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Triphenylmethanol Conjugates of Triptorelin as Anti-Lipid Peroxidation Prodrugs

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Abstract

Antioxidants are substances that can prevent or slow damage to cells caused by free radicals, unstable molecules that the body produces as a reaction to environmental and other pressures. Free radicals may play a role in heart disease, cancer and other diseases. If the body cannot process and remove free radicals efficiently, oxidative stress can result. This can harm cells and body function. Free radicals are also known as reactive oxygen species (ROS). In this research, Triptorelin® (TRP) conjugates of triphenylmethanol derivatives (TPMs) were synthesized to evaluate their *in vitro* lipid peroxidation potency. Comparative lipid peroxidation assays between TRP-TPMs conjugates and the corresponding TPMs derivatives were measured using thiobarbituric reactive substance (TBARS) in a dose- and time-dependent manner following the Fenton's pathway. Overall, TBARS decreased between 20% - 30% for the treated samples of synthesized conjugates compared to their respective control physical mixtures. These data suggest that TRP-TPMs derivatives can be used to improve the biological activity of TRP.

Keywords

Antioxidants, Prodrugs, Triptorelin, Polyphenols, Lipid Peroxidation, Triphenylmethanol

1. Introduction

Triptorelin® (TRP) is a synthetic analogue of the gonadotropin-releasing hormone (GnRH), first reported in 1976. The structure of TRP consists of ten amino acids (5-*oxo*Pro-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro-GlyNH$_2$) and is used to treat advanced prostate cancer and endometriosis. Initially TRP stimulates the pituitary secretion of FSH and LH, however, prolonged stimulation (constant concentration of TRP in the blood) of the pituitary causes insensitive to the ac-

tion of GnRH. This reduces the level of gonadotropin in the blood, resulting in decreased levels of sex hormones to postcastration or menopausal levels. These effects are reversible. In addition to the usual side effects of the agonist analogs of LH-RH, other reported adverse effects include transient hypertension, dry mouth, excessive salivation, paraesthesia and increased dysuria [1].

LHRH analogues are found in a variety of formulations and depending on the medication, can be administered every 1 to 12 months. Medications currently available in the United States include different formulations of triptorelin® leuprolide®, goserelin® and histrelin® in a variety of dosing intervals ranging from monthly to yearly. The associated side effects include hot flashes, decreased libido, erectile dysfunction, loss of bone mineral density, anemia and mood changes [2].

Efficacy and toxicity of anticancer drugs can be modified by using drug delivery systems and adjusting the physicochemical properties such as lipophilicity, cellular uptake and prolonging activity through chemical conjugation with various chemical moieties. Drug delivery systems avoid the P-glycoprotein and other multidrug resistance proteins (MRPs) that are involved in drug efflux to overcome the resistance problem and P-glycoprotein-mediated drug efflux [3] [4] [5].

Prodrug strategy is a drug delivery system through which chemical conjugation with the parent drug [6] [7] has been widely used in the delivery of anticancer drugs such as Doxorubicin® [8] [9]. For example, several conjugation methods have been used to improve the delivery of Doxorubicin®, including using gold nanoparticles [10], gold nanospheres [11], liposomes [12], peptides [13]-[18] and dendrimers [19]. The conjugation of TRP with agents that have optimal lipophilicity has yet to be explored. Therefore, the development of efficient and safe prodrug carriers to enhance the delivery and retention of TRP into drug-resistant tumor cells remains less explored.

Polyphenols are naturally occurring compounds found largely in fruits, vegetables, cereals and beverages. Fruits like grapes, apples, pears, cherries and berries contain up to 200 - 300 mg of polyphenols per 100 grams fresh weight [20] [21] [22]. In the last decade, there has been much interest in the potential health benefits of dietary plant polyphenols as antioxidants. The effect of polyphenols on human cancer cell lines is most often protective and induces a reduction in the number of tumors or rate of growth. These effects have been observed at various sites including the mouth, stomach, duodenum, colon, liver, lungs, mammary glands and skin. Many polyphenols, such as quercetin, catechins, isoflavones, lignans, flavanones, ellagic acid, red wine polyphenols, resveratrol and curcumin have been tested; all of them showed protective effects in some models although their mechanisms of action were found to be different [23] [24]. Polyphenols influence the metabolism of pro-carcinogens by modulating the expression of cytochrome P450 enzymes involved in their activation of carcinogens [25] [26].

To take advantage of the antioxidant properties of polyphenolic antioxidants, several polyphenolic derivatives were chosen for the chemical modification of

TRP. Dodecanedioic acid has been chosen as a lipophilic linker to attach TRP to polyphenolic derivatives.

In this study, we first report the synthesis of the antioxidant triphenylmethanol (TPMs) derivatives of TRP through the covalent conjugation with dodecanedioic acid as the linker. Second, we report the evaluation of their *in vitro* lipid peroxidation activities in cell lines. Prodrug conjugates were designed to improve cellular uptake, prolong biological activity and reduce therapeutic dosage of TRP. This study examines the antioxidant potential (ROS scavenging action) of TRP conjugate as a measure of their efficacy.

2. Experimental

TRP, DMSO and other chemicals and reagents were purchased from Fisher Scientific or Sigma-Aldrich Chemical Co. All coupling reactions (Figure 1) were carried out in Bio-Rad polypropylene columns by shaking and mixing using a Glass Col® small tube rotator in dry conditions at room temperature. TPMs **1a-c** which are the mimics of natural poly phenolic antioxidants, were synthesized in moderate yield (Figure 1) for covalent attachment to TRP via a hydrophobic linker, dodecanedioic acid. Tris(2-(hydroxymethyl) phenol) conjugates of TRP (**2a-c**) with optimal hydrophobicity were synthesized to carry TRP into the cells (Figure 1). All products were purified (≤95%) by a flash chromatography system (TeledyneCombiFlash® Rf-200) and the structures of all the final compounds were confirmed by ESI/TOF mass spectrometry. Figure 2 depicts the structure of the final TRP-TPMs conjugates **2a-c**.[1]

2.1. Preparation of TPMs 1a-c Derivatives

The TPMs were synthesized using a modified method based on literature [27] [28], (Figure 1). Accordingly, 1,3,5-trioxane (15 mmole) was added to 2-fluoroanisole, 2-notroanisole or 2-methylanisole (100 mmole) in 10 mL glacial acetic acid. The mixture was heated to 90°C - 95°C, then 1 mL of sulfuric acid: glacial acetic acid (1:5, v/v) was added to the solution. The mixture was stirred for 5 h at 90°C - 96°C. The reaction mixture was then cooled to 0°C using an ice bath and a homogenous solution of sodium nitrite (1.0 g, 15 mmole) and 2-fluoroanisole, 2-notroanisole or 2-methylanisole (15 mmole) in 10 ml concentrated sulfuric acid was added to the reaction mixture. The ice bath was removed and stirring continued at room temperature for an additional 24 hr the mixture was then poured into crushed ice (100 g) while stirring. The precipitate was filtered off and dried under vacuum and further purified on C_{18} column and hexanes/ethyl acetate as solvent using a TeledyneCombiFlash® Rf-200 chromatography machine with the gradient system set at a constant flow rate of 25 ml/min to yield pure products in 59% - 81%

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[1]There is a competition between phenol and primary alcohol to form ester with the linker. The alcohol has higher nucleophilicity in this reaction condition, however its nucleophilicity hampers by higher steric hindrance. Since the phenol is not the phenolate form, it is less reactive than alcohols for nucleophilic reactions, in contrast the lower steric hindrance around the phenol increases its nucleophilicity. Therefore, a mixture of esters is expected. We were not able to separate the mixture by chromatography technique.

Figure 1. Synthesis of triphenylmethanol (TPMs) derivatives **1a-c** and triphenylmethanol conjugates of TRP **2a-c**.
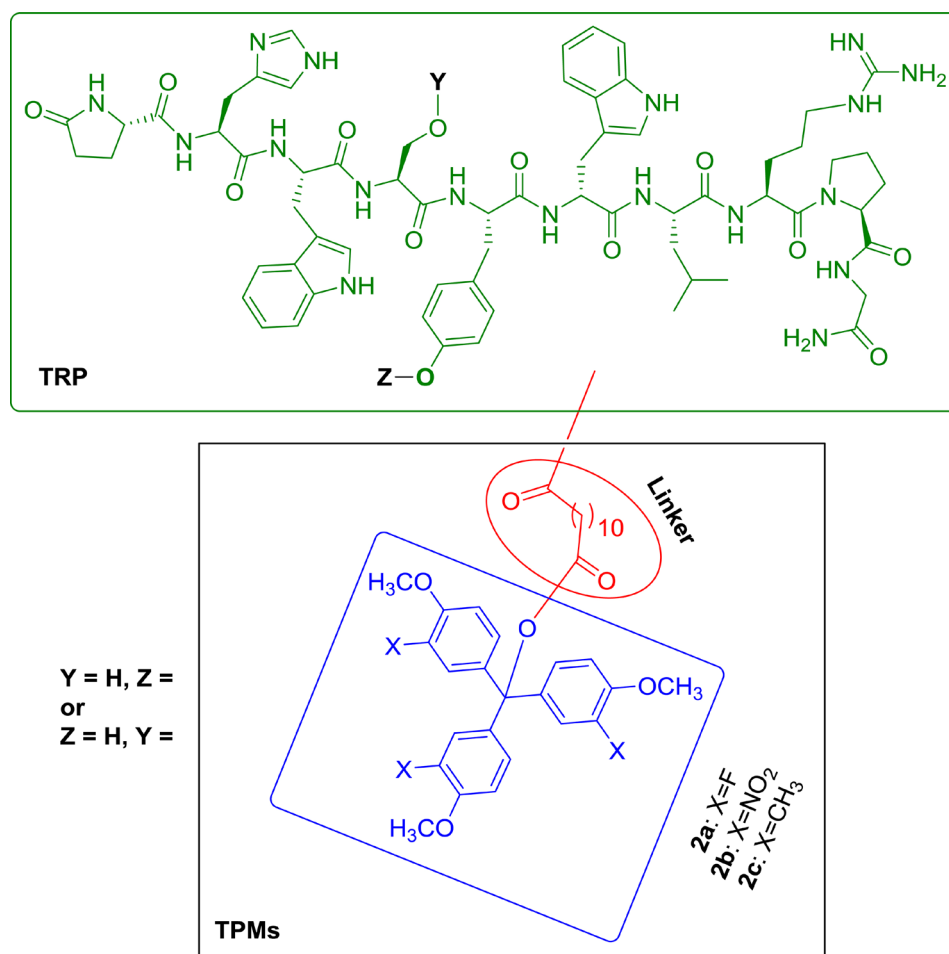
Figure 2. Structure of synthesized TRP-TPMs derivatives **2a-c**.

yield. Tris(3-fluoro-4-methoxyphenyl)methanol (**1a**), (3.59 g, 59%), MS (ESI-TOF) (*m/z*) for $C_{22}H_{19}F_3O_4$: calcd., 404.1, found 404.1 [M]$^+$; tris(3-nitro-4-methoxyphenyl) methanol (**1b**), (4.52 g, 63%), MS (ESI-TOF) (*m/z*) for $C_{22}H_{19}N_3O_{10}$: calcd, 485.1, found 485.2 [M]$^+$; tris(3-methyl-4-methoxyphenyl)methanol (**1c**), (4.77 g, 81%), MS (ESI-TOF) (m/z) for $C_{25}H_{28}O_4$: calcd. 392.2, found 392.1 [M]$^+$.

2.2. Preparation of Tris(2-(Hydroxymethyl) Phenol) Conjugates of TRP 2a-c

Tris(4-methoxyphenyl)methanol derivatives **1a-c** (0.05 mmol), TRP acetate (0.05 mmol), dodecanedioic acid, 12 mg, 0.05 mmol) and HBTU (19 mg, 0.05 mmol) were dissolved in dry NMP (3 mL). N,N'-diisopropylcarbodiimide (DIC, 8 µL, 0.05 mmol) and N,N-Diisopropylethylamine (DIPEA, 21 µL, 0.12 mmol) were added to the reaction mixture. The mixture was stirred at room temperature for 24 h. Afterwards, the solvent was evaporated and dried under vacuum. The final product was purified with a C_{18} column and hexanes/ethyl acetate as solvents using a TeledyneCombiFlash® Rf-200 chromatography machine. The gradient system set at a constant flow rate of 25 ml/min to yield pure TRP-TPMs conjugates **2a-c** (Figure 1). TRP-TPMs conjugate (**2a**), (61 mg, 62%), MS (ESI-TOF)

(*m/z*) for $C_{98}H_{120}F_3N_{18}O_{19}$: calcd, 1908.8, found 1908.7 $[M + H]^+$; TRP-TPMs conjugate (**2b**), (57 mg, 54%), MS (ESI-TOF) (*m/z*) for $C_{98}H_{120}N_{21}O_{25}$: calcd, 1989.9, found 1989.8 $[M + H]^+$; TRP-TPMs conjugate (**2c**), (67 mg, 65%), MS (ESI-TOF) (*m/z*) for $C_{101}H_{129}N_{18}O_{19}$: calcd, 1897.0, found 1896.9 $[M + H]^+$.

2.3. Antioxidant Evaluation of TRP-TPMs on Lipid Peroxidation.

The amount of 3 μl Canola vegetable oil in a total reaction volume of 1000 μl consistent of 0.05 M Tris-HCl pH 7.4, containing 0.15 M KCl, and 0.02 % Sodium dodecyl phosphate (SDS), to which was added to a freshly prepared solution of 5 μl hydrogen peroxide (H_2O_2) and Fe^{2+} (as Ferrous sulphate ($FeSO_4.7H_2O$) respectively. To different aliquots of this solution each containing, 5 μl H_2O_2, 5 μl of Fe^{2+} and 3 μl of the vegetable oil in Tris buffer, progressively increasing concentrations of different triptorelin derivatives were added. The samples were incubated at 37˚C for 4, 8 and 24 hr Control consisted of all the above reagents except the triptorelin derivatives.

2.4. Analysis of the Lipid Peroxidation Compounds in the Samples

2-Thiobarbituric acid (TBA, 2 ml of 67% solution), was added to the above samples and incubated at 60˚C for 1 hr in a water bath. The samples were then cooled down to room temperature and 2 ml of n-butanol was added. Then 220 μl each of the upper butanol layer was transferred into a-96 well plate. Absorbance was measured at 532 nm in a Synergy HT Microplate Reader (Winooski, VT).

2.5. Calculation of Lipid Peroxides in the Samples

The levels of lipid peroxide were calculated as thiobarbituric acid reactive substances (TBARS) using a molar extinction coefficient or molar absorptivity for the TBA (1.65×10^{-3} mM $cm^{-1} \cdot l^{-1}$) using the Beer's Law.

3. Results and Discussion

3.1. Measuring Lipid Peroxidation

Malondialdehyde (MDA), a major degradation product of lipid hydroperoxides, has attracted much attention as a marker for assessing the extent of lipid peroxidation [29] [30]. The compound is of particular concern since it has been shown to be mutagenic and carcinogenic and implicated in other pathological processes such as the formation of fluorescent pigments typical of cellular aging.

The most common method for measuring MDA in food products and biological samples seems to be the thiobarbituric acid (TBA) test, which is based on spectrophotometric quantitation of the pink complex formed after reaction of MDA with two molecules of TBA [31] [32]. The TBA test can be performed (a) by directly heating the sample with TBA followed by separation of the pink complex produced (b) by distillation of the sample followed by reaction of the distillate with TBA (c) by extraction of the lipid portion of the sample with organic solvents and reaction of the extract with TBA and (d) by extraction of

MDA using aqueous trichloroacetic or perchloric acid and reaction with TBA.

Although the distillation method is the most frequently used procedure and may be regarded as the standard method for MDA analysis, it is more cumbersome and requires more time than the aqueous acid extraction method. Furthermore, heating during distillation enhances the degradation of existing lipid hydroperoxides and, thus, additional MDA and other TBA-reactive substances (TBARS) may be formed even in the presence of metal chelators or phenolic antioxidants. The aqueous acid extraction method is also preferred by many workers because it is simple and gives results that are highly correlated with those of distillation and sensory evaluation methods.

3.2. Effects of TRP-TPMs Derivatives 2a-c on Lipid Peroxidation

Figures 3-5 show the results of several doses on concentrations (0, 5, 10, 15, 20, 25 µM) of TRP-TPMs derivatives on lipid peroxidation. For compound **2a**, the
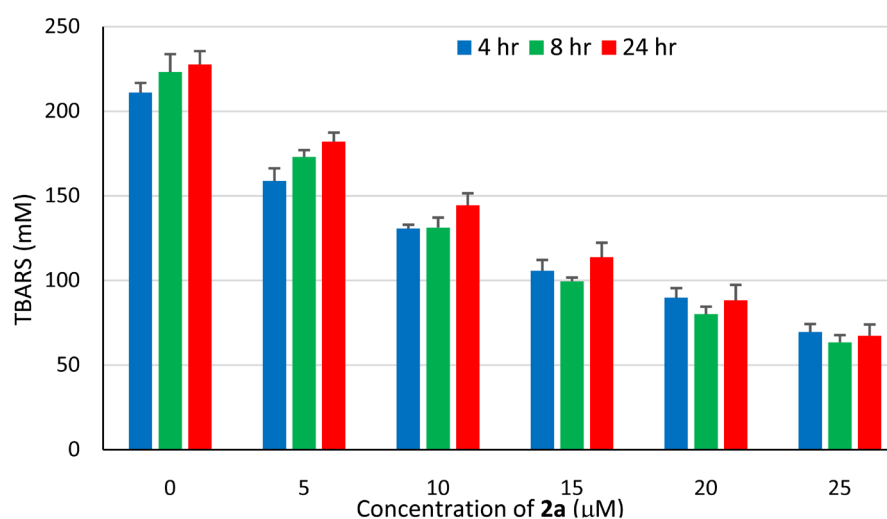

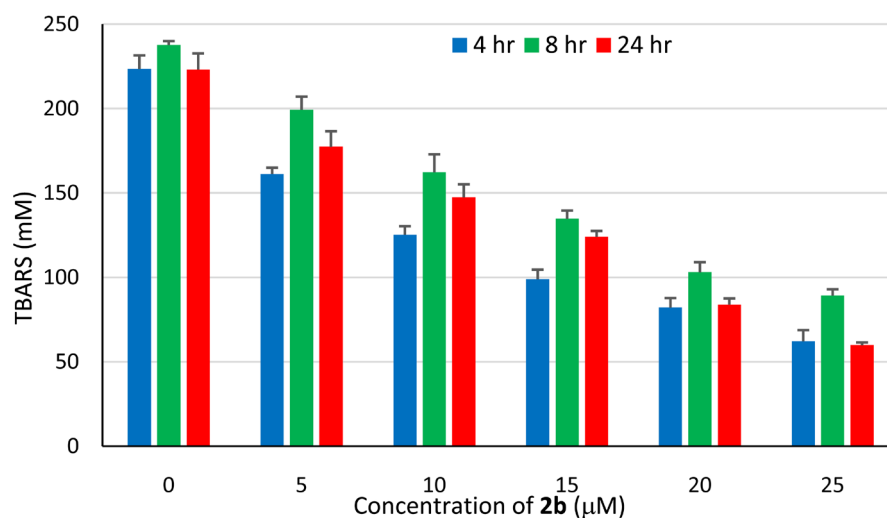**Figure 3.** Effect of **2a** on lipid peroxidation.

Figure 4. Effect of **2b** on lipid peroxidation.

results showed a similar trend for 4, 8, and 24 hrs. of incubation. But both 8 and the 24 hr incubations showed a remarkable decrease in lipid peroxides compared to the 4 hr (Figure 3). For compound **2b**, the results showed a similar trend for 4, 8, and 24 hrs. of incubation. However, the decrease in lipid peroxidation was more for the 24-hr compared to the 4 and 8 hr incubation with the above drugs (Figure 4). For compound **2c**, the results showed the trend for 4, 8, and 24 hrs. of incubation which indicates that the compounds **2c** reduced lipid peroxides in a dose and time dependent manner. The reduction in lipid peroxides were greater for the 24 hr at the high doses (20 and 25 μM) compared to the other two time periods (Figure 5).

3.3. Effects of TPM Derivatives 1a-c on Lipid Peroxidation

Figures 6-8 show the results of several doses on concentrations (0, 5, 10, 15, 20, 25 μM) of TPM derivatives on lipid peroxidation. A similar reduction in lipid
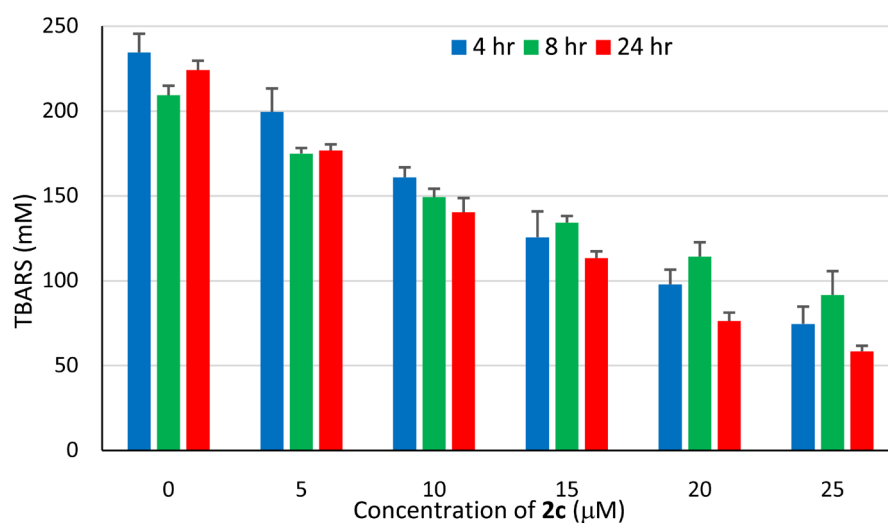

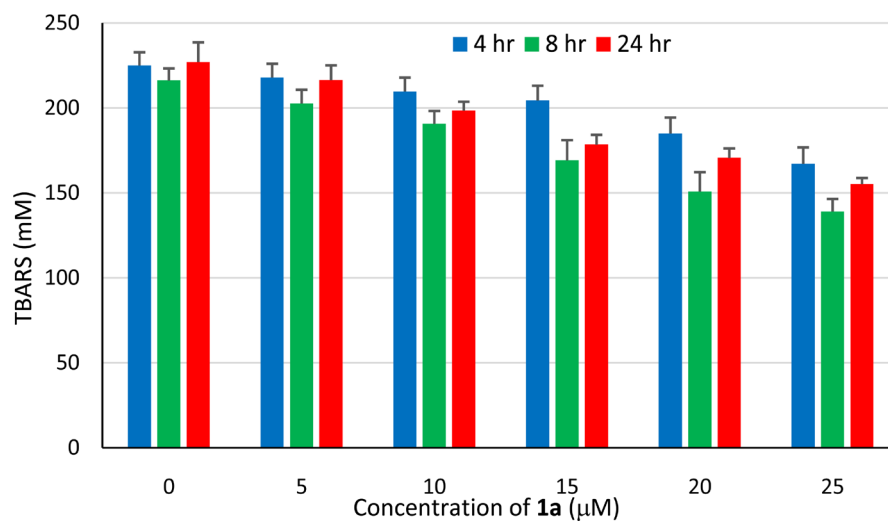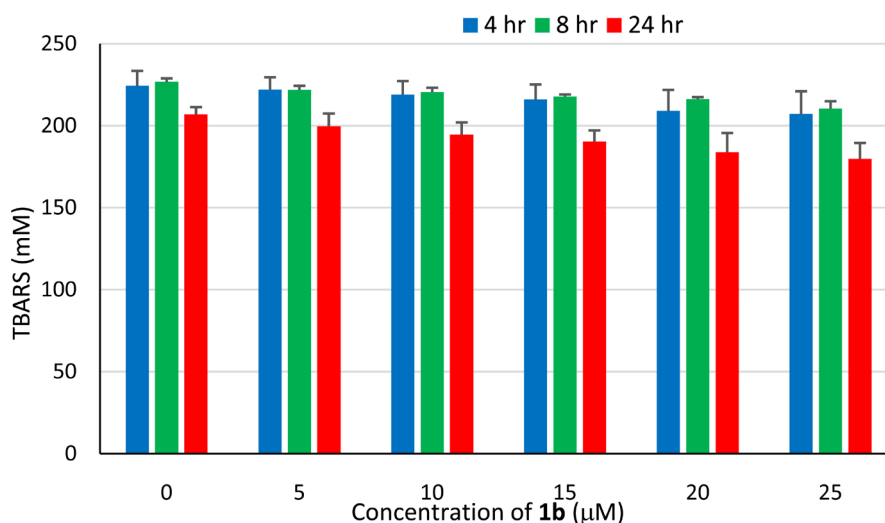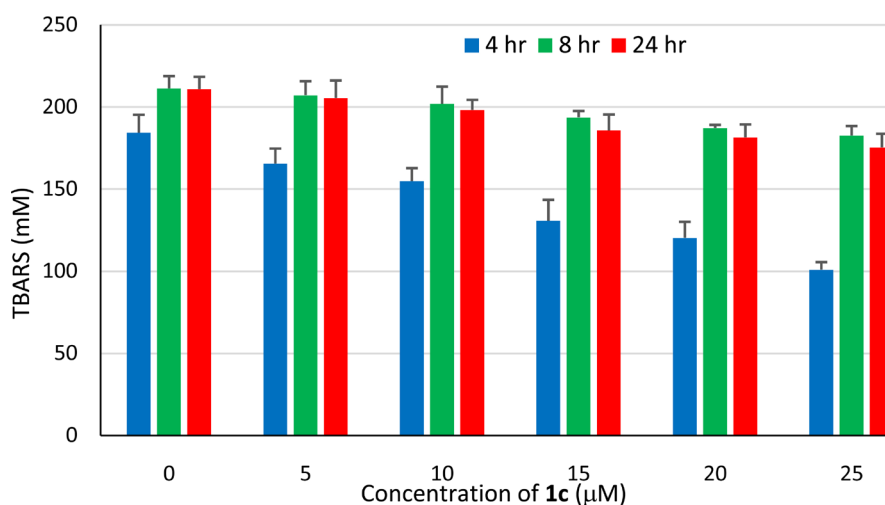Figure 5. Effect of **2c** on lipid peroxidation.

Figure 6. Effect of **1a** on lipid peroxidation.

Figure 7. Effect of **1b** on lipid peroxidation.

Figure 8. Effect of **1c** on lipid peroxidation.

peroxidation was observed for all the 4 and 8 hr incubation periods. The 24-hr incubation did result in the reduction of lipid peroxides for all the doses tested.

For compound **1a**, there was slight decrease in lipid peroxides from 15 - 25 µM dose levels. The slight decreases for the 4 and 24 hr incubations were not significantly different from each other (Figure 6). For compound **1b**, a similar reduction in lipid peroxidation was observed for all the 4 and 8 hr incubation periods. The 24-hr incubation did result in the reduction of lipid peroxides for all the doses tested (Figure 7). Finally, for compound **1c**, the results indicated that the 8 hr incubation reduced lipid peroxide compared to the 4 and 24 hr there were no differences in lipid peroxides between the various doses for the 24 hr incubation period (Figure 8).

3.4. Discussion

Notwithstanding remarkable progress in understanding the pathophysiology of

cancer and the discovery of several therapeutic prophylactics over the last few decades, cancer continues to be "the emperor of all maladies, the king of terrors". Cancer arises by accumulation of a number of mutations and epigenetic changes that endow fundamental alterations in genetic expression of a normal cell, driving it towards cancerous growth. Such cancerous growth is characterized by unregulated proliferation and tissue invasion. Years of studies have identified few specific properties of cancer cells including their metastatic property, property of unregulated cell proliferation, avoidance of apoptosis, escaping replicative senescence and avoiding differentiation, genetic instability, etc. It was therefore envisioned that modulating the lipophilicity of triptorelin might increase its cellular uptake, decrease the effect of drug efflux, thereby improving its bioefficacy and reduce the development of resistant cancer cells. Lipophilicity of triptorelin was increased by conjugating the drug with various tris (4-methoxyphenyl) methanol derivatives using long chain hydrophobic linkers. The linker used was dodecanedioic acid. The Tris (4-methoxyphenyl) Methanol were substituted at the third carbon of each of their three aromatic rings with F, NO_2, and CH_3.

In vitro lipid peroxidation assays using thiobarbituric acid (TBA) method revealed that the compound **2c** reduced lipid oxidation in a dose and time dependant manner. Interestingly there was greater reduction of the lipid oxidation during 4- and 24-hours incubation compared to 8 hours. The compound **2b** also effectively reduced the amount of lipid peroxidation in a dose and time dependant manner. Figure 4 indicates a progressive reduction in lipid peroxidation with increasing concentrations of the compound **2b** from 0 - 25 μm with maximal efficacy being at 25 micromolar concentration after 24 hrs of incubation. The results show similar trend for 4, 8, & 24 hrs of incubation of the compound **2b**. The decrease in lipid peroxidation, however, was more for the 24-hr compared to the 4 and 8 hr incubation with the above drugs. Similar to the compound **2c**, it is difficult to estimate the maximal effective concentration of tris (4-methoxy-3-methylphenyl) methanol triptorelin from this data, since none of the incubation period indicates saturation in decrease of lipid oxidation. Studies of lipid peroxidation as depicted in Figure 8 indicate that incubation with compound **2b** results in a progressive reduction in lipid peroxidation with increasing concentration of the prodrug from 0 - 25 μm with maximal efficacy being at 25 micromolar concentration after 8 hrs of incubation. The results however show similar trend for 4, 8, & 24 hrs of incubation. Similar to the other prodrugs, it is difficult to estimate the maximal effective concentration of tris (4-methoxy-3-methylphenyl) methanol Triptorelin from this data. The increase in efficacy at 8 hours of incubation compared to 4hrs and 24 hrs might be due to the fact that the particular time point of 8 hours may represent optimal incubation period for the compound **2a**, with lesser incubation providing reduced threshold of antioxidant activity and higher incubation rendering the pro-drug to increased areal oxidation.

Unconjugated compound **1a** by itself was only moderately efficacious in re-

ducing lipid peroxidation. The maximum reduction was seen at the dose of 25 μmol after 24 hours incubation. The compound **1b** was capable of significantly decreasing lipid peroxidation only at higher dosages under 24 hour's incubation. The compound **1c** could progressively reduce lipid peroxidation under 4 hrs, 8 hrs and 24 hours incubation but the 8-hr incubation was more effective.

4. Conclusions

The primary role of triptorelin conjugated pro-drug is to impart lipophilicity for increased uptake by the cell membrane. The data reported in the current studies indicate that the TPM derivatives have some antioxidant activity. This may be due to the fact that their attachment does not abrogate the antioxidant potential of triptorelin itself, and that these TPM derivatives at high concentrations also do contribute to the total antioxidant activity of the triptorelin conjugates.

It can therefore be envisioned that the triptorelin conjugates might have higher *in vitro* antioxidant activity than unconjugated triptorelin. Such hypothesis however remains to be tested in *in vitro* experiments, for example the use of several mammalian cell lines. The experimental design of such a prospective study will entail the evaluation of both the antioxidant potential of the triptorelin conjugates and unconjugated triptorelin in comparative studies. Thus, the measurement of lipid peroxides can again be used as a faithful marker in such a comparative study.

Acknowledgements

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Abbreviations

TBARS, thiobarbituric reactive substance

TRP, Triptorelin

TPMs, Triphenylmethanols

DCM, dichloromethane

NMP, (N-Methyl-2-pyrrolidone)

HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate

DIC, *N,N'*-Diisopropylcarbodiimide

DIPEA, *N,N*-Diisopropylethylamine

GnRH, gonadotropin-releasing hormone

FSH, Follicle-stimulating hormone

LH, Luteinizing hormone

LHRH, luteinizing hormone–releasing hormone.