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Original article

Synthesis and biological evaluation of a novel human stem/progenitor cells proliferation activator: 4-(4-(5-mercapto-1,3,4-oxadiazol-2-yl)phenyl) thiosemicarbazide (Stemazole)

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ABSTRACT

Stem/progenitor cells are crucial for cell-based therapy and regenerative medicine, and their application in clinical and basic research requires a large supply of cells. To identify effective stem/progenitor cell proliferation activators, we synthesised a series of new 4-(4-(5-mercapto-1,3,4-oxadiazol-2-yl)phenyl) thiosemicarbazide (named Stemazole) derivatives. Preliminary evaluation of the structure–activity relationship (SAR) and the biological activities of the compounds were determined with a luminescent cell viability assay. The identified leading compound, Stemazole, exhibited remarkable proliferation-promoting activity in human hippocampal stem/progenitor cells (HSCs) in a time-dependent and concentration-dependent manner. The proliferation-promoting activity of Stemazole was further confirmed against a panel of human stem/progenitor cells derived from each of the three blastoderm layers. In conclusion, Stemazole is a novel activator of stem cells proliferation.

1. Introduction

Stem cell research is an active and highly attractive field of research [1–3]. Diseases due to the loss or damage of functional cells, such as Alzheimer's disease and Parkinson's disease, can be treated by mobilising endogenous stem cells to proliferate and/or differentiate or by regenerative medicine through exogenous stem cell-based replacement therapies. The application of stem cells in clinical and basic research purposes currently requires a large quantity of stem/progenitor cells. However, the number of stem cells available in vivo is insufficient for research and clinical needs, and the propagation of stem/progenitor primary cultures in vitro is challenging. Effective activators of stem cell proliferation that can mobilise and expand stem cells are urgently needed. Consequently, their development and application is one of the most fundamental and important priorities in stem cell research [4,5].

The most frequently used stem cell proliferation activators are serum, feeder cells and exogenous growth factors [6,7]. Despite widespread use in cell culture, these activators have obvious shortcomings. For example, the composition of serum is complex and incompletely defined. Stem cells that are cultured long-term in the presence of serum tend to differentiate [8,9]. Irradiated feeder cells presumably secrete indistinct cell growth factors that encourage stem cell expansion but add undesirable complexity to the stem cell microenvironment. Exogenous growth factors, such as basic fibroblast growth factor (bFGF) or leukaemia inhibitory factor (LIF), can support adult neural stem cell expansion under serum-free conditions, but their ability to independently support long-term maintenance proliferation of other types of adult stem cells in the absence of serum or feeder cells is unclear [6,10].

Chemical approaches, including small molecules, have been the focus of increasing interest as an alternative to traditional stem cell proliferation activators. Chemical genetics has been used to identify several small molecules capable of promoting stem cell proliferation [11–13]. For example, a high-throughput screen of 50,000 compounds identified a heterocyclic compound, SC1, that propagated murine embryonic stem cells (ES) in an undifferentiated, pluripotent state under chemically defined conditions in the absence of feeder cells, serum and LIF [14]. The same author, S. Ding, later reported two small molecules, Tzv and Ptn, which promoted human ES cells survival and self-renewal, respectively [15]. Other significant small molecules have also been identified. A high-throughput screen of 1,400,000 compounds identified eight

Abbreviations: Stemazole, 4-(4-(5-mercapto-1,3,4-oxadiazol-2-yl)phenyl) thiosemicarbazide; Br-Stemazole, 4-(2-bromo-4-(5-mercapto-1,3,4-oxadiazol-2-yl)phenyl) thiosemicarbazide; HSCs, human hippocampal stem/progenitor cells; PSCs, human pancreatic stem/progenitor cells; CSCs, human cardiac stem/progenitor cells.

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compounds with unpublished structures that stimulated mouse adult SVZ progenitor cell proliferation [16]. The natural product 6-bromoindirubin-3′-oxime (BIO) maintains human or murine ES cells in an undifferentiated pluripotent state in conditioned medium via activation of the Wnt signalling pathway and promotes adult myocardial cell proliferation [17,18].

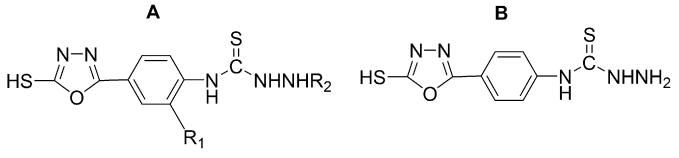
Although the number of known small molecule proliferation activators is small, natural or synthesised small molecule proliferation activators have distinct advantages [14,19–23]. First, small molecule compounds allow the proliferative culture conditions to be chemically defined, precisely controlling stem cell fate and avoiding the influence of indistinct exogenous factors in the stem cell culture environment [24]. Second, small molecules might simultaneously modulate multiple specific targets in a synergistic manner, potentially increasing the homogeneity of the harvested stem cells. Third, small molecules act in a temporal and adjustable way, making them useful tools for manipulating stem cells and investigating the complex molecular mechanisms that modulate stem cell fate. Fourth, small molecules have the greatest potential for development into new drugs targeted at stem cells [25]. Future stem cell drugs could treat or prevent diseases due to the loss or damage of functional cells by regulating the proliferation and/or differentiation of internal stem cells and rebuilding lost or damaged functional cells. Small molecule stem cell drugs not only resolve the limitations of adult stem cell resources and the ethical concerns associated with ES cells but also avoid immune rejection and operational sequelae resulting from stem cell transplantation. In light of these advantages, small molecules have become the subject of intense interest and research.

We performed a high-throughput chemical screen of approximately 25,000 compounds to identify small molecules that promoted stem cell proliferation. As a result of the screen, one compound, a 4-(4-(5-mercapto-1,3,4-oxadiazol-2-yl)phenyl) thiosemicarbazide (Stemazole), was used for subsequent research. Based on the skeleton structure of Stemazole (Scheme 1), we designed several novel heterocyclic small molecules and synthesised them in this study. The novel compounds were characterised by ^{1}H NMR, ^{13}C NMR and mass spectrometry. Preliminary evaluations of the structure–activity relationship (SAR) were performed to gain insight into the substitution pattern required for the greatest biological potency in human foetal-derived hippocampal stem/progenitor cells (HSCs) under serum-free culture conditions. From the SAR analysis, we identified the strongest proliferation-promoting compound **1**, Stemazole, as the lead compound, which was used in the subsequent time-response and dose-response studies. To further confirm the proliferation-promoting activity of Stemazole, we assessed its effect on a panel of human stem/progenitor cells derived from each of the three blastoderm layers (HSCs, ectoderm; PSCs, human pancreatic stem/progenitor cells, endoderm; and CSCs, human cardiac stem/progenitor cells, mesoderm).

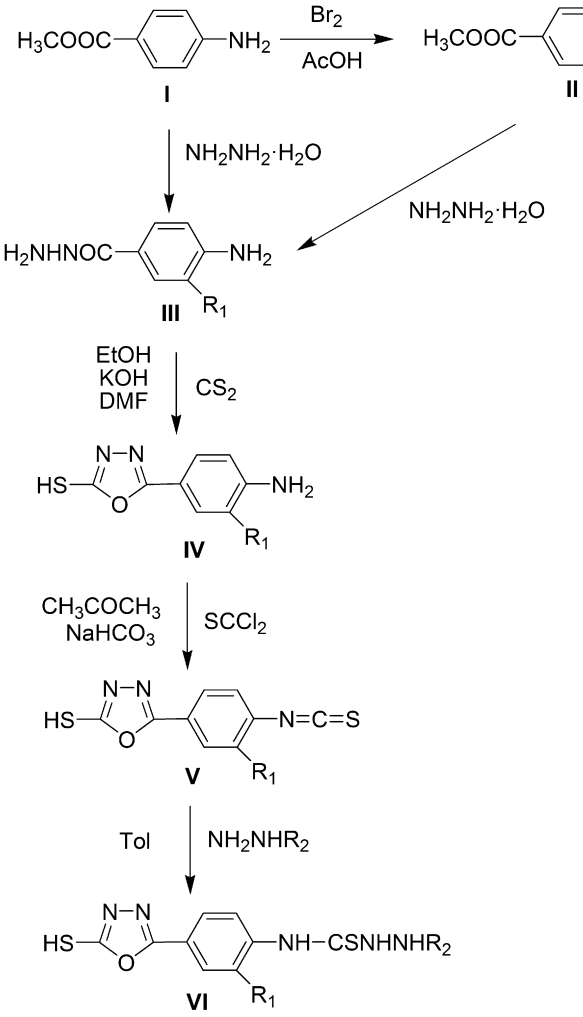
2. Results and discussion

2.1. Chemistry

The synthesis of the series of new Stemazole derivatives is illustrated in Scheme 2.



Scheme 1. Chemical structures of the 4-(4-(5-mercapto-1,3,4-oxadiazol-2-yl)phenyl) thiosemicarbazide scaffold (A) and Stemazole (B).



1: R_1=H , R_2=H
2: R_1=H, R_2=CH_3
3: R_1=H , R_2=-CH_2Ph
4: R_1=H, R_2=Ph
5: R_1=H , R_2=p-Cl-Ph
6: R_1=H , R_2=p-OMe-Ph
7: R_1=Br, R_2=H

Scheme 2. The synthetic route to Stemazole (**1**) and its derivatives (**2**–**7**).

Stemazole (**1**) was prepared in four steps. Compound **I** (methyl 4-aminobenzoate) was used as the starting material. Compound **III** (4-aminobenzohydrazide) was first synthesised from starting material **I** and hydrazine hydrate by hydrazinolysis. Compound **IV** was obtained by cyclisation of compound **III** and carbon disulphide. Compound **IV** plus thiophosgene afforded product **V** (5-(4-isothiocyanatophenyl)-1,3,4-oxadiazole-2-thiol). Lastly, Stemazole (**1**) was obtained from **V** by a thiosemicarbazone reaction with hydrazine hydrate. The series of Stemazole derivatives was synthesised by a similar procedure. All compounds were stable in the solid state and were soluble in dimethyl sulfoxide (DMSO).

2.2. Biological activities

2.2.1. Preliminary structure–activity relationship evaluation

To evaluate the SAR of Stemazole and its derivatives, we used HSCs as our stem cell scanning model. DMSO was used as the solvent for the tested compounds and as the negative control. The

effects of the small molecules were represented by cell viability (%). The cell viability (%) was defined as the percentage change in cell viability relative to the DMSO negative control: cell viability (%) = cell viability$_{sample}$/cell viability$_{DMSO}$ × 100%. The screening experiment identified obvious differences in the biological activities of the compounds (Fig. 1). The EC_{50} and IC_{50} values of the tested compounds promoting/inhibiting HSCs proliferation are shown in Table 1. Compounds **1** (Stemazole) and **7** (bromine-substituted Stemazole) promoted stem cell proliferation in vitro. Compound **3** (benzyl-substituted Stemazole) had no activity. Compounds **4** (phenyl-substituted Stemazole), **5** (para-chlorophenyl-substituted Stemazole) and **6** (para-anisyl-substituted Stemazole) were significantly cytotoxic. Compound **2** (methyl-substituted Stemazole) was weakly cytotoxic at high concentrations. The $\mathbf{R_1}$ position can likely tolerate a substituent; compound **7** (Br-Stemazole), which contains a bromine substituent at the $\mathbf{R_1}$ position, was similar to Stemazole with respect to its proliferation-promoting activity.

To identify a lead compound for subsequent research, we further compared the biological activity of Stemazole and Br-Stemazole in HSCs. Although the proliferation-promoting activity of Br-Stemazole was similar to that of Stemazole, its effective toxicity window was more narrow than Stemazole; at approximately 100 μM, Br-Stemazole exhibited weak cytotoxicity, which was not observed with Stemazole at this concentration. The maximum proliferation effect of Br-Stemazole was also lower than that of Stemazole (Fig. 1).

Based on its proliferation-promoting activity and relatively low cellular toxicity, compound **1**, Stemazole, was selected from this set of new compounds as the lead compound for subsequent research.

2.2.2. *The promoting-proliferation effects of Stemazole on HSCs are time- and dose-dependent*

Stemazole was evaluated by dose-response assays and time-response assays in HSCs under serum-free conditions without the growth factor bFGF. Stemazole had a very clear dose-response relationship between approximately 5 and 40 μM. The maximum effect was approximately three-fold higher relative to the DMSO negative control.

The proliferation-promoting activity of Stemazole was also distinctly time-dependent in HSCs. We performed a six-day proliferation experiment. The cells were measured every two days by three methods, including direct phase contrast microscope observation, cell number counting and a cell viability assay. As expected, cell proliferation increased with time after the addition of Stemazole, which continued until the end of the experiment. The cell number increased approximately three-fold after six days, which was similar to the bFGF positive control (Fig. 2).

Table 1
The EC_{50} and IC_{50} values of active compounds promoting/inhibiting HSCs proliferation.

No. of compounds	EC_{50}(μM)	IC_{50}(μM)
1	7.5	ND
2	ND	ND
3	ND	ND
4	ND	8.3
5	ND	3.2
6	ND	42.7
7	10.0	ND

"ND" means "Not Detected".
The EC_{50} (median effective concentration) represents the concentration of a compound required to induce a 50% promoting-proliferation effect. The IC_{50} (half-inhibitory concentration) represents the concentration of a compound required to induce a 50% against-proliferation effect.

2.2.3. *Stemazole showed similar promoting-proliferation activity with bFGF and no observed differentiation potential on HSCs*

To be useful, new activators should generally be equivalent or surpass existing activators. Therefore, we compared the effects of Stemazole and the frequently used stem cell growth factor bFGF (positive control). The experimental results revealed that both Stemazole (population doubling time$_{Stemazole}$ = 48.5 h, concentration$_{Stemazole}$ = 41.6 μM) and bFGF (population doubling time$_{bFGF}$ = 47.0 h, concentration$_{bFGF}$ = 20 ng/mL) significantly promoted HSCs proliferation relative to DMSO (negative control, population doubling time$_{DMSO}$ = 73.6 h, concentration$_{DMSO}$ = 0.08%). The proliferation-promoting effects of both molecules were similar whether cells were treated for four or six days. No significant differences were observed between Stemazole and bFGF (Fig. 3).

Additionally, the morphology of the cells treated with Stemazole and bFGF were evaluated. Again, the effects of Stemazole and bFGF were very similar. HSCs treated with the DMSO negative control appeared somewhat attached and inactive, whereas the cells treated with Stemazole or bFGF appeared clear, round

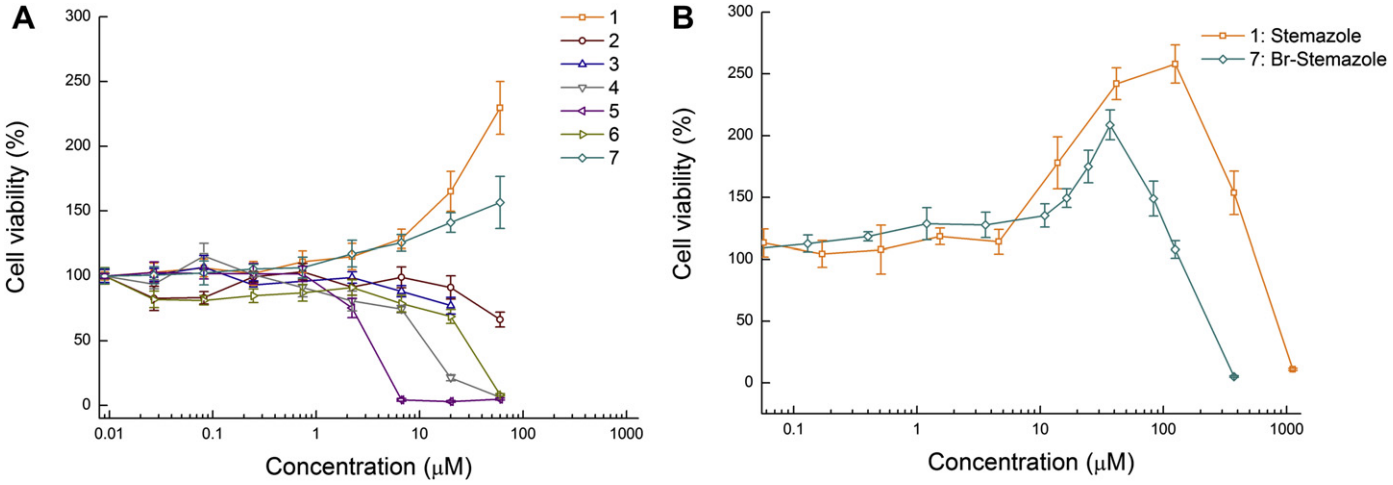


Fig. 1. The structure–activity relationship of Stemazole derivatives and identification of the lead compound. (A) Response of HSCs to Stemazole derivatives. HSCs were treated with Stemazole (**1**) and its derivatives (**2**–**7**) for four days, and their effects were analysed with a CellTiter-Glo luminescent cell viability assay. Compounds **1** and **7** promoted stem cell proliferation, while compounds **4**, **5** and **6** exhibited significant cytotoxicity. DMSO-treated cells were used as the control (100%). (B) Comparison of the biological activities of Stemazole (**1**) and Br-Stemazole (**7**) on HSCs. HSCs were treated with **1** or **7** for four days. Compounds **1** and **7** exhibited similar biological activities, but the effective toxicity window of **1** was wider than that of **7**, and the maximum proliferation effect of **1** was higher than that of **7**. The values presented are the mean ± SD.

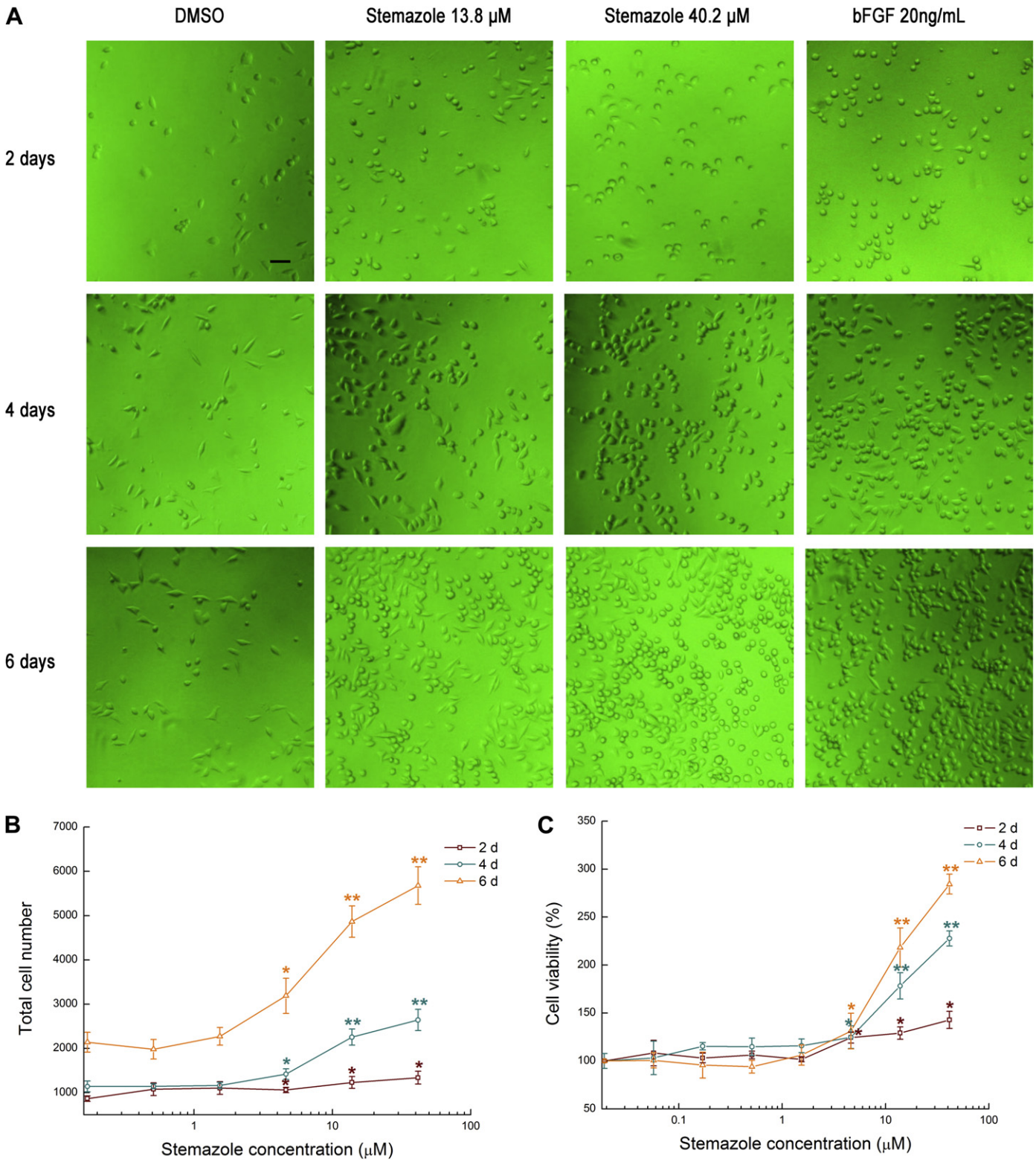


Fig. 2. The proliferation of HSCs treated with Stemazole. (A) Phase contrast images of HSCs treated with various concentrations of Stemazole, DMSO (negative control) and bFGF (positive control) at the indicated times. Bar: 50 μm. (B) The proliferation of HSCs treated with various concentrations of Stemazole as assessed by cell number counting at the indicated times. $^{*}P < 0.05$ compared to DMSO. $^{**}P < 0.001$ compared to DMSO. The values presented are the mean ± SD. (C) The proliferation of HSCs treated with various concentrations of Stemazole as assessed by cell viability assays at the indicated times. $^{*}P < 0.05$ compared to DMSO. $^{**}P < 0.001$ compared to DMSO. The values presented are the mean ± SD.

and compact in shape, indicating good cell viability and possible proliferation in progress. No morphological differentiation such as axonal growth or adipose drop appearance was visible (Fig. 2A).

2.2.4. Stemazole has similar proliferation-promoting effects on different stem/progenitor cells

In addition to HSCs, we also examined PSCs and CSCs. Humans develop from fertilised eggs through three embryonic germ layers,

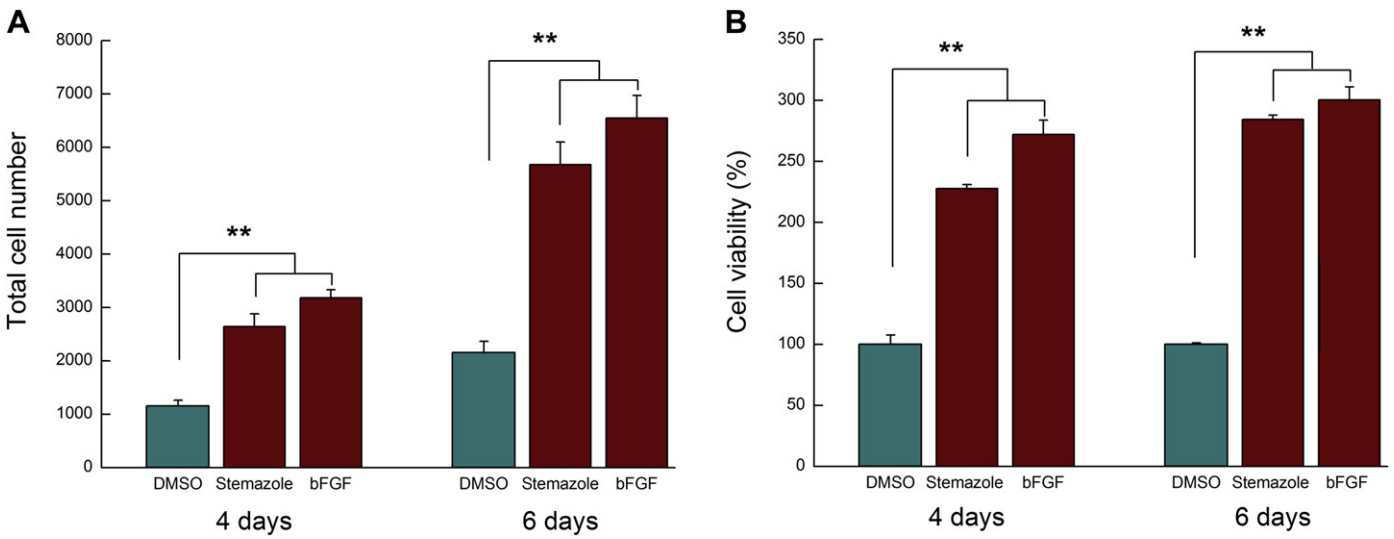


Fig. 3. Stemazole and bFGF have similar proliferation-promoting properties. (A) Comparison of the proliferation-promoting activities of Stemazole and bFGF in HSC cells as assessed by a cell number counting assay. Both Stemazole and bFGF significantly promoted proliferation relative to the DMSO negative control. There was no significant difference between Stemazole and bFGF. DMSO: 0.8%; Stemazole: 41.33 μM; bFGF: 20 ng/mL. The values presented are the mean ± SD. $^{**}P < 0.001$ compared to DMSO. (B) Comparison of the proliferation-promoting activities of Stemazole and bFGF in HSC cells as assessed by the CellTiter R-Pro luminescent cell viability assay. Both Stemazole and bFGF significantly promoted proliferation relative to the DMSO negative control. There was no significant difference between Stemazole and bFGF. DMSO: 0.8%; Stemazole: 41.33 μM; bFGF: 20 ng/mL. The values presented are the mean ± SD. $^{**}P < 0.001$ compared to DMSO.

the ectoderm, endoderm and mesoderm. When testing the promoting-proliferation activity of a compound on stem cells, the best and most comprehensive technique is to examine stem cells derived from the above three embryonic germ layers. In the presented study, we selected three representative stem cells, including hippocampal stem/progenitor cells (HSCs, representing ectoderm stem cells), human pancreatic stem/progenitor cells (PSCs, representing endoderm stem cells), and human cardiac stem/progenitor cells (CSCs, representing mesoderm stem cells), to evaluate the promoting-proliferation activity of Stemazole. These stem/progenitor cells were treated with Stemazole for four days. Cell viability assays showed that all the tested stem/progenitor cells had different but significant levels of proliferation. Among the progenitor cells, the ectoderm-derived HSCs appeared to be the most sensitive to Stemazole. The maximum proliferation effect was approximately three-fold higher relative to DMSO. The endoderm-derived PSCs responded to Stemazole in a similar fashion as the HSCs. The mesoderm-derived CSCs also responded to Stemazole, but the response was slightly weaker than in the other two cell lines (Fig. 4). The difference is likely because the different types and developing stages of the stem cells expand at different speeds, and their responses to the same regulator are often not identical. In summary, Stemazole exerted significant proliferative effects on stem/progenitor cells derived from the different germ layers.

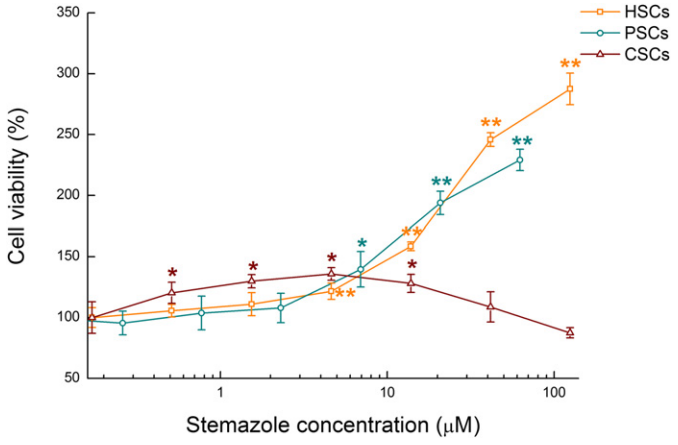


Fig. 4. The proliferation of multiple types of stem/progenitor cells derived from each of the three blastoderm layers treated with Stemazole for four days. HSCs: human hippocampal stem/progenitor cells, ectoderm; PSCs: Human pancreatic stem/progenitor cells, endoderm; CSCs: Human cardiac stem/progenitor cells, mesoderm. All three human blastoderm-derived stem/progenitor cells responded to Stemazole. HSCs were most sensitive to Stemazole, PSCs responded to Stemazole in a similar fashion. CSCs also responded to Stemazole, but the response was slightly weaker than in the other two cell lines. $^{*}P < 0.05$ compared to DMSO. $^{**}P < 0.001$ compared to DMSO. The values presented are the mean ± SD.

3. Conclusion

Seven Stemazole derivatives of previously unpublished structure were synthesised and characterised by ^{1}H NMR, ^{13}C NMR and mass spectrometry. Preliminary SAR studies revealed that Stemazole (**1**) and Br-Stemazole (**7**) possessed significant stem/progenitor cell proliferation-promoting activity. In contrast, compounds **4**, **5** and **6** were noticeably cytotoxic. Compound **2** was weakly cytotoxic, and compound **3** had no influence on the human HSCs tested. Stemazole was the strongest stem/progenitor cell proliferation activator among the set of tested compounds, and it stimulated human HSCs proliferation in a time-dependent and dose-dependent manner. The observed proliferation-promoting effect was reproduced in HSCs, PSCs and CSCs, which represent stem/progenitor cells from all three human blastoderm layers. The results suggest that Stemazole is a novel and potent general activator of stem/progenitor cell expansion and has the ability to play a critical role in the survival and expansion of multiple types of stem/progenitor cells in vitro. Thus, Stemazole has significant potential as a prospective stem cell drug. Identification of the effect of Stemazole on stem/progenitor cells in vivo and elucidation of the molecular mechanism underlying the proliferative phenomenon are in progress.

4. Experimental protocols

4.1. Materials and general methods

Chemical reagents and organic solvents were purchased from Sigma–Aldrich (MO, USA) unless otherwise mentioned. All commercial reagents and solvents for the reactions were of analytical grade. The structures of the novel synthetic compounds were identified by ^{1}H NMR, ^{13}C NMR and mass spectrometry. NMR spectra were obtained on a Bruker Avance III 400 in DMSO-d_6 (^{1}H: 400 MHz, ^{13}C: 100 MHz). Chemical shifts are reported in parts per million (ppm) downfield from tetramethylsilane (TMS). Spin multiplicities are described as s (singlet), d (doublet), t (triplet), q (quartet) and m (multiplet). Coupling constants are reported in Hertz (Hz). Mass spectra (ES) were determined on a high-resolution Micromass LCT Premier XE (Waters, UK). Thin-layer chromatography was performed on pre-coated Kieselgel 60 F254 plates (Merck, GER).

Dulbecco's Modified Eagle Media, Nutrient Mixture F-12 (DMEM/F12), Dulbecco's Modified Eagle Media (DMEM), low-glucose Dulbecco's Modified Eagle Media, Iscove's modified Dulbecco's medium (IMDM) and foetal bovine serum (FBS) were purchased from Gibco (CA, USA). Basic fibroblast growth factor (bFGF), B27 and epidermal growth factor (EGF) were purchased from Invitrogen (CA, USA). DMSO was purchased from Sigma–Aldrich (MO, USA). The CellTiter-Glo™ luminescent cell viability assay kit was purchased from Promega (WI, USA).

4.2. General procedures for the preparation of Stemazole ***1*** and derivatives ***2*** through ***7***

Compounds **1**–**7** were prepared according to Scheme 2. The details of the experimental protocol are provided below.

4.2.1. Synthesis of 4-(4-(5-mercapto-1,3,4-oxadiazol-2-yl)phenyl) thiosemicarbazide (***1***)

Compound **I** (45 g) was added in batches to a solution of 450 mL of hydrazine hydrate. The mixture was heated to reflux for 1 h and was monitored by TLC. After cooling to r.t., the excess hydrazine hydrate was evaporated under reduced pressure. The residue was poured into 50 mL of methanol, and the white solid compound **III** was filtered and dried to afford 45 g.

KOH (22 g) was added at 0 °C to a stirred solution of compound **III** (30 g) in anhydrous ethanol (400 mL) and DMF (50 mL). The reaction mixture was stirred for 10 min under N_2. To this mixture, CS_2 (45 g) was added dropwise at 0 °C. The mixture was warmed to ambient temperature for 0.5 h and heated to reflux overnight until no starting material remained. The pH was adjusted to pH 4 with 2 N HCl, and the solid was filtered and dried to afford 19 g of yellow solid compound **IV**.

To a stirred solution of compound **IV** (6.1 g) in acetone (40 mL), sulphur phosgene (2.9 mL) in acetone (11 mL) and saturated $NaHCO_3$ were added dropwise simultaneously at 0 °C. The mixture was then warmed to ambient temperature for 0.5 h. The mixture was poured into water to quench the reaction. The reaction was extracted with EtOAc, and the combined solution was dried with Na_2SO_4, filtered and evaporated under reduced pressure to give 5.7 g of yellow solid compound **V**.

Compound **V** (100 g) was suspended in toluene (1000 mL), and hydrazine hydrate (42.5 mL) was slowly added. The mixture was stirred at r.t. under N_2 until no starting material remained. The organic layer was separated, and the solid was filtered and washed with ethanol to afford 60 g of white solid compound **1**, Stemazole.

Total yield 14.6%, ^{1}H NMR (400 MHz, DMSO-d_6): δ 5.002 (s, 1H), 7.056 (s, 3H), 7.727 (d, J = 8.40 Hz, 2H), 7.871(d, 2H), 9.285 (S, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 179.44, 179.05, 160.68 (2C), 140.34 (2C), 124.73, 123.14, 121.01; HRMS (ESI) found: 268.0331, $[C_9H_9N_5OS_2 + H]^+$ calcd: 268.0327.

4.2.2. Synthesis of 4-(4-(5-mercapto-1,3,4-oxadiazol-2-yl)phenyl)-1-methythiosemicarbazide (***2***)

Compound **2** was obtained from compound **V** (235 mg) and 40% methyl hydrazine aqueous solution (233.5 mL) following the general procedure as a white solid (76 mg). Total yield 10.1%, ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.541 (s, 3H), 7.710 (d, J = 11.6 Hz, 2H), 7.787 (d, J = 11.2 Hz, 2H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 178.33, 177.64, 160.51 (2C), 142.98 (2C), 125.73, 123.14, 117.92, 42.22; HRMS (ESI) found: 282.0483, $[C_{10}H_{11}N_5OS_2 + H]^+$ calcd: 282.0483.

4.2.3. Synthesis of 1-benzyl-4-(4-(5-mercapto-1,3,4-oxadiazol-2-yl)phenyl)thiosemicarbazide (***3***)

Compound **3** was obtained from compound **V** (235 mg) and benzyl-hydrazine hydrochloride (319.2 mg) following the general procedure as a white solid (60 mg). Total yield 2.1%, ^{1}H NMR (400 MHz, DMSO-d_6): δ 5.327 (s, 2H), 7.333(m, 5H), 7.792 (d, 2H), 7.889 (d, 2H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 179.08, 177.25, 160.45 (2C), 143.42 (2C), 136.36, 128.45, 127.79, 127.31, 126.06 (2C), 123.55 (2C), 117.47, 56.20; HRMS (ESI) found: 358.0795, $[C_{16}H_{15}N_5OS_2+H]^+$ calcd: 358.0796.

4.2.4. Synthesis of 4-(4-(5-mercapto-1,3,4-oxadiazol-2-yl)phenyl)-1-phenylthiosemicarbazide (***4***)

Compound **4** was obtained from compound **V** (235 mg) and phenylhydrazine (216.28 mg) following the general procedure as a brownish yellow solid (122 mg). Total yield 13.2%, ^{1}H NMR (400 MHz, DMSO-d_6): δ 6.817–6.733 (m, 3H), 7.222–7.184 (m, 2H), 7.774 (d, J = 8.40 Hz, 2H), 7.866 (d, J = 8.80 Hz, 2H), 8.093 (s, 1H), 9.898 (s, 1H), 10.098 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 180.84, 177.25, 160.40 (2C), 147.69 (2C), 142.77, 128.86, 125.89, 124.77, 120.01, 117.99 (2C), 113.11 (2C); HRMS (ESI) found: 344.0646, $[C_{15}H_{13}N_5OS_2 + H]^+$ calcd: 344.0640.

4.2.5. Synthesis of 1-(4-chlorophenyl)-4-(4-(5-mercapto-1,3,4-oxadiazol-2-yl)phenyl)thiosemicarbazide (***5***)

Compound **5** was obtained from compound **V** (235 mg) and 4-Chloro-phenylhydrazine hydrochloride (358.1 mg) following the general procedure as a brownish yellow solid (78 mg). Total yield 7.7%, ^{1}H NMR (400 MHz, DMSO-d_6): δ 6.745 (d, J = 8.80 Hz, 2H), 7.260 (d, J = 8.80 Hz, 2H), 7.780 (d, J = 8.80 Hz, 2H), 7.845 (d, J = 8.80 Hz, 2H), 8.251 (s, 1H), 9.933 (s, 1H), 10.111 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 180.89, 179.26, 170.26 (2C), 160.59 (2C), 146.92 (2C), 140.61, 128.62, 124.96, 124.73, 124.16, 114.54 (2C); MS (EMS): m/z [M + H]+ 378.3 (100); HRMS (ESI) found: 378.0248, $[C_{15}H_{12}N_5OS_2Cl + H]^+$ calcd: 378.0250.

4.2.6. Synthesis of 4-(4-(5-mercapto-1,3,4-oxadiazol-2-yl)phenyl)-1-(4-methoxyphenyl)thiosemicarbazide (***6***)

Compound **6** was obtained from compound **V** (235 mg) and 4-methoxy-phenylhydrazine hydrochloride (349.26 mg) following the general procedure as a light brownish yellow solid (184 mg). Total yield 18.3%, ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.741 (s, 3H), 6.927(d, J = 8.80 Hz, 2H), 7.319 (d, J = 8.80 Hz, 2H), 7.792 (d, J = 9.20 Hz, 2H), 7.966 (d, J = 8.80 Hz, 2H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 178.92, 177.26, 160.45 (2C), 157.77 (2C), 143.21(2C), 138.71, 128.63, 126.04, 123.43, 117.49, 113.70 (2C), 55.32; HRMS (ESI) found: 374.0748, $[C_{16}H_{15}N_5O_2S_2+H]^+$ calcd: 374.0745.

4.2.7. Synthesis of 4-(2-bromo-4-(5-mercapto-1,3,4-oxadiazol-2-yl)phenyl)thiosemicarbazide (***7***)

To a stirred solution of compound **I** (1.51 g) in acetic acid Br_2 (1.59 g) was added at 0 °C. A large quantity of white solid was

obtained. TCL result showed that no starting material remained. The solid was purified by column chromatography on silica gel (DCM:CH_3OH = 20:1) to afford a white solid compound **II** (1.4g). The subsequent reaction followed the procedure outlined in Section 4.2.1.

Total yield 0.7%, brownish yellow, ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.034 (s, 3H), 7.731 (dd, J_1 = 11.4 Hz, J_2 = 1.60 Hz, 1H), 7.955 (d, J = 1.60 Hz, 1H), 8.647 (d, J = 8.80 Hz, 1H), 9.556 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 179.38, 178.73, 159.26, 138.54, 128.52, 124.86, 123.93, 121.62, 116.51; HRMS (ESI) found: 345.9439, $[C_9H_8N_5S_2BrO + H]^+$ calcd: 345.9432.

4.3. Biology

4.3.1. Preparation of stem/progenitor cells

Human stem/progenitor cells were kindly supplied by Prof. Lingsong Li (Peking University Stem Cell Research Center, Beijing, China). The stem/progenitor cells were maintained according to documented methods [26–29].

4.3.1.1. Maintenance of HSCs. The HSCs were maintained in DMEM/F12 supplemented with 20 ng/mL bFGF, 20 ng/mL EGF, 2% B27, 100 IU/mL penicillin and 100 IU/mL streptomycin at 37 °C in a 5% CO_2-humidified atmosphere. The medium was refreshed every 2–3 days. The cells were passaged by gently triturating the resulting neurospheres into a quasi-single cell suspension once per week. The sixth passage HSCs were used to evaluate the biological characteristics of Stemazole.

4.3.1.2. Maintenance of PSCs. PSCs were expanded in DMEM/F12 (7.33 mM glucose) with 10% FBS, 20 ng/mL bFGF, 100 IU/mL penicillin and 100 IU/mL streptomycin at 37 °C in a 5% CO_2-humidified atmosphere. The cells proliferated rapidly in the expansion medium. The sixth passage PSCs were used to evaluate the biological characteristics of Stemazole.

4.3.1.3. Maintenance of CSCs. CSCs were maintained in IMDM supplemented with 10% FBS, 2-mercaptoethanol, 100 IU/mL penicillin and 100 IU/mL streptomycin at 37 °C in a 5% CO_2-humidified atmosphere. The cells were passaged to the next generation upon reaching greater than 90% confluence. The third passage CSCs were used to evaluate the biological characteristics of Stemazole.

4.3.2. Measurement of proliferation-promoting activity

Exponentially growing cells were seeded on 384-well black wall clear bottom plates (Corning, USA) with a Multidrop Combi (Thermal, USA) at a density of 1×10^3 cells/well in basic culture medium and incubated for 24 h. Serial dilutions of the small molecules under investigation were added to the planted cells. DMSO was used as the negative control, and bFGF was used as the positive control. Cells were exposed to the synthetic compounds for 4 days (in the time-response assay, the exposure times were 2 days, 4 days and 6 days). The proliferation-promoting activities of the tested compounds were assessed by cell counting and a CellTiter-Glo™ luminescent cell viability assay.

4.3.3. Cell counting

Cell images were captured at the tested time points with a digital DP70 colour camera (Olympus, Japan) connected to a IX-51 microscope (Olympus, Japan). Three cell pictures were counted for each group. The total cell numbers in the pictures were counted with a haemocytometer.

4.3.4. Cell viability assay

The viability of the cells was quantitatively analysed by the CellTiter-Glo™ luminescent cell viability assay, which is based on the luciferase/luciferin reaction. In the presence of Mg^{2+} and ATP, the luminescence produced was proportional to the amount of ATP present, which is an indicator of cellular metabolic activity. The luminescent signal was directly proportional to the number of viable cells. Briefly, the assay was performed by adding CellTiter-Glo™ reagent directly to cells in culture. After mixing and incubating for 10 min at ambient temperature, luminescence was measured with a Multilabel Counter (PE, 1420, VICTOR$_3$™).

4.3.5. Statistical analysis

All experiments were performed at least in triplicate at each concentration level and repeated three times. Data are presented as the mean ± SD. Statistical comparisons were performed using a Student's t-test. A value of $P < 0.05$ was considered statistically significant. A value of $P < 0.001$ was considered markedly significant. The EC_{50} and IC_{50} values of the compounds were calculated based on the relationship of cell viability vs. the corresponding concentration by the SPSS software (version 13.0).

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