Homeopathy (2015) ■, 1–10

http://dx.doi.org/10.1016/j.homp.2015.08.007, available online at http://www.sciencedirect.com

ORIGINAL PAPER

Inhalation therapy with M1 inhibits experimental melanoma development and metastases in mice

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Background: **M1 is a homeopathic medicine with immunostimulatory properties used mainly by cancer patients to complement current therapies. Metastatic melanoma is a skin-originated form of cancer without a single therapy able to produce high rate and sustained responses, which attracts the use of complementary therapies such as M1. However, M1's anti-melanoma effects remain to be pre-clinically demonstrated. Therefore in the present work, we utilized a pulmonary metastatic melanoma model and a subcutaneous melanoma growth model to investigate the potential benefits of treatment with M1.**

Methods: **C57BL/6 mice were injected intravenously or subcutaneously with B16F10 mouse melanoma cells. After 24 h, mice were treated with either M1 or vehicle (water) for 14 days, euthanized and harvested for multi-parameter pulmonary and tumor analyses.**

Results: **Mice treated with M1 had significantly lower tumor burden in the lungs and subcutaneous tissue than control mice. Furthermore, tumors were impaired in proliferation and tumor related angiogenesis by the inhibition of myeloid derived suppressor cells (MDSC) positive for angiotensin II type 1 receptor (AT1R).**

Conclusion: **Altogether these data suggest M1 is an efficient candidate for melanoma therapy to be considered for future clinic studies as this study is the first supporting the idea that melanoma patients may benefit with the treatment. The treatment with M1 provides advantages considering the highly-diluted properties and a cost effective alternative to costly chemotherapeutic approaches with, if any, lower toxicity.** *Homeopathy* (2015) ■, 1—10.

Keywords: Melanoma; Homeopathy; M1; Angiotensin II receptor; Angiogenesis; Tumor growth

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Received 19 August 2014; revised 17 July 2015; accepted 13 August 2015

Please cite this article in press as: Ferrari de Andrade L, et al., Inhalation therapy with M1 inhibits experimental melanoma development and metastases in mice, Homeopathy (2015), http://dx.doi.org/10.1016/j.homp.2015.08.007

Introduction

Despite major advances in cancer therapy, resulting in increased survival and cure rates, cancer remains the leading cause of death worldwide. The most recent World Health Organization (WHO) estimate indicated that 8.6 million (M) people died of cancer in 2012.[1] For example in the United States an estimated 1.6 M new cancer cases and 0.6 M cancer deaths are projected to occur in 2014. In addition, skin cancer incidence included estimates of 76,100 of new melanoma cases with 12.7% of mortality.[2] Melanoma, a skin cancer resulting in 75% of skin cancer related death, originates from melanocytes located on the basal membrane of the epithelium tissue.[3] Early identification and diagnosis of melanoma can be treated with high effectiveness, yet contrastingly, if melanoma metastasizes to distal organs the median overall survival can be a brief 8−18 months. These consequences place metastatic melanoma as the most aggressive and dangerous form of skin cancer.[4]

Melanoma drug discovery currently represents the majority of effort in combating this deadly disease. Considering the high immunogenicity of melanoma tumors, therapies aimed to modulate or disarm tumor evading mechanisms and subsequently stimulate proficient anti-tumor immune response have gained considerable focus as of late.[5−7] It has been previously administered extensive and successful studies in trials of different formulations of highly diluted medicines following homeopathic approaches.[8−11] Following high throughput screening, a homeopathic medicine termed 'M1' was the most successful candidate based on several parameters of immune responses mediated by $CD8^+$ T lymphocytes in mice lymph nodes.[12] This commercial medicine has been indicated by homeopathic doctors in Brazil to complement the therapy of patients with immunologic-related disorders, such as cancer. Despite many cancer patients claim to benefit from homeopathic treatments,[13] M1 was not investigated yet in pre-clinical studies for melanoma.

Given M1 effects in the immune system, we now aimed to investigate whether this treatment affects tumor development and metastases. We chose to study melanoma because is an important clinical issue. There is no practical alternative to animal experiments in this case, therefore we used a mouse model. We chose to study B16F10 melanoma, an aggressive mouse tumor that can be treated with immunotherapies.[14] This pre-clinical study presented here provides data supporting the efficacy of M1 for melanoma therapy. Using a method based on combinatory and high occurrence rates and low doses of M1 inhaled, as previously described,[15] we show for the first time that M1 treatment reduced the tumor burden of an experimental pulmonary metastasis model and primary tumor formation of mouse melanoma. In summary, we identified physiologic factors leading to the control of initial pathways of tumor metastasis and growth.

Materials and methods

Derivation of M1

M1 was prepared according to 'Farmacopeia Homeopática Brasileira'.[16] M1 is composed by the following substances diluted in water, with vigorous shaking in-between dilutions, all together in the same flask: *Aconitum napellus*-20 Hahnemann's dilution (DH), *Arsenicum album*-18 DH, *Asa foetida*-20 DH, *Calcarea carbonica*-16 DH, *Chelidonium majus*-20 DH, *Cinnamon*-20 DH, *Conium maculatum*-17 DH, *Echinacea purpurea*-20 DH, *Gelsemium sempervirens*-20 DH, *Ipecacuanha*-13 DH, *Phosphorus* 20 DH, *Rhus toxicodendron*-17 DH, *Silicea*-20 DH, *Sulfur*-24 DH, and *Thuja occidentalis*-19DH, as previously described.[12]

Mice and cell line

C57BL/6 mice, 7−10 weeks of age (n = 69), were used in experiments according to the approval of the local ethics committee, which certified the procedures using mice in this study are in agreement with the Experimental Animal Brazilian Council and Canadian Council on Animal Care (CEUA certification no 653). Experiments were conducted using pool of mice of equal sex. B16F10 melanoma cell line, purchased from Rio de Janeiro Cell Bank (Rio de Janeiro, Brasil), was cultivated in Dulbecco's Modified Eagle Medium (DMEM) (Sigma−Aldrich) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Invitrogen) at 37°C with 5% CO_2. Cultures were sub-cultivated in strict accordance to the provided instructions and previous report.[17] Briefly, when the confluence neared 90%, cells were detached with 0.05% trypsin − 0.53 mM ethylenediamine tetraacetic acid (EDTA) (Sigma−Aldrich) for 5 min at 37°C; cellular suspension was subsequently diluted at a ratio of 1:10 for culture breeding. For each experiment a new vial of equivalent passage of cryopreserved B16F10 cells was thawed to minimize experimental variation.

Experimental pulmonary metastasis model

B16F10 melanoma cells were detached at 90% confluence as indicated above and resuspended in DMEM with 10% FBS followed by 3 consecutive washes with serum free DMEM. Cell viability was checked with 0.4% trypan blue (Sigma−Aldrich) and standardized at minimal of 90% viability for experimental follow up. After number adjustment by hemocytometer count, detached cells were maintained on ice for no more than 40 min prior injection. C57BL/6 mice were intravenously injected with 5×10^5 B16F10 melanoma cells via the tail vein. 24 h post-injection mice were treated with either M1 or its respective vehicle (ultra-pure water). Solutions were vigorously homogenized and immediate applied to animals via oral inhalation as previously described.[15] Briefly, animals were placed into oral inhalation chambers and treated for 10 min, twice a day, 12 h apart for a period of 14 days. Following 15 days of cell inoculation, mice were anesthetized with 100 mg/kg ketamine (Dopalen−Vetbrands) and 20 mg/kg xilazin (Anasedan−Vetbrands) followed by euthanasia by cervical dislocation. Lungs were subsequently perfused with phosphate buffered 0.9% NaCl solution (PBS) Metastasis foci in the entire lung surface was counted under a stereomicroscope and recorded for each mouse. Subsequently, lungs were analyzed either via: 1)

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