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Lack of long-lasting effects of mitotane adjuvant therapy in a mouse xenograft model of adrenocortical carcinoma

Mabrouka Doghman, Enzo Lalli *

Institut de Pharmacologie Moléculaire et Cellulaire CNRS, Valbonne, France
Associated International Laboratory (LIA) NEOGENEX CNRS, Valbonne, France
University of Nice-Sophia-Antipolis, Valbonne, France

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ABSTRACT

Mitotane is a widely used drug in the therapy of adrenocortical carcinoma (ACC). It is important to set up preclinical protocols to study the possible synergistic effects of its association with new drugs for ACC therapy. We assessed the efficacy of different routes of administration of mitotane (i.p. and oral) in inhibiting growth of H295R ACC cell xenografts in an adjuvant setting. Both formulations of mitotane could inhibit H295R xenografts growth only at short times after carcinoma cells inoculation, even though plasma mitotane levels approached or fell within the therapeutic range in humans. Our results show that mitotane adjuvant therapy is inadequate to antagonize long-term growth of H295R cancer cells xenografts and that care should then be taken in the design of preclinical protocols to evaluate the performance of new drugs in association with mitotane.

1. Introduction

Adrenocortical cancer (ACC) is a rare, life-threatening endocrine malignancy (Fassnacht et al., 2011). Mitotane (o,p′-DDD), a compound related to the insecticide DDT, is a drug widely used, alone or in combination with chemotherapeutic agents, in the therapy of advanced ACC because of its selective adrenolytic properties (Maluf et al., 2011; De Francia et al., 2012). To exert its activity, this drug requires metabolic transformation into an acyl chloride, which takes place in the mitochondria of steroidogenic cells. Mitotane is also used as an adjuvant in ACC patients who have undergone surgical resection of their primary tumor. A large retrospective study has shown that adjuvant mitotane may prolong recurrence-free survival in patients with radically resected ACC (Terzolo et al., 2007), although other studies have not confirmed a therapeutic benefit for it (Bertherat et al., 2007; Grubbs et al., 2010; reviewed in Maluf et al., 2011). Consequently, current therapeutic protocols envisage the use of adjuvant mitotane in patients with an incomplete (Rx/R1) resection, possibly in conjunction with tumor bed irradiation, and in high-risk (defined as having >10% Ki-67 labeling index) patients with complete (R0) surgical resection of their primary ACC (Fassnacht et al., 2011). A prospective, randomized clinical trial (ADIUVO; NCT00777244) is currently underway to assess the efficacy of adjuvant mitotane to prolong recurrence-free and overall survival in patients with low-intermediate risk (<10% Ki-67 labeling index) (https://www.epiclin.it/adiuvo). However, mitotane therapy is often associated to important secondary effects (especially neurological and gastrointestinal), which in some cases force the patients to suspend the treatment. Moreover, due to the peculiar pharmacokinetics of mitotane, only a subset of patients reach plasma mitotane levels in the therapeutic range (14–20 mg/L) (Haak et al., 1994).

Xenograft models of ACC have proven valuable to evaluate the efficacy of drugs targeting tumor cells (reviewed in Luconi and Mannelli, 2012). We have then set up to evaluate the effect of mitotane administered in an adjuvant setting in a xenograft model of the H295R ACC cell line implanted in immunodeficient mice. Two routes of administration of mitotane were compared: intraperitoneal (i.p.) and oral and plasma mitotane levels were measured in samples taken at the end of the experiment. The results of our study show that both formulations of mitotane lack long-lasting effect on H295R xenograft growth.

2. Materials and methods

2.1. Chemicals

Mitotane (o,p′-DDD) was purchased from Sigma–Aldrich (catalog number 25925). Stock solutions were prepared in ethanol at a concentration of 10 mM.

Abbreviations: ACC, adrenocortical carcinoma; NOD/SCID, non-obese diabetic/severe combined immunodeficiency.

* Corresponding author. Address: Institut de Pharmacologie Moléculaire et Cellulaire CNRS, 660 route des Lucioles, Sophia Antipolis, 06560 Valbonne, France. Tel.: +33 (0)4 93 95 77 55; fax: +33 (0)4 93 95 77 08.
E-mail address: ninino@ipmc.cnrs.fr (E. Lalli).

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2.2. Cell culture

H295R cells were cultured in a humidified atmosphere containing 5% CO_2 at 37 °C in DMEM/F12 additioned with 2% NuSerum (BD), 1% ITS+ (BD) and penicillin/streptomycin (Invitrogen).

2.3. In vitro proliferation tests

H295R cells were seeded in complete medium in 96-well plates (5×10^3 cells/well) and treated either with vehicle (ethanol) or with doses of mitotane ranging from 10^{-9} to 10^{-4} M. After 6 days, cell proliferation was measured with the MTS-based CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega). Data are representative of four experiments, each one performed in triplicate. IC50 for mitotane was calculated using the GraphPad Prism software.

2.4. Xenografts and treatments

For each animal, 6×10^6 H295R cells were inoculated subcutaneously into the right flank of four-week old female NOD/SCID/γ_c^{null} mice, as previously described (Doghman et al., 2010; Doghman and Lalli, 2012). Out of a total of 30 mice injected, 10 animals were randomly assigned to each of three groups receiving, respectively:

- Mitotane (440 mg/kg) i.p. (drug dissolved in 10% Tween-80 in PBS) (Barlaskar et al., 2009).
- Mitotane (440 mg/kg) by oral gavage (drug dissolved in olive oil).
- Placebo by oral gavage (olive oil) only.

Treatments were started the day after injection of tumor cells and continued for 5 days/week. No significant weight loss was noticed in any group of mice during the treatment period. Tumor growth was monitored (starting from day 13 after H295R cells inoculation) once per week by measuring with a Vernier caliper and calculating tumor volume by the formula: length × width × height × π/6 (Doghman et al., 2010; Doghman and Lalli, 2012). During the protocol period, two mice were lost in the control group and one in each of the mitotane-treated groups. All protocols were performed according to the institutional animal care and use committee guidelines. ANOVA with Bonferroni's correction for multiple testing was used to assess the significance of differences in xenografts growth among groups of animals.

2.5. Measurement of plasma mitotane levels

It was performed by HRA Pharma using HPLC chromatography on blood samples taken after animal euthanasia at the end of the protocol.

2.6. Steroid measurements in tissues

At the end of the treatment protocols, xenografts were excised, snap frozen in liquid nitrogen and stored at −20 °C. Steroids were ether extracted from the tissues, as described (Chao et al., 2011) and aldosterone, cortisol and DHEA-S measured in the extracts by enzyme-linked immunoassays, as described (Doghman et al., 2007).

3. Results

3.1. Mitotane inhibits proliferation of H295R cell in vitro

High concentrations of mitotane significantly reduced H295R cell proliferation *in vitro* (Fig. 1). The calculated IC50 for mitotane in these experiments was 22.8 μM, which closely matches the IC50 value (24 μM) previously calculated by Schteingart et al. (1993).

3.2. Effect of adjuvant mitotane administered to mice bearing H295R xenografts

We tested two routes of administration of mitotane (i.p. and oral) for their relative efficacy to reduce H295R xenografts growth in immunodeficient mice in an adjuvant setting. Mitotane administration was started the day after tumor cells inoculation in mice and continued 5 times/week at a dose of 440 mg/kg for a period of about 7 weeks. Another group of mice was treated with placebo only. At an early time point (day 13) after H295R cells inoculation both groups of mice treated with mitotane bore significantly smaller xenografts compared with the placebo-treated group (Fig. 2). However, the effect of oral mitotane treatment became non-significant by day 20 after H295R cells inoculation, while the effect of i.p. mitotane lasted until day 34. Starting from day 41 and up to conclusion of the protocol (day 48), xenograft volumes were similar in placebo-treated and in both groups of mitotane-treated animals.

3.3. Mitotane plasma levels

Mitotane plasma levels at the end of the treatment period reached a mean concentration of 12.3 ± 2.4 mg/L in the i.p. mitotane-treated group and of 20.1 ± 1.7 mg/L in the oral mitotane-treated group (Fig. 3).

3.4. Steroid measurements in H295R xenografts

H295R cells produce a variety of steroid hormones (Wang and Rainey, 2012). To assay for the effect of mitotane in inhibiting steroid synthesis in the H295R xenografts, we assayed their content in aldosterone, cortisol and DHEA-S by enzyme-linked immunoassays. No difference in steroid content was evident in xenografts from both mitotane-treated groups of animals compared to placebo (Fig. 4).

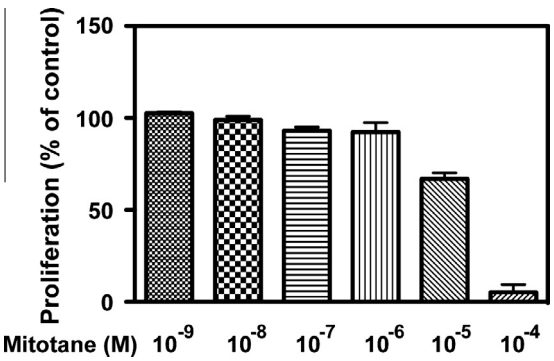

Fig. 1. Effect of varying doses of mitotane on H295R cells proliferation *in vitro*. Data are expressed as percentage of proliferation in mitotane-treated compared to vehicle (ethanol)-treated cells.