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PRECLINICAL STUDIES

Synthesis and in vitro cytotoxic evaluation of novel diazaspiro bicyclo hydantoin derivatives in human leukemia cells: A SAR study

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Summary To study the structure activity relationship (SAR) on the cytotoxic activity and probe the structural requirement for the potent antitumor activity, a series of novel diazaspiro bicyclo hydantoin derivatives were designed and synthesized. Their structures were confirmed by ^{1}H NMR, LCMS and IR analyses. The antiproliferative effect of these compounds were determined against human leukemia, K562 (chronic myelogenous leukemia) and CEM (T-cell leukemia) cells using trypan blue and MTT assay, and the SAR associated with the position of N-terminal substituents in diazaspiro bicyclo hydantoin have also been discussed. It has been observed that these compounds displayed strong, moderate and weak cytotoxic activities. Interestingly, compounds having electron withdrawing groups at third and fourth position of the phenyl ring displayed selectively cytotoxic activities to both the cell lines tested with IC_{50} value lower than 50 μM. In addition, the cytotoxic activities of the compounds 7(a–o) bearing the substituents at *N*–3 position of diazaspiro bicyclo hydantoin increases in the order alkene > ester > ether and plays an important role in determining their antitumor activities. The position and number of substituents in benzyl group attached to *N*–8 of diazaspiro bicyclo hydantoin nucleus interacted selectively with specific targets leading to the difference of biochemical and pharmacological effects.

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Introduction

Leukemia is a major type of cancer affecting a significant segment of the population, and especially children. In fact, leukemia is the most frequent childhood cancer, with 26% of all cases, and 20% mortality [1]. The American Cancer Society (ACS) estimated that 35,070 new cases of leukemia would be diagnosed in the United States in 2006, whereas about 22,280 adults and children would die of leukemia during 2006 [2]. Although the incidence rate for this disease remains relatively unchanged, some success has fortunately been attained in its treatment. But even if the success of clinical trials in identifying new agents and treatment modalities has been significant, current treatments have many limitations related to their side effects and the development of acquired drug resistance [3]. The new therapeutic agents thus needed should be more active and produce less side effects and they also should act through a mechanism different from that of cytotoxic agents already used.

Hydantoin derivatives possess a wide diversity of important biochemical effects and interesting pharmacological properties. In particular, spirohydantoin derivatives represent a potential starting point in discovering new and potent antitumoral agents with cytotoxic activity on ovarian and breast cancer [4]. Hydantoin derivatives also exhibit antidepressant, antiviral, antithrombotic activities as well as inhibitory activity against some enzymes (human aldose reductase and human leucocyte elastase) [5].

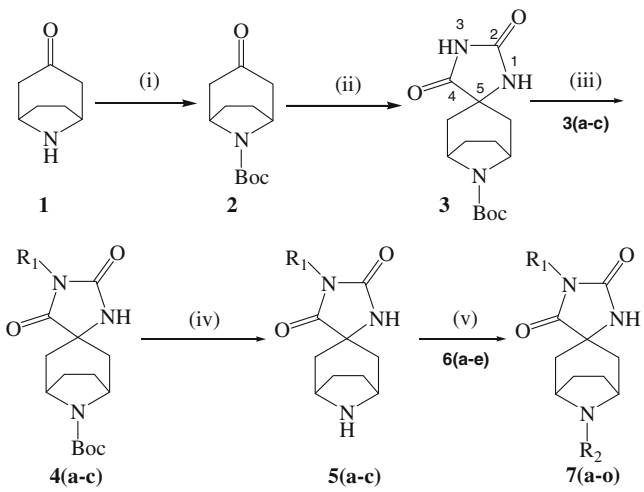
Some of bicyclic [5,5] hydantoin scaffold having fluorine and chlorine are found to be potent inhibitor of the LFA-1/CAM interaction and franesyl transferase [6, 7]. Recently, C. Carmi and co-workers investigated the effects of some of the 5-benzylidene-hydantoin derivatives on inhibition of the EGFR kinase activity and antiproliferative effects towards A431 cells [8]. They reported that, the exocyclic double bond is essential for both enzyme and cell growth inhibition, suggesting that, a rigid planar system is necessary to interact with the molecular target. To acquire more information about the structural requirements for the possible improvement of the cytotoxic potential and to elucidate SARs between substituent properties in hydantoin and antitumor activities, design and synthesis of novel hydantoin analogs with various substituents at different positions of the hydantoin nucleus are needed.

The unique character of fluorinated molecules in medicinal chemistry is well recognized [9–12]. Indeed, an increasing number of drugs on the market contain fluorine, the presence of which often is of major importance to biological activity. Possibly in no area of medicinal chemistry has fluorine been more important than in the development of anticancer agents [13, 14]. Fluorine substitution has been extensively practiced in the design of new biologically active compounds, including those with potential anticancer properties and they attributated a variety of attractive pharmacological effects [14].

Recently, we have investigated the synthesis of bioactive heterocycles and evaluation of their antiproliferative activity against different carcinoma cell lines [15, 16]. Our investigation on the synthesis of different substituted diazaspiro hydantoin derivatives and evaluation of their antitumor activities unraveled that the fluorine substituted hydantoin analogs possessed potent antitumor activity [16]. Inspired by these results, as part of our strategy towards the synthesis and biological evaluation of fluorinated and other bioactive heterocycles [17–19], we have designed a new series of more constrained derivatives of diazaspiro bicyclo hydantoin bearing fluorine and chlorine. The design of substituents at *N*−3 and *N*−8 position was based on previous results [16] and the choice of substituents in position *N*−3 was limited to alkene, ester and ether group. The purpose of the present study is to examine the effects of more constrained hydantoin derivatives having exocyclic double bond, ester and ether group against cell proliferation in leukemia cells, K562 and CEM. In addition, the focus of this investigation was to probe the optimal structural requirement of these compounds with regard to antitumor activities and further elucidate the SARs. To the best of our knowledge, all diazaspiro bicyclo hydantoin derivatives are novel, and herein, we report the synthesis and cytotoxic activities of novel diazaspiro bicyclo hydantoin derivatives.

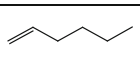
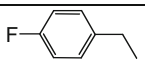
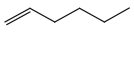
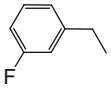
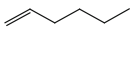
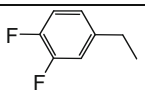
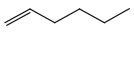
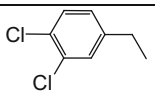
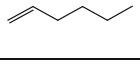
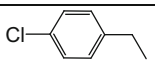
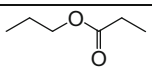
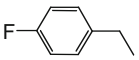
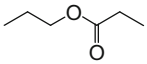
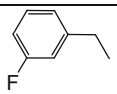
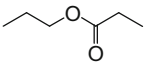
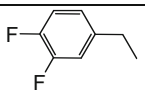
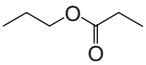
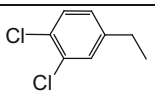
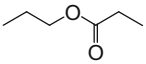
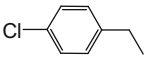
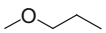
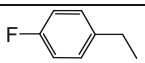
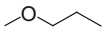
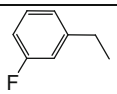
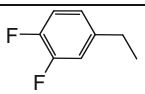
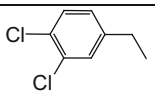
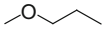
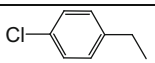
Chemistry

The chemistry of azaspiro undecane moiety has long attracted the interest of synthetic chemist due to their wide range of biological activity. Many syntheses of such compounds have been published in the literature in the recent years [20]. Compounds 7(a–o) were synthesized as outlined in the Scheme 1. The 8-azabicyclo[3.2.1]octan-3-one 1 was protected with Boc anhydride using dry THF as a solvent. Azaspiro bicyclo hydantoin 3 was synthesized under Bucherer-Bergs condition [21–23] using potassium cyanide and ammonium carbonate in 76% yield. The selective *N*-alkylation was carried at *N*−3 position of hydantoin ring using K_2CO_3 and DMF. Target key intermediates 5(a–c) were accomplished by deprotection of Boc group of 4(a–c) with ether in HCl. This sequence of reactions can be easily scaled up to give enough material 5(a–c) for further transformation. Substitution of alkene, ester or ether groups at the *N*−3 position of hydantoin ring system would be expected to enhance cytotoxicity, consequently a number of derivatives were designed and synthesized with electron withdrawing groups in phenyl ring (fluorine and chlorine) attached to *N*−8 of diazaspiro bicyclo hydantoin ring system. The amine functionalisation of the bicyclic ketone ring in the fifth step was carried out using substituted aryl halides 6(a–e) to lead the desired compounds 7(a–o), by normal condensation reaction with good yield and purity. Their structures were confirmed by ^{1}H NMR, LCMS and IR spectra. The chemical structures, purity and physical data of novel compounds are tabulated in Table 1.



Scheme 1 Schematic diagram for the synthesis of diazaspiro bicyclo hydantoin derivatives 7(a–o). Reagents and conditions: (*i*). $(Boc)_2$, TEA, dry THF, r.t. (*ii*). KCN, $(NH_4)_2CO_3$, ethanol/water, reflux (*iii*). 3(a–c), K_2CO_3, DMF, r.t. (*iv*). Ether in HCl, MDC (*v*). 6(a–e), K_2CO_3, DMF, r.t. **3a**: 5-bromo-pent-1-ene, **3b**: bromo-acetic acid propyl ester, **3c**: 1-bromo-2-methoxy-ethane, **6a**: 1-bromomethyl-4-fluorobenzene, **6b**: 1-bromomethyl-3-fluorobenzene, **6c**: 4-bromomethyl-1,2-difluorobenzene, **6d**: 4-bromomethyl-1,2-dichlorobenzene, **6e**: 1-bromomethyl-4-chlorobenzene

Table 1 Chemical structure, physical data and purity of the compounds 7(a–o)

Compound	R_1	R_2	M.P (°C)	Yield (%)	Purity (%)
7a			121-123	82	97.5
7b			162-164	80	97.7
7c			139-141	83	98.9
7d			168-170	80	98.6
7e			143-145	77	98.1
7f			191-193	79	97.6
7g			207-209	80	97.8
7h			213-215	72	99.1
7i			185-187	71	98.6
7j			173-175	72	97.3
7k			97-99	77	97.2
7l			110-112	75	98.3
7m			136-138	70	99.0
7n			91-93	68	98.5
7o			118-120	66	97.8

Experimental

Melting points were determined using SELACO-650 hot stage melting point apparatus and were uncorrected. IR spectra were recorded using a Jasco FTIR-2008 series. ^{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 parts per

million). 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. Silica gel column chromatography was performed using Merck 7734 silica gel (60–120 mesh) and Merck made TLC plates.

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 stir 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 over 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).

Synthesis of *tert*-butyl-2′,5′-dioxo-8-azaspiro[bicyclo[3.2.1] octane-3,4′-imidazolidin-*e*]-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) was taken in ethanol and water. Then potassium cyanide (16.4 g, 252 mmol) dissolved in water 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).

General procedure for the synthesis of *tert*-butyl-2′, 5′-dioxo-8-azaspiro [bicyclo [3.2.1]octane-3, 4′-imidazolidine]-8-carboxylate derivatives 4(a–c)

A solution of *tert*-butyl-2′,5′-dioxo-8-azaspiro[bicyclo [3.2.1]octane-3,4′-imidazolidine]-8-carboxylate 3 (1.0 eq) in *N*,*N*-dimethyl formamide was taken, anhydrous K_2CO_3 (3.0 eq) and alkyl halide (1–1.2 eq) were added to the solution. The reaction mixture was stirred at room temperature for 7–8 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 over anhydrous Na_2SO_4. The solvent was evaporated, crude product was purified by column chromatography using chloroform/ methanol (9:1) as an eluent.

Synthesis of *tert*-butyl-2′, 5′-dioxo-1′-(pent-4-enyl)-8-azaspiro[bicyclo[3.2.1] octane-3,4′-imidazolidine]-8-carboxylate (4a)

The general experimental procedure described afforded 4a (3.4 g) from *tert*-butyl-2′,5′-dioxo-8-azaspiro[bicyclo[3.2.1] octane-3,4′-imidazolidine]-8-carboxylate 3 (3.5 g, 11.8 mmol), 5-bromo-pent-1-ene 3a (1.93 g, 12.98 mmol) and K_2CO_3 (4.89 g, 35.4 mmol).

Synthesis of *tert*-butyl-2′, 5′-dioxo-1′-(2-oxo-2-propoxyethyl)-8-azaspiro [bicyclo [3.2.1]octane-3,4′-imidazolidine]-8-carboxylate (4b)

The general experimental procedure described afforded 4b (3.4 g) from *tert*-butyl-2′,5′-dioxo-8-azaspiro[bicyclo[3.2.1] octane-3,4′-imidazolidine]-8-carboxylate 3 (3.5 g, 11.8 mmol), bromo-acetic acid propyl ester 3b (2.34 g, 12.98 mmol) and K_2CO_3 (4.89 g, 35.4 mmol).

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

The general experimental procedure described afforded 4c (3.04 g) from *tert*-butyl-2′,5′-dioxo-8-azaspiro[bicyclo[3.2.1] octane-3,4′-imidazolidine]-8-carboxylate 3 (3.5 g, 11.8 mmol), 1-bromo-2-methoxy-ethane 3c (1.8 g, 12.98 mmol) and K_2CO_3 (4.89 g, 35.4 mmol).

General procedure for the synthesis of compound 5a, 5b and 5c

Compound 4a/4b/4c was taken in dry MDC, cooled to 0°C. Ether in HCl was added and allowed to stir at room temperature for 3 h. Deprotected salt compound was basified with $NaHCO_3$ solution and extracted with ethyl-acetate, organic layer was concentrated to get 5a/5b/5c.

General procedure for the synthesis of azaspiro bicyclo hydantoins 7(a-o)

A solution of target key intermediates (5a/5b/5c) (1.0 eq) in *N*,*N*-dimethyl formamide was taken, anhydrous K_2CO_3 (3.0 eq) was added to the solution and stirred for 10 min, then aryl halide 6(a–e) (1.0 eq) was added. The reaction mixture was stirred at room temperature for additional 6–8 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 over anhydrous Na_2SO_4. The solvent was evaporated, crude product was purified by column chromatography using chloroform/methanol (9:1) as an eluent.

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

The general experimental procedure described afforded 7a, the product obtained (0.231 g) was white pluffy solid from 1′-(pent-4-enyl)-8-azaspiro[bicyclo[3.2.1]octane-3,4′-imidazolidine]-2′,5′-dione 5a (0.20 g, 0.75 mmol), 1-bromomethyl-4-fluorobenzene (0.14 g, 0.75 mmol) and K_2CO_3 (0.314 g, 2.27 mmol). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 8.50 (s, 1H, –NH), 7.39–7.36 (m, 2H, Ar–H), 7.20–7.15 (m, 2H, Ar–H), 5.80–5.73 (m, 1H, =CH–), 5.03–4.93 (m, 2H, CH_2=), 3.54 (s, 2H, –CH_2–), 3.35–3.33 (t, 2H, –CH_2–), 3.19–3.12 (m, 2H, –CH_2–), 2.19–2.14 (m, 2H, –CH_2–), 1.96–1.89 (m, 6H, –CH_2–), 1.61–1.50 (m, 4H, –CH_2–). MS (ESI + ion): *m*/*z*=372. IR (KBr, cm^{-1}): 3301, 1657.

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

The general experimental procedure described afforded 7b, the product obtained (0.224 g) was glassy solid from 1′-(pent-4-enyl)-8-azaspiro[bicyclo[3.2.1]octane-3,4′-imidazolidine]-2′,5′-dione 5a (0.20 g, 0.75 mmol), 1-bromomethyl-3-fluorobenzene (0.14 g, 0.75 mmol) and K_2CO_3 (0.314 g, 2.27 mmol). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 8.52 (s, 1H, –NH), 7.38–7.33 (m, 1H, Ar–H), 7.19–7.15 (m, 2H, Ar–H), 7.07–7.02 (m, 1H, Ar–H), 5.82–5.71 (m, 1H, =CH–), 5.04–4.94 (m, 2H, CH_2=), 3.55 (s, 2H, –CH_2–), 3.37–3.33 (t, 2H, –CH_2–), 3.20–3.13 (m, 2H, –CH_2–), 2.20–2.15 (m, 2H, –CH_2–), 1.97–1.88 (m, 6H, –CH_2–), 1.62–1.52 (m, 4H, –CH_2–). MS (ESI + ion): *m*/*z*=372. IR (KBr, cm^{-1}): 3295, 1653.

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

The general experimental procedure described afforded 7c, the product obtained (0.244 g) was white crystalline solid from 1′-(pent-4-enyl)-8-azaspiro[bicyclo[3.2.1] octane-3,4′-imidazolidine]-2′,5′-dione 5a (0.20 g, 0.75 mmol), 4-bromomethyl-1,2-difluorobenzene (0.157 g, 0.75 mmol) and K_2CO_3 (0.314 g, 2.27 mmol). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 8.49 (s, 1H, –NH), 7.39–7.36 (m, 2H, Ar–H), 7.16–7.11 (m, 2H, Ar–H), 5.81–5.72 (m, 1H, =CH–), 5.03–4.94 (m, 2H, CH_2=), 3.56 (s, 2H, –CH_2–), 3.38–3.34 (t, 2H, –CH_2–), 3.21–3.14 (m, 2H, –CH_2–), 2.22–2.16 (m, 2H, –CH_2–), 1.98–1.88 (m, 6H, –CH_2–), 1.64–1.53 (m, 4H, –CH_2–). MS (ESI + ion): *m*/*z*=390. IR (KBr, cm^{-1}): 3305, 1649.

Synthesis of 8-(3,4-dichlorobenzyl)-1′-(pent-4-enyl)-8-azaspiro[bicyclo [3.2.1]octane-3,4′-imidazolidine]-2′, 5′-dione (7d)

The general experimental procedure described afforded 7d, the product obtained (0.256 g) was white crystalline solid from 1′-(pent-4-enyl)-8-azaspiro[bicyclo[3.2.1]octane-3,4′-imidazolidine]-2′,5′-dione 5a (0.20 g, 0.75 mmol), 4-bromomethyl-1,2-dichlorobenzene (0.182 g, 0.75 mmol) and K_2CO_3 (0.314 g, 2.27 mmol). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 8.47 (s, 1H, –NH), 7.59–7.57 (t, 2H, Ar–H), 7.36–7.34 (m, 1H, Ar–H), 5.81–5.74 (m, 1H, =CH–), 5.04–4.93 (m, 2H, CH_2=), 3.54 (s, 2H, –CH_2–), 3.35–3.32 (t, 2H, –CH_2–), 3.18–3.12 (m, 2H, –CH_2–), 2.18–2.14 (m, 2H, –CH_2–), 1.97–1.89 (m, 6H, –CH_2–), 1.60–1.50 (m, 4H, –CH_2–). MS (ESI + ion): *m*/*z*=422.1. IR (KBr, cm^{-1}): 3289, 1647.

Synthesis of 8-(4-chlorobenzyl)-1′-(pent-4-enyl)-8-azaspiro [bicyclo[3.2.1] octane-3,4′-imidazolidine]-2′, 5′-dione (7e)

The general experimental procedure described afforded 7e, the product obtained (0.226 g) was pale yellow crystalline solid from 1′-(pent-4-enyl)-8-azaspiro[bicyclo [3.2.1]octane-3,4′-imidazolidine]-2′,5′-dione 5a (0.20 g, 0.75 mmol), 1-bromomethyl-4-chlorobenzene (0.155 g, 0.75 mmol) and K_2CO_3 (0.314 g, 2.27 mmol). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 8.53 (s, 1H, –NH), 7.43–7.39 (m, 4H, Ar–H), 5.79–5.73 (m, 1H, =CH–), 5.02–4.93 (m, 2H, CH_2=), 3.52 (s, 2H, –CH_2–), 3.35–3.33 (t, 2H, –CH_2–), 3.19–3.12 (m, 2H, –CH_2–), 2.19–2.14 (m, 2H, –CH_2–), 1.96–1.89 (m, 6H, –CH_2–), 1.61–1.50 (m, 4H, –CH_2–). MS (ESI + ion): *m*/*z*=388.1. IR (KBr, cm^{-1}): 3286, 1645.

Synthesis of propyl-2-(8-(4-fluorobenzyl)-2′, 5′-dioxo-8-azaspiro[bicyclo [3.2.1] octane-3, 4′-imidazolidine]-1′-yl)acetate (7f)

The general experimental procedure described afforded 7f, the product obtained (0.215 g) was white pluffy solid from propyl-2-(2′,5′-dioxo-8-azaspiro[bicyclo [3.2.1]octane-3,4′-imidazolidine]-1′-yl)acetate 5b (0.20 g, 0.677 mmol), 1-bromomethyl-4-fluorobenzene (0.127 g, 0.677 mmol) and K_2CO_3 (0.280 g, 2.03 mmol). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 8.65 (s, 1H, –NH), 7.42–7.35 (m, 2H, Ar–H), 7.22–7.17 (m, 2H, Ar–H), 4.15 (s, 2H, –CH_2–), 4.03–4.01 (t, 2H, –CH_2–), 3.54 (s, 2H, –CH_2–), 3.16 (t, 2H, –CH_2–), 2.18–2.15 (m, 2H, –CH_2–), 1.97–1.92 (m, 4H, –CH_2–), 1.59–1.52 (m, 4H, –CH_2–), 0.88 (s, 3H, –CH_3). MS (ESI + ion): *m*/*z*=404. IR (KBr, cm^{-1}): 3308, 1741, 1652.

Synthesis of propyl-2-(8-(3-fluorobenzyl)-2′, 5′-dioxo-8-azaspiro[bicyclo [3.2.1] octane-3, 4′-imidazolidine]-1′-yl)acetate (7g)

The general experimental procedure described afforded 7g, the product obtained (0.218 g) was white pluffy solid from propyl-2-(2′,5′-dioxo-8-azaspiro[bicyclo [3.2.1]octane-3,4′-imidazolidine]-1′-yl)acetate 5b (0.20 g, 0.677 mmol), 1-bromomethyl-3-fluorobenzene (0.127 g, 0.677 mmol) and K_2CO_3 (0.280 g, 2.03 mmol). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 8.65 (s, 1H, –NH), 7.39–7.34 (m, 1H, Ar–H), 7.19–7.14 (m, 2H, Ar–H), 7.07–7.03 (m, 1H, Ar–H), 4.16 (s, 2H, –CH_2–), 4.04–4.01 (t, 2H, –CH_2–), 3.53 (s, 2H, –CH_2–), 3.15 (t, 2H, –CH_2–), 2.21–2.18 (m, 2H, –CH_2–), 2.0–1.9 (m, 4H, –CH_2–), 1.59–1.50 (m, 4H, –CH_2–), 0.88 (s, 3H, –CH_3). MS (ESI + ion): *m*/*z*=404. IR (KBr, cm^{-1}): 3302, 1744, 1652.

Synthesis of propyl-2-(8-(3,4-difluorobenzyl)-2′, 5′-dioxo-8-azaspiro[bicyclo[3.2.1]octane-3, 4′-imidazolidine]-1′-yl)acetate (7h)

The general experimental procedure described afforded 7h, the product obtained (0.20 g) was yellow crystalline solid from propyl-2-(2′,5′-dioxo-8-azaspiro[bicyclo[3.2.1] octane-3,4′-imidazolidine]-1′-yl)acetate 5b (0.20 g, 0.677 mmol), 4-bromomethyl-1,2-difluorobenzene (0.140 g, 0.677 mmol) and K_2CO_3 (0.280 g, 2.03 mmol). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 8.69 (s, 1H, –NH), 7.42–7.35 (m, 2H, Ar–H), 7.19–7.12 (m, 2H, Ar–H), 4.14 (s, 2H, –CH_2–), 4.06–4.02 (t, 2H, –CH_2–), 3.55 (s, 2H, –CH_2–), 3.16 (t, 2H, –CH_2–), 2.19–2.15 (m, 2H, –CH_2–), 1.98–1.92 (m, 4H, –CH_2–), 1.58–1.53 (m, 4H, –CH_2–), 0.88 (s, 3H, –CH_3). MS (ESI + ion): *m*/*z*=422.1. IR (KBr, cm^{-1}): 3996, 1748, 1653.

Synthesis of propyl-2-(8-(3,4-dichlorobenzyl)-2′, 5′-dioxo-8-azaspiro[bicyclo[3.2.1]octane-3, 4′-imidazolidine]-1′-yl)acetate (7i)

The general experimental procedure described afforded 7i, the product obtained (0.218 g) was white pluffy solid from propyl-2-(2′,5′-dioxo-8-azaspiro[bicyclo[3.2.1] octane-3,4′-imidazolidine]-1′-yl)acetate 5b (0.20 g, 0.677 mmol), 4-bromomethyl-1,2-dichloro benzene (0.162 g, 0.677 mmol) and K_2CO_3 (0.280 g, 2.03 mmol). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 8.66 (s, 1H, –NH), 7.64–7.58 (m, 2H, Ar–H), 7.38–7.35 (m, 1H, Ar–H), 4.17 (s, 2H, –CH_2–), 4.03–4.01 (t, 2H, –CH_2–), 3.54 (s, 2H, –CH_2–), 3.15 (t, 2H, –CH_2–), 2.21–2.17 (m, 2H, –CH_2–), 1.98–1.93 (m, 4H, –CH_2–), 1.61–1.52 (m, 4H, –CH_2–), 0.88 (s, 3H, –CH_3). MS (ESI + ion): *m*/*z*=454.1. IR (KBr, cm^{-1}): 3310, 1752, 1646.

Synthesis of propyl-2-(8-(4-chlorobenzyl)-2′, 5′-dioxo-8-azaspiro[bicyclo [3.2.1] octane-3, 4′-imidazolidine]-1′-yl)acetate (7j)

The general experimental procedure described afforded 7j, the product obtained (0.204 g) was white pluffy solid from propyl-2-(2′,5′-dioxo-8-azaspiro[bicyclo[3.2.1] octane-3,4′-imidazolidine]-1′-yl)acetate 5b (0.20 g, 0.677 mmol), 1-bromomethyl-4-chlorobenzene (0.139 g, 0.677 mmol) and K_2CO_3 (0.280 g, 2.03 mmol). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 8.64 (s, 1H, –NH), 7.42–7.38 (m, 4H, Ar–H), 4.18 (s, 2H, –CH_2–), 4.04–4.01 (t, 2H, –CH_2–), 3.53 (s, 2H, –CH_2–), 3.16 (t, 2H, –CH_2–), 2.19–2.16 (m, 2H, –CH_2–), 1.97–1.94 (m, 4H, –CH_2–), 1.59–1.51 (m, 4H, –CH_2–), 0.87 (s, 3H, –CH_3). MS (ESI + ion): *m*/*z*=420.1. IR (KBr, cm^{-1}): 3290, 1750, 1654.

Synthesis of 8-(4-fluorobenzyl)-1′-(2-methoxyethyl)-8-azaspiro[bicyclo [3.2.1] octane-3,4′-imidazolidine]-2′, 5′-dione (7k)

The general experimental procedure described afforded 7k, the product obtained (0.219 g) was white pluffy solid from 1′-(2-methoxyethyl)-8-azaspiro [bicyclo[3.2.1] octane-3,4′-imidazolidine]-2′,5′-dione 5c (0.20 g, 0.789 mmol), 1-bromomethyl-4-fluorobenzene (0.149 g, 0.789 mmol) and K_2CO_3 (0.327 g, 2.36 mmol). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 8.48 (s, 1H, –NH), 7.39–7.36 (m, 2H, Ar–H), 7.16–7.11 (m, 2H, Ar–H), 3.57 (s, 2H, –CH_2–), 3.52–3.49 (t, 2H, –CH_2–), 3.43–3.33 (t, 2H, –CH_2–), 3.20 (s, 3H, –OCH_3), 3.16–3.13 (m, 2H, –CH_2–), 2.19–2.15 (m, 2H, –CH_2–), 1.98–1.91 (m, 4H, –CH_2–), 1.55–1.47 (m, 2H, –CH_2–). MS (ESI + ion): *m*/*z*=362.1. IR (KBr, cm^{-1}): 3305, 1655.

Synthesis of 8-(3-fluorobenzyl)-1′-(2-methoxyethyl)-8-azaspiro[bicyclo [3.2.1] octane-3,4′-imidazolidine]-2′, 5′-dione (7l)

The general experimental procedure described afforded 7l, the product obtained (0.213 g) white glassy solid from 1′-(2-methoxyethyl)-8-azaspiro [bicyclo[3.2.1] octane-3,4′-imidazolidine]-2′,5′-dione 5c (0.20 g, 0.789 mmol), 1-bromomethyl-3-fluorobenzene (0.149 g, 0.789 mmol) and K_2CO_3 (0.327 g, 2.36 mmol). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 8.47 (s, 1H, –NH), 7.38–7.33 (m, 1H, Ar–H), 7.19–7.15 (m, 2H, Ar–H), 7.07–7.02 (m, 1H, Ar–H), 3.57 (s, 2H, –CH_2–), 3.50–3.47 (t, 2H, –CH_2–), 3.42–3.32 (t, 2H, –CH_2–), 3.21 (s, 3H, –OCH_3), 3.15–3.12 (m, 2H, –CH_2–), 2.20–2.15 (m, 2H, –CH_2–), 1.97–1.90 (m, 4H, –CH_2–), 1.56–1.47 (m, 2H, –CH_2–). MS (ESI + ion): *m*/*z*=362.0. IR (KBr, cm^{-1}): 3298, 1649.

Synthesis of 8-(3,4-difluorobenzyl)-1′-(2-methoxyethyl)-8-azaspiro[bicyclo [3.2.1]octane-3,4′-imidazolidine]-2′, 5′-dione (7m)

The general experimental procedure described afforded 7m, the product obtained (0.209 g) was white crystalline solid from 1′-(2-methoxyethyl)-8-azaspiro [bicyclo[3.2.1] octane-3,4′-imidazolidine]-2′,5′-dione 5c (0.20 g, 0.789 mmol), 4-bromomethyl-1,2-difluorobenzene (0.163 g, 0.789 mmol) and K_2CO_3 (0.327 g, 2.36 mmol). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 8.49 (s, 1H, –NH), 7.40–7.36 (m, 2H, Ar–H), 7.20–7.13 (m, 2H, Ar–H), 3.56 (s, 2H, $–CH_2–$), 3.49–3.48 (t, 2H, $–CH_2–$), 3.42–3.31 (t, 2H, $–CH_2–$), 3.20 (s, 3H, $–OCH_3$), 3.16–3.12 (m, 2H, $–CH_2–$), 2.22–2.15 (m, 2H, $–CH_2–$), 1.98–1.90 (m, 4H, $–CH_2–$), 1.58–1.48 (m, 2H, $–CH_2–$). MS (ESI + ion): m/z=380. IR (KBr, cm^{-1}): 3301, 1655.

Synthesis of 8-(3,4-dichlorobenzyl)-1′-(2-methoxyethyl)-8-azaspiro[bicyclo [3.2.1]octane-3,4′-imidazolidine]-2′, 5′-dione (7n)

The general experimental procedure described afforded 7n, the product obtained (0.211 g) was white pluffy solid from 1′-(2-methoxyethyl)-8-azaspiro [bicyclo [3.2.1]octane-3,4′-imidazolidine]-2′,5′-dione 5c (0.20 g, 0.789 mmol), 4-bromomethyl-1,2-dichloro benzene (0.189 g, 0.789 mmol) and K_2CO_3 (0.327 g, 2.36 mmol). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 8.50 (s, 1H, –NH), 7.62–7.58 (m, 2H, Ar–H), 7.38–7.33 (m, 1H, Ar–H), 3.57 (s, 2H, $–CH_2–$), 3.51–

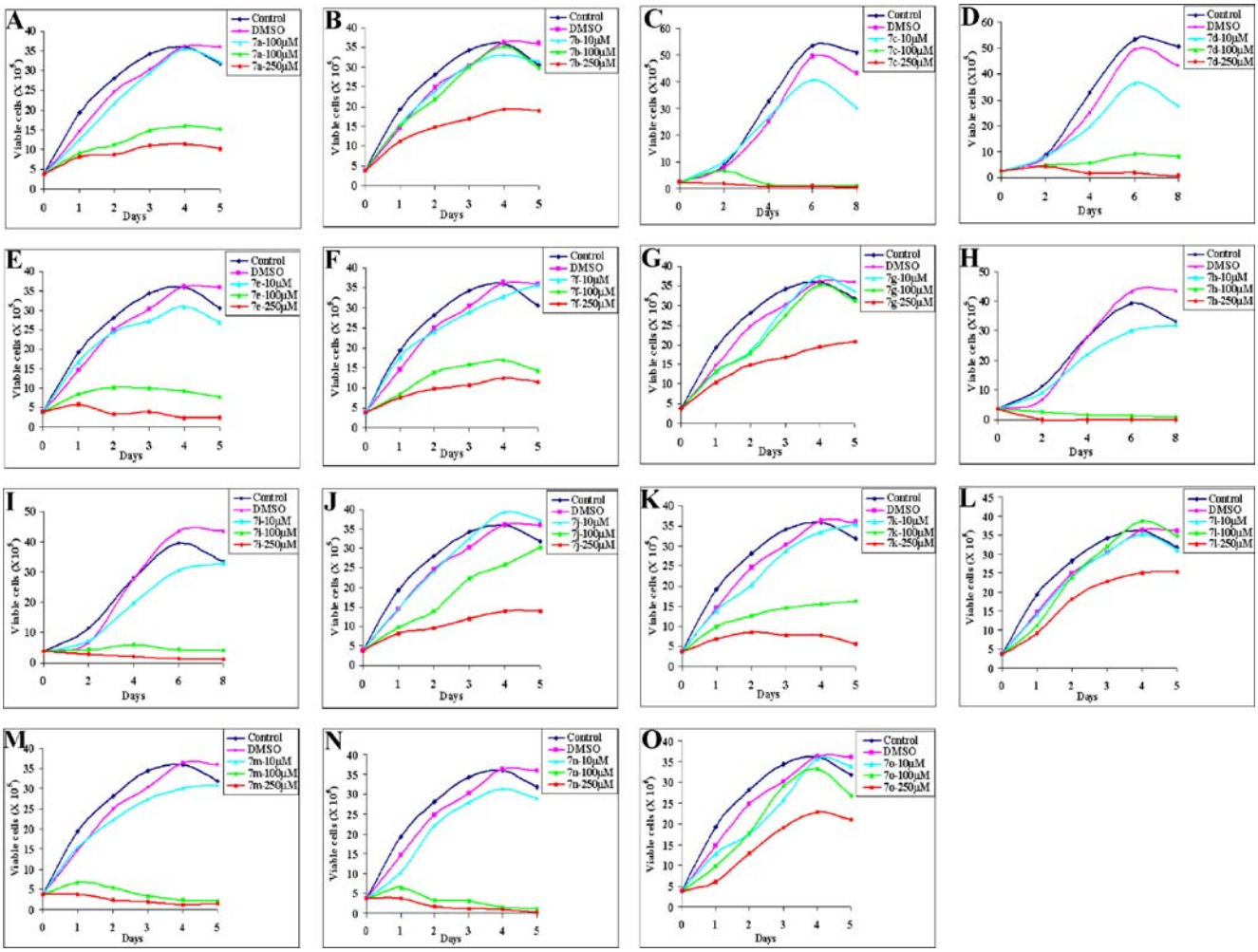


Fig. 1 Dose and time-dependent effect of diazaspiro bicyclo hydantoin analogs 7(a–o) on K562 cell proliferation. Approximately 0.75×10^5 cells/ml were cultured in a six-well sterilized tissue culture plate in RPMI supplemented with 10% FBS and incubated at 37°C. After 24 h, compound 7(a–o) were added at a concentrations of 10, 100 and 250 μM. Everyday proliferation was determined by trypan blue exclusion assay. Since the compound diluent is DMSO, the control treatment was performed with 0.6% final concentration of DMSO. **A**–**O** represents the treatment of compound 7(a–o) respectively

3.49 (t, 2H, $-CH_2-$), 3.43–3.31 (t, 2H, $-CH_2-$), 3.21 (s, 3H, $-OCH_3$), 3.15–3.12 (m, 2H, $-CH_2-$), 2.23–2.15 (m, 2H, $-CH_2-$), 1.97–1.90 (m, 4H, $-CH_2-$), 1.60–1.51 (m, 2H, $-CH_2-$). MS (ESI + ion): m/z=412.2. IR (KBr, cm^{-1}): 3307, 1645.

Synthesis of 8-(4-chlorobenzyl)-1′-(2-methoxyethyl)-8-azaspiro[bicyclo[3.2.1]octane-3,4′-imidazolidine]-2′, 5′-dione (7o)

The general experimental procedure described afforded 7o, the product obtained (0.196 g) was white pluffy solid from 1′-(2-methoxyethyl)-8-azaspiro [bicyclo[3.2.1] octane-3,4′-imidazolidine]-2′,5′-dione 5c (0.20 g, 0.789 mmol), 1-bromomethyl-4-chlorobenzene (0.162 g, 0.789 mmol) and K_2CO_3 (0.327 g, 2.36 mmol). ^1H NMR (DMSO-d_6, 400 MHz) δ: 8.48 (s, 1H, –NH), 7.40–7.37 (m, 4H, Ar–H), 3.55 (s, 2H, $-CH_2-$), 3.50–3.47 (t, 2H, $-CH_2-$), 3.44–3.31 (t, 2H, $-CH_2-$), 3.22 (s, 3H, $-OCH_3$), 3.16–3.12 (m, 2H, $-CH_2-$), 2.24–2.15 (m, 2H, $-CH_2-$), 1.96–1.90 (m, 4H, $-CH_2-$), 1.58–1.51 (m, 2H, $-CH_2-$). MS (ESI + ion): m/z=378.2. IR (KBr, cm^{-1}): 3298, 1657.

Biology

Inhibition of cell proliferation is an important potency indicator for chemotherapeutic drugs. Trypan blue assay was the first trial of our investigation, where we assessed the effect of diazaspiro bicyclo hydantoin analogs 7(a–o) on cell viability. To investigate this, the cells growing in log phase were treated with 10, 100 and 250 μM concentrations. The cells were counted after every 24 h till the control reached the stationary phase. Since the com-

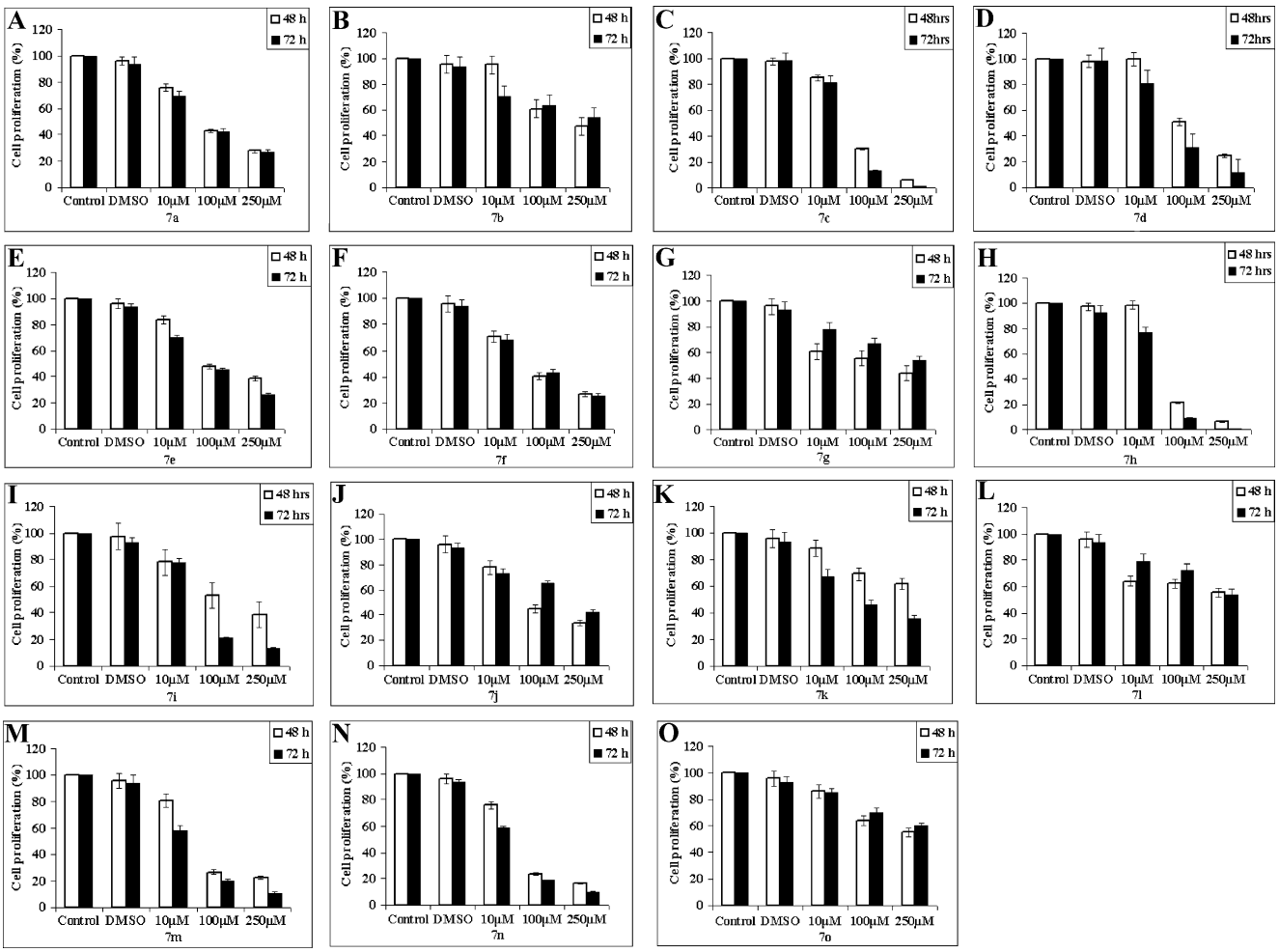


Fig. 2 Determination of the effect of diazaspiro bicyclo hydantoin analogs 7(a–o) on K562 cell viability by MTT assay. After 48 h and 72 h of exposure of cells with 7(a–o) at 10, 100 and 250 μM concentrations, cells were incubated with MTT (5 mg/ml) in duplicate for 4 h in 5% CO_2 at 37°C. Resulting blue formazan precipitate was dissolved in detergent and incubated for additional 2 h. Absorbance was measured at 570 nm. Results are presented as percentage of viable cells (the cell viability of vehicle cells were regarded as 100%). **A**–**O** represents the treatment of compound 7(a–o) respectively. Error bars are represented in the figures

pound diluent is DMSO, it was used as negative control. The amount of DMSO used was corresponding to the DMSO in highest concentration of compound tested, which did not have effect on the cell lines tested, as observed in Figs. 1 and 2.

Cell lines and culture

Human cell line, K562 (chronic myelogenous leukemia) and CEM (T-cell leukemia) was purchased from National Center for Cell Science, Pune, India. Cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 μg of streptomycin/ml and incubated at 37°C in a humidified atmosphere containing 5% CO_2.

In vitro cell proliferation and cell viability assay—Trypan blue exclusion assay

Trypan blue exclusion assay was performed to assess the effect of newly synthesized diazaspiro bicyclo hydantoin analogs 7(a–o) on viability of K562 and CEM cells. Approximately 0.75×10^5 cells/ml were seeded in a six-well tissue culture plate and different concentrations of compounds 7(a–o) were added after 24 h. For the determination of growth rate, smaller aliquots were collected in a 0.5 ml tubes, trypan blue (0.4%) was added to the cell suspension, the number of cells (viable-unstained and non viable-blue) was counted using a haemocytometer. The media was not changed during the induction period. Each experiment was repeated a minimum of three times and the results are presented as graphs (Fig. 1).

MTT assay

Cell survival was further assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye reduction assay [24], which is based on the ability of viable cells to metabolize a yellow tetrazolium salt to violet formazan product that can be detected spectrophotometrically. Exponentially growing cells (K562 and CEM) were plated in triplicate in 96-well sterilized plates at a density of 1×10^4 cells/well. After 24 h, cells were treated with escalating doses of 7(a–o) and incubated in 5% CO_2 atmosphere with high humidity. After 48 and 72 h of compound exposure, the cells were incubated with MTT (0.5 mg/ml) for another 4 h at 37°C. The blue MTT formazan precipitate was then solubilized in detergent (50% final concentration of *N*,*N*-dimethylformamide and 10% of sodium dodecyl sulphate) and incubated for an additional 2 h. Absorbance was measured at 570 nm on a multiwell ELISA plate reader. The mean absorbance of medium control was the blank and was subtracted. IC_{50} values (concentration of compound causing 50% inhibition of cell growth) were estimated after 72 h exposure of compound. The absorbance of control cells was taken as 100% viability and the values of treated cells were calculated as a percentage of control.

Results and discussion

The antiproliferative activity of all the newly synthesized compounds 7(a–o) have been evaluated in vitro against human leukemia (K562 and CEM) cells using trypan blue and MTT assay. As shown in Figs. 1 and 2, the tested compounds induce cell death in a dose and time dependent manner on K562 cells. Similar types of results are obtained with CEM (data not shown). It is found that the effect was improved linearly while prolonging the incubation time. Among the compounds 7(a–o), compounds 7c, 7d, 7h, 7i, 7m and 7n showed strong inhibition at IC_{50} values of 28, 35, 30, 35, 30 and 38 μM respectively (Table 2). The compounds 7a, 7e, 7f and 7k exhibited moderate inhibition. However, the remaining compounds 7b, 7g, 7j and 7l showed poor antiproliferation activity. The inhibition by compounds 7c, 7d, 7h, 7i, 7m and 7n could be attributed to the presence of electron withdrawing fluoro group at third and fourth position on the phenyl ring.

Initial structure–activity relationship can be drawn for these synthesized analogs. From our experimental results, it reveals that, the presence of pentene group in the 7(c–d) at the N-terminal of the hydantoin ring showed good

Table 2 The antiproliferative effects of diazaspiro bicyclo hydantoin derivatives 7(a–o) in K562 and CEM cells (IC_{50} value)

Compound	IC_{50}	
	K562 (in μM)	CEM (in μM)
7a	75	60
7b	>250	>250
7c	28	35
7d	35	40
7e	80	105
7f	70	75
7g	>250	>250
7h	30	32
7i	35	40
7j	180	>250
7k	70	80
7l	>250	240
7m	30	36
7n	38	48
7o	>250	>250

inhibition comparatively 7(h–i) having acetic acid propyl ester and 7(m–n) having methoxy ethane substitutions. It was also observed that the presence of a strong electron withdrawing group like fluoro in the phenyl ring of compound 7c, 7h and 7m enhances the antiproliferative activity, whereas 7d, 7i and 7n having chloro group decreases the activity on carcinoma cells. On the other hand it is observed that as the electro negativity increases, the activity also increases.

Another possible SAR study reveal that, replacement of the fluoro group in compounds 7a, 7f and 7k from *para* position to *ortho* position in compounds 7b, 7g and 7l causes the considerable decrease of activity. Similarly the presence of fluoro group in 7a, 7f, and 7k at *para* position increases the activity compare to the presence of chloro group in 7e, 7j and 7o at the same position. However, it is noteworthy to mention that hydantoin molecules with electron-withdrawing substituents on the phenyl ring showed good antiproliferation against leukemia cells.

From the SAR studies, it reveals that, the substitution at N-terminal of the hydantoin ring plays a key role in the antiproliferative activity. Although some of the diazaspiro bicyclo hydantoin derivatives presented here showed modest cytotoxic activities, the investigations of these structural modifications and preliminary SAR would be helpful to further design and development of more potent compounds. In this paper, we reported only preliminary results on the relationship between structure and cytotoxic activities. To acquire more information about the structural requirements for improving cytotoxic activities, the synthesis of more new diazaspiro bicyclo hydantoin derivatives with different substituents at other positions is needed. Further investigations and biological evaluations on these fluoro substituted hydantoin derivatives are in progress and the data will be reported later.

Conclusion

In summary, the diazaspiro bicyclo hydantoin derivatives 7(a–o) presented here showed strong, moderate and weak inhibitory activity against K562 and CEM cells. The derivatives 7c, 7d, 7h, 7i, 7m and 7n bearing two electron withdrawing groups (Fluorine and chlorine) showed significant inhibitory effects whereas compounds 7a, 7f and 7k with one electron withdrawing group (fluorine) showed moderate inhibitory activity against both the cell lines tested. The optimization of the nature of the linker on the *N*−3 position of hydantoin moiety and number of halogens on the benzyl group at *N*−8 position led to the identification of potent inhibitor for leukemia cells.

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