In vitro assessment of triterpenoids NVX-207 and betulinyl-bis-sulfamate as a topical treatment for equine skin cancer

Lisa Annabel Weber, Anne Funtan, Reinhard Paschke, Julien Delarocque, Jutta Kalbitz, Jessica Meißner, Karsten Feige, Manfred Kietzmann, Jessika-Maximiliane V. Cavalleri

1 Clinic for Horses, University of Veterinary Medicine Hannover, Foundation, Hannover, Germany, 2 Biozentrum, Martin-Luther-University Halle-Wittenberg, Halle (Saale), Germany, 3 BioSolutions Halle GmbH, Halle (Saale), Germany, 4 Department of Pharmacology, Toxicology and Pharmacy, University of Veterinary Medicine Hannover, Foundation, Hannover, Germany, 5 Equine Internal Medicine, University Equine Clinic, University of Veterinary Medicine Vienna, Vienna, Austria

* Jessika.Cavalleri@vetmeduni.ac.at

Abstract

Equine sarcoid (ES) is the most prevalent skin tumor in equids worldwide. Additionally, aging grey horses frequently suffer from equine malignant melanoma (EMM). Current local therapies targeting these skin tumors remain challenging. Therefore, more feasible topical treatment options should be considered. In order to develop a topical therapy against ES and EMM, betulinyl-bis-sulfamate and NVX-207, derivatives of the naturally occurring betulin and betulinic acid, respectively, were evaluated for their antiproliferative (crystal violet staining assay), cytotoxic (MTS assay) and apoptotic (AnnexinV staining, cell cycle investigations) effects on primary ES cells, EMM cells and equine dermal fibroblasts in vitro. The more potent derivative was assessed for its in vitro penetration and permeation on isolated equine skin within 30 min and 24 h using Franz-type diffusion cells and HPLC analysis. Betulinyl-bis-sulfamate and NVX-207 inhibited the proliferation and metabolism in ES cells, EMM cells and equine dermal fibroblasts in vitro. The more potent derivative was assessed for its in vitro penetration and permeation on isolated equine skin within 30 min and 24 h using Franz-type diffusion cells and HPLC analysis. NVX-207 had superior anticancer effects compared to betulinyl-bis-sulfamate. Both compounds led to the externalization of phosphatidylserines on the cell membrane and DNA fragmentation, demonstrating that the effective mode of action was apoptosis. After 48 h of treatment with NVX-207, the number of necrotic cells was less than 2% in all cell types. Detected amounts of NVX-207 in the different skin layers exceeded the half-maximal inhibitory concentrations calculated by far. Even though data obtained in vitro are auspicious, the results are not unconditionally applicable to the clinical situation. Consequently, in vivo studies are required to address the antitumoral effects of topically applied NVX-207 in ES and EMM patients.
Introduction

The skin is the organ in horses most frequently affected by tumors [1]. With a reported occurrence ranging from 35 to 90% of all cutaneous neoplasms [2–4], the equine sarcoïd (ES) is the most prevalent skin cancer in equids worldwide [5–7]. The pathogenesis of this coat-color independent tumor of the fibroblasts has been linked to an infection with the bovine papillomavirus type 1 and 2 [8–10], trauma [11, 12], and a genetic predisposition [13, 14]. According to their gross appearance and clinical behavior, sarcoïds are classified into six types: Mild occult or verrucous tumors and more severe nodular, fibroblastic, mixed and malevolent lesions [12]. Even though non-metastasizing and mostly not life-threatening, their locally invasive growth and predilection sites (e.g. head, saddle girth area) can seriously impair the equid’s welfare and compromise the use and economic value of the animal [1]. Multiple treatment modalities for the ES are described in the literature (e.g. surgery, radiation, chemotheraphy, immunotherapy) but universal effectiveness is not given and recurrence rates are high [11, 15].

Topical therapies generally seem particularly feasible as they are noninvasive and applicable, even on treatment sites that are difficult to access. However, the results regarding the efficacy of the acyclovir cream often used for mild-type ES treatment are contradictory [16, 17] and imiquimod may temporarily cause severe local side effects [18]. In addition, although a variety of other topical treatment options exists, mainly anecdotal evidence of their success is reported [1, 15, 19, 20]. Therefore, the development of a novel topical treatment approach for ES should be considered to take advantage of the benefits of topical therapies.

The equine malignant melanoma (EMM) is a frequently occurring, sex-independent skin neoplasm with a high prevalence in grey horses older than 15 years of age [21–25]. Melanomas are melanocytic tumors which typically occur as nodular in glabrous cutaneous regions (e.g. ventral surface of the tail, perineum, anus, external genitalia) [22, 26]. The dominant age-related phenotype of greying and the predisposition to melanoma are associated with a mutation in intron 6 of the syntaxin-17 gene [27, 28]. Most of the tumors show a slow growth pattern over years, however, more than 60% become malignant and cause clinical problems due to enlargement und widespread metastases [29–31]. Treatment options reported with varying outcomes include systemic and local approaches, such as immunotherapy [32–34], cimetidine application [35, 36], radiation [37], surgery [38, 39], and chemotherapy with cisplatin alone [40, 41] or in combination with electrochemotherapy [42, 43]. Although effective in many cases, surgical excision can be challenging due to the unfavorable localization of the tumors and the intratumoral injection of the mutagenic and carcinogenic cisplatin is linked to strict safety rules [44]. Therefore, a more practical treatment option for early stages of EMM, for example, in the form of a cream, would be useful.

Promising substances for topical ES and EMM treatment could be triterpenoids, such as betulinic acid (BA) and its derivatives [45, 46]. Betulinic acid, the oxidation product of betulin, is a pentacyclic lupane-type triterpenoid and can be extracted from various botanical sources [47]. Since first studies proved BA’s antitumor activity against human melanoma and other malignancies in cell culture and animal models [48, 49], a plethora of scientific work has verified the wide range of its biological capabilities in vitro and in vivo [50, 51]. Treatment with BA induces apoptosis in cancer cells due to a direct effect on the mitochondria [52] independent of CD95 ligand/receptor interaction [49]. Alterations in the mitochondrial membrane potential mediate a cytochrome c and apoptosis-inducing factor release, which results in the cleavage of caspasers and nuclear disintegration [53, 54]. Furthermore, the generation of reactive oxygen species [49, 55], the subsequent mitogen-activated protein kinase activation [56] and the inhibition of eukaryotic topoisomerase I [57], endothelial-to-mesenchymal-transition [58] and angiogenesis [59, 60] are suggested as BA-mediated antitumoral properties. The anticancer
effects of BA against EMM cells and its potent permeation in isolated equine skin have recently been reported [45]. However, based on a classification for the cytotoxicity of triterpenes [61], the half-maximal inhibitory concentrations (IC\textsubscript{50}) of BA for EMM cells and other human and animal cancer cell lines are considered to be only moderate. In addition, the compounds’ hydrosolubility is limited, which reduces the opportunities of medicinal use mainly to topical applications [62]. A variety of synthetically modified derivatives have been synthesized in the past few decades to enhance the pharmacological properties of BA and the closely related compound betulin [62]. Among these are betulinyl-bis-sulfamate ((3\(β\))-Lup-20(29)-ene-3,28-diol, 3,28-disulfamate; BBS) [63] and NVX-207 (3-acetyl-betulinic acid-2-amino-3-hydroxy-2-hydroxymethyl-propanoate) [64], from which, especially the latter substance, shows a higher cytotoxicity in various human and canine cancer cell lines compared to the parent BA [64–66]. It has been demonstrated that NVX-207 induces apoptosis in EMM cells [66]. In addition, the compound has already been successfully tested in a clinical study with canine cancer patients [64]. Within the frame of pilot safety studies, NVX-207 was well tolerated when applied topically in eight healthy horses [67] or injected intralesionally in two horses affected by EMM [66].

The objectives of this study were (1) to investigate the betulin derivative BBS and BA derivative NVX-207 for their antiproliferative, cytotoxic and apoptotic effects on ES cells, EMM cells and equine dermal fibroblasts and (2) to assess the more potent derivative for its penetration and permeation on isolated equine skin \textit{in vitro} with the aim of developing a topical therapy for the ES and EMM.

**Material and methods**

**Evaluation of the anticancer effects of BBS and NVX-207 on equine melanoma cells and equine dermal fibroblasts**

Compounds. Biosolutions Halle GmbH (Halle/Saale, Germany) synthesized BBS and NVX-207. The compounds were dissolved in dimethyl sulfoxide (WAK-Chemie Medical GmbH, Steinbach, Germany) to achieve 20 mM stock solutions.

**Cells and culture conditions.** All cells used for the experiments originate from different horses. Primary EMM cells (MelDuWi) and primary equine dermal fibroblasts (PriFi1, PriFi2) belong to the cell culture stock of the Clinic for Horses, University of Veterinary Medicine Hannover, Foundation, Hannover, Germany. The cells were cultured as monolayers at 37°C in a humified atmosphere with 5% CO\textsubscript{2} and maintained in RPMI1640 cell culture medium with stable glutamine (Biochrom GmbH, Berlin, Germany) supplemented with 15% fetal bovine serum superior (Biochrom GmbH) and 1% penicillin and streptomycin (10,000 international units (I.U.)/mL /10,000 \(\mu\)g/mL, Biochrom GmbH). Primary ES cells sRGO1 and sRGO2 (kindly provided by Dr. Sabine Brandt, University of Veterinary Medicine Vienna, Vienna, Austria) and primary EMM cells eRGO1 (kindly provided by Dr. Barbara Pratscher, University of Veterinary Medicine Vienna, Vienna, Austria) were cultured as monolayers at 37°C in a humified atmosphere with 5% CO\textsubscript{2} and kept in Dulbecco’s modified Eagle’s high glucose w/ Glutamax (4.5 g/L) cell culture medium (GIBCO-Invitrogen, Thermofisher, Darmstadt, Germany) supplemented with 10% fetal bovine serum superior (Biochrom GmbH) and 1% Antibiotic-Antimycotic (100x; GIBCO-Invitrogen), containing penicillin (10,000 units/mL), streptomycin (10,000 \(\mu\)g/mL) and amphotericin B (25 \(\mu\)g/mL).

**Proliferation assay.** The proliferation assay was performed as published [45]. Briefly, a modified crystal violet staining assay (CVS) was carried out to investigate the antiproliferative effects of BBS and NVX-207 on primary equine cells. The cells were exposed to BBS and NVX-207 at nine different concentrations ranging from 1–100 \(\mu\)mol/L for 5, 24, 48 and 96 h.
Proliferation and cytotoxicity experiments for this cell type were performed only for 5, 24 and 48 h as even untreated sarcoid cells showed an altered growth behavior in 96 h experiments. Control cells were treated with medium only. The proportion of cells treated relative to untreated controls was determined by crystal violet staining and photometric absorbance measurement at the incubation time points mentioned above. Proliferation assays were performed in six to eight biological replicates with two technical replicates for each combination of cell type, incubation time and compound concentration.

**Cytotoxicity assay.** The cytotoxicity of the compounds was assessed by the CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) (Promega GmbH, Mannheim, Germany) as reported [45]. In brief, in order to reach cell confluence within 48 h, cells were seeded into 96-well plates in adequate densities (MelDuWi 30,000 cells/well; PriFi1, PriFi2, eRGO1 20,000 cells/well; sRGO1 and sRGO2 15,000 cells/well). Incubation times and concentrations of BBS and NVX-207 were applied in accordance with the CVS assay. The formazan dye generated by the metabolic active cells was quantified photometrically. Cytotoxicity assays were performed in six to nine biological replicates with two technical replicates for each combination of cell type, incubation time and compound concentration.

**Cell cycle investigations.** Approximately 7.5 × 10^5 cells (MelDuWi) and 1.0 × 10^6 cells (PriFri2 and sRGO2) were seeded in 25 cm^2 cell culture flasks. After 24 h of incubation, the medium was replaced with medium containing either BBS or NVX-207 at their respective double IC_{50} concentration (measured after 96 h by sulforhodamine B [SRB] assay, analogous to [66]; see S1 and S2 Appendices). Following 24 and 48 h of incubation, the cells were harvested by mild trypsinization and washed twice with phosphate-buffered saline (PBS) buffer (containing Mg^{2+} and Ca^{2+}). Cells (1.0 × 10^6) were fixed with ethanol (70%, -20˚C, for 24 h). After discarding the ethanol, the cells were washed in 1 mL PBS buffer (containing Mg^{2+} and Ca^{2+}) and were centrifuged. The cell pellet was resuspended in 1 mL of staining PBS buffer (containing Mg^{2+} and Ca^{2+}, 10 μg/mL RNAse [Thermofisher] and 15 μg/mL propidium iodide [Sigma-Aldrich, Munich, Germany]) and was incubated for 30 min at room temperature. Analyses were performed using the Attune® FACS machine (Life Technologies, Darmstadt, Germany) collecting data from the BL-2A channel. Each sample was measured in duplicate.

**Annexin V staining.** Approximately 7.5 × 10^5 cells (MelDuWi) and 1.0 × 10^6 cells (PriFri2 and sRGO2) were seeded in 25 cm^2 cell culture flasks. After 24 h of incubation, the medium was replaced with medium containing either BBS or NVX-207 at their respective double IC_{50} concentration (measured after 96 h). Following 24 and 48 h of incubation, cells were harvested by mild trypsinization and washed twice with PBS buffer (containing Mg^{2+} and Ca^{2+}). Cells (1.0 × 10^6) were resuspended in AnnexinV binding buffer (BioLegend®, San Diego, US) to a concentration of 1.0 × 10^6 cells/mL. Approximately 100,000 cells were stained with propidium iodide solution (3 mL, 1 mg/mL) and FITC AnnexinV solution (5 mL, BioLegend®) for 15 min in the dark at room temperature. After the addition of Annexin V binding buffer (400 mL), the suspension was analyzed using the Attune® FACS machine (Life Technologies). After gating for living cells, the data from detectors BL-1A and BL-3A were collected. A total of 20,000 events were collected from each sample and technical duplicates were measured.

**Diffusion of NVX-207 into equine skin**

**Test formulations.** Two different pharmaceutical formulations were provided by Skinomics GmbH, Halle, Germany, for in vitro permeation studies. Based on previous permeation studies with BA [45], test formulation 1 consisted of “Basiscreme DAC” (pharmaceutical
amphiphilic cream as published in the German Drug Codex) with 1% NVX-207 and 20% medium-chain triglycerides. The formulation was modified because of an inhomogenous distribution of NVX-207 in test formulation 1 (oily sediments and overall recovery rate < 50% in Franz-type diffusion cells (FDC) experiments): Test formulation 2 contained "Basiscreme DAC" with 1% NVX-207.

**Skin sample preparation and Franz-type diffusion cell experiments.** Skin from six horses was used for each FDC experiment. The skin from the lateral thorax was dissected at the Institute of Pathology, University of Veterinary Medicine Hannover, Foundation, Hannover, Germany, after euthanasia of the horses at the Clinic for Horses, University of Veterinary Medicine Hannover, Foundation, for reasons unrelated to the present study. Therefore, a prospective approval of the experiments by an animal research ethics committee was not required. Skin samples were stored at -20°C until use (maximum five months). Table 1 provides information about the sex, breed and age of the different equine skin donors. Further skin sample preparation and diffusion experiments were performed as reported [45]. Skin samples were incubated with test formulation 1 for 24 h and with test formulation 2 for 30 min and 24 h, respectively.

**Sample processing and NVX-207 quantification.** Following diffusion experiments, skin sample processing and NVX-207 quantification were performed as published with a few modifications [45]. In short, in order to determine the concentration of NVX-207 in different skin layers, skin samples were cut with a cryostat (CryoStar™ NX70 Cryostat, Thermofisher, Darmstadt, Germany) in slices parallel to the skin surface starting from the epidermal side. The first slice had a thickness of 10 μm and, therefore, included the stratum corneum with potential residues of the test formulation, which had not been removed with the cotton swab. The following slices were 20-μm thick. Because of the short incubation time in the experiments (30 min), slices were pooled at 5 × 20 μm to investigate the concentration of NVX-207/100 μm skin depth and, therefore, increase the possibility of finding amounts of NVX-207/100 μm above the detection limit (0.1 μg/mL). A higher permeation rate of the compound was expected for 24-h experiments and, therefore, the 20-μm slices were stored and analyzed separately up to a depth of 310 μm. The slices were then pooled at 5 × 20 μm until a depth of a maximal 910 μm was reached. The cryostat blade was cleaned with tissues soaked in 80% methanol between each cut. The quantity of NVX-207 was determined by an analytic high-performance liquid chromatography (HPLC) method. Reverse phase analysis was performed using an Agilent 1100 system (Agilent, Waldbronn, Germany) on a Luna™ Omega column (3 μm, PS C18, 100 Å, 150 x 4.6 mm; Phenomenex, Torrance, US) at 30°C using a gradient method with acetonitrile (0.1% HCOOH)(A):water (0.1% HCOOH)(B) at 1.1 mL/min, (from 60 to 10% B within 7.50 min). The diode array detector was set at 200 nm.

**Statistical analysis**

Technical duplicates with a coefficient of variation of more than 20% were excluded from the cell assay analysis. IC_{50} values of BBS and NVX-207 from the proliferation and cytotoxicity

Table 1. Information about the equine skin donors used for Franz-type diffusion cell experiments.

| Incubation time | Number of horses | Sex | Breed | Median age in years (range min-max) |
|-----------------|------------------|-----|-------|-----------------------------------|
| 30 min          | 6                | 3 mares, 2 geldings, 1 unknown | 1 Hanoverian Warmblood, 1 Icelandic horse, 1 Arabian horse, 1 Clydesdale, 2 unknown | 19 (4–23) |
| 24 h            | 6                | 2 mares, 4 geldings           | 2 Warmblood horses, 1 Hanoverian Warmblood, 1 Holsteiner Warmblood, 1 Arabian horse, 1 Icelandic horse | 16 (6–25) |

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tests were calculated with the pharmacodynamic model 108 of Phoenix® WinNonlin® software (version 8.1, Certara, USA). Additional statistical data analysis was conducted with R 3.5.1. [68]. A generalized additive model was fitted for each test (MTS and CVS) and cell type comparison (primary EMM cells and fibroblasts, and primary ES cells and fibroblasts) using the ’mgcv’ package [69]. Compound concentrations and the duration of incubation were modeled as tensor product smooth interacting with compound and cell line. Cell passage was added as a random effect. An appropriate distribution was selected by the visual inspection of residuals. The p-values were obtained by performing a Wald test for each parameter. Statistical significance was set at 0.05.

Results

Proliferation inhibition and cytotoxicity of BBS and NVX-207 on equine cells

The antiproliferative and cytotoxic effects of NVX-207 and BBS on ES cells, EMM cells and equine dermal fibroblasts were assessed by the CVS and MTS assay. In general, both compounds had significant inhibitory effects on cell proliferation (p < 0.001 in CVS assay for every cell type) and cell viability (p < 0.001 in MTS assay for every cell type) compared to untreated controls. However, effects on the cells were dose- and time-dependent. Figs 1 and 2 show the results from the melanoma cell model. Results of the sarcoid cell model are attached as additional files S3 and S4 Appendices. First significant, dose-dependent antiproliferative effects on ES cells, EMM cells and fibroblasts were observed after 24 h of incubation with BBS and after 5 h of incubation with NVX-207. A significant, dose-dependent reduction in cell viability was observed in ES cells, EMM cells and fibroblasts after 5 h of treatment with BBS and NVX-207.

As assessed by determination of IC<sub>50</sub> values (Table 2) NVX-207 was more active against the investigated equine cells compared to BBS. When the cells were exposed to BBS for 5 h, the quantity of cells affected was too low to calculate the IC<sub>50</sub> values in both cytotoxicity and proliferation assays. After 48 h, NVX-207 exceeded BBS's antiproliferative effects about 23 and 29 times in ES cells sRGO1 and sRGO2, respectively, about 25 and 3 times in EMM cells eRGO1 and MelDuWi, respectively, and about 23 and 6 times in fibroblasts PriFi1 and PriFi2, respectively. NVX-207 was about 11 (sRGO1), 25 (sRGO2), 8 (eRGO1), 3 (MelDuWi), 34 (PriFi1) and 9 (PriFi2) times more cytotoxic than BBS.

Selectivity of both compounds towards the different cells varied. Compared to normal fibroblasts, BBS showed a selectivity to both sarcoid and EMM cells in the proliferation assay and a selectivity to eRGO1 and both sarcoid cell types in the cytotoxicity assay. Sarcoid cells were more sensitive to BBS than EMM cells. Normal fibroblasts did not show a better tolerance towards NVX-207 compared to EMM cells; by contrast, MelDuWi were revealed to be more resistant in both assays. A selectivity of NVX-207 towards fibroblasts was observed in the proliferation assay for sarcoid cells.

Cell cycle investigations. The cell death mechanisms of NVX-207 and BBS on ES cells, EMM cells and equine dermal fibroblasts were assessed by cell cycle investigations via flow cytometry. Condensation of chromatin and fragmentation of DNA and nuclei occurs in apoptotic cells, which can be detected by the SubG1 peak. In comparison to untreated cells (control), the treatment with BBS and NVX-207 caused an increase of subG1 cells after 48 h of treatment for all equine cells (Fig 3 and S5–S12 Appendices). The subG1 peak for the EMM cells MelDuWi arose after a treatment of 48 h to more than 40% for BBS and more than 60% for NVX-207. The equine dermal fibroblasts PriFi2 also showed an increased numbers of subG1 cells (> 80%) after 48 h of treatment with NVX-207 but only 14% after 48 h of
treatment with BBS (Fig 4 and S12 Appendix). Thus, a selectivity of BBS for the initiation of
the preferably programmed cell death in EMM cells could be shown. The effect of both active
substances on the sarcoid cells was noticeably lower compared to the other cell lines. After a
treatment time of 48 h, an enrichment of 20% subG1 cells was present.

AnnexinV staining. The externalization of phosphatidylserine s to the extracellular side of
the plasma membrane is a characteristic and early event in apoptosis [70, 71]. The change of
the extracellular plasma membrane composition was detected by using AnnexinV-FITC/ (pro-
pidium iodide) staining and analysis by flow cytometry (Figs 5 and 6 and S13–S20 Appendi-
ces). Untreated cells were used for control. After a treatment period of 24 h with BBS, 19%
of the sarcoid cells were early apoptotic and 39% were late apoptotic, while 2% of the control
cells were early and 14% were late apoptotic. After 48 h, the number of apoptotic cells further
increased and approximately 90% of the cells were apoptotic (8% early apoptotic; 82% late apo-
ptotic). NVX-207 had a weaker effect on the sarcoid cells and 40% were present as living cells
after 48 h. The equine dermal fibroblasts showed a slower increase of apoptotic cells after 24 h
of treatment with BBS. In this case, increases of 5% early apoptotic and 3% late apoptotic cells

Fig 1. Effects of betulinyl-bis-sulfamate and NVX-207 on primary equine cell proliferation at different time points. Proliferation dose-response
regression lines of betulinyl-bis-sulfamate (BBS) and NVX-207 on primary equine malignant melanoma (EMM) cells (eRGO1, MelDuWi) and
primary equine dermal fibroblasts (PriFi1, PriFi2) at four different time points (5, 24, 48 and 96 h) determined by CVS assay. Antiproliferative effects
of the compounds on primary equine cells increase with the concentration and time of drug exposition. Data represent regression lines and 95%
confidence intervals of 6–8 independent experiments for each combination of cell type, incubation time and concentration. Concentrations at which
the corresponding 95% confidence intervals do not cross the 100% line indicate a significant reduction of the proliferation rate.

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were present. However, an increase in late apoptotic cells after 48 h was observed (54% by treatment with BBS and 67% with NVX-207). Thus, it was shown that BBS had a better selectivity to sarcoid cells compared to fibroblasts.

Compared to BBS, NVX-207 had the stronger potential to induce apoptosis in EMM cells. After 48 h, 45% were late apoptotic cells and only 30% were living cells. In addition, 25% of cells were present in the early apoptotic phase. It was proven for all three equine cell lines that the necrosis rate after 48 h of treatment with NVX-207 was below 2%.

**Diffusion of NVX-207 into equine skin and overall NVX-207 recoveries**

When the skin samples were treated with test formulation 2 for 30 min, NVX-207 was detected in both the epidermis and dermis (Fig 7). An incubation time of 24 h led to an accumulation of the compound in the upper epidermis (11–30 μm) but did not increase the amount of NVX-207 in the other skin layers analyzed (Fig 7 and S21 Appendix). The detected concentrations exceeded the 24 h \( IC_{50} \) values of NVX-207 for ES cells, EMM cells and equine dermal fibroblasts determined in the proliferation and cytotoxicity assays even in the deeper skin layers examined (up to a depth of 810 μm). The overall NVX-207 recovery rate after 30 min of incubation was 89 ± 23% (mean ± SD; n = 6), from which 68 ± 18% of the substance was detected in the non-permeated proportion (cotton swabs) and 28 ± 17% in the skin. The overall recovery rate of NVX-207 in test formulation 2 after 24 h of incubation was 85 ± 14% (mean ± SD; n = 6). A quantity of 51 ± 9% of the NVX-207 amount applied was found in the
Table 2. IC$_{50}$ values (µmol/L) of betulinyl-bis-sulfamate (BBS) and NVX-207 for primary equine cells determined by CVS and MTS assay after 5, 24, 48 and 96 h of drug exposure.

| Cells   | CVS   | MTS   | CVS   | MTS   |
|---------|-------|-------|-------|-------|
| sRGO1   | -     | -     | 7 (5–10) | 9 (7–11) |
| sRGO2   | -     | -     | 6 (4–8)  | 8 (6–11) |
| eRGO1   | -     | -     | 10 (7–13) | 9 (4–15) |
| MelDuWi | -     | -     | 20 (13–26) | -     |
| PriFi1  | -     | -     | 11 (2–23) | 11 (9–13) |
| PriFi2  | -     | -     | 14 (4–24) | 20 (18–22) |

| Cells   | CVS   | MTS   | CVS   | MTS   |
|---------|-------|-------|-------|-------|
| sRGO1   | 40 (31–49) | -     | 7 (5–10) | 4 (2–5) |
| sRGO2   | 38 (33–43) | 45 (40–49) | < 1 (0–2) | 3 (2–4) |
| eRGO1   | 42 (36–48) | 47 (37–57) | 5 (3–7) | 7 (4–15) |
| MelDuWi | 50 (38–61) | 60 (30–91) | 16 (11–21) | 18 (15–21) |
| PriFi1  | 52 (41–62) | 59 (50–68) | 4 (2–6) | 4 (2–5) |
| PriFi2  | 62 (48–76) | 77 (35–118) | 8 (4–12) | 7 (5–9) |

| Cells   | CVS   | MTS   | CVS   | MTS   |
|---------|-------|-------|-------|-------|
| sRGO1   | 23 (16–31) | 25 (21–30) | < 1 (0–1) | 2 (1–4) |
| sRGO2   | 29 (19–31) | 28 (21–35) | < 1 (0–8) | 1 (1–2) |
| eRGO1   | 25 (7–44) | 32 (26–38) | < 1 (0 < < 1) | 4 (1–7) |
| MelDuWi | 36 (26–46) | 53 (41–65) | 12 (6–18) | 15 (12–19) |
| PriFi1  | 42 (32–51) | 35 (31–39) | 2 (1–3) | 1 (1–2) |
| PriFi2  | 39 (32–46) | 61 (48–74) | 7 (<0–15) | 7 (<1–7) |

| Cells   | CVS   | MTS   | CVS   | MTS   |
|---------|-------|-------|-------|-------|
| sRGO1   | n.a.  | n.a.  | n.a.  | n.a.  |
| sRGO2   | n.a.  | n.a.  | n.a.  | n.a.  |
| eRGO1   | 15 (5–25) 0.04 | 16 (13–18) | < 1 (<0–<1) | < 1 (<0–<1) |
| MelDuWi | 16 (4–29) | 32 (15–49) | 4 (3–5) | 8 (5–10) |
| PriFi1  | 17 (15–20) | 28 (26–31) | < 1 (<0–<1) | < 1 (<0–<1) |
| PriFi2  | 16 (11–21) | 20 (11–28) | < 1 (<0–<1) | 4 (<1–7) |

Antiproliferative (CVS assay) and cytotoxic (MTS assay) effects of BBS and NVX-207 on primary ES cells (sRGO1 and sRGO2), primary EMM cells (eRGO1 and MelDuWi) and primary equine dermal fibroblasts (PriFi1 and PriFi2) after a treatment duration of 5, 24, 48 or 96 h. Data represent mean IC$_{50}$ values (µmol/L) of 6–9 independent experiments with 95% confidence interval in parentheses. “-” = Quantity of cells affected was too low to calculate IC$_{50}$ values with the software applied; “n.a.” = data not available.
cotton swabs and 32 ± 12% of the NVX-207 amount applied was detected in the skin. No NVX-207 was detected in the acceptor medium in any of the FDC experiments.

Discussion

The ES is the dermatologic neoplasm in equids diagnosed most frequently. The EMM is also a common skin tumor, especially in aging grey horses. In order to develop a topical therapy
against the ES and EMM, the betulinic acid derivative NVX-207 and the betulin derivative BBS were assessed for their antiproliferative, cytotoxic and apoptotic effects on ES cells, EMM cells and fibroblasts in vitro. Both substances had significant anticancer effects on the cells and induced apoptosis. NVX-207 was revealed to be the more potent substance. Therefore, this

![Fig 5. AnnexinV staining of ES cells sRGO2.](https://doi.org/10.1371/journal.pone.0241448.g005)

![Fig 6. AnnexinV staining of equine cells.](https://doi.org/10.1371/journal.pone.0241448.g006)

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The compound NVX-207 was previously assessed for its cytotoxic effects on EMM cells "MelDuWi" with the sulforhodamine B assay and a 96-h IC$_{50}$ value of 5.6 μmol/L was reported [66]. Results of this first study on EMM cells "MelDuWi" could be replicated in the present study with different methodological approaches (CVS and MTS assay to assess the antiproliferative and cytotoxic effects, respectively) and widened by investigations with EMM cells "eRGO1," ES cells "sRGO1" and "sRGO2," and equine dermal fibroblasts "PriFi1" and "PriFi2." Three further treatment time points (5, 24 and 48 h) were included in the experiments to provide more information about the time-dependent efficacy of the drug. It was demonstrated that the antiproliferative and cytotoxic effects on ES cells, EMM cells and dermal fibroblasts enhanced with an increased treatment duration in a dose-dependent manner. After 48 and 96 h, very low concentrations of NVX-207 were sufficient to observe an inhibitory effect on the cells’ proliferation and survival rate (e.g. for EMM cells eRGO1 < 1 μmol/L in CVS and MTS assay after 96 h of incubation). In addition, after 5 h of drug exposure, the quantity of affected cells was already high enough to calculate IC$_{50}$ values, substantiating the potent effects of NVX-207 on equine cells. These data could be taken into account when prospective in vivo treatment regimens are designed.
This study is the first report on the influence of NVX-207 on ES cells and normal equine dermal fibroblasts. NVX-207 had cell viability reducing and antiproliferative effects on both cell types. The similar treatment response of the cells is not surprising, as the ES is addressed as a tumor of the fibroblasts [12]. However, compared to normal equine cells, a selectivity of the compound to ES cells could be demonstrated in the proliferation assay, suggesting that sarcoioid cells are even more sensitive. A selectivity of NVX-207 to EMM cells was not observed. The same was shown for EMM cells and fibroblasts when treated with the parent BA [45]. In contrast to these findings, it was reported that NVX-207 had little impact on the in vitro survival of normal human umbilical vein endothelial cells, fibroblasts and keratinocytes [64]. Furthermore, current in vivo data indicate a good systemic and local tolerability of 1% NVX-207 after topical application twice a day for seven consecutive days in eight healthy horses [67]. In addition, the intralesional injection of the compound in two EMM patients once a week for 19 consecutive weeks proved to be safe [66]. Intravenous application of the compound in mice did not lead to any side effects [64] and the intralesional treatment of different malignancies in five dogs was well tolerated and clinically beneficial tumor response was observed [64].

It has been demonstrated previously that NVX-207 triggers the mitochondrial-induced apoptotic pathway in human melanoma cell lines via activation of caspases-9, -3 and -7 and cleavage of poly (ADP-ribose) polymerase [64]. Furthermore, an increase of subG1 cells after treatment of various human cancer cell lines with BA and NVX-207 has been reported [72, 73]. An induction of both initiator caspases (caspase-8 and caspase-9) in EMM cells led to an activation of effector caspase-3 [66]. Comparable to a treatment with the parent BA, an accumulation of EMM cells in the subG1 phase and externalization of phosphatidylserines to the extracellular side of the plasma membrane, a characteristic feature of apoptosis, were observed after treatment with NVX-207 [66]. These preliminary investigations by Liebscher et al. on EMM cells MelDuWi could be reproduced in this study. However, up to now, no data on the molecular mechanisms in ES cells and normal equine cells after treatment with NVX-207 has existed. Cell cycle investigations and AnnexinV staining were performed to address this lack. Results from these apoptosis tests demonstrated that NVX-207 triggers apoptosis in ES cells sRGO2. However, the effects were less pronounced compared to EMM cells MelDuWi and equine dermal fibroblasts PriFi2. After 48 h, the number of apoptotic cells detected with AnnexinV staining was about 60%, of which 48% were late apoptotic. Only 17% of the cells were found to be in the subG1 phase with a fragmented DNA. The different quantity of apoptotic cells analyzed with different methods may be explained by the temporally staggered occurrence of characteristic cellular changes, which are made visible by the respective method. The results reported here further indicate that similar modes of action observed in equine cancer cells also take place in unaltered equine cells when treated with NVX-207. After an incubation of 48 h, about 85% of equine dermal fibroblasts PriFi2 were apoptotic and a clear shift to cells in the subG1 phase was already observed after 24 h of treatment. It is remarkable that the proportion of necrotic cells, whether in altered or normal cell types, was below 2% after a treatment of 48 h. Even though the results reported from in vitro experiments with equine skin cancer cells are promising, it must be emphasized that cells in their native microenvironment can be much more robust against (phyto)chemotherapeutic influences [74–76]. Therefore, no reliable conclusions can be drawn regarding the efficacy of a topical NVX-207 application in ES and EMM patients and prospective in vivo studies have made to address this question.

The betulin derivative BBS had significant antiproliferative and cytotoxic effects on all three cell types investigated in the present study, however, it was considerably less effective compared to NVX-207. In addition, the IC50 values of BBS calculated for EMM cells were higher than the ones reported for BA [45, 66]. Therefore, further permeation studies were performed with NVX-207. Nevertheless, in contrast to NVX-207, the compound was less toxic for normal
cells. In order to clarify the cellular pathways of BBS in ES cells, EMM cells and equine dermal fibroblasts, it was shown by AnnexinV staining and cell cycle investigations that BBS induced apoptosis in these cells. However, while the apoptotic impact of BBS was stronger in sarcoid cells compared to the effects of NVX-207 in these equine skin cancer cells, this was not the case for EMM cells and fibroblasts. In EMM cells, the amount of late apoptotic cells after 48 h was 44.8% (NVX-207) compared to 28.2% (BBS). Regarding the results from the proliferation test and the cell cycle analysis, there seems to be a selectivity of BBS towards equine skin cancer cells in comparison to unaltered equine dermal fibroblasts. In addition, AnnexinV staining revealed a preferred triggering of the programmed cell death for the sarcoid cells (82.1% late apoptotic after a 48-h treatment with BBS) when compared to the late apoptotic phase of fibroblasts (53.6%). However, only 28.2% of EMM cells were late apoptotic at this stage.

In addition to its apoptotic effects, it should be noted that BBS has been demonstrated to be an efficient inhibitor of human carbonic anhydrase isoenzymes I, II and IX [63]. Carbonic anhydrase IX is overexpressed in many tumors and involved in complex pathways leading to changes in the tumor microenvironment and subsequent tumor progression [77]. Human malignant melanoma cells also express this enzyme and a combination of proton pump and carbonic anhydrase IX inhibitors led to enhanced anticancer effects in these cells in vitro [78]. Further investigations are necessary to confirm and expand these results in equine malignancies, however, carbonic anhydrase inhibitors such as BBS could represent potential candidates as anti-tumor agents alone or adjunctive therapeutic drugs.

Except for ulcerated tumors, histopathologic examinations address the localization of melanocytic skin tumors in horses mostly as “dermal” or “subcutaneous” [79, 80]. The ES is regarded as a neoplasm of the dermal fibroblasts, which appear with an increased density and proliferation [81, 82]. Epidermal alterations, such as hyperplasia, hyperkeratosis or rete pig formation, vary between the different clinical ES types but are present in the majority of cases [82]. Due to the tumors’ microscopic appearance, the topically applied compound NVX-207 needs to liberate from the drug formulation, penetrate the body protective stratum corneum and permeate through the viable epidermal and dermal strata to reach the sarcoid and melanoma cells. A standardized use of ES or EMM skin was not possible for FDC experiments due to technical reasons, which is a limitation of the study. Therefore, normal thoracic equine skin was utilized, as described previously [45, 83].

It has been reported previously that high concentrations of BA could be reached in isolated equine skin when 1% of the compound was mixed in “Basiscreme DAC” with 20% medium-chain triglycerides [45]. Therefore, a drug formulation containing “Basiscreme DAC” with 20% medium-chain triglycerides and 1% of betulinic acid derivative NVX-207 (test formulation 1) was initially tested for in vitro permeation. A significant phase separation was already observed 24 h after the production of test formulation 1. The oily sediments were probably the 20% medium-chain triglycerides added, which coalesced as the emulsifier system combined with 1% NVX-207 was presumably not strong enough to form a stable emulsion with the additional fatty acids. The inhomogeneous distribution of NVX-207 suspected in test formulation 1 was confirmed when less than 50% of the substance, which had already been applied on the diffusion area, was detected in the HPLC analysis. The drug formulation was improved as such a low recovery rate in permeation studies and such high variations of active compound distribution in the cream are not acceptable for a topical medication. When 1% NVX-207 was mixed with “Basiscreme DAC” but without additional medium-chain triglycerides (test formulation 2), no phase separation was observed by visual inspection and the overall recovery rate was above 85%.

There was a nearly identical concentration profile of the compound in isolated equine skin when incubated for 30 min and 24 h, except for a considerable difference in the upper
epidermal layers. This indicates a rapid penetration of the lipophilic NVX-207 through the stratum corneum and accumulation in the viable epidermal skin layers, followed by a slower permeation into the subjacent, more hydrophilic dermal skin layers [84, 85]. As the blood circulation in in vitro FDC experiments is missing, no compound is absorbed by dermal capillary blood vessels, which could further explain the steady state between the 30-min and 24-h permeation studies. Regarding the in vitro data determined about antiproliferative and cytotoxic effects of NVX-207 towards ES cells and EMM cells reported here and formerly for EMM cells [66], the concentrations of the compound reached up to a depth of 810 μm in isolated equine skin after 30 min and 24 h of incubation in vitro would be sufficient to have an inhibitory or even cytotoxic impact on the cells’ metabolism. This might suggest that the proliferation and survival rate of ES and EMM cells especially in the superficial dermal skin layer could be reduced by NVX-207 in vivo. However, as mentioned previously, the epidermal nature in ES varies and epidermal thickening can influence the permeation rate of a topically applied drug negatively [86]. Furthermore, it should be considered that the in vitro permeation of acyclovir in ES skin differs significantly from epidermal to superficial dermal and deep dermal skin layers and that less acyclovir was found in the deep dermal layers of sarcoid skin compared to normal skin [87]. By contrast, the in vitro concentration profiles of NVX-207 in thoracic skin and hairless EMM predilection site skin (e.g. undersurface on the tail, perianal region) can be assumed to be comparable, as the concentrations of hydrocortisone, a lipophilic substance similar to NVX-207, did not differ significantly in the clipped thoracic equine skin and nearly glabrous groin skin [88]. However, an increased vascularization was described in some EMM [31, 79]. Compound elimination by dermal blood vessels cannot be evaluated by FDC experiments and, therefore, the permeated dose required to exert antitumoral effects in vivo can also be significantly higher. Furthermore, an encapsulation of the tumor could reduce the drug permeation rate at the treatment site. Because the in vitro anticancer effects were demonstrated to be concentration- and time-dependent, prospective in vivo treatment regimens with short application intervals and long treatment durations could favorably influence the concentration and efficacy of NVX-207 in the skin of ES and EMM patients.

Conclusion

In conclusion, the betulinic acid derivative NVX-207 has a superior antiproliferative and cell viability reducing effect on primary ES cells and EMM cells compared to BBS. Both compounds induced apoptosis. High concentrations of NVX-207 were reached in isolated equine skin—even after only 30 min of incubation—demonstrating a potent skin permeation. Although the in vitro data reported are promising, the results are not unconditionally applicable to the clinical situation. Therefore, in vivo studies are needed to assess the antitumoral effects of topically applied NVX-207 in equine patients suffering from ES or EMM.

Supporting information

S1 Appendix. IC_{50} values measured by SRB Assay after 96 h. IC_{50} values (μmol/L) of betulinyl-bis-sulfamate (BBS) and NVX-207 thereof on three equine cell types (equine sarcoid [ES] cells sRGO2, equine malignant melanoma [EMM] cells MelDuWi and equine dermal fibroblasts PriFi2) determined by SRB-Assay after 96 h of drug exposure. Measurements were carried out at least as thrice determination.

(SDOCX)

S2 Appendix. Cytotoxicity dose-response curves of BBS and NVX-207. ES cells sRGO2 (left), EMM (middle) and equine fibroblasts PriFi2 (right) determined by SRB Assay after 96
h (one representative of three independent experiments).

**S3 Appendix. Effects of BBS and NVX-207 on primary equine cell proliferation at different time points.** Proliferation dose-response regression lines of BBS and NVX-207 on primary ES cells (sRGO1, sRGO2) and primary equine dermal fibroblasts (PriFi1, PriFi2) at three different time points (5, 24 and 48 h) determined by crystal violet staining assay. Antiproliferative effects of the compounds on primary equine cells increase with concentration and time of drug exposition. Data represent regression lines and 95% confidence intervals of 6–8 independent experiments for each combination of cell type, incubation time and concentration. Concentrations at which the corresponding 95% confidence intervals do not cross the 100% line indicate a significant reduction of the proliferation rate.

**S4 Appendix. Effects of BBS and NVX-207 on primary equine cell viability at different time points.** Proliferation dose-response regression lines of BBS and NVX-207 on primary ES cells (sRGO1, sRGO2) and primary equine dermal fibroblasts (PriFi1, PriFi2) at three different time points (5, 24 and 48 h) determined by MTS assay. Cytotoxic effects of the compounds on primary equine cells increase with concentration and time of drug exposition. Data represent regression lines and 95% confidence intervals of 6–8 independent experiments for each combination of cell type, incubation time and concentration. Concentrations at which the corresponding 95% confidence intervals do not cross the 100% line indicate a significant reduction of the cell viability rate.

**S5 Appendix. Cell cycle distributions of ES cells sRGO2.** Cells were untreated (control) or treated with BBS and NVX-207 at their double IC$_{50}$ concentrations for 24 and 48 h (as indicated). The DNA was stained with propidium iodide and the cells were analyzed by flow cytometry. Red: SubG1 peak; light blue: G1/G0 phase peak; Yellow: S-phase peak; and dark blue: G2/M phase.

**S6 Appendix. Cell cycle percentage of ES cells sRGO2.** Cells were untreated (control) or treated with BBS and NVX-207 at their double IC$_{50}$ concentrations for 24 h.

**S7 Appendix. Cell cycle percentage of ES cells sRGO2.** Cells were untreated (control) or treated with BBS and NVX-207 at their double IC$_{50}$ concentrations for 48 h.

**S8 Appendix. Cell cycle distributions of EMM cells MelDuWi.** Cells were untreated (control) or treated with BBS and NVX-207 at their double IC$_{50}$ concentrations for 24 and 48 h (as indicated). The DNA was stained with propidium iodide and the cells were analyzed by flow cytometry. Red: SubG1 peak; light blue: G1/G0 phase peak; Yellow: S-phase peak; and dark blue: G2/M phase.

**S9 Appendix. Cell cycle percentage of EMM MelDuWi.** Cells were untreated (control) or treated with BBS and NVX-207 at their double IC$_{50}$ concentrations for 24 h.

**S10 Appendix. Cell cycle percentage of EMM MelDuWi.** Cells were untreated (control) or treated with BBS and NVX-207 at their double IC$_{50}$ concentrations for 48 h.
S11 Appendix. Cell cycle percentage of equine dermal fibroblasts PriFri2. Cells were untreated (control) or treated with BBS and NVX-207 at their double IC₅₀ concentrations for 24 h. (DOCX)

S12 Appendix. Cell cycle percentage of equine dermal fibroblasts PriFri2. Cells were untreated (control) or treated with BBS and NVX-207 at their double IC₅₀ concentrations for 48 h. (DOCX)

S13 Appendix. AnnexinV staining. Percentage of ES cells sRGO2 untreated (control) or treated with BBS and NVX-207 at their double IC₅₀ concentrations for 24 h. (DOCX)

S14 Appendix. AnnexinV staining. Percentage of ES cells sRGO2 untreated (control) or treated with BBS and NVX-207 at their double IC₅₀ concentrations for 48 h. (DOCX)

S15 Appendix. AnnexinV staining of equine dermal fibroblasts PriFri2. Cells were untreated (control) or treated with BBS and NVX-207 at their double IC₅₀ concentrations for 24 and 48 h (as indicated). After harvesting, the cells were stained and flow cytometry analysis was performed. Red: necrotic cells; green: late apoptotic cells; blue: early apoptotic cells; magenta: living cells. (PNG)

S16 Appendix. AnnexinV staining. Percentage of equine dermal fibroblasts PriFri2 untreated (control) or treated with BBS and NVX-207 at their double IC₅₀ concentrations for 24 h. (DOCX)

S17 Appendix. AnnexinV staining. Percentage of equine dermal fibroblasts PriFri2 untreated (control) or treated with BBS and NVX-207 at their double IC₅₀ concentrations for 48 h. (DOCX)

S18 Appendix. AnnexinV staining of EMM cells MelDuWi. Cells were untreated (control) or treated with BBS and NVX-207 at their double IC₅₀ concentrations for 24 and 48 h (as indicated). After harvesting, the cells were stained and flow cytometry analysis was performed. Red: necrotic cells; green: late apoptotic cells; blue: early apoptotic cells; magenta: living cells. (PNG)

S19 Appendix. AnnexinV staining. Percentage of EMM cells (MelDuWi) untreated (control) or treated with BBS and NVX-207 at their double IC₅₀ concentrations for 24 h. (DOCX)

S20 Appendix. AnnexinV staining. Percentage of EMM cells (MelDuWi) untreated (control) or treated with BBS and NVX-207 at their double IC₅₀ concentrations for 48 h. (DOCX)

S21 Appendix. Concentration profile of NVX-207 correlative to skin thickness after 24 h of incubation. The skin of six horses (two technical replicates each) were used to investigate the permeation of 1% NVX-207 in “Basiscreme DAC” within 24 h for the Franz-type diffusion cell experiment. The concentration of the compound was determined in 20 μm and 100 μm (deeper skin layers; pooled at 5 × 20 μm) cryostat skin slices at different skin depths by HPLC analysis. Figure data represent mean concentration of NVX-207 at the skin depth indicated and ± SD. Data for 10-μm skin depth (stratum corneum) with potential test compound.
residues were excluded from this figure.

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Author Contributions
Conceptualization: Reinhard Paschke, Jessica Meißner, Manfred Kietzmann, Jessika-Maximilian V. Cavalleri.
Formal analysis: Lisa Annabel Weber, Anne Funtan, Julien Delarocque.
Funding acquisition: Reinhard Paschke, Karsten Feige, Jessika-Maximilian V. Cavalleri.
Investigation: Lisa Annabel Weber, Anne Funtan, Jutta Kalbitz.
Methodology: Jutta Kalbitz.
Project administration: Lisa Annabel Weber, Reinhard Paschke, Jessika-Maximilian V. Cavalleri.
Supervision: Reinhard Paschke, Jessica Meißner, Karsten Feige, Manfred Kietzmann, Jessika-Maximilian V. Cavalleri.
Visualization: Lisa Annabel Weber, Anne Funtan, Julien Delarocque.
Writing – original draft: Lisa Annabel Weber.
Writing – review & editing: Lisa Annabel Weber, Anne Funtan, Reinhard Paschke, Manfred Kietzmann.

References
1. Scott DW, Miller WH. Equine dermatology. 2nd ed. Maryland Heights: Elsevier Saunders; 2011.
2. Scott D, Miller W. Equine dermatology I. S. Louis; 2003.
3. Valentine B. Survey of equine cutaneous neoplasia in the Pacific Northwest. J Vet Diagnostic Investig. 2006; 18:123–126. https://doi.org/10.1177/104063870601800121 PMID: 16566271
4. Goodrich L, Gerber H, Marti E, Antczak DF. Equine sarcoids. Vet Clin North Am Equine Pract. 1998; 14:607–623. https://doi.org/10.1016/S0749-0739(17)30189-X PMID: 9891727
5. Pascoe RR, Summers PM. Clinical survey of tumours and tumour-like lesions in horses in south east Queensland. Equine Vet J. 1981; 13:235–239. https://doi.org/10.1111/j.2042-3306.1981.tb03504.x PMID: 6459231
6. Baker JR, Leyland A. Histological survey of tumours of the horse, with particular reference to those of the skin. Vet Rec. 1975; 96:419–422. https://doi.org/10.1136/vet.96.19.419 PMID: 1173477
7. Marti E, Lazary S, Antczak DF, Gerber H. Report of the first international workshop on equine sarcoid. Equine Vet J. 1993; 25:397–407. https://doi.org/10.1111/j.2042-3306.1993.tb02981.x PMID: 8223371
8. Chambers G, Ellsmore VA, O’Brien PM, Reid SWJ, Love S, Campo MS, et al. Association of bovine papillomavirus with the equine sarcoid. J Gen Virol. 2003; 84:1055–1062. https://doi.org/10.1099/vir.0.18947-0 PMID: 12692268
9. Yuan ZQ, Gault EA, Saveria Campo M, Nasir L. Different contribution of bovine papillomavirus type 1 oncoproteins to the transformation of equine fibroblasts. J Gen Virol. 2011; 92:773–783. https://doi.org/10.1099/vir.0.028191-0 PMID: 21177927
10. Martens A, De Moor A, Ducatelle R. PCR detection of bovine papilloma virus DNA in superficial swabs and scrapings from equine sarcoids. Vet J. 2001; 161:280–286. https://doi.org/10.1053/tvjl.2000.0524 PMID: 11352485
11. Hainisch EK, Brandt S. Equine Sarcoid. Seventh Ed. Elsevier Inc.; 2014. https://doi.org/10.1016/B978-1-4557-4555-5.00099-6 PMID: 24467610
12. Knottenbelt DC. A suggested clinical classification for the equine sarcoid. Clin Tech Equine Pract. 2005; 4:278–295. https://doi.org/10.1053/j.ctep.2005.10.008.
13. Staiger EA, Tseng CT, Miller D, Cassano JM, Nasir L, Garrick D, et al. Host genetic influence on papillomavirus-induced tumors in the horse. Int J Cancer. 2016; 139:784–792. https://doi.org/10.1002/ijc.30120 PMID: 27037728
14. Angelos J, Oppenheim Y, Rebhun W, Mohammed H, Antczak DF. Evaluation of breed as a risk factor for sarcoid and uveitis in horses. Anim Genet. 1988; 19:417–425. https://doi.org/10.1111/j.1365-2052.1988.tb00833.x PMID: 3232865
15. Knottenbelt DC. The equine sarcoid: why are there so many treatment options? Vet Clin North Am—Equine Pract. 2019; 35:243–262. https://doi.org/10.1016/j.cveq.2019.03.008.
16. Stadler S, Kainzbauer C, Haralambus R, Brehm W, Hainisch E, Brandt S. Successful treatment of equine sarcoids by topical aciclovir application. Vet Rec. 2011; 168:1–4. https://doi.org/10.1136/vr.c5430 PMID: 21493530
17. Haspeslagh M, Jordana Garcia M, Vlaminck LEM, Martens AM. Topical use of 5% acyclovir cream for the treatment of occult and verrucous equine sarcoids: A double-blinded placebo-controlled study. BMC Vet Res. 2017; 13:1–6. https://doi.org/10.1186/s12917-017-1215-0 PMID: 28049469
18. Nogueira SAF, Torres SMF, Malone ED, Diaz SF, Jessen C, Gilbert S. Efficacy of imiquimod 5% cream in the treatment of equine sarcoids: A pilot study. Vet Dermatol. 2006; 17:259–265. https://doi.org/10.1111/j.1600-0749.2006.00526.x PMID: 16827669
19. Wilford S, Woodward E, Dunkel B. Owners' perception of the efficacy of Newmarket bloodroot ointment in treating equine sarcoids. Can Vet J. 2014; 55:683–686. PMID: 24982522
20. Taylor S, Haldorson G. A review of equine sarcoid. Equine Vet Educ. 2013; 25:210–216. https://doi.org/10.1111/j.2042-3292.2012.00411.x.
21. Teixeira RBC, Rendahl AK, Anderson SM, Mickelson JR, Sigler D, Buchanan BR, et al. Coat color genotypes and risk and severity of melanoma in gray quarter horses. J Vet Intern Med. 2013; 27:1201–1208. https://doi.org/10.1111/jvim.12133 PMID: 23875712
22. Seltenhammer MH, Simhofer H, Scherer S, Zechner P, Curik I, Sölkner J, et al. Equine melanoma in a population of 296 grey Lipizzaner horses. Equine Vet J. 2003; 35:153–157. https://doi.org/10.1076/evj.2003.35.5.153 PMID: 12638791
23. Fleury C, Béard F, Balme B, Thomas L. The study of cutaneous melanomas in Camargue-type gray-skinned horses (1): clinical-pathological characterization. Pigment Cell Res. 2000; 13:39–46. https://doi.org/10.1034/j.1600-0749.2000.130108.x PMID: 10761995
24. McDadeyan J. Equine melanomatosis. J Comp Pathol Ther. 1933; 46:186–204. http://dx.doi.org/10.1016/S0366-1742(33)80025-7.
25. Rodriguez M, Garcia-Barona V, Pena L, Castano M, Rodriguez A. Grey Horse Melanotic Condition: J Equine Vet Sci 1997; 17:677–81.
26. Valentine BA. Equine melanocytic tumors: a retrospective study of 53 horses (1988 to 1991). J Vet Intern Med. 1995; 9:291–297. https://doi.org/10.1111/j.1939-1676.1995.tb01087.x PMID: 8531173
27. Rosengren Pielberg G, Golovko A, Sundström E, Curik I, Lennartsson J, Seltenhammer MH, et al. A cis-acting regulatory mutation causes premature hair greying and susceptibility to melanoma in the horse. Nat Genet. 2008; 40:1004–1009. https://doi.org/10.1038/ng.185 PMID: 18641652
28. Sundström E, Komisarczuk AZ, Jiang L, Golovko A, Navratilova P, Rinkwitz S, et al. Identification of a melanocyte-specific, microphthalmia-associated transcription factor-dependent regulatory element in the intronic duplication causing hair greying and melanoma in horses. Pigment Cell Melanoma Res. 2012; 25:28–36. https://doi.org/10.1111/j.1755-148X.2011.00902.x PMID: 21863983
29. Macgillivray KC, Sweeney RW, Del Piero F. Metastatic melanoma in horses. J Vet Intern Med. 2002; 16:452–456.
30. Scott D. Neoplastic Diseases. In: Pedersen D, editor. Large animal dermatology. Philadelphia, USA: W.B. Saunders Company; 1988, p. 448–452.
31. Moore JS, Shaw C, Shaw E, Buechner-Maxwell V, Scarratt WK, Crisman M, et al. Melanoma in horses: current perspectives. Equine Vet Educ. 2013; 25:144–151. https://doi.org/10.1111/j.2042-3292.2011.00368.x.

32. Müller JMV, Feige K, Wunderlin P, Hödl A, Mehl ML, Seltenhammer M, et al. Double-blind placebo-controlled study with interleukin-12 and interleukin-12-encoding plasmid DNA shows antitumor effect in metastatic melanoma in gray horses. J Immunother. 2011; 34:58–64. https://doi.org/10.1097/CJI.0b013e3181f1997 PMID: 21150713

33. Phillips JC, Lembrecke LM. Equine melanocytic tumors. Vet Clin North Am—Equine Pract. 2013; 29:673–687. https://doi.org/10.1016/j.cveq.2013.08.008 PMID: 24267683

34. Mahlmann K, Feige K, Juhls C, Endmann A, Schuberth H-J, Oswald D, et al. Local and systemic effect of transfection-reagent formulated DNA vectors on equine melanoma. BMC Vet Res. 2015; 11:1–11. https://doi.org/10.1186/s12917-015-0422-9 PMID: 25582057

35. Laus F, Cerquetella M, Paggi E, Ippedico G, Argentieri M, Castellano G, et al. Evaluation of cimetidine as a therapy for dermal melanomatosis in grey horse. Isr J Vet Med. 2010; 65:47–52.

36. Goetz TE, Ogilvie GK, Keegan KG, Johnson PJ. Cimetidine for treatment of melanomas in three horses. J Am Vet Med Assoc. 1990; 196:449–452. PMID: 2298676

37. Bradley WM, Schilpp D, Khatibzadeh SM. Electronic brachytherapy used for the successful treatment of three different types of equine tumours. Equine Vet Educ. 2017; 29:293–298. https://doi.org/10.1111/eve.12420.

38. Groom LM, Sullins KE. Surgical excision of large melanocytic tumours in grey horses: 38 cases (2001–2013). Equine Vet Educ. 2018; 30:438–443. https://doi.org/10.1111/eve.12767.

39. Rowe EL, Sullins KE. Excision as treatment of dermal melanomatosis in horses: 11 cases (1994–2000). J Am Vet Med Assoc. 2004; 225:94–96. https://doi.org/10.2460/javma.2004.225.94 PMID: 15239480

40. Théon AP, Wilson WD, Magdesian KG, Pusterla N, Snyder JR, Galuppo LD. Long-term outcome associated with intratumoral chemotherapy with cisplatin for cutaneous tumors in equidae: 573 cases (1995–2004). J Am Vet Med Assoc. 2007; 230:1506–1513. https://doi.org/10.2460/javma.230.10.1506 PMID: 17504043

41. Hewes C, Sullins KE. Use of cisplatin-containing biodegradable beads for treatment of cutaneous neoplasia in equidae: 59 cases (2000–2004). J Am Vet Med Assoc. 2006; 229:1617–1622. https://doi.org/10.2460/javma.229.10.1617 PMID: 17107319

42. Scacco L, Bolaffio C, Romano A, Fanciulli M, Baldi A, Spugnini EP. Adjuvant electrochemotherapy increases local control in a recurring equine anal melanoma. J Equine Vet Sci. 2013; 33:637–639. https://doi.org/10.1016/j.jevs.2012.09.006.

43. Spugnini EP, D’Alterio GL, Dotsinsky I, Mudrov T, Dragonetti E, Murace R, et al. Electrochemotherapy for the treatment of multiple melanomas in a horse. J Equine Vet Sci. 2013; 33:637–639. https://doi.org/10.1016/j.jevs.2012.09.006.

44. Sanderson BJS, Ferguson LR, Denny WA. Mutagenic and carcinogenic properties of platinum-based anticancer drugs. Mutat Res—Fundam Mol Mech Mutagen. 1996; 355:59–70. https://doi.org/10.1016/0027-5107(96)00022-X PMID: 8781577

45. Weber LA, Meißner J, Delarocque J, Kalbitz J, Feige K, Kietzmann M, et al. Betulinic acid shows anti-cancer activity against equine melanoma cells and permeates isolated equine skin in vitro. BMC Vet Res. 2020; 16:1–9. https://doi.org/10.1186/s12917-020-2262-5 PMID: 31900161

46. Zalesińska MD, Borska S. Betulin and its derivatives—precursors of new drugs. World Sci News. 2019; 127:123–138.

47. Yogeeswar P, Srima D. Betulinic acid and its derivatives: a review on their biological properties. Curr Med Chem. 2005; 12:657–666. https://doi.org/10.2174/0929867053302214 PMID: 15790304

48. Pisha E, Chai H, Lee I-S, Chagwedera TE, Farnsworth NHS, Cordell GA, et al. Discovery of betulinic acid as a selective inhibitor of human melanoma that functions by induction of apoptosis. Nat Med. 1995; 1:1046–1051. https://doi.org/10.1038/nm1095-1046 PMID: 7489361

49. Fulda S, Friesen C, Los M, Scaffidi C, Mier W, Benedict M, et al. Betulinic acid triggers CD95 (APO-1/Fas)- and p53-independent apoptosis via activation of caspases in neuroectodermal tumours. Cancer Res. 1997; 57:4956–4964. PMID: 9354463

50. Ali-Seyed M, Jantan I, Vijayaraghavan K, Bukhari SNA. Betulinic acid: recent advances in chemical modifications, effective delivery, and molecular mechanisms of a promising anticancer therapy. Chem Biol Drug Des. 2016; 87:517–536. https://doi.org/10.1111/cbdd.12682 PMID: 26535952

51. Rios JL, Márquez S. New pharmacological opportunities for betulinic acid. Planta Med. 2018; 84:8–19. https://doi.org/10.1055/s-0043-123472 PMID: 29202513
52. Fulda S, Kroemer G. Targeting mitochondrial apoptosis by betulinic acid in human cancers. Drug Discov Today. 2009; 14:885–890. https://doi.org/10.1016/j.drudis.2009.05.015 PMID: 19520182

53. Fulda S, Scaffidi G, Susin SA, Krammer PH, Kroemer G, Peter ME, et al. Activation of mitochondria and release of mitochondrial apoptogenic factors by betulinic acid. J Biol Chem. 1998; 273:33942–33948. https://doi.org/10.1074/jbc.273.51.33942 PMID: 9852046

54. Mullauer FB, Kessler JH, Medema JP. Betulinic acid induces cytochrome c release and apoptosis in a Bax/Bak-independent, permeability transition pore dependent fashion. Apoptosis. 2009; 14:191–202. https://doi.org/10.1007/s10495-008-0290-x PMID: 19115109

55. Raghuvar Gopal D V., Narkar AA, Badrinath Y, Mishra KP, Joshi DS. Protection of Ewing’s sarcoma family tumor (ESFT) cell line SK-N-MC from betulinic acid induced apoptosis by α-DL-tocopherol. Toxicol Lett. 2004; 153:201–212. https://doi.org/10.1016/j.toxlet.2004.03.027 PMID: 15451550

56. Tan YM, Yu R, Pezzuto JM. Betulinic acid-induced programme mediated cell death in human melanoma cells involves mitogen-activated protein kinase activation. Clin Cancer Res. 2003; 9:2866–2875. PMID: 12856667

57. Chowdhury RA, Mandal S, Mittra B, Sharma S, Mukhopadhyay S, Majumder HK. Betulinic acid, a potent inhibitor of eukaryotic topoisomerase I: identification of the inhibitory step, the major functional group responsible and development of more potent derivatives. Med Sci Monit. 2002; 8:254–260. PMID: 12118187

58. Gheorgheosu D, Jung M, Ören B, Schmid T, Dehelean C, Muntean D, et al. Betulinic acid suppresses NGAL-induced epithelial-to-mesenchymal transition in melanoma. Biol Chem. 2013; 394:773–781. https://doi.org/10.1515/hsz-2013-0106 PMID: 23399635

59. Karna E, Szoka L, Palka JA. Betulinic acid inhibits the expression of hypoxia-inducible factor 1α and vascular endothelial growth factor in human endometrial adenocarcinoma cells. Mol Cell Biochem. 2010; 340:15–20. https://doi.org/10.1007/s10495-010-0395-8 PMID: 20174965

60. Ren W, Qin L, Xu Y, Cheng N. Inhibition of betulinic acid to growth and angiogenesis of human colorectal cancer cell in nude mice. Chinese-German J Clin Oncol. 2010; 9:153–157. https://doi.org/10.1007/s10330-010-0002-1

61. Gauthier C, Legault J, Lebrun M, Dufour P, Pichette A. Glycosidation of lupane-type triterpenoids as potent in vitro cytotoxic agents. Bioorganic Med Chem. 2006; 14:6713–6725. https://doi.org/10.1016/j.bmc.2006.05.075 PMID: 16787747

62. Csuk R. Betulinic acid and its derivatives: a patent review (2008–2013). Expert Opin Ther Pat. 2014; 24:913–923. https://doi.org/10.1517/13543776.2014.927441 PMID: 24909232

63. Winum JY, Pastorekova S, Jakublicova L, Montero JL, Scozzafava A, Pastorek J, et al. Carbonic anhydrase inhibitors: Synthesis and inhibition of cytosolic/tumor-associated carbonic anhydrase isozymes I, II, and IX with bis-sulfamates. Bioorganic Med Chem Lett. 2005; 15:579–584. https://doi.org/10.1016/j.bmcl.2004.11.058

64. Willmann M, Wacheck V, Buckley J, Nagy K, Thalhammer J, Paschke R, et al. Characterization of NVX-207, a novel betulinic acid-derived anti-cancer compound. Eur J Clin Invest. 2009; 39:384–394. https://doi.org/10.1111/j.1365-2362.2009.02105.x PMID: 19309323

65. Bache M, Bernhardt S, Passin S, Wichmann H, Hein A, Zschornak M, et al. Betulinic acid derivatives NVX-207 and B10 for treatment of glioblastoma—an in vitro study of cytotoxicity and radiosensitization. Int J Mol Sci. 2014; 15:19777–19790. https://doi.org/10.3390/ijms15111977 PMID: 25361208

66. Liebscher G, Vanchangiri K, Mueller T, Paschke R. In vitro anticancer activity of Betulinic acid and derivatives thereof on equine melanoma cell lines from grey horses and invivo safety assessment of the compound NVX-207 in two horses. Chem Biol Interact. 2016; 246:20–29. https://doi.org/10.1016/j.cbi.2016.01.002 PMID: 26772157

67. Weber LA, Puff C, Kalbitz J, Kietzmann M, Feige K, Bosse K, et al. Concentration profiles and safety of topically applied betulinic acid and NVX-207 in eight healthy horses—A randomized, blinded, placebo-controlled, crossover pilot study. J Vet Pharmacol Ther. 2020; 0:jvp.12903. https://doi.org/10.1111/jvp.12903 PMID: 32845519

68. Team RDC, R Development Core Team R. A Language and Environment for Statistical Computing. 2008. https://doi.org/10.1007/978-3-540-74866-7

69. Wood SN. Fast stable restricted maximum likelihood and marginal likelihood estimation of semiparametric generalized linear models. J R Stat Soc Ser B Stat Methodol. 2011; 73:3–36. https://doi.org/10.1111/j.1467-9868.2010.00749.x

70. Mourdjeva M, Kyurkchiev D, Mandinova A, Altankova I, Kehayov I, Kyurkchiev S. Dynamics of membrane translocation of phosphatidyserine during apoptosis detected by a monoclonal antibody. Apoptosis. 2005; 10:209–217. https://doi.org/10.1007/s10495-005-6076-5 PMID: 15711937
71. Fadok VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DL, Henson PM. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. J Immunol. 1992; 148:2207–2216. PMID: 1545126

72. Kommera H, Kaluderović GN, Bette M, Kalbitz J, Fuchs P, Fulda S, et al. In vitro anticancer studies of α- and β-d-glucopyranose betulin anomers. Chem Biol Interact. 2010; 185:128–136. https://doi.org/10.1016/j.cbi.2010.04.018 PMID: 20472329

73. Kommera H, Kaluderović GN, Kalbitz J, Dräger B, Paschke R. Small structural changes of pentacyclic lupane type triterpenoid derivatives lead to significant differences in their anticancer properties. Eur J Med Chem. 2010; 45:3346–3353. https://doi.org/10.1016/j.ejmech.2010.04.018 PMID: 20472329

74. Ferreira D, Adeg a F, Chaves R. The importance of cancer cell lines as in vitro models in cancer methylation analysis and anticancer drugs testing. Oncogenomics cancer proteomics—Nov. approaches Biomarkers Discov. Ther. Targets Cancer, vol. 3, InTech; 2013, p. 139–166. https://doi.org/10.5772/53110.

75. Kapalczyńska M, Kolenda T, Przybyła W, Zajączkowska M, Teresiak A, Filas V, et al. 2D and 3D cell cultures—a comparison of different types of cancer cell cultures. Arch Med Sci. 2016; 14:910–919. https://doi.org/10.5114/aoms.2016.63743 PMID: 30002710

76. van Staveren WCG, Solís DYW, Hébrant A, Detours V, Dumont JE, Maenhaut C. Human cancer cell lines: Experimental models for cancer cells in situ? For cancer stem cells? Biochim Biophys Acta—Rev Cancer. 2009; 1795:92–103. https://doi.org/10.1016/j.bbcan.2008.12.004 PMID: 19167460

77. Supuran CT. Carbonic anhydrase inhibitors as emerging agents for the treatment and imaging of hypoxic tumors. Expert Opin Investig Drugs. 2018; 27:963–970. https://doi.org/10.1080/13543784.2018.1548608 PMID: 30426805

78. Federici C, Lugini L, Marino ML, Carta F, Iessi E, Azzarito T, et al. Lansoprazole and carbonic anhydrase IX inhibitors synergize against human melanoma cells. J Enzyme Inhib Med Chem. 2016; 31:119–125. https://doi.org/10.1080/14756366.2016.1177525 PMID: 27142956

79. Seltenhammer MH, Heere-Ress E, Brandt S, Druml T, Jansen B, Pehamberger H, et al. Comparative histopathology of grey-horse melanoma and human malignant melanoma. Pigment Cell Res. 2004; 17:674–681. https://doi.org/10.1111/j.1600-0749.2004.00192.x PMID: 15541026

80. Smith SH, Goldschmidt MH, McManus PA. A comparative review of melanocytic neoplasms. Vet Pathol. 2002; 39:651–678. https://doi.org/10.1354/vp.39-6-651 PMID: 12450197

81. Tarwid J, Fretz P, Clark E. Equine sarcoi ds: a study with emphasis on pathologic diagnosis. Compend Contin Educ Pract Vet. 1985; 7:293–301.

82. Martens A, De Moor A, Demeulemeester J, Ducatelle R. Histopathological characteristics of five clinical types of equine sarcoma. Res Vet Sci. 2000. https://doi.org/10.1053/rvsc.2000.0432 PMID: 11124103

83. Stahl J, Kietzmann M. The effects of chemical and physical penetration enhancers on the percutaneous permeation of lidocaine through equine skin. BMC Vet Res. 2014; 10:1–6. https://doi.org/10.1186/1746-6148-10-138 PMID: 2483544

84. Luis A, Ruela M, Perissinato AG, Esselin M, Lino DS. Evaluation of skin absorption of drugs from topical and transdermal formulations. Brazilian J Pharm Sci. 2016; 52:527–544. http://dx.doi.org/10.1590/S1984-82502016000300018.

85. Guy RH, Hadgraft J. Prediction of Drug Disposition Kinetics. J Pharm Sci. 1984; 73:883–887. https://doi.org/10.1002/jps.2600730706 PMID: 6470948

86. Prausnitz MR, Elias PM, Franz TJ, Schmuth M, Tsai J-C, Menon GK, et al. Skin barrier and transdermal drug delivery. Med Ther. 2012; 5:2065–2073.

87. Haspeslagh M, Teavernier L, Maes AA, Vlaminck LEM, De Spiegeleer B, Croubels SM, et al. Topical distribution of acyclovir in normal equine skin and equine sarcomas: An in vitro study. Res Vet Sci. 2016; 106:107–111. https://doi.org/10.1016/j.rvsc.2016.03.021 PMID: 27234546

88. Mills PC, Cross SE. Regional differences in the in vitro penetration of hydrocortisone through equine skin. J Vet Pharmacol Ther. 2006; 29:25–30. https://doi.org/10.1016/j.jvp.2006.07.015 PMID: 16420298