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

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ARTICLE INFO

Article history:
Received 19 November 2015
Received in revised form 21 December 2015
Accepted 2 January 2016
Available online 7 January 2016

Keywords:
Betulinic acid derivatives
Equine melanoma cell lines
Cytotoxicity
Flow cytometry
NVX-207

ABSTRACT

Betulinic acid, a pentacyclic triterpene, and its derivatives are promising compounds for cancer treatment in humans. Melanoma is not only a problem for humans but also for grey horses as they have a high potential of developing melanoma lesions coupled to the mutation causing their phenotype. Current chemotherapeutic treatment carries the risk of adverse health effects for the horse owner or the treating veterinarian by exposure to antineoplastic compounds. Most treatments have low prospects for systemic tumor regression. Thus, a new therapy is needed. In this in vitro study, Betulinic acid and its two derivatives B10 and NVX-207, both with an improved water solubility compared to Betulinic acid, were tested on two equine melanoma cell lines (MelDuWi and MellJess/HoMelZh) and human melanoma (A375) cell line. We could demonstrate that all three compounds especially NVX-207 show high cytotoxicity on both equine melanoma cell lines. The treatment with these compounds lead to externalization of phosphatidylserines on the cell membrane (AnnexinV-staining), DNA-fragmentation (cell cycle analysis) and activation of initiator and effector caspases (Caspase assays). Our results indicate that the apoptosis is induced in the equine melanoma cells by all three compounds. Furthermore, we succeed in encapsulating the most active compound NVX-207 in 2-Hydroxyprolyl-β-cyclodextrine without a loss of its activity. This formulation can be used as a promising antitumor agent for treating grey horse melanoma. In a first tolerability evaluation in vivo the formulation was administered every one week for 19 consecutive weeks and well tolerated in two adult melanoma affected horses.

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1. Introduction

Triterpenoids are an important class of substances isolated from plant extracts. One of these compounds is Betulinic acid (1) (Fig. 1) Since, its anticancer activity on human melanoma cells was shown in 1995 the same effect was demonstrated for a variety of other cancer cell types in in vitro and in vivo models [1–10]. Furthermore, Betulinic acid(1) showed anti-HIV [11], anti-inflammatory [12], anti-malarial [13] and anthelmintic activities [14].

The anticancer activity is based on the induction of apoptosis in a p53-and CD95-independent fashion [15]. For the induction, the formation of pores in the mitochondrial membrane permeability (MMP) is necessary [16]. Recently it was published that Betulinic acid (1) inhibit the steroyl-CoA desaturase and consequently reduce the desaturation levels of cardiolipins in cancer cells. This leads to ultrastructural changes in mitochondrial membranes. As cancer cells depend on de novo lipogenesis, this would explain the selective effect of Betulinic acid (1) on cancer cells [17]. An direct interaction with cardiolipins with subsequent MMP formation seems also to be possible, because it was shown that Betulinic acid (1) interacts with the in human mitochondria most abundant Tetrailinoleolcardiolipin [18]. Additionally, the production of reactive oxygen species (ROS) plays a role in MMP formation since the co-treatment with the antioxidant alpha-DL-Tocopherol inhibited the induction of apoptosis [19,20]. As a consequence of the pore formation apoptogenic factors release from the mitochondria and caspases are activated [15,21]. Besides this the induction of other types of cell death are discussed [22] as well as the induction of
stress response signaling cascades such as mitogen-activated protein kinase [23] and cell specific NF-kappaB [24,25]. Additionally, the inhibition of other target proteins such as proalidase, proteins of collagen biosynthesis [26], the mammalian DNA topoisomerase I [27] and acyl CoA:cholesterol acyltransferase [28] were found. These multiple modes of action show the high anticancer efficacy of Betulinic acid (1), due to its poor water solubility it has not been used as a drug for cancer treatment. To solve this problem a plethora of new derivatives were synthesized in the last decade and two promising derivatives are the glycopyranoside derivate B-10 (2) and the Tris ester NVX-207 (3a) (Fig. 1) [29–31]. The latter agent showed already promising results in treatment of canine cancer patients [30]. Another way to improve the water solubility is the usage of a drug delivery system. In the last years different formulations were tested in vitro as well as in vivo in xenograft models, e.g. liposomes [6] or nanoparticles consisting of cellulose and Poly (l-lactate) and encapsulating Betulinic acid (1) [32]. The encapsulation in β-cyclodextrine was already used for encapsulating Betulinic acid (1) [33] and hydroxy propyl-β-cyclodextrine for encapsulating NVX-207 (3b). Cyclodextrine showed no effects on the activity of the encapsulated compounds [30] and is very likely degraded into non-toxic saccharides [34].

Cancer is a threat to equine health, especially grey horses suffer from cutaneous melanocytic tumors, since 80% of grey horses older than 15 years bear melanoma lesions [35]. Equine malignant melanoma (EMM) is reported to progress to malignancy and metastasize in the surrounding tissue, lymph nodes or other internal organs (EMM) is reported to progress to malignancy and metastasize. Despite promising local anti-tumor activity investigation. The aim of our project was to test Betulinic acid (1) and its derivatives B-10 (2), the Tris ester NVX-207 (3a) and cyclodextrine encapsulated NVX-207 (3b) (Fig. 1) thereof on equine melanoma cell lines and to show that they induce apoptosis comparable to human cancer cell line e.g. A375 (Melanoma) cell line. Additionally, the formulation with the highest prospects of clinical efficacy was evaluated for safety after intratumoral injection in two horses.

2. Materials and methods

2.1. Compounds

Betulinic acid (1), B-10 (2) and NVX-207 (3a) were obtained from BioSolutions Halle GmbH (Halle/Saale, Germany). The NVX-207 Cyclodextrine conjugate (3b) is prepared as follows: 1 mg of 3a in 1.25 ml of 96% ethanol added to 1.25 ml hydroxyl-β-cyclodextrine solution (0.05 M). After stirring, evaporation and lyophilization the conjugate can be dissolved in saline (0.9% w/v). All compounds were dissolved in dimethyl sulfoxide (DMSO) to achieve a 20 mM stock solution.

2.2. Cell lines and culture conditions

The equine melanoma cell lines MelDuWi (kindly provided by Dr. Saskia Willenbrock, University of Veterinary Medicine, Hannover, Foundation, Germany), MellJess/HoMelZh (kindly provided by Dr. Monika Seltenhammer, Veterinary University of Vienna) and A375 (kindly provided by BioSolutions Halle GmbH, Halle, Germany) were maintained as monolayers in RPMI 1640 (PAA Laboratories, Pasching, Austria) supplemented with 10% (A375) and 15% (MelDuWi & MellJess) heat inactivated fetal bovine serum (Sigma Aldrich, Steinheim, Germany) and penicillin/streptomycin (PAA Laboratories, Pasching, Austria) at 37 °C in a humidified atmosphere with 5% CO2.

2.3. Cytotoxicity assay

The cytotoxicity of the compounds was evaluated using the sulforhodamine-B (SRB) (Kiton-Red S, ABCR) micro culture colorimetric assay. In short, exponentially growing cells were seeded into 96-well plates on day 0 at the appropriate cell densities (A375-750 cells/well; MelDuWi-5000 cells/well; MellJess-4000 cells/well) to prevent confluence of the cells during the period of experiment. After 24 h, the cells were treated with serial dilutions of the compounds (0–100 μM) for 96 h. The final concentration of DMSO solvent never exceeded 0.5%, which was non-toxic to the cells. For time series the medium was replaced after 2, 5 and 24 h by medium with no compound. The percentages of surviving cells relative to untreated controls were determined 96 h after the beginning of drug exposure. After a 96 h treatment, the supernatant medium from the 96 well plates was discarded, and the cells were fixed with 10% trichloroacetic acid (TCA). For a thorough fixation, the plates were allowed to keep at 4 °C (minimum 2 h). After fixation, the cells were washed in a strip washer, the washing was done five times with water using alternate dispensing and aspiration procedures. Afterward the plates were dried with 100 μL of 0.4% SRB for about 40 min. The plates were washed with 1% acetic acid to remove the excess of the dye and allowed to air dry overnight. Tris base solution (100 μL of 10 mM) was added to each well and absorbance was measured at 570 nm (using a 96 well plate reader, Tecan Spectra, Crailsheim, Germany). The IC50 values were determined using three technical replicates each in triplicate applying a non-linear 4P Hillslope equation (software, GraphPadPrism6, US; top and bottom value were set to 100 and 0, respectively).

2.4. Cell cycle investigations

Approximately 1 × 106 cells (MelDuWi and MellJess) and 1.9 × 105 cells (A375) were seeded in 25 cm2 cell culture flasks.

Fig. 1. Structures of Betulinic acid (1), B-10 (2) and NVX-207 (3a).
After 24 h of incubation, the medium was replaced with medium with compounds 1, 2, 3a and 3b at their respective double IC50 concentration. Following 24 and 48 h of incubation, cells were harvested by mild trypsinization and washed twice with PBS buffer (with Mg2+ and Ca2+). Cells (1 × 106) were fixed with ethanol (70%, –20 °C, for 2 h). After discarding the ethanol the cells were washed in 1 ml staining buffer (PBS + 2% FCS + 0.01% NaN3) and centrifuged. The cell pellet was resuspended in 100 μl of RNase A (1 mg/ml). After 30 min incubation at 37 °C, the samples were stained with propidium iodide (20 μg/ml of staining buffer) and allowed to rest in dark for at least 30 min at room temperature. Analyses were performed using the Attune® FACS machine (Life technologies, Darmstadt, Germany); collecting data from the BL-2A channel. Doublet cells were excluded from the measurements by plotting BL-2A against BL-2H. For each cell cycle distribution 20,000 events were collected. Each sample was measured in duplicates (some triplicates). Cell Cycle distribution was calculated using ModFitLT™ (Verity Software House, Topsham, US).

2.5. Apoptosis test - Annexin-V-staining

Approximately 1 × 10⁶ cells (MelDuWi and MellJess) and 1.9 × 10⁶ cells (A375) were seeded in 25 cm² cell culture flasks. After 24 h of incubation the medium was replaced by medium with compounds 1, 2, 3a and 3b at their respective double IC50 concentration. Following 24 and 48 h of incubation, cells were harvested by mild trypsinization and washed twice with PBS buffer (with Mg2+ and Ca2+). The cell pellet was resuspended in AnnexinV binding buffer (BioLegend®, San Diego, US) to a concentration of 1 × 10⁶/ml. Approximately 100,000 cells were stained with PI solution (3 μl, 1 mg/ml) and FITC AnnexinV solution (5 μl, BioLegend®, San Diego, US) for 15 min in the dark at room temperature. After adding Annexin V binding buffer (400 μl) the suspension was analyzed using the Attune® FACS machine (life technologies, Darmstadt, Germany). After gating for living cells, the data from detectors BL-1A and BL-3A were collected. For each sample 20,000 events were collected and technical triplicates were measured.

2.6. Apoptosis test – caspase 3, 8 and 9 activation

Approximately 1 × 10⁶ cells (MelDuWi and MellJess) and 1.9 × 10⁶ cells (A375) were seeded in 25 cm² cell culture flasks. After 24 h of incubation the medium was replaced by medium with compounds 1, 2, 3a and 3b at their respective double IC50 concentration. Following 24 and 48 h of incubation, cells were harvested by mild trypsinization and washed twice with PBS buffer (with Mg2+ and Ca2+). The cell pellet was resuspended in PBS buffer (with Mg2+ and Ca2+) to a concentration of 1 × 10⁶/ml. Approximately 300,000 cells were treated with Caspase 3, 8 or 9 staining kit solution (1 μl, Promokine, Germany) for one hour at 37 °C and 5% CO2. Following the incubation the cell samples were washed twice in caspase washing solution (PromoKine, Germany), resuspended in 300 μl caspase washing solution (PromoKine, Germany) and analyzed using Attune® FACS machine (Life technologies, Darmstadt, Germany). For each sample 20,000 events were collected (detector BL-1A or BL-3A) and technical duplicates were measured. The following analysis of cell population with active caspase was performed using R and Bioconductor.

2.7. In vivo tolerability study

2.7.1. Horses

Two adult grey horses bearing at least one melanoma were treated intratumorally with the Betulinic acid (1) derivative NVX-207 (3a). Horse 1 was a 13 year old Warm blood mare with a weight of 695 kg and horse 2 was an 18 year old Warm blood mare weighing 587 kg. Both horses were housed in standard conditions with daily access to pasture and standard feeding with hay ad libitum. The experiment was approved by the German national authorities (LAVES 33.14-42502-04-14/1588). Horse 1 had a solitary melanotic lesion at the root of the tail, horse 2 had multiple melanoma lesions in predilection areas (underneath the tail, around the anus and vagina, in the head-neck area).

2.7.2. Study design

Each horse was clinically and clinical pathologically examined on each injection day and monitored daily for any clinical reaction. Horses were injected intratumorally in one (horse 1) respectively three dermal lesions (horse 2). NVX-207 was diluted in sterile saline to a concentration of 0.06 mg/ml. Horses were injected the maximum possible amount per tumor (approximately 1.5 ml in horse 1 and a total of approximately 6 ml in horse 2) every 7 days for 19 consecutive weeks. Monitoring of horses for adverse events included attitude, heart rate, respiratory rate, rectal temperature, mucus membrane color, capillary refill time and local signs such as swelling, puritus, pain, reddening, depigmentation and ulceration.

2.7.3. Sample collection

Blood was collected for hematology and blood biochemistry at each day of injection and 5 days after injection. Hematological parameters were determined using the ADVIA 120 (Siemens Healthcare Diagnostics, Eschborn, Germany). Blood biochemistry was assayed using the Cobas c311 (Roche Diagnostics GmbH, Mannheim).

3. Results

3.1. Cytotoxicity on equine cell lines

For Betulinic acid (1) and the two derivatives 2 and 3a an anticancer activity on variety of human cancer cell lines could be shown [2,29,30]. On the human melanoma cells (A375) or the compounds 1, 2 and 3a the IC50-values range between 13.3 μM, 10.3 μM and 2.6 μM respectively (Table 1).

The activity of the compounds 1, 2 and 3a on the equine melanoma cell lines (MelDuWi and MellJess) is lower, for which the IC50-values were determined as 33.1/33.4 μM and 5.6/6.5 μM (Fig. 2). NVX-207 (3a) showed the highest activity on all cell lines and was therefore selected for encapsulation to achieve higher water solubility. The encapsulated NVX-207 (3b) was diluted in physiological 0.9% sodium chloride solution as this formulation can be used for treatment of horses. The encapsulation has only a very slight effect on the cytotoxic activity as the IC50-values for the equine melanoma cells shift to 7.7 μM and 8.3 μM for MelDuWi and MellJess respectively. To assess the time period until NVX-207 enters and attach to the cells a time course was measured. For this the medium with substance was replaced by medium without drug after the indicated time points. The result is a clear shift of the IC50-value.

Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Horse</th>
<th>IC50 Value (μM)</th>
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<tbody>
<tr>
<td>A375</td>
<td>Horse 1</td>
<td>33.1 ± 3.3</td>
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<tr>
<td></td>
<td>Horse 2</td>
<td>11.6 ± 0.7</td>
</tr>
<tr>
<td>MelDuWi</td>
<td>Horse 1</td>
<td>33.4 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>Horse 2</td>
<td>11.8 ± 1.0</td>
</tr>
<tr>
<td>MellJess</td>
<td>Horse 1</td>
<td>33.1 ± 3.3</td>
</tr>
<tr>
<td></td>
<td>Horse 2</td>
<td>11.6 ± 0.7</td>
</tr>
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</table>

Values are the average from three independent experiments with standard error.
values for e.g. MelDuWi from 20.1 μM for the 2 h treatment to 14.6 μM, and 8.3 μM for 5 h and, 24 h treatments, respectively (Table 2 and Fig. 3). There is no substantial difference between the cytotoxic activities after 24h and 96 h.

3.2. Betulinic acid and the derivatives induce cell cycle perturbations

In previous studies an increase of subG1-cells after treatment of various human cancer cell lines with 1, 2 and 3a was shown [29,40]. The equine cancer cell lines were treated with the compounds 1, 2, 3a and 3b with their double IC50-values and analyzed after 24 h and 48 h via flow cytometry (Fig. 4 and Fig. S1). Compared to untreated cells (control) the treatment with all compounds leads to an increase of subG1-cells after 24 h treatment for A375 cells (Fig. 5). The amount of subG1-cells rises after 48 h of treatment to more than two thirds except for 3b treatment. The equine melanoma cell lines show a similar but slower increase of subG1-cells amount. After 24 h at least one fifth of the cells treated with 1, 3a and 3b are in subG1. After 48 h the portion of subG1 cells raise to more than two third for 1, 3a and 3b and for 2 to around 50%. The amount of cells in the different cell cycle phases decrease in the same ratios. There was no arrest in any of the cell cycle phases, instead of cell cycle arrest the accumulation of cells in subG1-phase was observed (Figs. S1 and S2).

3.3. Betulinic acid and the derivatives induce apoptosis (Annexin V/Propidium iodide (PI)-staining)

A characteristic feature of apoptosis is the externalization of phosphatidylserines to the extracellular side of the plasma membrane shortly after apoptosis induction [41,42]. The cha-nge of the extracellular plasma membrane composition was detected by using AnnexinV/(PI)-staining and analysis by flow cytometry (Fig. 6 and Fig. S3).

After 24 h treatment of MelDuWi cells with 3b an increase of apoptotic cells is visible or to put it more precisely half of the cells are late apoptotic and one third early apoptotic cells. After 48 h, the amount of early apoptotic cells is further increased so that around 90% of the cells are apoptotic (58% early apoptotic+32% late apoptotic). The same time dependent results were detected for all three melanoma cell lines treated with 1, 3a and 3b as well as for A375 treated with 2 (Fig. 7). The treatment of equine melanoma cell lines for 24 h with 2, results in a slight increase of apoptotic cells, but after 48 h the amount of apoptotic cells is comparable with a 3b treatment.

3.4. Betulinic acid and the derivatives induce activation of initiator and effector caspases

A hallmark of apoptosis is the activation of caspases. Thereby, two pathways of apoptosis induction could be discriminated – the intrinsic and the extrinsic pathway. In the first one, the initiator caspase 9 is involved and, in the latter one the initiator caspase 8. The executioner caspase 3 gets activated by both. Activation of the different caspases was analyzed by usage of fluorescent labeled inhibitors of caspases (FLICA) by flow cytometry (Fig. 8).

For A375 cell line all three analyzed caspases (caspase 3,8,9) were activated in one third to two thirds of the cells after 24 h when treated with 3b, 3a and 1, respectively. After 24 h all three caspases were activated in more than 80% of the cells. When treated with 2 more than 90% of the cells have activated caspases already after 24 h. Therefore, no measurements after 48 h were performed.

For the equine melanoma cells (MelDuWi and MelJess) the activation of the caspases is time shifted except for treatment with 3b which is similar to the above mentioned results. The treatment with 1, 2 and 3a result in a slight increase of the amount of cells with activated caspases after 24 h. After 48 h for MelDuWi cells treated with all tested drugs the number of cells with caspase activation accounts to 90%. After 48 h the MelJess cells treated with 1, 2, 3a and 3b leads to an activation of all three caspases was observed in two thirds, around 80% and more than 90% of the cells, respectively. No difference between the activation of the three caspases is visible and no conclusion can be drawn regarding the subsequent activation of the caspases.

Table 2

<table>
<thead>
<tr>
<th></th>
<th>2 h</th>
<th>5 h</th>
<th>24 h</th>
<th>96 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>A375</td>
<td>8.8 ± 0.9</td>
<td>6.7 ± 2.4</td>
<td>3.8 ± 1.1</td>
<td>2.9 ± 0.5</td>
</tr>
<tr>
<td>MelDuWi</td>
<td>20.1 ± 0.4</td>
<td>14.6 ± 1.5</td>
<td>8.3 ± 0.1</td>
<td>7.7 ± 0.7</td>
</tr>
<tr>
<td>MelJess</td>
<td>18.8 ± 1.4</td>
<td>16.2 ± 2.4</td>
<td>8.4 ± 0.2</td>
<td>8.3 ± 0.5</td>
</tr>
</tbody>
</table>

3.5. Intratumoral administration of NVX-207 is safe and well-tolerated in adult grey horses

Two adult female grey horses were administered with 0.06 mg/ml NVX-207 (100 μM) every one week for 19 consecutive weeks. Both horses tolerated the injections well and did neither show local signs of irritation or inflammation nor clinical signs of systemic illness. Hematologic and blood-biochemical evaluations revealed only slight deviations from the reference ranges of the laboratory
without any association to the application of the test substance.

Total white blood cell and differential counts basically remained within the reference ranges except for individual elevations of segmented granulocyte counts and a single decrease in lymphocyte proportions.

Clinical chemistry results unveiled no specific response to the weekly administration of NVX-207. However, the highly sensitive liver specific enzyme Gamma-glutamyl dehydrogenase (GLDH) was elevated at three time points and the Gamma-glutamyl transferase (GGT) was slightly elevated at the beginning of the study period in
the 13 year old mare. GGT elevation gradually decreased to normal values by week eight in this horse.

One horse was euthanized after the 19th injection of NVX-207 because of a colon torsion. At necropsy no specific findings associated with the administration of a chemotherapeutic agent were shown.

4. Discussion and conclusion

Betulinic acid (1) and their derivatives are known for about 20 years as efficient anticancer drugs [1]. The substances proved their efficacy on several human cancer cell lines and in vivo model systems [2–10]. Beside humans also grey horses suffer from melanoma as the mutation causing their grey hair phenotype likewise increase the risk for melanoma formation with a risk of
metastasizing [43]. For medical and ethical reasons a treatment is necessary. The local chemotherapy with cisplatin or a local excision show only for small tumors successful results. But due to the high number of melanoma and the risks when administering cisplatin, a new treatment strategy has to be found. A possible new drug is Betulinic acid (1) or one of its derivatives. In recent in vivo studies in mice no side effects were visible [1,3–10] and Betulinic acid (1) seems to have no effect on noncancerous cells [7]. In this in vitro study we could demonstrate that the derivative NVX-207 (3a) has compared to the other tested compounds holds the highest cytotoxicity on equine melanoma cell lines with an IC<sub>50</sub>-value of 5.6 μM and 6.5 μM (MelDuWi and MelJess) (Fig. 2 and Table 1). An encapsulation in HP-β-cyclodextrine (3b) resulted in a similar cytotoxic efficiency (IC<sub>50</sub>-value: 7.7 μM and 8.3 μM for MelDuWi and MelJess cells respectively) (Fig. 3 and Table 2). A time dependent administration of 3b revealed that the drug reaches and enter the cells or is attached to them within the first 24 h after treatment (Fig. 3 and Table 2). The subsequent experiments were performed after 24 h of treatment and later time points.

Betulinic acid (1) is inhibiting cardiolipin desaturation leading to ultrastructural changes in the mitochondrial membrane [17], possible reason could be a direct interaction with cardiolipins in the mitochondrial membrane [18]. As a consequence pores in the outer mitochondrial membrane are formed followed by release of Cyctochrome c from pores into the cytoplasm. The formation of the pores is an essential step in the apoptosis induction as apoptosis can be prevented by co-treatment with α-D-Tocopherol. The subsequent activation of the initiator caspase 9 and the effector caspasess e.g. caspase 3 was already demonstrated [15,21]. Nevertheless the caspase activation is under discussion since the inhibition of caspases with the broad range inhibitor zVAD-fmk could not prevent apoptosis induction. Betulinic acid (1) seems to activate also other pathways leading to controlled cell death in absence of caspase activity. For B10 it was recently demonstrated

Fig. 7. AnnexinV staining analyses of A375 (A), MelDuWi (B) and MelJess cells (C) untreated and treated with Betulinic acid (1) and its derivatives 2, 3a, 3b at their double IC<sub>50</sub> concentrations for 24 & 48 h (as indicated). Data shown are the percentages of early (black) and late apoptotic (light grey) cells as the mean values (±SD) of two independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).
that, it interrupts autophagy so that the cells die due to a massive autophagy [44]. In this study we could show that the DNA fragmentation takes place early stages of after 48 h treatment (Fig. 5).

No cell cycle arrest was detected. The AnnexinV-staining showed a huge increase of early and late apoptotic cells after 24 h and 48 h of treatment (Figs. 6 and 7). Especially there is an immense amount of

Fig. 8. Caspase activity measurement by usage of FLICA of A375, MelDuWi and MelJess cells untreated (yellow) and treated with Betulinic acid (1) (blue), and its derivatives 2 (green), 3a (red) and 3b (violet) at their double IC_{50} concentrations for 24 & 48 h (as indicated). The percentage of cells with active caspase 3, caspase 8, caspase 9 (as indicated) are displayed as the mean values (±SD) of two independent experiments.
early apoptotic cells after 48 h. The time shift between the amounts of apoptotic cells detected via annexinV staining and subG1-cell measurement is explained by the subsequent occurrence of the visualized characteristics of the apoptosis induction pathway.

Concurrently, the caspases 3, 8 and 9 get activated in a time dependent manner (Fig. 8). It is to mention that the action of B10 (2) is time shifted compared to that of the other three compounds 1, 3a and 3b. For the human cell line B10 (2) is already acting in between the first 24 h, whereas for the equine cell lines its action is delayed compared to the others. The effect after, it induces cell death is massive. It cannot be ruled out that this effect is also induced by immense autophagy in the B10 (2) treated cells. Furthermore, it seems as if there is a threshold concentration because the application of respective IC₃₀ concentrations does not induce apoptosis even after 72 h in all three cell lines (data not shown). This would also explain the steep slope of the dos–response curves.

The in vivo results of repeated administration of NVX-207 (3a) in two adult grey mares show a very good tolerability of the Betulinic acid (1) derivative NVX-207 (3a) after repeated intratumoral administration. Neither clinical nor clinicopathological adverse effects of the compound were noted. In conclusion, all three tested compounds induce apoptosis although the activation of other controlled cell death pathways cannot be excluded. Nevertheless, the results open up the possibility for first clinical in vivo studies on grey horses with melanoma.

Acknowledgments

We thank Prof. Reinhard Paschke, Dirk Menzel and the technical team from the clinical laboratory of the small animal clinic of the University of Veterinary Medicine Hannover, Foundation, for performing the clinicopathological assays of the in vivo study.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.cbi.2016.01.002.

References


