Keywords: 3,5-diethyl-1,3,5-thiadiazine-2-thione (DETT); NF κ B; multiple myeloma; apoptosis

An anti-leishmanial thiadiazine agent induces multiple myeloma cell apoptosis by suppressing the nuclear factor kappaB signalling pathway

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Background: Nuclear factor κ B (NF κ B) has a critical role in the pathophysiology of multiple myeloma. Targeting NF κ B is an important strategy for anti-myeloma drug discovery.

Methods: Luciferase assay was used to evaluate the effects of DETT on NF κ B activity. Annexin V–PI double staining and immunoblotting were used to evaluate DETT-induced cell apoptosis and suppression of NF κ B signalling. Anti-myeloma activity was studied in nude mice.

Results: DETT downregulated IKK α , β , p65, and p50 expression and inhibited phosphorylation of p65 (Ser536) and I κ B α . Simultaneously, DETT increased I κ B α , an inhibitor of the p65/p50 heterodimer, even in the presence of stimulants lipopolysaccharide, tumour necrosis factor- α , or interleukin-6. DETT inhibited NF κ B transcription activity and downregulated NF κ B-targeted genes, including *Bcl-2*, *Bcl-XL*, and *XIAP* as measured by their protein expression. Deregulation of NF κ B signalling by DETT resulted in MM cell apoptosis characterised by cleavage of caspase-3, caspase-8, and PARP. Notably, this apoptosis was partly blocked by the activation of NF κ B signalling in the presence of TNF α and IL-6. Moreover, DETT delayed myeloma tumour growth in nude mice without overt toxicity.

Conclusion: DETT displays a promising potential for MM therapy as an inhibitor of the NF κ B signalling pathway.

Multiple myeloma (MM) is a malignancy derived from plasma cells and is characterised by malignant monoclonal plasma cell proliferation, multiple bone lesions, and hypercalcemia. It accounts for 2% of all cancers and leads the second place in hematological malignancies (Raab *et al*, 2009). In the past decade, several novel drugs have been marketed for MM therapy, including proteasome inhibitors bortezomib and carfilzomib, and immunomodulators thalidomide and its analogs. However, MM remains incurable because of its complicated pathophysiology. Novel anti-MM drugs are in high demand.

Nuclear factor κ B (NF κ B) is a nuclear transcription factor that regulates the expression of a number of genes critical for cell

proliferation, viral replication, tumourigenesis, inflammation, and various autoimmune diseases (Aggarwal *et al*, 2006). In mammals, the NF κ B family is comprised of five different members: c-Rel, p65 (Rel A), Rel B, p50/p105 (NF κ B1), and p52/p100 (NF κ B2). The heterodimer p50/p65 is the most common complex in many cell types (May and Ghosh, 1998; Barkett and Gilmore, 1999). In non-stimulated cells, inactive NF κ B complexes are bound with a class of inhibitor proteins called I κ B, including I κ B α , I κ B β , I κ B γ , and the product of the putative proto-oncogene *bcl-3* (Whiteside *et al*, 1997; May and Ghosh, 1998). When I κ B is phosphorylated and subsequently degraded by the proteasome, NF κ B is released from I κ B and is translocated to the nucleus where it modulates gene

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expression (Barkett and Gilmore, 1999). Nuclear translocation of NF κ B can be induced by a variety of stimuli, including tumour necrosis factor- α (TNF α), lipopolysaccharide (LPS), and interleukins (Bitzer *et al*, 2000).

Recent evidence indicates that NF κ B and its signalling pathways are constitutively activated in both myeloma cell lines and primary myeloma cells (Annunziata *et al*, 2007; Demchenko and Kuehl, 2010). Inhibition of NF κ B signals by the proteasome inhibitor bortezomib induces MM cell apoptosis, suggesting that NF κ B is a potential target for anti-MM drug discovery. In the present study, we found that one of the tetrahydro-2H-1,3,5-thiadiazine-2-thione derivatives displayed potent anti-myeloma activity by inhibiting the NF κ B signalling pathway.

MATERIALS AND METHODS

Cell culture and chemicals. Human MM cell lines KMS11, LP1, OCI-My5, OPM2, RPMI-8226, and U266 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were grown in Iscove's Modified Dulbecco's Medium as described previously (Mao *et al*, 2007). DETT or 3,5-diethyl-1,3,5-thiadiazine-2-thione (Figure 2A) was purchased from Maybridge, Tintagel, UK.

Determination of apoptosis. Cell apoptosis was measured by flow cytometry (BD FACSCalibur, San Jose, CA, USA) with Annexin V-FITC/PI Apoptosis Detection Kit (BD Pharmingen, San Jose, CA, USA) as described previously (Ling *et al*, 2012).

Cell lysates preparation. Whole-cell lysates were prepared in an ice-cold lysis buffer containing 50 mM Tris, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 150 mM NaCl, 2 mM Na₃VO₄, 2 mM EGTA, 12 mM β -glycerol phosphate, 10 mM NaF, 16 mg ml⁻¹ benzamidine hydrochloride, and cocktail protease inhibitors (10 mg ml⁻¹ phenanthroline, 10 mg ml⁻¹ aprotinin, 10 mg ml⁻¹ leupeptin, 10 mg ml⁻¹ pepstatin, and 1 mM phenyl methyl sulfonyl fluoride; Mao *et al*, 2011). Cell lysates were then clarified at high speed at 4 °C.

To isolate cytoplasmic and nuclear proteins, cytoplasmic and nuclear extracts were prepared using the Nuclear and Cytoplasmic Extraction kit (Beyotime, Nantong, China) according to the manufacturer's instructions. Protein concentrations were determined by the BCA protocol (Beyotime).

Western blotting. Forty micrograms of total proteins were subject to fractionation on a SDS polyacrylamide gel electrophoresis, and followed by immunoblotting assay as described previously (Li *et al*, 2013). Primary antibodies, including PARP, CCND2, Bcl-2, XIAP, Bim, caspase-3, caspase-8, NF κ B, I κ B α , phospho-I κ B α , p105, p50, p65, phospho-p65, IKK α , IKK β , and Histone H3 were purchased from Cell Signaling Technologies Inc. (Danvers, MA, USA). The GAPDH antibody was obtained from Sigma (St Louis, MO, USA). Secondary horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit IgG were purchased from Beyotime. Signal detection was performed by the Enhanced Chemical Luminescence method (Beyotime) or by the SuperSignal West Pico Chemiluminescent kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

Transient transfection, luciferase, and β -galactosidase assays. HEK293T cells were seeded in 60-mm dishes (Nest Biotechnology Co., Wuxi, China). When cells were 60% confluent, the medium was replaced with serum-free Opti-MEM (Gibco BRL, Shanghai, China). Cells were cotransfected with the pNF κ B-Luciferase reporter plasmid (pNF κ B-Luc, Clontech, Mountain View, CA, USA) and the pGL4- β -galactosidase vector (Promega, Beijing, China) using 25KD PEI (Sigma) as the gene carrier. Twenty-four hours after transfection, cells were trypsinised, and equal numbers

of cells were plated in 24-well plates for 12 h. Cells were then treated with 0, 15, or 30 μ M of DETT for 9 h, followed by stimulation with or without LPS (5 μ g ml⁻¹) for 3 h. Luciferase assays and β -gal enzyme assays were performed 12 h after addition of DETT according to the manufacturer's protocol (Promega). Firefly luciferase activity was normalised to β -gal expression for each sample (Mao *et al*, 2007). All transfection experiments were performed in duplicate.

Multiple myeloma xenograft model. Female BALB/c nude mice (5–6 weeks old) were obtained from Shanghai Slac Laboratory Animal Co. Ltd, Shanghai, China (Zhang *et al*, 2013). All animal studies were conducted according to the protocols approved by the Ethical Committee of Experimental Animals of Soochow University. All mice were subcutaneously inoculated with RPMI-8226 cells (3×10^7 cells per injection) in the right flank in 200 μ l of sterile PBS containing 100 μ l of Matrigel (BD Pharmingen). When tumours were measurable, mice were randomly assigned into two groups, one group was orally administrated DETT (50 mg kg⁻¹ day⁻¹, 1/12 LD₅₀) and the other received vehicle. Tumour sizes and mice body weights were monitored every other day as described previously (Mao *et al*, 2011). Mice were killed 20 days after treatment with DETT, and all tumours were excised. After weighing and size measurement, tumour samples were snap-frozen in liquid nitrogen and then stored at -80 °C for further study. To examine the NF κ B signals in tumour tissues after DETT treatment, tumour tissues were subject to western blotting assay against p65, p50, and PARP as described previously (Mao *et al*, 2008).

Statistical analysis. When it was applicable, statistical significance was analyzed by using the Student's *t*-test.

RESULTS

DETT inhibits the NF κ B signalling pathway in MM cells. NF κ B is critical for MM cell proliferation and survival (Annunziata *et al*, 2007; Demchenko and Kuehl, 2010), but there was little evidence to visualise NF κ B expression in MM cells, therefore we first evaluated NF κ B components in a panel of MM cells. Immunoblotting analysis on six MM cell lines revealed that both p105 and p65 were universally highly expressed in all the cell lines. IKK α , β , p50, I κ B α , and phosphorylated p65 were highly expressed in four of the six cell lines examined (Figure 1), suggesting that NF κ B were important for MM cell proliferation and survival.

Because NF κ B is a ubiquitous transcription factor, we next designed a NF κ B responsive element-driven luciferase reporter to evaluate DETT activity. A luciferase reporter specifically responsive to NF κ B was transfected into HEK293T cells followed by DETT treatment in the presence or absence of 5 μ g ml⁻¹ LPS. As shown in Figure 2B, DETT suppressed baseline activity of NF κ B-driving luciferase. More impressively, it suppressed LPS-induced NF κ B activity by 80% compared with the control. To further understand how DETT blocked the NF κ B pathway, we measured the changes of protein expression of the NF κ B-associated members in MM cell lines OCI-My5 and RPMI-8226 after treatment with increasing concentrations of DETT for 24 h. As shown in Figure 2C, all components, including IKK α , β , p65, phospho-p65, and phospho-I κ B α , were downregulated by DETT. In contrast, total I κ B α was significantly increased following DETT treatment (Figure 2C).

Phosphorylation of p65 (Ser536) mediated by IKKs (Sakurai *et al*, 1999) facilitates p65 nuclear translocation and DNA binding (Zhong *et al*, 2002). Because both IKK α and β were downregulated by DETT (Figure 2C), we wondered whether DETT could suppress p65 phosphorylation in the presence of NF κ B signalling stimulants TNF α and LPS. To determine this, MM cells were treated with DETT for 24 h followed by addition of TNF α (20 min) or LPS (3 h).

Immunoblotting assays revealed that both TNFα and LPS induced p65 phosphorylation, but it was completely abolished by DETT (Figure 2D). Notably, consistent with the change of phospho-p65,

phospho-IκBα was also decreased, whereas total IκBα was increased by DETT (Figure 2D).

DETT suppressed NFκB activity in a manner different from bortezomib. Bortezomib is a potent anti-MM drug by inhibiting the proteasomes, stabilising IκBα, and suppressing p65/p50 activation. Because DETT was also able to inhibit NFκB signals, we wondered whether these two agents act in a similar manner. To this end, OCI-My5 and RPMI-8226 were treated with DETT or bortezomib followed by analysis of the NFκB signalling. Immunoblotting revealed that DETT and bortezomib exerted similar effects on p65, p50, and phospho-p65. Both agents decreased phospho-p65, p65, and p50 in the cytosol and suppressed p65 phosphorylation in the nuclear fragment, while total p65 and p50 were not affected (Figure 3A). However, their effects on IκBα were different. As shown in Figure 3B, bortezomib stabilised phospho-IκBα, while DETT decreased phospho-IκBα in a concentration-dependent manner (Figure 3B); accordingly, total IκBα was increased by DETT but not by bortezomib (Figure 3B). This result suggested bortezomib stabilises IκBα protein by inhibiting proteasomes (Murray and Norbury, 2000), while DETT probably inhibits IKKs, thus decreasing phosphorylation of IκBα and preventing it from degradation (Figure 2).

DETT significantly induces MM cell apoptosis. As a ubiquitous transcription factor, NFκB modulates a broad panel of signal transduction involved in cell proliferation, survival, and anti-apoptosis, therefore inhibition of NFκB can lead to MM cell death (Yinjun *et al*, 2005; Fabre *et al*, 2012). To check whether DETT was able to induce MM cell apoptosis, six MM cell lines, including KMS11, LP1, OCI-

Figure 1. Constitutive expression of the NFκB signalling components in MM cells. MM cell lines LP1, RPMI-8226 (8226), OCI-My5 (My5), U266, OPM2, and KMS11 were prepared for the whole-cell lysates in the RIPA lysis buffer containing 1% SDS. Immunoblotting analyses were performed against specific antibodies as indicated.

Figure 2. DETT inhibits NFκB activity. (A) The structure of the DETT. (B) HEK293T cells were co-transfected with NFκB-luciferase reporter and β-gal expression plasmids. Twenty-four hours later, cells were trypsinised, and equal numbers of cells were plated in 24-well plates and cultured for 12 h. Cells were then treated with 0, 15, or 30 μM of DETT for 9 h, followed by stimulation with or without LPS (5 μg ml⁻¹) for 3 h. Then luciferase activity was measured using Bright-Glo reagents (Promega). #*P* < 0.05, ##*P* < 0.01 compared with the control. (C) RPMI-8226 and OCI-My5 cells were treated with DETT (0, 15, or 30 μM) for 24 h and analyzed by western blotting against specific antibodies as indicated. (D) RPMI-8226 cells were pre-treated with DETT at 0, 15, or 30 μM for 24 h, followed by addition of TNFα (T, 50 ng ml⁻¹, 20 min) or LPS (L, 5 μg ml⁻¹, 3 h). Whole-cell lysates were then prepared for NFκB analysis against specific antibodies.

analysis revealed that PARP was markedly cleaved by DETT in LP1, OCI-My5, OPM2, and RPMI-8226 cells but less cleaved in U266 and KMS11 (Figure 5A), which was consistent with the apoptotic analysis by flow cytometry (Figure 4). Therefore, these data further demonstrated that DETT-induced MM cell apoptosis is highly associated with NF κ B signalling. To further characterise DETT-induced MM cell apoptosis, we evaluated activation of caspase-3 and -8 in OCI-My5 and RPMI-8226 cells. As shown in Figure 5B, DETT induced cleavage of PARP, caspase-3, and -8 in a concentration-dependent manner. Concomitantly, anti-apoptotic proteins XIAP and Bcl-2 were

Figure 5. DETT activates cell apoptotic pathway and suppresses expression of anti-apoptotic proteins. (A) Six MM cell lines were treated with 0 or 20 μ M of DETT for 24 h, followed by evaluation of PARP. (B) OCI-My5 and RPMI-8226 cells were treated with 0, 2.5, 5, 10, or 20 μ M of DETT for 24 h, and cell lysates were subject to caspase-3, caspase-8, PARP, and GAPDH analysis. (C) OCI-My5 and RPMI-8226 were treated with DETT for 24 h, followed by western blotting assay for pro-apoptotic (Bim) and anti-apoptotic (Bcl-2 and XIAP) proteins using specific antibodies. Abbreviations: Pro-casp3: pro-caspase 3; cle-casp8: cleaved caspase 8.

decreased, while pro-apoptotic Bim was induced by DETT (Figure 5C).

Activation of NF κ B partly suppresses DETT-induced MM cell apoptosis. The aforementioned studies demonstrated that DETT deregulated NF κ B signalling, especially suppressed phosphorylation of both p65 and I κ B α , and induced MM cell apoptosis. It seemed that sensitivity of MM cells to DETT was associated with NF κ B signalling, especially the status of p65 phosphorylation. To verify this hypothesis, RPMI-8226 cells were treated with DETT alone or in combination with NF κ B stimulants TNF α or IL-6 in a series of incubation periods. As shown in Figures 6A and B, p65 phosphorylation was induced by TNF α and IL-6 but was markedly decreased by DETT within 4 h. Without TNF α or IL-6, DETT could markedly inhibit p65 phosphorylation and induced PARP cleavage within 2 h. Addition of IL-6 or TNF α activated p65 phosphorylation, and it partly attenuated DETT-induced MM cell apoptosis along with the suppression of NF κ B signals (Figure 6). This finding and above studies therefore further demonstrated that DETT induced MM cell apoptosis, at least partly, by suppressing the NF κ B signalling.

DETT delays human MM tumour growth in nude mice models. All the above studies have provided reliable evidence that DETT inhibits NF κ B signals and induces MM cell apoptosis. To further investigate the effect of DETT against human MM *in vivo*, a myeloma xenograft model was established by subcutaneous injection of RPMI-8226 cells in the right flanks of nude mice. When the tumours were palpable (around 50 mm³), mice were orally administrated DETT with the dosage of 1/12 LD50 or 50 mg kg⁻¹ on a daily base for 20 days, and tumour sizes

Figure 6. Activation of NF κ B partly suppresses DETT-induced MM cell apoptosis. RPMI-8226 cells were treated with DETT (40 μ M), (A) 50 ng ml⁻¹ of TNF α or (B) IL-6 for the indicated periods. Whole-cell lysates were prepared for western blotting assays against specific antibodies.

and mice body weights were monitored every other day. The results indicated that DETT significantly inhibited tumour growth on the seventh day of administration (Figure 7A). At the end of the experiment, the tumour weights in the vehicle group and DETT treatment group were 1.55 ± 0.438 and 0.48 ± 0.163 g, respectively. Tumour growth was significantly decreased by DETT with the P value = 0.000234 (Figure 7B). There were no adverse effects or aberrant behaviour or gross organ damage in DETT-treated mice, which suggested that DETT was well tolerated (Figure 7C). In western blotting analysis, phospho-p65, p65, and p50 were decreased in tumours from the DETT-treated mice but not in those from untreated mice (Figure 7D). Moreover, PARP was also cleaved in the DETT-treated group, suggesting DETT also induced apoptosis *in vivo*. Taken together, these results supported that DETT displayed significant anti-MM activity *in vivo* by inhibiting the NF κ B signals.

DISCUSSION

Recent studies found that thiadiazine derivatives represent a class of potential anti-leishmanial agents, in addition to their antibacterial, antifungal, and antimicrobial activity (Monzote Fidalgo *et al*, 2004). These compounds showed cytotoxic properties against cervical cancer cell line HeLa and colon adenocarcinoma cell line HT-29 but did not display activity against Hep G2 cells (Perez *et al*, 2000). This selective cytotoxicity of thiadiazine derivatives for certain cancer cell lines suggests that these agents have a potential for the treatment of some specific cancers. Recently, some such derivatives have been reported to inhibit proliferation of chronic myelogenous leukemia cell line K562 and breast cancer cell line MDA-MB-468 as cell cycle inhibitors (Radwan *et al*, 2012). But the detailed mechanisms of anti-tumour activity have not been defined. In the current work, we found DETT, one of the thiadiazine derivatives, displays potent activity against MM in both *in vitro* and *in vivo* models. At a concentration of 5 μ M, DETT markedly activates caspase signals in MM cells. In the presence of MM cell activators such as IL-6, DETT still displays potent efficacy in inducing MM apoptosis. Notably, oral administration of DETT at 50 mg kg⁻¹ suppresses MM tumour growth by >70% within 3 weeks. All these results suggest DETT is potent for the treatment

Figure 7. DETT delays myeloma tumour growth in nude mice models. Three million of RPMI-8226 cells were subcutaneously injected in the right flank of each mouse. When tumours were palpable, mice were randomly distributed into two groups, one was orally given DETT (50 mg kg^{-1}) every day, while the other was received the same volume of vehicle. Mice weight and tumour sizes were monitored every other day. **(A)** The curves of tumour sizes (*, $P = 0.000444$). **(B)** Tumour weight was measured at the end of the experiment after tumours were excised. **(C)** The body weights were not markedly changed by DETT based on a measurement every other day. **(D)** DETT suppressed the expression of p65, phospho-p65, and p50 and cleaved PARP in the tumour tissues from DETT-treated mice.

of MM. Mechanistically, anti-MM activity of DETT is associated with the NF κ B signalling pathway, especially the phosphorylation status of p65, because those MM cell lines such as U266 and KMS11 with only a faint phospho-p65 level are not sensitive to DETT.

There are two NF κ B signalling pathways, one is canonical in which the I κ B α /p65/p50 complex is the key player, the other one is non-canonical in which p100/RelB is most important (Tully *et al*, 2012). The canonical pathway contains several strictly regulated steps, including extracellular stimulation, IKK activation, I κ B α phosphorylation and degradation, p65/p50 nuclear translocation, NF κ B-DNA binding, and NF κ B transactivation (Gilmore and Herscovitch, 2006). The traditional concept in the NF κ B signalling is that NF κ B is inhibited by association with I κ B α . Once phosphorylated by IKK upon signalling triggers such as TNF α or IL-6 stimulation, I κ B α is subsequently degraded by the 26S proteasomes, and the p65/p50 heterodimer is then liberated and activated followed by nuclear translocation. I κ B α is the key negative regulator of the NF κ B activation (May and Ghosh, 1998; Kim *et al*, 2006). Many NF κ B inhibitors such as bortezomib induce cancer cell apoptosis by suppressing I κ B α degradation thus suppressing NF κ B activation (Murray and Norbury, 2000). Bortezomib is an inhibitor of proteasomes thus stabilising hyperphosphorylated I κ B α and maintaining its inhibitory effects on p65/p50. However, different from bortezomib, DETT decreases I κ B α phosphorylation and increases total I κ B α level (Figure 3). Although the effects of these two agents on NF κ B are distinct in terms of phosphorylated and total I κ B α proteins, the final effects are probably the same, because bortezomib stabilises phospho-I κ B α from proteasomal degradation, while DETT suppresses I κ B α phosphorylation, which prevents I κ B α from degradation by proteasomes. In DETT-treated MM cells, this is dramatic, because total I κ B α was increased by DETT.

In addition to I κ B α phosphorylation, more and more studies demonstrated that p65 is also phosphorylated by stimulants such as TNF α (Sakurai *et al*, 1999). The subunit p65 of NF κ B, also called RelA, is the key component of the functional NF κ B that is translocated into nuclei where it binds to DNA and modulates gene transcription (Nolan *et al*, 1991). Phosphorylation of p65 could occur at Ser276, Ser311, Ser529, and Ser536 (Sakurai *et al*, 1999; Wang *et al*, 2000; Zhong *et al*, 2002; Duran *et al*, 2003; Vermeulen *et al*, 2003), but the blockage of p65 phosphorylation at Ser536 rather than at Ser276 or Ser529 abolishes p65 transcription activity (Hu *et al*, 2004). It is believed that phospho-p65 (Ser536) facilitates p65 nuclear translocation, improves its DNA binding, recruits p300 to the p65 complex, and releases p65 from HDAC1 and HDAC3, thereby regulating downstream gene expression (Buss *et al*, 2004; Hu *et al*, 2004). Our study showed that, similar to bortezomib, DETT also suppresses p65 phosphorylation in cytoplasm. Because DETT can downregulate the expression of IKK α and β , as well as p65 and I κ B α phosphorylation, IKK α / β are probably the major target of DETT. We noticed that DETT leads to concentration- and time-dependent decrease of p65 phosphorylation in both cytoplasmic and nuclear fragments. However, total p65 protein level is only decreased in the cytoplasm but not changed in the nuclei (Figure 3). Moreover, DETT-induced MM cell apoptosis is dependent on p65 phosphorylation level. U266 and KMS11 cells with less phosphorylated p65 are resistant to DETT compared with the other cell lines expressing phospho-p65 (Figures 1, 4, and 5). These findings suggest that NF κ B activation with p65 phosphorylation is required for cancer cell survival and could be a target by some chemicals, such as DETT.

The IKKs are key kinases that phosphorylate both p65 and I κ B α (Yang *et al*, 2003; Viatour *et al*, 2005). Several studies indicate that PI3K/Akt-dependent signalling activates NF κ B activity but depends on the relative levels of the IKK α subunit (Gustin *et al*,

2004). By reviewing the effects of DETT on NF κ B signalling nodes, we can conclude that DETT might suppress NF κ B activation by inhibiting p65 and I κ B α phosphorylation.

Therefore, in the present study, we found that anti-leishmanial thiadiazine-derivative DETT could be a potential anti-myeloma agent by targeting the NF κ B signalling. The low toxicity and high potency in MM cell apoptosis and delaying MM tumour growth *in vivo* merits DETT for further evaluation.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

GC, BC, and XM designed the research. XM and GC wrote the manuscript. GC, KH, XX, XD, ZZ, JT, MS, MW, and JL performed the experiments.

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