, suggesting that PPARα signaling is involved in the synergistic action. When used alone, the IC_{50} of clofibrate was 513 μM in A2780 cells. However, the addition of 5 μM clioquinol to clofibrate-treated cells led to a dramatic reduction of its IC_{50} value (148 μM). The combination effects index (CI) analysis confirmed the synergy of clioquinol and clofibrate on inhibiting cancer cell viability. Using inhibitors to block PPARα signaling diminished the synergistic cytotoxicity of clioquinol and DHA. These results provide pharmacological evidence that the synergistic anticancer action of clioquinol and DHA is mediated by PPARα signaling in human cancer cells.

1. Introduction

The biological differences between normal and cancer cells are too subtle to easily achieve selective cancer cell targeting [1]. Recently, drug combinations have been utilized in an attempt to more effectively kill cancer cells. Combinations of drugs with different modes of action may enhance individual drugs' anticancer action yet protect the host from side effects. Such synergistic interactions of anticancer drugs have been reported in studies of cultured tumor cell lines, animal models, and cancer patients [2–8].

The mechanisms by which a combination of drugs may selectively target tumor cells have not been well understood, although different modes of action are proposed to be responsible for the observed synergy [1]. Several cellular signaling molecules or pathways are proposed to be involved in the drug synergy on cancer cells, including Akt, NF-κB, apoptosis-related proteins, and the COX-2 pathway [9]. It is apparent that each drug combination may have distinct mechanisms of action because they may target different molecules or signaling pathways [1]. Since anticancer drugs are often toxic to normal cells, drug combinations, in some cases, may also be synergistically toxic to the host, parallel to their enhanced anticancer effects [1]. Therefore, more insight into the cellular mechanisms responsible for the synergistic action is critical to the development of an effective drug combination against cancer.

DHA is a long-chain n-3 polyunsaturated fatty acid, and it has been recognized to have anticancer activity in cultured tumor cells and various animal models [10–15]. Its synergy with chemotherapeutics has also been reported [16–19]. Since DHA is an essential fatty acid and protects neuronal cells from apoptosis [20,21], the combination of DHA and chemotherapeutics may achieve enhanced toxic effects selectively towards cancer cells. Indeed, DHA has been shown to enhance the cytotoxicity of paclitaxel in experimental models and in human clinical trials without seeing enhanced side effects in patients [3,4]. These observations justify

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Abbreviations: CI, combination effects index; Clioquinol, 5-chloro-7-iodo-8-hydroxyquinoline; DHA, docosahexaenoic acid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; PPARα, peroxisome proliferator activated receptor alpha; PPARγ, peroxisome proliferator activated receptor gamma.

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the use of DHA as combination therapy with chemotherapeutics. We have recently demonstrated that DHA acts synergistically with clioquinol, a metal binding compound, to kill tumor cells [22]. Clioquinol has been recently tested for the treatment of Alzheimer's disease in clinical trials [23,24] and is a newly discovered anticancer agent [25–28]. Understanding the mechanisms of the synergistic action is essential for further development of DHA and clioquinol as a novel drug combination for chemotherapy. Because DHA is a well-established peroxisome proliferator activated receptor (PPAR) ligand [29], the present study examined the involvement of PPAR signaling in the synergistic anticancer action. We report here that PPARα, but not PPARγ, mediates the synergistic anticancer action of DHA and clioquinol in human cancer cells.

2. Materials and methods

2.1. Materials

3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was purchased from Promega (Madison, WI). Antibodies for PPARα and PPARγ were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was obtained from ProMab Biotechnologies, Inc. (Albany, CA). The PPRE-lu reporter construct was obtained from Dr. Bruce Spiegelman at the Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA [30], and the RXRα cDNA construct from Dr. Ronald C. Kahn at the Joslin Diabetes Center, Harvard Medical School, Boston, MA. The Vybrant Apoptosis Assay kit 3 was from Molecular Probes (Invitrogen, Carlsbad, CA). Chemicals including clofibrate, troglitazone, clioquinol, DHA, tetrathiomolybdate, pyrithione, disulfiram, propidium iodide, GW9662, and GW6471 were analytic grade and purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Cell lines and cell viability assay

The human ovarian cell lines, A2780 and A2780CP, were obtained from Dr. Stephen Howell (University of California, San Diego). Other cell lines, including the human B-cell lymphoblastoid line Raji, the human cervical cancer line SiHA, the human breast cancer lines MCF-7 and MDA MB-231, the ovarian cancer cell line OVCAR-3, and the pancreatic cancer cell lines MiaPaCa-2 and Panc-1, were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultivated in appropriate media according to the ATCC, under a humid environment at 37 °C, 5% CO_2. Cells were plated into 96-well plates at approximately 3000–10,000/well in 100 μL medium, which led to a 40–60% confluence of each individual cell line after 24 h of dividing. Various compounds were added to the cells at indicated concentrations and durations. Cell viability was assessed with the MTS assay following the manufacturer's protocol. In short, 20 μL of MTS solution was added to each well and cells were incubated at 37 °C for about 1 h before the optical density of each well was read at 490 nm. Data were presented as a percentage of the values obtained from untreated control cells.

2.3. The combination effects index (CI) calculation

The IC_{50} values for clioquinol and clofibrate on cell viability were determined using the MTS cell viability assay. The ratio of these values was chosen to establish a molar ratio of clioquinol and clofibrate at different dose combinations (1:30) subsequently used. The cells were then treated with increasing concentrations of both compounds and a new concentration-dependent curve was constructed based on the viability assay. The CI was calculated as previously indicated [31]. In short, CI = percentage inhibition by clioquinol plus clofibrate/(percentage inhibition by clioquinol + -percentage inhibition by clofibrate). A CI value of more than one indicates synergy, a CI equal to one indicates addition, and a CI of less than one indicates antagonism.

2.4. Apoptosis assay

The Vybrant Apoptosis Assay kit was used to analyze apoptosis and necrosis. A2780 cells were plated in 6-well plates at 40,000 cells per well. 24 h after plating, cells were treated with clioquinol and clofibrate, used alone or in combination, for 20 h. Cells from the medium were collected and combined with adherent cells that were harvested by trypsinization. The cells were pelleted and resuspended in 100 μL of 1× annexin-binding buffer. 5 μL of Annexin V conjugated fluorescein (Annexin V-FITC) and 1 μL of 100 μg/mL propidium iodide were added to the cells and incubated for 15 min at room temperature. An additional 400 μL of 1× annexin-binding buffer was added to the solution and the samples were analyzed with flow cytometry (excitation wavelength of 488 nm and observation wavelengths of 530 and 575 nm).

2.5. DNA transfection and luciferase activity assay

A PPRE-luciferase plasmid construct (3 μg) and a RXRα cDNA construct (1 μg) were transfected into A2780 cells growing in 100 mm dishes with lipofectamine reagent (Invitrogen, Carlsbad, CA) as we previously described [26]. On the next day, cells were lifted and plated into 24-well plates at 200,000/well. After 48 h of transfection, cells were treated with troglitazone, clofibrate, and DHA at indicated concentrations and durations. The cells were then lysed using reporter lysis buffer and the luciferase activity assayed using a Turner TD/20E luminometer. The assay was performed by mixing 30 μL of luciferase assay reagent (Promega, Madison, WI) with 50 μL of protein extract. The relative light units were normalized for the amount of protein in each extract, and the results were reported as relative changes in luciferase activity.

2.6. Western blot analysis

Western blot analysis was performed as we previously described [22,26]. Cells were harvested at appropriate time points. Cell lysates were centrifuged at 15,000 × *g* to remove insoluble material. 50 μg of protein from each sample was resolved in 10% SDS PAGE gel, transferred to PVDF membrane, and blotted with antibodies against PPARα, PPARγ, and GAPDH.

2.7. Statistical analysis

All statistical analysis was done with Graphpad Prism software (San Diego, CA). One-way ANOVA with Dunnett's post-test or student *t*-test were used to determine differences among groups of data, with $p < 0.05$ as the level of statistical significance.

3. Results

3.1. Effects of DHA and clioquinol on cell viability in human cancer cells

We first determined the effects of clioquinol and DHA on the viability of nine different human cancer cell lines. Treatment of the cells with clioquinol and DHA for three days inhibited cell viability in a concentration-dependent manner. The IC_{50}s of clioquinol and DHA used alone or in combination were calculated (Table 1). The combination of these two compounds significantly

Table 1
Sensitivities of human cancer cell lines to the treatment with clioquinol plus DHA. Cells were treated with increasing concentrations of clioquinol (CQ) and DHA for 72 h, and the viability was assessed by the MTS assay. IC_{50} values were calculated using the Graphpad Prism software ($n = 3$).

Cell lines	IC_{50}, (μM)		
	CQ	DHA	CQ + DHA
Raji (Lymphoma)	14	94	5 + 33
A2780 (Ovarian)	13	236	5 + 42
A2780CP (Ovarian)	15	270	5 + 51
OVCAR-3 (Ovarian)	18	190	5 + 66
MCF-7 (Breast)	19	266	10 + 80
MDA-MB-231 (Breast)	22	216	7 + 59
MiaPaCa-2 (Pancreas)	16	296	9 + 88
Panc-1 (Pancreas)	28	296	10 + 88
SiHA (Cervical)	38	>300	15 + 93

lowered the IC_{50} value in each of the cell lines examined but the magnitude of the change differed among cell lines, indicating that some cell lines are more sensitive to the drug combination than others. The A2780 cell line seemed to be the most sensitive among adhesion cells and was chosen for further pharmacological characterization.

3.2. Effects of metal binding compounds on DHA-induced cytotoxicity

Clioquinol is a metal binding compound that binds to zinc and copper [32]. To understand whether metal binding contributes to its synergistic action with DHA, three other metal binding compounds were tested, including tetrathiomolybdate and disulfiram, two established copper binding compounds [33,34], and pyrithione, a zinc binding compound [26]. DHA did not enhance the cytotoxicity of these three compounds in A2780 cells, although these compounds alone are toxic to the cells in a concentration-

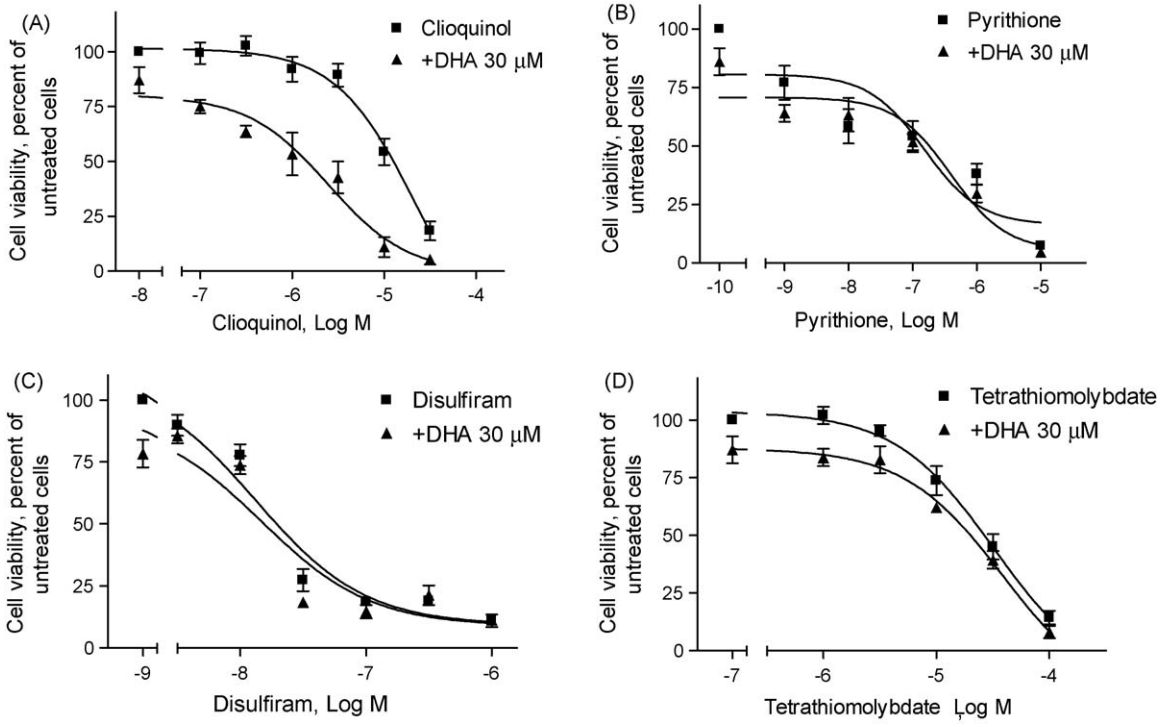

Fig. 1. Effects of metal binding compounds on DHA-induced cytotoxicity in A2780 cells. Cells were treated with different metal binding compounds at indicated concentrations in the presence or absence of 30 μM DHA for 72 h. Cell viability was analyzed with the MTS assay. Data ($n = 3$, bar: SE) are expressed as percentages of untreated cells. (A) Clioquinol; (B) Pyrithione; (C) Disulfiram; (D) Tetrathiomolybdate.

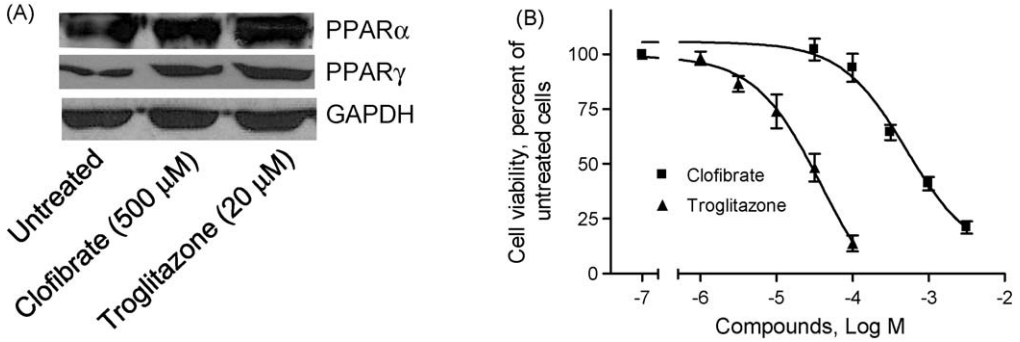

Fig. 2. Effects of PPARα and PPARγ ligands on the viability of A2780 cells. (A) Total protein was extracted from A2780 cells that had been treated with 500 μM clofibrate or 20 μM troglitazone for 4 h. The proteins were separated on SDS PAGE gel, transferred, and immunoblotted with specific antibodies against PPARγ, PPARα, and GAPDH. (B) A2780 cells were treated with clofibrate or troglitazone at indicated concentrations for 72 h. Cell viability was analyzed with the MTS assay. Data are expressed as percentages of untreated control cells ($n = 3$, bar, SE).

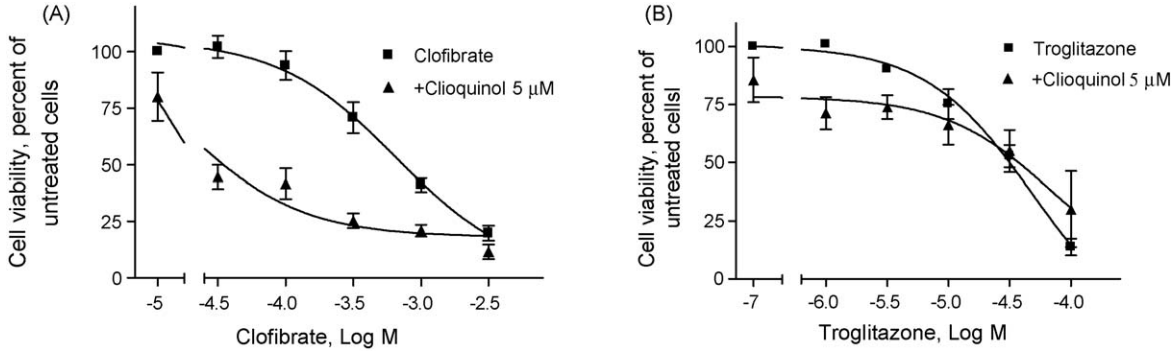

Fig. 3. Effects of PPAR agonists on clioquinol-induced cytotoxicity in A2780 cells. Cells were treated with clofibrate (A) and troglitazone (B) at indicated concentrations in the presence or absence of 5 μM clioquinol for 72 h. Cell viability was analyzed with the MTS assay. Data (n = 3, bar: SE) are expressed as percentages of the values detected in untreated control cells.

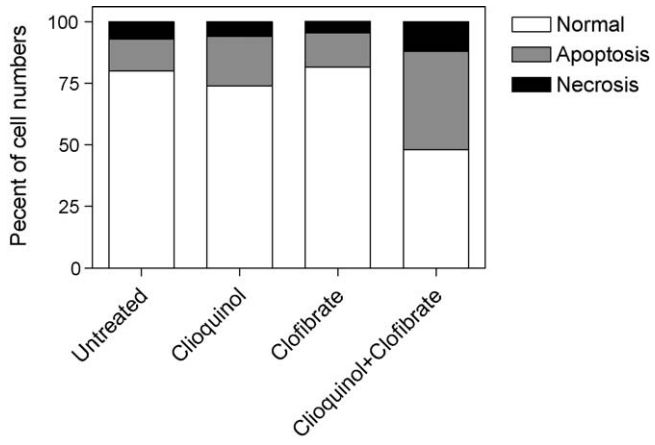

Fig. 4. Clofibrate enhances clioquinol-induced apoptosis in A2780 cells. Cells were treated with 500 μM clofibrate and 5 μM clioquinol for 20 h. Apoptosis and necrosis were determined using flow cytometry after the cells were stained with Annexin V-FITC and propidium iodide. Data are representative of two independent experiments.

dependent manner (Fig. 1). These results are consistent with our recent report [35], suggesting that the synergistic action of clioquinol and DHA is not associated with the metal binding capability of clioquinol.

3.3. Effects of PPAR agonists on clioquinol-induced cytotoxicity

DHA is an established ligand to PPAR isoforms [29,36,37], a family of transcription factors known to play an important role in cancer progression [38]. Western blot analysis confirmed that both PPARα and PPARγ were expressed in A2780 cells (Fig. 2A), both of which were moderately up-regulated upon ligand stimulation. Both clofibrate, a PPARα ligand [39], and troglitazone, a PPARγ ligand [40], inhibited cell viability in a concentration-dependent manner (Fig. 2B), with IC_{50} values of 513 μM for clofibrate and 39 μM for troglitazone. To understand whether the PPAR signaling is involved in the synergistic anticancer action of clioquinol and DHA, clofibrate and troglitazone were each applied to the cells together with clioquinol. The concentrations of clofibrate and troglitazone were chosen based on previous studies [41–44] and our experimental results. As shown in Fig. 3, clofibrate significantly enhanced clioquinol's cytotoxicity (Fig. 3A), while troglitazone did not shift the curve to the left (Fig. 3B), suggesting that PPARα signaling is involved in the synergistic action of DHA and clioquinol. Cell death analysis revealed that clofibrate enhances clioquinol-induced apoptosis of A2780 cells (Fig. 4). The synergistic inhibitory action of clioquinol and clofibrate on the viability of A2780 cells was further confirmed by the combination effects index (CI) analysis (Table 2). At concentrations used in the present study, all CI values were greater than 1, indicating a synergistic inhibitory action of these two compounds on cancer cell viability. A

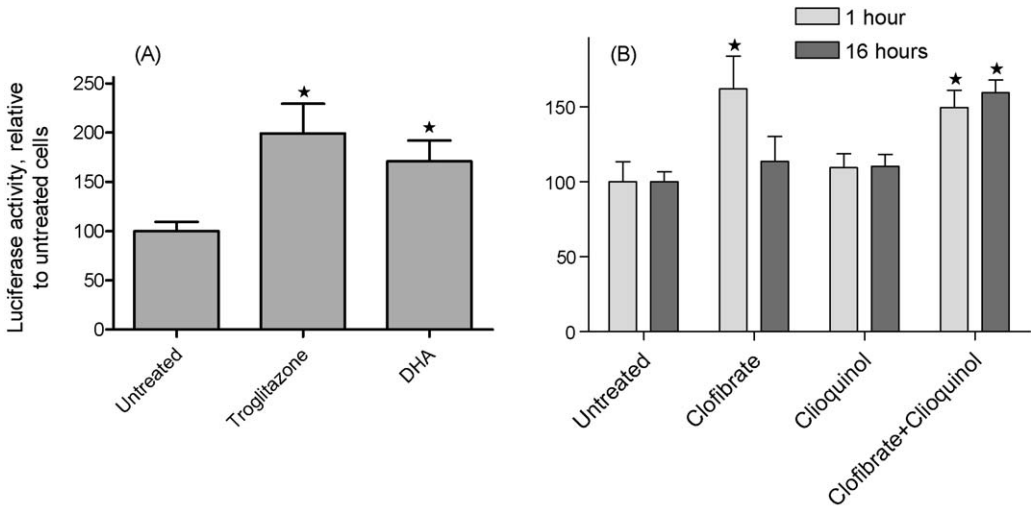

Fig. 5. Effects of troglitazone, DHA, and clofibrate on PPAR signaling in A2780 cells. Cells were transfected with the PPRE-lu reporter and RXRα cDNA constructs and treated with 20 μM troglitazone, 100 μM DHA for 4 h (A) or 500 μM clofibrate and 5 μM clioquinol for 1 and 16 h (B). Luciferase activity was assayed and expressed as percentages of untreated control cells (n = 3, bar, SE).

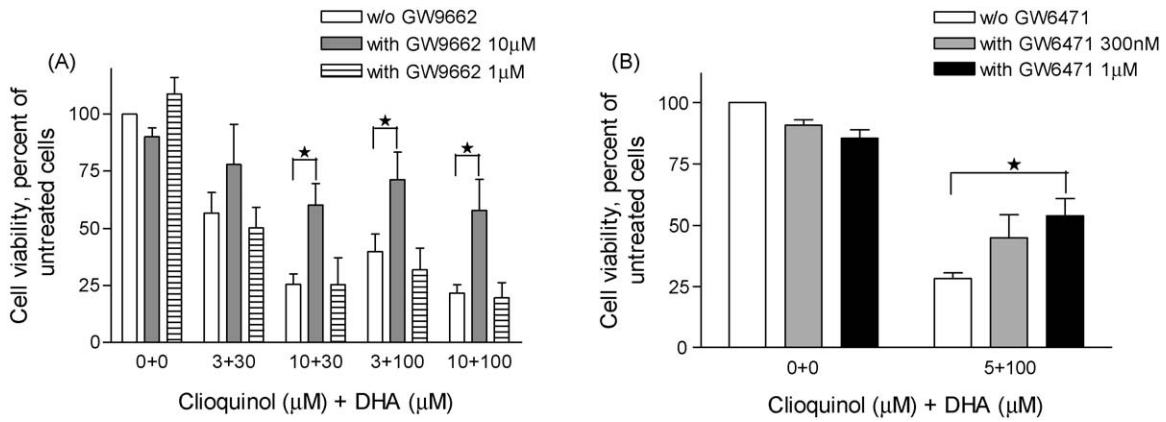

Fig. 6. Effects of PPAR antagonists on the synergistic action of clioquinol and DHA. Cells were pre-treated with GW9662 (A, PPARγ antagonist) or GW6471 (B, PPARα antagonist) 15 min prior to the addition of clioquinol and DHA at indicated concentrations. After 72 h of incubation, cell viability was analyzed with the MTS assay. Data (n = 3–5, bar: SE) are expressed as percentages of the values detected in untreated control cells.

PPRE-lu reporter construct was used to verify that troglitazone and clofibrate activate PPAR signaling in A2780 cells (Fig. 5). Interestingly, clioquinol did not activate PPAR signaling by itself but sustained clofibrate-induced promoter activity in this model system (Fig. 5B). Since clioquinol is a zinc binding compound and PPARα contains zinc finger motif, whether this effect of clioquinol directly relates to the structure and activation of PPARα remains to be investigated.

3.4. Effects of PPAR antagonists on the synergistic cytotoxicity of clioquinol and DHA

To pharmacologically establish the role of PPARα in the synergistic anticancer action of clioquinol and DHA, two well-characterized PPAR antagonists, GW9662 (PPARγ) and GW6471 (PPARα), were applied to A2780 cells prior to the addition of DHA and clioquinol. As shown in Fig. 6A, at a relatively high concentration (10 μM, a concentration that could inhibit both PPARγ and PPARα function [45]), GW9662 significantly attenuated the synergistic cytotoxicity of DHA and clioquinol, whereas at a lower concentration (1 μM, which selectively inhibits PPARγ [45]), GW9662 did not alter the synergy of clioquinol and DHA. Conversely, addition of the PPARα antagonist, GW6471 [46] at a low concentration (1 μM), significantly counteracted the synergistic action (Fig. 6B). These results further support our conclusion that PPARα signaling mediates the synergistic anticancer action of DHA and clioquinol.

4. Discussion

The present study demonstrates, through pharmacological characterization, that PPARα signaling mediates the synergistic anticancer action of clioquinol and DHA in human cancer cells.

Table 2
Effects of clioquinol and clofibrate on the viability of A2780 cells.

Clofibrate (μM)	Clioquinol (μM)				
	0	0.1	1	3	10
0	100	99	103	89	55
3	98	96 (CI = 1.03)			
30	96		84 (CI = 1.46)		
90	79			38 (CI = 2.00)	
300	59				11 (CI = 1.02)

Cells were treated with clioquinol, clofibrate, or in combination at indicated concentrations for 72 h. Cell viability was analyzed with the MTS assay. Data (n = 3) are expressed as percentages of the value detected in untreated control cells. CI was calculated as described in Section 2. CI value >1 indicates synergistic action.

These observations provide novel information on the synergistic anticancer action of clioquinol and DHA and suggest that PPARα ligands could potentially be used to enhance anticancer activity of chemotherapeutics.

Both DHA and clioquinol have been recognized to have anticancer activity [25–28,47,48]. However, only recently have the synergistic anticancer action of these two compounds been described in selected model systems [22]. Considering the fact that clioquinol has been used in human clinical trials in recent years [23,24] and that DHA is a natural product having both anticancer activity [47,48] and protective action against nerve cell death [20,21], the combination of these compounds holds a great potential for cancer therapy that might enhance cancer cell killing and minimize host toxicity. The present study extends our previous findings in selected cell model systems to additional human cancer cell lines. In all cell lines examined, DHA and clioquinol showed a synergistic cytotoxicity towards cancer cells, indicating that the enhanced cytotoxicity is not cell line-specific. However, cell lines differ in their sensitivity to clioquinol and DHA with the order of magnitude of the differences being significantly different between the most sensitive and most resistant cell lines. This suggests that the mechanisms of the synergistic anticancer action merits further investigation which may determine whether a novel drug combination for cancer therapy can be developed based on the cellular mechanisms revealed. The results from the present study demonstrate that while DHA does not work in synergy with other metal binding compounds in killing cancer cells, clioquinol does act in synergy with a PPARα agonist to enhance its cytotoxicity towards cancer cells. This indicates that the synergistic anticancer action of clioquinol and DHA is not related to the metal binding properties of clioquinol, but associated with PPAR signaling.

It has been well-established that PPAR signaling mediates cancer progression and may be a target for cancer therapy [38]. It has been recently reported that PPARγ ligands act synergistically with chemotherapeutic drugs to inhibit tumor growth *in vivo* [49,50], strongly suggesting the use of PPARγ ligands together with chemotherapeutics to treat cancer patients. On the other hand, PPARα ligands, such as clofibrate, have been reported to have anticancer activity in experimental model systems [51], but the involvement of PPARα in anticancer drug synergy has not been previously reported. Several lines of evidence from the present study allude to the involvement of PPARα in the synergistic anticancer action of clioquinol and DHA. First, both PPARα and PPARγ are expressed in A2780 cells, and the addition of PPAR ligands results in an activation of a luciferase reporter containing the PPRE element; second, the PPARα agonist, clofibrate, but not

the PPARγ agonist, troglitazone, acts in synergy with clioquinol to inhibit the viability of A2780 cells; third, inhibition of the PPARα signaling by selective inhibitors attenuates the synergistic toxicity of clioquinol and DHA. These findings provide pharmacological evidence indicating that PPARα mediates the synergistic cytotoxicity of clioquinol and DHA in human cancer cells. This suggests that PPARα ligands may be applied to enhance the anticancer action of chemotherapeutic agents, such as clioquinol. *In vivo* cancer model systems are required to directly test this assumption.

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