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Optimising intratumoral treatment of head and neck squamous cell carcinoma models with the diterpene ester Tigilanol tiglate

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Summary
The five-year survival rate for patients with head and neck squamous cell carcinoma (HNSCC) has remained at ~50% for the past 30 years despite advances in treatment. Tigilanol tiglate (TT, also known as EBC-46) is a novel diterpene ester that induces cell death in HNSCC in vitro and in mouse models, and has recently completed Phase I human clinical trials. The aim of this study was to optimise efficacy of TT treatment by altering different administration parameters. The tongue SCC cell line (SCC-15) was identified as the line with the lowest efficacy to treatment. Subcutaneous xenografts of SCC-15 cells were grown in BALB/c Foxn1nu and NOD/SCID mice and treated with intratumoral injection of 30 μg TT or a vehicle only control (40% propylene glycol (PG)). Greater efficacy of TT treatment was found in the BALB/c Foxn1nu mice compared to NOD/SCID mice. Immunohistochemical analysis indicated a potential role of the host’s innate immune system in this difference, specifically neutrophil infiltration. Neither fractionated doses of TT nor the use of a different excipient led to significantly increased efficacy. This study confirmed that TT in 40% PG given intratumorally as a single bolus dose was the most efficacious treatment for a tongue SCC mouse model.

Keywords · Head and neck squamous cell carcinoma · Protein kinase C · Diterpene ester · Intratumoral injection · Tigilanol tiglate · EBC-46

Introduction
Over 600,000 new patients are diagnosed with cancer of the head and neck annually, the fifth most common cancer worldwide [1]. Head and neck squamous cell carcinoma (HNSCC) is responsible for 90% of all head and neck cancers. Decisions regarding the treatment of a patient with HNSCC involve a large multidisciplinary team of surgeons, radiation and medical oncologists, radiologists, pathologists, speech therapists, dietitians and dentists. Treatment usually includes a combination of surgical resection followed by radiation therapy and/or chemotherapy tailored specifically to the patient’s preference, disease characteristics and co-morbidities. Despite sophisticated advances in surgical technique, targeted radiotherapy and drug discovery, the 5-year survival rate has remained low at 50% for the past 30 years with the exception of human papilloma virus (HPV)-related oropharyngeal cancer [2]. Unfortunately, the median time of survival following loco-regional recurrence or metastatic disease is only six months [3]. Consideration of further surgical resection or radiation therapy following recurrence is precluded at times due to their associated high morbidity on previously treated patients. It has therefore been imperative to investigate novel therapies that can offer increased survival for HNSCC patients with low associated morbidity.

Derived from the seed of the native Northern Queensland plant Fontainea picrosperma, Tigilanol tiglate (also known as EBC-46 [12-Tigloyl-13-(2-methylbutanoyl)-6,7-epoxy-4,5,9,12,13,20-hexahydroxy-1-tigliaen-3-one; 562.65 g/mol]) is a novel diterpene ester and activator of Protein
Kinase C (PKC). Exploratory case studies (over 300 dogs, cats and horses with solid tumors) and veterinary clinical trials (110 dogs with mast cell tumors or soft tissue sarcomas) showed very promising results, with the majority of tumors eradicated without recurrence (measured at 12 months) by intratumoral injection of TT. TT has also recently completed Phase I human clinical trials. We previously demonstrated that a single intratumoral dose of TT caused haemorrhagic necrosis and tumor ablation in melanoma, HNSCC and other tumor mouse models [4]. TT was also found to induce a respiratory burst from human polymorphonuclear cells, and cause increased permeability of human umbilical vein endothelial cells. Furthermore, its action was found to be at least in part PKC-dependent, by partial inhibition with the pan-PKC inhibitor bisindolylmaleimide-1 (BIS-1).

Experiments with TT so far have used buffered propylene glycol as the standard vehicle for intratumoral injection. Little is known whether efficacy is improved with alternative suitable excipients, such as 2-hydroxypropyl-β-cyclodextrin. In addition, the impact of the host on TT efficacy has not previously been addressed. This study aimed to enhance efficacy of intratumoral TT injection of HNSCC xenografts by altering different administration parameters in mouse models with different innate immune systems.

Materials and methods

Human cell lines

The cell lines used in this project [SCC-9 (Tongue SCC, ATCC No. CRL-1629), SCC-15 (Tongue SCC, ATCC No. CRL-1623) and SCC-25 (Tongue SCC, ATCC No. CRL-1628)] were all of human origin. Routine mycoplasma tests were performed using PCR and were always negative. Cell line identity was routinely checked by Short Tandem Repeat (STR) profiling with the GenePrint® 10 System (Promega, Madison, WI) according to the manufacturer’s instructions.

Cell culture and passaging

All cell lines were cultured in Roswell Park Memorial Institute (RPMI-1640) media containing 10% (v/v) foetal bovine serum (FBS; Life Technologies, Scoresby, Victoria, Australia), 100 μg/ml streptomycin, 60 μg/ml penicillin (Life Technologies), 1 mM pyruvate, 0.2 mM nicotinamide, and 3 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulphonic acid). Cell lines were incubated at 37°C with 5% CO₂ and 95% humidity. Cultured cell lines were passaged biweekly to maintain log phase growth of cells. Once a cell line had reached 80% confluence in the containing flask, media was removed, cells washed with PBS, then detached using trypsin with versene. Cells were then sub-cultured at a 1:5 or 1:10 dilution into new flasks with RPMI-1640.

Research involving animals

This study was performed in strict accordance with the recommendations in the Australian Code for the Care and Use of Animals for Scientific Purposes 8th Edition (2013), of the National Health and Medical Research Council of Australia. All protocols were reviewed and approved by the QIMR Berghofer Medical Research Institute Animal Ethics Committee (QIMR-B AEC; approval numbers A0106-042 M and A0404-606 M).

Subcutaneous inoculation of mice with human cancer cell lines

Five week old BALB/c Foxn1nu and NOD/SCID mice were housed under pathogen-free conditions in the QIMR Berghofer Animal Facility on a 12 h light/dark cycle, with freely available sterile water and food. All procedures were performed with aseptic technique in a biohazard flow hood in accordance with institutional standard operating protocols. Cultivated SCC-9, SCC-15 and SCC-25 cells were collected from culture and cell density was determined using a haemocytometer. Cells were collected by centrifugation at 1500 rpm for 5 min at 4 °C; the resulting cell pellet was resuspended in RPMI-1640 media with 10% FBS to achieve a density of 40 × 10⁶ cells/ml. Cells remained stored on ice for the minimal amount of time until the point of injection. For experiments using NOD/SCID mice, the fur over the hind-quarters was removed with electric clippers (Wahl) prior to inoculation. Subcutaneous inoculation was performed using Terumo® U-100 Insulin 29G x ½” needles to inject 50 μl (2 × 10⁶ cells) of the cell suspension, below the dorsal skin of the mouse’s hindquarter on the left and right side. Mice were ear tagged for future identification and housed in a filtered cage. Mice were monitored bi-weekly for tumor size and daily for overall wellbeing.

Intratumoral injection with Tigilanol tiglate

Tumors were monitored until they reached the desired volume of approximately 100 mm³. Mice in the treated groups received 50–100 μl of 0.3–0.6 mg/ml TT (30 μg; dissolved in 40% propylene glycol (PG) in 30 mM sodium acetate buffer, pH 4.2 or 2-hydroxypropyl-β-cyclodextrin) to each tumor site using a Terumo® U-100 Insulin syringe with a 29G x ½” needle. Mice in the control groups received 50–100 μl of 40% PG or cyclodextrin. Mice were assessed for clinical signs according to a QIMR Berghofer Animal Ethics Committee approved clinical score sheet during the period of the
experiment to determine whether the treatments (i.e., tumor burden and effects of drugs) were causing distress to the mice.

**Mouse monitoring and tumor measurements**

Individual tumor length and width was measured bi-weekly with digital vernier callipers (Kinachrome) and the resulting tumor volume (mm$^3$) estimated using the formula (length/2) × width$^2$. Once total tumor burden reached 1000 mm$^3$ per mouse, mice were euthanized by CO$_2$ inhalation. The raw measurement data was recorded and the average volume calculated using Microsoft Excel. The average volume and associated standard error of the mean (SEM) were then plotted against days since treatment. Kaplan-Meier survival curves were prepared in GraphPad Prism 6. Mice were monitored for up to 10 months after tumor treatment to assess long-term efficacy of TT. In addition, digital photographs were taken to document tumor progression. Qualitative measures of tumor characteristics and overall mouse distress were also made.

**Immunohistochemistry**

Mice were euthanised by CO$_2$ inhalation at 0–24 h following a single intratumoral injection of TT or 40% PG to their tumors or normal skin. The treated sites were excised using sterile surgical instruments and fixed in 10% phosphate buffered formalin for 24 h at 4 °C and transferred into 70% ethanol until further processing. Tumors were paraffin embedded, sectioned and stained for haemotoxylin and eosin, neutrophils (LyG6, MPO), macrophages (F4/80), and endothelial cells (CD31) by the QIMR Berghofer MRI Histology Facility. Slides were scanned at 40× magnification using the Aperio AT Scanscope.

**Results**

**Identification of a head and neck cancer cell line with lowest efficacy to intratumoral treatment with Tigilanol tiglate**

Intratumoral treatment with 30 μg of TT successfully ablated FaDu and CAL-27 xenografts in BALB/c Foxn1nu mice [4]. In an attempt to identify a cell line that displayed a weaker response to TT treatment, three additional tongue SCC cell lines were obtained and tested: SCC-9, SCC-15 and SCC-25. Xenografts of the three cell lines were established by subcutaneously injecting ten five-week old BALB/c Foxn1nu mice for each group with $2 \times 10^6$ cells at two sites. SCC-15 xenografts required 8 days of growth to reach the appropriate treatment volume of approximately 100 mm$^3$. SCC-9 and SCC-25 xenografts were slower growing and reached treatment size at 17 days post-injection. Of the ten mice in each group, five were treated with 30 μg of TT (50 μl of 600 μg/ml TT in 40% propylene glycol (PG)) at both tumor sites, whilst the remaining five mice were injected with 50 μl of 40% PG only (Fig. 1).

In all three xenograft groups treated with TT, the previously described localized haemorrhagic response and subsequent eschar formation occurred. This was not seen in the groups treated with the vehicle control. Average tumor volumes showed a steady decline following treatment with TT. SCC-15 xenografts were completely ablated by day six post-treatment with TT, compared to day 14 post treatment for SCC-9 and SCC-25. In contrast, the control groups for all three xenografts continued to have tumor growth following intratumoral injection with the vehicle control. Mice were euthanized once total tumor burden had reached approximately 1000 mm$^3$ per mouse. TT treatment led to a statistically significant increase in survival time in all three xenograft models (SCC-9, $P < 0.001$; SCC-15, $P < 0.0001$; SCC-25, $P < 0.0001$; Fig. 1a-c).

Recurrences were seen at two tumor sites (2/10; 20%) for SCC-9 xenografts (Fig. 1a), and one tumor site (1/10; 10%) for SCC-25 xenografts (Fig. 1c). Three recurrences (3/10; 30%) in the SCC-15 group were identified by day 21 post-initial treatment with TT (Fig. 1b). Of the total six recurrences seen across the groups, three tumors (one per group) were retreated with 30 μg TT resulting in complete ablation. Mice were monitored for ten months and no further recurrences were seen. Given the greatest proportion of recurrences, SCC-15 was identified as the cell line with lowest efficacy to treatment with TT and therefore selected to be the cell line utilized for further optimisation of TT administration.

**The role of the host in Tigilanol tiglate efficacy**

Ten-five-week old male NOD/SCID mice were subcutaneously inoculated with $2 \times 10^6$ SCC-15 cells/site, with two sites per mouse. Tumor growth occurred very quickly in comparison to the BALB/c Foxn1nu mice xenografts, with an average tumor volume of 159 mm$^3$ ($\pm$ 20 mm$^3$ SEM) seven days following initial tumor cell injection. On day seven, five mice were intratumorally injected with the previously established treatment of 30 μg TT and five mice with 50 μl of the vehicle control 40% PG. The mice injected with the vehicle control continued to display exponential tumor growth, as seen in the BALB/c Foxn1nu cohort (Fig. 2a). In comparison, tumor growth in the group treated with the previously established dosing regimen of 30 μg TT did not mirror that seen in the nude mice. On average, tumor growth was suppressed by approximately 50%, and no tumors were successfully ablated (0/10). Mice reached maximum tumor burden and were all culled by day 14 and day 21, for the control and treated groups, respectively (Fig. 2b).
The survival time difference between the control and treatment groups was however statistically significant \((P = 0.0028)\).

Intratumoral injection of Tigilanol tiglate to small SCC xenografts in NOD/SCID mice

The reduced efficacy of TT treatment seen in the larger SCC-15 xenografts in NOD/SCID mice potentially could have been attributed to the larger average tumor size at the time of treatment \((159 \text{ mm}^3 \pm 20 \text{ mm}^3 \text{ SEM})\). The dose of TT may have been inadequate for the number of tumor cells, and/or local spread of tumor cells may have already occurred to a site outside the treatment field. In order to understand the reduced efficacy of TT treatment, NOD/SCID mice were inoculated with \(2 \times 10^5\) cells/site and treated at a reduced mean tumor volume \((67 \text{ mm}^3 \pm 3 \text{ mm}^3 \text{ SEM})\). Within the mice with
smaller tumors, ten tumors were treated with 50 μl of 40% PG vehicle control and ten treated with 30 μg TT in a single bolus injection (Fig. 3a). Of the ten tumors treated with TT in 40% PG, 7 were successfully ablated at day 35 post treatment. The remaining tumors showed initial clearance but developed local recurrence two weeks following TT treatment.

**Single versus divided dose administration of Tigilanol tiglate**

To investigate whether more tumor cells could be targeted with a dose given in each tumor quadrant, compared to the single dose, five mice were treated with a total 30 μg TT given in four divided doses (7.5 μg TT × 4). No tumors were successfully ablated as seen in Fig. 3a. Mice treated with divided doses rather than the single dose of TT had significantly reduced survival \( (P = 0.0001; \text{Fig. 3b}) \). Upon administration of the divided doses, there was a significant amount of leakage of the solution out of the preceding needle puncture sites, reducing the overall concentration of TT the tumor tissue would have otherwise retained. Solution leakage was also seen in tumors that appeared to be particularly necrotic/ulcerated.

**Propylene glycol compared to 2-hydroxypropyl-β-cyclodextrin as an excipient for Tigilanol tiglate administration**

We wished to assess a different excipient for TT, and its effect on treatment efficacy. Cyclohexadrenes are a family of cyclic oligosaccharides used widely in the food, agricultural, and pharmaceutical industries. The ring-like structure of these compounds provides a hydrophilic inner and a hydrophilic outer region, facilitating the inclusion of hydrophobic/lipophilic compounds and potentially increasing their bioavailability when used as a drug excipient [5]. We therefore used 4% 2-hydroxypropyl-β-cyclodextrin as the vehicle for TT (Fig. 3a). There was no difference in the survival of mice with tumors treated with 40% PG or 4% 2-hydroxypropyl-β-cyclodextrin alone (Fig. 3b). All tumors continued to grow after injection of either excipient with all reaching endpoint at day 13 post treatment. Treatment with TT lead to significant increases in survival of mice with either excipient (TT in 40% PG versus 40% PG alone, \( P = 0.0003 ; \) TT in 4% 2-hydroxypropyl-β-cyclodextrin versus 4% 2-hydroxypropyl-β-cyclodextrin alone, \( P < 0.0001 ) \). Further, TT in 4% 2-hydroxypropyl-β-cyclodextrin did not significantly improve number of recurrences compared to TT in 40% PG \( (P = 0.9603, \text{Fig. 3b}) \). A total of 6 sites had no recurrence with TT in 4% 2-hydroxypropyl-β-cyclodextrin while treatment with TT in 40% PG showed no recurrence in 7 sites. These results suggest that 2-hydroxypropyl-β-cyclodextrin was no more effective as an excipient compared to 40% PG, and importantly show that the anti-tumor activity of TT is maintained in different vehicles.

**Impact of delivery volume on efficacy of Tigilanol tiglate**

As described above, during administration of 30 μg of TT in 50 μl of vehicle to some xenografts in NOD/SCID mice, significant leakage of the solution from necrotic areas of the tumor occurred, resulting in the observed reduction of tumor
regression. Those particular tumors often were not successfully ablated likely due to the overall reduced concentration of TT at the tumor site or reduced treatment field area. It was therefore proposed that increasing the solution volume, with the same dose (30 μg) could potentially minimize dose wastage from leaking and improve tumor infiltration. SCC-15 xenografts were established in NOD/SCID mice and treated with 40% PG alone (n = 5), 30 μg TT in 50 μl with 40% PG (n = 5), or 30 μg TT in 100 μl with 40% PG (n = 5) (Fig. 4a). Following TT treatment, the resulting haemorrhagic region surrounding the tumor appeared moderately more extensive for those treated with 30 μg TT in 100 μl with 40% PG. No additional adverse effects were noted during routine mouse and tumor monitoring. No significant difference in tumor growth or survival was apparent between the two TT treated groups (Fig. 4b).

**Immunohistochemistry on tumors from different mouse hosts show variation in neutrophil recruitment**

To investigate the differences in response that occurred following treatment of SCC-15 xenografts in BALB/c Foxn1nu and NOD/SCID mice, and whether a recruitment of macrophages and/or neutrophils also occurred, immunohistochemistry was performed for general morphology and salient markers following TT treatment. Immunohistochemical staining was performed for LyG6, a myeloid differentiation antigen present on peripheral neutrophils in addition to myeloperoxidase (MPO), a lysosomal protein expressed in neutrophil granules. Both stains identified the presence of neutrophils in the tumor; however, MPO could also potentially identify sites of recent neutrophil degranulation. A substantial infiltration of neutrophils into tumor tissue was apparent 24 h after intratumoral treatment with TT in BALB/c Foxn1nu (Fig. 5). Evidence of this infiltration was supported with a similar response seen in MPO staining at 24 h (data not shown). No neutrophil infiltrations or areas of degranulation were demonstrated in tumor tissue treated with 40% PG. In contrast to the patterns seen in the BALB/c Foxn1nu mice, no infiltration of neutrophils or evidence of increased sites of neutrophil degranulation was identified in NOD/SCID mice at any time point following treatment with TT (Fig. 5). LyG6 and MPO staining across all time points were similar to that seen in the untreated tumor tissue. No differences in staining patterns for CD-31 or F4/80 were observed (data not shown).

**Discussion**

There have been previous attempts to treat HNSCC with intralesional delivered therapies, such as injection of anticancer agents including cisplatin [6, 7], interleukins [8] or oncolytic viruses [9, 10]. The aim of the current research was to identify an HNSCC model that was inherently difficult to treat with intralesional-delivered TT and try to overcome this using variations of dose administration, volume or excipient. SCC-15, a tongue SCC cell line, was found to be the most difficult to treat with to intratumoral TT administration with ~30% relapse rate in BALB/c Foxn1nu mice, however a single repeated dose with TT led to cure with no sign of
recurrence ten months later. Less efficacy was observed in SCC-15 xenografts grown on NOD/SCID mice, which indicated potential involvement of the host’s innate immune system in TT’s mechanism of action. BALB/c Foxn1nu mice are known to be more immunocompetent than their NOD/SCID mice counterparts. Whilst both strains have impaired B-cell and T-cell lymphocyte development, NOD/SCID mice have an additional impairment of their macrophages, natural killer (NK) cells and complement components [11]. The differences in the comparative infiltration of neutrophils between the NOD/SCID and Foxn1nu mice was of particular interest. This observation appears to be first objective evidence for the NOD/SCID strain of having defective homing of proinflammatory cells, alluded to in several descriptions of the strain [11, 12]. The lack of neutrophil recruitment in the NOD/SCID mice could potentially contribute to the reduced efficacy of TT seen in this mouse model. With a reduced number of neutrophils present in the tumor tissue following injection with TT, the concentration of neutrophil-produced cytokines and chemokines in the tumor microenvironment may be substantially lower, thus leading to reduced tumor cell killing.

Using the identified tongue SCC mouse model, TT administration parameters were then adjusted in an attempt to overcome this inherent resistance. It was shown that a single bolus dose of 30 μg TT in 50 μl 40% PG, to a tumor site led to a greater ablation rate compared to divided doses of the novel drug. Potentially this was due to additional leakage of the solution out of preceding needle holes in a relatively small tumor volume, thus reducing the overall concentration of the drug at the tumor site with divided dosing.

Despite the hypothesis that 2-hydroxypropyl-β-cyclodextrin could potentially carry TT in its hydrophobic centre and remain at the tumor site for an extended period of time, the use of this compound as an excipient yielded no improved survival or further reduction in tumor growth. This study confirmed that 50 μl 40% PG as an excipient for TT appeared to be the most efficacious. However, with the
gaining popularity of other excipients such as nanoparticles, it may be prudent to investigate these compounds in future experiments.

Overall, this study confirmed that 30 μg TT in 50 μl 40% PG given intratumorally as a single bolus dose without prior priming was the most efficacious treatment for a tongue SCC mouse model. The greater efficacy of TT treatment in the BALB/c Foxn1nu mice potentially indicates the additional role of the host’s innate immune system. At the time of writing, a Phase I clinical trial for TT in a palliative subset of patients with head and neck cancer, amongst other indications, has been completed in Victoria, New South Wales, South Australia and Queensland, Australia with data analysis currently underway. It is hoped that TT will provide a treatment option for selected patients with head and neck cancer as an adjunct or stand-alone therapy.

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Compliance with ethical standards

Conflict of interest Peter G. Parsons declares consulting payments and contract research funding from QBiotics Ltd. Benedict J. Panizza declares consulting payments from QBiotics Ltd. Glen M. Boyle declares he was a recipient of a fellowship co-sponsored by QBiotics Ltd. and contract research funding from QBiotics Ltd. For the remaining authors no conflicts of interest were declared.

Ethical approval This article does not contain any studies with human participants performed by any of the authors.

Informed consent This article does not contain any studies with human participants performed by any of the authors.

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References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D (2011) Global cancer statistics. CA Cancer J Clin 61:69–90
2. Bose P, Brockton NT, Dort JC (2013) Head and neck cancer: from anatomy to biology. Int J Cancer 133:2013–2023
3. Murdoch D (2007) Standard, and novel cytotoxic and molecular-targeted, therapies for HNSCC: an evidence-based review. Curr Opin Oncol 19:216–221
4. Boyle GM, D’Souza MM, Pierce CJ, Adams RA, Cantor AS et al (2014) Intra-lesional injection of the novel PKC activator EBC-46 rapidly ablates tumors in mouse models. PLoS One 9:e108887
5. Kurkov SV, Loftsson T (2013) Cyclodextrins. Int J Pharm 453:167–180
6. Castro DJ, Sridhar KS, Garewal HS, Mills GM, Wenig BL, Dunphy FR, Costantino PD, Leavitt RD, Stewart ME, Orenberg EK (2003) Intratumoral cisplatin/epinephrine gel in advanced head and neck cancer: a multicenter, randomized, double-blind, phase III study in North America. Head Neck 25:717–731
7. Paiva MB, Publik M, Castro DJ, Udewitz M, Wang MB, Kowalski LP, Sercarz J (2005) Intratumor injections of cisplatin and laser thermal therapy for palliative treatment of recurrent cancer. Photomed Laser Surg 23:531–535
8. van Herpen CM, van der Voort R, van der Laak JA, Klasen IS, de Graaf AO et al (2008) Intratumoral rhIL-12 administration in head and neck squamous cell carcinoma patients induces B cell activation. Int J Cancer 123:2354–2361
9. Nemunaitis J, Khuri F, Ganly I, Arsenau J, Posner M, Vokes E, Kuhn J, McCarty T, Landers S, Blackburn A, Romel L, Randlev B, Kaye S, Kim D (2001) Phase II trial of intratumoral administration of ONYX-015, a replication-selective adenovirus, in patients with refractory head and neck cancer. J Clin Oncol 19:289–298
10. Fujimoto Y, Mizuno T, Sugita S, Goshima F, Kohno S et al (2006) Intratumoral injection of herpes simplex virus HF10 in recurrent head and neck squamous cell carcinoma. Acta Otolaryngol 126:1115–1117
11. Shultz LD, Schweitzer PA, Christianson SW, Gott B, Schweitzer IB et al (1995) Multiple defects in innate and adaptive immunologic function in NOD/LtSz-scid mice. J Immunol 154:180–191
12. Juan TY, Roffler SR, Hou HS, Huang SM, Chen KC, Lee YL, Prijovich ZM, Yu CP, Wu CC, Sun GH, Cha TL (2009) Antiangiogenesis targeting tumor microenvironment synergizes glucuronide prodrug antitumor activity. Clin Cancer Res 15:4600–4611