Bioorganic & Medicinal Chemistry 18 (2010) 6109–6120

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

CLEFMA—An anti-proliferative curcuminoid from structure–activity relationship studies on 3,5-bis(benzylidene)-4-piperidones

Pallavi Lagisetty [a], Prachi Vilekar [a], Kaustuv Sahoo [a], Shrikant Anant [b], Vibhudutta Awasthi [a,*]

[a] Department of Pharmaceutical Sciences, University of Oklahoma Health Sciences Center, 1110 N. Stonewall Avenue, Oklahoma City, OK 73117, USA
[b] Department of Medicine, University of Oklahoma Health Sciences Center, 1110 N. Stonewall Avenue, Oklahoma City, OK 73117, USA

ARTICLE INFO

Article history:
Received 21 April 2010
Revised 14 June 2010
Accepted 17 June 2010
Available online 22 June 2010

Keywords:
Bis(benzylidene) derivatives
Lung adenocarcinoma
Cell proliferation
Curcuminoid
Autophagy
CLEFMA

ABSTRACT

3,5-Bis(benzylidene)-4-piperidones are being advanced as synthetic analogs of curcumin for anti-cancer and anti-inflammatory properties. We performed structure–activity relationship studies, by testing several synthesized 3,5-bis(benzylidene)-4-piperidones for anti-proliferative activity in lung adenocarcinoma H441 cells. Compared to the lead compound **1**, or 3,5-bis(2-fluorobenzylidene)-4-piperidone, five compounds were found to be more potent (IC_{50} <30 μM), and 16 compounds possessed reduced cell-killing efficacy (IC_{50} >50 μM). Based on the observations, we synthesized 4-[3,5-bis(2-chlorobenzylidene-4-oxo-piperidine-1-yl)-4-oxo-2-butenoic acid] (**29** or CLEFMA) as a novel analog of **1**. CLEFMA was evaluated for anti-proliferative activity in H441 cells, and was found to be several folds more potent than compound **1**. We did not find apoptotic cell population in flow cytometry, and the absence of apoptosis was confirmed by the lack of caspase cleavage. The electron microscopy of H441cells indicated that CLEFMA and compound **1** induce autophagic cell death that was inhibited by specific autophagy inhibitor 3-methyladenine. The results suggest that the potent and novel curcuminoid, CLEFMA, offers an alternative mode of cell death in apoptosis-resistant cancers.

1. Introduction

Despite the growing understanding about the molecular basis of oncogenesis, cancer remains a challenging health care problem. Chemotherapeutic drugs are the mainstay in managing patients diagnosed with any form of cancer. The emergent chemo-resistance, morbid toxicities and overall inefficacy of current drug portfolios in many cancers necessitate the development of new drugs with novel mechanism of action and selective action on cancer cells. Taking a cue from the recent findings that curcumin has tumor suppressive activity in a variety of cancers,[1,2] our laboratory preformed a structure–activity relationship on synthetic diphenyldihaloketone analogs.[2,3] As a class, such compounds belong to chalcone group of chemicals. Chalcones are open-chain molecules where two aromatic rings flank a three-carbon enone fragment on either side. Curcumin, (1*E*,6*E*)-1,7-bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione, is a naturally occurring bis-chalcone derivative present in *Curcuma longa Linn*, a commonly used Indian spice turmeric. Studies have shown that curcumin has potent anti-angiogenic, anti-cancer properties.[4] Although several in vitro investigations and pre-clinical studies have demonstrated immense potential of curcumin in cancer treatment, its clinical application has been found limited by its instability and poor bioavailability.[5] As such, to improve the spectrum of activity as well as to modify pharmaceutical properties, several structurally-related compounds have been synthesized and evaluated as anti-proliferative and anti-infective agents.[6–9] A few curcumin analogs act as anti-inflammatory molecules by inhibiting cyclooxygenase-2 (COX-2) activity. Incidentally, COX-2 is also over-expressed in many malignant tissues.[10] In Alzheimer's disease also curcumin has been found to have beneficial effects.[11]

3,5-Bis(2-fluorobenzylidene)-4-piperidone (also known as EF24) is a synthetic analog of curcumin that was first reported by Adams et al.[6] It has been shown to possess potent anti-proliferative activity against a number of cancer cell lines such as colon,[2] breast[12] and ovarian.[13] Like curcumin, the exact mechanism of action of EF24 is unclear, but it appears to suppress cancer cell proliferation and angiogenesis by downregulating various cancer promoting genes such as COX-2, IL-8 and VEGF.[2] It has also been found to induce G2/M cell cycle arrest and apoptosis in cisplatin-resistant human cancer cells.[13] A recent study suggests that EF24 suppresses NF-κB signaling by directly inhibiting I-κB kinase.[14] Chemically, it has been proposed that conjugated enones inhibit glutathione-*S*-transferase, which enhances the cytotoxicity of these compounds.[15] The enones permit an Michael addition of intracellular thiol compounds, such as glutathione, to the olefinic double bond. The addition products are capable of releasing the conjugated drug based on the reversible equilibrium existing between the conjugate and free drug.[16–19]

* Corresponding author. Tel.: +1 405 271 6593x47331; fax: +1 405 271 7505.
E-mail address: vawasthi@ouhsc.edu (V. Awasthi).

0968-0896/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2010.06.055

Among various malignancies, lung cancers are the leading cause of cancer deaths in the world. For instance, in the United States of America an estimated 160,390 deaths in 2007 were attributed to lung cancer.[20] About 6 out of 10 people with lung cancer die within 1 year of being diagnosed with the disease. In non-small cell lung carcinoma (NSCLC), which histologically includes adenocarcinoma, squamous cell carcionoma, and large cell carcinoma, surgery is the only curative treatment modality.[21] Meta-analysis of clinical data suggests that up to 85% of the NSCLC patients depend on systemic chemotherapy as part of the overall management.[22] The current standard of care for lung cancer produces unsatisfactory responses and inadequate improvement in survival.[23] Since anti-cancer drugs remain the mainstay in the post-diagnosis management of lung cancer, broadening of the chemotherapeutic options is of contemporary interest.

In this article, we report the results of structure–activity relationship studies on 3,5-bis(benzylidene)-4-piperidones. The compounds were systematically evaluated for anti-proliferative activity in cultured lung adenocarcinoma cells. The lead compound, named CLEFMA, was preliminarily investigated for the molecular basis of anti-proliferative action. The information provides a sound basis for further chemical modifications of the core structure resulting in potentially more bioavailable and potent compounds amenable to improved formulation pharmaceutics.

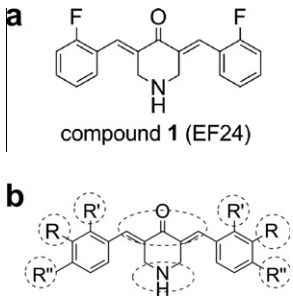


Figure 1. (a) The structure of 3,5-bis(2-fluorobenzylidene)-4-piperidone and (b) the general diagram of 3,5-bis(benzylidene)-4-piperidones showing regions (dotted lines) that were chemically modified.

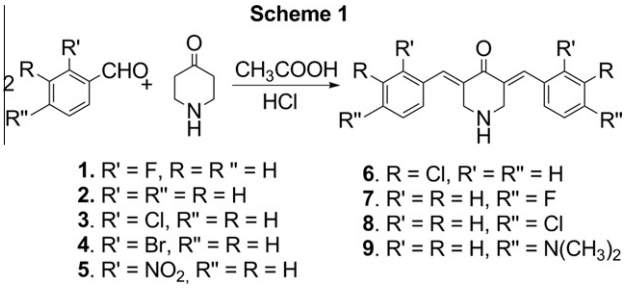


Figure 2. Synthetic scheme for various 3,5-bis(benzylidene)-4-piperidones.

2. Results and discussion

2.1. Chemistry

We synthesized several analogs of 3,5-bis(2-fluorobenzylidene)-4-piperidone, **1** (Fig. 1a), and classified them into five series, namely A, B, C, D and E as shown in Table 1 (Supplementary data) and Fig. 1b. The basic chemical reaction was Claisen–Schmidt condensation between 4-piperidone hydrochloride and substituted aromatic aldehydes (Fig. 2).[6] Single X-ray crystallography of **1** revealed that the olefinic double bonds adopts *E* configuration, and the central piperidone ring remains in a sofa conformation.[3] This is consistent with the previously reported crystal structures for 3,5-bis(benzylidene)-4-piperidones and 2,6-bis(arylidene)cyclohexanones.[24–26]

2.1.1. Series A compounds (aromatic ring substitutions)

3,5-Bis(benzylidene)-4-piperidones containing different substitutions on the two aromatic rings were synthesized in good yields (Fig. 2). Compounds **2–5** carry different *ortho*-substituents on their aromatic rings. Compound **6** has the substitution on *meta*-position, and compounds **7–9** bear substitution on *para*-position of the aromatic ring.

2.1.2. Series B compounds (reduction of unsaturated vinyl bonds and ketone group)

We modified the conjugated enone (in **1**) by selectively reducing either the double bonds, or the ketone group. First, the ketone functional group of 3,5-bis(2-fluorobenzylidene)-4-piperidone was exclusively reduced to the corresponding alcohol using $NaBH_4$ (Fig. 3). Sodium borohydride is a selective reductant for reducing ketone functional group to hydroxyl derivative without affecting the -ene part of enone.[8,27,28] We observed that the ketone reduction resulted in disappearance of the characteristic yellow color in the precursor compound. The structure of 3,5-bis(2-fluorobenzylidene)-4-hydroxy-piperidine (**10**) was confirmed by the appearance of a C-4 proton, obtained by reduction of carbonyl group, at δ 4.66 ppm (^{1}H NMR).

The selective reduction of olefin functional group of 3,5-bis (2-fluorobenzylidene)-4-piperidone was accomplished by Pd/C/H_2 at atmospheric pressure to obtain 3,5-bis(2-fluorobenzyl)-4-piperidone, **11** (Fig. 3).

2.1.3. Series C compounds (*N*-acyl monocarboxylic acid derivatives)

One of our goal was to impart additional reactive sites to the purported 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore of **1**. The additional reactive site could facilitate addition of a linker for targeting, or improving physicochemical properties by enabling conjugation to molecules, such as poly(ethylene glycol). We modified piperidinyl nitrogen by using anhydrides of various dicarboxylic acids to form *N*-acyl monocarboxylic acid derivatives. The reaction of 3,5-bis(2-fluorobenzylidene)-4-piperidone (**1**) with dicarboxylic acid anhydrides in presence of triethylamine in methylene chloride provided the corresponding acid derivatives **12–17** in high yields within 2–3 h at room temperature (Fig. 4). The anhydrides were chosen based on the varying carbon chain-length, aromaticity and unsaturation. For instance, compound **13** (maleic) represents an unsaturated counterpart of compound **12** (succinic).[12] Similarly, **14** (diglycolic) is a congener of compound **15** (glutaric). For exploring the effects of saturated versus unsaturated rings of *N*-acyl monocarboxylic acid derivatives, compounds **16** and **17** were synthesized.

The piperidine methylene (C-2) protons of acid derivatives (**12–17**) of compound **1** appeared at δ 4.26–4.70 and δ 4.70–4.84 ppm as two singlets. On the other hand, in the precursor compound **1**, these protons were at δ 4.40 ppm as one singlet. This difference observed is more likely due to the tertiary amide rotamers in compounds **12–17**.

2.1.4. Series D (*N*-substitutions on compound 1)

The secondary nitrogen of piperidone ring in **1** could be modified to alter the anti-proliferative properties. As such, we performed formylation, acetylation, tosylation and glycation at this nitrogen. Formylation of **1** by a 2:1 mixture of formic acid/acetic anhydride[29,30] afforded us an aldehyde derivative, *N*-formyl-3,

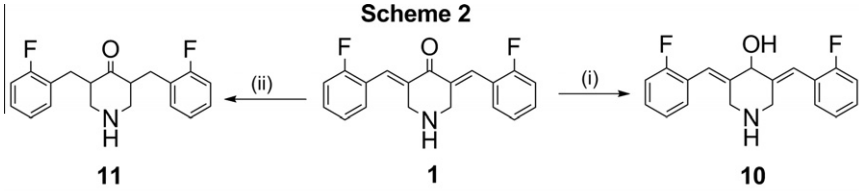


Figure 3. The selective reduction of—ene and—carbonyl functionalities in 3,5-bis(2-fluorobenzylidene)-4-piperidone.

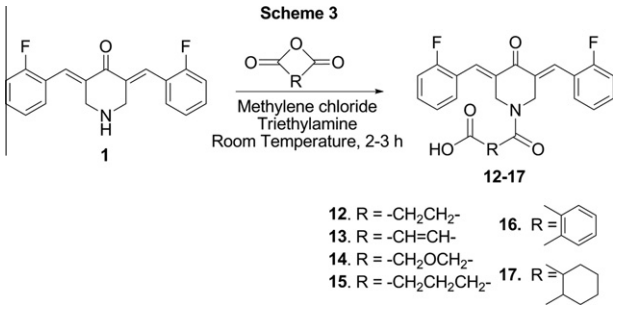


Figure 4. A general scheme for the synthesis of *N*-acyl derivatives of 3,5-bis (2-fluorobenzylidene)-4-piperidone. See Table 2 (Supplementary data) for details of the numbered compounds **12**–**17**.

5-bis(2-fluorobenzylidene)-4-piperidone (**18**). The same compound was also obtained by the formylation of compound **1** with ammonium formate, and refluxing in acetonitrile for 16 h (Fig. 5).

N-Acetyl derivative of compound **1** was obtained by reacting compound **1** with acetic anhydride in pyridine. Similarly, an *N*-tosyl derivative, **20**, was synthesized by a reaction with tosyl chloride in pyridine.

An addition of glucose moiety at the piperidinyl nitrogen was performed with a goal to achieve higher aqueous solubility, and a possibility of enhanced trans-cellular transport via glucose transporters. This chemical modification was accomplished in a two-step process. First, a reactive succinimidyl ester intermediate of compound **1** was synthesized. Briefly, **12** (succinic acid derivative of **1**) was activated to an *N*-hydroxy succinimide ester derivative (**21**) by reaction with *N*-hydroxy succinimide in dichloromethane in presence of dicyclohexyl carbodiimide (DCC). The *N*-hydroxy esters are known to be reactive towards primary amine group. To add glucose moiety, 2-glucosamine was allowed to react with **21** in pyridine solvent at 90 °C. After 3 h, compound **22** was obtained in good yield.

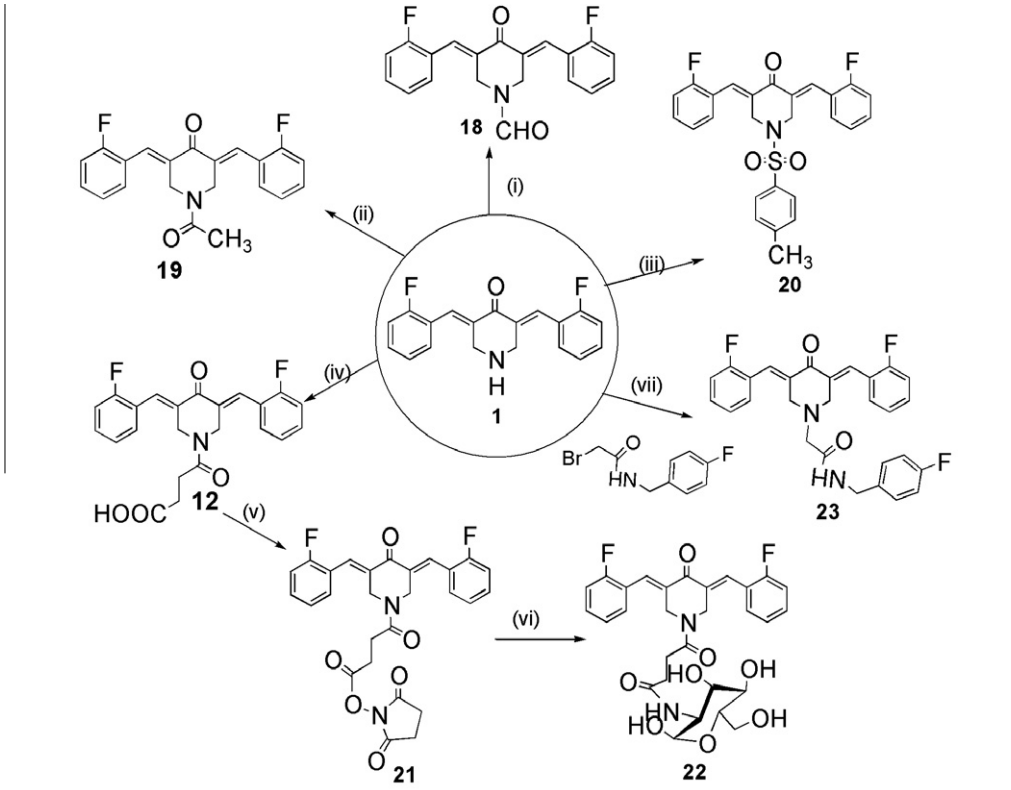
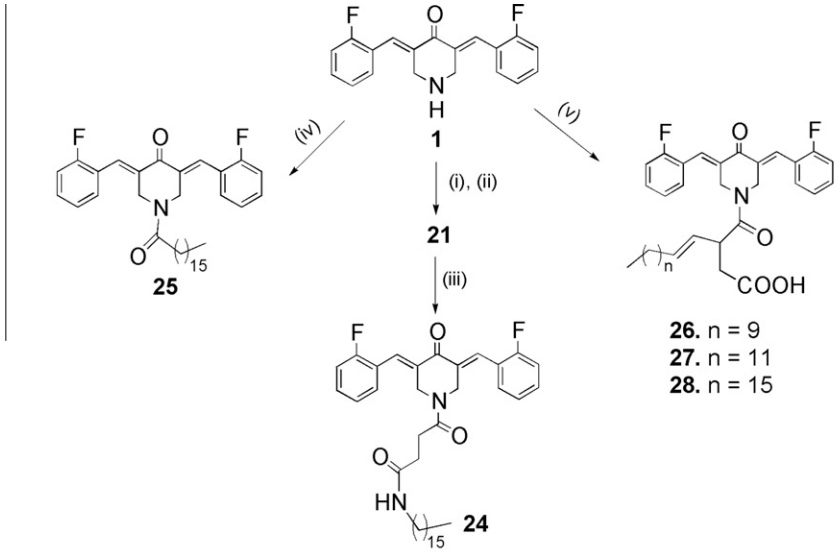


Figure 5. Synthetic schemes for various *N*-substituted derivatives of 3,5-bis(2-fluorobenzylidene)-4-piperidone. See Table 3 (Supplementary data) for details of the numbered compounds **18**–**23**.



Conditions: (i) Succinic anhydride, Triethylamine Room Temp, 2 h
(ii) Dicyclohexylcarbodiimide, *N*-hydroxysuccinimide, Room Temp, 16 h
(iii) Hexadecylamine, Py, 90 °C, 3h (iv) Stearoyl chloride, Triethylamine, Room Temp, 16 h
(v) long chain dicarbocylicacid anhydride, Triethylamine, Room Temp, 2 h

Figure 6. Synthetic scheme for the lipid derivatives of 3,5-bis(2-fluorobenzylidene)-4-piperidone.

2.1.5. Series E (lipid derivatives of compound 1)

Our laboratory has an additional interest in the incorporation of cytotoxic drugs in lipid drug carriers, such as liposomes. In order to stably incorporate the cytotoxic analogs in such formulations, we synthesized five lipid derivatives of compound **1**. The reaction schemes for these modifications are described in Figure 6. The hexadecylamine conjugate, **24** was a product of a reaction between **21** and hexadecylamine. To obtain *N*-stearoyl derivative (**25**), stearoyl chloride in dichloroethane was allowed to react with compound **1**. After 16 h and usual work up, we obtained *N*-stearoyl-3,5-bis(2-fluorobenzylidene)-4-piperidone, **25** in 67% yield. The other lipid derivatives, **26–28** were obtained by treating **1** with long chain dicarboxylic acid anhydrides, such as dodecenyl succinic anhydride, tetradecenyl succinic anhydride and octadecenyl succinic anhydride. The reactions were accomplished within 2 h, and lipidoyl monocarboxylic acid derivatives were obtained in good yields.

2.2. Biology

2.2.1. Cell proliferation studies

In order to assess the anti-proliferative activity of the synthesized compounds, an in vitro cell culture system of lung adenocarcinoma cell line H441 was used. The cell detachment and anti-proliferative activity was measured as a decrease in hexosaminidase enzyme activity associated with remainder of the adhered cells.[31] The concentration of various compounds to inhibit 50% of H441 cell proliferation (IC_{50}) was evaluated after 24 h of treatment (1–100 μM). The concentration versus cell proliferation plots were analyzed by an exponential fit (Table 1, Supplementary data). The results were compared with the anti-proliferative activity of compound **1**. As shown in Table 1 (Supplementary data), only five of the synthesized compounds **2**, **3**, **4**, **13** and **29** showed anti-proliferative potency exceeding that of compound **1** (IC_{50} <30 μM). Compounds **5**, **8**, **9**, **10**, **11**, **12**, **14**, **15**, **16**, **17**, **19**, **20**, **22**, **24**, **26** and **28** showed significantly lower activity (IC_{50} >50 μM), and the rest of the compounds demonstrated more or less no change in activity as compared to that shown by compound **1** (30 μM < IC_{50} < 50 μM).

It has been shown that electron-withdrawing substitutions in the aromatic rings enhances the cytotoxicity of 3,5-bis(benzylidene)-4-piperidones.[18,32,33] For instance, chalcones with electron withdrawing groups in the 2- and 6-positions of aromatic rings have been reported to be potent inhibitors for endothelial cell proliferation.[9] Similarly, the compounds with fluorine atoms at *ortho*-position have been reported to be potent anti-cancer compounds in breast, ovarian and colon cancers.[2,6,13,34] Contrary to these observations, we found that 2-chloro substituted compound **3** is more anti-proliferative than the 2-fluoro substituted compound **1** (Fig. 7a). When a strong electron withdrawing nitro substitution was performed at 2-position of the aromatic rings, **5**, the activity remained more or less the same. On the other hand, 3-chloro-substitution (**6**) showed little change in IC_{50} as compared to that of compound **1**. There is only one report about the 2-substituted compound in the published literature.[35]

There are a few accounts of 3,5-bis(benzylidene)-4-piperidones where the aromatic ring carries a substitution at *para*-position. It has been recently shown that 2-, 3- and 4-fluoro substitutions have good inhibitory effects against the Fanconi anemia pathway responsible for DNA repair in cancer cells.[36] Electron-withdrawing substitutions at *para*-position have been reported to diminish anti-cancer activity of compounds as compared to those that carry no substitution at *para*-position.[18,32,33] However, another report found that 3,5-bis(4-chlorobenzylidene)-*N*-methyl-4-piperidone possessed most active anti-HIV activity; the activity decreased with *para*-substitutions with CH_3 and OCH_3, or without any substitution.[27] In yet another report, 4-hydroxy and 3,4-dihydroxy derivatives of 3,5-bis(benzylidene)-4-piperidones were found to be inhibitors of α-glucosidase and HIV integrase enzymes.[7,37] In our experience, 4-chloro substitution resulted in no change in the anti-proliferative activity of **8** compared to that of **7** possessing a 4-fluoro substitution. Taken together the results imply that *para*-substitution with strong electron-withdrawing groups may increase the anti-proliferative activity.

In compounds **10** and **11** (series B), the enone unsaturation is selectively reduced. Both these compounds showed significantly less anti-proliferative activity as compared to compound **1**

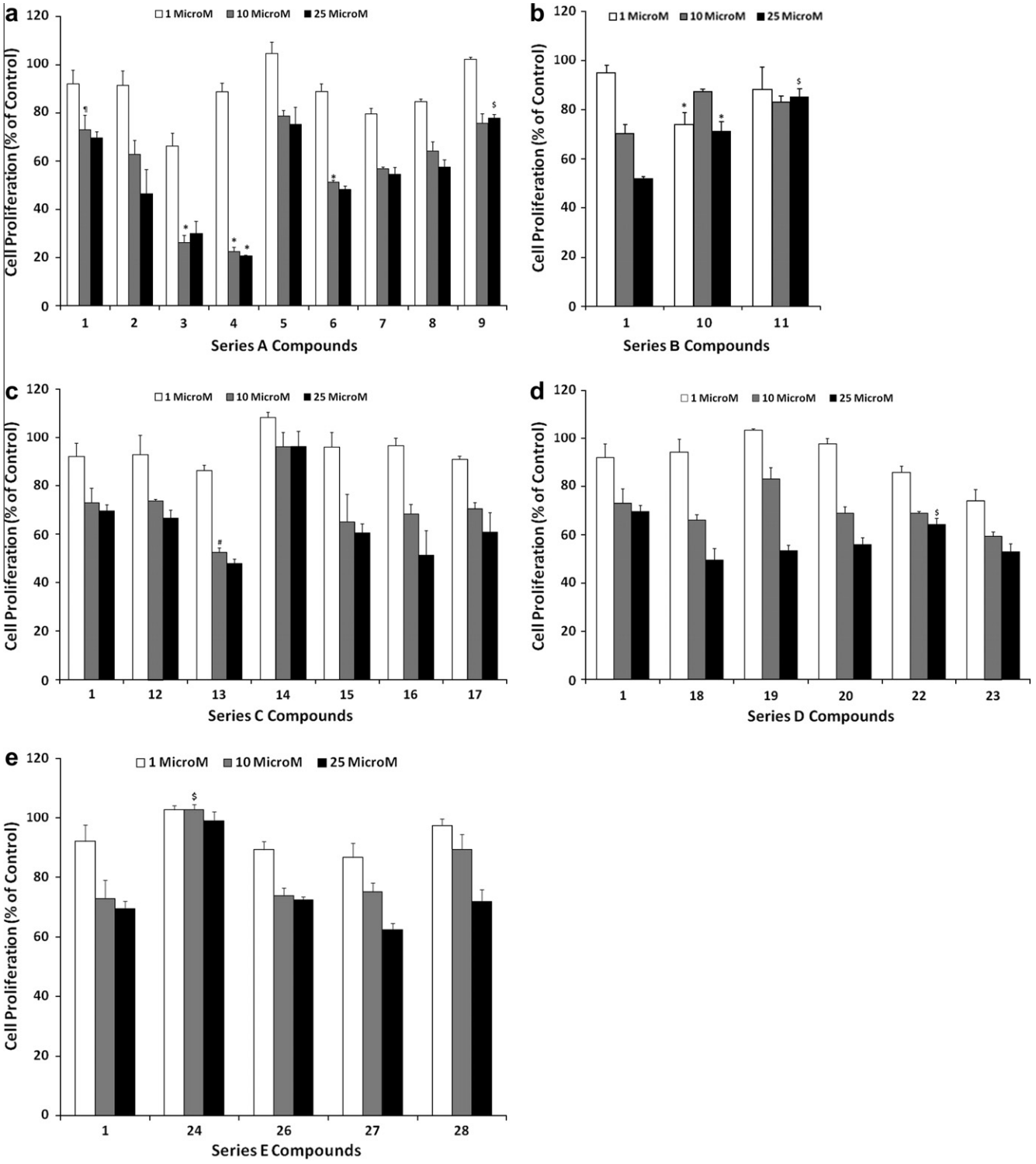


Figure 7. Anti-proliferative action of various compounds was studied by hexosaminidase assay in lung adenocarcinoma H441 cells. Compound belonging to series A (a), series B (b), series C (c), series D (d), and series E (e) were tested. Symbols ¶, ⋆, # and $ indicate p <0.001 compared to control, p <0.05 compared to compound **1**, p <0.01 compared to compound **1**, and p <0.001 compared to compound **1**, respectively.

(Fig. 7b). Apparently, the unsaturation is important for potent activity of compound **1**. Our results are consistent with previous reports where it has been shown that this structural feature mediates interaction with thiols inside the cell.[8,18,27,33]

We extended the structural modifications of 3,5-bis(benzylidene)-4-piperidones to the substitutions on piperidinyl nitrogen. In general, *N*-substituted derivatives have been reported to be less cytotoxic than their precursors. Only, *N*-acroyl analogs have been shown to be more potent than their precursors.[38–40] It has been suggested that this may be due to the double bond in acroyl moiety which provides an additional site of interaction with intracellular constituents, such as glutathione.[18,38–40] We synthesized *N*-substituted carboxylic acid derivatives (series C). All the members of this series were anti-proliferative, and compared to compound **1**, the differences in activity at 25 μM dose were not significant. Compound **14** is an oxy-linked congener of compound **15**, but is found

to be less potent as compared to compound **15**. Compound **13** (maleic acid derivative) showed a significant enhancement in potency. Compound **16** was found to be a very potent analog possessing anti-proliferative activity at lower doses similar to that shown by the higher doses of compound **1** (Fig. 7c). These results suggest that by appropriately choosing a carboxylic acid chain, compound **1** can be modified at piperidinyl nitrogen to carry additional functional features, such as an imaging radionuclide, or a cancer-specific ligand for targeted cytotoxicity.

In series D, piperidinyl nitrogen was substituted with more short chain moieties. All compounds, except compound **22**, showed anti-proliferative activity comparable to that shown by compound **1**. In fact, compounds **18** and **19** performed slightly better than compound **1** (Fig. 7d). This observation re-affirmed our previous observation with carboxylic acid derivatives that short chain substitutions at piperidinyl nitrogen do not compromise anti-proliferative activity of 3,5-bis(benzylidene)-4-piperidones. Compound **22** is a glucosylated analog that was synthesized to impart selectivity to the anti-proliferative action in cells over-expressing GLUT transporters. It remains to be seen if in spite of reduced anti-proliferative activity, this compound shows any selectivity in cell killing. Compound **23** contains a third fluorine atom in the substitution at piperidinyl nitrogen. We are working towards using this compound to obtain a radioactive positron-emitting F-18-labeled analog of compound **23** for imaging with positron emission tomography.

Since short chain *N*-substitutions demonstrated minimal impact on the activity of compound **1**, we desired to link longer lipid chains at piperidinyl nitrogen (series E). Our goal was to synthesize compounds that can be inserted into lipid carriers, such as liposomes. It was clear that compounds **26** and **27** containing dodecenyl- and tetradecenyl-modifications had an acceptable anti-proliferative activity, but the substitution with 18-carbon octadecenyl lipid (compound **28**) resulted in a total loss of activity. All these compounds were soluble in dimethyl sulfoxide, but another *N*-stearoyl analog (not shown) was insoluble in all common solvents except chloroform, and was not tested for activity in cell culture. The retention of activity by compounds with 12–14 carbon unsaturated a chain modification is encouraging, for it enables the incorporation of these compounds in a lipid carrier system. It is interesting to note that *N*-substitution with a saturated hexadecyl amine chain (compound **24**) also resulted in a loss of anti-proliferative activity. This appears to be the first report where piperidinyl nitrogen of chalcones has been modified with lipid chains.

From the results described above we determined a few structural requirements for anti-proliferative action of 3,5-bis(benzylidene)-4-piperidones. A few important characteristics of the synthesized compounds are included in Table 2 (Supplementary data). It is clear from the results of cell proliferation experiments in lung adenocarcinoma cells that the structural requirements for retaining (or enhancing) anti-proliferative properties in 3,5-bis-(benzylidene)-4-piperidones are not rigid. As such, the observations supported the following general conclusions:

1. An *ortho*-substitution on the aromatic rings with less electronegative halogens compared to fluorine, such as chloro- may increase the activity.
2. The *meta*-position substitutions have minimal impact on anti-proliferative activity.
3. A *para*-substitution with electron-donating group, such as $(CH_3)_2NH$-group reduces the activity.
4. The enone moiety is critical for the growth inhibitory activity.
5. The unsaturated short-chain carboxylic substitutions at piperidinyl nitrogen may result in more active compounds.
6. The short lipid modifications at piperidinyl nitrogen do not adversely affect anti-proliferative activity.

Based on these premise we synthesized a compound (**29**, or CLEFMA) carrying *N*-maleic acid functional group, and 2-chloro substitution on the aromatic rings (Fig. 8). From the cell proliferation studies in cancer cell lines, H441 (lung adenocarcinoma), PC-3 (prostate cancer), MiaPaCa-2 and PANC-1 (both pancreatic cancer), it was clear that the predicted structural modifications enhanced the potency of anti-proliferative action (Fig. 9). The light microscopic observations clearly demonstrated the impact of drug treatments on cell number and morphology (not shown). Although the exact mechanism is unclear, the introduction of an additional α,β-unsaturated carbonyl unit could have aided in the enhanced anti-proliferative activity of CLEFMA.

2.2.2. CLEFMA (29) induce autophagic death in H441 cells

From the above qualitative cell-cycle analysis of Sub-G1 cell population, it appeared that these compounds do not induce apoptosis in H441 cells. This was also confirmed by immunoblotting of H441 cell lysates for caspase-3 activation where no change in its activation level was observed after CLEFMA treatment. Further, we also definitively established that CLEFMA does not induce apoptosis by performing Apo-One homogeneous caspase-3/7 assay (Promega, Madison WI). Again, we could not see any cleavage-based caspase activation (data not shown). Incidentally, the literature provides several reports that the majority of lung cancer cells, including H441 cells are resistant to apoptosis because of mutations in tumor suppressor p53 and pro-oncogenic *k*-Ras.[41–43] Next, we investigated if these compounds cause cell death in H441 cells by inducing autophagy. In transmission electron micrographs (Fig. 10), compound **1** and CLEFMA induced a substantial change in cellular architecture. In few views, double-membrane pre-autophagosomal structures (PAP) containing cell organelles existed; large single-walled autophagic vacuoles were widely seen. There was peri-nuclear concentration of cell organelles, including mitochondria. Blebbing of nuclear membrane was evident in few cells, but no chromatin condensation (a sign that apoptosis was not induced) was observed.

In a separate fluorescence microscopy-based experiment, we found that the treatments with compound **1** and CLEFMA

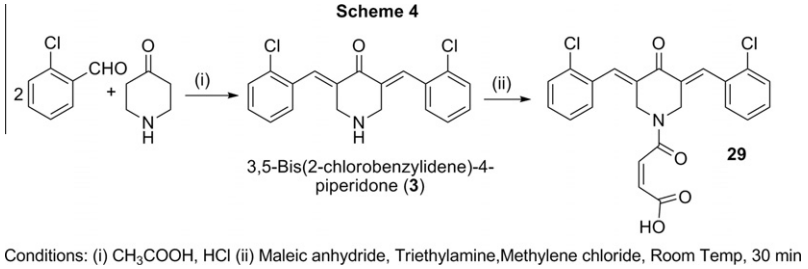


Figure 8. Synthesis scheme for the predicated compound **29** or CLEFMA.

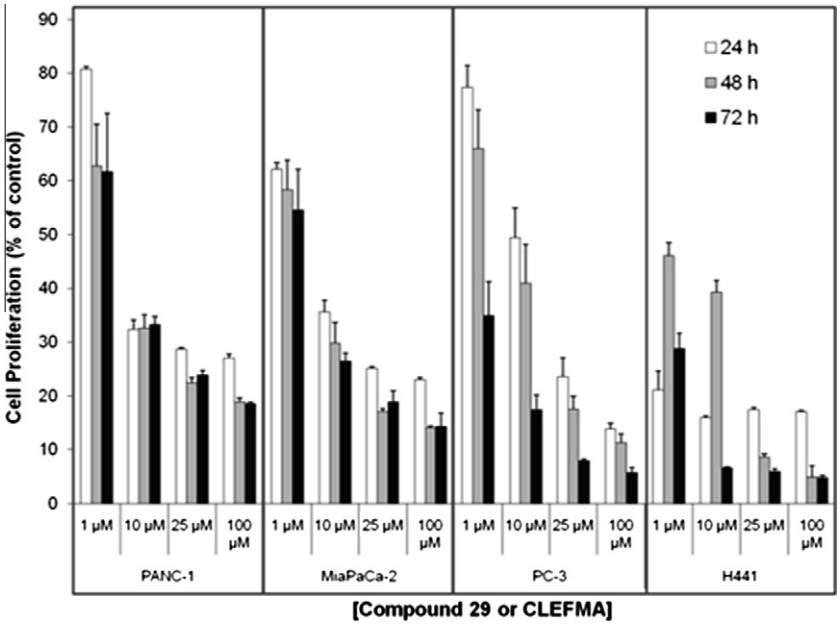


Figure 9. Cell proliferation studies of CLEFMA in pancreatic cancer PANC-1 and MiaPaCa-2 cells, prostate cancer PC-3 cells, and lung adenocarcinoma H441 cells.

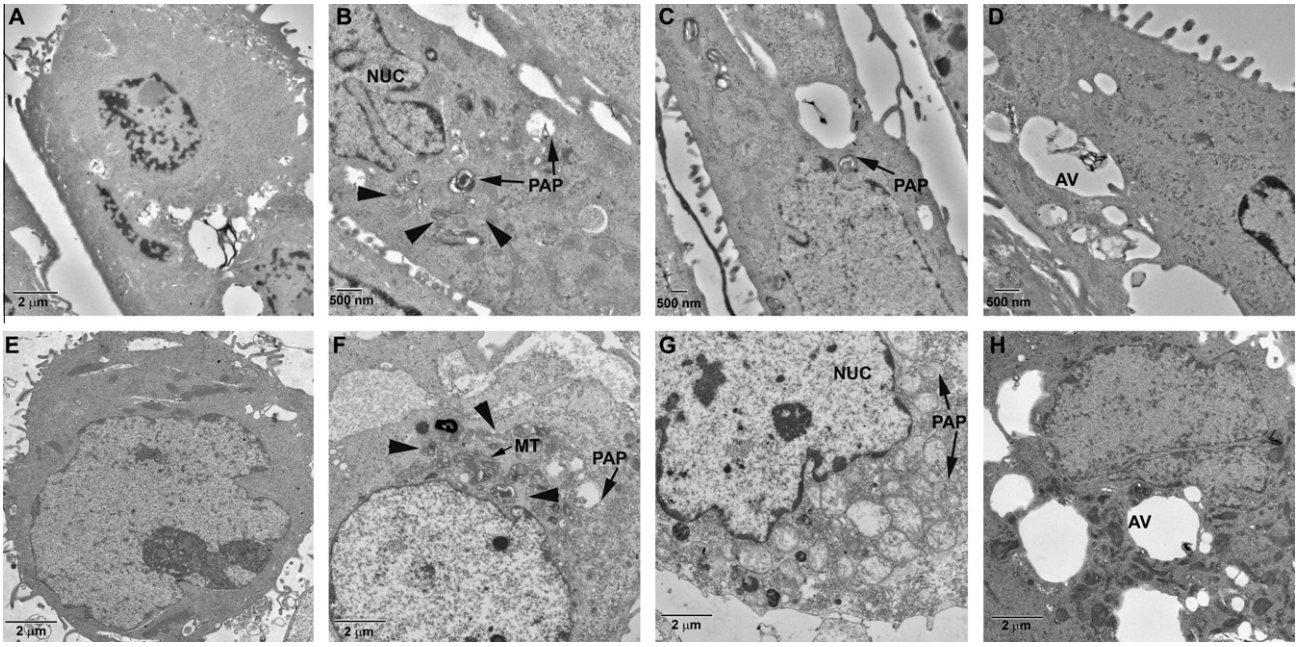


Figure 10. Transmission electron micrographs of H441 cells treated with compound **1** (B–D) and compound **29** or CLEFMA (F–G). For comparison, the untreated cells were also micrographed as controls (A and E). The typical features of autophagy were observed. The cells showed nuclear (Nuc) blobbing, and peri-nuclear accumulation of organelles (arrow head) as well as mitochondria (MT). There were numerous pre-autophagosomes or PAPs, and autophagosomes or AVs. The PAPs and AVs containing cell organelles and cytoplasmic material were widely seen.

were followed by the appearance of intracellular vacuoles or autophagosomes (Fig. 11). Monodansylcadaverine (MDC) is an autofluorescent marker for autophagic vacuoles.[44] We found the emergence of intense MDC-fluorescence in the treated cells. The fluorescence vanished when the cells were treated with 3-methyl adenine (3-MA). It has been shown that 3-MA specifically inhibits autophagic/lysosomal pathway by suppressing the formation of autophagosomes.[45]

The original inventors of compound **1** (EF24) have shown that it causes apoptosis in MDA-MB-231 human breast cancer cells and DU-145 human prostate cancer cells via a redox-dependent mechanism.[16] EF24 and its analogs containing dienone moiety serve as Michael acceptors; the involvement of cellular redox status in EF24's action was confirmed in a study comparing EF24 with its water-soluble glutathione adducts.[19] That EF24 causes apoptotic cell death was also observed in cisplatin-resistant ovarian cancer cells.[13] Evidently, this activity in ovarian cancer cells was mediated by induction of PTEN and inhibition of AKT activities. In a more elaborate in vivo study, Subramaniam et al., EF24 was found to induce caspase-mediated apoptosis in HCT-116 colon cancer xenografts.[2] At the same time, marked reduction in AKT activity, as well as decreased cyclooxygenase-2, interleukin-8, and vascular endothelial growth factor mRNA and protein expression was reported.[2] A common theme in these studies showing mechanistic

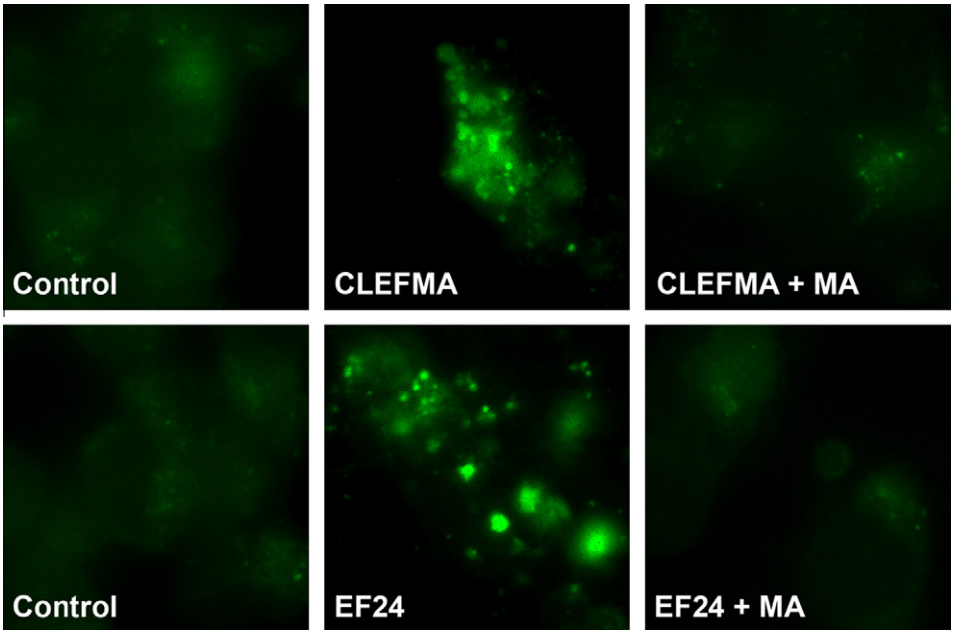


Figure 11. Fluorescent microscopic pictures of compound **29** (CLEFMA)- and compound **1** (EF24)-induced autophagosome formation in H441 cells. The cells were labeled with an autophagosome marker monodansylcadaverine. The autophagy was inhibited by 3-methyladenine (3-MA).

details of EF24 action has been the induction of apoptosis following G2-M cell cycle arrest. At molecular level, EF24 has been shown to suppress NF-κB signaling pathway through direct action on I-κB kinase in lung, breast, ovarian and cervical cancer cells.[14] Evidently, NF-κB plays a pivotal role in linking inflammation and oncogenesis,[46] and an understanding is developing that the anti-cancer activity of curcumin and its analogs may be mediated primarily by inhibition of NF-κB activity.[47]

Contrary to the observation that EF24 induces apoptotic cell death, we present data that in H441 lung cancer cells, CLEFMA induces autophagic cells death. By flowcytometric analysis and caspase-3 immunoblotting also, we could not find characteristic signatures of apoptotic cell death (data not shown). Although apoptosis is believed to be the primary mechanism of chemotherapy-induced cell death, there is considerable merit in designing drugs that induce a mode of cell death alternative to apoptosis, especially in cells that may be deficient in cellular mediators of apoptosis. For instance, lung cancers are resistant to therapeutic induction of apoptosis because of the mutation in apoptosis regulators p53, bcl-2 and p21WAF1 genes.[48–50] Interestingly, H441 cells have recently been reported to carry p53 as well as k-Ras mutation to gain survival advantage.[43] Altered expression of these genes renders many of the apoptosis-inducing drugs ineffective in lung cancer. Therefore, therapies that promote other types of death, such as autophagy, may be preferential for use in treating lung cancer. At the same time, current scientific evidence is suggestive of a more heterogeneous model of tumor response to therapy wherein multiple modes of cell death combine to generate an overall tumor response. Perhaps the eventual mechanism(s) of cell death is determined by the drug, the dosing regimen, and the genetic background of the tumor cells.[51] This conjecture is supported by the observation that the therapeutic response does not correlate with apoptosis, and that anti-apoptotic mutations or altered expression of genes, such as bcl-2, p21, and p53, are not negative predictors of therapeutic benefits.[52–54]

3. Experimental

All reagents were obtained from commercial sources, and used directly without further purification. The reactions were monitored by thin layer chromatography (TLC) on 250 μm silica plates. ^{1}H NMR spectra and ^{13}C NMR spectra (DMSO-d_6 and $CDCl_3$) were recorded at 300 and 75 MHz on Mercury-VX 300 and Varian VNMRS-400 NMR Spectrometers. Spectra were referenced to the residual protonated solvents. Abbreviations s, d, t, m, br, dd and dt used in the description of NMR spectra denote singlet, doublet, triplet, multiplet, broad, double doublet, and double triplet, respectively. Chemical shifts and coupling constants were reported in δ parts per million (ppm) and hertz (Hz), respectively. Mass spectra were recorded by Finnigon Mat LCQ mass spectrometer (San Jose, CA). Samples for IR spectroscopic measurements were finely ground, and prepared as KBr pellets in a Bruker IFS 66v spectrometer with a KBr beam splitter. Sixty four scans at a spectral resolution of 1 cm^{-1} were averaged for each spectrum. Melting points were recorded on an Electrothermal Mel-Temp melting point apparatus (Thermo Scientific, Waltham, MA). The reported melting points (degree Celsius) are uncorrected. Where applicable, the compounds were purified by column chromatography using 200–300 mesh silica gel columns.

3.1. Chemistry

3.1.1. General method for the synthesis of series A compounds

Hydrochloric Acid gas (generated in situ) was bubbled into a solution of 4-piperidone hydrochloride monohydrate (1 equiv) in glacial acetic acid until a clear solution was obtained (about 15 min). Aromatic aldehyde (2 equiv) was added to the solution, and left at room temperature for 48 h. In the case of compounds **3**, **4** and **5**, solids were separated by scratching the glass surface for 5 min. The crystals formed were filtered on a Buchner funnel, washed with absolute ethanol (50 ml) and ether (50 ml). The hydrochloride salts of various 3,5-bis(benzylidene)-4-piperidones were obtained as yellow crystalline solids. The free bases were generated by the treating the solids with 10% K_2CO_3. Specific details of synthesis of series A compounds are described in Table 2 (Supplementary data).[6,18,32,35,38,39,55–61]

3.1.1.1. 3,5-Bis(2-fluorobenzylidene)-4-piperidone (1). From 4-piperidone hydrochloride monohydrate (3 gm, 19.5 mmol) and 2-fluorobenzaldehyde (6 ml, 56.5 mmol), we obtained compound

1 as a yellow crystalline solid (5.71 gm, 94% yield). R_f (60:40 ethyl acetate/hexanes) = 0.46. ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.94 (s, 1H, NH), 7.88 (s, 2H, C=CH), 7.61–7.54 (m, 2H, Ar-H), 7.51 (t, 4H, Ar-H, J = 7.8), 7.39 (q, 2H, Ar-H, J = 7.2), 4.37 (s, 4H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 172.09, 160.37 (d, J = 249.1), 132.55 (d, J = 8.5), 131.79 (d, J = 3.8), 131.03 (d, J = 1.5), 129.89, 124.95 (d, J = 3.2), 121.52 (d, J = 13.1), 116.09 (d, J = 21.0), 43.85 (d, J = 3.1). FT-IR (cm^{-1}): 797, 1201, 1243, 1453, 1483, 1616, 1715, 2977, 3025. ESI mass calcd for $C_{19}H_{16}F_2NO$ $(M+H)^+$ 312.12, found 312.13.[6,35,58–60]

3.1.2. Synthesis of series B compounds

In this series, we selectively reduced the enone unsaturation in 3,5-bis(2-fluorobenzylidene)-4-piperidone.

3.1.2.1. 3,5-Bis(2-fluorobenzylidene)-4-hydroxy-piperidine (10). Sodium borohydride (36 mg, 0.96 mmol) was added to a solution of compound **1** (300 mg, 0.96 mmol) in ethanol at 0 °C, in portions. The reaction mixture was stirred at room temperature for 1 h. The solvent was evaporated to dryness. The residue was dissolved in chloroform, and the organic phase was washed with brine and water. The organic layer was dried over sodium sulfate, and concentrated to dryness. The crude product was passed through silica column, and eluted with 70% ethyl acetate in hexanes. The title compound **10** was obtained as colorless thick syrup (296 mg, 98% yield). R_f (60:40 ethyl acetate/hexanes) = 0.10. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.25 (s, 1H), 7.00 (q, 2H, Ar-H, J = 7.4), 6.93 (t, 2H, Ar-H, J = 7.8), 6.86 (t, 2H, Ar-H, J = 7.0), 6.81 (t, 2H, Ar-H, J = 7.6), 6.39 (s, 2H, C=CH), 4.66 (s, 1H), 3.61 (d, 2H, J = 14.8), 3.25 (d, 2H, J = 14.8). ^{13}C NMR (75 MHz, $CDCl_3$): δ 161.42 (d, J = 246.2), 144.36, 130.74, 128.50, 128.60, 128.49, 124.31, 124.12, 123.64, 115.43, 115.14, 75.62, 45.68. ESI Mass calcd for $C_{19}H_{17}F_2NO$ $(M)^+$ 313.13, found 313.87.

3.1.2.2. 3,5-Bis(2-fluorobenzyl)-4-piperidone (11). To a solution of compound **1** (300 mg, 0.96 mmol) in ethanol, 10 mg of 5% Pd/C was added. The reaction mixture was stirred for 16 h under hydrogen gas at atmospheric pressure. Pd/C was filtered, and the solvent was evaporated to dryness. Compound **11** was obtained as a white solid (285 mg, 95% yield). R_f (30:70 methanol/chloroform) = 0.20. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.25–6.90 (m, 8H, Ar-H), 3.60–2.50 (m, 10H, NCH_2CHCH_2). ^{13}C NMR (75 MHz, $CDCl_3$): 175.10, 161.05 (d, J = 250.0), 131.34 (d, J = 4.3), 128.68 (d, J = 8.3), 124.28 (d, J = 15.5), 115.48 (d, J = 21.6), 48.55, 46.54, 25.52. ESI Mass calcd for $C_{19}H_{20}F_2NO$ $(M+H)^+$ 316.15, found 316.20.

3.1.3. General procedure for the synthesis of series C compounds

Dicarboxylic acid anhydrides (1.00–1.20 mmol) and triethylamine (2 mmol) were added to a solution of compound **1** (1 mmol) in dry methylene chloride (10 ml). The reaction mixtures were stirred at room temperature for 2–4 h. The reactions were monitored by TLC, and upon consumption of the starting material, the reaction mixtures were diluted with methylene chloride (10 mL). The diluted mixture was washed with saturated sodium bicarbonate followed by a water-wash. The organic layer was dried with anhydrous sodium sulfate and concentrated. The crude reaction products were purified by column chromatography on silica gel (200–300 mesh) using (10:90 methanol/chloroform) system solvent system, except for compound **17** where 5:95 ratio was used. Fractions containing pure product were combined, evaporated and dried under vacuum. TLC was developed in methanol/chloroform system (10:90). The synthetic details about individual compounds belonging to series C are described in Table 3 (Supplementary data).[12]

3.1.4. Synthesis of series D compounds

In series D we synthesized four *N*-substituted 3,5-bis(2-fluorobenzylidene)-4-piperidones. The substitutions were performed with functionalities containing relatively short carbon chain.

3.1.4.1. *N*-Formyl-3,5-bis(2-fluorobenzylidene)-4-piperidone (18). Compound **18** was synthesized by two methods. In the first method, acetic anhydride (3 ml) was added drop-wise to an ice-cold solution of compound **1** (300 mg, 0.96 mmol) in formic acid (6 ml), and the reaction mixture was stirred at room temperature for 16 h. The solvent was distilled-off under vacuum. The solid obtained was recrystallized from chloroform and hexanes to obtain **18** as a yellow solid (148 mg, 45% yield). In the second method, ammonium formate (121 mg, 1.92 mmol) was added to a solution of compound **1** (300 mg, 0.96 mmol) in dry acetonitrile. The mixture was refluxed for 24 h, before evaporating to dryness. The solid was recrystallized from methylene chloride and hexanes to obtain pure **18** as a yellow solid (190 mg, 57% yield). R_f (60:40 ethyl acetate/hexanes) = 0.60. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.16 (s, 1H, CHO), 8.09, 8.06 (2s, 2H, C=CH), 7.60–7.22 (m, 8H, Ar-H), 4.87, 4.66 (2s, 4H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 185.34, 162.14 (d, J = 45.6), 160.95, 159.87 (d, J = 45.9), 132.85, 132.19 (d, J = 25.7), 131.12 (d, J = 25.4), 130.78, 124.46 (d, J = 3.1), 116.49 (d, J = 22.1), 116.18 (d, J = 22.3), 46.38 (d, J = 5.4), 41.11 (d, J = 5.2). ESI Mass calcd for $C_{20}H_{15}F_2NNaO_2$ $(M+Na)^+$ 362.10, found 362.07.

3.1.4.2. *N*-Acetyl-3,5-bis(2-fluorobenzylidene)-4-piperidone (19). Pyridine (1 ml) and acetic anhydride (110 μl, 1.15 mmol) was added to a solution of compound **1** (300 mg, 0.96 mmol) in anhydrous methylene chloride (5 ml). The reaction mixture was stirred at room temperature for 16 h, diluted with methylene chloride, washed with water. The organic phase was separated, dried with anhydrous sodium sulfate, concentrated, and separated on a silica column using 60% ethyl acetate in hexanes. *N*-Acetyl derivative, **19**, was obtained as a yellow solid (311 mg, 91% yield). R_f (60:40 ethyl acetate/hexanes) = 0.45. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.89, 7.84 (2s, 2H, C=CH), 7.37 (q, 2H, Ar-H, J = 7.6), 7.30–7.05 (m, 6H, Ar-H), 4.77, 4.54 (2s, 4H), 1.88 (s, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 185.97, 169.16, 162.42 (d, J = 40.3), 159.08 (d, J = 39.1), 133.39 (d, J = 13.8), 131.47 (d, J = 8.9), 131.14 (d, J = 4.0), 130.21, 124.40 (d, J = 3.7), 124.32 (d, J = 3.7), 122.55 (d, J = 13.5), 122.22 (d, J = 14.4), 116.20 (d, J = 21.6), 115.95 (d, J = 21.6), 47.16 (d, J = 5.1), 45.32 (d, J = 3.7), 21.01. ESI Mass calcd for $C_{21}H_{18}F_2NO_2$ $(M+H)^+$ 354.13, found 354.13.

3.1.4.3. *N*-(4-Methylbenzenesulfonyl)-3,5-bis(2-fluorobenzylidene)-4-piperidone (20). Tosyl (4-methylbenzenesulfonyl) chloride (68 mg, 0.35 mmol) and pyridine (1 ml) were added to a solution of compound **1** (100 mg, 0.32 mmol) in anhydrous methylene chloride (3 ml). The reaction mixture was stirred at room temperature for 16 h, diluted with methylene chloride, and washed with water. The organic phase was dried with anhydrous sodium sulfate, and concentrated to obtain a yellow solid. The crude compound was purified on a silica column using 50% ethyl acetate in hexanes (128 mg, 86% yield). R_f (30:70 ethyl acetate/hexanes) = 0.60. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.72 (s, 2H, C=CH), 7.46–7.32 (m, 4H, Ar-H), 7.24–7.10 (m, 8H, Ar-H), 4.49 (s, 4H), 2.40 (s, 3H, CH_3), ^{13}C NMR (100 MHz, $CDCl_3$): δ 184.00, 160.69 (d, J = 251.6), 144.28, 134. 65, 131.81 (d, J = 4.3), 131.76 (d, J = 4.3), 130.72, 129.75, 127.58, 124.40 (d, J = 4.1), 122.25 (d, J = 13.8), 116.16 (d, J = 21.8), 47.38 (d, J = 4.8), 21.60. ESI Mass calcd for $C_{26}H_{21}F_2NNaO_3S$ $(M+Na)^+$ 488.11, found 488.07.

3.1.4.4. NHS ester of compound 12, 4-oxo-4-[3,5-bis(2-fluorobenzylidene-4-piperidone-1-ylcarbonyl)-N-hydroxysuccinimidyl butanoic ester] (21). Compound **21** was synthesized as an intermediate in the synthesis of **22** and **24**. *N*-Hydroxy succinimide (88 mg, 0.77 mmol) and dicyclohexyl carbodiimide (158 mg, 0.84 mmol) were added to a solution **12** (290 mg, 0.70 mmol) in dry methylene chloride (4 ml). The reaction was allowed to occur with stirring at room temperature for 16 h. Dicyclohexyl urea

was filtered-off on a Buchner funnel, and the filtrate was concentrated to dryness to obtain the NHS ester as a yellow solid (295 mg, 81% yield). R_f (10:90 methanol/chloroform) = 0.80. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.92, 7.88 (2s, 2H, C═CH), 7.39 (t, 4H, Ar-H, J = 7.0), 7.27–7.10 (m, 4H, Ar-H), 4.81 (s, 2H), 4.57 (s, 2H), 2.86 (t, 2H, J = 6.7), 2.90–2.75 (m, 6H, CH_2, NHS). ESI Mass calcd for $C_{27}H_{22}F_2N_2NaO_6$ $(M+Na)^+$ 531.13, found 531.00.

3.1.4.5. Glucosamine conjugate, 4-oxo-4-[3,5-bis(2-fluorobenzylidene-4-piperidonylcarbonyl)-2-glucose butanamide] (22). Glucosamine (13 mg, 0.06 mmol) was added to a solution (30 mg, 0.06 mmol) of **21** in dry pyridine (0.3 ml), and stirred at 90 °C for 3 h. The compound was eluted with (20:80 methanol/chloroform) on a silica column to obtain **22** as a yellow solid (23 mg, 68% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.67 (br, 1H, NH), 7.73 (s, 2H, C═CH), 7.65–7.50 (m, 4H, Ar-H), 7.40–7.30 (m, 4H, Ar-H), 7.20 (d, 1H, H-1', J = 4.5), 5.59 (br, 1H, OH), 5.17 (t, 1H, H-2', J = 4.3), 4.75 (s, 4H), 3.80–3.50 (m, 4H, H-3′, 4′, CH_2), 3.15 (t, 2H, CH_2, J = 5.8), 2.90–2.75 (m, 5H, H-5′, 6′, 6″, 2 OH). ESI Mass calcd for $C_{29}H_{31}F_2N_2O_8$ $(M+H)^+$ 573.20, found 573.00.

3.1.4.6. 2-[3,5-Bis(2-fluorobenzylidene-4-piperidone-1-yl)-*N*-(4-fluorobenzyl)acetamide] (23). Commercially available 4-fluorobenzylamine (500 mg, 3.99 mmol) was reacted with bromoacetyl bromide (380 μl, 4.39 mmol) in presence of triethylamine (600 μl, 4.39 mmol) at room temperature for 20 min. The progress of the reaction was monitored by a faster moving spot in silica TLC (60% ethyl acetate in hexanes). At the end of the reaction, the mixture was filtered, and the solvent was dried to obtain 2-bromo-*N*-[4-fluorobenzyl]acetamide. Potassium iodide (166 mg, 1 mmol) and Cs_2CO_3 (325 mg, 1 mmol) and 2-bromo-*N*-(4-fluorobenzyl)acetamide (270 mg, 1.20 mmol) were added to the compound **1** (311 mg, 1 mmol) in DMF (2 ml). The reaction mixture was heated at 95 °C for 60 min. The solvent was evaporated to dryness, and the residue was dissolved in chloroform. After brine and water wash, the organic phase was dried over anhydrous sodium sulfate, and concentrated to obtain a crude yellow solid. The crude compound was recrystallized from chloroform and hexanes to get the title compound **23** as a yellow crystalline solid (252 mg, 53% yield). ^{1}H NMR (300 MHz, $CDCl_3$): 7.93 (s, 1H), 7.83 (s, 2H, C═CH), 7.75–7.02 (m, 8H, Ar-H), 6.97 (dd, 2H, Ar-H, J = 7.2, 2.1), 6.68 (t, 2H, Ar-H, J = 7.2), 4.19 (s, 2H, benzylic), 4.17 (s, 2H, benzylic), 3.76 (s, 4H). ESI Mass calcd for $C_{28}H_{23}F_3N_2NaO_2$ $(M+Na)^+$ 499.16, found 499.91.

3.1.5. Synthesis of series E compounds

In this series we synthesized a few conjugates of compound **1** containing a long lipid chain at piperidinyl nitrogen.

3.1.5.1. 4-[3,5-Bis(2-fluorobenzylidene)-4-oxo-piperidin-1-yl]-*N*-hexadecyl-4-oxo-butyramide (24). Hexadecylamine (14 mg, 0.06 mmol) was added to a solution of compound **21** (30 mg, 0.06 mmol) in dry pyridine (0.3 ml) and stirred at 90 °C for 3 h. After completion of the reaction, the solvent was dried, and the crude compound was eluted with (20:80 methanol/chloroform) on a silica column to obtain compound **24** as a yellow solid (22 mg, 59% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.89, 7.87 (2s, 2H, C═CH), 7.53–7.10 (m, 8H, Ar-H), 6.11 (t, 2H, J = 4.9), 4.75, 4.62 (2s, 4H), 3.11 (q, 2H, J = 6.6), 2.48 (t, 2H, J = 5.6), 2.36 (t, 2H, J = 6.2), 1.87 (br, 2H), 1.71–1.64 (m, 2H), 1.40–1.08 (m, 22H), 0.85 (t, 3H, J = 6.6, CH_3). ESI Mass calcd for $C_{39}H_{52}F_2N_2O_3$ $(M+H)^+$ 635.40, found 635.20.

3.1.5.2. *N*-Stearoyl-3,5-bis(2-fluorobenzylidene)-4-piperidone (25). Triethylamine (0.4 ml, 2.85 mmol) was added to a solution of compound **1** (300 mg, 0.96 mmol) in ice-cold 1,2 dichloroethane. A solution of stearoyl chloride (450 mg, 1.49 mmol) in 1,2-dichloroethane was added drop-wise to the ice-cold solution of compound **1**. The reaction mixture was stirred at room temperature for 16 h. After completion of the reaction, 10% potassium carbonate solution (10 ml) was added, and stirred for 30 min. The organic phase was separated, dried with anhydrous sodium sulfate, and concentrated to obtain a shiny solid of **25** (373 mg, 67% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.92, 7.89 (2s, 2H, C═CH), 7.50–7.35 (m, 4H, Ar-H), 7.30–7.14 (m, 4H, Ar-H), 4.79, 4.55 (2s, 4H), 2.44 (td, 2H, J = 7.3, 2.3), 2.09 (t, 2H, J = 7.6), 1.65 (t, 2H, J = 7.0), 1.41 (t, 2H, J = 7.3), 1.40–1.05 (m, 24H), 0.87 (t, 3H, J = 6.7, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 172.05, 133.77, 133.40, 133.48, 130.76, 130.23, 124.29, 116.39, 116.09, 115.81, 46.45, 43.48, 35.26, 33.08, 31.92, 29.69, 29.37, 28.86, 25.06, 24.21, 22.69, 14.12. ESI Mass calcd for $C_{37}H_{50}F_2NO_2$ $(M+H)^+$ 578.38, found 578.40.

3.1.5.3. 3-[3,5-Bis-(2-fluorobenzylidene)-4-oxo-piperidine-1-carbonyl]-pentadec-4-enoic acid (26). Dodecenyl succinic anhydride (179 mg, 0.67 mmol) and triethylamine (0.18 ml, 1.29 mmol) were added to a solution of compound **1** (200 mg, 0.64 mmol) in dry methylene chloride and stirred at room temperature for 2 h. The mixture was chromatographed on a silica column, and the title compound **26** was eluted from the column using 10% methanol/chloroform as eluant. The compound **26** was obtained as yellow thick syrup (352 mg, 95% yield). R_f (10:90 methanol/chloroform) = 0.26. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.90–7.80 (m, 3H, 2C═CH, vinyl-H), 7.42–7.00 (m, 9H, Ar-H, vinyl-H), 5.30–5.05 (m, 4H), 5.00–4.95 (m, 2H), 4.82–7.78 (m, 2H), 4.65–4.40 (m, 4H), 2.85–2.70 (m, 2H), 2.50–2.40 (m, 2H), 2.30–2.10 (m, 4H), 2.08–1.90 (m, 4H), 1.90–1.70 (m, 2H), 0.91 (t, 3H, J = 6.4, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 185.81, 173.27, 170.15, 161.96 (d, J = 55.2), 159.45 (d, J = 53.7), 135.38, 134.29, 133.30, 133.10, 131.52, 130.78, 130.51, 125.33, 124.78, 124.23 (d, J = 3.1), 122.47 (d, J = 13.3), 122.22 (d, J = 14.8), 116.32 (d, J = 18.9), 116.22 (d, J = 21.8), 115.93 (d, J = 25.7), 46.45 (d, J = 3.2), 46.48 (d, J = 3.1), 35.27,33.08, 31.92, 29.9, 29.19, 29.44, 29.16, 28.86, 25.05, 24.21, 22.67, 14.13. ESI Mass calcd for $C_{35}H_{41}F_2NNaO_4$ $(M+Na)^+$ 600.29, found 600.20.

3.1.5.4. 3-[3,5-Bis-(2-fluorobenzylidene)-4-oxo-piperidine-1-carbonyl]-heptadec-4-enoic acid (27). Tetradecenyl succinic anhydride (198 mg, 0.67 mmol) and triethylamine (0.18 ml, 1.29 mmol) were added to a solution of compound **1** (200 mg, 0.64 mmol) in methylene chloride. The reaction mixture was stirred at room temperature for 2 h. The crude compound was passed through silica column, and using 10% methanol/chloroform as eluant, compound **27** was obtained as yellow thick syrup (357 mg, 92% yield). R_f (10:90 methanol/chloroform) = 0.31. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.90–7.80 (m, 3H, 2C═CH, vinyl-H), 7.45–7.00 (m, 9H, Ar-H, vinyl-H), 5.40–4.50 (m, 9H), 2.70–1.70 (m, 9H), 1.40–1.20 (m, 12H), 0.93 (t, 3H, J = 6.6, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 186.10, 174.03, 170.76, 162.6 (d, J = 51.2), 133.75, 133.55, 132.36, 130.75, 126.68, 124.22 (d, J = 9.8), 115.98 (d, J = 21.2), 44.75, 32.46, 31.90, 22.67, 14.12. ESI Mass calcd for $C_{37}H_{45}F_2NNaO_4$ $(M+Na)^+$ 628.32, found 628.27.

3.1.5.5. 3-[3,5-Bis-(2-fluorobenzylidene)-4-oxo-piperidine-1-carbonyl]-heneicos-4-enoic acid (28). Octadecenyl succinic anhydride (235 mg, 0.67 mmol) and triethylamine (0.18 ml, 1.29 mmol) were added to a solution of compound **1** (200 mg, 0.64 mmol) in methylene chloride. Compound **28** was purified as described above, and was obtained as yellow syrup (395 mg, 93% yield). R_f (10:90 methanol/chloroform) = 0.32. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.88–7.82 (m, 3H, 2C═CH, vinyl-H), 7.40–7.05 (m, 9H, Ar-H, vinyl-H,), 5.21–5.14 (m, 2H), 5.01–4.90 (m, 2H), 4.68–4.50 (m, 8H), 2.80–2.70 (m, 2H), 2.66–2.41 (m, 4H), 2.28–2.19 (m,

2H), 1.85–1.77 (m, 2H), 1.30–1.05 (m, 16H), 0.78 (t, 3H, J = 6.7, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 186.09, 179.02, 176.63, 174.01, 170.76, 162.41 (d, J = 30.8), 159.08 (d, J = 30.5), 133.63 (d, J = 9.2), 133.53 (d, J = 9.3), 131.58, 130.18, 126.45, 125.53, 122.48, 115.98, 46.32, 44.77, 43.33, 41.79, 37.98, 35.08, 33.58, 32.47, 32.42, 31.90, 29.70, 29.29, 22.67. ESI Mass calcd for $C_{41}H_{53}F_2NNaO_4$ $(M+Na)^+$ 684.38, found 684.40.

3.1.5.6. 4-[3,5-Bis-(2-chlorobenzylidene)-4-oxo-piperidin-1-yl]-4-oxo-2-butenoic acid (29). This compound was synthesized in a manner similar to that of the series C compounds. From compound **3** (547 mg, 1.59 mmol), maleic anhydride (171 mg, 1.75 mmol) and triethylamine (670 μl, 4.76 mmol), we obtained the title compound **29** as yellow solid (661 mg, 94% yield). R_f (10:90 methanol/chloroform) = 0.42. ^1H NMR (300 MHz, $CDCl_3$): δ 10.42 (br s, 1H, COOH), 7.97, 7.89 (2s, 2H, C=CH), 7.50–7.05 (m, 8H, Ar-H), 6.28 (d, 1H, CH=CH, J = 11.7), 5.86 (d, 1H, CH=CH, J = 11.7), 4.77, 4.55 (2s, 4H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 185.67, 167.20, 167.17, 135.92, 135.18, 135.04, 134.75, 132.51, 130.76, 129.99, 126.93, 118.13, 110.00, 46.79, 42.87. ESI Mass calcd for $C_{23}H_{18}Cl_2NO_4$ 442.06 $(M+H)^+$, found 442.08.

3.2. Biology

3.2.1. Cell culture and drug treatment

Human lung adenocarcinoma cell line NCI-H441 (ATCC Number: HTB-174) was obtained from the American Type Culture Collection (Manassas, VA). H441 cells were maintained at 37 °C with 5% CO_2 in McCoy's 5A Medium (Invitrogen, Carlsbad, California) supplemented with 5% heat-inactivated fetal bovine serum (FBS). All media contained gentamicin at 50 μg/ml (GIBCO Laboratories, Grand Island, NY). Other cells lines (PANC-1, MiaPaCa-2 and PC-3) were also similarly maintained, except that the cell culture medium was RPMI 1640 instead of McCoy's.

To evaluate the anti-proliferative activity of synthesized bis (2-fluorobenzylidene)-4-piperidone and its derivatives, the cells were seeded in 96-well flat-bottom tissue culture plates at a density of 10×10^3 cells per well. The cells were allowed to attach and grow overnight. The test compounds were solubilized in dimethyl sulfoxide (DMSO), filtered through 0.2 μm nylon filter and added to cells at 1, 10, 25 and 100 μM concentrations in culture medium supplemented with 5% FBS. The DMSO concentration was maintained at 0.1% per well. Control wells received equivalent volume of DMSO without any test compounds. The cells were allowed to remain in the treatment medium for 24, 48 and 72 h.

3.2.2. Cell proliferation

The total number of cells after the treatment period was estimated by the hexosaminidase assay.[31] Briefly, the medium was removed and hexosaminidase substrate solution in citrate buffer pH 5 (7.5 mM), *p*-nitrophenol-*N*-acetyl-β-D-glucosaminidase (Calbiochem, San Diego, CA) was added at 60 μl per well. The plate was incubated at 37 °C in 100% humidity for 30 minutes, before stopping the reaction with 90 μl of 50 mM glycine containing 5 mM of EDTA (pH 10.4). The absorbance was measured at 405 nm. The experiments were conducted in triplicate, and repeated at least twice. The data were analyzed as percent of control, where the control wells were treated with equivalent amounts of DMSO alone, and the analyzed data was presented as average ± standard error of mean. The differences among mean values were deemed significant at p <0.05.

For IC_{50} calculations, a plot between the drug concentration and hexosaminidase activity was generated and the data was fitted either linearly or exponentially. The best fit was used for further processing of data. IC_{50} was obtained by determining the concentration of compounds (μM) resulting in 50% of cell death after 24 h of treatment.

3.2.3. Transmission electron microscopy (TEM)

TEM was performed after 24 h treatment with 1 and 10 μM of compound **1** or compound **29** (CLEFMA). The cells were fixed in 0.1 M sodium cacodylate buffer containing 4% paraformaldehyde (PFA) and 2% glutaraldehyde for 4 h at room temperature. The samples were post-fixed in 1% osmium tetroxide for 1.5 h and washed with 0.1 M sodium cacodylate buffer, followed by dehydration in an ethanol series of 50%, 60%, 75%, 85%, and 95% for 15 min each. The cells were washed twice in 100% Ethanol and passed through a series of epon–araldite (6.2 g epon + 4.4 g araldite + 12.4 g of dodecenyl succinic anhydride + 0.8 g *N*,*N*-dimethylbenzylamine) solution in ethanol,[62] before embedding the cells in epon–araldite resin. Finally, 100 nm sections were cut using a microtome, and the sections were placed on glow-discharged 300 mesh Copper grids. The ultrasections were stained with Sato's lead (mixture of calcinated lead citrate, lead nitrate, lead acetate and sodium citrate), and observed by a Hitachi H-7600 Transmission Electron Microscope at 2500× (Oklahoma Medical Research Foundation Imaging and core facility, Oklahoma City).

3.2.4. Monodansylcadaverine (MDC) staining

H441 cells at a density of 1×10^6 were allowed to adhere overnight on a sterile cover-glass placed in 6-well tissue culture plates. The cells were treated with 10 μM compound **29** (CLEFMA) or compound **1** (EF24) for 24 h in the presence or absence of 2 mM of autophagy inhibitor 3-methyladenine (Sigma, St. Louis, MO). After washing the cells once with PBS, 50 μM of MDC (Sigma, St. Louis, MO) was added to each well. After 5 min of incubation, the cover-glass was washed once with PBS and mounted on a glass-slide using Vectashield® (Vector Laboratories Inc, Burlingame, CA). The green fluorescence was visualized on the Leica DM400B upright microscope (Leica Microsystems, Bannockburn, IL) using filter cube L5 (excitation 480 ± 40 nm, suppression filter 527 ± 30 nm). Images were captured using the Spot® camera with exposure time of 32 s.

Acknowledgments

The authors acknowledge the funding from the National Cancer Institute grant 1R03 CA143614-01. We are thankful to Dr. HK Lin, Associate Professor (Department of Urology, University of Oklahoma Health Sciences Center) for providing prostate cancer PC-3 cells.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.06.055.

References and notes

1. Lev-Ari, S.; Starr, A.; Vexler, A.; Karaush, V.; Loew, V.; Greif, J.; Fenig, E.; Aderka, D.; Ben-Yosef, R. *Anticancer Res.* **2006**, *26*, 4423.
2. Subramaniam, D.; May, R.; Sureban, S. M.; Lee, K. B.; George, R.; Kuppusamy, P.; Ramanujam, R. P.; Hideg, K.; Dieckgraefe, B. K.; Houchen, C. W.; Anant, S. *Cancer Res.* **2008**, *68*, 1962.
3. Lagisetty, P.; Powell, D. R.; Awasthi, V. *J. Mol. Struct.* **2009**, *936*, 23.
4. Leyon, P. V.; Kuttan, G. *J. Exp. Clin. Cancer Res.* **2003**, *22*, 77.
5. Anand, P.; Kunnumakkara, A. B.; Newman, R. A.; Aggarwal, B. B. *Mol. Pharmacol.* **2007**, *4*, 807.
6. Adams, B. K.; Ferstl, E. M.; Davis, M. C.; Herold, M.; Kurtkaya, S.; Camalier, R. F.; Hollingshead, M. G.; Kaur, G.; Sausville, E. A.; Rickles, F. R.; Snyder, J. P.; Liotta, D. C.; Shoji, M. *Bioorg. Med. Chem.* **2004**, *12*, 3871.
7. Du, Z. Y.; Liu, R. R.; Shao, W. Y.; Mao, X. P.; Ma, L.; Gu, L. Q.; Huang, Z. S.; Chan, A. S. *Eur. J. Med. Chem.* **2006**, *41*, 213.
8. Modzelewska, A.; Pettit, C.; Achanta, G.; Davidson, N. E.; Huang, P.; Khan, S. R. *Bioorg. Med. Chem.* **2006**, *14*, 3491.

9. Robinson, T. P.; Hubbard, R. B. t.; Ehlers, T. J.; Arbiser, J. L.; Goldsmith, D. J.; Bowen, J. P. *Bioorg. Med. Chem.* **2005**, *13*, 4007.
10. Eberhart, C. E.; Coffey, R. J.; Radhika, A.; Giardiello, F. M.; Ferrenbach, S.; DuBois, R. N. *Gastroenterology* **1994**, *107*, 1183.
11. Roberson, E. D.; Mucke, L. *Science* **2006**, *314*, 781.
12. Sun, A.; Shoji, M.; Lu, Y. J.; Liotta, D. C.; Snyder, J. P. *J. Med. Chem.* **2006**, *49*, 3153.
13. Selvendiran, K.; Tong, L.; Vishwanath, S.; Bratasz, A.; Trigg, N. J.; Kutala, V. K.; Hideg, K.; Kuppusamy, P. *J. Biol. Chem.* **2007**, *282*, 28609.
14. Kasinski, A. L.; Du, Y.; Thomas, S. L.; Zhao, J.; Sun, S. Y.; Khuri, F. R.; Wang, C. Y.; Shoji, M.; Sun, A.; Snyder, J. P.; Liotta, D.; Fu, H. *Mol. Pharmacol.* **2008**, *74*, 654.
15. O'Dwyer, P. J.; LaCreta, F. P.; Haas, N. B.; Halbherr, T.; Frucht, H.; Goosenberg, E.; Yao, K. S. *Cancer Chemother. Pharmacol.* **1994**, *34*, S46.
16. Adams, B. K.; Cai, J.; Armstrong, J.; Herold, M.; Lu, Y. J.; Sun, A.; Snyder, J. P.; Liotta, D. C.; Jones, D. P.; Shoji, M. *Anticancer Drugs* **2005**, *16*, 263.
17. Costi, R.; Santo, R. D.; Artico, M.; Massa, S.; Ragno, R.; Loddo, R.; La Colla, M.; Tramontano, E.; La Colla, P.; Pani, A. *Bioorg. Med. Chem.* **2004**, *12*, 199.
18. Pati, H. N.; Das, U.; Quail, J. W.; Kawase, M.; Sakagami, H.; Dimmock, J. R. *Eur. J. Med. Chem.* **2008**, *43*, 1.
19. Sun, A.; Lu, Y. J.; Hu, H.; Shoji, M.; Liotta, D. C.; Snyder, J. P. *Bioorg. Med. Chem. Lett* **2009**, *19*, 6627.
20. Tsuboi, M.; Kato, H. *Gan To Kagaku Ryoho* **2007**, *34*, 1538.
21. Burdett, S. S.; Stewart, L. A.; Rydzewska, L. *Cochrane Database Syst. Rev.* **2007**, CD006157.
22. Akerley, W.; Choy, H. *Semin. Radiat. Oncol.* **1999**, *9*, 85.
23. Dubey, S.; Powell, C. A. *Am. J. Respir. Crit. Care Med.* **2009**, *179*, 860.
24. Dimmock, J. R.; Kandepu, N. M.; Nazarali, A. J.; Kowalchuk, T. P.; Motaganahalli, N.; Quail, J. W.; Mykytiuk, P. A.; Audette, G. F.; Prasad, L.; Perjesi, P.; Allen, T. M.; Santos, C. L.; Szydlowski, J.; De Clercq, E.; Balzarini, J. *J. Med. Chem.* **1999**, *42*, 1358.
25. Dimmock, J. R.; Padmanilyam, M. P.; Zello, G. A.; Quail, J. W.; Oloo, E. O.; Prisciak, J. S.; Kraatz, H. B.; Cherkasov, A.; Lee, J. S.; Allen, T. M.; Santos, C. L.; Manavathu, E. K.; De Clercq, E.; Balzarini, J.; Stables, J. P. *Eur. J. Med. Chem.* **2002**, *37*, 813.
26. Jia, Z.; Quail, J. W. *Acta Crystallogr., Sect. C* **1988**, *44*, 2114.
27. El-Subbagh, H. I.; Abu-Zaid, S. M.; Mahran, M. A.; Badria, F. A.; Al-Obaid, A. M. *J. Med. Chem.* **2000**, *43*, 2915.
28. Rahman, M. A.; Kelly, D. R.; Srivastava, R. M.; Fraser-Reid, B. *Carbohydr. Res.* **1985**, *136*, 91.
29. Reddy, P. G.; Kishore Kumar, G. D.; Baskaran, S. *Tetrahedron Lett.* **2000**, *41*, 9149.
30. Westerhoff, O.; Lutzen, A.; Maison, W.; Kosten, M. *J. Chem. Soc., Perkin Trans. 1* **2001**, 508.
31. Landegren, U. *J. Immunol. Methods* **1984**, *67*, 379.
32. Dimmock, J. R.; Arora, V. K.; Wonko, S. L.; Hamon, N. W.; Quail, J. W.; Jia, Z.; Warrington, R. C.; Fang, W. D.; Lee, J. S. *Drug Des. Delivery* **1990**, *6*, 183.
33. Pati, H. N.; Das, U.; Das, S.; Bandy, B.; De Clercq, E.; Balzarini, J.; Kawase, M.; Sakagami, H.; Quail, J. W.; Stables, J. P.; Dimmock, J. R. *Eur. J. Med. Chem.* **2009**, *44*, 54.
34. Thomas, S. L.; Zhong, D.; Zhou, W.; Malik, S.; Liotta, D.; Snyder, J. P.; Hamel, E.; Giannakakou, P. *Cell Cycle* **2008**, *7*, 2409.
35. Snyder, J. P.; Davis, M. C.; Adams, B.; Shoji, M.; Liotta, D. C.; Ferstl, E. M.; Sunay, U. B. U.S. Patent WO 200,10,40,188, 2003.
36. Landais, I.; Hiddingh, S.; McCarroll, M.; Yang, C.; Sun, A.; Turker, M. S.; Snyder, J. P.; Hoatlin, M. E. *Mol. Cancer* **2009**, *8*, 133.
37. Artico, M.; Di Santo, R.; Costi, R.; Novellino, E.; Greco, G.; Massa, S.; Tramontano, E.; Marongiu, M. E.; De Montis, A.; La Colla, P. *J. Med. Chem.* **1998**, *41*, 3948.
38. Das, U.; Alcorn, J.; Shrivastav, A.; Sharma, R. K.; De Clercq, E.; Balzarini, J.; Dimmock, J. R. *Eur. J. Med. Chem.* **2007**, *42*, 71.
39. Das, U.; Das, S.; Bandy, B.; Stables, J. P.; Dimmock, J. R. *Bioorg. Med. Chem.* **2008**, *16*, 3602.
40. Das, U.; Molnar, J.; Barath, Z.; Bata, Z.; Dimmock, J. R. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 3484.
41. Herbst, R. S.; Heymach, J. V.; Lippman, S. M. N. *Engl. J. Med.* **2008**, *359*, 1367.
42. Huncharek, M.; Muscat, J.; Geschwind, J. F. *Carcinogenesis* **1999**, *20*, 1507.
43. Meylan, E.; Dooley, A. L.; Feldser, D. M.; Shen, L.; Turk, E.; Ouyang, C.; Jacks, T. *Nature* **2009**, *462*, 104.
44. Biederbick, A.; Kern, H. F.; Elsasser, H. P. *Eur. J. Cell Biol.* **1995**, *66*, 3.
45. Seglen, P. O.; Gordon, P. B. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 1889.
46. Mantovani, A. *Curr. Mol. Med.10*, 369.
47. Singh, S.; Khar, A. *Anti-Cancer Agents Med. Chem.* **2006**, *6*, 259.
48. Huang, C. L.; Yokomise, H.; Miyatake, A. *Future Oncol.* **2007**, *3*, 83.
49. Lee, J. S.; Yoon, A.; Kalapurakal, S. K.; Ro, J. Y.; Lee, J. J.; Tu, N.; Hittelman, W. N.; Hong, W. K. *J. Clin. Oncol.* **1995**, *13*, 1893.
50. Niklinski, J.; Niklinska, W.; Laudanski, J.; Chyczewska, E.; Chyczewski, L. *Lung Cancer* **2001**, *34*, S53.
51. Morse, D. L.; Gray, H.; Payne, C. M.; Gillies, R. J. *Mol. Cancer Ther.* **2005**, *4*, 1495.
52. Lock, R. B.; Stribinskiene, L. *Cancer Res.* **1996**, *56*, 4006.
53. Rein, D. T.; Schondorf, T.; Breidenbach, M.; Janat, M. M.; Weikelt, A.; Gohring, U. J.; Becker, M.; Mallmann, P.; Kurbacher, C. M. *Anticancer Res.* **2000**, *20*, 5069.
54. Wouters, B. G.; Giaccia, A. J.; Denko, N. C.; Brown, J. M. *Cancer Res.* **1997**, *57*, 4703.
55. Das, S.; Das, U.; Selvakumar, P.; Sharma, R. K.; Balzarini, J.; De Clercq, E.; Molnar, J.; Serly, J.; Barath, Z.; Schatte, G.; Bandy, B.; Gorecki, D. K.; Dimmock, J. R. *ChemMedChem* **2009**, *4*, 1831.
56. Dimmock, J. R.; Padmanilayam, M. P.; Puthucode, R. N.; Nazarali, A. J.; Motaganahalli, N. L.; Zello, G. A.; Quail, J. W.; Oloo, E. O.; Kraatz, H. B.; Prisciak, J. S.; Allen, T. M.; Santos, C. L.; Balzarini, J.; De Clercq, E.; Manavathu, E. K. *J. Med. Chem.* **2001**, *44*, 586.
57. Mosley, C. A.; Liotta, D. C.; Snyder, J. P. *Adv. Exp. Med. Biol.* **2007**, *595*, 77.
58. Snyder, J. P.; Davis, M., C.; Adams, B.; Shoji, M.; Liotta, D. C.; Ferstl, E. M.; Sunay, U. B. U.S. Patent WO/2001/040,188, 2001; WO/2001/040188.
59. Snyder, J. P.; Davis, M., C.; Adams, B.; Shoji, M.; Liotta, D. C.; Ferstl, E. M.; Sunay, U. B. U.S. Patent 66,64,272, 2008.
60. Snyder, J. P.; Davis, M., C.; Adams, B.; Shoji, M.; Liotta, D. C.; Ferstl, E. M.; Sunay, U. B. U.S. Patent 2008/02,34,230 A1, 2008.
61. Youssef, D.; Potter, E.; Jha, M.; De Clercq, E.; Balzarini, J.; Stables, J. P.; Jha, A. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 6364.
62. Matsko, N.; Mueller, M. *J. Struct. Biol.* **2005**, *152*, 92.