A comparative study regarding melanoma activity of Betulinic acid on topical ointment vs. systemic nanoemulsion delivery systems

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Abstract
Betulinic acid is an important antitumor compounds that is involved in preclinical and toxicological tests. It proved an selective antitumor activity and also it is an effectiv e compound in nevi treatment. Melanoma is a fatal disease and intervention in early phases could be helpful to reduce its evolution. The most important tests are non invasive type for topical evaluation and histopathological for systemic administration. Mexameter can appreciate the evolution of melanin and haemoglobin. Histopathological evaluation underline pathological events such as metastatic process. The aim of present work was the observations regarding betulinic acid activity on topical and systemic application. The main conclusion is that it is effective both on topical experimental nevi and metastatic melanoma in early stages.

Keywords: betulinic acid, nevi, murine melanoma, ointment, nanoemulsion

1. Introduction
The interest of researchers on plant triterpenoids from lupan series as antitumor agents in last years has increased considerably [1]. Betulinic acid (BA) is a pentacyclic titerpene, lupan derivate with a relevant antitumor potential, more specific with an in vitro and in vivo antimelanoma activity.

Metastatic melanoma is a multistep process that develops from melanocytes to nevi, radial growth phase and vertical growth phase [2]. The anticancer activity of betulinic acid was reviewed in detailed recent studies [3, 4]. It induces apoptosis in melanoma cells in vitro and in vivo but detailed mechanism about the antimelanoma activity could be detailed [1, 5]. Its apoptotic activity involved caspas (Komera N 2009). BA was selected in a Programme that includes RAID (Rapid Access to Intervention in Development) [6]. It is considered a selective compound on melanoma cells, a lack toxicity compound and its activity involves higher doses in vivo, 250mg/kg body weight, on athymic mice [7]. An applicable experimental melanoma model used C57BL/6J mouse and B16F10 cells [8].
The therapeutic activity of BA determines 60% reduction in the number of skin tumors following initiation and promotion with 7,12-dimethylbenzanthracene and 12-O-tetradecanoylphorbol-13-acetate (TPA). These chemical compounds are also used to obtain murine melanoma [9].

As an in vivo agent betulinic acid functions as a selective inhibitor of apoptosis on a xenograft melanoma model in athymic mice [11]. BA is a therapeutic agent that developed an immunomodulatory activity [12]. BA can be an interesting antitumor candidate alone or combined with radiotherapy [13]. Melanin evolution and skin pigmentation could be measured even experimentally by non-invasive devices such as Mexameter [14].

The aim of our study was the evaluation of betulinic acid on topical and systemic formulations on murine models. The experimental models were chemical and by inoculation of tumor cells. The mice were a type known as susceptible to malignant melanoma C57BL/6J.

2. Materials and methods

Materials. Betulinic acid was purchased from Sigma Aldrich (Taufkirchen, Germany). Components of ointment: 2-propanol, isopropyl myristate and PEG 4000 (Ph.Eur. 6), diethylene glycol monoethyl ether (Transcutol®) and caprylocaproyl macrogolglycerides (Labrasol®) were form Gattefossé (France). Flax-seed oil, which is rich in alpha-linolenic acid (an omega-3 polyunsaturated fatty acid precursor), was kindly provided by Jedwards International (Quincy, MA). Egg phosphotidylcholine (Lipoid® E80) was obtained from the Lipoid GmbH (Ludwigshaffen, Germany).

The carcinogenic agents were 7,12-dimethylbenzanthracene (DMBA) as tumour initiator and 12-O-tetradecanoylphorbol-13-acetate (TPA) as tumour promoter. Both substances were obtained from Fluka and Sigma and prepared as solutions in acetone as is mentioned in the literature [10, 15] and the quantity of applied substance was 200 nmol in 100 µl acetone and 5 nmol TPA in 100 µl acetone. The protocol of application was correlated with a reproducible one also mentioned in previous studies [16].

B16 melanoma 4A5 (ECACC and Sigma Aldrich, origin Japan stored UK) cells were grown in DME media, supplemented with 10% foetal bovine serum, penicillin (100 U/ml) and streptomycin (100 mg/ml) in specific cells culture plates. Cells were incubated with 1 ml of media at 37°C in an incubator for cell culture with 95% air and 5% CO₂. For the subcutaneous injection with B16 cells on mice were used 0.5 ml of cells without media, in saline solution. Animals. C57BL/6J mice of eight weeks were purchased from Charles River (Sulzfeld, Germany). The work protocol followed all NIAH-National Institute of Animal Health rules: animals were maintained during the experiment in standard conditions: 12h light-dark cycle, food and water ad libidum, temperature 24 º C, humidity above 55%. The total number of mice taken into study was 8.

For topical test was applied a long term application of DMBA and TPA as was describe in Yafan Li et al. protocol in 2007, a double DMBA initiation in alternation with TPA. After 25 days of application the ointment with BA was applied 2 times/day on group B mouse skin.

For intraperitoneal application mice were divided into two groups: group A, formed by four mice received food, water and B16 melanoma cells and group B similar with group A but with the application of 0.5 ml nanoemulsion of betulinic acid for 10 days, intraperitoneally. The cells were inoculated in spleen area. After housing for four weeks the mice from group A and B were inoculated with 0.5 ml B16 melanoma cell suspension prepared in the moment of using with saline solution as is described in cell culture protocol. For the group B after 2 days from the inoculation of B16 cells was administered the nanoemulsion of betulinic acid for other 10 days. From both groups A and B, mice with most representative skin pathology were chosen for analyses.

Preparation of betulinic acid nanoemulsion. BA containing oil-in-water nanoemulsion was prepared by high pressure homogenization method with a Microfluidizer® M-110EH processor as previously described. Flax-seed oil was used as the internal oil phase component of the nanoemulsion. BA (0.15 g) was dissolved in chloroform and added to the flax-seed oil (3.0 g). Nitrogen gas was blown in the sample to evaporate all of the chloroform resulting in oil phase with the dissolved drug.
The aqueous phase was prepared by dissolving the egg phosphatidylcholine (0.36 g) in 13 ml of deionized distilled water. The aqueous phase was added to the oil phase at 70°C under constant stirring. The mixture was pre-homogenized for 5 cycles at 1,000 psi with a Microfluidizer® processor M-110EH (Microfluidics Corporation, Newton, MA) to form coarse emulsion. Finally, the coarse emulsion was homogenized for another 5 cycles at 12,000 psi until an equilibrium size was reached. Control nanoemulsion sample without the drug (Blank NE) was also prepared using a similar process.

**Particle Size and Zetapotential:** Droplet size and zeta potential (surface charge) values of nanoemulsions were determined using dynamic light scattering technique with a Zetasizer (Malvern Instruments, UK) at a 90° fixed angle and at 25°C temperature. For this, nanoemulsions were diluted in deionized distilled water, and the numbered average oil droplet hydrodynamic diameter and the polydispersity index were determined. For the zeta potential, nanoemulsions diluted in deionized distilled water were placed in the electrophoretic cell of the Zetasizer and the average surface charge was determined.

**Preparation of topical ointment formulation.** Only the one part of betulinic acid dissolves in the mixture of liquid components (2-propanol, Transcutol®, isopropyl myristate and Labrasol®). After mixing this suspension with the melted PEG 4000, it was stirred (2000 rpm) with a Heidolph Diax (Heidolph Instruments GmbH &Co.) mixer until cooling (solidification).(Table I)

**Rheology.** Rheological measurements were carried out with a Physica MCR101 rheometer (Anton Paar, Austria). A cone-plate measuring device was used in which the cone angle was 1 degree, and the thickness of the sample was 0.046 mm in the middle of the cone. The measurements were performed at 25 °C. Flow curves and viscosity curves of the samples were also determined. The shear rate was increased from 0.1 to 150 1/s (up curve), and then decreased from 150 to 0.1 1/s (down curve) in the CR mode. The shearing time was 300 s in case of both segments.

**Histology.** Tissue samples (skin) were fixed in 10% formalin solution and were embedded in paraffin and cut at 4 microns.

Finally after deparaffinized the samples were stained with H&E (hematoxylin-eosin) and microscopically analysed. The biopsy for sample 1 was obtained in the day 12 after inoculation and for sample 2 after 10 days from inoculation and 10 days of treatment for intraperitoneal application and after 45 days (25 days of chemical damage and 20 of treatment).

**Mexametric measurements.** The mexametric measurements were performed using a research device from Courage Khazaka, with a mexameter MX18 (Courage&Khazaka Electronics, Cologne, Germany).

The maximum units for Mexameter are 999 (interval 0-999) and the measurement is based on the absorption/reflexion. For the measurements, the mice were anesthetised with xylazine and ketamine. The time of measuring was continuous, for 20s. The protocol followed the observations on melanin evolution and haemoglobin status (pigmentation and erythema).

The device was applied on most obvious affected areas and maintain on the skin for 20 seconds. The data were registered by the specific soft from the Mexameter MX18 device and then expressed as units. All data were processed as initial and final measurements values on the same area.

**Table 1.** Semisolid formulation and its composition for100g formulation (5% betulinic acid)

<table>
<thead>
<tr>
<th>Active agent</th>
<th>5g</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-propanol</td>
<td>37.3g</td>
</tr>
<tr>
<td>Transcutol®</td>
<td>9.3g</td>
</tr>
<tr>
<td>Isopropyl myristate</td>
<td>9.3g</td>
</tr>
<tr>
<td>Labrasol®</td>
<td>0.3g</td>
</tr>
<tr>
<td>PEG 4000</td>
<td>38.8g</td>
</tr>
</tbody>
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**3. Results and Discussions**

The cream applied on the skin had proper rheological properties. Both of the ointments, the blank and the active agent containing, have a well characterized thixotropy, which helps the spreadability of the ointment on the skin.

The rheological properties presented in Figures 1 and 2 shown the good quality of semisolid formulations. These data indicate the applicability of betulinic acid ointment because of acceptable rheological properties that confers a good spreadability.
The topical application of betulinic acid could change the skin aspect when there is a continuous application minimum 2 times/day. The most relevant macroscopic aspects are presented in Figure 3.

The data observed on macroscopic level were confirmed by HE staining on the skin as is shown in Figure 4.

For a detailed evaluation of skin recovery a non-invasive device was use (Mexa meter) that could detect the changes of melanin (pigmentation) and haemoglobin (irritation, lesions). The results can show even sensitive changes. The actual data included in Figures 5 and 6 presented quantifications on the beginning and the end of experiment from the same area of a representative mouse.

The mexametric measurements indicated the intervention of betulinic acid as active compound on skin level. Its intervention is very intense on haemoglobin but it is also present on melanin level. These data suggest the intervention of BA in pathologies like skin burn, beginning of skin carcinoma, nevi.
All these data confirm the topical activity of betulinic acid. Previous studies presented the activity of betulinic acid on dysplastic nevi [3]. It is a compound involved in clinical trials for treatment of dysplastic nevi with a potential transformation in melanoma, phase I/II.

Topical application of DMBA and TPA can determine the apparition of skin carcinoma and compounds like betulinic acid can influence the pathology if is applied topical.

Reducing of the intensity for pathologic aspects proved by histological evaluation is an indicator of the intervention of topical activity of betulinic acid on cutaneous area.

These aspects are also important for proving the intervention of BA on melanoma development because the same protocol with DMBA and TPA is used to induce melanoma on long term. The beginning of pathology can be dysplastic nevi but they are able to develop melanoma [2].

For the intraperitoneal (i.p.) administration of BA was used the nanoemulsion and the main observations are that in group 1 is a beginning of liver metastasis for melanoma cells as is observable in Figure 7A. The administrations of betulinic acid nanoemulsion reduce and maybe delay the metastatic process to the liver (Figure 7B).

The correlation of macroscopic observations with the microscopic ones leads to the same opinion of presence of metastasis on liver level in group 1. (Figure 8A) On group 2 in day 10 after 8 days of treatment with betulinic acid there is no evidence of metastasis on liver (Figure 8B).

For better observation as is known that B16 cells are specific for mouse melanoma with development on skin level skin biopsies from both 2 groups were prepared (Figure 9 A and B).

The main observations are that as is presented in other data [3] betulinic acid is an important antimelanoma agent. These previous studies proved its intervention on delaying the metastasis process for melanoma cells inside the body aspect correlated with the previous data that precise the activity of BA on metastasis melanoma. Our work proved also its early intervention.

The liver is a first station or a very close station for melanoma cells [17]. In order to obtain reproducible models the use of animal models for melanoma and screening of therapeutic agents are important aim. It could be relevant for studying organs of interest and possible metastasis to them [17].

The concentration of $10^5$ cells was used for preparing a murine metastatic melanoma [18]. The metastasis to liver are not difficult to produce if is used an intrasplenic inoculation of tumour cells because of the vicinity of organs and the intense blood circulation on liver level.
4. Conclusion

Betulinic acid is an active compound in metastatic melanoma. This pentacyclic triterpene is active also on local treatment as ointment and reduce the erythema and intense pigmentation. It could intervene even in first steps of pathologic evolution and it can reduce proliferation to important organs such as liver.

Experimental murine model offers important background for studying possibilities of treatment and therapeutic screening. Absence of metastasises on organs well irrigated and close to the place of inoculation suggested the strong antitumoral activity of betulinic acid.

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References


