RESEARCH ARTICLE

Biological Screening of Novel Derivatives of Valproic Acid for Anticancer and Antiangiogenic Properties

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Abstract

Background: Valproic acid (VPA) is a potent anticancer and antiangiogenic agent. However, design and synthesis of chemical derivatives with improved antiangiogenic and anticancer activities are still necessary. In this study a library of novel derivatives of VPA was synthesized and tested. Methods: A human liver cancer cell line (HepG2) and a human normal embryonic kidney cell line (HEK 293) were exposed to various concentrations of VPA derivatives for 24 hours and cell viability was checked by MTT colorimetric assay. Anti-angiogenic properties were evaluated in transgenic zebrafish embryos. Results: N-valproylglycine derivatives suppressed survival almost 70% (p value 0.001) in HepG2 cells but only 10-12% in HEK 293 cells (p value 0.133). They also suppressed angiogenic blood vessel formation by 80% when used between 2-20 μM in zebrafish embryos. Valproic acid hydrazides showed moderate level of anticancer activity by affecting 30-50% (p value 0.001) of cell viability in HepG2 cells and 8-10% in HEK293 cells (p value 0.034). Conclusion: The majority of compounds in this study showed potent and stronger antiangiogenic and anticancer activity than VPA. They proved selectively toxic to cancer cells and safer for normal cells. Moreover, these compounds inhibited developmental angiogenesis in zebrafish embryos. Based on the fact that liver is a highly vascularized organ, in case of liver carcinoma these compounds have the potential to target the pathological angiogenesis and could be an effective strategy to treat hepatocellular carcinoma.

Keywords: Valproic acid derivatives - hydrazides - glycine - hepatocellular carcinoma cells - angiogenesis

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Introduction

Hepatocellular carcinoma (HCC) is the primary cause of mortalities among liver cancers. The distribution of HCC varies among worldwide but high prevalence has been noticed in Asian population (Teo Fock, 2001; Can et al., 2014; Poortahmasebi et al., 2014; Somboon et al., 2014). Keeping in view of high mortality rate associated with liver cancer, tremendous efforts are going on to design effective therapeutic for successful treatment.

Valproic acid (VPA) has been shown to inhibit the growth of a variety of cancer cells including human hepatocellular cancer cells in vitro and in vivo (Kawagoe et al., 2002; Kaiser et al., 2006; Machado et al., 2011; Shan et al., 2012; Wang et al., 2013; Can et al., 2014). Beside an effective epileptic drug VPA also inhibits angiogenesis both in vitro and in vivo (Michaelis et al., 2004; Zgouras et al., 2004; Farooq et al., 2008; Kitazoe et al., 2009; Osuka et al., 2012). Being a smaller in size and remarkable therapeutic potential, several synthetic compounds have been designed as structural derivatives of VPA with improved bioactivities. But no attempt has been made so far, to synthesize VPA derivatives with improved antiangiogenic potential.

Substituted hydrazine has found many scientific and commercial applications (Schmidt, 2001; Rothgery 2005). Hydrazones have been demonstrated to posses, antimicrobial, anticonvulsant, analgesic, anti-inflammatory, antiplatelet, antitubercular and antitumor activities (Loupy et al., 1998; Kucukguzel, 1999; Kucukguzel et al., 2002; Rollas et al., 2002).

In order to study angiogenesis, variety of in vitro and in vivo models has been used. The mostly used in vitro model for this purpose is a cell line derived from human umbilical cord "HUVEC" (human umbilical cord vein endothelial cells) (Jaffe et al., 1973). In vivo models of angiogenesis

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have also been developed using chick, rabbit and mouse embryos (Staton et al., 2009). Each of these experimental models has merits and demerits. For example the in vitro model of angiogenesis using the HUVEC cells, often lacks information about how endothelial cells interact with their neighboring cells. The animal models more accurately represents physiological model of angiogenesis. However, large-scale chemical screening with these models is difficult due to the cost and space needed for husbandry facilities. Drug screening and development could be cost effective and save lot of time if relevant in vivo models are used. Zebrafish become an important model organism for the study of development, genetics, immunity, cancer and other diseases (Amatruda Patton, 2008; Huang Zon, 2008; Ingham, 2009; Chan Mably, 2011; Littleton Hove, 2013; Terriente Pujades, 2013) and at the same time variety of potent natural and synthetic antiangiogenic molecules have been identified using this animal model (Crawford et al., 2011; Liu et al., 2011a; Lin et al., 2012; Bohni et al., 2013; Yang et al., 2014)

The objective of this study was to synthesize hydrazide- hydarzone derivatives of VPA which could have improved antiangiogenic and anticancer potential. Among the synthesized compounds, the valproylglycine hydrazide-hydrazone derivatives (compounds 12 a-c, Table 1) exhibited strong anti-angiogenic and anti cancer activity. These compounds suppressed significant proportion of inter-segmental vessels and sub-intestinal vein formation in all treated zebrafish embryos. The time window treatment of Tg (fli-1:EGFP) embryos with compounds 12a at 52 and 96 hours post fertilization (hpf) showed that these compounds affected the blood vessels formation process at the time of endothelial cells differentiation. Valproylglycine hydrazide-hydrazone derivatives also exhibited strong anti cancer activity by suppressing 60-80% cell survivals in HepG2 cancer cell line and mildly affected the cell viability of normal human embryonic kidney cells (HEK293).

Materials and Methods

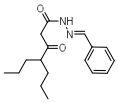
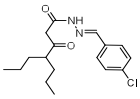
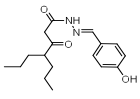
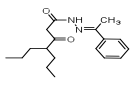
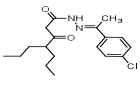
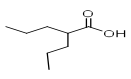
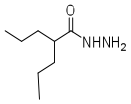
Animals

Wild type (AB/Tuebingen TAB-14) and Tg (fli1:EGFP) zebrafish were obtained from zebrafish international resource center (ZIRC University of Oregon, Oregon, USA) and maintained in our facility under recommended conditions. The embryos were obtained by natural spawning and staged as described by Kimmel et al (Kimmel et al., 1995).

Treatment of Zebrafish Embryos with VPA and its Derivatives

Stock solutions: VPA and newly synthesized derivatives were dissolved in molecular biology grade

Table 1. Comparative Antiangiogenic Profile of Valproylglycine Hydrazide-Hydrazone Derivatives and Valproic Acid in Transgenic Zebrafish Embryos at 3dpf

Compounds ID	Compound Structure	% age of affected embryos	Anti-angiogenic activity	Anticancer activity			
			concentration μM	% ISV** inhibition	% SIV** inhibition	% survival in HepG2 cells treated with 40 μM***	% survival in HEK 293 treated with 40 μM***
12a		120/150 (80%)	2	72±1.667	100±0 p value (0.0014)	36.88±0.008 p value (0.1336)	83.99±0.009
12b		100/150 66.66%	7	72±0.666	100±0 p value (0.0108)	71.34±0.009 p value (0.161)	91.456±0.013
12c		120/150 80%	10	83±1.527	100±0.333 p value (0.002)	62.17±0.017 p value (0.056)	92.418±0.003
12d		150/150 100%	32	0±0	100±0 p value (0.021)	78.62±0.009 p value (0.979)	100.120±0.005
12e		150/150 100%	50	0±0	0±0 p value (0.047)	84.76±0.010 p value (0.433)	96.750±0.004
VPA		150/150 100%	50	17±0.5773	100±0 p value (0.087)	87.26±0.004	ND
VALPH		150/150 100%	80	0±0	0±0 p value (0.039)	83.47±0.010	ND
control			1% DMSO v/v	0±0	0±0	100±021	98.47812±0.017

*ISV: inter-segmental vessels,*SIV; Subintestinal vein, **Values are standard error of three different experiments

dimethyl sulfoxide (DMSO) Sigma Aldrich Cat#D8418 to make a stock concentration of 20 mg/mL. The compounds were further diluted in Embryo Medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$ & 0.33 mM $MgSO_4$) to obtain required working dilutions. The mock (0.5% DMSO v/v) treated embryos served as control.

Animal treatment: Synchronized AB wild type embryos were raised to shield stage: (-6 hours post fertilization). Any unfertilized and developmentally abnormal embryos were excluded. 50 embryos were placed in 60 mm glass Petri dishes; containing 10 ml embryo medium with desired quantity of compound. The embryos were incubated in refrigerated air incubator at 28.5°C overnight. On the following day any dead embryos either in control or treated groups were recorded or removed. The embryos were subsequently raised in embryo medium without the compounds up to 5 days post fertilization (dpf) and hence the embryos were exposed to compounds only for 12-15 hours.

Antiangiogenic assay in zebrafish embryos

We have scored the antiangiogenic activity of the compounds in live transgenic zebrafish embryos by observing the reduction in the outgrowth of blood vessels at two time points i) Inter-segmental blood vessels (ISV) at 48 hpf and then ii) subintestinal vein (Bohni et al., 2013) out growth at 72 hpf. The embryos were anesthetized using 0.003% tricane (Sigma Aldrich cat#E10521) for counting the blood vessels and subsequently photographed. A scoring method was used to assess the level of antiangiogenic activity of compounds by counting total number of blood vessels in the trunk area of control embryos and any missing or un-developed blood vessels in treated embryos at same developmental stage. The percentage was calculated by using following equation.

Three biological replications with different clutches of embryos were conducted.

Cell culture and proliferation assay

Human liver cancer cell lines (HepG2) and human embryonic kidney cell line (HEK293) were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM: Life technologies cat#11995073) supplemented with 10% Fetal bovine serum (FBS: Life technologies cat#16000044) in a humidified incubator with 5% CO2 at 37°C. Around $2x10^3$ cells were seeded in each well of a 96-well cell culture plate in triplicate and were allowed to adhere and spread for 24 hours. The compounds were added to a final concentration of 40 μM. The cells were allowed to expose to the compounds for 24 hours. The cell viability was determined using MTT 1-(4,5-Dimethylthiazol-2-yl)-3, 5-diphenylformazan colorimetric assay. Briefly, the media was removed from the treated or untreated cells and were washed for three times with 1X PBS. The cells were trypsenized, centrifuged and the resulting pellet was re-suspended in 100 μL of DMEM serum free medium. 20μL of MTT solution (5 mg/mL in PBS: Sigma Aldrich cat#M2003) was added to each well and incubated for 2 hours at 37°C. The plate was centrifuged at 40000 rpm for 10 minutes and medium was removed. Isopropanol containing 0.04 M HCI was added and optical density of the formazan product in solution was measured with a microplate reader at 540 nm. The experiment was conducted in triplicate. Data were calculated as percent of cell viability by the following formula: % cell viability = Mean absorbance in test wells Mean absorbance in control wells 100.

Chemistry

The solvents used were of HPLC reagent grade. Melting points were determined with a Mel-Temp apparatus and are uncorrected. Infrared (IR) spectra were recorded on a Perkin-Elmer 1600 series Fourier transform instrument as KBr pellets. Nuclear Magnetic resonance spectra (^{1}H NMR and ^{13}C NMR spectra) were recorded on a JOEL 500 MHz and on a Mercury 400 MHz spectrometer with chemical shift values reported in δ units (ppm) relative to an internal standard. Elemental analyses were performed on Perkin-Elmer 2400 elemental analyzer, and the values found were within ±0.3% of the theoretical values. Follow-up of the reactions and checks of the purity of the compounds was done by TLC on silica gel-protected aluminum sheets (Type 60 GF254, Merck) and the spots were detected by exposure to UV-lamp at λ 254 nm for a few seconds. The compounds were named using Chem. Draw Ultra version 11, Cambridge soft Corporation. The detailed chemistry of the compounds will be reported somewhere else.

Microscopy and imaging: Images were acquired using Olympus fluorescence stereomicroscope SZX10 fitted with Olympus DP 72 camera

Results

Valproylglycine hydrazide-hydrazone derivatives exhibited strong antiangiogenic and anticancer activities

The valproylglycine hydrazide-hydrazone derivatives compounds synthesized in this study (12a-e; Table 1), showed significant level of anti-angiogenic activity in transgenic zebrafish embryos (Tg: fli1:EGFP)y1. The zebrafish embryos were treated with serial dilution of the compounds in order to evaluate a certain range of concentration in which these compounds are do not kill the embryos but rather induced specific antiangiogenic affect. As shown in Figure 1E&F and Table 1, 12a was most potent in term of inhibition of angiogenic blood vessels at minimum concentration. It suppressed 72% of inter-segmental (Se) and 100% of subintestinal vein formation process at 2μM in all treated embryos. The compound 12c inhibited more than 83% of intersegmental (Se) and 100% of subinntestinal vein (Bohni et al., 2013) formation but with at 10μM (table1). The intersegmental blood vessels (Se) formed normally in compound 12d treated embryos; however, subinntestinal vein (Bohni et al., 2013) did not form at all in these embryos (table 1). The compound 12e failed to show any significant level of antiangiogenic activity in treated zebrafish embryos even using a 10X (50μM) more concentration as compared to 12a. Valproic acid alone inhibited only 17% of ISV but at tenfold higher concentration (i.e 50μM) than 12a (Table1). Besides disrupting the blood vessels formation, a cardiac edema and disruption of circulation was also

seen in treated embryos. The compound 12a specifically induced a curvature in the posterior tail in the treated embryos (Figure 1D).

We also screened valproylglycine hydrazide-hydrazone derivatives for anti-cancer activity in invitro in two cell lines; the human liver cancer cell line (HepG2) and human normal embryonic kidney cell line (HEK293). Valproylglycine hydrazide-hydrazone derivatives were generally more toxic towards cancer cells compared to normal. As shown in table 1, the compound 12a suppressed a significant level (p value 0.001) of cell viability in HepG2 by suppressing 60% of cell survival at 40μM. Compound 12a suppressed 16% cell survival in HEK293 cells. The statistical analyses showed that the cell survival among control and compound 12a treated cells were not significantly different (p value of 0.133). The compound 12c, suppressed 40% cell survival in HepG2 (p value 0.002) and less than 10% in HEK 293 (p value 0.056) cells while 70% cell viability of HepG2 was observed with compound 12b (p value 0.010) and it suppressed around 10% cell survival in HEK 293 (p value 0.461). The compounds 12d and 12e exhibited moderate level of anticancer activity by suppressing 20% and 15% of HepG2cell survival at 40μM (Table 1).

The second series of compounds which were synthesized in this study were valproic acid hyrazide hydrazones derivatives. The compounds 10a-g showed moderate level of antiangiogenic activity and anticancer activities. As shown in Figure 2 B&D and Table 2, most of these compounds failed to affect the inter-segmental blood vessels formation process. However, three compounds in this series (10a-d) completely blocked the formation of sub-intestinal vein (Bohni et al., 2013) within the concentration range of 40-50μM in 100% of treated embryos (Table 2). The remaining three compounds 10e-g

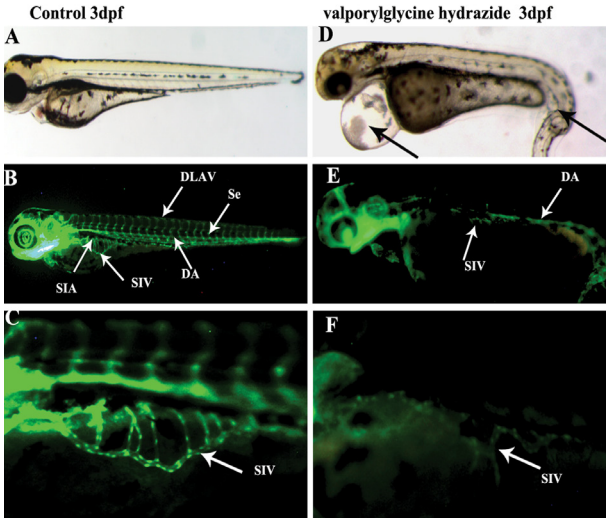


Figure 1. Valproylglycine Hydrazide-Hydrazone Derivatives of Valproic Acid Inhibited Angiogenesis at Minimum Concentration. Represented live images of Tg (fli-1:EGFP) embryos at 72 hpf. **A&D** are the bright field images of mock (1% DMS v/v) treated or valporylgycine hydrazide-hydrazone compound 12a (2μM) treated embryos. A large cardiac edema and a curvature in the posterior tail (black arrows) are evident. Inter-segmental blood vessels (**B**), supraintestinal artery and subinntestinal vein formed normally in mock treated embryos (white arrows), whereas, there was 72% reduction in intersegmental blood vessels (**E**), and 100% of supraintestinal artery and subinntestinal vein (**F**) in 12a treated embryos. The abbreviations used are: Se; intersegmental blood vessels, SIV: subintestinal vein, SIA supraintestinal artery, DLAV; Dorsal Longitudinal Anastomotic Vessel. All the images are taken at same magnification and at same exposure length. The anterior is towards left

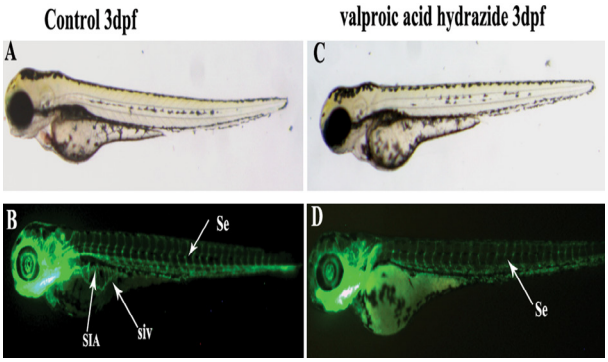


Figure 2. Hydrazide-Hydrazone Derivatives of Valproic Acid Perturbed only Subintestinal Vein Formation Process in Zebrafish Embryos. Represented live images Tg (fli-1:EGFP) embryos at 72 hpf either mock 1% DMSO (**A&B**) treated OR with one of the hydrazide-hydrazone derivative series (compound 10a 40 μM) C&D.Inter-segmental blood vessels (**B**), supraintestinal artery and subinntestinal vein formed normally in mock treated embryos (A&B), whereas the intersegmental blood vessels formed normally in compound 10a treated embryos but supraintestinal artery and subinntestinal vein did not form at all (white arrow in **D**). It is clear from the bright field images that there these compounds did not induce any teratogenic phenotype (**C**). All the images are taken at same magnification and at same exposure length. The anterior is towards left

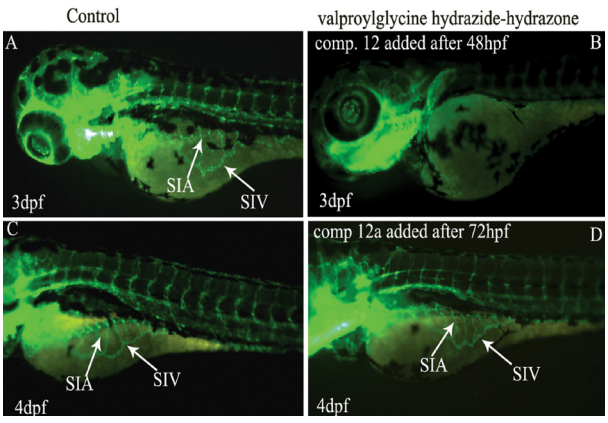
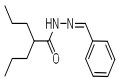
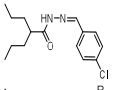
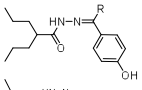
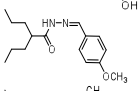
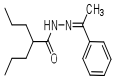
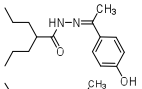
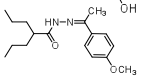


Figure 3. Valproylglycine Hydrazide-Hydrazone Derivatives of Valproic Acid Disrupted The Angiogenesis Blood Vessels Formation at Endothelial Cells Differentiation Stage. In order to analyze the specificity of newly synthesized valproic acid derivatives, the zebrafish embryos were treated with compound 12a (2 μM) either at 48 hpf (when all the inter-segmental blood vessels have been formed; top panel) or at 72 hpf (a stage at which all the inter-segmental and subintestinal vein blood vessels have been formed; lower panel). The intersegmental blood vessels were not affected in embryos treated with 12a at 48 hpf but subintestinal vein formation was severely affected in these embryos (B), similarly, treating the embryos after 72 hpf did not affected any of angiogenic blood vessels formation (D)

Table 2. Comparative Antiangiogenic and Anticancer Profile of Valproic Acid Hydrazide Derivatives in Transgenic Zebrafish Embryos at 3dpf

Compounds ID	Compound Structure	% age of affected embryos	Anti-angiogenic activity			Anticancer activity	
			concentration μM	% ISV inhibition	% SIV inhibition	% survival in HepG2 cells treated with 40 μM of compounds	% survival in HEK 293 treated with 40 μM of compounds
10a		98/150 65.33%	40	0±0	100±0	57.58±0.025 p value (0.00315)	97.531±0.034 p value (0.65246)
10b		100/150 66.66%	45	0±0	100±0	68.30±0.044 p value (0.051533)	92.158±0.040 p value (0.34629)
10c		110/150 73.33%	50	0±0	100±0	75.25±0.004 p value (0.023920)	92.496±0.019 p value (0.143513)
10d		100/150 66.66%	50	0±0	100±0	72.96±0.009 p value (0.012787)	98.710±0.058 p value (0.97822)
10e		87/150 58%	40	0±0	50±1.66	52.46±0.001 p value (0.001995)	98.478±0.017 p value (0.116232)
10f		100/150 66.66%	80	0±0	0±0	75.85±0.007 p value (0.019893)	92.12±0.036 p value (0.23586)
10g		100/150 66.66%	80	0±0	0±0	78.08±0.014 p value (0.015785)	85.478±0.041 p value (0.11623)

did not show significant level of antiangiogenic activity except 10e which inhibited around 50% sub-intestinal vein (Bohni et al., 2013) formation at 40μM.

Valporic acid hydrazide-hydrazone derivatives (10a-g) suppressed almost 25-40% of cell survival in HepG2 at 40μM. However the anticancer activity of these compounds was better than valproic acid. VPA suppressed only 13% of cell survival in HepG2 cells at 40μM (Table 2).

Discussion

Solid tumors are supplied with nutrients by diffusion from nearby blood vessels. In order to grow larger, they create their own blood vessels. Drugs that interrupt that process show promise in treating cancer (Molema Griffioen 1998). The most successful approach to modulate angiogenesis, to date, is the use of agents that specifically inhibit the growth of the endothelial cells. The pivotal role of angiogenesis in tumor progression and metastasis has urged researchers to test newly developed angiogenesis inhibitors in a broad variety of animal tumor growth models. The short chain fatty acid valproic acid (VPA) and VPA-analogs have been reported as an affective anticancer molecules by inducing differentiation, inhibiting proliferation, increasing apoptosis, and immunogenicity and by decreasing metastatic and angiogenetic potential (Regan, 1985; Kawagoe et al., 2002; Michaelis et al., 2004; Blaheta et al., 2005; Rocchi et al., 2005; Kaiser et al., 2006; Rezacova et al., 2006; Kostrouchova Kostrouch, 2007). Valproic acid is emerging as a potential anticancer drug and may also serve as a powerful molecular tool for targeting of pathways that regulate the behavior of cancer cells (Kostrouchova Kostrouch 2007). Keeping in view the simple structure and remarkable biological activity which valproic acid posses, there is tremendous interest to synthesize new derivatives with improved pharmacokinetic or safety profiles. However, valproic acid has received much less attention from synthetic chemists and biologists to explore its tremendous antiangiogenic activity and to design; synthesize and test valproic acid derivatives with improved anti-angiogenic potential.

The zebrafish transgenic line (Tg: fli1:EGFP) y1 is an establish in vivo model to assess the antiangiogenic activity of synthetic compounds and natural products (Farooq et al., 2008; Lin et al., 2012; Tse et al., 2012; Yeh et al., 2012; Zhong et al., 2012; Hollenbach et al., 2013). The formation of inter-segmental blood vessels (Se) and sub-intestinal vein (Bohni et al., 2013) are the result of angiogenic process in zebrafish embryo (Isogai et al., 2003; Ellertsdottir et al., 2010). However care should be taken to interpret the results of zebrafish antiangiogenic assay. The effect could be due to toxicity of the compounds rather than specific effect. In this study the care has been taken to make sure that phenotype which was observed is due to the specific activity of the compound and not its toxicity. In order to minimize interpreting potential false positive results (which are mostly due to the embryonic developmental delay caused by the exposure of embryos to certain chemicals), two strategies have been adopted. The routine practice is to expose the zebrafish embryos to antiangiogenic compounds at 6 hpf and observe the effect of compound at 48 hpf. While in this study the effect of compounds on modulation of angiogenesis was monitored at later stage at 72 hpf (a stage at which all inter-segmental blood vessels have been formed and majority of

sub-intestinal veins also developed (Isogai et al., 2003)). The second criterion was to treat the embryos with serial dilution of the compounds to attain a concentration of the compound which affects only blood vessels formation with minimum teratological abnormalities. Thirdly only those compounds were taken into consideration which showed at least 50% antiangiogenic activity with synchronized phenotype criterion (showing the same affect in 60% of treated embryos) and could be replicable in least three different clutches of embryos. Although, the bioactivity guided screening using zebrafish helped to identify natural antiangiogenic compounds as well as screening of synthetic libraries (Wu et al., 2006; Crawford et al., 2011; Liu et al., 2011a; Bohni et al., 2013; Dasari et al., 2013; Gong et al., 2013), but most of these studies reported their findings by observing only one of angiogenic developmental process either formation of inter-segmental blood vessels (Se) and sub-intestinal vein formation. In contrast to earlier studies the effect was evaluated by observing both of angiogenic events in this study. This strategy not only helped us to identify potent compounds but at the same time, some "active" compounds were saved from being excluded for the reason of being non active only at one developmental stage.

In this study, novel valproic acid derivatives compounds were synthesized. The prepared compounds were screened in transgenic zebrafish embryos (Tg flil-1: EGFP) for antiangiogenic activity and their anti-cancer activity was checked in HepG2 liver carcinoma cell line and HEK293 normal human embryonic kidney cell line. The newly synthesized valproic acid derivatives in this study target specifically the developing angiogenesis process in developing zebrafish embryos. In order to evaluate whether valproylglycine hydrazide-hydrazone derivative affected the differentiation or proliferation of endothelial cells; the zebrafish embryos were exposed to 2μM of compound 12a at two different developmental stages i.e one after long pec stage (48 hpf) at which all inter-segmental blood vessels have been formed and then second at protruding mouth (72 hpf) stage at which majority of sub-intestinal vein have been developed. The formations of blood vessels in treated embryos were observed later at 4 dpf. A significant perturbation of inter-segmental blood vessels was not observed when the embryos were treated after 48hpf, but indeed specific disruption of sub-intestinal vein formation was observed (Figure 4 B&D). All the blood vessels formed normally in those embryos which were treated with compound 12a after 72 hpf. This time window treatment clearly indicates that these compounds were affective only when added to the embryos at a stage when endothelial cells were at the time of differentiation.

The tested valporic acid hydrazide-hydrazone derivatives showed a significant improvement in antiangiogenic and anti cancer activities as compared to valproic acid alone. The introduction of the glycine moiety showed a remarkable performance of the valproylglycine hydrazide-hydrazone derivatives compounds 12a-e. The inhibitions of developmental angiognesis by valproylglycine hydrazide is not surprising, as quite recently, the tumor growth reduction by selectively inducing tumor blood vessels thrombosis and tumor necrosis in tumor bearing mouse has been reported by synthetic polypeptides having glycine as one of the residue (Liu et al., 2011b; Huang et al., 2013; Zhao et al., 2013). Besides interfering with angiogenic blood vessels formation, the compounds from valproylglycine hydrazide series also induced severe cardiac edema (Figure 1 black arrow in D). The cardiac edema has also been shown in previous studies which are HDAC mutant (Pillai et al., 2004; Yamaguchi et al., 2005) or embryos were treated with VPA or any other HDAC inhibitor (Farooq et al., 2008; Cao et al., 2009; Johnson et al., 2013). We have not analyzed the HDAC enzymatic activity with these compounds but most likely that the cardiac edema which is result of newly synthesized VPA derivatives could be due to their HDAC inhibition activity.

The improved bioactivity in term of modulation of angiogenesis could be due to following structure modification: The valproylglycine hydrazide-hydrazone derivatives 12a-e showed significant level of antiangiogenic and anticancer activity, which varied with each compound. The benzaldehyde derivative 12a was the most potent in term of inhibition of angiogenic blood vessels at minimum concentration and also suppressed maximum level of cell survival (60%) in HepG2 cells at 40μM. Followed by the para chloro- and para hydroxyl benzaldehyde derivatives 12c and 12b respectively. The acetophenone derivatives 12d and 12e exhibited moderate to low level of anticancer and antiangiogenic activity. The hydrazide-hydrazone derivatives 10a-g showed moderate level of antiangiogenic activity, most of these compounds failed to affect the inter-segmental blood vessels formation process in treated embryos however they affected the sub-intestinal vein formation with concentation value ranging from 40-50μM. The benzaldehyde derivative 10a showed also the best performance, followed by the p-chloro, p-hydroxy and p-methoxy benzaldehyde derivatives 10b, 10c and 10d respectively. As observed for the valproylglycine hydrazide-hydrazone derivatives, the acetophenone derivatives of the hydrazide-hydrazones 10e-g, gave lower activity. The anticancer activity of the hydrazide-hydrazone derivatives 10a-g have improved significantly as compared to valproic acid 1, where compounds 10a and 10e (the un-substituted phenyl ring derivatives) showed the highest levels of suppression of cell proliferation in HepG2 cell.

The introduction of the aldehyde and ketone moieties through their condensation with valporic hydrazide and N-valporic benzoic hydrazide showed a significant improvement in the efficiency of the prepared hydrazide hydrazones. In term of antiangiogenic and anticancer activity, the hydrazide-hydrazone derivatives 10a-g have improved significantly as compared to valproic acid and valproic hydrazide (Valph) the first building block to synthesis derivatives.

In conclusion, various structural derivatives of VPA have been synthesized and their antiangiogenic and anticancer profile have been studied in this study. As liver is one of the highly vascularized organ and metastases of cancer cell is most frequent in liver cancer, so a compound having dual biological activity i.e suppressing the cancer

cell survival and at the same time suppressing the tumor angiogenesis activity would specifically benefit the HCC patient. The exact mechanism by which Valproylglycine hydrazide-hydrazone derivatives suppressed HepG2 cell survival is not known but inhibition of HDAC enzymatic activity could be one of possible mechanism attributed to this activity. VPA is also a potent histone deacetylase (HDAC) inhibitor (Sriraksa Limpaiboon 2013; Abaza et al., 2014; Ganai et al., 2014), so it could be quite possible that newly synthesized compounds have better HDAC inhibition activity as compared to VPA. Relevant studies are ongoing in the lab to check the potential of these compounds in term of HDAC inhibition.

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