European Journal of Medicinal Chemistry 46 (2011) 4878–4886

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Synthesis and characterization of novel PUFA esters exhibiting potential anticancer activities: An *in vitro* study

Azmat Ali Khan [a], Mahboob Alam [b], Saba Tufail [a], Jamal Mustafa [c], Mohammad Owais [a,*]

[a] Interdisciplinary Biotechnology Unit, Aligarh Muslim University, Aligarh 202002, India
[b] Department of Chemistry, Faculty of Science, Aligarh Muslim University, Aligarh 202002, India
[c] University Polytechnic, Aligarh Muslim University, Aligarh 202002, India

ARTICLE INFO

Article history:
Received 13 January 2011
Received in revised form
26 July 2011
Accepted 26 July 2011
Available online 3 August 2011

Keywords:
Linoleic acid
Arachidonic acid
Propofol
Anticancer
Apoptosis

ABSTRACT

Polyunsaturated fatty acids (PUFAs) have been reported to play a regulatory role in tumour growth progression. In the present study, we have synthesized ester derivatives of two important PUFA *viz.*, linoleic acid (LA) and arachidonic acid (AA) with propofol, a widely used general anaesthetic-sedative agent. The novel propofol ester analogues have been found to inhibit various cancer cell lines in a dose-dependent manner. Moreover, the compounds have been found to induce apoptotic cell death by enhancing the release of cytochrome *c* and expression of caspase-3. The data of the present study suggest that novel propofol–PUFA esters have strong potential to emerge as effective anticancer agents.

1. Introduction

Polyunsaturated fatty acids (PUFAs) perform multiple tasks in cell functioning and intracellular signalling [1–4]. Their role as dietary supplements in the prevention and treatment of chronic disorders have attracted due interest in recent past [2,3]. Preclinical studies demonstrate effectiveness of PUFA derivatives in growth inhibition and cytotoxicity against cancer cells *in vitro* [1–4]. The possible role of PUFA in inhibition of tumour cell proliferation, induction of apoptosis and improved sensitivity against cancer cells at par with other chemotherapeutic agents [5,6] has led to the development of fatty acid based anticancer compounds [7–11]. Although several PUFA derivatives have been thoroughly investigated in experimental models, however, to the best of our knowledge derivatives of the two important fatty acids *viz.*, arachidonic acid and linoleic acid have till date not been explored for their anticancer potential.

While mammalian hepatocyte can readily introduce double bonds at Δ^9 position of fatty acid but cannot introduce additional double bonds between C-10 and the methyl terminal end. Being necessary precursors for the synthesis of other products, linoleic as well as linolenic acids can be considered essential fatty acids for mammals. In general, linoleic acid (LA) is obtained from plant based dietary sources. LA by itself has been reported to show anti-tumour activity on various neoplastic cells in culture [12,13]. It has also been reported to exert differential effects on the kinetics of various anticancer drugs [14].

LA may be converted to certain other PUFAs including arachidonic acid. Arachidonate inturn is precursor of regulatory lipids, the eicosanoids, which are very potent biological signalling molecules that can act as short-range messengers. Arachidonic acid (AA) metabolism has apparently been observed to play a key role in cancer biology where its ability to induce cancer cell apoptosis has become an interesting strategy in cancer therapy. Arachidonic acid, is basically an ω-6 fatty acid and is primarily found in fatty parts of red meat and fish. The presence of four *cis* double bonds are primarily responsible for its flexibility, keeping the pure AA in liquid state, even at subzero temperatures. It is also supposed to help mammalian cell membranes to attain correct fluidity at physiological temperature [15]. It is one of the essential PUFAs and contrary to other more abundant PUFAs, level of unesterified AA is stringently controlled within mammalian cells [16]. Interestingly, the anticancer potential of AA has been established against various cell lines and animal models [17–19].

Propofol (Diisopropylphenol) is a low molecular weight phenolic compound that is widely used as an intravenous

* Corresponding author. Tel.: +91 571 2720388; fax: +91 571 2721776.
E-mail address: owais_lakhnawi@yahoo.com (M. Owais).

0223-5234/$ – see front matter © 2011 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.ejmech.2011.07.044

sedative-hypnotic agent in humans and animals. Preliminary studies demonstrated that LD50 of propofol in mouse was 170 mg/kg [20], while it was 386 mg/kg in human beings [21]. It has a structural analogy with the vitamin E, an antioxidant vitamin, a feature that partly explains its antioxidant properties [22]. Interestingly, clinically relevant concentrations of propofol are reported to decrease the metastatic potential of human cancer cells [23] and have also been shown to induce apoptosis through both extrinsic and intrinsic pathways [24]. The propofol also entertains certain advantages such as rapid clearance, minimal side effects and non-toxicity to humans at high doses that render it suitable as a future anticancer drug [25].

In the present study, we have synthesized various ester analogues of LA and AA with propofol. Both LA and AA have been conjugated with one of the two isomers of propofol *viz*. 2,4-Diisopropylphenol (2,4-propofol) or 2,6-Diisopropylphenol (2,6-propofol) separately. To establish the structure of the synthesized compounds; various analytical methods such as UV spectra, FT-IR, ^{1}H NMR, ^{13}C NMR and high-resolution FAB-MS have been exploited. The cytotoxic effect of novel propofol–PUFA analogues was assessed against various cancer cell lines. *In vitro* anticancer potential was further established by assessing lipid peroxidation level and induction of apoptosis in treated cancer cells.

2. Chemistry

The two isomers of propofol *viz*. 2,4-propofol and 2,6-propofol were conjugated with LA and AA separately, employing synthesis pattern introduced by Siddiqui et al. [10] with some modifications. The four novel compounds were obtained by esterification of hydroxyl (^{1}C–OH) group of propofol with the terminal carboxylic group of PUFA in the presence of *N*,*N*-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP). The reaction sequences are outlined in Scheme 1 and 2.

3. Results

3.1. Synthesis of propofol–PUFA analogues

The chemical synthesis setup resulted in synthesis of ester derivatives of propofol (Scheme 1 and 2). The synthesis of novel drug product was confirmed by assessing R_f value using solvent system with composition 1:1 *n*-hexane and diethyl ether. The new product was observed at locations having a new R_f value on TLC plates when compared to both PUFA and propofol as substrates (Supplementary Fig. 1). The compounds synthesized in good yield were colourless viscous liquid (oily) at room temperature.

3.2. Characterization of propofol–PUFA analogues

3.2.1. UV spectroscopy

3.2.1.1. Propofol–LA analogues. The spectra of parent LA (Supplementary Fig. 2) revealed absorption peak at 267 nm. Whereas 2,6-propofol and 2,4-propofol showed absorption peaks at 292 and 281 nm, respectively. These peaks were shifted to 268 and 267 nm, for **2,6P-LA** and **2,4P-LA** compounds, respectively.

3.2.1.2. Propofol–AA analogues. The spectra of parent AA (Supplementary Fig. 2) showed absorption peak at 272 nm whereas for 2,6-propofol and 2,4-propofol the absorption peaks were found to be centred at 293 and 281.5 nm, respectively. These peaks were shifted to 282 and 280 nm, for **2,6P-AA** and **2,4P-AA** compounds, respectively.

3.2.2. FT-IR spectroscopy

The infrared absorption spectra of propofol–PUFA analogues are provided as supplementary file (Supplementary Fig. 3). In the following write-up, respective resulting spectroscopic values for 2,4-propofol–PUFA analogues are given in bracket.

3.2.2.1. Propofol–LA analogues. FT-IR spectra of **2,6P-LA** showed two strong absorption bands at 1757.85 (1755.51) and 1137.00 (1147.02) cm^{-1} that are attributable to ν(C=O) and ν(C–O) bond, respectively, indicating the presence of an ester. The spectra also revealed a short band at 2931.26 (2925.99) cm^{-1}, a characteristic of an aromatic C–H bond (propofol), and the band at 2866.02 (2858.87) cm^{-1}, a characteristic of aliphatic C–H bonds. No hydroxyl (–OH) absorption band was seen, indicating the absence of non-esterified propofol.

3.2.2.2. Propofol–AA analogues. The FT-IR spectra showed two strong absorption bands at 1750.23 (1756.21) and 1143.30 (1134.42) cm^{-1} that are attributable to ν(C=O) and ν(C–O) bond, respectively, indicating the presence of an ester group in newly synthesized compounds. The spectra also revealed a short band at 2957.69 (2949.86) cm^{-1} a characteristic of an aromatic C–H bond (propofol), and the band at 2908.39 (2913.53) cm^{-1}, a characteristic of aliphatic C–H bonds. No hydroxyl (–OH) absorption band was seen, indicating the absence of non-esterified propofol.

3.3. Cytotoxic effect of propofol–PUFA analogues against cancer cells

The cytotoxic effect of propofol–PUFA derivatives against various cancer cell lines was examined by evaluating the metabolic status of cells after exposing them with increasing concentrations of various synthesized compounds (0–15 μM). Inhibition of cancer cell growth was observed to be in a dose-dependent manner (Supplementary Figs. 6a and 6b). All tested compounds showed significant ($p < 0.05$) anticancer activity in comparison to vehicle control. The anticancer property of the synthesized compounds was compared with standard drug (Table 1). It was observed that esterification with the 2,4-propofol/2,6-propofol resulted in an increase in the anticancer activity of the parent PUFA. The effectiveness of compounds, however, varied with respect to cell lines where inhibition was seen in the order:

2,4P-LA/2,6P-LA: MDA-MB-361 > HepG2 > SK-MEL-1 > A549 > HL-60 (Supplementary Fig. 6a)

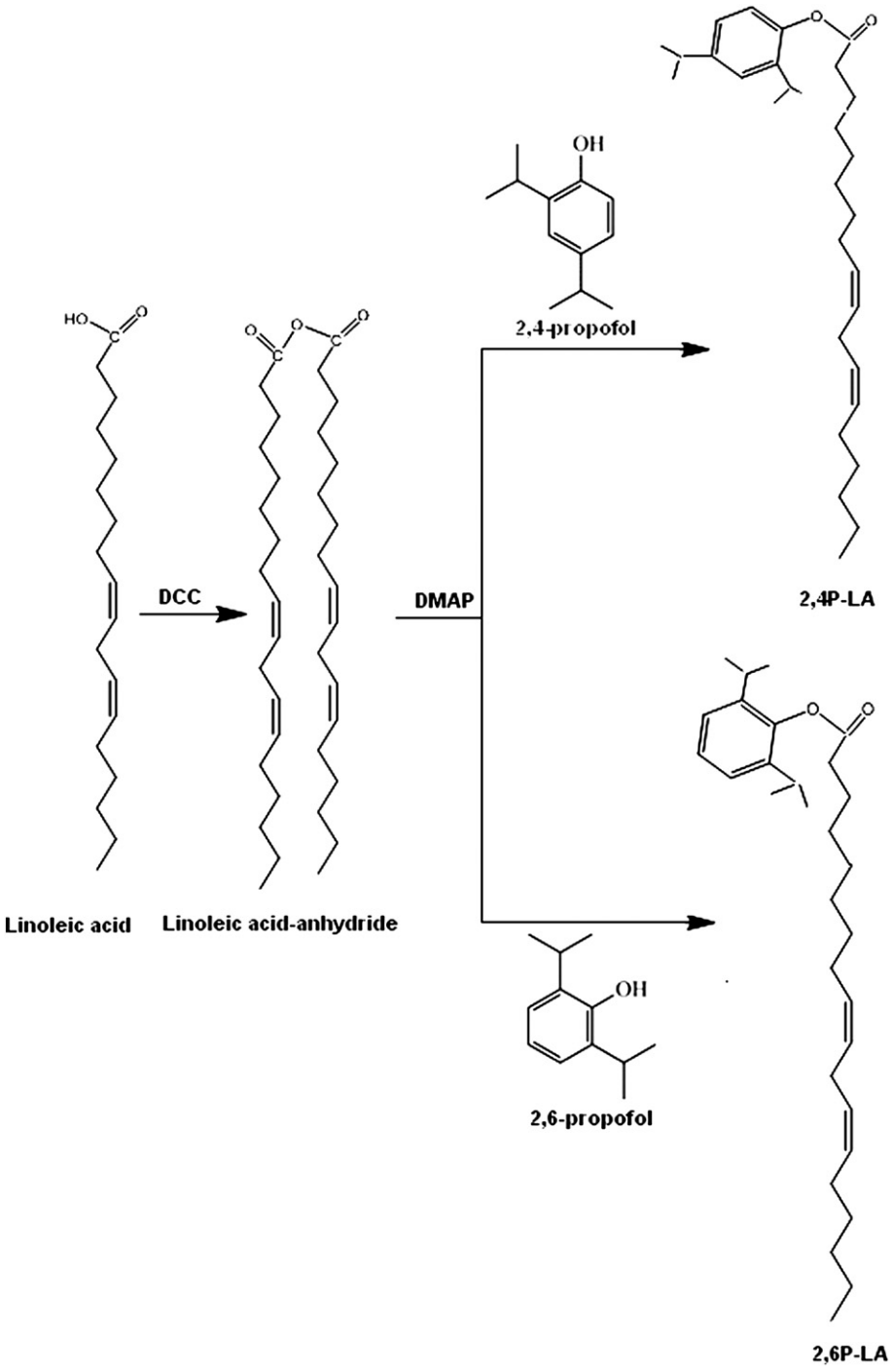
2,4P-AA/2,6P-AA: HepG2 > HL-60 > A549 > MDA-MB-361 > SK-MEL-1 (Supplementary Fig. 6b)

Results on normal HFL1 cell line were observed to be quite distinct where propofol–LA analogues were found to spare killing of normal cells. In contrast, propofol–AA analogues showed ~15% killing thus seemed to be less potent than parent AA.

Note: Further *in vitro* evaluation of anticancer efficacy of newly synthesized compounds was done on the cell lines that were found to be most susceptible to the cytotoxic effect. Therefore, MDA-MB-361 (human ductal carcinoma, breast) cell line for propofol–LA analogues and HepG2 (human liver hepatocellular carcinoma) cell line for propofol–AA analogues were used.

3.4. Evaluation of lipid peroxidation

Treatment of HepG2 cells with propofol–AA analogues (Fig. 1) increased the malondialdehyde (MDA) production after 24 h in a dose-dependent manner. The compounds significantly caused more lipid peroxidation even at relatively low doses (2.27–3.40 nmol MDA produced per 10^6 cells). Among parent reactant controls, AA showed increase in MDA production with

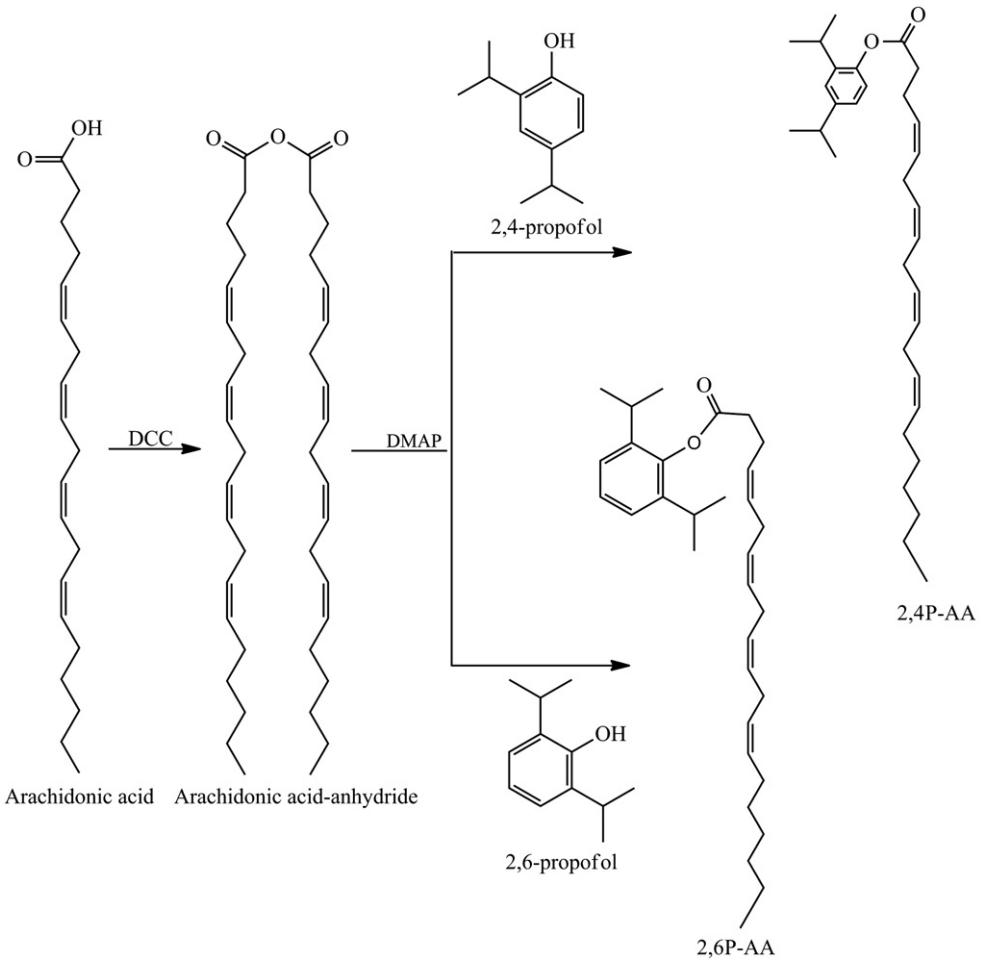


Scheme 1. Schematic representation of chemical synthesis of propofol–LA analogues.

increase in concentration whereas no effect was observed with propofol alone treatment. Propofol–LA analogues were unable to increase the lipid peroxidation level (0.25–0.34 nmol MDA produced per 10^6 cells) significantly in MDA-MB-361 cells (Fig. 1A). Similar effect was recorded for other parent reactant molecules.

3.5. Apoptosis induction by propofol–PUFA analogues

Both HepG2 and MDA-MB-361 cancer cells were incubated with novel propofol derivatives for 24 h. The expression of various apoptotic proteins in two cell lines was screened by Western blotting after 24 h of treatment. Exposure with Propofol–LA analogues induced upregulation of apoptosis in MDA-MB-361 cancer cells at 15 μM concentration as evident from significant increase ($p < 0.01$) in expression of cytochrome *c* and caspase-3 (Fig. 2, lanes 4 and 5). Although propofol–AA analogues were able to initiate the release of cytochrome *c* but no expression of caspase-3 was recorded (Fig. 3). Mean densitometric values of individual bands of cytochrome *c* and caspase-3 were quantified using UVI doc software. Each value was mean of three experiments. In propofol–LA analogues; 35.2% and 36.6% cytochrome *c* release and 32.6% and 33.7% caspase-3 expression was observed after treatment with



Scheme 2. Schematic representation of chemical synthesis of propofol–AA analogues.

2,4P-LA and **2,6P-LA**, respectively (Fig. 2B,C). The propofol–AA analogues induced 24.7% and 24.2% cytochrome *c* release after treatment with **2,4P-AA** and **2,6P-AA**, respectively (Fig. 3B).

4. Discussion

Most of the presently available anticancer drugs are associated with toxicity and other related adverse effects. Besides, some other limitations such as widespread systemic distribution in the recipient, as well as their rapid elimination, further aggravate toxicity related issues and eventually restrict their usage as potential chemotherapeutic agents. Keeping into consideration various untoward manifestations associated with most of the presently available anticancer drugs, it is desirable to opt for suitable substitutes for them. There have been many reports concerning role of dietary PUFAs in relation to tumour suppression [26,27]. Recently, clinical applications of such compounds in cancer treatment have also been reported [28]. For example, the ω-hydroxy PUFAs are reported to prevent cancer metastasis and also inhibit cancer [6]. It has also been suggested that exogenous unsaturated PUFA may increase anticancer activity of various existing cancer chemotherapeutic agents [29]. Earlier studies involving chemically modified PUFA molecules have demonstrated their specific and potent biological activity against a range of therapeutic targets. Since last decade, several attempts have been made to synthesize novel anticancer compounds by conjugating fatty acids with various anticancer drugs [9,28].

In the present study, synthesis of four novel propofol–PUFA analogues was achieved by coupling of the hydroxyl function (^{1}C–OH) present in the propofol with the terminal carboxylic group

Table 1
Cytotoxicity of propofol–PUFA analogues (1–4) in a panel of cell lines.

Propofol–PUFA analogues	Cell lines (IC_{50}, μM)					
	HepG2	MDA-MB-361	SK-MEL-1	A549	HL-60	HFL1
2,4-Diisopropylphenol-linoleic acid (**2,4P-LA**)	4.1	3.2	4.9	LA	LA	NA
2,6-Diisopropylphenol-linoleic acid (**2,6P-LA**)	3.3	2.76	4.1	LA	LA	NA
2,4-Diisopropylphenol-arachidonic acid (**2,4P-AA**)	3.7	7.5	LA	5.3	4.4	LA
2,6-Diisopropylphenol-arachidonic acid (**2,6P-AA**)	3.1	7.21	LA	4.6	3.9	LA
Doxorubicin	2.17	2.03	3.092	3.6	3.137	NA

The highest concentration tested was 15 μM. Values are means of three observations. LA, less active; NA, not active; SK-MEL-1, human skin malignant melanoma; HepG2, human liver hepatocellular carcinoma; MDA-MB-361, ductal breast carcinoma; A549, human lung carcinoma; HL-60, human leukaemia; HFL1, human lung fibroblast.

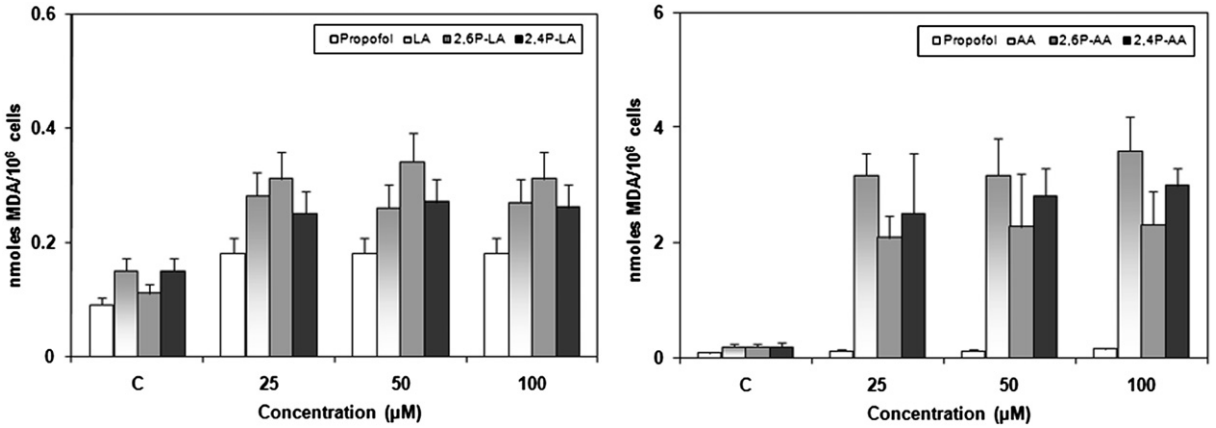


Fig. 1. Modulation of lipid peroxidation by propofol–LA analogues in MDA-MB-361 breast cancer cells and propofol–AA analogues in HepG2 liver cancer cells. Cells were exposed to vehicle alone (control or C) or 25, 50 or 100 μM of individual compounds for 24 h. Lipid peroxidation is expressed as nmol malondialdehyde (MDA) produced/10^6 cells. Data are presented as means ± SD from five experiments.

of PUFA to synthesize a specific ester. Quantitative formation of the compounds was achieved with the help of DCC as a coupling reagent and DMAP as a catalyst (Scheme 1 and 2). As a catalyst, DMAP is essential for esterification, especially in a concentration range of 10 mol%. The DCC/DMAP method is a more suitable synthetic route with mild reaction conditions, higher yield and convenient product purification. Spectrophotometric characterization involving UV absorption, infrared, NMR and mass spectroscopy established synthesis of the ester based compounds. In infrared absorption spectra, the presence of an ester bond, aromatic C–H absorbance, and absence of free –OH group absorbance, confirmed the formation of the specific ester. Elemental analysis of compounds showed the values for 'CHN' within ±0.4% of the theoretical ones. The presence of carbon and hydrogen ions in structure of newly formed compounds was determined by ^{1}H NMR and ^{13}C NMR spectra. When NMR signal of the compounds were compared with the parent propofol [10], the hydroxyl proton signal at about 4.82 ppm in 2,6-propofol was not observed in the spectra of compounds. The absence of signal of hydroxyl group in ^{1}H spectra of the compounds confirms the synthesis of new products, which differ from parent compounds. Overall shift in signals indicated that the ester bond was formed at C1′ hydroxyl group of the propofol (Supplementary Figs. 4a–d). Results of mass spectra identified products with molecular mass values which were very close to the calculated molecular mass values of 440 Da/464.47 Da for propofol–LA/propofol–AA analogues, respectively (Supplementary Figs. 5a–d).

Keeping in view the fact that PUFAs play a regulatory role in tumour growth suppression on one hand, and propofol enhances the

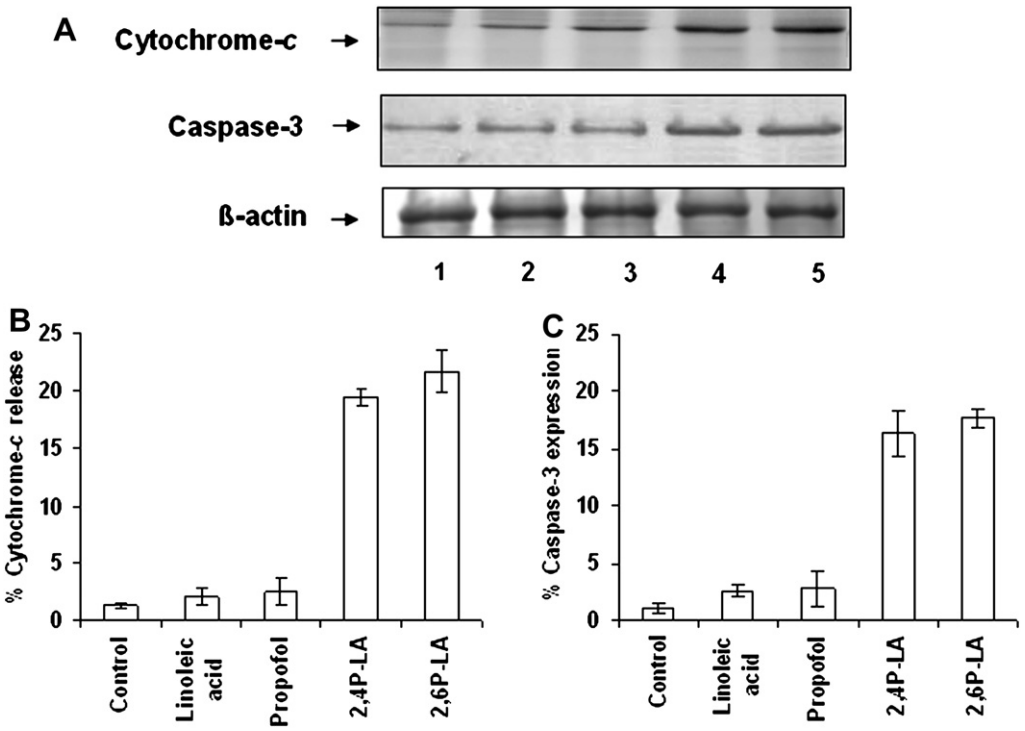


Fig. 2. A: Immunoblots showing relative distribution of cytochrome *c*, caspase-3 and β-actin (loading control) in MDA-MB-361 cells after treatment with (1) control; (2) linoleic acid; (3) propofol; (4) **2,4P-LA**; (5) **2,6P-LA**. B,C: Densitographs showing relative density of cytochrome *c* and caspase-3 respectively, in the nitrocellulose blot. Results are mean values ± SD in triplicates.

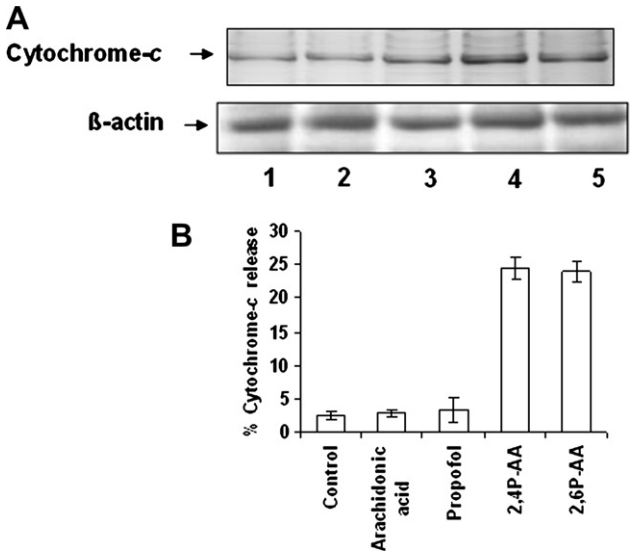


Fig. 3. A: Immunoblots showing relative distribution of cytochrome *c* and β-actin (loading control) in HepG2 cells after treatment with (1) control; (2) arachidonic acid; (3) propofol; (4) **2,4P-AA**; (5) **2,6P-AA**. B: Densitographs showing relative density of cytochrome *c* in the nitrocellulose blot. Results are mean values ± SD in triplicates.

cytotoxicity of anticancer agent on the other; all the four novel propofol–PUFA analogues were evaluated for their anticancer efficacy against various cancer cell lines. The cytotoxic potential of various PUFA and propofol based esters were evaluated against a panel of cancer cells. The compounds showed significant growth inhibition of cancer cells in a dose-dependent manner. The difference in inhibition activity was observed within a given PUFA derivative where both 2,6-propofol–PUFA were found to be more potent than 2,4-propofol analogues. Nevertheless, the novel compounds were able to inhibit the cancer cells at concentration where parent reactants alone were not effective, thereby, establishing their potent anticancer properties. Interestingly, the synthesized test compounds (except slight toxicity shown by propofol–AA analogues) did not induce any structural alterations in non-cancerous HFL1 cells under experimental conditions while they were able to inhibit cancerous cells, indicating that toxicity is not due to PUFA metabolism *per se* but rather due to the effect of the ester based compounds. Overall, the inhibitory effects of novel compounds were more or less similar to the results obtained for docosahexaenoic acid–propofol esters recently reported by Harvey et al. [11]. However, considering wide availability of both PUFAs used in the present study, the synthesized compounds are likely to be more economical when used as anticancer agent in clinical settings.

In order to identify the mechanism likely to be involved in test compounds-induced cell growth inhibition, the possibility of involvement of lipid peroxidation pathway was investigated. For propofol–LA analogues, negligible MDA production was counted on MDA-MB-361 cells at 24 h. Interestingly, results are in accordance with those reported for exogenous AA [30]. Both newly synthesized propofol–AA analogues induced a dose-dependent increase of MDA production at 24 h time period in HepG2 cells. However, the presence of propofol, an antioxidant, in the newly synthesized test compounds, seems to down-regulate lipid peroxidation level in comparison to parent AA (Fig. 1). The data of the present study suggest that in contrast to LA, AA influences the growth of cancer cells by increasing lipid peroxidation products [31].

The anticancer effects of PUFA upon tumour cells have been attributed to their ability to trigger programmed cell death by apoptosis. The induction of apoptosis by ω-6 PUFAs has been extensively studied against various tumour types [32]. In the present study, role of these compounds on induction of apoptotic factors was therefore analyzed by determining the release of cytochrome *c* and activation of caspase-3. Cytochrome *c* has a function in the intrinsic pathway of apoptosis and leads to the activation of caspase-3, which is a downstream enzyme in the apoptosis process and is involved in the execution phase of the death pathway. In propofol–LA analogues; **2,6P-LA** showed increased expression of both cytochrome *c* (22.5%) and caspase-3 (17.3%) when compared with **2,4P-LA** (Fig. 2B–C). On the other hand, **2,4P-AA** upregulated the release of cytochrome *c* slightly more as compared to **2,6P-AA** (Fig. 3B). Surprisingly no signals were recorded for caspase-3 expression in HepG2 cells when treated by propofol–AA analogues. Effective induction of apoptosis by propofol–AA and propofol–LA analogues is similar to the effects shown by other PUFA derivatives *viz*. propofol–DHA and propofol–EPA [10]. Overall, the compounds were found to induce caspase-3 mediated apoptosis in cancer cell lines that eventually led to the death of cancer cells. As apoptotic cell death is accompanied by the release of cytochrome *c* from mitochondria to the cytosol followed by activation of caspase-3 that is suggestive of mitochondrial mode for observed programmed cell death.

Conclusively, chemical synthesis followed in the present study has introduced four novel propofol–PUFA analogues. The novel compounds are colourless viscous liquid (oily) at room temperature, conforming to the common physical state of PUFA esters. The structural characterization of novel compounds has also been achieved successfully. The *in vitro* studies suggest that novel compounds possess potential anticancer efficacy. The increased anti-proliferating effect of novel compounds can be attributed to their unique structures wherein PUFA with strong hydrophobic nature is rapidly translocated across the plasma membrane, besides propofol, being a partly lipophilic agent, also facilitates uptake of PUFA–propofol based compounds by cancer cells. It seems volatile nature and a very short plasma half-life of propofol also help in its rapid removal from membranes. It is speculated that conjugation of propofol with LA and AA will result in increased lipid-solubility, bioavailability, activity, and decreased side-effects of the novel compounds. Moreover, the two structural moieties of the compound (propofol/PUFA), which should be released in the cell via enzymatic actions, were expected to have synergistic effect so that an optimum increase in their chemotherapeutic index is possible. Experiments are under way to establish efficacy of the compounds against various types of cancer in animal models.

5. Conclusions

The high abundance of PUFA in various natural sources offers an economic and cost effective strategy for synthesis of propofol–PUFA derivatives. The present study focuses on synthesis of four novel propofol–PUFA analogues. The synthesized novel compounds reveal dose-dependent growth inhibition against a panel of cancer cell lines. The mechanism underlying the antiproliferative effect of LA and AA appears to be involving apoptotic pathways despite the production of MDA derived from lipid peroxidation by propofol–AA analogues. From the data of the present study it could be suggested that the PUFA-propofol based ester compounds are more active, possess unique anticancer activities than the parent reactant controls and are able to inhibit tumour growth.

6. Experimental protocols

6.1. Materials

Linoleic acid, arachidonic acid, 2,6-Diisopropylphenol, 2,4-Diisopropylphenol, *N*,*N*-dicyclohexylcarbodiimide (DCC), 4-

dimethylaminopyridine (DMAP), Rosewell Park Memorial Institute (RPMI)-1640 medium, Eagle's Minimum Essential Medium (EMEM); 2-[4-(-2 hydroxyethyl)piperazine-1-yl]ethane sulfonic acid (HEPES); phenylmethylsulfonyl fluoride (PMSF); EDTA; dithiothreitol (DTT); 3-4, 5-dimethylthiazol-2-yl-2, 5-diphenyl-tetrazolium bromide (MTT), thiobarbituric acid (TBA), sodium dodecyl sulphate (SDS) were acquired from Sigma–Aldrich. Foetal calf serum (FCS) was procured from Bio-Whittaker. Thin-layer chromatographic plates (60A, 0.2 mm thick) and silica gel (60–120 mesh) were purchased from Fisher Scientific. Dichloromethane (DCM), methanol, ethanol, chloroform, *n*-hexane, diethyl ether, sucrose, KCl, $MgCl_2$, dimethyl sulphoxide (DMSO) and acetic acid were procured from Merck.

Monoclonal antibodies such as anti-caspase-3, anti-cytochrome *c*, anti-β-actin were procured from BD Biosciences (San Diego, CA). The secondary anti-mouse peroxidase-conjugated antibody was from Amersham Pharmacia Biotech. Chemiluminescence detection kit was purchased from GE healthcare.

6.2. Cell lines and culture conditions

Cell lines SK-MEL-1 (ATCC# HTB-67), HepG2 (ATCC# HB-8065), MDA-MB-361 (ATCC# HTB-27), A549 (ATCC# CCL-185), HL 60 (ATCC# CCL-240) and non-cancerous HFL1 (ATCC# CCL-153) were obtained from American Type Culture Collection (Rockville, MD). SK-MEL-1 and HepG2 cell lines were maintained in EMEM whereas MDA-MB-361, A549, HL-60 and HFL1 were maintained in RPMI medium. The complete growth mediums were supplemented with 10% (v/v) heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. All cells were maintained at 37 °C in a 95% humidified atmosphere containing 5% CO_2. Cells were screened periodically for mycoplasma contamination.

6.3. Synthesis and purification of propofol–PUFA analogues

Synthesis of the compounds was accomplished using method of Siddiqui et al. [10] with some modifications. Firstly, 1 mmol of a respective PUFA was dissolved in 5 ml dichloromethane (DCM). Subsequently, 0.45 mmol of coupling reagent *N*,*N*-dicyclohexylcarbodiimide (DCC) was added and the reaction mixture was stirred for 10 min at room temperature (23–25 °C). Finally, 1 mmol of one of the propofol isomer (2,6-propofol/2,4-propofol) was dissolved and reaction mixture was esterified in the presence of a catalyst, 0.152 mmol 4-dimethylaminopyridine (DMAP). The reaction mixture was stirred for a period of 10 h in dark. Reaction was stopped by filtration and the filtrate was concentrated under reduced pressure to get the product. The progression of reaction and synthesis of product was visualized by thin layer chromatography (TLC) followed by exposure to iodine vapour. Finally, the synthesized compound was purified by silica gel column chromatography with solvent system *n*-hexane and diethyl ether (1:1).

6.3.1. Elemental analysis

The values found for CHO of synthesized compounds using elemental analysis, were within ±0.4% of the theoretical ones. Values are as follows: **2,4P-LA**: analysis calculated for $C_{30}H_{48}O_2$: C, 81.76; H, 10.97; O, 7.27. Found: C, 81.75; H, 10.99; O, 7.26 **2,6P-LA**: analysis calculated for $C_{30}H_{48}O_2$: C, 81.76; H, 10.97; O, 7.27. Found: C, 81.73; H, 11.01; O, 7.26, **2,4P-AA**: analysis calculated for $C_{32}H_{48}O_2$: C, 82.69; H, 10.42; O, 6.89. Found: C, 82.75; H, 10.50; O, 6.75 and **2,6P-AA**: analysis calculated for $C_{32}H_{48}O_2$: C, 82.69; H, 10.42; O, 6.89. Found: C, 82.72; H, 10.43; O, 6.85.

6.3.2. 2,4-Diisopropylphenol-linoleic acid (*2,4P-LA*)

Yield 84%; ^{1}H NMR ($CDCl_3$, δ_H; ppm; *J*, Hz): 0.88 (3H, q, *J* 6.8 Hz, H20), 1.22 (12H, m, 2Ar–C –$(CH_3)_2$), 1.44–1.33 (14H, m, 7× CH_2), 1.78–1.75 (2H, t, *J* 7.6 Hz, –$COCH_2CH_2$–), 2.07 (4H, q, *J* 6.8 Hz, 2–CH_2–CH=), 2.58 (2H, t, *J* 6.4 Hz, –$COCH_2$–), 2.79 (2H, t, *J* 6.4 Hz, =CH–CH_2–CH=), 3.00 and 2.90 (2H, t and t, Ar–2CH–), 6.87 (1H, d, *J* 8.1, Ar–H6′) and 7.05 (1H, dd, *J* 6.0 and 2.4 Hz, Ar–H5′) and 7.13 (1H, d, *J* 2.2 Hz, Ar–H3′); ^{13}C NMR ($CDCl_3$; *δ*, ppm): 25.0 (C3*), 29.1–29.7 (C4, C5, C6, C7, C14, C15,), 27.2 (C8), 130.2 (C9), C10 (128.1), C11 (25.6), C12 (127.9), C13 (130.0), C16 (31.5), C17 (22.6), 14.1 (C18), 33.8 (Ar–C–(C)2–)*, 27.5 and 23.0 (Ar–2C)*, 121.9 (Aromatic carbon C6′), 124.3 (Aromatic carbon C5′), 124.6 (Aromatic carbon C3′), 139.5 (Aromatic carbon C2′), 146.0 (Aromatic carbon C4′) 146.5 (Aromatic C1′) and 172.2 (CO).

Note: *Asterisks denote assignments that may be interchanged due to merging of peaks.

6.3.3. 2,6-Diisopropylphenol-linoleic acid (*2,6P-LA*)

Yield 86%; ^{1}H NMR ($CDCl_3$, δ_H; ppm; *J*, Hz): 0.88 (3H, q, *J* 6.3 Hz, H20), 1.20 (12H, d, *J* 6.8, 2Ar–C–$(CH_3)_2$), 1.35–1.26 (14H, m, 7× CH_2), 1.88 (2H, q, *J* 7.5 Hz –$COCH_2CH_2$–), 2.06 (4H, q, *J* 6.8 Hz, 2–CH_2–CH), 2.63 (2H, t, *J* 7.6 Hz, –$COCH_2$–), 2.24 (2H, d, *J* 6.8 Hz, = CH–CH_2–CH=), 3.18 (2H, m, Ar–CH–), 7.15 (1H, t, *J* 6.4, Ar–H4′) and 7.20 (2H, d, *J* 6.0 Hz, Ar–H3′ and H5′; Ar–H6′) and 7.05 (1H, dd, *J* 6.0 and 2.4 Hz, Ar–H5′) and 7.13 (1H, d, *J* 2.2 Hz, Ar–H3′); ^{13}C NMR ($CDCl_3$; *δ*, ppm): 25.0 (C3*), 29.2–29.7 (C4, C5, C6, C15, C17*), 31.9 (C7), 27.2 (C8), 130.6 (C9), 128.1 (C10), C11 (27.1), C12 (127.9), C13 (130.2), C14 (29.2), 14.1 (C18), 27.5 (Ar–C–(C)2–)*, 26.5 (Ar–C)*, 123.4–123.8 (Aromatic carbon C3′ and C5′), 126.7 (Aromatic carbon C4′), 140.3 (Aromatic carbon C2′ and C6′), 145.6 (Aromatic C1′) and 172.4 (CO).

6.3.4. 2,4-Diisopropylphenol-arachidonic acid (*2,4P-AA*)

Yield 82%; ^{1}H NMR ($CDCl_3$, δ_H: ppm): 0.88 (q, *J* 6.8 Hz, 3H), 1.22 (m, 2Ar–C–$(CH_3)_2$, 12H), 1.25–1.30 (m, 6H), 1.32–1.35 (m, 2H), 1.85 (t, *J* 7.4 Hz, 2H), 2.06 (dt, *J* 6.6 Hz, 2H), 2.22 (dd, *J* 6.2 Hz, 6.8 Hz, 2H), 2.58 (t, *J* 7.6 Hz, 2H), 2.84 (m, 6H), 2.87 (m, Ar–CH–, 2H), 5.35–5.44 (m, 8H), 6.87 (d, *J* 8.1 Hz, Ar–H6′, 1H) and 7.05 (dd, *J* 6.0 Hz, 2.4 Hz, Ar–H5′, 1H) and 7.13 (d, *J* 2.2 Hz, Ar–H3′, 1H); ^{13}C NMR ($CDCl_3$; δ_C: ppm): 14.1 (C20), 22.6 (C19), 23.0 (Ar–C–(C)2–)*, 24.1 (C3), 24.9 (C4), 25.6 (C7), 26.6 (C10), 27.2 (C13) *, 27.5 (Ar–C), 29.3 (C16), 29.3 (C17), 31.5 (C18), 33.7 (Ar4′–C), 33.6 (C2), 121.8 (Aromatic carbon C6′), 124.4 (Aromatic carbon C5′), 124.6 (Aromatic carbon C3′) 127.8–130.7 (C5, C6, C8, C9, C11, C12, C14 and C15), 139.5 (Aromatic carbon C2′), 146.0 (Aromatic carbon C4′) 146.5 (Aromatic C1′) and 172.2 (CO). FAB-MS: m/z = 487 (6%), m/z = 439 (63%), m/z = 263 (78%), m/z = 261 (16%), m/z = 245 (9%), m/z = 205 (6%), m/z = 178 (100%), m/z = 163 (97%), m/z = 137 (8%), m/z = 135 (28%), m/z = 95 (66%), 91 (53%), 82 (34%) and lower mass fragments (less than 6%).

Note: *Asterisks denote assignments that may be interchanged due to unhindered behaviour of 2,4-propofol as comparison of 2,6-propofol.

6.3.5. 2,6-Diisopropylphenol-arachidonic acid (*2,6P-AA*)

Yield 85%; ^{1}H NMR ($CDCl_3$, δ_H: ppm): 0.88 (q, *J* 7.0 Hz, 3H), 1.20 (d, *J* 6.8 Hz, 2Ar–C–$(CH_3)_2$, 12H), 1.25–1.36 (m, 6H), 1.88 (t, *J* 7.3 Hz, 2H), 2.06 (dis, *J* 6.8 Hz, 2H), 2.24 (d, *J* 6.0 Hz, 6.8 Hz, 2H), 2.63 (t, *J* 7.4 Hz, 2H), 2.85 (m, 6H), 2.88 (m, Ar–CH–, 2H), 5.35–5.91 (m, 8H), 7.15 (t, *J* 6.4 Hz, Ar–H4′, 1H) and 7.20 (d, *J* 6.0 Hz, Ar–H3′ and H5′, 2H); ^{13}C NMR ($CDCl_3$; δ_C: ppm): 14.1 (C20), 22.6 (C19), 22.7, (C3), 24.9 (C4), 25.6 (C7), 26.7 (C10), 27.1 (C13), 27.2 (Ar–C–(C)2–), 27.5 (C16), 29.3 (Ar–C), 29.7 (C17), 30.9 (C18), 33.6 (C2), 123.4–123.9 (Aromatic carbon C3′ and C5′), 126.7 (Aromatic carbon C4′) 127.8–130.7 (C5, C6, C8, C9, C11, C12, and C14), 133.6 (C15), 140.3 (Aromatic carbon C2′ and C6′), 145.5 (Aromatic C1′) and 172.2 (CO).

FAB-MS: m/z = 510 $(M+2Na)^+$ (3%), m/z = 418 (74%), m/z = 401 (13%), m/z = 375 (68%), m/z = 373 (6%), m/z = 333 (29%), m/z = 285 (6%), m/z = 271 (100%), m/z = 269 (48%), m/z = 239 (65%), m/z = 237 (13%), m/z = 219 (6%), m/z = 178 (87%), m/z = 163 (42%), m/z = 137 (35%), m/z = 135 (13%), m/z = 111 (10%), 95 (16%), 91 (Characteristics peak), 82 (13%) and lower mass fragments (less than 6%).

6.4. Spectroscopic characterization of propofol–PUFA analogues

The formation of propofol–PUFA analogues was confirmed by various spectrophotometric studies. The presence of propofol in the synthesized compounds was assessed by UV spectroscopy on UV Mini-1240 spectrophotometer. The absorption spectra were measured between 200 and 600 nm. The absorbance was read and spectral scanning curves were made. The infrared spectra of the compounds were recorded on Nikolet-6700 FT-IR. Ten microlitre (1 μg/μl) of the compound was deposited exactly within the cell limit and was run as a thin film after evaporation of solvent. For data acquisition, a resolution of 32 was used. The analysis was performed in triplicate with data spacing of 15.428 cm^{-1}. Elemental analyses of compounds were carried out with a Carlo Erba EA-1108 analyzer. The formation of new compounds was elucidated with ^1H and ^{13}C NMR spectra on BRUKER AVANCE II 400 NMR spectrometer. The molecular mass of the compounds was determined by FAB-MS spectrum on a JEOL SX 102 Mass Spectrometer/Data System using Argon/Xenon (6 kV, 10 mA) as the FAB gas.

6.5. Growth inhibition of cancerous cell lines by propofol–PUFA analogues

The novel compounds were examined for their cytotoxicity against five different types of cancer cell lines *viz*., SK-MEL-1 (human skin malignant melanoma), HepG2 (human liver hepatocellular carcinoma), MDA-MB-361 (human ductal carcinoma, breast), A549 (human lung carcinoma), HL-60 (human leukaemia, acute promyelocytic) as well as one non-cancerous HFL1 (human lung fibroblast) using a standard 3-4, 5-dimethylthiazol-2-yl-2, 5-diphenyl-tetrazolium bromide (MTT) reduction assay. Cells in exponential growth were seeded into 96-well plates at a concentration of 5 x 10^5 cells/200 μl/well and allowed to grow in specific medium containing 5% FCS. After 24 h, cells were treated with various concentrations of test compound or parent reactant controls (PUFA only/propofol only) at a concentration range of 0–15 μM. Vehicle control (ethanol only) and positive control (doxorubicin) cells were cultured using the same conditions. Following 94 h incubation, the medium was removed and replaced with fresh medium. MTT reagent (5 mg/ml in PBS) was added to each well at a volume of 1:10 and incubated for 2–3 h at 37 °C. After treatment, 100 μl of DMSO was added to each well after carefully aspirating the supernatants. Absorbance was measured at 620 nm in a multi-well plate reader. Triplicate wells were prepared for each individual concentration. Dose-response curves were plotted as percentages of the cell absorbances. Drug sensitivity was expressed in terms of the concentration of drug required for a 50% reduction of cell viability (IC_{50}).

6.6. Lipid peroxidation

Lipid peroxidation was measured spectrophotometrically by determining malondialdehyde (MDA) production by the thiobarbituric acid assay [33]. Data were expressed as nmol of MDA produced per 10^6 cells.

6.7. Preparation of post-nuclear fraction for apoptosis assay

Cancer cells (1×10^7 cells per well) were grown in 6-well plates in serum free culture medium (respective to the cell line used) in a humidified CO_2 incubator at 37 °C. After 24 h, confluent cells were treated with control (ethanol only) or parent control (PUFA only/propofol only) or test compound and further incubated for 24 h. After stipulated time period, the cells were harvested by trypsinization and washed twice in PBS. The cells were suspended in 50 μl of ice-cold TNN buffer containing 50 mM Tris–HCl pH 7.4, 100 mm NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 1 μg/ml pepstatin, 0.5 mM EGTA, 200 μM PMSF, 0.5 mM DTT and 1 μg/ml of leupeptin and homogenized in a Teflon homogenizer. A post-nuclear fraction was prepared by centrifugation for 5 min at 2000 rpm at 4 °C. The supernatant was further centrifuged for 20 min at 10,000 *g* at 4 °C and the resultant cytosolic fraction was used for detecting the expression of two apoptotic factors; *viz*. cytochrome *c* and caspase-3.

6.8. Protein determination

Protein content was determined with the BCA method [35]. The mixture of solutions A and B (1:49) of BCA reagent was added to the protein sample and further incubated at 37 °C for 45 min. The absorbance was measured at 562 nm and the protein concentration was calculated using a standard curve of BSA.

6.9. Western blotting

The samples with equal amounts of protein were subjected to 10% SDS-polyacrylamide gel electrophoresis [34]. Immunoblotting of resolved proteins was achieved using nitrocellulose membrane. Non-specific binding on the nitrocellulose membrane was minimized by its blocking for 1 h at room temperature with PBS-T [PBS (pH 7.5) and 0.05% Tween-20] containing 5% (w/v) non-fat skimmed milk. The treated membrane was washed in PBS-T and then incubated overnight at 4 °C with specific primary antibodies (monoclonal anti-cytochrome-*c* or monoclonal anti-caspase-3) in PBS-T containing 5% (w/v) BSA. The membranes were again washed in PBS-T, anti-mouse peroxidase-conjugated secondary antibodies in PBS-T were added for 2 h, and immunoreactive bands were detected by enhanced chemiluminescence detection kit. Blots were re-probed with an antibody for β-actin to control for equal protein loading and transfer. Densitometric values of protein bands were quantified using UVI-doc Imaging Software.

6.10. Statistical data analysis

Results are expressed as the mean ± SD of three experiments for each treatment and were plotted accordingly. Individual treatments were tested against the control by using Student-*t* tests. Significant differences from control were considered at $p < 0.05$. Analyses were conducted with SPSS version 13.0.

Acknowledgements

We are thankful to Prof. M. Saleemuddin, Co-ordinator, Interdisciplinary Biotechnology Unit, Aligarh Muslim University, Aligarh, India for providing the central instrumentation facility to carry out the experiments. We also thank Sophisticated Analytical Instrumentation facility (SAIF) of Central Drug Research Institute, Lucknow, India for providing NMR and mass spectra facility. The study was conducted in financial support to Mr. Azmat Ali Khan from University Grants Commission (India).

Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejmech.2011.07.044.

References

[1] M.E. Begin, G. Ells, D.F. Horrobin, J. Natl. Cancer Inst. 80 (1988) 188–194.
[2] U.N. Das, Cancer Lett. 56 (1991) 235–243.
[3] H.S. Finstad, C.A. Drevon, M.A. Kulseth, et al., Biochem. J. 336 (1998) 451–459.
[4] R.A. Hawkins, K. Sangster, M.J. Arends, J. Pathol. 185 (1998) 61–70.
[5] A.P. Albino, G. Juan, F. Traganos, L. Reinhart, J. Connolly, D.P. Rose, Z. Darzynkiewicz, Cancer Res. 60 (2000) 4139.
[6] N. Ito, H. Tsuji, Y. Fukuda PCT Int. Appl.: WO 0238148 A1 20020516, (2002) p. 41.
[7] M. P. Moyer, W. E. Hardman, I. Cameron, U.S. Patent Application 2002-102907 200220322, Priority: US 2001-278138, (2003) p. 27.
[8] J.A. Menendez, S. Ropero, R. Lupu, R. Colomer, Oncol. Rep. 11 (2004) 1241–1252.
[9] J. Mustafa, S.I. Khan, G. Ma, L.A. Walker, I.A. Khan, Lipids 39 (2004) 167.
[10] R.A. Siddiqui, M. Zerouga, M. Wu, A. Castillo, K. Harvey, G.P. Zaloga, W. Stillwell, Breast Cancer Res. 7 (2005) R645–R654.
[11] K.A. Harvey, Z. Xu, P. Whitley, V.J. Davisson, R.A. Siddiqui, Bioorg. Med. Chem. 18 (2010) 1866–1874.
[12] Y. Hayashi, S. Fukushima, T. Hirata, S. Kishimoto, T. Katsuki, M. Nakano, J. Pharmacobiodyn. 13 (11) (1990) 705–711.
[13] J.A. Menéndez, M. del Mar Barbacid, S. Montero, E. Sevilla, E. Escrich, M. Solanas, H. Cortés-Funes, R. Colomer, Eur. J. Cancer 37 (3) (2001) 402–413.
[14] S. Ikushima, F. Fujiwara, S. Todo, S. Imashuku, Anticancer Res. 10 (4) (1990) 1055–1059.
[15] A.R. Brash, J. Clin. Investig. 107 (2001) 11.
[16] F.H. Chilton, A.N. Fonteh, M.E. Surette, M. Triggiani, J.D. Winkler, Biochim. Biophys. Acta 1299 (1996) 1–15.
[17] A.H. Conney, Y.R. Lou, J.G. Xie, T. Osawa, H.L. Newmark, Y. Liu, R.L. Chang, M.T. Huang, Proc. Soc. Exp. Biol. Med. 216 (1997) 234–245.
[18] T.A. Chan, P.J. Morin, B. Vogelstein, K.W. Kinzler, Proc. Natl. Acad. Sci. U S A 95 (1998) 681–686.
[19] D. Dymkowska, J. Szczepanowska, M.R. Więckowski, L. Wojtczak, Biochim. Biophys. Acta 1763 (2006) 152–163.
[20] R.B. Moffett, P.H. Seay, J. Med. Pharm. Chem. 2 (1960) 201–212.
[21] M. el-Ebiary, A. Torres, J. Ramirez, A. Xaubet, R. Rodriguez-Roisin, Crit. Care Med. 23 (11) (1995) 1928–1930.
[22] L. Aarts, R. Van der Hee, I. Dekker, J. De Jong, H. Langemeijer, A. Bast, FEBS Lett. 357 (1995) 83–85.
[23] T. Mammoto, M. Mukai, A. Mammoto, Y. Yamanaka, Y. Hayashi, T. Mashimo, Y. Kishi, H. Nakamura, Cancer Lett. 184 (2002) 165–170.
[24] M. Tsuchiya, A. Asada, K. Arita, T. Utsumi, T. Yoshida, E.F. Sato, K. Utsumi, M. Inoue, Acta Anaesthesiol. Scand. 46 (2002) 1068–1074.
[25] R.D. Miller, Local Anesthetics, fifth ed. Churchill Livingstone, New York, 2000, pp. 491–521.
[26] R.K. Severson, A.M.Y. Nomura, J.S. Grove, G.N. Stemmermann, Cancer Res. 49 (1989) 1857–1860.
[27] C.W. Welsch, D.H. O'Connor, Cancer Res. 49 (1989) 5999–6007.
[28] C.F. Van der Merwe, J. Booyens, E. Katzeff, Br. J. Clin. Pract. 41 (1987) 907–915.
[29] J.A. Menendez, S. Ropero, M.D.M. Barbacid, S. Montero, M. Solanas, E. Escrich, H. Cortes-Funes, R. Colomer, Cancer Res. Treat. 72 (2002) 203–219.
[30] L. Maehle, E. Lystad, E. Eilertsen, E. Einarsdottir, A.T. Hostmark, A. Haugen, Anticancer Res. 19 (1999) 1649–1655.
[31] W.-C. Chang, T.-W. Liu, C.-C. Ning, H. Suzuki, T. Yoshimoto, S. Yamamoto, J. Biol. Chem. 268 (25) (1993) 18734–18739.
[32] S. Serini, E. Piccioni, N. Merendino, G. Calviello, Apoptosis 14 (2009) 135–152.
[33] R.A. Canuto, G. Muzio, A.M. Bassi, M. Maggiora, G. Leonarduzzi, R. Lindahl, M.U. Dianzani, M. Ferro, Free Radic. Biol. Med. 18 (1995) 287–293.
[34] U.K. Laemmli, Nature (London) 227 (1970) 680–685.
[35] C.M. Stoscheck, Methods Enzymol. 182 (1990) 50–69.