Cancer Chemother Pharmacol (1993) 32: 392–395

Cancer Chemotherapy and Pharmacology

Topical toremifene: a new approach for cutaneous melanoma?

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Received: 3 November 1992/Accepted 19 March 1993

Abstract. The distribution of topically applied toremifene (0.5–1 mg/day for 5 days) in the ultraviolet B (UVB)-induced *Monodelphis domestica* opossum melanoma model was examined. The mean concentration of toremifene measured in the skin was 1200 nmol/g, or >500 times that detected in any other tissues (blood, brain, liver, testicles, heart, uterus, eyes). In plasma, toremifene could be detected in only one animal of six (0.04 nmol/ml). Intraperitoneal administration of 0.5 mg toremifene daily for 5 days in three female animals resulted in a mean uterus concentration of 22.9 nmol/g, or 400-fold that achieved by topical administration of 0.5 mg/day in three other female *Monodelphis* (0.05 nmol/g). The cytostatic effect of toremifene was studied in three human melanoma cell lines and three experimental cell lines derived from UVB-induced melanocytic nevi in *M. domestica*. Toremifene had a cytostatic effect on all cell lines (50% growth-inhibitory concentrations, 5.8–9.6 μ*M*). Topical toremifene administration yields high local concentration with minimal systemic distribution. In addition, toremifene has a cytostatic effect at achievable concentrations in a variety of melanomatous cell lines.

Introduction

At present, there is no effective therapy for malignant melanoma except surgery for the early stages of the disease [12]. Dacarbazine has been the drug of choice, with a response rate of 15% [3]. Biologic response modifiers are currently being studied as single agents and in combination chemotherapy in the treatment of melanoma [25, 26]; their ultimate significance remains to be elucidated. Because a possible link exists between steroid hormones and melanoma, tamoxifen has been examined for its benefits as an alternative to cytotoxic drugs [4, 16, 21–24]. More recently, the main emphasis has been placed on combining tamoxifen with cytotoxic drug therapy, with the knowledge that the effects are not necessarily based on a hormonal mechanism [3, 7, 18].

Toremifene is a new triphenylethylene with activity in patients with breast and endometrial cancer [10, 20, 28]. Toremifene has also been shown to have multidrug resistance (MDR)-reversing properties [2, 5, 19]. Quite recently, we have found in nude mice and in baboons that toremifene can be applied topically to obtain very high concentrations in superficial tumors while minimizing systemic exposure (submitted for publication).

The purpose of the present study was to evaluate the cytostatic effects of toremifene on cultured human and animal melanomatous cells and to study the distribution of toremifene given topically or intraperitoneally in an in vivo ultraviolet (UV) light-induced melanoma model, *Monodelphis domestica* [15, 17; VandeBerg JL et al, submitted for publication].

Materials and methods

Drugs. Toremifene (molecular weight 406) was obtained from Orion Corporation Farmos (Turku, Finland) in the form of toremifene citrate (molecular weight 598). For topical therapy, toremifene was first dissolved in dimethylsulfoxide (DMSO), after which it was added to 3% methylcellulose in ethanol to a final concentration of 1 mg/ml. For intraperitoneal (i.p.) therapy, toremifene was dissolved in peanut oil to a final concentration of 5 mg/ml.

Animals. The *Monodelphis domestica* colony at the Southwest Foundation for Biomedical Research is a large colony (steady state, about 2,000 individuals) the genetic background of which is well documented [29]. Six male animals aged 12–24 months and weighing 119–132 g were given toremifene topically for 5 days. The dose was 0.5 mg/day in three animals and 1 mg/day in the other three. Toremifene was applied topically to shaved skin on a surface area of 1 cm^2 of the lower back. At 2 h after the last dose on day 5 the animals were killed, and the concentrations of toremifene and its metabolites were measured in the plasma, skin, testicles, liver, eyes, brain, and heart. Six female animals aged 12–18 months and weighing 83–104 g were given toremifene at

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Table 1. Mean (±SD) concentrations of toremifene (TOR) measured in plasma (nmol/ml) and various tissues (nmol/g) following topical TOR administration at 0.5 ($n = 3$) and 1 ($n = 3$) mg/day for 5 days in 6 male *Monodelphi domestica*

Site	0.5 mg/day	1 mg/day
Plasma	0	0[a]
Skin	1296.40±1003.93	1237.35±768.90
Liver	1.07± 0.69	3.60± 2.23
Testicles	1.52± 2.13	0.52± 0.05
Brain	0.82± 0.99	0.82± 0.62
Eyes	0	0.17± 0.05
Heart	0	0.21± 0.16

[a] 0.04 nmol/ml in one sample

Table 2. Mean (±SD) concentrations of toremifene (TOR) measured in plasma (nmol/ml) and various tissues (nmol/g) following topical ($n = 3$) and i.p. ($n = 3$) TOR administration at 0.5 mg/day for 5 days in 6 female *Monodelphi domestica*

Site	Topical application	Intraperitoneal injection
Plasma	0	0.03[a]
Skin	150.98±149.83	0.06± 0.05
Liver	1.07± 0.31	21.62±14.05
Uterus	0.05± 0.01	22.91±19.50
Brain	0.16[a]	1.09± 0.52
Eyes	0.65± 0.12	0.58± 0.29
Heart	0.67± 0.15	1.26± 0.39

[a] Mean of only two values

0.5 mg/day intraperitoneally (three animals) or topically (three animals) for 5 days. The topical therapy was carried out as described above. For i.p. administration, 0.1 ml toremifene solution was given with a 1-ml syringe and a 22-gauge needle. At day 5 the animals were killed, and the concentrations of toremifene and its metabolites were measured in the plasma, uterus, skin, eyes, heart, brain, and liver.

To induce melanocytic lesions, 4- to 5-month-old *M. domestica* were exposed to UV light (UVB) with a wavelength of 302 nm for 45 weeks (3 times per week, each dose being 125 J/m^2) [30]. This procedure induces dermal melanocytic nevi in approximately 14% of animals; these nevi have been identified histologically as melanocytic hyperplasias and benign melanomas [15]. Three cell lines were developed from these lesions as described below.

Toremifene analysis in tissue and blood samples. At day 5, the *M. domestica* were anesthesized with metafane, blood was obtained by cardiac puncture and collected into heparinized tubes, and plasma was collected and frozen at –20° C. Tissue samples from the uterus/testes, skin (including subcutaneous tissue up to the depth of 5–10 mm) from the site of application, eyes, heart, brain, and liver were weighed and also frozen at –20° C. Analysis of toremifene and its metabolites by high-performance liquid chromatography (HPLC) was done as previously described [6]. The limit of detection of toremifene by HPLC is 8 ng/ml. In tissue samples, the concentrations were expressed in nanomoles per gram, and in plasma, in nanomoles per milliliter.

Cell cultures. The human melanoma cell lines were grown in vitro to assess the cytostatic 50% growth-inhibitory concentrations (IC_{50} values) of toremifene. The metastatic melanoma cell line SK-MEL-31 was obtained from the American Type Culture Collection (Rockville, Md.). This line was grown in vitro in Eagle's minimum essential medium (EMEM) supplemented with 15% fetal bovine serum (FBS) at 37° C in an atmosphere containing 5% CO_2. Two additional human cell lines, TD 30A and TD 36, were derived from portions of surgically excised large-diameter, superficial spreading melanomas. The biopsy specimens were minced and plated on plastic at 37° C in an atmosphere containing 5% CO_2 in a medium (BMGM [9]) supplemented with 50% melanocyte growth medium (MGM; Clonetics Inc.) plus 50% Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 160 n*M* 12-o-tetra-decanoyl-phorbol-13-acetate (TPA), 1 n*M* cholera toxin, and 0.2% penicillin-streptomycin (pen-strep).

Three cell lines were derived from UVB-induced dermal melanocytic nevi in *M. domestica* [8]. TD 1.4 was developed from a benign melanoma, whereas TD 7.2 and TD 8 were derived from melanocytic hyperplasias. The cell lines were grown at 35° C in an atmosphere containing 5% CO_2 in DMEM supplemented with 10% FBS, 160 n*M* TPA, 1 n*M* cholera toxin, 5 µg insulin/ml, 5 µg transferrin/ml, 5 ng selenite/ml, 2 m*M* L-glutamine, and 0.2% pen-strep.

All lines except SK-MEL-31 were assayed in 24-well plastic plates using the crystal violet staining method [9]. The wells were seeded with 1×10^5 cells and were continuously exposed to a titration of toremifene (1–16 µ*M*) for a total of 4 days. All samples were run in triplicate. At the end of the treatment period, the mean values for adherent cell number were derived spectrophotometrically using a 0.1% crystal violet/10% ethanol stain. Untreated control cells were included on the plates for comparison and to permit calculation of the percentage of cell survival. Then, the IC_{50} values were determined from semilogarithmic plots of the percentage of cell survival versus the concentration of toremifene citrate. The IC_{50} value for toremifene in SK-MEL-31 was determined by cell counting using a Coulter Model ZM Counter.

Table 3. IC_{50} values determined for toremifene in human and experimental melanomatous cell lines

Cell lines		IC_{50}
Human:		
	TD 36	5.8 µ*M*
	TD 30A	7.7 µ*M*
	SK-MEL-31	8.1 µ*M*
Experimental:		
	TD 8	7.7 µ*M*
	TD 1.4	9.3 µ*M*
	TD 7.2	9.6 µ*M*

Results

The mean plasma and tissue concentrations of toremifene measured in six animals following topical administration at 0.5 and 1 mg/day for 5 days are given in Table 1. The toremifene concentration measured in the skin was more than 500-fold that detected in other tissues, among which the highest concentration was found in the liver and testicles. In the eyes and heart, a very low amount of drug was found only in the higher-dose group. In the other tissues, the concentrations of toremifene were in the same range in both groups. In plasma, toremifene was detected in a trace amount, or 0.04 nmol/ml, in a single animal treated with 1 mg/day.

Toremifene metabolites were found in moderate amounts (individual data not shown), suggesting systemic metabolism. The difference between the skin and other tissue concentrations of 4-OH-toremifene was not as evident as was the case for toremifene. The amounts of *N*-desmethyltoremifene were quite low.

In a comparison of the concentrations resulting from i.p. and topical administrations (Table 2), the most striking difference was noted between the uterus and skin concentrations. Following i.p. administration, the mean uterus

concentration was 22.91 nmol/g, or 400-fold that obtained by topical administration (0.05 nmol/g). Also, the liver concentration was quite high after i.p. administration as compared with the topical route.

The growth-inhibitory effect of toremifene was very similar in all six cell lines of melanocyte origin. The IC_{50} values ranged from 5.8 to 9.6 μM (Table 3). In the skin, these concentrations were easily achieved by topical administration.

Discussion

It has long been known that pregnancy has a deteriorating effect on the natural course of melanoma [1]. A possible explanation for this might be the presence of estrogen receptors in part of the melanomas [11]. These findings led to clinical trials of tamoxifen in the treatment of melanoma. The oral dose of tamoxifen used in the therapy of malignant melanomas has been 20 mg in three studies [16, 21, 22], 20–40 mg in one study [23], 40 mg in another investigation [4], and 100 mg in yet another study [24]. The combined response rate of 97 patients has been 12% and the complete response rate, 2%. The majority of the responders have had superficial lesions. Neither the dose of tamoxifen, sex, nor the estrogen-receptor status influenced the response rate [4, 16, 21–24].

More recent findings suggest that tamoxifen appears to be clinically synergistic in melanoma with other chemotherapeutic agents such as dacarbazine, carmustine, and cisplatin [3, 7, 18]. This effect is probably not mediated via steroid receptors but has more to do with other biochemical pathways (e.g., protein kinase C, calcium-channel blocking). Toremifene is a structurally related triphenylethylene that appears to be well tolerated at doses of up to 460 mg daily [14]. Toremifene has been shown to have hormone-dependent and -independent antitumor activity in experimental animals [13] and also clinically in breast and endometrial cancers and desmoid tumors [10, 20, 28, 31]. Similarly to tamoxifen [27], toremifene has been shown to reverse multidrug resistance [2, 5, 19].

In the present study, we demonstrated that toremifene has a clear cytostatic effect on human and experimental melanomatous cell lines in vitro. Although a cytostatic effect was observed in all cell lines, the concentrations needed to produce this effect were relatively high. However, topical administration of toremifene produces skin concentrations that are far beyond these in vitro concentrations, suggesting that a clear cytostatic effect could be obtained on cutaneous melanoma in vivo. Furthermore, topical treatment of the margins of surgically excised cutaneous melanomas to prevent subsequent growth of cryptic melanoma cells is also possible. In conclusion, it appears that a new strategy in the treatment of melanoma would be to treat cutaneous lesions (or the margins following surgery) with topical toremifene and, in more disseminated cases, to combine cytotoxic therapy with both oral and topical toremifene.

Acknowledgements. This work was supported in part by grants from the Robert J. Kleberg, Jr., and Helen C. Kleberg Foundation (to T. D., J. V. B., and E. R.), the Samuel Roberts Noble Foundation, Inc. (to T. D., J. V. B., and E. R.), and Orion Corporation Farmos (to J. M. and M. D.), which also kindly provided the drug. The authors gratefully acknowledge the technical assistance of Patricia Porter.

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