Original Article

Antiproliferative and antimetastatic activity of DM-1, sodium 4-[5-(4-hydroxy-3-methoxyphenyl)-3-oxo-penta-1,4-dienyl]-2-methoxy-phenolate, in B16F10 melanoma

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Abstract

Melanoma causes 75% of skin cancer deaths mainly due to its high potential to progress to metastasis and by its recognized resistance to conventional therapies. Compound DM-1, sodium 4-[5-(4-hydroxy-3-methoxyphenyl)-3-oxo-penta-1,4-dienyl]-2-methoxy-phenolate, presents structural and biological similarity to curcumin, exhibiting properties such as potent antitumoral and antioxidant activities. In this work, the antitumoral and antiproliferative effects of this compound in *in vitro* assays with tumor and normal cell lines have been evaluated. Also evaluated was the *in vivo* antitumoral potential against B16F10 melanoma-bearing mice. Normal and tumor cells were treated with different concentrations of compound DM-1 and the cellular viability was determined by MTT colorimeter assay. The half maximal inhibitory concentration (IC_{50}) found was 30 µg/mL in B16F10 melanoma cells, while no toxic activity was verified against normal human fibroblastic cells. When DM-1 was administrated by intraperitoneal and endovenous routes to melanoma-bearing animals the survival rate increased by 40% when compared to the control group. Tumor load was reduced by 84% when administered via endovenous and by 54% via intraperitoneal. In conclusion, compound DM-1 acts as selective antitumoral agent inducing cytotoxicity in B16F10 melanoma cells, reducing the tumor load in the treated animals, as well as increasing the survival rate of the animal bearing this neoplasia.

Keywords: Melanoma; Antiproliferative; Antimetastatic, Antitumoral, Pentadienone derivatives

Introdution

Cancer is basically a disease of cells, characterized by a loss in the mechanisms which drive the proliferation and cellular differentiation. The cells under neoplastic transformation proliferate excessively, producing local tumors which may compress or invade normal adjacent structures.[1]

Melanoma is a kind of cancer which represents about 1% of all tumors and is responsible for 75% of skin cancer deaths.[2] The incidence of cutaneous melanoma has improved drastically for decades worldwide and, only in the last two decades, this number has grown three-fold, with an annual increase rate of 3 to 7%.[3-4]

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The main problem related to its treatment is the existence of multiple interactions among the tumors cells and the lost of homeostatic control mechanisms if metastasis occurs.

Malignant melanoma in its advanced metastatic stage of disease presents severe prognostic with the average survival rate around nine months.[5] Furthermore, melanoma is the third most common cause of metastasis in the brain.[6]

The 1,5-bis(4-hydroxy-3-methoxyphenyl)-penta-1,4-dien-3-one and its derivatives can be obtained by ultrasonic irradiation [7], starting from vanillin, a natural product existent in Brazilian propolis and in plants. Due to the fact that 1,5-bis(4-hydroxy-3-methoxyphenyl)-penta-1,4-dien-3-one presents structural similarity with curcumin, it is also believed that their biological profiles are similar as potential antitumoral, antioxidant and anti-inflammatory.[8]

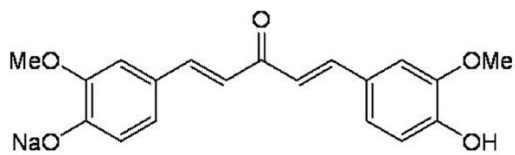

Figure 1- Structural formula of compound DM-1

Antiproliferative studies *in vivo* with 1,5- bis(4-hydroxy-3-methoxyphenyl)-penta-1,4-dien-3-one were limited; however with problems of compound solubility in water. For this reason, the hydrosoluble derivative, sodium 4-[5-(4-hydroxy-3-methoxyphenyl)-3-oxo-penta-1,4-dienyl]-2-methoxy-phenolate, here denominated DM-1 (Figure 1), was obtained to accomplish the pharmacological and toxicological studies.[9]

It is important to highlight that previous toxicological studies have demonstrated that DM-1 has exhibited a lethal dose (LD_{50}) of 700 mg/Kg of body weight in *Balb-c* mice, demonstrating, therefore, low toxicity in acute pharmacological studies.[9]

The *in vitro* trials in cell cultures are used to evaluate the relationship between therapeutic effects versus toxicity. Numerous *in vitro* techniques have been developed for fast assessment of cytotoxicity and genotoxicity of compounds as well as to comprehend their mechanisms in cellular and subcellular levels. [10,11]

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction method is the most employed viability test applied to cell cultures and analyzes the mitochondrial integrity, based on the enzymatic reduction of the reagent by the mitochondrial dehydrogenase.[12]

This work evaluated the cytotoxicity of DM-1 compound by means of *in vitro* tests with melanoma cells and normal fibroblasts. Also studied was the antitumoral potential of the compound regarding *in vivo* tests with B16F10 melanoma-bearing animals after the treatment with DM-1administered by endovenous and intraperitoneal routes.

Materials and methods

Compound DM-1

Compound DM-1 was obtained by synthetic approach as previously described by Quincoces *et al.* [9]

Tumor and Normal Cell Lines

Tumor cell lines of B16F10 murine melanoma and normal human fibroblasts were cultivated in cell culture flasks of 75 cm^2 in medium Roswell Park Memorial Institute (RPMI) 1640 (RPMI-1640), supplemented with 10% of inactive fetal bovine serum, 2mM of L-glutamine and antibiotics.

After 24 hours of adhesion and confluence, the cells were treated with different concentrations (50 mg/mL to 9.5 x 10^{-5} mg/mL) of compound DM-1 using 0.9% physiologic saline solution as diluent. Control plates (tumors and normal cell lines) were cultivated in the same conditions with supplemented culture medium RPMI-1640.

The cellular viability of B16F10 melanoma cells and normal fibroblasts in presence of different concentrations of compound DM-1 was determined by MTT colorimetric assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide].[13] After 24 hours of culture, 10 μL of MTT (5 mg/mL) were added, and the plates incubated for 3 hours in incubator contained 5% of $C0_2$ at 37°C. After this period, the medium was removed and 100 μL of absolute methanol was added to dissolve the formed formazan crystals and precipitates. Absorbance was measured in Enzyme-

Linked Immunosorbent Assay (ELISA) reader with 540 nm wavelength and the inhibitory concentration 50% (IC_{50}) was determined.

Animals and Experimental Conditions

Thirty mice of C57BL/6J strain, males and females, with weigh around 25g each and age from 6 to 8 weeks were used in the *in vivo* experiments and were maintained with a diet of water and ration *ad libitum* during entire experiment period. The animals were inoculated by subcutaneous dorsal administration of 5 x 10^4 tumors cells in sterile conditions following the protocol of Butantan Institute ethics commission on the use of animals (CEUIAB): 479/08.

The compound DM-1 was administered, daily, by endovenous (EV) and intraperitoneal (IP) routes for the treatment of melanoma in the concentration of 3.0 μg in 100μL of saline solution. This dose was calculated considering the IC_{50} value determined in the *in vitro* assays.

The animals were observed daily for 14 days and tumor growth observed until reaching the average diameter of 0.5 cm^2. Three experimentation groups were used:

- Group 1: Ten dorsal melanoma-bearing animals treated with 100 μL of saline solution - control;
- Group 2: Ten dorsal melanoma-bearing animals treated with 100 μL of compound DM-1 diluted in saline solution via intraperitoneal;
- Group 3: Ten dorsal melanoma-bearing animals treated with 100 μL of compound DM-1 solution diluted in saline solution via endovenous.

Evaluation of Dorsal Tumor Growth

Tumor growth was measured, with a pachymeter, through longitudinal and transverse dimensions daily after the implantation of B16F10 cells. The entire process was monitored by photo documentation. The average of area (A) and the tumor load (C) were calculated using the following equations, respectively:

$$A = \varpi R^2$$

C = 100 - (average tumors mass of treated animals - average tumors mass of controls group animals) / 100

Statistical Analysis

Data analysis was done by the comparisons of two or more groups with non-parametric distribution using analysis of variance (ANOVA) followed by the Tukey-Kramer test of multiple comparison.

All values were expressed as average ± standard deviation, and the average survival rate in days, considering as critical level for significance values $p<0.05$.

Results

In vitro evaluation of the cytotoxic and antiproliferative activity of DM-1

The experiments showed that compound DM-1 induced significant cytotoxicity in B16F10 melanoma cells, presenting IC_{50} value of 30 μg/mL obtained by straight line equation with high correlation ($r^2 = 0.978$). On the other hand, this activity pattern was not observed in normal human fibroblasts (Figures 2 and 3).

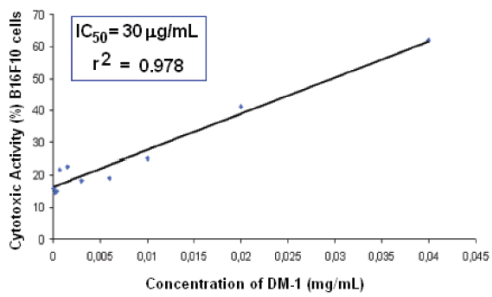

Figure 2 - Cytotoxic activity in B16F10 melanoma cells, in the presence and absence of compound DM-1. Straight line and linear regression curve equations were calculated with the Graph Pad Prism Instat program. IC_{50} of the composition was 30μg/mL

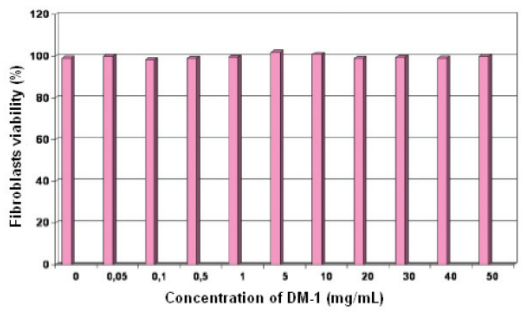

Figure 3 - Cytotoxic activity in normal human fibroblasts in the presence and absence of compound DM-1. Note the absence of toxic activity of the compound on proliferative activity after 24 hours of treatment

In vivo *evaluation results*

After the eleventh day of the tumor cells implantation, 100% of the animals presented small neoplastic nodules with around 0.5cm^2 of diameter and brownish coloration.

The tumors of the animals treated with DM-1 through EV and IP routes presented pigmented dorsal nodules, non-ulcerated and with small variations of volume. No significant neovascularization nor any signs of anemia and cachexia were observed. On the other hand, the control group presented pigmented dorsal tumors and extreme ulceration. After surgical dissection, the implanted tumor was verified to be located in the subcutaneous tissue, presenting grown dark coloration

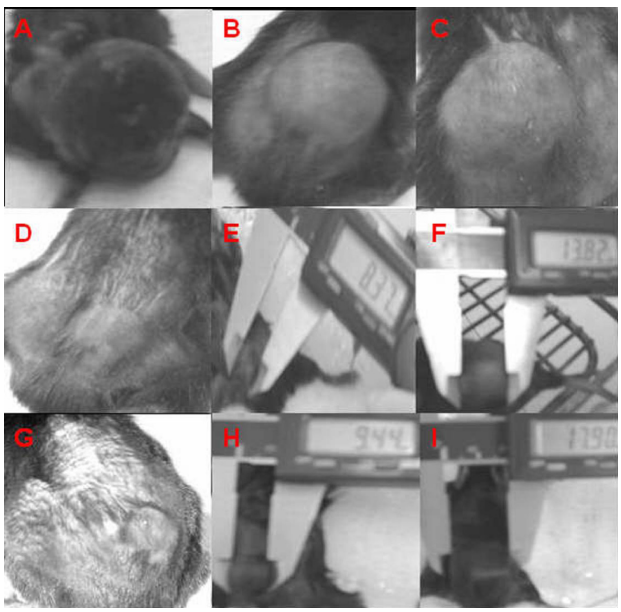

Figure 4 - Macroscopic aspect of the melanoma dorsal tumor of the control group (A, B and C) and of the groups treated with compound DM-1, administered via intravenous (D, E and F) and via intraperitoneal (G, H and I)

due to the production and excessive liberation of melanin (Figure 4).

Survival Rate Analysis

The survival rate, obtained by Kaplan-Meier test, showed that DM-1 expressively increased the survival of dorsal melanoma-bearing animals. The treated mice obtained survival 40% higher than the control group. This parameter was evaluated during the entire

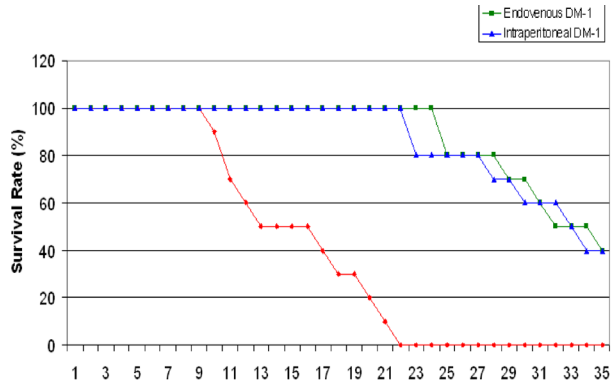

Figure 5 - Survival analysis (Kaplan-Meier) of survival time of animals treated with compound DM-1 and control group

experiment, from tumors inoculation until treatment conclusion (Figure 5).

Tumoral growth analysis

The growth's curves - surface, mass and tumor load - are presented in Figures 6, 7 and 8, respectively, and correspond to the tumor treatment after the eleventh day of melanoma implantation. The treated group presented a significant reduction in tumor growth, while the rate obtained in tumor growth for the control group was vast and expressive.

The treated groups presented a decrease in the dorsal tumor surface, with average values ranging from

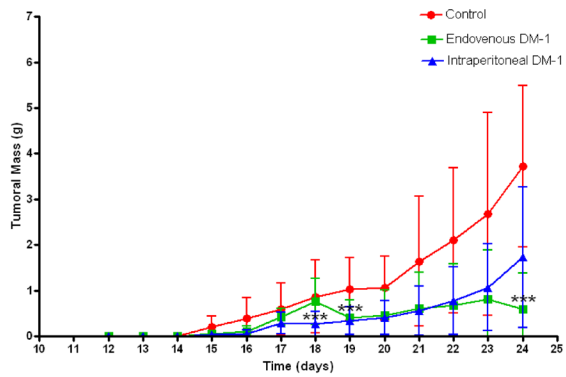

Figure 6 - Analysis of tumours area during 14 days of treatment of B16F10 melanoma, with compound DM-1 administered via intravenous and intraperitoneal, as compared to the control group

5.98 ± 2.1 cm^2 to DM-1 administrated IP and 2.36 ± 1.4 cm^2 to DM-1 EV route, while the control group exhibited average values of 9.47 ± 2.9 cm^2.

The tumors mass of the treated animals also presented significant reduction: 1.72 ± 0.7 cm^2 and 0.58 ± 0.4 cm^2 via to IP and EV administrations when compared to the average values of 3.72 ±1.4 cm^2 of the control group.

The effectiveness of treatment parameters were calculated and it was observed that compound DM-1 reduced tumor load by 84.3% when administered EV, and by 53.6% when administered IP.

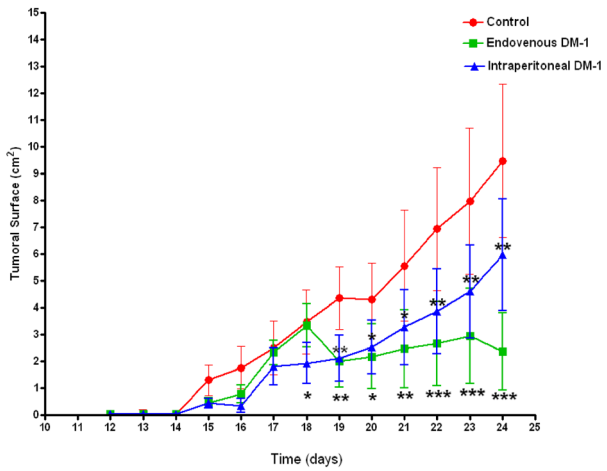

Figure 7 - Analysis of tumours mass during 14 days of treatment of B16F10 melanoma, with compound DM-1 administered via intravenous and intraperitoneal, as compared to control group

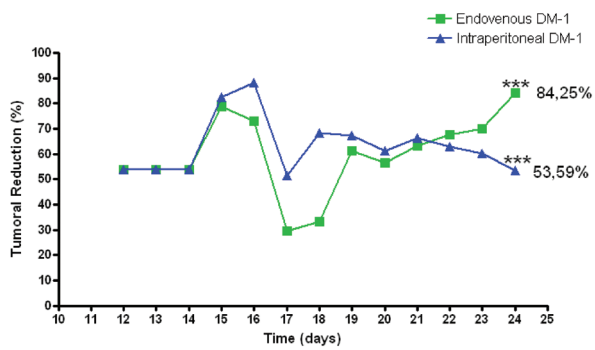

Figure 8 - Evaluation of therapeutic effectiveness (tumor load) of treatment of B16F10 melanoma, with compound DM-1 administered via intravenous and intraperitoneal, during 14 days of treatment

Metastasis Quantification

Metastasis quantification was done through macroscopic analysis, digital photo documentation and histopathologic analysis of all internal organs and primary dorsal tumors.

Results showed that in all therapeutic schemes where compound DM-1 was administered, the percentage of internal metastasis diminished significantly. In the IP treated animals group, a 60% decrease was observed in the number of renal and splenic metastasis. To the EV treated group, the decrease was also extremely significant, reducing renal metastasis by 50% and splenic metastasis by 60%, while the control group presented 90% of internal metastatic lesions distributed in the kidneys and 80% in the spleen. These data are presented in Figure 9.

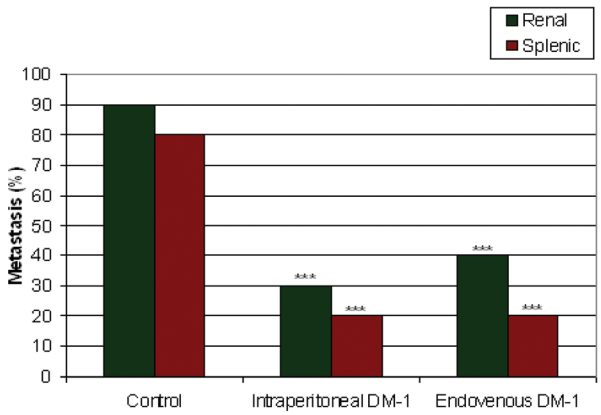

Figure 9 - Distribution of the number of internal metastases in B16F10 melanoma-bearing C57BL/6J mice of the groups control and treated with compound DM-1

Discussion

The emergence of selective antitumoral drugs targeting each acquired capacity of cancer behavior such as metastasis, angiogenesis, etc. and their use in appropriate associations; combined with sophisticated technology to detect and to identify all of the disease stages, can contribute to the profilaxy of many tumors,

and even, to the cure of pre-existent tumors.[14]

No progress, however, has been achieved toward compounds with high efficacy against one or more tumor types but with low systemic toxicity profiles.

Moreover, many of the more potent cytotoxic substances act in specific phases of the cellular cycle and, consequently, only perform their activity against cells that are found in high division processes.[15] Solid tumors that present relatively slow cellular division, such as lung, colon and breast carcinomas, constitute more than 90% of all the types of human cancer and, for this reason, they are in urgent need for new drugs.[16]

Our results suggested that compound DM-1 can fulfill these necessities. The cytotoxicity curve obtained is a typical example of the dose-response curve. The IC_{50} value was 30 μg/mL to B16F10 melanoma cells which indicates a significant inhibitory activity. Besides, no toxic or inhibitory effect was observed toward normal fibroblasts. Consequently, DM-1 exhibits good antiproliferative action toward melanoma cells, with high specificity and selectivity.

As earlier mentioned, DM-1 exhibited LD_{50} of 700 mg/Kg of body weight in *Balb-c* mice, which can be interpreted as an almost atoxic compound.

The *in vivo* treatment of B16F10 melanoma with compound DM-1 administered IP and EV confirmed the efficacy in reducing of tumor growth. Furthermore, it was observed an increase in the survival rate of the animals. Non-treated B16F10 melanoma-bearing C57BL/6J mice died, on average, 35 days after the start of the experiment as reported in literature [17] confirming results found with the control group.

The tumor load decreased significantly with the treatment with DM-1, both through endovenous (84%) or intraperitoneal (54%) routes.

Cell cycle studies have been carried out to elucidate more about the molecular mechanisms involved in the DM-1 action, but these findings, only, already suggest good in vitro and in vivo potency combined to the low toxicity profile, which are relevant parameters taken into account for the approval of new compounds by the regulatory agencies around the world.

Conclusion

The results reveal DM-1 as an efficient agent with high selectivity and specificity, evidenced by the *in vitro* and *in vivo* assays. The administration of compound DM-1 increased the survival rate of animals without causing collateral effects on the hematopoietic system and without causing cachexia. The tumor load observed in the several treatment protocols evidenced the antiproliferative effect of compound DM-1, reducing by 84% in via endovenous administration and by 54% when administered via intraperitoneal, the tumor load of the B16F10 melanoma-bearing animals, besides significantly reducing the number of internal metastasis.

It is probable that new therapies will come to substitute or to combine with what already exists, increasing treatment effectiveness and reducing the incidence of collateral effects. DM-1 is a promising alternative to accomplish this goal.

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