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Original article

In vitro characterization of arylhydrazones of active methylene derivatives

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ABSTRACT

Arylhydrazones of active methylene compounds (AHAMCs) are potent chemotherapy agents for the cancer treatment. AHAMCs enhance the apoptotic cell death and antiproliferation properties in cancer cells. In this study, a series of AHAMCs, 13 compounds, was assayed for cytotoxicity, apoptosis, externalization of phosphatidylserine, heterogeneity and cellular calcium level changes. The *in vitro* cytotoxicity study against HEK293T cells suggests that AHAMCs have significant cytotoxic effect over the concentrations. Top 5 compounds, 5-(2-(2-hydroxyphenyl) hydrazono)pyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione (**5**), 4-hydroxy-5-(2-(2,4,6-trioxo-tetrahydro-pyrimidin-5(6H) ylidene)hydrazinyl)benzene-1,3-disulfonic acid (**6**), 5-chloro-3-(2-(4,4-dimethyl-2,6-dioxocyclohexylidene)hydrazinyl)-2-hydroxybenzenesulfonic acid (**8**), 5-(2-(4,4-dimethyl-2,6-dioxocyclohexylidene)hydrazinyl)-4-hydroxybenzene-1,3-disulfonic acid (**9**) and 2-(2-sulfophenylhydrazo)malononitrile (**10**) were chosen for the pharmacodynamics study. Among these, compound **5** exhibited the better cytotoxic effect with the IC_{50} of 50.86 ± 2.5 mM. DNA cleavage study revealed that **5** induces cell death through apoptosis and shows more effects after 24 and/or 48 h. Independent validation of apoptosis by following the externalization of phosphatidylserine using Annexin-V is also in agreement with the potential activity of **5**. Single cell image analysis of Annexin-V bound cells confirms the presence of mixture of early, mid and late apoptotic cells in the population of the cells treated with **5** and a decreased trend in cell-to-cell variation over the phase was also identified. Additionally, intracellular calcium level measurements identified the Ca^{2+} up-regulation in compound treated cells. A brief inspection of the effect of the compound **5** against multiple human brain astrocytoma cells showed a better cell growth inhibitory effect at micro molar level. These systematic studies provide insights in the development of novel AHAMACs compounds as potential cell growth inhibitors for cancer treatment.

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1. Introduction

Arylhydrazones of active methylene compounds (AHAMCs) and their metal complexes have emerged as potential chemotherapeutic drugs for cancer treatment (Hamid et al., 2004; Sambasivarao, 2013; Jimenez et al., 2009; Jones and Senft, 1985; Nitsch et al., 2000; Ozdemir et al., 2003). AHMACs are linking β-diketones and aryldiazonium salt that have been identified as potential antibacterial, antifungal, analgesic and antipyretic drugs (Hamid et al., 2004; Sambasivarao, 2013; Jimenez et al., 2009; Jones and Senft, 1985; Nitsch et al., 2000; Ozdemir et al., 2003; Arndt-Jovin and Jovin,

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1989; Zhang et al., 1997). Recently, Di- and tri-organotin(IV) complexes of arylhydrazones of methylene active compounds are evaluated as potential antiproliferative compounds against in HCT116 and HEPG2 tumor cell lines (Nitsch et al., 2000). Besides, curcumin is a one of the family of β-diketone derivatives extracted from Curcuma longa exhibits tumor suppressive and preventive characteristics in many cancerous models (Demchenko, 2013). Curcumin induces the apoptosis by Bid cleavage, caspase 9 and 3 activation, and by downregulating anti-apoptotic proteins BCl-2 and BclXL (Zhang et al., 1997; Hingorani et al., 2011). The analogues of Curcumin also effectively inhibit the growth of prostate cancer cells. Lanthanide chelation with β-diketones derivatives have also been applied in the treatment of colon cancer (Hagan and Zuchner, 2011). Polyphenol 3 compound is shown to have more toxicity effect on HEK293T cell lines at 48 h than any other compounds that are being used commonly in the cancer treatment (Hamid et al., 2004; Sambasivarao, 2013; Jimenez et al., 2009; Jones and Senft, 1985; Nitsch et al., 2000; Ozdemir et al., 2003; Arndt-Jovin and Jovin, 1989; Zhang et al., 1997; Doan et al., 2016).

However, the mechanism such as transportation of the drug through plasma membrane, defence against drug, enhanced DNA repair, alterations in target molecules, access to target cells, metabolic effects and growth factors, are common barrier that reduce the development of efficient anti cancerous drugs (Helmy and Abdel, 2012). Ultimately, cancer cells protect themselves in a distinctive microenvironment resulting in disrupting the apoptotic mechanism in response to the drugs. This results in the failure of chemotherapy and development of multi-drug resistivity. Thus, the main objective of the chemotherapeutic agents is to progress therapeutic efficacy, selectivity and incapacitating drug resistance for enhanced cancer treatment (Tian et al., 2009).

The chemotherapeutic drugs are having potential's to induce apoptosis in many cancer cells. Severe damage to the genomic DNA by drugs rather leads to the activation of specific regulatory gene that causes apoptosis in a cell (Mattana et al., 1997). Initiating the apoptosis regulatory genes cause the cell to undergo cell morphological changes resulting in programmed cell death. Apoptosis is an active regulatory mechanism, energy-dependent process, plays a vital role in the cancer cell death (Grynkiewicz et al., 1985). It is clearly observable that inducing the apoptotic responsible gene is one of the efficient ways for the cancer cure. The cytotoxicity and apoptosis associated damage are the two significant actions result in chemotherapy induced apoptosis. Thus, the enhanced treatment in chemotherapy helps to develop drugs and to target the essential regulators for the apoptotic activation (Hahnel et al., 1999; Oruç et al., 2006).

Here, we investigate the possibility of cytotoxic effects of novel AHMACs against human embryonic kidney cell line (HEK293T) and multiple glial tumor cells lines. HEK293T cells are suitable for investigating the potential toxicity of nanoparticles too. HEK293 are immortalized already by known oncogene but not malignant yet (Rauf et al., 2008; Küçükgüzel et al., 1999). HEK293T cells were generated by transformation of normal HEK cells with sheared human adenovirus type 5 DNA that results in decreased senescence. At first, we studied the effect of 13 novel AHMACs compounds and top 5 compounds were selected for the evaluation of cytotoxicity over the concentrations and time. To test the induction of apoptosis the Propidium iodide and Annexin-V staining methods were performed. Further, the effect of top compound on cell-to-cell variation and intracellular calcium also measured using microscopic imaging analysis. Additionally, to observe the potential growth inhibitory effect of the top compound on multiple glial tumor cell lines we used 1321N1 and U87-MG. Therefore, this study extends the possibilities to use novel AHMACs against the growth of many cancer cells.

2. Materials and methods

2.1. Synthesis of novel compound azo derivatives of AHMACs

Thirteen novel AHMACs were synthesized and used in this study. The list of compounds are as follows: (*E*)-3-(2-(1-ethoxy-1, 3-dioxobutan-2-ylidene)hydrazinyl)-2-hydroxy-5-nitrobenzene sulfonic acid (**1**), 2-(2-(2-hydroxy-4-nitrophenyl)hydrazono)-2*H*-indene-1,3-dione (**2**), (*Z*)-5-chloro-2-hydroxy-3-(2-(4,4,4-trifluoro-1,3-dioxo-1-(thiophen-2-yl)butan-2-ylidene)hydrazinyl)benzenesulfonic acid (**3**), 5-chloro-2-hydroxy-3-(2-(2,4,6-trioxo-tetrahydropyrimidin-5(6*H*)-ylidene)hydrazinyl)benzenesulfonic acid (**4**), 5-(2-(2-hydroxyphenyl) hydrazono)pyrimidine-2,4,6 (1*H*,3*H*,5*H*)-trione (**5**), 4-hydroxy-5-(2-(2,4,6-trioxo-tetrahydro-pyrimidin-5(6H) ylidene)hydrazinyl)benzene-1,3-disulfonic acid (**6**), 5-(2-(2-hydroxy-4-nitrophenyl)hydrazono)pyrimidine-2,4,6 (1*H*,3*H*,5*H*)-trione (**7**), and 5-chloro-3-(2-(4,4-dimethyl-2,6-dioxo cyclohexylidene)hydrazinyl)-2-hydroxybenzenesulfonic acid (**8**) 5-(2-(4,4-dimethyl-2,6-dioxocyclohexylidene)hydrazinyl)-4-hydroxybenzene-1,3-disulfonic acid (**9**), 2-(2-sulfophenylhydrazo)malononitrile (**10**), 2-(2-carboxyphenylhydrazo)malononitrile (**11**), 2-(2-(2,4-dioxopentan-3-ylidene)hydrazinyl)phenylarsonic acid (**12**) and 5-(2-(2,4-dioxopentan-3-ylidene)hydrazinyl)-2,3-dihydrophthalazine-1,4-dione (**13**) were used. The compounds **1**–**8** were synthesized by reaction between the diazonium salt of substituted anilines and β-diketones following Japp-Klingemann reaction. The compounds **9**–**13** were synthesized by reaction between the respective aromatic diazonium salt and methylene active compounds in a water solution containing sodium acetate or sodium hydroxide (Oruç et al., 2006; Rauf et al., 2008; Küçükgüzel et al., 1999; Kandhavelu et al., 2012; Mahmudov et al., 2014; Shukla et al., 2012; Viswanathan et al., 2014; Cells et al., 2011). The synthesis and characterization of AHAMCs (Scheme 1) were reported earlier by us (Kandhavelu et al., 2012).

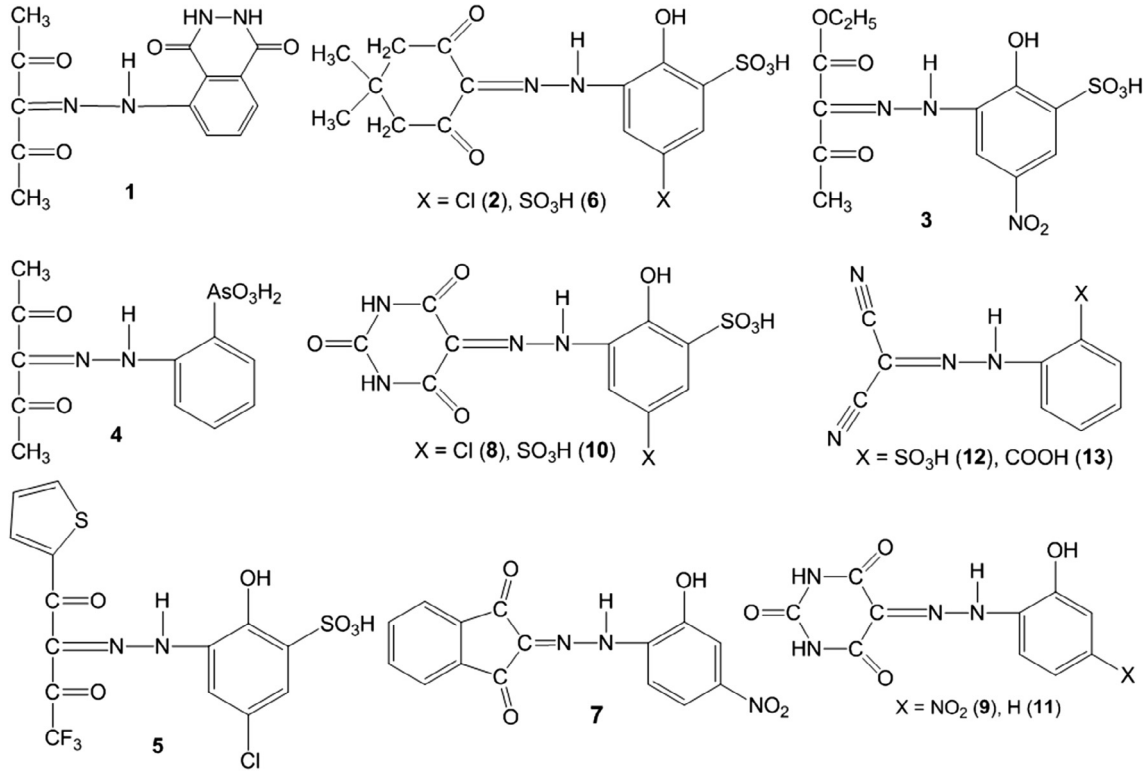
2.2. Cell culture

Human Embryonic Kidney cells (HEK293T) (ATCC, crl-3216, Sigma-Aldrich, UK) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) of fetal bovine serum (FBS), penicillin and streptomycin (100 U/ml), sodium pyruvate (1mM), and amphotericin B (250 μg/ml) (Sigma-Aldrich, St. Louis, MO) and grown at 37 °C in a humidified 95% air and 5% CO_2. To characterize cytotoxicity of the novel compounds, 1×10^6 cells per well were seeded in a 6-well plate and incubated at 37 °C in the CO_2 incubator (passage 20). After incubation for 24 h, the medium was removed and replaced with fresh medium.

Human brain astrocytoma cell lines U-87 MG and 1321N1 were used for screening the activity of top compounds. Minimum Essential Medium Eagle (MEM) with L-glutamine was used for culturing U-87 MG cells (passage 19). For 1321N1 cells (passage 23), to Dulbecco's Modified Eagle Medium (DMEM) with L-glutamine, 2 mM sodium pyruvate was added. In addition, 10% (v/v) Fetal Bovine Serum (FBS), penicillin and streptomycin (100 U/ml), and 0.025 mg/mL amphotericin B, were added in Both U-87 MG and 1321N1 media. The cells maintained in the incubator with humidified conditions at 37 °C and 5% CO_2.

2.3. Cytotoxicity of AHAMCs

The compounds were dissolved in DMSO (>99.7%) to obtain a concentration of 100 mM. Initial dilution of the compounds was made with DMSO and further dilutions were made using dH_2O. To determine cytotoxicity, first, cells were treated with a maximum concentration of 25 mM of each compound and incubated



Scheme 1. Arylhydrazones of active methylene compounds (AHAMCs)[22].

for 24 h. After incubation, the cells were trypsinised (0.5%) and cytotoxicity was determined using trypan blue exclusion assay (Sigma Aldrich, WGK, Germany). The number of live and dead cells was enumerated using a Bürker hemocytometer (Heinz Herenz, Germany) under bright field microscope. Top compounds, **5**, **6**, **8**, **9** and **10**, which exhibited better cytotoxicity, were selected for further analysis.

2.4. Dose-dependent response

To determine the inhibitory concentration (IC_{50}) of each drug, a dose response curve was plotted by treating the drugs at five different concentrations, namely 6.25 mM, 12.5 mM, 25 mM, 50 mM, and 100 mM. Briefly, 1×10^6 cells per well were seeded in 6 well plates and grown for 24 h. After incubation, the old medium was replaced with fresh medium containing the different concentrations of each compound and incubated for 24 h. Cytotoxicity was determined using trypan blue exclusion assay. The percentage of cell death was calculated (% cell death = Number of dead cells/ Total number of cells $\times$ 100). A dose response curve was plotted using concentration-effect curve function of Graphpad Prism 6. Three technical and biological repeats were performed for each condition.

2.5. Analyses of cell death

To elucidate whether cell death occurs by apoptosis or necrosis in response to treatment with the selected compounds, PI staining and Annexin-V assay were performed. For PI staining (Life Technologies, USA), 1×10^6 cells were seeded in 6-well plates and incubated at 37 °C. After 24hrs, cells were resuspended in fresh medium containing the IC_{50} of each compound. After 24 and 48 h post-treatment with the compounds, the media were removed and washed with DBPS for 3 times and the cells were trypsinized and washed with DBPS for 3 times and re-suspended in 2 X SSC buffer containing 1 mg/mL PI stain and incubated for 5 min. The samples were then placed on the microscope slides and DNA cleavage was imaged using a confocal microscope, with excitation at 535 nm and the emission 617 nm. To ascertain the mode of cell death, Annexin-V Alexa Fluor® 350 Conjugate (Life Technologies, USA) staining was performed. Briefly, after 24 and 48 h of treatment with the drugs, cells were trypsinized and washed 3 times with DBPS and resuspended in annexin-binding buffer. 10 μl of Annexin-V conjugate was added to the cell suspension and incubated for 15 min at 37 °C. Cells were then washed with Annexin-V-binding buffer and placed on the microscope slides and covered with coverslips and imaged using confocal microscope by excitation at 346 nm and emission 442 nm. For both PI staining and Annexin-V assay, approximately 100 cells were analyzed. Three technical and biological repeats were performed.

2.6. Study of cell death effect at single cell level

To investigate the cell death effect over the time at single cell level, the fluorescence images of Annexin-V stained cells acquired using confocal microscope and images were analyzed using imageJ software. The intensity variation of the cells at various stages of the apoptosis was analyzed using histogram analysis in imageJ. The standard deviation and mean after subtracting the background were calculated for the images. The coefficient of variation was calculated using the formula: $CV = \sigma/\mu$, where CV is the coefficient of variance, σ is the standard of the intensity, and μ is the mean of the intensity.

2.7. Measurement of calcium

To analyze changes in mitochondrial calcium levels, cells were treated with IC_{50} of the top compound for 24 h. Fura 2-AM (Sigma

Aldrich, WGK, Germany), a chelator of calcium was used to measure the changes in the mitochondrial calcium according to the manufacturer's protocol. The fluorescent signal was measured using a fluorescence plate reader (Ascent) by excitation at 355 nm and emission at 495 nm. Three technical and three biological repeats were performed.

2.8. Validation of lead compound activity in multiple glioma cells

To study cytotoxicity of compound 5 in multiple glioma cell lines, U-87 MG and 1321N1 cells were seeded on 12 well plates with the density 1×10^5 cells per well. After 48 h incubation, the cells were treated with 100 μM of compound 5 and with controls, for 24 h. The concentration 0.1% of DMSO and 100 μM of temozolomide were used as negative control and positive control (PC), respectively. After incubation, the cells were trypsinized and cytotoxicity was determined using trypan blue assay as described above. Biological and technical repeats were performed to obtain statistically significant results.

3. Results and discussion

3.1. Cytotoxic effect of AHMACs derivatives

AHAMCs have been shown to have cytotoxic effect against cancer cells. In this work, 13 AHAMCs were synthesized and tested for their cytotoxic activity. For that, HEK293T cells were treated with 25 mM of the compounds and cytotoxicity was determined using trypan blue exclusion assay, after 24 h (Hamid et al., 2004). Among the compounds tested, compounds **5, 6, 8, 9** and **10** (Scheme) exhibited better cytotoxicity compared to the other compounds (Fig. 1), with cytotoxicity of 46%, 36%, 45%, 42%, and 37% respectively. The cytotoxic activity of the other compounds such as **1**, **2**, **3**, **4**, **7**, **11**, **12**, and **13** was ≤30%. Only the top 5 compounds were selected for further analysis (Hamid et al., 2004).

3.2. Dose-dependent effect of top compounds

The IC_{50} of the selected compounds was determined from a dose-response curve. Cells were treated with different concentrations, as mentioned in the methods, and the percentage of cell death was plotted. As expected, all the five compounds exhibited strong dose-response relationship (Fig. 2). Compounds **5**, **6**, **8**, **9** and **10** exhibit an IC_{50} of 50.86 ± 2.5 mM, 77.08 ± 0.5 mM, 75.50 ± 2.3 mM, 104.2 ± 5.4 mM and 73.27 ± 4.5 mM, respectively. Compounds **5**, **8** and **10** produced better cytotoxicity effects even at a lower concentration. Treatment with 100 mM concentration of compounds **5** and **8** showed a cell death of nearly 80%. Interestingly, compounds **6** and **9** which produced the similar cytotoxicity effect at lower concentrations (6.25, 12.5 and 25 mM), did not elicit significant cytotoxicity at higher concentrations, 50 and 100 mM.

3.3. Differentiation of apoptosis and necrosis cell death

Cytotoxic compounds induce cell death by two different mechanisms namely apoptosis and necrosis. To differentiate the mechanism of cell death in the population of cells treated with the compounds, we used the PI staining method. Apoptosis is typically characterized by cytoplasmic and nuclear fragmentation, cell shrinkage, membrane blobbing adjacent to the nuclear envelope. Upon staining with PI, the fragmented nucleus appears as multiple spots under the microscope (Sambasivarao, 2013). Whereas, in the case of necrosis, cells typically exhibit cytoplasmic vacuolization nuclear swelling and rupture of nuclear and plasma membrane (Jimenez et al., 2009; Jones and Senft, 1985; Nitsch et al., 2000; Ozdemir et al., 2003). Upon staining with PI, the nucleus appears intact and rounded. Also, the rate of PI stain diffusion is greater in necrotic cells than the apoptotic cells. In order to distinguish the two populations of cells, we performed image based analysis of PI-stained nuclei (Arndt-Jovin and Jovin, 1989). Cells treated with IC_{50} of compound 5 produced apoptosis of about 40% and necrosis of about 20%, after 24 h of exposure (Fig. 3 A). After 48 h, nearly 80% of cell death was observed with nearly 60% of the cells undergoing apoptosis (≈60%) and 20% undergoing necrosis

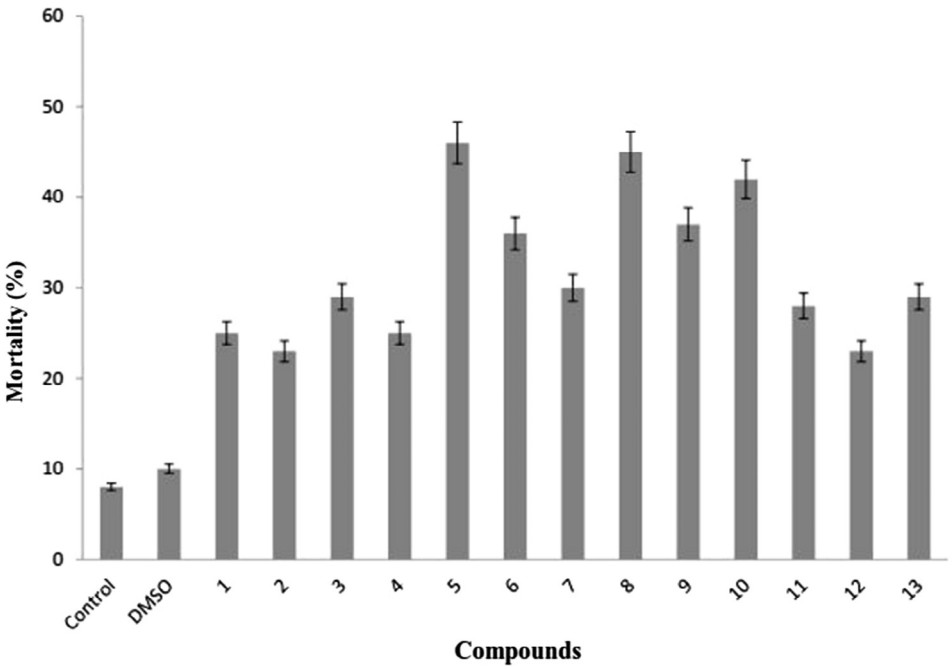


Fig. 1. The effect of AHMACs after 24 h incubation on HEK293T cells. The mortality percentage of the cells was calculated using trypan blue assay. Control - untreated; Negative control - DMSO; AHMACs-1-13.

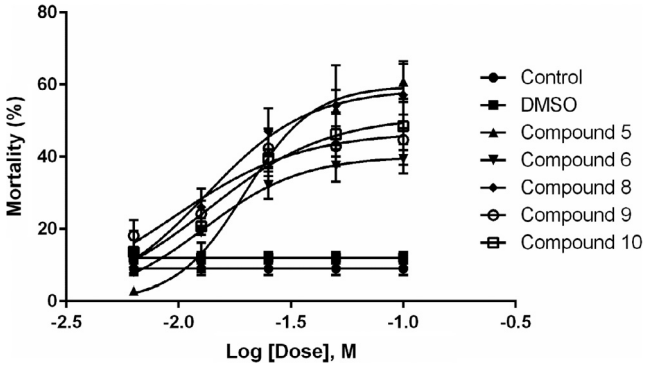


Fig. 2. Dose Response Curve *vs.* Mortality: Dose-response curve of mortality at 24 h following the exposure of Compound **5**, **6**, **8**, **9**, and **10**. The HEK293T cells were treated with different concentration 6.25 mM, 12.5 mM, 25 mM, 50 mM and 100 mM. Data are presented as means of 3 independent ± standard error of the mean.

(Fig. 3B). A similar response was observed in case of compounds 8 and 10 24 h post-treatment (Fig. 3 A), while 48 h of treatment showed the lower response (Fig. 3B). Compounds **6** and **9** produced less cell death compared to the other compounds.

3.4. Detection of externalized phosphatidylserine

Annexin V assay was performed to confirm apoptosis following treatment with the compounds, since apoptotic cells exclude PI stain during early apoptosis. Apoptosis is characterized by several changes in the surface of the outer membranes of the cells (Zhang et al., 1997; Demchenko, 2013). One of the hallmarks of induction of apoptosis is the translocation of phosphatidylserine, located at the inner membrane of the viable cells, to the outer membrane. Annexin-V, human anticoagulant, has a high affinity for phospholipid phosphatidylserine and it can detect early phases of apoptosis (Hingorani et al., 2011). Here, we observed the externalization of phosphatidylserine using Annexin-V conjugate under the microscope after treatment with compounds **5**, **6**, **8**, **9**, and **10**. The results were in agreement with the PI staining method: Compound **5** exhibited more apoptotic effect compared to the other compounds at both 24 h and 48 h. The trend of apoptosis level was similar at 24 h for compounds **5**, **8**, and **10**; however, compounds **8** and **10** exhibits lower level of apoptosis after 48 h of treatment. Fig. 4B is the example fluorescence images of apoptotic cells labelled with Annexin-V post 24 h and 48 h of treatment with the selected compound. Noteworthy, it is not able to distinguish between early, mid and late apoptotic cells using manual observation.

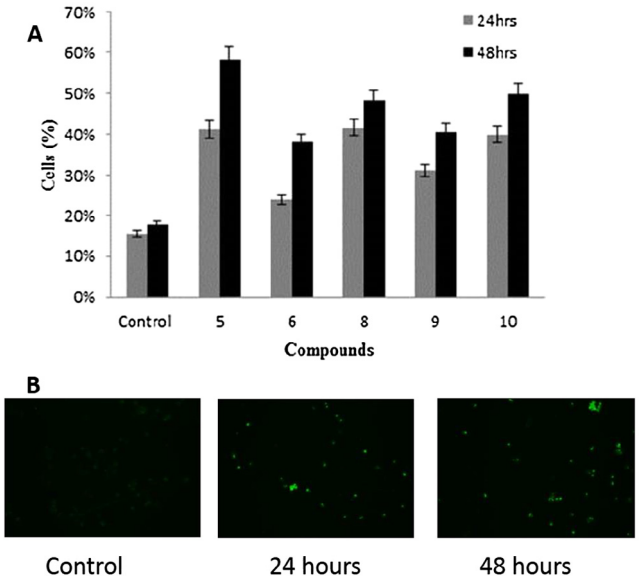


Fig. 4. Measurement of externalized phosphatidylserine: (A) After 24 h and 48 h exposure of compounds with the HEK293T cells, the apoptosis was determined using Annexin-V Staining. Data are presented as means of 3 independent ± standard error of the mean. (B) Representative images of bright field and Annexin-V stained fluorescence images of HEK293T cells: control, treated with Compound 5 at 24 h and 48 h.

3.5. Drug-induced cell-to-cell variation at single cell level

Cells undergoing apoptosis were analyzed at the single cell level to detect the phase of apoptosis after treatment with the compounds, as manual counting does not provide insight into the phase of apoptosis in cells. For this, we used image, an image analysis tool. The fluorescence intensity variation between the control cell and the apoptotic cell has been observed after 24 h of incubation. The intensity variations in cells at the different stage of apoptosis were extracted by performing histogram analysis (Helmy and Abdel, 2012; Sonka et al., 2007). It showed that: (1) non-apoptotic cells (control) expressed no fluorescence intensity; (2) early apoptosis has expressed less fluorescence intensity; (3) mid-apoptosis has expressed average fluorescence intensity; (4) late apoptosis

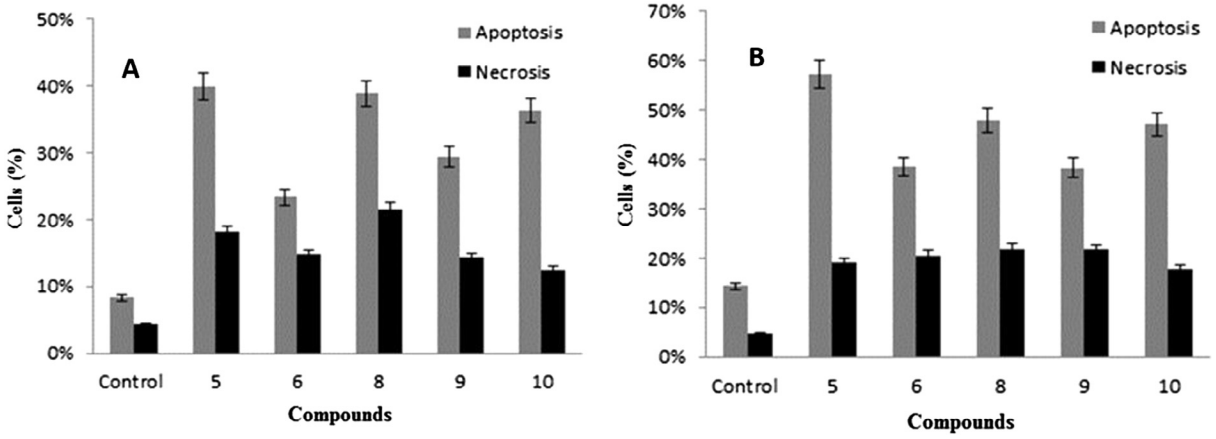


Fig. 3. Quantification of apoptotic and necrotic cell death: (A) Treatment of HEK293T cells with compounds 5, 6, 8, 9 and 10 for 24 h and (B) 48 h caused the high level of apoptosis and necrosis. Data are presented as means of 3 independent ± standard error of the mean.

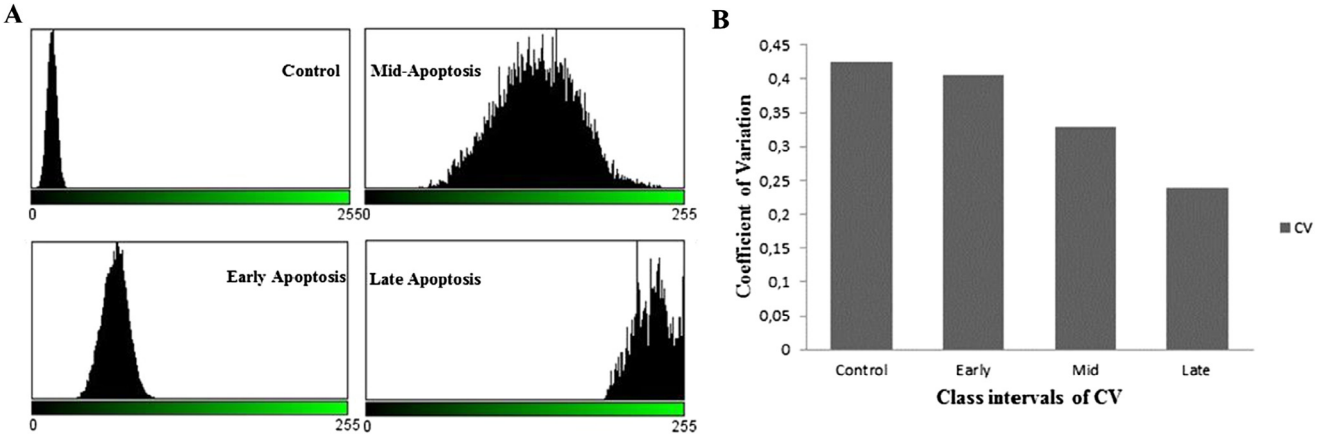


Fig. 5. Single cell analysis of drug-induced apoptosis process: (A) Intensity distribution of Annexin-V bound cells treated with compound **5** at 24 h. Variation in intensity distribution is observed in control, early-apoptotic, mid-apoptotic and late-apoptotic cells. (B) The coefficient of variation for the different stages of apoptosis was calculated and plotted in the bar graph.

has expressed high fluorescence intensity (Fig. 5A). The coefficient of variation of the apoptotic cells is inversely proportional to the fluorescence intensity (Fig. 5B). This analysis was performed only for the best lead compound **5**. It suggests that compound **5** reduces the immortal cells heterogeneity through the sequential step process of apoptosis and pushes the population to reach death phase.

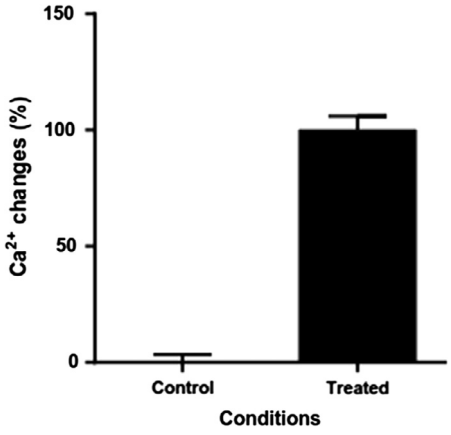


Fig. 6. Measurement of Calcium Changes in untreated and treated cells: The graph shows the percentage of calcium changes in the cells treated with compound **5** and the untreated cells after 24 h incubation. Data are presented as means of 3 independent ± standard error of the mean.

3.6. Compound-induced calcium changes

Calcium is a secondary metabolite messenger, which plays an important role in cell signalling of the biological systems (Tian et al., 2009). The biological responses such as cell proliferation, programmed cell death, cellular responses due to extrinsic compounds alter the calcium level in the cells (Mattana et al., 1997). The elevated level of intra cellular calcium was measured during necrosis and apoptosis condition upon the induction of anti-cancer compounds. Here, we used Fura 2 as a calcium chelator that contains a membrane-permeable derivative called Fura 2-AM (Grynkiewicz et al., 1985) to measure the changes of calcium level in different experimental conditions. This measurement allowed investigating the level of calcium changes at single cells upon treatment of compound **5**. Fig. 6 shows that the control expresses the low level of calcium accumulation than the treated. Notably, when HEK293T cells were treated with 100 mM of compound **5** we observed that 100% of increased calcium level when compared to the control. It is noted that the intracellular calcium of Syrian Hamster Embryo (SHE) cells treated with 60 μM of Ochratoxin A

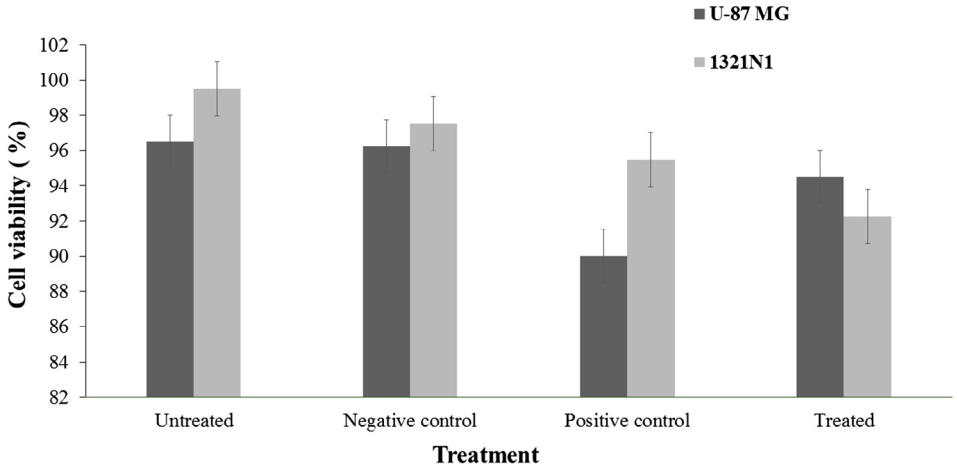


Fig. 7. Validation of activity of lead compound in U-87 MG and 1321N1 glioma cell lines. The viability percentage of the tumor cells were calculated for the following condition after 24hrs incubation. (1) Untreated, (2) Negative control-DMSO 0.1%, (3) Positive control-temolozolomide, 100 μM, (4) Treated - Compound 5, 100 μM.

increased 45% compared to the untreated cells (Hahnel et al., 1999). Our results suggest that compound **5** can induce the cell death through the cellular calcium regulation (see Fig. 6).

3.7. Antitumor activity

Preliminary screening of several AHMACs showed **5** as lead compound. This compound was selected to test the cytotoxicity effect on multiple glial tumor cell lines, U-87 MG and 1321N1. For the comparison, we also used well-known anti-glioma drug temozolomide as a positive control. From all the tested conditions, compound **5** showed slightly higher cytotoxicity effect, 20% mortality of 1321N1 while 10% in U-87 MG cells at 100 μM concentration. Compared to temozolomide induction condition 5 showed 10% higher inhibitory effect in 1321N1 while 10% lower in U-87 MG cells at 100 μM concentration (see Fig. 7.). Interestingly, **5** showed better cytotoxicity effect in 1321N1 and U-87 MG cells compered to HEK-293T, suggesting the cell line specific cytotoxicity action of **5**. In case of HEK-293T cells the lead compound inhibited the growth at millimolar range while it affects glioma cells at micromolar range. A weaker mortality effect on HEK-293T cells than the glioma cells further supporting to consider AHMACs as a future potential compound for tumor treatment.

4. Conclusions

We found that the AHAMCs induce apoptosis in the Human Embryonic Kidney cells. Among the 13 compounds, compounds **5**, **6**, **8**, **9**, and **10** showed more anticancer activity than the other ones, **1**, **2**, **3**, **4**, **7**, **11**, **12** and **13**. DNA cleavage measurement and Annexin-V binding assay shows the significant cytotoxic effects for the compound **5**. The resulting of calcium level changes due to induction of apoptosis suggests the potential activity of compound **5**. The higher cytotoxicity effect of compound **5** on multiple glioma cells provide insight on AHMACs compound activity and specially to consider **5** as a potential lead compound for the development of anticancer agent.

Authorship contributions

M.K. supervised the project. M.K. and O.Y. designed the experiments. F.I.G., K.T.M. and A.J.L.P. performed the chemical synthesis experiments. S.P., A.Z. and P.D. performed all other experiments. S.P., A.Z., P.D., J.G., F.I.G., K.T.M., A.J.L.P., O.Y. and M.K. analyzed the data. All authors wrote the main manuscript reviewed the manuscript.

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Conflict of interest

We also declare that we do not have any financial interest and conflict of interest.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jsps.2017.12.018.

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