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Targeting AMPKa1 Gene in PC3 Cells by Triphenylmethanol Derivatives

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ABSTRACT

Epidemiological studies indicate that treatment with metformin, an AMP-activated protein kinase (AMPK) activator, reduces the incidence of cancers. Activation of AMPK has also been reported to oppose tumor progression in diverse types of cancers and offers promising cancer therapy. Furthermore, AMPK is a primary regulator of energy metabolism and has also been implicated in cell cycle progression, angiogenesis, cell transformation, migration, and cancer. We have recently synthesized novel flavonoids, namely, triphenylmethanol derivatives (TPMs), but the effectiveness of the TPMs on the activity of AMPK remains unclear. We hypothesized that the novel TPMs would inhibit cancer cell proliferation through the activation of AMPK isoforms in cells. The effects of TPMs on prostate cells (PC-3) were investigated. Cells were exposed to TPMs for either 12 or 24 hr. at the respective doses of 0, 25, 50 100, and 200 μM based on the cell viability studies by the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) (MTT) assay. The results indicate that cells exposed to the respective doses of TPMs increased both *phospho*- and *total*-AMPK$_{a1}$ in a dose- and time-dependent manner. The effects of the increases for the *phospho*- and *total*-AMPK*a* in cells were greater for the 24-hr than the 12-hr. incubation. Further studies are currently going on to elucidate the specificities of the said insults in increasing the *phospho*- and *total*-AMPK*a* activities and for the other respective isoforms.

1. INTRODUCTION

AMPK is important in cells and the protein has been reported to be primarily found in eukaryotic cells where it exists in the respective heterotrimeric forms [1, 2]. Isoforms of the gene have also been reported in humans. In yeast it has been reported that the AMPK, genes have similar function in varied species and are required for the response to glucose starvation [3, 4]. Studies have also shown that the gene

can be activated over a 100-fold through phosphorylation of threonine 172 within the activation loop of the α subunit [5]. It is also known that the primary upstream kinase phosphorylation of the activation loop is a complex process and involves the protein kinase LKB1 and its corresponding accessory subunits [6] where it functions as a tumour suppressor and acts upstream of AMPK [7, 8].

Cancer is one of the devastating diseases that affect humans and has been reported to be the main causes of death globally [9]. Despite the surge in the incidence of the disease, the rate of mortality over the years has decreased due to advances in several therapeutic treatments [10]. The etiology of cancer has been attributed both to environmental as well as genetic factors [11]. Many studies in the literature have indicated that diet in relation to certain lifestyles is one of the factors that affect cancer incidence and mortality [12]. For example, diets based on high intakes of vegetables and fruits have had some strong association with a significant reduction in cancer risk [13, 14]. Analyses of these food consumptions have revealed the presence of some bioactive compounds such as the presence of flavonoids, carotenoids, lignans and other phenolic acids that have potential health benefits [15, 16]. These compounds act by protecting cells through their antioxidative properties against several environmental conditions, such as exposure to radiation, metals, and other microorganisms that could trigger the oncogenic processes leading to cancer development [17-19]. Furthermore, several studies have been reported elsewhere and have indicated that some of these active compounds have demonstrated their anti-mutagenic and carcinogenic effects against several types of cancers [13, 20-22].

Studies on AMPK have primarily been involved with metabolism and only limited work unravels its direct role in inflammatory processes and how it relates to the anti-cancer activities with drugs such as metformin, NSAIDs, TCM and other AMPK activators. Additionally, AMPK activation on some oncogenic signaling and biological functions are still unclear and needs further studies. We have synthesized a wide range of phenolic compounds, namely the triphenyl methanol derivatives (TPMs). We have evaluated their respective antioxidative and antiproliferative properties using both lipid and cell models [23-25]. Nevertheless, the use of our synthesized compounds on AMPK activation in humans is limited in literature. Thus, the current studies investigate the potential effect of the TPMs on *total-* and *phospho*-AMPKal levels in PC-3 cells. We believe additional studies are necessary to generate more data on the mechanisms and effects of TPMs in modulating the AMPK gene and for any future consideration as effective cancer treatment drug.

2. EXPERIMENTAL

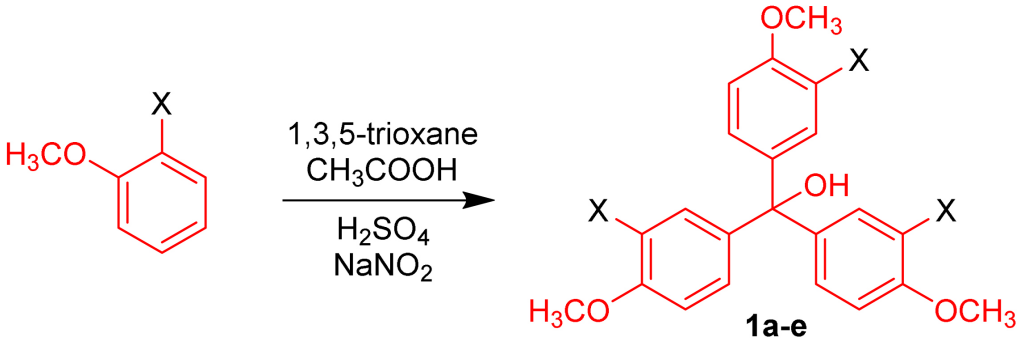
2.1. Preparation of TPMs 1a-e

The TPM derivatives were prepared according to a modified protocol as described in the literature [26-28] (Scheme 1). The method involved reaction of 1,3,5-trioxane with 2-chloroanisole, 2-nitroanisole, 2-methylanisole, 1,2-dimethoxybenzene, or methyl 2-methoxybenzoate, followed by the addition of a mixture of sulfuric acid and glacial acetic acid. The products were then purified by flash chromatography, resulting in pure products with yields ranging from 63% to 87% [28].

Tris(3-chloro-4-methoxyphenyl)methanol (**1a**), (4.51 g, 66%), MS (ESI-TOF) (*m*/*z*) for $C_{22}H_{19}C_{l3}O_4$: calcd., 453.0, found 453.0 $[M + H]^+$; 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 485.4 $[M]^+$; tris(3-methyl-4-methoxyphenyl)methanol (**1c**), (4.77 g, 81%), MS (ESI-TOF) (m/z) calcd. 415.2, found 415.2 $[M + Na]^+$; tris(3,4-dimethoxyphenyl)methanol (**1d**), (5.75 g, 87%), MS (ESI-TOF) (*m*/*z*) for $C_{25}H_{28}O_7$: calcd, 441.2, found 441.4 $[M + H]^+$; trimethyl 5,5',5"-(hydroxymethanetriyl)tris(2-hydroxybenzoate) (**1e**), (5.57 g, 77%), MS (ESI-TOF) (m/z) for $C_{28}H_{28}O_{10}$: calcd. 525.2, found 525.4 $[M + H]^+$.

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

The coupling of Tris(4-methoxyphenyl) methanol derivatives **1a-e** with Triptorelin acetate and sebacic acid as a lipophilic linker were accomplished according to our previously reported method [24]. Purification of the final products yielded pure TRP-TPMs conjugates **2a-e** (Scheme 2). TRP-TPMs



(**1a**) X=Cl, (**1b**) X=NO_2, (**1c**) X=CH_3, (**1d**) X=OCH_3, (**1e**) X=CO_2CH_3

Scheme 1. **Synthesis of triphenylmethanol (TPMs) derivatives 1a-e.**

conjugate (**2a**), (64 mg, 66%), MS (ESI-TOF) (*m*/*z*) for $C_{96}H_{116}Cl_3N_{18}O_{19}$: calcd, 1929.8, found 1929.8 $[M + H]^+$; TRP-TPMs conjugate (**2b**), (71 mg, 72%), MS (ESI-TOF) (*m*/*z*) for $C_{96}H_{115}N_{21}O_{25}$: calcd, 1961.9, found 1978.9 $[M + OH]^+$; TRP-TPMs conjugate (**2c**), (64 mg, 68%), MS (ESI-TOF) (*m*/*z*) for $C_{99}H_{122}N_{18}O_{18}$: calcd, 1850.9, found 1850.9 $[M - H_2O]^+$; TRP-TPMs conjugate (**2d**), (84 mg, 87%), MS (ESI-TOF) (*m*/*z*) for $C_{99}H_{124}N_{18}O_{22}$: calcd, 1917.9, found 1917.9 $[M + H]^+$; TRP-TPMs conjugate (**2e**), (77 mg, 77%), MS (ESI-TOF) (*m*/*z*) for $C_{102}H_{125}N_{18}O_{25}$: calcd, 2001.9, found 2001.9 $[M + H]^+$.

2.3. Antiproliferative Studies with TPMs by the MTT Assay

Human prostate cancer (PC3) cell line was from ATCC. Cells were cultured as previously reported [29]. Cell viability and antiproliferative studies were also conducted by the MTT assay [30].

3. CULTURE OF PC3 AND TREATMENT WITH TPMS

3.1. Cell Culture & Treatment

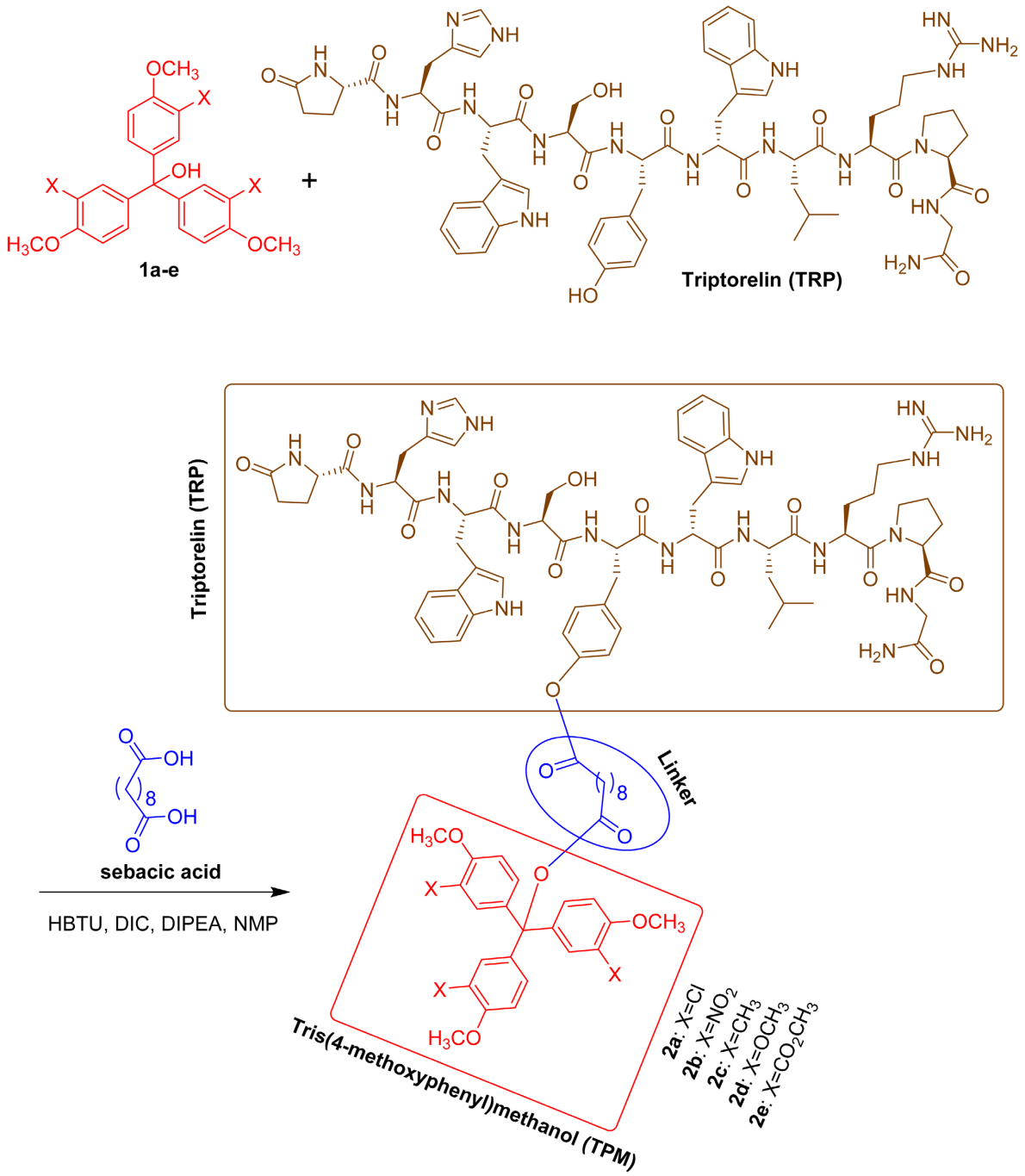
Prostate cells (PC3) upon reaching 80% confluency, were trypsinized for 2 minutes and counted using a hemocytometer. Cells were seeded and cultured overnight for 24 hr. in 96 well plates. Following the incubation, the media was aspirated, and treated with the respective concentrations of 0, 25, 50, 100, and 200 µM of the respective TPMs and incubated for either 12 or 24 hr.

3.2. Analyses of Total- and Phospho-AMPKα1

The total- and phospho-$AMPK_{\alpha 1}$ were analyzed according to the Phosphorylation assay kit (LSBio) with modifications. Briefly, adherent cells on amine coated plates were allowed to attach for 24 hr. Cells in a 200 µl volume of the same media was used to maintain the cells in bulk culture. 100 µl of 1X primary antibody solution were added, and following incubation overnight in the cold were washed with PBS. Aliquots of the 1X secondary were then added and samples left at room temperature and in the dark for 2 hr. Secondary antibody was decanted, and the wells washed 3 times with 200 µl of 1X PBS. Following the treatment with the primary and secondary antibodies the fluorescence for the respective treated samples were measured in a fluorescence plate reader.

3.3. Calculations

Fluorescence intensities for controls and treated samples were calculated according to the manufacturer's instructions. Normalized phosphorylated AMPK (pAMPK) to controls were quantified using the formula below as described by the manufacturer [31].



Scheme 2. Synthesis of triphenylmethanol conjugates of TRP 2a-e.

$$\text{Normalized pAMPK} = \frac{\Delta\bar{F}pAMPK / \Delta\bar{F}Prot}{\Delta\bar{F}pAMPK / \Delta\bar{F}Prot}$$

4. RESULTS AND DISCUSSION

4.1. Cytotoxicity and Antiproliferative Activity of TRP-TPMs

TRP, TPMs 1a-d at the respective dose of 100 and 200 μM did not have any toxicity in PC3 following

treatment for 24 h. Bioactivities of compounds 2a-e were also evaluated and compared to the noncovalent mixtures of (TPMs 1a-e + TRP) and TRP, respectively.

4.2. Effects of TRP-TPMs on Total-AMPK$_{a1}$ Following Treatment for 12 and 24 hr

Figure 1 and Figure 2 show the expression profiles of total-AMPK levels normalized to controls in PC3 cells following incubation for 12 and 24 hr respectively.

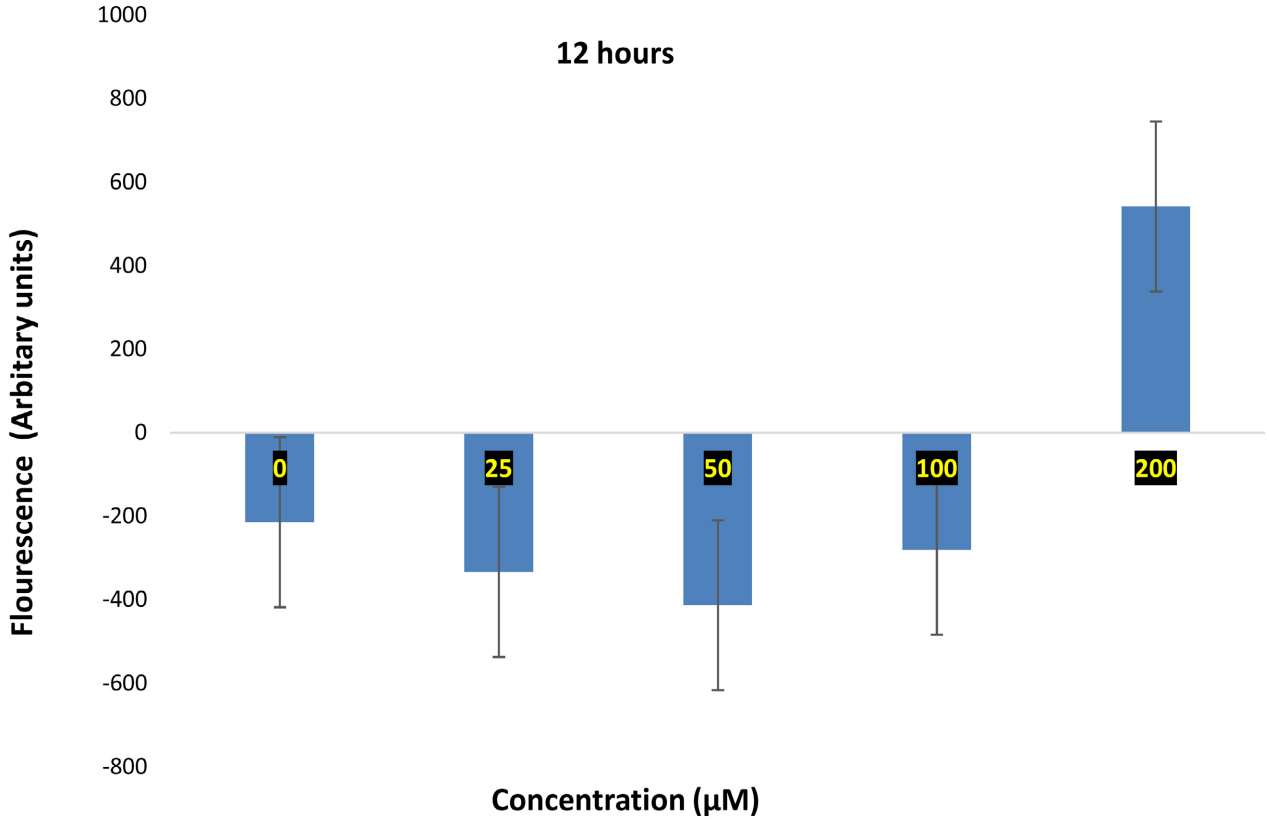


Figure 1. Effects of TPMs (2a-e) on total-AMPK levels, expressed by fluorescence, in (Arbitary units) in PC3 cells following incubation for 12 hr.

Levels of total-AMPK expression was significantly higher for the respective doses for the 24-hr to the 12 hr. AMPK total expression for the 12-hr. incubation was abysmal and the same for all the doses evaluated except at the 200 μM. There was, a significant decrease, in total AMPK for the 24-hr. incubation at the 200 μM compared to the other respective doses. The above observation seems to suggest that the effects of the differences may be due to the dose- and time-dependent processes.

4.3. Effects of TRP-TPMs on Phospho-AMPK$_{a1}$ Following Treatment for 12 and 24 hr

Phospho-AMPK$_{a1}$ levels normalized to their respective control levels are shown in Figure 3 and Figure 4, respectively. The results indicate a higher and significant increase, $p < 0.05$, for 24 hr. treated samples compared to the 12 hr. The increases in phosphorylated levels for the 24-hr. incubation were sturdy and slightly higher over the controls for the 25, 50, 100 μM and increased significantly, $p < 0.01$, at the 200 μM levels. The increased *phospho*-levels indicate that the TPMs are remarkably effective in modulating and causing increases in AMPK levels. Such increases can increase in the antioxidant capacity of PC3 cells to better cope with oxidative processes thereby reducing and or preventing prostate cancer in men.

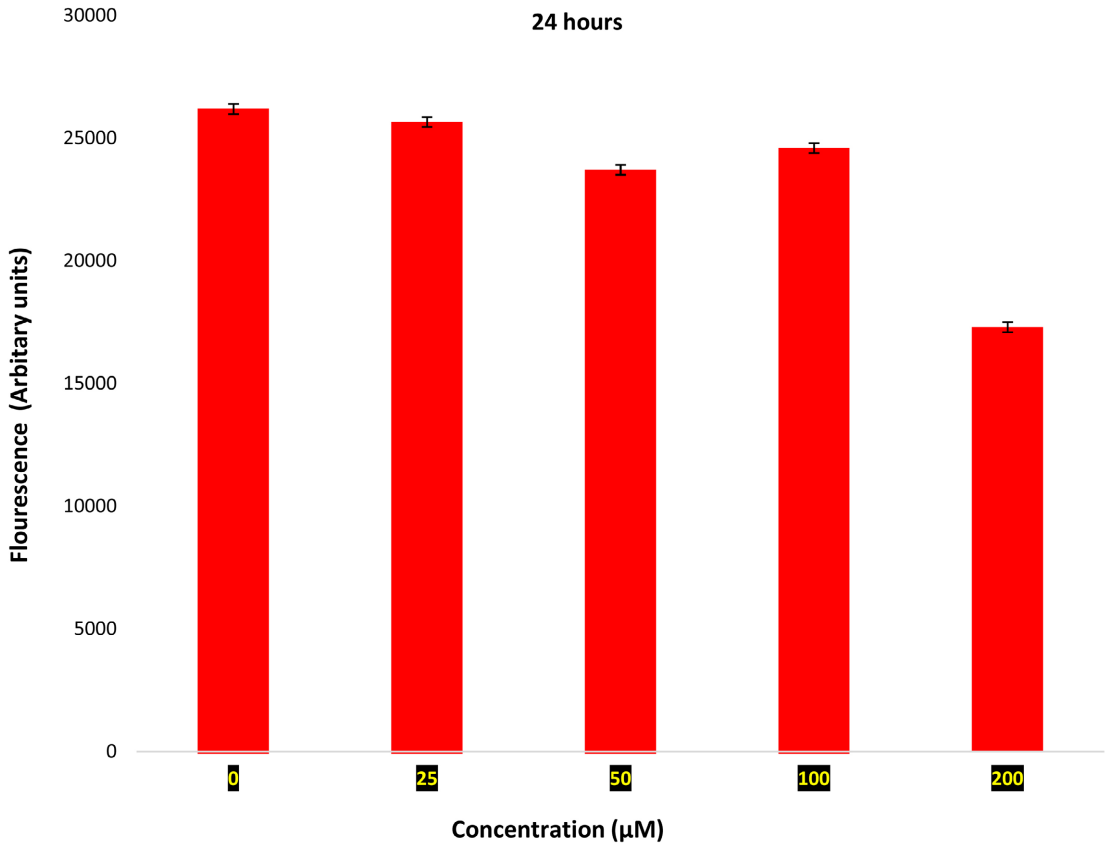


Figure 2. Effects of TPMs (2a-e) on total-AMPK levels, expressed by fluorescence, in (Arbitary units) in PC3 cells following incubation for 24 hr.

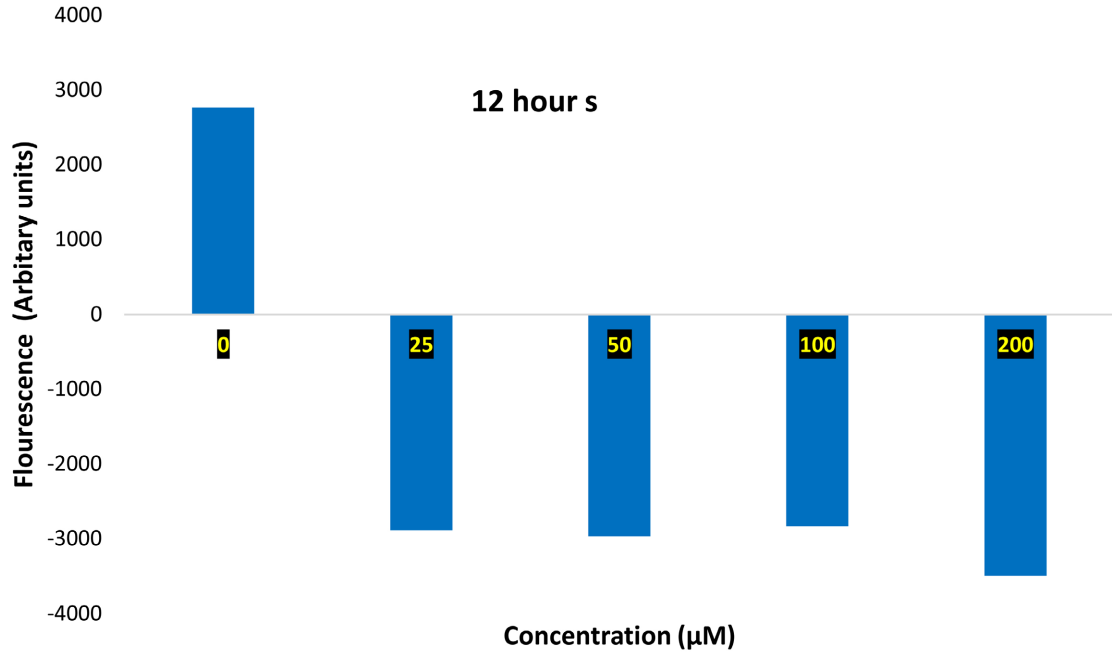


Figure 3. Effects of TPMs (2a-e) on phospho-AMPK levels, expressed by fluorescence, in (Arbitary units) in PC3 cells following incubation for 12 hr.

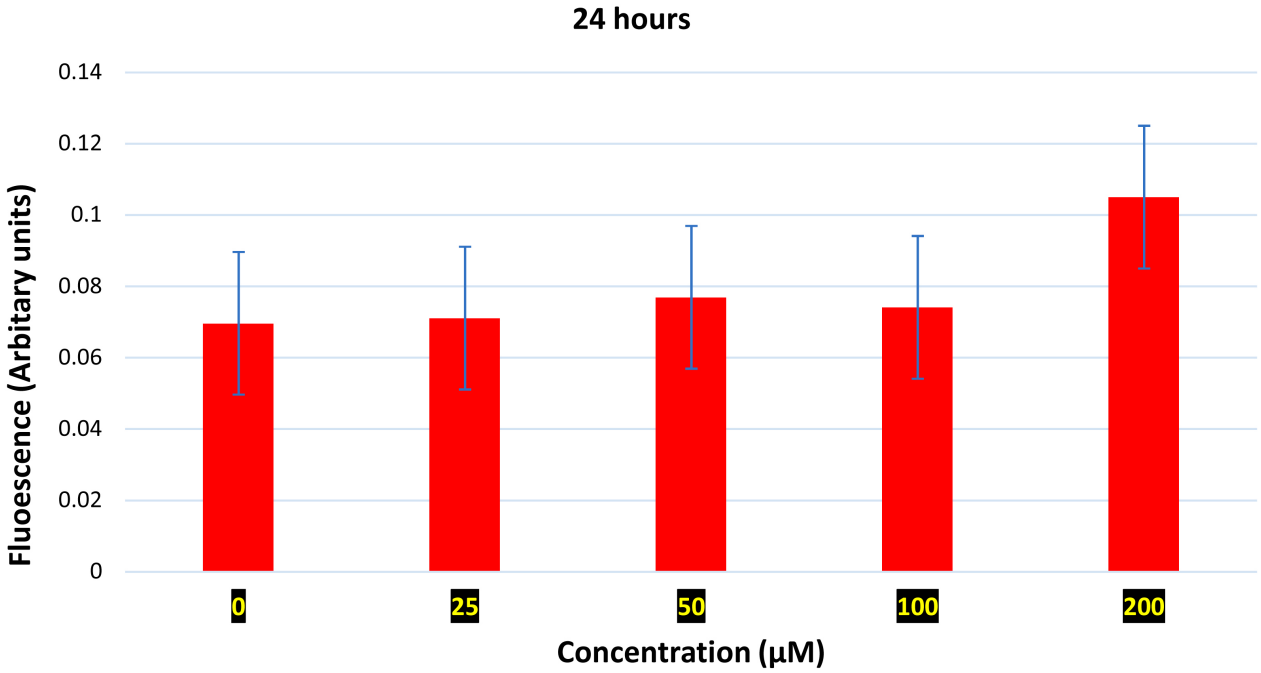


Figure 4. Effects of TPMs (2a-e) on phospho-AMP levels, expressed by fluorescence, in (Arbitary units) in PC3 cells following incubation for 24 hr.

5. CONCLUSION

TPMs derivatives were evaluated for their antiproliferative activities in PC3 Cells. TRP-TPMs showed comparable antiproliferative activity against PC3, when compared to TRP alone as previously reported [22]. We then sought in the current studies to determine the efficiencies and optimum dosages of TPMs on *total*- and *phospho*-AMPK$_{a1}$ in PC3 cells. The cell viability studies indicate that the TPMs at the respective doses did not significantly affect the PC3 cells and beyond the 100 μM following the treatment of cells with the synthesized compounds. TRP-TPMs 2a-e (see **Scheme 2**) showed higher antiproliferative activity compared to the TRP alone. Nevertheless, the results indicate that exposure of PC3 to TPMs at the respective doses had significant effects on *total*- and *phospho*-AMPK$_{a1}$ in cells. The increases in both *total*- and *phospho*-AMPK levels following the exposure were significantly higher for the 24-hr. The findings indicate that TPMs could increase the antioxidative capabilities of the cells to better cope with oxidative stress and/or to prevent and reduce the incidence of prostate cancer. We observed disparities in the controls for total- and phospho-AMPK$_{a1}$ (**Figures 1-4**) proteins for the 12 and 24 studies. This could be due to the differences in incubation times with regards to protein expression [32]. Secondly, the control values for **Figure 3** and **Figure 4** reflect the actual quantitative levels of phospho-AMPK$_{a1}$ which though were not significantly different from each other but reflect the quantitative gains following the 24 hrs. incubation to the TPMs normalized to the controls. Furthermore, our future studies on the other isoforms of the AMPK gene which are ongoing will help define the ultimate relationship between AMPK and the PI3K/AKT signaling pathway following exposure to the TPMs. We believe such findings lend credence to the mechanisms underlying the effects of TPMs on PC3 cells and may contribute to the scientific knowledge base for any novel treatment strategies targeting AMPK and the PI3K/AKT signaling pathway. We will also seek to use normal cells such as human fibroblasts or HeK293 from kidneys, which may be helpful to elucidate the potential effects and benefits of TRP-TPMs in the said cells in comparison to cancer.

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CONFLICTS OF INTEREST

There are no conflicts of interests by the authors associated with the publication of this paper.

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