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Synthesis and *in vitro* antitumor activity of novel acylspermidine derivative N-(4-aminobutyl)-N-(3-aminopropyl)-8-hydroxy-dodecanamide (AAHD) against HepG2 cells

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ABSTRACT

Naturally occurring polyamines like Putrescine, Spermidine, and Spermine are polycations which bind to the DNA, hence stabilizing it and promoting the essential cellular processes. Many synthetic polyamine analogues have been synthesized in the past few years, which have shown cytotoxic effects on different tumours. In the present study, we evaluated the antiproliferative effect of a novel, acylspermidine derivative, (N-(4-aminobutyl)-N-(3-aminopropyl)-8-hydroxy-dodecanamide) (AAHD) on HepG2 cells. Fluorescence staining was performed with nuclear stain (Hoechst 33342) and acridine orange/ethidium bromide double staining. Dose and the time-dependent antiproliferative effect were observed by WST-1 assays, and radical scavenging activity was measured by ROS. Morphological changes such as cell shrinkage & blebbing were analyzed by fluorescent microscopy. It was found that AAHD markedly suppressed the growth of HepG2 cells in a dose- and time-dependent manner. It was also noted that the modulation of ROS levels confirmed the radical scavenging activity. In the near future, AAHD can be a promising drug candidate in chalking out a neoplastic strategy to control the proliferation of tumour cells. This study indicated that AAHD induced anti-proliferative and pro-apoptotic activities on HCC. Since AAHD was active at micromolar concentrations without any adverse effects on the healthy cells (Fibroblasts), it is worthy of further clinical investigations.

1. Introduction

Hepatocellular carcinoma (HCC) is the sixth most common type of cancer worldwide with a reputation of third deadliest cancers causing 600,000 deaths every year globally [1,2]. In the regions of East Asia and sub-Saharan Africa, the incidence rate is much higher with 20 per 100,000 persons [3]. This could be primarily due to the Hepatitis B virus infections, which can be timely controlled by efficient immunization drives. However, the incidence rate of HCC has raised alarmingly in the USA in the recent few years with a 5-year survival rate of 15% [4]. The burgeoning obesity epidemic, chronic liver disease and cirrhosis are the contributing factors in such high incidence of HCC in the USA [4]. Despite the technological improvements in the development of novel therapies, currently, there is just single FDA approved

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drug—Sorafenib to treat HCC that acts as a pan-kinase inhibitor [5]. Hence, it is highly desirable to develop effective therapeutic approaches which can be employed to treat HCC.

Polyamine metabolism and the biosynthetic pathway have been studied extensively for the last three decades. Naturally occurring polyamines like Spermine, Spermidine and Putrescine play a crucial role in the proliferation and maintenance of healthy cells [6,7]. However, the demand of these biogenic polyamines rises manifold in the rapidly increasing HCC cells. In order to maintain adequate supply, the import of polyamines from the external sources increases with decreased specificity [8]. Hence synthetic polyamine analogues can be supplied from the external source that has a similar structure as biogenic polyamines but differs in the function leading to cell death and apoptosis.

Many synthetic analogues have been developed and tested in a panel of diverse cancer cell lines. Over the last few years, the development of polyamine analogues as chemotherapeutic agents against different cancers has fascinated many researchers [7,9,10]. Many polyamine analogues have been extensively studied as LSD1 inhibitors leading to modulation of the chromatin [11,12]. Moreover, many previous studies have also evaluated polyaminobenzamides (PABAs) and polyaminohydroxamic acid (PAHA) derivatives as HDAC inhibitors [13,14]. In our earlier study, we demonstrated the pro-apoptotic effect of the novel acylspermidine analogues on Jurkat and MCF7 [15]. But the off-target impact and the dose-limiting cytotoxicity still remains a challenge in the way of designing new therapeutic strategies.

Building upon the data obtained on acylspermidine analogues from our earlier studies, we evaluated the *in vitro* anticancer effect of a novel acyl spermidine derivative-N-(4-aminobutyl)-N-(3-aminopropyl)-8-hydroxy-dodecanamide (AAHD) on HepG2 cells.

2. Materials and methods

2.1. Chemical synthesis of the acylspermidine derivative-AAHD

2.1.1. N1,N8-bis-tert-butoxycarbonyl-N4-(8-hydroxylauroyl)spermidine

Under the nitrogen atmosphere, 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (665 mg, 3.47 mmol) was added to a solution of N1,N8-bis-tert-butoxycarbonylspermidine (1.00 g, 2.89 mmol), 8-hydroxylauric acid (626 mg, 2.89 mmol) and dimethylaminopyridine (424 mg, 3.47 mmol) in dichloromethane (20 mL), and stirred for 2 days at room temperature. Then, a solution of 10% (w/w) citric acid (10 mL) was added to the reaction mixture and stirred for 10 min.

The reaction mixture was extracted with dichloromethane (10 mL × 3), dried with Na_2SO_4 and concentrated *in vacuo*. The residue was purified with silica gel column chromatography (*n*-hexane: ethyl acetate = 1:1) to give N^1,N^8-bis-*tert*-butoxycarbonyl-N^4-(3-hydroxylauroyl)spermidine as a colorless oil (1.05 g, 1.94 mmol, 67.1%), which was sufficiently pure for further use.

2.1.2. N^4-(8-hydroxylauroyl) spermidine

Under nitrogen atmosphere, N^1,N^8-bis-*tert*-butoxycarbonyl-N^4- (8-hydroxylauroyl) spermidine (1.05 g, 1.94 mmol) was dissolved into 50% (v/v) trifluoroacetic acid solution in dichloromethane (10 mL) and stirred for 20 min. After the addition of methanol (30 mL), the solvent was removed *in vacuo*. Then 1 M NaOH (10 mL) was added and the aqueous solution was extracted with dichloromethane (10 mL × 3). The combined organic layers were dried with Na_2SO_4 and concentrated *in vacuo* to give N^4-(3-hydroxylauroyl) spermidine as a colourless oil (182 mg, 0.53 mmol, 18.3%). The general scheme of the synthesis has been described in Fig. 1.

2.2. ^1H NMR data of AAHD

^{1}H NMR (500 MHz, $CDCl_3$) δ 3.57 (m, 1H, C<u>H</u>(OH)), 3.43–3.24 (m, 4H, CH_2NCH_2), 2.75–2.66 (m, 4H, 2 × C<u>H</u>$_2$NH$_2$), 2.40–2.20 (m, 2H, COC<u>H</u>$_2$), 1.70–1.50 (m, 8H, NCH$_2$C<u>H</u>$_2$C<u>H</u>$_2$CH$_2$NH$_2$, NCH$_2$C<u>H</u>$_2$CH$_2$NH$_2$), 1.34 (m, 10*H*, (C<u>H</u>$_2$)$_3$, (C<u>H</u>)$_2$CH$_3$), 0.91 (t, J = 7.0 Hz, 3H, CH_3). The data was generated by using the NMR Instrument JNM ECA500II, Jeol Ltd, Tokyo, Japan.

2.3. Cell culture and treatment

Human liver hepatocellular carcinoma cell line (HepG2) and baby hamster kidney cell line (BHKSINLU2, BHK-21) were purchased from ATCC (CRL-11997 and CRL-12071 respectively). The cells were maintained in Dulbecco's Eagle's Modified Medium (DMEM) (HyClone™, catalog # SH30022.01) supplemented with 10% Fetal Bovine Serum (FBS), (HyClone™, catalog # SH30910.03HI) and 1% Penicillin-streptomycin antibiotics (HyClone™, catalog # SV30010) in a humidified incubator with 5% CO2 and 37 °C.

2.4. Hoechst 33258 and acridine orange/ethidium bromide staining

The fluorescent staining was performed as described earlier [16]. Briefly, the HepG2 cells were seeded in clear flat-bottom 24-well plates at a density of 2 × 10^4 cells/well. After treating the cells with the desired concentrations for suitable time durations, the spent media was removed and a fresh 200 µl solution of Hoechst 33258 Stain (Sigma-Aldrich) was added and incubated for 30 min at room temperature. For Acridine orange (AO) and Ethidium Bromide (EtBr) staining (Sigma-Aldrich), after the desired treatment conditions, 5 µl of AO and 5 µl of EtBr were added to the media and incubated for 20 min. After incubation, the cells were directly observed under a fluorescence microscope at 400× magnification (Leica, Wetzlar, Germany).

2.5. Cell viability assay (WST-1)

The antiproliferation of HepG2 cells was done by using WST-1 (Sigma-Aldrich, USA) reagent according to the manufacturer's instructions. Briefly, the HepG2 cells were seeded on a transparent, flat-bottom 96 well plate at a density of 6 × 10^3 cells/well and incubated for 24 h and then treated with different concentrations (0.05 mM, 0.1 mM, 0.2 mM, 0.3 mM) for required time periods. After incubation for the desired time durations, 10 µl of the WST-1 reagent was added to the wells and incubated for additional 4 h at 37 °C. Finally, the absorbance was recorded at 450 nm by microplate ELISA reader (ELx800TM Biotek, USA); Gen5 software (Biotek, USA. The control/un-treated cells were considered as 100% viable, and the cell viability was calculated as reported earlier [17].

2.6. Assessment of mitochondrial potential

The mitochondrial potential (ΔΨm) was measured by using 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzamidazolylcarbocyanine iodide (JC-1; Sigma Aldrich-molecular probe T3168) dye using the manufacturer's instructions [16,18]. In brief, the HepG2 cells were seeded in 96-well plate at a density of 10^3 cells/well and incubated for 24 h. Then the cells were treated with desired concentrations for required time periods. The treated cells were trypsinized and suspended in 1 mL PBS and 10 µg/mL JC-1 dye was added and incubated for 15 min. Finally, the red and green fluorescence was observed with a flow cytometer at an excitation wavelength of 488 nm. The red fluorescence was observed at 585 nm and the green at 530 nm.

2.7. Wound healing assay

The HepG2 cells were seeded in a clear 6-well flat bottom plate at a density of 2 × 10^4 cells/well. The spent media was replaced with fresh DMEM media after an incubation of 24 h. After incubation, the cells were treated with different concentrations (0.1, 0.2 and 0.3 mM) of AAHD for 24 h. Then, a scratch was made on the monolayered cells with a 10 µl pipette tip in each well. Phase contrast microscope (Leica,

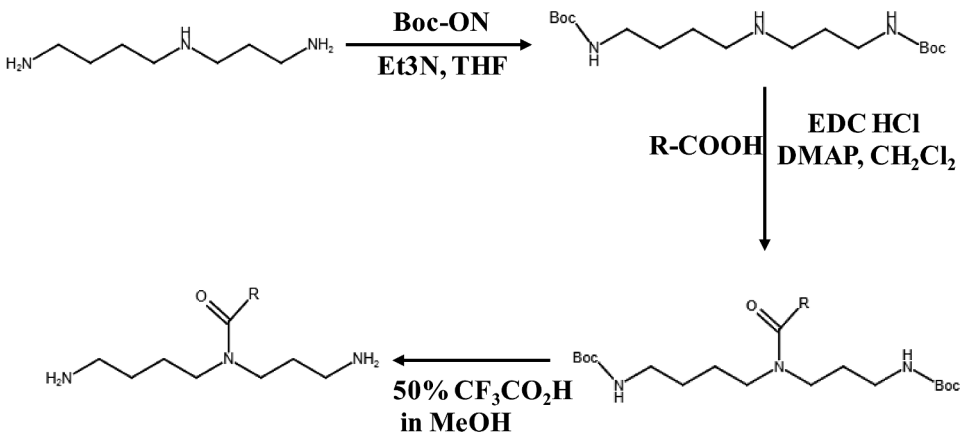

Fig. 1. The overall scheme of synthesis in the preparation of AAHD is represented as a flow chart with 'R' moiety as an acyl side chain.

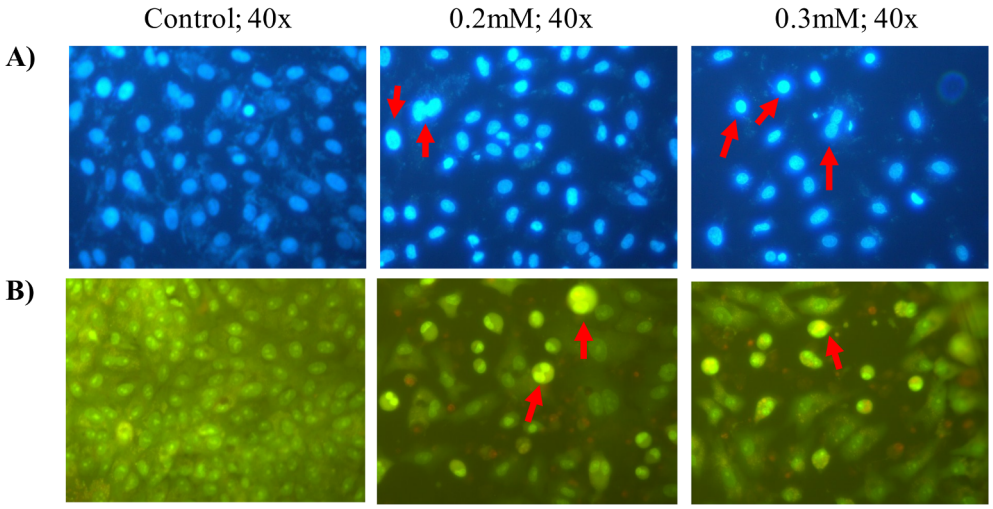

Fig. 2. (A) The HepG2 cells were treated with different concentrations of AAHD (0.2 mM & 0.3 mM) and Hoechst 33342 stain was used to observe the alterations in the morphology. There was a considerable change in the morphology of the cells as compared to the control group. (B) Nuclear and morphological changes are depicted after Acridine Orange/Ethidium Bromide staining. The significant changes are indicated by red arrows. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Wetzlar, Germany) with 500× magnification was used to record the images at 0 h and the images of the same scratch were taken after an incubation of 24 h [19,20].

2.8. Statistical analysis

The data is represented as the mean ± S.E.M of 3 independent experiments performed in triplicate. The significance of the treated and the untreated samples was calculated by student's 't' test using GraphPad Prism 6 software (GraphPad, San Diego, USA). The statistical significance is denoted by *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$.

3. Results

The scheme of synthesis of the novel acylspermidine derivative-AAHD has been summarized in Fig. 1 and the ^{1}H NMR data can be viewed under Section 2.2 in the material and method section.

3.1. AAHD altered the morphology of the nucleus as detected by fluorescent staining

To observe the morphological alterations of the nucleus and the cytoplasm, we treated HepG2 cells with different concentrations of AAHD and subsequently stained them with fluorescent stains like Hoechst 33342 and AO/EtBr dyes. When the cells were observed under 400× magnification, it revealed that the cells with compromised membranes showed deep blue stained nuclei, while the intact cells were blue in colour on staining with Hoechst 33342 (Fig. 2A). Moreover, when the cells were stained with AO/EtBr stain, the untreated cells showed green nuclei while the treated cells showed orange/red colour nuclei (Fig. 2B) indicating the breach of the cell membrane. There was a considerable shrinkage of the cell membrane and blebbing which could be a possibility of an initiated cell death process.

3.2. AAHD reduced the cell viability of HepG2 in a dose-dependent manner

Thereafter, we performed an antiproliferative study on HepG2 cells to assess the cytotoxic potential of the AAHD in a dose- and time-dependent manner through WST-1 assay. Interestingly, as shown in the Fig. 3A, there was a decrease in the cell viability starting from 0.05 mM (88.2%), which significantly decreased to 71.8%, 39.4% and 30.7% at a concentration of 0.1 mM ($p < 0.01$), 0.2 mM ($p < 0.001$) and 0.3 mM ($p < 0.001$) respectively. In contrast to the tumor cells, the normal BHK cells showed slight inhibitory effect with the cell viability of 90.6%, 81.5%, 82.6% and 80.7% with the corresponding AAHD concentrations of 0.05 mM, 0.1 mM, 0.2 mM, 0.3 mM respectively (Fig. 3B). The IC_{50} value of AAHD on HepG2 cells was found to be 0.176 mM and, while the IC_{50} value of AAHD on normal BHK cells was calculated as 0.421 mM.

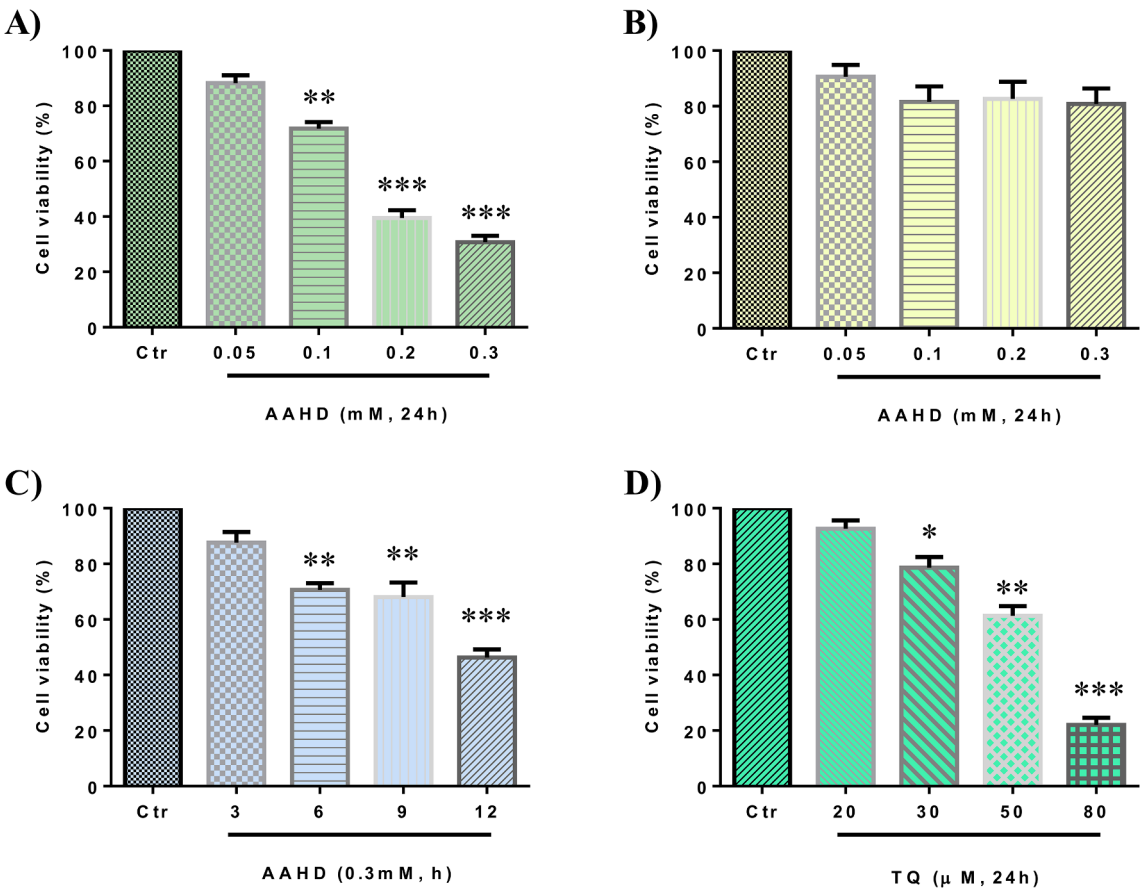

Fig. 3. (A) Dose-dependent effect of AAHD on cell viability of HepG2. HepG2 cells were treated with several concentrations of AAHD for 24 h. The cell viability was assessed using WST-1 assay. (B) Dose-dependent effect of AAHD on cell viability of BHK (non-cancerous) cells. (C) Time-dependent effect of AAHD on cell viability of HepG2. (D) Thymoquinone was used as a positive control to assess the dose-dependent effect on HepG2. The values here are represented as mean ± S.E.M. of two experiments; statistically significant (unpaired 't' test; two-tailed): *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; versus the corresponding untreated control.

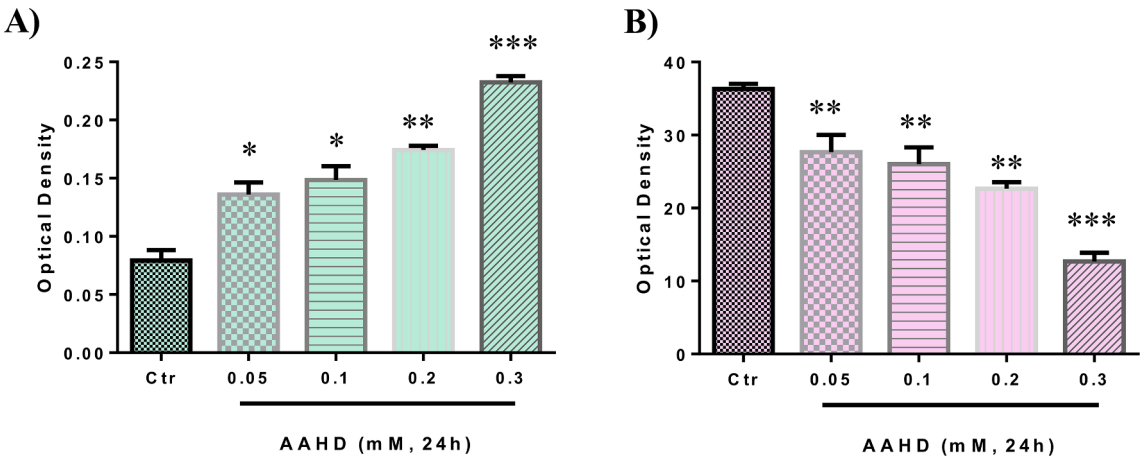

Fig. 4. (A) AAHD induced the production of ROS in HepG2 cells as a consequence of the decrease in the mitochondrial potential. (B) The mitochondrial potential of AAHD-treated HepG2 cells was decreased significantly with the increase in the concentration. The values are represented as mean ± S.E.M. of two experiments; statistically significant (unpaired 't' test; two-tailed): *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; versus the corresponding untreated control.

3.3. The antiproliferative effect of AAHD on HepG2 was time-dependent

We next investigated the time-dependent antiproliferative impact of AAHD on HepG2 cells through WST-1 assay. The cell viability decreased to 87.6% after a time duration of 3 h, after a span of 6 h, it was 70.6% ($p < 0.01$). When we further increased the time of incubation for 9 h, the cell viability was significantly reduced ($p < 0.01$) to 68% which also further decreased to 46.3% ($p < 0.001$) at 12 h, as depicted in Fig. 3C. The decrease in the cell viability from 6 h to 9 h was minimal (1.4%), but altogether, the result showed that the sequential program of cell death could have been activated. Thymoquinone was used as a positive control to compare the cytotoxic effect of AAHD (Fig. 3D).

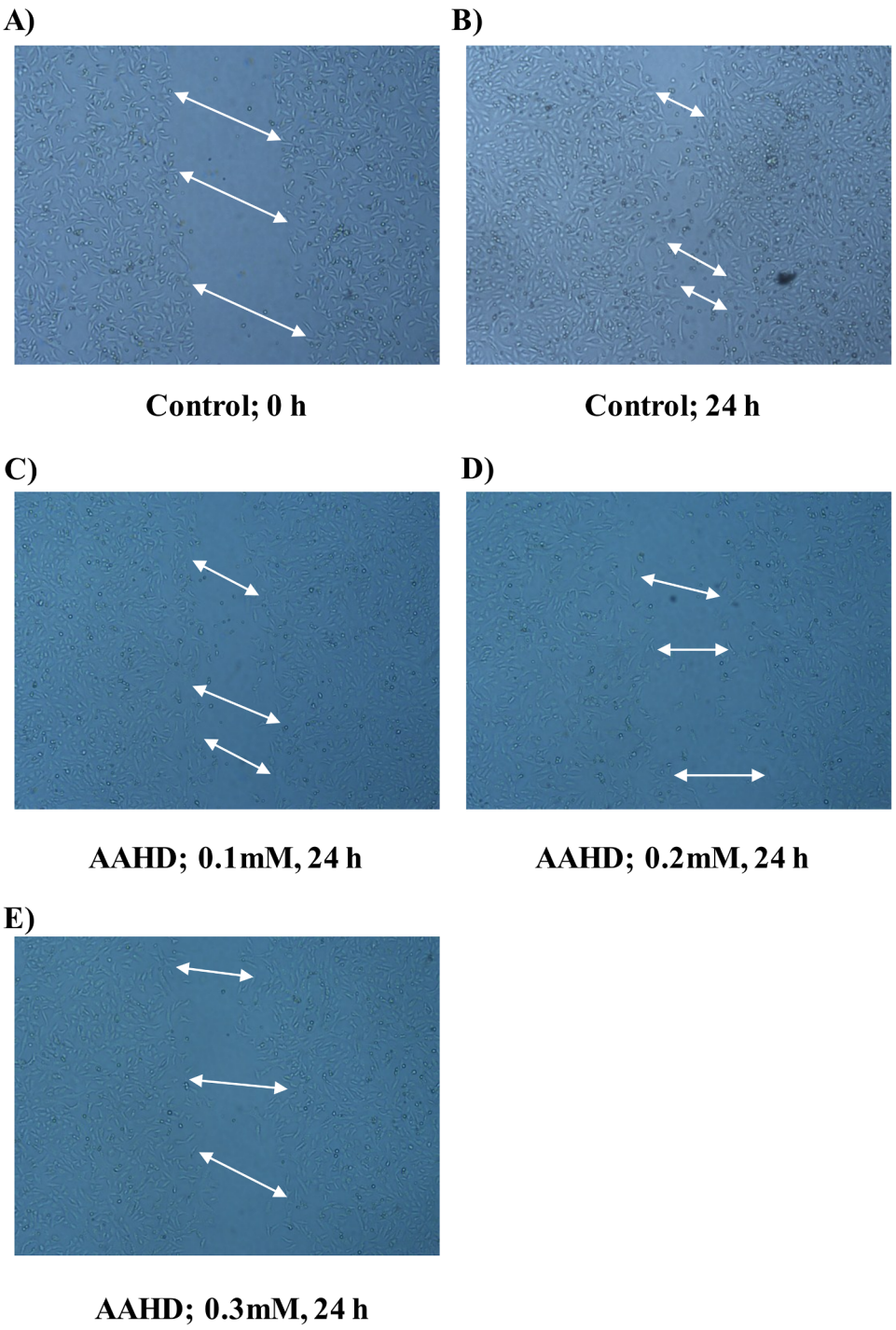

Fig. 5. Inhibitory effect of AAHD on HepG2 cell migration. (A) The scratch is quite prominent in the control at 0 h. (B) The wound scratch has been healed in the control with the migration of cells after 24 h. (C) AAHD reduced the migration of cells when used at a concentration of 0.1 mM after 24 h (D) Effect of AAHD at a concentration of 0.2 mM after 24 h. (E) Maximum inhibition of cell migration was observed after an incubation of 24 h with AAHD at a concentration of 0.3 mM.

3.4. AAHD induced the production of ROS and decreased the mitochondrial potential in HepG2 cells

It has been well documented that the accumulation of ROS and mitochondrial damage inside the tumor cells triggers apoptosis leading to activation of a nonreversible cascade of cell-death events. To investigate the role of mitochondria and ROS levels in cell death, we measured the ROS levels and evaluated the magnitude of the decrease in the mitochondrial potential (ΔΨm). As the concentration of AAHD increased, there was a significant increase in the ROS levels with the increase in the concentration of the drug ranging from 0.05 mM to 0.3 mM as shown in the Fig. 4A. Interestingly, there was a significant decrease ($p < 0.05$) in the ΔΨm commencing from the initial dose of 0.05 mM as shown in the Fig. 4B. However, the decrease in the ΔΨm was not much prominent from 0.05 mM (OD-27.6) to 0.1 mM (OD-26); but was quite significant from 0.2 mM (O.D-22.6; $p < 0.01$)) to 0.3 mM (O.D-12.6; $p < 0.001$).

3.5. AAHD inhibits wound healing in HepG2 cells.

Finally, we sought to investigate the potential of AAHD to impede the movement of HepG2 cells and prevent the healing of wound-induced in the form of a scratch. It was observed that the scratch/wound which was quite apparent at 0 h (Fig. 5A) was healed in the control group after an incubation of 24 h as depicted in Fig. 5B. Whereas, when AAHD was used to treat HepG2 cells with concentrations of 0.1, 0.2 and 0.3 mM (Fig. 5C, D & E respectively), the wound healing was inhibited significantly. This demonstrates the potential of AAHD in controlling the cell migration and could be a candidate to regulate metastasis in hepatocellular carcinoma which needs to be further investigated.

4. Discussion

Natural polyamines like spermidine, spermine and their precursor putrescine play a vital role in the regular maintenance and cell proliferation of the eukaryotic cell. However, in cancer and other hyperproliferative diseases, the requirement and the metabolism of polyamines is dysregulated. Therefore, the polyamine pathway can be an attractive therapeutic target for designing an anticancer chemotherapeutic drug regimen. In our previous *in vitro* study with other polyamine analogues, we demonstrated the pro-apoptotic effect of the acylspermidine analogues on MCF7 and Jurkat cells [17]. It is quite evident from the nuclear and cytoplasmic staining results that AAHD induces chromatin condensation accompanied by the formation of perinuclear apoptotic bodies. Moreover, the dose- and time-dependent antiproliferative assays demonstrate a sequential decrease in the cell viability suggesting a possible activation of the apoptotic cascade. Several previous studies with different synthetic polyamine analogues have demonstrated both caspase-dependent and caspase-independent mechanisms [21–24]. Interestingly, the analogue AAHD showed minimal cytotoxicity towards the non-carcinogenic, healthy BHK cells expressing its specific action on tumour cells. There is considerable evidence that the polyamine analogues specifically target the mitochondria as potential sites of activity [25,26]. Hence, we evaluated the modulatory effect of the AAHD on the ΔΨm. It was observed that with the increase in the concentration, the ΔΨm decreased gradually which could be due to the depolymerization of the mitochondrial membrane. Many studies have implicated the role of cytochrome *c* in the apoptotic cell death with tumour cells treated with specific polyamine analogues [27,28]. The significant loss of ΔΨm and accumulation of ROS leads to the cell death which is a prominent biomarker to be measured to know the pathological state of a cell [29]. Our results are in line with the above mentioned previous studies reported on the role of a decrease in the ΔΨm and hence resulting in the release of the pro-apoptotic factors which could be further confirmed by protein and gene studies. However, it may be possible that the mitochondrial pathway would not be the primary target of all the polyamine analogues, as the antiproliferative effect occurs prior to the initiation of mitochondrial activity. Moreover, the cells lacking mitochondria also show positive antiproliferative response with the polyamine analogues [30]. Hence it could be professed that the acylspermidine analogue-AAHD targets mitochondria and initiates cell death which must be further confirmed by molecular studies. Additionally, the results obtained from the cell migration assay have shown that this derivative could also inhibit the migration of cancer cells significantly which needs to be further studied.

In conclusion, the acylspermidine analogue-AAHD could be an excellent pharmacological lead in the development of novel chemotherapeutic drug targeting hepatocellular carcinoma. AAHD could also be a part of the current treatment regimens as it shows minimal toxicity towards non-cancerous cells. We recommend the future '*Omics*' approach in deciphering the actual mechanism and pathways involved which could give us a clear understanding of the mechanism of action and the gene alteration pattern induced by AAHD.

Conflict of interest

The authors declare that they have no financial conflict of interest.

6. Consent

All the authors have carefully read the manuscript and have approved it for publication.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.102937.

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