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Olive Oil-derived Oleocanthal as Potent Inhibitor of Mammalian Target of Rapamycin: Biological Evaluation and Molecular Modeling Studies

Mohammad A. Khanfar^{1,*}, Sanaa K. Bardaweel¹, Mohamed R. Akl², and Khalid A. El Sayed²

¹Department of Pharmaceutical Sciences, Faculty of Pharmacy, The University of Jordan, Amman 11942, Jordan

²Department of Basic Pharmaceutical Sciences, School of Pharmacy, University of Louisiana at Monroe, Monroe, Louisiana, USA

Abstract

The established anticancer and neuroprotective properties of oleocanthal combined with the reported role of mammalian target of rapamycin (mTOR) in cancer and Alzheimer's disease development encouraged us to examine the possibility that oleocanthal inhibits mTOR. To validate this hypothesis, we docked oleocanthal into the adenosine triphosphate binding pocket of a close mTOR protein homologue, namely, PI3K- γ . Apparently, oleocanthal shared nine out of ten critical binding interactions with a potent dual PIK3- γ /mTOR natural inhibitor. Subsequent experimental validation indicated that oleocanthal indeed inhibited the enzymatic activity of mTOR with an IC₅₀ value of 708 nM. Oleocanthal inhibits the growth of several breast cancer cell lines at low micromolar concentration in a dose-dependent manner. Oleocanthal treatment caused a marked downregulation of phosphorylated mTOR in metastatic breast cancer cell line (MDA-MB-231). These results strongly indicate that mTOR inhibition is at least one of the factors of the reported anticancer and neuroprotective properties of oleocanthal.

Keywords

oleocanthal; mTOR; breast cancer; docking; antiproliferative

INTRODUCTION

Mammalian target of rapamycin (mTOR) is a serine/threonine kinase and member of the PI3K-related kinase family (Kim *et al.*, 2002). It has a crucial role in integrating signals from energy homeostasis, metabolism, stress response, and cell cycle (Kim *et al.*, 2002; Hay and Sonenberg, 2004). Abnormal PI3K/mTOR activation is frequently observed in cancers (Engelman, 2009; Meric-Bernstam and Gonzalez-Angulo, 2009). mTOR plays a substantial role in supporting cell survival and proliferation of cancer under metabolic stress conditions.

*Correspondence to: Mohammad A. Khanfar, Department of Pharmaceutical Sciences, Faculty of Pharmacy, The University of Jordan, Amman 11942, Jordan. m_khanfar@ju.edu.jo.

Conflict of Interest

The authors declare that there is no conflict of interest.

mTOR activates HIF-1 α under hypoxic condition to support tumor cell survival (Land and Tee, 2007). Inhibition of mTOR arrests mitotic cells in G1 phase and may ultimately end in cell death by apoptosis, feasibly through downregulation of cyclin D1 translation (Gao *et al.*, 2004). Accordingly, mTOR is a therapeutically valid target for the treatment of cancer (Don and Zheng, 2011).

Additionally, mTOR is involved in other pathogenesis. It is overexpressed in brains of Alzheimer's disease patients and is involved in the development of amyloid beta (A β) and tau proteins (Caccamo *et al.*, 2010; Chano *et al.*, 2007). Furthermore, hyperactivation of the mTOR pathway by excessive food consumption is thought to be a critical factor underlying diabetes (Zoncu *et al.*, 2011). Hyperactivation of mTOR during hyper-feeding leads to insulin desensitization and, consequently, results in reduction of glucose uptake and glycogen synthesis and increased gluconeogenesis and glucose release in liver. Mutually, these effects lead to deteriorating of the hyperinsulinemia and hyperglycemia (Zoncu *et al.*, 2011; Di Paolo *et al.*, 2006).

The incidence of cancer and neurodegenerative diseases in the Mediterranean countries is lower than the European countries and the United States (Nuwer, 2013; Panza *et al.*, 2004; Akl *et al.*, 2014). Moreover, Mediterranean diet, rich in olive oil and monounsaturated fats, protects against age-related cognitive decline. The principal source of dietary fat in the Mediterranean diet is virgin olive oil, and this has partly been recognized as a contributing factor towards the favorable health profile of the Mediterranean population (Nuwer, 2013; Panza *et al.*, 2004; Akl *et al.*, 2014). Oleocanthal (Fig. 1) is a naturally occurring secoiridoid from olive oil (*Olea europaea*, family: Oleaceae), which was shown to exhibit potent antiinflammatory, anticancer, and neuroprotective activities. Recently, there has been an increasing interest in the biological effects of oleocanthal in inflammation, Alzheimer's disease, and cancer (Akl *et al.*, 2014; Abuznait *et al.*, 2013; Beauchamp *et al.*, 2005; Elnagar *et al.*, 2011; Parkinson and Keast, 2014).

Oleocanthal treatment demonstrates an inhibition of proliferation, migration, and invasion of various human breast, prostate cancer, and multiple myeloma cells (Akl *et al.*, 2014; Elnagar *et al.*, 2011; Parkinson and Keast, 2014). Additionally, oleocanthal prevents tumor-induced cell transformation in mouse epidermal JB6 Cl41 cells and encourages cell apoptosis (Khanal *et al.*, 2011). Interestingly, oleocanthal was found to significantly reduce two heat shock (Hsp90) proteins, Akt and Cdk4, and chaperone proteins that stabilize a number of proteins critical for tumor growth (Margarucci *et al.*, 2013).

In recent studies, oleocanthal has been demonstrated to exhibit potential neuroprotective properties and thus contribute to preventing cognitive decline because of neurodegenerative diseases (Abuznait *et al.*, 2013; Li *et al.*, 2009; Monti *et al.*, 2011). One cohort study performed on 1880 elders in the United States showed a 40% decrease in the incidence of Alzheimer's disease in populations consuming Mediterranean style diet (Scarmeas *et al.*, 2009). Oleocanthal inhibits the formation of neurofibrillary tangles, a key hallmark in the pathogenesis of Alzheimer's disease (Li *et al.*, 2009). Moreover, oleocanthal reduces the formation of A β senile plaques in the brain, which is another pathological hallmark of Alzheimer's disease (Abuznait *et al.*, 2013).

Based on the established anticancer and neuroprotective properties of oleocanthal combined with the reported role of mTOR in cancer and Alzheimer's disease development, we propose that oleocanthal activity maybe in part mediated by mTOR inhibition. Therefore, we decided to study the inhibitory potential of oleocanthal against mTOR. Our recent interest in mTOR inhibitors allowed the identification of several nanomolar and low micromolar inhibitors (Khanfar and Taha, 2013; Khanfar *et al.*, 2013).

Our study commenced by virtual docking of oleocanthal structure into the adenosine triphosphate (ATP) binding pocket of PIK3- γ . We were forced to dock into PIK3- γ because of the lack of crystallographic structure for mTOR and the close homology between mTOR and PIK3- γ , particularly their kinase domains (Khanfar *et al.*, 2013). Subsequently, we validated the docking results through *in vitro* assay against mTOR, antiproliferative assays against several cancer cell lines, and western blot analysis of mTOR expression.

MATERIALS AND METHODS

Extraction and isolation of oleocanthal

Oleocanthal was isolated from extra-virgin olive oil (*O. europaea*) as described before (Busnena *et al.*, 2013). Briefly, 1 kg of olive oil was mixed with 2L of *n*-hexane followed by addition of 1L of CH₃CN–MeOH (20:80). The dried organic layer (24 g) was subjected to repeated medium pressure liquid chromatography (MPLC) in a 50 × 3 cm column on lipophilic Sephadex LH20 using *n*-hexane-CH₂Cl₂ (1:9), isocratic elution, followed by MPLC (10 g, 25 × 1 cm column) on C-18 reversed-phase silica gel to afford 13.3-mg oleocanthal with >99% purity. Identification and purity of oleocanthal were also based on comparison of its ¹H and ¹³C NMR data with the literature (Smith *et al.*, 2005).

Docking studies

The crystal structure for the PI3K- γ (PDB code 3LJ3) was used. Docking experiments were conducted employing LigandFit docking engine (which considers the flexibility of the ligand and considers the receptor to be rigid). The binding site was generated from the “Find sites as volume of selected ligands” option in DiscoveryStudio 2.5, employing the following docking configurations:

1. Monte Carlo search parameters were as follows: number of trials = 30 000; and search step for torsions with polar hydrogens = 30.0°.
2. The root mean square threshold for ligand-to-binding-site shape matching was set to 2.0 Å, employing a maximum of 1.0 binding-site partitions.
3. The interaction energies were assessed employing the CFF force field (v. 1.02) with a nonbonded cutoff distance of 10.0 Å and distance-dependent dielectric. An energy grid extending 5.0 Å from the binding site was implemented. The interaction energy was estimated with a trilinear interpolation value using soft potential energy approximations.

Rigid body ligand minimization parameters: 40 steepest descent iterations followed by the 80 Broyden–Fletcher–Goldfarb–Shannon minimization iterations were applied to every

orientation of the docked ligand. The proposed inhibitors were further energy minimized within the binding site by implementing the “Smart Minimization” option for a maximum of 1000 iterations (Khanfar and Taha, 2013; Khanfar *et al.*, 2013).

The oleocanthal was scored using seven scoring functions: Jain, LigScore1, LigScore2, PLP1, PLP2, PMF, and DOCK_SCORE. LigScore1 and LigScore2 scores were calculated employing the CFF force field (v.1.02) and using grid-based energies with a grid extension of 7.5 Å across the binding site. PMF scores were calculated employing cutoff distances of 12.0 Å for carbon–carbon interactions and other atomic interactions (Khanfar and Taha, 2013; Khanfar *et al.*, 2013).

Cell proliferation assay

Cell lines and culture conditions—The human breast adenocarcinoma cell line MCF-7, the human ductal breast epithelial tumor cell line T47D, the human colorectal adenocarcinoma cell line Caco-2, and the human adenocarcinoma cell line HeLa were purchased from ATCC. Cell lines were cultured in high glucose Dulbecco’s modified eagle medium (Invitrogen, USA) containing 10% heat inactivated fetal bovine serum (FBS) (Invitrogen), 2 mmol/L of L-glutamine, 50U/mL of penicillin, and 50 µg/mL of streptomycin. Cell lines were maintained at 37 °C in a 5% CO₂ atmosphere of 95% humidity.

MTT assay

Viable cell count was determined using the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay. The yellow tetrazolium dye was reduced by metabolically active cells into an intracellular purple formazan product. The quantity of formazan product, as determined by the absorbance at 490 nm, is directly proportional to the number of living cells in the culture. To ensure exponential growth throughout the experimental period, as well as a linear relationship between absorbance and cell number, cells were seeded at 1×10^4 per well for each cell line in all experiments.

Cell growth and viability studies

Oleocanthal was first dissolved in a volume of DMSO to provide a final 1 mM stock solution. Then, the stock solution was used to prepare various concentrations of oleocanthal diluted in culture media. Final concentration of DMSO was maintained constant in all treatment groups within a given experiment and never exceeded 0.1%. Cells were plated at a density of 1×10^4 cells per well in 96-well culture plates, maintained in Dulbecco’s modified eagle media, and allowed to adhere overnight. After 24 h, the cells were treated with various concentrations, in three triplicates for each concentration, of oleocanthal and incubated at 37 °C in a 5% CO₂ incubator, for 48 h. At the end of the treatment period, MTT assay was carried out as previously described. Absorbance at 490 nm was read on a plate reader (Tecan Group Ltd., Switzerland). Control wells were prepared under the same experimental conditions. Wells containing culture media without any treatment were regarded as negative controls, whereas wells containing 10% of DMSO were considered as positive controls.

Western blot analysis

Antibodies against mTOR and p-mTOR were purchased from Cell Signaling Technology (Beverly, MA). Goat anti-rabbit secondary antibody was purchased from PerkinElmer Biosciences (Boston, MA). The human breast cancer cell line MDA-MB-231 was purchased from American Type Culture Collection (Rockville, MD). The cell lines were maintained in RPMI-1640 supplemented with 10% FBS, 100 U/mL penicillin G, and 0.1 mg/mL streptomycin in a humidified atmosphere of 5% CO₂ at 37 °C. Oleocanthal was first dissolved in a volume of DMSO to provide final 25 mM stock solutions, which was used to prepare treatment media. To study the effect of oleocanthal treatments, MDA-MB-231 cells were plated at a density of 1×10^6 cells/100-mm culture plate and maintained in RPMI-1640 media supplemented with 10% FBS and allowed to adhere overnight. The next day, cells were divided into different treatment groups and then given various treatments in RPMI-1640 medium containing 40 ng/mL hepatocyte growth factor (HGF) as the mitogen for 72 h. At the end of treatment period, cells were lysed in radio immunoprecipitation assay buffer (Qiagen Sciences Inc., Valencia, CA), and protein concentration was determined by the bicinchoninic acid assay (Bio-Rad Laboratories, Hercules, CA). Equivalent amounts of protein (30 µg) were electrophoresed on sodium dodecyl sulfate (SDS)–polyacrylamide gels. The gels were then electroblotted onto polyvinylidene fluoride (PVDF) membranes. These PVDF membranes were then blocked with 2% bovine serum albumin (BSA) in 10 mM Tris–HCl containing 50 mM NaCl and 0.1% Tween 20, pH 7.4 (TBST) and then incubated with specific primary antibodies overnight at 4 °C. At the end of incubation period, membranes were washed five times with TBST and then incubated with respective horseradish peroxidase-conjugated secondary antibody in 2% BSA in TBST for 1 h at room temperature followed by rinsing with TBST for five times. Blots were then visualized by chemiluminescence according to the manufacturer's instructions (Pierce, Rockford, IL). Images of protein bands from all treatment groups within a given experiment and scanning densitometric analysis were acquired using Kodak Gel Logic 1500 Imaging System (Carestream Health Inc, New Haven, CT). The visualization of β-tubulin was used to ensure equal sample loading in each lane. All experiments were repeated at least three times (Akl *et al.*, 2014).

RESULTS AND DISCUSSION

Molecular modeling studies

The reported observations that oleocanthal exhibits significant anticancer properties and the fact that inhibition of mTOR decreases cancer cell growth prompted us to test the hypothesis that oleocanthal might inhibit mTOR activity.

Consequently, we commenced our studies by computer-aided docking of oleocanthal into the ATP binding pocket of a close mTOR homologue, PI3K-γ. There are more than 80 PI3K-γ crystallographic structures available at the protein data bank (PDB). Therefore, we searched for a particular protein based on two criteria: it should have a co-crystallized potent and dual PI3K-γ/mTOR inhibitor and it should express relatively high resolution. Twenty-eight PI3K-γ proteins were found to have dual mTOR/PI3K-γ; however, only 11 of them have acceptable resolution limit of 2.7 Å. To select the optimal one, Tanimoto chemical similarity (Maggiora *et al.*, 2014) was applied to select PI3K-γ protein that has the most

similar co-crystallized ligand to that of oleocanthal. Accordingly, the protein with PDB code of 3LJ3 and a resolution of 2.43 Å was selected for the docking studies.

However, in order to validate our docking configurations, the co-crystallized inhibitor was red-docked after being extracted from 3LJ3 using the same docking settings intended for oleocanthal. Fig. 2 compares the docked pose with the corresponding experimental co-crystallized structure. The docking settings closely reproduced the co-crystallized structure with RMSD value of 1.93 Å, thus allowing us to assertively proceed to oleocanthal docking experiment.

Fig. 3 compares the molecular interactions tying docked oleocanthal with those of co-crystallized inhibitor within PI3K- γ binding pocket. Clearly in Fig. 3A, the phenolic hydroxyl of oleocanthal is hydrogen-bonded to the carboxylate side chain of ASP841 and hydroxyl of TYR841, in a comparable fashion to hydrogen-bonding interactions connecting one of the resorcinol hydroxyls of co-crystallized inhibitor with the same amino acid residues within PI3K- γ ATP binding site (Fig. 3C). However, the second resorcinol hydroxyl of co-crystallized inhibitor has an extra hydrogen-bonding interaction with the carboxylate of ASP964.

On the other hand, the benzofuranone carbonyl of co-crystallized ligand is hydrogen-bonded to ammonium of LYS833 (Fig. 3A). Comparably, the same amino acid is hydrogen-bonded to the ester oxygen of oleocanthal via the same ammonium moiety (Fig. 3C).

Similarly, the critical hinge Val882 is hydrogen-bonded via its amidic NH to the terminal aldehyde oxygen of docked oleocanthal. Comparably, the same amino acid is hydrogen-bonded to the pyrrolopyridine nitrogen of co-crystallized inhibitor via the same amidic NH.

A similar analogy can be drawn by comparing the hydrophobic interactions anchoring co-crystallized ligand within PI3K- γ with respective interactions binding oleocanthal. For example, the central backbone of docked oleocanthal stacks against the sulfide and aliphatic side chains of MET953 and ILE879, respectively. Additional hydrophobic interaction anchored the terminal acrolein of docked oleocanthal in a hydrophobic pocket of MET804, ILE831, and TRP812 (Fig. 3A). Similar hydrophobic interactions can be seen in the co-crystallized pose, albeit anchoring the pyrrolopyridine and piperazine fragments of co-crystallized ligand in the two hydrophobic pockets (Fig. 3C).

The fact that docked oleocanthal shared nine out of ten critical binding interactions with co-crystallized dual inhibitor of mTOR and PI3K- γ (Fig. 3A and C) supported our confidence in the docking settings and results and provided impetus to proceed to *in vitro* testing.

Biological evaluation of oleocanthal

The anti-mTOR inhibitory potential of oleocanthal was tested using the Invitrogen Z'-LYTE™ Kinase Assay kit. Five oleocanthal concentrations spanning over three logarithmic folds were selected. Fig. 4 shows mTOR inhibition as a function of oleocanthal concentration. The IC₅₀ value was calculated using GraphPad Prism 5.0 and applying nonlinear regression of the log(concentration) versus percent inhibition values and found to be 708 nM. The dose–response curves of captured hits exhibit Hill slope value of 0.70 and

excellent correlation coefficient (R^2) value of 0.96, which strongly suggest the authenticity (i.e., non-promiscuousity) of oleocanthal (Shoichet, 2006).

To validate our bioassay procedure, we measured the inhibitory profile of a standard mTOR inhibitor, PF-04691502 under identical assay conditions. The measured IC_{50} value was found to be 76.8 nM, which is within reasonable range compared with the reported value (4 nM) (Yuan *et al.*, 2011).

The activation of mTOR is known to play an important role in cell proliferation in many kinds of cancer cells. Therefore, the antiproliferative activity of oleocanthal was measured against the human breast adenocarcinoma cell line MCF-7, the metastatic breast adenocarcinoma cell line MDA-MB-231, the human ductal breast epithelial tumor cell line T47D, the human colorectal adenocarcinoma cell line Caco-2, and the human adenocarcinoma cell line HeLa. These cell lines were selected because of mTOR's significant role in initiation and development of breast, colorectal, and cervical cancers (Zaytseva *et al.*, 2012). A recent published study demonstrated that mTOR is intimately involved in epithelial–mesenchymal transition, motility, and metastasis in colorectal cancer (Faller *et al.*, 2015). Inhibition of mTOR leads to a significant decrease in proliferation of colorectal cancer cells and attenuates cell cycle progression in both rapamycin-sensitive and rapamycin-resistant cell lines (Gulhati *et al.*, 2009). The critical nature of mTOR has also been demonstrated in breast cancer. Several randomized trials have shown that the use of mTOR inhibitors could improve patient outcome with hormone receptor-positive or human epidermal growth factor receptor-2-positive breast cancer (Vicier *et al.*, 2014). For example, everolimus is an oral, selective mTOR inhibitor recently approved by the US FDA in combination with exemestane for treatment of hormone receptor-positive advanced breast cancer. On the hand, mTOR signaling pathway is a promising targeted-therapy for cervical carcinoma. mTOR was identified in 53% of adenocarcinoma of the cervix, and expression of phosphorylated mTOR may have a role as a marker to predict response to chemotherapy and survival of cervical cancer patients (Hussein-zadeh and Hussein-zadeh, 2014). Single agent temsirolimus, a specific inhibitor of mTOR, has modest activity in cervical carcinoma with about two-thirds of patients exhibiting stable disease (Tinker *et al.*, 2013).

Clearly in Table 1, oleocanthal showed potent anti-proliferative activities against all breast cancer cell lines, with superior activity towards the highly metastatic breast cancer cell line (MDA-MB-231) with IC_{50} value of 18.5 μ M. However, oleocanthal showed poor antiproliferative activities against the colorectal (Caco-2) and cervical cancer cell lines (HeLa). The possible explanation for the discrepancy in the antiproliferative activities of oleocanthal is the level of mTOR signaling and expression between tested cell lines. mTOR is highly expressed in several breast cancer cell lines, particularly in the invasive one (MDA-MB-231) (Albert *et al.*, 2006; Bakarakos *et al.*, 2010; Zhou *et al.*, 2004).

Similar antiproliferative behavior was observed with Rapamycin, a potent inhibitor of mTOR (IC_{50} = 0.1 nM) marketed by Pfizer. Rapamycin was highly active against MDA-MB-231, MCF7, and T47D (Noh *et al.*, 2004; Shapira *et al.*, 2006); however, it was inactive against several colorectal cancers (e.g., Caco-2 cell) (Gulhati *et al.*, 2009). mTOR exists in two distinct functional complexes: mTORC1 and mTORC2. mTORC1 is sensitive to

rapamycin treatment; mTORC2 is thought to be rapamycin insensitive (Gulhati *et al.*, 2009). Wan *et al.* demonstrated that inhibition of mTORC1 by rapamycin leads to a negative feedback activation via S6K and insulin-like growth factor-1 receptor, which results in feedback activation of Akt pathway. Akt activation promotes cell survival and resistance to the therapeutic benefits of mTORC1 inhibition. This paradoxical activation is probably associated with oleocanthal-resistant cancer cell lines (Wan *et al.*, 2007). Therefore, selective targeting of mTORC2 may represent a novel therapeutic strategy for treatment rapamycin-sensitive and rapamycin-resistant cancer cells.

Activation of mTOR is associated with phosphorylation of one of its amino acid residues, specifically Ser2448 (Sekulic *et al.*, 2000). Previous reports demonstrated that mTOR phosphorylation is blocked by rapamycin, a potent and selective mTOR inhibitor. Phosphorylation was consistently suppressed in cells treated with rapamycin. Furthermore, the phosphorylation of Ser2448 was dependent on mTOR kinase activity; that is, mTOR phosphorylation is a reasonable indicator of the level of mTOR signaling in cells or tissues (Chiang and Abraham, 2005).

In order to test whether oleocanthal will inhibit phosphorylation of mTOR, western blotting analysis was performed on HGF-induced mTOR phosphorylation on MDA-MB-231 cells at 0 or 10 μ M oleocanthal for 72 h. Results revealed that oleocanthal treatment resulted in suppression of mTOR phosphorylation by more than 60% without affecting its total level (Fig. 5). These findings confirmed the inhibitory activity of oleocanthal in suppressing mTOR phosphorylation and inhibiting mTOR signaling pathway in breast cancer cells.

Serum-free medium containing HGF (defined medium) was used in western blotting analysis to specifically study the effects of oleocanthal on mTOR activation and phosphorylation. HGF is a potent mitogen and morphogen for a broad spectrum of tissues and cell types. It triggers resistance in cancer cells by activating the Met/PI3K/Akt/mTOR pathway. PI3K mediates signals from receptor tyrosine kinase Met, phosphorylating Akt, and activating mTOR (Akl *et al.*, 2014; Guertin and Sabatini, 2009). Therefore, HGF was applied to induce and activate mTOR signaling in MDA-MB-231 breast cancer cell line. Animal serum is an extremely complex mixture of a large number of constituents, low and high molecular weight biomolecules, with different growth-promoting and growth-inhibiting activities; thus, it is an ambiguous factor in cell culture. Moreover, serum batches display unknown quantitative and qualitative variations in their composition and introduce a serious lot-to-lot variability; therefore, it was avoided in the current western blotting analysis.

CONCLUSIONS

Molecular docking and *in vitro* kinase assay experiments show that oleocanthal binds and significantly inhibits mTOR enzyme with an IC₅₀ value of 708 nM. Oleocanthal showed strong antiproliferative against several breast cancer cell lines and downregulate the expression of phosphorylated mTOR in metastatic breast cancer cell line (MDA-MB-231). These results suggest that the reported anticancer and neuroprotective activity of oleocanthal might be in part mediated by mTOR inhibition.

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Figure 1.
The chemical structure of oleocanthal.

Figure 2. Comparison between the docked poses of the dual mTOR/PI3K- γ inhibitor (red) as produced by docking simulation and its crystallographic structure within PI3K- γ (blue, PDB code: 3LJ3). This figure is available in color online at wileyonlinelibrary.com/journal/ptr.

Figure 3.

(A) Docked pose of oleocanthal into PI3K- γ binding pocket (PDB code: 3LJ3, resolution 2.43 Å), (B) docked pose of oleocanthal (red) aligned over the co-crystallized ligand (blue), (C) co-crystallized ligand within the binding pocket of PI3K- γ (PDB code: 3LJ3, resolution 2.43 Å), and (D) the chemical structure of the co-crystallized ligand. Hydrogen-bonding interactions are shown as green lines. This figure is available in color online at wileyonlinelibrary.com/journal/ptr.

Figure 4.

Inhibitory effect of oleocanthal on the activity of mTOR. The enzyme was preincubated with oleocanthal at a concentration range of 0.1, 0.5, 1.0, 10.0, and 50.0 μM . The results show the % inhibition of mTOR activity at the (log) concentration of oleocanthal. Values were expressed as mean $\pm$ SD ($n = 2$). Standard deviation values are shown as error bars on the curves.

Figure 5.

Effects of oleocanthal treatment on the levels of mTOR proteins in the human breast cancer cell line, MDA-MB-231. Oleocanthal treatment (10 μM) caused a marked downregulation of the phosphorylated mTOR (p-mTOR). Scanning densitometric analysis was performed on all blots carried out in triplicate, and the integrated optical density of each band was normalized with corresponding β-tubulin, as shown in bar graphs beside their respective western blot images. Vertical bars in the graph indicate the normalized integrated optical density of bands visualized in each lane ± standard error of the mean, **p* 0.05 as compared with vehicle-treated controls.

Table 1

The antiproliferative activities of oleocanthal against multiple cancer cell lines

Cancer cell line	IC ₅₀ (μM) ± SD ^a
MCF-7	28.0 ± 3
T47D	20.0 ± 1
MDA-MB-231	18.5 ± 2
Caco	>150.0
HeLa	>150.0

^aValues are the mean ± SD; each experiment was conducted in triplicate.