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Anti-tumor and anti-angiogenic activity of novel hydantoin derivatives: Inhibition of VEGF secretion in liver metastatic osteosarcoma cells

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ABSTRACT

A series of new azaspiro bicyclic hydantoin derivatives has been designed and synthesized. Initially, the anti-proliferative effect of the hydantoin derivatives was evaluated against human ovarian cancer cells (SKOV-3 and OVSAHO) and murine osteosarcoma cells (LM8 and LM8G7). Among the tested compounds, 8-(3-fluorobenzyl)-1′-(4-(methylsulfonyl)benzyl)-8-azaspiro[bicyclo[3.2.1]octane-3,4′-imidazolidine]-2′,5′-dione (**7h**) and 8-(3,4-difluorobenzyl)-1′-(4-(methylsulfonyl)benzyl)-8-azaspiro[bicyclo[3.2.1]octane-3,4′-imidazolidine]-2′,5′-dione (**7i**) showed a significant anti-proliferative activity against the OVSAHO and LM8G7 cells. The real-time monitoring of the effect of the compounds **7h** and **7i** against the proliferation of LM8G7 was revealed that resulting IC_{50} values were 102 μM and 13 μM, respectively. We reasoned that the presence of fluorine atom at the 3rd position of the phenyl ring of the hydantoin side chain may determine the potency of the molecule. Furthermore, the compound **7i** inhibited the tube formation of the mouse endothelial cells. Finally, the treatment of the compound **7i** against the proliferation of LM8G7 cells demonstrated the down regulation of the secretion of VEGF, indicate the potential angioinhibitory effects. In conclusion, our findings demonstrate the suppression of the secretion of VEGF by LM8G7 cells by the compound **7i** might contribute at least in part to the antitumor action.

1. Introduction

With a limited improvement in diagnosis, surgical techniques, patient care, and adjuvant therapies, most of the death from cancer is due to metastases.[1] When cancer has metastasized, it may be treated with radiosurgery, chemotherapy, radiation therapy, biological therapy, hormone therapy, or a combination of these. Mainly, the macroscopic growth of tumor masses requires not only tumor cell proliferation but also concomitant angiogenesis.[2] Angiogenesis has been described as one of the hallmarks of cancer.[3] The stimuli that promote tumor angiogenesis may be provided directly by the tumor cells themselves or indirectly by host inflammatory cells that are attracted to the tumor site. Endothelial cell activation and proliferation is regulated by multiple proangiogenic factors, of which the most widely studied is vascular endothelial growth factor (VEGF). VEGF, a potent endothelial cell mitogen in vivo whose function is critical for angiogenesis in both normal development and in tumour cell expansion and invasion.[4,5] Thus, the inhibition of tumor angiogenesis affords attractive targets for the development of novel antitumor agents. The present invention is to explore the effect of novel hydantoin derivatives on the proliferation of human ovarian tumor cells such as SKOV-3, OVSAHO and as well as murine osteosarcoma cells like LM8 and LM8G7, which have metastatic potential to lung and liver, respectively.[6] Hydantoin lesions have been demonstrated to be highly mutagenic both in vitro and in vivo.[7] The derivatives of hydantoin have attracted much interest in drug discovery because of their wide range of biological activities and therapeutic applications depending on the nature of substitution on the hydantoin ring.[8] In continuation of our ongoing research,[9] herein we report the antitumor and anti-angiogenic activity for the newly synthesized compounds **7 (a–i).** The possible mechanism for the anti-tumor activity was examined under in vitro condition.

2. Results

2.1. Chemistry

The synthetic pathway utilized in the preparation of the azaspiro bicyclic hydantoin derivatives is outlined as shown in Scheme 1. A variety of combinatorial approaches have been described by

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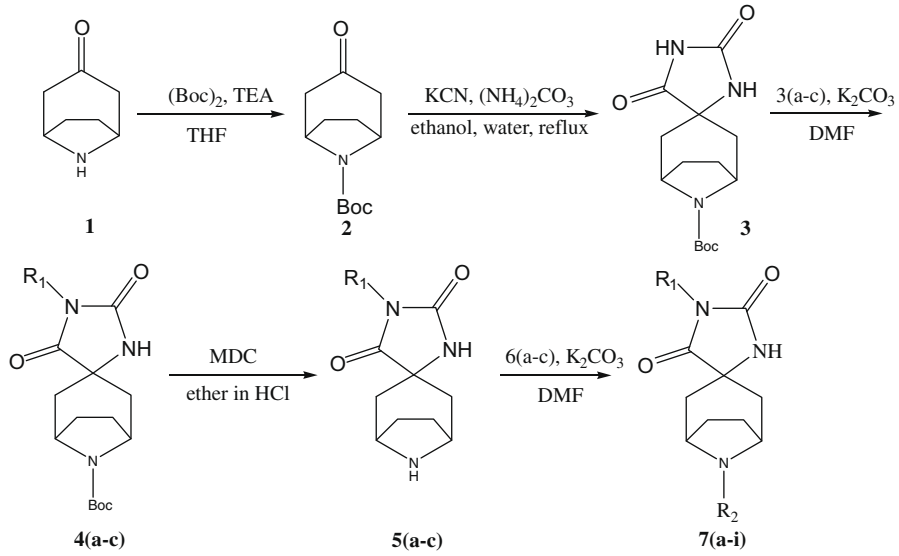


3a: 1-(bromomethyl)benzene

3b: 3-bromoprop-1-ene

3c: 1-(bromomethyl)-4-(methylsulfonyl)benzene

6a: 1-(bromomethyl)-4-fluorobenzene

6b: 1-(bromomethyl)-3-fluorobenzene

6c: 4-(bromomethyl)-1,2-difluorobenzene

Scheme 1.

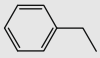
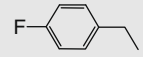
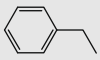
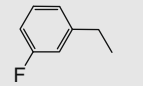
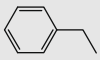
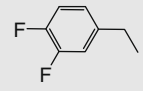
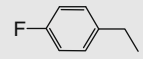
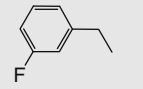
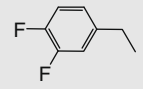
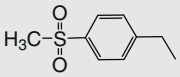
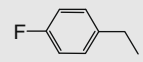
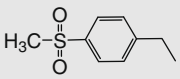
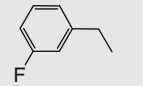
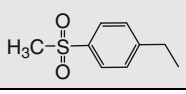
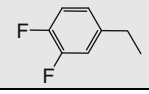
which pharmacophoric groups were attached to such a relatively rigid scaffold. The synthesis begins with the protection of 8-azabicyclo[3.2.1]octan-3-one **1** with Boc anhydride using dry THF as a solvent. Under Bucherer-Bergs condition,[10] construction of azaspiro bicyclic hydantoin ring **3** was made. The reaction was carried out in aqueous ethanolic media by using potassium cyanide and ammonium carbonate at reflux temperature. The introduction of the substituted alkyl/aryl groups at N-3 position of hydantoin ring was achieved via selective N-alkylation reaction by reacting alkyl/aryl halides in presence of K_2CO_3 and DMF solvent. Target key intermediates **5 (a–c**) was accomplished by deprotection of Boc group from the compound **4 (a–c**) with ether in HCl and followed by basification with $NaHCO_3$ solution. The aim of the fifth step was to introduce respective fluoro substituted aryl halides at the 8th position of azaspiro bicyclic moiety to lead the desired compounds 7 (**a–i**) for SAR study (Table 1). This was furnished by normal condensation reaction with good yield. The formation of the hydantoin ring was confirmed by ^1H NMR and IR spectra.

2.2. Biology

2.2.1. Effects of hydantoin derivatives against the proliferation of normal and tumor cells

The anti-proliferative activity of hydantoin derivatives **7 (a–i)** against four adherent tumor cells was studied. The inhibitory effects of the compounds **7 (a–i)** against the human ovarian tumor cells like SKOV-03 and OVSAHO are summarized (Figs. 1 and 2). Similarly, the inhibitory effects of the compounds **7 (a–i)** against the murine osteosarcoma cells such as LM8 and LM8G7 are summarized (Figs. 3 and 4). Among the tested compounds, **7h** and **7i** were significantly inhibited the proliferation of tumor cells. Further, we dynamically monitored the effects of the compound **7h** or **7i** on the proliferation of LM8G7 cells using RT-CES™

Table 1
Chemical structures, physical data and purity of compounds **7(a–i)**

Compound	R_1	R_2	Mp (°C)	Yield (%)	Purity (%)
7a			168–170	82	97.9
7b			142–144	77	98.7
7c			181–183	75	99.1
7d			101–103	78	98.40
7e			139–141	80	97.39
7f			156–158	65	97.9
7g			117–119	80	98.6
7h			119–121	79	99.2
7i			132–134	75	97.87

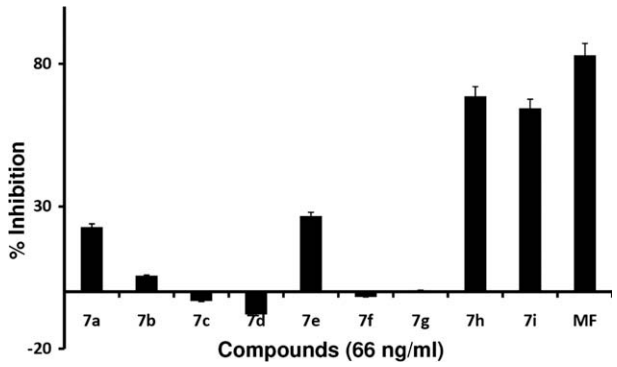


Figure 1. The inhibitory effects of hydantoin derivatives on the proliferation of SKOV-3 cells (MF, mipefristone).

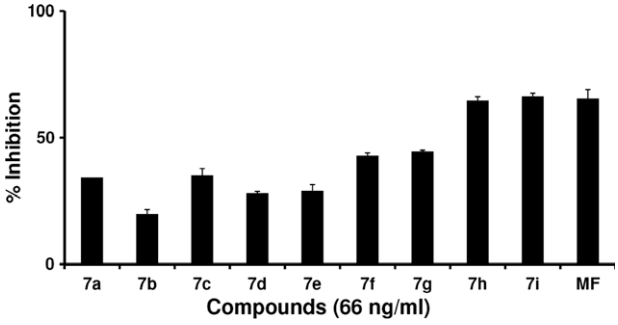


Figure 2. The inhibitory effects of hydantoin derivatives on the proliferation of OVSAHO cells.

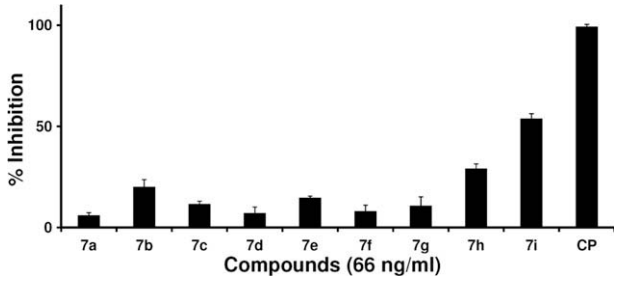


Figure 3. The inhibitory effects of hydantoin derivatives on the proliferation of LM8 cells (CP, cisplatin).

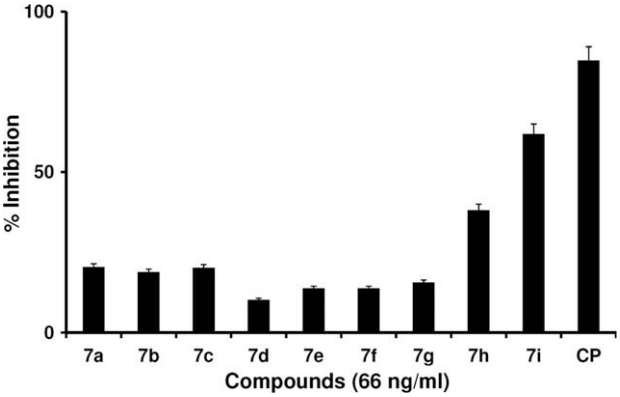


Figure 4. The inhibitory effects of hydantoin derivatives on the proliferation of LM8G7 cells.

(Real-Time Cell Electronic Sensing) system.[11] The kinetic values (IC_{50}) were calculated from concentration–response curves by a non-linear regression analysis using Prism software (GraphPad software, Sandiego, CA). Compounds **7h** and **7i** inhibited the proliferation of VEGF-expressing LM8G7 cells dose-dependently with an IC_{50} value of 102 and 13 μM, respectively (Figs. 6 and 7). Therefore, the above results indicated that the compound **7i** markedly inhibits the growth of tumor cells. The cytotoxicity of the compounds **7 (a–i)** were tested using mouse endothelial cells (Fig. 5).

2.2.2. The compound 7i inhibits angiogenesis in vitro

Since the compound **7i** showed anti-tumor activity, we tested the anti-angiogenic effects of compound **7i** using an in vitro angiogenesis model. The cultured mouse endothelial cells (40,000 cells/well) on Matrigel™ in the presence of DMSO (0.1%) and VEGF (2 ng/mL) rapidly align and form a hollow tube like structures (Fig. 8A). Compound **7i** selectively inhibited the tube formation

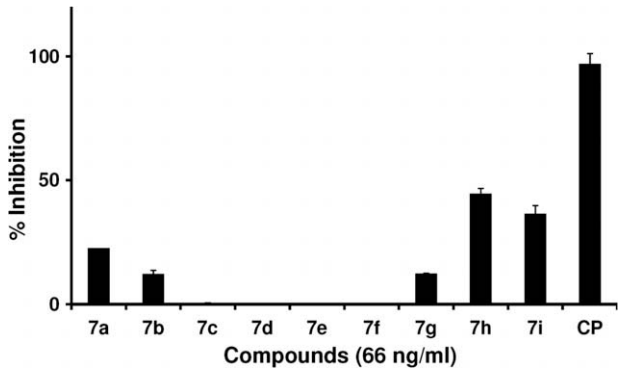


Figure 5. The inhibitory effects of hydantoin derivatives on the proliferation of UV♀2 cells.

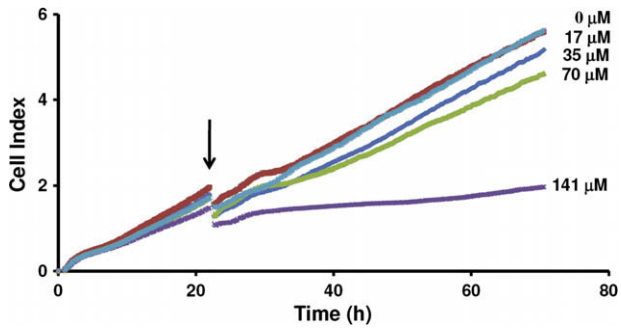


Figure 6. Real-time monitoring of the effects of compound **7h** on the proliferation of LM8G7 cells (the arrow indicates the time of the addition of compound **7h**).

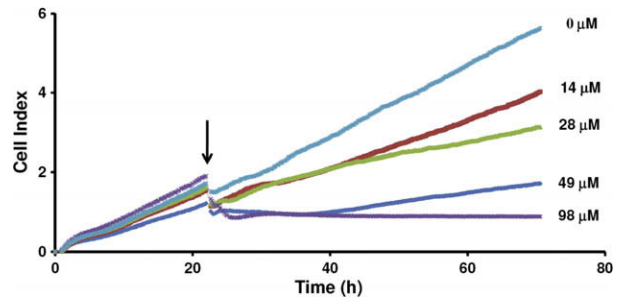


Figure 7. Real-time monitoring of the effects of compound **7i** on the proliferation of LM8G7 cells (the arrow indicates the time of the addition of compound **7i**).

on Matrigel™ (Fig. 8B) indicates the anti-angiogenic property of the molecule.

2.2.3. Effects of the compound 7i on the secretion of VEGF by LM8G7 cells

Since the molecule, **7i** showed the potent inhibition against the proliferation of LM8G7 cells, which express high amount of VEGF mRNA.[6c] Using the Quantikine mouse VEGF immunoassay kit, we measured the amount of VEGF secreted by the LM8G7 cells.[12] LM8G7 (1×10^6) cells were cultured in 6-well plates for 24 h. Then the cells were treated with or without the compound **7i** at various concentrations. After 48 h, the cells supernatant was taken, centrifuged, and estimated the protein content. Using the known amount of protein, the VEGF content in the cells supernatant was measured. Compound **7i** inhibited the secretion of VEGF in a dose-dependent manner (Fig. 9). Compound **7i** at 56, 102 and 204 μM effectively inhibited the secretion of VEGF by 23.8%, 46.7% and 63.9%, respectively.

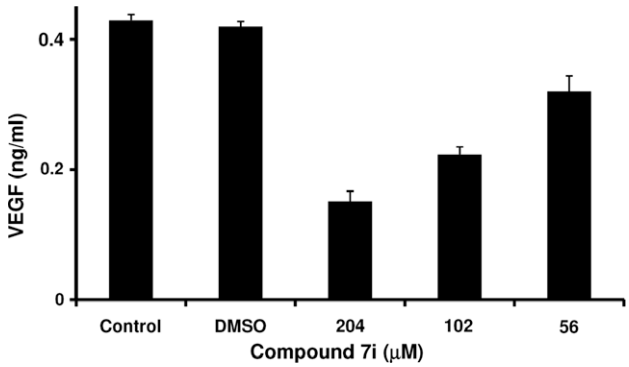


Figure 9. Determination of the amount of VEGF secreted by LM8G7 cells after the treatment with the compound **7i** at various concentrations.

3. Discussion

The inhibitory effects of hydantoin derivative on the proliferation of tumor cells were compared with cisplatin and mifepristone. The anti-proliferative activity of **7i** is considerably higher than the activity of all other hydantoin derivatives tested. The experiment clearly demonstrates the presence of 4-methylsulfonyl benzyl group and 3,4-difluoro benzene moiety, which has an influence on the anti-proliferative activity of hydantoin derivatives. The real-time monitoring of the effect of the compound **7i** against the proliferation of LM8G7 cells confirms the potency of the molecule.

Further, VEGF is considered to be one of the major stimulators of tumor angiogenesis.[13] The compound **7i** was evaluated for its ability to inhibit angiogenesis in vitro. Endothelial cells (UV♀2) are induced to undergo tube formation and form cell–cell contacts that lead to branched networks, which are similar to capillary-like blood vessels. However, when UV♀2 cells were cultured on polymerized Matrigel™ in the presence of **7i** (50 μM), the cells failed to organize into capillary-like structures. At these concentrations no other compounds tested inhibited tube formation.

In order to understand the mechanism of action, the compound **7i,** was treated with LM8G7 cells at different concentration to check its effects on the secretion of VEGF. The compound **7i** effectively inhibited the secretion of VEGF in a dose-dependent manner. These results indicate that the compound **7i** might be inhibited the growth factor-induced proliferation of LM8G7 cells.

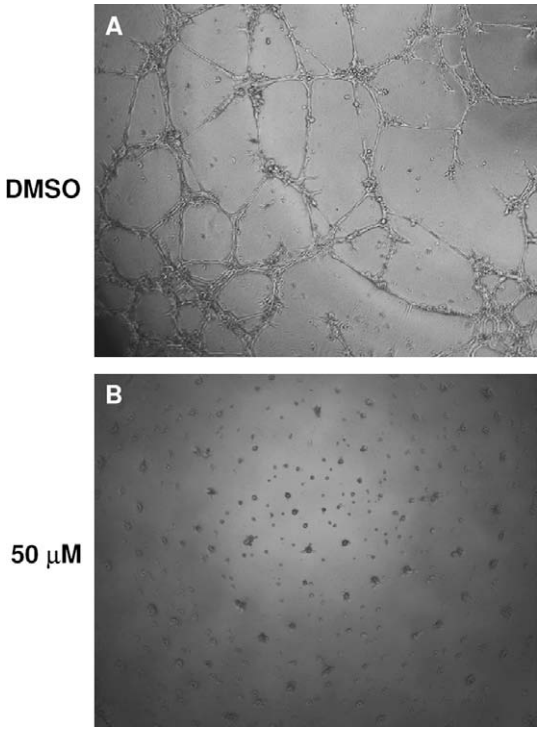


Figure 8. Compound **7i** inhibits the tube formation in vitro. The UV♀2 cells were seeded in 6-well plates coated with Matrigel™ and the cells were incubated with or without compound **7i** (50 μM). After 18 h of culture, the reorganization of the subconfluent monolayer of UV♀2 cells in 3-dimensional Matrigel™ was monitored and photographed in a light microscope attached to a 3CCD camera.

4. Conclusion

In conclusion, our current findings are consistent with the data concerning the inhibitory effects of the compound **7i** on the growth of human/mouse tumor cells. Further, the compound **7i** inhibited the tube formation of UV♀2 cells. Treatment with compound **7i** demonstrated the down regulation of the secretion of VEGF in LM8G7 cells indicating the potential angioinhibitory effect of the compound. In conclusion, our findings demonstrate the suppression of the secretion of VEGF by LM8G7 cells might contribute at least in part to the antitumor action of compound **7i**. However, further experimental study and preclinical trials could be useful to fully elucidate mechanism of their anti-proliferative action and possible pharmacological strategy for the future treatment of patients with osteosarcoma cancer.

5. Experimental

Melting points were determined using SELACO-650 hot stage melting point apparatus and were uncorrected. Infrared (IR) spectra were recorded using a Jasco FTIR-4100 series. Nuclear magnetic resonance (^{1}H NMR) spectra were recorded on Shimadzu AMX 400-Bruker, 400 MHz spectrometer using $CDCl_3$ as a solvent and TMS as internal standard (chemical shift in δ ppm). Spin multiplets are given as s (singlet), d (doublet), t (triplet) and m (multiplet). Mass and purity were recorded on a LC-MSD-Trap-XCT. Elemental (CHNS) analyses were obtained on Vario EL III Elementar. Silica gel column chromatography was performed using Merck 7734 silica gel (60–120 mesh) and Merck made TLC plates.

5.1. Synthesis of *tert*-butyl 3-oxo-8-azabicyclo[3.2.1]octane-8-carboxylate 2

A solution of 8-azabicyclo[3.2.1]octan-3-one **1** (15 g, 119 mmol) was taken in dry THF, cooled to 0 °C. Triethyl amine (30.0 g,

297 mmol) and Boc anhydride (25.9 g, 119 mmol) were added. The reaction mixture was allowed to stirred at room temperature for 6 h. The progress of the reaction was monitored by TLC. Upon completion, the solvent was removed under reduced pressure and residue was taken in water and extracted with ethyl acetate. Finally organic layer was washed with water and dried with anhydrous Na_2SO_4. The organic layer was evaporated to get *tert*-butyl 3-oxo-8-azabicyclo[3.2.1]octane-8-carboxylate in good yield (77%, 20.6 g).

5.2. Synthesis of *tert*-butyl 2′,5′-dioxo-8-azaspiro [bicyclo[3.2.1]octane-3,4′-imidazolidine]-8-carboxylate 3

A solution *tert*-butyl 3-oxo-8-azabicyclo[3.2.1]octane-8-carboxylate **2** (19.0 g, 84 mmol), ammonium carbonate (24.2 g, 252 mmol) were taken in ethanol and water. Then potassium cyanide (16.4 g, 252 mmol) dissolved in water and it was added the above solution. The reaction mixture was refluxed for 40 h. The progress of the reaction was monitored by TLC. Upon completion, the reaction mixture was filtered and washed with water to get hydantoin product 3 in good yield (76%, 18.8 g).

5.3. Synthesis of *tert*-butyl 1′-benzyl-2′,5′-dioxo-8-azaspiro [bicyclo[3.2.1]octane-3,4′-imidazolidine]-8-carboxylate 4a

A solution of *tert*-butyl 2′,5′-dioxo-8-azaspiro[bicyclo[3.2.1] octane-3,4′-imidazolidine]-8-carboxylate **3** (2.0 g, 6.77 mmol) in dimethyl formamide was taken, anhydrous K_2CO_3 (2.8 g, 20.30 mmol) and 1-(bromomethyl) benzene **3a** (1.15 g, 6.77 mmol) were added to the solution. The reaction mixture was stirred at room temperature for 7 h and progress of the reaction was monitored by TLC. Upon completion, the solvent was removed under reduced pressure and residue was taken in water and extracted with ethyl acetate. Finally water wash was given to the organic layer and dried with anhydrous Na_2SO_4. The solvent was evaporated, crude product was purified by column chromatography over silica gel (60–120 mesh) using chloroform/methanol (9:1) as an eluent (80%, 2.0 g).

5.4. Synthesis of *tert*-butyl 1′-allyl-2′,5′-dioxo-8-azaspiro [bicyclo[3.2.1]octane-3,4′-imidazolidine]-8-carboxylate 4b

The general experimental procedure described above afforded 4**b** (1.7 g) from *tert*-butyl 2′,5′-dioxo-8-azaspiro[bicyclo[3.2.1]octane-3,4′-imidazolidine]-8-carboxylate **3** (2.0 g, 6.77 mmol), 3-bromoprop-1-ene (0.98 g, 8.12 mmol) and K_2CO_3 (2.8 g, 20.3 mmol).

5.5. Synthesis of *tert*-butyl 1′(4-(methylsulfonyl)benzyl-2′,5′-dioxo-8-azaspiro [bicyclo[3.2.1]octane-3,4′-imidazolidine]-8-carboxylate 4c

The general experimental procedure described above afforded 4**c** (2.26 g) from *tert*-butyl 2′,5′-dioxo-8-azaspiro[bicyclo[3.2.1]octane-3,4′-imidazolidine]-8-carboxylate **3** (2.0 g, 6.77 mmol), 1-(bromomethyl)-4-(methylsulfonyl)benzene (1.68 g, 6.77 mmol) and K_2CO_3 (2.8 g, 20.3 mmol).

5.6. Synthesis of 1′-benzyl-8-azaspiro[bicyclo[3.2.1]octane-3,4′-imidazolidine]-2′,5′-dione 5a

Compound **4a** was taken in dry MDC, cooled to 0 °C. Ether in HCl was added and allowed to stirred at room temperature for 3 h. Deprotected salt compound was basified with $NaHCO_3$ solution and extracted with ethylacetate, organic layer was concentrated to get **5a**. Similar experimental procedure was followed for the compounds **5b** and **5c.**

5.6.1. Synthesis of 1′-benzyl-8-(4-fluorobenzyl)-8-azaspiro [bicyclo[3.2.1]octane-3,4′-imidazolidine]-2′,5′-dione 7a

A solution of 1′-benzyl-8-azaspiro[bicyclo[3.2.1]octane-3,4′-imidazolidine]-2′,5′-dione **5a** (0.20 g, 0.70 mmol) in dimethyl formamide was taken, anhydrous K_2CO_3 (0.48 g, 3.5 mmol) was added to the solution and stirred for 10 min 1-(bromomethyl)-4-fluorobenzene **6a** (0.13 g, 0.70 mmol) was added. The reaction mixture was stirred at room temperature for 8 h and progress of the reaction was monitored by TLC. Upon completion, the solvent was removed under reduced pressure and residue was taken in water and extracted with ethyl acetate. Finally water wash was given to the organic layer and dried with anhydrous Na_2SO_4.The solvent was evaporated, crude product was purified by column chromatography over silica gel (60–120 mesh) using chloroform/methanol (9:1) as an eluent to get **7a** (**0.22 g**) as pale yellow crystalline solid. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 8.53 (s, 1H, –NH), 7.24–7.36 (m, 5H, Ar-H), 7.07–7.1 (m, 3H, Ar-H), 4.63 (s, 2H, $-CH_2-$), 3.61 (s, 2H, $-CH_2-$), 2.42–2.46 (m, 2H, –CH–), 2.0–2.15 (m, 4H, $-CH_2-$), 1.77–1.84 (m, 4H, $-CH_2-$). MS (ESI + ion): *m/z* = 394.1. IR (KBr, cm^{-1}): 3360, 1653.

5.6.2. Synthesis of 1′-benzyl-8-(3-fluorobenzyl)-8-azaspiro [bicyclo[3.2.1]octane-3,4′-imidazolidine]-2′,5′-dione (7b)

The general experimental procedure described above afforded **7b**, the product obtained (0.21 g) was pale yellow crystalline solid from 1′-benzyl-8-azaspiro[bicyclo[3.2.1]octane-3,4′-imidazolidine]-2′,5′-dione **5a** (0.20 g, 0.70 mmol), 1-(bromomethyl)-3-fluorobenzene (0.13 g, 0.70 mmol), and K_2CO_3 (0.48 g, 3.5 mmol). ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 8.58 (s, 1H, –NH), 7.24–7.36 (m, 5H, Ar-H), 7.07–7.1 (m, 3H, Ar-H), 4.65 (s, 2H, $-CH_2-$), 3.63 (s, 2H, $-CH_2-$), 2.41–2.46 (m, 2H, –CH–), 2.0–2.17 (m, 4H, $-CH_2-$), 1.75–1.85 (m, 4H, $-CH_2-$). MS (ESI + ion): *m/z* = 394. IR (KBr, cm^{-1}): 3360, 1653.

5.6.3. Synthesis of 1′-benzyl-8-(3,4-difluorobenzyl)-8-azaspiro [bicyclo[3.2.1]octane-3,4′-imidazolidine]-2′,5′-dione (7c)

The general experimental procedure described above afforded **7c**, the product obtained (0.21 g) was pale yellow crystalline solid from 1′-benzyl-8-azaspiro[bicyclo[3.2.1]octane-3,4′-imidazolidine]-2′,5′-dione **5a** (0.20 g, 0.70 mmol), 4-(bromomethyl)-1,2-difluorobenzene (0.14 g, 0.70 mmol), and K_2CO_3 (0.48 g, 3.5 mmol). ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 8.51 (s, 1H, –NH), 7.24–7.36 (m, 5H, Ar-H), 7.07–7.1 (m, 3H, Ar-H), 4.65 (s, 2H, $-CH_2-$), 3.60 (s, 2H, $-CH_2-$), 2.43–2.47 (m, 2H, –CH–), 2.0–2.17 (m, 4H, $-CH_2-$), 1.76–1.82 (m, 4H, $-CH_2-$). MS (ESI + ion): *m/z* = 412.2. IR (KBr, cm^{-1}): 3354, 1649.

5.6.4. Synthesis of 1′-allyl-8-(4-fluorobenzyl)-8-azaspiro [bicyclo[3.2.1]octane-3,4′-imidazolidine]-2′,5′-dione (7d)

The general experimental procedure described above afforded **7d**, the product obtained (0.22 g) was white crystalline solid from 1′-allyl-8-azaspiro[bicyclo[3.2.1]octane-3,4′-imidazolidine]-2′,5′-dione **5b** (0.20 g, 0.85 mmol), 1-(bromomethyl)-4-fluorobenzene (0.16 g, 0.85 mmol), and K_2CO_3 (0.58 g, 4.25 mmol). ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 8.52 (s, 1H, –NH), 7.35–7.39 (m, 2H, Ar-H), 7.15 (t, 2H, Ar-H), 5.73–5.86 (m, 1H, =CH), 5.0–5.09 (m, 2H, CH_2=), 3.94 (d, 2H, $-CH_2$), 3.53 (s, 2H, $-CH_2$), 2.44–2.50 (m, 2H, –CH–), 2.1–2.2 (m, 4H, $-CH_2-$), 1.92–1.99 (m, 4H, $-CH_2-$). MS (ESI + ion): *m/z* = 344.1. IR (KBr, cm^{-1}): 3355, 1653.

5.6.5. Synthesis of 1′-allyl-8-(3-fluorobenzyl)-8-azaspiro [bicyclo[3.2.1]octane-3,4′-imidazolidine]-2′,5′-dione (7e)

The general experimental procedure described above afforded **7e**, the product obtained (0.23 g) was white crystalline solid from 1′-allyl-8-azaspiro[bicyclo[3.2.1]octane-3,4′-imidazolidine]-2′,5′-dione **5b** (0.20 g, 0.85 mmol), 1-(bromomethyl)-3-fluorobenzene (0.16 g, 0.85 mmol), and K_2CO_3 (0.58 g, 4.25 mmol). ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 8.50 (s, 1H, –NH), 7.30 (m, 1H, Ar-H), 7.17

(d, 2H, Ar-H), 6.97 (m, 1H, Ar-H), 5.77–5.86 (m, 1H, =CH), 5.16–5.20 (m, 2H, CH_2=), 4.1 (d, 2H, $-CH_2$), 3.65 (s, 2H, $-CH_2$), 2.46–2.51 (m, 2H, –CH–), 2.17–2.20 (m, 4H, $-CH_2-$), 1.77–1.82 (m, 4H, $-CH_2-$). MS (ESI + ion): m/z = 344.1. IR (KBr, cm^{-1}): 3350, 1660.

5.6.6. Synthesis of 1′-allyl-8-(3,4-difluorobenzyl)-8-azaspiro [bicyclo[3.2.1]octane-3,4′-imidazolidine]-2′,5′-dione (7f)

The general experimental procedure described above afforded **7f**, the product obtained (0.19 g) was white crystalline solid from 1′-allyl-8-azaspiro[bicyclo[3.2.1]octane-3,4′-imidazolidine]-2′,5′-dione **5b** (0.20 g, 0.85 mmol), 4-(bromomethyl)-1,2-difluorobenzene (0.17 g, 0.85 mmol), and K_2CO_3 (0.58 g, 4.25 mmol). ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 8.52 (s, 1H, –NH), 7.32–7.39 (m, 2H, Ar-H), 7.17–7.2 (m, 1H, Ar-H), 5.72–5.79 (m, 1H, =CH), 4.93–5.08 (m, 2H, CH_2=), 3.94 (d, 2H, $-CH_2$), 3.52 (s, 2H, $-CH_2$), 2.44–2.49 (m, 2H, –CH–), 2.15–2.2 (m, 4H, $-CH_2-$), 1.92–1.98 (m, 4H, $-CH_2-$). MS (ESI + ion): m/z = 362.2. IR (KBr, cm^{-1}): 3371, 1658.

5.6.7. Synthesis of 8-(4-fluorobenzyl)-1′-(4-(methylsulfonyl) benzyl)-8-azaspiro[bicyclo [3.2.1]octane-3,4′-imidazolidine]-2′,5′-dione (7g)

The general experimental procedure described above afforded **7g**, the product obtained (0.20 g) was off-white crystalline solid from 1′-(4-(methylsulfonyl)benzyl)-8-azaspiro[bicyclo[3.2.1]octane-3,4′-imidazolidine]-2′,5′-dione **5c** (0.20 g, 0.55 mmol), 1-(bromomethyl)-4-fluorobenzene (0.10 g, 0.55 mmol), and K_2CO_3 (0.38 g, 2.75 mmol). ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 8.60 (s, 1H, –NH), 7.91 (d, 2H, Ar-H), 7.55 (d, 2H, Ar-H), 7.27–7.38 (m, 2H, Ar-H), 7.02 (t, 2H, Ar-H), 4.72 (s, 2H, $-CH_2-$), 3.60 (s, 2H, $-CH_2-$), 3.04 (s, 3H, $-CH_3$), 2.44–2.47 (m, 2H, –CH–), 2.17–2.19 (m, 4H, $-CH_2-$), 1.74–1.80 (m, 4H, $-CH_2-$). MS (ESI + ion): m/z = 472.1. IR (KBr, cm^{-1}): 3357, 1650, 1340, 1285.

5.6.8. Synthesis of 8-(3-fluorobenzyl)-1′-(4-(methylsulfonyl) benzyl)-8-azaspiro[bicyclo [3.2.1]octane-3,4′-imidazolidine]-2′,5′-dione (7h)

The general experimental procedure described above afforded **7h**, the product obtained (0.19 g) was off-white crystalline solid from 1′-(4-(methylsulfonyl)benzyl)-8-azaspiro[bicyclo[3.2.1]octane-3,4′-imidazolidine]-2′,5′-dione **5c** (0.20 g, 0.55 mmol), 1-(bromomethyl)-3-fluorobenzene (0.10 g, 0.55 mmol), and K_2CO_3 (0.38 g, 2.75 mmol). ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 8.66 (s, 1H, –NH), 7.89 (d, 2H, Ar-H), 7.45 (d, 2H, Ar-H), 7.32–7.38 (m, 1H, Ar-H), 7.19 (t, 2H, Ar-H), 7.07 (t, 1H, Ar-H), 4.64 (s, 2H, $-CH_2-$), 3.57 (s, 2H, $-CH_2-$), 3.15 (s, 3H, $-CH_3$), 2.49–2.50 (m, 2H, –CH–), 2.19–2.23 (m, 4H, $-CH_2-$), 1.93–2.0 (m, 4H, $-CH_2-$). MS (ESI + ion): m/z = 472. IR (KBr, cm^{-1}): 3355, 1648, 1333, 1280.

5.6.9. Synthesis of 8-(3,4-difluorobenzyl)-1′-(4-(methylsulfonyl) benzyl)-8-azaspiro[bicyclo [3.2.1]octane-3,4′-imidazolidine]-2′,5′-dione (7i)

The general experimental procedure described above afforded **7i**, the product obtained (0.195 g) was off-white crystalline solid from 1′-(4-(methylsulfonyl)benzyl)-8-azaspiro[bicyclo[3.2.1]octane-3,4′-imidazolidine]-2′,5′-dione **5c** (0.20 g, 0.55 mmol), 4-(bromomethyl)-1,2-difluorobenzene (0.11 g, 0.55 mmol), and K_2CO_3 (0.38 g, 2.75 mmol). ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 8.64 (s, 1H, –NH), 7.87 (d, 2H, Ar-H), 7.46 (d, 2H, Ar-H), 7.30–7.36 (m, 1H, Ar-H), 7.19 (t, 1H, Ar-H), 7.04 (t, 1H, Ar-H), 4.60 (s, 2H, $-CH_2-$), 3.55 (s, 2H, $-CH_2-$), 3.17 (s, 3H, $-CH_3$), 2.45–2.50 (m, 2H, –CH–), 2.19–2.25 (m, 4H, $-CH_2-$), 1.95–2.0 (m, 4H, $-CH_2-$). MS (ESI + ion): m/z = 490. IR (KBr, cm^{-1}): 3351, 1653, 1340, 1284.

5.6.10. In vitro cell proliferation assay

SKOV-3, OVSAHO, LM8, LM8G7, and UV♀2 cells were seeded at a density of 15,000 cells/well in a 96-well plate and incubated overnight at 37 °C. The medium was changed to the new medium containing 66 μg/mL concentrations of hydantoin derivatives. Incubated the plates for 48 h at 37 °C under 5% CO_2. After the indicated time, 5 μL of TetraColor One was added to each well and the mixture was incubated for an additional 2 h. The viability of cells treated with DMBO was measured using TetraColor One.[14] Absorbance at 450 nm was monitored and the percentage viability of the cells was calculated. The % inhibition of the proliferation of normal or tumor cells by the hydantoin derivatives was presented. Results are reported as the average four replicates.

5.6.11. Real-time proliferation assay

LM8G7 (10,000 cells/well) were seeded in ACEA's 96X e-plate™ in a final volume of 150 μL.[11] Approximately, 24 h after seeding, when the cells were in the log growth phase, they were incubated with 150 μL of medium containing various concentrations of compound **7h** or **7i** or DMSO as a solvent control. The effect of the compounds on the proliferation of LM8G7 cells was monitored dynamically for every 10 min. The cell index (quantitative measurement of cells) against the time was plotted. The IC_{50} values were calculated from concentration–response curves by a non-linear regression analysis using Prism software (GraphPad Software, San Diego, CA).

5.6.12. In vitro angiogenesis assay

Matrigel™ (ECM 625, Chemicon) was added to a 24-well plate in a final volume of 100 μL and allowed to solidify at 37 °C for 30 min. Single cell suspensions (100 μL) of UV♀2 were seeded at a density of 4×10^5 cells/mL, to the Matrigel™ coated well, with or without compound **7i** (50 μM) and VEGF (2 ng/mL). After 18 h of culture, the reorganization of the sub-confluent monolayer of UV♀2 cells in 3-dimensional Matrigel™ was monitored and photographed in a light microscope attached to a 3CCD camera (Olympus, FX380-model, JAPAN).

5.6.13. VEGF quantification assay

LM8G7 (1×10^6) cells were seeded into a 6-well plates and incubated overnight. After 24 h, the media was replaced with fresh serum-free medium containing 56 μM, 102 μM, and 204 μM of compound **7i.** The cells were further incubated for 48 h at 37 °C. The cell supernatant was collected, centrifuged at 5000 rpm for 5 min at 4 °C and the protein concentrations were determined by BCA (bicinchoninic acid) assay kit (Thermo Fisher Scientific Inc. USA).[15] The quantification of the mouse VEGF was done using the Immuno Assay Kit (Quantikine, R&D systems). Briefly, the known amount of recombinant VEGF as standard and the samples to be tested were added to a 96-well microplate, which was precoated with the polyclonal antibody specific for mouse VEGF and incubated for 2 h. The bound VEGF was detected by adding horseradish peroxidase conjugated polyclonal antibody against mouse VEGF. After 2 h, the substrate chromogen was added and allowed to react for 30 min with the peroxidase conjugate, the reaction was stopped and the VEGF content was quantified in a micro-plate reader at 450 nm (BIORAD, Model 680). The decrease in the amount of VEGF secretion by the compound **7i** was presented.

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